Phenotypic characterization of a *Candida albicans* strain deficient in its major exoglucanase

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Both alleles of the *XOG1* gene of *Candida albicans*, which encodes a protein with exoglucanase activity, were sequentially disrupted. Enzymic analysis of either cell extracts or culture supernatants of disrupted strains revealed that this gene is responsible for the major exoglucanase activity in *C. albicans*, although residual exoglucanase activity could still be detected. *xog1* null mutants showed similar growth rates in both rich and minimal liquid medium as compared to the wild-type strain, indicating that the enzyme is not essential for *C. albicans* growth. In addition, no differences were observed between wild-type and *xog1* null mutants with respect to their ability to undergo dimorphic transition. However, small but repeatable differences were found between the wild-type and the null mutant with respect to susceptibility to chitin and glucan synthesis inhibitors. Using a murine model of experimental infection, no significant differences in virulence were observed. The *xog1* null strain is thus a suitable recipient for studying *Candida* gene expression using the exoglucanase as a reporter gene.

Keywords: *Candida albicans*, exoglucanase, gene disruption, reporter gene, pathogenesis

INTRODUCTION

The fungal cell wall is an essential structure involved in many crucial aspects of fungal cell physiology. β -Glucan, a cell-wall polymer of β -glucose linked by 1,3- β -bonds with occasional 1,6- β -branching, is one of its major components, contributing significantly to cell wall mechanical strength and being partially responsible for the generation of a network in which other cell-wall components are assembled. In *Saccharomyces cerevisiae*, a set of different 1,3- β -glucanases involved in β -glucan degradation have been characterized. Glucanases play an important role in cell wall construction (Cid *et al.*, 1994; Nombela *et al.*, 1988) although individual members within this group are redundant. The *EXG1* gene, which was cloned by complementation of a mutant strain deficient in glucanase activity (Nebreda *et al.*, 1986, 1987; Santos *et al.*, 1979b) using a strategy involving the overproduction of glycosidases (Kuranda

& Robbins, 1987), codes for a 51 kDa polypeptide (Vázquez de Aldana *et al.*, 1991) responsible for most of the glucanase activity in *S. cerevisiae*, although with no evident role (Santos *et al.*, 1979a, 1982) in morphogenesis. The *EXG2* gene (Cid *et al.*, 1995) displays significant homology with *EXG1* but has an additional C-terminal region that is apparently responsible for its correct location and assembly in the cell wall (Correa, 1993; del Rey *et al.*, 1994). The third exoglucanase gene (or activity), *SSG1*, encodes a sporulation-specific exoglucanase (Muthukumar *et al.*, 1993; San Segundo *et al.*, 1993). Our understanding of the role of glucanases in *S. cerevisiae* is far from complete and there is an evident lack of knowledge about their role in pathogenic fungi.

Candida albicans is an opportunistic pathogenic yeast responsible for a steadily increasing incidence of fungal diseases among industrialized countries (Fox, 1993). Genetic manipulation of this yeast has been difficult since it is diploid and lacks a sexual cycle (Kurtz *et al.*, 1990). However, in recent years, some of the tools necessary for gene isolation (Cannon *et al.*, 1990, 1992; Herreros *et al.*, 1992; Pla *et al.*, 1995) have been developed. Biochemical work in *C. albicans* has identi-

Abbreviations: FDGP, fluorescein di-(β -D-glucopyranoside); 5-FOA, 5-fluoroorotic acid; MUG, 4-methylumbelliferyl β -D-glucoside; PNPG, p-nitrophenyl β -D-glucopyranoside.

fied a major glucanase activity located in the periplasm of the cell (Molina *et al.*, 1987); this was later shown to be due to a non-glycosylated 40 kDa protein (Luna Arias *et al.*, 1991). Recently, the *XOG1* gene from *C. albicans* has been cloned and shown to encode a 438 amino acid exo-1,3- β -glucanase which has both sequence (58% identity) and antigenic homologies with *S. cerevisiae* Exg1p (Chambers *et al.*, 1993). This gene has been assigned to chromosome I (Dr P. Sullivan, <http://www.alces.med.umn.edu/candida.html>). However, the physiological relevance of the *XOG1* gene has not been explored. In this work we constructed *C. albicans* *xog1* mutant strains using the strategy recently developed by Fonzi & Irwin (1993) to address its functional implications and its role in virulence.

