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Role of Trehalose-6-Phosphate Phosphatase Encoding Gene in Cell Wall Homeostasis of *Talaromyces marneffei*

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ABSTRACT

Fungal cell walls are complex and incredibly dynamic during growth of the dimorphic fungus that grows in both forms of filament and yeast such as *Talaromyces marneffei* (formerly *Penicillium marneffei*). The role of the trehalose biosynthesis pathway on cell wall regulation has never been studied in this particular human pathogen. Moreover, a critical reason makes the trehalose biosynthesis pathway under the investigation due to the absence of genes involving in this pathway in humans. The roles of *orlA* gene encoding for trehalose-6-phosphate phosphatase on the morphological phenotypes, conidiation, and the involvement of cell wall regulation were determined by the characterization of the generated mutant strain lacking an *orlA* gene. The results showed that lack of *orlA* has an impact on morphology including the conidiation defect on media for fungal cultivation including the minimal media containing ammonium sulfate (ANM+AS), brain heart infusion agar, malt extract agar, and potato dextrose agar at 25^{o}C. Interestingly, a nitrogen source such as gamma-Aminobutyric acid (GABA) dramatically restores the conidiation of the *orlA* mutant strain. Finally, loss of *orlA* significantly inhibits growth on the media containing cell wall perturbing agents (50 µg/ml Calcofluor white and 0.5 mg/ml Congo red) similar to the results found in other human pathogenic fungi which are not dimorphic fungi. In conclusion, the results show the importance of *orlA* gene and trehalose pathway in the cell wall homeostasis and conidiation of dimorphic fungus which confirms previous suggestions that this catalytic enzyme is a promising target for antifungal drug development.

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Keywords: Cell wall; Dimorphic; *Penicillium marneffei*; *Talaromyces marneffei*; Trehalose-6-Phosphate Phosphatase

1. Introduction

Penicilliosis is a fungal infection caused by the dimorphic mold *Talaromyces marneffei* (formerly *Penicillium marneffei*). It has been recognized as an emerging infection among Human Immunodeficiency Virus (HIV) infected patients in Southeast Asia and the southern part of China [1, 2]. Significant increases in the proportions of bloodstream *T. marneffei* infection are correlated with increasing HIV prevalence in southern Vietnam [3]. In Thailand, it has high prevalence of infection and is the third most common opportunistic infection following tuberculosis and cryptococcosis that causes a fatal systemic mycosis in patients with AIDS [4]. More importantly, penicilliosis has also been observed in non-HIV infected patients, in particular other immunocompromised patients, and is also considered as a primary pathogen in a group of people with normal immunity [2, 5]. Following medical technology advances, there has been a substantial increase in the number of immunocompromised patients who are at high risk for certain zoonotic infections [6]. *T. marneffei* is recognized as thermally dimorphic fungi whose principal virulence factor is an ability to switch its morphology from mycelial to yeast, known as the dimorphic phase transition. Studies on *T. marneffei* have largely been focused on identifying regulatory mechanisms and phase-specific genes as potential targets for the development of a new antifungal agent.

T. marneffei, has evolved multifactorial mechanisms to survive stress conditions encountered in *in vivo* host cells during infection. The trehalose biosynthesis pathway is one fungal metabolic pathway that is unique to fungi and absent in humans. Synthesis of trehalose in fungi has been extensively studied in the model yeast *Saccharomyces cerevisiae* [7]. Yeast synthesizes trehalose in a two-step reaction by a protein complex consisting of four subunits: Tps1 (Trehalose-6-Phosphate Synthase; TPS), Tps2 (Trehalose-6-Phosphate Phosphatase; TPP), Tsl1, and Tps3 (two regulatory proteins). The second reaction is catalyzed by Tps2 (OrlA in filamentous mold i.e. *Aspergillus nidulans*, and *A. fumigatus*) and has been reported as having an essential role in the cell wall integrity and virulence of *A. fumigatus* [8]. Importantly, the trehalose biosynthesis pathway has been well characterized in the pathogenesis of various fungal pathogens. The trehalose biosynthesis pathway is required for virulence in *Cryptococcus neoformans*, *C. gattii*, *Candida albicans*, and *A. fumigatus* [1, 9-13]. Recently, a mechanism of population fitness related to the trehalose pathway has been shown to be involved in the stress resistant cell state of *S. cerevisiae* under high temperature stress. These data suggest that trehalose synthesis is part of a complex and multifactorial mechanism contributing to fungal survival in harsh environments [8]. Despite the promise of the trehalose biosynthesis pathway as an antifungal drug target, the key catalytic enzymes encoding gene, *orlA*, has never been characterized in *T. marneffei*.

