Published in final edited form as:
Curr Opin Microbiol. 2007 August ; 10(4): 320–325. doi:10.1016/j.mib.2007.05.014.

Cryptococcus neoformans, a fungus under stress

Sarah M. Brown[1], **Leona T. Campbell**[1], and **Jennifer K. Lodge**[1,2]

1*Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, 1402 S. Grand Boulevard, Saint Louis, Missouri 63104*

2*Department of Molecular Microbiology and Immunology, Saint Louis University School of Medicine, 1402 S. Grand Boulevard, Saint Louis, Missouri 63104*

Summary

Cryptococcus neoformans is a human fungal pathogen that survives exposure to stresses during growth in the human host, including oxidative and nitrosative stress, high temperature, hypoxia, and nutrient deprivation. There have been many genes implicated in resistance to individual stresses. Notably, the catalases do not have the expected role in resistance to external oxidative stress, but specific peroxidases appear to be critical for resistance to both oxidative and nitrosative stresses. Signal transduction through the *HOG1* and calcineurin/calmodulin pathways has been implicated in the stress response. Microarray and proteomic analyses have indicated that the common responses to stress are induction of metabolic and oxidative stress genes, and repression of genes encoding translational machinery.

Introduction

The ubiquitous environmental fungus, *Cryptococcus neoformans*, can cause morbid meningioencephalitis in the mammalian host [1]. The disease progresses after inhalation into the lung, followed by evasion of the innate immune system, replication within phagocytes and tissues, and systemic dissemination. Stressors include those that impede growth, such as temperature, pH, anoxia and nutrient deprivation, and those that are potentially toxic, such as reactive oxidative, nitrosative and chlorinating species. It is unlikely that the stress response pathways were developed specifically for survival in a mammalian host, but are the result of stress that the fungus encounters in its primary ecological niche. How *C. neoformans* is able to survive the diverse stressors it encounters within the host is the focus of research over the last three years.

Substantial progress has been made in the field of stress response, but the regulation and mechanisms behind the overall stress responses are not fully understood. Research has continued to utilize studies of individual genes and pathways, but the recent sequencing of the genome [2•] has led to the development of *C. neoformans*-specific microarrays and the application of proteomic techniques. These research strategies have contributed to the field by providing genome-wide data that has led to hypotheses about global responses, such as translation repression, metabolic changes, or cell wall rearrangements. This review will discuss the genes that are important for stress resistance, the signal transduction pathways that have

Corresponding author: Jennifer K. Lodge, Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, Saint Louis, MO 63104, Phone: (314) 977-9217, Fax: (314) 977-9205, email: lodgejk@slu.edu.

been implicated in stress response, and the trends revealed by genome-wide studies (see Figure 1).

Increased temperature is likely the first stressor that *C. neoformans* encounters after it gains entry to the host

Two diverse temperature resistance mechanisms employed by *C. neoformans* include the apparent protective properties of the sugar, trehalose, and the mechanistic link between antioxidant protection and the adaptation to growth at human body temperature.

Accumulation of the disaccharide trehalose in *S. cerevisiae* has been shown to prevent denaturation of important proteins as well as to aid heat-shock protein chaperones in re-naturation by preventing aggregation of denatured proteins [3]. In *C. neoformans* , null mutants of trehalose-6-phosphate synthase (*TPS1*) and trehalose-6-phosphate phosphatase (*TPS2*) revealed they are both required for growth at 37°C, but the protective mechanism of trehalose remains to be elucidated. As expected, these T^s mutants were avirulent in mammalian infection models, however the *tps1*Δ strain also had attenuated virulence in a *C. elegans* model at 25° C, suggesting that trehalose biosynthesis has a role in virulence independent from its role in growth at 37°C [4].

Recent data implies that higher temperatures may stimulate the rate of mitochondrial respiration, leading to an increase of production of superoxide. Mitochondrial manganese superoxide dismutase (SOD), which promotes the rapid degradation of superoxide into hydrogen peroxide and O_2, has been suggested to augment adaptation of *C. neoformans* to human host body temperature [5] by regulating steady-state concentrations of oxygen radicals in the mitochondria and contributing to the integrity of the electron transport chain.

