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For publication

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

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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.

Key words: 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 have 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 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 *Clavicepspurpurea*, 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 *Magnaportheoryzae*[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 1h [14]. Acid phosphatase expression was induced by growing cells in YNB medium for 18 h at 28ºC 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% CO_2.

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).

2.3. Plasmid construction

An 8,569bp PCR fragment was first amplified from the *C. guilliermondiiCHK1* locus (Genbank accession numberXM_001483618) (Fig. 1A) with the primer pair CHK1S5'-TGGTTTCCTGATTCAGTTCGCTATACCC-3' and CHK1R 5'-AGTACCCACGACCAGTACCAGGATAAGG-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 4,041 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*Ito 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 *Not*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

126 random hexamer primers (0.5 µM) with 15 units of Superscript III reverse transcriptase (Invitrogen).
127 The *C. guilliermondii CHK1* mRNAs were detected by PCR using primers CHK1S and CHK1R
128 under the following conditions: 95°C for 10 min and 30 cycles with denaturation at 95°C for 30 s
129 followed by annealing at 60°C for 30 s and extension at 72°C for 4 min. The *C. guilliermondii* Actin
130 1 gene (*ACT1*) was used as a housekeeping gene and amplified by using primers qACT1-F 5'-
131 CCGACTTGGGATTGGAAGCCGC-3' and qACT1-R 5'-
132 CTCAGGAGGAGCGATGATCTTAACC-3' with the following conditions: 95°C for 10 min and
133 30 cycles with denaturation at 95°C for 30 sec followed by annealing at 60°C for 30 s and extension
134 at 72°C for 1 min.
135
136 *2.6. Sensitivity test for stress responses, drugs and antifungal compounds*
137 EUCAST testing was performed according to EUCAST EDef 7.1 methodology[18]. All
138 strains were cultured on YNB before susceptibility testing. RPMI (Sigma Aldrich) was
139 supplemented with antifungal agents, including 0.03-16.0 µg/mL amphotericin B or caspofungin,
140 0.125-64.0 µg/mL flucytosine, 0.25-128.0 µg/mL fluconazole, and 0.015-8.0 µg/mL voriconazole;
141 or with other chemical compounds, including 0.125-1.0 mM sodium dodecyl sulfate (SDS, Thermo
142 Fisher), 0.25-2.0 mM caffeine (Sigma-Aldrich), 0.25-2.0 M NaCl (Thermo Fisher) or sorbitol
143 (AppliChem), 1.0-11.5mM H_2O_2, 0.003-0.375 mM menadione (Sigma Aldrich), 0.003-0.375mg/mL
144 Congo red (Sigma Aldrich). Inoculated plates were incubated at 32°C and read with a
145 spectrophotometer after 48 h. According to EUCAST EDef 7.1 recommendations, MIC 90 was
146 determined for amphotericin B and MIC 50 for other drugs and compounds. The EUCAST MICs
147 for the quality control strain *C. parapsilosis* ATCC 22019 were all within the recommended ranges
148 for the licensed compounds. Due to the low solubility of calcofluor white (CFW) and the high
149 natural resistance of *C. guilliermondii* towards this compound[19], comparison of susceptibility of
150 wild type (WT) and mutant strains to CFW was achieved using a standard drop plate assay protocol.
151 For this purpose, each strain was incubated overnight at 35°C in YPD broth, washed, serially diluted

(10^2 to 10^5dilutions) 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 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 mMTris-HCl, pH 6.8 and disrupted with glass beads in a Braun homogenizer. The homogenate was centrifuged at 20,000 x 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 x 10^6 PBMCs/ml was added to U-bottom 96-well microplates along with 100 µL containing 1x10^5fungal cells. The interaction was incubated for 24 h at 37 °C with 5% (v/v) CO_2. Supernatants were collected by centrifuging and used to quantify levels of IL-1α, TNFα, IL-6, IL-10 by ELISA (Preprotech). The IL-1β levels were measured using a commercial kit from R&D. For IL-1α quantification, stimulated PBMCs were disrupted by three sequential freezing cycles as reported[23].

