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Candida albicans UPC2 is transcriptionally induced in response to antifungal drugs and anaerobicity through Upc2p dependent and independent mechanisms.

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Abstract

Many genes in the *Candida albicans* ergosterol biosynthetic pathway are controlled by the transcriptional activator Upc2p, which is upregulated in the presence of azole drugs and has been suggested to regulate its own transcription by an autoregulatory mechanism. The *UPC2* promoter was cloned upstream of a luciferase reporter gene (*RLUC*). *UPC2-RLUC* activity is induced in response to ergosterol biosynthesis inhibitors and in response to anaerobicity. In both conditions, induction correlates with the magnitude of sterol depletion. Azole inducibility in the parental strain was approximately 100-fold, and in a *UPC2* homozygous deletion strain was 17-fold, suggesting that in addition to autoregulation, *UPC2* transcription is controlled by a novel, Upc2p-independent mechanism(s). Curiously, basal *UPC2-RLUC* activity is 5-fold higher in the deletion strain, which may be an indirect consequence of the lower sterol level in this strain, or a direct consequence of repression by an autoregulatory mechanism. These results suggest that transcriptional regulation of *UPC2* expression is important in the response to antifungal drugs, and that this regulation occurs through Upc2p-dependent as well as novel Upc2p-independent mechanisms.

Keywords

Candida albicans; UPC2; azoles; resistance; ergosterol biosynthesis; autoregulation

INTRODUCTION

The pathogenic yeast *Candida albicans* causes oral, vaginal and systemic disease in immunocompromised hosts, and vaginal infection in immune competent hosts. Significant mortality is seen with systemic disease, which is most commonly seen in neutropenic patients, such as those receiving transplant chemotherapy. *Candida* infections are one of the most common opportunistic infections associated with AIDS, and usually manifests as oral disease in these patients (Pfaller & Diekema, 2004).

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The most frequently used antifungals for treatment of oral candidiasis are the azoles which inhibit ergosterol biosynthesis. Resistance to the azoles has emerged due to the fungistatic nature of these drugs and their frequent use for prophylaxis (Pfaller & Diekema, 2004). The azoles, such as fluconazole (FLC) and clotrimazole (CLO), act by targeting the ergosterol biosynthesis enzyme lanosterol 14-α-demethylase which is encoded by the gene *ERG11* (White *et al.*, 1998). Other ergosterol biosynthesis inhibitors act either up or downstream of Erg11p. These include terbinafine (TER) that inhibits the *ERG1* gene product, fenpropimorph (FEN) that inhibits Erg2p, and lovastatin (LOV) that inhibits Hmg1p. Inhibition of sterol synthesis at any of these points results in upregulation of many genes within the pathway (Arthington-Skaggs *et al.*, 1996; Dimster-Denk & Rine, 1996; Henry *et al.*, 2000; Song *et al.*, 2004). Expression of many of these genes has recently been shown to be controlled by the master sterol transcriptional regulator Upc2p (MacPherson *et al.*, 2005; Silver *et al.*, 2004).

C. albicans Upc2p (*Ca*Upc2p) is a Zn_2Cys_6 cluster transcription factor and is homologous at the sequence and functional levels to the *Saccharomyces cerevisiae* paralogs *UPC2* and *ECM22* (*ScUPC2* and *ScECM22*) (MacPherson *et al.*, 2005; Silver *et al.*, 2004). *Ca*Upc2p is required for upregulation of *ERG11* (Oliver *et al.*, 2007) and other sterol biosynthesis genes in response to sterol depletion (MacPherson *et al.*, 2005; Silver *et al.*, 2004), and it activates transcription of target genes by binding to a conserved core sequence known as the sterol response element (SRE) (MacPherson *et al.*, 2005). The *CaUPC2* homozygous deletion is hypersensitive to ergosterol biosynthesis inhibitors as well as to certain drugs that target the cell wall, demonstrating that this transcription factor is central to the response to many antifungal drugs (MacPherson *et al.*, 2005; Silver *et al.*, 2004).

Interestingly, the *CaUPC2* promoter itself contains a putative SRE (MacPherson *et al.*, 2005), suggesting transcriptional self-regulation. It is generally accepted that transcriptional self-activation accounts for the majority of control of *ScUPC2* expression, but this hypothesis has previously only been supported by indirect experimental evidence. Transcriptional profiling of a mutant containing a hyperactive allele of *ScUPC2* (*UPC2-1*) revealed an increase in *ScUPC2* mRNA when compared to wild-type, suggesting that *ScUPC2* was self-activated in the *UPC2-1* strain (Wilcox *et al.*, 2002). Another study using a *ScUPC2-lacZ* fusion showed that deletion of the SRE causes a significant, although not complete reduction in the anaerobic inducibility of the reporter, some of which appears to be due to an increase in basal activity of the promoter lacking the SRE (Abramova *et al.*, 2001). Both of these studies were conducted using *S. cerevisiae* strains containing the *ScUPC2* paralog *ScECM22*, and inducibility of *ScUPC2* may be affected by the presence of *ScECM22*. Studies showing that *ScUPC2* expression is induced by azole drugs have not shown whether inhibition of the ergosterol biosynthetic pathway with other antifungal drugs also results in a *ScUPC2* transcriptional response. The work in this study characterizes the transcriptional activation profile of Ca*UPC2* in response to sterol depletion mediated by sterol synthesis inhibitors and anaerobicity, and investigated the hypothesis that *CaUPC2* expression is self-regulated.

MATERIALS AND METHODS

Abbreviations

Abbreviations are used throughout the text for drugs used in this study as follows: clotrimazole (CLO), fenpropimorph (FEN), fluconazole (FLC), lovastatin (LOV), nikkomycin Z (NKZ), nourseothricin (NAT) and terbinafine (TER).

Strains and growth conditions

C. albicans strain BWP17 (*ura3*::λ*434/ura3*::λ*imm434his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG*) and its derivative D-6 (*upc2*::*URA3/upc2*::*ARG4*) were transformed with *UPC2-RLUC* expression constructs containing the nourseothricin resistance marker *SAT1* (generously provided by Dr. Joachim Morchauser) to create strains CaUPC2-750WT (strain TW16201) and CaUPC2-750D (strain TW16202). Strains were maintained on YEPD (10 g Difco yeast extract, 20 g Bacto peptone, and 20 g dextrose per liter) containing 200 μg/ml nourseothricin (NAT). Innocula prepared for luciferase assays and ergosterol quantitation were grown in CSM (0.75 g CSM (Bio101 Inc, Vista, CA) 5.0 g ammonium sulfate, 1.7 g yeast nitrogen base without amino acids or ammonium sulfate, and 20 g dextrose per liter) with 200 μg/ml NAT to provide selection. Growth during assays was carried out in CSM lacking nourseothricin to avoid pleiotrophic effects of the selective agent.

