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Heat Shock Proteins in Dermatophytes: Current Advances and Perspectives

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Abstract: Heat shock proteins (HSPs) are proteins whose transcription responds rapidly to temperature shifts. They constitute a family of molecular chaperones, involved in the proper folding and stabilisation of proteins under physiological and adverse conditions. HSPs also assist in the protection and recovery of cells exposed to a variety of stressful conditions, including heat. The role of HSPs extends beyond chaperoning proteins, as they also participate in diverse cellular functions, such as the assembly of macromolecular complexes, protein transport and sorting, dissociation of denatured protein aggregates, cell cycle control, and programmed cell death. They are also important antigens from a variety of pathogens, are able to stimulate innate immune cells, and are implicated in acquired immunity. In fungi, HSPs have been implicated in virulence, dimorphic transition, and drug resistance. Some HSPs are potential targets for therapeutic strategies. In this review, we discuss the current understanding of HSPs in dermatophytes, which are a group of keratinophilic fungi responsible for superficial mycoses in humans and animals. Computational analyses were performed to characterise the group of proteins in these dermatophytes, as well as to assess their conservation and to identify DNA-binding domains (5′-nGAAn-3′) in the promoter regions of the *hsp* genes. In addition, the quantification of the transcript levels of few genes in a *pacC* background helped in the development of an extended model for the regulation of the expression of the *hsp* genes, which supports the participation of the pH-responsive transcriptional regulator PacC in this process.

Keywords: pH regulation, Heat shock proteins, HSPs, Phosphate regulation, Antifungal resistance, Transcription factor Hsf1, Fungal pathogenicity, Heat shock elements (HSE).

INTRODUCTION

The heat shock response (HSR) was first observed in the early 1960s, by Ferruccio Ritossa, in the salivary glands of *Drosophila melanogaster* subjected to a temperature shift. He observed a new chromosomal puffing pattern in the salivary gland, representing active RNAs that accumulated under heat stress within a few minutes [1]. These chromosomal puffs indicated a strong transcriptional activity when the cells were exposed to higher temperatures. The newly synthesised proteins were identified about a decade later, and named heat shock proteins (HSPs). These proteins are now known in all organisms [2, 3], and act as molecular chaperones or transcription regulators in a myriad of physiological functions. Owing to their ubiquitous nature and thermal responsiveness, the HSPs have been widely studied. The HSR is a highly conserved mechanism among different organisms, and appears to be an emergency response for cell survival since transcription of its constituent proteins occurs within a few minutes of stress exposure [4]. Although the HSR was first described as activated by heat, the *hsp* genes are now known to be up- or down-regulated in response to a large array of stressful situations, such as oxidative and osmotic stress; exposure to drugs, heavy metals, or toxins; nutrient deprivation; bacterial infection; several human diseases such as in cancer; and host-pathogen interaction [4, 5].

HSPs fulfil a protective role in the cell by enabling the proper folding and unfolding of proteins, which led to their designation as molecular chaperones. It has been proposed that ubiquitin and HSPs play complementary roles in protecting cells against the production of denatured protein aggregates after exposure to temperature shifts. While ubiquitin tags these proteins for removal by proteolysis, the HSPs prevent the aggregates from forming or disaggregate them once they are formed [4, 6]. However, the role of HSPs goes beyond the process of protein folding, since these proteins are also involved in the assembling of protein complexes, transport and sorting of proteins into the proper cellular compartments, and cell-cycle control. Moreover, they have also been found in the extracellular environment and on the surface of tumour cells [2, 7].

Some HSPs, such as Hsp60, Hsp70, and Hsp90, display immunogenic features, stimulating macrophages and dendritic cells through the activation of Toll-like receptors and thereby inducing the production of several cytokines. HSPs also play a role in the immune response by chaperoning and transferring antigens to the class I and II molecules of the

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major histocompatibility complex, allowing antigen presentation [8]. In fungi, HSPs are implicated in several processes, including pathogenicity, phase transition in dimorphic fungi, and antifungal drug resistance. The synthesis of HSPs is an adaptive response to stress that contributes to the survival of pathogenic microorganisms in mammalian hosts [5, 9].

THE HSP FAMILIES

HSPs are classified into several families based on their molecular weight and function: the ATP-dependent Hsp100, Hsp90, Hsp70, and Hsp60/Hsp10 (chaperonin) families, the small HSPs, and the ATP-independent HSPs [3]. The most studied HSPs are Hsp60, Hsp70, and Hsp90. In general, HSPs contain a conserved ATPase amino-terminal domain and a carboxy-terminal substrate-binding domain. In some HSPs, conformational changes in the substrate-binding domain are driven by the hydrolysis of ATP. These changes increase affinity for the substrate and allow proper folding of the client protein/peptide. In addition, the activation of several HSPs is regulated by co-chaperones, such as members of the Hsp40 family. Hsp40s are subdivided into two classes [10]. Class I HSPs contain three conserved domains: a centrally located cysteine-rich domain, a zinc finger-like domain, and a carboxy-terminal protein-binding domain. In Class II HSPs, the central cysteine-rich domain is replaced by a glycine- and phenylalanine-rich sequence. In *Saccharomyces cerevisiae*, several Hsp40s have been identified, showing similarity to the DnaJ protein from *Escherichia coli*. The J domain present in Hsp40s seems to be involved in the interaction and recruitment of the appropriate member of the Hsp70 family [5, 11].

The Hsp70s are a highly conserved family of at least 10 proteins, all of which contain two functional domains – a 44 kDa ATP-binding domain and an 18 kDa substrate-binding domain. Some Hsp70 members are constitutively expressed (Ssa1 and Ssa2), while others are modulated in response to stress (Ssa3 and Ssa4) [5]. When *S. cerevisiae* cells are exposed to high temperatures, Hsp104 acts by disaggregating proteins, generating substrates for proper folding by Hsp70 and Hsp40 [12]. Hsp104 is also involved in the accumulation and degradation of cytoplasmic trehalose, which occurs in response to heat and assists in the refolding of glycoproteins in the endoplasmic reticulum [13].

