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New technology and resources for cryptococcal research

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Abstract

Rapid advances in molecular biology and genome sequencing have enabled the generation of new technology and resources for cryptococcal research. RNAi-mediated specific gene knock down has become routine and more efficient by utilizing modified shRNA plasmids and convergent promoter RNAi constructs. This system was recently applied in a high-throughput screen to identify genes involved in host-pathogen interactions. Gene deletion efficiencies have also been improved by increasing rates of homologous recombination through a number of approaches, including a combination of double-joint PCR with split-marker transformation, the use of dominant selectable markers and the introduction of Cre-Loxp systems into *Cryptococcus*. Moreover, visualization of cryptococcal proteins has become more facile using fusions with codon-optimized fluorescent tags, such as green or red fluorescent proteins or, mCherry. Using recent genome-wide analytical tools, new transcriptional factors and regulatory proteins have been identified in novel virulence-related signaling pathways by employing microarray analysis, RNA-sequencing and proteomic analysis.

Keywords

Cryptococcus neoformans; *Cryptococcus gattii*; cryptococcosis; pathogenic fungus; RNAi; yeast; technology; microscopy; experimental model

Cryptococcus neoformans is a life-threatening opportunistic pathogen that causes both pulmonary disease and meningoencephalitis not only in immunocompromised patients, but in healthy individuals, as well. Cases of cryptococcosis have increased dramatically over the past 30 years and currently results in approximately half a million deaths per year (Park et al., 2009). In survivors, the disease is a major cause of neurological sequelae in HIV patients, causing substantial morbidity (Banerjee et al., 2001). Because of its significant impact on human health as well as the recent development of robust genetic and disease models, *C. neoformans* has become an ideal model system for researching mechanisms of fungal pathogenesis. Two varieties of *C. neoformans* have been recognized. *C. neoformans* var. *neoformans* and var. *grubii*, the latter representing the dominant strain in clinical

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isolates. A former variety of *C. neoformans* var. *gattii* was reclassified as a separate species *Cryptococcus gattii* (Kwon-Chung et al., 2002). As a second major pathogenic *Cryptococcus*, it is capable of causing infections in previously healthy individuals although a subgroup has been recently shown to possess immunosuppressing autoantibodies to granulocyte-monocyte stimulating factor, GM-CSF (Rosen et al., 2013). The outbreak of *C. gattii* in Vancouver Island, BC, Canada, has led to increased recognition of cryptococcosis in non-HIV infected individuals.

C. neoformans grows in culture as haploid yeast forms and reproduces by budding. During mating, *C. neoformans* generates hyphae, fuses with opposite or, rarely, the same mating types (Fraser et al., 2005; Lin et al., 2005) and produces spores. One of the unique features of *Cryptococcus* is the polysaccharide capsule that enlarges during infection or under infection-related conditions, such as low glucose, low nitrogen, serum and low iron (O'Meara and Alspaugh, 2012). Based on antigenic differences in the polysaccharide capsule, *Cryptococcus* is traditionally subdivided into 4 serotypes, A, B, C, and D as well as a hybrid serotype, AD.

Formerly, molecular studies of *C. neoformans* were challenging, due to the lack of genetic tools and the low efficiency of homologous recombination. To overcome these difficulties, researchers have made progress in the areas of genome sequencing, gene silencing and disruption, transformation and imaging. The completion of genome sequencing of two related strains of *C. neoformans*-- serotype D (JEC21 and B3501A) was first reported by Loftus and his colleagues in 2005 (Loftus et al., 2005) and has been extended to the reference serotype A isolate, H99 (www.broad.mit.edu) (Janbon et al., 2014), as well as *C. gattii* (<http://www.bcgsc.ca/project/cryptococcus>). The genome of *C. neoformans* is approximately 19Mb and consists of 14 chromosomes. The genome of *C. gattii* strains WM276 and R265 are also assembled into 14 chromosomes with a size of 18.4Mb. When compared with the genome of *C. neoformans* strain B3501A, the WM276 and R265 genomes show 87% and 85.6% identity respectively (Janbon et al., 2014). In addition, many chromosomal rearrangements were observed between *C. gattii* and *C. neoformans* especially in chromosomes 4, 9, 10 in the two strains (D'Souza et al., 2011). These advances in the sequencing field have provided researchers an important resource to apply newly available molecular technologies to silence, delete or mutate specific genes by homologous recombination or random insertion of exogenous DNA (Edman and Kwon-Chung, 1990; Erickson et al., 2001b; Lodge et al., 1994; Toffaletti et al., 1993). In this article, we review new molecular and genetic tools that have been applied to the regulation of gene and protein expression as well as protein localization and interactions, which have become useful experimental methods for *Cryptococcus* research.

