Group X hybrid histidine kinase Chk1 is dispensable for stress adaptation, host–pathogen interactions and virulence in the opportunistic yeast Candida guilliermondii


Abstract

Hybrid histidine kinases (HHKs) progressively emerge as prominent sensing proteins in the fungal kingdom and as ideal targets for future therapeutics. The group X HHK is of major interest, since it was demonstrated to play an important role in stress adaptation, host–pathogen interactions and virulence in some yeast and mold models, and particularly Chk1, that corresponds to the sole group X HHK in Candida albicans. In the present work, we investigated the role of Chk1 in the low-virulence species Candida guilliermondii, in order to gain insight into putative conservation of the role of group X HHK in opportunistic yeasts. We demonstrated that disruption of the corresponding gene CHK1 does not influence growth, stress tolerance, drug susceptibility, protein glycosylation or cell wall composition in C. guilliermondii. In addition, we showed that loss of CHK1 does not affect C. guilliermondii ability to interact with macrophages and to stimulate cytokine production by human peripheral blood mononuclear cells. Finally, the C. guilliermondii chk1 null mutant was found to be as virulent as the wild-type strain in the experimental model Galleria mellonella. Taken together, our results demonstrate that group X HHK function is not conserved in Candida species.

Keywords: Histidine kinases; Stress adaptation; Cell signaling; Virulence; Candida

1. Introduction

In bacteria, archaea, slime molds, plants and fungi, hybrid histidine kinases (HHKs) sense and transduce many intra- and extracellular signals, regulating a broad palette of physiological processes. Although some HHKs appear to be present in humans, typical HHK-like sensor proteins have not yet been reported in mammals, promoting these proteins as ideal targets for future therapies [1].

Since their pioneering identification in fungal cells, a limited number of fungal HHKs has been studied over the last fifteen years, and their functions in fundamental cellular processes are not yet clearly identified [2,4]. HHKs are involved in cell signaling systems referred to as His-to-Asp

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phosphorelays and several canonical schemes depicting transduction pathways involving HHKs in fungi have emerged. To date, HHKs act as primary sensors for various environmental stimuli, and upon activation, initiate phosphate transfer events between various proteins, leading to an adaptive response [4]. The number of HHK-encoding genes differs sharply from one fungal clade to another, and the N-terminus sensing domain is highly variable among HHK structures. Considering these observations, several classifications of fungal HHKs were established, and currently, they are categorized into sixteen groups [4–6].

Group X gathers some of the first described fungal HHKs; thus, it is one of the most thoroughly studied groups in the fungal kingdom. In the dimorphic fungus *Penicillium marneffei*, the group X HHK PmHhk1 was shown to regulate polarized growth, sporulation and cell wall composition [7]. In *Claviceps purpurea*, the agent of ergot of grasses, homologous HHK was involved in spore germination, sensitivity to oxidative stress and fungicide resistance, and virulence of the mutant strains was attenuated in both *C. purpurea* and *Magnaporthe oryzae* [8,9]. Most available data concerning the role of fungal group X HHKs were gained from functional characterization of *Candida albicans* Chk1, which was shown to play a crucial role in virulence, morphogenesis, peroxide adaptation, cell wall composition, quorum sensing, biofilm formation and triazole resistance [3]. Furthermore, during fungal infection, *C. albicans* Chk1 was required for survival within human neutrophils and adherence to *ex vivo* human esophageal cells [3].

In the present work, we investigated the role of the *C. albicans* CHK1 homolog in a low-virulence-related yeast species, namely *Candida guilliermondii*, in order to gain insight into putative conservation of the role of group X HHKs in the fungal CTG clade [10]. Indeed, although *C. guilliermondii* is an infrequent agent of candidiasis, this species has been described as an emerging pathogen intrinsically poorly susceptible to fluconazole and caspofungin, with a propensity for causing treatment failure [11]. Recent studies specified that *C. guilliermondii* accounts for 1–3% of all candidemia, but most cases of *C. guilliermondii* infection occur in immunocompromised patients with hematological malignancies [12,13].

