Talaromyces marneffei laccase modifies THP-1 macrophage responses

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ABSTRACT
Talaromyces (Penicillium) marneffei is an emerging opportunistic pathogen associated with HIV infection, particularly in Southeast Asia and southern China. The rapid uptake and killing of T. marneffei conidia by phagocytic cells along with the effective induction of an inflammatory response by the host is essential for disease control. T. marneffei produces a number of different laccases linked to fungal virulence. To understand the role of the various laccases in T. marneffei, laccase-encoding genes were investigated. Targeted single, double and triple gene deletions of laccases encoding lacA, lacB, and lacC showed no significant phenotypic effects suggesting redundancy of function. When a fourth laccase-encoding gene, pbrB, was deleted in the ΔlacA ΔlacB ΔlacC background, the quadruple mutant displayed delayed conidiation and the conidia were more sensitive to H2O2, sodium dodecyl sulfate (SDS), and antifungal agents than wild-type and other transformants. Conidia of the quadruple mutant showed marked differences in their interaction with the human monocyte cell line, THP-1 such that phagocytosis was significantly higher when compared with the wild-type at one and 2 hours of incubation while the phagocytic index was significantly different from 15 to 120 minutes. In addition, killing of the quadruple mutant by THP-1 cells was more efficient at 2 and 4 hours of incubation. The levels of the proinflammatory cytokines TNF-α, IL-1β and IL-6 from THP-1 cells infected with the quadruple mutant were also significantly increased in comparison with wild-type. The results demonstrate that production of laccases by T. marneffei actually promotes the pathogen’s resistance to innate host defenses.

Introduction
Talaromyces marneffei, formerly known as Penicillium marneffei, is a thermally dimorphic fungus and the etiologic agent of the human systemic disease penicilliosis marneffei, which affects individuals in endemic areas throughout Southeast Asia and southern China.1-3 T. marneffei has emerged as an opportunistic fungal pathogen associated with HIV infection in Northern Thailand, where penicilliosis is the third most common AIDS-associated disease, after tuberculosis and cryptococcosis.4 Although the majority of T. marneffei infections in immunocompromised hosts have been found in HIV-infected patients, the incidence of penicilliosis marneffei has recently increased among non-HIV patients with other underlying diseases such as diabetes, cancer or systemic lupus erythematosus as putative susceptibility conditions.5 T. marneffei is a member of an evolutionary diverse group of fungi that are thermally dimorphic.6 In its environmental form, the fungus grows as saprophytic mycelium that produce the infective propagules (conidia). Conidia are inhaled into the host’s lungs where they subsequently transform into yeast cells that replicate by binary fission.7 The initial interactions of the T. marneffei conidia with the host phagocytic cells and the degree of activation of the host’s innate immune responses to the fungus are critical parameters determining the host’s ability to control disease.8 Macrophages appear to be the initial immune cells that phagocytose conidia and secrete the pro-inflammatory cytokines responsible for inducing responses that subsequently arrest infection.7,9 Phagocytosis of conidia and subsequent maturation of the

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phagosome exposes the fungal cells to an oxidative burst, acidic condition (pH 4–5), enzymatic attack and nutrient-limitation, all of which are critical mechanisms for the macrophage to kill ingested microbes.10 T. marneffei yeast cells are also susceptible to being killed by human neutrophils stimulated with granulocyte macrophage colony-stimulating factor (GM-CSF).12 In penicilliosis marneffei patients, phagocytosed conidia can germinate into yeast cells that reside as intracellular parasites within macrophages.13 Previous studies have demonstrated that some fungal laccases are upregulated during H₂O₂-mediated oxidative stress and nutrient starvation, especially glucose limitation, in order to support fungal growth and stress defense.14–17

To understand the pathogenesis of T. marneffei, studies of the fungal determinants associated with virulence are needed. The putative virulence attributes of T. marneffei include adhesion,18,19 dimorphism,20–22 production of melanin or melanin-like substance23,24 and metabolic factors such as the glyoxylate cycle.25 Laccases have been previously linked to fungal development, morphogenesis, detoxification processes, stress resistance, pigment formation, nutrient acquisition and pathogenicity.26–28 Laccase is an important virulence factor in many pathogenic microorganisms including fungi and has been best characterized in Cryptococcus neoformans.15,29 Expression of fungal laccase has been shown to be induced by environmental stress such as oxidative stress, acidic environments, and nutrient deprivation.14,28 A laccase-like activity has been detected in protein extracts of dimorphic fungi including T. marneffei,23 Paracoccidioides brasiliensis,30 and Histoplasma capsulatum,31 and laccase has been implicated in the enzymatic synthesis of melanin in these fungi.

In this study we sought to understand the role of the various laccases encoded by T. marneffei and determine how these laccases affect vegetative growth and H₂O₂-mediated oxidative stress. We have previously shown that the pbrB gene of T. marneffei encodes a laccase specifically required for pigment formation in conidia.32 Three additional laccase-encoding genes of interest were investigated for their expression and roles in stress response and during growth in host cells. Gene deletion strains were generated that either lacked a single gene or combinations of genes. Notably, we only identified defects in the quadruple mutant that lacked all 4 laccase encoding genes (lacA, lacB, lacC and pbrB) suggesting significant redundancy, despite the fact that these genes show differential expression patterns. Conidiation in the ΔlacA ΔlacB ΔlacC ΔpbrB strain was delayed and the conidia were more susceptible to oxidative stress (H₂O₂), cell wall stress (SDS) and antifungal agents such as itraconazole, fluconazole and clotrimazole compared to wild-type and non-quadruple mutant conidia. Examination of the role of these laccases altered the fate of T. marneffei conidia with the THP-1 cells, a human monocytic cell line. The results demonstrate that laccase production significantly affects phagocytosis and intracellular survival of conidia in THP-1 cells as well as alters the release of cytokines by these host effector cells. Hence, we provide strong evidence for the role of laccases in the host immune response to T. marneffei.