RNA interference

Progress in molecular biology has enabled the silencing of gene expression in a regulated and specific way through introduction of double-stranded small interfering RNA (siRNAs) to target complementary messenger RNA (mRNA) for degradation. The RNAi machinery is evolutionary conserved in most eukaryotes, including the pathogenic fungus *C. neoformans* (Nakayashiki et al., 2006). In this highly-conserved regulatory process, siRNA duplexes of

20–25 nucleotides are produced from long double-stranded RNAs (dsRNAs) or small hairpin RNAs (shRNAs) by an endoribonuclease Dicer. The duplexes of siRNA activate and bind the RNA-induced silencing complex (RISC), in which the sense strand is cleaved by endonucleases, and the remaining antisense-RISC complex can target homologous mRNA sequences for RNA degradation. Since shRNA can be synthesized intracellularly from a plasmid, one of the valuable molecular tools for *C. neoformans* is to silence targeted genes via shRNA.

In 2002, Gorlach and his colleagues provided the first evidence that RNA-mediated silencing functions in *C. neoformans* (Gorlach et al., 2002). They were able to repress the expression of calcineurin A (*CNA1*) in serotype D and laccase (*LAC1*) in serotype A using plasmids carrying genes' cDNA sequence in an antisense orientation. In the same year, a successful shRNA-mediated silencing of *CAP59* and *ADE2* was reported by Liu et al. (Liu et al., 2002), which was later widely used in the *Cryptococcus* field. An additional advance consisted of using a cryptococcal intron in a 'one step' plasmid construction approach, rather than a random fragment of DNA to bridge the sense and anti-sense strands (Fig.1) (Hu et al., 2008; Panepinto et al., 2009; Panepinto et al., 2005; Reese and Doering, 2003). This allows efficient formation of dsRNA hairpins after cryptococcal intron splicing and more effective eukaryotic silencing but successful recovery of the shuttle vector from *E. coli* due to lack of splicing in prokaryotes. However the application of these methods has been limited by the efficiency of RNAi constructs transformation and time required for generation of silencing plasmids; a simpler RNAi construct with two cryptococcal promoters in opposing orientation was thus developed. The two convergent promoters initiate the transcription of both sense and antisense strands of the target gene sequences. Each newly synthesized transcript anneals with its complementary strand to form a double-stranded RNA which activates the RNAi system (Bose and Doering, 2011). *Cryptococcus* researchers have used both shRNAi and convergent promoter RNAi constructs to study the function of genes involved in *C. neoformans* virulence.

RNAi mediated gene silencing has several advantages over gene disruption. For example, different promoters can be used to regulate the synthesis of dsRNA in *C. neoformans*. They can be either constitutively expressed, under promoters such as *ACT1* (Cox et al., 1995; Varma and Kwon-Chung, 1999) or inducibly expressed or repressed under galactose-inducible *GAL7* promoters, which allows conditional gene silencing for targeted genes (Ory et al., 2004; Ruff et al., 2009; Wickes and Edman, 1995). Moreover, combinations of dsRNA can be used to silence multiple genes at the same time, or to silence the expression of a gene family or paralogous genes using a single antisense sequence (Zhao et al., 2005). Stability of multiple RNAi plasmids also allows simultaneous suppression of a regulator or trafficking gene and localization of its target protein through epitope tagging (Panepinto et al., 2009). Specific transcript variants can also be targeted by a unique sequence which is not present in other transcript isoforms (Celotto et al., 2005). In addition, constitutive RNAi can be used for the study of essential genes since expression is merely reduced rather than eliminated and has a distinct advantage over conditional modalities such as temperature-sensitive mutations in that retained phenotypes after RNAi suppression are likely to be unrelated to the targeted mutation because of continual suppression during all stages of