2. Materials and methods

2.1. Strains and media

Yeast strains (Table 1) were routinely cultivated in liquid YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30 °C under agitation (150 rpm). YNB (0.67% yeast nitrogen base with ammonium sulfate and without amino acids, 2% sucrose) agar (2%) plates were used for selection of transformants following electroporation experiments. For heat inactivation, cells were resuspended in phosphate-buffered saline (PBS) and incubated at 56 °C for 1 h [14]. Acid phosphatase assay was induced by growing cells in YNB medium for 18 h at 28 °C with constant shaking (200 rpm).

The murine macrophage cell line J774A.1 (ATCC TIB-67) was cultured in DMEM (Gibco) containing 10% decomplemented fetal bovine serum (FBS, Gibco) and 1 mM sodium pyruvate, or in cRPMI (complete RPMI corresponding to RPMI-1640 (Sigma) without phenol red and supplemented with 10% decomplemented FBS, 1 mM sodium pyruvate and 2 g/l sodium bicarbonate) for the infection experiments, at 37 °C in 5% CO2.

2.2. Nucleic acids purification and PCR amplification

Genomic DNA from *C. guilliermondii* strains was extracted using the Plant Nucleospin II kit (Macherey–Nagel). DNA purification was performed using the Nucleospin Extract II kit (Macherey–Nagel). PCR assays were performed with Phusion DNA polymerase (New England Biolabs).

### Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Genotype</th>
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<tbody>
<tr>
<td><em>Candida guilliermondii</em></td>
<td>ATCC 6260&lt;sup&gt;a&lt;/sup&gt;</td>
<td>wild type</td>
</tr>
<tr>
<td></td>
<td>U312&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ura3</td>
</tr>
<tr>
<td></td>
<td>chkh&lt;sub&gt;Δ&lt;/sub&gt;</td>
<td>ura3, chk1::REP-URA3-REP</td>
</tr>
<tr>
<td><em>Candida parapsilosis</em></td>
<td>ATCC 22019&lt;sup&gt;a&lt;/sup&gt;</td>
<td>wild type</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>SC5314&lt;sup&gt;b&lt;/sup&gt;</td>
<td>wild type</td>
</tr>
<tr>
<td><em>Candida lusitaniae</em></td>
<td>ATCC 38533&lt;sup&gt;a&lt;/sup&gt;</td>
<td>wild type</td>
</tr>
<tr>
<td><em>Candida glabrata</em></td>
<td>ATCC 90030&lt;sup&gt;a&lt;/sup&gt;</td>
<td>wild type</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reference strain from American Type Culture Collection, ATCC Manassas, USA.

<sup>b</sup> From [16].

2.3. Plasmid construction

An 8,569 bp PCR fragment was first amplified from the *C. guilliermondii* CHK1 locus (Genbank accession number XM_001483618) (Fig. 1A) with the primer pair CHK1S 5’-TGTTTCTCATCTCCTGGATCC-3’ and CHK1R 5’-AGTACCCGACCGTGATCTACCC-3’ [15]. This amplicon was cloned into the pGEM-T easy vector (Promega) to yield plasmid pG-CHK1 (Fig. 1B). The pG-CHK1 plasmid was digested with *Cla*I (Fig. 1B) to delete a 4041 bp central fragment in the cloned CHK1 coding sequence, and the resulting digested plasmid was ligated to the REP-URA3-REP fragment released after digestion of the pG-REP-URA3-REP plasmid [16] (Fig. 1C) with *Cla*I to yield plasmid pG-5’CHK1-REP-URA3-REP-3’ CHK1 (Fig. 1D). The 5’CHK1-REP-URA3-REP-3’ CHK1 disruption cassette was released from pGEM-T vector after digestion of the pG-5’CHK1-REP-URA3-REP-3’ CHK1 with *Nor*I (Fig. 1E).

2.4. Yeast transformation

Transformation of *C. guilliermondii* cells was performed by electroporation as described [17].

2.5. mRNA detection

Total RNA was extracted from *C. guilliermondii* cultures (YPD) with Nucleospin RNA Plant (Macherey–Nagel). First-strand cDNAs were synthesized from 1 μg of total RNA using...
random hexamer primers (0.5 μM) with 15 units of Super-script III reverse transcriptase (Invitrogen). The C. guilliermondii CHK1 mRNAs were detected by PCR using primers CHK1S and CHK1R under the following conditions: 95 °C for 10 min and 30 cycles with denaturation at 95 °C for 30 s followed by annealing at 60 °C for 30 s and extension at 72 °C for 3 min. The C. guilliermondii Actin 1 gene (ACT1) was used as a housekeeping gene and amplified by using primers qACT1-F 5'-CCGACTTGGATGGAAGCCGC-3' and qACT1-R 5'-CTCAGGAGGACGATCCCTAACC-3' with the following conditions: 95 °C for 10 min and 30 cycles with denaturation at 95 °C for 30 s followed by annealing at 60 °C for 30 s and extension at 72 °C for 1 min.