**Results**

Talaromyces marneffei laccases are upregulated during oxidative stress at 37°C

T. marneffei produced and secreted laccase during vegetative growth at 28°C and 37°C (Fig. 1A). We assessed whether oxidative stress and/or acidic condition could induce T. marneffei laccase expression by measuring enzymatic levels of cytoplasmic laccases. Cytoplasmic laccase activity was increased significantly (p < 0.01) after T. marneffei encountered with acidic (pH 5) or H₂O₂-mediated oxidative stress (1 mM H₂O₂ at pH 5; phagolysosome-like condition)10 conditions (Table 1). However, the addition of glucose (5%) to the acidic and oxidative stress treatments reduced the level of T. marneffei laccase, particularly when coupled with oxidative stress conditions (p < 0.01). These data are consistent with the hypothesis that T. marneffei laccases are expressed in phagolysosomes of macrophages.

In this study, we focused on the expressions of 3 genes (lacA; PMAA072680, lacB; PMAA085520, and lacC; PMAA055370) because of their similarity24 with Cryptococcus neoformans lac1; a defined virulence factor.15 Amplification of lac transcripts in RNA extracts indicated different patterns of lac expression during growth under standard and oxidative stress conditions. During vegetative growth, lacA and lacC mRNA was readily detectable while lacB expression was not detected at either temperature. At 37°C lacA expression was about 2-fold higher than that of lacC but there was no difference at 28°C (Fig. 1B, C). When T. marneffei was subjected to exogenous H₂O₂ for 30 minutes, lacB expression was induced. An induction of lacA and lacB due to exogenous H₂O₂ was verified by decreasing cDNA template (2-fold lower). LacA, but not lacC, transcripts were readily detectable. Also noteworthy was the expression of the lacB mRNA under these conditions. Upregulation of lacA and lacb was consistent with accumulation of LacA::GFP and LacB::GFP fusion proteins within fungal cells found during H₂O₂-mediated oxidative stress at 37°C (data not shown).
**T. marneffei laccases are essential for cell integrity and stress resistance**

To assess the role of these laccases, deletion strains for the lacA, lacB and lacC genes were generated. Levels of cytoplasmic laccase activity were significantly decreased when compared with wild-type (p < 0.01) (Table 2) whereas secreted laccase activity was not detected in ΔlacA and ΔlacC mutants at 28°C (Fig. S1) and 37°C (data not shown). In vitro growth testing of these strains during hyphal growth at 28°C and yeast growth at 37°C under various conditions including H2O2 oxidative stress failed to detect any discernible differences from the wild-type control (data not shown). Since we found that lacA and lacB were expressed during H2O2-mediated oxidative stress at 37°C and lacA and lacC were expressed at both 28°C and 37°C under standard growth conditions, we generated ΔlacA ΔlacB double mutant and ΔlacA ΔlacB ΔlacC triple mutant strains to assess its sensitivity to stress. Growth testing of these strains at 28°C showed no discernible differences on standard medium (Table 3). Conidia from 2 independent colonies of each deletion strain were tested under H2O2-mediated oxidative stress at acidic condition, sodium dodecyl sulfate (SDS)-mediated cell wall stress and antifungal agent-mediated growth inhibition (see Materials and Methods). Each stressor was added to BHI medium where higher level of laccase activity was detected in the wild-type (data not shown). None of these deletion strains showed any detectable difference compared to the wild-type (Fig. 2).

Conidia of triple deletion exhibited green color indicating the presence of DHN-melanin. We hypothesized that alteration of DHN-melanin synthesis in a ΔlacA

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**Table 1. Measurement of laccase activity in normal and stress conditions.**

| Conditions | Mean of absorbance (N = 3) | SEM | Statistical significance |
|------------|----------------------------|-----|-------------------------|
| Cytoplasmic extracts |                           |     |                         |
| pH 7 at 1h  | 0.232                     | 0.009 |                        |
| pH 5        | 0.332                     | 0.014 | *p = 0.004              |
| pH 5 + 5% glucose | 0.211                     | 0.010 | **p = 0.002             |
| pH 5 H2O2   | 0.440                     | 0.020 | **p = 0.001             |
| pH 5 H2O2 5% glucose | 0.148                     | 0.015 | **p < 0.001             |

*Relative to pH 7 control
**Relative to the same condition without 5% glucose

**ΔlacB ΔlacC triple mutant strains to assess its sensitivity to stress. Growth testing of these strains at 28°C showed no discernible differences on standard medium (Table 3). Conidia from 2 independent colonies of each deletion strain were tested under H2O2-mediated oxidative stress at acidic condition, sodium dodecyl sulfate (SDS)-mediated cell wall stress and antifungal agent-mediated growth inhibition (see Materials and Methods). Each stressor was added to BHI medium where higher level of laccase activity was detected in the wild-type (data not shown). None of these deletion strains showed any detectable difference compared to the wild-type (Fig. 2). Conidia of triple deletion exhibited green color indicating the presence of DHN-melanin. We hypothesized that alteration of DHN-melanin synthesis in a ΔlacA

