The Candida glabrata putative sterol transporter gene CgAUS1 protects cells against azoles in the presence of serum

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Received 9 March 2007; returned 21 April 2007; revised 31 July 2007; accepted 1 August 2007

Objectives: The uptake of endogenous sterol from serum may allow Candida glabrata to survive azole treatment. This study aims to determine the contribution of a sterol transporter that alters fluconazole sensitivity in the presence of serum.

Methods: Bioinformatic analysis predicted CgAUS1 as the C. glabrata orthologue of the Saccharomyces cerevisiae transporters AUS1 and PDR11. To investigate whether the CgAUS1 gene has sterol transporter activity, we investigated the effects of an AUS1 deletion on the growth of a tetracycline-regulatable ERG9 strain (tet-ERG9aus1), wherein ERG9 expression is turned off giving rise to a sterol requirement. Tetracycline-dependent repression of CgAUS1 in the tet-AUS1 strain was used to determine the fluconazole susceptibility of CgAUS1 in the presence and absence of serum.

Results: The tetracycline-treated tet-ERG9aus1 strain failed to grow in the presence of serum, whereas the parental tet-ERG9AUS1 strain grew by incorporating sterol from exogenously supplied serum. Serum cholesterol protected cells against the antifungal effects of fluconazole and this protection was lost by repressing CgAUS1 gene expression. Furthermore, such protection was also observed during itraconazole treatment, but not observed in cells treated with non-azole antifungals.

Conclusions: CgAUS1 appears to function as a sterol transporter that may contribute to lower azole susceptibility in the presence of serum and to protect C. glabrata against azole toxicity in vivo.

Keywords: antifungal resistance, sterol uptake, tetracycline-regulatable strain

Introduction

Azole antifungals are fungistatic inhibitors of ergosterol biosynthesis and have been widely used for the treatment of both systemic and superficial fungal infections. Prolonged use can select for azole-resistant clinical isolates. It has become a concern that azole-resistant isolates are recovered from immunocompromised and immunosuppressed patients with increased frequency. Therefore, the mechanism of azole resistance in Candida albicans has been well studied; resistance may be due to overexpression of drug efflux pumps, overexpression or alteration in theazole target Erg11p or mutation of a gene such as ERG3 that participates later in the ergosterol biosynthesis pathway.1–5

The pathogenic fungus Candida glabrata is the second or third most common cause of candidosis. Infections by this organism have been linked to the death of immunocompromised and immunosuppressed patients.6–7 Unlike C. albicans, C. glabrata grows only as the yeast form in the host and lacks certain...
virulence attributes: secreted hydrolases are minimal and adhesion is relatively weak. A potential explanation of why *C. glabrata* infections are now prevalent is its intrinsically low susceptibility to azoles. In a four and one-half year global surveillance study, ~12% of 8013 *C. glabrata* clinical isolates were found to be resistant to fluconazole, whereas only 0.95% of 52,987 *C. albicans* isolates and 3.4% of 4,852 *Candida tropicalis* isolates were found to be resistant.

Saccharomyces cerevisiae cells, which are closely related to *C. glabrata* cells, do not take up exogenous steroids, a phenomenon called ‘aerobic sterol exclusion’. Under anaerobic conditions or in mutants that lack haem, this yeast requires sterols and unsaturated fatty acids, as the synthesis of these lipids requires molecular oxygen. Recent studies indicated that even under aerobic conditions, growth of *C. glabrata* cells depleted of cellular ergosterol was rescued by adding human or mouse serum to the medium. In addition, it has been reported that mutant clinical isolates unable to synthesize ergosterol could take up cholesterol from the growth medium. Thus, sterol uptake by *C. glabrata* cells could contribute to their resistance to azoles. In order to investigate this hypothesis, we searched for a predicted sterol transporter gene in *C. glabrata* and studied its contribution to azole resistance. Herein, we report that expression of a *C. glabrata* sterol transporter gene *CgAUS1* could overcome growth inhibition by fluconazole in the presence of serum, suggesting a novel mechanism to reduce the susceptibility to azoles.

