Role of the yakA gene in morphogenesis and stress response in *Penicillium marneffei*

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*Penicillium marneffei* is a thermally dimorphic fungus and a highly significant pathogen of immunocompromised individuals living in or having travelled in south-east Asia. At 25 °C, *P. marneffei* grows filamentously. Under the appropriate conditions, these filaments (hyphae) produce conidiophores bearing chains of conidia. Yet, when incubated at 37 °C, or upon infecting host tissue, *P. marneffei* grows as a yeast that divides by binary fission. Previously, an *Agrobacterium*-mediated transformation system was used to randomly mutagenize *P. marneffei*, resulting in the isolation of a mutant defective in normal patterns of morphogenesis and conidiogenesis. The interrupted gene was identified as yakA. In the current study, we demonstrate that the yakA mutant produced fewer conidia at 25 °C than the wild-type and a complemented strain. In addition, disruption of the yakA gene resulted in early conidial germination and perturbation of cell wall integrity. The yakA mutant exhibited abnormal chitin distribution while growing at 25 °C, but not at 37 °C. Interestingly, at both temperatures, the yakA mutant possessed increased chitin content, which was accompanied by amplified transcription of two chitin synthase genes, *chsB* and *chsG*. Moreover, the expression of yakA was induced during post-exponential-phase growth as well as by heat shock. Thus, yakA is required for normal patterns of development, cell wall integrity, chitin deposition, appropriate *chs* expression and heat stress response in *P. marneffei*.

INTRODUCTION

*Penicillium marneffei* is the only dimorphic species of the genus *Penicillium*. It is one of the common opportunistic infections in patients with AIDS, especially in south-east Asia (Boyce & Andrianopoulos, 2013; Vanittanakom et al., 2006). Penicilliosis due to *P. marneffei* is fatal if untreated. The growth of *P. marneffei* can be divided into two phases based on natural growth and during infection (Andrianopoulos, 2002; Cooper & Vanittanakom, 2008; Vanittanakom et al., 2006). At 25 °C, *P. marneffei* grows as a filamentous (mould) form and displays a yellowish green powdery to velvety colony with red pigment diffused into the medium. The mould form produces hyaline, septate hyphae with conidiophores bearing phialides and chains of conidia. *P. marneffei* converts to the yeast form at 37 °C both in vitro and in vivo. The latter cells, which divide by binary fission, grow as a yeast-like colony that secretes a reddish brown pigment.

Thermal dimorphism of *P. marneffei* plays an important role in its pathogenesis. Once conidia are inhaled into the lungs of a mammalian host, they are typically engulfed by alveolar macrophages. These macrophages serve as the primary defence response of the host. In immunocompromised patients, *P. marneffei* conidia develop as yeast cells within the macrophage. Subsequently, following colonization of the host cell, the macrophage is killed allowing the yeast cells to disseminate throughout the host. Hence, the pathogenicity of this fungus appears to be intimately related to the dimorphic transition from the mycelial phase in the environment to the yeast phase in the human host.

Many investigations have focused on morphogenetic mechanisms and other dimorphism-associated attributes that contribute to pathogenicity of this fungus. Recently, by using an *Agrobacterium tumefaciens*-mediated transformation system for random mutagenesis via T-DNA integration, mutants were derived possessing defects in various genes associated with morphogenesis (Kumphamsook et al., 2010). One such mutant exhibited an abnormal colony
phenotype. Subsequently, the defect was identified as an interruption in the yakA gene, which encodes a protein kinase.

YakA from *P. marneffei* belongs to a member of the dual-specificity tyrosine phosphorylation-regulated protein kinases (Aranda et al., 2011). These include Yak1 from the industrial yeast *Saccharomyces cerevisiae* and the human pathogenic yeast *Candida albicans*. In *S. cerevisiae*, Yak1 was initially identified as a growth antagonist of the protein kinase A pathway (Garrett & Broach, 1989). Yak1 is a type of Ser/Thr protein kinase whose activity is moderated by autophosphorylation on the second Tyr residue in the YXY motif for full kinase activity (Kassis et al., 2005). In *S. cerevisiae*, the kinase activity of Yak1 is low during exponential growth and increases when the cells are induced to arrest early in the cell cycle (Garrett et al., 1991). The Yak1 kinase in yeast is a component of the glucose-sensing system that is involved in growth control in response to available carbon source. The localization of this protein kinase is altered in response to glucose, as Yak1 accumulates in the nucleus upon glucose starvation (Moriya et al., 2005). Also, Yak1 activates two stress-responsive transcription factors, Hsf1 and Msb2, by phosphorylation upon nutrient deprivation (Lee et al., 2008). In addition, Yak1 in *S. cerevisiae* has been reported to be involved in thermotolerance (Hartley et al., 1994).