Hsp60 participates in protein folding in the mitochondrial matrix. Its release from its substrate is assisted by Hsp10, which also plays a role in protein sorting. The presence of Hsp70 is also required for proper protein folding in the mitochondrial matrix [5]. In the human pathogen *Histoplasma capsulatum*, Hsp60 is localised in the cell surface and interacts with proteins in the cell membrane of phagocytes [14]. It governs the traffic of proteins to the cell wall and interacts with several proteins to allow cell survival during stress, including small chaperones, Hsp70, Hsp90, and proteins involved in energy and metabolism [15]. Recently, it has been demonstrated that cytosolic Hsp60 is able to modulate proteasome activity in *S. cerevisiae* [16].

Hsp90 constitutes about 2% of the protein repertoire in eukaryotic cytosol. It is also essential for fungal survival, and forms a complex with Hsp70 to promote protein folding [17]. Hsp90 functions as a dimer, and contains three structural domains: an ATP-binding domain, a dimerisation domain, and the client protein-binding domain. Hsp90 plays a role in protein folding in an ATP-dependent manner, and serves a diverse set of client proteins involved in signal transduction, protein trafficking, and the maturation of cell receptors [17, 18]. In *S. cerevisiae*, the two Hsp90 homologues, Hsc82 and Hsp82, present different gene expression patterns. Hsc82 is constitutively expressed, while Hsp82 is induced by heat [19]. Hsp90 is involved in a variety of cellular functions in fungi, including drug resistance, morphogenesis, cell wall remodelling, and virulence [20-22].

Small HSPs (Hsp12, Hsp20, Hsp21, and Hsp30) play several roles in the cell which, in general, involve retaining client proteins for further folding by other molecular chaperones [23]. Hsp12 is required for growth, survival, cell morphology, virulence, and evasion of immune response [24-26]. In *S. cerevisiae*, Hsp12 is found in both the cytosol and the plasma membrane. The *S. cerevisiae hsp12* gene is up-regulated in response to stress and is important for fungal survival and maintaining viability and morphology during heat, osmotic, and oxidative stress [26]. The Hsp21 of *C. albicans* is required for thermotolerance, adaptation to oxidative and heat stress, hyphal extension, and pathogenicity [25]. In the phytopathogen *Ustilago maydis*, the small HSPs Hsp12, Hsp21, and Hsp30 are up-regulated during plant infection, and strains with defects in these genes are sensitive to oxidative stress and exhibit attenuated virulence [24].

The heat shock genes have been found scattered throughout the genome, with some related genes clustered while others are interspersed with independently regulated genes [4]. Under heat stress, *Drosophila* spp. cells undergo a drastic decrease in translational activity, and within ten minutes of a temperature shift, there is a decrease in the number of polysomes. However, heat shock mRNAs are translated with high efficiency, and pre-existing mRNAs are retained in the cell and reactivated after recovery [4]. In general, the heat shock genes are activated by the heat shock transcription factor (HSF1), which is activated by phosphorylation and trimerisation under stress conditions. Heat shock transcription factors (HSFs) contain a helix-turn-helix domain that binds to the heat shock elements (HSE) in the promoter region of target genes, a coiled-coil hydrophobic domain required for trimerisation, and a carboxy-terminal activation domain. HSF1 from *S. cerevisiae* also contains an amino-terminal activation domain [11].

Fungal cutaneous infections are prevalent in healthy humans, and they can become invasive in immunocompromised patients. Dermatophytes are a group of keratinophilic filamentous fungi that typically infect the cornified layers of the skin, hair, and nails, but there are some case reports of invasion into the dermis, subcutaneous tissue, or internal tissues [27-30]. Treatment of fungal infections is still difficult due to the side effects of the currently available drugs, which act against a limited number of molecular targets that are mostly involved in the synthesis or function of ergosterol in the fungal cell membrane [31, 32]. Thus, the search for new targets is necessary to enable the discovery of new drugs to control fungal infections. The genomes of several dermatophyte species have been completely sequenced, enabling the search for different genes and metabolic path-

ways and comparison among the species. HSPs are highly conserved among different pathogens and play a crucial role in fungal pathogenicity, survival under stress, and antifungal drug resistance. Therefore, this review focuses on recent advances in understanding the relationship between structural and functions of the HSPs in dermatophytes, including an evaluation of the broad range of highly conserved HSPs that can be identified in the genomes of different species of dermatophytes.

REGULATION OF *hsp* GENES AND INTERACTION WITH pH SIGNALLING

Transcription of HSPs is linked to a number of stressing conditions, including phosphate (Pi) deprivation and changes in extracellular pH. Induction of heat shock genes in response to heat stress is mediated by two regulatory proteins, the binding of heat shock factor 1 (HSF1) to the heat shock elements (HSE) and the binding of zinc finger Msn2/4 transcription factors to the stress response elements (STRE) [33-38]. HSF1 is encoded by a single gene in yeasts [39] and in the dermatophytes we analysed [40]. HSE consists of multiple inverted adjacent repeats of the 5 base pair (bp) DNA-binding domain (5′-nGAAn-3′) in the promoter region of the *hsp* genes. HSF1s are highly conserved regulators that recognise continuous and discontinuous repeats. Three repeats are thought to be the minimum required for transcriptional activation of the target genes, with the following ideal consensus sequences: Perfect HSE (nGAAnnTTCnnGAAn), Gapped HSE (nTTCn{5nt}nTTCnnGAAn), and Stepped HSE (nTTCn{5nt}nTTCn{5nt}nGAAn) [34, 41-48].

Although transcription of heat shock genes has been well characterised in many filamentous fungi, little is known about the expression of HSPs in response to Pi and ambient pH changes in these organisms, including in dermatophytes. The highly conserved molecular mechanisms controlling the responses to Pi deprivation and ambient pH changes seem to be well established in non-pathogenic fungi such as *Neurospora crassa* and *Aspergillus nidulans*.

