Phenotype in *Candida albicans* of a disruption of the *BGL2* gene encoding a 1,3- β -glucosyltransferase

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The *BGL2* gene encodes a unique 1,3- β -glucosyltransferase (Bgl2p) present in the cell wall of *Candida albicans* and other fungi. Although believed to be involved in cell wall assembly, disruption of the gene in *Saccharomyces cerevisiae* showed no apparent phenotype. We performed sequential disruptions of the *BGL2* loci in a homozygous *ura3* clinical isolate of *C. albicans* using the *URA3* blaster method, in order to investigate the role of Bgl2p in this dimorphic, pathogenic fungus. Strain CACW-1 contained disruptions of both homologues of the *BGL2* gene and lacked Bgl2p, as assessed by protein extraction, SDS-PAGE and Western blot analysis, and enzyme assay; however, residual non-Bgl2p transferase activity was detected. CACW-1 was attenuated in virulence for mice when compared to an isogenic parent strain, and fewer organisms were recovered from the kidneys of infected animals. Additional phenotypic changes included: (1) a dramatic increase in the sensitivity to the chitin synthesis inhibitor nikkomycin Z when CACW-1 cells were incubated at 37 or 42 °C; (2) an $8.7 \pm 1.6\%$ slower growth rate at 37 °C for CACW-1 when compared to its isogenic parent; and (3) aggregation of CACW-1 cells during stationary phase and/or incubation of stationary phase cells in phosphate buffer. Characterization of SDS-extracted cell walls did not reveal any significant differences in the levels of 1,3- β - or 1,6- β -glucan. These data reveal that loss of Bgl2p does have a phenotype in *C. albicans*, and indicate that (1) loss of Bgl2p function renders cells more dependent on chitin for wall integrity, and attenuates virulence (probably due to subtle changes in wall structure), and (2) that additional 1,3- β -glucosyltransferases are present in the *C. albicans* *BGL2* disruptant.

Keywords: *Candida albicans*, *BGL2*, transglycosylation

INTRODUCTION

The biochemistry and structure of the fungal cell wall represent some of the few and clear differences between fungal and mammalian cells. The cell wall provides many functions, including maintenance of cell shape, protection from damage by extracellular agents, protection from osmotic forces, and expression of virulence properties facilitating interaction of the pathogen with the host (Reiss *et al.*, 1992; Shepherd, 1987; Shepherd *et al.*, 1985). The cell wall is therefore an attractive target for antifungal drug development, and specific lipopeptide (echinocandin class), glycolipid (papulacandin class) and peptidyl-nucleotide (polyoxin class) com-

pounds are either in preclinical or clinical development as antifungal agents which affect cell wall synthesis. In addition, a study has shown that reduction of the chitin content of the cell wall of *Candida albicans* via disruption of the *CSD2/CAL1/DIT101/KTI2* gene (the *C. albicans* homologue of the *Saccharomyces cerevisiae* *CHS3* gene) resulted in attenuation of virulence of this pathogen (Bulawa *et al.*, 1995), demonstrating that non-lytic alterations in fungal cell wall synthesis can decrease fungal virulence *in vivo*. The magnitude of this effect may be in question based on a separate study (Mio *et al.*, 1996).

A large number of mannoproteins as well as an extensive

glucan and chitin network make up the architecture of the cell wall of *S. cerevisiae* and other fungi (Shepherd, 1987; Shepherd *et al.*, 1985). Recent data have confirmed the existence of covalent linkages between various structural components of the fungal cell wall, including chitin and glucan (Elorza *et al.*, 1989; Sietsma & Wessels, 1981; Surarit *et al.*, 1988; Kollar *et al.*, 1995). The action of wall-associated glycosyltransferases is the most likely mechanism for formation of such covalent linkages. A novel 35 kDa glucosyltransferase encoded by the *BGL2* gene, first described in *C. albicans* (Hartland *et al.*, 1991), catalyses the following transferase reaction with 1,3- β -glucan:

where E is the enzyme, G_n is the donor ($n \geq 5$), $E:G_{n-2}$ is the enzyme:glucosyl intermediate, G_2 is the released disaccharide laminaribiose, G_y is the acceptor glucan ($y \geq 4$) and G_{n+y-2} is the transferase product which contains a 1,6- β -linkage at the transfer site (Hartland *et al.*, 1991; Goldman *et al.*, 1995). The *BGL2*-encoded enzyme is present in *S. cerevisiae* (Goldman *et al.*, 1995; Klebl & Tanner, 1989; Mrsa *et al.*, 1993) and the pathogenic fungi *C. albicans* (Hartland *et al.*, 1991) and *Aspergillus fumigatus* (R. Hartland, personal communication). The transferase reaction catalysed by the Bgl2p enzyme is not essential for growth in *S. cerevisiae* (Klebl & Tanner, 1989). However, we previously reported that a Bgl2p-like enzyme probably was functional in *S. cerevisiae* (Coen *et al.*, 1994), as one of the products, laminaribiose (G_2), was released from cell wall glucan during growth. The *BGL2* gene product was originally described as an exoglucanase (Klebl & Tanner, 1989), then as an endoglucanase (Mrsa *et al.*, 1993), but is now known to have active transglycosylase function (Hartland *et al.*, 1991; Goldman *et al.*, 1995). Structural analysis of Bgl2p reveals that it is uniquely specialized for transferase function (C. Hutchins & R. Goldman, unpublished).

