

Single allele knock-out of *Candida albicans* *CGT1* leads to unexpected resistance to hygromycin B and elevated temperature

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Almost all eukaryotic mRNAs are capped at their 5'-terminus. Capping is crucial for stability, processing, nuclear export and efficient translation of mRNA. We studied the phenotypic effects elicited by depleting a *Candida albicans* strain of mRNA 5'-guanylyltransferase (mRNA capping enzyme; *CGT1*). Construction of a *Cgt1*-deficient mutant was achieved by URA-blaster-mediated genetic disruption of one allele of the *CGT1* gene, which was localized on chromosome III. The resulting heterozygous mutant exhibited an aberrant colony morphology resembling the 'irregular wrinkle' phenotype typically obtained from a normal *C. albicans* strain upon mild UV treatment. Its level of *CGT1* mRNA was reduced two- to fivefold compared to the parental strain. Proteome analysis revealed a large number of differentially expressed proteins confirming the expected pleiotropic effect of *CGT1* disruption. The disrupted strain was significantly more resistant to hygromycin B, an antibiotic which decreases translational fidelity, and showed increased resistance to heat stress. Proteome analysis revealed a 50-fold overexpression of *Ef-1 α* p and a more than sevenfold overexpression of the cell-wall heat-shock protein *Ssa2p*. Compared to a reference strain, the *cgt1/CGT1* heterozygote was equally virulent for mice and guinea pigs when tested in an intravenous infection model of disseminated candidiasis.

Keywords: *Candida albicans*, mRNA capping enzyme, *CGT1*, real-time RNA quantitation, proteome

INTRODUCTION

The incidence of fungal infections continues to increase, particularly in immunocompromised patients where these infections can be life-threatening. *Candida albicans* remains the most common pathogen causing deep mycosis in humans (Groll *et al.*, 1998). *C. albicans* is a commensal, dimorphic fungus with no known sexual cycle. Resistance of fungal pathogens to clinically used antifungal agents used to be a rare occurrence, but has been increasingly documented around the world since the early 1990s. The increase in infections combined with the reduced efficacy of the currently available drugs lends importance to the discovery of new at-

tractive molecular targets for antifungal drugs. A starting point for finding novel molecular targets for such drugs is to study processes that are critical for growth and/or survival of the fungal pathogen. One such process is the acquisition of the 5' cap, the earliest modification event during eukaryotic mRNA synthesis. The 7-methyl-5'-guanosinetriphosphate-5'-N (m7GpppN) cap was first elucidated in 1975 (Wei & Moss, 1975; Furuichi *et al.*, 1975; Furuichi & Miura, 1975). The cap structure has been shown to be essential for protecting mRNA from untimely degradation (Schwer *et al.*, 1998) and to have a positive, though not essential, role in *Saccharomyces cerevisiae* pre-mRNA splicing (Fresco & Buratowski, 1996; Schwer & Shuman, 1996).

The mRNA capping occurs by three sequential enzymic steps in which the 5' triphosphate terminus of a primary

Abbreviations: GTase, 5' guanylyltransferase; TPase, 5' triphosphatase; DIG, digoxigenin; MALDI, matrix-assisted laser desorption/ionization; TEF, translation elongation factor.

transcript is first cleaved to a diphosphate-terminated RNA by mRNA 5' triphosphatase (TPase; mRNA capping enzyme, β subunit), then capped with GMP by mRNA 5' guanylyltransferase (GTase; mRNA capping enzyme, α subunit) and finally methylated at the N7 position of guanine by mRNA cap methyltransferase. Of the three catalytic steps involved in cap formation, the GTase reaction has been studied in most detail (Schwer & Shuman, 1994, 1996; Schwer *et al.*, 1998; Yamada-Okabe *et al.*, 1998a). As both the GTase and mRNA cap methyltransferase genes are essential in *S. cerevisiae* (Shibagaki *et al.*, 1992; Mao *et al.*, 1995), it is likely that every step of mRNA capping in *S. cerevisiae* is essential and that yeasts have no isozymes for enzymes synthesizing the mRNA cap structure (Yamada-Okabe *et al.*, 1996).

Genes encoding GTases have been identified in *Can. albicans*, *Sac. cerevisiae*, *Schizosaccharomyces pombe*, *Mus musculus*, *Homo sapiens* and *Caenorhabditis elegans*. Previous studies have shown that the GMP-binding site of GTase lies within a conserved KXDG motif with the lysine (K) residue being absolutely essential (Schwer & Shuman, 1994). The hallmark of the RNA capping reaction is the formation of an enzyme-guanylate intermediate in which GMP is covalently linked to the lysine residue via a phosphoamide bond. Multiple conserved sequence motifs have now been shown to be essential for the function of mRNA capping enzyme (Shuman *et al.*, 1994; Yamada-Okabe *et al.*, 1998a).

The *C. albicans* mRNA GTase gene (*CGT1*) was cloned by functional complementation of a *S. cerevisiae* GTase (*ceg1*) null mutant and was found to exist as a single copy in the genome of *C. albicans* strain IFO 1060 (Yamada-Okabe *et al.*, 1996).

Lower and higher eukaryotes clearly differ with respect to the physical linkage of the GTase and TPase functions (Ping Wang *et al.*, 1997). The yeast capping enzymes consist of two subunits (in *S. cerevisiae* there is a 52 kDa α subunit responsible for GTase activity and 80 kDa β subunit responsible for TPase activity), but in higher eukaryotes both GTase and TPase activities are present on a single polypeptide. It has been demonstrated that in higher eukaryotic capping enzymes, the N-terminal part is responsible for TPase activity and the C-terminal part is essential for GTase activity (Yamada-Okabe *et al.*, 1998b).

S. cerevisiae GTase mutants accumulate unspliced mRNA (Fresco & Buratowski, 1996). Coupling of pre-mRNA splicing to the presence of the cap offers several potential benefits to the cell. First, it provides a mechanism for the splicing machinery to discriminate RNA polymerase II transcripts from those produced by other polymerases. A second benefit is that the splicing machinery will not assemble on partially degraded heterogeneous nuclear RNAs (pre-mRNAs) lacking an m7GpppN cap.

Furthermore, the rate of protein synthesis and the steady-state levels of multiple individual mRNAs are

decreased in *S. cerevisiae* GTase mutants (Fresco & Buratowski, 1996; Schwer *et al.*, 1998). However, mRNA polyadenylation and nuclear export remain unaffected.

Because of the potential importance of *C. albicans* *CGT1* as an antifungal target we wanted to explore the impact that an inhibitor of *CGT1* activity might have on a pathogen and we mimicked this effect by generating a *C. albicans* strain depleted of *CGT1*. This was achieved by disrupting one allele of the *CGT1* gene in *C. albicans* strain CAI-4 and subsequent investigation of the resulting phenotypic effects.

METHODS

Strains and media. *C. albicans* CAI-4 (*ura3::imm434/ura3::imm434*) and its parental strain SC5314 were kindly provided by Dr W. Fonzi, Georgetown University, USA (Fonzi & Irwin, 1993). *C. albicans* B2630 was obtained from the American Type Culture Collection (Manassas, VA; no. 44858).

Synthetic selective medium contains 0.67% yeast nitrogen base without amino acids (Difco), 0.2% Ura dropout powder (Bufferad) and 2% glucose. Uridine was added at a final concentration of 20 $\mu\text{g ml}^{-1}$ to ensure growth of CAI-4. YPD medium contains 2% peptone (Difco), 1% yeast extract (Difco) and 2% glucose (Sigma).

C. albicans Ura⁻ revertants were selected upon excision of the *C. albicans* *URA3* gene from an integrative transformant strain (see below), on the basis of the resistance of the Ura⁻ revertants to 5'-fluoroorotic acid (Sigma) and following the procedure of Fonzi & Irwin (1993).

