Virulence associated with fluconazole (FL) resistance in *Candida glabrata* is a global problem and has not been well characterized at the proteome level. In this study, a stable FL-resistant (MIC >256 μg ml⁻¹) strain of *C. glabrata* was generated on agar containing FL. Eight phenotypic mutants were characterized by contour-clamped homogeneous electrophoretic field analysis and two-dimensional PAGE. The secondary derivatives of *C. glabrata* yielded four distinct genotypes with varying chromosomal profiles. Proteomic analysis performed by tandem mass spectrometry for two of the mutants, CGL2 and CSG3, demonstrated a total of 25 differentially regulated proteins of which 13 were upregulated and 12 were downregulated or were similar compared with the parental isolate. The mRNA transcript levels of significantly (P<0.001) upregulated genes were determined by quantitative RT-PCR analysis, and their physiological relevance in terms of phenotypic expression of virulence attributes was verified by conventional laboratory methodologies. The data showed that the FL resistance (MIC >256 μg ml⁻¹) of CSG3 was associated with significantly upregulated (P<0.001) mRNA transcript levels of several genes – ERG11, CDR1, CDR2, MFS, MTI, TPR, VPS and EFT2 – in addition to a number of other potentially virulent genes expressed differentially at a lower level. The results demonstrated accentuated phenotypic expression of bud formation of yeast and metallothionein production associated with FL resistance in *C. glabrata*, which may help the fungus to colonize the host.

**INTRODUCTION**

*Candida glabrata* is a common fungal commensal of humans. During the past two decades, the species has evolved into a potent opportunistic pathogen that causes life-threatening infections in an ever-expanding population of immunosuppressed hosts (Li et al., 2007). *C. glabrata* is also the most common non-*Candida albicans* species recovered from the oral cavities of human immunodeficiency virus-infected patients and is considered to be a leading cause of candidaemia in bloodstream infections (Hachem et al., 2008). *C. glabrata* infections were found to occur at a relatively high level (nearly 46% of 114 non-*C. albicans Candida* species) among cancer patients with organ and haematological malignancies (Safdar et al., 2001). Furthermore, there is an increasing rate of colonization and infection by *C. glabrata* in leukaemic or bone-marrow transplant recipients (Raad et al., 2004).

Of the currently available antifungal drugs, fluconazole (FL) is the most widely used agent for prophylaxis and therapy of candidiasis in patients with AIDS and organ or bone-marrow transplant recipients, as well as in patients in intensive care units (Hollenbach, 2008). The positive effects of FL prophylaxis such as a decrease in *Candida* colonization and infection and a decrease in fungus-related mortality have been reported in compromised patients (Fidel et al., 1999). However, reports of FL failure in similar groups of patients have also been documented (Marr et al., 2002; Saiman et al., 2000). Additionally, on exposure to FL, the levels of resistance can increase dramatically, which selects for drug-resistant subpopulations, leading to clinically significant FL resistance (Pfaller et al., 2006). *C. glabrata* is thought to acquire drug resistance as a result of its haploid nature and its ability to mutate rapidly following exposure to triazole-derived agents (Safdar et al., 2002).

Acquired resistance to the antifungal FL by *C. glabrata* has been ascribed to the upregulation of genes encoding ATP-binding cassette (ABC) transporters encoded by *CDR1* and

**Abbreviations:** 2D, two dimensional; 2DE, 2D gel electrophoresis; CHEF, contour-clamped homogeneous electrophoretic field; FCS, fetal calf serum; FL, fluconazole; IPG, immobilized pH gradient; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MFS, major facilitator superfamily; TFA, trifluoroacetic acid; TTC, 2,3,5-triphenyltetrazolium chloride; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.
CDR2 (also known as PDH1). Proteomic studies of C. glabrata have also confirmed transcriptional regulation of the ABC-type drug efflux transporter Cdrp1p (Rogers et al., 2006).

Despite the considerable body of data showing that C. glabrata is highly resistant to FL both in vitro (Brun et al., 2003) and in vivo (Safdar et al., 2002), its phenotype and associated virulence attributes once it has acquired FL resistance are incompletely understood. Therefore, the aims of the present study were as follows: (i) to determine chromosomal changes in two stable C. glabrata phenotypic mutants with acquired FL resistance in comparison with the parental strain, (ii) to evaluate qualitatively and quantitatively the protein expression profiles of C. glabrata after acquiring FL resistance, and (iii) to evaluate the possible role of some of the differentially expressed proteins in relation to metallothionein and bistaspore formation.

**METHODS**

**Parental C. glabrata strain and growth conditions.** The reference laboratory strain C. glabrata ATCC 2001 was selected for this study. Identification of the organism was confirmed by a germ-tube test and an API 20C Aux identification kit (Analytical Profile Index; bioMérieux). Stock cultures were maintained at −70 °C. After recovery, these were maintained on Sabouraud’s dextrose agar (SDA; Oxoid) and these isolates were maintained at Etest methodology as recommended by the manufacturer (AB Biodisk), and these isolates were maintained at 4 °C during the experimental period. The purity of cultures was ensured by regular random identification.

**FL preparation.** A stock solution of 1 mg FL ml⁻¹ (Pfizer-Roerig) was prepared as a suspension in dimethyl formamide and stored in aliquots at −20 °C.

**Invoking FL resistance in C. glabrata.** The parental C. glabrata ATCC 2001 strain was subjected to sequential FL exposure in our laboratories to invoke drug resistance. In brief, the parental C. glabrata strain was freshly cultured on SDA for 20 h at 37 °C and suspended in PBS (pH 7.2) to yield a cell concentration of 10⁶ c.f.u. ml⁻¹. The yeast cells were then harvested by centrifugation at 2665 g for 5 min at 4 °C and resuspended in 60 ml RPMI broth. FL was added to a final concentration of 1.6 μg ml⁻¹ (2 × MIC) to a volume of 40 ml of this yeast suspension; 20 μl dimethyl formamide was added to the remaining 20 ml of the yeast suspension as the control. Both test and control tubes were incubated aerobically at 37 °C for 18–20 h. The yeast cells were then harvested by centrifugation at 2665 g for 5 min at 4 °C, washed in PBS and resuspended in RPMI broth/agar containing 1.6 μg FL ml⁻¹ or in RPMI broth/agar devoid of FL. This procedure was repeated daily for a period of 46 days, and thereafter with incremental FL concentrations as follows: 10 × MIC (8 μg ml⁻¹) for days 47–54 and 15 × MIC (12 μg ml⁻¹) for days 55–61.