A YAK1 orthologue has been identified in the dimorphic fungus *C. albicans*. The *C. albicans* YAK1 gene is necessary for pseudohyphal growth. Strains lacking YAK1 showed defects in the yeast-to-hypha transition and in maintaining hyphal growth. These strains also could not form biofilms. However, *C. albicans* strains with mutated YAK1 alleles remained virulent in murine models of systemic and oropharyngeal candidiasis (Goyard et al., 2008). These results suggest that YAK1 is dispensable for *C. albicans* virulence. Additionally, YAK1 is not only necessary for the upregulation of a subset of hypha-specific genes in *C. albicans*, but also it may be involved in regulating the differential expression of genes during the yeast-to-hypha transition (Goyard et al., 2008).

In this study, we sought to characterize the role of the *P. marneffei* yakA gene in the growth and development of this dimorphic fungus. We demonstrated that a *AyakA* mutant exhibits abnormal morphogenesis characterized by reduced colony expansion, diminished conidiation and more rapid conidial germination. Furthermore, our investigations show that the *yakA* gene plays a role in cell wall chitin deposition, chitin synthase gene expression and heat stress response. The collective results suggest that the *yakA* gene of *P. marneffei* plays a prominent role in normal growth and development by influencing mechanisms affecting cell wall integrity.

### METHODS

**Fungal strains, media, reagents and inocula.** Three strains of *P. marneffei* were employed in this study: the wild-type [F4 (yakA); CBS No. 119456], I231 (AyakA; Kummassook et al., 2010) and a genetically complemented transformant, CY21 (AyakA + yakA). The derivation of strain CY21 is described below. All three strains were maintained at 25 °C on potato dextrose agar [PDA (Difco brand); Becton Dickinson].

We also used a nutrient-rich medium, brain heart infusion agar [BHA (Difco brand); Becton Dickinson], to promote the formation of the yeast phase of *P. marneffei*. Experiments involving liquid cultures employed Sabouraud dextrose broth [SDB (Difco brand); Becton Dickinson]. The latter medium supports both hyphal and yeast development.

Unless otherwise noted, all chemical reagents and antibiotics were purchased from Amresco. Oligonucleotide primers were obtained from Integrated DNA Technologies. Primers employed in the various amplification experiments were designed using the Primer 3 software (Table 1).

For preparing inocula consisting of conidial spore suspensions, the strains were grown on PDA supplemented with 8 % glucose at 25 °C. Conidial suspensions were isolated and quantified as previously described (Gifford & Cooper, 2009).

**Complementation of strain I231 (AyakA).** The DNA extraction methods, inverse PCR procedures and Southern blot analyses used to identify the yakA gene have been previously described (Kummassook et al., 2010). Analyses of DNA sequences were carried out using the BLAST PROGRAM (Altschul et al., 1990, 1997; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

For complementation of the *AyakA* mutant (strain I231), a 1584 bp upstream fragment together with the entire sequence of the yakA gene and a 154 bp downstream region was amplified from the *P. marneffei* wild-type (strain F4) with primers yakAF-NgoMIV (5'-CAGCGCCGGCATGATGGAGACACCTTGTA-3'; NgoMIV restriction site sequence underlined) and yakAR-HindIII (5'-CAATGGAAGCTTGTCATATGCTGACATACCGGA-3'; HindIII restriction site underlined). A PCR was conducted using Phusion Hot Start High-Fidelity DNA Polymerase (Finzymes) in accordance with the supplier’s instructions. Briefly, the PCR conditions were as follows: 98 °C for 30 s; 35 cycles of 98 °C for 10 s, 60 °C for 30 s and 72 °C for 3 min; 72 °C for 10 min; and hold at 4 °C. The PCR products were precipitated and then digested with restriction enzymes NgoMIV and HindIII. The digested PCR products were excised from 1 % gel agarose gel and purified with the QIAquick Gel Extraction kit (Qiagen). The gel-purified fragments were ligated into NgoMIV-/HindIII-treated pAN7-1 plasmid (Punt et al., 1997) containing the hph gene for resistance to the antibiotic hygromycin B. The complete complementing plasmid pAN7.1-yakA was transformed in Escherichia coli DH5α (Riley et al., 2008) and isolated as described by Sambrook & Russell (2001).

