

In Vitro and In Vivo Effects of 14 α -Demethylase (*ERG11*) Depletion in *Candida glabrata*

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Sterol 14 α -demethylase (*ERG11*) is the target enzyme of azole antifungals that are widely used for the treatment of fungal infections. *Candida glabrata* is known to be less susceptible to fluconazole than most *Candida albicans* strains, and the incidence of *C. glabrata* infection has been increasing mostly in conjunction with the use of azole antifungals. Recently, it has been reported that *C. glabrata* can rescue the defect of ergosterol biosynthesis by incorporating cholesterol from serum. To explore the effect of inactivating Erg11p in *C. glabrata*, we generated mutant strains in which the *ERG11* gene was placed under the control of tetracycline-regulatable promoters. In these mutants, expression of the *ERG11* gene can be repressed by doxycycline (DOX). All mutants showed a growth defect in the presence of DOX. The numbers of CFU of the mutants were lowered by only 1/10 with DOX treatment. In these mutants, accumulation of 4,14-dimethylzymosterol, which differs from an accumulated abnormal sterol detected in *C. albicans* and *Saccharomyces cerevisiae* treated with fluconazole, was observed by DOX treatment. Although such phenotypes were also observed in serum-containing media by DOX treatment, they were alleviated. Furthermore, the mutant could grow in DOX-treated mice without a severe reduction in the number of cells. Thus, depleting the expression of the *ERG11* gene lowered the number of CFU by only 1/10 due to the accumulation of 4,14-dimethylzymosterol in vitro, and it did not result in the defective growth of fungal cells in mice. These results suggested that Erg11p is not an ideal target molecule of antifungals for *C. glabrata*.

The incidence of life-threatening fungal infections has been increasing, particularly among patients who are immunocompromised by human immunodeficiency virus infection and those who are receiving immunosuppressive therapy for organ transplantation or chemotherapy for cancer. Azole antifungals are widely used in current therapies against such infections. Fluconazole, a water-soluble triazole with greater than 90% bioavailability after oral administration, has been used extensively to treat a wide range of *Candida* infections (30). Azole antifungals, including fluconazole, selectively inhibit the sterol 14 α -demethylase gene (*ERG11*), which is an essential participant in ergosterol biosynthesis. The nitrogens in an azole ring form a complex with a heme iron component of the cytochrome group, resulting in the inhibition of the enzyme (46). Ergosterol is an essential component of the fungal plasma membrane. In *Saccharomyces cerevisiae* and *Candida albicans*, sterols have been shown to be important in membrane fluidity, membrane permeability, cell morphology, enzyme activity, and cell cycle progression (3, 16–18).

It has been a matter of concern that an increasing number of azole-resistant isolates are recovered in many immunocompro-

mised and immunosuppressed patients (39). The mechanisms of azole resistance have been identified and classified mainly into three categories at this time. Mutation or disruptions of *ERG3*, which encodes $\Delta 5,6$ sterol desaturase, are associated with increased resistance to azole in *S. cerevisiae* (44) since the loss of function of Erg3p exerts a suppressor effect on the phenotype strain harboring *erg11* mutations (2, 38). Increased efflux of drugs, mediated by multidrug pumps, including the major facilitators and the ATP-binding cassette (ABC) transporters, also confers resistance to azole antifungals (1, 21, 33, 35–37, 42, 43, 45). In addition, alteration of the target enzyme Erg11p, including point mutations (15, 19, 20, 34, 40) and overexpression (13, 45), causes resistance to azole antifungals.

Candida glabrata, which always grows as a haploid yeast cell, is a common pathogen in immunocompromised persons or those with diabetes mellitus. Although *C. albicans* is the best known of the pathogenic *Candida* group, the frequency of other *Candida* species that are isolated from clinical infections has been increasing during the past few years. Among the infections of the non-*albicans* *Candida* species, the incidence of *C. glabrata* infection has been increasing, mostly in conjunction with the use of azole antifungals (25, 27, 28). Consistently, this organism has been reported to be an intrinsically resistant *Candida* species, as is *Candida krusei* (5, 29). Other recent studies reported that *C. glabrata* is often the second or third most common cause of candidiasis after *C. albicans* and that *C. glabrata* infections have been linked to the death of compromised, at-risk hospitalized patients (4). Recently, we reported that squalene synthase (*ERG9*), which is also essential for ergosterol biosynthesis, is not required for the growth of *C. glabrata* in mice since *C. glabrata* has the ability to incorpo-

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TABLE 1. Strains used in this study

Strain	Parent strain	Genotype ^a	Reference
ACG4	ATCC 2001	<i>his3 trp1 PScADH1::tetR::GAL4AD::TRP1</i>	23
97ERG11	ACG4	<i>his3 trp1 PScADH1::tetR::GAL4AD::TRP1 97t::ERG11-HIS3</i>	This study
98ERG11	ACG4	<i>his3 trp1 PScADH1::tetR::GAL4AD::TRP1 98t::ERG11-HIS3</i>	This study
99ERG11	ACG4	<i>his3 trp1 PScADH1::tetR::GAL4AD::TRP1 99t::ERG11-HIS3</i>	This study

^a *PScADH1*, *S. cerevisiae ADH1* promoter; *GAL4AD*, *GAL4* activation domain.

rate sterol from serum even under aerobic conditions (24). However, since a disruption study has proven that the *ERG11* gene is also required for the aerobic growth of *C. glabrata* (6), the following question is raised: how can the inactivation of Erg11p, which may mimic fluconazole treatment, affect fungal growth in mice? To answer the question, we studied the effect of diminishing the expression of the *ERG11* gene on growth in both in vitro and in vivo settings. For this study, we used tetracycline-regulatable promoters (23) to repress expression of the *ERG11* gene. Although the generated strains showed a growth defect by repressing the expression of the *ERG11* gene by using doxycycline (DOX), severe reduction of the number of viable cells could not be observed in either in vitro or in vivo culture settings. We also showed that abnormal sterol, which is different from the accumulated abnormal sterol detected in fluconazole-treated *C. albicans* and *S. cerevisiae* (11, 12), accumulated in such strains. Based on these results, Erg11p is not an ideal target molecule of antifungals for *C. glabrata* infection.

