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Thermotolerance and virulence of *Aspergillus fumigatus*: role of the fungal nucleolus

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The ability to thrive at 37°C is characteristic of all human pathogens and has long been suspected to play a role in the pathogenesis of aspergillosis. As a thermotolerant fungus, *Aspergillus fumigatus* is capable of growth at temperatures that approach the upper limit for all eukaryotes, suggesting that the organism has evolved unique mechanisms of stress resistance that may be relevant to its ability to adapt to the stress of growth in the host. High temperature is a strain on many biological systems, particularly those involved in complex macromolecular assemblies such as ribosomes. This review will discuss the relationship between thermotolerance and virulence in pathogenic fungi, emphasizing the link to ribosome biogenesis in *A. fumigatus*. Future work in this area will help determine how rapid growth is accomplished at elevated temperature and may offer new avenues for the development of novel antifungals that disrupt thermotolerant ribosome assembly.

Keywords *Aspergillus fumigatus*, CgrA, ribosome biogenesis, thermotolerance, virulence

Introduction

Aspergillus fumigatus is an opportunistic fungal pathogen that has become the leading infectious mould in the immunocompromised patient population [1–3]. The prevalence of *A. fumigatus* conidia in the environment does not completely account for the high frequency of infections with this fungus [4], so it is generally assumed that some aspect of the physiology of *A. fumigatus* allows the organism to be a more efficient opportunistic pathogen than other commonly encountered environmental moulds. Thermotolerance is often cited as a potential virulence factor in this regard. As a major component of the biomass in a self-heating compost pile, *A. fumigatus* is capable of growing rapidly at 37°C and tolerating temperatures above 50°C [5,6]. This is in contrast to most other fungi which are mesophilic, displaying temperature optima in the range of 25–35°C [7]. The ability to thrive at a high temperature is a unique characteristic shared by the thermotolerant and thermophilic fungi. Although species that fall into either category can thrive at temperatures above 50°C, they are distinguished by the fact that thermotolerant forms maintain growth at temperatures below 20°C, whereas true thermophilic fungi have growth minima at or above 20°C [8]. This arbitrary classification places *A. fumigatus* in the thermotolerant group of fungi. However, since *A. fumigatus* can tolerate 60°C, which is the upper temperature limit for eukaryotic organisms [9], it has likely evolved mechanisms of thermal resistance that are similar to thermophilic fungi. The absence of eukaryotic species from extreme environments, where thermophilic bacteria or *archaea* can flourish [10], is thought to reflect the inability of any eukaryote to maintain functional organelle membranes at high temperature [9]. Nevertheless, it is interesting to speculate that mechanisms of thermotolerance in *A. fumigatus* may involve temperature-induced expression of stress resistance genes that confer unique virulence properties to the organism, similarly to what has been

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reported for some bacterial pathogens [11]. An understanding of how *A. fumigatus* can sustain growth at high temperatures may thus provide important insights that are relevant to the pathogenesis of aspergillosis in humans.

Thermotolerance and virulence

The ability to grow at 37°C is universal among human pathogens. In bacteria, the transition from a vector or environmental reservoir into a human host is associated with global changes in gene expression that contribute to pathogenesis [11]. A number of these changes are temperature-regulated and include the induction of virulence-related genes, examples of which have been reported in diverse human pathogens such as *Shigella flexneri*, *Bordetella pertussis*, *Yersinia pestis* and *Borrelia burgdorferi* [11]. Some of the gene products encoded by bacterial temperature-regulated genes are considered to be true virulence factors, whereas others are thought to affect pathogenesis indirectly by enhancing overall fitness at higher temperatures [12]. As for bacterial pathogens, the ability to grow at 37°C is a prerequisite for fungal pathogenesis, and failure to grow at 37°C is predictive of attenuated virulence. For example, UV-induced mutants of *Cryptococcus neoformans* that are unable to grow *in vitro* at 37°C are avirulent in mice [13]. Variability in the thermal tolerance of different clinical isolates of *Sporothrix schenckii* also correlates with virulence: isolates obtained from fixed cutaneous lesions grew poorly at 37°C and were unable to multiply in the internal organs of mice, whereas isolates obtained from cases of lymphatic spread grew well at 37°C and were able to cause deep infections in mice [14]. Sensitivity to elevated temperature has also been shown to correlate with strain-specific variations in virulence in the thermally dimorphic fungal pathogen *Histoplasma capsulatum* [15] and, in one thermosensitive strain, is associated with altered expression of the 70 kD heat shock protein (hsp70) [16] and a Δ^9-fatty acid desaturase involved in membrane fluidity [17]. Similar correlations have been obtained in *Saccharomyces cerevisiae*, where the ability to grow at temperatures up to 42°C distinguishes clinical isolates from standard laboratory and industrial strains [18–20]. Genetic characterization of such pathogenic *S. cerevisiae* isolates showed that the ability to grow at 42°C is a complex polygenic trait; segregants of hybrids between clinical isolates and laboratory strains showed that various genetic backgrounds contain different sets of alleles which govern thermotolerant growth, although specific loci were not identified in that study owing to the genetic complexity of the high temperature growth phenotype [20,21]. Such complexity is consistent with the fact that many genes have been reported to affect thermotolerant growth in *S. cerevisiae* [22–27], in addition to diverse physiological conditions such as growth phase, growth medium, colonial organization and cell cycle position [28–32].