We initiated a study in *C. albicans* to determine the effects of loss of the Bgl2p enzyme activity on growth, wall structure/function and/or pathogenicity. Strains deficient in the Bgl2p enzyme can also be used to characterize other glucosyltransferase activities that may be essential for cell wall assembly. The present study describes the disruption of both copies of the *BGL2* gene in a clinical isolate of *C. albicans*. The strain was further evaluated for the presence of glucosyltransferase activity and virulence using a mouse model system. Low levels of non-Bgl2p glucosyltransferase activity were detectable in this strain. The strain also showed a slight attenuation of virulence as compared to the *BGL2* parent, and fewer *C. albicans* were recovered from the kidneys of infected mice.

METHODS

Yeast strains, plasmids and media. The *C. albicans* strains used are listed in Table 1. Strain CAI-4 was used to disrupt the *BGL2* gene homologues in the construction of CACW-0 and

CACW-1. The *hisG-URA3-hisG* and *C. albicans BGL2* sequences are contained in pMB7 (Fonzi & Irwin, 1993) and pDS14 (provided by P. A. Sullivan, Massey University, New Zealand), respectively, and were used to construct strains containing disruptions in the *BGL2* gene. Yeast strains were maintained on YPD medium (Sherman *et al.*, 1982) supplemented with 25 μ g uridine ml⁻¹. Synthetic depleted (SD) medium contained 0.67% Yeast Nitrogen Base (Difco) and 2% (w/v) glucose (Sherman *et al.*, 1982). Uridine auxotrophs were selected by the 5-fluoroorotic acid method as described by Boeke *et al.* (1984) and maintained on medium supplemented with uridine. SD medium was supplemented with 25 μ g uridine ml⁻¹ when required.

Plasmid construction, and transformation. A 930 bp PCR fragment was amplified from pDS14 such that the *BGL2* coding sequence was flanked by a unique *Sall* site at the 5' end and unique *Bgl*II and *Bam*HI sites at the 3' end. PCR amplification was performed using the Perkin Elmer Cetus thermal cycler and GeneAmp kit with the primers 5' TGTGT-AGTCGACTACTCTCGCAACTGTTCT 3' and 5' AGAG-TGGATCCAGATCTAGACAATCAAAAAACAC 3'. Thirty cycles of 94 °C/30 s melting, 55 °C/1 min annealing and 72 °C/1 min extension steps were used for the amplification. The PCR product was digested with *Bam*HI and *Sall* and cloned into the *Bam*HI and *Sall* sites of pBluescript KS (Stratagene) to create pBSCaBGL2. Plasmid pMB7 (Fonzi & Irwin, 1993) was digested with *Sall* and *Bgl*II to release a 3.9 kb fragment containing the *hisG-URA3-hisG* cassette. This fragment was blunt-end ligated into the flush-ended *Eco*RI site in the *BGL2* gene of pBSCaBGL2 to generate pCBHUH1. A 5 kb *Bgl*II-*Sall* fragment containing the *hisG-URA3-hisG* cassette inserted into the *BGL2* gene was isolated from pCBHUH1 and transformed into *C. albicans* as described by Burgers & Percival (1987). Uridine prototrophs were selected on SD medium lacking uridine.

Southern blot analysis. Yeast genomic DNA was extracted as described by Hoffman & Winston (1987). Approximately 5 μ g DNA was digested with *Eco*RI and electrophoresed on an agarose gel which was then transferred onto Hybond-N paper (Amersham). Southern blot analysis was done as described by Maniatis *et al.* (1982) using a ³²P-labelled fragment containing the *Candida BGL2* gene as probe.

Growth rate measurements. Strains were grown overnight at 37 °C in SD medium with limiting glucose (0.1%) and transferred to SD medium containing 0.5% glucose to a final OD₄₂₀ of 0.05 (measured using a Bausch & Lomb Spectronic 1001 spectrophotometer). Cells were grown at 37 °C with shaking, and mildly sonicated for 10 s in a Bransonic model 220 sonicator bath to disrupt clumps prior to reading the optical density value. Cultures were diluted before reading to obtain optical density values below 1.0 when required, in order to avoid non-linear readings. The generation time was determined in triplicate, and converted to *k* (the instantaneous growth rate constant) by the formula $g = \ln 2/k$, where *g* is expressed in hours.

Aggregation measurements. The tendency towards aggregation was monitored in both exponential- and stationary-phase cells grown in SD medium plus 0.5% glucose. Exponential-phase cells were harvested at an optical density value of 2.0–3.0, while stationary-phase cells were from cultures grown for 16 h. Cells were washed once with PBS (0.137 M NaCl; 2.7 mM KCl; 0.10 M sodium phosphate), once with 0.066 M sodium/potassium phosphate buffer (pH 7.5) and resuspended in 0.066 M phosphate buffer to an OD₈₀₀ value of about 1.0. Cells were incubated with shaking at 200 r.p.m. in a New

Table 1. *C. albicans* strains

Strain	Genotype	Source
CAF2	Δ ura3::imm434/URA3	W. A. Fonzi (Georgetown University Medical Center, Washington, DC, USA)
CAI-4	Δ ura3::imm434/ Δ ura3::imm434	As above
CACW-0	bgl2::hisG-URA3-hisG/BGL2, Δ ura3::imm434/ Δ ura3::imm434	This study
CACW-1	bgl2::hisG/bgl2::hisG-URA3-hisG, Δ ura3::imm434/ Δ ura3::imm434	This study

Brunswick Aquatherm water-bath shaker at 37 °C, and the optical density value was determined over time. Alternatively, cells were examined microscopically and the frequency distribution of aggregates was determined by triplicate counting of 100–150 aggregates. Aggregates of size two or more may represent mother–daughter associations that had not separated, or more distantly related cells which had aggregated together when cells were examined directly from culture.