MATERIALS AND METHODS

Strains and growth media. The *C. glabrata* strains used in this study are shown in Table 1. The *C. glabrata* strains were grown at 37°C on yeast extract-peptone dextrose (YEPD) complex medium containing 2% (wt/vol) glucose, 2% (wt/vol) Bacto peptone (Difco Laboratories), and 1% (wt/vol) yeast extract (Difco). The YEPD agar plates contained 2% (wt/vol) agar (Difco) as a supplement. Yeast nitrogen base (0.67%; Difco) with 2% (wt/vol) glucose, 2% (wt/vol) agar (Difco), and appropriate amino acids and bases was used as the selective medium after the transformation of ACG4. Yeast transformations were carried out by the modified lithium acetate method (8, 10). *Escherichia coli* DH5 α was used as the host strain for all plasmid constructions and was grown on standard media.

Construction of plasmids and strains. All primers in this study are shown in Table 2. Plasmids p97ERG11, p98ERG11, and p99ERG11 were constructed by introducing region A (nucleotides [nt] -417 to -156) or region B (nt -6 to 330) of *CgERG11* (6) into *SacII* and *XbaI* sites or *EcoRI* and *SalI* sites, respectively, of p97CGH, p98CGH, and p99CGH (23). Region A or region B of the *C. glabrata ERG11* gene was amplified with PCR using the primer pairs ERG11AF and ERG11AR or P5ERG11 and P3ERG11, respectively. To replace the endogenous promoter of *ERG11* with tetracycline-regulatable promoters (97t, 98t, and 99t) by homologous recombination (Fig. 1A), p97ERG11, p98ERG11, and p99ERG11 linearized with *SacII* and *SalI* were used to transform ACG4, yielding strains 97ERG11, 98ERG11, and 99ERG11 (designated controllable strains), respectively. Approximately 0.2 μ g of genomic DNA and the primer pair ERG11CH5 (nt -548 to -529) and ERG11CH3 (nt 430 to 450) were used for

PCR to confirm correct integration of tetracycline-regulatable promoters in these controllable strains.

Northern blot analyses. Total RNA was extracted by the glass bead lysis method (32). Ten micrograms of total RNA was separated on an agarose gel, transferred onto a nylon membrane (Hybond-N; Amersham-Pharmacia Biotech), and hybridized with a radiolabeled probe. The DNA fragments used for hybridization were *ERG11* region B and the 0.5-kb *MunI* fragment of *S. cerevisiae ACT1* exon 2. The signal obtained from *ACT1* was used to normalize the mRNA signals. Radiolabeling of DNA was carried out by the random priming method using [α -³²P]dCTP. The RNA bands that hybridized with the radiolabeled probe were visualized by autoradiography or with a BAS 1000 image analyzer (Fuji film). The intensity of each *ERG11* signal was measured with an analyzing program installed in BAS 1000.

Effect of DOX on growth and the number of viable cells. To investigate the effect of DOX on growth, approximately 10⁵ of the cells were inoculated in YEPD medium with or without 10 μ g of DOX per ml and cultured at 37°C. After a 14-h culture, their growth was monitored by measuring optical density at 660 nm. In order to determine the number of viable cells at several time points, 10⁵ 98ERG11 cells were inoculated into YEPD with or without DOX (10 μ g/ml). The number of viable cells was determined by counting the number of colonies on an agar plate in which 20 μ l of diluted cultures had been spread after a 24-h incubation at 37°C.

Analyses of sterol composition of *C. glabrata* cells. We prepared samples for analyzing the sterol content as described previously (24). Approximately 10⁶ cells/ml were inoculated into YEPD medium or YEPD medium containing 5% (vol/vol) human serum and cultured at 37°C with or without DOX (10 μ g/ml). The cells were harvested at the times indicated in Table 3. We used 4,4-diphenyl-1-benzyl-piperidine as the internal control for the analyses.

Effect of serum on the growth defect produced by DOX. Approximately 10⁶ 98ERG11 cells were inoculated into YEPD medium with or without DOX (10 μ g/ml) and cultured at 37°C with or without 5% (vol/vol) human serum (Irvine Scientific). Their growth was monitored by measuring optical density at 660 nm at the indicated times.

Determination of the number of viable *C. glabrata* cells in mice. To generate immunocompromised mice, male CD-1 mice were treated as described previously (23, 24). Each mouse was intravenously inoculated with 10⁵ ATCC 2001 or 98ERG11 cells after having been given 5% (wt/vol) sucrose solution with or without DOX (2 mg/ml) as drinking water from 3 days before the infection. In this dose regimen, each mouse drank approximately 5 ml of sucrose solution every day. Results show that the concentrations of DOX in serum, liver, and kidney were maintained at more than 2 μ g of serum per ml, 8 μ g of liver per g, and 10 μ g of kidney per g. Such concentrations can be enough to repress the gene expression regulated by tetracycline-regulatable promoters (23, 24). The five pairs of mouse kidneys per group were sacrificed and homogenized 5 h, 7 days, and 14 days after the infections. The homogenates were spread on YEPD plates containing penicillin G (200 U/ml) and streptomycin (200 μ g/ml), which were used for preventing bacterial infection. The number of colonies that had appeared after culturing the cells for 24 h at 37°C was counted.