Construction of *CGT1* disruption cassette. A DNA fragment containing the entire coding region of *C. albicans* *CGT1* was obtained from Dr Yamada-Okabe (Nippon Roche Research Center, Japan). The coding region of *CGT1* was PCR amplified and cloned into a *Sma*I-linearized pGEX-2T vector (Smith & Johnson, 1988) yielding *CGT1/pGEX-2T*. The coding region of *CGT1* was fused in-frame to the GST part of pGEX-2T. The HISG-URA3-HISG cassette was released from pMB7 (a gift from Dr W. Fonzi, Georgetown University, USA; Fonzi & Irwin, 1993) using *Bgl*II/*Sal*I and extended at one end using *Bgl*II/*Sal*I linkers. Double-stranded *Bgl*II/*Sal*I linkers were obtained through annealing of oligonucleotides A (5'-CCGAATTCTAGAGTCGACA-3') and B (5'-GATCTGTC-GACTCTAGAATTCCGG-3'). The intermediate construct *CGT1/pGEX-2T* was cleaved with *Xho*I to accept the *Sal*I-digested HISG-URA3-HISG cassette. Finally, the plasmid *CGT1-URAcassette/pGEX-2T* was cleaved with *Sca*I to release a 6.3 kb *CGT1* disruption cassette (with *CGT1* flanking recombinogenic ends of 0.4 and 1 kb) to be used for transformation.

Transformation of *C. albicans*. CAI-4 was transformed by using a modified spheroplast transformation method (M. Logghe and others, unpublished). Transformants were plated on synthetic selective medium using sterile 0.4 cm glass pearls (Glaverbel) and incubated for 3–5 d at 30 °C.

Southern blot analysis. *C. albicans* strains were grown in 2 ml synthetic selective medium overnight. Genomic DNA was isolated using the Nucleon MI Yeast kit (Amersham) and the concentration of genomic DNA was estimated by analysing a sample on a 0.7% agarose gel in 0.5 × TBE and comparison to a known standard molecular mass marker. Approximately 200 ng genomic DNA was digested overnight with *Xba*I,

treated with RNase and incubated for 20 min at 65 °C. Samples were resolved on a 0.7% agarose gel in 0.5× TBE and run for 6 h at 4 V cm⁻¹. DNA was visualized using ethidium bromide staining and subsequent UV transillumination, and blotted onto Hybond-N+ membrane (Amersham) as described by Sambrook *et al.* (1989). Digoxigenin (DIG)-labelled probes were prepared in a 50 µl reaction using DIG-dUTP (Boehringer) at a 1:3 dTTP: DIG-dUTP ratio, 500 pg template plasmid DNA, 1× PCR buffer II (Perkin Elmer Cetus), 0.4 µM each primer (Eurogentec), 0.2 mM each dATP, dCTP and dGTP (Perkin Elmer Cetus), 2.5 mM MgCl₂ (Perkin Elmer Cetus), and 1.25 U *Taq* polymerase with *TaqStart* antibody (Boehringer). A 1.6 kb URA-blaster-cassette-specific probe fragment was amplified using pMB7 as a template and using primers P3 (5'-CAGCAATTCTCGTGAATCATCG-3') and P5 (5'-TGATCAATTCCTGAGCAACAAC-3'). A 0.5 kb *CGT1*-specific probe fragment was amplified using *CGT1*/pGEX-2T as a template and using primers P6 (5'-AGGAATTACGATTGATGGTGGC-3') and P7 (5'-AGGGGAGTTGACTATATCAGG-3'). PCR was performed on denatured (10 min at 94 °C) DNA for 30 cycles, each consisting of 1 min at 94 °C, 1 min at 57 °C and 2 min at 72 °C. A final elongation step at 72 °C for 7 min completed the reaction.

The membrane was prehybridized at 42 °C in DIG Easy Hyb (Boehringer) for a minimum of 1 h. Hybridization was performed using 1 µl denatured PCR product (= 1/50 of the total volume) ml⁻¹ DIG Easy Hyb solution. The probes were denatured by heating the PCR reaction for 10 min at 96 °C, then quick-chilling on ice. The probe was kept on ice for 5 min, centrifuged briefly and diluted in prewarmed DIG Easy Hyb solution. The entire probe solution was filtered through a 0.45 µm filter (Millex HV, Millipore) prior to use. Hybridizations were carried out overnight at 42 °C. After hybridization, membranes were washed twice with 2× SSC, 0.1% SDS at ambient temperature for 15 min and twice with 0.1× SSC, 0.1% SDS at 68 °C for 15 min. Detection was performed using the DIG Wash and Block Buffer Set as described by the manufacturer (Boehringer) and the blot was exposed to Kodak XAR-5 film for 1 h at ambient temperature.

PCR analysis. PCR analyses were used to verify the disruption of the *C. albicans CGT1* gene. Primers P1 (5'-TAGAAATTCAGGACCCTATACAC-3') and P2 (5'-CAGAAACCGAGAAAGAATCAAAC-3') were used to distinguish disrupted from wild-type *CGT1* genes on the basis of size and are within the *CGT1* gene itself. Primers P3 and P4 lie within the URA-blaster disruption cassette. P1 was used in combination with P2 and P3 (5'-CAGCAATTCTCGTGAATCATCG-3'). Primer P2 was used in combination with P4 (5'-CTAACGCAGTCAGGCACCG-3'). PCR was performed on 10 ng denatured (10 min at 94 °C) genomic DNA using the primer combinations described above (all primers were from Eurogentec) for 30 cycles, each consisting of 1 min at 94 °C, 1 min at 57 °C (or 59 °C for the P1/P2 primer combination) and 3 min at 72 °C (or 6 min for the P1/P2 primer combination). A final elongation step at 72 °C for 7 min completed the reaction. In the reaction mixture 2.5 U *Taq* polymerase (Boehringer) with *TaqStart* antibody (Clontech) (1:1) were used, and the final concentrations were 0.2 µM each primer, 2 mM MgCl₂ (Perkin Elmer Cetus) and 200 µM dNTPs (Perkin Elmer Cetus). PCR was performed in a Perkin Elmer model 9600 PCR machine.

PFGE. Liquid starter cultures were set up by inoculating each colony into 1 ml synthetic selective medium and incubating overnight at 30 °C at 300 r.p.m. These cultures were used to inoculate 20 ml synthetic selective medium and incubated overnight at 30 °C at 300 r.p.m.

Ten millilitres of each culture was centrifuged for 10 min at 2890 g and resuspended in filter-sterilized, cold 50 mM EDTA. The cell density was determined using a Coulter Counter (Coulter Z1, Coulter Electronics), a total of 1.4 × 10⁸ cells was transferred to a 1.5 ml Eppendorf tube and centrifuged for 5 min at 2890 g. The pellet was used to make plugs according to the protocol enclosed with the CHEF genomic DNA plug Kit (Bio-Rad). Electrophoresis of the plugs was performed using a 0.8% agarose (Seakem gold agarose for PFGE, FMC BioProducts) gel in 1× TAE buffer. As a size marker, Bio-Rad *Hansenula wingei* chromosomes were used. The Bio-Rad CHEF Mapper II was used for electrophoresis set at the following parameters: switch time, 120 s; final switch time, 480 s; run time, 40 h; voltage, 3 V cm⁻¹; angle, 106°. The gel was stained using ethidium bromide at a final concentration of 0.5 µg ml⁻¹ for 15 min at ambient temperature and blotted overnight by capillary action (Sambrook *et al.*, 1989) onto a positively charged nylon membrane (Cat. No. 1417240, Boehringer). The 1.6 kb URA-blaster-cassette-specific probe fragment was amplified as described above and purified using the Qiagen PCR purification kit according to the manufacturer's instructions. Twenty nanograms were labelled with [α -³²P]dCTP using the Rediprime II labelling system (Amersham Pharmacia Biotech). The chromosomal blot was hybridized and washed as described by Sambrook *et al.* (1989). The blot was exposed to a Kodak Biomax MS film at -70 °C for 1.5 h with two intensifying screens and developed using the Kodak X-OMAT M43 processor.