Creation of *UPC2* constructs containing the *Renilla reniformis* luciferase reporter

The plasmid pCRW3 containing the *Renilla* luciferase reporter plasmid was generously provided by D.R. Soll (Srikantha *et al.*, 1996). To construct the reporter plasmid containing the nourseothricin resistance marker, the plasmid pA83 (Reuss *et al.*, 2004) was used to amplify the *SAT1* marker with the oligonucleotides SAT1Kpn and SAT1EcoRV (Table 1). The resulting PCR fragment was cloned into the vector pCR-Topo (Invitrogen, Carlsbad, CA), after which the *SAT1* marker was excised and ligated into *EcoR*V and *Kpn*I digested pCRW3 to create pCRW3-SAT1. This was done such that the *SAT1* marker would be transcribed in the opposite direction of the reporter gene, to avoid potential *RLUC* activity that could result from incomplete termination of *SAT1* transcription. To create the *CaUPC2-RLUC* fusion, 750bp of *CaUPC2* sequence upstream of the initiating ATG were amplified from the plasmid pGEM-HIS-UPC2 (Silver *et al.*, 2004) using oligonucleotides UPC2Kpn and UPC2Sma. The resulting fragment was cloned into *Kpn*I-*Xma*I digested pCRW3-SAT1 to create pUPC2-RLUC. This plasmid was then linearized using *Nsi*I and integrated at the *ADE2* locus of *C. albicans* strains according to an integration strategy previously used in this laboratory (Song *et al.*, 2004).

C. albicans transformation

C. albicans strains were transformed using the lithium acetate-heat shock method described previously (Sanglard *et al.*, 1996) with modifications. Briefly, 500 uL of YEPD overnight cultures were diluted into 50 ml of fresh YEPD and grown for 5 h at 30°C with shaking at 180 RPM. Cells were prepared by pelleting, washing once with sterile water, and resuspending in 0.1M Lithium acetate in TE pH 7.5 (Li-TE). 5-10 ug of *Nsi*I linearized plasmid DNA and 10 ug carrier DNA (sheared herring sperm DNA, Invitrogen) was used for

each transformation. Cells were incubated overnight with DNA in 40% PEG 3350 in Li-TE, heat shocked for 30 m at 42°C and washed once in 1M sorbitol. This was followed by 4 h of growth at 30°C with shaking at 180 RPM in YEPD in the absence of nourseothricin selection after which cells were plated on YEPD plates containing 200 μg/ml nourseothricin as described previously (Reuss *et al.*, 2004).

PCR and Southern blot screening of transformants

Genomic DNA from pUPC2-RLUC transformed BWP17 and D6 was prepared from cells grown overnight in YEPD-NAT using glass bead lysis as described previously (Hoffman & Winston, 1987). Transformants were initially screened for positive integration of *Nsi*I digested pUPC2-RLUC at the *ADE2* locus using the oligonucleotides ADE2 and RLUC (Table 1). PCR positive transformants were then confirmed by Southern blotting to contain the pUPC2-RLUC construct in single copy. Briefly, approximately 10 ug of genomic DNA was digested with *Kpn*I overnight and run on a 0.7% agarose gel and blotted overnight to a nitrocellulose membrane. The blot was probed with ^{32}P end-labeled RLUC oligonucleotide probe (Table 1). Transformants containing pUPC2-RLUC in single copy at the *ADE2* locus were used for luciferase assay.

Drugs and conditions for *UPC2-RLUC* activity

Drugs used for induction of the *UPC2* reporter construct include the azoles FLC (Pfizer, New York, NY, stock concentration of 3 mg/ml in water) at final concentrations of 0.1-100 μg/ml, and CLO (Sigma-Aldrich, stock concentration 10 mg/ml in DMSO) at a final concentration of 10 μg/ml. TER (Novartis, Vienna, Austria, stock concentration of 10 mg/ml in DMSO) was used at a final concentration of 100 μg/ml, FEN (Sigma-Aldrich, stock concentration of 10 mg/ml in DMSO) at a final concentration of 100μg/ml, nikkomycin Z (NKZ) (Sigma-Aldrich, stock concentration 5 mg/ml in water) at final concentration of 10-100 μg/ml, and LOV (Calbiochem, San Diego, CA, stock concentration 10 mg/ml in ethanol) at a final concentration of 20 μg/ml. In assays where non-water vehicle was used, the no-drug controls were also treated with vehicle. For anaerobic conditions, AnaeroPack anaerobic catalysts (Mitsubishi Gas Chemical, New York, NY) were used in GasPack anaerobic jars (BD, Franklin Lakes, NJ). All drug or anaerobicity induction experiments were carried out in 5 ml volumes in 50 ml conical tubes at 30°C with shaking at 180rpm for 6, 24, or 48 h.

Luciferase assay of *UPC2-RLUC* activity

Luciferase assays were performed as described previously (Srikantha *et al.*, 1996) with modifications described in (Song *et al.*, 2004). Because of inter-assay variability, data presented are representative of three independent experiments. Intra-assay variability was assessed by growing three independent colonies of both *UPC2-RLUC* strains in the presence and absence of 100 μg/ml FLC. This experiment confirmed that within a given assay, variability is low (under 10%).

Ergosterol quantitation experiments

Total ergosterol levels were measured as described previously (Arthington-Skaggs *et al.*, 1999). Cultures were inoculated such that the optical density at 600 nm was 0.2 in a total volume of 25 ml CSM. Cells were grown in the presence and absence of drug, or grown anaerobically for 48 h before harvesting. Equivalent OD units of each culture were used to allow for direct comparison of ergosterol levels between strains and conditions. Wavelength scans of samples were performed from 210 nm to 340 nm.

RESULTS

Creation of *C. albicans* strains expressing *CaUPC2-RLUC* fusions

Previous work has shown that exposure to azoles and other ergosterol biosynthesis inhibitors induces the expression of ergosterol biosynthetic genes as well as their transcriptional activator *Ca*Upc2p (Silver *et al.*, 2004). In order to study the effect of these drugs over prolonged exposure, however, it was necessary to create reporter fusions to avoid difficulties in extracting high quality RNA at late time points. Additionally, to address the role of *Ca*Upc2p self-activation in *CaUPC2* transcriptional induction, it was necessary to express the *CaUPC2-RLUC* fusion in our Δ*upc2*/Δ*upc2* strain D6. This allowed for monitoring changes between wild type and the deletion strain to test the effect of endogenous *Ca*Upc2p on *CaUPC2* transcriptional inducibility. The *Renilla reniformis* luciferase reporter from plasmid pCRW3 (Srikantha *et al.*, 1996) was used to monitor changes in *CaUPC2* transcriptional activation. To express *CaUPC2-RLUC* in both strains tested in this study, the nourseothricin resistance marker *SAT1* was cloned into pCRW3, and 750 bp of the *CaUPC2* promoter was fused to the *RLUC* gene in this plasmid (see Materials and Methods for details).