In *N. crassa,* the response to Pi consists of a highly conserved and hierarchical relationship among at least five genes: *nuc-2*, *preg*, *pgov*, *mak-2*, and *nuc-1*. These genes function to effect the translocation of the transcription regulator NUC-1 into the nucleus, thereby activating the transcription of Pi repressible phosphatase genes, among others [49-57]. In *A. nidulans*, the response to pH changes involves the highly conserved PacC signal transduction pathway, which senses acidic or alkaline pH and mediates many metabolic events, including virulence [58-63]. Thus, the ambient pH-signalling pathway ensures that nutritional enzymes or proteins will be secreted or synthesised at pH values at which they can function effectively. The PacC regulatory system consists of a conserved signalling cascade composed of six *pal* genes (*pal*A, B, C, F, H, and I). The system senses the ambient pH and transmits information about the acidity or alkalinity via a protein-protein interaction between PalA and PacC. This interaction is necessary for the proteolytic activation of the transcription regulator PacC by the calpain-like enzyme PalB.[61, 64-67]. Once activated, PacC regulates a variety of physiological events, including the glycosylation, catalytic activity, and secretion of enzymes in filamentous fungi, e.g., the keratinolytic activity in the dermatophyte *Trichophyton rubrum* [63, 68].

We have shown that the genes encoding the 70 kDa class of HSPs in *N. crassa* are modulated in response to extracellular pH changes and exhibit complex patterns of expression [69-71]. Interestingly, the *hsp70-1* (NCU09602) and *hsp70-2* (NCU08693) genes are transcribed preferentially in cultures with sufficient Pi at pH 5.4 and 30°C, which are optimal conditions for fungal growth. There is no significant accumulation of these transcripts at 30°C, at alkaline pH. When mycelia are incubated at 45°C, transcripts of both genes accumulate to high levels, regardless of the Pi and ambient pH changes. Transcription of these two genes (*hsp70-1* and *hsp70-2*) is strongly reduced in a *pacC*$^-$ background regardless of culture conditions, indicating that transcription is positively regulated by PacC at both 30°C and 45°C [71]. Heat shock induction of these two *hsp* genes is apparently not altered in low-Pi cultures in a *nuc-1*$^-$ background. Transcription of the *hsp70-3* (NCU01499) gene is depressed in low-Pi cultures at either acidic or alkaline pH but not induced by heat shock. This gene is an interesting molecular target since it is probably unique to ascomycetes [72]. Expression of the Pi-repressible phosphatases genes and other genes involved in responding to Pi stress is dependent directly or indirectly on both NUC-1 and PACC transcription factors [57], suggesting a possible interaction between the pH and Pi responsive circuits in the regulation of the *hsp* genes.

Transcripts of the *pacC* and *hsf1* genes accumulate in response to temperature shifts when *T. rubrum* and *Trichophyton interdigitale* are grown in keratin cultures. Heat shock induction has also been observed for the genes *hsp70-like*, *hspSsc1*, and *hspcdc37* at 37°C in a *pacC*$^+$ *hsf1*$^+$ background. Interestingly, transcription of the *hsf1* gene is dramatically reduced in a *pacC*$^-$ background whereas transcription of the *hsp70*, *hspSsc1*, and *hspcdc37* genes is constitutively de-repressed independent of heat induction in a *pacC*$^-$ background (Fig. **1**). These findings suggest that low transcription levels of these *hsp* and the *cdc37* genes at normal growth temperature is dependent on a concomitant *hsf1*$^+$ and *pacC*$^+$ background. Heat shock induction of these genes may be a consequence of the loss of the interaction between the transcriptional regulators PacC and Hsf1 seen under optimal growth temperature (28°C), a loss that is mimicked by the *pacC*$^-$ background. Thus, cooperation between PacC and Hsf1 may determine the degree to which the quantity of *hsp* transcripts is modulated in support of these important regulatory circuits (Fig. **1**).

The number of the consensus binding sequences for the regulators Hsf1, Nuc-1, and PacC in the promoter regions (up to 1000 bp) of the *hsf1*, *nuc-1*, and *pacC* genes in the sequenced genomes of eight dermatophytes is shown in (Table **1**). The consensus for HSF1 is fully represented in the promoter region of the *nuc-1* and *pacC* genes, but poorly represented in its own promoter, except for in *Microsporum canis*. The consensus for PacC [65] is fully represented in the promoter region of the *hsf1* and *pacC* genes, but in the *nuc-1* promoter it is present only in four dermatophyte species. The consensus for Nuc-1 [56, 73] is practically absent in the promoter region of the *pacC* and *hsf1* genes, but is

