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Expression of Heat Shock Protein 30 in *Talaromyces marneffei* during Phase Transition and in Response to Heat and Oxidative Stresses

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ABSTRACT

OBJECTIVE Heat shock protein 30 (Hsp30) has been identified as an immunogenic, yeast phase-specific protein in *Talaromyces marneffei*. The purpose of this study was to investigate how the *hsp30* gene and Hsp30 protein are expressed during phase transition and in response to heat and oxidative stress exposure.

METHODS Several sequence analysis tools were employed to predict *hsp30* control elements and to determine the subcellular localization of Hsp30. In the phase transition experiment, *Talaromyces marneffei* conidia were cultivated at two different temperatures, 25 °C and 37 °C. Subsequently, stress response tests were conducted by subjecting the yeast cells to heat at 42 °C and by treating them with hydrogen peroxide. The levels of the *hsp30* transcript and its protein were measured using real-time RT-PCR and western immunoblot analysis, respectively.

RESULTS The sequence analysis revealed the presence of heat response element (HRE), stress responsive element (STRE), and xenobiotic responsive element (XRE), which are typically involved in regulating hsp genes. A web-based tool predicted that Hsp30 protein is localized in cytoplasm, nucleus, and cell membrane. The *hsp30* transcript and Hsp30 protein were highly clearly detected in both yeast cells and conidia. Furthermore, the *hsp30* transcript in yeast cells was upregulated following heat shock at 42° C and exposure to hydrogen peroxide. These findings indicate that Hsp30 plays a crucial role in assisting the yeast phase of *T. marneffei* to cope with heat and oxidative stresses.

CONCLUSIONS Hsp30 is a protein specific to the conidial and yeast phases of *T. marneffei*. It likely performs a conserved chaperone function during yeast growth and plays a significant role in stress response by mitigating protein aggregation issues.

KEYWORDS *Talaromyces marneffei*, heat shock protein 30, expression

INTRODUCTION

Talaromyces marneffei is a thermally dimorphic fungus. At 25 °C, it grows as the mold phase, producing hyaline septate hyphae, conidiophores, phialides, and conidia. When exposed to a temperature of 37 °C in an artificial medium or in human tissue, the fungus grows in a yeast phase, producing arthroconidium cells or fission yeasts (1). The fungus can cause an opportunistic infection called talaromycosis in immunocompromised patients, especially in people residing in an endemic area and travelers visiting Southeast Asian countries (2-4).

Body temperature at 37 °C is one of the major initiation factors for the mycelial to yeast phase conversion of *T. marneffei*. The ability of the fungus to tolerate high temperatures and propagate inside a human host is well recognized as the essential virulence contributing factor. To overcome heat stress, the organisms usually make use of proteins in the heat shock protein (Hsp) superfamily. Hsps are divided into several families based on molecular weights and functions (5). They perform diverse functions, including transcription, translation, posttranslational modification and nascent protein folding as well as prevention of aggregation and disaggregation of the misfolded protein (chaperone function) (6). This protein family plays a key role in coping with various changing environmental stresses such as pH fluctuation, starvation and osmotic pressure as well as oxidative and xenobiotic stress also plays a role in the morphogenesis and dimorphism of fungi (7).

In studies of heat shock proteins in *T. marneffei*, only *hsp70* and *hsp30* genes have been characterized (8,9). The *hsp70* gene was first isolated from a cDNA library using a DNA hybridization approach. Expression of *hsp70* was found to be constitutively expressed in both the mold and yeast phases of *T. marneffei*. It is also upregulated during the mold-to-yeast phase transition and heat shock condition, revealing the chaperone role of Hsp70 during heat stress (8). A later study isolated and characterized the *hsp30* gene from using an antibody screening approach (9). In that study, Hsp30 was shown to be an immunogenic protein eliciting antibody response in *T. marneffei*-infected patients' sera. Unlike the *hsp70* expression, the *hsp30* transcript has been found to be expressed only in conidia and yeasts. Another proteomic analysis using a different strain of *T. marneffei* supports this finding (10). Since this protein appears to be yeast-phase specific, understanding its biological role is necessary to enhance knowledge regarding this pathogen. This study aimed to examine the expression pattern of this protein to develop an initial understanding of its role during phase transition and during different stress exposures.