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**Table 2. Cytoplasmic laccase activities in the wild-type and mutants.**

| Strains | Mean of absorbance (N = 3) | SEM |
|---------|-----------------------------|-----|
| Wild-type | 0.409                      | 0.003 |
| ΔlacA    | 0.308**                    | 0.003 |
| ΔlacB    | 0.332**                    | 0.007 |
| ΔlacC    | 0.330**                    | 0.004 |
| ΔpbrB    | 0.375**                    | 0.003 |
| ΔlacA ΔlacB | 0.209**                 | 0.005 |
| ΔlacA ΔlacB ΔlacC | 0.241*                  | 0.003 |
| ΔlacA ΔlacB ΔlacC ΔpbrB | 0.228*                | 0.003 |
| ΔlacA ΔlacB ΔlacC ΔpbrB::pbrB | 0.235*            | 0.002 |

*By comparing with quadruple lac deletions, these differences are not statistically significant (p > 0.05).
**By comparing with quadruple lac deletions, these differences are statistically significant (p < 0.01).
\(\Delta lac B \Delta lac C\) background may increase the sensitivity of conidia to oxidative stress. To test this, we produced \(\Delta lac A \Delta lac B \Delta lac C \Delta pbr B\) quadruple deletion transformants. Macroscopic examination of \(\Delta lac A \Delta lac B \Delta lac C \Delta pbr B\) transformants resembled that of the other deletion mutant transformants and wild-type except for conidial coloration at 28°C (Fig. 3A). In addition, it appeared that conidial production was reduced in the \(\Delta lac A \Delta lac B \Delta lac C \Delta pbr B\) strains (data not shown). To ensure these phenotypic observations were due to loss of \(pbr B\) in the triple \(lac\) deletion background, we generated a complemented strain in which the \(pbr B\) gene was reintroduced into the \(\Delta lac A \Delta lac B \Delta lac C \Delta pbr B\) transformant at the native locus. The resulting transformants (\(\Delta lac A \Delta lac B \Delta lac C \Delta pbr B::pbr B\)) produced green conidia similar to \(\Delta lac A \Delta lac B \Delta lac C\) mutant and wild-type (Fig. 3A). The conidial phenotype of the quadruple mutant could be due to a reduced growth rate, frequency of conidiophores or rate of conidial production on conidiophores. The radial growth rate data revealed that all of the strains had equivalent growth rates (Table 3). Colonies growing on the slides were observed microscopically for defects in conidiation and conidia production for 5, 6, 7, and 10 days at 28°C (Fig. 4). The conidiophores of all mutant strains were morphologically similar to those of the wild-type with respect to the various cell types and their abundance. In contrast, the density of conidiophores was greatly reduced in the \(\Delta lac A \Delta lac B \Delta lac C \Delta pbr B\) mutants, especially at the early time points. This suggested that conidiation of \(\Delta lac A \Delta lac B \Delta lac C \Delta pbr B\) transformant is delayed. Besides, we found that conidia of quadruple mutant (\(\Delta lac A \Delta lac B \Delta lac C \Delta pbr B\)) are more sensitive to various stressors. When compared with the other mutants, it was clear that the \(\Delta lac A \Delta lac B \Delta lac C \Delta pbr B\) mutant was more sensitive to \(H_2O_2\), SDS and antifungal agents, including clotrimazole, itraconazole, and fluconazole (Fig. 2). Increasing of sensitivity to cell wall stressor (SDS) suggests that cell wall integrity

### Table 3. Growth rate of transformants.

| Transformants | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
|--------------|------|------|------|------|------|
| \(\Delta pbr B\) | 0.18 + 0.02 | 0.57 + 0.01 | 1.03 + 0.01 | 1.68 + 0.02 | 1.94 + 0.01 |
| \(\Delta lac A \Delta lac B\) | 0.16 + 0.02 | 0.54 + 0.01 | 0.95 + 0.01 | 1.55 + 0.02 | 1.80 + 0.01 |
| \(\Delta lac A \Delta lac B \Delta lac C\) | 0.18 + 0.02 | 0.54 + 0.02 | 0.97 + 0.02* | 1.54 + 0.03 | 1.88 + 0.02 |
| \(\Delta lac A \Delta lac B \Delta lac C \Delta pbr B\) | 0.20 + 0.01 | 0.52 + 0.01 | 0.93 + 0.01* | 1.59 + 0.02 | 1.95 + 0.02 |
| \(\Delta lac A \Delta lac B \Delta lac C \Delta pbr B::pbr B\) | 0.16 + 0.01 | 0.56 + 0.02 | 0.96 + 0.02 | 1.57 + 0.01 | 1.90 + 0.02 |

*The value difference is considered to be not statistically significant (\(p = 0.1\)).

Figure 2. Stress susceptibility tests showing stress sensitive phenotype of \(\Delta lac A \Delta lac B \Delta lac C \Delta pbr B\) transformant. Ten-fold serial dilutions of conidia were pipetted onto BHI agar containing 2.1 mM \(H_2O_2\) (oxidative stress), 20 \(\mu g/ml\) SDS (cell wall stress), or antifungal agents (0.1 \(\mu g/ml\) clotrimazole, 0.04 \(\mu g/ml\) itraconazole, and 40 \(\mu g/ml\) fluconazole). Plates were placed in 37°C incubator for 1 week. Growth of the \(\Delta lac A \Delta lac B \Delta lac C \Delta pbr B\) strain was only evident in spots containing the higher concentrations of conidia showing that this strain is more sensitive to certain stressors compared with wild-type G681 and other mutants.
may be affected. This is consistent with observations that show melamins are deposited at the cell wall and increase cell integrity, thus protecting fungal cells against various stressors.\textsuperscript{37,38} Laccase activity in cytoplasmic extracts of mutants grown in BHI broth at 37°C for 3 days was determined and this showed a significant decrease in activity for the \( \Delta \text{lacA} \Delta \text{lacB} \Delta \text{lacC} \Delta \text{pbrB} \) strain compared to wild-type, single gene deletion and double gene deletion strains (\( p < 0.05 \)) (Table 2). Statistic differences were shown significantly (\( p < 0.05 \)) when compared between laccase activity of each mutant with wild-type or \( \Delta \text{pbrB} \) mutant. Among triple mutant, quadruple mutant and \( \Delta \text{pbrB} \)-complemented quadruple mutant, their cytoplasmic laccase activities in 37°C cultures were not different (\( p > 0.05 \)). \( \Delta \text{lacA} \Delta \text{lacB} \Delta \text{lacC} \Delta \text{pbrB} \) mutant could produce melamins detected by using anti-melanin monoclonal antibody\textsuperscript{23} developed for \( T. \text{marneffei} \) and confirmed by melanin extraction.\textsuperscript{23}