Zymolyase sensitivity. The sensitivity towards lysis by Zymolyase 100T (Seikagaku America) was determined for both exponential- and stationary-phase cells prepared as given above for aggregation measurements. Cells suspended in 0.02 M phosphate buffer with varying amounts of Zymolyase 100T were incubated at 37 °C with shaking, and the optical density value was determined with time. Data were plotted and the rate of lysis was determined from the linear portion of the curve (time vs optical density value).

Germ tube formation upon glucose starvation. Cells were grown overnight at 37 °C in SD medium plus 0.1 % glucose. Coded samples were analysed in a cell counting chamber for total cell number and percentage germ tubes. Microscopy fields (10 per sample) were counted, and the standard deviation was determined.

MIC profiles. The MIC values for various drugs were determined by micro broth dilution in 96-well microtitre trays, using cells grown in SD medium plus 0.5 % glucose. Cells were inoculated to about 2×10^5 cells ml⁻¹ in 100 µl media containing twofold serial dilutions of drugs. Plates were incubated at the indicated temperatures and monitored for growth on days 1, 2 and 4. MIC profiles were determined three to five times, depending on the drug, with similar results, and representative data are reported.

Bgl2p extraction and analysis. Glucosyltransferase activity was extracted from walls as previously described (Goldman *et al.*, 1995). Briefly, 16 g cell paste was thawed in 5 ml 25 mM Tris/HCl (pH 7.5) and mixed with 45 ml acid-washed 0.5 mm glass beads in a Braun 70 ml glass cylinder. Cells were maintained at 4 °C with CO₂ cooling and broken using a Braun type 853030 cell disrupter. The cell wall pellet was washed once with cold distilled H₂O by centrifugation (10000 g for 10 min) and the pellet wet weight was recorded. The pellet was resuspended in cold distilled H₂O (1.8 × wet wt) and transferred to a glass Corex tube. The pellet was extracted with butanol (1.2 × wet wt) at 4 °C for 15 min with

gentle rocking, then centrifuged at 10000 g for 5 min at 4 °C and the supernatant was discarded. The extraction and centrifugation steps were repeated two more times. The cell wall pellet was washed once with cold distilled H₂O by centrifugation, and the pellet wet weight was recorded. The pellet was resuspended in 5 mM Tris/HCl (pH 7.5) (2.0 × wet wt) and heated at 70 °C for 10 min. The sample was cooled by addition of 1 × volume of 5 mM Tris/HCl (pH 7.5) and centrifuged at 30000 g for 30 min at 4 °C. The supernatant was concentrated to approximately 500 µl and assayed for protein concentration and glucosyltransferase activity. Protein extracts were separated by SDS-PAGE (4–20 %, w/v, acrylamide) and analysed by Western blotting using antiserum raised against Bgl2p (1:5000 dilution) isolated from *S. cerevisiae*, and goat anti-rabbit IgG (peroxidase-conjugated; 1:5000 dilution).

Glucosyltransferase assay. Reagents were prepared and reactions were conducted as previously described (Goldman *et al.*, 1995). Briefly, this included preparation of labelled glucan using *C. albicans* microsomes in a final volume of 2 ml 2 mM UDP-[³H]Glc [Amersham TRK.385; 12.3 Ci (4.55 × 10¹¹ Bq) mmol⁻¹; final specific activity of 62–124 mCi (3.7 × 10⁷ Bq) mmol⁻¹], 1 mM EDTA, 8 % (v/v) glycerol, 20 µM GTPγS, 0.5 % Brij-35, 80 mM Tris/HCl (pH 7.75) and microsomes (2 mg protein). Glucan was precipitated with 2 vols cold 95 % ethanol, and the dried glucan was digested with laminarioligohydrolase prepared from Zymolyase 100T. The soluble glucan oligosaccharides were purified on Sephadex G50 and then by HPLC as described by Goldman *et al.* (1995), and stored at –20 °C in H₂O containing 1 % ethanol. Laminarioligosaccharides and glucosyltransferase reaction products were separated on a Dynamax-60A 8 µm NH₂ column, 4.6 mm internal diameter × 25 cm (Rainin Instrument), using 60 % (v/v) acetonitrile as the mobile phase and a flow rate of 1 ml min⁻¹. Radioactivity was monitored with a Radiomatics flow detector (United Packard).

Characterization of cell walls. Cells from 20 ml culture were mixed with an equal volume of acid-washed 0.5 mm glass beads and broken by vortexing four times for 1 min. Walls were collected by centrifugation, resuspended in 3 ml 2 % (w/v) SDS in 10 mM Tris/HCl (pH 7.5) and heated to 100 °C for 5 min. Walls were centrifuged as above and the pellets were washed twice with 5 ml H₂O to remove the SDS. Walls (0.5 ml; 2 mg dry wt in 20 mM ammonium acetate, pH 5.6) were digested with 100 units Zymolyase 100T in 0.02 %

sodium azide overnight at 37 °C with rotation. Following digestion, the reaction mixture was heated to 60 °C for 5 min to inactivate the Zymolyase enzymes. Particulate matter was removed by centrifugation and the supernatants were frozen at -20 °C for subsequent total hexose determination using the phenol-sulfuric acid method (Dubois *et al.*, 1956). Zymolyase-soluble material was fractionated on Sephadex G50 columns (10 ml bed volume in 20 mM ammonium acetate, pH 5.6), collecting 0.75 ml fractions. The material from peak 1 (void volume) was pooled, lyophilized and resuspended in 300 µl 25 mM sodium acetate (pH 4.2).