METHODS

Media and culture conditions. Yeast and bacterial strains are listed in Table 1. Yeast strains were routinely grown in YED medium (20 g glucose l⁻¹, 10 g yeast extract l⁻¹) or minimal medium (6.7 g yeast nitrogen base without amino acids l⁻¹, 20 g glucose l⁻¹) supplemented with 40 mg uridine l⁻¹ when necessary. *C. albicans* Ura⁻ revertants were selected at 30 °C upon excision of the *C. albicans* *URA3* gene from integrative transformants (see below) on the basis of the resistance of the Ura⁻ revertants to 5-fluoroorotic acid (5-FOA, SCM Specialty Chemicals). The 5-FOA plates were prepared as described previously (Boeke *et al.*, 1987) but 25 µg uridine ml⁻¹ was used instead of uracil. Measurements of yeast conversion to hyphae were performed in Lee's medium (Lee *et al.*, 1975) at 37 °C. Cell growth was estimated by optical density at 600 nm. *Escherichia coli* strains were grown at 37 °C in Luria-Bertani (LB) broth or Terrific broth (TB) supplemented with 100 µg ampicillin ml⁻¹ for plasmid selection.

Molecular biology techniques. All DNA manipulations were carried out using standard protocols (Sambrook *et al.*, 1989). Restriction enzymes were supplied by Boehringer Mannheim. PCR amplification reactions used a 100 µl reaction volume containing 10 mM Tris/HCl pH 8.3, 1.5 mM MgCl₂, 50 mM

KCl, 0.1 g gelatin l⁻¹, 50 µM dNTPs, 1.5 µM of each oligonucleotide primer and approximately 1 ng plasmid DNA. The reaction was started by the addition of 1 unit *Taq* polymerase (Boehringer Mannheim). After an initial incubation step at 94 °C for 3 min, the samples were subjected to 30 cycles (2 min at 92 °C, 1.5 min at 58 °C, and 2 min at 72 °C) using the following oligonucleotide primers: Olihis, 5'-cgccagctcgattc-tggactgc-3' (23 oligonucleotides), which hybridizes with the fragment included between positions 854 and 877 of the *hisG* gene; Oliura, 5'-agatccagatattgaaggtaaaagg-3' (25 oligonucleotides), which hybridizes with the fragment included between positions 1211 and 1236 of the *URA3* gene; and Olixog, 5'-AGCAGACCATTACCAGCGACG-3' (22 oligonucleotides), which hybridizes with the fragment included between positions 1451 and 1473 of the *XOG1* gene. The Ura⁺ transformants were analysed using the Oliura-Olixog primer pair; for Ura⁻ analysis, the Olihis-Olixog primer pair was used.

***C. albicans* strain construction.** The *C. albicans* strains used are listed in Table 1. To obtain a *C. albicans* *xog1* null strain, we used the standard strategy devised by Fonzi & Irwin (1993). Plasmid pE1 was obtained by insertion of a 3.8 kb *Bam*HI-*Bgl*II from the plasmid pCUB-6 (provided by Dr W. A. Fonzi, Georgetown University Medical Center, USA) containing the *hisG-URA3-hisG* cassette in the *StyI* site of plasmid BS-XOG1, which is a pBluescript derivative containing an *Eco*RI-*Sal*I fragment from plasmid pXAG1 (provided by Dr P. A. Sullivan, University of British Columbia, Canada) subcloned in the *Eco*RI-*Sal*I fragment of pBluescript KS (Stratagene). This 8.3 kb plasmid was then digested with *Bst*XI and *Pst*I and used to transform the CAI-4 strain, thus promoting homologous recombination at the *XOG1* locus (the disruption strategy is shown in Fig. 1a). In this way, strains CNC30-a and CNC30-b were selected from among the positive Ura⁺ transformants and shown both by PCR (data not shown) and Southern blot (Fig. 1b) analysis to display the required integration event in both alleles [the alleles of this strain display restriction fragment polymorphism for the restriction enzyme *Bgl*II (Chambers *et al.*, 1993)]. Strains CNC31-a and CNC31-b were isolated as spontaneous Ura⁻ mitotic recombinants of strains CNC30-a and CNC30-b, respectively.