**Phagocytosis assay**

To assess whether any of these laccase-encoding genes play a role in the interaction of \( T. \text{marneffei} \) with host cells, the frequency with which \( T. \text{marneffei} \) wild-type, laccase mutant conidia and \( \Delta \text{pbrB} \)-complemented quadruple mutant are phagocytosed by THP-1 cells was measured by counting the number of conidia ingested over time. The percentages of conidia ingested increased over time for all strains examined (Fig. 5A). For the early time points at 15 and 30 minutes post-inoculation, the percentages were similar (approximately 5–6 %) for all strains. However, at the later time points of 60 and 120 minutes post-inoculation there were significant differences between the quadruple \( \text{lac} \) gene deletion strain and the other strains. There were no significant differences in the wild-type conidia, the single \( \text{lac} \) gene mutants as well as the \( \Delta \text{pbrB} \)-complemented quadruple mutant at any of the intervals examined. Interestingly, the phagocytic index for each of the strains remained fairly constant over the different time intervals assessed (Fig. 5B). The phagocytic index for the quadruple \( \text{lac} \) gene deletion (\( \Delta \text{lacA} \Delta \text{lacB} \Delta \text{lacC} \Delta \text{pbrB} \)) strain was significantly higher than the other strains at each time interval (\( p < 0.05 \)). Phagocytosis rates of the \( \Delta \text{pbrB} \)-complemented quadruple mutant were similar to those of the wild-type and the single \( \text{lac} \) gene mutants at each time interval.

**Killing assay**

To compare the percentages of intracellular killing of \( T. \text{marneffei} \) wild-type and laccase mutant strains by THP-1 cells, CFUs were determined at different time intervals post-infection (Fig. 6). As early as 2 hours after inoculation, \( T. \text{marneffei} \) CFUs for the quadruple mutant dropped compared to conidia of the other strains, although the difference at this early time interval was not significant. This suggested that THP-1 cells killed quadruple \( \text{lac} \) gene mutant conidia more readily. At 4 and 8 hours, the THP-1 macrophages killed significantly more of the quadruple \( \text{lac} \) gene deletion conidia compared to any other strains. The percentages of single \( \text{lac} \) gene deletion strains and the reconstituted strain killed at each time interval were similar to the wild-type.

**Cytokine response to wild-type and laccase deleted mutants of \( T. \text{marneffei} \) conidia**

To determine the role of laccases on cytokine production, we examined the capacity of the different mutant
conidia to induce TNF-α, IL-6 and IL-1β after incubation with THP-1 cells. The quadruple lac gene deletion strain elicited a significantly higher release of TNF-α as compared to the wild-type, any single lac gene deletion strains and the reconstituted strain after 8 and 24 hours of incubation (Fig. 7A). Similarly, the quadruple lac gene deletion strain elicited a significantly stronger IL-6 and IL-1β response at 8 and 24 hours of incubation (Fig. 7B, 7C). There were no significant differences in the production of these cytokines when comparing the wild-type to the single lac gene mutants as well as the pbrB-complemented quadruple mutant.

Discussion

Laccase-encoding genes are widely distributed in the fungal kingdom, especially among the ascomycete and basidiomycete species. Fungal laccases function in various metabolic processes including nutrient acquisition, growth and development, and protection against life-threatening stressors. The presence of paralogous laccase-encoding genes, which presumably originated from gene duplication events, may be to fulfill a variety of targeted functions during the life cycle and to respond to environmental conditions. Multiplicity of laccase