The Hsp30 protein is a member of the small heat shock proteins (sHsps). This family of proteins shares the common characteristic of containing an alpha-crystallin domain (ACD). The function of sHsps involves maintaining non-native proteins in folding-competent states for subsequent degradation and reactivation by ATP-dependent chaperones (11). In *Saccharomyces cerevisiae*, the Hsp30 could can inhibit proton-ATPase to conserve cellular energy and maintain the fluidity of the plasma membrane during thermal stress (12). Additionally, Hsp30 plays a role in heat shock at 45 °C and in nutrient starvation in *Neurospora crassa* (13). Recently, an additional role of Hsp30 was observed, as an assisting molecule in hemoglobin uptake in the dimorphic fungus *Paracoccidioides brasiliensis* (14). The Hsp30 is likely involved in the pathogenesis of this fungus as evidenced by the Hsp30 knockdown strain having a decreased ability to recognize hemoglobin. As *T. marneffei* is one of the dimorphic fungi, it is possible that Hsp30 could be involved in pathogenesis. To initially investigate the role of Hsp30 in *T. marneffei*, we employed bioinformatic tools to predict its cellular localization and function. Additionally, the expression at both transcript and protein levels was examined during phase transition and when under stress.

METHODS

Fungal strain and culture conditions

Talaromyces marneffei FRR2161 (ATCC18224) strain was maintained on a potato dextrose agar (PDA) (Difco, Inc., NJ, USA) for 10 days. Collection of conidia was performed by scraping the colony surface with a sterile spatula and then suspended suspending the conidia in a 5-10 ml sterile normal saline-tween solution (0.1% v/v tween 40 in 0.85% w/v NaCl). The conidial suspension was filtered through a sterile Miracloth (Calbiochem, Darmstadt, Germany) and enumerated by counting the number of conidia in using a hemacytometer.

The conidia were prepared to a final concentration of 10^8/mL in an *Aspergillus* minimal medium (ANM). The fungus was subjected to cultivation at 25 and at 37 °C to generate mold and yeast forms, respectively. In the phase transition experiment, the 3-day-old mold culture was incubated at 37 °C, and the cells were harvested at 6, 12, 24, 48, and 72 h after

the temperature shift. For the stress exposure experiments, the 3-day-old yeast culture was treated with either heat at 42 °C or 1 mM hydrogen peroxide for 1 h. The fungal cells were then harvested and used in total RNA extraction.

Bioinformatic analysis

DNA sequence (approximately 1 kb) upstream of the Hsp30 open reading frame (PMAA_014600) was used to identify the consensus sequence for control elements. The deduced amino acid of Hsp30, TmHsp30 (Genbank ABF82266.1), was used to predict the subcellular localization using a DeepLoc-1.0 tool (https://services.healthtech.dtu.dk/service.php?DeepLoc-1.0, accessed on 28 Dec 2022) (15). The signal peptide was detected via a Signal P 5.0 program (https://services.healthtech.dtu.dk/service.php?SignalP-5.0, accessed on 28 Dec 2022) (16). The glycosylphosphatidylinositol anchoring sequence was detected using a big-PI predictor tool (https://mendel.imp.ac.at/gpi/cgi-bin/gpi_pred.cgi, accessed on 28 Dec 2022) (17). Finally, the epitopes of TmHsp30 were predicted with an ABCpred tool (https://webs.iiitd.edu.in/raghava/) (18).

Western blot analysis

The conidia of *T. marneffei* were inoculated into a Sabouraud dextrose broth to a final concentration of 10^8 conidia/mL and cultivated at either 25 or 37 °C with 200 rpm continuous shaking. Total protein was extracted from cells harvested at 0, 24, 48, 72, and 96 h after cultivation. Approximately 0.5 g wet-weight cells were mechanically lysed with 0.5-mm glass beads (Biospec, lnc., Bartlesville, OK, USA) in a Bead beater homogenizer (Biospec Inc., Bartlesville, OK, USA). The cell lysate was resuspended with in 500 µL of phosphate-buffered saline (PBS) containing the protease inhibitors 10 µM iodoacetic acid (IAA), 10 µM phenyl-methylsulfonyl fluoride (PMSF), and 5 mM ethylenediamine tetra-acetic acid (EDTA). After centrifugation, the supernatant containing protein was collected. The protein concentration was determined with a Bradford assay reagent kit (Bio-Rad Protein Assay dye reagent concentrate, Bio-Rad, Hercules, CA, USA). Fifty micrograms of the total proteins were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was visualized by silver staining (Boster's silver staining kit, Boster Biological Technology, Pleasanton, CA, USA). Western blotting was performed to transfer proteins to a 0.45-µm nitrocellulose membrane (Amersham Pharmacia Biotech, London, UK). The membrane was then blocked in a PBS containing 5% (w/v) skim milk (Bio Basic, Amherst, NY, USA) either overnight at 4 °C or for at least 2 h at room temperature before performing the immunoreaction procedures.