2.10. Macrophage infection with yeasts

Yeast cells were labelled 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, 2×10^5 macrophages per well were adhered overnight in 96-well plates at 37°C in 5% CO_2 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 multiplication 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 calcein-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 (calcein and anti-CD16) in an infection assay versus the uninfected macrophage control. Phagocytosing macrophages were quantified as the number of macrophages positive for calcein, 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) (λ_{ex} 535 nm, λ_{em} 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, 26's 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 2,900 bp and 1,600 bp of the 5' and 3' *CHK1*homologous arms, respectively, was generated. This disruption cassette was used to transform the *C. guilliermondii* U312 ($ura3_{\Delta 290}$) 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*, *chk1*Δ*::REP-URA3-REP* (abbreviated *chk1*Δ, 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 *chk1*Δ 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 chk1*Δ null mutant was generated.

3.2. Growth, stress tolerance, and drug susceptibility of the C. guilliermondii chk1*Δ null mutant*

Since the *chk1*Δ null mutant was Ura$^+$, we first compared its growth kinetics with WT strain ATCC 6260 (the parental strain of U312)[16]: both exhibited similar doubling times in liquid YPDor 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.

Since previous works demonstrated the involvement of HHK in the capacity of adaptation to hyperosmotic conditions in several fungal species[3], we next examined the osmotolerance of the *chk1*Δ 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 impactof *C. guilliermondii CHK1* in resistance to oxidant stresses was evaluated n media supplemented either with H_2O_2 or with menadione. Neither hypersensitivity nor resistance of the *chk1*Δ mutant towards these oxidant conditions was observed (Table 2).

Finally, because it was previously shown that the *C. albicans chk1*Δ null mutant is hypersensitive to fluconazole and voriconazole[35], we monitored the effect of a series of clinical antifungals (amphotericin B, flucytosine, fluconazole, voriconazole, and caspofungin) against both *C. guilliermondii* reference strain ATCC 6260 and the *chk1*Δ null mutant. Neither hypersensitivity nor resistance to these antifungals of the *chk1*Δ 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 chk1*Δ null mutant*

It was previously reported that disruption of *CHK1* in *C. albicans* led to shortening in cell wall mannans[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 control cells and the *chk1*Δ null mutant, indicating no obvious defects in this biosynthetic pathway (Fig. 3). In order to confirm this result, we quantified cell wall phosphomannan content, an indirect indicator of the status of protein glycosylation pathways[14,20,38,39], and found no significant differences in the content of this moiety attached to both *N*-linked and *O*-linked mannans (Table 3)[14]. We next determined the cell wall composition of the null mutant by HPAEC-PAD and we could not find significant differences in the content of chitin, glucan and mannan (Table 3). Moreover, protein content within the cell wall was similar in both the WT control strain and the *chk1*Δ null mutant. These results are in line with the fact that drugs interfering with the biosynthesis and structure of the fungal cell wall, such as caffeine, Congo red (Table 2) and calcofluor white (not shown) displayed similar effects on WT and mutant strains. Furthermore, neither flocculation at the macroscopic level nor aggregation of cells observed by microscopy, as described in the *C. albicanschk1*Δ 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.

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 evaluation of cellular interactions of the *C. guilliermondii* reference strain or the *chk1*Δnull mutant with macrophages. For this purpose, we used a recently developed in vitro model allowing multiparametric 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, C. glabrata* and *C. 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 chk1*Δ null mutant with macrophages, we next evaluated the ability of this mutant to stimulate cytokines by

human PBMCs. Our results showed that the WT control strain and the *chk1*Δ 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 *chk1*Δ null mutant were detected. Therefore, *CHK1* does not affect the *C. guilliermondii*-human PBMC interaction.