The fungal cell wall is a unique component of fungi which is absent in humans. Defects in cell wall integrity have important implications for fungal virulence [14]. It has been shown that dysregulation of cell wall biosynthesis can affect the initial stages of infection in *A. fumigatus* and *C. neoformans* [15, 16]. Defects in cell wall integrity can also increase recognition by the innate immune system [17, 18]. The fungal cell wall is a complex dynamic structure that continuously changes during

growth depending on the culture conditions and environmental stresses [14, 19]. The aim of this investigation was to test the hypothesis that the trehalose biosynthesis is required for the regulation of cell wall homeostasis as a virulent factor of *T. marneffei*.

2. Materials and Methods

2.1 Strains and media

T. marneffei wild type and *pyrG* auxotroph strains (kind gift of Andrianopoulos *et al*. [20]) utilized in this study were routinely grown in malt extract agar (MEA; HIMEDIA) at 25°C. The *orlA* mutant was specially grown in sorbitol minimal media containing GABA for conidiation (SMM; 1.2 M sorbitol, 1% glucose, 10 mM gamma-Aminobutyric acid (GABA), Sigma-Aldrich). SMM+GABA media was used as standard media in all experiments requiring conidia due to the restricted ability of Δ*orlA* to generate conidia on other media. The conidia from each strain were collected by 0.01% Tween-80 after 3 weeks of incubation at 25°C. Fresh conidia were used in all experiments. Macroscopic and microscopic morphology analysis was performed on all fungal strains at 25°C by measuring colony diameter and slide culture technique. Various media utilized for morphology analysis were ANM+AS (containing 10 mM Ammonium sulfate) [48], Potato Dextrose Agar (PDA; HIMEDIA) and Brain Heart Infusion Agar (BHI; Difco).

2.2 Strain construction

Mutant strains were generated by standard fungal protoplast transformation. To enhance successful gene deletion, the latest parental *T. marneffei* strain lacking non-homologous DNA integration plus uracil auxotroph strain (Δ*ligD pyrG*-) was provided in collaboration with the Andrianopoulos *et al*. [20] group and used to generate the *orlA* deleted strain. PCR generated gene replacement or complementation constructs were utilized. Gene replacement constructs contain approximately 1 kb of homologous sequence flanking the *A. nidulans pyrG* gene. All transformations include a "no DNA" control to exclude the possibility of wild-type contamination. Transformants were initially screened with PCR to identify potential transformants with homologous recombination events at the specified gene locus. Deletion of *orlA* was verified by Southern blot analysis and a strain carrying single insertion of the disruption *pyrG* construct into the specified gene locus was selected for further identification and characterization.

2.3 Cell wall integrity assay

The radial growth rates of mutant, complemented, and wild type strains were measured on malt extract media at 25°C. Strains were tested with potent cell wall inhibitors to examine cell wall integrity; Calcofluor white (CFW; Fluorescent Brightener 28, Sigma-Aldrich), and Congo red (CR, Sigma-Aldrich) [8, 21]. Colony diameters of a 5 µl drop of an equal amount of $1x10^6$ spores were measured every 24 h. for a period of 10 days for each fungal strain in triplicate. The mean value of each reliable experiment was reported and Student's T tests performed to measure statistical significance ($P \leq 0.05$).

