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Research Article

An Antigenic Protein Mplp6 is Dispensable in *Talaromyces marneffei*

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Abstract

MPLP6 is one of the genes in the mannoprotein superfamily of a human pathogenic fungus *Talaromyces marneffei*. It encodes for a highly immunogenic, yeast phase specific protein Mplp6. In this study, to analyze the function of *MPLP6*, the deletion and reconstitution strains were constructed and characterized phenotypically. The mutant strain demonstrated the same phenotypes as those of the wild type isolate; the morphology of the colony, growth rate and conidiation, indicating the dispensability of the *MPLP6* gene. Growth of the mutant on fatty acid containing media was similar to that observed in the wild type, implying that Mplp6 is not required for fatty acid utilization. Moreover, the mutant showed cell wall, hyper-osmotic and oxidative stress tolerance similar to the wild type; therefore it is reasonable to conclude that Mplp6 is not the component that takes responsibility in cell wall integrity. In addition, the mutant was phagocytosed and killed in equal measures when compared with the wild type and complementary strains when observed in the THP-1 monocyte-derived macrophage infection model. Interestingly, attachment of the yeast cells of the mutant to fibronectin and laminin was slightly reduced when compared to the parental strain, suggesting that the Mplp6 could serve as one of the molecules that contribute to the host-pathogen interaction. Additionally, the recombinant Mplp6 could bind to both extracellular matrix molecules in a dose-dependent manner. These results suggest that the Mplp6 could be a putative adhesin.

Keywords: *MPLP6*; Mplp6; Mannoprotein; Function; *Talaromyces marneffei*

Abbreviations

LBD: Lipid Binding Domains; 5-FOA: 5-Fluoro-Orotic Acid; *MPLP6*: Mannoprotein-Like Protein 6 (Gene); Mplp6: Mannoprotein-Like Protein 6 (Protein); Δ*MPLP6: MPLP6* Gene Deletion; rec-*MPLP6: MPLP6* Gene Reconstitution; rMplp6: Recombinant Mannoprotein-Like Protein 6; DAPI: 4',6-Diamidino-2-Phenylindole; SDS: Sodium Dodecyl Sulfate; PMA: Phorbol 12-Myristate 13-Acetate; MOI: Multiplicity of Infection; CFU: Colony Forming Unit; ANM: *Aspergillus nidulans* Minimal Medium; SDA: Sabourauds's Dextrose Agar; BHA: Brain Heart Infusion Agar; PDA: Potato Dextrose Agar; BMGY: Buffered Glycerol-Complex Medium; ELISA: Enzyme Immunosorbent Assay; PCR: Polymerase Chain Reaction; RT-PCR: Reverse Transcription Polymerase Chain Reaction; GPI: Glycosyl-Phosphatidyl-Inositol

Introduction

The *Talaromyces marneffei* genome contains at least 14 homologous genes in the mannoprotein superfamily [1]. This gene family is highly lineage specific in so far as it has only been reported in species of *Aspergillus* and *Penicillium* [2-5]. However, there is nucleotide diversity that makes the orthologous genes different and unique in both species. Some of the genes in this family have high levels of nucleotide substitution exhibiting high discriminatory powers. Thus they are used in molecular typing because of their fast evolutionary rates [6].

The best-characterized gene in the mannoprotein superfamily of *T. marneffei* is an *MP1*. Initially, the gene *MP1* was isolated and characterized. It encoded for a cell wall mannoprotein antigen, Mp1p [2]. The recombinant Mp1p was used as a diagnostic marker in the development of serodiagnostic assay for *T. marneffei* infection [7,8]. The *MP1* gene was also tested successfully as a DNA vaccine in a mouse model [9]. Recently, the Mp1p protein has been shown to be one of the virulence factors when it comes to binding to arachidonic acid pro-inflammatory molecules through the lipid binding domains (LBDs) and thus modulating the immune system [10,11].

In our previous study, a gene encoding for a mannoprotein-like protein 6 (*MPLP6*) was isolated and characterized. The Mplp6 was demonstrated as an immunogen to elicit antibody response in *T. marneffei*-infected patients [12]. It is speculated to play a role in the yeast phase of the life cycle of *T. marneffei* since it is specifically expressed in this form. The expression is induced during transition from the conidial to yeast form, but its expression could not be induced by heat or oxidative stresses [12]. Recently, a knockdown mutant of *MPLP6* showed that this gene is not involved in the survival of mice in the infection model [10]. However, the function of the *MPLP6* has not been fully investigated. To investigate the biological role of Mplp6, the deletion and reconstitution strains were constructed and characterized in this study.