The Hsp30 protein was detected using a rabbit polyclonal anti-*T. marneffei* Hsp30 (TmHsp30) primary antibody. The polyclonal antibody was generated by a service from GenScript, Inc. (Catalog No. U650XFG020, GenScript, Piscataway, NJ, USA). The rabbit was immunized with a 14-amino acid (ENENPHAPKPASVE), a polypeptide part of TmHsp30. One µg/mL of the primary antibody was prepared and incubated to the membrane for 1 h at room temperature. After washing with a 0.05% PBS-tween20 washing buffer, the membrane was incubated with a horseradish peroxidase (HRP) conjugated anti-rabbit IgG (GE Healthcare, Amersham, UK) at 1:1,000 dilution for 1 h. Finally, the membrane was immersed in a tetramethylbenzidine (TMB) substrate solution for western blot blotting (BioFX® TMB Component HRP Membrane Substrate, Surmodics, Street Eden Prairie, Eden Prairie, MN, USA) for 5 minutes to generate the signal.

RNA extraction, real-time RT-PCR, and RT-PCR

Conidia of *T. marneffei* (ATCC18224) were prepared to 10^8 conidia/mL in ANM broth and cultured at either 25 or 37 °C for 24 h. The cells were harvested and mechanically broken in the Beat Beater (Biospec, Bartlesville, OK, USA). The total RNA was extracted using a Trizol® reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA). The RNA concentration was measured using a NanoDrop (NanoDrop 2000: Thermo Scientific, Waltham, MA, USA). One microgram of the total RNA was converted to a cDNA using ReverTra Ace® qPCR RT Master Mix (TOYOBO, Osaka, Japan).

Real-time RT-PCR was performed with an SYBR Green qPCR mix (Thunderbird SYBR Green Chemistry, TOYOBO, Osaka, Japan), and the signal

was read using a 7500 Real-Time PCR System (Applied Biosystem, Foster City, CA, USA). Forward primer, P23-RT-F (5'-CGAGTCCAA GGATGCTTACC-3') and reverse primer, P23-RT-R (5'-GGCTTAGGAGCATGAGGGTT-3') were used to detect part of the *hsp30* transcript. An actin gene was used as the internal control gene and for normalization. The Act1F (5' TGAT-GAGGCACAGTCTAAGC-3') and Act1R (5'-CT-TCTCTCTGTTGGACTTGG-3') primers were used in the reaction. The real-time PCR condition was 95 °C for 60 s (1 cycle), and then 95 °C for 60 s (40 cycles). Relative expression was calculated using the formula 2-(ΔCt) (where ΔCt = Ct actin - Ct target).

RT-PCR was used to examine the *hsp30* mRNA in the total RNA extracted from the cells in the stress exposure experiments. Primers were designed to bind an entire open reading frame that amplified a 510-bp product (*hsp30*-F; 5'-GTCTCCTCGACGACTATG-3' and *hsp30*-R; 5'-TGTGATCTTGCGACTAGT-3'). A ribosomal RNA (rRNA) gene was used as an internal control gene. Primers used in the amplification were RRF1 (5'-ATCTAAATCCCTTAACGAGGAA-CA-3') and RRH1 (5'-CCGTCAATTTCTTTAA-GTTTCAGCCTT-3'). Reverse transcription was performed as described above, and 2 µL of the cDNA was used in the PCR reaction. Fifty µL of the reaction mixture contained 3 mM MgCl2, 200 µM each dNTP, 0.5 mM each primer, and 1 U Taq DNA polymerase (Qiagen). The PCR parameter was 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min.

Statistical analysis

Student's t-test was used in the analysis of the *hsp30* expression with statistical significance set at $p \leq 0.05$ for comparison of the level of co-nidial expression. All statistical analysis was performed using Prism software (GraphPad, version 7.0).