Strain I231 (AyakA) was transformed with plasmid pAN7.1-yakA using the protocol of Borneman et al. (2001) with the following modifications. To obtain transformants, protoplasts exposed to pAN7.1-yakA were plated onto BHA containing 200 μg hygromycin B ml⁻¹ and 1.2 M sorbitol. The plates were incubated at 25 °C for 5 days, and then at 37 °C for 5 days. Resulting hygromycin-resistant colonies were selected and purified, then subjected to confirmation using PCR and Southern blot analysis (Kummassook et al., 2010).

**Microscopy.** Samples of *P. marneffei* cells grown in SDB at 25 and 37 °C were collected by centrifugation (4 °C for 5 min at 3000 g) prior to fixation for microscopy and staining. Cells were fixed by suspension in 4 % (w/v) p-formaldehyde in PBS (pH 7.4) for at least 1 h at room temperature. The fixed cells were washed with PBS, then mounted on slides and viewed using the differential interference contrast optics of an Olympus IX51 microscope. Digital photomicrographs were taken with an RKE Spot Digital Camera and the associated Spot Software (version
hydrochloric acid benzaldehyde in a 1:1 mixture of ethyl alcohol and concentrated incubated at 90°C (0.5 M sodium carbonate) was added to each sample, which was millilitre of Ehrlich's reagent milligram dry weight of the cells extracted. with a spectrophotometer. The final chitin content was calculated per incubation at room temperature. Optical densities were read at 530 nm as a standard. Briefly, 1 ml of acetyl acetone reagent (2% (w/v) acetyl acetone in 0.5 M sodium carbonate) was added to each sample, which was incubated at 90°C for 45 min, then cooled to room temperature. One millilitre of Ehrlich’s reagent (2.67% (w/v) solution of p-dimethylamino- benzaldehyde in a 1:1 mixture of ethyl alcohol and concentrated hydrochloric acid) was added to the solution, followed by 1 h of incubation at room temperature. Optical densities were read at 530 nm with a spectrophotometer. The final chitin content was calculated per milligram dry weight of the cells extracted.

Colony growth, conidiation and conidial germination. For quantification of radial growth and conidiation, conidial inocula were harvested from 7-day-old PDA culture plates either supplemented with glucose or lacking glucose. Conidia (3 × 10⁶) of each strain were placed in the centre of a plate containing PDA or PDA supplemented with 1 M sorbitol as an osmotic stabilizer, then incubated at 25°C. The diameters of the colonies were measured over a period of 2–7 days. The resulting conidia from these colonies were quantified as previously described (Kummasook et al., 2013). These experiments were repeated in triplicate.

A modified germination assay was performed based upon a previously described method (Boyce & Andrianopoulos, 2007). Approximately 10⁶ conidia were inoculated into 300 μl SDB and incubated for 12 h at either 25 or 37°C. Germination rates were measured microscopically by counting the numbers of conidia with a visible germ tube in a population of 100 or more fungal cells. The mean and SD for germination rates were calculated using SPSS (version 17.0; SPSS; http://www-01.ibm.com/software/analytics/analytics/spss/). The experiments were repeated in triplicate.

**Isolation of RNA and quantitative PCR analysis.** Conidia (1 × 10⁶) of each strain were inoculated into 50 ml SDB, then incubated for 2 days at 25 and 37°C with agitation at 150 r.p.m. To induce a heat shock response, similarly prepared 2-day-old cultures were incubated at 39°C for 20 min. Fungal cells were harvested by centrifugation to remove the supernatant. Total RNA was isolated from the cellular material by mechanical disruption with bead beating and isolation using an RNeasy Plant Mini kit (Qiagen) according to the manufacturer’s protocol. Subsequently, the isolated RNA was treated with DNase (Qiagen) prior to cDNA synthesis. cDNA was synthesized by reverse transcription of purified total RNA using the iScript cDNA synthesis kit (Bio-Rad). The mRNA of each gene was normalized to the mRNA level of the *P. marneffei* benA gene (Boyce et al. 2009).

### Table 1. Primers used in this study

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<td>hyg-R</td>
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<td><strong>For qPCR experiments:</strong></td>
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<td>yakAq-R</td>
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<td>benA-R</td>
<td>AGTTGTTACCCAGCCGGAC</td>
<td>Boyce et al. (2009)</td>
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4.6; Diagnostic Instruments). Photomicrographs were also taken of *P. marneffei* slide cultures. These cultures, grown on PDA (without additional glucose) or BHA for 7–10 days at 25 or 37°C, were prepared as previously described (Harris, 1986). For staining with Calcofluor White (fluorescent brightener 28; Sigma-Aldrich), fixed cells were suspended in a 0.1% (w/v) solution of the fluorescent dye at room temperature for at least 15 min. Subsequently, samples were mounted onto slides prior to being viewed using the above optical system and UV light illumination in conjunction with appropriate wavelength filters. Digital photomicrographs were taken as described above.