CaUPC2 expression is highly regulated at the transcriptional level in response to azole drugs

To assess the role of *CaUPC2* transcriptional activation in response to ergosterol depletion in a WT strain, CaUPC2-750WT was grown in the presence and absence of various antifungal drugs that target the ergosterol biosynthesis pathway. The effect of a range of FLC concentrations (0.1-100 μg/ml) on *CaUPC2-RLUC* expression was tested at 6, 24 and 48 h. The drug concentrations were chosen to test the effect of concentrations of drug below, at and above the MIC of FLC for the wild-type strain. This analysis revealed that *CaUPC2* transcriptionally responds to FLC exposure at concentrations near or above the MIC (1.0 μg/ml, Fig 1). Exposure to a sub-inhibitory concentration of FLC (0.1 μg/ml) does not induce *UPC2* transcription to a significant degree (Fig. 1). The presence of 1.0, 10 or 100 μg/ml of FLC, however, causes activation of *CaUPC2-RLUC* activity, and this induction increases with time, to a maximum of about 100-fold inducibility at 48 h (Fig 1). Interestingly, prolonged incubation with more than 1.0 μg/ml FLC causes a greater induction at earlier time points, but by 48 h, 1.0 μg/ml of FLC is sufficient to induce a maximal transcriptional response. The effect of another azole, CLO, was similar to that of FLC at the concentration tested (10 μg/ml, Fig 2). The concentration of CLO used was based on previous work (Song *et al.*, 2004) and is reflective of the MIC of CLO for the *C. albicans* strains used in this study.

CaUPC2 expression is transcriptionally induced in response to multiple ergosterol biosynthesis inhibitors

*Ca*Upc2p has been shown to be a global regulator of sterol biosynthesis genes in response to levels of ergosterol or other late stage intermediates of the sterol pathway (MacPherson *et al.*, 2005; Silver *et al.*, 2004). Because of this, it was of interest to test whether *CaUPC2* is transcriptionally induced in the presence of inhibitors of ergosterol biosynthesis enzymes other than the azoles which target *ERG11*. To test this, CaUPC2-750WT was grown in the presence or absence of FEN, TER, and LOV. These drugs were chosen because they target enzymes of both the early (LOV targets Hmg1p,) and late (TER targets Erg1p, which is upstream of Erg11p; FEN targets Erg2p, downstream of Erg11p) parts of the ergosterol biosynthetic pathway. As shown in Figure 2, TER and FEN (solid triangles and solid squares, respectively) treatment induces transcription of *CaUPC2* to levels similar to those seen with azole exposure (Fig. 1), and this induction increases with prolonged growth in the presence of drug. Incubation with 20 μg/ml LOV also induced a *CaUPC2* transcriptional response, although the overall fold induction was lower than that seen with other ergosterol biosynthesis inhibitors (approximately 5-fold, Fig 2, open squares).

CaUPC2 expression is induced in response to ergosterol biosynthesis inhibitors in the absence of endogenous *Ca*Upc2p

To test the hypothesis that *CaUPC2* expression is transcriptionally self-regulated, CaUPC2-750D6, the *Δupc2/Δupc2* strain expressing *CaUPC2-RLUC*, was grown in the presence and absence of ergosterol biosynthesis inhibitors. This allowed for monitoring of the *CaUPC2* reporter activity in the absence of endogenous *Ca*Upc2p. Interestingly, growth of CaUPC2-750D6 in the presence and absence of 0.1-100 μg/ml of FLC resulted in *UPC2* transcriptional induction, albeit to a lower level than that seen in CaUPC2-750WT (Fig. 3). The concentration of FLC required to induce a maximal transcriptional response beginning at early time points in this strain was 1.0 μg/ml. Incubation of CaUPC2-750D6 with CLO, FEN, TER (Fig. 4 solid diamonds, solid squares and solid triangles, respectively) or LOV (Fig. 4, open squares) also resulted in induction of the *CaUPC2-RLUC* fusion, but with overall inducibility lower than that seen in the wild-type strain.

CaUPC2-RLUC basal activity is higher in the absence of endogenous *Ca*Upc2p

The observation that *CaUPC2* fold induction is lower in the *CaUPC2* homozygous deletion suggested either that *CaUPC2* is autoregulated, or that there are pleiotrophic effects due to the genetic deletion. To address this, the basal *CaUPC2-RLUC* activities of both strains were compared, as well as the absolute level of activity in the presence of azoles, so that the two strains could be directly compared to each other. Interestingly, the decrease in fold induction seen in CaUPC2-750D6 is due primarily to an increased basal level of expression in the absence of drug (Fig. 5). The *CaUPC2* deletion strain exhibits approximately 5-fold higher basal activity when compared to the wild-type strain. Additionally, the *CaUPC2-RLUC* activity is higher in the deletion strain at the lowest concentration of FLC tested (0.1 μg/ml). Most importantly, the maximal level of promoter activity in the presence of FLC is the same in both strains (Fig. 5), indicating that the *CaUPC2* promoter is activated transcriptionally in response to drug to the same degree in both strains. The relationship of the *CaUPC2-RLUC*

activities between both strains was highly reproducible despite variation in absolute levels of luciferase activity between experiments. Additionally, this phenomenon was consistent in assays using other antifungal drugs (data not shown).

CaUPC2 expression is induced in response to anaerobicity

It has previously been shown that a *CaUPC2* homozygous deletion is impaired for anaerobic growth (MacPherson *et al.*, 2005). Additionally, it has been shown that *CaERG11*, which is under the control of *Ca*Upc2p, is anaerobically induced (Song *et al.*, 2004), and it is known that *Ca*Erg11p requires oxygen to be functional. These data suggest that *CaUPC2* is transcriptionally activated in the absence of oxygen. To test this, CaUPC2-750WT and CaUPC2-750D6 were grown aerobically and anaerobically in the presence and absence of 100 μg/ml FLC. Although the strain D6 is impaired for anaerobic growth compared to BWP17, it is able to undergo several generations of growth, presumably due to ergosterol stores. After 48 h of anaerobic growth, *CaUPC2-RLUC* activity was increased approximately 23—fold and 2.3—fold for the WT and deletions strains, respectively (Fig.6). The lack of an additional effect when 100 μg/ml FLC is added during anaerobic growth indicates that the effects of FLC and anaerobicity are not additive (Fig 6).

CaUPC2 transcriptional induction correlates with sterol depletion

Because *CaUPC2* transcription increases over time with drug exposure, it was of interest to test whether this response may be linked to total cellular ergosterol levels. To measure ergosterol content in response to various inhibitors, cells were grown for 48 h (the time-point when the transcriptional response is maximal) and total sterols were extracted and measured spectrophotometrically as described previously (Arthington-Skaggs *et al.*, 1999). Sterol levels were shown to correlate well with the degree of *CaUPC2* induction over a range of FLC concentrations, (Table 2) as increasing amounts of FLC resulted in a decrease in total sterol in CaUPC2-750WT, with the maximal degree of ergosterol depletion being seen with 100 μg/ml FLC (5.85% of total ergosterol in cells grown in the absence of FLC). Similarly, with other ergosterol biosynthesis inhibitors as well as anaerobicity, a decrease in ergosterol production was seen (Table 2).