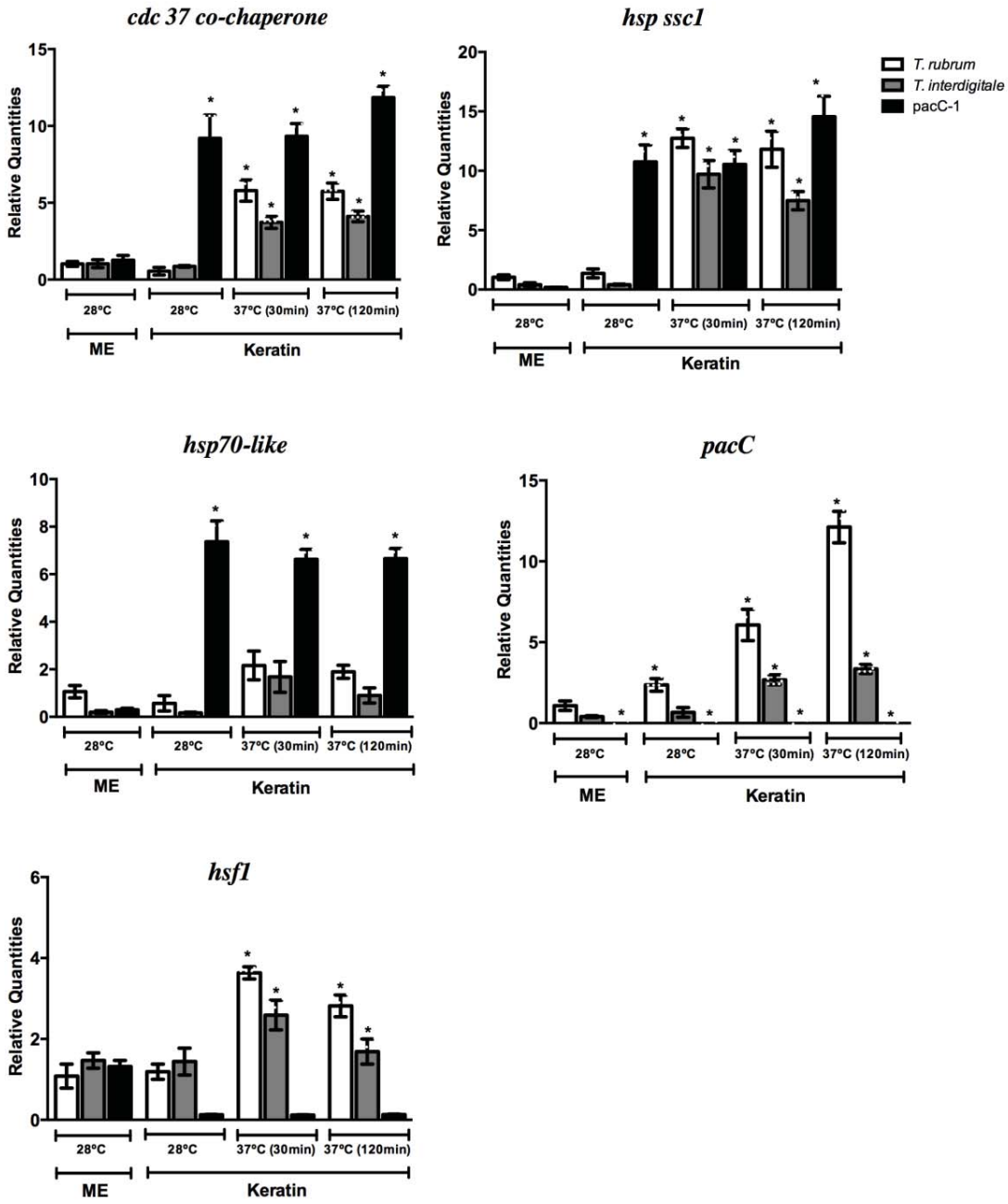

Fig. (1). Transcript levels of differentially expressed genes in dermatophytes. Real-time reverse transcription PCR (qRT–PCR) analyses of the *cdc37* co-chaperone, *hspssc1*, *hsp70-like*, *pacC*, and *hsf1* transcript levels in *T. rubrum*, *T. interdigitale*, and the *pacC-1* mutant of *T. interdigitale*. These genes were amplified from cDNA obtained from mycelia of these species grown in keratin cultures at 28°C and shifted to 37°C for 30 min or 120 min. The gene expression levels are represented by the relative quantities in each condition relative to the control (malt extract, ME). The qRT-PCR data shown are representative of the average values ± standard deviation (SD) from three independent experiments with reactions performed in triplicate. Statistical analyses were performed in comparison to malt extract cultures at 28°C. Statistical significance was determined using Bonferroni's *ad hoc* test and is indicated by an asterisk: $*P < 0.05$.

fully represented in its own promoter region (Table **1**). In addition, *in silico* prediction of the putative DNA-binding sites for the transcription factors Hsf1, Nuc-1, and PacC identified in the DNA sequences upstream (1000 bp) of the promoter region of the *hsps* and related genes is presented in (Table **S1**).

(Fig. **2**) shows the proposed model for the heat shock response in dermatophytes. This model is based on previously published data, computational analyses, and experimental results described here, focusing on the regulation of the expression of the transcriptional regulator Hsf1 under conditions of pH, Pi, and heat stresses. We observed that Hsf1 is potentially competent to induce the expression of the *pacC* and *nuc-1* genes as well as a number of other *hsp* genes. In addition, transcription of the *hsf1* gene is dependent on a $pacC^{+}$ background and transcription of the *pacC* and

Table 1. ***In silico*** **prediction of DNA-binding sites for the regulators Hsf1, Nuc-1, and PacC. The number of putative consensus binding sites was determined by identifying the following DNA sequences upstream (1000-bp) from the promoter region of the *hsf1*, *nuc-1*, and *pacC* genes: Hsf1, Stepped HSE (nTTCn{5nt}nTTCn{5nt}nGAAn); Nuc-1, CACGTG; PacC, GCCARG.**

		Dermatophytes*								Other
Gene	**Consensus for Protein Regulators**	***A. benhamiae***	***T. interdigitale***	***M. canis***	***M. gypseum***	***T. equinum***	***T. rubrum***	***T. tonsurans***	***T. verrucosum***	***A. fumigatus***
hsf1		ARB_00838	H101_05876	MCYG_02012	MGYG_04072	TEQG_06155	TERG_04406	TESG_06833	TRV_07496	Afu5g01900
	Hsf1	1		4	1				1	
	Nuc-1				1					1
	PacC	3	3	2	2	4	2	4	3	1
nuc-1		ARB_04187	H101_05990	MCYG_05354	MGYG_06409	TEQG_03386	TERG_06537	TESG_03753	TRV_06019	Afu1g07070
	Hsf1	1	1	3	5	2	1	2	3	2
	Nuc-1	3	1	2	3	3	2	3	3	1
	PacC		1		2	2		2		4
pacC		ARB_07827	H101_07360	MCYG_01012	MGYG_00974	TEQG_01042	TERG_00838	TESG_04572	TRV_01305	Afu3g11970
	Hsf1	3	1	2	6	3	3	3	2	3
	Nuc-1									1
	PacC	10	1	15	9	16	12	14	3	2

* Broad Institute of Harvard and MIT

http://www.broadinstitute.org/annotation/genome/dermatophyte_comparative/MultiHome.html