Specific genes that have been demonstrated to influence thermotolerance in pathogenic fungi also have diverse functions and often participate in pathways that are linked to the stress response. For example, although the *RAS1* gene is dispensable for normal growth of *C. neoformans* at 24°C, it is required for growth at 37°C [33], emphasizing the importance of *RAS1* signaling to the thermotolerant phenotype. A *ras1* mutant was avirulent in a rabbit model of cryptococcal meningitis, and since this mutant displayed no alterations in well-established virulence factors for *C. neoformans*, such as capsule, melanin and prototrophy, it was concluded that the avirulent phenotype was directly attributable to the temperature-sensitive growth defect [33]. Similar findings of thermosensitive phenotypes in *C. neoformans* have been reported for mutants in calcineurin, a Ca^{2+}-calmodulin-reglated protein phosphatase that is the target for the immunosuppressive drugs cyclosporine A and FK506. Mutations in either the catalytic or regulatory subunits of calcineurin blocked 37°C growth *in vitro* and abrogated virulence in animal models [34,35]. The link between thermotolerance and calcineurin is not universal, however, since inactivation of calcineurin in *C. albicans* attenuated virulence without causing thermosensitivity [36].

Wangiella dermatitidis is a dematiaceous fungal pathogen that expresses high levels of the class V chitin synthase WdChs5p at high temperatures [37]. Disruption of the WdCHS5 gene produced mutants that grew normally at 25°C but showed cell wall integrity defects at 37°C that were associated with attenuated virulence in a mouse model [37]. This suggests that the cell wall undergoes temperature-dependent modifications that are necessary to support growth *in vivo*. Cell wall stress is known to signal through the mitogen activated protein (MAP) kinase cascade in *S. cerevisiae* [38], so the fact that a *C. neoformans* Mpk1 mutant showed impaired cell integrity in response to high temperature and was attenuated for virulence [39] is consistent with the idea that pathways controlling the cell wall may influence fungal pathogenesis. Remodeling of the fungal membrane may also be necessary for optimal growth at high temperature; disruption of the *C. neoformans* Mga2 transcription factor, which is thought to regulate genes

encoding fatty acid biosynthesis, is induced by 37°C and is required for optimal growth at this temperature [40].

Thermal dimorphism is perhaps the best example of temperature-regulated gene expression that is relevant to fungal pathogenesis. For example, the mycelium-yeast transition in *B. dermatitidis* is induced by shifting mycelia to 37°C, and is associated with the expression of virulence factors such as the surface protein *BAD1* [41]. Temperature-regulated virulence factors have yet to be identified in *A. fumigatus*, although one report of temperature-regulated expression of elastase activity in *A. fumigatus* [42] raises the possibility that networks of temperature-induced genes that could be relevant to pathogenesis may be uncovered by genome-wide expression technology.

Since the ability to grow at high temperature distinguishes *A. fumigatus* from most other environmental moulds, it is conceivable that mechanisms of thermal resistance used by *A. fumigatus* could also be activated as part of the general response to *in vivo* stress and thus contribute to survival in the host. To examine this possibility, a chemical mutagenesis approach was employed to identify *A. fumigatus* mutants that grow normally up to 42°C but fail to grow at 48°C [43]. A total of seven mutants were isolated that met this criterion, one of which was complemented by the introduction of DNA from a cosmid genomic library. Characterization of the complementing DNA fragment identified *THTA*, a gene of unknown function that was required for growth at 48°C when subsequently deleted from wild type *A. fumigatus*. Neither the *thtA* mutant, nor any of the remaining six mutants that failed to grow at 48°C showed a reduction in virulence in a mouse infection model, suggesting that the genes affected by these mutations are dispensable for wild type virulence at 37°C. However, since thermotolerance is likely to be polygenic, the possibility remains that other genes that contribute to high temperature growth could still be involved in pathways that affect virulence.