3.6. The C. guilliermondii chk1*Δnull 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 *chk1*Δ null mutant; both strains killed the entire insect population in less than a week (**Fig. 6**). Therefore, the *C. guilliermondii chk1*Δ 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 in 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. albicansCHK1* 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 chk1*Δ mutant strain and carried out a series of phenotypic analyses that were previously investigated in the *C. albicans chk1*Δ 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 chk1*Δ 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 chk1*Δ 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 *Alternariabrassisicola* and *Alternarialongipes*[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 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.

Acknowledgments

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References

1. Bem AE, Velikova N, Pellicer MT, BaarlenPv, Marina A, Wells JM. Bacterial histidine kinases as novel antibacterial drug targets. ACS ChemBiol2015;10:213-224.
2. Chauhan N, Latge JP, Calderone R. Signalling and oxidant adaptation in *Candida albicans* and *Aspergillus fumigatus*. Nat Rev Microbiol 2006;4:435-444.
3. Li D, Agrellos OA, Calderone R. Histidine kinases keep fungi safe and vigorous. CurrOpinMicrobiol2010;13:424-430.
4. Defosse TA, Sharma A, Mondal AK, Dugé de Bernonville T, Latgé JP, Calderone R, *et al*.Hybrid histidine kinases in pathogenic fungi. MolMicrobiol2015;95:914-924.

5. Catlett NL, Yoder OC, Turgeon BG. Whole-genome analysis of two-component signal transduction genes in fungal pathogens. Eukaryot Cell 2003;2:1151-1161.
6. Lavín JL, Ramírez L, Ussery DW, Pisabarro AG, Oguiza JA. Genomic analysis of two-component signal transduction proteins in basidiomycetes. J MolMicrobiolBiotechnol 2010;18:63-73.
7. Wang F, Tao J, Qian Z, You S, Dong H, Shen H,*et al.* A histidine kinase PmHHK1 regulates polar growth, sporulation and cell wall composition in the dimorphic fungus *Penicillium marneffei*. Mycol Res 2009;113:915-923.
8. Nathues E, Jörgens C, Lorenz N, Tudzynski P. The histidine kinase CpHK2 has impact on spore germination, oxidative stress and fungicide resistance, and virulence of the ergot fungus *Clavicepspurpurea*. Mol Plant Pathol2007;8:653-665.
9. Jacob S, Foster AJ, Yemelin A, Thines E. Histidine kinases mediate differentiation, stress response, and pathogenicity in *Magnaportheoryzae*. Microbiologyopen 2014;3:668-687.
10. Papon N, Savini V, Lanoue A, Simkin AJ, Crèche J, Giglioli-Guivarc'h N, *et al.Candida guilliermondii*: biotechnological applications, perspectives for biological control, emerging clinical importance and recent advances in genetics. Curr Genet2013;59:73-90.
11. Pfaller MA, Diekema DJ, Mendez M, Kibbler C, Erzsebet P, Chang SC, *et al.Candida guilliermondii*, an opportunistic fungal pathogen with decreased susceptibility to fluconazole: geographic and temporal trends from the ARTEMIS DISK antifungal surveillance program. J ClinMicrobiol 2006;44:3551-3556.
12. Girmenia C, Pizzarelli G, Cristini F, Barchiesi F, Spreghini E, Scalise G, *et al.Candida guilliermondii*fungemia in patients with hematologic malignancies. J ClinMicrobiol. 2006;44:2458-2464.
13. Savini V, Catavitello C, Onofrillo D, Masciarelli G, Astolfi D, Balbinot A, *et al.* What do we know about *Candida guilliermondii*? A voyage throughout past and current literature about this emerging yeast. Mycoses 2011;54:434-441.
14. Mora-Montes HM, Bates S, Netea MG, Díaz-Jiménez DF, López-Romero E, Zinker S, *et al.* Endoplasmic reticulum alpha-glycosidases of *Candida albicans* are required for N glycosylation, cell wall integrity, and normal host-fungus interaction. EukaryotCell 2007;6:2184-2193.