Materials and methods

**Strains and media**

*Escherichia coli* DH5α (F-, Φ80, lacZΔM15, Δ(lacZY-argF) U169, hsdR17(λ’-m5’), recA1, endA1, deoR, thi-1, supE44, gyrA96, relA1 λ-) was used in plasmid propagation. Bacterial strains were grown in Luria-Bertani medium (LB) with ampicillin. The *U169*, tet-*AUS1* same as *ACG4* but additional adenine and 60 mg/L additional histidine. Solid medium was supplemented with 2% agar (Nacalai Tesque, Kyoto, Japan). Doxycycline and bovine serum were purchased from Sigma-Aldrich Chemical Co. (St Louis, USA). Bile and cholesterol were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

**Sequence analysis**

Nucleotide/amino acid sequence analyses and homology searches were performed using the BLAST search, the CLUSTAL W (1.83) multiple/pairwise sequence alignment program and the T-Coffee (3.27) multiple/pairwise sequence alignment program. The synteny and transcriptional factors were analysed using our scripts implemented in Ruby (http://www.ruby-lang.org/) and BioRuby (http://biourby.org/). The synteny around a targeted gene was analysed by comparing chromosomal genes near the target gene with adjacent genes close to homologues corresponding to the target gene.

**Northern analysis for CgAUS1 transcript**

Cells grown on YPD medium at 30°C overnight were inoculated at ~1 × 10^8 cells/mL and cultured for 4 h in CSM (pH 5.8) medium with and without 10% bovine serum in the absence or presence of 20 mg/L doxycycline or 5 mg/L fluconazole. Total RNA samples were prepared as described previously. Total RNA was separated with a 0.9% formamide agarose gel and transferred to Hybond-N+ nylon membrane (GE Healthcare UK Ltd, Buckinghamshire, UK). Using ACG4 chromosomal DNA as template, DNA probes for detection of *CgAUS1*-5′-GCTTATACGGAGCGAACCCTGAG-3′ and *CgAUS1*-3′-5′-ACCTGCAAGGACAATCAATTC-3′ were prepared as described previously.

**Effect of AUS1 on sterol uptake in Candida glabrata**

Strain tet-ERG9AUS1, in which the endogenous *ERG9* promoter in AGC22 was replaced with the tetracycline-regulatable promoter 97t, was generated by using homologous recombination to sandwich the *CgURA3-97t* DNA fragment between the 5′ non-coding region of the *ERG9* gene (nt −503 to −133) and the 5′-portion of the *ERG9* ORF (nt −6 to 314). The integration of the expected locus in transformants was confirmed by PCR using primers E9CHA (5′-GCTTATACGGAGCGAACCCTGAG-3′) and E9CHB (5′-ACCTGCAAGGACAATCAATTC-3′) (data not shown).

The *Cgaus1* deletion strain, tet-ERG9aus1, was obtained by transforming tet-ERG9AUS1 with a fragment in which the *CgHIS3* gene was flanked by the 5′ non-coding (nt −479 to −46) and the 3′ non-coding regions (nt +4194 to +4594) of the *CgAUS1* gene. The integration of the *HIS3* gene at the expected locus was confirmed in transformants by PCR using primers AUSICH and AUSICHB (5′-GCTTATACGGAGCGAACCCTGAG-3′) (data not shown).

The *Cgaus1* conditional strain, tet-AUS1, was obtained by transforming ACG4 with a fragment in which the *HIS3-97t* gene was flanked by the 5′ non-coding (nt −479 to −46) and the 3′ coding sequence regions (nt −6 to +464) of the *CgAUS1* gene. The integration of the *HIS3-97t* gene at the expected locus was confirmed in transformants by PCR using the primers AUSICH and AUSICHB (5′-GCTTATACGGAGCGAACCCTGAG-3′) (data not shown).