**Chitin content.** Cellular chitin content was determined based upon the amount of glucosamine (GlcN) detected in 2-day-old cultures of each strain incubated at 25 or 37°C. Cells from 50 ml SDB cultures were harvested, then dried and weighed. Dry samples were suspended in 1 ml 4 M HCl and boiled for 4 h. The hydrolysates were diluted with sterile distilled water. The amount of hexosamine contained in 1 ml of diluent was determined by the Elson-Morgan method (Boas, 1953), using GlcN as a standard. Briefly, 1 ml of acetyl acetone reagent (2% (v/v) acetyl acetone in 1 M sodium carbonate) was added to each sample, which was incubated at 90°C for 45 min, then cooled to room temperature. One millilitre of Ehrlich’s reagent (2.67% (w/v) solution of p-dimethylamino-benzaldehyde in a 1:1 mixture of ethyl alcohol and concentrated hydrochloric acid) was added to the solution, followed by 1 h of incubation at room temperature. Optical densities were read at 530 nm with a spectrophotometer. The final chitin content was calculated per milligram dry weight of the cells extracted.

et al., 2009), which encodes β-tubulin. Quantitative reverse-transcription PCR (qRT-PCR) was used for determination of mRNA expression levels of each gene using the iQ5 system and the iQ SYBR Green Supermix (Bio-Rad). All experiments were performed in triplicate and statistical analyses were conducted using SPSS software (version 17.0).

Cell-wall-disrupting agents and antifungal sensitivity assays. Fresh spore suspensions of strains F4 (yakA), I231 (AyakA) and CY21 (ΔyakA + yakA) were serially diluted prior to spotting 5 μl of each dilution (10−10−1 cells ml−1) onto PDA plates containing the following cell wall stressors: Congo red (CR; Sigma; item number C 6767), 40 and 70 μg ml−1; SDS, 0.0175 and 0.02 %; and caspofungin (CAS; Sigma; item number SML 0425), 70 and 120 μg ml−1. PDA plates lacking the cell-wall-disrupting agents were used for controls. All plates were incubated at 25 or 37 °C for up to 4 days.

RESULTS

The yakA gene was disrupted in strain I231

Strain I231 is a T-DNA random insertional mutagenesis transformant. This strain was originally isolated as a bleomycin-resistant, yeast-like colony growing on BHA containing bleomycin at 37 °C (Kummasook et al., 2010). At 25 °C, the mutant appeared defective in colony growth and conidiation on PDA when compared with the wild-type.

Inverse PCR of XhoI-treated DNA from strain I231 generated a 3.5 kb product, whereas there was no amplification product from the wild-type, indicating T-DNA integration (Fig. 1a). Southern blot analysis using a fragment of the bleomycin-resistance gene (ble) as a probe verified a single inserted copy of T-DNA (Fig. 1b). Subsequent sequence analysis of the nucleotides flanking the integration region demonstrated identity with the gene encoding the putative protein kinase, Yak1 (GenBank accession no. XM_002148368.1). So as to be consistent with the genetic nomenclature used in other reports involving genes from P. marneffei, we have designated the gene encoding this kinase as yakA and the encoded protein as YakA. The sequence data also showed that T-DNA inserted within the coding sequence of the yakA gene resulting in the deletion of 24 nt (data not shown). Thus, strain I231 was given the genetic designation ΔyakA.

Genetic complementation of strain I231

A fragment containing the native promoter, the full-length ORF and the terminal sequence of the P. marneffei yakA gene was cloned into the plasmid pAN7.1. This construct was used to transform protoplasts of strain I231 by ectopic integration of the hybrid vector. One of the hygromycin B-resistant colonies, CY21 (designated ΔyakA + yakA), was selected for comparison with the wild-type and strain I231. This colony possessed both the yakA (Fig. 1c) and the hph (Fig. 1d) genes. The CY21 strain contained only one copy of the complementing plasmid (Fig. 1e).