growth. Recently the RNAi system has been used for high-throughput screens in cryptococcal genome analysis and to identify genes involved in host-pathogen interactions (Falschlehner et al., 2010; Prudencio and Lehmann, 2009).

In addition to exogenous RNAi introduced into cells, endogenous RNAi has also been reported in *C. neoformans*, such as RNAi-mediated transposon regulation and specific gene silencing during sexual reproduction (Janbon et al., 2010; Wang et al., 2010). Recently miRNAs (miR1 and miR2) were identified in transgene silencing in *C. neoformans* (Jiang et al., 2012). The authors found that miR1 or miR2 fused *URA5* and *CLC1* reporter genes (*URA5-miRs* and *CLC1-miRs*) were successfully silenced via RNAi pathway in *C. neoformans*, then these miRNAs can be useful tools to fuse with a target gene for gene silencing in cryptococcal research.

Gene disruption

Since RNAi typically does not eliminate a targeted gene's function, a complementary approach for the study of non-essential genes is to completely disrupt a gene's sequence by replacing it with a gene-disruption cassette (Lodge et al., 1994; Nelson et al., 2001; Salas et al., 1996). However the homologous recombination rate in *C. neoformans* is much lower than in *Saccharomyces cerevisiae* and it requires longer flanking sequences (preferentially 500bp or more). The H99 serotype A strains has a rate of up to 50% in homologous integration using biolistic transformation; however, the rate dramatically decreases to a reported 1–4% with serotype D (Davidson et al., 2000). The integration rates for serotype B and C are about 3.4% and 8–12% respectively (Fraser et al., 2003; Narasipura et al., 2003). The soil bacterium *Agrobacterium tumefaciens* has also been used to increase transformation efficiency of *C. neoformans* (Idnurm et al., 2009; McClelland et al., 2005). Another way to increase the frequency of homologous recombination is to reduce the rate of non-homologous recombination by deleting the Ku proteins which are important for non-homologous end joining processes, although Ku mutations may reduce virulence of the strains (Goins et al., 2006).

In most laboratories, overlap-PCR methods have been used routinely to amplify a gene-disruption cassette with selectable makers, such as nourseothricin acetyltransferase (NAT) (Fig.2) (Davidson et al., 2002). However this type of PCR requires multiple templates and generates long lengths of the final PCR products, which lowers its efficiency. In addition, all insertional constructs have high rates of non-homologous recombination in *C. neoformans*. To improve the frequency of homologous recombination in recovered transformants, a split-marker transformation method was developed (Fu et al., 2006). It uses a mix of double-joined PCR products including overlapping truncations of the selectable marker for homologous recombination (Fig.2). The homologous recombination rates are reported to be as high as 60% depending on the target gene. However because it uses orotidine monophosphate pyrophosphorylase (OMPPase/*URA5*) as a selectable marker and *ura5* auxotrophic strains for transformation, it may be less applicable to the direct transformation of clinical isolates. Recently, this method was modified by Kim MS *et al* by replacing the *URA5* with a NAT dominant selectable marker in combination with double-Joint PCR (DJ-PCR) (Fig. 2) (Kim et al., 2009). The modified method requires fewer templates and

generates shorter PCR products, so it is more convenient and efficient with higher targeted-integration frequencies for gene disruption in *C. neoformans*.