2.6. Sensitivity test for stress responses, drugs and antifungal compounds

EUCAST testing was performed according to EUCAST EDef 7.1 methodology [18]. All strains were cultured on YNB before susceptibility testing. RPMI (Sigma Aldrich) was supplemented with antifungal agents, including 0.03–16.0 μg/mL amphotericin B or caspofungin, 0.125–64.0 μg/mL fluconazole, and 0.015–8.0 μg/mL voriconazole; or with other chemical compounds, including 0.125–1.0 mM sodium dodecyl sulfate (SDS, Thermo Fisher), 0.25–2.0 mM caffeine (Sigma Aldrich), 0.25–2.0 M NaCl (Thermo Fisher) or sorbitol (AppliChem), 1.0–11.5 mM H2O2, 0.003–0.375 mM menadione (Sigma Aldrich), 0.003–0.375 mg/mL Congo red (Sigma Aldrich). Inoculated plates were incubated at 32 °C and read with a spectrophotometer after 48 h. According to EUCAST EDef 7.1 recommendations, MIC 90 was determined for amphotericin B and MIC 50 for other drugs and compounds. The EUCAST MICs for the quality control strain Candida parapsilosis ATCC 22019 were all within the recommended ranges for the licensed compounds. Due to the low solubility of calcofluor white (CFW) and the high natural resistance of C. guilliermondii towards this compound [19], comparison of susceptibility of wild type (WT) and mutant strains to CFW was achieved using a standard drop plate assay protocol. For

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**Fig. 1. Different steps in construction of the CHK1 disruption cassette.**

this purpose, each strain was incubated overnight at 35 °C in YPD broth, washed, serially diluted (10² to 10⁵ dilutions) in distilled water and spotted (4 µL) onto solid YPD medium supplemented or not with 100–600 µg/mL CFW. Plates were incubated for 3 days at 32 °C.

2.7. Analysis of cell wall composition

Quantification of cell wall mannan, β-glucan and chitin was performed by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD), measuring the levels of the corresponding monosaccharides: mannose, glucose and N-acetylglucosamine, respectively. Cells grown in YPD broth were washed twice with PBS and broken with glass beads. Cell walls were pelleted by centrifuging and purified as described [14]. The samples were then acid-hydrolyzed with trifluoro-acetic acid, and analyzed by HPAEC-PAD in a Dionex equipment as previously reported [14].

To determine the content of cell wall proteins, walls were alkali-hydrolyzed before quantifying using a standard colorimetric method, while the levels of cell wall phosphomannan were quantified by cell ability to bind the cationic dye Alcian blue, as described [20].

2.8. Zymogram analysis

Acid phosphatase was used as a marker of the status of the N-linked glycosylation pathway, as previously reported [21]. Cells grown overnight in YNB broth were collected by centrifuging, washed with 10 mM Tris–HCl, pH 6.8 and disrupted with glass beads in a Braun homogenizer. The homogenate was centrifuged at 20,000 g for 10 min, the soluble fraction saved and loaded onto native 8% (w/v) polyacrylamide gels. Electrophoresis was run at 110 V for 1.5 h, the gel rinsed with 100 mM sodium acetate, pH 5.8 and enzyme activity was visualized by incubating with 0.4 mM 4-methylumbellyferyl-phosphate (Sigma) in 100 mM sodium acetate, pH 5.8 for 30 min at 37 °C.

2.9. Cytokine production by human peripheral blood mononuclear cells

The Ethics Committee of the Universidad de Guanajuato approved the use of human cells in this study (permission number 17082011). Healthy adult volunteers provided blood samples after a written informed consent was signed. Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll gradient, as previously reported [22]. A 100 µL aliquot of 5 × 10⁶ PBMCs/mL was added to U-bottom 96-well microplates along with 100 µL containing 1 × 10³ fungal cells. The interaction was incubated for 24 h at 37 °C with 5% (v/v) CO₂. Supernatants were collected by centrifuging and used to quantify levels of IL-1α, TNFα, IL-6 and IL-10 by ELISA (Preprotech). The IL-1β levels were measured using a commercial kit from R&D. For IL-1z quantification, stimulated PBMCs were disrupted by three sequential freezing cycles as reported [23].