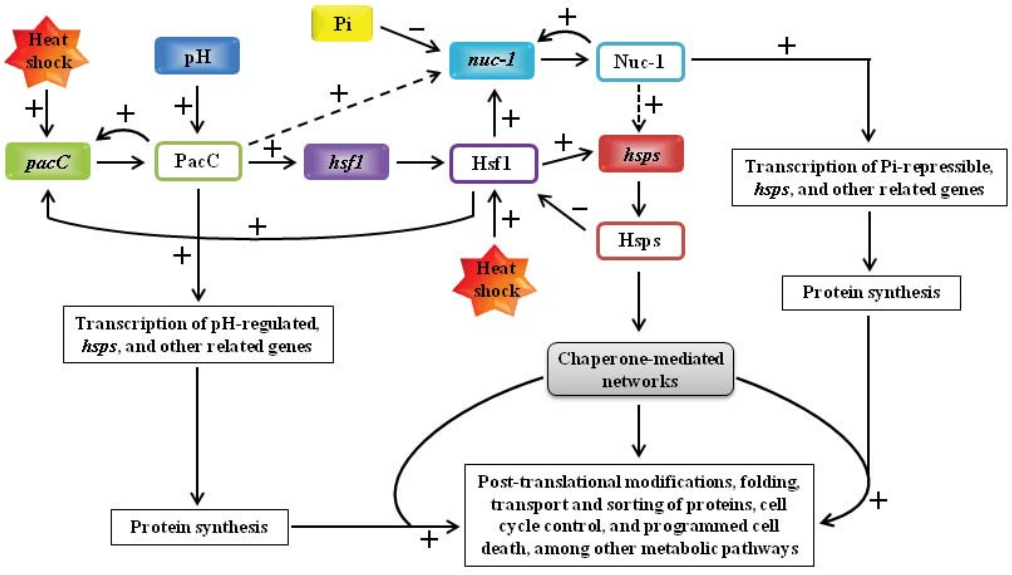

Fig. (2). Model proposed for the regulation of heat shock protein genes (*hsp*) in dermatophytes under the action of transcriptional regulators Hsf1, Nuc-1, and PacC. Activation and inhibition are marked by positive and negative signs, respectively. Broken lines represent the action of PacC and Nuc-1 through presumed metabolic pathways that have not yet been fully elucidated. Other signalling pathways in which the Hsf1, Nuc-1, and PacC proteins are probably involved are not represented in the proposed model.

nuc-1 genes may be auto regulated, while transcription of *hsf1* does not seem to be. Interestingly, Hsf1 exhibits positive feedback induction for the transcription of the *pacC* gene.

THERMOTOLERANCE AND FUNGAL PATHOGENICITY

It is estimated that thousands of fungal species infect plants or insects, while only a few hundred are pathogenic to mammals. The potential of most fungal isolates to infect mammals is affected by their capacity to survive at high temperatures [74]. For thermally dimorphic fungi, such as *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Penicillium marneffei*, and *Paracoccidioides brasiliensis*, a morphological transition between yeast and hyphae is induced by the host temperature and is fundamental for the organism's life cycle, virulence, and pathogenicity [75, 76]. Elevated temperature is also a requirement for the morphological transition and hyphal growth in *Candida albicans*, which is crucial in causing disease [77]. The ability to grow at 37°C or higher temperatures is a determinant for the virulence of human pathogens. Impaired growth at 37°C correlated to attenuated virulence has been reported for the most common fungal pathogens of humans, including *C. albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Wangiella dermatitidis*, and *H. capsulatum*. On the other hand, increased thermotolerance associated with a virulent phenotype was reported for *S. cerevisiae* [5, 25, 78, 79].

The ability to sense and adapt to thermal conditions is crucial for pathogen survival in host microenvironments. The induction of proteins involved in adaptive responses is fundamental for the success of pathogenesis. Members belonging to the heat shock family of proteins have been reported to be induced not only upon thermal stress, but also in diverse conditions.

ROLE OF HSPs IN FUNGAL PATHOGENICITY

In *C. albicans,* the essential heat shock transcription factor Hsf1 is phosphorylated upon heat stress, regulating the expression of heat shock element (HSE) containing genes. Hsf1 is required for the expression of chaperones, including Hsp104, Hsp90, and Hsp70. Activation of Hsf1 by phosphorylation at specific sites in the CE2 domain is required for full virulence [80, 81]. Hsp90 governs the morphogenetic transition of *C. albicans* from yeast to filamentous growth and its depletion attenuates virulence [82]. Hsp90 interacts with Hsf1, and plays a critical role in the regulation of the Hsf1-HSE regulon. Hsp90 activity is controlled post-translationally by an autoregulatory loop involving Hsf1. Hsp90 also modulates the activities of key MAP kinase signalling pathways that mediate cell wall remodelling [17, 83]. Sfl1p and Sfl2p are two homologous heat shock factor-type transcriptional regulators that carry a putative Hsf-type DNA binding domain and antagonistically control morphogenesis [84]. Ssa1, a member of the 70 kDa HSP family, is expressed on the surface of *C. albicans* cells and mediates the invasion of epithelial and endothelial cells *in vitro*. The null mutant presents significantly attenuated virulence in murine models of oropharyngeal candidiasis and disseminated candidiasis [85]. Members of the HSP family were also reported to be immunogenic and recognised by serum samples from patients infected by *C. albicans* [86]. The small heat shock protein Hsp21 is involved in thermal and oxidative stress responses and glycerol and glycogen regulation, and plays a major role in trehalose homeostasis. Deletion of *hsp21* reduces the filament size and impairs the organism's ability to damage human-derived endothelial and oral epithelial cell lines and survive attack by human neutrophils. Hsp21 is also required for full virulence of *C. albicans* in mice [25].