The scheme for exposure of the parental C. glabrata strain to sequentially increasing doses of FL to invoke resistance is shown in Fig. 1. C. glabrata cultured on RPMI agar containing 12 μg FL ml⁻¹ (15 × MIC) produced drug-resistant (MIC >256 μg ml⁻¹) morphologically variable (small to very small) colonies on day 61. Eight of these phenotypic mutants were randomly picked (CGG1–CGG4 and CGG5–CGG8) and tested for stability by culturing the yeast in RPMI agar without FL for 24 h. The resistance to FL, ketoconazole, itraconazole, voriconazole and amphotericin B was measured using Etest methodology as recommended by the manufacturer (AB Biodisk), and these isolates were maintained at −70 °C for further molecular studies.

**Molecular typing.** Molecular typing of the eight C. glabrata phenotypic mutants CGG1–CGG4 and CGG5–CGG8 was performed by using the contour-clamped homogeneous electrophoretic field (CHEF) method described by Espinel-Ingroff et al. (1999) with modifications. The electrophoretic conditions used were as follows: two-state mode with linear ramping factors; run time, 72 h; initial and final switching times, 3 and 17 min, respectively; constant voltage of 3 V cm⁻¹; and switching angle, 120°. Saccharomyces cerevisiae chromosomes (Bio-Rad) were used as molecular size standards. The resulting gel was stained with ethidium bromide and destained in distilled water for 3 h. The DNA bands were visualized and photographed under a gel documentation system. Each specimen was analysed on two separate occasions.

Two of the phenotypic mutants, CGG1 and CGG5, were randomly selected for proteomic and phenotypic analysis. As these mutants were morphologically very small, both isolates were also tested for mitochondrial deficiency by the following three methods: a 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay, growth in glycerol and the tetrazolium overlay technique to detect respiratory deficiency of yeast.

**XTT assay.** An XTT reduction assay was performed as described previously (Jin et al., 2003). Briefly, XTT (Sigma) was dissolved in PBS to a final concentration of 1 mg ml⁻¹. The solution was filter-sterilized using a 0.22 μm pore-size filter and stored at −70 °C until required. Menadione (Sigma) solution (0.4 mM) was also prepared and filtered immediately before each assay. Prior to each assay, XTT solution was thawed and mixed with menadione solution at a ratio of 20:1 (v/v).

The cell pellets were washed four times with 200 μl PBS to remove loosely adherent cells. Next, 158 μl PBS, 40 μl XTT and 2 μl menadione were added to each of the pre-washed wells. After incubation in the dark for 3 h at 37 °C, 100 μl of the solution was decanted to a new well and the colorimetric change in A₆₅₀ of the solution was determined using a microtitre plate reader (Spectra-Max 340 Tunable Microplate Reader; Molecular Devices).

**Characterization of respiratory capacity.** The respiratory status of the two FL-resistant mutant isolates was investigated on yeast extract-peptone-glycerol medium containing 1 % (w/v) yeast extract, 2 % (w/v) peptone and 3 % (w/v) glycerol as the sole carbon source (Hutter & Oliver, 1998). Thus, cells unable to respire were negatively selected on this medium.

**Tetrazolium overlay technique for population studies of respiration deficiency of yeast.** The test medium for this assay consisted of 1.5 % Bacto agar in 0.067 M phosphate buffer (pH 7.0) with 0.1 % 2,3,5-triphenyltetrazolium chloride (TTC). Both TTC and Bacto agar were prepared as stock solutions and autoclaved separately. The test was performed by pouring 20 ml TTC agar over 3–4-day-old SDA plates bearing 50–100 yeast colonies per plate. After 1 h of overlay, the colonies were scored for colour development: white colonies indicated respiratory-deficient colonies, whereas red colonies indicated respiratory-sufficient colonies (Ogur et al., 1957).

**Protein extraction.** Soluble proteins were extracted as described previously by Casanova & Chaffin (1991) with slight modifications. C. glabrata cells grown in RPMI agar with or without FL were collected by scraping the agar plates with a sterile wire loop. The cells were washed twice with PBS, harvested by centrifugation at 4500 g for 10 min at 4 °C and resuspended in 700 μl extraction buffer containing ammonium carbonate (1.89 g l⁻¹) and 1 % (v/v) β-mercaptoethanol (Casanova & Chaffin, 1991). After adding 1 g glass beads (0.5 mm diameter; Roth), the cells were vortexed for 1 min on ice for seven cycles using a Ribolysor (Hybaid-AGS). Cell debris and the glass beads were removed by centrifugation at 16 100 g for 15 min at 4 °C.
The resulting supernatant contained the soluble protein fraction. The protein fraction was stored at \(-70^\circ C\) and the protein content was measured using a Pierce-modified Bradford assay (Bradford, 1976).

**Image analysis.** Two-dimensional (2D) gels were prepared from four independent replicate cultures for each of the test yeast isolates. For 2D-PAGE, gel images were analysed and spots were detected with PDQuest (Advanced) 2D gel analysis software (Bio-Rad Laboratories) as described previously (Rogers *et al.*, 2006). To refine automatic spot matching, mismatched spots were corrected manually and protein spots that were reproducible in at least three of four replicate gels (run from four independent experiments) were selected for subsequent analysis. Protein spots that showed statistically significant differences were selected for further analysis.

**Fig. 1.** Flow diagram depicting the sequential exposure of *C. glabrata* ATCC 2001 to FL to invoke resistance.
at least twofold in their mean spot volume on at least three of four gels (t-test, P<0.5) were excised from the gels.

**Isolation of total fungal RNA.** A loopful of the stock culture of yeast was streaked onto RPMI agar (with/without FL for control C. glabrata) and incubated at 37 °C for 18 h. The resultant cultures were harvested and washed three times in PBS. Total RNA was extracted using an SV Total RNA Isolation system (Promega) according to the manufacturer’s manual. RNA was quantified using a Beckman spectrophotometer: A_{260/280} readings of 1.8–2.0 confirmed the purity. Additionally, gel electrophoresis was performed to verify intact RNA.

**PCR amplification of C. glabrata genes.** Primers used for the amplification of the C. glabrata genes ERG11, MFS, CDR1, CDR2, MTI, MTIIa, TPR, STE20, EF2 and VPS are listed in Table 1.

**cDNA synthesis.** Reverse transcription was performed on 5 μl oligo(dT) primer (Gibco-BRL, Life Technologies) at 70 °C for 10 min as described previously by Samaranayake et al. (2005). The product was chilled on ice and mixed with 4 μl first-strand buffer, 2 μl 0.1 M dithiothreitol and 1 μl 10 mM dNTPs. After incubation at 43 °C for 2 min, 1 μl 200 U μl⁻¹ SuperScript II reverse transcriptase was added, making a final volume of 20 μl, and the mixture was incubated at 43 °C for 90 min for cDNA synthesis (Samaranayake et al., 2005).