TABLE 2. Linkers and primers used in this study

Name	Designed <i>C. glabrata ERG11</i> region (orientation)	Sequence (5'-3') ^a
ERG11AF	5'-end flanking (sense)	TTTTTTCGCGG ATTATCGCTTCTTCTCTAATCCA
ERG11AR	5'-end flanking (antisense)	TTTTTTTCTAGA TGAGTATACGGGTCTCTCG
P5ERG11	N terminus (sense)	TTTTTTGAATTC AATAACATGTCCACTGAAAAC
P3ERG11	N terminus (antisense)	TTTTTTGTGCGAC GTGACCCCTTTGGACCCAAG
ERG11CH5	5'-end flanking (sense)	CCAACTACAATCGAGTGGAGC
ERG11CH3	N terminus (antisense)	TTCCATTAGTCTGTGGTTTGG

^a Italic letters indicate restriction enzyme sites.

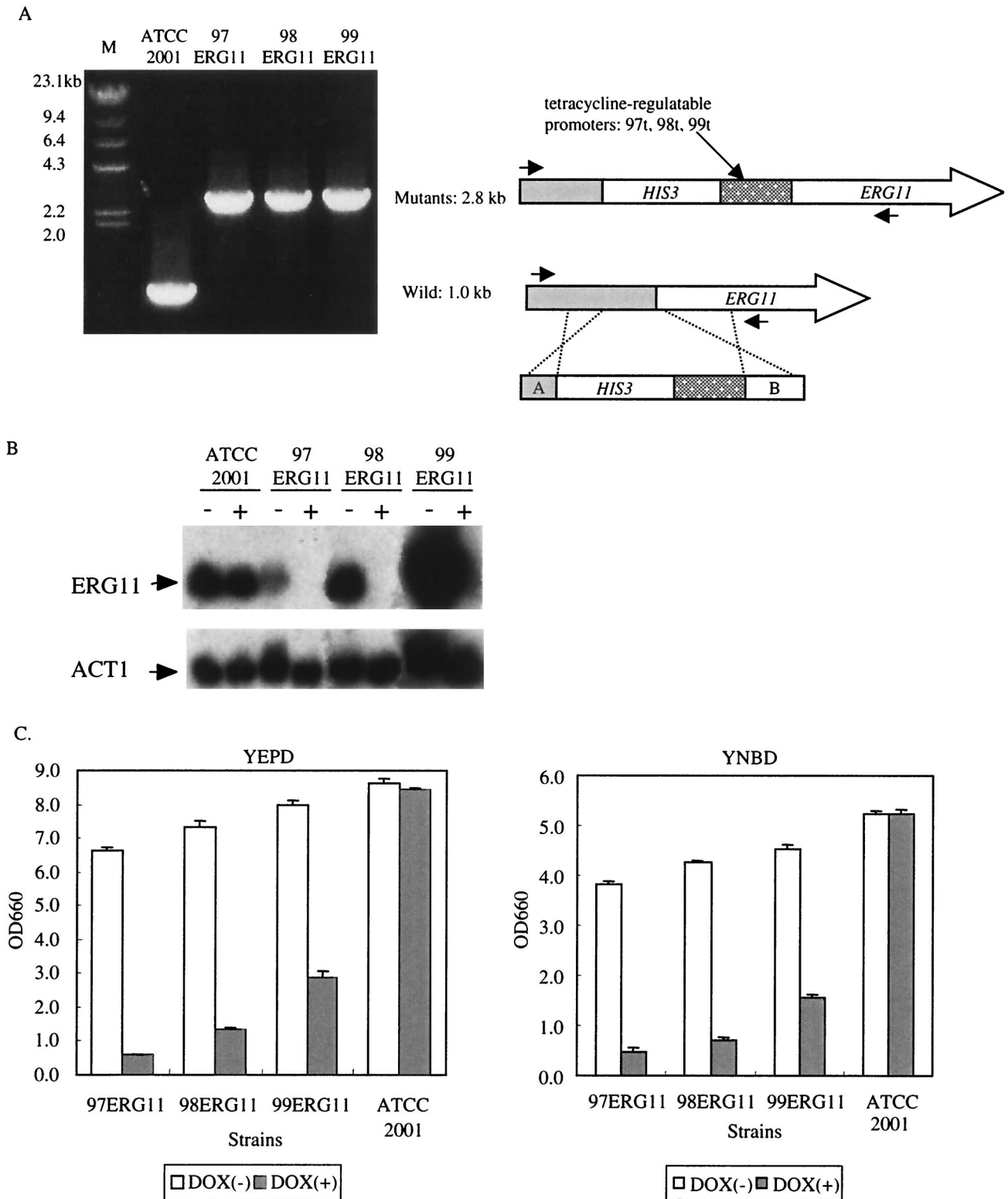


FIG. 1. Effect of overexpression and repression of the *ERG11* gene. (A) Construction of the controllable strains 97ERG11, 98ERG11, and 99ERG11. We generated 97ERG11, 98ERG11, and 99ERG11 by replacing the endogenous *ERG11* promoter with a tetracycline-regulatable promoter as shown on the right. Its replacement was confirmed by PCR (on the left). (B) Northern blot analysis of 97ERG11, 98ERG11, and 99ERG11. The actin gene (*ACT1*) was used as an internal control to quantify the amount of RNAs. Minus signs show total RNA extracted from cells not treated with DOX; plus signs show total RNA extracted from DOX-treated cells. (C) Growth defects of 97ERG11, 98ERG11, and 99ERG11 produced by DOX. 97ERG11, 98ERG11, 99ERG11, and ATCC 2001 cells (10^5 cells each) were inoculated into YEPD medium and cultured at 37°C with or without DOX (10 μ g/ml). The results for three independent experiments were averaged. OD660, optical density at 660 nm.