The *C. neoformans* Hsp70 homologue Ssa1 acts as a co-activator, interacting with Hsf to form a regulatory complex that drives the transcription of the virulence factor laccase in response to glucose starvation, iron, copper, calcium, and stressing temperature. Deletion of Ssa1 causes reduced laccase and melanin production, as well as attenuated virulence in a murine model of meningoencephalitis [87]. The chaperone Hsp70 is both surface-associated and distributed within the fungal cytoplasm. The interaction of *C. neoformans* cells with human alveolar epithelial cells has been shown to be positively modulated in the presence of recombinant Hsp70. Phagocytosis was not affected by recombinant Hsp70, however increased fungal survival was observed when macrophages were pre-incubated with this protein prior to incubation with fungal cells. Hsp70 was also found to compete with glucuronoxylomannan, the major capsular antigen, in binding to host cells [88]. In addition, Hsp70 is recognised by serum samples from individuals with cryptococcosis [89, 90].

In the thermotolerant *A. fumigatus*, Hsp30/Hsp42 and Hsp90 proteins were the most abundant among several heat shock proteins induced under thermal upshift [91]. Hsp90 plays a role in cell wall integrity and morphogenetic processes. Its repression causes defects in germination and sporulation, affecting the production and pigmentation of conidia [92, 93]. Hsp90 was also detected by serum samples from patients with invasive aspergillosis [94].

H. capsulatum is a facultative intracellular fungus that binds to CD11/CD18 receptors on macrophages. Hsp60 is localised on the surface of *H. capsulatum* yeasts, in the cell wall. This protein was found to act as a ligand that binds *H. capsulatum* to macrophages [14]. Furthermore, vaccination with Hsp60 from *H. capsulatum* induces a protective immune response in mice [95, 96].

In *P. brasiliensis*, vaccination with recombinant PbHsp60 confers protection against pulmonary paracoccidioidomycosis in mice [97]. The Hsp90-encoding gene is transcriptionally up-regulated during the mycelium to yeast transition and oxidative stress and its inactivation by specific Hsp90 inhibitors is lethal [98]. Yeast cells from a strain with reduced expression of Hsp90 were found to be more susceptible to acidic conditions and oxidative stress, and also showed reduced viability upon interaction with macrophages [99].

The Hsp60-encoding gene is upregulated during thermal stress in the keratinolytic fungi *Trichophyton mentagrophytes*. [100]. Indeed, *hsp60* has been reported as a potential vaccine. The protective effect of a DNA vaccine that expresses the immunodominant *hsp60* antigen has been confirmed on calves and guinea pigs, as it was found to reduce the clinical course of skin mycoses caused by *T. mentagrophytes* [101]. In *Arthroderma benhamiae* (the teleomorph of *T. mentagrophytes*) a gene encoding a putative Hsp70 was found to be up-regulated during keratin degradation [102]. In addition, transcriptional profiling in *T. rubrum* grown on soy and keratin-soy media revealed the accumulation of transcripts encoding secreted proteases and other proteins poten-

tially involved in soy and keratin utilization, such as Hsp70, Hsp90 and Hsp98 [103]. The gene encoding Hsp30 has been identified in clinical isolates from skin and nail mycoses [104] and has been reported to be transcriptionally up-regulated during degradation of keratin [105-107]. In this fungus, transcription analyses also reveal that the gene encoding for an Hsp70 is up-regulated upon heat shock [108], after shifting the pH of the culture medium from acid to alkaline [90], and during interaction with human skin [109]. The *hsp90* gene is also up-regulated during interaction with human skin. The genes encoding Hsp70, Hsp90, and the heat shock binding protein Sti1 also have been reported to be up-regulated during the declination phase in cultures grown *in vitro* [110]. We also have evaluated transcriptional levels of members of the HSP family in *T. rubrum* during *ex vivo* infection of human nails and skin (Fig. **3**). The accumulation of *hsf1, ssc1,* and *cdc37* transcripts increased to similar levels in both infection models. However, there was a significant difference in the accumulation of *hsp70* transcripts, which increased during nail infection but not during skin infection (Fig. **3**).

THE ROLE OF HSPs IN THE RESISTANCE AND RESPONSE TO ANTIFUNGAL AGENTS

Fungi use signal transduction pathways to sense environmental stresses and ensure adaptive physiological responses, which are characterised by the modulation of cell stress genes [31]. These organisms respond to sub-lethal doses of chemical and physical agents by synthesising a variety of specific proteins and low molecular weight compounds, and by activating chaperones and signal transduction cascades that promote the development of defensive reactions or tolerance [111, 112]. Antifungal drugs induce cell stress responses that can overcome the toxic effects of these drugs and allow fungal survival. The identification of transcripts differentially expressed during exposure of fungi to cytotoxic drugs contributes to our understanding of the mechanisms of action of these drugs and of the organism's adaptation to stress [31].

The exposure of dermatophytes to sub-inhibitory concentration of cytotoxic drugs modulates the expression of several genes involved in lipid metabolism, molecule transport, multidrug resistance, defence mechanisms, signal transduction mechanisms, translation, post-translation modification, secondary metabolite biosynthesis, and protein turnover, as well as genes that code for chaperones [107, 111, 113-116].

The antifungal drug terbinafine inhibits the enzyme epoxidase, which is involved in the conversion of squalene-to-squalene epoxide. This inhibition causes the accumulation of squalene and the depletion of fungal ergosterol. Molecular mechanisms contributing to terbinafine resistance in dermatophytes and other fungi have been described [114, 117, 118]. The exposure of *T. rubrum* to a sub-inhibitory concentration of terbinafine increases the accumulation of transcripts encoding HSPs such as Hsp70-1, Hsp104 [119], Hsp20, HspSsc1, Hsp70, and Hsf1 (Fig. **3**; Table **2**). In addition, Amphotericin B, a polyene antifungal drug which binds to ergosterol in the fungal cell membrane, increases the transcription of *T. rubrum* genes that encode Hsp30 [107] and HspClpA and decreases the accumulation of *hsp10* transcripts [120]. In *C. albicans,* some genes encoding members of the Hsp70 family are involved in amphotericin B and fluconazole resistance [121]. Three different genes encoding molecular chaperones, including Hsp70, were also up-regulated when *T. rubrum* was exposed to PHS11A, a fungal fatty acid synthase inhibitor [122].