Similar decreases in total ergosterol levels were seen with the CaUPC2-750D6 strain, in that exposure to ergosterol biosynthesis inhibitors results in reduced ergosterol production (Table 2). The CaUPC2-750D6 strain exhibits ergosterol depletion over a range of FLC concentration, which parallels the *CaUPC2* transcriptional induction shown in Figure 3. It is important to note that while the *Δupc2Δ/upc2* strain has lower total ergosterol than does the wild-type strain following FLC treatment, the intrinsic level of ergosterol in the deletion strain is lower even in the absence of drug treatment. The deletion strain shows a decrease in total ergosterol following exposure to FEN, LOV or anaerobicity (39.07%, 43.5%, and 44.81% of ergosterol level in BWP17 in absence of drug, respectively). Interestingly, the ergosterol level in the *Δupc2Δ/upc2* strain did not decrease in response to TER. As previously reported (Silver *et al.*, 2004), the deletion strain exhibits a lower level of ergosterol than the wild-type (74.66%, Table 2) in the absence of drug, which correlates with the difference between wild-type and deletion strain basal *CaUPC2* reporter activity in the absence of drug as shown in Figure 5.

Chemical inhibition of cell wall biosynthesis does not affect *CaUPC2* induction

Based on altered susceptibility of *C. albicans* Δ*upc2*/Δ*upc2* to the chitin synthase inhibitor NKZ (Silver *et al.*, 2004), as well as the role of *ScUPC2* in regulation of cell wall gene (Abramova *et al.*, 2001), it was hypothesized that *CaUPC2* may transcriptionally respond to NKZ treatment. Growth of CaUPC2-750WT or CaUPC2-750D in the presence of 10 or 100 μg/ml of NKZ did not alter *CaUPC2* expression (data not shown). In parallel, NKZ exposure did not alter total ergosterol levels in either strain (data not shown).

DISCUSSION

The goal of this study was to characterize the role of transcriptional inducibility of *CaUPC2* in response to antifungal drugs that target ergosterol biosynthesis. An important component of *ScUPC2* transcriptional regulation has previously been hypothesized to be positive auto-regulation (Abramova *et al.*, 2001; Wilcox *et al.*, 2002). Therefore, this study also addressed whether *CaUPC2* regulates its own transcription by using a *CaUPC2* homozygous deletion strain and a *CaUPC2* luciferase reporter construct.

Initial characterization of the *CaUPC2* transcriptional response to ergosterol depletion showed that in the wild-type strain CaUPC2-750WT, *CaUPC2* is highly regulated at the transcriptional level. With prolonged exposure to many ergosterol biosynthesis inhibitors, *CaUPC2-RLUC* activity increased up to 100-fold. This suggests that in addition to other potential mechanisms of regulation of the ergosterol biosynthetic pathway, transcriptional control of the major sterol regulator *CaUPC2* may play an important role in the response to antifungal drugs. Previous work has suggested a post-translational control mechanism regulating *Sc*Upc2p in *S. cerevisiae* (Davies *et al.*, 2005; Vik & Rine, 2001). Data suggested a model in which *Sc*Upc2p is present in the nucleus and is bound by a repressor during sterol-rich conditions. Upon sterol depletion, however, the repression is released and *Sc*Upc2p binds to and activates sterol biosynthesis genes (Davies *et al.*, 2005). An alternative model is based in analogy to the mammalian sterol regulator SREBP and was proposed in a recent review (White & Silver, 2005). In this model, the N-terminal *Ca*Upc2p DNA binding domain is anchored to a membrane via four predicted transmembrane spans found in the C-terminal portion of the protein. Upon sterol depletion, it is proposed that a cleavage event liberates the DBD which can then translocate to the nucleus and activate target genes. This model is consistent with *S. cerevisiae* localization experiments that show that the C-terminally tagged Upc2p is not nuclear localized (C. Marie and T. White unpublished observations). The current evidence cannot determine which model is correct, but either model suggests an important post-translational regulatory mechanism. This work demonstrates that in addition to these proposed mechanisms, transcriptional activation of *CaUPC2* likely plays an important role in regulating downstream genes via a large increase in abundance of *CaUPC2* mRNA and, putatively, its subsequent protein product.

It was also shown that transcriptional induction became maximal after 48 h of growth in the presence of drug. This observation is consistent with the hypothesis that it is depletion of sterols that triggers *CaUPC2* upregulation, as sterol depletion will only become severe after prolonged exposure to inhibitors.

The observation that inhibition of multiple steps in the ergosterol biosynthetic pathway results in an increase in *CaUPC2-RLUC* activity is consistent with evidence that *Ca*Upc2p acts as a global regulator of sterol biosynthesis genes. These data are consistent with previous results from this laboratory showing that *CaERG11-RLUC* activity is responsive to inhibition of multiple steps in the ergosterol biosynthetic pathway (Song *et al.*, 2004). It is important to note that all of the genes for enzymes that were inhibited in this study (*HMG1, ERG1, ERG11, ERG2*) contain putative *Ca*Upc2p binding sites within their promoters (MacPherson *et al.*, 2005; Silver *et al.*, 2004). In addition, microarray analysis suggests that transcriptional activation of each of these drug targets in response to FLC is *Ca*Upc2p dependent (unpublished results). When these data are taken together with the level of sterol depletion caused by these inhibitors, it seems likely that the signal that induces *CaUPC2* expression and subsequent upregulation of *ERG* genes may be the lack of ergosterol or a late sterol pathway intermediate. Indeed, this work has shown that inhibition of multiple steps in the biosynthetic pathway results in a decrease in the end product ergosterol, and therefore it seems likely that this decrease serves as a signal to induce expression of the transcriptional activator of the pathway, *Ca*Upc2p. This hypothesis is supported by the observation that ergosterol depletion (Table 2) correlates with induction of *CaUCP2-RLUC* activity (Figures 1-4).