2.10. Macrophage infection with yeasts

Yeast cells were labeled overnight in liquid YPD medium supplemented with 5 µg/mL CFW. Macrophages were infected as previously described in cRPMI medium [24]. We used a multiplicity of infection (MOI) of 1 macrophage to 1 yeast (1M:1Y) for all experiments, except for yeast viability, where we used 10M:1Y. Briefly, 2 × 10⁵ macrophages per well were adhered overnight in 96-well plates at 37 °C in 5% CO₂ and infected with the required number of CFW-labeled yeasts in cRPMI in the presence of 5 µg/mL CFW to allow the continuous labeling of newly replicated yeasts outside the macrophages.

2.11. Fluorimetry and flow cytometry assays

Fluorimetry and flow cytometry assays were conducted as previously described using a Fluostar Optima fluorimeter (BMG Labtech) and a FACSCanto II (Becton Dickinson), respectively [24]. Fluorimetry was used to determine multiplications of the fungal biomass and the ratio of ingested fungal cells after 1 h, 5 h and 24 h of infection. Briefly, we measured the total fluorescence of CFW-labeled yeasts in an infection well. Then, a final concentration of 250 µg/mL of trypan blue was used to quench the fluorescence of non-ingested CFW-labeled yeasts, and we measured the fluorescence of internalized CFW-labeled yeasts. Flow cytometry was used to measure macrophage and yeast mortality rates and the ratio of macrophages engaged in phagocytosis. Briefly, macrophages were double-stained with anti-mouse CD16-APC (membrane stain) and calcine-AM (a marker of active metabolism) after 24 h of incubation with CFW-labeled yeasts. The percentage of macrophage viability was calculated as the number of macrophages positive for both fluorescence (calcine and anti-CD16) in an infection assay versus the uninfected macrophage control. Phagocytosing macrophages were quantified as the number of macrophages positive for calcine, anti-CD16 and CFW fluorescence. To determine yeast mortality inside macrophages, macrophages were infected with CFW-labeled yeasts at a MOI of 10 M:1Y in a 96-well plate. After 24 h, wells were washed, macrophages were detached and lysed with 0.1% Triton X-100 (Acros Organics) to release ingested yeast cells. We used 1 µg/mL propidium iodide (PI) (Sigma) (λex 535 nm, λem 617 nm) to stain collected yeasts for flow cytometry analysis. PI only enters dead cells. Heat-killed yeasts were used as positive controls and live yeasts were used as negative controls for PI staining. For heat inactivation, yeast cells were incubated for 30 min at 90 °C. For analysis, the CFW population was selected and the ratio of the population positive for the PI signal was then measured to determine yeast mortality.

2.12. Virulence assays in the insect species Galleria mellonella

Groups of 10 G. mellonella larvae of at least 1 cm length and uniform color were inoculated in the last left proleg with the fungal suspension using a Hamilton syringe (701N, 26s gauge, 10 µL capacity) [25]. Infection was achieved by
injecting 10 µL inoculum into the hemocoel. Animal groups were kept at 37 °C and mortality and phenotypical changes were recorded for 15 days. As a control, a group of 10 animals was injected with PBS.

2.13. Statistical analysis

Data are cumulative results of all experiments performed and are shown as mean ± SD or SE. The Mann–Whitney U test was used to establish statistical significance, which was set at P < 0.05. Mortality results were analyzed using the Log-rank test and shown in Kaplan–Meier charts, with a significance level set at P < 0.05.

3. Results

3.1. Disruption of the C. guilliermondii CHK1 gene

We previously provided a complete bioinformatics characterization of both the C. guilliermondii CHK1 gene and Chk1 deduced protein in a global study deciphering the subcellular localization of the series of HHKs found in this species [15]. Briefly, the C. guilliermondii Chk1 predicted protein is composed of 2428 amino acids and shares 30% identity with Chk1 from C. albicans. C. guilliermondii Chk1 displays all subdomains specifically found in C. albicans Chk1, thus belonging to the group X HHK [4]. Importantly, no further homologous sequences were found in the genome of C. guilliermondii, indicating that, as observed in C. albicans, a unique copy of the CHK1 gene is present and thus encodes the sole member of group X HHK in C. guilliermondii.