Each peak 1 sample was digested with 0.04 units (1 unit = 1 µmol reducing sugar released min⁻¹) of endo-1,6- β -exoglucanase for 1 h at 50 °C, followed by an additional 0.04 units for another 1 h digestion. Each sample was centrifuged through a Microcon 3 membrane (Filtron; 3000 Da cut-off) to near-dryness and the membrane was washed three times with 100 µl 25 mM sodium acetate (pH 4.2). The filtrates were combined and assayed for hexose content. In addition, the retentate was resuspended in 1 ml buffer and assayed for hexose content. The amount of hexose passing through the membrane as filtrate was used to quantify the amount of material in peak 1 which was susceptible to 1,6- β -exoglucanase digestion.

Virulence studies. Cells were grown overnight in Sabouraud broth at 37 °C with shaking. Cultures were diluted in phosphate buffer and groups of ten mice (CF-1) were inoculated with 0.2 ml of each dilution via a lateral tail vein. Mice were monitored for 28 d. Colonization and survival in kidney tissue were monitored by inoculating mice as above. At days 1 and 4 post-inoculation, kidney homogenates from 10 mice from each group were plated on Sabouraud Dextrose Agar (Difco) and colony counts were determined after 24 h incubation at 37 °C.

Reagents. Cilofungin was obtained from Eli Lilly & Co.; fusaricidin A was isolated at Abbott Laboratories; Calcofluor white and miconazole were purchased from Sigma. Nikkomycin Z, tunicamycin and staurosporine were purchased from CalBiochem. Amphotericin B was purchased from Gibco-BRL. Zymolyase 100T was from Seikagaku America. Antibody to the *S. cerevisiae* Bgl2p, and purified endo-1,6- β -glucanase from *Eupenicillium brefeldianum* were kindly provided by Dr P. A. Sullivan, Massey University, New Zealand.

RESULTS AND DISCUSSION

Sequential disruption of the *BGL2* gene in *C. albicans* CAI-4

A homozygous *ura3* mutant clinical isolate, *C. albicans* CAI-4, was used to sequentially disrupt the *BGL2* loci using the method described by Fonzi & Irwin (1993). A 3.9 kb DNA fragment containing the *Candida* *URA3* gene flanked by repeats of the *Salmonella* *hisG* gene was first inserted into the *EcoRI* site located in the coding region of the *C. albicans* *BGL2* gene. The restriction map of the wild-type *BGL2* gene containing the insertion of the *hisG-URA3-hisG* cassette at the *EcoRI* site is shown in Fig. 1. The parental strain CAI-4 was then transformed with the *BglII-SalI* fragment containing the *hisG-URA3-hisG* cassette flanked by *BGL2* sequences. Southern analysis of three *URA*⁺ transformants indi-

Fig. 1. Southern blot analysis of *C. albicans* *BGL2* disruptants. The coding sequence for Bgl2p (hatched) is contained on a *DraI-XbaI* fragment (upper panel). Insertion of the *hisG-URA3-hisG* cassette is at the *EcoRI* site. Lower panel: Southern blot analysis of genomic DNA from CAI-4 and its transplacements. Lanes 1-4 contain DNA digested with *EcoRI* from CAI-4, heterozygote CACW-0 containing the primary *bgl2* disruption, the uracil-requiring recombinant of CACW-0 and the *bgl2* double disruptant CACW-1, respectively. The blot was probed with a 0.9 kb PCR fragment (hatched) labelled with ³²P (MegaPrime; Amersham).

cated that one transformant, CACW-0, contained a disruption of one of the two *BGL2* genes by insertion of the *hisG-URA3-hisG* DNA construct.

ura3 mutant derivatives were obtained from CACW-0 by first growing in non-selective (YEPD) medium to saturation and then plating on SD medium containing 5-fluoroorotic acid plus uridine to select for uridine auxotrophs. Southern analysis of six uridine auxotrophs showed that all contained *bgl2::hisG*, which should have arisen by intragenic recombination between the two copies of the *hisG* sequences. A homozygous disruption at the *BGL2* locus was obtained by transformation with the same *BglII-SalI* fragment containing the *hisG-URA3-hisG* sequences inserted into the *BGL2*

Fig. 2. SDS-PAGE analysis of protein extracted from cell walls. Extracts of wall proteins were prepared from CAI-4 and CACW-1 and protein (50 µg) was electrophoresed on 12.5% acrylamide gels and either stained for protein (lanes 1–4) or Western blotted and probed with antibody to *S. cerevisiae* Bgl2p (lanes 5–7). Lanes: 1, molecular mass markers; 2 and 7, 1 µg and 100 ng purified *S. cerevisiae* Bgl2p, respectively; 3 and 6, extract from CAI-4 (parent strain); 4 and 5, extract from CACW-1 (*BGL2* double disruptant). Note that the *C. albicans* Bgl2p migrates slightly more slowly compared to Bgl2p from *S. cerevisiae*.