Growth monitoring. For liquid cultures, starter cultures were set up by inoculating each colony in 1 ml synthetic selective medium and incubating overnight at 30 °C at 300 r.p.m. Cell densities were determined using a Coulter counter (Coulter Z1; Coulter Electronics). Subsequently, 2.5 × 10⁵ cells were inoculated into synthetic selective medium in a final volume of 1 ml and cultures were incubated for 24 h at 30 °C at 300 r.p.m. Cultures were washed in minimal medium without glucose (S medium) and the pellet was resuspended in 650 µl S medium. Eight microlitres of this culture was used for inoculating 400 µl cultures in a Honeywell-100 plate (Bio-screen Analyzer; Labsystems). Each strain was grown at 30 °C for 48 h in synthetic selective medium; shaking was at high intensity and was carried out every 3 min for 20 s. Optical densities were measured every hour. Growth curves were generated automatically using the BioLINK software (Labsystems).

For plate cultures, strains were streaked out on synthetic selective medium/agar plates using an inoculating loop and incubated for 2–3 d at 30 °C in a static incubator (Memmert).

Spot test. Starter cultures were set up from each strain and grown to a final density of 2 × 10⁷ cells ml⁻¹ (OD₆₀₀ = 1.0). Cells were plated by transferring 5 µl of the original suspension as well as 10⁻¹, 5 × 10⁻¹, 10⁻², 5 × 10⁻², 10⁻³, 5 × 10⁻³, 10⁻⁴, 5 × 10⁻⁴, 10⁻⁵ and 5 × 10⁻⁵ dilutions onto plates of synthetic selective medium. Spotting was in duplicate and one series was incubated at 30 °C and the other series at 37 °C for 3 d. Plates used were synthetic selective medium, synthetic selective medium + 400 µg hygromycin B ml⁻¹ (Sigma), synthetic selective medium + 0.08 M hydroxyurea (Serva), synthetic selective medium without glucose + 2% galactose, synthetic selective medium without glucose + 2% galactose + 400 µg hygromycin B ml⁻¹ and synthetic selective medium without glucose + 2% galactose + 0.08 M hydroxyurea.

Real-time quantitation of the *CGT1* mRNA transcript. PCR quantitations using specific primers and probes were performed according to the *TaqMan* procedure (Livak *et al.*,

1995; Orlando *et al.*, 1998) using the ABI Prism 7700 sequence detector (Applied Biosystems). Primers and probes for *C. albicans* *ACT1*, encoding β -actin, and *CGT1* genes were designed using the PrimerExpress software system (Perkin Elmer Cetus). Cells were grown to $OD_{600} \sim 1.0$ and total RNA was prepared using the RNeasy midi kit (Qiagen; mechanical disruption protocol) according to the manufacturer's instructions. All RNA samples were DNaseI (Boehringer, RNase-free)-treated at $20 \text{ U } \mu\text{g}^{-1}$ in $50 \mu\text{l}$ solution for 40 min at ambient temperature, phenol/chloroform extracted and precipitated. Pellets were dissolved in $20 \mu\text{l}$ MilliQ water (Millipore) and RNA concentrations were determined spectrophotometrically by measuring OD_{260} in a UV-1601 UV/visible spectrophotometer (Shimadzu). First-strand cDNA synthesis was performed in a final volume of $20 \mu\text{l}$ containing $1 \times$ Superscript RT buffer (Life Technologies), 10 mM DTT, 125 μM each dNTP, 50 μM hexamer primers (Life Technologies) and 1 μg RNA. Mixtures were incubated for 10 min at ambient temperature and 1 μl was removed and diluted 1:4 for the non-amplification control; 20 U Superscript reverse transcriptase (Life Technologies) was added and the reaction was incubated for 1 h at 42°C . The enzyme was inactivated for 10 min at 70°C .

PCR reactions were set up in triplicate for all genes and contained 5 μl PCR buffer A, 4 mM MgCl_2 , 200 μM each dATP, dGTP, dCTP and 400 μM dUTP, 250 nM fluorogenic probe (for *CGT1*: 5'-CTGAATGTCGCCAATCTACAACTAAGAAGGGA-3') labelled at the 5' end with 6-carboxyfluorescein and at the 3' end with 6-carboxytetramethylrhodamine (Genset), 1.25 U AmpliTaq Gold, 16.75 μl H_2O , 300 nM of appropriate FORWARD (for *CGT1*: 5'-AGCAACCATTACAAGGCAGGA-3') and REVERSE (for *CGT1*: 5'-CTGAATCGAAGCATTTCCTCAA-3') PCR primers, 1 μl of the reverse transcription reaction mixture. For the non-amplification control, 1 μl of the 1:4 diluted RTase-negative sample was added while 1 μl H_2O was added to each non-template control sample. The ABI PRISM 7700 was run for 50 cycles of 15 s at 95°C , 1 min at 60°C . These cycles were preceded by 10 min at 95°C . Data were analysed using the ABI PRISM 7700 software package. Data were normalized according to *ACT1* CT (threshold cycles) values (User bulletin 2, PE Applied Biosystems).

Experimental disseminated *C. albicans* infections. *C. albicans* SC5314, B2630 and dCGT1 were maintained on Sabouraud glucose agar (Oxoid). For the preparation of inocula, a yeast colony was lightly touched with a wet inoculating loop and the material on the loop was transferred to 5 ml Sabouraud broth (Oxoid) in a sterile 15 mm glass test tube. The test tube cultures were rotated at 20 r.p.m. and an angle of 5° to the horizontal for 18–20 h at $30 \pm 1^\circ\text{C}$ (Odds, 1991). The cell concentration was estimated by means of haemocytometer counts and appropriate dilutions were made in physiological saline for intravenous injection in mice and guinea pigs. Specific pathogen-free albino guinea pigs and NMRI mice were purchased from Charles River. Guinea pigs were housed individually and mice in groups of up to ten animals. They were fed food and water *ad libitum*. Individual animals were weighed and infected intravenously with *C. albicans* via either the lateral tail vein (mice) or the lateral vein of the penis (guinea pig). Different test inocula were administered in separate experiments. In the first experiment, the animals were challenged with 10^4 *C. albicans* cells per g body weight. This dose usually leads to a mean survival time of 5–7 d when the infecting strain is a fresh clinical isolate. On the basis of the survival times at this level of challenge, higher or lower infecting doses were used in subsequent experiments to

establish a dose-survival relationship for each of the three *C. albicans* strains.

The animals were observed daily for 21 d. Animals surviving at this time were humanely killed. At a post-mortem examination, the right kidney of each animal was homogenized in 15 ml physiological saline and 10-fold dilutions of the homogenate were plated on Sabouraud agar to determine the organ burden of *C. albicans* (\log_{10} c.f.u. per kidney for mice, \log_{10} c.f.u. per gram kidney weight for guinea pigs). Mean survival times, percentage of survivors on day 21, percentage of culture-negative kidneys and mean organ burden for culture-positive kidneys were the parameters used to determine the relative virulence of the infecting strains.

Proteome analysis. The proteomics strategy included high-resolution two-dimensional gel electrophoresis, in-gel trypsin digestion followed by identification of a limited set of differentially expressed proteins using different approaches including narrow-bore HPLC followed by N-terminal sequencing, MALDI (matrix-assisted laser desorption/ionization)-MS or nanospray-MS/MS.

C. albicans starter cultures were set up by inoculating each colony in 1 ml synthetic selective medium and incubating overnight at 30°C and 300 r.p.m. Cell densities were determined using a Coulter counter (Coulter Z1; Coulter Electronics). Approximately 2.5×10^5 cells in 1 ml were inoculated in 200 ml synthetic selective medium and cultures were incubated at 30°C and 300 r.p.m. until a final OD_{600} of 1.0 was reached.