Materials and Methods

Fungal strains and culture conditions

T. marneffei uracil auxotrophic strain G816 (Δ*ligD niaD pyrG*$^{-}$) [13], a strain that facilitates efficient homologous integration and a *MPLP6* deletion strain (Δ*MPLP6*) were grown and maintained on an *Aspergillus nidulans* medium (ANM) containing 5 mM uracil and 10 mM $(NH_4)_2SO_4$. A parental G809 strain (Δ*ligD niaD pyrG*$^{+}$) and a *MPLP6* reconstituted strain (rec*MPLP6*) were maintained on an ANM with 10 mM $(NH_4)_2SO_4$. All fungal strains were grown on ANM with uracil to facilitate the production of the conidia for 10 days at 25°C. A conidial suspension was prepared by scraping the surface of fungal cultures with a sterile cotton swab and suspended in a sterile normal saline containing 0.1% tween-40. The suspension was then filtered through a Miracloth (Calbiochem, Germany) or glass wool and the conidia were enumerated under a microscope using a hematocytometer. Preparation of the swollen conidia was performed by inoculation of the conidia into a BHI broth, culturing being facilitated at 37°C for 18 - 20 h in an orbital shaker.

Generation of the *MPLP6* deleted (Δ*MPLP6*) and reconstituted strains (rec*MPLP6*)

To generate a clone containing the *MPLP6* gene and flanking regions, the PCR fragment containing the *MPLP6* flanks with 2-kb of upstream (5') and downstream (3') regions was amplified from genomic DNA of *T. marneffei* (ATCC18224) using primers KK3 (5'-TCATCATTCCAAGCAGCGAG-3') and KK4 (5'-CTCCCAGCCAGTGCGTTCTA-3'). The reaction mixture contained 1.5 mM $MgCl_2$, 0.5 μ M of each primer, 0.2 mM of each dNTP and 1 U of Phusion® Hot start High-Fidelity DNA polymerase (Finnzyme) and 100 ng of DNA template. A 4.8-kb amplified product was purified and cloned into a pGEM-T® easy plasmid (Promega) which enabled the generation of a pMP7487 plasmid. Inverse PCR was then performed using pMP7487 as the template. An inverse PCR product (6.6-kb) containing 5' and 3' flanking regions of the *MPLP6* and pGEM-T® easy plasmid as a backbone was amplified and digested with *Sma*I and *Eco*RV to generate ends for ligation. Then a deletion construct, pMP7488 was generated by ligation of the inverse PCR product with a 2.3-kb *Sma*I/*Eco*RV digested pAB4626 (kindly provided by Andrianopoulos A., The University of Melbourne, Australia) fragment containing an *Aspergillus nidulans pyrG* (*AnpyrG*) blaster cassette. The knockout cassette was then prepared by *Not*I digestion of the pMP7488 deletion construct to release a fragment containing upstream and downstream flanking regions of the *MPLP6* (to allow homologous recombination) interrupted by the *AnpyrG* blaster. This cassette was then transformed into the *T. marneffei* G816 strain (*ligD* $^{-}$*niaD*$^{-}$ *pyrG*$^{-}$) by using the protoplast transformation method as described previously [14-18]. The transformants were selected on the ability to grow on a protoplast medium without uracil. They were subjected to PCR and Southern blot analysis to confirm the successful deletion of the *MPLP6* gene.

To generate a complementing plasmid, a fragment containing a promoter region and open reading frame of the *MPLP6* gene was amplified and ligated into a *pyrG* targeting plasmid (kindly provided by Andrianopoulos A., The University of Melbourne, Australia). To generate a recipient strain for reconstitution, the Δ*MPLP6* was prepared for recycling of the selectable marker. The mutant was forced into looping out of the *AnpyrG* by growing the conidia on a medium containing 5-fluoro-orotic acid (5-FOA). The uracil auxotrophic Δ*MPLP6* strain was then selected and used for the transformation of a complementing plasmid. This plasmid allowed homologous integration at the silent *pyrG* gene locus into the *T. marneffei* genome and enabled the generation of the uracil prototrophy transformants (denoted as rec*MPLP6*). The reconstituted strain was confirmed as being effective for the recovery of *MPLP6* expression by RT-PCR.

RT-PCR

The total RNA was extracted from 3-day-old yeast or mycelium culture by using TRIzol® reagent (Gibco-BRL) and treated with DNase. Approximately 1.5 mg RNA was used in the reverse transcription reaction using the Omniscript® RT kit (QIAGEN) and poly (dT) primer. Two microliters of reverse transcribed product was used in PCR with *MPLP6* specific primers. The PCR reaction was performed in a reaction containing 1.5 mM $MgCl_2$, 0.5 μ M of each primer, 0.2 mM of each dNTP and 1 U of DyNAzyme™ EXT DNA polymerase (Finnzymes). The PCR conditions were 94°C for 2 minutes; 35 cycles of 94°C for 30s, 55°C for 30s, 72°C for 35s and a final extension at 72°C for 10 minutes.

Morphological study

In the colony morphological study, 5 x 10^5 conidia of *T. marneffei* G809 strain (Δ*ligD niaD pyrG*$^{+}$), Δ*MPLP6*, and rec*MPLP6* strains were spotted onto the surface of several mycological media and incubated at either 25°C or 37°C.