Table 2. Analysis of 1,3-β-glucosyltransferase activity in *C. albicans*

Radiolabelled G_5 (laminaripentaose) was incubated with wall extract protein for 3 h, and products were separated by HPLC analysis. G_8 and G_{11} are the expected Bgl2p reaction products arising from G_5 by the enzyme equation $2G_n \rightarrow G_{2n-2} + G_2$ followed by the reaction $G_n + G_{2n-2} \rightarrow G_{3n-4} + G_2$, where $n = 5$. G_x represents transferase products of unknown structure.

HPLC run	CAI-4 (parent)			CACW-1 (<i>BGL2</i> disruptant)		
	G_8 c.p.m.*	G_{11} c.p.m.*	G_x c.p.m.	G_8 c.p.m.	G_{11} c.p.m.	G_x c.p.m.
Run 1	50725 (4.10)	25144 (1.48)	75785	0	0	63395
Run 2	47645 (3.85)	25106 (1.48)	74725	0	0	129588
Mean	49185 (3.98)	25125 (1.48)	75255 (4.8)†	(<0.2)‡	(<0.1)‡	963661 (6.2)†

* Values in parentheses are nmol product produced.

† Value in parentheses is the percentage of total labelled G_5 converted to product.

‡ No G_8 or G_{11} product was detected within the nmol limits given.

gene. Only two out of 28 URA⁺ transformants contained insertion of the cassette at the second *BGL2* locus as determined by Southern analysis (Fig. 1). One of the transformants, CACW-1, was used for further studies.

Biochemical characterization of CACW-1

Bgl2p protein was missing from walls of CACW-1, as assessed by SDS-PAGE and Western blot analysis (Fig. 2). The Bgl2p from *S. cerevisiae* runs slightly faster on SDS-PAGE compared to the homologous protein from *C. albicans* CAI-4 (Fig. 2, lanes 2 and 3, respectively). No comparable band was found in the extract prepared from CACW-1. Antibody raised against Bgl2p from *S. cerevisiae* cross-reacted with Bgl2p from *C. albicans* (Fig. 2, lanes 6 and 7). No Bgl2p cross-reacting material was detected in the extract from CACW-1 (Fig. 2, lane 5). Consistent with these findings, G_8 and G_{11} transferase products were not detected when G_5 (laminaripentaose) was incubated with cell wall extracts from *C. albicans* CACW-1 (Table 2). As expected, G_8 and G_{11} transferase products were detected when extracts of parental strain CAI-4 were incubated with G_5 . However, transferase products of unknown structure, which did not comigrate

with G_8 or G_{11} , were detected as reaction products using extracts of both CAI-4 and CACW-1.

The growth rate of CACW-1 ($\mu = 1.23 \pm 0.039$ h, $k = 0.563 \pm 0.018$) was slightly slower than the growth rate of CAF2 ($\mu = 1.13 \pm 0.019$ h, $k = 0.613 \pm 0.010$) when grown in SD medium plus 0.5% glucose. We noted that the OD₄₂₀ value of overnight cultures of CACW-1 was always lower by 30–40% compared to CAF2. Although there was a slight tendency for CACW-1 to aggregate during growth at 37 °C, extensive aggregation of stationary-phase cells occurred (Fig. 3). Aggregation was observed microscopically, and followed by monitoring the OD₈₀₀ value of cells incubated in phosphate buffer (pH 7.5). Vigorous mixing or mild sonication disrupted aggregates, i.e. aggregation as observed microscopically was reduced, and the OD₈₀₀ reading increased. However, no significant difference in cell aggregation was observed when CAF2 and CACW-1 were grown to stationary phase in Sabouraud Dextrose Broth (Fig. 4), nor were any observable hyphae formed by either strain under these conditions. There was no difference in the number of yeast ($2.96 \pm 0.13 \times 10^7$ yeast ml⁻¹) or the number of hyphal forms ($2.14 \pm 0.93 \times 10^6$ ml⁻¹) produced when two individual colonies of

Fig. 3. Aggregation of CACW-1 cells. Cells grown in SD medium plus 0.5% glucose were washed and resuspended in phosphate buffer (pH 7.5) at 37 °C. Aggregation of CAF2 and CACW-1 exponential- and stationary-phase cells was measured over time by monitoring the OD₈₀₀ value. (a) Exponential- and stationary-phase cells of both strains were compared. □, CAF2 exponential-phase cells; ○, CACW-1 exponential-phase cells; ■, CAF2 stationary-phase cells; ●, CACW-1 stationary-phase cells. (b) Two independent colonies of CAF2 (□, ■) and CACW-1 (○, ●) were grown to stationary phase and aggregation was examined. Stationary-phase cells of CACW-1 were prone to aggregation.

each strain were compared by growth in glucose-limited SD (0.1% glucose).