RESULTS

Analysis of control elements located near the translation start sites and prediction of TmHsp30 cellular localization

To predict the conditions that induce the expression of *hsp30*, the promoter-proximal elements that help regulate the gene expression were determined at 977 bp upstream of the ATG start site (+1). The stress-responsive element (STRE) CCCCT was found at -955. Additional control elements, the heat shock element (HSE) TGAATTCCGAAT, and the xenobiotic-responsive element (XRE) CACGCT were found at -949 and -720, respectively (Figure 1). The presence of these control elements at the 5'-UTR of the *hsp30* gene indicated that the *hsp30* expression

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                          STRE          HSE
-970  CGTATCGTCT GGACGCCCCT ATGAATTCCG AATACGGTCG TGCTCGGCGT TAGAGGACGC ATAAAGCGAA -901
-900  ACCACACCAT TACATAGTAA AACACTACAA CATATGACAC TTGGTGATCG TGGAAATGTA ACGAATCTGC -831
-830  AAAAGACGGT TGCTGGTGGA AGATCCGCTC GACTTCAGTC GATTCCCGGT CAAGTCAAGC ACTGTCGTTA -761
                                                 XRE
-760  TCGTACGGAG CATGTCAGGA TTGAATAGAA TGACGTTGTG CACGCTGTAG ACTGGAATTT TCTACAATGC -691
-690  CCATTGGTTG TTGATCGAAT GATATGGGCA CATTGAGCGA AACAACCGTA TGATTTGTCT ACATTCAGCT -621
-620  GGAAATTCTA GTCTACAAGG TGCGTGTTGA CCAATACAGAA CATAAATGAC GTCAGCATT GATCGATCAT -551
-550  TGGGGGATGA ATGGTACTCT CAACTAAGTT ACACAGTACT CAGATGTTGG TTTCACTCGA TAATCTCCAA -481
-480  GCCGATAGTG ACTGAAAGCG CGGATATTTT CTTTTCTTTT GCTTTTCTTT CTTTTCTTTA TGTAACTGCG -411
-410  ACTTAATTGA GTAGAAGATG TGTCTCGACT TAAGCCGCAC TGATTTCCTC TTTCGTCTGG TTTTGCAGGT -341
-340  TGGATTAGCG GGCCCCACTC GGCCGCCACC CGAGGATTCT GGAACGCTCC ACTAATGTCT AGCTAACCAT -271
-270  CAAATCGACT GGAAGCATCG TCGCGAATCT CTCGAGTATT CTGCAATGAC CGCGACATTT CATCATCACC -201
-200  ACCATGGCAC GTTCCAGAAT ACCAGGTAAT GGGTATATAT ATCCCCTCTG GTCTCTGGTC GAATTACTTT -131
-130  CTCTTCATCA GAAATCACAC TCAAAGCATC ATCCAGCTGA CTACCATTGG AACCCAACTA CCCAACAACT  -61
-60   TAAAACATCA TCACTTCTTC ACCCTTCTTA AATCCTTTAC TACCAAATCA ACCAATTGAA ATG
                                                                        +1
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Figure 1. Summary of the transcription-control sequences for the *hsp30* gene in *T. marneffei*. The TATA promoter (bolded, and underlined) at which transcription initiates locates at -167 base pairs upstream from the ATG start site (+1). Green boxes show the STress Responsive Elements (STRE) locate at -955 upstream of the ATG start site. Heat Shock Element (HSE) (grey box) and Xenobiotic Responsive Element (XRE) (yellow box) at -949 and -720, respectively

can be induced by heat, abiotic and xenobiotic stresses in *T. marneffei*.

The deduced amino acid sequence of TmHsp30 (ABF82266.1) was analyzed to find the cellular localization and functional implications. Eukaryotic protein subcellular localization prediction using a DeepLoc 1.0 suggested that the TmHsp30 contained a high solubility score (0.76), an indication that it is a soluble protein. Additionally, a nuclear localization signal (NLS) was found, meaning that it can be transferred into the nucleus (Figure 2). This prediction suggests that TmHsp30 can be translocated between the cytoplasm and the nucleus to perform its functions; this assumption needs to be further investigated. However, the training set of the DeepLoc analysis tool does not include the membrane-associated proteins normally found in the fungal cell wall- proteins; therefore, Signal P and big-PI programs were also included in the analysis. As a result, signal peptide and potential GPI-anchoring signals were detected in the TmHsp30 sequence (Figure 3). This result implies that the TmHsp30 can also be delivered to the cell surface and incorporated as part of the cell membrane via GPI-linkage. It can be excreted as an extracellular protein as well. This prediction is supported by a recent study that reported TmHsp30 was present in an extracellular vesicle (10). Taken together, these results suggest that the TmHsp30 can have diverse functions because of its wide cellular distribution. The exact role of this protein should be further investigated to better understand its biological role in the yeast pathogenic phase of *T. marneffei* and to provide additional insight into its specific roles in the heat shock protein family and in different fungi.