TABLE 3. Effect of DOX and human serum on sterol composition of controllable strains and ATCC 2001 cells^a

Strain	Incubation time (h)	Medium supplementation			Amt of sterol ($\mu\text{g/g}$ [dry weight] of cell)			
		DOX ($\mu\text{g/ml}$)	Fluconazole ($\mu\text{g/ml}$)	Serum (%)	Cholesterol	Ergosterol	4,14-Dimethyl-zymosterol	Lanosterol
97ERG11	8	0	0	0	0	180.6 \pm 26.2	58.7 \pm 7.0	221.0 \pm 19.7
	8	10	0	0	0	26.1 \pm 3.2	359.8 \pm 33.2	1405.8 \pm 103.2
99ERG11	8	0	0	0	0	963.6 \pm 65.5	42.3 \pm 6.4	25.2 \pm 6.8
	8	10	0	0	0	108.5 \pm 12.3	337.6 \pm 7.2	1201.2 \pm 17.9
98ERG11	8	0	0	0	0	277.5 \pm 25.2	12.1 \pm 2.9	12.1 \pm 3.0
	8	10	0	0	0	33.2 \pm 2.3	376.7 \pm 43.0	1380.1 \pm 100.3
	8	0	0	5	1101.9 \pm 118.9	671.4 \pm 63.9	30.5 \pm 2.4	41.8 \pm 14.4
ATCC 2001	8	0	0	0	0	301.4 \pm 27.9	12.0 \pm 0.6	5.9 \pm 0.8
	8	0	30	0	0	18.3 \pm 1.6	183.7 \pm 16.1	630.8 \pm 46.5
	8	0	0	5	808.1 \pm 43.3	254.5 \pm 16.1	7.9 \pm 2.5	5.0 \pm 2.0
98ERG11	16	0	0	0	0	591.1 \pm 19.5	20.3 \pm 1.4	11.2 \pm 2.1
	16	10	0	0	0	28.1 \pm 2.8	246.8 \pm 9.5	1202.0 \pm 97.9
	16	0	0	5	93.0 \pm 1.5	562.3 \pm 33.1	22.6 \pm 4.9	32.8 \pm 16.2
ATCC 2001	16	10	0	5	388.7 \pm 19.8	45.9 \pm 9.8	187.1 \pm 11.2	906.0 \pm 56.2
	16	0	0	0	0	265.8 \pm 12.3	10.2 \pm 1.0	9.4 \pm 2.2
	16	0	0	5	48.4 \pm 2.4	245.0 \pm 6.3	8.3 \pm 1.5	6.6 \pm 0.6

^a Values are means \pm standard deviations (three independent samples per group).

Fluconazole sensitivity. Approximately 10^5 cells (each) of strains 97ERG11, 98ERG11, and 99ERG11 were inoculated into YEPD medium containing the concentrations of fluconazole indicated in Fig. 5 in the absence or presence of DOX (10 $\mu\text{g/ml}$). After 14 h at 37°C, optical density at 660 nm was determined.

RESULTS

Effect of altering expression of the *ERG11* gene on cell growth. To investigate the effect of deactivating Erg11p on *C. glabrata* growth, we generated three *ERG11*-controllable strains in which the endogenous *ERG11* promoter was replaced with three tetracycline-regulatable promoters, 97t, 98t, and 99t (23), and which were designated 97ERG11, 98ERG11, and 99ERG11, respectively. The correct replacement from the endogenous promoter to the tetracycline-regulatable promoter in each strain was confirmed by PCR (Fig. 1A). Since it had been shown that the promoters 97t, 98t, and 99t have different activities (22, 23), we first compared the expression level of the *ERG11* gene in each controllable strain with that of their isogenic wild-type strain ATCC 2001. Northern analysis showed that the expression level of the *ERG11* gene in the absence of DOX varied among the three controllable strains. The 98ERG11 cells could express the *ERG11* gene at almost the same level as that in ATCC 2001, and the level of 98ERG11 cells was approximately fivefold higher than that of 97ERG11 and was one-fifth lower than that of 99ERG11 (Fig. 1B and see Table 4). We next investigated the effect of DOX on the expression of the *ERG11* gene. The expression of the *ERG11* gene was almost completely repressed in all controllable strains (Fig. 1B). The growth defect was consistently observed in all three controllable strains when they were cultured with DOX both in YEPD and a synthetic medium, yeast nitrogen base and dextrose (YNBD). In both media, 97ERG11 showed a slight growth defect even in the absence of DOX (Fig. 1C and data not shown). Thus, the decrease in *ERG11* gene expression was able to affect growth.