In addition, preliminary analysis of the 1,3- β - and 1,6- β -glucan and mannan fractions did not reveal any significant differences (Table 3 and Fig. 5). Equivalent amounts of hexose were released by Zymolyase 100T treatment of walls isolated from exponential- or stationary-phase cells of CAF2 and CACW-1 (Table 3). Zymolyase-released material separated into two fractions by size exclusion chromatography on Sephadex G50 (Fig. 5). Peak 1 (void volume) material contained approximately equal amounts of 1,6- β -glucan (glucose) and mannan (mannose) as assessed by gas chromatographic analysis of peracetylated alditol acetates, and methylation analysis (data not shown). Peak 2 contained 1,3- β -oligosaccharides as assessed by TLC, HPLC, methylation analysis and NMR (data not shown). Between 60 and 80% of the hexose in peak

Fig. 4. Aggregation profile of stationary phase cells in Sabouraud Dextrose Broth. Cells were grown overnight to stationary phase at 37 °C, and analysed microscopically using a slide counting chamber. Triplicate preparations were analysed for aggregation state by counting 100–150 aggregates, and the mean and standard deviation are given. □, CAF2; ▨, CACW-1.

1 samples was digested by endo-1,6- β -glucanase. Thus the levels of 1,3- β - and 1,6- β -glucan and mannan in cell walls of CACW-1 are equivalent to the parent CAF2. Stationary-phase cells were slightly more sensitive to Zymolyase 100T lysis on several occasions of testing, but we are not confident that the slight increase (5–10% in increased lysis rate) is significant (data not shown).

Virulence studies on CACW-1

The virulence of CACW-1 was slightly attenuated (Fig. 6) when compared to the isogenic parental strain CAF2 (experiment performed twice with similar results, and one set of data is depicted in Fig. 6). In addition, fewer c.f.u. were recovered from the kidneys of animals infected with CACW-1 compared to CAF2 (Table 4). It is difficult to predict what effect the slight decrease in generation time of CACW-1 (see above) might play in attenuating virulence, as compared to some alteration in the cell wall due to lack of the Bgl2p glucosyltransferase (see below). Regardless, the lack of Bgl2p glucosyltransferase reproducibly attenuated virulence in the standard mouse model. There was no significant difference in the aggregation state of CACW-1 compared to CAF2 when grown to stationary phase (Fig. 4, and see above) in the Sabouraud Dextrose Broth used to grow infecting inoculums. Thus it is unlikely that differences in aggregation caused the attenuation in virulence.

MIC profiles

The MIC values of several antifungal drugs, especially those acting by way of perturbation of cell wall synthesis or assembly, were measured on strains CAF2 and

Table 3. Analysis of wall glucan

Walls prepared from exponential- and stationary-phase cells were digested with Zymolyase 100T and released material was fractionated on Sephadex G50.

Strain	Zymolyase-released hexose (μg glucose equivalents)*	Hexose in peak 1 (%)†	Hexose in peak 2 (%)‡
CAF2 exponential	1116 $\pm$ 120	49.1	50.9
CAF2 stationary	1303 $\pm$ 147	43.4	56.6
CACW-1 exponential	1453 $\pm$ 87	45.2	54.8
CACW-1 stationary	1133 $\pm$ 108	48.8	51.2

* Assayed in triplicate with mean and standard deviation given.

† Peak 1 (void volume) contained 1,6- β -glucan and mannan as assessed by gas chromatography of peracetylated alditol acetate derivatives and methylation analysis. Data are the mean of two measurements.

‡ Peak 2 (included fraction) contained 1,3- β -oligosaccharides as assessed by methylation analysis. Data are the mean of two measurements.

Fig. 5. Analysis of wall glucan and mannan by size exclusion chromatography. SDS-extracted walls from exponentially growing cells were digested with Zymolyase 100T and the released hexose was fractionated on Sephadex G50. Peak 1 (void volume) contained mannan and 1,6- β -glucan; peak 2 (included volume) contained 1,3- β -oligosaccharides released by the 1,3- β -glucanases in Zymolyase 100T. $\square$, CAF2; $\circ$, CACW-1. Profiles for CAF2 and CACW-1 stationary-phase cells gave similar patterns (data not shown).

CACW-1 (Table 5). The most striking observations were: (1) the 30–250-fold increase in sensitivity of CACW-1 to the chitin synthase inhibitor nikkomycin Z compared to CAF2 at 37 and 42 °C; and (2) the increased sensitivity of both CAF2 and CACW-1 to nikkomycin Z when compared over the incubation temperatures 30, 37 and 42 °C. CACW-1 also showed a slight increase in sensitivity to Calcofluor white, which perturbs cell wall synthesis and assembly. In contrast, no significant

Fig. 6. Virulence of CAF2 ($\square$) and CACW-1 ($\bullet$) in the mouse model. Mice (10 for each group) were inoculated with (a) 1×10^7 , (b) 1×10^6 or (c) 1×10^5 c.f.u. of each strain via the tail vein, and the time of death was recorded. Mean survival times (d) are: (a) 1.0 (CAF2) and 1.4 (CACW-1); (b) 2.3 (CAF2) and 9.1 (CACW-1); (c) 10.5 (CAF2) and 12.6 (CACW-1).

differences were observed in the MIC values for ciclofungin and fusacandin A (inhibitors of glucan synthesis), staurosporine (inhibitor of wall synthesis via protein

Table 4. Colonization of mouse kidney tissue following intravenous inoculation

Twenty female CF-1 mice were inoculated with *C. albicans* strains, and at days 1 and 4 kidney homogenates from 10 mice from each group were plated on Sabouraud Dextrose Agar. *C. albicans* colony counts were determined after 24 h incubation.

Strain	Inoculum dose (c.f.u.)	c.f.u. recovered from kidneys/c.f.u. inoculated*	
		Day 1	Day 4
CAF2	9.3×10^4	0.796 (± 0.334)	1.29 (± 1.183)
	9.3×10^3	0.161 (± 0.075)	0.280 (± 0.086)
CACW-1	5.0×10^4	0.070 (± 0.026)	0.032 (± 0.008)
	5.0×10^3	0.074 (± 0.028)	0.182 (± 0.064)

* Mean values with standard deviation in parentheses.