Among the series of selectable markers available for C. guilliermondii genetics [26–30], we chose to use the recently developed URA3 blaster system to disrupt C. guilliermondii CHK1 [16]. This was done to recreate the genetic modifications performed in C. albicans, where the URA3 blaster system was initially used to generate C. albicans chk1 mutants [31,32]. For this purpose, a 5′CHK1-REP-URA3-REP-3′ CHK1 fragment with 2900 bp and 1600 bp of the 5′ and 3′ CHK1 homologous arms, respectively, was generated. This disruption cassette was used to transform the C. guilliermondii U312 (ura3Δ290) recipient strain (auxotroph for uracil) to prototrophy. Ura+ transformants were selected on minimal medium plates. The efficiency of transformation was roughly 5–6 transformants per µg of DNA. A series of 30 randomly selected Ura+ transformants were analyzed by colony PCR as previously described [33]. Homologous integration of the 5′CHK1-REP-URA3-REP-3′ CHK1 cassette at the CHK1 locus (Fig. 2A) occurred in 3 of the analyzed transformants and was derived from gene replacement, resulting in disruption of the target gene and in the genotype ura3, chk1Δ::REP-URA3-REP (abbreviated chk1Δ, see a representative clone in Fig. 2B). For the remaining Ura+ transformants, it is highly likely that they were derived from gene replacement at the ura3 locus (not studied). Loss of intact C. guilliermondii CHK1 was confirmed by both PCR and RT-PCR analyses (Fig. 2B). Lack of amplification of CHK1 cDNA in the chk1Δ mutant was unlikely to be related to poor quality of the RNA preparations, as these were successfully used to synthesize and amplify the ACT1 cDNA (Fig. 2B). Therefore, a C. guilliermondii chk1Δ null mutant was generated.

3.2. Growth, stress tolerance, and drug susceptibility of the C. guilliermondii chk1Δ null mutant

Since the chk1Δ null mutant was Ura+, we first compared its growth kinetics with the WT strain ATCC 6260 (the parental strain of U312) [16]: both exhibited similar doubling times in liquid YPD or YNB media (data not shown). Furthermore, no differences were observed in colony growth (diameter and aspect) of either the WT or mutant strains when cultured on solid YNB or YPD media (data not shown). These results suggest that CHK1 deletion does not affect C. guilliermondii overall development.

![Disruption of C. guilliermondii CHK1](image)

Fig. 2. Disruption of C. guilliermondii CHK1. A. Schematic representation of the wild-type and disrupted CHK1 locus. B. PCR analysis of the CHK1 locus in the ATCC 6260 wild-type strain and a representative chk1Δ mutant using CHK1S and CHK1R primers (upper panel). The presence of CHK1 mRNAs in the ATCC 6260 wild-type strain and the representative chk1Δ mutant was detected with reverse transcriptase-PCR (middle panel). The C. guilliermondii actin 1 gene (ACT1) mRNAs (lower panel) were detected as a control of RNA integrity.
Since previous works demonstrated the involvement of HKs in the capacity of adapting to hyperosmotic conditions in several fungal species [3], we next examined the osmotolerance of the chk1Δ null mutant. Both WT and null mutant strains displayed similar growth rates in high-osmolarity media containing NaCl or sorbitol (Table 2). These results demonstrate that the CHK1 deletion has no effect on the growth and the capacity of adaptation of C. guilliermondii yeast cells to hyperosmotic conditions.

It is now well described that Chk1 plays a major role in the perception of oxidant conditions in C. albicans [34]. Thus, the impact of C. guilliermondii CHK1 in resistance to oxidant stresses was evaluated in media supplemented either with H2O2 or with menadione. Neither hypersensitivity nor resistance of the chk1Δ mutant towards these oxidant conditions was observed (Table 2).