Effect of DOX on sterol composition. We anticipated that altering the sterol composition by deactivating Erg11p resulted in a growth defect, since it was shown that abnormal sterol,

14 α -methylergosta-8,24(28)-dien-3 β ,6 α -diol, was produced in azole-treated *S. cerevisiae* and *C. albicans* and that this accumulation leads to cell death (11, 12). We therefore investigated the sterol compositions of the controllable strains and ATCC 2001 cells in the absence or presence of DOX. As shown in Table 3, in the absence of DOX, the amount of ergosterol correlated with the activity of tetracycline-regulatable promoters and the composition of 98ERG11 resembled that of ATCC 2001. Although the amount of ergosterol in all controllable strains was dramatically reduced in the presence of DOX, the amount of ergosterol in 99ERG11 treated with DOX was obviously much larger than that of 97ERG11 and 98ERG11 treated with DOX, suggesting that the DOX-dependent repression activity of the tetracycline-regulatable promoter 99t was lower than that of 97t or 98t. In addition to decreasing ergosterol, large amounts of lanosterol and 4,14-dimethylzymosterol were accumulated in the strains treated with DOX. Furthermore, even in the absence of DOX, we could also detect 4,14-dimethylzymosterol in 97ERG11, which showed a slight growth defect. Thus, sterol analysis implied that accumulation of the abnormal sterol 4,14-dimethylzymosterol might cause a growth defect, since other sterol fractions excluding these three sterols could not be detected in this analysis. In the absence of DOX, we could also detect this sterol in 99ERG11 without the accumulation of lanosterol, although 99ERG11 could grow as well as ATCC 2001 (data not shown). This result implied that the toxicity of 4,14-dimethylzymosterol can be alleviated by the overproduction of ergosterol and that the accumulation of lanosterol can also participate in a growth defect by DOX.

Effect of DOX on cell viability and sterol composition at several time points. We used 98ERG11 for further experiments since it showed almost the same sterol composition and expression level of the *ERG11* gene in the absence of DOX as ATCC 2001. Sterol analysis implied that DOX-dependent depletion of Erg11p could be complete, as the amount of ergosterol of 98ERG11 treated with DOX in a 16-h culture showed no increase from the amount in an 8-h culture. When we

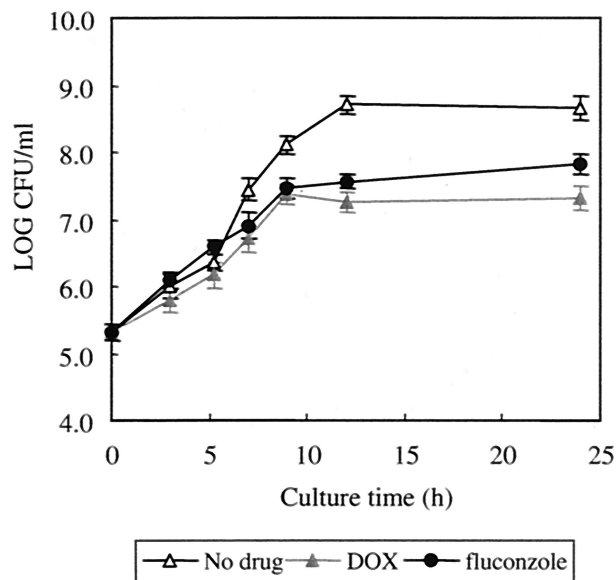


FIG. 2. Effect of DOX or fluconazole on the number of viable cells of 98ERG11. The cells (10^5 each) were cultured with YEPD containing DOX ($10 \mu\text{g/ml}$) or fluconazole ($30 \mu\text{g/ml}$) at 37°C . The cells were harvested and diluted on an agar plate at the indicated times. After a 24-h incubation at 37°C , the number of colonies was counted. Error bars show standard deviations for the averages of the results of three independent experiments.

investigated the number of viable cells cultured with DOX at several time points, the number of CFU of DOX-treated cells was smaller than that of DOX-untreated cells at each time point. The maximum difference was about 1/10. However, the numbers of CFU of 98ERG11 treated with DOX (Fig. 2) in a 9-h culture and a 24-h culture were not significantly different. These results suggest that growth inhibition by diminishing the expression of the *ERG11* gene had reached a plateau.

Investigation of the importance of the *ERG11* gene on cell growth in serum-containing media. It was recently demonstrated that the *C. glabrata* squalene synthase (*ERG9*) gene, which also participates in ergosterol biosynthesis, is not essential for cell growth in either serum-containing media or mice due to the incorporation of exogenous sterol (24). We therefore investigated the importance of the *ERG11* gene for the growth of *C. glabrata* cells in human serum-containing media. As shown in Fig. 3, the addition of serum resulted in the alleviation of the growth defect by DOX in 98ERG11 cells. A growth defect, however, was still observed. We then analyzed the sterol composition of serum-treated 98ERG11 and ATCC 2001 cells after cultivation for 8 and 16 h. Incorporation of cholesterol was observed independent of DOX treatment, although the amount of cholesterol in cells cultured for 16 h decreased compared to that in the cells cultured for 8 h. In 98ERG11 treated with DOX and human serum, the amount of ergosterol was slightly increased compared to that of 98ERG11 treated with DOX at both culturing times. However, the addition of serum could not affect depletion of the *ERG11* gene by DOX, since the amount of ergosterol did not change in 8- or 16-h cultures. In 98ERG11 cultured with DOX and serum, ergosterol slightly increased and 4,14-dimethylzymosterol decreased compared to levels in 98ERG11 treated with DOX

only (Table 3), presumably due to the incorporation of cholesterol from serum. Such a change in sterol composition may result in the alleviation of the growth defect by DOX.