15. Foureau E, Clastre M, Montoya EJ,Besseau S, Oudin A, Glévarec G,*et al.* Subcellular localization of the histidine kinase receptors Sln1p, Nik1p and Chk1p in the yeast CTG clade species *Candida guilliermondii.* Fungal Genet Biol 2014;65: 25-36.
16. Foureau E, Courdavault V, Simkin AJ, Pichon O, Crèche J, Giglioli-Guivarc'h N, *et al.*Optimization of the URA-blaster disruption system in *Candida guilliermondii*: efficient gene targeting using the URA3 marker. J Microbiol Methods 2012;91:117-120.
17. Millerioux Y, Clastre M, Simkin AJ, Marais E, Sibirny AA, Noël T, *et al.*Development of a URA5 integrative cassette for gene disruption in the *Candida guilliermondii* ATCC 6260 strain. J Microbiol Methods 2011;84:355-358.
18. Rodriguez-Tudela JL, Arendrup MC, Barchiesi F, Bille J, Chryssanthou E, Cuenca-Estrella M, *et al.* EUCAST Definitive Document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts: Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST). Clin Microbiol Infect2008;14:398-405.
19. Walker LA, Gow NA, Munro CA. Elevated chitin content reduces the susceptibility of *Candida* species to caspofungin. Antimicrob Agents Chemother 2013;57:146-154.
20. Bates S, MacCallum DM, Bertram G, Munro CA, Hughes HB, Buurman ET, *et al. Candida albicans* Pmr1p, a secretory pathway P-type Ca2+/Mn2+-ATPase, is required for glycosylation and virulence. J BiolChem 2005;280:23408-23415.
21. Hobson RP, Munro CA, Bates S, MacCallum DM, Cutler JE, Heinsbroek SE, *et al.*Loss of cell wall mannosylphosphate in *Candida albicans* does not influence macrophage recognition. J BiolChem2004;279:39628-39635.
22. Endres S, Ghorbani R, Lonnemann G, van der Meer JW, Dinarello CA. Measurement of immunoreactive interleukin-1 beta from human mononuclear cells: optimization of recovery, intrasubject consistency, and comparison with interleukin-1 alpha and tumor necrosis factor. ClinImmunolImmunopathol1988;49:424-438.
23. Mora-Montes HM, Netea MG, Ferwerda G, Lenardon MD, Brown GD, Mistry AR, *et al.* Recognition and blocking of innate immunity cells by *Candida albicans* chitin. Infect Immun. 2011;79:1961-1970.

24. Dementhon K, El-Kirat-Chatel S, Noël T. Development of an in vitro model for the multi-parametric quantification of the cellular interactions between *Candida* yeasts and phagocytes. PLoS One 2012;**7**:e32621.

25. Gago S, García-Rodas R, Cuesta I, Mellado E, Alastruey-Izquierdo A. *Candida parapsilosis, Candida orthopsilosis,* and *Candida metapsilosis* virulence in the non-conventional host *Galleria mellonella.* Virulence 2014;5:278-285.

26. Obando Montoya EJ, Mélin C, Blanc N, Lanoue A, Foureau E, Boudesocque L, *et al*. Disrupting the methionine biosynthetic pathway in *Candida guilliermondii*: characterization of the *MET2* gene as counter-selectable marker. Yeast 2014;31:243-251.

27. Foureau E, Courdavault V, Rojas LF, Dutilleul C, Simkin AJ, Crèche J, *et al*.Efficient gene targeting in *a Candida guilliermondii* non-homologous end-joining pathway-deficient strain.Biotechnol Lett 2013;35:1035-1043.

28. Foureau E, Courdavault V, Simkin AJ, Sibirny AA, Crèche J, Giglioli-Guivarc'h N, *et al*.Transformation of *Candida guilliermondii* wild-type strains using the *Staphylococcus aureus* MRSA 252 ble gene as a phleomycin-resistant marker. FEMS Yeast Res 2013;13:354-358.