The yakA gene is required for appropriate colony expansion, conidiation and correct abaA expression in P. marneffei

At 25 °C during filamentous growth, wild-type colonies are composed of vegetative hyphae, aerial hyphae and conidiophores. The colonies are green due to the pigmentation of asexual spores (conidia) on conidiophores. To measure the colony diameter expansion rate, PDA plates were point inoculated with 3 × 105 conidia of each strain. There was a significant reduction in colony diameter of the ΔyakA mutant in comparison with the wild-type over a 7-day incubation period (Fig. 2a). By comparison, the colonies of the complemented strain, CY21 (ΔyakA + yakA), grew in diameter similar to that of the wild-type. A significant difference in colony diameters among the three strains also occurred upon culture on an osmotically stabilized medium (1 M sorbitol). Yet, the reduced growth of the ΔyakA mutant recovered slightly compared with growth on non-stabilized medium, suggesting that strain I231 possesses a weakened cell wall due to altered chemical composition, structural integrity or both (Fig. 2a).

In addition, the colonies of the ΔyakA mutant were sporadically grey–white on PDA plates, indicating a reduction in conidia production via loss of the green–yellow colour on the colony surface as compared with the wild-type and strain CY21 (Fig. 2b). When analysed, colonies of the ΔyakA strain produced approximately one-ninth the amount of conidia compared with the wild-type and complemented strains (Table 2).

Because Yak1 in S. cerevisiae is a component of the glucose-sensing system, we sought to determine if glucose would increase the production of conidia in the P. marneffei ΔyakA mutant. With the addition of glucose to the PDA medium, the surface of ΔyakA colonies appeared more green–yellow, suggesting that more conidia were produced than on non-glucose-supplemented PDA (Fig. 2b). Yet, although higher than on normal PDA, the number of conidia on glucose-supplemented PDA was still lower than produced by the wild-type and complemented strain grown on the same medium (data not shown). Thus, increased glucose in this medium can partially restore conidial production in the ΔyakA mutant. Microscopically, the ΔyakA strain appeared to be morphologically similar to the wild-type and complemented strain except that fewer conidia and phialides were observed (Fig. 2c). These phenotypic affects were largely reversed upon culture on glucose-supplemented media. These collective results indicate that the mutation in the yakA gene impacts proper asexual development in P. marneffei.

Interestingly, morphological defects due to the ΔyakA mutation seem to be restricted to the mycelial phase of P. marneffei. When cultured at 37 °C, the wild-type (F4; yakA), the complemented (CY21; ΔyakA + yakA) and the ΔyakA mutant (I231) all grew as yeast-like cells on BHA or 1 % peptone after 4 or 3 days of incubation, respectively.
Therefore, at the gross phenotypic level, the \textit{yakA} gene appears to be involved in the morphogenesis of \textit{P. marneffei} only at 25 °C.

As the \textit{abaA} gene plays a significant role in phialide development and conidial production in \textit{P. marneffei} (Borneman et al., 2000), we examined the level of \textit{abaA} mRNA in the \textit{ΔyakA} mutant, the wild-type and the complemented strain on PDA with or without supplementary glucose. On PDA alone, the \textit{abaA} transcript was present at significantly low levels in the \textit{ΔyakA} mutant compared with expression levels in the wild-type and complemented strains (Fig. 2d). In contrast, the expression of \textit{abaA} in the \textit{ΔyakA} mutant was upregulated in cultures grown on PDA supplemented with glucose, but not in the wild-type and complemented strains (Fig. 2d). Similar results were noted for two other genetic regulators of conidial development, \textit{brlA} (Fig. 2e) and \textit{wetA} (Fig. 2f), thus, the \textit{yakA} gene appears to be involved in proper expression of \textit{abaA} and perhaps other genes in the conidial developmental pathway.

**The \textit{yakA} gene participates in conidial germination at 25 and 37 °C**

Under appropriate conditions, conidia germinate by initially growing isotropically, which is followed by a switch to apical, polarized growth to produce a germ tube. To determine whether the disruption of \textit{yakA} affected conidial germination, the rates of germination were assessed. The germ tubes in the \textit{yakA} mutant were apparently longer than those of both the wild-type and complemented strains after 12 h in liquid media at both 25 and 37 °C (data not shown). These results were accompanied by a significant increase in germination in...
comparison with the wild-type and complemented strains (Fig. 3). Thus, the yakA gene affects conidial germination kinetics. The possible explanation for these results is that interruption of the yakA gene results in the generation of a softer, more pliable cell wall, leading to more rapid germination.