Table 1. Strains used in this study

Strain*	Relevant genotype or phenotype	Source
<i>C. albicans</i>		
SC5314	Wild-type	Gillum <i>et al.</i> (1984)
CAI-4	Δ ura3::limm434/ Δ ura3::limm434	Fonzi <i>et al.</i> (1993)
CNC30-a	<i>xog1</i> :: <i>hisG-URA3-hisG</i> / <i>XOG1</i> Δ ura3::limm434/ Δ ura3::limm434	This work
CNC30-b	<i>XOG1/xog1</i> :: <i>hisG-URA3-hisG</i> Δ ura3::limm434/ Δ ura3::limm434	This work
CNC31-a	<i>xog1</i> :: <i>hisG/XOG1</i> Δ ura3::limm434/ Δ ura3::limm434	This work
CNC31-b	<i>XOG1/xog1</i> :: <i>hisG</i> Δ ura3::limm434/ Δ ura3::limm434	This work
CNC32-a	<i>xog1</i> :: <i>hisG/xog1</i> :: <i>hisG-URA3-hisG</i> Δ ura3::limm434/ Δ ura3::limm434	This work
CNC32-b	<i>xog1</i> :: <i>hisG-URA3-hisG/xog1</i> :: <i>hisG</i> Δ ura3::limm434/ Δ ura3::limm434	This work
CNC33-a	<i>xog1</i> :: <i>hisG/xog1</i> :: <i>hisG</i> Δ ura3::limm434/ Δ ura3::limm434	This work
CNC33-b	<i>xog1</i> :: <i>hisG/xog1</i> :: <i>hisG</i> Δ ura3::limm434/ Δ ura3::limm434	This work
<i>E. coli</i>		
DH5 α F'	K-12 Δ (<i>lacZYA-argF</i>)U169 <i>supE44 thi-1 recA1endA1 hsdR17 gyrA</i> <i>relA1</i> (ϕ 80 <i>lacZ</i> Δ M15) F'	Hanahan <i>et al.</i> (1988)

* The letter a or b in *C. albicans* CNC strain designations refers to which chromosomal homologue has been affected in the gene replacement.

Fig. 1. Strategy used for disruption of both alleles of the *XOG1* gene in *C. albicans* strain CAI-4. (a) The *hisG-URA3-hisG* construction was inserted at the *StyI* site in the *XOG1* gene. *BstXI-PstI* sites were used to force the integration at the *XOG1* locus. Genomic DNA was digested with *BglII* and *PstI* and analysed by Southern blotting using a probe which contained almost the entire *XOG1* gene (*EcoRI-BamHI*). (b) Southern hybridization analysis of DNA from strains obtained during the disruption process. Lanes: 1, CAI-4 (wild-type, *Ura*⁻); 2, CNC30-a (*xog1::hisG-URA3-hisG/XOG1*); 3, CNC31-a (*xog1::hisG/XOG1*); 4, CNC32-a (*xog1::hisG/xog1::hisG-hisG-URA3-hisG*); 5, CNC33-a (*xog1::hisG/xog1::hisG*); 6, CNC33-b (*xog1::hisG/xog1::hisG*); 7, CNC32-b (*xog1::hisG-URA3-hisG/xog1::hisG*); 8, CNC31-b (*XOG1/xog1::hisG*); 9, CNC30-b (*XOG1/xog1::hisG-URA3-hisG*); 10, CAI-4 (wild-type, *Ura*⁻); 11, 1001 (wild-type, *Ura*⁺). The 2.4 and 1.9 kb bands correspond to the wild-type *xog1* gene, the 5.7 and 6.2 kb bands to the *xog1::hisG-URA-hisG* mutation, and the 3.0 and 3.5 kb bands to the *xog1::hisG* mutation.

Repetition of this procedure allowed us to isolate strains CNC32-a and CNC32-b (*Ura*⁺ background) and CNC33-a and CNC33-b (*Ura*⁻ background) using both CNC31-a (CNC32-a and CNC33-a) and CNC31-b (CNC32-b and CNC33-b) as starting strains for transformation. Southern blot analysis was carried out using a 1.1 kb *EcoRI-BamHI* internal fragment as probe (Fig. 1a).

Flow cytometry. Preparation of cells for flow cytometry assays of exoglucanase activity was performed as described by Cid *et al.* (1994). Briefly, cells were incubated in minimal medium supplemented with 40 mg uridine l⁻¹ until the late-exponential phase. Two 0.1 ml culture samples were transferred to two Eppendorf tubes. Cells were centrifuged and the supernatant was removed. The pellet was washed once with 0.05 M acetate buffer pH 5.3, resuspended in 0.05 ml of the same buffer and 2.5 µl of a 1 mg ml⁻¹ dilution of fluorescein di-(β-D-glucopyranoside) (FDGP, Sigma) was added. The remaining tube was left as a control without fluorogenic substrate. Both tubes were incubated at 30 °C for 3 h in darkness. A 0.5 ml aliquot of 50 mg l⁻¹ propidium iodide in PBS (0.145 M sodium chloride, 0.15 M sodium phosphate) was added to each sample