The agar-coated technique was used in the microscopic study. ANM or BHI agar containing 5 mM uracil was used to coat a tilted slide and was allowed to solidify. The conidia of *T. marneffei* strains were inoculated on the agar-coated slides and were allowed to grow for 3 days at 25°C or for 5 days at 37°C. The slides were fixed in a fixative solution (4% paraformaldehyde, 50 mM PIPES, 25 mM EGTA, 5 mM $MgSO_4$, pH 6.7) for 30 min. The fungus was then stained with calcofluor white (0.1 mg/ml) and 4',6-diamidino-2-phenylindole (DAPI) (1 μ g/ml). Fungal morphology was observed under a fluorescence microscope (AW70, Olympus) at an excitation wavelength of 358 nm.

Stress tolerance assays

A serial dilution (10^1 - 10^4 conidia/drop) of the conidia from *T. marneffei* strains were spotted onto an ANM medium containing stressors. One to three molar sorbitol, 10-100 μ g/ml sodium dodecyl sulfate (SDS) and 1 - 15 μ g/ml calcofluor white and 1 - 5 mM hydrogen peroxide were used as the osmotic, cell membrane, cell wall, and oxidative stressors, respectively. The cultures were incubated at either 25°C or 37°C for 6 days.

Macrophage infection for phagocytosis and killing assay

The THP-1 human monocyte cell line (10^6 cells/well) was cultured on a circular cover slip in a 24-well plate containing an RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco-BRL) at 37°C and 5% CO_2. For monocyte to macrophage differentiation, the THP-1 cells were induced with 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) and incubated at 37°C, 5%CO_2 for 48 h. In the phagocytosis assay, the media was removed from the wells and replaced with the media containing conidia of the G809, Δ*MPLP6*, and rec*MPLP6* strains at the ratio of 10 conidia per macrophage (MOI = 10). The mixture was incubated for 2 hours. The glass coverslips were removed, and then the samples were fixed with 1% paraformaldehyde and subjected for examination for the determination of percentages of phagocytosis. These experiments were repeated in triplicated. Efficiency of phagocytosis was calculated as the percentage of intracellular conidia from the total number of all cell-associated conidia.

In the killing assay, THP-1 monocytes were seeded into a 24-well tissue culture plate at a concentration of 1 x 10^6 cells/well and stimulated with phorbol 12-myristate 13-acetate (PMA). After differentiation, the culture medium was removed and the 500- μ l RPMI medium containing 10^7 of conidia was added to each well (MOI = 10). The mixture was incubated for 2 h to allow adhesion and phagocytosis. After 2 h, each well was washed with an RPMI medium containing 240 U/ml of nystatin (Sigma-Aldrich, St. Louis, USA) to remove extracellular conidia. The THP-1 cells were lysed immediately (time point = 0 h) or incubated for 2 h (time point = 2 h) to allow macrophages to kill the engulfed conidia. Macrophage cells were lysed with 1% Triton X-100 (Sigma-Aldrich, St. Louis, USA) and the cell lysates were diluted and spread on Sabouraud's dextrose agar to enable the determination of the colony forming units (CFU). The percentage of killing was assessed using the equation [(CFU at 0 h-CFU at 2 h)/CFU at 0 h] x 100.

Production of His-tagged Mplp6 recombinant protein

The histidine-tagged Mplp6 protein was produced in *Pichia pastoris*. The first step, an amplicon containing the *MPLP6* open reading frame (encoding amino acid number 19-219) with *Eco*RI and *Xho*I ends was in-flame cloning into the corresponding enzyme digested pPICZαB plasmid (Invitrogen). The amplicon was prepared using PCR with the forward primer (5'-CCGGAATTCACAGCTCTTACGTCGATTAT-3') and reverse primer (5'-ATTCTCGAGGGTTAGCACTAGCAGCATC-3'). The PCR product (627 bp) was purified and cloned into the pPICZαB plasmid downstream of the sequence encoding six histidine residues. The ligation reaction was transformed into calcium chloride treated DH5α competent cells and the transformants were selected on a LB medium containing 25 μg/ml zeocin (Invitrogen). A recombinant plasmid of 4,196 bp was purified and sequenced to confirm in-frame cloning and named pPICZαB-*MPLP6*. Then the recombinant plasmid was transformed into the *P. pastoris* X33 strain by an electroporation method as recommended in the manufacturer's protocol (Invitrogen). Briefly, 5 μg of the *Sac*I-linearized pPICZαB -*MPLP6* recombinant plasmid was electrotransformed into the 80 μl of competent *P. pastoris* using a Bio-Rad Genepulser apparatus at 1.5 kV, 25 μF, 400 ohm and 10 ms). The transformants were selected on an YPD agar plate containing 100 μg/ml zeocin. The transformants with mut^+ phenotype were selected for PCR using 5' and 3' *AOX1* primers (5'-GACTGGTTCCAATTGACAAGC-3' and 5'-GCAAATGGCATTCTGACATCC-3') and sequenced to confirm the chromosomal integration and orientation of the recombinant plasmid. Then a verified transformant was subjected to protein expression by being grown at 30°C in a 10 ml BMGY medium for 24 h until reaching an OD_{600} of 10. The cells were harvested and resuspended in a 50-ml BMGY medium containing 0.5% (v/v) methanol. After incubating the culture at 30°C for 72 h, methanol was added at 1% (v/v) once a day to maintain the induction. The culture supernatant was collected, concentrated and analyzed using SDS-PAGE. The his_6-tagged rMplp6 with the size of 32 kDa was subsequently purified. For purification of a His-Mplp6 fusion protein, the supernatant was applied to a nickel-immobilized chelating sepharose fast flow column (Amersham, Biosciences). The purification process was performed according to the manufacturer's protocol.

