Original article

Stress adaptation in *Talaromyces marneffei*

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Talaromyces marneffei (*T. marneffei*) (previously named *Penicillium marneffei*) is a thermal dimorphic fungus that causes disseminated infection, which is common in AIDS patients, especially in northern Thailand. This fungus has the ability to survive in a host macrophage, which is the virulence factor attributed to promoting pathogenicity. At present, understanding of *T. marneffei* stress response *in vivo* is limited. Therefore, this study analyzed genes that are responsible for stress. They are highly expressed during the pathogenic yeast phase and encoded antigenic proteins of *T. marneffei*. Expression patterns of genes encoded for heat shock protein 30, glutathione peroxidase, cytochrome c oxidase and NADH-ubiquinone oxidoreductase were different in the mold, conidial and yeast phase. Interestingly, genes responsible for heat shock and nutrient starvation had lowest activity in the mold phase instead of the conidial phase. This result suggested that, even in the dormant stage, the conidia of this fungus prepared the genes responsible for several stresses, and they may play a role in the germination and survival process during infection. This study hypothesized that this pathogen has the ability to resist complex and dynamic host niches by occupying alternative carbon sources and having simultaneous response to heat and oxidative stresses inside the host. **Chiang Mai Medical Journal 2016;55(Suppl 1):23-30.**

keywords: *Talaromyces marneffei*, pathogenicity, heat shock, oxidative stress, starvation, adaptation

Introduction

Talaromyces marneffei (*T. marneffei*) can survive in the macrophage of individuals with severely compromised immune systems such as neutropenia or AIDS patients, which leads to disseminated infection[1,2]. *T. marneffei* is currently the third most common cause of opportunistic fungal infection in AIDS patients in Thailand[3,4]. The high incidence of infection in northern Thailand reflects the challenge of early diagnosis, since delay in treatment results in a high mortality rate. It is clear that *T. marneffei* can adapt itself effectively to macro-phage host niches by transition from the conidia to yeast phase inside the phagosomes[2,5]. Several studies have demonstrated that stress responsible genes and proteins are up-regulated in the pathogenic yeast phase[6-15]. However, significant progress has to be made in elaborating on stress-adaptive responses and their regulation in *T. marneffei*.

Previously, the authors constructed a cDNA library from the pathogenic yeast phase of *T. marneffei*[13]. Clones containing genes that encode antigenic proteins were isolated from antibody screening of the cDNA library and partially characterized for their putative functions.

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Seventeen genes were isolated and sequenced. Seven genes are not homologous to any known ones and were therefore excluded from this analysis. Ten genes had known functions, including catalase-peroxidase, heat shock protein 30, fructose-1,6-bisphosphatase, 60S ribosomal protein, cytochrome C oxidase, NADH-ubiquinone oxidoreductase, mannoprotein like protein 6, glutathione peroxidase, thymine synthase and stearic acid desaturase. This study observed the relevance of these ten genes to stress adaptation, and determined their expression levels in this fungus. It also proposed a mechanism on the main theme of stress adaptation in the context of host niches during *T. marneffei* infection.

Materials and methods

Fungal strain and growth condition

Talaromyces marneffei F4 strain was obtained in January 1999 from the hemoculture of an AIDS-patient at the Central Laboratory, Maharaj Nakorn Chiang Mai Hospital. The fungus was maintained in mold form on an SDA slant at 28 °C. The 5-day-old conidia were harvested, and 10^{10} conidia were used to inoculate 500 ml of Sabouraud'd dextrose broth (SDB) or brain-heart infusion broth (BHIB) in a 2-liter Erlenmeyer flask (2x 10^7 conidia/mL). They were cultured at either 25 °C or 37 °C in an orbital shaker bath and shaken moderately at a speed of 140 rpm for 3 days.