to discriminate dead cells (de la Fuente *et al.*, 1992) and the samples (1 × 10⁴ cells) were immediately analysed on a FACScan flow cytometer (Becton-Dickinson). The fluorescence emitted by the fluorescein released was measured in fluorescence mean units (FL1) on a log scale (*x* axis) against the relative cell number (*y* axis). A rough estimation of the amount of chitin was accomplished by flow cytometry: approximately 1 × 10⁴ cells were recovered from exponentially growing cultures, washed once with 0.5 ml PBS and stained with calcofluor white at 10 µg ml⁻¹ for 30 min at 30 °C in darkness. The fluorescence emitted by calcofluor bound to chitin was measured on a BRYTE HS (Bio-Rad) flow cytometer equipped with a Xe-Hg lamp. Flow cytometry analyses were performed twice using three different clones each time.

Confocal microscopy. Preparation of cells for primuline staining and confocal analysis was performed as follows. Cells from a culture in the late-exponential phase were washed with PBS and resuspended in 0.5 ml PBS with 1 µl of a 1 mg ml⁻¹ dilution of primuline. The samples were observed after 30 min of incubation at room temperature in darkness and were analysed on a MRC1000 confocal microscope (Bio-Rad). The images obtained were selected from z-reconstruction on the plane where bud-scars were observed. Confocal analyses were performed twice using three different clones each time.

Glucanase activity assays. For the detection of β-glucanase activity in solid cultures, cells were grown on solid minimal medium supplemented with amino acids for 2–3 d and then covered with an overlay containing the substrate 4-methylumbelliferyl β-D-glucoside (MUG, Sigma). The overlay was prepared by mixing equal volumes of melted agar (10 g l⁻¹) and a solution of 0.3 g l⁻¹ MUG in 0.05 M acetate buffer pH 5.3, pouring the mixture over the plate surface and allowing it to solidify at room temperature. The plates were then further incubated at 30 °C for 3 h and observed under UV light (λ = 260 nm). Positive colonies (i.e. producing exoglucanase) show a white luminescence. This protocol is a slight modification of a previous one (Santos *et al.*, 1979b).

Cell-free extracts were prepared from freshly harvested cells after disruption in a Braun homogenizer as previously described (Fleet & Manners, 1976). Assays of 1,3-β-glucanase activity were based on the release of reducing sugars from laminarin, which were quantified using previously described methods (Ashwell, 1957; Somogyi, 1952). The reaction mixtures contained the enzyme preparation in a total of 250 µl 0.001 M sodium succinate buffer pH 5.3. The corresponding substrate was added to a final concentration of 2.5 g l⁻¹ (w/v). Incubations were performed at 37 °C for 30 min. One unit of enzyme activity (EU) was defined as the amount of enzyme which releases 1 nmol of the measured reaction product per min under the reaction conditions. Protein concentrations were determined either by the method of Bradford (1976) (in both cases using BSA as standard) or spectrophotometrically at OD₅₉₅ (Beckman spectrophotometer DU 640). Specific activity was defined as units of enzyme activity (mg protein)⁻¹.

Activity against *p*-nitrophenyl β-D-glucopyranoside (PNPG, Sigma) was measured spectrophotometrically (λ = 410 nm) after incubation of 0.125 ml of cell-free extract or culture supernatants with 0.125 ml of a 5 g l⁻¹ solution of PNPG in acetate buffer pH 5.3 for 60 min at 37 °C. An aliquot (2.25 ml) of a solution of 40 g l⁻¹ Na₂CO₃ in distilled water was added to stop the reaction and samples were analysed on a spectrophotometer. Enzymic activity units were calculated as previously described (Cenamor *et al.*, 1987). The assays were performed three times for each strain.

Culture supernatants were obtained as previously described (Molina *et al.*, 1987). Glucanase activity was measured in 0.05 ml samples by addition of 2.5 µl of a 1 mg ml⁻¹ dilution of FDGP. Absorbance ($\lambda = 510$ nm) was measured after incubation at 30 °C for 3 h in darkness. The assays were performed three times with each strain.

Virulence assays. Male and female inbred Balb/c and DBA/2 mice (Charles River) weighing 18–22 g were used. Yeast strains were grown at 30 °C on minimal medium agar plates (6.7 g Difco yeast nitrogen base l⁻¹, 20 g glucose l⁻¹, 20 g agar l⁻¹). After 24 h, cells were harvested, washed twice with PBS and diluted to the desired density in 0.5 ml of the same buffer for injection into the lateral tail vein of the mice. Kidneys and brains from individual mice were removed aseptically and placed in a tissue homogenizer with 5 ml or 3 ml PBS, respectively. The number of viable yeast units in the specimens (six to eight mice per group) was determined by a plate dilution method on YED/chloramphenicol solid media. Results (means $\pm$ standard deviation) are expressed as log c.f.u. per organ.