Figure 4. Representation of differences in density of conidiophores observed on slide cultures. Conidia of wild-type G681, ΔpbrB, ΔlacA ΔpbrB, ΔlacA ΔpbrB ΔlacC, ΔlacA ΔpbrB ΔlacC ΔpbrB, ΔlacA ΔpbrB ΔlacC ΔpbrB ΔpbrB were cultured on ANM agar and incubated in a moist chamber at 28°C for 5, 6, 7, and 10 days. Unlike other strains, ΔlacA ΔpbrB ΔlacC ΔpbrB produces very few conidiophores on day 5 to 6. Slide cultures were examined under the microscope for conidiophores. Images shown are at 100X magnification. White triangles point to conidiophores. The data represents the results of 2 independent experiments performed in duplicate.
Figure 5. THP-1 macrophages more effectively ingested *T. marneffei* quadruple laccase mutant conidia. The percentages of phagocytosis (A) and phagocytic index (B) of THP-1 macrophages co-cultured with *T. marneffei* conidia from wild-type, quadruple *lac* gene deletion strain, the single gene disruptants, ΔlacA, ΔlacB, ΔlacC, ΔpbrB, and the *pbrB* complemented quadruple mutant at 15, 30, 60 and 120 min. Each bar represents the mean ± SEM of 3 sets of experiments, each performed in duplicate. The * denotes a *p* < 0.05 when comparing the wild-type and quadruple *lac* gene disruptant conidia.
genes allows broader substrate recognition which confers an advantage in the competition for space and nutrients. Expression of the various laccases ranges from constitutive to inducible triggered by exogenous regulators such as pH, H$_2$O$_2$, temperature, a variety of aromatic compounds related to lignin or its derivatives, metal ions (e.g., copper and iron) and concentrations of carbon (e.g., glucose and sucrose) and nitrogen sources. The mechanism by which laccases scavenge H$_2$O$_2$ is not clearly understood; however, the catalyzed product of this enzyme, melanin, is described as a scavenger of oxidative radicals. Melanin acts as a free radical trap and stabilizes harmful reactive oxygen species. The expression of laccase acting indirectly to scavenge intracellular H$_2$O$_2$ and protect cells from lipid oxidative damage has been demonstrated in the heterologous expression system. Trametes sp. 5930 lac gene expressed in Pichia pastoris responds to exogenous H$_2$O$_2$ and the latter enhances transcriptional gene products of the glutathione-dependent antioxidative system of P. pastoris.

T. marneffei genome possesses a family of laccase-encoding gene based on sequence similarity. The genetic redundancy of lac genes may be to provide the robustness of biological functions. According to the previous report, LacA and LacC are classified into ascomycete laccase clade I together with uncharacterized T. marneffei laccases (PMAA100410, PMAA050860 and PMAA008350). PbrB laccase is in ascomycete laccase clade II involving in conidial pigment synthesis. LacB is quite similar to ferroxidases than laccase clades but LacB is not grouped into ascomycete laccases nor ferroxidases. Similarity search of lacB yielded 78% sequence homology to C. neoformans lac1. LacB might be ferroxidase-laccase enzyme. Analysis of subcellular localizations by ProtComp 9.0 suggested that LacA and LacC are membrane bound or secreted enzymes while LacB localizes at plasma membrane. The transcripts of lacA and lacC were presented in hyphal (28°C) and yeast (37°C) cells and induction of lacA and lacB expression was evident during H$_2$O$_2$-mediated oxidative stress.

Figure 6. THP-1 macrophages kill T. marneffei quadruple laccase mutant conidia more effectively. The percentages of killing of conidia from T. marneffei wild-type, quadruple lac gene deletion strain, the single gene disruptants, ΔlacA, ΔlacB, ΔlacC, ΔpbrB, and the pbrB complemented quadruple mutant at 2, 4 and 8 hours. Each bar represents the mean ± SEM of 3 sets of experiments, each performed in duplicate. The * denotes a p < 0.05 when comparing the wild-type and quadruple lac gene disruptant conidia.
Figure 7. T. marneffei quadruple laccase mutant conidia induced significantly more proinflammatory cytokine production by THP-1 macrophages. A) Tumor necrosis factor α (TNF-α), B) interleukin-6 (IL-6), and C) interleukin-1β (IL-1β) levels in THP-1 cells co-cultured with conidia from T. marneffei wild-type, quadruple lac gene disruptant, the single gene disruptants, ΔlacA, ΔlacB, ΔlacC, ΔpbrB, and the pbrB complemented quadruple mutant. Measurements of TNF-α, IL-6, and IL-1β were achieved using supernatants pooled from 3 sets of experiments and expressed as mean ± SEM. The * denotes a p < 0.05 in TNF-α, IL-6 and IL-1β production between wild-type and quadruple lac gene disruptant conidia.
Since the localization of fungal laccases associate to physiological functions, the laca and lacC expressions are possibly to support growth. The low level of laca and lacC transcripts could be from the use of brain heart infusion (an enrichment media) as a culture medium. In contrast, extracellular of ABTS catalytic activity depletes in mutants with laca or lacC deletions (see Fig. S1) suggesting their co-function to oxidize extracellular substance. When fungal cells encountered with exogenous H2O2, transcripts of laca and lacC were increased. LacA is expected to be secreted while LacB is at plasma membrane. These data suggest that LacA and LacB may be involved in extracellular and/or intracellular oxidative stress defense. The presence of PbrB is found in asexual development structures and this laccase participates in DHN melanin production during asexual development. The melanin product provides fitness and a nonspecific protection against various stressors. The different expression patterns suggest the T. marneffei lac gene redundancy with divergent function. Glucose-repressible expression has been demonstrated in fungi such as Cryptococcus neoformans, Cerrena unicolor, and Trametes versicolor. Especially in C. neoformans, a virulence factor lac1 expression is extremely sensitive to glucose. Similarly, T. marneffei laccase activity increased in both acidic and H2O2-treated conditions, whereas it decreased in the presence of high glucose (5%).

Generation of T. marneffei lac mutants via target gene deletion reveals the distinct roles of laccases but their functions involve in the fitness of the airborne infectious propagules. Deletion of lac genes expressed at 37°C and during oxidative stress did not affect stress resistance, growth or development. However when coupled with a pbrB gene deletion, encoding a laccase required for DHN-melanin production in conidia, conidiation in this quadruple mutant (ΔlacA ΔlacB ΔlacC ΔpbrB) was delayed. This delay of conidiation occurs only in quadruple mutant suggesting their necessary functions during asexual development. Since laca and lacC express at 28°C whereas lacB respond to oxidative stress, it is possible that these genes participate in conidiation by manipulate stressors occurred during melanin synthesis, melanin deposition and/or involve in morphogenesis of conidial cell wall. Even though laccase activities of single, double and triple lac deletions were lower than wild-type significantly (p < 0.05), these strains were not stress sensitive unlike the quadruple mutant. Since conidia were used to test stress susceptibility and pbrB is expressed during conidiation, the data reflect laca, lacB and/or lacC expression during asexual development and their functions contribute to conidial fitness. These explanations need further study about conidial cell wall architecture of quadruple mutant. Moreover, not only was conidiation affected but an increasing sensitivity to stress conditions such as H2O2-mediated oxidative stress, SDS-mediated cell wall stress, and antifungal agents (e.g. clotrimazole, itraconazole, and fluconazole) was also evident. These findings support the hypothesis that laccases are required in conidia to tolerate various kinds of stressors.