The yakA mutant displays an increased sensitivity to SDS and CAS, but greater resistance to CR

We compared the sensitivity of the ΔyakA mutant to different cell-wall-disturbing compounds with that of both the wild-type and the complemented strains (Fig. 4). The yakA mutant...
showed hypersensitivity to the cell-membrane-perturbing agent, SDS, at both 25 and 37 °C. A slight increase in sensitivity of the ΔyakA mutant to CAS, a specific inhibitor of β-(1,3)-glucan synthesis, was observed at 25 °C, whereas susceptibility to this agent increased moderately at 37 °C. Interestingly, however, the mutant strain displayed increased resistance to CR at both 25 and 37 °C. CR is a compound that interferes with glucan structure (Kopecká & Gabriel, 1992). Although the susceptibility to CAS and CR appear somewhat in opposition, they nonetheless suggest that the cell wall of the ΔyakA mutant is structurally altered.

The yakA gene is important for chitin deposition in the mould phase, normal levels of chitin synthesis, and appropriate chsB and chsG expression

To investigate whether yakA affects patterns of cell wall chitin deposition, cells were stained using Calcofluor White, a fluorescent dye that binds to chitin-like microfibrils. When stained, the septa of the wild-type and complemented strains appear to be laid down at regular intervals along the hyphae and in developing yeast cells.

![Image 361x451 to 552x706](http://mic.sgmjournals.org)

**Fig. 4.** Disruption of yakA results in increased sensitivity to SDS and CAS, but increased resistance to CR. For each dilution in the spore suspension (10^7–10^3 cells ml⁻¹), 5 μl was spotted onto PDA plates containing: SDS, 0.02 and 0.0175 %; CR, 70 and 40 μg ml⁻¹; CAS, 120 and 70 μg ml⁻¹. The growth of each strain was observed after 4 days of incubation at 25 and 37 °C.

Side-wall chitin appears similar in both strains as well. In contrast, although the side-wall and septum staining patterns appeared similar to that of the wild-type and complemented strains, the hyphae of the ΔyakA mutant possess patchy areas of Calcofluor White staining material while growing at 25 °C (Fig. 5a). These patches appeared to be localized within the cytoplasm. However, no such staining patterns were noted in developing yeast cells of the ΔyakA mutant at 37 °C (Fig. 5b). The Calcofluor White staining patterns appeared similar for all three strains at 37 °C. Hence, these results suggest that yakA is required for the proper localization of chitin in the mould phase of *P. marneffei*, but not in the yeast phase.

The aberrant chitin deposition observed in the ΔyakA mutant suggested that the actual chitin content may differ from that of the wild-type and complemented strains. Therefore, we determined the changes in the level of chitin from that of the wild-type and complemented strains. The actual chitin content may differ from that of the wild-type and complemented strains after 2 days of growth at both 25 and 37 °C. Thus, the increase in chitin content of the ΔyakA mutant may occur as a cellular response to the defects of a weakened cell wall.

![Image 86x147 to 278x263](http://mic.sgmjournals.org)

**Fig. 3.** The ΔyakA mutant plays a role in conidial germination. The germination rates of the indicated strains are depicted after incubation in SDB for 12 h at 25 and 37 °C. The results are expressed as the percentage (+ s.d) of germinated conidia in three independent experiments. *P<0.05.

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**Table 2.** Conidial production among *P. marneffei* strains

Data are shown as means ± s.e. Conidia were counted after incubation for 7 days on PDA.

<table>
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<tr>
<th>Strain</th>
<th>No. of conidia (10⁴ conidia cm⁻²)</th>
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<tr>
<td>F4 (yakA)</td>
<td>197.3 ± 0.07</td>
</tr>
<tr>
<td>I231 (ΔyakA)</td>
<td>21.3 ± 0.20*</td>
</tr>
<tr>
<td>CY21(ΔyakA+yakA)</td>
<td>187.0 ± 0.19</td>
</tr>
</tbody>
</table>