**Table 1.** Strains used in this study

<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACG4</td>
<td>his3 trp1::PscADH1::tetR::ScGAL4AD::TRP1</td>
<td>Nakayama et al. 21</td>
</tr>
<tr>
<td>ACG22</td>
<td>his3 ura3 trp1::PscADH1::tetR::ScGAL4AD::TRP1</td>
<td>Nakayama et al. 21</td>
</tr>
<tr>
<td>tet-ERG9AUS1</td>
<td>same as ACG22 but erg9::URA3-97t-ERG9</td>
<td>this study</td>
</tr>
<tr>
<td>tet-ERG9aus1</td>
<td>same as tet-ERG9AUS1 but CgAUS1::CgHIS3 Cgaus1::CgHIS3</td>
<td>this study</td>
</tr>
<tr>
<td>tet-AUS1</td>
<td>same as ACG4 but CgAUS1::CgHIS3-97t-AUS1</td>
<td>this study</td>
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</table>
(GE Healthcare UK Ltd). After hybridizing with either CgAUS1 or ACT1 probes for 18 h, signals were detected according to manufacturer’s instructions with CDP-Star Detection Reagent (GE Healthcare UK Ltd).

**Analysis of sterol content**

The sterol content of *C. glabrata* strains was determined according to previously described procedures. Cells were grown on CSM with or without 10% bovine serum (~53 mg/L total cholesterol after saponification). Sterols were analysed by gas chromatography. An HP5890 series II equipped with the Hewlett-Packard CHEMSTATION software package (CA, USA) was used to analyse sterol content. The capillary column (DB-5) was 15 m × 0.25 mm × 0.25 µm film thickness (J&W Scientific, CA, USA) and was programmed from 195 to 280°C (1 min at 195°C, then an increase to 240°C at 20°C/min, followed by 2°C/min until a final temperature of 280°C was reached). The linear velocity was 30 cm/s using nitrogen as the carrier gas, and all injections were run in the splitless mode. Sterol analysis showed that cholesterol was the only sterol in bovine serum used in this study.

**Cell viability assay**

Approximately 5 × 10^5 cells were used to inoculate CSM medium (2 mL) and were then cultured with or without 10% bovine serum in the presence or absence of doxycycline (100 mg/L). The culture 

**Antifungal susceptibility**

Approximately 2 × 10^5 cells were used to inoculate CSM medium (200 µL) and were then cultured with serially diluted fluconazole (from 0 to 400 mg/L; Pfizer), itraconazole (from 0 to 40 mg/L; Astellas Pharma Inc., Osaka, Japan) or amphotericin B (from 0 to 400 mg/L; Pfizer), itraconazole (from 0 to 40 mg/L; Sigma-Aldrich Chemical Co.), terbinafine (from 0 to 40 mg/L; Sigma-Aldrich Chemical Co.), micafungin (from 0 to 4 mg/L; Astellas Pharma Inc., Osaka, Japan) or amphotericin B (from 0 to 4 mg/L; Sigma-Aldrich Chemical Co.) in the presence or absence of doxycycline (20 mg/L) and 10% bovine serum. After incubation at 30°C for 24 or 48 h, the OD at 595 nm of the cells was measured.

**Results**

**Identification of a sterol transporter in *C. glabrata***

The sterol transporter genes, *AUS1* and *PDR11*, which are expressed anaerobically in *S. cerevisiae* were selected as queries for homology searches to identify sterol transporters in *C. glabrata*. A BLAST search against the *C. glabrata* genome [http://cbi.labri.fr/Genolevures/eltl/CAGL.html](http://cbi.labri.fr/Genolevures/eltl/CAGL.html) predicted CAGL0F01419 g to be a functional homologue of both *S. cerevisiae AUS1* and *S. cerevisiae PDR11*. As a result of this syntenic analysis, some homologues adjacent to CgAUS1 on *C. glabrata* chromosome F were found to be located adjacent to *S. cerevisiae AUS1* on chromosome XV in a conserved order. In contrast, no homologues adjacent to CgAUS1 were located adjacent to *S. cerevisiae PDR11* on chromosome IX (Figure 1). In addition, the predicted amino acid identity of CAGL0F01419 g with *S. cerevisiae AUS1* (71.3%) was greater than that with *PDR11* (64.4%) (Table 2). The predicted gene was therefore named CgAUS1 (*C. glabrata* ABC transporter involved in the uptake of sterol, accession no. AB 297452). The