Virulence and the translational machinery

Pathogenic fungi must adapt to multiple adverse environmental conditions during the transition to a mammalian host, and meeting these challenges requires an adequate supply of ribosomes so that appropriate defenses can be rapidly mobilized. Indeed, upregulation of the translational apparatus in *Candida albicans* is one of the earliest transcriptional events to occur in response to exposure to human blood [44] suggesting that an increase in translational capacity is part of the initial adaptive response to *in vivo* growth. Since clinical isolates of *C. albicans* are known to exhibit considerable variability in the number of rDNA repeats [45–47], it is likely that *in vivo* stress continues to exert selective pressure throughout the course of the infection, forcing the organism to modulate rRNA synthesis in proportion to the physiological demand for new protein synthesis. Genome-wide expression profiling of *C. albicans* exposed to itraconazole has also revealed upregulation of the translational machinery, suggesting that increased translation may be part of the general response to environmental stress [48].

The transcriptional profiling of temperature-induced changes in gene expression has been reported for *C. neoformans* using serial analysis of gene expression (SAGE). A comparison of the transcriptomes of strains grown at either 25°C or 37°C *in vitro* showed increased abundance of the translational machinery at 37°C [49], emphasizing the importance of increased translation to meet the demands of high temperature growth. A similar study performed on *C. neoformans* isolated from a rabbit model of cryptococcal meningitis also showed high levels of SAGE tags representative of the translational apparatus [50]. Interestingly, 73% of these translation-related SAGE tags were more abundant in the SAGE library constructed from the rabbit model than the *in vitro* temperature libraries [49], suggesting that the host environment is a more potent inducer of translational capacity than temperature alone. Other screens to identify temperature-regulated screens in *C. neoformans* have identified COX1 (cytochrome c oxidase subunit 1), ICL1 (isocitrate lyase) and AOX1 (alternative oxidase) [51–53]. Mutants in COX1 or ICL1 showed no defects in 37°C growth or virulence, whereas a mutant in AOX1 had attenuated virulence in the absence of a temperature-sensitive growth phenotype, indicating that temperature-regulated genes are not always required for 37°C growth or virulence.

Ribosome biogenesis and virulence of *A. fumigatus*

Mutations in a number of bacterial heat shock proteins cause ribosome assembly defects at elevated temperature, indicating that chaperone activity facilitates ribosome synthesis at temperatures that are unfavorable to the stability of large macromolecular complexes such as ribosomes [54–57]. In fungi, as in all eukaryotes, ribosome biogenesis takes place predominantly in the nucleolus. The process is highly complex, and much of what is known about the steps involved has been