Expression of *hsp30* transcript and Hsp30 protein

A northern blot analysis showed that the *hsp30* transcript was accumulated inside the conidia and yeast cells of *T. marneffei* (9). Another study

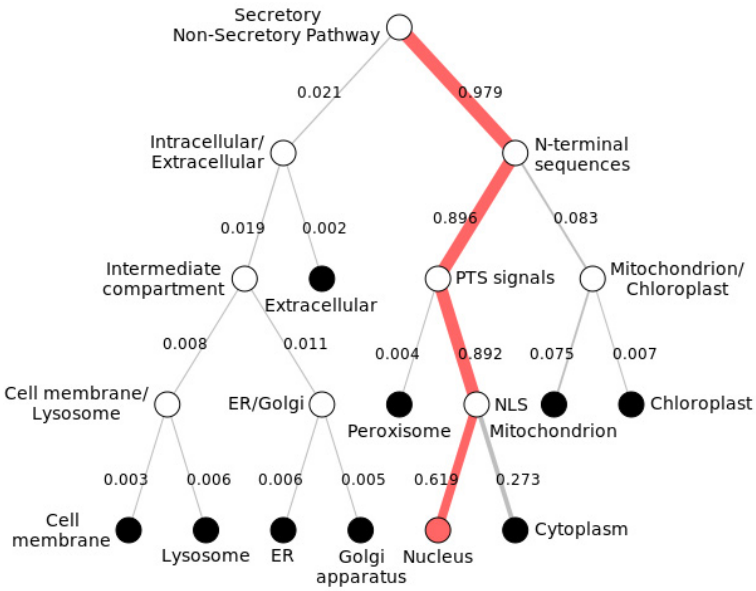

Figure 2. Subcellular localization prediction using a DeepLoc-1.0 suggested the Hsp30 of *T. marneffei* can localize in the cytosol and nucleus

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1   MSLFHRSGDF APLFRLLDDY DLHRSGRDGQ TPASSSISSF APRFDVRESK DAYHLDGEL PGIAQKDVEI   70