Investigation of the effect of diminishing the expression of the *ERG11* gene on cell growth in mice. As the next step, we investigated the effect of diminishing the expression of the *ERG11* gene on growth in mice. Five hours, 7 days, and 14 days after the infections, we determined the numbers of CFU in mouse kidneys treated with or without DOX (see Materials and Methods). As shown in Fig. 4, the number of CFU of 98ERG11 recovered from DOX-treated mice was not significantly different from that of DOX-untreated mice at each time point (*P* values were 0.329 [5 h], 0.175 [7 days], and 0.169 [14 days]). This result suggested that depleting the *ERG11* gene would not affect cell growth in mice. On the other hand, the number of CFU of ATCC 2001 recovered from DOX-treated mice was higher than that of DOX-untreated mice 14 days after the infections (the *P* value was 0.009). At 7 and 14 days after the infection, the number of CFU of ATCC 2001 recovered from DOX-treated mice was higher than that of CFU of 98ERG11 recovered from both the DOX-treated and untreated mice. Furthermore, the number of CFU of 97ERG9 recovered from DOX-treated mice was higher than that of 97ERG9 recovered from mice not treated with DOX (24; data not shown). Taken together, these results suggested that it might be an indirect effect of DOX that ATCC 2001 in the DOX-treated mice yielded CFU numbers higher than those for the mice not treated with DOX, although we did not rule out the possibility that the difference in numbers of CFU was caused by strain differences. If DOX treatment indirectly decreased the number of CFU of *C. glabrata* in mice, the effect

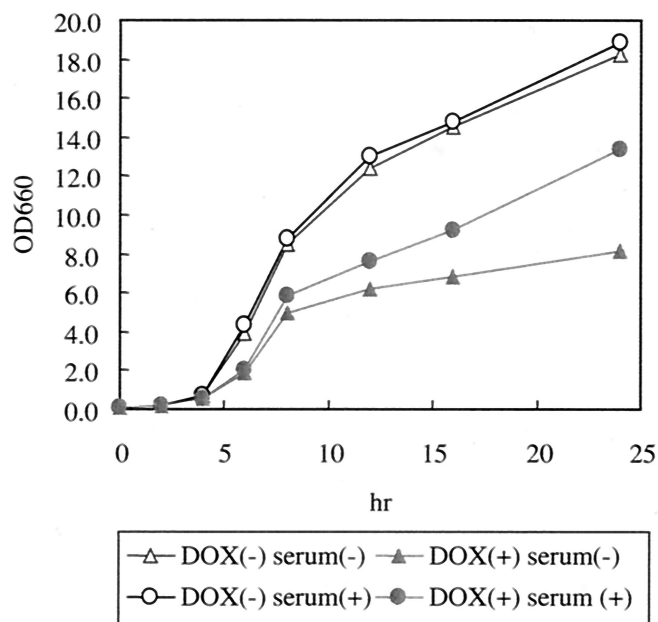


FIG. 3. Alleviation of the growth defect with the addition of serum. 98ERG11 cells (10^6 cells each) were inoculated into YEPD or 5% human serum containing YEPD and cultured with or without DOX ($10 \mu\text{g/ml}$). Optical densities at 660 nm (OD660) were measured at the indicated times. The values are averages of results for two independent experiments.

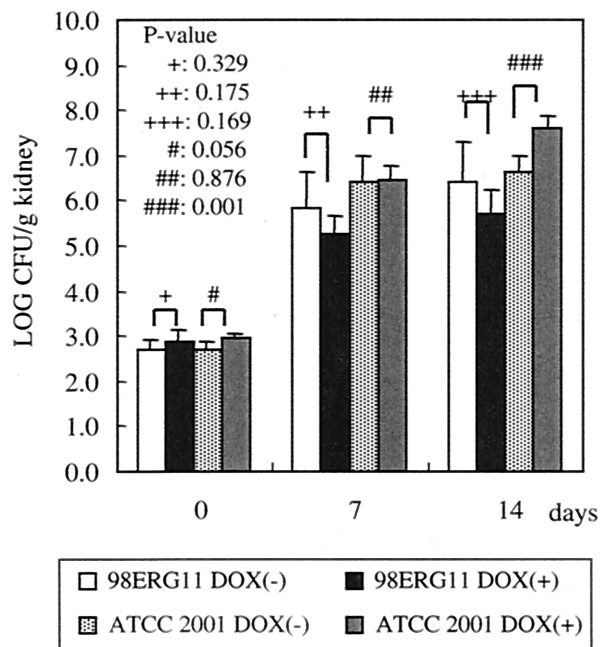


FIG. 4. Effect of DOX on the growth of 98ERG11 and ATCC 2001 in mouse kidneys. The mice, infected with these cells, were sacrificed, and the *C. glabrata* cells in their kidneys were recovered. Each bar represents the average number of cells recovered from five mice. *P* values were calculated by the paired *t* test. The same results were obtained in two independent experiments.

was marginal. Therefore, diminishing the expression of the *ERG11* gene could not result in defective growth in mice.

Effect of altering the expression of the *ERG11* gene on fluconazole sensitivity. Since it was reported that overexpression of the *ERG11* gene conferred fluconazole resistance in *C. albicans* and *S. cerevisiae* (13, 45), we investigated the fluconazole sensitivities of the three controllable strains in the absence of DOX. As shown in Fig. 5A, 99ERG11, in which the *ERG11* gene is overexpressed, was most resistant to fluconazole among the tested strains. On the other hand, 97ERG11 was the most sensitive. Thus, the fluconazole sensitivity of each controllable strain was well correlated to the level of *ERG11* mRNA and the amount of ergosterol (Table 4). We then investigated whether or not a further growth defect by fluconazole was observed in DOX-treated controllable strains. A fluconazole amount below 100 μg per ml could not cause a further growth defect in 97ERG11 and 98ERG11. On the other hand, a further growth defect was observed in 99ERG11 treated with DOX by adding 0.1 μg of fluconazole per ml or more (Fig. 5). However, the percentages of 99ERG11 cells showing growth, which were calculated by dividing the value for the optical density at 660 nm of fluconazole-treated 99ERG11 by that of 99ERG11 not treated with fluconazole, could not be lowered below 50%. The level of growth inhibition of 99ERG11 treated with DOX and fluconazole was the same as that of 97ERG11 or 98ERG11 treated with DOX. These results were reproducibly observed with YNBD and RPMI medium (data not shown). Although we did not mea-