29. Foureau E, Clastre M, Millerioux Y,Simkin AJ, Cornet L, Dutilleul C, *et al.TRP5*/5-fluoroanthranilic acid counter-selection system for gene disruption in *Candida guilliermondii*. Curr Genet 2012;58:245-254.

30. MilleriouxY, Clastre M, Simkin AJ, Courdavault V, Marais E, Sibirny AA, *et al*.Drug-resistant cassettes for the efficient transformation of *Candida guilliermondii* wild-type strains. FEMS Yeast Res 2011;11:457-463.

31. Calera JA, Calderone R. Flocculation of hyphae is associated with a deletion in the putative CaHK1 two-component histidine kinase gene from *Candida albicans*. Microbiology 1999;145:1431-1442.

32. Yamada-Okabe T, Mio T, Ono N. Roles of three histidine kinase genes in hyphal development and virulence of the pathogenic fungus *Candida albicans*. J Bacteriol 1999;181:7243-7247.

33. Akada R, Murakane T, Nishizawa Y. DNA extraction method for screening yeast clones by PCR. Biotechniques 2000;28:668-670,672,674.

34. Li D, Gurkovska V, Sheridan M, Calderone R, Chauhan N. Studies on the regulation of the two-component histidine kinase gene CHK1 in Candida albicans using the heterologous lacZ reporter gene. Microbiology 2004;150:3305-3313.
35. Chauhan N, Kruppa M, Calderone R. The Ssk1p response regulator and Chk1p histidine kinase mutants of *Candida albicans* are hypersensitive to fluconazole and voriconazole. Antimicrob Agents Chemother2007;51:3747-3751.
36. Li D, Williams D, Lowman D, Monteiro MA, Tan X, Kruppa M,*et al*. The Candida albicans histidine kinase Chk1p: signaling and cell wall mannan. Fungal Genet Biol. 2009;46:731-741.
37. Klippel N, Cui S, Groebe L, Bilitewski U. Deletion of the *Candida albicans* histidine kinase gene CHK1 improves recognition by phagocytes through an increased exposure of cell wall beta-1,3-glucans. Microbiology 2010;156:3432-3444.
38. Bates S, Hughes HB, Munro CA, Thomas WP, MacCallum DM, Bertram G, *et al*. Outer chain N-glycans are required for cell wall integrity and virulence of *Candida albicans*. J BiolChem. 2006;281:90-98.
39. Lopes-Bezerra LM, Lozoya-Perez NE, Lopez-Ramirez LA, Martinez-Alvarez JA, Teixeira MM, Felipe MS, *et al*. Functional characterization of *Sporothrixschenckii*glycosidases involved in the N-linked glycosylation pathway. Med Mycol 2015;53:60-68.
40. Torosantucci A, Chiani P, De Bernardis F, Cassone A, Calera JA, Calderone R. Deletion of the two-component histidine kinase gene (CHK1) of *Candida albicans* contributes to enhanced growth inhibition and killing by human neutrophils in vitro. Infect Immun2002;70:985-987.
41. Gow NA, Netea MG, Munro CA, Ferwerda G, Bates S, Mora-Montes HM,*et al*. Immune recognition of *Candida albicans* beta-glucan by dectin-1. J Infect Dis 2007;196:1565-1571.
42. Arendrup M, Horn T, Frimodt-Møller N. In vivo pathogenicity of eight medically relevant Candida species in an animal model. Infection 2002;30:286-291.
43. Cook SM, McArthur JD. Developing *Galleria mellonella* as a model host for human pathogens. Virulence 2013;4:350-353.