Cultures were centrifuged for 10 min at 1470 g and the supernatant was discarded. The cell pellets were frozen immediately at -70°C . The following steps were performed at the Wittmann Institute of Technology and Analysis of Biomolecules (WITA, Germany). Each pellet was crushed in a mortar under liquid nitrogen. The crude extract was dissolved in 588 μl AP buffer (9 M urea, 70 mM DTT, ampholyte mix) and 10 μl protease inhibitor mix. The protein extracts were concentrated four-fold prior to application onto the gels. Proteins were separated by a large gel high-resolution two-dimensional electrophoresis technique (Klose & Kobalz, 1995; Brockstedt *et al.*, 1998). All equipment and ready-to-gel solutions were from WITA. Gels were silver stained as described by Heukeshoven & Dernick (1985). For the identification of individual proteins, preparative high-resolution two-dimensional gels were run and stained with Coomassie blue (Klose & Kobalz, 1995; Eckerskorn *et al.*, 1988) and the relevant protein spots were excised from the gel manually. In-gel digestion, extraction of the peptides from the crushed acrylamide gel fragment, desalting and concentration by a reversed-phase technique were performed according to Otto *et al.* (1996). MALDI-MS was performed with a VG Tof Spec (Fisons) equipped with a nitrogen laser and a VAX 400 VLC station using OPUS software, version 3.1. Spectra were obtained in the linear mode by summing 20–50 laser shots. A saturated solution of α -cyano-4-hydroxycinnamic acid in aqueous 50% (v/v) acetonitrile and 0.1% liquid trifluoroacetic acid was used as the matrix. The matrix (1.2 μl) and sample (0.8 μl) were mixed on the target and air-dried. For mass determination, the dried target was inserted into the mass spectrometer; measurements were under vacuum at 22 and 24 kV.

Peptide mixtures were separated on a narrow-bore reverse-phase HPLC column (Smart-HPLC system, Pharmacia) employing gradients made from 0.1% liquid trifluoroacetic acid in water and 85% (w/v) acetonitrile/0.08% liquid trifluoroacetic acid in water. The fractions were dried and used for Edman micro-sequencing (Wittman-Liebold, 1992). The

dried peptides were dissolved in acetonitrile/water/TFA (50:45:5 by vol.) and applied to pre-washed Biobrene-coated glass fibre filters and run in a sequencer (Applied Biosystems Instruments; Procise 492 and on-line PTH-amino acid analyser).

Mass fingerprinting by MS was performed using a Q-ToF (Micromass). Operation was at 30 °C with a nitrogen drying gas flow of about 180 l h⁻¹. A potential of 1.4 kV was applied to the nanoflow borosilicate glass capillary. The peptide mixture of each spot, without pre-separation, was desalted and concentrated in a microtip filled with a few RP-C18 beads (Vydag) and eluted from the reverse phase material with acetonitrile/water/trifluoroacetic acid (50:45:5 by vol.) in a 1 µl volume. This solution was introduced into a microcapillary and sprayed into the Z-spray ionic source of the nanospray mass spectrometer. The individual peptide masses were recorded in the time-of-flight analyser of the instrument. For nanospray-MS/MS analysis, the peptide ions were fragmented in the collision hexapole of the instrument. The fragments of each peptide were analysed in the time-of-flight analyser of the instrument, yielding partial sequences. An aliquot of 1 µl, obtained from half to one-fifth of the Coomassie-stained spot sufficed for one mass-fingerprint spectrum and five to six MS/MS experiments were simultaneously recorded, employing a flow rate of 30 nl min⁻¹. A collision energy of 28–35 V (depending on the charge state of the daughter ions) was applied; the gas pressure in the collision cell was regulated to 6.0 × 10⁻⁵ bar. Calibration of the instrument was done with Glu-fibrinopeptide.

Peptide analysis. The peptide masses of the mass fingerprints obtained from each protein were searched in the SWISS-PROT database using the FRAGMOD program (E.-Ch. Muller, Berlin), a modified version of the FRAGFIT program (Henzel *et al.*, 1993). Several other search programs as described by Mueller *et al.* (1999) were used in case identification was not possible using the above-described strategy.

DNA sequencing. Reactions were performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit according to the instructions of the manufacturer (PE Applied Biosystems) except for the following modifications. The total reaction volume was reduced to 15 µl and volumes of individual reagents were changed accordingly. The 6 µl Terminator Ready Reaction Mix was replaced by a mixture of 3 µl Terminator Ready Reaction Mix + 3 µl Half Term (GENPAK). After cycle sequencing, reaction mixtures were purified over Sephadex G50 columns prepared on Multiscreen HV opaque Microtitre plates (Millipore) and dried in a Speedvac. Reaction products were resuspended in 3 µl loading buffer. Following denaturation for 2 min at 95 °C, a 1 µl sample was applied to a 5% (w/v) Long Ranger Gel (36 cm well-to-read) prepared from Singel Packs according to the supplier's instructions (FMC BioProducts). Samples were run for 7 h with run module 2X on an ABI 377XL DNA sequencer. Data collection version 2.0 and Sequence analysis version 3.0 (for basecalling) software packages were from PE Applied Biosystems.

RESULTS

Creation of a strain with diminished levels of mRNA capping enzyme

To generate a strain with low levels of mRNA capping enzyme, a *CGT1* single allele knock-out strain was constructed. Genetic disruption of *C. albicans* genes is particularly difficult since this fungus (unlike *S. cere-*

visiae, for example) has no known sexual cycle or haploid form (although individual genes can be haploid or polyploid). To accomplish disruption of the first allele of the *CGT1* gene, the URA-blaster system was used (Fonzi & Irwin, 1993). Disruption of the *CGT1* allele was confirmed in the transformants by PCR and Southern blot analysis. A schematic representation of the wild-type and disrupted alleles of *CGT1* is shown in Fig. 1(a).

Confirmation of the disruption of one copy of the *C. albicans CGT1* gene was obtained by PCR using three different primer combinations. PCR amplification from wild-type CAI-4 genomic DNA (not shown) using primer pair P1/P2 yielded a 1.4 kb fragment, as expected. Primer pairs P1/P3 and P2/P4 did not yield any bands. As one primer in each of these pairs is situated within the URA-blaster cassette which is absent in non-transformed CAI-4 strain, this absence of PCR product is as expected.

PCR amplification from *cgt1/CGT1* heterozygote CAI-4 genomic DNA using primer pair P1/P2 yielded the expected fragment of 1.4 kb (Fig. 1b); the second expected fragment of 5.5 kb from the allele disrupted with the URA-blaster cassette was not observed presumably because of the large size of the fragment and the preferential amplification of the smaller 1.4 kb fragment. A PCR fragment of 0.5 kb was obtained with primer pair P1/P3, while primer pair P2/P4 yielded a PCR fragment of 2.6 kb (Fig. 1b), both as expected.

The correct integration of the URA-blaster cassette into the *CGT1* locus was also confirmed by Southern blot analysis. In Southern blots of genomic DNA hybridized with a *URA3*-specific probe, no signal was detected in the wild-type while 1.5 and >3.7 kb *XbaI* fragments corresponding to the disrupted allele were detected in the *cgt1/CGT1* heterozygote (Fig. 1c). In identical blots hybridized with a *CGT1*-specific probe, a 2 kb *XbaI* fragment representing the wild-type allele and 0.5 kb and >3.7 kb fragments corresponding to the disrupted allele were found in the *cgt1/CGT1* heterozygote (Fig. 1c).

Attempts were made to construct a homozygous *cgt1/cgt1* null mutant. The *URA3* marker in the *cgt1/CGT1* heterozygote was removed by growth on 5'-fluoroorotic acid and loss of this marker was checked by Southern hybridization (data not shown). One Ura⁻ revertant was selected for transformation using the disruption cassette previously used for the generation of the single allele knock-out. A total of 13 transformants were checked by Southern blot analysis for homozygous disruption of the *CGT1* gene (data not shown). Homozygous null strains were not recovered (8 integration events occurred at the already disrupted allele; 5 integrations occurred outside of the *CGT1* locus).