Currently, there are only five dominant selectable markers available from gene deletion cassettes, which limit the number of genes that can be deleted from the genome in the same strain. However the introduction of Cre-Loxp systems in *Cryptococcus* allows the reuse of the selectable marker (G418), enabling multiple gene deletions in one strain (Baker and Lodge, 2012) (Fig. 2). This Cre-Loxp system is a powerful tool for gene disruption in *Cryptococcus*. Cre recombinase plasmids can be introduced into strains containing loxp flanked target genes for single, multiple or inducible gene deletions, improving the efficiency of the deletion system.

The genome of *C. neoformans* encodes an estimated 6,967 genes based on the results of gene prediction programs (Janbon et al., 2014), making the identification of mutant genes created through chemical or ultraviolet radiation difficult. Thus, to improve identification of mutagenized genes responsible for a given phenotype, a signature-tagged mutagenesis (STM) approach was developed for screening avirulent mutants of *C. neoformans*. The STM strategy was first developed in bacteria, relying on transposon random insertion into the genome of bacteria (Hensel et al., 1995). Each transposon has a unique tag sequence for identification. Researchers have utilized STM to discover virulence-associated genes by injecting a mixture of different random mutants into an animal model for virulence analysis. However there are no active transposon systems present in *Cryptococcus*. Thus, to isolate virulence mutants of *C. neoformans*, a plasmid vector, pKB-STM was designed and used for insertional mutagenesis through ectopic integration of foreign DNA into the genome of fungus (Nelson et al., 2001; Saenz and Dehio, 2005). Recently, this approach was modified and used in a high-throughput in vivo screen for pulmonary infectivity (Liu et al., 2008). The new method utilized fusion PCR to create deletion constructs with 48 unique barcodes in a high throughput manner. These constructs was transformed using enhanced biolistic transformation for higher rate of homologous recombination. A total of 1180 gene deletion mutants were created in the screen. Another approach consists of insertional mutagenesis of a pBSK-derived *E. coli* plasmid containing a cryptococcal *URA5* transformation marker. Identification of a mutagenized gene is made by restriction nuclease digestion of fungal genomic DNA, using an enzyme that cuts outside of the insertional vector, followed by religation and recovery as an *E. coli* shuttle vector. This approach has an advantage that insertions have tended to be single site insertions and has been extensively used in the identification of laccase-associated virulence genes including a vacuolar proton pump, *VPH1* and a global regulator, *VAD1* (Erickson et al., 2001a; Panepinto et al., 2005).

More recently, targeted genome-wide mutant libraries have being developed with approximately 1500 deletion mutants thus far and the number is increasing every year (Idnurm et al., 2005; Liu et al., 2008). This collection is currently available through the fungal genetic stock center (www.fgsc.org) or ATCC (www.atcc.org) and has been successfully utilized for several screens (He et al., 2012; Tseng et al., 2012). The library of *C. neoformans* deletion mutants is expected to facilitate the screening of virulent factors important for fungal pathogenicity and the dissection of multiple signaling pathways in *Cryptococcus*'s. However, other methods of mutagenesis will continue to be valuable as

production of high throughput deletion libraries often require parental strains that have higher transformation efficiencies, but may have lost certain virulence features.

Expression plasmids used in *C. neoformans*

Plasmids have provided an important workhorse for molecular studies in *C. neoformans*, including protein expression, RNAi gene silencing, protein localization and genomic library constructions (Fox et al., 2003; Vallim et al., 2005; Varma et al., 2006). The first group of plasmids used in *C. neoformans* carried either a uracil, adenine or lysine synthetic gene as a selected marker, which could complement the corresponding auxotrophic mutant (Chang and Kwon-Chung, 1994; Kwon-Chung et al., 1992; Toffaletti et al., 1993). This method was modified by several groups to construct a new set of plasmids with dominant drug resistant markers such as hygromycin B, NAT, neomycin, geneticin or phleomycin (Cox et al., 1996; Hu and Kronstad, 2006; Hua and et al., 2000; McDade and Cox, 2001).