44. Calera JA, Zhao XJ, De Bernardis F, Sheridan M, Calderone R. Avirulence of Candida albicans CaHK1 mutants in a murine model of hematogenously disseminated candidiasis. Infect Immun 1999;67:4280-4284.
45. Kruppa M, Goins T, Cutler JE, *et al*. The role of the *Candida albicans* histidine kinase CHK1 gene in the regulation of cell wall mannan and glucan biosynthesis. FEMS Yeast Res. 2003;3:289-299.
46. Kruppa M, Jabra-Rizk MA, Meiller TF, Calderone R. The histidine kinases of *Candida albicans*: regulation of cell wall mannan biosynthesis. FEMS Yeast Res 2004;4:409-416.
47. Kruppa M. Quorum sensing and *Candida albicans*. Mycoses2009;52:1-10.
48. Li D, Bernhardt J, Calderone R. Temporal expression of the *Candida albicans* genes CHK1 and CSSK1, adherence, and morphogenesis in a model of reconstituted human esophageal epithelial candidiasis. Curr Top Med Chem 2002;2:939-971.
49. Chapeland-Leclerc F, Paccallet P, Ruprich-Robert G, Reboutier D, Chastin C, Papon N. Differential involvement of histidine kinase receptors in pseudohyphal development, stress adaptation, and drug sensitivity of the opportunistic yeast *Candida lusitaniae*. Eukaryot Cell 2007;6:1782-1794.
50. Cho Y, Kim KH, La Rota M, Scott D, Santopietro G, Callihan M, *et al*. Identification of novel virulence factors associated with signal transduction pathways in *Alternariabrassicicola*. Mol Microbiol 2009;72:1316-1333.
51. Luo YY, Yang JK, Zhu ML, Liu CJ, Li HY, Lu ZB, *et al*. The group III two-component histidine kinase AlHK1 is involved in fungicides resistance, osmosensitivity, spore production and impacts negatively pathogenicity in *Alternarialongipes*. CurrMicrobiol2012;64:449-456.
52. Papon N, Courdavault V, Clastre M, Bennett RJ. Emerging and emerged pathogenic *Candida* species: beyond the *Candida albicans* paradigm. PLoSPathog 2013;9:e1003550.

FIGURE LEGENDS

Fig.1. Different steps in construction of the *CHK1* disruption cassette. A.Schematic representation of the *C. Guilliermondii CHK1* locus and position of primers CHK1S and CHK1R. **B.** Representation of plasmid pG-*CHK1*. **C.** Structure of the pG-*RU3R* plasmid. **D.** Representation

of plasmid pG-*5'CHK1-REP-URA3-REP-3'CHK1*. **E.** Structure of the *5'CHK1-REP-URA3-REP-3'CHK1*disruption cassette.

Fig. 2. Disruption of *C. guilliermondiiCHK1*. 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 *chk1*Δ mutant using CHK1S and CHK1R primers (upper panel). The presence of *CHK1* mRNAs in the ATCC 6260 wild-type strain and the representative *chk1*Δmutant 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.

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 *chk1*Δ 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.

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

570 the survival of infected macrophages after 24 h of infection. The number of surviving macrophages,
571 positive for both calcein and anti-CD16 fluorescence, was determined in the presence and in the
572 absence of yeast cells to calculate percent survival. Macrophages associated with yeast, i.e,
573 phagocytosing macrophages, were identified as macrophages positive for CFW fluorescence. Non-
574 phagocytosing macrophages were negative for CFW fluorescence. Results are shown as mean ± S.E.
575 of three independent experiments performed in triplicate or quintuplet.
576
577 **Fig. 5. Loss of *CHK1* does not affect the ability of *C. guilliermondii* to stimulate cytokine**
578 **production by human PBMCs.** Live or heat-killed (HK) cells either from strain ATCC 6260
579 (closed bars) or the *chk1*Δ null mutant (open bars) were co-incubated with human PBMCs at 37°C
580 and 5% CO_2. Upon 24 h incubation, supernatants were collected and used to measure cytokine
581 levels by ELISA. Results are shown as mean ± SD of three independent experiments performed in
582 duplicate, with a total of six donors. *P< 0.05.
583
584 **Fig. 6. Virulence of *C. guilliermondii chk1*Δ in the insect model *G. mellonella*.** Groups containing
585 10 larvae were infected with 10 µL of 1 x 10^5 yeast/µL of strains ATCC 6260 (WT) or the *chk1*Δ
586 null mutant, and mortality was recorded daily for 15 days. As a control, one animal group was
587 injected with 10 µL of PBS. Kaplan-Meier plots were generated with cumulative data of three
588 independent experiments, and each survival curve contained 30 animals.