Chromosomal localization of *CGT1*

The location of the *CGT1* gene was assigned by separating the chromosomes of the *cgt1/CGT1* heterozygote by PFGE (Gerring *et al.*, 1991; Magee, 1994a,

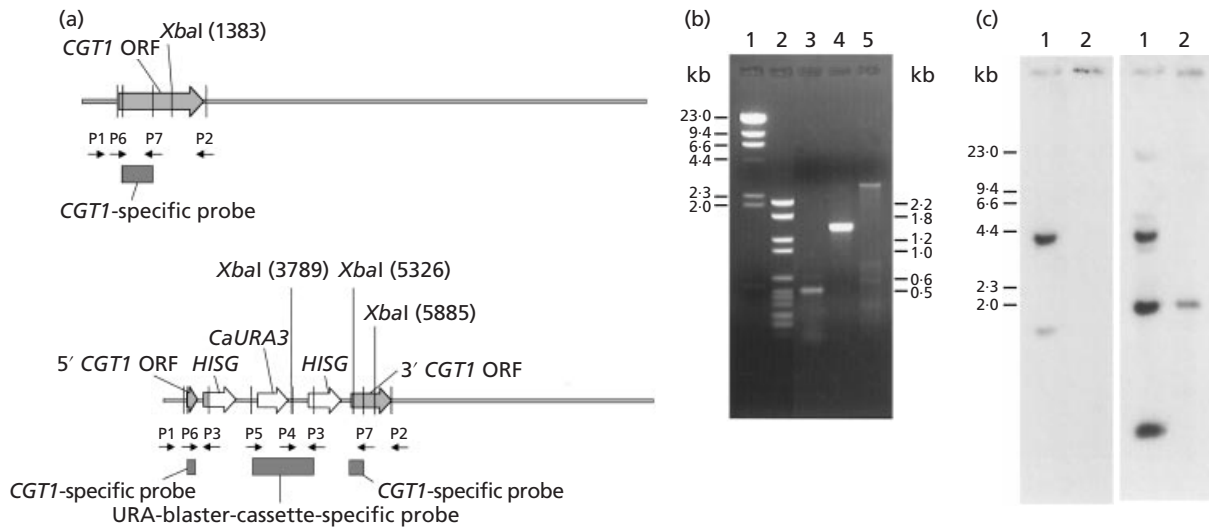


Fig. 1. Targeted disruption of *C. albicans CGT1*. (a) Genomic arrangement of the *CGT1* locus in wild-type strain CAI-4 (top) and *cgt1/CGT1* heterozygote (bottom). Locations of the primers (Pn) used for PCR analysis and probes used for Southern blot analysis are indicated on the map. (b) PCR confirmation of the disruption of one copy of the *C. albicans CGT1* gene. Lane 1, molecular mass marker II (Boehringer); lane 2, molecular mass marker VI (Boehringer); lane 3, PCR amplification with *cgt1/CGT1* genomic DNA using primer pair P3/P4; lane 4, PCR amplification with *cgt1/CGT1* genomic DNA using primer pair P1/P2; lane 5, PCR amplification on *cgt1/CGT1* genomic DNA using primer pairs P2/P5. (c) Confirmation of the disruption of one copy of the *C. albicans CGT1* gene by Southern blot analysis. Lane 1, genomic DNA from *cgt1/CGT1* heterozygote cleaved with *Xba*I; lane 2, genomic DNA from the parental CAI-4 strain cut with *Xba*I. The probe in the left panel was a 1.6 kb fragment of the *URA*-blaster cassette and in the right panel was a 0.5 kb *CGT1*-specific gene fragment.

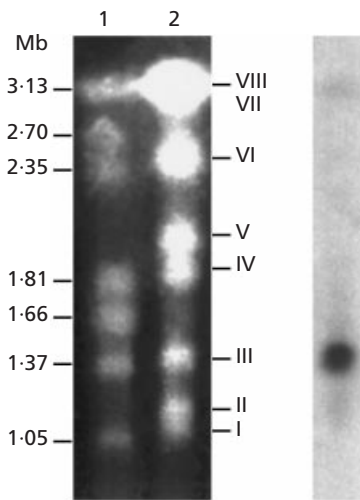


Fig. 2. Localization of the disrupted *C. albicans CGT1* gene to chromosome III. Left panel: lane 1, *Hansenula wingee* chromosomes (Bio-Rad); lane 2, *C. albicans cgt1/CGT1* heterozygote chromosomes. Right panel: Southern blot analysis showing hybridization of the *CGT1*-*URA3*-specific probe to chromosome III.

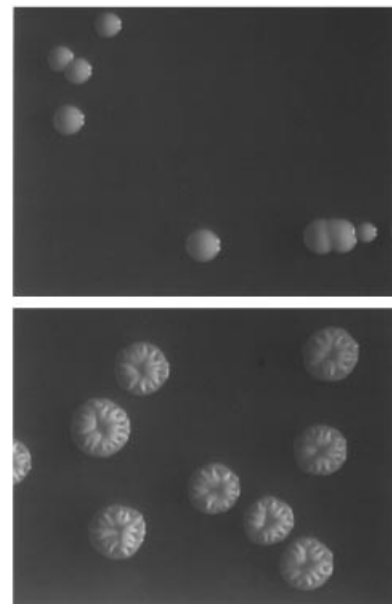


Fig. 3. Colony morphology of the *cgt1/CGT1* heterozygote (bottom) and the parental strain (top). Plates were incubated for an equal length of time.

b) followed by blotting and hybridization to a *CGT1/URA3*-specific probe. The *CGT1* gene was localized to chromosome III (Fig. 2; chromosome numbering is as described by Rustchenko-Bulgac *et al.*, 1990). Comparing the electrophoretic karyotypes of the

parental strain and a *cgt1/CGT1* heterozygote (disruption confirmed by both PCR and Southern blot analysis) did not reveal any obvious genetic aberrations (data not shown).

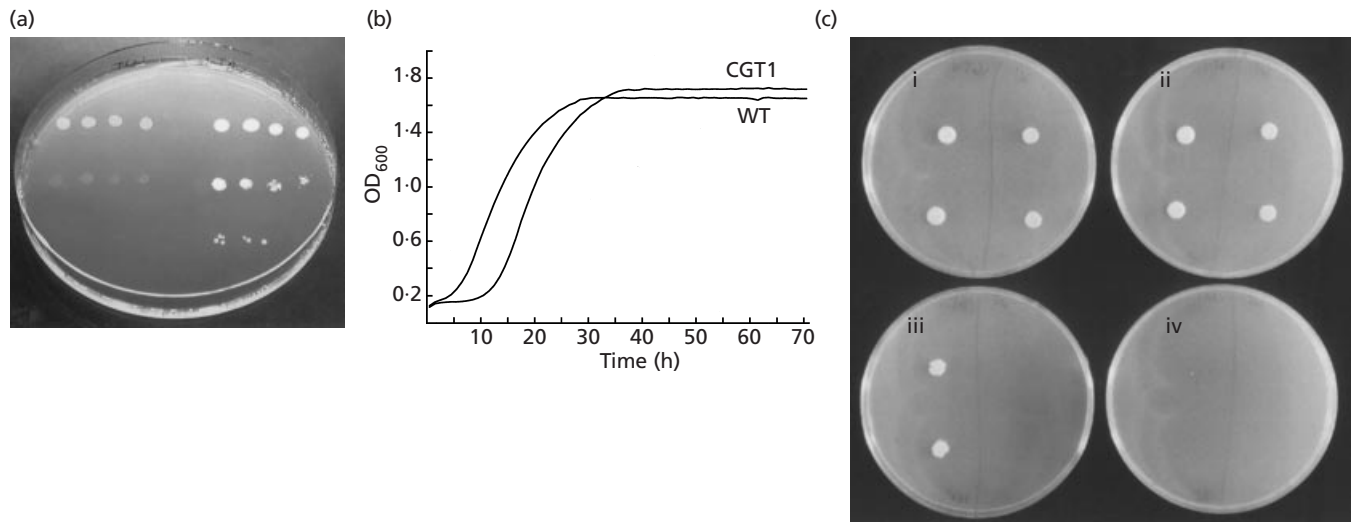


Fig. 4. (a) Spot test: two-fold dilutions of an inoculum of each strain were spotted onto the plate. *cgt1/CGT1* heterozygote (right) and parental strain (left) were tested for growth in the presence of hygromycin B ($400 \mu\text{g ml}^{-1}$). (b) Growth curve of the *cgt1/CGT1* heterozygote and its parental strain with optical densities measured every hour over 70 h while incubating at $30 \text{ }^\circ\text{C}$ in a Bioscreen Analyzer (Labsystems). (c) Growth after heat shock at $55 \text{ }^\circ\text{C}$ of 0 (i), 90 (ii), 120 (iii) or 180 (iv) min and subsequent incubation at $30 \text{ }^\circ\text{C}$ for a total incubation time of 48 h of the *cgt1/CGT1* heterozygote (left; duplicate spots) and the parental strain (right; duplicate spots).