Constitutively-expressed or regulated plasmids are used to control protein expression under certain circumstances. For example, genes driven by glyceraldehyde-3-phosphate dehydrogenase (*GDP*) and actin (*ACT1*) promoters can be constitutively overexpressed (Varma and Kwon-Chung, 1999). Inducible promoters including *CTR4* and *GAL7* are regulated by copper and galactose respectively (Ory et al., 2004; Wickes and Edman, 1995). Two other galactose-inducible promoters have also been successfully used in *C. neoformans* var. *grubii*, which is useful for the analysis of gene functions in the most prevalent serotype A reference strain, H99 (Ruff et al., 2009).

Plasmid transformation in *C. neoformans* can be achieved by electroporation which tends to retain the plasmid as an extra-chromosomal plasmid (Edman and Kwon-Chung, 1990), but *Cryptococcus* has low transformation efficiency compared to model yeasts. Alternatively, transformation can be accomplished by biolistic transformation, which delivers DNA more efficiently to *Cryptococcus* (Davidson et al., 2000; Toffaletti et al., 1993). When co-incubated with *C. neoformans*, *A. tumefaciens* cells containing a modified Ti plasmid can inject its plasmid into host cells and become integrated into the host genome. Unlike other transformation methods, this method allows host genome integration, but does not mediate homologous recombination. The *A. tumefaciens* mediated transformation efficiency is comparable to either electroporation or biolistic transformation and shows higher efficiency in serotype B and C with stable transformants and a low integration rate (McClelland et al., 2005).

Stability and transformation efficiencies are keys to successful plasmid construction. Previous studies in *C. neoformans* showed that foreign DNA could be transformed with higher efficiency by adding telomeric repeats to both ends of the DNA. These elements can be inserted into a plasmid as inverted repeats, which when cleaved, generate linearized plasmid telomeric ends that facilitate plasmid transformation and stabilize transformants (Edman, 1992; Edman and Kwon-Chung, 1990). Another feature that stabilizes plasmids of the pPM8 and the pKUTAP class of plasmids is an *E.coli*-derived *STAB1* sequence that allows retention of the plasmid even under non-selective conditions (Mondon et al., 2000). Such plasmids have sufficient stability to enable retention even during mouse passage,

enabling virulence studies to be conducted after altered gene dosing by RNAi suppression (Panepinto et al., 2009) or overexpression (Hu et al., 2014).

Microscopy

Microscopy has been used extensively in the diagnostic and research fields of *Cryptococcus*. For example, capsules in cerebral spinal fluid (CSF) can be visualized by India ink stain, which produces a distinctive halo appearance around the cell wall due to capsular exclusion of the microparticulate dye. Mucicarmine stain, derived from pigments produced by scale insects of certain *Porphyrophora* species, is used for *Cryptococcus* diagnostic visualization in tissues (Lazcano et al., 1991). In frozen or paraffin-embedded tissue sections, cryptococcal cell wall can be detected with calcofluor white, a cotton whitener that fluoresces under ultraviolet light (Giles et al., 2009; Monheit et al., 1984). Lucifer yellow (Srikanta et al., 2011) and eosin Y (Baker et al., 2007) are also used to stain cryptococcal cell walls but none except mucicarmine are specific to *Cryptococcus*. In addition, ultrathin-section, quick-freeze, deep-etch scanning electron microscopy and differential interference microscopy can be used for characterizing the three-dimensional ultrastructure of *C. neoformans* (Cleare and Casadevall, 1999; Edwards et al., 1967; Feldmesser et al., 2001) and freeze fracturing methods are especially useful for studying the fine ultrastructure of yeast capsular mutants (Fig.3) (Park et al., 2014).