Finally, because it was previously shown that C. albicans chk1Δ null mutant is hypersensitive to fluconazole and voriconazole [35], we monitored the effect of a set of clinical antifungals (amphotericin B, flucytosine, fluconazole, voriconazole [35], we monitored the effect of a set of clinical antifungals (amphotericin B, flucytosine, fluconazole, voriconazole, and caspofungin) against both antifungals (amphotericin B, flucytosine, fluconazole, voriconazole, and caspofungin) against both C. guilliermondii reference strain ATCC 6260 and the chk1Δ null mutant. Neither hypersensitivity nor resistance to these antifungals of the chk1Δ mutant was observed. Indeed, the MICs of both strains were similar to those previously described for C. guilliermondii (Table 2) [11].

3.3. Protein glycosylation and cell wall composition in the C. guilliermondii chk1Δ null mutant

It was previously reported that disruption of CHK1 in C. albicans led to shortening in cell wall mannan content [36], and this allowed exposure of β1,3-glucan at the cell surface, resulting in increased phagocytosis by phagocytic cells [37]. Thus, we assessed whether loss of CHK1 affected the glycosylation pathways in C. guilliermondii. Using zymogram analysis of secreted acid phosphatase, a protein reporter of the status of the N-linked glycosylation pathway [20], we found no differences in the mobility of the enzyme extracted from WT and mutant strains. Furthermore, neither flocculation at the macroscopic level nor aggregation of cells in several fungal species [3] were observed by microscopy, as described in the C. albicans chk1Δ mutant [31], were observed in liquid cultures of the corresponding C. guilliermondii mutant. Therefore, loss of CHK1 has no obvious impact on C. guilliermondii cell wall composition or protein glycosylation.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>MIC determination of C. guilliermondii wild type strain and chk1Δ null mutant towards various antifungal drugs and other chemicals.</th>
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<td><strong>Antifungals</strong></td>
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<tr>
<td>Amphotericin B (µg/mL)</td>
<td>0.5 (0.25–0.5)</td>
</tr>
<tr>
<td>Flucytosine (µg/mL)</td>
<td>0.25 (0.12–0.5)</td>
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<td>Fluconazole (µg/mL)</td>
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<tr>
<td>Voriconazole (µg/mL)</td>
<td>0.06</td>
</tr>
<tr>
<td>Caspofungin (µg/mL)</td>
<td>2 (1–2)</td>
</tr>
<tr>
<td><strong>Other chemicals</strong></td>
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<tr>
<td>Congo Red (mM)</td>
<td>0.03</td>
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<tr>
<td>SDS (mM)</td>
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<td>Caffeine (mM)</td>
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<tr>
<td>NaCl (M)</td>
<td>1.25 (1–1.25)</td>
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<tr>
<td>Sorbitol (M)</td>
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<tr>
<td>H2O2 (mM)</td>
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<td>Menadione (mM)</td>
<td>2 (1–2)</td>
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<table>
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<th>Table 3</th>
<th>Cell wall composition of C. guilliermondii chk1Δ null mutant.</th>
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<td><strong>Strain</strong></td>
<td><strong>Cell wall abundance</strong></td>
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<td><strong>ATCC</strong></td>
<td>1.8 ± 1.2</td>
</tr>
<tr>
<td><strong>chk1Δ</strong></td>
<td>1.6 ± 1.3</td>
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</table>

* µg of Alcian Blue bound/OD600 = 1.

µg of protein/mg of cell wall.

Fig. 3. Loss of CHK1 does not affect protein N-linked glycosylation in C. guilliermondii. Cell homogenates were obtained from either the wild type or chk1Δ null mutant and used to perform a zymogram to reveal acid phosphatase activity, a reporter of the N-linked mannosylation pathway, as described in Materials and methods. Similar electrophoretic mobility of the enzyme was observed in both samples.
3.4. Multi-parametric monitoring of interactions between C. guilliermondii cells and macrophages