3. Results and Discussion

3.1 The PMAA_004960 is a single homolog of *orlA* gene in *T. marneffei*

Preliminary studies have identified the putative components of this pathway in *T. marneffei*, and the functional role of this gene in *T. marneffei* will be characterized for fungal morphology and integrity of cell wall. Accordingly, this study was seeking to

determine the role of the known catalytic *orlA* gene involved in trehalose biosynthesis in *T. marneffei*. A putative single homolog of *tps2* gene, PMAA_004960, was found closely related to true filamentous fungi (Fig. 1) and designated *orlA* consistent with previous nomenclature from *A. nidulans*, and *A. fumigatus* [8, 22]. Two homologs of genes encoding the first key catalytic enzyme (Tps1) were also clustered with high similarity to *A. fumigatus* (Fig. 1). In order to test hypothesis that the enzyme involved in the trehalose pathway is important in regulation of fungal metabolic pathways and cell wall homeostasis of *T. marneffei*, a mutant lacking the homolog of *S. cerevisiae* Tps2 was generated.

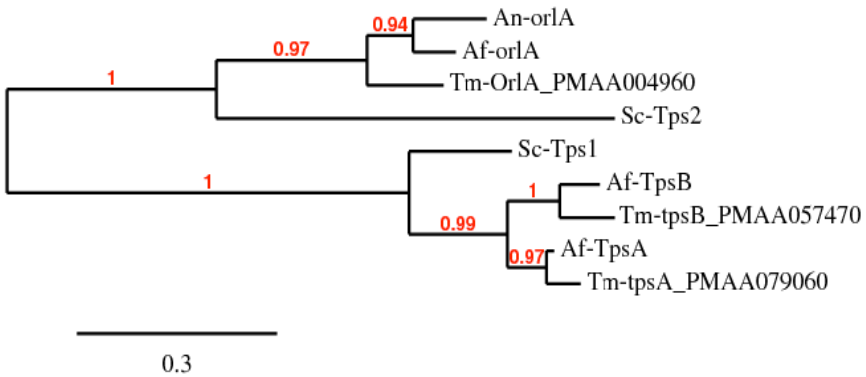

Fig. 1. Phylogenetic tree showing two clades: trehalose-6-phosphate synthase (Tps1), and trehalose-6-phosphate phosphatase (Tps2), among yeast and molds. An; *Aspergillus nidulans*, Af; *Aspergillus fumigatus*, Tm; *Talaromyces marneffei*, and Sc; *Saccharomyces cerevisiae*. Note the duplication of Tps1 in *A. fumigatus* and *T. marneffei*.

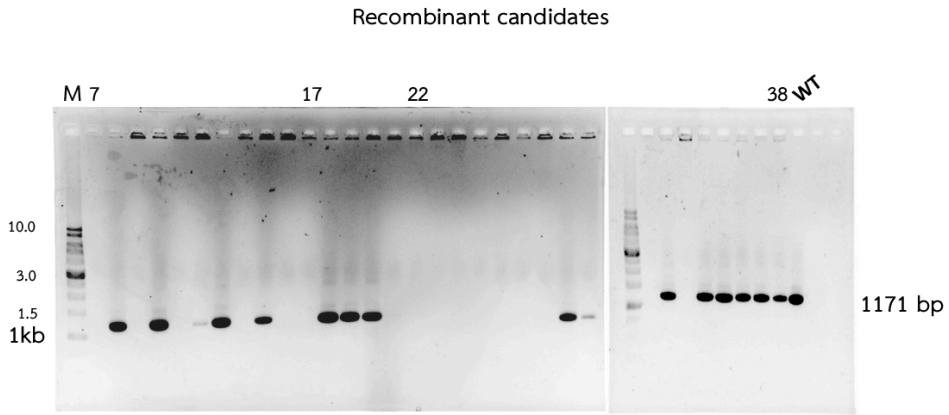

Fig. 2. Gel electrophoresis analysis of an inside *orlA* gene-1171 bp PCR product on 1% agarose gel. Presence of *orlA* gene was amplified from *T. marneffei* (WT) and some representative transformants. Among the transformants #7-38, the candidate 17 and 22 showing an absence of the inside *orlA* gene were selected for further confirmation analysis. Lane M; 1Kb DNA ladder marker.

