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RESEARCH ARTICLE

Deletion of *GLX3* in *Candida albicans* affects temperature tolerance, biofilm formation and virulence

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One sentence summary: Deletion of the glyoxalase-encoding gene GLX3 in *Candida albicans* affects temperature tolerance, biofilm formation and virulence.
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ABSTRACT

Candida albicans is a predominant cause of fungal infections in mucosal tissues as well as life-threatening bloodstream infections in immunocompromised patients. Within the human body, *C. albicans* is mostly embedded in biofilms, which provides increased resistance to antifungal drugs. The glyoxalase Glx3 is an abundant proteomic component of the biofilm extracellular matrix. Here, we document phenotypic studies of a *glx3*Δ null mutant concerning its role in biofilm formation, filamentation, antifungal drug resistance, cell wall integrity and virulence. First, consistent with its function as glyoxalase, the *glx3* null mutant showed impaired growth on media containing glycerol as the carbon source and in the presence of low concentrations of hydrogen peroxide. Importantly, the *glx3*Δ mutant showed decreased fitness at 37°C and formed less biofilm as compared to wild type and a reintegrant strain. At the permissive temperature of 28°C, the *glx3*Δ mutant showed impaired filamentation as well as increased sensitivity to Calcofluor white, Congo red, sodium dodecyl sulfate and zymolyase, indicating subtle alterations in wall architecture even though gross quantitative compositional changes were not detected. Interestingly, and consistent with its impaired filamentation, biofilm formation and growth at 37°C, the *glx3*Δ mutant is avirulent. Our results underline the role of Glx3 in fungal pathogenesis and the involvement of the fungal wall in this process.

Keywords: *C. albicans*; candidiasis; Glx3 protein; biofilms; cell wall

INTRODUCTION

Candida albicans is an opportunistic pathogenic yeast present in the mucous membranes of many humans, and is usually isolated from oral cavities and gastrointestinal and urogenital tracts (Brown, Baker and Barker 2007). In healthy individuals, *C. albicans* may form part of the human microbiota as a commensal without causing any detrimental effects. However, a disturbance in the balance between the normal microbiota and the host immune system may cause outgrowth of *C. albicans* and

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colonization of human tissues, which can develop into different forms of candidiasis including life-threatening invasive infections, the latter especially occurring in individuals with weakened immune systems (Fidel, Vazquez and Sobel 1999).

Switching between yeast, pseudohyphal and hyphal growth morphologies, depending on nutritional as well as environmental conditions, is considered an important factor contributing to *C. albicans*' success as a pathogen (Sellam and Whiteway 2016). In addition, *C. albicans* has the ability to firmly adhere and form biofilms on mucosal tissues as well as implanted medical devices, especially implanted intravascular catheters (Yousif, Jamal and Raad 2015). As most *Candida* cells in the human body are present in biofilms, these structures play a very important role in pathogenesis (Soll 2008). Within biofilms, cells are embedded in an extracellular matrix (ECM) containing proteins and polysaccharides, which provides protection against antifungal compounds and decreases their efficacy in therapeutic treatments (Sardi *et al.* 2013). Other factors considered important for *C. albicans* virulence are, for example, candidalysin, a secreted cytolytic peptide toxin that is critical for mucosal infection (Moyes *et al.* 2016), and secreted hydrolytic enzymes involved in tissue damage and invasion of tissues, wall composition, cell differentiation and antigenic variability (Cutler 1991).

Proteomic analysis of ethanol extracts from mature (48 h) *C. albicans* biofilms formed on polystyrene resulted in the identification of 86 ECM proteins, one of the most abundant being the glutathione-independent glyoxalase Glx3 (Martínez *et al.* 2016). This protein was also detected in biofilms onto polymethylmethacrylate upon extraction with ammonium bicarbonate/β-mercaptoethanol buffer. Yet, its expression appeared to increase due to filamentation rather than directly being related to the formation of the biofilm (Martínez-Gomariz *et al.* 2009). In accordance with the above-mentioned proteomic studies and its theoretical mass of 26 kDa, Glx3 had been first documented as a 29-kDa protein that showed binding to IgE antibodies in sera from asthmatic patients (Chou *et al.* 2003), and was suggested to be related to allergic conditions in humans such as bronchial asthma, allergic rhinitis or atopic dermatitis (Savolainen *et al.* 1993; Akiyama *et al.* 1994).