Resistance to reactive oxidative and nitrosative species is important for successful colonization of mammalian hosts

C. neoformans is phagocytosed by alveolar macrophages during the initial stages of infection, and must protect itself from reactive nitrogen and oxygen intermediates. Activated alveolar macrophages can produce up to 14mM hydrogen peroxide and 57μM nitric oxide (NO) [6], however *C. neoformans* can prevent macrophage activation to avoid exposure to these reactive molecules. The inhibition of activation is mediated by the extracellular polysaccharide capsule [7]. *C. neoformans* does not appear to be unusually resistant to reactive oxidative and nitrosative stresses, compared to other fungi. However, resistance is clearly important for survival in mammalian hosts, since impairment of several resistance pathways reduces survival in macrophages and attenuates virulence. Furthermore, these pathways are upregulated by oxidative and nitrosative stresses.

Cu,Zn superoxide dismutase has been shown to be important for resistance to oxygen radicals generated by epinephrine and for growth in macrophages [8]. Unexpectedly, catalases do not seem to play a major role in detoxification of exogenous reactive molecules [9••], since deletion of the entire family of catalases has no effect on resistance to oxidative stress or virulence. Instead, several peroxidases have critical roles in resistance to oxidative stress and, in some cases, overlap with resistance to nitrosative stress.

The glutathione system, which ultimately depends on the pools of reduced glutathione (GSH) maintained by glutaredoxin, is critical for resistance to oxidative and nitrosative stress. Glutathione peroxidase (GPX) removes hydrogen peroxide, and as expected, null gpx mutants are sensitive to peroxides [10]. Another protein in the glutathione system that impacts resistance to nitrosative but not oxidative stress is glutathione reductase [11]. The precise role of Glr in

nitrosative stress is puzzling because in other systems, Glr has been shown to destroy alkyl peroxides and remove glutathione from protein thiols [12].

Laccases are important for production of the pigment melanin, which is a free radical scavenger and thus plays a protective role in stress resistance. Laccase also sequesters iron during infection, which interferes with the oxidative burst of phagocytes. Laccase expression decreases as fungal burden increases [13], although the mechanism of regulation of laccase during *C. neoformans* infection has yet to be fully described. *In vivo* regulation of laccase proceeds, at least in part, via the co-activator Ssa1, a member of the Hsp70 family [14].

The role of Tsa1 in stress resistance is particularly intriguing. A *tsa1*Δ strain has shown that Tsa1 is needed for normal resistance to oxidative and nitrosative stress [15]. Reduction is required for recycling of the enzyme to its active form, and in other systems this is catalyzed by thioredoxin (Trx). If Trx alone were responsible for the reduction of CnTsa1, then a Trx deficient strain would be at least as sensitive to stress as is a *tsa1*Δ strain. In *C. neoformans*, a strain deleted for both *TRX* genes is less sensitive to oxidative stress than a *tsa1*Δ strain. It is possible that components of the glutathione system, such as Glr and perhaps glutathione itself, may be involved in reduction of Tsa1. The existence of such cooperation between oxidative stress systems like the glutathione system and the thioredoxin system has not yet been explored. Conversely, Tsa1 may not be recycled, but may be overoxidized and degraded. Modified forms of Tsa1 are detected on 2D protein gels that may represent overoxidized protein [11].

The *tsa1*Δ strain is also sensitive to NO stress, yet it is unclear whether Tsa1 is directly involved in NO detoxification. Modified forms of Tsa1 were detected after exposure to NO [11], providing initial support for the direct involvement of Tsa1 in detoxification of NO. The delineation of the amino acids involved in NO resistance, and nature of the modifications will clarify the role of Tsa1 in NO resistance. Alternatively, Tsa1 may serve to propagate a signal that is essential for resistance to nitrosative stress. Tsa1 has already been shown to regulate the laccase genes during nitrosative stress, lending credence to a signaling role [16].