**Table 1. Sequences of primers used in this study**

<table>
<thead>
<tr>
<th>GenBank accession no. (gene)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Generation of standard curve</strong></td>
<td>Primer name</td>
<td>Sequence (5’→3’)</td>
<td>Primer name</td>
</tr>
<tr>
<td>FJ167409</td>
<td>ERG11-F</td>
<td>ACGGTACCAAGGCAATCAGAG</td>
<td>ERG11-R</td>
</tr>
<tr>
<td>XM_447641</td>
<td>MFS-F</td>
<td>TACCGACAGCCAACCAAGTA</td>
<td>MFS-R</td>
</tr>
<tr>
<td>AF109723</td>
<td>CDR1-F</td>
<td>CCGTGTGCTTGGTCCAAAT</td>
<td>CDR1-R</td>
</tr>
<tr>
<td>AF251023</td>
<td>CDR2-F</td>
<td>GaggGATACGAGGAGGAGG</td>
<td>CDR2-R</td>
</tr>
<tr>
<td>J05133</td>
<td>MTI-F</td>
<td>TcTACCAAGGGTCGGCTTTC</td>
<td>MTI-R</td>
</tr>
<tr>
<td>J05398</td>
<td>MTIIa-F</td>
<td>CGACTGACTCTCCGTGATTT</td>
<td>MTIIa-R</td>
</tr>
<tr>
<td>XM_448621</td>
<td>TPR-F</td>
<td>GAAGGTGTCCTCCACGATT</td>
<td>TPR-R</td>
</tr>
<tr>
<td>CR380957</td>
<td>STE20-F</td>
<td>AACACTCTGCTGGCACTCC</td>
<td>STE20-R</td>
</tr>
<tr>
<td>AF196836</td>
<td>HEMO-F</td>
<td>AGGCTACCTGGCATGCTGCT</td>
<td>HEMO-R</td>
</tr>
<tr>
<td>AF107287</td>
<td>EF2-F</td>
<td>GACTGCTGACGCGGAACAT</td>
<td>EF2-R</td>
</tr>
<tr>
<td>XM_447320</td>
<td>VPS-F</td>
<td>GAATGAGGAGGATTTGGTCC</td>
<td>VPS-R</td>
</tr>
<tr>
<td>CR380955.2</td>
<td>URA-F</td>
<td>CAAAGTCATGCGCCTTAT</td>
<td>URA-R</td>
</tr>
</tbody>
</table>

| **Quantitative real-time PCR** | Primer name | Sequence (5’→3’) | Primer name | Sequence (5’→3’) |
| FJ167409 | ERG11-R | CGGTTTTTGATTCGTCTTCTT | ERG11-R | CGGTTTTTGATTCGTCTTCTT |
| XM_447641 | MFS-R | TAGGGATTTGACAGGGTTC | MFS-R | TAGGGATTTGACAGGGTTC |
| AF109723 | CDR1-F | AGGGTGACAGGAGGAA | CDR1-R | AGGGTGACAGGAGGAA |
| AF251023 | CDR2-F | GTATGCAAGGAGGTAG | CDR2-R | GTATGCAAGGAGGTAG |
| J05133 | MTI-R | ATCTGAGGCGGCTTTC | MTI-R | ATCTGAGGCGGCTTTC |
| J05398 | MTIIa-R | TGGCACTGGACCTTTCG | MTIIa-R | TGGCACTGGACCTTTCG |
| XM_448621 | TPR-R | AGGGATCGCCTCCACTCTC | TPR-R | AGGGATCGCCTCCACTCTC |
| CR380957 | STE20-R | TGTAGCGCCACCAACCTG | STE20-R | TGTAGCGCCACCAACCTG |
| AF196836 | HEMO-R | TCAGGGCAAGTGTGTTGA | HEMO-R | TCAGGGCAAGTGTGTTGA |
| AF107287 | EF2-R | CAGCACCTGCTGAGTAC | EF2-R | CAGCACCTGCTGAGTAC |
| XM_447320 | VPS-R | CAGACCCACGACATACT | VPS-R | CAGACCCACGACATACT |
| CR380955.2 | URA-R | GCTGACCCTGATGAG | URA-R | GCTGACCCTGATGAG |

**Standard curve preparation.** PCR was performed with the primers listed in Table 1. Initial denaturation was carried out at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 2 min and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were analysed by 1.5% agarose gel electrophoresis and purified using a QIAquick Gel Extraction kit (Qiagen). The purified PCR products were quantified by the measurement of A_{260} and A_{280} and used for the standard curve for real-time PCR (Trama et al., 2005). The gene copy number of PCR products was calculated as described by Luo et al. (2004).

**Quantitative real-time PCR analysis.** The primers for real-time PCR were designed using Primer Express Software (Applied Biosystems) (Table 1). The reaction has been described previously (Samaranayake et al., 2006). The reaction was carried out on ABI Step One Real-time PCR System using 2 × SYBR Green Master Mix (Applied Biosystems). The conditions for real-time PCR were optimized as 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Quantification standards, composed of tenfold serial dilutions of PCR products, were run in conjunction with each set of samples, and a negative master-mix control (water) was included. Each experimental condition was performed in duplicate (Pabinger et al., 2009) and repeated at least once on different days for reproducibility. A melting-curve assay was carried out for each experiment to confirm the specificity of the primers (Lachke et al., 2000).
**2D gel electrophoresis (2DE).** For protein separation in the first dimension by isoelectric focusing (IEF), 120 μg protein extract was loaded onto an immobilized pH gradient (IPG) strip, with a non-linear pH gradient range of 3–10, 3–6 or 4–7 (Bio-Rad Laboratories). The proteins were then lyzed overnight in lysis buffer containing 7 M urea, 2 M thiourea, 2 % CHAPS, 10 mM dithiothreitol and 0.5 % IPG buffer (Bio-Rad Laboratories). IEF was performed in a Protein IEF Cell (Bio-Rad Laboratories) using the following conditions: step 1, 250 V for 15 min; step 2, 4000 V for 120 min; step 3, ramp from 4000 to 70 000 V; hold at 400 V until the second separation (Kusch et al., 2008).

Proteins were separated in the second dimension by PAGE (12.5 % acrylamide) (Kusch et al., 2008) at 200 V in a Protein II X1 cell (Bio-Rad Laboratories). To visualize the larger-molecular-mass proteins, an 8 % acrylamide gel and IPG strip of pH 3–10 with active hydration was also used. The protein spots were stained with silver nitrate (Yan et al., 2000) or SYBR Ruby (Molecular Probe) (Gygi et al., 2000). Gel images were captured using a Chemi-doc system (Bio-Rad Laboratories) or a Typhoon 9410 scanner (GE Healthcare) and quantified and analysed with PDQuest (Advanced) 2-D gel analysis software (Bio-Rad Laboratories), as described previously (Rogers et al., 2006). Experiments were conducted on three separate occasions.

**Protein identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).** For peptide mass fingerprinting using MALDI-TOF MS, freshly prepared reagents and a modification of previously published methods (Law et al., 2009; Wang et al., 2007) were used.