Various antibodies are also used to image *Cryptococcus* by immunofluorescence staining, such as anti-capsule antibodies (Belay et al., 1997; Casadevall et al., 1994; Feldmesser et al., 2000). The fluorescein or FITC labeled lectins or wheat germ lectin were used to visualize cryptococcal cells (Botts et al., 2009; Fonseca et al., 2013; Rodrigues et al., 2008). Fluorescent-tagged cryptococcal proteins can also be detected under fluorescent microscopy (Ding et al., 2013; Haynes et al., 2011). Several fluorescent-tagged proteins have been codon-optimized and are highly expressed in *Cryptococcus*, such as green fluorescent protein (GFP, Fig. 4, top panel) (Adler et al., 2011; Liu and Balasubramanian, 2001; Liu et al., 2006; Panepinto et al., 2005; Verma and Idnurm, 2013; Wang et al., 2010), mCherry (Fig.4 bottom panel) (Kozubowski et al., 2013; Park et al., 2014; Waterman et al., 2012) and Dsred (Xue et al., 2006).

Genome-wide analysis

Whole genome analytical techniques were greatly facilitated by the acquisition and development of multiple cryptococcal genomic sequence annotations and expressed sequence tag (EST) databases. Initial methods for whole genome transcriptional analysis consisted of serial analysis of gene expression (SAGE), developed for rapid and detailed analysis of the overall gene expression profiles between cell populations (Velculescu et al., 1995). Sage was used in *Cryptococcus* to examine the transcriptome changes in response to temperature and iron concentration manipulations (Hu et al., 2008; Steen et al., 2002). Recently, progress in transcriptional techniques has allowed researchers to analyze gene expression profiling changes to antifungal drugs, iron depletion, CO₂ and oxidative stress by DNA microarray analysis in *Cryptococcus* (Florio et al., 2011; Kim et al., 2010; Upadhyay et al., 2013). These microarray analytic techniques have resulted in the identification of new

transcription factors and regulatory proteins in signaling pathways (Chun et al., 2011; O'Meara et al., 2010). Transcriptional analysis has also been combined with RNA sequencing (RNA-seq) to explore regulatory networks in cell wall remodeling and capsule regulation (Haynes et al., 2011; O'Meara et al., 2013). RNA-seq has an advantage in being an 'open method' in that it is not restricted in sequence output, like microarrays, and can identify novel genes, or compare specific transcript regions or non-coding RNAs. In addition, transcriptional analysis can be used to compare microevolutionary changes within the mammalian host (Hu et al., 2014) and comparative transcriptomics can be used to identify novel signaling pathways in evolutionarily diverse transcriptomes (Adler et al., 2011). By using RNA-seq and transcriptome analysis at the site of human cryptococcal meningitis, Chen and his colleagues identified genes and pathways uniquely regulated by exposure to CSF, which could be important for the survival of *C. neoformans* in the central nervous system (Chen et al., 2014). Recently, a high-throughput small RNA (sRNA) sequencing technique enabled the discovery of a novel RNAi dependent antifungal drug resistance mechanism (Calo et al., 2014).

In addition to genome-wide transcriptional analysis, proteomics has been also increasingly utilized for cryptococcal research. A recent proteomics study revealed that in *C. neoformans*, calcineurin interacts with endoplasmic reticulum and Golgi trafficking proteins during heat stress (Kozubowski et al., 2011), suggesting potential mechanisms by which these fungi could be targeted. A global phosphoproteome analysis of *C. neoformans* var. *grubii* recovered 1089 phosphopeptides derived from 648 proteins, providing bases for understanding the functions of phosphoproteins in virulence and infection (Selvan et al., 2014). Moreover, Vu and his colleagues identified a secreted metalloprotease (Mpr1) required for central nervous system infection of *C. neoformans* through a proteomic analysis of the extracellular proteome (Vu et al., 2014). This approach was also used for identifying the substrate of a virulence associated enzyme, components important for the interactions of extracellular vesicles with the cell wall, and metabolic changes in cryptococcal biofilms (Liu and Xue, 2014; Santi et al., 2014; Wolf et al., 2014). An important outcome of proteome analysis has been the identification of potential new drug targets for the treatment of fungal infection. The rapid progress of proteomics in cryptococcal research will certainly shed light on discovering proteins associated with fungal virulence.