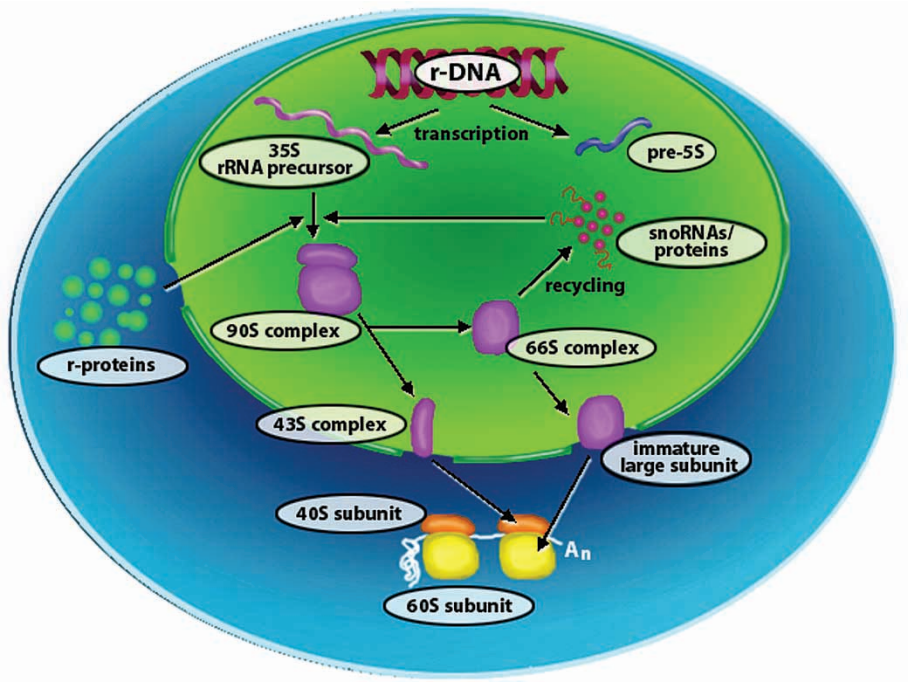

Fig. 1 Schematic overview of the basic steps involved in ribosome biogenesis in *Saccharomyces cerevisiae*. Each ribosomal DNA (rDNA) repeat unit is transcribed by RNA polymerase I as a single 35S precursor ribosomal RNA (rRNA) that undergoes processing and modification within the nucleolus to generate the mature rRNAs: 18S, 5.8S and 25S rRNA. The fourth rRNA, 5S rRNA, is transcribed independently by RNA polymerase III. Small nucleolar RNAs (snoRNAs) function in the modification and processing of the precursor rRNA. Ribosomal proteins (r-proteins) synthesized on cytoplasmic ribosomes move into the nucleolus where they assemble with rRNA before export and final maturation in the cytoplasm. For clarity, the process is expanded in this schematic to include the whole nucleus whereas in reality it occurs predominantly in the nucleolus. For reviews, see [79–82].

elucidated in *S. cerevisiae* (Fig. 1). Numerous accessory proteins guide the processing and assembly of maturing ribonucleoprotein complexes [58], and their presence in the nucleolus is tightly regulated by the metabolic needs of the cell [59]. The high concentration of proteins in the nucleolus is considered to be the most concentrated mass of proteins per unit volume in the cell, particularly during period of active ribosome assembly [60]. Such a high concentration of macromolecules predisposes proteins to aggregation and denaturation [61]. It is not surprising, therefore, that nucleolar chaperones with functions in the maintenance of nucleolar stability and ribosome biogenesis have also been identified in eukaryotic cells [61–68], and it is interesting to speculate that such proteins in *A. fumigatus* contribute to thermostable ribosome assembly in the host environment.

Since the number of ribosomes in an ungerminated spore is insufficient to support hyphal growth, new ribosome production is a major synthetic process during the early minutes of germination in filamentous fungi. This process results in a 10-fold increase in the number of ribosomes actively engaged in translation within the first few minutes [69–71]. The initial induction of ribosome synthesis in *A. fumigatus* conidia is thus likely to be one of the first steps to occur during the infection process. In immunocompromised patients, the failure of pulmonary defenses would allow the inhaled conidia to generate sufficient ribosomes to support rapid growth and to disseminate to other

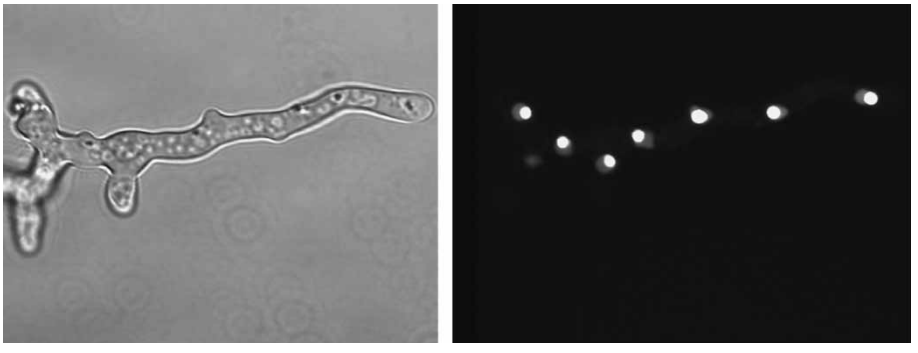

Fig. 2 Morphology of the *Aspergillus fumigatus* nucleolus revealed by localization of a green fluorescent protein (GFP)-tagged CgrA fusion protein. CgrA-GFP localizes predominantly to the nucleolar compartment, with some presence also in the nucleoplasm. Left panel: differential interference contrast image. Right panel: fluorescent image.