Adherence assays

Adherence assays were performed as previously described with slight modification [19-21]. In the assays, yeast form was used to determine the adhesion in a scenario of dimorphic conversion during the *T. marneffei* infection. The conidial culture at 37°C was shown to have the *MPLP6* gene expression after 12 h of incubation and reach the maximum level at 24 h in the previous study [12]. To generate the yeast cells for the assay, the conidia of *T. marneffei* were grown in brain heart infusion broth for 24 hours with 150 rpm continuous shaking. At this time point, the conidia turned into the yeast phase by showing isotropic growth as the swollen cells. For adherence assays, wells of microtiter plates (Maxisorp, Nunc) were coated with 100 μl of a 100 μg/ml solution of laminin or fibronectin (Sigma-Aldrich) in PBS and incubated for 1 h at 37°C. The plates were blocked overnight at 4°C with PBS-1% bovine serum albumin (BSA). For an adhesion assay, 10^5 of 24-h yeast cells of G809 or Δ*MPLP6* were incubated in the coated plate for 2 h at 37°C. Nonadherent yeasts were then removed by three washes with PBS-0.05% tween 20, and the adhered yeasts were fixed with 2.5% glutaraldehyde for 15 minutes and counted at 400 times magnification using an Olympus CK40 inverted microscope (Olympus, Tokyo, Japan). The adhered yeasts were counted in 10 fields for each well. An average count of yeasts in control wells containing BSA was used to background subtract from each tested well. The data obtained were analyzed for average and standard deviation including two-tailed unpaired t-test analysis by using Microsoft excel.

Enzyme-linked immunosorbent assay (ELISA)

One hundred microliters of 100 μg/ml recombinant Mplp6 (rMplp6) protein was added in a 96-well microtiter plate (Nunc, Denmark) for 1h at 37°C. After blocking overnight with 1% BSA, 100 μl of 100 μg/ml fibronectin (Sigma-Aldrich) and laminin derived from Engelbreth-Holm-Swarm mouse sarcoma (Sigma-Aldrich) at various concentrations (10, 25 and 50 μg/100 μl in PBS) were added. Then 1:5000 anti-fibronectin or anti-laminin antibody (Abcam) were added and incubated for 1 h at 37°C, followed by 1:10,000 of HRP-labeled goat anti-rabbit IgG (H+L) antibody (Abcam). A TMB one component HRP Microwell substrate mixture (BioFx®, SurModics, USA) was used for color development, and the absorbance at 450 nm was measured using a Multi-detection plate reader (Synergy H4, US Biotek, USA). Wells without rMplp6 were used as a control. The data were analyzed by using microsoft excel.

Results and Discussion

Expression of *MPLP6* in wild type, Δ*MPLP6*, and *MPLP6* complemented strains

MPLP6 is expressed only in the yeast phase of the *T. marneffei* G809 strain (Figure 1A). The transcript was absent in the mutant strain but was recovered in the reconstituted strain as shown in the figure 1B. This result confirmed the accomplished construction of *MPLP6* mutant and complemented strains.

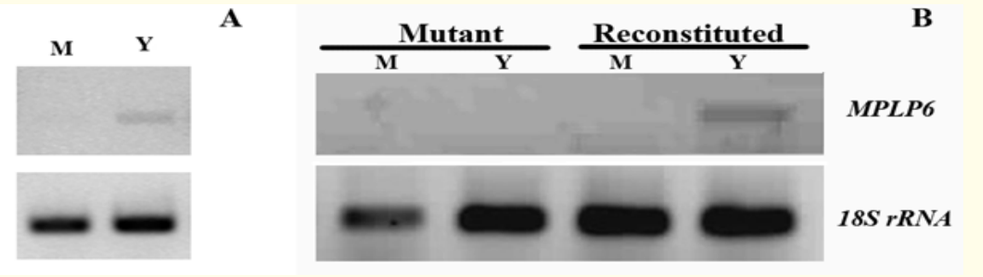

Figure 1: RT-PCR result shows the level of *MPLP6* transcript. One hundred nanograms of cDNA were used in PCR with *MPLP6* gene specific primers. (A) *MPLP6* expression is found only in the yeast phase of wild type G809 strain (Δ*ligD niaD pyrG*$^{+}$) (B) The *MPLP6* expression was absent in the mutant strain and re-expressed in the reconstituted strain. M depicts mycelial phase and Y depicts yeast phase. The result shows a 722-bp band of expectation only in yeast phase of the complemented strain. The 400-bp product of 18S rRNA was shown as the PCR control.