Table 1. Strains used in this study

Species	Strain	Genotype
Candida guilliermondii	ATCC 6260 [a]	wild type
	U312 [b]	*ura3*
	chk1Δ	*ura3, chk1::REP-URA3-REP*
Candida parapsilosis	ATCC 22019 [a]	wild type
Candida albicans	SC5314 [a]	wild type
Candida lusitaniae	ATCC 38533 [a]	wild type
Candida glabrata	ATCC 90030 [a]	wild type

[a] Reference strain from American Type Culture Collection, ATCC Manassas, USA.

[b] From [16].

Table 2. MIC determination of *C. guilliermondii* wild type strain and *chk1*Δ null mutant towards various antifungal drugs and other chemicals.

	WT	*chk1Δ*
Antifungals		
Amphotericin B (µg/mL)	0.5 (0.25-0.5)	0.25 (0.25-0.5)
Flucytosine (µg/mL)	0.25 (0.12-0.5)	0.25 (0.12-0.5)
Fluconazole (µg/mL)	4	2 (2-4)
Voriconazole (µg/mL)	0.06	0.06 (0.03-0.06)
Caspofungin (µg/mL)	2 (1-2)	2
Other chemicals		
Congo Red (mM)	0.03	0.03 (0.03-0.06)
SDS (mM)	0.375 (0.375-0.5)	0.5 (0.5-0.625)
Caffeine (mM)	0.75	0.75 (0.5-0.75)
NaCl (M)	1.25 (1-1.25)	1.25 (1-1.25)
Sorbitol (M)	1.25 (1-1.25)	1 (0.75-1.25)
H_2O_2 (mM)	2.5 (2.5-4)	4 (2.5-4)
Menadione (mM)	2 (1-2)	2 (1-2)

Table 3. Cell wall composition of *C. guilliermondii chk1*Δ null mutant

	Cell wall abundance				
Strain	Chitin (%)	Mannan (%)	Glucan (%)	Phosphomannan content (μg)[a]	Protein (μg)[b]
ATCC6260	1.8 ± 1.2	43.7 ± 3.2	54.5 ± 4.3	58.8 ± 9.0	165.7 ± 14.6
*chk1*Δ	1.6 ± 1.3	42.7 ± 3.5	55.7 ± 3.7	59.8 ± 10.9	157.7 ± 15.6