71  EFSDPQTLTI KGRSVREYHT LPENENPHAP KPASVEDAPE SSDETAVQKS SDKKEVSKA QGNGYKYWVS  140

141 ERSVGEFHRS FNFPSRVDHN GVKASLKNGV LTVTVPKAAP PTSRKITIE                        187
```

Figure 3. Deduced amino acid sequence of Hsp30 of *T. marneffei* (TmHsp30). The green box indicates signal peptide, a signal in the classical protein secretion pathway. The yellow highlight shows a potential GPI modification site. The selected epitope region (14 amino acids) used in polyclonal antibody production is shown in red alphabets.

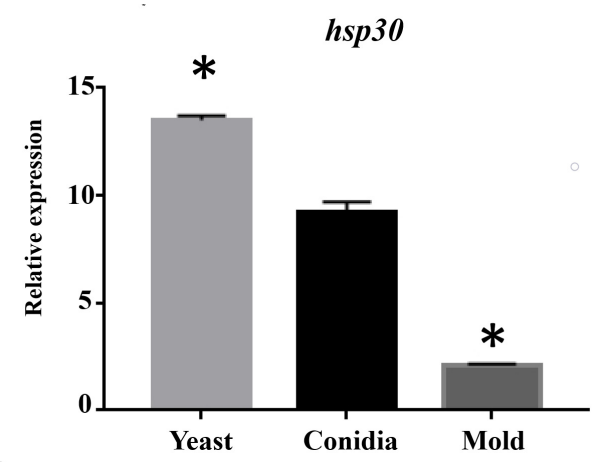

Figure 4. Expression of *hsp30* in the mold and yeast phases of *T. marneffei*. Total RNA was isolated from the 1-day-old mycelia and yeast cells of *T. marneffei* and subjected to a real-time RT-PCR to determine the *hsp30* transcript levels. Each bar indicates the relative expression of the *hsp30* transcript normalized in comparison to the actin gene. The experiments were performed with triplicates. Asterisks show a significant level of $p \leq 0.05$ when compared to the conidial level

found that the level of *hsp30* mRNA was three times higher in the yeast phase than in the mold phase as verified by RNA-Seq analysis (10). The present study aimed to examine how the *hsp30* transcript and protein are expressed in each phase of *T. marneffei*.

Quantitative RT-PCR was used to detect the *hsp30* transcript level inside conidia, mycelia, and yeast cells. As shown in Figure 4, the *hsp30* transcript is accumulated in the conidia. During the 24 h after transition from the conidial to the mold phase, the transcript level was significantly decreased. In contrast, the expression level in 1-day-old yeasts was highly elevated. This result indicates that *hsp30* played a role during the conidial to yeast phase transition.

Expression of the TmHsp30 protein was observed in this study. A rabbit polyclonal antibody (pAb) to TmHsp30 was produced. Using the ABCpred tool, several epitopes of TmHsp30 were predicted (18). An epitope sequence of 14 amino acids (Figure 3) was selected for peptide synthesis and rabbit immunization (GenScript service). The anti-TmHsp30 pAb was used as a primary antibody in the Western immunoblot assay. A positive band at 21-kDa of the expected size was detected in the conidia and yeast cells (Figure 5). However, positive signals at other protein sizes were also found in the yeast phase. The presence of multiple signals could be the result of the binding of TmHsp30 to the misfolded proteins, brought on by heat stress at 37 °C. Our sample buffer did not contain the denaturing agent 2-mercaptoethanol and the treatment of samples was performed at 37 °C.

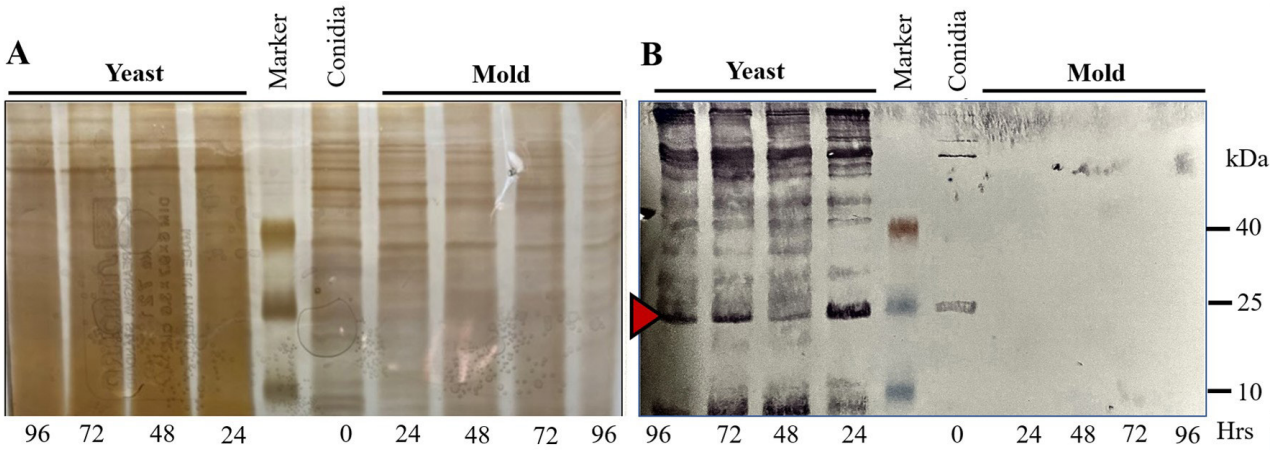

Figure 5. Expression of Hsp30 protein during conidial germination to mold and yeast phases. *T. marneffei* ATCC18224 conidia were cultured in ANM broth (10^8 conidia/ml) and subjected to growth at either 25 °C or 37 °C to generate the mold and the yeast form. The fungal cells were collected at 0, 24, 48, 72, and 96 h after incubation, and total proteins were isolated. Fifty micrograms of each protein sample were used in SDS-PAGE. (A) Silver staining shows the protein profile (B) Western immunoblot assay using polyclonal rabbit anti-TmHsp30 antibody shows the 21-kDa band of the expected size of TmHsp30 (red triangle) in the conidia and yeast cells at every time point. The result is representative of triplicate experiments. M, Marker (Spectra Multicolor Low range protein ladder, Thermo Scientific Baltics UAB, Vilnius, Lithuania).

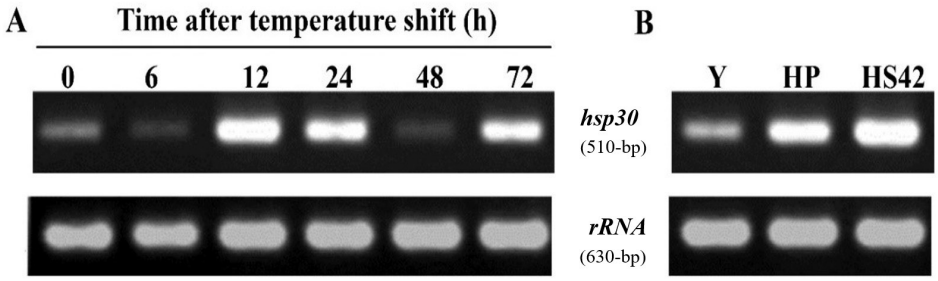

Figure 6. Expression of *hsp30* transcript during mycelial to yeast transition and stress-induced condition. (A) Three-day-old mold phase culture of *T. marneffei* FRR2161 was changed to incubate at 37 °C with continuous shaking. The fungal cells were collected at time points 0, 6, 12, 24, 48, and 72 h after the temperature shift. (B) Three-day-old yeast phase culture (Y) was subjected to treatment with 1 mM hydrogen peroxide (HP) and heat shock at 42 °C (HS42). Total RNA was isolated, and an RT-PCR experiment was performed using primers specific to *hsp30* to verify the expression level of the *hsp30* transcript (510-bp amplicon). The internal control rRNA gene was amplified from each sample to serve as an internal control (630-bp amplicon). The result is representative of triplicate experiments