Total RNA isolation

Total RNA was isolated using the Chomczynski and Sacchi method with TRIzol® reagent (Gibco BRL, Gaithersburg, MD, USA). Conidia, yeasts or mold cells were pelleted and washed once in sterile PBS. The fungal pellet (about 5-g in wet weight) was flash-frozen in liquid nitrogen and ground to powder using a pestle and mortar. The cell powder was homogenized in TRIzol® reagent (1 mL of TRIzol® reagent per 100 mg of pellet). The lysate was transferred to a polypropylene tube and 0.2 volume of chloroform was added sequentially. The mixture was shaken thoroughly for 30 sec and the tube centrifuged at 10,000xg for 20 min at 4 °C. The upper aqueous phase, containing total RNA, was collected. The RNA was precipitated with an equal volume of ice-cold isopropanol, kept at room temperature for 5 min, and centrifuged at 12,000 x g for 10 min at 4 °C. The RNA pellet was washed with 1 mL of ice-cold RNase-free 70% ethanol and dried. Finally, the pellet was resuspended in 600 µL of DEPC-treated water. The RNA concentration and purity was determined by measuring absorption at 260 and 280 nm, respectively (Spectronics Genesys2, Spectronics, London, UK). The suspension was then stored at -80 °C until used.

Construction and antibody screening of the cDNA library

The SuperScript®cDNA synthesis system (Gibco BRL) was used in the *T. marneffei* cDNA library construction. The procedure was carried out following to the protocol of Gibco BRL. The experimental details were described previously[13].

The constructed cDNA library was screened with purified IgG from a pooled sera (n = 5) derived from *T. marneffei*-infected patients. The positive phage clones were then isolated and purified by repeating the antibody screening process until the homogeneous positive signal was generated.

DNA sequencing and sequence analysis

Phage-to-plasmid conversion of positive clones was performed by *in vivo* excision using the *E. coli* DH10B strain (Gibco BRL), based on the *cre-loxP* recombination process, which generated pZL1 plasmids containing the cDNA inserts of interest. Subsequently, the plasmids were isolated by using a plasmid mini kit (Qiagen GmbH, Germany). DNA sequencing of the cDNA was performed with the dideoxynucleotide chain termination method (Sanger, Nicklen& Coulson, 1977) using the CEQ Dye terminator cycle sequencing (DTCS) quick start kit or BigDye Terminator sequencing kit (Beckman Coulter, Fullerton, CA, USA). The sequenced products were analyzed on a CEQ2000XL automated sequencer (Beckman Coulter, Fullerton, CA, USA) or Genetic analyzer Model 310 (Beckman). The DNA sequences obtained were analyzed by using the web-based analysis programs on NCBI (www.ncbi.nlm.nih.gov) in order to find similarity to DNA or protein in the database. Multiple sequence alignment and protein analyses were performed by using ClustalW and other related programs (http://www. ebi.ac/uk/clustalw/index.html).

Real-time polymerase chain reaction (PCR)

The RNA was converted into cDNA by using the ReverTra Ace®qPCR RT kit (Toyobo, Osaka, Japan). The reaction contained 100 ng of RNA, 0.5 µM Oligo dT, and 2 mM dNTP mix. The cDNA synthesis was performed at 37 °C for 1 h. Real-time PCR was performed by using a Thunderbird™ SYBR®qPCR mix (Toyobo Inc.) in the ABI7500 real-time PCR system (Applied Biosystems, USA). Relative expression was calculated

from each gene and normalized with an actin transcript from each condition. The transcript was measured in duplicate for each experiment. The average expression levels from two independent experiments were demonstrated in this study.

Results

Complementary DNA (cDNA) library construction and isolation of antigenic protein-encoding genes

The λZipLox-based cDNA library was constructed from about 10 μg of poly(A)+ RNA isolated from the yeast cells of *T. marneffei*. The primary cDNA library titer was $2.5x10^5$ pfu/mL and cloning efficiency 98%. Subsequent library amplification raised the titer to $5x10^9$ pfu/mL, with the same percentage of recombination. Five *T. marneffei*-infected sera with different patterns of immunoreactivity were pooled and used in the cDNA library screening assay. Up to 100,000 pfu of the cDNA library were screened with the pooled sera. Seventeen different positive clones were isolated, sequenced, and analyzed for their putative functions. Seven genes were not homologous to any known ones, and therefore excluded from analysis. Ten genes had known functions, including catalase-peroxidase, heat shock protein 30, fructose-1,6-bisphosphatase, 60S ribosomal protein, cytochrome C oxidase, NADH-ubiquinone oxidoreductase, mannoprotein like protein 6, glutathione peroxidase, thymine synthase and stearic acid desaturase. This study summarized key stress signaling pathways and highlighted their relevance to infection, as described below.