Glx3 is a member of the DJ-1 superfamily and is the ortholog of Hsp31 in *Saccharomyces cerevisiae*. These proteins have enzymatic glyoxalase activity, converting the cytotoxic carbonyl compound methylglyoxal into D-lactate. The crystal structure of Glx3 has been resolved, and its glutathione-independent glyoxalase activity has been demonstrated (Hasim *et al.* 2014). Consistent with this, mutants lacking *GLX3* displayed increased levels of intracellular methylglyoxal and growth defects when grown on glycerol as the sole carbon source (Hasim *et al.* 2014).

In this study, we focus on the role of Glx3 in biofilm formation, filamentation and virulence. We show that a homozygous *glx3*Δ null mutant displays a temperature-sensitive phenotype, alterations in cell wall organization, reduced biofilm formation, failure to develop germ tubes, and is avirulent, altogether stressing the importance of Glx3 in the establishment of *C. albicans* infections.

MATERIALS AND METHODS

Strains, growth conditions and fitness

Candida albicans strains used in this study are listed in Table 1. Yeast cells were grown at 28°C in liquid YPD (1% yeast extract, 2% peptone, 2% dextrose) medium or on YPD agar unless stated otherwise. Growth in the presence of glycerol as the carbon source was performed in yeast extract/peptone medium containing 2% (v/v) of glycerol (YPG).

Fitness of yeast strains was determined by monitoring their growth in YPD or YPG during exponential growth as described (St Onge *et al.* 2007). Given values are means (normalized to wild type [WT]) and standard deviations (SD) of three independent experiments. Statistical significance of data was analyzed by one-way ANOVA and post hoc Bonferroni tests.

Disruption of the *GLX3* gene

The *GLX3* gene (C3_02610C_A) was disrupted using the *Sat1*-flipper technique described by (Reuß *et al.* 2004). The *SAT1* flipper cassette in plasmid pSFS2 employs a *CaSAT1* marker, which confers resistance to the antibiotic nourseothricin (NT), a *C. albicans*-adapted version of the site-specific flippase *FLP1* from *S. cerevisiae* for recycling of the deletion cassette, and Flp1 recognition target sequences (*FRT*) on either end of the cassette. To facilitate homologous recombination at the *GLX3* locus, flanking 5′ and 3′ regions of *GLX3* were cloned between the vector backbone and the *FRT* sequences after PCR amplification of the respective regions from the genome of reference strain SC5314. PCR amplification was performed with proofreading KAPA polymerase (KAPA BIOSYSTEMS) and primers GLX3up_F (CACAGGGCCCTCAATCTTGTTGGTGTATAAACTTTC) and GLX3up_R (CGGCCTCGAGTCCACACCGGGATATTGTCTTGC), generating an upstream fragment of 428 bp (positions –468 to –40 with respect to start codon), and primers GLX3down_F (CACACCGCGGTAGGGCATACAAAAGGTGTGGAT) and GLX3down_R (ATCCGAGCTCCCACCACCCACAAGGGAGAC), generating a 485-bp downstream fragment (positions +815 to +1300). Restriction enzyme recognition sites incorporated in the primers for cloning are underlined. The amplified up- and downstream fragments were cloned in a two-step process in the *Apa*I and *Xho*I (upstream fragment) and *Sac*II and *Sac*I (downstream fragment) sites of pSFS2 using standard methods, yielding the *GLX3* deletion construct, which was verified by sequencing.

Table 1. *Candida albicans* strains used in this study.

Strains	*GLX3* genotype	Parental strain	Source
SC5314	*GLX3/GLX3*		Gillum, Tsay and Kirsch (1984)
*GLX3/glx3*Δ	*GLX3/glx3*Δ::*FRT*	SC5314	This work
*glx3*Δ/*glx3*Δ	*glx3*Δ::*FRT*/*glx3*Δ::*FRT*	*GLX3/glx3*Δ	This work
*glx3*Δ/*glx3*Δ, *GLX3*	*glx3*Δ::*FRT*/*glx3*Δ::*FRT*, *RPS1*/*rps1*::*pGLX3*	*glx3*Δ/*glx3*Δ	This work

Candida albicans transformation was performed as described (Reuß *et al.* 2004; Sánchez-Fresneda *et al.* 2015). Transformants were grown on YPD plates containing 200 μg/ml NT (Jena Biosciences). Selected transformants were grown in liquid YPM (1% yeast extract, 2% peptone, 2% maltose) to induce excision of the deletion cassette, and then spread on YPD plates containing 10 μg/ml NT to select NT-sensitive colonies. After verification of the heterozygous mutants, the whole procedure was repeated to obtain homozygous mutants. Correct integration of the disruption cassette in both alleles of the *GLX3* locus was verified by southern blot analysis, detailed in Supplementary Fig. S1.