A consumer of nitric oxide is the hemoprotein flavohemoglobin. Deletion of the flavohemoglobin gene (*FHB1*) in *C. neoformans* resulted in hypersensitivity to NO and attenuated virulence in a murine model [17]. The reduction in virulence was directly related to NO sensitivity, since the *fhb1*Δ strain behaved as wild type in an iNOS deficient mouse model.

Does resistance to reactive species produced by cells other than macrophages play a role in pathogenesis?

Most of the research on phagocytosis has focused on macrophages, but *C. neoformans* can be phagocytosed by other cells, including neutrophils and endothelial cells, which produce additional stressors. A considerable amount of the microbicidal activity of neutrophils depends on the production of hypochlorous acid by the myeloperoxidase system, which produces HOCl from Cl and hydrogen peroxide via myeloperoxidase (MPO). In other organisms, like *S. aureus* and *E. coli*, genes that impart resistance to H_2O_2 are also important for resistance to hypohalous acids and superoxide [18], but the oxidative stress mutants of *C. neoformans* have not been tested for sensitivity to HOCl. Enticingly, MPO knock out mice are more susceptible to *C. neoformans* infection compared to wild-type mice [19].

Iron deprivation provokes major responses in *C. neoformans*

Iron acquisition during growth in a host is known to be a limiting factor for most pathogens. Low iron conditions have been associated with major phenotypic changes in *C. neoformans*, including induction of the large polysaccharide capsule and repression of laccase activity. Iron

deprivation has been shown to induce a siderophore iron transporter (*SIT1*) and an iron permeases (*FTR1*), which function to increase intracellular pools of iron available to the fungus [20,21]. Deletion of the permease or transporter results in very slow growth in low iron conditions.

C. neoformans employs multiple sensing and signaling systems to detect local environmental stresses enabling it to affect a response

Recent work has focused on a histidine kinase (HK), Protein Kinase C (*PKC1*), and the calmodulin/calcineurin pathways. A *C. neoformans* HK system activates the Hog1 MAPK signaling pathway [22•], leading to recruitment of downstream effectors that mount the appropriate stress response [23]. Most fungi, with the exception of *S. cerevisiae*, possess multiple hybrid HK sensors, allowing them to sense a variety of environmental cues including osmotic shock, UV irradiation, oxidative damage, and high temperature. HK sensor systems in the ascomycete fungi have been classified into 11 distinct groups [24]. However, Bahn et al. [22•] identified previously unclassified HK sensor systems in the basidiomycete *C. neoformans*, suggesting that this sensing system may be even more diversified than previously observed. Furthermore, this system is involved in *C. neoformans*' response to low environmental oxygen stress. Both the HK pathway and the SREBP (sterol-response element protein binding) pathway, which activates genes involved in sterol biosynthesis, are involved in *C. neoformans* ability to adapt to the hypoxic conditions encountered within infected host tissues and are required for virulence [25•]. Both pathways were demonstrated to be required for proliferation of the pathogen within the host tissues, suggesting a link between adaptation to hypoxia and pathogenesis.

Another conserved protein found to be involved in regulating stress responses is calmodulin. The Ca^{2+} sensor calmodulin is a small protein that upon binding Ca^{2+}, activates the serine threonine-specific protein phosphatase, calcineurin. Calcineurin is critical for growth at 37°C in *C. neoformans* [26;27] and calmodulin (*CAM1*) is essential for viability [28]. Isolation of a Cn*CAM1*-T^{s}mutant allele led to the delineation of a bifurcated calmodulin/calcineurin signaling system with both a calcium-dependent and -independent mechanism regulating *C. neoformans* growth at host body temperature [29•]. The downstream pathway activated by the Ca^{2+} -independent calmodulin remains to be determined.

The *PKC1* pathway is a MAP kinase pathway believed to be involved in both sensing stress and maintaining cell wall integrity during stress [30]. This pathway likely includes transmembrane receptors or sensors, and several potential sensor/receptors have been predicted in *C. neoformans* using a bioinformatic approach and searching for specific stress sensing domains [Gerik *et al*. abstract 07-GM-A-4356-ASM, ASM General 107th Meeting 2007]. This pathway may be involved in fungal stress resistance [31], and cell wall genes are often regulated during various stresses. The calcineurin pathway can also affect cell wall biosynthesis by intersecting with the *PKC1* pathway [32].