Antifungal susceptibility assays. The procedure of Anaissie *et al.* (1994) was used to determine MICs. Microtitre plates were inoculated with 1×10^4 cells ml⁻¹ and incubated at 35 °C for 48 h. The antifungal assay was repeated three times only against those antifungal agents for which differences were observed in the corresponding initial MIC assay.

RESULTS

XOG1 is not essential in *C. albicans*

Disruption of the two alleles of the *XOG1* gene was performed in a sequential manner following the strategy described elsewhere (Fonzi & Irwin, 1993). As shown in Fig. 1, a 4.03 kb *Bgl*III–*Bam*HI fragment from pCUB-6 carrying the *URA3* gene flanked by the *Salmonella hisG* gene was inserted into the *Sty*I site of the *C. albicans* *XOG1* gene. Like ATCC strain 10261, CAI-4 was found to be heterozygous at the *XOG1* locus (Chambers *et al.*, 1993), thus allowing tracing of the chromosomal homologue in which gene replacement had taken place. Transformation with this linearized construction into strain CAI-4 yielded strains CNC30-a and CNC30-b (depending on which chromosome was affected). The estimated frequency of homologous integration at the *XOG1* locus was 60%. Excision of *URA3* selected on 5-FOA plates yielded strains CNC31-a and CNC31-b, which were then used to select the *xog1* null allele strains CNC32-a and CNC32-b and the corresponding CNC33-a and CNC33-b strains (*Ura*⁻ background). For some of the subsequent functional studies, both the heterozygous and homozygous null allele strains CNC31-a and CNC31-b (relevant genotype *xog1/XOG1*) and CNC33-a and CNC33-b (relevant genotype *xog1/xog1*) were used.

CNC30-a, CNC30-b, CNC32-a and CNC32-b showed no obvious growth differences as compared to wild-type strain SC5314 when growing exponentially at different temperatures (30, 37 and 42 °C) and in different synthetic minimal or rich media (data not shown). The final absorbances reached in stationary-phase cultures were also similar, indicating that deletion of the *XOG1* gene does not induce obvious defects even in the stationary

Fig. 2. Exoglucanase activity in intact cells of *C. albicans* strains SC5314 (wild-type, i); CNC30-a (*xog1::hisG-URA3-hisG/XOG1*, ii); and CNC32-a (*xog1::hisG/xog1::hisG-URA3-hisG*, iii). (a) Exoglucanase activity as observed by fluorescence of UV-irradiated cells after MUG hydrolysis on solid medium. (b) Exoglucanase quantification by flow cytometry using the substrate FDGP. Controls (lacking substrate) are represented by the bold line. FL1-H was 14.85 for strain SC5314 (i), 8.3 for CNC30-a (ii) and 3.59 for CNC32-a (iii).

phase of growth. In addition, careful and exhaustive phase contrast microscopic observation of the three strains was performed, but no appreciable morpho-

Fig. 3. Assay of exoglucanase activity in cell-free extracts (a) and supernatants (b) of *C. albicans* wild-type strain SC5314 and disrupted strains CNC30-a and CNC32-a. Activity in extracts was assayed using laminarin and PNPG as substrates, and in supernatants using FDGP as substrate. Bars represent standard deviations.

logical differences among them were observed, nor even when scanning and transmission electron microscopy were used (data not shown). Finally, neither the homozygous nor the heterozygous null strains showed appreciable differences with regard to the timing and morphological appearance of filaments when dimorphic transition was induced either in Lee's medium or serum (24 h at 37 °C, data not shown). Collectively, these data indicate that the *XOG1* gene is not essential in *C. albicans* and is apparently not involved in dimorphic transition.

XOG1 encodes the major exoglucanase activity in *C. albicans*

Exoglucanase activity was measured in the wild-type strain SC5314, and in *xog1::hisG-URA3-hisG/XOG1* and *xog1::hisG/xog1::hisG-URA3-hisG* strains on solid medium using MUG and in liquid medium using FDGP as substrates (Fig. 2). A significant reduction was observed in the fluorescence emitted by *xog1::hisG-URA3-hisG/XOG1* cells when irradiated under UV light compared to the wild-type control strain. This result was clearly confirmed using cell-free extracts and supernatants from exponentially growing liquid cultures (see Methods), using laminarin and FDGP as substrates (Fig. 3). Using PNPG as substrate, no significant

differences were detected. Thus, deletion of *XOG1* did not result in a reduction in PNPG activity, in agreement with previous reports (Molina *et al.*, 1987; Luna Arias *et al.*, 1991) and indicating that the major glucanase activity from *C. albicans* does not show significant activity against PNPG.