The response of *CaUPC2* to anaerobicity is also consistent with previous data. In *S. cerevisiae,* anaerobic growth is not possible in the absence of exogenous ergosterol, and this appears to be largely due to the dependence of Erg11p on molecular oxygen as a cofactor, as well as the heme requirement of this enzyme (Setiadi *et al.*, 2006; White *et al.*, 1998). *C. albicans*, however, can grow anaerobically in the absence of exogenous ergosterol, and this growth is accompanied by an increase in *ERG11* expression (Setiadi *et al.*, 2006; Song *et al.*, 2004). The anaerobic induction of *ERG11* is likely due to the oxygen dependence of the sterol pathway, which utilizes 12 molecules of O_2 for every 1 molecule of ergosterol synthesized (Hughes *et al.*, 2005). This study shows that the amount of ergosterol biosynthesis under anaerobic conditions is clearly decreased when compared to aerobically grown cells, although not to the same degree as seen with drug inhibition. This intermediate degree of anaerobic sterol depletion is paralleled with an increase in *CaUPC2-RLUC* activity that is somewhat lower than what is seen with direct chemical inhibition of ergosterol biosynthesis. Previous studies have also shown that a *CaUPC2* deletion strain is deficient in anaerobic growth (MacPherson *et al.*, 2005), suggesting that transcriptional activation of the ergosterol biosynthetic pathway by *Ca*Upc2p is essential in anaerobicity, which is consistent with the data presented in this study. It is important to note that the anaerobic induction experiment in this study was performed using aerobically grown innocula, so that luciferase activity reflects the adaptation to anaerobicity, not true anaerobic growth.

The *CaUPC2* deletion was previously shown to exhibit hypersensitivity to cell wall perturbing agents such as NKZ (Silver *et al.*, 2004), but it is unclear whether this sensitivity is a direct result of *Ca*Upc2p activation of cell wall associated genes, or a pleitrophic effect resulting from altered membrane sterol composition. *Sc*Upc2p transcriptionally activates some cell wall associated proteins in response to anaerobicity, such as those in the *DAN/TIR* family (Abramova *et al.*, 2001). This evidence suggested that perhaps the effect of *ScUPC2*

deletion on cell wall sensitivity is due to a direct effect of *Sc*Upc2p on cell wall gene expression. If the effect was the result of transcriptional activation by *Ca*Upc2p, it was expected that *CaUPC2* would transcriptionally respond to treatment with NKZ. When this was tested, however, there was no change in *CaUPC2-RLUC* activity in the presence of NKZ. Additionally, NKZ did not alter total ergosterol levels. These data suggest that *CaUPC2* transcriptional activation is specific to alterations in the sterol biosynthetic pathway. The NKZ susceptibility of the *CaUPC2* deletion mutant may due to pleitrophic effects of the lower sterol level of the mutant rather than direct control of expression of cell wall associated genes by *Ca*Upc2p.

The comparison of *CaUPC2-RLUC* activity between the wild-type and *CaUPC2* deletion mutant suggests either direct autoregulation by *Ca*Upc2p or an indirect consequence of the lower basal level of total sterol in the deletion strain. The difference in fold-induction between the two strains clearly demonstrates that the *CaUPC2* mutant has an altered regulation of *CaUPC2* promoter activity (Figures 1-4). The lower fold inducibility of *CaUPC2-RLUC* in Δ*upc2*/Δ*upc2* suggests an important component of transcriptional self-regulation, which is consistent with the limited evidence reported for *S.cerevisiae* (Abramova *et al.*, 2001; Davies *et al.*, 2005) as well as the presence of a putative *Ca*Upc2p binding site within the *CaUPC2* promoter. The increase in basal activity of the Δ*upc2*/Δ*upc2* strain when compared to wild-type, however, suggests more than one possibility. It is possible that *Ca*Upc2p acts as a transcriptional repressor at its own promoter, but there has been no previous evidence to support this. Alternatively, the intrinsically lower level of ergosterol in the Δ*upc2*/Δ*upc2* mutant (Silver *et al.*, 2004) may account for the increased *CaUPC2-RLUC* activity in the absence of drug. This indirect effect may mask direct consequences of *CaUPC2* deletion on *CaUPC2-RLUC* activity, and further study including nested deletions and direct binding to the putative SRE is needed to definitively address autoregulation. These studies are currently underway in this laboratory. While the intrinsically lower level of sterol in the deletion strain may explain the higher level of *UPC2-RLUC* activity in the absence of drug, recent work has demonstrated the *Ca*Upc2p indeed does bind to the *CaUPC2* promoter (Znaidi *et al.*, 2008). This evidence, along with the data presented in the present study, suggests that at least some component of *UPC2* inducibility is transcriptionally self-regulated. The *CaUPC2-RLUC* inducibility that remains in the Δ*upc2*/Δ*upc2* strain strongly suggests that in addition to *Ca*Upc2p, a novel sterol responsive transcription factor also controls *CaUPC2* expression. This is consistent with previous work in which deletion of the *Sc*Upc2p binding site within the *ScUPC2* promoter reduced, but did not eliminate *ScUPC2* inducibility (Abramova *et al.*, 2001). Identification of additional transcription factors controlling *CaUPC2* expression is currently being addressed in this laboratory and will contribute to our understanding of how *C. albicans* cells respond to antifungal drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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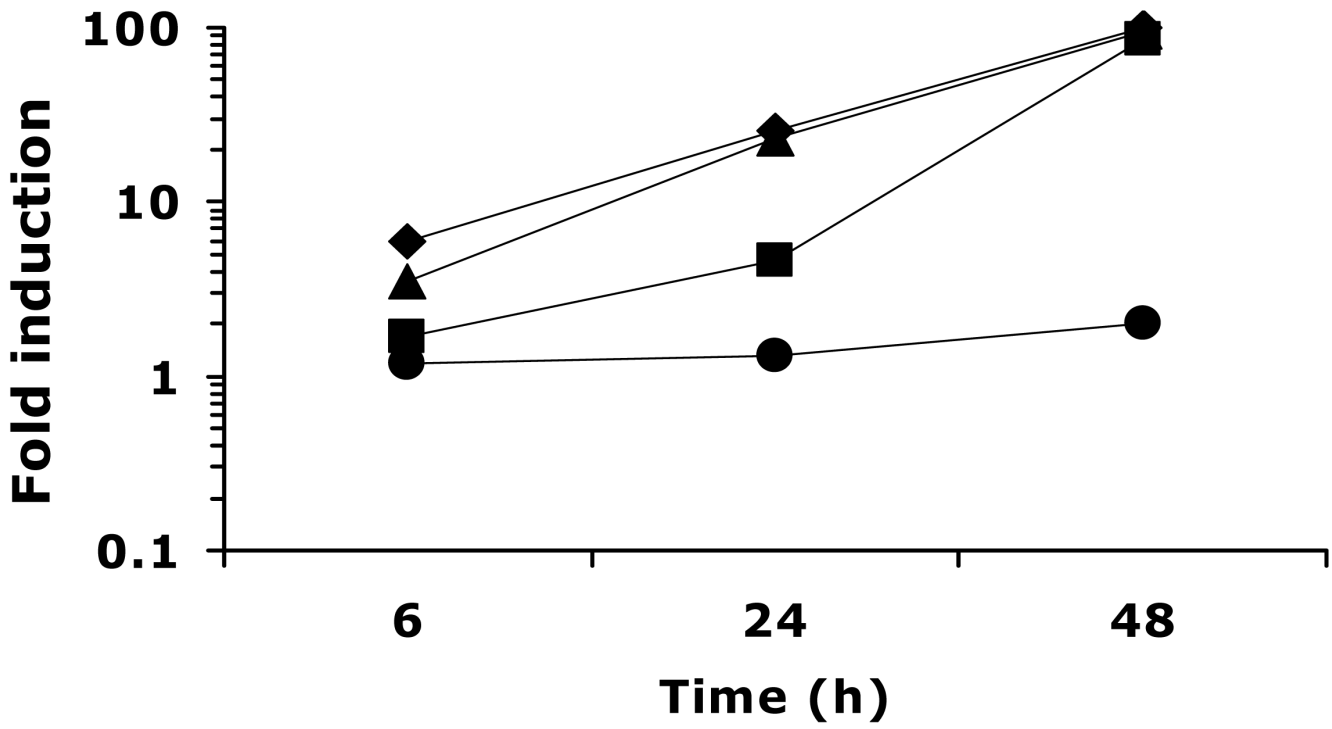