Construction of a *GLX3* reintegrant strain

A reintegrant strain was constructed to complement the loss of *GLX3* in the *glx3*Δ/*glx3*Δ null mutant. The *GLX3* gene plus promoter and terminator regions (positions −935 to +1536) were PCR amplified using primers GLX3p_F (5′-GGTACCTGACCAATACTGCTCACTACTAG-3′) and GLX3t_R (5′-GTCGACTGTGTGATGTGTGTGTTGAGTGG-3′) containing engineered *Kpn*I and *Sal*I restriction sites, respectively, and cloned in plasmid CIpSAT2 (Moreno-Ruiz *et al.* 2009), a derivative of the CIp10 plasmid (Murad *et al.* 2000) in which the *URA3* marker was substituted by *CaSAT1*. After sequence verification, the generated plasmid pGLX3 (CIpSAT2::*GLX3*) was linearized by digestion with *Bst*BI and transformed into the *glx3*Δ/*glx3*Δ mutant. NT-resistant transformants containing a complete *GLX3* gene were identified by PCR using primers GLX3F (ATGGTCAAAGTTTTACTCGCTC) and GLX3R (ATTACATTCAAAAGCAGCAATTAC).

Cell wall composition and drug sensitivity testing

Cell walls from cultures grown to log phase at 28°C were isolated as described (Pedreño *et al.* 2004; Castillo *et al.* 2008). The methodology used for determination of the cell wall composition (protein, glucan, mannan, chitin) can be found in (Laforet *et al.* 2011) and references therein.

Sensitivity to Calcofluor white (CFW), Congo red (CR), sodium dodecyl sulfate (SDS) and hydrogen peroxide (H_2O_2) was tested using spot assays. In short, 5 μl of cell suspensions of OD_{600nm} = 1.0 and 10-fold serial dilutions thereof were spotted on YPD plates containing different concentrations of CFW (12 and 15 μg/ml), CR (50 and 100 μg/ml), SDS (0.015 and 0.025%) or H_2O_2 (11 and 12 mM). Growth was monitored after 2–3 days of incubation at 28°C and 37°C.

Sensitivity to zymolyase was measured as described (Laforet *et al.* 2011) with small modifications. Cell cultures that were grown to the exponential phase were adjusted to an OD_{600nm} of 1 in 10 mM Tris-HCl, pH 7.5, containing 100 μg/ml of zymolyase 20T. The decrease in OD_{600nm} as a measure of zymolyase sensitivity was monitored over a 120-min period.

Minimal inhibitory concentration (MIC) values of clinical antifungal drugs (fluconazole, voriconazole, posaconazole, caspofungin, micafungin, anidulafungin, amphotericin B) were determined following CLSI guidelines (CLSI 2008).

Biofilm formation

Yeast cultures were grown overnight in liquid YPD, harvested, and washed in sterile 10 mM phosphate-buffered saline (PBS), pH 7.4. Cells were resuspended at a concentration of 1×10^6 cells/ml in RPMI-1640 medium supplemented with L-glutamine (2 mM) and buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (25 mM), and 100 μl of the cell suspensions were pipetted into sterile polystyrene 96-well plates (Nalgene). Plates were incubated for 24 h at 37°C with gentle shaking (75 rpm) to allow biofilm formation after which the medium was aspirated and non-sessile cells were removed by washing the wells three times with sterile PBS. Biofilm formation was quantified by staining with 0.05% crystal violet (CV) solution for 15 min at room temperature. Unbound CV stain was removed by gently washing with water until no more stain was coming off the wells. The remaining bound CV in each well was solubilized by adding 150 μl of 33% acetic acid, and quantified by reading the OD_{595nm} using a microtiter plate reader (Bio-Rad). Metabolic activity of biofilms was determined by the XTT (2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)-reduction assay (Ramage *et al.* 2001). XTT (Sigma) and menadione were added to 24-h biofilms, incubated in the dark for 2 h at 37°C, followed by measuring the $OD_{480\ nm}$. Statistical significance of the data was analyzed by one-way ANOVA and post hoc Bonferroni tests. Microscopical inspection of biofilm cell morphology was performed by phase-contrast microscopy using a Leica DM1000 microscope.