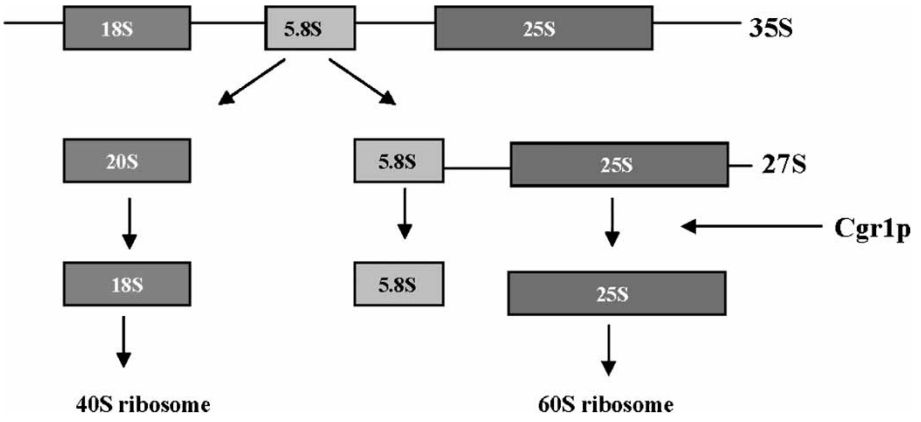

Fig. 3 A simplified schematic of the major rRNA processing pathways in *Saccharomyces cerevisiae*. The 35S pre-rRNA is processed to form the 18S rRNA (found in the 40S ribosome subunit) and the 25S and 5.8S rRNAs (found in the 60S subunit). Current evidence suggests that Cgr1p has a role in the pathway leading to the synthesis of the mature 25S rRNA (arrow) that is incorporated into the 60S ribosomal subunit [78].

organs, so the ability to block this process at 37°C would have considerable merit as a potential therapeutic strategy. Recent evidence suggests that the synthesis of ribosomes during conidial germination requires the activity of CgrA, a nucleolar protein that provides a link between thermotolerance, ribosome biogenesis and virulence of *A. fumigatus* [72].

The CgrA protein is highly conserved among fungi, with readily identifiable homologs in both filamentous fungi and yeasts. The protein has a distinct nucleolar localization in both yeast [73] and *A. fumigatus* [72], and the ability of the *A. fumigatus* protein to localize to the nucleolus when heterologously expressed in yeast [74] indicates that mechanisms of nucleolar localization are highly conserved between these species. Fig. 2 shows a strain of *A. fumigatus* that expresses a green fluorescent protein (GFP)-tagged CgrA protein, showing the typical morphology of the *A. fumigatus* nucleolus at one edge of the nucleus. Disruption of the *cgrA* gene in *A. fumigatus* produced a mutant that grew normally at 25°C but was unable to achieve the rapid growth rates that are characteristic of *A. fumigatus* at higher temperatures. The growth rate of the Δ*cgrA* strain progressively decreased as the temperature increased above 25°C and conidia were unable to germinate at temperatures above 48°C. By comparison, wild type *A. fumigatus* was able to grow optimally between 37°C and 42°C and retained the ability to grow at 48°C [72].

The Δ*cgrA* mutant was attenuated in an immunosuppressed mouse model of invasive aspergillosis; mice inoculated with the wild type strain died within the first week (median survival 5–6 days), whereas all of the mice infected with the Δ*cgrA* strain survived the first week with over 60% survival to day 14 [72]. Histologic examination of lung tissue showed extensive growth of the wild type strain around bronchioles within two days post inoculation, but only sporadic germlings were evident in the mice inoculated with the Δ*cgrA* strain. The ability to sustain rapid growth at 37°C is thus an important characteristic of the virulence phenotype for *A. fumigatus*, and is consistent with recent evidence showing that the growth rate of different clinical isolates of *A. fumigatus* at 37°C correlates with virulence in animal models [75]. When the virulence of the Δ*cgrA* mutant was compared in *Drosophila melanogaster*, an invertebrate infection host that can be maintained at 25°C [76,77], the Δ*cgrA* mutant was comparable in virulence to wild type [72]. Since the Δ*cgrA* mutant grows at the same rate as wild type at 25°C, the attenuated virulence of the Δ*cgrA* mutant in mice is most likely due to impaired growth at mammalian body temperature.

The ability of *A. fumigatus cgrA* to complement a yeast *cgr1* mutant indicates that CgrA and Cgr1 are orthologous proteins with overlapping functions [74]. To gain insight into this function, yeast *cgr1* mutants were analysed for defects in ribosome biogenesis. The Cgr1 protein was shown to be necessary for pre-rRNA processing and 60S ribosomal subunit assembly (Fig. 3), thereby establishing a role for this protein in ribosome biogenesis [78]. Further understanding of CgrA and the mechanism by which it contributes to ribosome synthesis at elevated temperature may offer new insights into pathways of thermotolerant ribosome assembly that might be amenable to therapeutic targeting.

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