Fig. 1.

Time course of *UPC2-RLUC* induction in WT by a range of FLC concentrations. CaUPC2-750WT was grown in the presence and absence of FLC at 0.1 (circles), 1.0 (squares), 10 (triangles), or 100 (diamonds) μg/ml. Luciferase activity was assayed at 6, 24 and 48 h. Data are presented as fold induction, or the luciferase activity in the presence of drug relative to the luciferase activity in the absence of drug. The results are representative of three independent experiments.

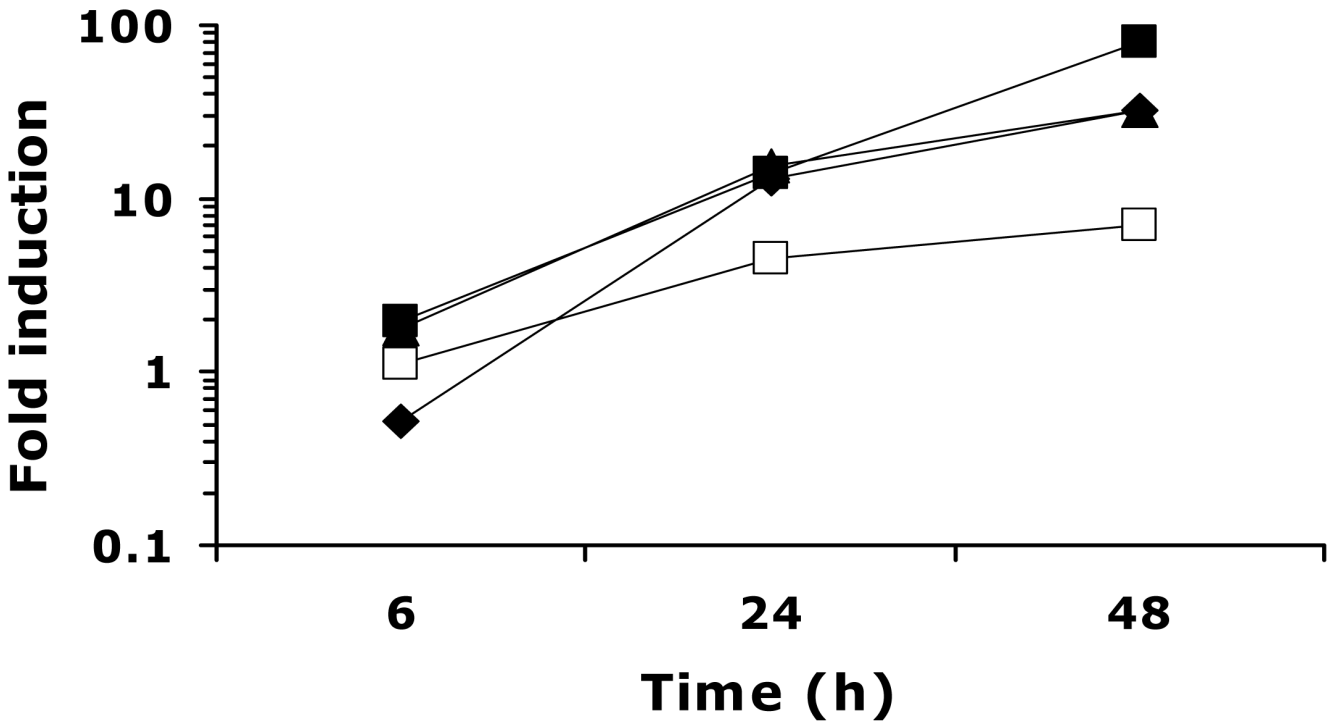

Fig. 2.

Time course of *UPC2-RLUC* induction by additional ergosterol biosynthesis inhibitors. CaUPC2-750WT was grown in the presence and absence of CLO at 10 μg/ml (solid diamonds), TER at 100 μg/ml (solid triangles), FEN at 100 μg/ml (solid squares) or LOV at 20 μg/ml (open squares). Luciferase activity was assayed at 6, 24 and 48 h. Data are presented as fold induction, or the luciferase activity in the presence of drug relative to the luciferase activity in the absence of drug. The results are representative of three independent experiments.