Acridine derivatives, such as acriflavine, act at the level of DNA-coiling enzymes (topoisomerases) by stabilising the enzyme-DNA cleavable complex [123, 124]. Acriflavine has been used as a topical antiseptic due to its antibiotic activity against fungi, bacteria, viruses, and parasites [125, 126]. It is also noteworthy that acriflavine is apparently oxidised *in vivo* by catalase and probably by other enzymes to generate its biologically active form [127]. The mode of action of acriflavine against dermatophytes has been investigated by evaluating the time-dependent effects of this compound on the *T. rubrum* transcriptome using high-throughput RNA-sequencing (RNA-seq) technology. Several genes were differentially expressed after exposure to acriflavine. Some genes belonging to the hsp70 family were up-regulated and the chaperone dnaK and Hsp90 co-chaperone Cdc37 were down-regulated [128]. We have shown using another experimental assay that the *hsp20*, *hspSsc1*, *hsp70*, and even the *hsf1* and *pacC* transcripts accumulated after exposure of *T. rubrum* to acriflavine (Fig. **3**; Table **2**). Indeed, the accumulation of *hsf1* and *pacC* transcripts during exposure of *T. rubrum* to cytotoxic drugs suggests that the transcription factors encoded by these genes are involved in the drug stress response. Interestingly, *hsp70-like* transcripts of *T. rubrum* do not seem to accumulate under any stress conditions (Fig. **3**).

Transcripts of the gene encoding a 12 kDa heat shock protein accumulate when *S. cerevisiae* is challenged with ketoconazole, amphotericin B, caspofungin, or 5-fluorocytosine, which are representatives of four classes of antifungal agents [129]. This gene is likely involved in the survival mechanism employed by yeast cells when exposed to toxic agents. Hsp20/Hsp30 (TERG_01659) seems to play a similar role when *T. rubrum* is exposed to terbinafine, amphotericin B, itraconazole, or acriflavine (Table **2**). Also, transcripts of *hsp70* (TERG_01883) accumulate when *T. rubrum* is exposed to terbinafine, acriflavine, or PHS11A (Table **2**). However, the precise role of these modulated genes remains unclear. Further study concerning these genes and their respective gene products in the context of antifungal resistance is necessary.

Hsp90 is probably the most thoroughly studied heat shock protein. Hsp90 directly or indirectly controls the function of at least 10% of the entire proteome of yeasts [130, 131], via roles in folding proteins, modulating the activities of key regulators, and triggering adaptive responses to stress conditions [17, 93, 132]. Dermatophytes, *A. fumigatus*, and other fungi each have a single Hsp90 protein, while *S. cerevisiae* has two Hsp90 homologues (Table **1** Supplementary). The fungal Hsp90 has numerous client proteins, including protein kinases, transcription factors, and receptors, and can interact with calcineurin [17, 133]. Hsp90 plays a crucial role in the development of resistance of *C. albicans* to two major classes of antifungals, the azoles and echinocandins [21, 134, 135]. Under normal