Induction of germ tube formation

Cells from exponentially growing cultures (in YPD at 28°C) were washed with distilled water and resuspended at an OD_{600nm} of 0.3 in prewarmed YPD containing 10% (v/v) filter-sterilized human serum to allow filamentation. After 2 h of incubation at 28°C, cells were stained with CFW and germ tube formation was monitored by fluorescence microscopy as described (Guirao-Abad *et al.* 2013).

Virulence tests in mice

Candida albicans cells were grown overnight in Sabouraud broth at 28°C. Cells were harvested, washed twice and then resuspended in sterile PBS to obtain suspensions of 5×10^6 CFU/ml. For each yeast strain, groups of at least 10 mice of 6- to 8-week-old female immunocompetent BALB/c mice with a weight of about 20 g were injected with 200 μl of the cell suspensions through the lateral tail vein. Survival was monitored over a period of 30 days. Survival times were calculated using the Kaplan–Meier method and compared using the Mantel-Cox log-rank test.

RESULTS

Candida albicans Glx3 (Candida Genome Database systematic name C3_02610C_A) is a glutathione-independent methylglyoxalase (Hasim *et al.* 2014) that has been detected in ECM samples from biofilms (Martínez *et al.* 2016) as well as in cell wall extracts (Castillo *et al.* 2008; Gil-Bona *et al.* 2015). In this study, we therefore focus on the importance of Glx3 for biofilm formation, cell wall integrity and virulence. *GLX3* deletion mutants in wild-type strain SC5314 background were constructed using the *SAT1*-flipper method (Reuß *et al.* 2004), as well as *GLX3* reintegrant strains expressing (one copy of) the gene from its own promoter at the neutral *RPS1* locus. Obtained *glx3*Δ mutants were validated by southern blotting (Supplementary Fig. S1). Phenotypic validation of the *glx3* null mutants was achieved by monitoring growth on YPG media containing 2% glycerol as carbon source. With growth rates of 53% (at 28°C) and 51% (at 37°C) as compared to WT, the null mutant showed the same carbon source-dependent growth defect when lacking this methylglyoxalase as described by Hasim and colleagues (Hasim *et al.* 2014).

Table 2. Relative fitness of *glx3*Δ mutant strains.

	Relative fitness[a]	
Strain	28°C	37°C
SC5314	1.00 ± 0.01	1.00 ± 0.02
*GLX3/glx3*Δ	0.99 ± 0.01	1.04 ± 0.03
*glx3*Δ/*glx3*Δ	0.76 ± 0.02**	0.40 ± 0.03**
*glx3*Δ/*glx3*Δ, *GLX3*	0.88 ± 0.02**	0.89 ± 0.02*

[a]Relative fitness is calculated as growth rate in YPD normalized to SC5314. Values are means of at least three independent experiments. Mutant values with statistically significant differences compared to WT are indicated by asterisks (*, $P < 0.05$; **, $P < 0.001$).

Deletion of *GLX3* leads to temperature-dependent growth defects

Fitness of *glx3*Δ mutants was studied by growing in YPD medium at 28°C and 37°C (Table 2). At 28°C, the homozygous null mutant strain grew well and showed only a slight reduction in fitness as compared to the parental strain. However, when grown at 37°C, the growth rate of the null mutant was reduced by more than 50%. This same temperature-dependent growth defect was also observed in the drop assay on YPD agar (Fig. 2A). The temperature-sensitive growth phenotype was not observed for the heterozygous mutant, and was almost completely alleviated by reintroduction of the *GLX3* gene in the *glx3*Δ/*glx3*Δ null mutant strain. Because of the thermosensitivity of the null mutant, when appropriate, most of the follow-up experiments were performed at 28°C instead of (or in addition to) 37°C.