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**Figure 1.** Synteny analysis of sterol transporter genes. The upper panel shows synteny between CAGL0F01419g (CgAUS1) and YOR011W (AUS1) and the lower panel shows synteny between CAGL0F01419g and YIL013C (PDR11). CAGL0F01419g (CgAUS1) extends from 127 to 157 kb on chromosome F. *S. cerevisiae* YOR011W (AUS1) and YIL013C (PDR11) genes extend from 334 to 364 kb on chromosome XV and from 313 to 343 kb on chromosome IX, respectively. The lines in the upper panel shows the relationship between homologues. Solid arrowheads pointing towards the coding sequences indicate the positions of introns.
predicted ORF is 4197 bp with no intron and encodes a putative protein, CgAus1p, of 1398 amino acid residues (158 kDa). The sequence of CgAus1p suggests a typical fungal ABC transporter, arranged in two halves, each half comprising an N-hydrophilic domain (containing the ATP-binding cassette) and a C-hydrophobic domain with six transmembrane domains.27

Effect of CgAUS1 gene expression on the growth of a sterol auxotrophic mutant

As described previously, 18 repression of ERG9 expression using the tetracycline-regulated 97t21 promoter in the tet-ERG9AUS1 strain blocked squalene biosynthesis and growth.18 Inclusion of serum in the medium rescued the growth defect in a tet-off ERG9 strain (97SQS).18 To determine whether CgAus1 encodes a putative sterol transporter, the CgAus1p deletant strain, tet-ERG9aus1, was generated from tet-ERG9AUS1. When the ERG9 gene products of both tet-ERG9AUS1 and tet-ERG9aus1 were depleted by doxycycline, cells showed a severe growth defect in the absence of bovine serum because of the inability to synthesize squalene. In contrast to near-normal growth of the tet-ERG9AUS1 strain grown in the presence of doxycycline and bovine serum, tet-ERG9aus1 cells were not rescued under the same conditions, apart from a barely significant increase in cfu (Figure 2). These results are consistent with the sterol uptake in the CgAus1p strain, compensating effectively for endogenous sterol depletion.

Manipulation of sterol content in the tet-AUS1 strain

To confirm this finding, we generated the conditional mutant tet-AUS1 from ACG4,21 in which the CgAUS1 gene was placed under the control of a tetracycline-regulatable promoter.21 When tet-AUS1 cells were cultured with doxycycline, growth was unaffected both in the presence or in the absence of bovine serum, indicating that expression of the CgAUS1 gene did not affect growth under these conditions (data not shown). The CgAUS1 gene in tet-AUS1 was constitutively expressed in the absence of doxycycline whether the cells were cultured with or without bovine serum, but completely repressed in the presence of doxycycline (Figure 3). The effects of CgAUS1 expression on sterol uptake were determined by measuring the sterol composition of tet-AUS1 cells. When tet-AUS1 cells were cultured with bovine serum and without doxycycline, more than one half of the cellular sterol was cholesterol (Table 3). Cholesterol accumulation in tet-AUS1 cells grown with bovine serum decreased with increasing doxycycline concentrations. Furthermore, no cholesterol was detected in tet-AUS1 cells grown in medium not containing serum either with or without doxycycline (Table 3), supporting the hypothesis that the CgAus1p facilitates cholesterol uptake from serum.

Effects of CgAUS1 gene knockdown on fluconazole susceptibility

Because cholesterol uptake from serum alleviated the growth defect of ERG11-depleted cells,28 we hypothesized that this uptake would reduce the susceptibility of C. glabrata to fluconazole, even though Erg11p was inhibited by the drug. Measurement of theazole susceptibility of the tet-AUS1 strain and its parent ACG4 provided a test for this hypothesis.