Expression of *hsp30* transcript during heat and oxidative stress-inducing conditions

To examine how the *hsp30* expression changed in response to heat stress, three-day-old mycelia were exposed to a temperature increase to 37 °C and were harvested at 6, 12, 24, 48, and 72 h after incubation. RT-PCR results found that the mRNA level had increased at 12 h after the temperature shift, then continually decreased from 24-48 h, and re-elevated at 72 h (Figure 6A). This result implies that the *hsp30* directly responded to the heat stresses in *T. marneffei*. Additionally, when the three-day-old yeast cells were treated with 1 mM hydrogen peroxide (HP) and severe heat shock at 42 °C (HS42) for 60 minutes, the *hsp30* expression was prominently elevated (Figure 6B). This indicates that *hsp30* could also play a role in the oxidative response. These results support the bioinformatics analysis of the expression control of *hsp30* in this fungus.

DISCUSSION

The promiscuous interactions and pleiotropic functions of small heat shock proteins (sHsps) and the underlying molecular mechanism are not completely understood. In most organisms, sHsps play a role as one of the chaperones which prevent nascent protein folding. They also help protect other proteins against heat-induced denaturation and aggregation, especially in stress-inducing conditions, presumably via an alpha-crystallin conserved domain (ACD) (19, 20). Protein similarity searches of *Talaromyces marneffei* heat shock protein 30 (TmHsp30) found significant matches with the homolog proteins among Ascomycetous filamentous fungi in the genera *Talaromyces*, *Aspergillus*, *Paecilomyces*, *Histoplasma* and *Blastomyces*. Unlike in yeasts and humans, the role of Hsp30 in dimorphic fungi has not yet been investigated. Since the TmHsp30 also contains ACD, it is probable that it has a chaperone function. In contrast to its previously identified functions, Hsp30 plays a unique role in hemoglobin binding in *Paracoccidioides* species (14), indicating that the upregulated expression of *hsp30* in *T. marneffei*'s yeast pathogenic phase may have some significance for pathogenesis. It has previously been demonstrated that the plant's ACD domain also regulates transcription and responds to sodium and lithium salt stress (21,22). It remains to be determined whether these roles are also present in *T. marneffei*.

Analysis of the control elements at the upstream region of the *hsp30* gene in *T. marneffei* found several that respond to heat and stress conditions. One, HRE, a heat-responsive element accompanies the binding of heat-shock transcriptional factor. This element is usually found at the upstream region of most Hsp-encoding genes (23). Another, STRE, a stress-responsive element, normally plays a role in responding to several stresses such as osmotic and oxidative stresses. Finding these elements in *T. marneffei* supports the assumption that TmHsp30

can play a role in response to heat and other stresses. Bioinformatic analysis for prediction of cellular localization indicated that TmHsp30 may be located inside the nucleus, cytoplasm, and cell membrane. Determination of the actual exact subcellular location will require further experimentation, e.g., the generation of chimeric protein with fluorescent reporter. TmHsp30 is also predicted to be able to be transported to the cell surface and to link to the cell membrane. Taken together, the data indicate that TmHsp30 is a multi-localized protein which may have multiple additional functions that have yet to be identified.