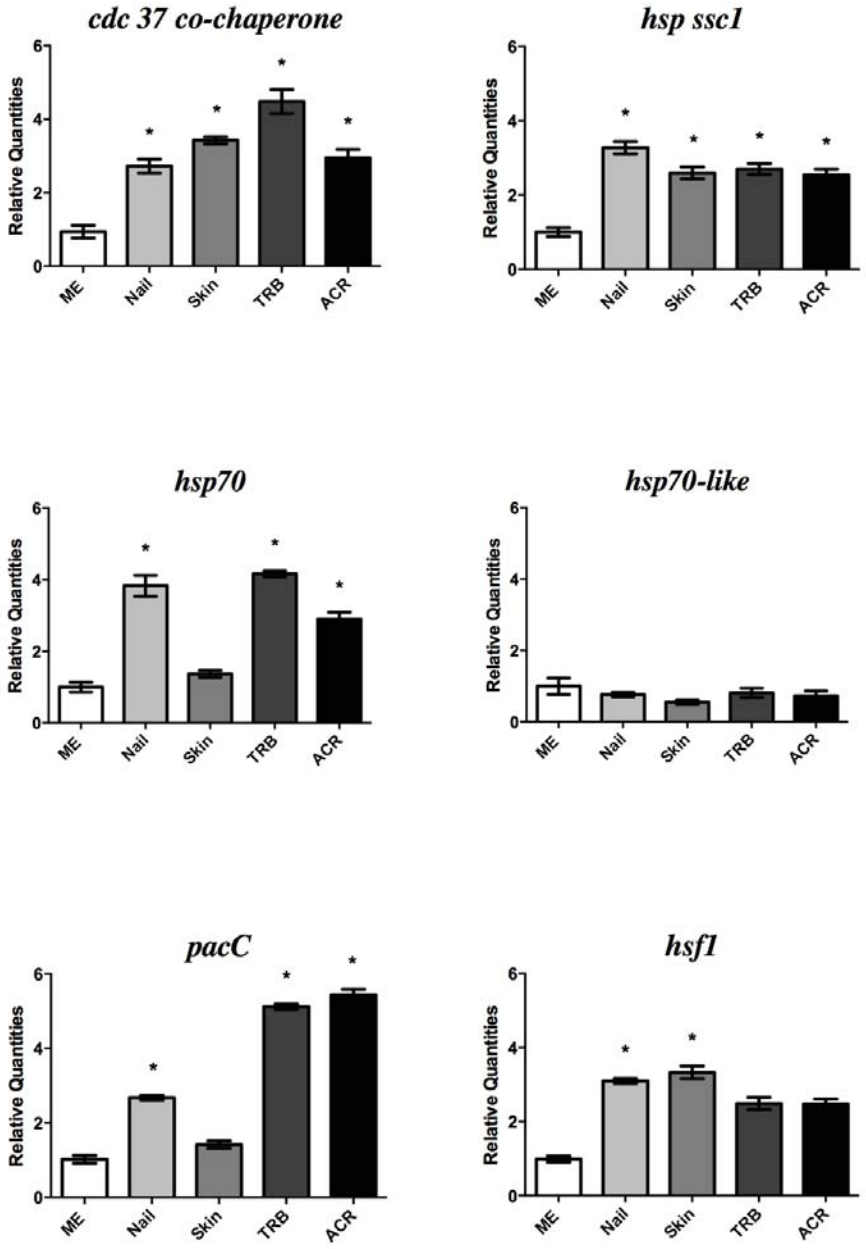

Fig. (3). Transcript levels of differentially expressed genes in *Trichophyton rubrum*. Real-time reverse transcription PCR (qRT–PCR) analyses of the transcript levels of various *hsps* and related genes. These genes were amplified from cDNA obtained from mycelia grown in malt extract (ME), in human nail fragments incubated in water and human skin at 28°C for 96 h. Drug responses were observed in mycelia grown in ME after exposure to terbinafine or acriflavin for 3h. The gene expression levels are represented by the relative quantities in each condition relative to the control (ME). The qRT-PCR data shown are representative of the average values ± standard deviation (SD) from three independent experiments with reactions performed in triplicate. Statistical analyses were performed in comparison to malt extract cultures at 28°C. Statistical significance was determined using Bonferroni's *ad hoc* test and is indicated by an asterisk: $*P < 0.05$.

physiological conditions, ergosterol is present in fungal cell membranes and the calcineurin-dependent stress response is not active. However, when fungi are submitted to an azole antifungal, blockage of ergosterol synthesis occurs, and a toxic sterol intermediate that disrupts membrane integrity accumulates. Calcineurin chaperoned by Hsp90 initiate the signal transduction networks that are required for the emergence and maintenance of resistance to these drugs [21].

Genetic depletion of Hsp90 increases susceptibility to echinocandins and prevents the emergence of azole resistance [93, 134, 136]. Considering this, the importance of Hsp90 for cell physiology, and calcineurin's dependence on an interaction with Hsp90 to modulate resistance to antifungal drugs, the inhibition of Hsp90 could be a promising treatment for dermatophytosis and other fungal infections, especially in combination therapies [22, 92].

Table 2. A representative list of *hsp*s and related genes of *T. rubrum* up-regulated upon exposure to antifungal drugs.

Antifungal	Accession Number*	ID	Reference
Terbinafine	TERG_01659	Hsp20/Hsp30	Unpublished results
	TERG_03037	Hsp70	[119]
	TERG_01883	Hsp70	Fig. 3
	TERG_03206	HspSsc1 (70/DnaK)	Fig. 3
	TERG_06398	Hsp90 co-chaperon Cdc37	Fig. 3
	TERG_04406	Hsf1	Fig. 3
	TERG_ 00838	PacC	Fig. 3
Amphotericin B	TERG_01659	Hsp20/Hsp30	[107]
	TERG_07049	Heat shock ClpA	[120]
Itraconazole	TERG_01659	Hsp20/Hsp30	[115]
PHS11A	TERG_03037	dnaK-chaperonin	[122]
	TERG_01002	dnaK	[122]
	TERG_01883	Hsp70	[122]
Acriflavine	TERG_01659	Hsp20/Hsp30	[128]
	TERG_03206	HspSsc1 (70/DnaK)	Fig. 3
	TERG_01883	Hsp70	Fig. 3
	TERG_05615	hsp70-like	[128]
	TERG_06505	Hsp70-like protein	[128]
	TERG_03062	DnaJ domain-containing protein	[128]
	TERG_03037	dnaK- chaperonin	[128]
	TERG_06398	Hsp90 co-chaperone Cdc37	Fig. 3
	TERG_04406	Hsf1	Fig. 3
	TERG_00838	Zinc finger transcription factor pacC	[128]; Fig. 3

* Broad Institute of Harvard and MIT http://www.broadinstitute.org/annotation/genome/dermatophyte_comparative/MultiHome.html

CONCLUSION AND PERSPECTIVES

The genomes of 13 of these human/animal pathogens have been sequenced, and show similar genome organisation and genic content. The *hsp* and related genes are also quite similar among the dermatophytes. Most of these genes are represented in the genomes of all dermatophytes analysed in this work, and the few that are not universally found do not appear to characterise a specific gender or niche. We have shown that several *hsps* from these dermatophytes are putative pathogenicity/virulence and antifungal response factors by comparing them to orthologous proteins in other pathogenic fungi. Our findings suggest the presence of crosstalk among the pH-, Hsf1-, and Pi-regulatory circuits. Interestingly, the transcripts of some small HSPs accumulate in response to different classes of antifungal drugs, suggesting a nonspecific response to the stress triggered by these drugs. This response is probably involved in detoxification and cell maintenance. The essentiality of Hsp90 for cell physiology and its role in antifungal resistance has led some authors to consider this protein as a therapeutic target to treat *Candida* and *Aspergillus* infections, a proposal that can be extrapolated to dermatophytes due to the high degree of conservation of this protein among fungal species. Although the high degree of conservation between fungal and host Hsp90 proteins is a challenge for this strategy, minor structural and functional differences between them can be explored for the development of new drugs or for the modification of pre-existing ones. Inhibitors of the molecular chaperone HSP90 have been used in clinical development as anticancer agents since 1999 [137]. To date, a number of active Hsp90 inhibitor oncology trials have been undertaken. Furthermore, some of these inhibitors have made their way into the clinic in the past few years, thus confirming the tolerance of the human body to these drugs [138].

High-throughput next-generation sequencing technology is being used for transcription profiling and gene expression quantification in numerous fungal growth conditions. When these data are associated with proteome analyses, they will

surely reveal novel aspects of the physiological functions of the HSPs or their specific function in each species or specific niche. Fungi can also serve as models for understanding human diseases caused by HSP disorders and can contribute to the elucidation of the mechanisms underlying the roles of specific genes in the manifestation of these diseases [5, 139, 140]. Thus, a better understanding of the complexity of fungal metabolism will facilitate its application in biotechnology, medicine, and other fields.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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