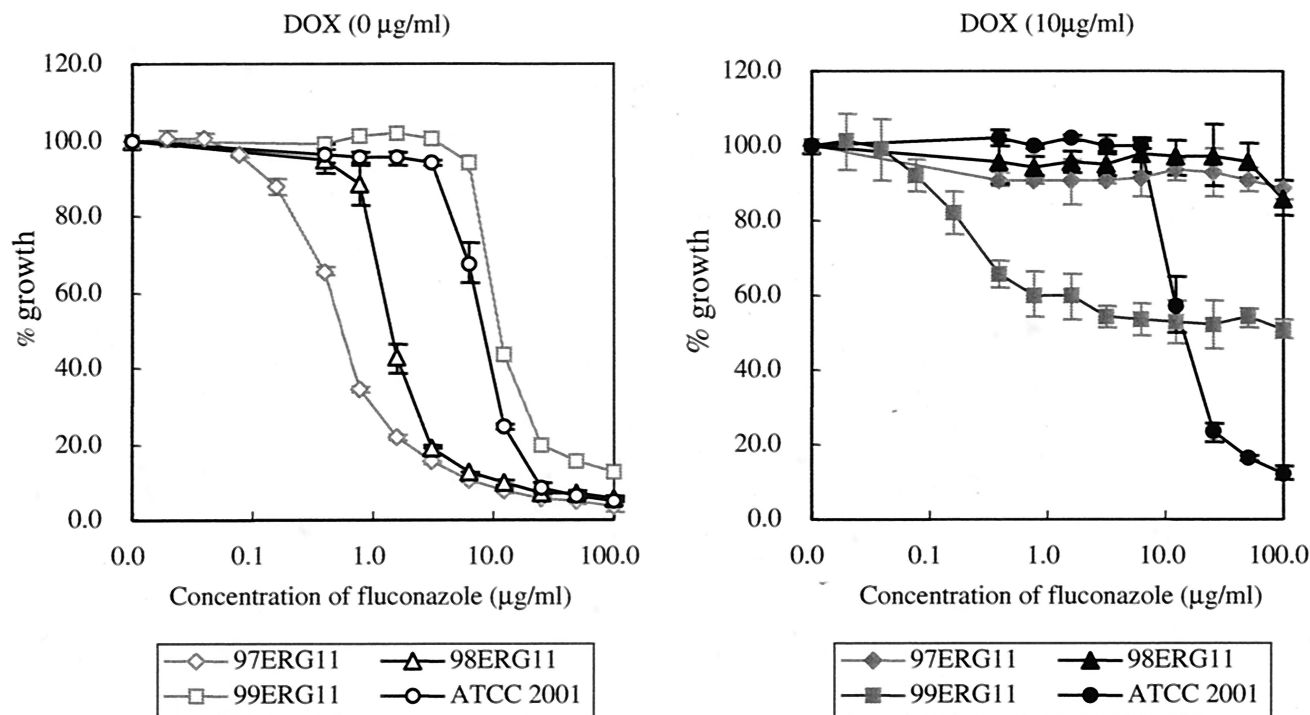


FIG. 5. Fluconazole sensitivity. The controllable strains and ATCC 2001 cells were inoculated into YEPD and were cultured for 14 h with the indicated concentrations of fluconazole at 37°C in the absence (left panel) or presence (right panel) of DOX (10 $\mu\text{g}/\text{ml}$). The y axis shows the growth rate relative to that of each strain without fluconazole treatment. Therefore, each value was calculated by dividing the optical density value of the fluconazole-treated strain with that of the untreated strain. Error bars show standard deviations for the averages of results of three independent experiments.

TABLE 4. Comparison of fluconazole sensitivities and expression levels of the *ERG11* gene in controllable strains

Presence of DOX	Strain	Relative % of mRNA ^a	IC ₅₀ of fluconazole (μg/ml) ^b
Yes	97ERG11	18.9	0.4 (27.0)
	98ERG11	100.0	1.4 (100.0)
	99ERG11	536.6	9.7 (681.2)
No	97ERG11	ND ^c	>100
	98ERG11	ND	>100
	99ERG11	ND	>100

^a Each value shows the percentage of mRNA relative to the amount in 98ERG11 in the absence of DOX after normalization by the ACT1 signal.

^b IC₅₀, 50% inhibitory concentration for the cells not treated with fluconazole. Values in parentheses show percentages of the 50% inhibitory concentration for 98ERG11 in the absence of DOX.

^c ND, not detected.

sure these DOX-dependent levels of depletion at the protein level, these results strongly suggested that the depletion of Erg11p by DOX could be almost complete in 97ERG11 and 98ERG11 but that some amounts of Erg11p remained in 99ERG11. In addition, these results also suggested that the mutants, in which expression of the *ERG11* gene is maintained at a very low level, show fluconazole resistance.