The differentially expressed proteins in SDS-PAGE gels, selected for identification (after DeCyder software analysis of the differential gel electrophoresis images), were subsequently excised from the gels and in-gel trypsin digestion was performed using an experimental protocol described by Wang et al. (2007). The gel plugs were first washed with 50 mM ammonium bicarbonate and then 50 % (v/v) acetonitrile (ACN) in water, followed by 100 % (v/v) ACN for dehydration. After overnight digestion with trypsin (Promega) in 50 mM ammonium bicarbonate (pH 8.0) at room temperature, the peptides were extracted using sequential steps of 0.2 % (v/v) trifluoroacetic acid (TFA) in water, followed by 50 % (v/v) ACN in 0.1 % (v/v) TFA. Some of the peptide extracts were desalted using a Zip Tips C18 kit (Millipore) according to the manufacturer’s protocol. The peptides were eluted with 5 μl 50 % (v/v) ACN and 0.1 % (v/v) TFA.

For MALDI-TOF peptide mass fingerprinting protein identification, MALDI mass spectra were recorded with a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems) that operated in the positive ion reflectron mode at 20 kV accelerating voltage. x-Cyano-4-hydroxy cinnamic acid [10 mg ml⁻¹ in 50 % (v/v) ACN and 0.1 % (v/v) TFA] was used as the matrix. Spectra were calibrated externally using a Calmix standard (Applied Biosystems). One missed cleavage per peptide was allowed, and an initial mass tolerance of 50 p.p.m. was used in all searches. Partial carbamidomethylation for cysteine and oxidation for methionine were assumed.

**Database searching.** The MASCOT program (http://www.matrixscience.com/search_form_select.html) was used to identify C. glabrata proteins and to assign their potential functions against the NCBI/frm fungal database (http://www.ncbi.nlm.nih.gov) and the S. cerevisiae database (http://www.yeastgenome.org). One missed cleavage per peptide was allowed, and an initial mass tolerance of 20 p.p.m. was used in all database searches. Partial carbamidomethylation for cysteine and oxidation for methionine were assumed.

**Phenotypic tests.**

Detection of metallothionein production. Metallothionein production by switch phenotypes of C. glabrata was detected by the method described by Lachke et al. (2002). Cells from a primary colony (cultured on RPMI agar containing 120 μg FL ml⁻¹) and visually homogenous for a single switch phenotype were inoculated into liquid yeast-peptone-glucose medium containing 1 mM CuSO₄ (YPG-CuSO₄) and grown at 25 °C until they reached a concentration of ~5 x 10⁸ c.f.u. ml⁻¹. The cells were then diluted and distributed evenly on agar plates containing YPG-CuSO₄ medium at a density of 50–70 c.f.u. per plate. The plates were incubated at 25 °C for 5–7 days, observed for the diverse switch phenotypes with varying colours and the results were captured using an acolyte colony counter (Synbiosis).

**Induction of germ-tube formation.** The parental C. glabrata and mutant isolates CGL2 and CGS3 were first grown on RPMI agar containing 120 μg FL ml⁻¹ at 37 °C for 18–20 h. A yeast inoculum at a concentration of ~10⁸–10⁹ c.f.u. ml⁻¹ was prepared in glass tubes with medium containing 10 or 15 % fetal calf serum (FCS). For examination of filamentous growth, these cultures were incubated at 37 °C for 2 h. A drop of the yeast suspension was then placed on a clean microscope slide and examined under a microscope (40× magnification) (Hilmioglu et al., 2007). Formation of hyphal elements or changes in cell morphology were observed and recorded using a confocal laser-scanning microscope (Fluoview FV1000; Olympus).

**Assay of adherence to silicone surfaces.** The parental C. glabrata and mutant isolates CGL2 and CGS3 were first grown on RPMI agar containing 120 μg FL ml⁻¹ (15 × MIC) at 37 °C for 18–20 h. A yeast inoculum at a concentration of ~10⁹ cells ml⁻¹ (MacFarland standard no. 4) was prepared in RPMI. A volume of 2 ml of this yeast suspension was added to the wells of a microtitre plate and incubated with a silicone catheter disc (0.5 cm diameter) for 90 min at 37 °C in a shaker at 75 r.p.m. After adhesion, the cell suspensions were aspirated and each well was washed twice with 150 μl PBS to remove loosely adherent cells. The adhesion of yeasts to the catheter discs was quantified by an XTT reduction assay, as described previously (Samaranayake et al., 2009).

**RESULTS**

**Characterization of FL-resistant colonies**

CHEF analysis revealed that three of the large colonies (CGL2, CGS2 and CGS4) and two each of the small colonies (CGS1 and CGS4, and CGS2 and CGS3) had similar chromosomal profiles with both major and minor chromosome-size polymorphisms yielding four distinct genotypes. The chromosomal DNA of CGL2, CGS3 and CGS4 separated into eight bands ranging in size from 610 to 2200 kb, whereas bands in the ranges of 1125–1600 and 825–1020 kb were found for CGS1. Similarly, two groups of banding patterns were observed for the smaller-sized mutant isolates: CGS5 and CGS6 demonstrated bands of 1125–1600 kb, whereas CGS2 and CGS3 had bands of 1020–1600 kb (Fig. 2). Two of the FL-resistant phenotypes that were stable in the absence of FL (CGL2 and CGS3) were selected randomly for proteomic analysis. The characterization of these two phenotypes is shown in Table 2.

**2DE analysis**

To increase the sensitivity and resolution of the 2DE analysis, IEF strips of three pH ranges were used. For pH range 3–10, the parental C. glabrata, CGL2 and CGS3 strains
yielded a mean total of 236 ± 34, 223 ± 51 and 188 ± 55 proteins, respectively (Fig. 3a). For the pH range 4–7, the mean totals were 262 ± 50, 183 ± 71 and 173 ± 76, respectively (Fig. 3b). Separation at a pH range of 4–7 was thus advantageous for the C. glabrata parental strain, because 26 more proteins were visualized than at a pH range of 3–10. However, the pH range of 3–10 allowed better separation for both the CGL2 and CGS3 strains, demonstrating an extra 35 and 15 proteins, respectively, than at pH 4–7. Because >90 % of protein spots were located within the pH range 4–7, IEF strips with a pH range of 3–6 were used to reveal additional proteins located within this acidic region. A total of 446, 209 and 179 protein spots were observed for the parental C. glabrata, CGL2 and CGS3, respectively, in this range (Fig. 3c).

SYBRO Ruby staining patterns were similar to those obtained after silver staining of gels. In an attempt to visualize larger-molecular-mass proteins, an 8 % polyacrylamide gel and an IPG strip with a pH range of 3–10 were used to reveal additional proteins located within this acidic region. A total of 446, 209 and 179 protein spots were observed for the parental C. glabrata, CGL2 and CGS3, respectively, in this range (Fig. 3c).