3.2 Construction of *orlA* mutant

To enhance successful gene deletion, the latest parental *T. marneffei* strain lacking non-homologous DNA integration (Δ*ligD pyrG*+) was provided in collaboration with the Andrianopoulos *et al.* group [2 0] and used to generate the *orlA* deleted strain. PCR generated gene replacement or complementation constructs were utilized. There were 90 recombinant candidates obtained from protoplast transformation. Only 62 candidates showed an absence of 1171 bp product of the inside portion of the *orlA* gene as screened with PCR as shown in a group of selected recombinant candidates compared to wild type *T. marneffei* positivecontrol (Fig. 2). Following the PCR result, candidates were grouped by colony morphology and another PCR analysis was performed to confirm the presence of the *pyrG* selective marker. Altogether, the mutant candidates that lack *orlA* gene showed conidiation defects on the ANM+AS media compared to wild type.

Positive candidates of the *orlA* mutant were subsequently verified by Southern blot analysis with left flank (LF) probe of *orlA* gene (Fig. 3). Strain carried single insertion of the *pyrG* construct into the specified gene locus, the Δ17 was selected for further identification and characterization.

3.3 Defective conidiation of the *orlA* mutant.

Several candidates of *orlA* mutant were similar in both hybridized band and morphological properties of abolished conidiation on the ANM+AS media. The candidate Δ17 was selected as the representative mutant for further studies. A series of experiments was performed to characterize the phenotypes of the mutant strain to confirm their role in trehalose biosynthesis and *T. marneffei* biology. The *orlA* mutant was different from the wild type in growth rate and asexual reproduction. Less growth of the mutant was observed in ANM+AS, MEA, PDA, and BHI media at 25°C than in the wild type. The conidiation defect was partially rescued on media containing an osmotic stabilizer (1.2M sorbitol) and gamma-Aminobutyric acid (GABA) as shown in Figure 4. Microscopic analysis showed that the *orlA* mutant has changed hyphal morphology and conidiation on all tested media (Fig. 5) and in particularly we observed fragmentation of hyphae on brain heart infusion agar at 25°C.

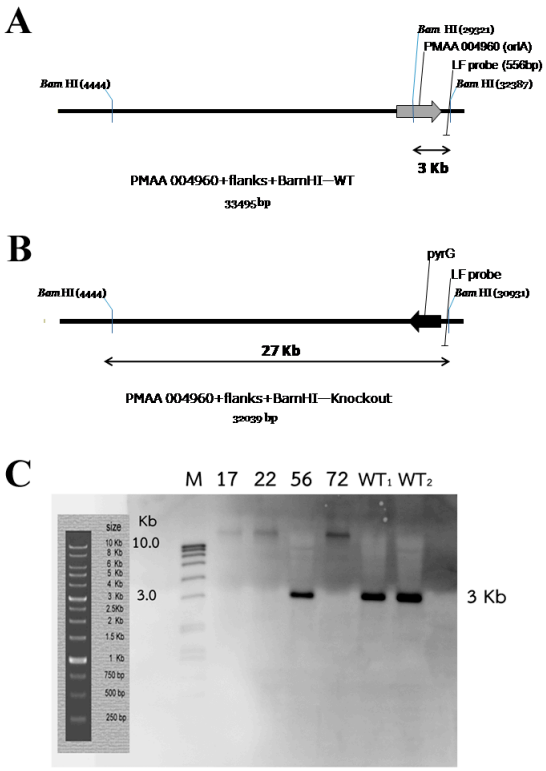

Fig. 3. Generation of mutant lacks the *orlA* gene (PMAA004960) of *T. marneffei*. Illustration of *orlA* gene locus in wild type strain (A) and a knockout strain (B) after successful replacement of *orlA* gene with *pyrG* selective marker. Southern blot analysis of wild type and the *orlA* mutants (C). The *Bam*HI digested genomic DNA of all strains were separated on a 1 % agarose gel, blotted and hybridized with 5 5 6 bp LF probe. The expected fragment sizes of the *orlA* locus in the wild type and *orlA* mutant were observed at bands of 3 kb and 2 7 kb, respectively.