Morphology of Δ*MPLP6*

Colony morphology of the wild type G809 (Δ*ligD niaD pyrG*$^{+}$), Δ*MPLP6* and reconstituted strains were studied on various mycological media including *Aspergillus nidulans* medium (ANM), Sabouraud's dextrose agar (SDA), potato dextrose agar (PDA) at 25°C and brain heart infusion agar (BHA) at 37°C. All media contained uracil to act as the growth supplement factor of the mutant strain. Figure 2A shows representative pictures of the mold colonial morphology from all strains. They were almost identical in growth and conidiation rate. Microscopic morphology in figure 2B demonstrates normal penicillus type conidiation of Δ*MPLP6* which was indistinguishable from the wild type strain. Yeast growth on the BHI medium also showed the same yeast-like morphology in all strains as seen in figure 2C. Similar to the mycelium form, the Δ*MPLP6* produced the normal appearance of yeast morphology (Figure 2D). These results indicated that the deletion of *MPLP6* did not affect the morphogenesis of this fungus.

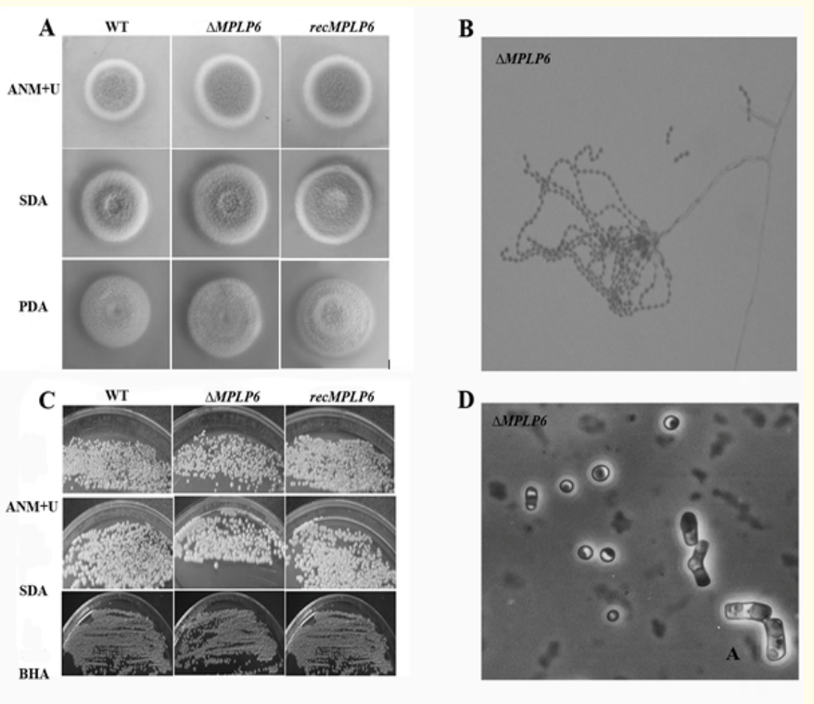

Figure 2: Morphology of G809 (WT), Δ*MPLP6* and reconstituted (rec*MPLP6*) strains on different media at either 25°C or 37°C for 7 days. No difference between strains was observed. (A) Mold colony morphology on ANM with uracil, Sabouraud's dextrose (SDA) and potato dextrose agar (PDA) at 25°C (B) Microscopic characteristic of the mold form (magnification 400) of Δ*MPLP6* in ANM broth with uracil (C) Yeast-like colony morphology on ANM with uracil, SDA and brain heart infusion agar (BHA) (D) Arthroconidia and yeast of Δ*MPLP6* stain in brain heart infusion broth at 37°C.

To check whether the deletion of *MPLP6* could cause a defect in cell division and septation, the hyphae were stained at the septa with calcofluor white and at the nucleus with DAPI. Under fluorescent microscopy, the fungus showed normal chitin deposition at the septa and nuclear arrangement. The conidiation appeared to be regular when compared to the wild type (Figure 3). Therefore *MPLP6* is not required for cell division in *T. marneffei.*

Osmotic, oxidative and cell wall stress tolerance of Δ*MPLP6*

The tolerance of Δ*MPLP6* was tested on media containing stress-inducing agents. Sorbitol (1 - 3 M) was used to generate hyperosmotic stress; sodium dodecyl sulfate (40 - 90 μg/ml) and calcofluor white (3 - 15 μg/ml) were used as the cell membrane and cell wall stressors, and hydrogen peroxide (2 - 5 mM) was used as the oxidative stress generating agent. Conidia of *T. marneffei* strains were dropped on the media with various concentrations of stressors and incubated at either 25°C or 37°C for 7 days. The mycelial tolerance of all tested strains showed no difference when exposed to all stresses (Figure 4). The tolerance in the yeast phase also showed the same results (data not shown). Therefore, the experimental evidence indicates that the deletion of *MPLP6* did not affect osmotic, oxidative stress tolerance and cell wall integrity.