In summary, the ΔlacA ΔlacB ΔlacC ΔpbrB mutant is stress sensitive due to the cumulative loss of laccase activities in vegetative cells that can in part be masked by the conidial laccase encoded by pbrB. Loss of pbrB in the background of the 3 lac mutants uncovers the stress resistance phenotype, demonstrating partial redundancy due to overlapping spatial and temporal expression patterns of these 4 laccase-encoding genes in T. marneffei.

T. marneffei laccases also play a role in the host-pathogen interaction. Laccases have been associated with virulence in many fungal pathogens and the varied activities of the enzyme have been well documented in C. neoformans. Laccase itself directly protects C. neoformans from the antifungal activity of macrophages by functioning as an iron scavenger during infection, which is a distinctly different role from the key role in melanin biosynthesis. As a defense against host immune cells, laccase is believed to oxidize iron, which decreases the production of hydroxyl radicals in alveolar macrophages. Since host effector cells first interact with inhaled resting conidia, we have begun to examine the roles of laccases in the pathobiology of T. marneffei. To investigate the different activities of laccases in T. marneffei we compared the wild-type to single and compound lac gene mutants. Laccase activity was significantly reduced in the quadruple lac gene deletion strain compared to the wild-type and the single lac gene deletion mutants. Despite deletion of 4 lac genes in the quadruple mutant, residual activity on the 2,6 DMP substrate used in the assay was still evident. This may be bona fide laccase activity from one of the other more diverged laccase-like genes in but may equally be the result of other enzymes such as peroxidases. The comparisons of the phagocytic and killing activities of THP-1 macrophage cells against conidia from the T. marneffei quadruple lac gene deletion strain and the wild-type strain revealed clear differences in these processes. The phagocytic index was significantly higher in the quadruple mutant as early as 15 min after engaging THP-1 cells and the phagocytosis percentages notably different by 60 min. Moreover, we determined that there were significant differences in killing between the quadruple deletion strain and the other strains by 4 and 8 hours of co-incubation, with the quadruple deletion strain showing increased susceptibility to the fungicidal responses of the THP-1 cells. Hence, it seems that laccases protect the fungal cells from the cytotoxic interactions with THP-1 cells. This result is consistent with observations that
showed that infection of mice with a laccase-deficient strain of *C. neoformans* resulted in significantly lower pulmonary fungal burden in comparison to mice infected with wild-type yeast cells. In summary, our data support a role for *T. marneffei* laccases in the protection of conidia against phagocytic cells early after infection.

Interestingly, the data showed that a single deletion of a *lac* gene was not sufficient to alter the characteristics assessed in our assays. The *T. marneffei* quadruple *lac* gene deletion strain was more sensitive to oxidative stress (H$_2$O$_2$), cell wall stress (SDS), and antifungal agents compared to the wild-type, whereas strains with single, double and triple *lac* gene deletions behaved similar to the wild-type. The quadruple mutant was also more susceptible to antifungal activity of THP-1 cells. Similarly, a study of LAC genes in *C. neoformans* revealed that deletion of both *lacA* and *lacB* was required to alter susceptibility to H$_2$O$_2$ or nitric oxide. In addition, the deletion of both laccases reduced the survival of *C. neoformans* in primary macrophages.

A recent study in *C. neoformans* isolates from patients found a significant positive correlation between laccase activity with *ex vivo* survival in cerebrospinal fluid (CSF) macrophages co-cultures and the *in vivo* rate of fungal clearance. Interestingly, higher laccase activity in *C. neoformans* enhanced survival *ex vivo* and was correlated with increased resistance to clearance following antifungal treatment in patients. Our data is in accord with these findings, as the wild-type *T. marneffei* conidia were significantly more resistant to phagocytosis and killing that the quadruple *lac* mutant, which suggests that laccase may play a prominent role *in vivo*.

The comparison of the inflammatory responses to the laccase quadruple mutant compared to the wild-type revealed significant differences in pro-inflammatory cytokine responses by the THP-1 cells. Consistent with this finding, the levels of TNF-α in mice infected with a *C. neoformans* laccase-deficient strain was significantly increased compared with TNF-α levels in mice infected with wild-type *C. neoformans*. Similarly, albin (alb1, conidial polyketide synthase deficient) *A. fumigatus* conidia induced significantly more IL-6, TNF-α and IL-10 in PBMC compared to melanized wild-type conidia. In principal, increased levels of pro-inflammatory cytokines like TNF-α, IL-6 and IFN-γ have been related to an efficient response to *A. fumigatus* infection, through induction of potent antifungal cellular responses to clear the pathogen. A less robust host pro-inflammatory response, as seen with the *T. marneffei* wild-type conidia in comparison to that produced with the quadruple disruptant, may obstruct the effectiveness of the antifungal defense mechanisms, especially in an immunocompromised host. To our knowledge, this is the first study showing that conidial laccases of *T. marneffei* modulate the pro-inflammatory cytokine response.