Table 5. Effect of antifungal agents on CAF2 and CACW-1 strains at 30, 37 and 42 °C

MIC values were determined by micro broth dilution in SD medium plus 0.5% glucose at the indicated incubation temperatures, and results were scored on days 1, 2 and 4. Values in parentheses are the fold difference in MIC between CAF2 and CACW-1 under the given conditions.

Compound and temperature	MIC day 1 ($\mu\text{g ml}^{-1}$)		MIC day 2 ($\mu\text{g ml}^{-1}$)	
	CAF2	CACW-1	CAF2	CACW-1
30 °C				
Fusacandin A	1.95	3.9	3.9	7.8
Cilofungin	1.95	1.95	1.95	1.95
Calcofluor white	15.6	3.9	15.6	7.8
Staurosporine	0.98	0.5	15.6	7.8
Amphotericin B	0.5	0.5	0.5	0.5
Tunicamycin	15.6	15.6	15.6	31.25
Nikkomycin Z	>250	>250	>250	>250
Miconazole	7.8	7.8	15.6	15.6
37 °C				
Fusacandin A	0.98	1.95	7.8	7.8
Cilofungin	1.95	1.95	3.9	3.9
Calcofluor white	3.9	0.98 (4)	15.6	1.95 (8)
Staurosporine	<0.03	<0.03	0.98	1.95
Amphotericin B	0.24	0.24	0.98	0.98
Tunicamycin	7.8	15.6	31.25	15.6
Nikkomycin Z	250	0.98 (255)	250	7.8 (32)
Miconazole	0.5	0.5	0.5	0.98
Compound and temperature	MIC day 2 ($\mu\text{g ml}^{-1}$)		MIC day 4 ($\mu\text{g ml}^{-1}$)	
	CAF2	CACW-1	CAF2	CACW-1
42 °C				
Fusacandin A	7.8	1.95	7.8	3.9
Cilofungin	1.95	0.98	3.9	1.95
Calcofluor white	1.95	0.98	7.8	0.98 (8)
Staurosporine	0.5	0.5	0.98	0.98
Amphotericin B	0.24	0.24	0.5	0.98
Tunicamycin	7.8	3.9	15.6	15.6
Nikkomycin Z	15.6	0.5 (31)	125	1.95 (64)
Miconazole	1.98	0.5	1.98	0.98

kinase C), amphotericin B (binding to ergosterol), tunicamycin (inhibitor of glycoprotein assembly) or miconazole (inhibitor of ergosterol synthesis).

Nikkomycin Z affected cell morphology even at sub-MIC levels. CAF2 cells appeared as pseudo-hyphal or hyphal forms in the presence of sub-MIC levels of nikkomycin Z at all three temperatures. This morphology may be related to partial inhibition of one of the three known chitin synthases. The sensitivity to nikkomycin Z does vary between the three forms of chitin synthase in *S. cerevisiae* (Gaughran *et al.*, 1994). CAF2 demonstrated approximately 72% pseudo-hyphal or hyphal forms after 24 h growth with the sub-MIC dose of 62.5 µg nikkomycin Z ml⁻¹ at 37 °C. Untreated cells showed only about 12% hyphal growth, and the yeast form appeared as oblong, unicellular cells (7.5 µm long by 4 µm wide) at 37 °C.

In contrast, the CACW-1 strain grown at 37 °C had a different proportion of hyphal growth when treated with sub-MIC levels of nikkomycin Z. Approximately 70% of the cells were yeast forms (10 µm long by 7.5 µm wide) whereas only 30% were hyphal or pseudo-hyphal when observed after 24 h treatment with a sub-MIC dose of 0.24 µg nikkomycin Z ml⁻¹ at 37 °C. Extensive lysis of strain CACW-1 occurred at the MIC value of 7.8 µg nikkomycin Z ml⁻¹ at 37 °C after 48 h. Increasing the temperature to 42 °C did not change the nikkomycin Z effects on the CAF2 or CACW-1 strains. However, a higher ratio of hyphal/pseudo-hyphal growth (20–30%) was seen in both untreated strains at 42 °C.

The dramatic increase in sensitivity to nikkomycin Z is of particular interest, and indicates that loss of Bgl2p glucosyltransferase renders cells more dependent on chitin as a structural element in the wall. We have previously proposed repair functions for the Bgl2p glucosyltransferase in that it catalyses a 'strand-rejoining' reaction using a free reducing and a free non-reducing 1,3-β-glucan strand (Goldman *et al.*, 1995). Free ends would be generated not only by the action of endoglucanase activity, perhaps activated during bud emergence or cell septation/cell separation, but also following initiation and completion of new 1,3-β-glucan strands. There is a well-known relationship between chitinase activity and chitin repair during cell septation and separation (Kuranda & Robbins, 1991; Cabib *et al.*, 1992). Loss of chitin repair (*CHS1*) function leads to lysis of separating cells due to chitinase action at the septum. Similar biochemical events probably balance glucan synthesis and hydrolysis during bud emergence and cell septation/separation. Thus it is feasible that some aspect of bud emergence and or septation/separation is compromised in the Bgl2p-deficient CACW-1 strain (perhaps strand rejoining of glucan nicked by endoglucanase), rendering the cells more dependent on chitin when grown at 37 and 42 °C.