Biofilm formation and filamentation is affected in *glx3*Δ null mutants

The ability of *C. albicans* to form biofilms is considered an important feature contributing to its virulence (Ramage, Martínez and López-Ribot 2006; Kniemeyer *et al.* 2011). As formation of biofilms is very limited at 28°C, the aptitude of *glx3*Δ mutants to form biofilms onto polystyrene was analyzed at 37°C. In two different assays, monitoring CV staining and XTT reduction of biofilms, respectively, the *glx3*Δ/*glx3*Δ homozygous null mutant showed a more than 2-fold reduction in biofilm formation (Fig. 1A). Reintroduction of *GLX3* restored the ability to form biofilms almost to the level of the heterozygous mutant, supporting that the reduction in biofilm formation is caused by the lack of *GLX3*.

Within the human host, *C. albicans* biofilms normally present a mixture of different morphologies including yeast, pseudohyphae and hyphae (Sudbery, Gow and Berman 2004). Filamentation therefore is considered to contribute significantly to biofilm formation. Thus, apart from the reduced growth rate at 37°C, a second reason for the decreased biofilm formation of the homozygous mutant may be an altered pattern of filamentation. Supporting this idea, microscopical examination of the cells in the biofilms cells showed extensive filamentation of the parental WT strain, whereas only pseudohyphal-like structures and yeast cells were observed for the homozygous *glx3*Δ mutant (Fig. 1B). Germ tube formation of *glx3*Δ mutants was also tested at the permissive temperature of 28°C in YPD supplemented with 10% (v/v) of human serum. Under these conditions, after 2 h of induction, WT cells had started to develop germ tubes but this was not the case for any of the *glx3*Δ mutants (Fig. 1C). Thus, full Glx3 activity seems to be required to trigger filamentation under these conditions.

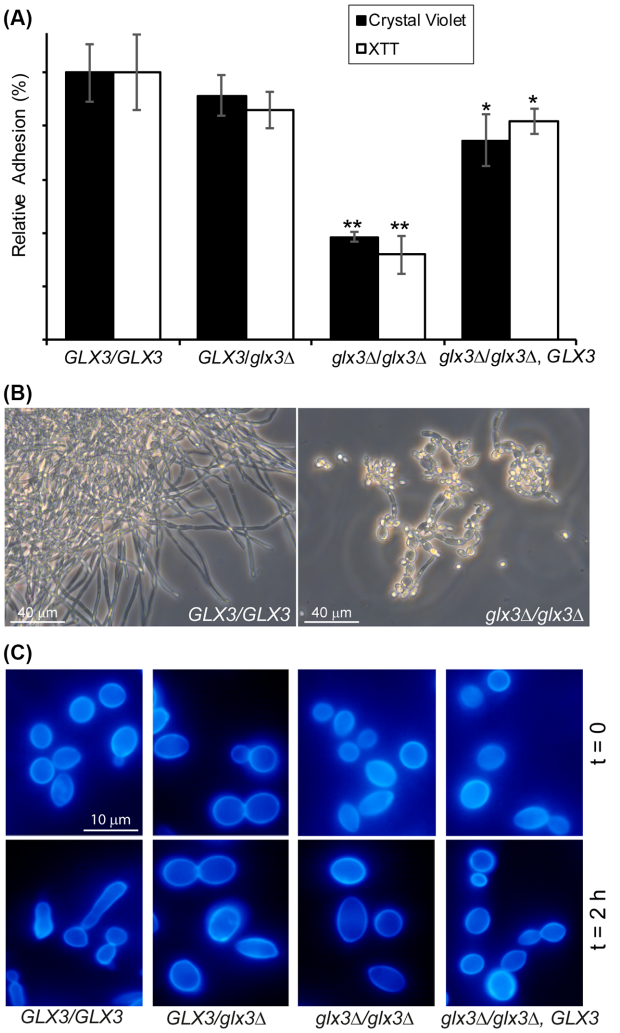

Figure 1. Deletion of *GLX3* affects biofilm formation and filamentation. (**A**) Biofilm formation onto polystyrene after 24 h in RPMI at 37°C was measured by CV staining and XTT reduction. Values are mean ± SD of three independent experiments. Statistically significant differences between WT and mutants are indicated (*, $P < 0.05$; **, $P < 0.001$). (**B**) Cellular morphology of WT and *glx3*Δ null mutant in biofilms. (**C**) Induction of germ tube formation by human serum. After 2 h at 28°C, cells were stained with CFW and observed by fluorescence microscopy.