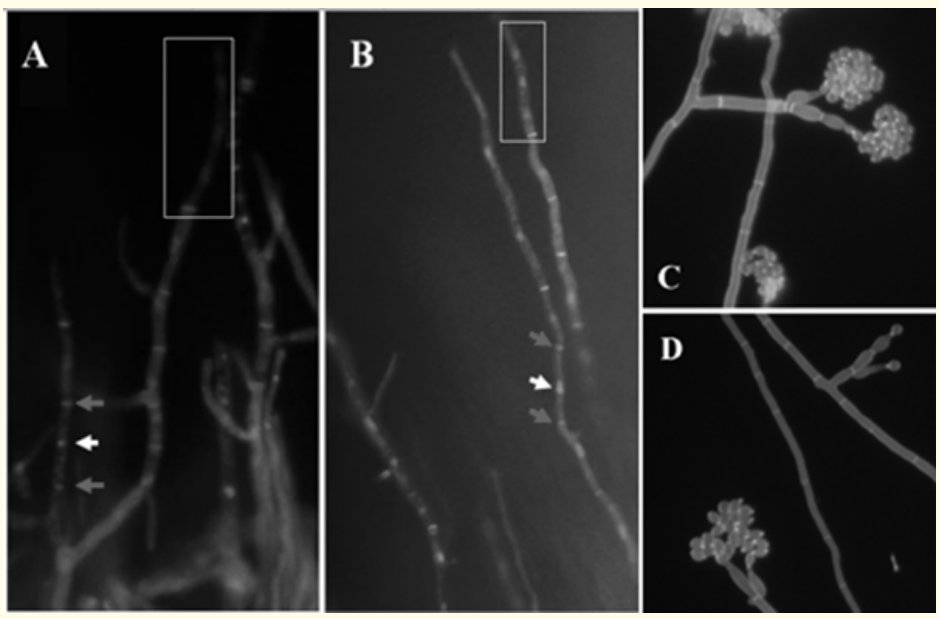

Figure 3: Fluorescent staining of G809 and Δ*MPLP6* strains of *T. marneffei.* Calcofluor white (CFW) (grey arrows) and DAPI (white arrows) staining of yeast phase of G809 (A) and Δ*MPLP6* (B) shows normal septation. Box indicates regular multiple nuclei at the hyphal tip. CFW staining demonstrates normal penicillus pattern in G809 (C) and Δ*MPLP6* (D).

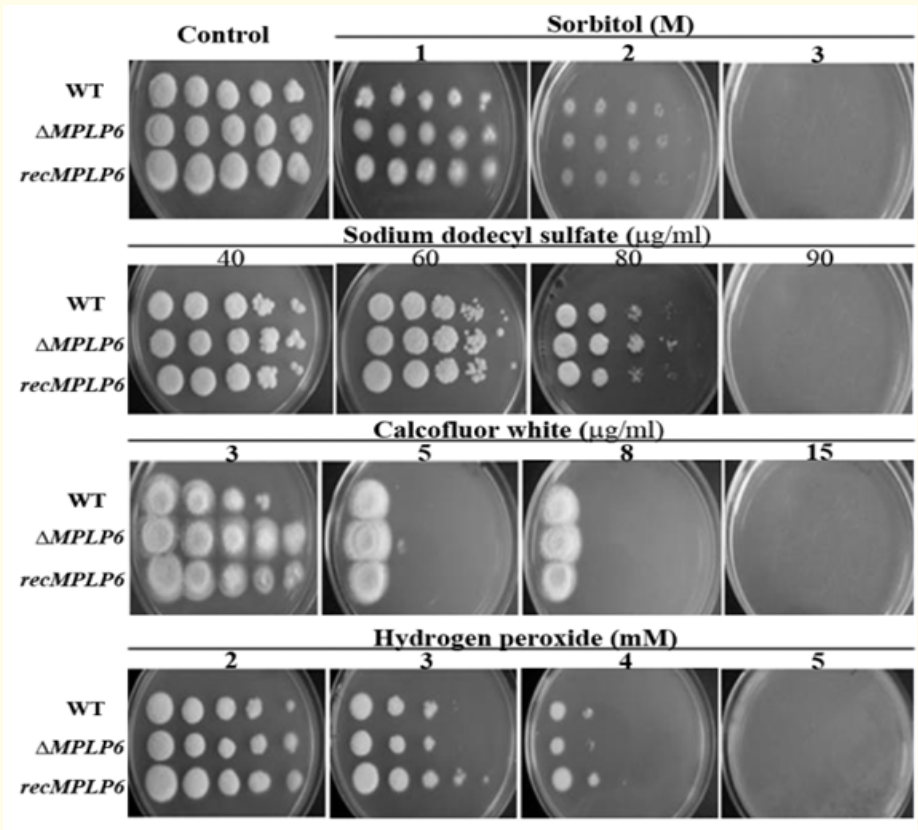

Figure 4: The stress tolerance assays of G809 (WT), Δ*MPLP6* and reconstituted strains. One week-old mold colony was observed at 25°C. Fungal strains were grown on ANM containing various stressors and concentrations as indicated. Control is the growth on ANM without stressor. The result showed no difference in stress tolerance among all tested strains.