*P<0.01.
Fig. 5. The ΔyakA strain shows abnormal chitin deposition. Strains were grown for 4 days in SDB at 25 °C (a) or 37 °C (b), then stained with Calcofluor White to visualize chitin deposition in the cell wall and septa. Abnormal deposits of chitin were observed within the hyphae of the ΔyakA mutant at 25 °C, but not in developing yeast cells at 37 °C. Bars, 20 μm. (c) The chitin content of the wild-type, ΔyakA mutant and the complemented strain. Conidia were inoculated in SDB and incubated at 25 and 37 °C for 2 days. Developing cells were harvested and the chitin levels were chemically determined. Results were derived from three independent experiments. (d) Induction of chitin synthase transcript levels in the ΔyakA mutant. Conidia of each strain were grown for 48 h in SDB at 25 and 37 °C. RNA isolated from cultured cells was subjected to qRT-PCR analysis to determine the expression levels of chitin (chsB and chsG), 1,3-β-glucan (fksP), α-1,3-glucan (ags2) and β-1,6-glucan (kre6) synthase genes. Expression data from five other chitin synthase genes show no significant differences (data not shown). The data are representative of three different experiments. In (c) and (d), *P<0.05.
We further hypothesized that the increase in the mutant’s chitin content may occur via changes in expression of genes encoding different chitin synthases. To test this hypothesis, total RNA was isolated from *P. marneffei* grown in SDB medium at 25 and 37 °C. Expression of the seven known *P. marneffei* chitin synthase genes was determined by qRT-PCR. Curiously, the levels of *chsB* and *chsG* mRNA increased significantly in the Δ*yakA* mutant at both 25 and 37 °C (Fig. 5d), but no expression level changes were noted for the remaining five chitin synthase genes (data not shown).

The susceptibility results described above also suggest that alterations in cell wall glucan structure exist in the Δ*yakA* strain of *P. marneffei*. Therefore, we examined the mRNA expression levels of the 1,3-β-glucan synthase (*fksP*), α-1,3-glucan synthase (*ags2*) and β-1,6-glucan synthase (*kre6*) genes of *P. marneffei* at 25 and 37 °C (Fig. 5d). However, there were no significant differences in transcriptional levels of these mRNAs between the Δ*yakA* mutant, the wild-type and the complemented strains.

**yakA** expression is involved in cell growth and heat stress response

It has been reported in *S. cerevisiae* that Yak1 activity increases in the late-exponential growth phase (Moriya et al., 2001). Therefore, to investigate whether the level of *yakA* expression depends on cell growth, wild-type cells were grown to early (2 days) and post-exponential phases (6 days) at 25 and 37 °C. At both temperatures, the transcription of *yakA* is present at high levels at the late-exponential phase when compared with those at the early-exponential phase, especially at 37 °C (Fig. 6a). Furthermore, because the YAK1 gene plays a role in the resistance of *S. cerevisiae* to heat, we also assessed the transcription level of *yakA* upon heat shock. Changes of *yakA* expression in cells grown at 37 °C were significantly increased after exposure to heat at 39 °C for 20 min (Fig. 6b).

**DISCUSSION**

In the industrial yeast *S. cerevisiae*, Yak1 was first identified to play a role as a growth antagonist because deletion of the YAK1 gene can suppress loss of function of the cAMP–protein kinase A pathway (Garrett & Broach, 1989). Recently, the function of YAK1 has been characterized in the human pathogenic yeast *C. albicans*. The YAK1 gene product is necessary for biofilm formation and hyphal emergence in vitro, but not for virulence in vivo (Goyard et al., 2008). The aim of this study was to characterize an orthologous YAK1 mutant of *P. marneffei*, a significant fungal pathogen of immunocompromised individuals in South-East Asia. The mutant strain of *P. marneffei*, designated Δ*yakA*, was demonstrated to possess a disruption of the *yakA* gene through a T-DNA insertion (Kummasook et al., 2010). To confirm that all mutant phenotypes were due to the specific targeting of the *yakA* gene, we reintroduced the entire *yakA* sequence into the mutant to generate a wild-type-like phenotype. Hence, the results of all subsequent experiments were demonstrated to be the consequence of a defect in the *P. marneffei* *yakA* gene.

Initial characterization of the role of the *yakA* gene in *P. marneffei* revealed that this gene was involved in colony expansion and conidial development. In this study, we observed that the Δ*yakA* mutant grown on PDA produced fewer phialides and conidia. However, the addition of glucose to PDA can partially restore conidial production in the Δ*yakA* strain. In addition, we observed that the level of *abaA* mRNA in the *P. marneffei* Δ*yakA* mutant increased on medium supplemented with glucose. The *abaA* gene is known to be required for conidial production in *P. marneffei* (Borneman et al., 2000). We hypothesize that the high glucose concentration in the medium induces the transcription of *abaA* in the Δ*yakA* mutant of *P. marneffei*, thereby serving as a bypass for conidiogenesis.