Laccase is a critical enzyme in melanin biosynthesis and we have previously shown that *pbrB* mutants have a defect in conidial pigmentation. This phenotype was not exacerbated by the quadruple mutants suggesting that the *pbrB* gene is specific for conidial pigmentation in *T. marneffei* and the other genes are not redundant for this role. As only the quadruple mutant displayed significantly increased susceptibility to innate host defenses, it is clear that each of these genes and their specific expression patterns play an overlapping but coordinated role in *T. marneffei*. Similarly, in *A. fumigatus*, mutation of the *abr2* gene encoding a conidial laccase resulted in poor conidial pigmentation, but this deletion did not alter virulence or impact the susceptibility of the mutant to ROS or diamide compared to the wild-type.

In conclusion, these studies reveal that *T. marneffei* has a number of laccase-encoding genes that produce laccases with both specific and redundant activities during normal growth condition and under stress conditions. Low level of *lacA* and *lacC* transcripts were present in both hyphal (28°C) and yeast (37°C) vegetative cell types. Expression of *lacA* and *lacB* is upregulated in response to oxidative stress at 37°C during yeast cell growth. Deletion of *lacA*, *lacB*, *lacC*, and *pbrB* genes not only altered DHN-melanin biosynthesis during asexual development, but delayed conidiation as well. The ΔlacA ΔlacB ΔlacC ΔpbrB mutants were sensitive to various stressors including H$_2$O$_2$, SDS, and antifungal agents (e.g., clotrimazole, itraconazole, and fluconazole). The results indicate that laccases play a role in protecting *T. marneffei* conidia against phagocytic cells such as THP-1. In addition, this study identified a laccase-dependent effect during the innate phase of initial engagement of the immune system, suggesting that laccase contribute to virulence by promoting *T. marneffei* resistance to macrophage killing, through a reduction in phagocytosis and intracellular death as well as modulating the cytokine milieu. Further studies are required in order to elucidate how laccases are capable of protecting *T. marneffei* against these host responses. Analysis of the role of this group of enzymes may clarify the pathogenicity mechanisms used by this fungal pathogen and to validate laccase and/or melanization as a target for combating the disease.

**Materials and methods**

**Fungal strains and media.** Experiments were conducted using *T. marneffei* strain F4 (CBS no. 119456), wild-type G681 (ΔpkuA::pyrG+) and *lac* deletion strains (ΔlacA::pyrG+, ΔlacB::pyrG+, ΔlacC::MT1612 pyrG+, ΔpbrB::
Expression of lac genes

To examine the expression of the laccase-encoding genes, *T. marneffei* F4 cells were grown in BHI at 28°C and 37°C for 3 days, harvested and washed with cold PBS before extracting RNA. Yeast cells from the 37°C culture were treated with 1 mM H₂O₂ at pH 5. At 0, 30 and 60 minutes of incubation, treated cells were washed with cold PBS before performing RNA extraction. RNA was extracted from each sample using Nucleospin Extract II kit. The extracted RNA samples were checked for gDNA contamination by PCR before performing cDNA synthesis using 200 ng (28°C and 0 min at 37°C samples) or 100 ng (H₂O₂ treatment for 30 minutes) of RNA (Omniscript RT kit). As a control for RT-PCR, cDNA of 18s RNA transcript was amplified from 50 ng RNA using Pm1/Pm2 primers. RT-PCR was performed in duplicate. Primers used to amplify lac transcripts are shown in Table S1.

Generation of transformants

The lac genes containing the 5′ and 3′ untranslated regions were amplified from genomic DNA of the *T. marneffei* G681 strain (Δ*pykA::pyrG*) using primers listed in Table S1. PCR-amplified lac fragments were ligated into pGEM-T Easy (Promega) to generate the constructs pASlac19 (lacA), pASlac42 (lacB) and pASlac7 (lacC). To generate the deletion constructs, pASlac19 was digested with EcoRV/XhoI to remove lacA sequences from +20 to +1,840 (relative to the ATG) and the non-coding flanking regions ligated to a SmaI/XhoI fragment containing the pyrG selectable marker cassette (pAB4342). For lacB, pASlac42 was digested with EcoRV/Clai to remove lacB sequences from −449 to +1,746 and the non-coding flanking regions ligated to a SmaI/Clai fragment containing the pyrG selectable marker cassette. For lacC, pASlac7 was digested with HindIII/NruI to remove lacC sequences from −1,095 to +3,049 and the non-coding flanking regions ligated to a HindIII/EcoRV fragment containing glufosinate resistant cassette (pMT1612). The deletion constructs were linearized by digestion with NotI and purified before transformation. DNA-mediated transformation of *T. marneffei* G526 strain (Δ*pykA::pyrG*) was performed as described previously.

Transformants of lacA and lacB deletions were selected on medium without uracil and genomic DNA samples were extracted from 2 isolated colonies to check the replacement of target gene by transformation cassette. Deletion of lacC produced ΔlacC::MT1612 pyrG− uracil auxotrophic strain. To remove this auxotrophy, the ΔlacC::MT1612 pyrG− strain was transformed with the *T. marneffei* pyrG targeting vector gene (pLS7413) to generate ΔlacC::MT1612 pyrG+ transformants. PCR and southern blot analysis of genomic DNA from each strain was performed to
confirm homologous recombination and target gene loss. Amplification of T. marneffei fetC is an internal PCR control. Primers used to amplify each lac are described in Table S1.