Phagocytosis and killing of Δ*MPLP6* in THP-1 cell line

The conidia from Δ*MPLP6*, reconstituted strains and G809 strains were used to inoculate the differentiated human monocytic cell line (THP-1). The percentages of phagocytosis and killing in each strain were observed. At the 2 hour assessment after incubation of the conidia with the macrophages, 2 - 7 conidia of all strains were internalized within a macrophage and approximately 80% of the macrophages had the fungus inside. The percentage of phagocytosis in wild type (G809), mutant and reconstituted strains showed no difference. The percentages of killing were also similar in all stains (Figure 5). These results indicated that the *MPLP6* was not involved in the intracellular survival of *T. marneffei.*

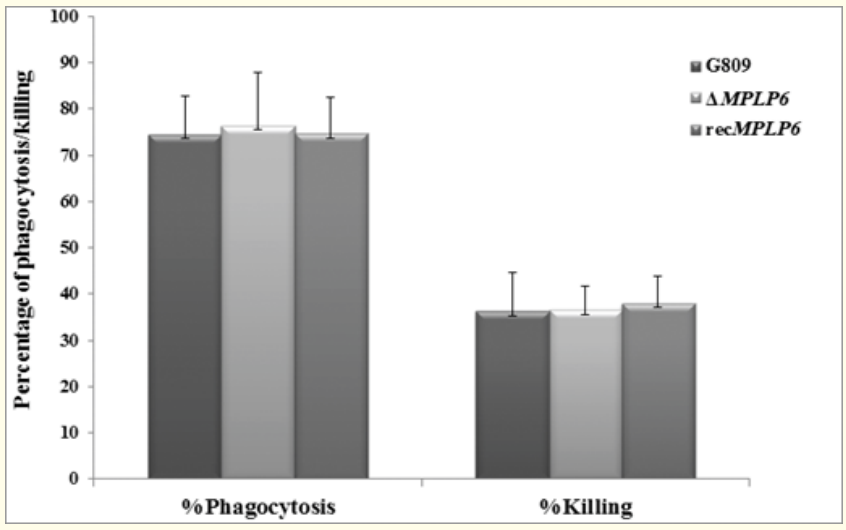

Figure 5: Phagocytosis and survival of *T. marneffei* conidia in the THP-1 cell line. The conidia of G809, Δ*MPLP6* and rec-*MPLP6* strains were inoculated into the differentiated THP-1 cell lines at MOI = 10. Percentages of phagocytosis and killing were determined at 2 hours after infection. The data showed the average numbers from three independent experiments.

Mplp6 is a putative adhesin

As the Mplp6 is a cell wall mannoprotein, we hypothesized that its oligosaccharidic component may contribute to the host-fungus interactions. To prove that the Mplp6 functioned as an adhesin, we used adherence assay techniques to compare the binding ability of the *MPLP6* mutant and G809 strain containing wild type *MPLP6* with two major extracellular matrix molecules, fibronectin and laminin. The 24-h-yeast cells of *T. marneffei* (24 h cultivation in brain heart infusion broth at 37°C) were used in the assay for the reason that the expression of the *MPLP6* transcript was detected after incubation of the conidia at 37°C for at least 12 h [12]. The results of the adherence assay showed that the numbers of adherence yeast cells of Δ*MPLP6* to both molecules was reduced slightly when compare to the wild type (Figure 6) whereas no adherence was observed in the wells coated with BSA (data not shown). Even the significant reduction of the adherence activity was not detected, but the data suggested that the *MPLP6* could possibly be one of the molecules that contribute to the association between the fungal cells and host extracellular matrix molecules. A slight decrease in adherence activity of the *MPLP6* mutant could be due to the dominant activities of other adhesion molecules on the surface of the fungal cells.