Table 2. Characterization of the FL-resistant (MIC > 256 µg ml⁻¹) phenotypic mutants CGL2 and CGS3

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>MIC (µg ml⁻¹)*</th>
<th>Colony size (mm)</th>
<th>Tetrazolium result</th>
<th>Metallothionein detection</th>
<th>Protein expression</th>
<th>mRNA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG (parent strain before FL exposure)</td>
<td>0.002</td>
<td>8</td>
<td>1</td>
<td>16</td>
<td>0.38</td>
<td>4</td>
</tr>
<tr>
<td>CGL2 (FL-resistant phenotype)</td>
<td>0.064</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>3</td>
</tr>
<tr>
<td>CGS3 (FL-resistant phenotype)</td>
<td>0.125</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>2</td>
</tr>
</tbody>
</table>

*AmB, Amphotericin B; Ke, ketoconazole; Itra, itraconazole; Vori, voriconazole. Red indicates mitochondrial sufficient, whilst white indicates mitochondrial deficient.
contrast, CGS3 showed upregulation of 32 and downregulation of 29 protein spots. When 8% PAGE included active rehydration and a pH range of 3–10, CGL2 showed a total of 56 upregulated and 49 downregulated spots, whereas CGS3 showed 62 upregulated and 66 downregulated spots. After IEF with a pH range of 4–7 and 12.5% PAGE, a lower number of differentially expressed proteins was found for both mutants: CGL2 showed upregulation of 21 and downregulation of 15 proteins, whilst CGS3 showed upregulation of 13 and downregulation of 14 proteins. After IEF with a pH range of 3–6 and 12.5% PAGE, 34 spots were upregulated and 69 were downregulated for CGL2, whereas 28 were upregulated and 34 were downregulated for CGS3. A summary of the differentially expressed proteins among the three C. glabrata phenotypes (parental C. glabrata, CGL2 and CGS3) is given in Table 3.

Identification of differentially expressed proteins and mRNA

Our findings indicated that the proteome of the stable FL-resistant mutants differed noticeably from that of the FL-susceptible parent. The proteomes of the two mutant strains were compared using 2DE analysis to determine proteins with pI values between pH 3–10 (a), pH 4–7 (b) and pH 3–6 (c) for C. glabrata ATCC 2001 (CG), (CGL2) and (CGS3). Proteins were detected by silver staining. Numbers indicate spots that are significantly changed compared with CG.
Table 3. Description of the FL-induced proteins identified by 2DE and MALDI-TOF peptide mass fingerprinting that were differentially expressed between the two phenotypes CG\textsubscript{L2} and CG\textsubscript{S3}

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein accession no.</th>
<th>Protein description</th>
<th>C. glabrata gene</th>
<th>S. cerevisiae gene</th>
<th>Mol. mass (Da)</th>
<th>pI</th>
<th>Matched peptide</th>
<th>Protein coverage (%)</th>
<th>CG\textsubscript{L2} Fold change</th>
<th>P value</th>
<th>CG\textsubscript{S3} Fold change</th>
<th>P value</th>
<th>Fold change CG\textsubscript{L2}/CG\textsubscript{S3}</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>pH 3–10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1901</td>
<td>gi/50284721</td>
<td>Leu1p (highly similar to 3-isopropylmalate dehydratase)</td>
<td>CAGLOA0 0363 g</td>
<td>LEU1</td>
<td>86469</td>
<td>5.74</td>
<td>10</td>
<td>↓</td>
<td>0.30</td>
<td>0.126</td>
<td>0.99</td>
<td>0.874</td>
<td>0.30</td>
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</tr>
<tr>
<td>5001</td>
<td>gi/50287353</td>
<td>Sod1p (highly similar to Cu–Zn superoxide dismutase)</td>
<td>CAGLOC 04741 g</td>
<td>SOD1</td>
<td>15740</td>
<td>5.81</td>
<td>7</td>
<td>↑</td>
<td>4.22</td>
<td>0.179</td>
<td>1.61</td>
<td>0.021</td>
<td>2.61</td>
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<td>5101</td>
<td>gi/50286955</td>
<td>Etf2p (highly similar to elongation factor 2)</td>
<td>CAGLE 04356 g</td>
<td>EFT2</td>
<td>15738</td>
<td>5.64</td>
<td>6</td>
<td>↑</td>
<td>3.52</td>
<td>0.317</td>
<td>3.21</td>
<td>0.391</td>
<td>1.09</td>
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<td>5201</td>
<td>gi/50290419</td>
<td>Glk1p (similar to hexokinase)</td>
<td>CAGL07099.1 g</td>
<td>GLK1</td>
<td>55434</td>
<td>5.38</td>
<td>16</td>
<td>↓</td>
<td>0.70</td>
<td>0.110</td>
<td>0.22</td>
<td>–</td>
<td>3.18</td>
<td></td>
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<tr>
<td>5301</td>
<td>gi/50291147</td>
<td>DnaJ p (similar to DnaJ domain/heat-shock protein 40)</td>
<td>CAGLOJ 06908 g</td>
<td>DBP1</td>
<td>66901</td>
<td>7.72</td>
<td>10</td>
<td>↓</td>
<td>0.25</td>
<td>0.017</td>
<td>0.27</td>
<td>0.044</td>
<td>0.90</td>
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<td>5401</td>
<td>gi/50285353</td>
<td>Tdh3p (similar to glyceraldehyde 3-phosphate dehydrogenase)</td>
<td>CAGLOG 09383 g</td>
<td>TDH3</td>
<td>35985</td>
<td>6.46</td>
<td>10</td>
<td>↑</td>
<td>1.33</td>
<td>0.500</td>
<td>1.58</td>
<td>0.286</td>
<td>0.84</td>
<td></td>
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<tr>
<td>5501</td>
<td>gi/50291453</td>
<td>Dhlp1p (similar to dihydrolipoamide dehydrogenase)</td>
<td>CAGLOM 0134 g</td>
<td>GND1</td>
<td>53876</td>
<td>6.16</td>
<td>9</td>
<td>↑</td>
<td>1.16</td>
<td>0.500</td>
<td>1.22</td>
<td>0.437</td>
<td>0.95</td>
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<tr>
<td>5601</td>
<td>gi/50292377</td>
<td>Vps5p (similar to tetratricopeptide repeat domain)</td>
<td>CAGLOK 09240 g</td>
<td>YCR060W</td>
<td>16681</td>
<td>9.35</td>
<td>4</td>
<td>→</td>
<td>1.00</td>
<td>1.000</td>
<td>0.72</td>
<td>0.295</td>
<td>1.35</td>
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<tr>
<td>pH 3–10</td>
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<td>1001</td>
<td>gi/50288881</td>
<td>Tdhlp1p (similar to glyceraldehyde 3-phosphate dehydrogenase)</td>
<td>CAGLOG 09383 g</td>
<td>TDH3</td>
<td>35985</td>
<td>6.46</td>
<td>10</td>
<td>↑</td>
<td>1.33</td>
<td>0.500</td>
<td>1.58</td>
<td>0.286</td>
<td>0.84</td>
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<td>1003</td>
<td>gi/50295024</td>
<td>Gndp (6-phosphogluconate dehydrogenase)</td>
<td>CAGLOM 0134 g</td>
<td>GND1</td>
<td>53876</td>
<td>6.16</td>
<td>9</td>
<td>↑</td>
<td>1.16</td>
<td>0.500</td>
<td>1.22</td>
<td>0.437</td>
<td>0.95</td>
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<tr>
<td>1006</td>
<td>gi/50287255</td>
<td>Lpd1p (highly similar to dihydrolipoamide dehydrogenase)</td>
<td>CAGLOF 01947 g</td>
<td>LPD1</td>
<td>53329</td>
<td>6.88</td>
<td>9</td>
<td>↑</td>
<td>1.55</td>
<td>0.437</td>
<td>1.55</td>
<td>0.291</td>
<td>1.00</td>
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<tr>
<td>pH 4–7</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>gi/50289777</td>
<td>Vps5p (vacuolar protein sorting 55)</td>
<td>CAGLOM 01518 g</td>
<td>VPSS5</td>
<td>15570</td>
<td>5.0</td>
<td>1</td>
<td>↑</td>
<td>1.06</td>
<td>0.889</td>
<td>1.00</td>
<td>1.000</td>
<td>1.06</td>
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<tr>
<td>2002-a</td>
<td>gi/50294496</td>
<td>Egd2p (similar to transcription factor homologous to NACs-BTF3)</td>
<td>CAGLOM 07161 g</td>
<td>EGD2</td>
<td>17883</td>
<td>5.14</td>
<td>2</td>
<td>↓</td>
<td>0.45</td>
<td>0.170</td>
<td>0.61</td>
<td>0.016</td>
<td>0.73</td>
<td></td>
</tr>
</tbody>
</table>
isolates resembled each other for a few proteins, although the magnitude of change differed (Fig. 4). Twenty proteins were common to both CG12 and CG33 phenotypic mutants, six of which (spot numbers S002, 1006, 7001, 8101-3, 9602 and S003) were upregulated by >1.5-fold for both mutant isolates (Table 4). In addition, eight of the 20 proteins were upregulated by different degrees (three by >1-fold; two by >1.5-fold and four by >2-fold) when compared with the parental C. glabrata (Fig. 4).