Experimental Models of Cryptococcosis for the Fungal Geneticist

The vertebrate models are most commonly used to study cryptococcal pathogenesis since they possess both innate and adaptive immune systems. Among infection models, the mouse infection model is a robust model to study mammalian immunological responses to cryptococcosis, since it is well established and provides numerous genetic backgrounds and mutant strains. Immunological modeling is important to the fungal geneticist as it allows the study of the role of fungal virulence genes in modulating the mammalian immune system beyond simple survival studies. For example, deletion of the global regulator, *VAD1* was recently shown to result in infections inducing a more robust innate immune response (Qiu et al., 2013). The cryptococcal infection routes used in mice models includes intranasal, intraperitoneal, intravenous and intratracheal inoculation, the latter introduced either surgically or via pharyngeal inoculation (Clemons et al., 1996; Qiu et al., 2013; Zaragoza et

al., 2007). These multiple infection methods allow the study of cryptococcal pathogenesis from different perspectives. This model has been used to identify numerous virulent factors of *Cryptococcus*, including *CAP59*, *LAC1*, *VPH1*, *CLC1*, *VAD1* et al. (Chang and Kwon-Chung, 1994); (Erickson et al., 2001a; Idnurm et al., 2004; Panepinto et al., 2005; Salas et al., 1996). Recently this model was employed to identify *FRE3* as a virulence adaptation gene, so called because of its role in the microevolution of cryptococcal virulence during the environmental to mammalian transition (Hu et al., 2014). Furthermore, an immunocompromised rabbit model of CNS infections has been used for the study of meningitis (Perfect et al., 1980). This model is unique in that it allows serial sampling of the infective organism during meningeal infection (Steen et al., 2003). In addition, a guinea pig model has recently been employed in antifungal drug testing (Kirkpatrick et al., 2007). All these in vivo models are very useful for revealing the varied and unique aspects of cryptococcal host-pathogen interactions.

In addition to mouse models, several In vitro cellular models have been developed for evaluating the function of innate immune cells and have been adapted in the high-throughput multistrain analysis of fungal mutants. Monocytes and macrophages play important roles in innate immune responses to cryptococcal infections. Macrophages are the dominant phagocytic cells that interact with *Cryptococcus*. They can recognize, phagocytose and digest extracellular cryptococcal cells followed by presentation of antigen to T cells (Mansour et al., 2002). Macrophage killing functions in cryptococcal infections have been analyzed using this model. For example, it has been shown that macrophages from HIV/AIDS patients were defective in cryptococcal phagocytosis and failed to induce T cell proliferation when co-cultured with lymphocytes (Monari et al., 1997). This model was also used to evaluate the ability of mutant strains to grow in macrophages (Hu et al., 2008; Tucker and Casadevall, 2002). In addition, genetic markers to measure growth rates of *Cryptococcus* in macrophages have been identified using live imaging and gene-expression methods in macrophage cell lines (J774 and RAW) as well as primary cells (Alvarez and Casadevall, 2006; Fan et al., 2005; Johnston and May, 2010; Ma et al., 2006).

Similar to the phagocytosis of microbes by human macrophages, soil amoebae feed by phagocytosis of microorganisms. *Cryptococcus* can cause amoebae to lyse following phagocytosis (Steenbergen et al., 2001). The popular theory for the amoebae model is that the ability of fungi to escape the predator has been adapted to the escape from mammalian phagocytes. This theory was supported by the evidence that factors promoting *Cryptococcus* survival in *Acanthamoeba castellanii* were also identified as virulence factors that increased *Cryptococcus* survival in macrophages (Steenbergen et al., 2001). Therefore, the amoebae model is widely used to identify virulence factors in *Cryptococcus* (Casadevall, 2012; Chrisman et al., 2011; Derengowski Lda et al., 2013).