Figure 2. Effect of CgAUS1 gene expression on the growth of the sterol auxotroph conditional mutant (tet-ERG9). Cells (5×10⁵) were inoculated into CSM media (pH 5.8) (2 mL) and cultured with or without 10% bovine serum in the absence and presence of doxycycline (DOX) (100 mg/L). After cultivation at 30°C for 14 h, the cfu was determined by obtaining colony counts on YPD plates. Mean values and error bars are indicated for three independent experiments. Open bars, without serum; grey bars, with 10% bovine serum.

Figure 3. CgAUS1 mRNA expression in tet-AUS1 and ACG4 treated in the absence (a) or presence of fluconazole (5 mg/L) (b). Cells were cultured for 4 h in CSM medium (pH 5.8) with the indicated supplements. Total RNA samples were separated on 0.9% formamide agar gels and hybridized with CgAUS1 or ACT1 probes. ACT1 was used as an internal control. BS, bovine serum; DOX, doxycycline.
functions as a sterol transporter that ameliorates the growth susceptibility to fluconazole at ACG4 and tet-AUS1 cells without doxycycline treatment. When doxycycline also repressed (Figure 4a and b). Northern analysis revealed that 20 mg/L doxycycline in the presence of fluconazole did not alter by adding doxycycline in the presence of fluconazole (Figure 4a and b). Northern analysis revealed that 20 mg/L doxycycline also repressed CgAUS1 expression in tet-AUS1 cells and that the CgAUS1 gene was constitutively expressed in the presence of fluconazole. In contrast, CgAUS1 expression was up-regulated by adding bovine serum in strain ACG4 that has the native promoter of CgAUS1 (Figure 3).

We further tested substitutes of bovine serum to rescue growth inhibition of tet-AUS1 by fluconazole. Although addition of free cholesterol (54 mg/L) did not alter the growth inhibition profile of fluconazole, the addition of 0.8% bile reduced fluconazole susceptibility of both ACG4 and tet-AUS1 to a greater degree than bovine serum (Figure 4c–f).

Table 3. Sterol accumulation profiles for tet-AUS1 cells cultured with or without doxycycline (DOX) in the absence or presence of bovine serum

| Bovine serum (10%) | DOX (mg/L) | Squalene | Cholesterol | Zymosterol | Ergosterol | Other sterols
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<tr>
<td>−</td>
<td>0</td>
<td>1.2 ± 1.1</td>
<td>0.0 ± 0.0</td>
<td>16.4 ± 3.7</td>
<td>53.7 ± 6.8</td>
<td>27.2 ± 5.9</td>
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<td></td>
<td>100</td>
<td>2.5 ± 1.8</td>
<td>0.0 ± 0.0</td>
<td>15.5 ± 3.4</td>
<td>58.3 ± 5.8</td>
<td>24.0 ± 2.7</td>
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<td>200</td>
<td>2.8 ± 1.8</td>
<td>0.2 ± 0.3</td>
<td>14.5 ± 2.6</td>
<td>62.2 ± 3.8</td>
<td>20.9 ± 3.1</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>1.8 ± 1.1</td>
<td>52.3 ± 17.0</td>
<td>1.8 ± 1.6</td>
<td>15.5 ± 11.2</td>
<td>26.1 ± 8.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.9 ± 1.0</td>
<td>27.6 ± 7.7</td>
<td>7.3 ± 2.8</td>
<td>35.1 ± 8.2</td>
<td>24.6 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>1.1 ± 1.0</td>
<td>19.0 ± 12.8</td>
<td>6.1 ± 2.4</td>
<td>40.1 ± 8.9</td>
<td>28.3 ± 7.8</td>
</tr>
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</table>

*Included in this group are fecosterol, ergosta-5,7-diene-ol, 4-methylfecosterol, lanosterol and 4,4-dimethylzymosterol.