To give more evidence for the putative adhesion function of Mplp6, the recombinant Mplp6 protein was used to coat the wells of the plate in an indirect ELISA assay and detect its direct binding to fibronectin and laminin. The rMplp6 bound to both molecules in a dose-dependent manner (Figure 7). This result suggested that the Mplp6 could serve as one of the adhesion molecules in *T. marneffei.*

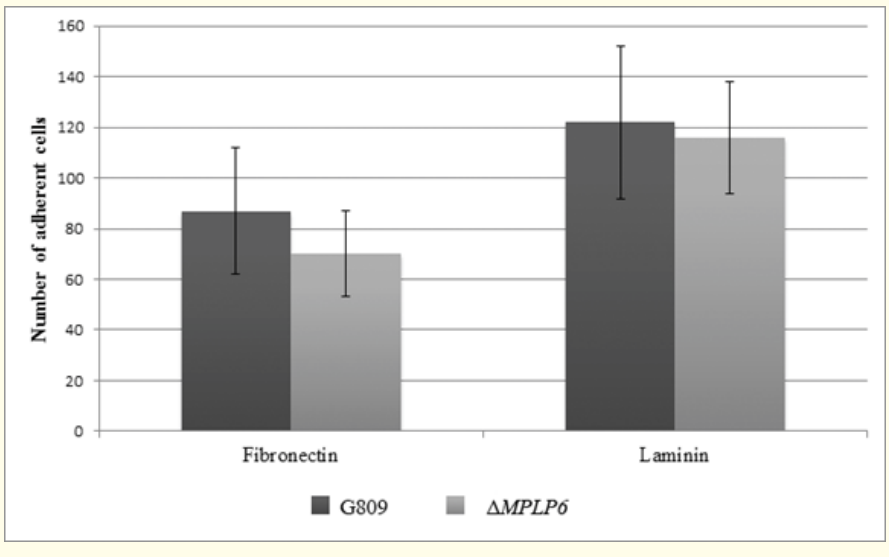

Figure 6: Adherence assay of *T. marneffei* yeasts to the fibronectin and laminin. One hundred micrograms of fibronectin and laminin were coated in the wells of 96-well plates. A hundred microliters of 10^6 yeast cells/ml of G809 or Δ*MPLP6* strains of *T. marneffei* were added to the well. The adherent yeasts were counted after washing. The results show the average numbers of five experiments.

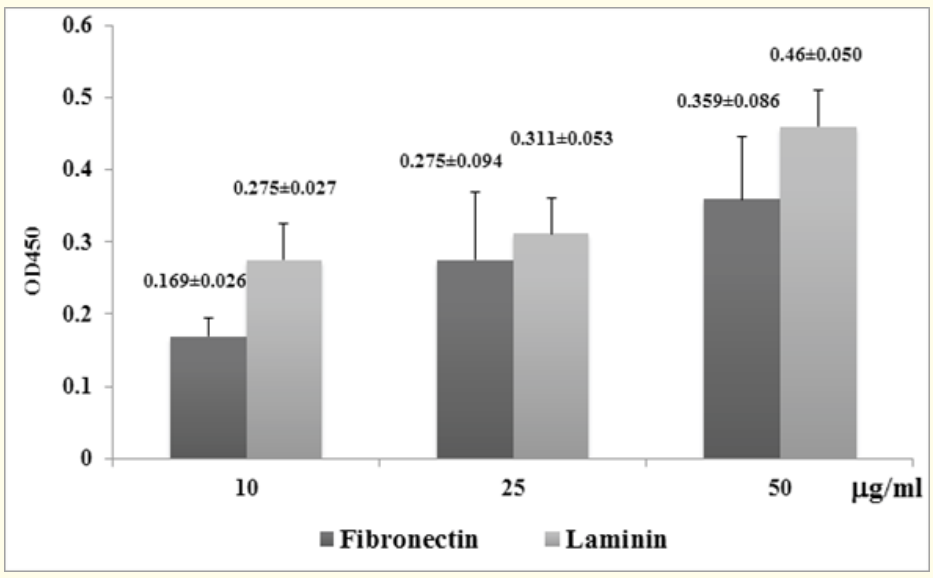

Figure 7: ELISA assay for determination of the binding of recombinant Mplp6 to fibronectin and laminin. The rMplp6 was used to coat the wells of the microtiter plate. Fibronectin and laminin were added at various concentrations. The color development was performed and measured at OD_{450}. The experiments were repeated three times. Each bar shows the average values with standard deviation.

Conclusion

The *MPLP6* gene is dispensable in *T. marneffei*. The *MPLP6* mutant showed no phenotypic change in macroscopic (colony) and microscopic morphology, cell division process, conidiation and hyphal formation. Additionally, the tolerance to cell wall and other stressors were comparable to the parental strain indicated that the *MPLP6* is not involved in cell wall integrity. Also, the mutant showed no detectable sensitivity to phagocytosis and killing in the macrophage infection model, suggesting that the *MPLP6* is not necessary for intracellular survival of *T. marneffei*. There were evidences that suggest a partial involvement of Mplp6 in adhesion to the host extracellular matrix. Yeast cells of the Δ*MPLP6* showed slightly decrease in adherence to laminin or fibronectin in the adherence assay. Additionally, the recombinant Mplp6 bound to both molecules in the dose-dependent manner. These results suggested the putative function of Mplp6 to be one of the adhesion molecules in *T. marneffei.*

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Conflict of Interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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