Comparison of fluconazole treatment and DOX-dependent depletion of *ERG11*. Well-correlated fluconazole sensitivity to the expression level of the *ERG11* gene in the controllable strains implied that the results of the depletion of Erg11p could mimic some of the physiological effects of fluconazole treatment. Therefore, we examined whether or not the effects of fluconazole treatment are similar to those of DOX-dependent *ERG11* depletion. When the effect of fluconazole on the growth of 98ERG11 was investigated at several time points, the number of CFU of fluconazole (30 μg/ml)-treated 98ERG11 at each time point was similar to that of 98ERG11 treated with DOX. This result suggested that depletion of the *ERG11* gene by DOX can mimic the growth defect produced by fluconazole with a similar time dependency (Fig. 3). Furthermore, the sterol composition of 98ERG11 treated with DOX resembled that of ATCC 2001 treated with fluconazole (Table 3). The abnormal sterol 4,14-dimethylzymosterol, detected as a unique abnormal sterol, was also detected in the other *C. glabrata* strains treated with fluconazole or voriconazole (14). Thus, the effects of DOX-dependent diminishment of the expression of *ERG11* were quite similar to those of fluconazole treatment.

DISCUSSION

In this report, by using tetracycline-regulatable promoters, we investigated the effect of diminishing the level of expression of the *ERG11* gene on the growth of *C. glabrata* cultured in both in vitro and in vivo settings. Sterol analysis showed that lanosterol and an abnormal sterol, 4,14-dimethylzymosterol, were accumulated by diminishing the expression of the *ERG11* gene by DOX. Consistently, under all tested culture conditions, the growth defect was observed after diminishing the expression of the *ERG11* gene. However, the extent of this growth defect was small: the number of CFU was lowered by only 1/10. More than a few authors have suggested that the accumulation of the abnormal sterol 14α-methylergosta-8,24

(28)-dien-3β,6α-diol leads to cell death in *C. albicans* and *S. cerevisiae* (2, 11, 12, 26, 44). However, as shown here, 4,14-dimethylzymosterol, which accumulated after the level of expression of the *ERG11* gene was diminished, was found to be a unique abnormal sterol in *C. glabrata*. The accumulation of this methylated sterol was observed together with that of other abnormal sterols in *Cryptococcus neoformans* treated with azole antifungals. The treatment with azole antifungals of *C. neoformans* showed that the extent of the growth defect was different from that in *C. glabrata*, presumably due to further alterations of sterol composition compared to that of *C. glabrata* (7, 41). These results suggest that the growth defect by accumulation of 4,14-dimethylzymosterol may be marginal. Since production of 14α-methylergosta-8,24(28)-dien-3β,6α-diol is necessary for further modifications of 4,14-dimethylzymosterol, it is anticipated that *C. glabrata* late genes in the ergosterol biosynthesis pathway cannot recognize 4,14-dimethylzymosterol. This speculation suggests that the late genes in the ergosterol biosynthesis pathway of *C. glabrata* do not resemble those of *C. albicans* or *S. cerevisiae*.

As shown in Fig. 5, the expression level of the *ERG11* gene correlates well with the fluconazole sensitivities of controllable strains. However, fluconazole sensitivities obviously differed between 98ERG11 and ATCC 2001 in spite of showing almost the same expression level of the *ERG11* gene in the absence of DOX. This difference in sensitivity may depend on the kinds of promoters that regulate expression of the *ERG11* gene in each strain. As Henry et al. recently reported (9), the endogenous *ERG11* promoter can be up-regulated by fluconazole treatment, resulting in a two- to fourfold increase in *ERG11* RNA levels. On the other hand, it can be expected that its expression in 98ERG11 cannot be affected by fluconazole treatment since the expression from a tetracycline-regulatable promoter is constitutive.

Sterol analyses suggested new insights into ergosterol biosynthesis regulation. In the absence of DOX, the amount of ergosterol of 98ERG11 cells increased by further culture whereas that of ATCC 2001 was maintained at the same level, suggesting that the Erg11p activity can be down-regulated at a late log phase, at least at a transcriptional level. In addition, the amount of 4,14-dimethylzymosterol of DOX-treated 98ERG11 in a 16-h culture was decreased compared to that in an 8-h culture, suggesting that Erg24p, which can convert from lanosterol to 4,14-dimethylzymosterol, was also down-regulated. With serum-containing medium, the following observations could not correlate with artificial expression or repression of the *ERG11* gene (1). The amount of ergosterol increased when the 98ERG11 cells were cultured with serum, whereas the amount of ergosterol in ATCC 2001 was not altered (2). In spite of continuous depletion of the *ERG11* gene, the amount of ergosterol in 98ERG11 treated with DOX and human serum was slightly increased compared to that in 98ERG11 treated with DOX. Although further studies are needed, these results suggested that *C. glabrata* cells could also incorporate precursors of ergosterol, such as episterol, fecosterol, lanosterol, and squalene, from serum.

Depletion of the level of expression of the *ERG11* gene could not be a direct corollary of the result of fluconazole treatment since many other factors, such as export via efflux pumps, come into play with regard to the effect of fluconazole

treatment on cell growth. However, the physiological effects of DOX-dependent diminishment of the expression of the *ERG11* gene were quite similar to those of fluconazole treatment, as far as was investigated in this study. Furthermore, it has been reported that the pattern of expression observed after treatment with fluconazole most closely resembles that seen after *ERG11* inhibition in *S. cerevisiae* (31). Therefore, the efficacy of fluconazole against *C. glabrata* infection might be estimated by our model, namely, depletion of the level of *ERG11* expression by the tetracycline-regulatable promoter. As shown in this study, the *ERG11* gene is not an ideal target molecule of antifungals for *C. glabrata* infection, presumably because diminishment of its level of expression results in the accumulation of a unique abnormal sterol, 4,14-dimethylzymosterol, which might not be highly toxic. This conclusion supports evidence of an intrinsic resistance of *C. glabrata* to fluconazole, although multiple mechanisms of azole resistance are suggested.

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