### Identification of differentially expressed genes

For some of these proteins, the corresponding genes were also expressed differentially, indicating a positive correlation between expression of both genes and proteins. Thus, following MS analysis, ten proteins of the two resistant phenotypes were selected for mRNA analysis and compared with the control C. glabrata. ERG11, CDR1, CDR2, MTI, MTIIa, TPR, VPS and EFT2 gene expression was significantly increased (P<0.001) in CG12, whereas in CG33, ERG11, MFS, CDR1, CDR2, MTI, TPR, EFT2 and VPS gene expression was significantly upregulated (P<0.001), whilst MTIIa gene expression was significantly downregulated (P<0.01) (Fig. 5). However, a protein highly similar to GTPases (STE20), important in mediating the regulation of various aspects of morphogenesis, mating and the pathogenicity in eukaryotes was moderately expressed in both mutants.

### Phenotypic analyses

#### Metallothionein production

Phenotypic switching in the mutants CG12 and CG33 was observed in the presence of extracellular copper ions. FL-resistant CG12 produced colonies with brown centres, in contrast to the control C. glabrata strain (Fig. 6). This phenomenon is known to be associated with the production of metallothioneins, which are encoded by two distinct gene subfamilies: MTI and MTII (Mehra et al., 1989). Confirming the latter, the mRNA levels of MTI and MTIIa of the two FL-resistant mutants grown on medium containing FL were significantly altered compared with the control strain, with MTI being significantly upregulated (P<0.001) and MTIIa being significantly downregulated (P<0.01) (Fig. 5).

#### Induction of germ-tube formation

Both phenotypic mutants, when subcultured in growth medium supplemented with 10 or 15 % (v/v) FCS and incubated at 37 °C, induced bud formation of yeast cells. A highly significant increase in yeast cells was seen for cells grown in both concentrations of calf serum compared with in medium devoid of calf serum (P<0.01 for CG33 in 10% FCS, P<0.001 for other comparisons) (Fig. 7a). These yeast cells formed a large number of blastospores that resembled bunches of grapes. The extent of cell clumping or co-aggregation was greater for CG12 than for CG33 compared with the parental strain (Fig. 8).

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein accession</th>
<th>C. glabrata</th>
<th>S. cerevisiae</th>
<th>Mol. mass (Da)</th>
<th>pI</th>
<th>Matched peptide</th>
<th>Protein coverage (%)</th>
<th>Fold change</th>
<th>P-value</th>
<th>Fold change</th>
<th>P-value</th>
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<tr>
<td>2002-2</td>
<td>gi</td>
<td>52098067</td>
<td>CAGL0E</td>
<td>YLL050c</td>
<td>15986</td>
<td>4.9</td>
<td>Adfp (similar to actin depolymerization factor)</td>
<td>20</td>
<td>0.65</td>
<td>0.000</td>
<td>0.59</td>
</tr>
<tr>
<td>3002</td>
<td>gi</td>
<td>53009175</td>
<td>CAGL0I</td>
<td>Q</td>
<td>32001</td>
<td>4.3</td>
<td>But2p (similar to ubiquitin 3-binding protein But2)</td>
<td>33</td>
<td>0.74</td>
<td>0.351</td>
<td>0.80</td>
</tr>
<tr>
<td>3302</td>
<td>gi</td>
<td>52099221</td>
<td>CAGL0I</td>
<td>YGR135W</td>
<td>27378</td>
<td>4.9</td>
<td>pH 3–6</td>
<td>27</td>
<td>0.74</td>
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<td>0.80</td>
</tr>
<tr>
<td>S002</td>
<td>gi</td>
<td>50285177</td>
<td>TFS1</td>
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<td>23012</td>
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<td>Highly similar to phosphatidyl-ethanolamine-binding protein</td>
<td>27</td>
<td>1.91</td>
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<td>gi</td>
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<td>27378</td>
<td>5.13</td>
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<td>1.91</td>
<td>0.463</td>
<td>1.14</td>
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<tr>
<td>S005</td>
<td>gi</td>
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<td>CAGL0B</td>
<td>YAL049c</td>
<td>27378</td>
<td>5.13</td>
<td>Highly similar to phosphatidyl-ethanolamine-binding protein</td>
<td>27</td>
<td>1.91</td>
<td>0.463</td>
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<tr>
<td>S006</td>
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<td>CAGL0B</td>
<td>YAL049c</td>
<td>27378</td>
<td>5.13</td>
<td>Highly similar to phosphatidyl-ethanolamine-binding protein</td>
<td>27</td>
<td>1.91</td>
<td>0.463</td>
<td>1.14</td>
</tr>
</tbody>
</table>

*CG, C. glabrata ATCC 2001.*
Assay of adherence to silicone surfaces. The results of the in vitro adherence assay indicated that the adherence of the FL-resistant isolates CGS2 and CGL2 to silicone surfaces was significantly ($P < 0.05$) higher than that of the parental C. glabrata strain (Fig. 7b). There was no significant difference between adherence of the two FL-resistant isolates.