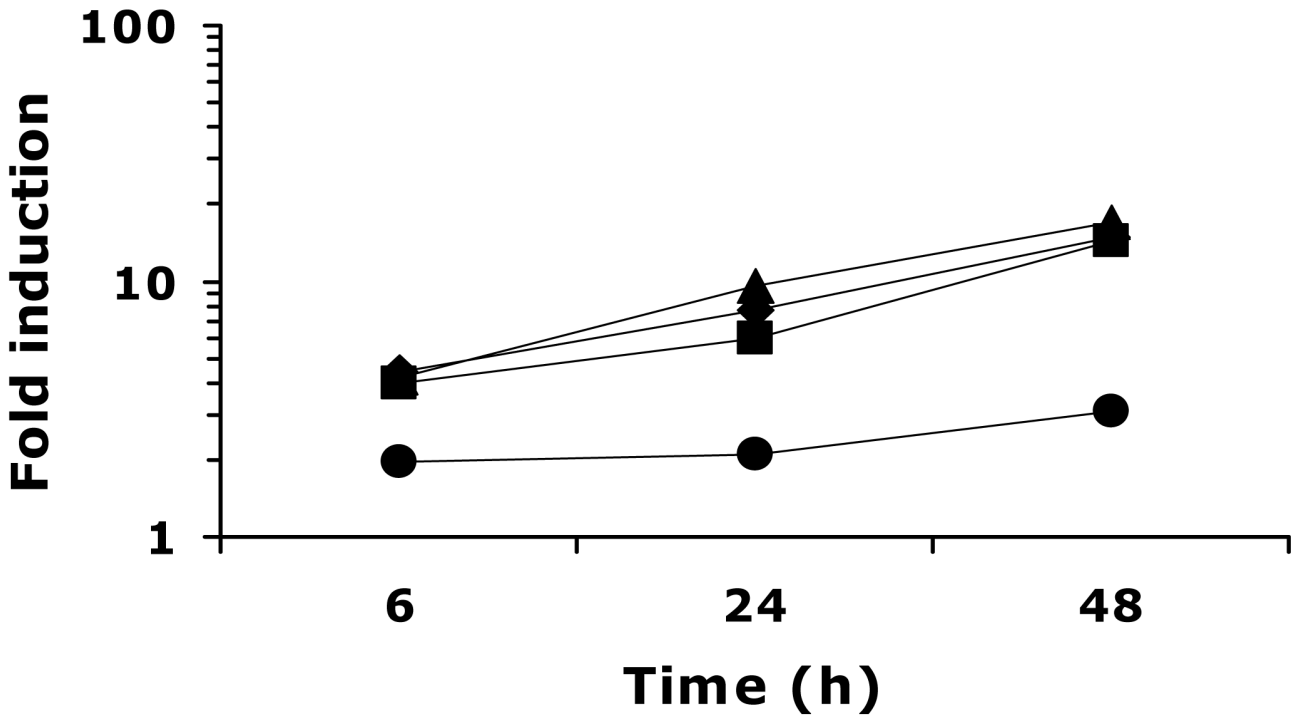

Fig. 3.

Time course of *UPC2-RLUC* induction in *Δupc2/Δupc2* by a range of FLC concentrations. CaUPC2-750D6 was grown in the presence and absence of FLC at 0.1 (circles), 1 (diamonds), 10 (squares), or 100 (triangles) μg/ml. Luciferase activity was assayed at 6, 24 and 48 h. Data are presented as fold induction, or the luciferase activity in the presence of drug relative to the luciferase activity in the absence of drug. The results are representative of three independent experiments.

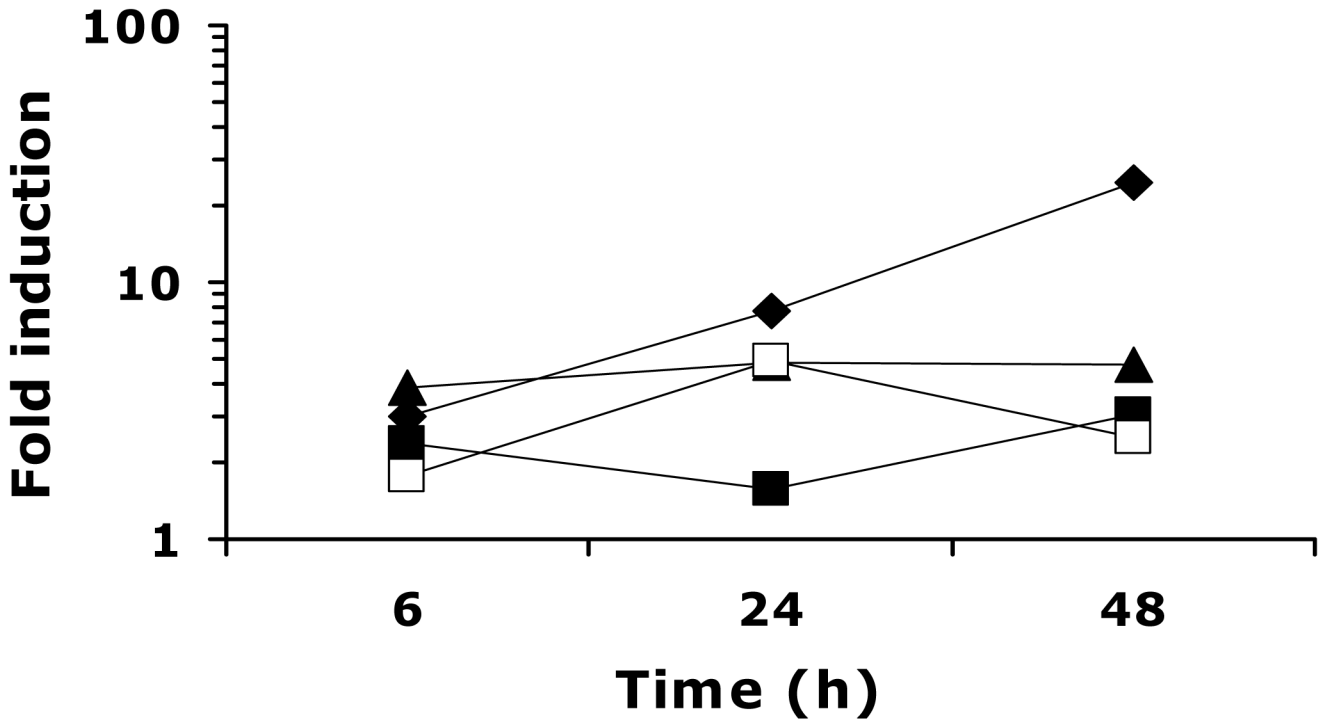

Fig.4.

Time course of *UPC2-RLUC* induction in *Δupc2/Δupc2* by additional ergosterol biosynthesis inhibitors. CaUPC2-750D6 was grown in the presence and absence of CLO at 10 μg/ml (solid diamonds), TER at 100 μg/ml (solid triangles), FEN at 100 μg/ml (solid squares) or LOV at 20 μg/ml (open squares). Luciferase activity was assayed at 6, 24 and 48 h. Data are presented as fold induction, or the luciferase activity in the presence of drug relative to the luciferase activity in the absence of drug. The results are representative of three independent experiments.