Since it was recently demonstrated that deletion of C. albicans CHK1 improves recognition by phagocytes [37,40], we were thus interested in evaluating of cellular interactions of the C. guilliermondii reference strain or the chk1Δ null mutant with macrophages. For this purpose, we used a recently developed in vitro model allowing multi-parametric monitoring [24]. The J774 macrophages were infected with the C. guilliermondii reference strain, and the behavior of both cell types was monitored over time. Multiplication of the C. guilliermondii fungal biomass was inhibited in the presence of J774 macrophages, as shown by total CFW fluorescence which did not increase between 1 h and 24 h of infection (Fig. 4A). In the case of both strains, the rate of internalized yeast cells was 40% after 1 h of infection, and reached a plateau of 80% at 5 h (Fig. 4B). C. guilliermondii cells remained intramacrophagic at 24 h post-infection, as confirmed by observation under the microscope (data not shown), with a mortality rate of roughly 20% (Fig. 4C). The vast majority of the macrophages (nearly 90%) survived after 24 h of infection, and 63% were engaged in phagocytosis (Fig. 4D). Finally, we compared the cellular interactions of C. guilliermondii with those of other Candida species, including C. albicans, Candida glabrata and Candida lusitaniae (Table 4). Globally, C. guilliermondii behaved similarly to C. glabrata and appeared to be the least aggressive species, as indicated by the lowest macrophage killing and the highest proportion of fungal cells internalized.

3.5. Loss of CHK1 does not affect C. guilliermondii ability to stimulate cytokine production by human PBMCs

Since we did not find obvious differences in the interaction of the C. guilliermondii chk1Δ null mutant with macrophages, we next evaluated the ability of this mutant to stimulate cytokines by human PBMCs. Our results showed that the WT

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Fig. 4. Multi-parametric analysis of the interaction between J774 macrophages and yeast cells over 24 h time course experiments. Macrophages were infected with yeast as described in the Materials and methods. A. Fluorimetry analysis of the multiplication of fungal biomass in the presence of macrophages. CFW was added to the well at the beginning of the infection, and total fluorescence of CFW-labeled yeast was measured over time. B. Fluorimetry analysis of the uptake of yeast cells by macrophages. Trypan blue was used to quench the CFW fluorescence of non-ingested yeast cells. Residual CFW fluorescence reflected internalized yeasts. C. Flow cytometry analysis of the mortality of intramacrophagic yeast cells after 24 h of phagocytosis. Ingested CFW-labeled yeast cells were released from macrophages using Triton X-100 and were stained with propidium iodide. Dead cells showed both CFW and PI fluorescence. D. Flow cytometry analysis of the survival of infected macrophages after 24 h of infection. The number of surviving macrophages, positive for both calcein and anti-CD16 fluorescence, was determined in the presence and in the absence of yeast cells to calculate percent survival. Macrophages associated with yeast, i.e., phagocytosing macrophages, were identified as macrophages positive for CFW fluorescence. Non-phagocytosing macrophages were negative for CFW fluorescence. Results are shown as mean ± S.E. of three independent experiments performed in triplicate or quintuplet.
control strain and the chk1Δ null mutant had a similar ability to stimulate production of TNFα, IL-6, IL-1α, IL-1β, and IL-10 (Fig. 5). Artificial exposure, by heat-killing, of inner wall components at the wall surface of C. albicans stimulated differential cytokine production by human PBMCs [41]. Therefore, we performed experiments using heat-killed C. guilliermondii cells. Despite observing a significant increment in cytokine production upon inactivation of cells by heating (Fig. 5), no significant differences between WT control cells and the chk1Δ null mutant were detected. Therefore, CHK1 does not affect the C. guilliermondii-human PBMC interaction.

### Table 4

Comparative analysis of the interactions involving J774 macrophages and different Candida species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Fungal biomass multiplication at 24 h</th>
<th>Internalized yeast cells (%)</th>
<th>Macrophage (%) survival at 24 h (phagocytosing, %)</th>
<th>Yeast (%) mortality at 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 h</td>
<td>5 h</td>
<td>24 h</td>
</tr>
<tr>
<td>C. albicans SC5314</td>
<td>6.8 ± 0.1</td>
<td>18 ± 2</td>
<td>10 ± 1</td>
<td>0</td>
</tr>
<tr>
<td>C. glabrata ATCC 90030</td>
<td>1.4 ± 0.1</td>
<td>55 ± 5</td>
<td>76 ± 2</td>
<td>67 ± 9</td>
</tr>
<tr>
<td>C. lusitaniae ATCC 38533</td>
<td>3.6 ± 1.0</td>
<td>54 ± 5</td>
<td>64 ± 2</td>
<td>37 ± 4</td>
</tr>
<tr>
<td>C. guilliermondii ATCC 6260</td>
<td>1.1 ± 0.1</td>
<td>41 ± 4</td>
<td>77 ± 10</td>
<td>81 ± 21</td>
</tr>
</tbody>
</table>

Results are shown as mean ± S.E. of three to six independent experiments performed by triplicates or quintuplets.