[a]μg of Alcian Blue bound/OD_{600}=1

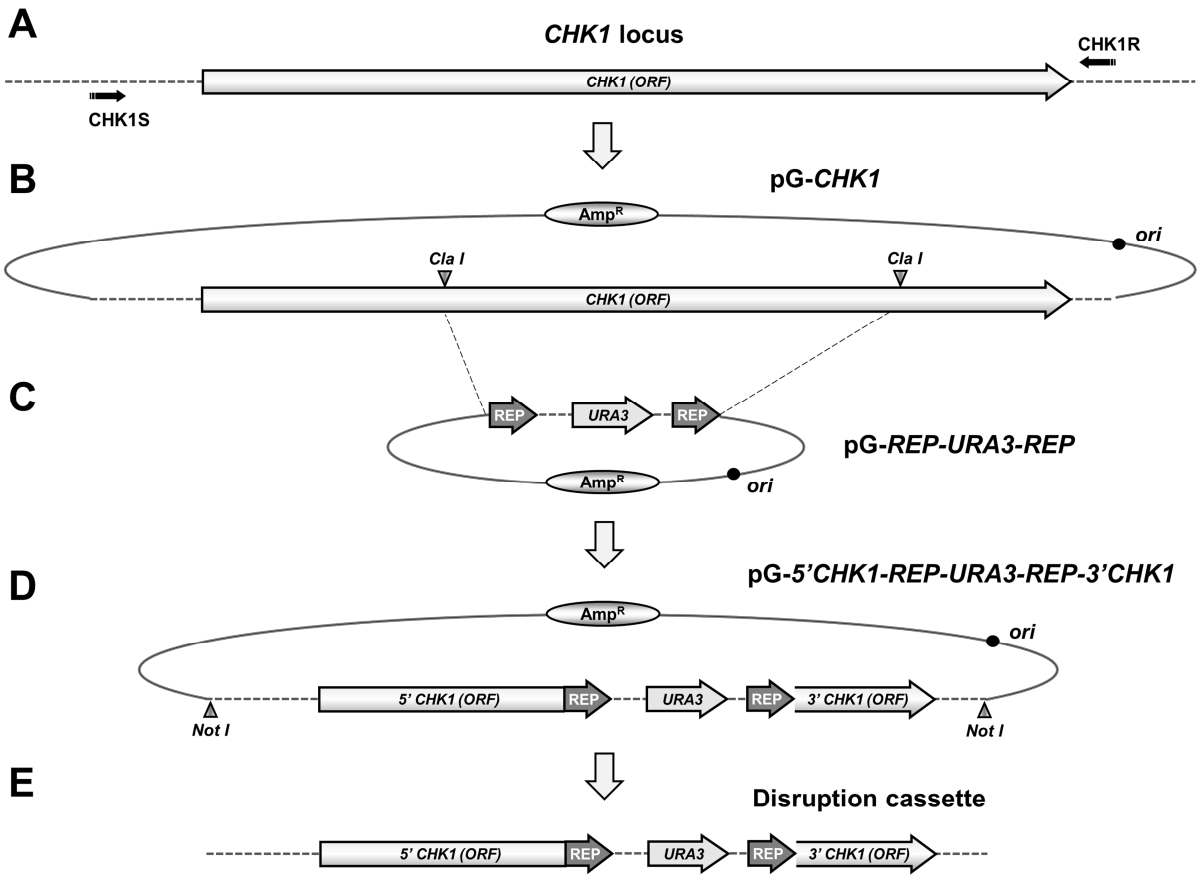
[b]μg of protein/mg of cell wall

Table 4. Comparative analysis of the interactions involving J774 macrophages and different *Candida* species

Species	Fungal biomass multiplication at 24 h[a]	Internalized yeast cells (%)			Macrophage (%) survival at 24 h (phagocytosing, %)	Yeast (%) mortality at 24 h
		1h	5 h	24 h		
C. albicans SC5314	6.8 ± 0.1	18 ± 2	10 ± 1	0	5 ± 3 (67 ±17)	0
C. glabrata ATCC 90030	1.4 ± 0.1	55 ± 5	76 ± 2	67 ± 9	79 ± 12 (47 ± 11)	15 ± 5
C. lusitaniae ATCC 38533	3.6 ± 1.0	54 ± 5	64 ± 2	37 ± 4	73 ± 7 (57 ± 6)	26 ± 1
C. guilliermondii ATCC 6260	1.1 ± 0.1	41 ± 4	77 ± 10	81 ± 21	90 ± 24 (63 ± 1)	18 ± 1

[a]compared to initial fungal biomass at time = 1h.

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


A
CHK1 locus
CHK1R
CHK1 (ORF)
CHK1S
B
pG-CHK1
Amp^R
ori
Cla I
Cla I
CHK1 (ORF)
C
REP
URA3
REP
pG-REP-URA3-REP
Amp^R
ori
D
pG-5'CHK1-REP-URA3-REP-3'CHK1
Amp^R
ori
5' CHK1 (ORF)
REP
URA3
REP
3' CHK1 (ORF)
Not I
Not I
E
Disruption cassette
5' CHK1 (ORF)
REP
URA3
REP
3' CHK1 (ORF)