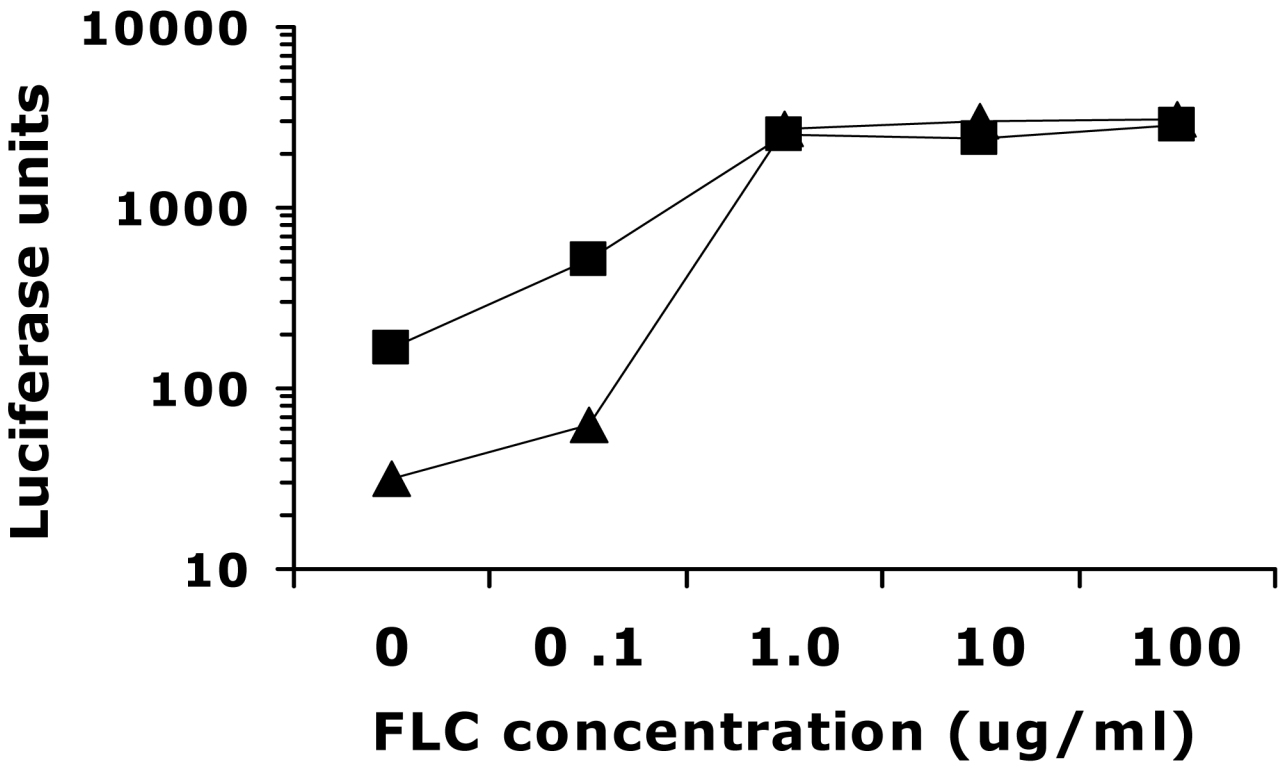

Fig. 5.

Levels of *UPC2-RLUC* activity in WT and *Δupc2/Δupc2* over a range of FLC concentrations. CaUPC2-750WT (triangles) CaUPC2-750D6 (squares) were grown for 48 hours in various fluconazole concentrations. Luciferase activity is expressed as the specific activity of luciferase corrected for total protein content. The results are representative of three independent experiments.

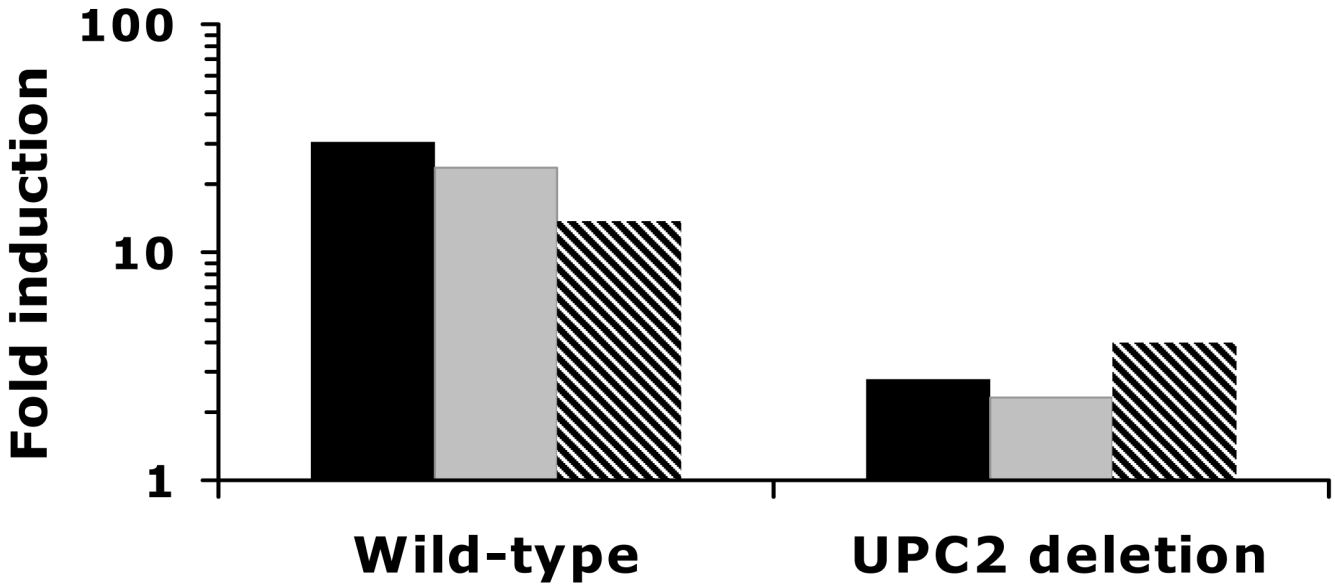

Fig. 6.

Anaerobic inducibility of *UPC2-RLUC* activity in WT and *Δupc2/Δupc2.* Data were collected after 48 hrs of growth. *UPC2-RLUC* induction in 100 μg/ml FLC is shown in black bars, induction during anaerobic growth is shown in grey bars, and induction in the presence of 100 μg/ml FLC during anaerobicity in hatched bars. Data are presented as fold induction, or the luciferase activity in the presence of drug relative to the luciferase activity in the absence of drug. The results are representative of three independent experiments.

TABLE 1

Oligonucleotides used for reporter plasmid construction and transformant screening

Oligonucleotide name	Sequence (5'- 3')
SAT1Kpn	TACAACGGTACCCAGCGTCAAAACTAGAGAATAATAAG
SAT1EcoRV	TACAACGATATCGATTTCTAGAAGGACCAC
UPC2Kpn	CTCTCGGTACCATGGATGTTGGTATATCAGG
UPC2Sma	CTCTCCCCGGGAAATGGCTTTTTTGTTGAAAAA
RLUC	CACCACTGCGGACCAGTTATCATCCGTTTCC
ADE2	CAGTTAAATAGTCTTCATATC

TABLE 2

Ergosterol content of cells grown in sterol depleting conditions

	CaUPC2-750WT		CaUPC2-750D6	
	ERG content[a]	**Induction level**[b]	**ERG content**[a]	**Induction level**[b]
No Drug	100.00	N/A	74.66	N/A
FLC (ug/ml):				
0.1	80.36	2.02	65.21	3.09
1	52.06	86.58	27.49	14.97
10	4.74	95.72	12.19	14.44
100	5.85	99.66	3.07	17.02
LOV 20 ug/ml	60.32	7.08	43.51	2.48
TER 100 ug/ml	55.20	31.97	85.77	4.78
FEN 100 ug/ml	23.04	79.57	39.07	3.06
Anaerobicity	21.72	23.49	44.81	2.31

[a] Expressed as percent of WT in absence of drug.

[b] *UPC2-RLUC* induction level at 48 h expressed as fold induction.