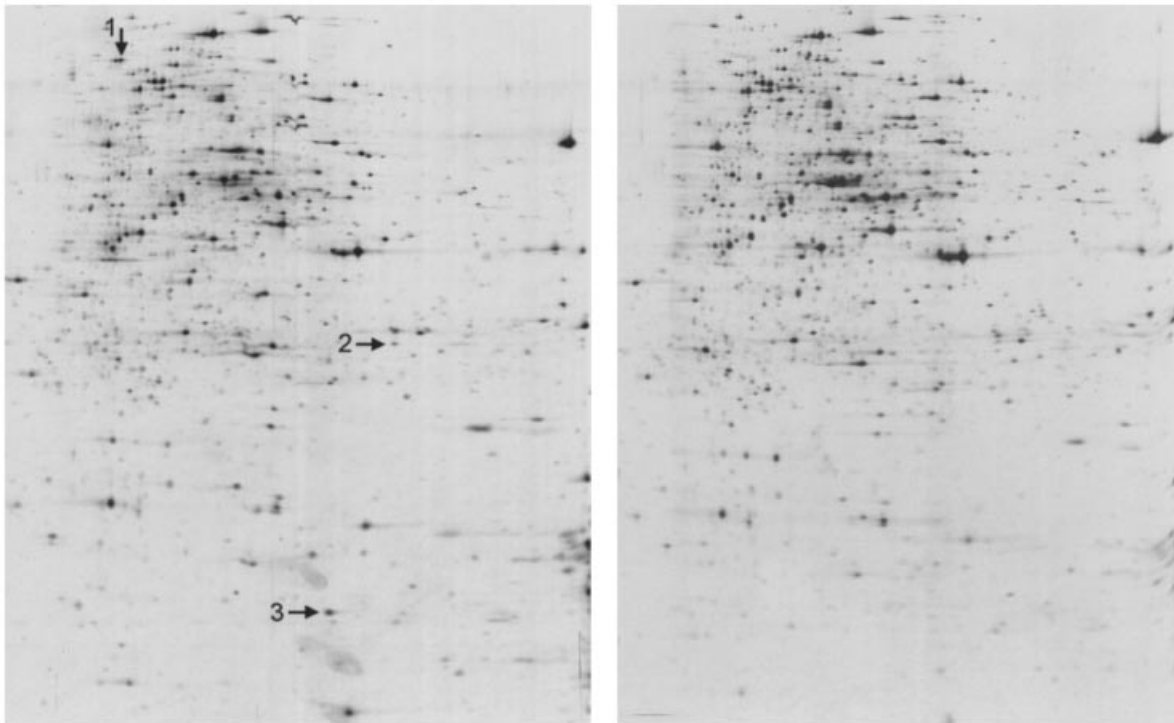


Fig. 5. Two-dimensional gel electrophoresis of whole protein extracts from the *cgt1/CGT1* heterozygote (left) and its parent strain CAI-4 (right). Spots of three proteins upregulated in the *cgt1/CGT1* heterozygote and identified by MS are marked by arrows (1 = Ssa2p, 2 = Rps5p, 3 = Ef1 α p).

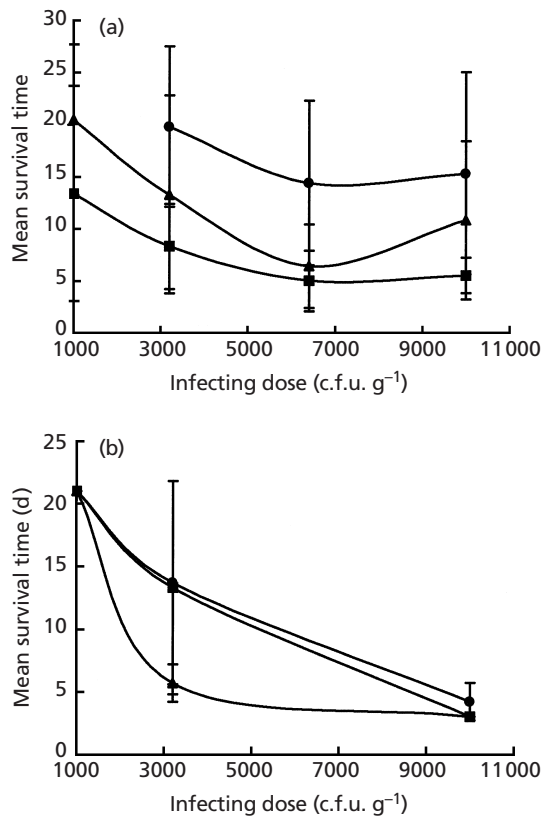


Fig. 6. Mean survival times \pm SD for mice (a) and guinea pigs (b) infected intravenously with *C. albicans* B2630 (●), SC5314 (■) and dCGT1 (▲) (*cgt1/CGT1* heterozygote).

Real-time quantitation of the *CGT1* mRNA transcript

To determine whether disruption of one *CGT1* allele leads to reduced levels of *CGT1* transcript, we performed a real-time mRNA quantitation by PCR. Real-time quantitative PCR measures PCR product accumulation with a fluorogenic probe and allows accurate quantitation of transcripts (Heid *et al.*, 1996; Lie & Petropoulos, 1998). Four independent quantitation experiments were performed using two independently isolated RNA samples from the parental CAI-4 strain and the *cgt1/CGT1* heterozygote. Relative *CGT1* mRNA levels were determined using the comparative C_T method (user bulletin 2, PE Applied Biosystems) in which β -actin mRNA levels are used to normalize the data and differences are expressed in relation to the wild-type controls. Three independent quantitation experiments using identical RNA preparations showed 4.8-, 3.8- and 2.6-fold less *CGT1* transcript in the *cgt1/CGT1* heterozygote than in the parental CAI-4 strain. A fourth quantitation experiment using an independently isolated RNA preparation showed 2-fold less *CGT1* transcript in the *cgt1/CGT1* heterozygote than in the parental CAI-4 strain. These results demonstrated a clear reduction in the expression of the *CGT1* mRNA in the heterozygote, although to a varying extent (2–5-fold).

Colony morphology

A very striking difference in colony morphology and size was observed between the parental CAI-4 strain and the *cgt1/CGT1* heterozygote when grown on minimal medium with glucose as a carbon source. While the parental strain produced perfectly circular, smooth colonies, the *cgt1/CGT1* heterozygote produced much larger, irregularly shaped, colonies with a tentacle-like structure growing from the inside of the colony, resembling the so-called ‘irregular wrinkle’ phenotype (Fig. 3). A second, independently derived *cgt1/CGT1* heterozygote displayed an identical phenotype.

Microscopic examination of samples cut from the lower smooth part of the colony and the upper tentacle-like structure showed ellipsoidal blastoconidial (‘yeast-like’) and true hyphal (‘hyphal-like’) growth, respectively (data not shown).

Growth analysis

The parental CAI-4 and the *cgt1/CGT1* heterozygote strains were grown at 30 °C for 70 h in synthetic selective medium. Optical densities were measured every hour and growth curves were generated (Bioscreen analyser; Labsystems). These demonstrate that the *cgt1/CGT1* heterozygote strain had a somewhat longer lag phase compared to the parental strain (Fig. 4b) but it eventually reaches slightly higher optical densities at stationary phase.

Drug susceptibility and temperature sensitivity

One way to address the possible role of a potential drug target is to observe the phenotypes elicited by genetic or pharmacological interference with that target (Giaever *et al.*, 1999). However, to date no compounds have been reported to act specifically on the mRNA capping enzyme. One might expect pleiotropic effects upon decreases in Cgt1p levels due to the universal role of Cgt1p in the synthesis of all mRNAs. We therefore tested the parental CAI-4 and the *cgt1/CGT1* heterozygote strains for possible differences in susceptibility to hygromycin B (an antibiotic which decreases translational fidelity) and hydroxyurea (a ribonucleotide reductase inhibitor). Surprisingly, the *cgt1/CGT1* heterozygote strain was clearly more resistant to hygromycin B (Fig. 4a). No difference was found in susceptibility to hydroxyurea. Both strains grew equally well on medium containing galactose as the carbon source (data not shown). Growth at 42 °C, however, unexpectedly revealed an increased resistance of the *cgt1/CGT1* heterozygote to elevated temperature (data not shown). Resistance to heat stress was therefore further examined by studying the effects of heat-shock treatments at 55 °C. Inocula of 10⁵ exponentially growing cells ml⁻¹ were spotted onto YPD agar to be challenged at 55 °C for periods of 0 to 180 min and further incubated at 30 °C for a total incubation time of 48 h. The results (Fig. 4c) show that *cgt1/CGT1*

Table 1. Virulence testing of *cgt1/CGT1* heterozygote

ND, not done.