Interestingly, the single knock-out strains CNC30-a and CNC30-b displayed an intermediate phenotype, indicating that deletion of a single allele results in a decrease in the total glucanase activity of the cell. No differences were observed between CNC30-a and CNC30-b, suggesting that this effect may be the result of a different gene dosage rather than functional differences between the *XOG1* alleles.

XOG1 null mutants are virulent

To determine whether the glucanase *XOG1* gene plays a role in pathogenicity, both the parental SC5314 strain and the *xog1::hisG/xog1::hisG-URA3-hisG* strains were tested for virulence in systemic infections. Two inbred strains of mice were used: Balb/c mice (less susceptible to this kind of infection) develop T_H1-associated immunity while DBA/2 animals (more susceptible) are characterized by a predominance of T_H2 response (Hector *et al.*, 1982; Romani *et al.*, 1992, 1993). To evaluate the patterns of susceptibility of Balb/c and DBA/2 mice to systemic infection by *C. albicans* SC5314 and the *xog1* null mutant, the animals were injected in the lateral tail vein with four different doses of yeast cells: 1×10^7 , 1×10^6 , 1×10^5 and 5×10^4 . The course of the infection was monitored by mortality parameters: median survival time (MST) and number of dead mice out of the total (D/T), as well as the level of fungal load in representative organs, i.e. kidney and brain. Table 2 shows that the inoculation of high doses (1×10^7 and 1×10^6 cells) of both strains of *C. albicans* killed all the mice in few days. Inoculation of lower doses led to the appearance of chronic infection in Balb/c mice, whilst in DBA mice all the animals died, even with the lowest dose. A difference in MST was only observed in Balb/c mice inoculated with 1×10^6 cells, but without statistical significance ($P > 0.05$, ANOVA test) when all the mice were considered. The degree of fungal load in the kidneys and brains of the infected mice was also measured (Table 2). As can be seen, both *C. albicans* strains behaved in a similar fashion: the log c.f.u. recovered from the kidneys was high (between 4 and 6) in both strains of mice, whilst renal clearance was observed in some of the Balb/c mice when the 1×10^5 and 5×10^4 doses were used. Statistical analysis (ANOVA test) was applied and the differences in renal load between both strains of *C. albicans* were found to be not significant ($P = 0.230$). By contrast, the number of cells recovered from brain was significantly different between the parental and the mutant strains ($P < 0.001$). As mentioned above, DBA/2 mice were more susceptible, as reflected in a high log c.f.u., even at the lower doses whereas Balb/c mice inoculated with these doses showed brain clearance. Taken together, these results indicate that *xog1* null mutants are as virulent as the

Table 2. Course of *C. albicans* infection in Balb/c and DBA/2 mice

Age-matched mice of either strain were challenged with various doses of *C. albicans* SC5314 or CNC32-a cells as described in Methods.

Challenge dose	<i>C. albicans</i> strain	Balb/c mice			DBA/2 mice		
		MST (d)*	log C.f.u. per kidney†	log C.f.u. per brain†	MST (d)*	log C.f.u. per kidney†	log C.f.u. per brain†
10 ⁷	SC5314	1	6.03 ± 0.84	4.47 ± 0.13	1	NT	NT
	CNC32-a	1	5.73 ± 0.13	2.19 ± 0.51	1	NT	NT
10 ⁶	SC5314	3	5.93 ± 0.18	4.04 ± 0.22	1	6.02 ± 0.92	3.81 ± 0.16
	CNC32-a	7	4.79 ± 0.47	2.56 ± 0.91	1	5.52 ± 0.17	3.64 ± 0.09
10 ⁵	SC5314	> 60	4.32 ± 1.51‡	—‡	4	5.17 ± 0.76	3.70 ± 0.19
	CNC32-a	> 60	4.58 ± 1.12‡	—‡	4	5.78 ± 0.20	3.26 ± 0.52
5 × 10 ⁴	SC5314	> 60	4.87 ± 0.96‡	—‡	7	6.45 ± 0.56	4.53 ± 0.50
	CNC32-a	> 60	4.35 ± 1.49‡	—‡	7	5.54 ± 0.59	2.99 ± 0.66

NT, Not tested.

* MST, median survival time.

† Parallel groups of mice were killed the day before that indicated in the MST column for quantification of c.f.u. in their kidneys and brains. Values shown are means ± SD of six to eight mice. Statistically significant differences were not found between values for kidney ($P = 0.230$) but were found for brain ($P < 0.001$), as determined by ANOVA tests by *C. albicans* strain with mice and dose as covariates.