* Compared to initial fungal biomass at time = 1 h.

#### 3.6. The C. guilliermondii chk1Δ null mutant is as virulent as the wild type strain

Finally, we aimed to assess the impact of CHK1 disruption on the virulence of C. guilliermondii. Since no apparent defect in growth, wall composition or interaction with immune cells was observed, we hypothesized that the virulence of the null mutant was not affected. Thus, we did not perform experiments in mice, but in the alternative host G. mellonella, which has been successfully used to assess the virulence of other Candida species, providing results similar to those generated in the gold standard model, i.e., the mouse [25,42,43]. Again,
we did not observe any significant differences in mortality associated with the WT strain and the chk1Δ null mutant; both strains killed the entire insect population in less than a week (Fig. 6). Therefore, the *C. guilliermondii* chk1Δ null mutant does not display virulence attenuation.

4. Discussion

*C. albicans* group X HHK Chk1 plays a crucial role in morphogenesis (especially the yeast to hyphae switch), virulence, peroxide adaptation, cell wall composition, quorum sensing, biofilm formation and triazole resistance [31,32,34,35,44–48]. Furthermore, during fungal infection, *C. albicans* Chk1 is required for survival within human neutrophils and adherence to *ex vivo* human esophageal cells [37–40]. Therefore, all these studies suggest that group X HHK represents an interesting fungal target for the discovery of new antifungal drugs. Via this perspective, it remains essential to explore the conservation of the role of such potential targets in related species. In the present work, we thus investigated the role of the *C. albicans* CHK1 homolog in the low-virulence related yeast species *C. guilliermondii* in order to gain insight into putative conservation of the role of these proteins in other members of the *Candida* CTG clade. For this purpose, we generated a *C. guilliermondii* chk1Δ mutant strain and carried out a series of phenotypic analyses that were previously investigated in the *C. albicans* chk1Δ mutant (above mentioned). We demonstrated that disruption of the CHK1 ortholog does not influence growth, stress tolerance, drug susceptibility, protein glycosylation or cell wall composition in *C. guilliermondii*. In addition, it was found that loss of CHK1 does not affect *C. guilliermondii* ability to interact with macrophages and to stimulate cytokine production by human PBMCs. Finally, the *C. guilliermondii* chk1Δ null mutant was shown to be as virulent as the WT strain in the experimental model *G. mellonella*. Importantly, it was previously demonstrated that the CHK1 homolog plays no obvious role in the low-virulence related yeast species *C. lusitaniae* [49]. Therefore, although experimental conditions used in the present study were not strictly identical to those reported for *C. albicans* chk1Δ mutant phenotypic analysis, taken together, and in line with what was previously suggested in *C. lusitaniae* [49], our results suggest that group X HHK function is not conserved in *C. albicans*, *C. lusitaniae* or *C. guilliermondii*. This divergence in the function of several HHK groups was already reported in molds [3,4]. For instance, group III HHK could play an opposing role in virulence in the two closely related species of plant pathogens *Alternaria brassicicola* and *Alternaria longipes* [50,51]. While *C. albicans*, *C. lusitaniae* and *C. guilliermondii* commonly belong to the particular fungal CTG clade, it is now accepted that the genetic distance between any *Candida* species is considerable [52]. Thus, in agreement with low conservation of the amino acid sequence of *C. albicans*, *C. lusitaniae* and *C. guilliermondii* Chk1, it is likely that the differences observed in the function of these crucial sensing proteins could underlie, at their own scale, prominent traits leading to specific strategies of these yeast species to adapt to their specific environmental niches and hosts. Finally, it is important to remember that, as observed in other closely related *Candida* species [49], *C. guilliermondii* harbors two additional HHK genes, namely SLN1 and NIK1, which encode HHKs belonging to groups VI and III, respectively [15]. Although the structures of the sensing domains in Chk1, Sln1 and Nik1 deduced proteins are highly divergent, it is not excluded that functional redundancy could occur between these three HHKs, and further efforts will be needed to address this pending hypothesis.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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References