**DISCUSSION**

**FL-induced antifungal resistance**

The emergence of rapid resistance to azole antifungals in C. glabrata is of great concern in patients on prolonged azole prophylaxis (Bennett et al., 2004; Hajjeh et al., 2004; Pfaller et al., 2004). A number of studies have also documented increased FL resistance in native C. glabrata strains, as well as the acquisition of new resistant strains in cohorts of the latter (Bennett et al., 2004; Marichal et al., 1997). In C. glabrata, multiple mechanisms of azole resistance have been reported. These include increased expression of the ABC transporter genes $CDR1$ and $CDR2$ ($PDH1$), which results in decreased accumulation of intracellular FL (Bennett et al., 2004; Miyazaki et al., 1998; Sanglard et al., 1999), and the increased expression of $ERG11$ (also known as $CYP51$) encoding the FL target lanosterol 14α-demethylase (Henry et al., 2000; Marichal et al., 1997; Redding et al., 2003). There is also evidence that the upregulation of SNQ2 transcripts is correlated with the acquisition of azole antifungals (Sanguinetti et al., 2005).

The lack of a complete database for the C. glabrata genome sequence has hampered the comprehensive characterization of molecular mechanisms that regulate FL-induced changes in C. glabrata (Dujon et al., 2004). Nevertheless, using available databases for C. glabrata, earlier studies have documented the overexpression of a number of genes and their gene products using 2DE and MALDI-TOF MS in azole-resistant planktonic-phase C. glabrata strains (Niimi et al., 1999; Pitarch et al., 2003). In the present study, to ensure that the genomic and proteomic changes
Fluconazole exposure upregulates \textit{C. glabrata} virulence

in the yeast strains were mainly the result of FL exposure, we selected a laboratory \textit{C. glabrata} ATCC strain that had had no previous exposure to antifungal agents.

**Azole resistance and morphologically variable mutants**

Azole-resistant petite mutants of \textit{C. glabrata} have been described previously. The resistance or decreased susceptibility to azoles in \textit{C. glabrata} petite mutants is associated with increased expression of \textit{CDR1} and, to a lesser extent, of \textit{CDR2}, two genes encoding ABC transporters (Brun et al., 2004). The latter petite mutants of \textit{C. glabrata} obtained by FL exposure or induced by ethidium bromide are respiratory deficient and their mitochondrial origin has been confirmed by transmission electron microscopy and restriction endonuclease analysis of the mitochondrial DNA (Brun et al., 2004).

The two morphologically variable, FL-resistant (MIC >256 \text{ \mu g \text{ ml}^{-1}}) \textit{C. glabrata} isolates \text{CGL}_2 and \text{CGS}_3 observed in the current study demonstrated increased resistance to polyenes and cross-resistance to ketoconazole, voriconazole and itraconazole. Most importantly, both isolates were not respiratory deficient or mitochondrial DNA deficient, as confirmed by XTT analysis, growth in glycerol medium and a tetrazolium overlay technique. Moreover, these petite mutants demonstrated increased expression of the gene encoding the azole target \textit{ERG11}, and its corresponding protein in the mutant phenotype \text{CGS}_3.

Azole resistance in \textit{C. glabrata} may arise from increased expression of the gene encoding the azole target (\textit{ERG11}) due to gene duplication (Miyazaki et al., 1998; vanden Bossche et al., 1992). \textit{ERG11} overexpression has also been reported in another laboratory-derived FL-resistant \textit{C. glabrata} isolate (Marichal et al., 1997). Upregulation of \textit{ERG11} mRNA is a widely documented mechanism that confers azole resistance in \textit{C. albicans} (Lopez-Ribot et al., 1998; White, 1997). Our observation of \textit{ERG11} mRNA and protein upregulation in the \text{CGL}_2 mutant confirms previous findings that decreased susceptibility to FL in \textit{C. glabrata} is associated with markedly increased mRNA expression levels of cytochrome P450 lanosterol 14\textalpha-demethylase (\textit{ERG11}) (Geber et al., 1995; Henry et al., 2000; Nii et al., 2002; Vermitsky & Edlind, 2004).

The major drug efflux pumps belong to the major facilitator superfamily (MFS) of membrane transport proteins or to the ABC superfamily. Transcriptional upregulation of multidrug transporter genes has frequently been identified in \textit{C. glabrata} (Vermitsky & Edlind, 2004), other \textit{Candida} species (Katiyar & Edlind, 2001; Perea et al., 2002) and non-\textit{Candida} yeasts (Posteraro et al., 2003). In this investigation, we isolated the total proteins, not just the drug-resistant proteins. The soluble protein fraction was separated by SDS-PAGE and identified by MALDI-TOF peptide fingerprinting. As we specifically did not study plasma-bound proteins, we may not have detected the more commonly documented drug resistance (multidrug transporter) protein profiles of

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**Fig. 5.** Histograms showing the gene expression of significantly upregulated or downregulated genes in \text{CGL}_2 and \text{CGS}_3 relative to \textit{C. glabrata} ATCC 2001 (CG) in RPMI agar in the presence of FL. ***\textit{P}<0.001; **\textit{P}<0.01; NS, not significant.
Cdr1p and Cdr2p. Nevertheless, we were able to detect both Erg11p (cytochrome P450 lanosterol 14α-demethylase) and Mfs1p (similar to MFS), widely known to cause FL drug resistance in C. glabrata. Therefore, we also evaluated the mRNA transcript levels of CDR1 and CDR2, the two representative FL-resistant genes found in C. glabrata, and observed that CGL2 and CGS3 produced adequate amounts of CDR1 and CDR2 mRNA.

Phosphatidylethanolamine-binding protein (Pebpp) was identified in the present study. This protein is found in the outer leaflet of the Candida plasma membrane. Dogra et al. (1999) has presented evidence to show that the availability of Pebpp in the plasma membrane of C. albicans correlates with the expression of the multidrug transporter gene CDR1 of C. albicans. Interestingly, in our study, the amount of Pebpp in the two FL-resistant isolates showed upregulation (by 2.53- and 2.29-fold for CGL2 and CGS3, respectively), whilst also depicting more pronounced mRNA levels for CDR1 and CDR2 for both CGL2 and CGS3 when compared with the parental C. glabrata strain.