Strain	Dose (c.f.u. g ⁻¹)	No. animals	Survival (d)*		Survival (%)*	Negative kidneys (%)	Organ burden†	
			Mean	SD			Mean	SD
Mouse experiments								
B2630	3200	10	19.8	7.7	30	0	7.3	0.3
	6400	10	14.4	7.9	0	0	7.2	0.4
	10000	10	15.3	9.7	20	0	7.0	0.6
SC5314	1000	10	13.4	10.3	30	0	6.2	1.1
	3200	10	8.3	4.1	0	0	6.3	0.4
	6400	10	5.0	2.9	0	0	6.5	0.6
dCGT1	10000	10	5.5	1.7	0	0	6.1	0.6
	1000	10	20.5	7.2	30	0	6.6	0.5
	3200	10	13.3	9.5	10	0	6.6	0.4
	6400	10	6.4	4.0	0	0	6.3	0.6
	10000	10	10.8	7.6	0	0	6.1	0.6
	Guinea pig experiments							
B2630	1000	6	21.0	0.0	100	100	ND	ND
	3200	6	13.7	8.1	50	0	3.8	1.2
	10000	6	4.2	1.5	0	0	5.2	0.3
SC5314	1000	6	21.0	0.0	100	100	ND	ND
	3200	6	13.3	8.5	50	50	4.7	0.4
	10000	6	3.0	0.0	0	0	5.5	0.3
dCGT1	1000	6	21.0	0.0	100	100	ND	ND
	3200	6	5.7	1.5	0	0	4.6	0.5
	10000	6	3.0	0.0	0	0	5.6	0.5

* Mean survival times \pm SD and percentage of survival in mice (at day 28) and guinea pigs (at day 21) for animals infected intravenously with *C. albicans* B2630, SC5314 and dCGT1 are shown.

† Organ burdens (log₁₀ c.f.u. per kidney for mice, log₁₀ c.f.u. per g kidney weight for guinea pigs) of *C. albicans* in mice and guinea pig kidney after intravenous challenge are given. The organ burden was calculated only for kidneys with detectable *C. albicans* in culture.

heterozygosity leads to significantly increased thermal resistance.

Two-dimensional gel electrophoresis and analysis

The universal role of *CGT1* in the synthesis of all mRNAs prompted us to compare overall protein expression between the parental CAI-4 and the *cgt1/CGT1* heterozygote.

Total protein extracts from parental CAI-4 and *cgt1/CGT1* heterozygote strains were separated by high-resolution two-dimensional gel electrophoresis. In general, the wild-type sample yielded a denser pattern and showed more intense staining of the proteins, which suggests that the protein concentration was higher on average in the wild-type than in the mutant (Fig. 5). Although the pattern of protein spots was strikingly similar, a very large number of differentially expressed proteins could be detected (Fig. 5). Approximately 1600 protein spots were identified for the parental strain while the *cgt1/CGT1* heterozygote showed almost 1500 spots. As expected, most differentially expressed pro-

teins were more abundant in the parental strain; however, a few proteins strikingly more abundant in the mutant were identified. Underexpression in the mutant varied from 1.2- to 77-fold, with most proteins falling in the 2- to 10-fold range. Seven spots representing proteins overexpressed in the *cgt1/CGT1* heterozygote were excised from the gels and subjected to in-gel digestion, extraction of the peptide mixture from the gel, desalting and peptide concentration determination. The total peptide mixture was either used directly for analysis by MALDI- or nanospray-MS, or the mixture was separated by HPLC in order to sequence individual peptide fragments by Edman degradation. These approaches were used in combination to identify the protein and assign some function to it (based on similarities of the sequence to sequences of other proteins with reported function). Three spots were identified unambiguously. Translation elongation factor 1- α (Sundstrom *et al.*, 1990; Dinman & Kinzy, 1997; Kovalchuk *et al.*, 1998) was found to be overexpressed in the mutant (\sim 50-fold) as were a heat-shock protein, Ssa2p (\sim 7.4-fold; Lopez-Ribot *et al.*, 1996) and a ribosomal protein, Rps5p (\sim 5.4-fold; Ignatovich *et al.*, 1995) (Fig. 5).

Experimental disseminated *C. albicans* infections in mice and guinea pigs

Three strains of *C. albicans*, SC5314, B2630 and the *CGT1* single allele knock-out (d*CGT1*), were injected intravenously at different concentrations into guinea pigs or mice (Fig. 6). In mice, SC5314 showed statistically (*t*-test, $p < 0.05$) higher lethality than B2630 (lower mean survival time for a given inoculum size). B2630 and d*CGT1* were equally lethal. In guinea pigs, no difference in lethality was observed between the three strains (Table 1). The organ burden of *C. albicans* (\log_{10} c.f.u. per kidney for mice, \log_{10} c.f.u. per g kidney weight for guinea pigs) was comparable for all administered infective doses in one animal type (Table 1).

DISCUSSION

In our search for novel molecular targets for antifungal drugs we chose to study processes that are critical for growth and/or survival of the fungal pathogen. One such process is the acquisition of the 5' cap during mRNA synthesis, with GTase (*CGT1*) as one of the key enzymes. To get an idea of the impact that an inhibitor of *CGT1* activity might have on a pathogen, we mimicked this effect by generating a *C. albicans* derivative that expressed reduced levels of Cgt1p.

To achieve this we disrupted one allele of the *CGT1* gene in *C. albicans* strain CAI-4 by integrative transformation with a URA-blaster cassette containing *CGT1* flanking recombinogenic ends. Correct and unique integration of the disruption cassette was confirmed by PCR and Southern blot analysis. Attempts to generate a homozygous *cgt1/cgt1* null strain failed, indicating that *CGT1* is probably essential for growth of the organism. This was previously shown to be the case in *S. cerevisiae* (Shibagaki *et al.*, 1992). The disrupted gene was localized to chromosome III and the disruption did not induce any obvious chromosomal aberrations.

As expected, we found a clear reduction in *CGT1* mRNA in the *cgt1/CGT1* heterozygote compared to the parental strain. Although a 50% reduction in *CGT1* transcript might be expected when knocking out one allele of a gene from a diploid organism, we found more pronounced effects, with a reduction of *CGT1* transcript level to up to 80%. This might indicate either that the second intact allele is transcribed less or that both copies work together to achieve efficient transcription. This also shows that *C. albicans* can survive with lower than wild-type levels of *CGT1* mRNA.