1. Heat shock

Heat shock response involves the induction of heat shock proteins (HSPs), which promote damaged protein folding[16]. Previously, antigenic heat shock protein 30 (*Hsp30*) gene was shown to be expressed abundantly in conidia, yeasts, and cells during conidial to yeast phase transition by Northern Blot analysis[15]. In contrast, the expression level was very low in the mold phase. The quantitative real-time PCR result (Figure 1) corresponded to the Northern Blot result, in that the transcript accumulated highly in the conidia and yeast cells, while being almost absent in the mycelium. Accumulation of the *hsp30* transcript in the conidia and yeasts suggested an important function of *hsp30* in the germination stage and anti-heat condition during yeast survival.

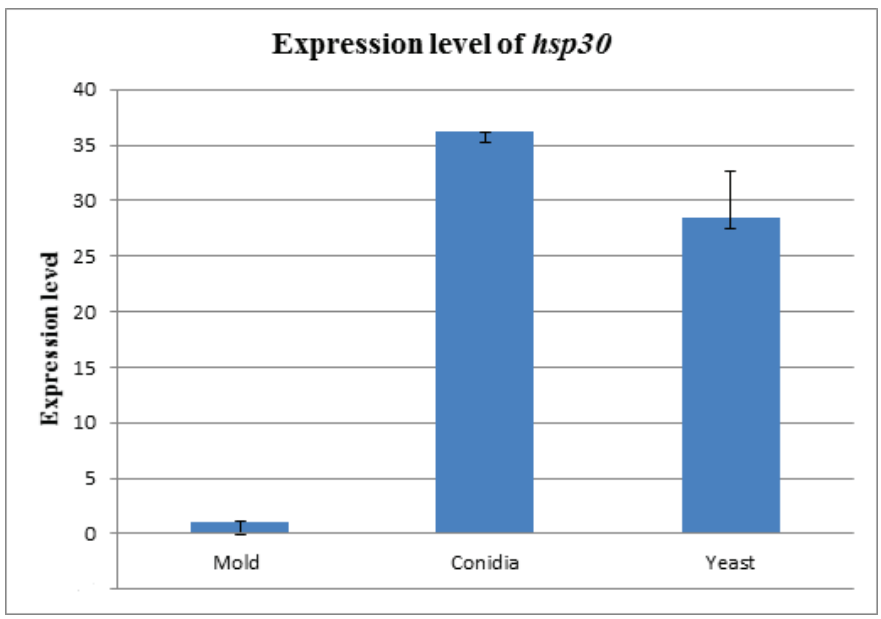

Figure 1. Expression level of heat shock protein 30 (*hsp30*) transcript in *Talaromyces marneffei*. Quantitative RT-PCR was performed using 100 ng of RNA, which was isolated from each phase of *T. marneffei* (as indicated). The expression level shown in the figure is the average level from 2 independent experiments

Thus, deletion of this gene and phenotypic characterization should be performed further in order to prove this hypothesis.

Expression pattern of *hsp30* demonstrated a yeast phase specific characteristic. This is contrast to *hsp70* that is constiitutively expressed in all phase of *T. marneffei*[7]. However, both *hsp30* and *hsp70* are up-regulated during heat shock response. Additionally, the small heat shock protein in *T. marneffei* was demonstrated for its antigenic property for the first time[15]. The expression level was high in the pathogenic phase implying that *hsp30* involvement in pathogenicity needed further verification. Although *hsp30* is conserved and ubiquitous, previous studies on its function were performed in nonpathogenic fungi, and there is no evidence of its contribution in the pathogenicity of fungi. Therefore, the function of *hsp30* in *T. marneffei* needs further study in order to obtain a better understanding of the heat shock response mechanism and host-fungus relationship.

2. Oxidative stress

Talaromyces marneffei is rather sensitive to reactive oxygen species (ROS), with only 3 mM of hydrogen peroxide treatment able to kill yeast cells *in vitro* (compared to 20 mM in *Candida albicans*)[17]. Presumably, the ROS concentration in the macrophage of advanced AIDS patients is too low to kill this fungus, or there are other factors that help this fungus to tolerate a lethal concentration. In support of the latter case, this study found genes encoding ROS detoxifying proteins, i.e. superoxide dismutase (SOD)[14] and catalase-peroxidase (Cpe)[13]. The gene encoding glutathione peroxidase (GPx) also was shown to be up-regulated in this analysis (Figure 2). This gene was almost absent in the mold and conidial phase. BlastX showed high similarity to the GPx from *Shizosaccharomyces pombe* (64%) and *Saccharomyces cerevisiae* (60%), but no similarity to human GPx was found. This antioxidant protein possibly protects the fungus against oxidative stress by reacting directly with OH^-. Together, these proteins could detoxify and mediate cellular adaptation to oxidative stress. Subsequently, inactivation of *cpeA* diminished the expression of catalase-peroxidase, rendering *T. marneffei* sensitive to hydrogen peroxide[12]. Depletion of the genes encoding glutathione peroxidase and superoxide dismutase also could attenuate the oxidative resistance of this fungus.