Transcription factors have been identified that regulate stress responses

Two putative transcription factors have been found to regulate the thioredoxin system under oxidative and nitrosative stress. Yap4, which is homologous to an AP1 transcription factor, is required for induction of *TRX* under nitrosative stress, and a *yap4*Δ mutant is sensitive to NO. An ARF/CREB-like gene, *ATF1*, is needed for induction of *TRX* under oxidative stress, and an *aft1*Δ mutant is sensitive to oxidative stress [33]. Neither of these transcription factors are the major response regulator, since expression of other critical genes such as *TSA1* or *FHB1* are not regulated by *YAP4* or *ATF1*.

Another transcription factor, Skn7, homologous to a stress response regulator in *S. cerevisiae*, was upregulated in *C. neoformans* recovered from endothelial cells. Although its exact role it still unclear, disruption of *SKN7* leads to sensitivity to oxidative stress and susceptibility to killing in endothelial cells [34].

Excitingly, the central regulator for the iron response in *C. neoformans* has been identified as Cir1 [35••]. By microarray analysis, this transcription regulator was shown to impact expression of the iron regulon, as well as the virulence factors laccase, phopholipase, and capsule. Cir1 also regulates cell wall biosynthesis genes possibly by influencing expression of the Ca^{2+}/calmodulin and *PKC1* signaling pathways.

Microarrays and proteomic analysis have recently been employed to acquire snapshots of genome and proteome wide changes under a variety of stresses

Microarray analysis of changes in transcript levels have been done using cells with and without exposure to low oxygen, high temperature, reactive species, and phagocytosis.

The transcriptional profile of *C. neoformans* cells grown under normal oxygen conditions was compared to those grown under low oxygen conditions. Changes were observed in the levels of 347 transcripts with up-regulation of genes involved in stress regulation, carbohydrate metabolism and respiration and down-regulation of those involved in translation, vesicle trafficking, and cell wall and capsule synthesis [25].

Kraus et al [28] conducted microarray analysis comparing transcripts of cells exposed to 25 degrees to those exposed to 37 degrees. Temperature regulated genes included SOD, trehalose synthase, and a putative cell wall stress sensor. Interestingly, a transcription factor ortholog, Mga2, was induced at 37°C found to be important for high temperature growth.

A recent study used microarrays to investigate changes in gene expression during murine macrophage infection [36]. Results showed that while several hundred genes were upregulated, many fewer were downregulated after phagocytosis. Upregulated genes included those involved in membrane transport, oxidative stress response, signaling pathways, metabolism, autophagy, and mating type. Most of the downregulated genes were involved in translation machinery, suggesting that translation is likely repressed as a general stress response which allows the cell to adapt to the environment while conserving energy by reducing protein synthesis. Interestingly, the number of repressed translation genes increased substantially between 2 and 24hr, but this seems paradoxical since *C. neoformans* efficiently replicates eight hours after being ingested [37]. It is not clear these changes result in non-specific or regulated repression of translation. Proteomic analysis suggests that there is very little proteomic change following peroxide treatment [Brown and Lodge, unpublished], but there is clearly a transcriptional response to oxidative stress as evidenced by analysis of several oxidative stress genes. This suggests that there may be a global repression of translation during oxidative stress. However, serial expression of gene analysis (SAGE) of the transcriptome of *C. neoformans* in a model of rabbit meningitis, suggested that the translational machinery was induced compared to *in vitro* growth [38].

The transcriptional and translational response to nitrosative stress was described together showing how these two analyses could complement each other [11]. Upregulated proteins included those involved in stress pathways, signaling, and metabolism. These results were compared to and supported by the microarray analysis of mRNA from the same cells. Modified forms of at least three proteins, including Tsa1, were found by 2D gel analysis of the protein.