3.4 Lack of *orlA* gene has impaired cell wall integrity

Dysfunction in the trehalose biosynthesis pathway and incorporation with a defective glycolysis will be proposed as the alteration of cell wall integrity against tested cell wall perturbing agents. Measurement of colony diameter for fungal growth rate study showed that the *orlA* mutant grew significantly slower than the wild type on malt extract agar (p value = 0.0034). We next tested the ability to grow on the cell wall perturbing agents; calcofluor white (CFW) and congo red (CR). Loss of *orlA* gene resulted in increased susceptibility to CFW and CR compared with the wild type strain (Fig. 6).

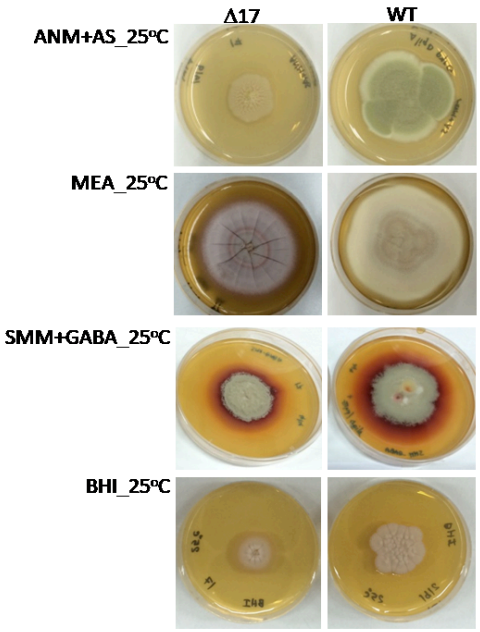

Fig. 4. Macroscopic morphology of wild type *T. marneffei* and the *orlA* mutant strain (Δ*orlA*; Δ17) on different media. Conidiation of the *orlA* mutant was restored on SMM+GABA media after 3 weeks of incubation at 25°C.

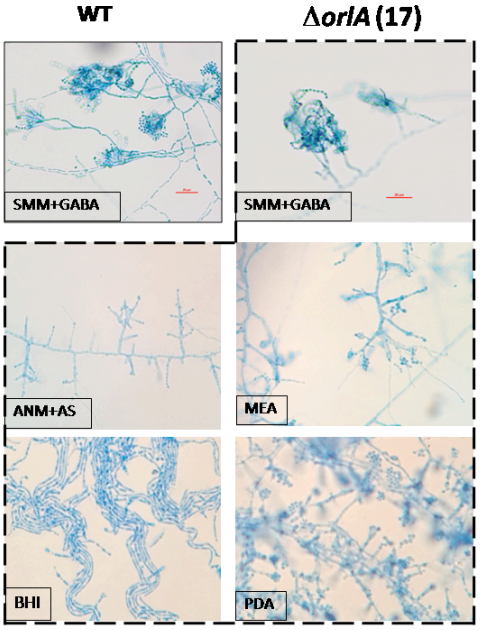

Fig. 5 . Microscopic morphology of wild type *T. marneffei* and the *orlA* mutant strain (Δ*orlA*; Δ17) on different media. Conidiation of the *orlA* mutant was restored on SMM+GABA media after 3 weeks of incubation at 25°C.