In conclusion, we have described several phenotypic changes in a *C. albicans* strain lacking the Bgl2p glucosyltransferase due to disruption of the two *BGL2* alleles. This strain lacks cell-wall-associated Bgl2p

glucosyltransferase activity, but contains glucosyltransferase activity of an as yet unknown type. Changes indicating that cell wall alterations have occurred in strain CACW-1 include (1) extensive aggregation of CACW-1 stationary-phase cells, (2) increased sensitivity of CACW-1 to nikkomycin Z, and possibly (3) the attenuated virulence in the mouse infection model. The function of other non-*BGL2*-encoded glucosyltransferase activities in *C. albicans* and *S. cerevisiae* is currently being investigated.

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REFERENCES

- Boeke, J. D., LaCrute, F. & Fink, G. R. (1984). A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol Gen Genet* 197, 345–346.
- Bulawa, C. E., Miller, D. W., Henry, K. L. & Becker, J. M. (1995). Attenuated virulence of chitin-deficient mutants of *Candida albicans*. *Proc Natl Acad Sci USA* 92, 10570–10574.
- Burgers, P. M. J. & Percival, K. J. (1987). Transformation of yeast spheroplasts without cell fusion. *Anal Biochem* 163, 393–397.
- Cabib, E., Silverman, S. J. & Shaw, J. A. (1992). Chitinase and chitin synthase 1: counterbalancing activities in cell separation of *Saccharomyces cerevisiae*. *J Gen Microbiol* 138, 97–102.
- Coen, M., Lerner, C. & Goldman, R. C. (1994). Synthesis of yeast wall glucan and evidence for glucan metabolism in a *Saccharomyces cerevisiae* whole cell system. *Microbiology* 140, 2229–2237.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal Chem* 28, 350–356.
- Elorza, V., Mormeneo, S., Garcia de la Cruz, F., Gimeno, C. & Sentandreu, R. (1989). Evidence for the formation of covalent bonds between macromolecules in the domain of the wall of *Candida albicans* mycelial cells. *Biochem Biophys Res Commun* 162, 1118–1125.
- Fonzi, W. A. & Irwin, M. Y. (1993). Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* 134, 717–728.
- Gaughran, J. P., Lai, M. H., Kirsch, D. R. & Silverman, S. J. (1994). Nikkomycin Z is a specific inhibitor of *Saccharomyces cerevisiae* chitin synthase isozyme Chs3 in vitro and in vivo. *J Bacteriol* 176, 5857–5860.
- Goldman, R. C., Sullivan, P. A., Zakula, D. & Capobianco, J. O. (1995). Kinetics of β-1,3 glucan interaction at the donor and acceptor sites of the fungal glucosyltransferase encoded by the *BGL2* gene. *Eur J Biochem* 227, 372–378.
- Hartland, R. P., Emerson, G. W. & Sullivan, P. A. (1991). A secreted β-glucan-branching enzyme from *Candida albicans*. *Proc R Soc Lond B Biol Sci* 246, 155–160.
- Hoffman, C. S. & Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* 57, 267–272.
- Klebl, F. & Tanner, W. (1989). Molecular cloning of a cell wall exo-β-1,3-glucanase from *Saccharomyces cerevisiae*. *J Bacteriol* 171, 6259–6264.

- Kollar, R., Petrokova, E., Ashwell, G., Robbins, P. W. & Cabib, E. (1995). Architecture of the yeast cell wall. The linkage between chitin and β (1,3)-glucan. *J Biol Chem* **270**, 1170–1178.
- Kuranda, M. J. & Robbins, P. W. (1991). Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. *J Biol Chem* **266**, 19758–19767.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Mio, T., Yabe, T., Sudoh, M., Satoh, Y., Nakajima, T., Arisawa, M. & Yamada-Okabe, H. (1996). Role of three chitin synthase genes in the growth of *Candida albicans*. *J Bacteriol* **178**, 2416–2419.
- Mrsa, V., Klebl, F. & Tanner, W. (1993). Purification and characterization of the *Saccharomyces cerevisiae* BGL2 gene product, a cell wall endo- β -1,3-glucanase. *J Bacteriol* **175**, 2102–2106.
- Reiss, E., Hearn, V. M., Poulain, D. & Shepherd, M. G. (1992). Structure and function of the fungal cell wall. *J Med Vet Mycol* **30**, 143–156.
- Shepherd, M. G. (1987). Cell envelope of *Candida albicans*. *Crit Rev Microbiol* **15**, 7–25.
- Shepherd, M. G., Poulter, R. T. & Sullivan, P. A. (1985). *Candida albicans*: biology, genetics, and pathogenicity. *Annu Rev Microbiol* **39**, 579–614.
- Sherman, F., Fink, G. R. & Hicks, J. B. (1982). *Methods in Yeast Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sietsma, J. H. & Wessels, J. G. H. (1981). Solubility of (1 $\rightarrow$ 3)- β -D/(1 $\rightarrow$ 6)-D-glucan in fungal walls: importance of presumed linkage between glucan and chitin. *J Gen Microbiol* **125**, 209–212.
- Surarit, R., Gopal, P. K. & Shepherd, M. G. (1988). Evidence for a glycosidic linkage between chitin and glucan in the cell wall of *Candida albicans*. *J Gen Microbiol* **134**, 1723–1730.
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