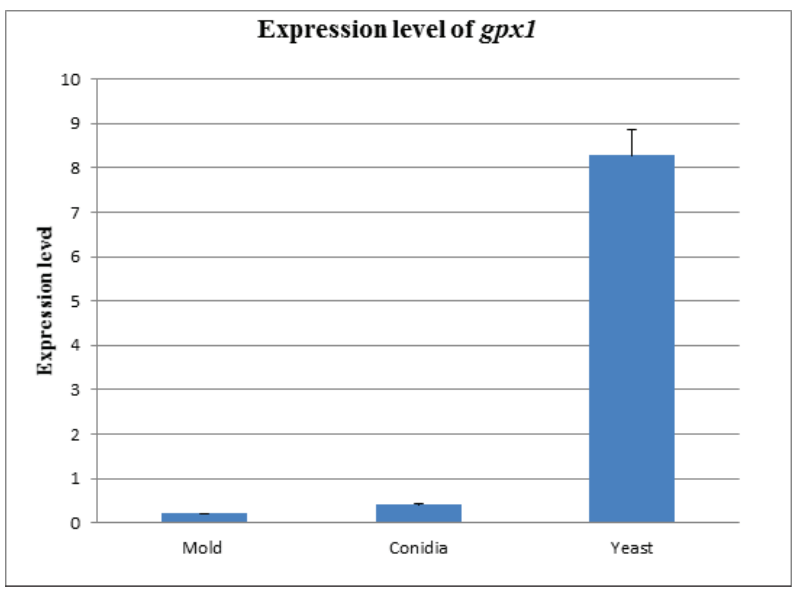

Figure 2. Expression level of glutathione peroxidase (*gpx1*) transcript in *Talaromyces marneffei*. Quantitative RT-PCR was performed using 100 ng of RNA, which was isolated from each phase of *T. marneffei* (as indicated). The expression level shown in the figure is the average level from 2 independent experiments.

3. Starvation stress

Many host niches either lack sugars, such as glucose, or contain glucose at low concentrations. Instead, these niches contain complex mixtures of alternative carbon sources, for example, amino acids, and carboxylic acids such as lactate and fatty acids. After *T. marneffei* had been phagocytosed by the host macrophages, it adapted itself to starvation stress by activating the pathway for alternative carbon utilization. The glyoxylate cycle shunt pathway was shown to induced in the yeast phase of *T. marneffei*[18], and concomitant reduction of glyceralde-3-phosphate dehydrogenase (*gpdA*), encoding an enzyme involving glycolysis, was down-regulated during macrophage infection[19]. The analysis from this study found another glycolytic enzyme, i.e. fructose-1,6-bisphosphatase (FBP), among antigenic protein-encoding genes. This enzyme shares similarity to the *fbpA* of several fungi, including *Saccharomyces cerevisiae* (60%) and *Kluyveromyces lactis* (60%), and plants such as *Brassica napus* (56%) and *Orysa sativa* (50%). There was no similarity to human *fbp* found. The quantitative real-time PCR result showed induction of this gene in the yeast phase of *T. marneffei* (Figure 3).

The attempt to retrieve *P. marnefei* in nutrient for use as an energy source is visualized. This study found that the yeast phase increased the expression levels of genes en-coding 2 enzymes, cytochrome c oxidase and NADH-ubiquinone oxidoreductase, in ATP production (Figure 3). Similar to *hsp30*, cytochrome c oxidase transcript accumulated highly in the conidia of *T. marneffei*, suggesting that it could function by adapting to the early stage of infection. The amount of NADH-ubiquinone oxidoreductase transcript showed much lower presence in the conidial and up-regulated yeast phase, suggesting that it uses energy production after phase transition. Interestingly, the activity of both enzymes is rather low in the mold phase.