‡ Some or all the mice showed infection clearance.

Table 3. Susceptibility of *C. albicans* strains SC5314 and CNC32-a to different antifungals

Values shown are means of three determinations (± SD) where applicable

Antifungal	MIC (µg ml ⁻¹)	
	SC5314	CNC32-a
Papulacandin B	0.52 ± 0.22	0.26 ± 0.11
Cilofungin	0.157 ± 0.068	0.08 ± 0.03
Nikkomycin	0.0087 ± 0.0057	0.072 ± 0.048
Calcofluor	33 ± 14.43	41.6 ± 14.43
5-fluorcytosine	0.08 ± 0.034	0.16 ± 0.069
Amphotericin B	0.24	0.24
Miconazole	0.48	0.48
Trichodermine	0.078	0.078
Canavanine	32	32
Caffeine	485.5	485.5
2-Deoxyglucose	200	200

parental strain, in spite of having a reduced capacity to colonize the brain.

Deletion of *XOG1* and effects on the cell wall

Although deletion of *XOG1* did not involve major phenotypic changes, we further characterized the mutant cells as regards their susceptibility to a wide variety of antifungals to identify putative underlying differences.

We used a set of unrelated compounds that affect different processes in fungal physiology. No differences were observed for antibiotics that interfere with processes such as protein synthesis (trichodermin), ergosterol biosynthesis (miconazole), membrane functionality (amphotericin B) or that behave as toxic metabolites (2-deoxyglucose, canavanine) (Table 3). However, null mutant cells were more susceptible to antifungals that inhibit 1,3-β-glucan biosynthesis, such as cilofungin and papulacandin B (only a slight variation in the sensitivity shown by the wild-type strain with respect to the mutant was observed). Since the differences were repeatable, even though they were very small, these results appear to be consistent with a slight difference in the cell wall between the wild-type and mutant strains. The antifungals act at the same level interfering with cell wall synthesis and/or assembly; therefore, although not very prominent, we believe that the aforementioned differences must have some functional meaning. In parallel, cells were found to be more resistant to nikkomycin, an antibiotic that inhibits chitin biosynthesis in *C. albicans* (Chapman *et al.*, 1992). This suggests subtle differences in cell wall architecture. To visualize chitin septa, the fluorochromes calcofluor (Pringle, 1984) and primuline (Duffus *et al.*, 1984) were used. Calcofluor and primuline are fluorochromes that show high affinity for yeast cell wall chitin and, in addition, calcofluor shows strong antifungal activity when used at high concentrations (Roncero & Duran, 1985). This different susceptibility between the mutant and the wild-type cells may reflect differences in the cell wall composition. This could affect the permeability – and hence the accessibility of the antifungal – and the

Fig. 4. Relative chitin content in *C. albicans* strains SC5314 (wild-type, i) and CNC32-a (*xog1* null mutant, ii). (a) Distribution of chitin in progressive sagittal sections of infected mice separated by 0.5 μ m using confocal microscopy and primuline as fluorochrome. The signal detected corresponds to primuline binding to chitin-rich zones. In the wild-type strain (i) the chitin is associated with the bud scars, whereas in the mutant strain (ii) it is distributed randomly throughout the cell. (b) Chitin quantification by flow cytometry using calcofluor as fluorochrome. The value in the lower left corner represents the mean intensity of fluorescence (FL1).

different susceptibilities observed may therefore reflect a difference, perhaps subtle, at the level of cell wall composition or organization. Mutant cells emitted more fluorescence due to primuline excitation than wild-type strain cells (Fig. 4a). Similar results were obtained when the fluorescence emitted by calcofluor was analysed by flow cytometry (Fig. 4b).

DISCUSSION

The recent development of an efficient gene disruption system in *C. albicans* (Fonzi & Irwin, 1993) has enabled an assessment of the role of individual genes in *C. albicans* physiology. In this work, we characterized a

strain deficient in *XOG1*, a gene recently cloned using a reverse genetic approach (Chambers *et al.*, 1993). Our results confirm previous studies from our laboratory and others (Chambers & Sullivan, 1993; Luna Arias *et al.*, 1991; Molina *et al.*, 1987), pointing to the existence of a major glucanase activity in *C. albicans*, and identified *XOG1* as responsible for this 1,3- β -glucanase activity. However, they also suggest the existence of other glucanases, since *xog1* null mutants cells still have residual glucanase activity. Removal of the major exoglucanase activity in the *C. albicans xog1* null strain may allow, as in the case of *S. cerevisiae*, cloning of other minor glucanases (Correa, 1993; Muthukumar *et al.*, 1993; San Segundo *et al.*, 1993).