It has been demonstrated that *C. albicans* is highly adaptive, suggesting it can rapidly modify its own physiology and phenotype in response to altered conditions. The pleiotropic effects that can be expected upon a decrease in *CGT1* activity might thus explain the aberrant phenotype of the *cgt1/CGT1* heterozygote strain. Although we did not introduce the wild-type *CGT1* allele back into the heterozygote derivative to show restoration of the phenotype, we could demonstrate that independently derived heterozygotes ex-

hibited the same aberrant phenotype. The 'irregular wrinkle' phenotype exhibits both yeast-like and true hyphal growth in one colony. There was no evidence of switching in subcultures of the *cgt1/CGT1* heterozygote, implicating a stable phenotype. Radford *et al.* (1994) reported that wrinkled colonies are composed almost entirely of branched hyphal cells with very few blastospores. Hyphal growth is encouraged by high temperature, a high ratio of CO₂ to O₂, neutral pH and nutrient-poor media (Lee *et al.*, 1975; Buffo *et al.*, 1984; Soll, 1986). Conversely, low temperature, aeration, acidic pH and enriched media promote blastospore growth (Lee *et al.*, 1975; Buffo *et al.*, 1984; Soll, 1986). Each strain of *C. albicans*, in addition to being capable of differentiating in a reversible fashion into a budding or a hyphal growth form (Soll, 1986) is also capable of undergoing high-frequency switching of colony phenotype. The 'irregular wrinkle' phenotype is one of a limited number of general phenotypes described to occur during this switching process (Slutsky *et al.*, 1985; Soll, 1992; Radford *et al.*, 1994). The frequency of switching is influenced by environmental conditions. Switching is influenced by stress conditions such as heat, UV irradiation and ageing (Slutsky *et al.*, 1985; Rikkerink *et al.*, 1988; Morrow *et al.*, 1989; Soll, 1992) and is controlled by the *SIR2* gene (Pérez-Martin *et al.*, 1999). As *C. albicans* has no known sexual cycle, the ability to switch phenotype could be an alternative way to obtain the variability required to survive in certain environments. Cells in the middle of *cgt1/CGT1* heterozygote colonies are presumably exposed to a different micro-environment from cells in the middle of a wild-type colony or have acquired restricted capabilities to cope with certain environmental stress factors. In the middle of the colony, nutrients are depleted earlier; this could be exacerbated by the reduced mRNA capping activity and subsequent decrease in a number of proteins responsible for the transport or metabolism of nutrients.

The pleiotropic effects that can be expected upon a decrease in Cgt1p activity are expected to lead to all sorts of defects and decreased function. Indeed, the *cgt1/CGT1* heterozygote showed a clearly extended lag phase compared to the parental strain, although eventually growth catches up. Apparently this initial backlog in growth does not allow for the immune system of the host (tested here in mice and guinea pigs) to combat the infection more efficiently. Decreased virulence observed in strains constructed using the URA-blaster cassette cannot unambiguously be attributed in all cases to the disrupted gene itself (Lay *et al.*, 1998) because expression of the *URA3* (orotidine-5'-phosphate decarboxylase) gene in the derivatives might affect virulence. For this reason the Ura⁺ parental strain of CAI-4, SC5314, was used for comparison with the *cgt1/CGT1* mutant. However, various strains carrying the URA-blaster cassette have been tested for Ura3p activity and showed reduced activities compared to that of the wild-type. As the activity levels vary from strain to strain, positional effects have been suggested (Lay *et al.*, 1998). Altered expression of *URA3* in gene-disrupted *C. albicans* strains

might thus complicate the interpretation of results obtained in virulence studies. The *cgt1/CGT1* mutant strain and the parental strain (SC5314) were equally virulent for mice and guinea pigs when tested in an intravenous infection model of disseminated candidiasis. Some observations (Sandbaken *et al.*, 1990; Hinnebusch & Liebman, 1991) suggest that under- or overexpression of any component of a process (e.g. translation) could lead to altered sensitivity to an inhibitor of a relevant step in that process. Such an inhibitor is expected to be more potent against a cell limited by a deficiency in this component and less potent against a cell containing an excess of that component, compared to the wild-type cell. Following this line of reasoning, we expected the *cgt1/CGT1* heterozygote to be more susceptible to certain drugs and tested the parental CAI-4 strain and the *cgt1/CGT1* heterozygote for possible differences in susceptibility to hygromycin B and hydroxyurea. Unexpectedly, the *cgt1/CGT1* heterozygote was found not to be more, but significantly less, sensitive to hygromycin B. This antibiotic inhibits peptide chain elongation by yeast polysomes by preventing translation elongation factor 2-dependent translocation (Gonzalez *et al.*, 1978). Sensitivity to this drug has been shown to correlate with reduced translational fidelity (Palmer *et al.*, 1979; Singh *et al.*, 1979). Another unexpected finding was the increased resistance of the *cgt1/CGT1* heterozygote to high-temperature stress.

To elucidate possible mechanisms behind these observations we looked at the effects of *CGT1* depletion on global changes in protein expression by two-dimensional gel electrophoresis and MS coupled with searches of protein and EST databases (Blackstock & Wier, 1999). The amount of protein loaded on each two-dimensional gel was derived from an equal number of *Candida* cells. The wild-type has a denser pattern and more strongly staining protein spots compared to the *cgt1/CGT1* mutant. The pleiotropic effects that one might expect upon Cgt1p depletion are reflected in the large number of differentially expressed proteins. Sequencing of peptides derived from a number of proteins whose level was decreased in the mutant led to the identification of proteins in different pathways (data not shown), confirming pleiotropic effects. Surprisingly, the level of a number of proteins was clearly and significantly increased.

One of these proteins is Rps5p. Little is known about the function of Rps5p in higher eukaryotes but its counterpart in *E. coli*, S7, has been extensively studied. S7 is crucial for the assembly of the 30S ribosomal subunit, it contacts residues in the anticodon stem-loop of ribosome-bound tRNAs and it is the primary mRNA-binding protein in ribosomes. In *S. cerevisiae*, *RPS5* exists as a single copy, intronless gene and is essential for viability. It is known that production of ribosomal proteins and rRNAs increases and decreases according to requirement (Woolford & Warner, 1991). As *CGT1* has such a vast impact on all RNAs, its depletion might lead to altered expression of ribosomal proteins as a means to enhance efficiency of translation.

A second protein was expressed at a significantly higher level (~50-fold) in the disruptant was Ef-1 α p, a translation elongation factor catalysing the critical step of delivering aminoacyl-tRNAs to the ribosome (Sundstrom *et al.*, 1990; Dinman & Kinzy, 1997; Kovalchuk *et al.*, 1998). This overexpression might explain the significantly reduced sensitivity to hygromycin B. Dinman & Kinzy (1997) described a series of *S. cerevisiae* strains containing mutant *TEF1* alleles showing increased sensitivity to hygromycin B and other compounds affecting translational elongation. The mechanism by which such a vast overexpression of a protein can be explained remains speculative. It could be envisaged that upon Cgt1p depletion, the level of nearly all mRNA transcripts is lowered. If Cgt1p is a repressor protein, genes solely regulated by this protein might become deregulated and thus overexpressed. This is working on the assumption that intact mRNA would be available for processing and that proteins involved in the translational machinery are sufficiently present. The latter could be assumed as eukaryotic ribosomal protein mRNAs have been described to have a long half-life (Simonin *et al.*, 1997; Liu & Fallon, 1998).

A third protein also clearly up-regulated in the *cgt1/CGT1* heterozygote is the cell-wall-associated protein Ssa2p (HS72), a member of the heat-shock HSP70 family. HSP70 family members are highly conserved proteins that contribute to the protection and repair of cells after exposure to stress conditions and they are found in different cellular locations (Ellis & van der Vies, 1991; Mager & Ferreira, 1993). Eukaryotic cells subjected to a 42 °C heat-shock respond by rapidly increasing the synthesis of a set of evolutionarily conserved heat-shock proteins. However, in contrast to the heat-shock proteins Ssa1p, Ssa3p and Ssa4p, which accumulate to high levels in *S. cerevisiae* subjected to high temperature (42 °C), Ssa2p was found to be constitutively expressed (Nicolet & Craig, 1991; Schwer *et al.*, 1998). Heat-shock proteins in *C. albicans* have been detected throughout the range of 41 °C to 46 °C (Zeuthen & Howard, 1989). The *C. albicans* heat-shock protein Ssa2p was demonstrated to be present in the cell wall and was found to be equally expressed by *C. albicans* at both 25 °C and 37 °C (Lopez-Ribot *et al.*, 1996). Ssa2p expression studies at elevated temperatures (41 °C to 46 °C) have not been reported. The enhanced expression of Ssa2p in the *CGT1* mutant might well explain its increased resistance to heat stress, although the precise mechanism of action remains unclear.

In summary, lowering of *CGT1* mRNA in *C. albicans* to below 50% of the normal level leads to an aberrant colony morphology, a slight increase in the lag phase of growth and a globally reduced level of protein expression, but the overall functionality of the pathogen and its ability to cause infection remain surprisingly intact. Its increased resistance to hygromycin B and heat stress might even allow it to cope under less favourable growth conditions. Therefore, it might not prove easy to develop a sufficiently effective drug for this molecular target.

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