Deletion of *GLX3* affects cell wall organization

Several components of the *C. albicans* cell wall have been shown to play a role in biofilm formation (Nobile *et al.* 2008; Soll 2008; Moreno-Ruiz *et al.* 2009; Liu and Filler 2011). Glx3 has been detected as a so-called moonlighting protein in cell wall extracts (Castillo *et al.* 2008; Gil-Bona *et al.* 2015); we therefore set out to assess a possible link between Glx3 function and cell wall structure. Absolute quantification of the cell wall macromolecular components in *C. albicans*, mostly yielding values of 50%–60% for glucan, 25%–35% for mannan, 2.5%–4% for chitin and 3%–5% for protein, has been reported in various papers (Kapteyn *et al.* 2000; De Groot *et al.* 2008; Thevissen *et al.* 2012). With relative amounts of 99% glucan, 98% mannan, 106% chitin and 99% protein (standard errors ranging from 5% to 8%), no significant differences

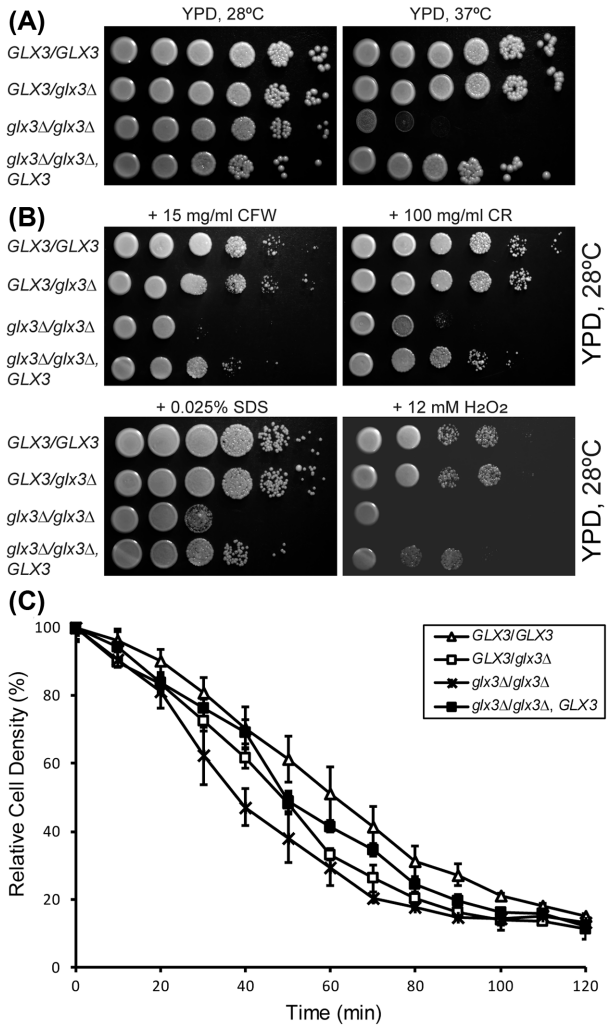

Figure 2. Lack of *GLX3* results in a temperature-dependent growth defect, alterations in cell wall organization and hypersensitivity to oxidative stress. (**A**) Growth on YPD agar at 28°C and 37°C. (**B**) Sensitivity to CFW, CR, SDS and H_2O_2 oxidative stress. Serial 10-fold dilutions of cells were spotted on YPD agar containing the compounds at the indicated concentrations. Lower concentrations of compounds (see Materials and Methods) gave similar results and are therefore not shown. (**C**) Sensitivity to zymolyase. Exponentially growing cells were incubated with 100 μg/ml of zymolyase 20T and the decrease of OD_{600nm} over time was monitored. Data represent the average ± SD of three independent experiments.

in the quantities of these cell wall components were observed in the homozygous *glx3*Δ mutant as compared to the parental strain. To reveal possible alterations in cell wall architecture rather than wall composition, sensitivity to the cell wall-perturbing compounds CFW and CR was assayed using drop tests (Fig. 2B). The homozygous but not heterozygous *glx3*Δ mutant showed increased sensitivity to both CFW and CR compared to the parental strain. In both cases, the increased sensitivity was partially alleviated by reintroduction of *GLX3*. The homozygous mutant also showed increased sensitivity to the presence of the membrane-perturbing detergent SDS at a concentration of 0.025% (Fig. 2B). The effect of *GLX3* disruption on cell wall organization was also investigated by analyzing sensitivity to zymolyase (a β-1,3-glucanase enzyme preparation). Absence of *GLX3* resulted in faster cell lysis upon adding zymolyase (Fig. 2C).