A relevant and additional conclusion from our results is that the *XOG1* gene is not essential in *C. albicans*. This observation is in accordance with the role of exoglucanases in *S. cerevisiae*, since none of the three exoglucanase genes cloned to date – *EXG1* (Nebreda *et al.*, 1986), *EXG2* (Correa, 1993) and *SSG1* (Muthukumar *et al.*, 1993; San Segundo *et al.*, 1993) – have been implicated in morphogenic processes. The existence of different genes with an apparently similar function points to an important (although hitherto cryptic) role of glucanases in cell physiology. However, it should be stressed that disruption of either of the *XOG1* alleles elicits an intermediate decrease in exoglucanase activity of about 40%. This suggests that the reduction in activity may be due to gene dosage and not allelic differences. Furthermore, disruption of both alleles elicits an 80% decrease in exoglucanase activity.

Since the timing and morphological appearance of mycelia were similar in the wild-type and single and double *XOG1* disruptants, another important observation of this work is that the *XOG1* gene is not involved in dimorphism. Furthermore, the *XOG1* gene can be used as a reporter to identify the genes involved in dimorphic transition.

However, it is also evident that disruption of *XOG1* leads to subtle but consistent changes in the cell wall, as deduced from the effect of its disruption in antifungal susceptibility and the fluorescence pattern with the cell wall fluorochromes primuline and calcofluor. Nevertheless, since the *xog1* null mutants show enhanced susceptibility to β -glucan inhibitors and since this mutant also shows more resistance to nikkomycin, a chitin inhibitor, another interpretation could be that chitin and glucan might play compensatory roles in cell wall architecture, as has been suggested for *S. cerevisiae* (Kollár *et al.*, 1995; Popolo *et al.*, 1997). If the level of chitin synthesis in the mutant strain were higher than in the wild-type it would appear logical that higher doses of the antifungal nikkomycin would be required to inhibit growth. The data presented in Fig. 4 can be explained using two different lines of discussion: (1) elimination of the exoglucanase could have produced an overall change in cell wall permeability, allowing a more efficient staining with the dyes used; (2) the increase in fluorescence afforded by primuline and calcofluor could have been due to an increase in the amount of chitin in the cell wall of the mutant. However, it should be stressed that neither calcofluor nor primuline stains are specific for chitin in all contexts (Duffus *et al.*, 1984; Pringle, 1984). Despite this, it has been observed that these fluorochromes show strong affinity for the chitin present in the cell wall of yeasts (Roncero & Duran, 1985). Since we only used these fluorochromes for comparative purposes, we believe that it is not very relevant that they are not exclusively specific for chitin. The aim of this work is merely to pinpoint some subtle differences between the mutant and wild-type strains, knowing that more detailed studies would be required to define such differences at a molecular level. Due to the opportunistic pathogenic nature of *C.*

albicans, we undertook a detailed analysis of the role of the *XOG1* gene in virulence. A *C. albicans* mutant disrupted in a cell wall biosynthetic enzyme (*chs2*) showed no differences in dimorphism or in virulence (Gow *et al.*, 1994). However, a recent report indicates that another chitin-deficient mutant (*chs3*) and a calcofluor-resistant mutant are less virulent (Bulawa *et al.*, 1995). Despite this, Toshiyuki *et al.* (1996), analysing the virulence of *chs3* mutants found no differences in virulence as compared with the wild-type strain and attributed this to the strains and assay methods used. In this study we show that in an experimental murine model the *xog1* null mutant is as pathogenic as the wild-type strain, as shown by similar median survival time (MST) and number of dead mice out of the total (D/T) values. Concerning the ability to colonize organs, statistically significant differences were only found in the log c.f.u. recovered from brain.

The non-essential nature of *XOG1* gene rules out its use as an antifungal target. However, it could be a very attractive reporter gene. Although in *C. albicans* there are some recently developed systems (Leuker *et al.*, 1992; Myers *et al.*, 1995; Srikantha *et al.*, 1996; Cormack *et al.*, 1997), none of them displays the versatility of the exoglucanase system for its use in flow cytometry, biochemical measures and agar plate detection in gene reporter assays. Another important point is that since deletion of the *XOG1* gene does not affect virulence, it would be possible to set up a strategy aimed at the isolation and identification of *C. albicans* genes involved in virulence using the exoglucanase as a reporter. A similar conceptual approach has been described by Mahan *et al.* (1993a, b) for the selection of bacterial virulence genes.

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