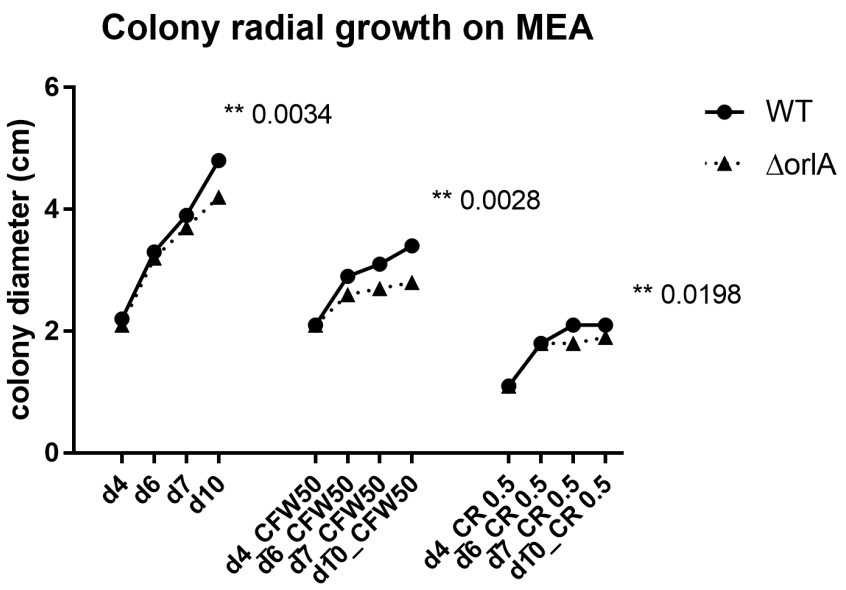

Fig. 6. The *orlA* mutant strain is sensitive to cell wall perturbing agents. Wild type and mutant were tested on Malt extract agar contained 50 µg/ml CFW and 0.5 mg/ml CR. The experiment was repeated in biological triplicates with identical results.

4. Conclusion

T. marneffei is a unique dimorphic fungus causing penicilliosis in HIV infected patients and other immunocompromised patients. *T. marneffei* is capable of survival and differentiation into the fission yeast form under nutrient deprivation conditions in host macrophages. Tight regulation mechanisms of key metabolic pathways and cell wall homeostasis are likely required to make this transition. The trehalose pathway is a stress protectant pathway that has been studied in several model plant and animal pathogenic fungi. In particular, the *orlA* encoding gene has been observed to be involved in the regulation of glycolysis and cell wall integrity. The conidiation defect observed in the *orlA* mutant of *T. marneffei* corresponds with another important human fungal pathogen *Aspergillus fumigatus* [8]. Abolished conidia production and altered hyphal morphology of mutant were observed on ANM+AS, MEA, and PDA media which generally are utilized for cultivation of wild type strain. Interestingly, normal sorbitol media with ammonium sulfate was unable to reconstitute the conidiation of the *orlA* mutant but the addition of gamma-Aminobutyric acid (GABA) successfully rescued conidia formation in the mutant lacking *orlA* gene (Fig. 4. and data not shown). Hyphal fragmentation was observed at 25^{o}C in both wild type and mutant in the brain heart infusion agar (BHI).

Results of the cell wall integrity assay confirmed that loss of *orlA* gene caused the alteration of the cell wall in which sensitive to grow on the malt extract media containing inhibitors of cell wall component synthesis such as chitin and glucan. Accordingly, the susceptible phenotype of the *orlA* mutant is similar to those observed in other fungal pathogens. This data

suggested that not only yeast including *Cryptococcus neoformans*, *C. gattii*, *Candida albicans*, and filamentous fungus such as *Aspergillus fumigatus*, but the dimorphic fungus; *T. marneffei* also has similar aberrant results in the absence of the *orlA* gene. Taken together, the trehalose tps1/tps2 pathway is essentially implicated in the cell wall formation and defects in cell wall integrity have important implications for fungal pathogenicity. It has been shown that the *T. marneffei drkA* mutant strain has increased rate of phagocytosis corresponding to its severe cell wall defects [20]. The recognition of β-1,3 glucan by the innate immune system is found to be inhibited in *Histoplasma capsulatum*, by masking of α-1,3 glucan composition on the cell wall [23]. These data suggested that the trehalose pathway and critical enzymes are required for *T. marneffei* cell wall homeostasis and further investigation on ability to survive inside macrophages and cause disease in the immunocompromised murine model is needed.

In conclusion, our results show the importance of *orlA* gene and trehalose pathway in the cell wall homeostasis and conidiation of dimorphic fungus which confirms previous suggestions that this catalytic enzyme is a promising target for antifungal drug development. Therefore, further insights into this fungal biology and virulence will provide an elucidated inclusive data on the therapeutic strategies to develop broad spectrum antifungal therapy on human mycoses.

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