The mutation of the *yakA* gene also resulted in more rapid conidial germination, suggesting that the conidial cell wall of the Δ*yakA* mutant is markedly weak and more pliable. Patterns of early germination have been noted in *Aspergillus fumigatus* cell wall mutants (Δ*ags3* and Δ*ecm33*) (Maubon et al., 2006; Romano et al., 2006). Therefore, we assessed the integrity of the cell wall in the Δ*yakA* mutant using the ionic detergent SDS, the glucan-binding dye CR and the β-1,3-glucan synthase inhibitor CAS (Hill et al., 2006). In addition, we not only determined the chitin content of the mycelial- and yeast-phase cell walls of *P. marneffei*, but also employed the chitin-binding dye Calcofluor White to assess the pattern of cell wall chitin deposition. The results demonstrated that *yakA* plays a prominent role in determining cell wall ultrastructure and cellular development in the mycelial phase of the fungus, but the phenotypic impact of this gene in development of the yeast phase is not pronounced.

The phenotypic differences of the mutant strain compared with the wild-type and complemented strains at both 25 and 37 °C prompted an examination of the expression of particular genes involved in chitin and glucan biosynthesis. We observed no significant differences in the expression of the three glucan synthase genes studied (Fig. 5d) or for five of the seven chitin synthase genes examined (data not shown). However, two chitin synthase genes, *chsB* and *chsG*, were expressed at significantly higher levels at both 25 and 37 °C in the Δ*yakA* mutant as compared with the wild-type and complemented strains (Fig. 5d). Interestingly, the *A. nidulans* orthologue of *P. marneffei* *chsB* is required for conidia formation, whereas the *A. nidulans* orthologue of *P. marneffei* *chsG* plays an important role in normal hyphal growth (Borgia et al., 1996; Lee et al., 2004). It is possible that the Δ*yakA* mutant responds to cell wall weakening by activation of the chitin biosynthetic pathway as a compensatory mechanism. The relationship between the mutations in the genes involved in cell wall synthesis and
increased chitin levels has also been observed in *S. cerevisiae* and *A. nidulans* (Bulik et al., 2003; Guest et al., 2004).

Our present results show that yakA expression was increased at the late-exponential phase, suggesting that transcription of yakA may change in response to glucose depletion. These results suggest that yakA is a growth-related gene. In addition, although there are no differences in yakA expression between 25 and 37 °C after 2 days of incubation, yakA expression appears to be upregulated in response to heat shock at 39 °C (a condition mimicking human fever) compared with cells grown at 37 °C (representing the normal homeostatic condition). These data indicate that the yakA gene is involved in response to heat shock stress. Collectively, these observations suggest that yakA has a role in response to heat stress during yeast development, but not mycelial growth.

Clearly, the *P. marneffei* yakA gene plays a significant role in normal development of the mould phase of this pathogenic fungus. Its role in the growth and development of the yeast phase is not as obvious from a gross morphological perspective. The yeast phase of all three strains examined in this study appear similar. Nonetheless, there are notable phenotypic effects in the ΔyakA mutant with regard to the composition and structure of the cell wall. Hence, one might expect differences in virulence between the wild-type and yakA mutant. However, in a moth larvae model (Cotter et al., 2000; Kavanagh & Fallon, 2010), we have observed no difference (data not shown), indicating that the yakA gene may have a role in morphogenesis, but not in pathogenesis. This is similar to the observations in another dimorphic, pathogenic fungus, *C. albicans* (Goyard et al., 2008). Although yakA-like mutants of *C. albicans* exhibit defects in the phase transition, hyphal growth and biofilm formation, they are virulent.

While yakA does not appear to contribute to virulence when the fungus is directly used to infect a model organism, a role for this gene in pathogenesis may yet exist. Given that infections by *P. marneffei* are believed to be initiated via the inhalation of conidia, any gene that functions in conidiogenesis can be considered a potential virulence factor. Hence, because the ΔyakA mutation markedly reduces conidiogenesis, the gene is indirectly associated with the virulence potential of *P. marneffei*. Therefore, studies directed towards those molecular mechanisms that regulate conidiogenesis are as critical as those investigations more focused on the genetic basis of dimorphism in *P. marneffei*. Thus, more efforts are warranted in understanding asexual development in this and other pathogenic fungi.

In summary, we have characterized a conidiation mutant of *P. marneffei* that possesses a defect in the yakA gene. This study illustrates the importance of yakA in *P. marneffei* not only in conidiogenesis, but also in germination and cell wall integrity. It is curious that the kinase encoded by yakA appears to have a role in the regulation of chitin synthesis and the deposition of this cell wall constituent. Exactly how yakA exerts this influence is unknown. However, this affect is noted only in the mycelial phase of *P. marneffei*, suggesting that the abnormal synthesis and deposition of chitin is a reaction to aberrations in the hyphal cell wall.

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