Besides macrophages, functions of other phagocytes, innate and adaptive immune cells have been also evaluated by in vitro cellular models when co-cultured with the fungi including dendritic cells, neutrophils, eosinophil and lymphocytes (Chaturvedi et al., 1996; Feldmesser et al., 1997; Goulart et al., 2010; Kelly et al., 2005; Mambula et al., 2000; Murphy et al., 1993; Qin et al., 2011). The application of these in vitro cellular models sheds light on the

role of the fungus and virulence factors in the innate and adaptive immune responses to *Cryptococcus* infection in vivo.

Cryptococcal infection models have also been established in invertebrate models using *Caenorhabditis elegans*, *Acanthamoeba castellanii* and the insects *Galleria mellonella* and *Drosophila melanogaster* (Apidianakis et al., 2004; Mylonakis et al., 2002; Swanson and Hammer, 2000). These have advantages in studying fungal mutants which do not grow at mammalian host temperatures.

Concluding remarks

The persistent burden of severe cryptococcosis has prompted researchers to develop more effective tools to study its pathogenicity and characterize its unique features during host-pathogen interactions. In the past decade, the availability of advanced molecular biology tools and the whole genome sequences of *C. neoformans* and *C. gattii* have enabled the generation of new technology and resources for cryptococcal research. RNAi-mediated specific gene knock down has become routine and more efficient and has been applied in high-throughput screens for identifying new genes involved in host-pathogen interactions. Gene disruption is a valuable tool in exploring the functions of non-essential genes and its usefulness has been facilitated by increasing rates of homologous recombination and minimizing non-homologous recombination. Modified STM-mediated gene deletion has also been employed in a high-throughput screen to discover virulent associated genes. In addition, new approaches for cellular localization of cryptococcal proteins during infection have been facilitated by the development of codon-optimized fluorescent tags, such as GFP, mCherry and RFP. Recently, multiple genome wide analysis have identified new transcriptional factors and regulatory proteins involved in novel signaling pathways, which sheds light on cryptococcal pathogenesis. Finally the application of multiple experimental infection models allows us to reveal the mechanisms of host-pathogen interactions from different perspectives.

However, many questions remain to be answered about *Cryptococcus*' unique biology, its pathogenesis and its interactions with the mammalian host. For example, how do virulence genes cooperate to produce the virulence composite of this formidable pathogen? How do cryptococcal genes help resist innate or adaptive immune defenses and facilitate its neurotropism in animals and in human patients? As homologous recombination rates are still relatively low for *Cryptococcus*, more efficient tools are required to increase plasmid transformation efficiency, stability and homologous recombination rates and apply these to effective animal modeling and clinical studies to further facilitate the *Cryptococcus* research field and apply these insights for our patients.

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Highlights

- The availability of the whole genome sequences have enabled the generation of new tools for cryptococcal research.
- RNAi has become more efficient and been applied in screens for identifying new genes involved in virulence.
- Gene disruption efficiencies have been increased. Modified STM has also been employed in screens for virulence genes.
- Cellular localization of cryptococcal proteins has been facilitated by optimized tags, such as GFP, mCherry and RFP.
- Multiple genome-wide analyses have identified new factors involved in the pathogenesis of *Cryptococcus*.

Figure 1. The cryptococcal shuttle vector, KUTAP

It contains a *URA5* selective marker. After linearized by *SceI*, it can be transformed for effective RNAi-mediated suppression of target genes.

Figure 2. Generation of knockout strains by homologous recombination

Schematic drawing of overlapping PCR (A), double-joint PCR with a split marker (B), and Cre-lox system (C).

Figure 3. Three dimensional ultrastructure of *C. neoformans* capsule

Cryptococcus neoformans wild-type (H99) and lactonohydrolase knock out (*lhc1*) cells were subjected to Cryo-Scanning Electron Microscopy (Cryo-SEM). Bar = 500 nm by the method of Park et al., 2014.

Figure 4. Localization of Atg8-GFP and Lhc1-mCherry fusion proteins

Cryptococcus neoformans cells were grown in the ASN for 2 days at 30°C. Atg8-GFP and Lhc1-mCherry fusion proteins were observed by differential interference contrast microscopy (DIC) or fluorescence microscopy. Bar = 10 μm.