How well *C. neoformans* is able to adapt to external stress likely depends on how well it is able to maintain metabolic homeostasis under stress

The importance of SOD for high temperature growth suggests that at least one stressor, temperature, results in an increased rate of mitochondrial respiration. Since many metabolic pathways, such as the pentose phosphate pathway, are regulated by ATP, these pathways are likely upregulated under stress as well. At the same time, upregulation of genes involved in carbohydrate and lipid transport and autophagy [11,25,28,36], indicate that there is a significant need for uptake of nutrients and energy production after host infection and ingestion by phagocytes.

Since metabolism involves the production of molecules that are important for fueling stress pathways, metabolic flux would directly influence the ability to maintain the redox state of the cell under stressful conditions. For example, the reducing equivalent NADPH produced by the pentose phosphate pathway is essential for maintenance of functional glutathione and thioredoxin systems and is also bound to proteins like Fhb1. Missall [11] reported that transaldolase, a key enzyme in the pentose phosphate pathway, is upregulated and modified during nitrosative stress, and several members of the pentose phosphate pathway were highly expressed in the rabbit model of meningitis [38]. This indicates the pathway may be induced by stress to increase the production of NADPH, as shown in other systems [39].

Conclusions

The stress response in *C. neoformans* is an intricate network of proteins and pathways that involve sensing stress, reacting to stress, and maintaining homeostasis in a stressful environment. Studies of individual genes and pathways have revealed unique features of stress resistance in *C. neoformans*. It was anticipated that the catalases would play major roles in stress resistance, and yet they are dispensable. Peroxidases, especially the peroxiredoxin Tsa1, have surprising roles in resistance to nitrosative stress. Two signaling pathways have been implicated in transducing the stress response, but the mechanisms of how the stress is sensed, and how the pathways propagate the signal is still unknown

The application of systems biology approaches, that incorporates microarray, proteomic and metabolomic data, will generate hypotheses about the common and specific stress responses. Similar themes have already emerged from genome-wide analyses of the stress responses, one of which is repression of translation. Whether this repression is specific to a subset of genes is undetermined. The induction of metabolism and oxidative stress genes in several stresses implies that respiration and thus the demand for reductive metabolic intermediates is increased as a common response to stress. These hypotheses are ready to be tested.

Acknowledgements

The authors wish to thank Dr. John Corbett for helpful discussions. Work that contributed this manuscript was supported by National Institute of Health grants RO1AI051209 and R01HL088905 to JKL.

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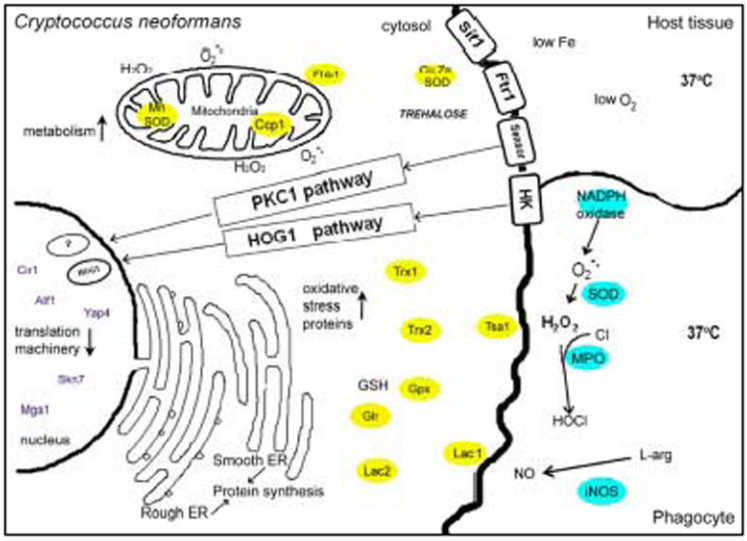

Figure 1.

A schematic of the stresses that *C. neoformans* encounters in the human host, and the genes and pathways involved in the stress responses. The *C. neoformans* proteins are defined in the text.