Table 3. *In vitro* susceptibility of the *glx3*Δ deletion mutant to antifungal drugs.

	MICS (μg/ml)[a]	
Antifungal compound	SC5314 (WT)	*glx3*Δ/*glx3*Δ
Caspofungin	0.125	0.250
Micafungin	0.016	0.016
Anidulafungin	0.016	0.016
Fluconazole	0.250	0.500
Voriconazole	0.016	0.016
Posaconazole	0.016	0.016
Amphotericin B	0.400	0.050

[a]Values are representative outcomes of three independent experiments determined following CLSI guidelines (CLSI 2008).

Mutants lacking *GLX3* are hypersensitive to H_2O_2 and amphotericin B

In a previous work, it has been proposed that, in addition to its role in detoxifying glyoxals, Glx3 may have other functions in stress response (Hasim *et al.* 2014). Therefore, sensitivity to oxidative stress was tested by the presence of H_2O_2 in the growth medium (Fig. 2B). Growth of the *glx3*Δ null mutant was clearly affected by the presence of 12 mM H_2O_2 (at 11 mM the effect was less strong, not shown), and this hypersensitivity to H_2O_2 was partially alleviated when a copy of *GLX3* was restored.

Sensitivity of the homozygous *glx3*Δ mutant to different pharmaceutical antifungal drugs was assayed by determining their MICs (Table 3). Echinocandins affect the synthesis of the major cell wall component β-1,3-glucan. But, despite the observed altered sensitivity to cell wall-perturbing agents, only small 2-fold (caspofungin) or no (micafungin and anidulafungin) alterations were detected in sensitivity to this class of antifungals. Also no dramatic alterations were detected in the MICs for the azoles fluconazole, voriconazole and posaconazole. However, the MIC value of the ergosterol-binding drug amphotericin B for the *glx3*Δ mutant was 8-fold lower than for WT.

*glx3*Δ mutants are avirulent in mice

Involvement of *GLX3* in virulence was tested using the mouse model of systemic infections. Eight days after infection, all mice that were injected with the parental strain had died, whereas all mice injected with the homozygous *glx3*Δ/*glx3*Δ mutant stayed alive until the end of the experiment at day 30 (Fig. 3). Mice infected with the heterozygous mutant and the reintegrant strain yielded intermediate results. These data indicate that in this *in vivo* mouse model, *GLX3* is required for full virulence of *C. albicans* in a gene dose-dependent manner.

DISCUSSION

Glx3 in *C. albicans* is a glutathione-independent glyoxalase that plays an important role in managing metabolically generated methylglyoxal (Hasim *et al.* 2014). Because Glx3 and its fungal homologs are not redundant with glutathione-dependent glyoxalase of the *GLO1*/*GLO2* system, it has been proposed that in addition to its role in detoxifying glyoxals, Glx3 may have other important physiological functions, such as in stress response (Hasim *et al.* 2014). The observed temperature-sensitive growth defect at 37°C and increased sensitivity to oxidative stress of homozygous *glx3*Δ mutants support this hypothesis.

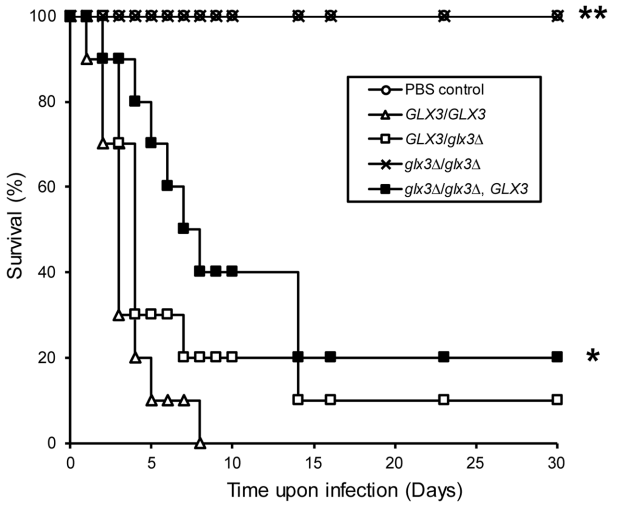

Figure 3. Glx3 is required for full virulence. Kaplan–Meier survival graph for mice infected with *C. albicans* cells. The control group was injection with PBS buffer only. Significant differences in survival times compared to the parental strain are indicated (*, $P < 0.05$; **, $P < 0.001$).