Effects of CgAUS1 gene knockdown on antifungal susceptibility

To confirm whether CgAUS1 expression could affect susceptibility to other azoles and antifungals, we tested whether depleting the CgAUS1 gene product would alter susceptibility to the other antifungals itraconazole (Figure 5a and b), amphotericin B (Figure 5c and d), terbinafine (Figure 5e and f) and micafungin (Figure 5g and h). The susceptibility of tet-AUS1 cells for all drugs was decreased when the cells were cultivated with bovine serum in the absence of doxycycline (Figure 5). The cells of tet-AUS1 cultured with doxycycline and bovine serum were only more susceptible to itraconazole. Thus, tet-AUS1 did not show increased susceptibility to non-azole compounds even when CgAUS1 expression was repressed (Figure 5), suggesting that serum uptake only alleviated azole sensitivity.

Discussion

Molecular genetic manipulation of the expression of the ERG9 and CgAUS1 genes demonstrates that the CgAUS1 gene product functions as a sterol transporter that ameliorates the growth inhibition caused by the loss of ERG9 expression or fluconazole-mediated inhibition of Erg11p. Three different experiments support these conclusions. First, the growth of doxycycline-treated tet-ERG9AUS1 cells was rescued in serum-supplemented medium when ERG9 was repressed, whereas serum did not rescue the severe growth defect of a doxycycline-treated tet-ERG9aus1 strain. Secondly, the tet-AUS1 did not accumulate cholesterol from serum-supplemented media under CgAUS1-repressed conditions. Thirdly, expression of CgAUS1 ameliorated the growth inhibitory effects ofazole in the presence of serum.

Although the drug susceptibility tests demonstrated that CgAUS1 expression would affect azole susceptibility, two unexpected results were observed. One was residual growth of tet-AUS1 cultured with serum in the presence of doxycycline as growth inhibition did not fall to <20% (Figure 4). Another was trailing of the growth inhibition curve of tet-AUS1 cultured with serum in the presence of doxycycline at higher concentrations of itraconazole (Figure 5a and b). In this case, growth inhibition gradually diminished at concentrations >12.5 mg/L. Although further studies are required to explain such phenomena, some possibilities are as follows. Overexpression of efflux pumps such as CDR1 might be raised as an explanation for trailing, as previous studies have reported that higher concentration of azoles induces overexpression of efflux pumps such as CDR1. As for residual growth, two possibilities can be suggested: (i) constituents from serum that control a key function affecting azole susceptibility might be transported by some other transporters; and (ii) a small amount of sterol might support residual growth. As shown in Table 3, cholesterol was detected in tet-AUS1 cells cultured in the presence of doxycycline. These results suggest that sterol transport might not be completely inhibited as any gene knockdown system may not completely abolish target gene expression. An alternative reason for cholesterol detection might be that other transporters may recognize cholesterol as a lower affinity substrate [transporters such as SNQ2 (CAGL0f04862g) homologue are highly similar to CgAUS1].

The susceptibility testing of antifungals in serum-containing media demonstrated that CgAus1p did not affect susceptibility to non-azole antifungals such as amphotericin B, terbinafine and micafungin (Figure 5c–h). Our previous studies demonstrated that C. glabrata cells still continue to synthesize ergosterol even while taking up exogenous sterol. The effective concentration of terbinafine may have decreased because of binding to...
constituents of serum both in ACG4 and in tet-AUS1 cells (Figure 5e and f). Furthermore, terbinafine susceptibility was altered by adding 20 mg/L doxycycline both in ACG4 and in tet-AUS1 in the presence of bovine serum, suggesting that doxycycline may affect drug permeability. In fact, 100 mg/L doxycycline also lowered fluconazole susceptibility of ACG4 in the presence of serum, and expression in ACG4 was repressed under these conditions (data not shown); however, 20 mg/L doxycycline did not affect ACG4 fluconazole susceptibility or CgAUS1 expression (Figure 3a and b).