Heat shock protein 30 seems to posses a yeast-phase specific role in *T. marneffei*. Normally the mold life cycles do not include confronting heat shock stress conditions, so, sHsps are rarely expressed (24,25). The expression of sHsps is observed only in the presence of stressors. The expression pattern of *T. marneffei* during the yeast growth phase, one of its physiological features, is considered unique. Since the ability to prevent the aggregation of proteins is the most important function of many sHsps, especially under conditions of stress that can lead to the unfolding of cellular proteins, a high level of expression of TmHsp30 in the yeast phase likely occurs to help the proteins from becoming heat-denatured. This ability may be the source of the virulence of *T. marneffei* inside the host. However, we noticed that the expression of *hsp30* transcript and TmHsp30 in the yeast cells were upregulated during conidial to yeast phase transition process. It elevated at the first 6-12 h of germination , temporarily reduced after 24-48 h, and re-elevated at 72 h after germination (Figure 5, 6). Interestingly, this pattern of shifting transient reduction and re-elevation has also been observed in another stress-responsive gene, *cpeA*. That gene encodes a catalase-peroxidase, an antioxidative bifunctional enzyme, in *T. marneffei* (27,28). This pattern of expression could be explained by fact that the yeast growth phase has a unique metabolism which responds to environmental change cultivation. At first, the fungus produces high levels of anti-stress responsive proteins to provide resistance ability. After the fungus has adapted well to the potentially harmful conditions, protein production is temporarily halted. Finally, the division cycles of yeast cells can help maintain the basal level of expression that best suits the cellular metabolism. The exact explanation, however, will require further investigation. Additionally, we observed an accumulation of TmHsp30 in *T. marneffei* conidia. The preparation of heat shock protein genes and transcripts inside the conidia was also observed in *Aspergillus* species. Mycologists believe that the fungi accumulate some important proteins in preparation for germination and in response to the surrounding environment during the conidiation process (30). Our data suggested that *T. marneffei* must prepare its conidia to be ready to respond to any stress conditions encountered during germination.

The stress conditions investigated in this study imitated severe heat shock at 42 °C (high fever) and oxidative stress inside the phagosome of the macrophages. Hsp30 is highly activated upon exposure to danger, suggesting the role of Hsp30 in responding to stress conditions. TmHsp30 could play a vital role in coping with the stress conditions endured by the fungus during infection as well. Thus, generation of the *hsp30* null mutant and testing it in a macrophage infection model could help researchers understand the role of Hsp30 in the yeast growth phase and its contribution to the pathogenic process.

CONCLUSION

Expression of *hsp30* was demonstrated at both the transcript and protein levels in a dimorphic opportunistic fungus, *Talaromyces marneffei*, in this study. Both were highly expressed in the conidia and the yeast phase and upregulated in response to heat and oxidative stresses. The mycelia maintained a low basal level of the transcript, but no detectable amount of protein was observed, suggesting post-transcriptional control of *hsp30* under non-stress conditions. Our data suggest that Hsp30 plays a role in supporting cell survival while coping with stress and may possibly be involved in the pathogenesis of *T. marneffei*.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ADDITIONAL INFORMATION

Author contributions

Conceptualization, M.P.; methodology, N.J., A.K., M.P.; validation, M.P.; writing—original draft preparation, N.J. and M.P.; writing—review and editing, N.J. and M.P.; visualization, M.P.; supervision, M.P. All authors have read and agreed to the published version of the manuscript.

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