**Stress susceptibility test**

To prepare conidial suspension, conidia were harvested from ANM plates incubated at 28°C for 2 weeks. Ten-fold serial dilutions of conidia were pipetted onto BHI (Difco, USA) medium pH 5.4 containing each kind of stressors such as 2.1 mM H2O2 (oxidative stressor), 20 μg/ml SDS (cell wall stressor) and antifungal agents (0.1 μg/ml clotrimazole, 0.04 μg/ml itraconazole, and 40 μg/ml fluconazole). Plates were incubated in 37°C incubator for 1 week before observation of transformant growth.

**Growth assay**

To assay growth rates, 2 independent colonies of each transformant were used to inoculate solid ANM agar medium at a density such that approximately 10 conidia were seeded per plate. The plates were incubated at 28°C for 2 weeks. Ten-fold serial dilutions of conidia were pipetted onto BHI (Difco, USA) medium pH 5.4 containing each kind of stressors such as 2.1 mM H2O2 (oxidative stressor), 20 μg/ml SDS (cell wall stressor) and antifungal agents (0.1 μg/ml clotrimazole, 0.04 μg/ml itraconazole, and 40 μg/ml fluconazole). Plates were incubated in 37°C incubator for 1 week before observation of transformant growth.

**Melanin detection**

Approximately 10⁸ conidia of each mutant were inoculated into 200 ml BHI broth and incubated in 37°C shacking incubator for 5 days. Fungal cells were washed with PBS before performing melanin immunolabeling and extraction described in previous work. Positive staining and particles left after extraction process indicate that melanin can be produced.

**THP-1 infected with T. marneffei**

The human monocytic cell line THP-1 (ATCC TIB-202) was cultured in RPMI 1640 medium (Gibco, USA) containing 10% (v/v) heat-inactivated FBS (Gibco). For the induction of cellular differentiation, cells (2 × 10⁶ per ml) were seeded into 24-well culture plates (Costar, Corning, NY) in 1 ml of RPMI 1640 medium with 10% (v/v) FBS and 100 ng/ml phorbol myristate acetate (PMA) (Sigma, St. Louis, Mo) for 72 hours. After incubation, non-attached cells were removed by aspiration and the adherent cells were washed with RPMI 1640 3 times. THP-1 cells in RPMI 1640 without PMA were used as control (undifferentiated) cells. The conidia from wild-type or mutant strains suspended in RPMI 1640 medium with 10% (v/v) FBS were added to the THP-1 monolayers.

**Phagocytosis assay**

The phagocytosis assay was initiated by adding 4 × 10⁶ conidia to 2 × 10⁶ macrophage cells in each well (MOI = 2). THP-1 cells were allowed to interact with conidia for 15, 30, 60, and 120 minutes. At each time interval, supernatants were discarded, and the wells were washed gently 3 times with PBS pH 7.2 to remove unbound conidia. THP-1 were removed from the wells after treatment with 0.25% trypsin-EDTA (Gibco) for 5 min at 37°C and then washed twice to remove trypsin-EDTA. The cells were fixed by adding 0.5% paraformaldehyde in PBS (Sigma-Aldrich GmbH, Germany). The phagocytosis was assessed by light microscopy, and the percentage of phagocytosis was the total number of macrophage cells from a 100 cell count that internalized fungal conidia. The phagocytic index was determined by calculating the average number of intracellular conidia per macrophage as follows:

\[
\text{Phagocytic index} = \frac{\text{Total number of intracellular conidia}}{\text{Number of THP-1 containing conidia}}
\]

**Measurement of conidial survival in THP-1**

THP-1 cells were infected with wild-type, mutant or complemented strains of T. marneffei conidia at an MOI=2 for 2 hours. Unbound conidia were removed by washing the wells 3 times with PBS/0.05% Tween 20 (PBS-T). Extracellular conidia were killed with 50 μg/ml of nystatin as described. THP-1 cells were then supplemented with fresh media for an additional 2, 4 and 8 hours at 37°C prior to lysis by the addition of 1.0% Triton X-100 followed by serial dilutions of the released conidia and plating onto PDA medium and incubating at 25°C (3 replicate plates/well). The colony forming unit (CFU) of wild-type T. marneffei from cell lysates after 2 hours of phagocytosis was used to establish a baseline value (CFU control) for comparison with the CFUs at subsequent time intervals. The percentage of killing in CFU was calculated as follow:

\[
\text{Percentage of killing} = \frac{1 - \text{CFU test}}{\text{CFU control}}
\]

**Induction of cytokines in human monocye cell line THP-1**

To assess cytokine production, THP-1 cells were infected with wild-type, mutant or complemented strains of T. marneffei conidia at an MOI = 5 and incubated for 2, 8 or 24 hours at 37°C. After incubation, the supernatants were collected and kept at −20°C until the cytokine assays were performed. All of the experiments were repeated at least 3 times and each set was done in duplicate. TNF-α, IL-6 and IL-1β were measured by commercial ELISA kits (BioLegend, San Diego, CA), according
to the manufacturer’s instructions. The concentrations of cytokines in the experimental samples were calculated according to the optical densities at 450 nm obtained from wells containing cytokine standard. The measurable concentrations of TNF-α and IL-6 ranged from 7.8 to 500 pg/ml, while IL-1β ranged from 2.0 to 125 pg/ml.

**Statistical analysis**

All data are expressed as mean ± standard error of the mean (SEM) of the number of determinations carried out in triplicate for the percentage of phagocytosis, phagocytic index and percentage of killed CFU. Variables were tested for normality and then the different groups were compared using the One-Way ANOVA, where P < 0.05 was considered as statistically significant between the groups.

Differences in the cytokine production between wild-type and laccase mutants were analyzed by One-Way ANOVA. The level of significance was set at P < 0.05.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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