Xiong et al. recently demonstrated that Aspergillus fumigatus also imports exogenous cholesterol from serum and that this property counteracted the toxicity of sterol biosynthetic inhibitors. Interestingly, it is noted that both C. glabrata and A. fumigatus can take up exogenous sterols under aerobic conditions, whereas S. cerevisiae, which is phylogenetically closely related to C. glabrata, takes up sterols under anaerobic conditions or in mutants lacking haem. As shown in Figure 3, the CgAUS1 gene was up-regulated by serum in strain ACG4 that has the native CgAUS1 promoter, suggesting that C. glabrata may have an enhanced growth response to constituents in serum. However, the aerobic uptake of cholesterol appears to require a serum/bile factor, as free cholesterol is not taken up by tet-AUS1 strain constitutively expressing CgAus1p (Figures 3 and 4). For example, lipoproteins may need to associate with the transporter for effective cholesterol transport, or alternatively, other uncharacterized factors

**Figure 4.** Susceptibility of ACG4 (a, c and e) and tet-AUS1 (b, d and f) to fluconazole. Cells (2 × 10^5 cells/well) were inoculated in CSM medium (pH 5.8) with and without 10% bovine serum (a and b), cholesterol (c and d) or bile (e and f) in the presence or absence of doxycycline. After cultivation at 30°C for 24 h, the OD at 595 nm was measured, and the growth ratio of fluconazole-treated cells to the cells cultured without fluconazole was calculated. Similar results were obtained when cells were cultured at 30°C for 48 h. Filled circles, without supplement; open circles, with supplements; filled triangles, with 20 mg/L doxycycline; open triangles, with 20 mg/L doxycycline and supplement.
may play a role similar to the cell wall protein Danlp in *S. cerevisiae* that is specifically produced under anaerobic conditions and required for sterol uptake along with Auslp and Pdr11lp. Promoter analysis for *AUS1*, *PDR11* and *CgAUS1* also supports different gene regulation of sterol transporter genes in *C. glabrata* and *S. cerevisiae*. The Ecm22p promoter-binding motif, which is

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**Figure 5.** Susceptibility of ACG4 (a, c, e and g) and tet-AUS1 (b, d, f and h) to various antifungals. Cells (2 x 10^5 cells/well) were inoculated in CSM medium (pH 5.8) with and without bovine serum in the presence or absence of doxycycline. After cultivation at 30°C for 24 h, the OD at 595 nm was measured, and the growth ratio of antifungal-treated cells to cells cultured without antifungal was calculated. Similar results were obtained when cells were cultured at 30°C for 48 h. Filled circles, without supplement; open circles, with 10% bovine serum; filled triangles, with 20 mg/L doxycycline; open triangles, with 10% bovine serum and 20 mg/L doxycycline.
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the major regulator of the S. cerevisiae AUS1 gene, \(^{33}\) is not present in the promoter region of CgAUS1, which suggests that CgAUS1 is not an anaerobically expressed gene in C. glabrata as in S. cerevisiae.

Our results, together with studies of some other pathogenic fungi, suggest that sterol transporters can counteract ergosterol deficiency and azole toxicity. It may also be possible that mutations in regulatory factors of various pathogenic fungi, whose sterol transporter(s) is repressed in vivo, might allow the development of azole resistance. Thus, the study of sterol transporters in pathogenic fungi, including their physiological functions, regulation and mutation, can provide insights into novel mechanisms of azole resistance and could promote the development of improved antifungals.

Acknowledgements

We thank Drs Masayuki Sudoh and Kunio Kitada for providing strains ACG4 and ACG22, Astellas Pharma Inc., for providing micafungin, Mr Tetsuo Iwata, Takuya Nakanishi and Makoto Okano for experimental help and Professor Richard Cannon and Maureen Bard for critical reading of the manuscript.

Funding

This work was supported by grant-in-aid from SFIF (Systemic Fungus Infection Forum). K. T. is supported by grants KH33310 and SH24405 from the Japan Health Sciences Foundation. M. N. is supported by the Health Science Research Grants for Research on Emerging and Re-emerging Infectious Diseases of the Ministry of Health, Labour and Welfare of Japan. M. B. acknowledges the support of NIH grant GM62104 and Burroughs Wellcome Fund grant.

Transparency declarations

We declare no conflicting financial interests.

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