Discussion

This study reported cloning, isolation and analysis of some antigenic protein-encoding genes from the yeast phase of *T. marneffei*. Study of the antigenic factors can be important in large areas such as the purpose of diagnosis.

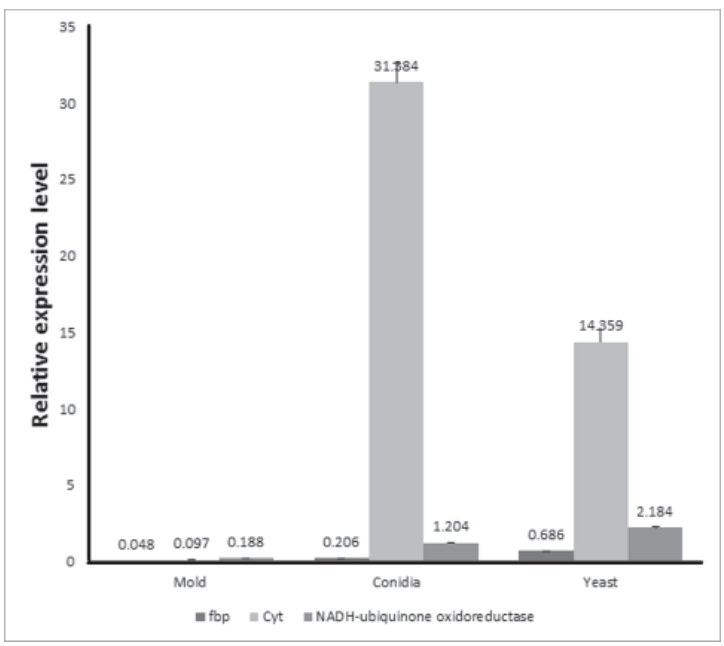

Figure 3. Expression levels of fructose-1,6-bisphosphastase (fbp), cytochrome c oxidase (Cyt) and NADH-ubiquinone oxidoreductase transcripts in *Talaromyces marneffei*. Quantitative RT-PCR was performed using 100 ng of RNA, which was isolated from each phase of *T. marneffei* (as indicated). The expression level shown in the figure is the average level from 2 independent experiments.

Another is the discovery of molecules that may have biologically important functions such as virulence factors. Discoveries of these molecules enable the possibility of using these factors as targets for the design of drugs, vaccines and/or specific treatment against fungal infections. The design of tests is based on ELISA and PCR, which detect specific compo-nents of *T. marneffei* or its genes, and have provided promising results in detecting infection.

Besides the benefits of obtaining antigenic protein-encoding genes, this study found the relationship of these genes in stress response. Most of the genes with a known function are associated with heat and oxidative and nutrient deprived stresses. In linking the intracellular nature of this fungus during infection, with its phase transition-related pathogenesis, these proteins should be essential for pathogenicity. Therefore, this study expanded its analysis by looking at expression levels. By focusing on the pathogenic yeast phase, all stress responsive genes were expressed at a higher level, when compared to the saprophytic mycelia phase. Transcript profiling experiments have demonstrated that genes with oxidative stress response are up-regulated in either conidia or yeast form.

Little is known about the exact interrelationship of host-*T. marneffei* during an infection. Although this study does not provide direct evidence, the clones were isolated by using the antibody produced from naturally infected patients, and they could provide the relationship in which proteins express during real infection. After the passage of conidia through the lung of infected patients, the fungus must encounter a combination of stresses. Heat stress is possibly the first signal of phase transition induced in this fungus. Usually, heat shock proteins are the main arm for coping with heat when cells encounter high temperature[20]. Finding of HSPs in this study suggested that they could be involved in pathogenesis. The authors of this study are particularly interested in *hsp30*, since the function of this protein is unknown in dimorphic fungus. The aim is to answer the questions how a small heat shock protein is important in thermal dimorphic fungus and why it expresses in the conidial and yeast phase. Exact function and virulence studies need to be carried out further in *T. marneffei* in order to answer these questions. In addition to heat stress, the molecular armory of macrophage cells includes ROS that contributes to killing *T. marneffei* conidia[21]. Antioxidant systems are very effective in pathogenic fungi[22-30]. The *T. marneffei* genome contains several antioxidant systems, which might help in its survival inside the macrophage[31]. Also, antioxidative proteins were reported in *T. marneffei* previously[12,14]. This study reported glutathione peroxidase as an additional antioxidant. Additionally, nutritional stress elicited an adaptive response in *T. marneffei*. An alternative pathway for energy production has been shown in this study.