Although expression of the gene SNQ2 has been shown to change in FL-resistant C. glabrata (Sanguinetti et al., 2005),

![Fig. 6. C. glabrata ATCC 2001 and FL-resistant (MIC <256 µg ml⁻¹) mutant CGL2 cultured on agar containing CuSO₄. Note the copper-induced brown coloration that results from the reduction in CuSO₄ to CuS (a rapid adaptation in response to toxic concentrations of copper ions) in the CGL2 mutant.](image)

![Fig. 7. (a). Effect of 10 and 15 % FCS on yeast bud formation of FL-resistant C. glabrata isolates. The results represent the mean number of buds formed in four independent assays carried out on separate days. Results are shown as means ± SD. (b) Adherence to silicone catheter discs by FL-resistant C. glabrata isolates. The values represent the mean of A₄₉₀ values for a total of eight readings. Results are shown as means ± SD. *P<0.05; **P<0.01; ***P<0.001.](image)
such differences in gene upregulation or protein expression were not observed in CGL2 and CGS3, suggesting that it is unlikely that the SNQ2 gene is closely involved in azole resistance of these two C. glabrata isolates. In addition to the proteins described above, we observed the upregulation of an abundance of proteins that have not been reported previously on 2DE maps of C. glabrata, probably because previous authors primarily aimed to identify key genes responsible for drug inhibition and drug resistance mechanisms.

More recently, the overexpression of 17 gene products and the downregulation of seven proteins in C. glabrata biofilms formed in the wells of polystyrene culture plates was shown by our group as a result of antifungal exposure of the biofilm phase of C. glabrata (Seneviratne et al., 2010). However, in the present study, where changes in the proteome following FL exposure of the planktonic phase of C. glabrata were evaluated, not one of these (up- or downregulated) proteins was observed, which demonstrates the versatility of this fungus in adapting to different environmental conditions.

**Phenotypic characterization of upregulated virulence attributes**

In addition to the above-described genes that were upregulated, other significantly upregulated or downregulated proteins associated with virulence in FL-resistant C. glabrata phenotypes were detected. These were the MTI and MTIIa genes involved in metallothionein production, Tprp (similar to tetratricopeptide repeat domain), which induces germ-tube formation, and 14-3-3 proteins (at least in part via an interaction with Ste20) required for filamentation.

**Metallothionein production.** Phenotypic switching in the mutants CGL2 and CGS3 was observed in the presence of extracellular copper ions, indicating that exposure to FL is associated with morphological changes in C. glabrata. This phenomenon is known to be associated with the production of metallothionineins, which in C. glabrata are encoded by two distinct gene subfamilies, MTI and MTIIa (Mehra et al., 1989). Confirming the latter, the mRNA levels of MTI and MTIIa of the two FL-resistant mutants grown on medium containing copper were far greater than that of the control parental strain (P<0.05) without copper supplements. It has also been demonstrated that genes other than those involved in copper assimilation, such as HLP1, may be induced in response to rapid environmental challenge (Lachke et al., 2000). Indeed, in C. albicans, high-frequency phenotypic switching involves the combinatorial regulation of phase-specific genes (Soll, 1997), several of which appear to facilitate pathogenesis, including secreted proteinases and drug resistance genes.

**Induction of yeast bud formation and co-aggregation.** Adherence to tissue is a pre-requisite for colonization and infection, and C. glabrata interacts with a variety of host
extracellular matrix molecules that promote adhesion to host surfaces (Hawser & Douglas, 1994). In the host environment, Candida exists predominantly as biofilms rather than as planktonic cells, especially on indwelling medical devices such as catheters and prostheses (Jain et al., 2007).

C. glabrata is known to switch to pseudothalgal growth when starved of nitrogen (Csank & Haynes, 2000), but the fungus does not form hyphae during host invasion (Lachke et al., 2002) and only budding growth has been observed in animal tissues (Fidel et al., 1999). In this study, highly FL-resistant C. glabrata mutants formed a higher number of budding yeasts and also larger aggregates than the parental control when cultured in FCS, indicating that this attribute may be associated with high FL resistance. Concomitantly, we noted upregulation of the germ-tube-promoting gene Vps55p (vacuolar protein sorting 55) and other hypha-associated genes, TPR (tetra-tri-coperpetico repeat domain), two Eft2p proteins and 14-3-3p, in the FL-resistant CG12 and CGS3 isolates.

What we observed in particular was increased co-aggregation of C. glabrata blastospores in both FL-resistant isolates. The in vitro studies indicated that adherence of the FL-resistant isolates CGS3 and CG12 to silicone surfaces was significantly higher than that of the parental C. glabrata strain. There was no significant difference between the adherence of the two FL-resistant isolates. These results may explain to some degree the increased number of catheter-associated C. glabrata infections in bloodstream infections.

Other functions. Phenotypes associated with other differentially expressed and upregulated proteins in the two mutants deserve mention. Sod1p (very similar to Cu–Zn superoxide dismutase), Sod2p (manganese superoxide dismutase), Pebp and Eft2p (spot no. 9602) were commonly upregulated in both CG12 and CGS3. Of particular interest, two proteins, Erg11p and Eft2p (spot no. 5808), which are both involved in drug resistance and protein synthesis (Vicente et al., 2009), were differentially regulated (more than twofold in CGS3 and more than onefold for CG12). Furthermore, the 14-3-3 proteins were moderately enhanced for both mutants, consistent with their multifunctionality and the fact that they are required for filamentation via an interaction with Ste20p (Roberts et al., 1997) and have been reported to play a key role in Candida virulence (Lo et al., 1997).

In addition to the abovementioned proteins, transcriptome analysis showed that C. glabrata exposed to FL differentially expressed a number of other proteins involved in a variety of different physiological pathways currently associated with putative virulence attributes: Lpd1p, RPS2p (associated with virulence), Tdh3p (similar to glyceraldehyde 3-phosphate dehydrogenase), Gndp (6-phosphogluconate dehydrogenase), proteins involved in physiological processes involved in carbohydrate metabolism and in reversible oxidative decarboxylation cell viability, GTPase protein (similar to Ste20) and genes involved in the stress responses such as cell-wall integrity. However, to date, the importance and impact of these proteins on the pathobiology of drug-resistant C. glabrata have not been evaluated, and further work, possibly in animal studies, is required to clarify this mechanism.

Conclusions

Our investigation has shown that FL resistance in C. glabrata, a means of combating the inhibitory effects of the antifungal, leads to significant changes in an array of virulence genes that are upregulated simultaneously to overcome the stress imposed by the azole. Although the virulence genes were upregulated in vitro as a result of FL exposure, it can be hypothesized that this transition of adaptation to environmental cues may prevail at sites of infection. Further characterization of proteomic changes should prove invaluable in aiding our understanding of the biological complexities associated with FL resistance in C. glabrata. Such data may help the development of novel strategies or antifungals to target these proteins effectively and to manage an increasingly important clinical infection.

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