Here, we focused on the possible role of Glx3 in biofilm formation, cell wall organization and virulence. Formation of biofilms is considered an important factor in developing *Candida* infections. In *C. albicans,* biofilms usually consist of a mixture of hyphae, pseudohyphae and yeast cells, embedded in an ECM. For *C. albicans*, filamentation seems to be important in the development of biofilm structures (Garcia-Sanchez *et al.* 2004; Laforet *et al.* 2011). Identified as an abundant protein species in *C. albicans* biofilm extracts (Martínez-Gomariz *et al.* 2009; Martínez *et al.* 2016), Glx3 might be an important element in the formation of biofilms as component of the ECM. The 2-fold reduction in biomass formation observed in *glx3*Δ null mutants supports this idea. As *glx3*Δ mutants did not show initiation of germ tube formation, our results suggest that both the reduced fitness at 37°C as well as the aberrant filamentation are factors that caused the decreased biofilm formation of the homozygous *glx3*Δ mutant.

Glx3 is predicted to be localized in the cytosol (PSORT II, https://psort.hgc.jp/form2.html) and exerts intracellular activity (Hasim *et al.* 2014). Therefore, the fact that the protein has been detected in cell wall extracts in different studies (Castillo *et al.* 2008; Gil-Bona *et al.* 2015) is rather controversial (Klis *et al.* 2007). Nevertheless, the phenotypes observed in our study suggest that deletion of *GLX3* does affect cell wall integrity through architectural rather than compositional alterations. First, the *glx3*Δ null mutant showed increased sensitivity to CFW and CR. CFW and CR are compounds that perturb the correct assembly of chitin and β-1,3-glucan (Cabib and Bowers 1971). Therefore, mutants with abnormal cell wall structures often show alterations in sensitivity to these compounds. Second, the *glx3*Δ null mutant also showed increased sensitivity to zymolyase, an enzyme preparation that causes cell lysis by hydrolysis of the major cell wall component β-1,3-glucan. As for echinocandins, which inhibit β-1,3-glucan synthesis, a slightly decreased susceptibility was observed towards caspofungin but not towards micafungin and anidulafungin. However, quantitative differences of the main cell wall components glucan, chitin, mannan and protein were not observed. Also frequently observed in cell wall mutants, mostly as pleiotropic phenotypes, are altered sensitivities to conditions that affect the biosynthesis or integrity of the plasma membrane. Indeed, the *glx3*Δ null mutant presented increased sensitivity to the detergent SDS and to the antifungal drug amphotericin B. On the other hand, except a slightly higher MIC value for fluconazole, sensitivity to azole antifungals was mostly unaltered. Altogether, our results indicate that the absence of *GLX3* leads to subtle differences at the cell surface that were not measurable as quantitative differences in cell wall components but that do affect the architecture of the cell wall when challenged with surface-perturbing compounds.

Finally, our results demonstrate that Glx3 plays an important role in the virulence of *C. albicans*. The homozygous *glx3*Δ mutant was completely avirulent in a mouse model of disseminated candidiasis. Such attenuated infectivity resembles that of knockout mutants of *ECM33* (Martinez-Lopez *et al.* 2004). In the heterozygous *GLX3/glx3*Δ mutant and the reintegrant, strains that contain a single copy of *GLX3*, a reduction but not complete absence of virulence was observed, indicating a gene-dose dependent effect. Fitness, cell wall organization, morphological switching and biofilm formation are all factors that are implicated in *C. albicans* pathogenesis. As we have shown, the Glx3 protein is important for fitness at 37°C, affects cell wall integrity as well as filamentation and biofilm formation. By analogy to the protective vaccine effect that has been shown for *ecm33* mutants (Martínez-López *et al.* 2008), we propose that it might be worth investigating if *glx3*Δ null mutants could be used in the future as an immunological tool.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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