In summary, *T. marneffei* must encounter a combination of stresses inside the host cells. Gene encoding proteins, which are involved in heat, oxidative and starvation stresses, were recovered from the cDNA library produced from the pathogenic yeast phase, implying that they could be expressed during an infection. Real-time PCR analysis showed the up-regulation of these genes in either conidia (infectious propagules) or yeast (pathogenic form), thus revealing that they might play a role in adaptation and survival of this fungus during an infective stage. An *in vivo* virulence assay should be applied further in a pathogenesis study of this fungus.

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Declaration of interest: The authors report no conflicts of interest, and they alone are responsible for the content and writing of this paper.

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การปรับตัวเข้ากับสภาวะเครียดในเชื้อ *Talaromyces marneffei*

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Talaromyces marneffei (ชื่อเดิม *Penicillium marneffei*) จัดเป็นราสองรูปขึ้นกับอุณหภูมิซึ่งสามารถก่อโรคแบบแพร่กระจายทั่วร่างกาย พบการติดเชื้อได้มากในผู้ป่วยโรคเอดส์โดยเฉพาะอย่างยิ่งในแถบภาคเหนือของประเทศไทย ปัจจัยที่ช่วยให้เชื้อราชนิดนี้สามารถก่อโรคได้ในร่างกายของผู้ป่วย ได้แก่ความสามารถในการมีชีวิตรอดได้ในเซลล์แมคโครฝาจ ในปัจจุบันยังมีความเข้าใจเกี่ยวกับกลไกที่เชื้อใช้ในการต้านสภาวะเครียดในร่างกายผู้ป่วยอยู่อย่างจำกัด เราจึงได้ทำการวิเคราะห์ยีนที่รับผิดชอบต่อสภาวะเครียด ซึ่งมีการแสดงออกสูงในระหว่างที่เชื้ออยู่ในรูปแบบยิสต์ ซึ่งจัดเป็นรูปแบบที่ก่อโรคในเชื้อ *T. marneffei* และยีนเหล่านี้กำหนดการสร้างโปรตีนแอนติเจน ได้แก่ ยีนที่กำหนดการสร้าง heat shock protein 30, glutathione peroxidase, cytochrome c oxidase และ NADH-ubiquinone oxidoreductase พบว่ายีนเหล่านี้มีรูปแบบการแสดงออกแตกต่างกันเมื่อเชื้ออยู่ในรูปของราสาย โคนิเดีย และยีสต์ เป็นที่น่าสนใจว่า ยีนที่มีการสนองตอบต่อความร้อน และสภาวะขาดแคลนอาหารนั้น มีการแสดงออกต่ำมากที่สุดในเชื้อรูปแบบราสายแทนที่จะเป็นรูปแบบโคนิเดีย ผลนี้น่าจะบ่งบอกให้ทราบว่า แม้แต่ในรูปแบบที่เชื้ออยู่ในภาวะหยุดการเจริญชั่วคราวนั้น ก็ยังมีการเตรียมยีนที่จะช่วยในการตอบสนองต่อภาวะเครียดชนิดต่าง ๆ ไว้ ซึ่งยีนเหล่านี้น่าจะทำหน้าที่ในการงอกและกระบวนการอยู่รอดของเชื้อในระหว่างการติดเชื้อในร่างกาย เราตั้งสมมุติฐานว่า เชื้อก่อโรคชนิดนี้มีความสามารถในการทนต่อสภาวะที่เปลี่ยนแปลงและซับซ้อนโดยอาศัยการใช้พลังงานจากคาร์บอนทางเลือก ร่วมด้วยความสามารถในการปรับตัวต่อความร้อน และความเครียดจากออกซิเดทีฟในร่างกายผู้ป่วยได้ **เชียงใหม่เวชสาร 2559;55(ฉบับเสริม 1):23-30.**

คำสำคัญ: *Talaromyces marneffei* กลไกการก่อโรค ความร้อน ความเครียดจากสารออกซิแดนซ์ ภาวะขาดอาหาร การปรับตัว