



Loss of C-5 Sterol Desaturase Activity in *Candida albicans*: Azole Resistance or Merely Trailing Growth?

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ABSTRACT Increased expression of drug efflux pumps and changes in the target enzyme Erg11p are known to contribute to azole resistance in *Candida albicans*, one of the most prevalent fungal pathogens. Mutations that inactivate *ERG3*, which encodes sterol $\Delta^{5,6}$ -desaturase, also confer *in vitro* azole resistance. However, it is unclear whether the loss of Erg3p activity is sufficient to confer resistance within the mammalian host, and relatively few *erg3* mutants have been reported among azole-resistant clinical isolates. Trailing growth (residual growth in the presence of the azoles) is a phenotype observed with many *C. albicans* isolates and, in its extreme form, can be mistaken for resistance. The purpose of this study was to determine whether the growth of Erg3p-deficient *C. albicans* mutants in the presence of the azoles possesses the characteristics of azole resistance or of an exaggerated form of trailing growth. Our results demonstrate that, similar to trailing isolates, the capacity of an *erg3* Δ/Δ mutant to endure the consequences of azole exposure is at least partly dependent on both temperature and pH. This contrasts with true azole resistance that results from enhanced drug efflux and/or changes in the target enzyme. The *erg3* Δ/Δ mutant and trailing isolates also appear to sustain significant membrane damage upon azole treatment, further distinguishing them from resistant isolates. However, the insensitivity of the *erg3* Δ/Δ mutant to azoles is unaffected by the calcineurin inhibitor cyclosporin A, distinguishing it from trailing isolates. In conclusion, the *erg3* mutant phenotype is qualitatively and quantitatively distinct from both azole resistance and trailing growth.

KEYWORDS *Candida albicans*, *ERG3*, azoles, resistance, trailing growth

Mortality rates associated with invasive fungal infections (IFIs) remain alarmingly high, despite the availability of three major classes of antifungal drugs (1). The azoles remain the most widely used antifungal treatments, acting through inhibition of lanosterol demethylase (Erg11p), an enzyme required for ergosterol biosynthesis. Depletion of cellular ergosterol and the accumulation of abnormal sterol species, such as 14 α -methylergosta-8,24(28)-dien-3 β ,6 α -diol, are both thought to cause plasma membrane dysfunction and ultimately to arrest fungal growth following azole treatment (2). In *Candida albicans*, one of the most prevalent fungal pathogens, several well-described mechanisms are known to contribute to azole resistance, including increased expression of drug efflux pumps belonging to the major facilitator and ABC transporter superfamilies, as well as the target enzyme (Erg11p) itself (3, 4). Mutations that reduce the azole binding affinity of the target enzyme are also important determinants of susceptibility (5–7). However, a combination of the aforementioned mechanisms is usually necessary to confer clinically relevant levels of azole resistance (3, 4, 8, 9).

A less common mechanism of azole resistance arises through inactivation of sterol $\Delta^{5,6}$ -desaturase (Erg3p), the enzyme responsible for converting the lanosterol/14 α -methylfecosterol that accumulates upon azole-mediated inhibition of Erg11p into the toxic diol species (2). As a result, *ERG3*-deficient mutants of *C. albicans* accumulate

Citation Luna-Tapia A, Butts A, Palmer GE. 2019. Loss of C-5 sterol desaturase activity in *Candida albicans*: azole resistance or merely trailing growth? *Antimicrob Agents Chemother* 63:e01337-18. <https://doi.org/10.1128/AAC.01337-18>.

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Received 25 June 2018

Returned for modification 25 July 2018

Accepted 10 October 2018

Accepted manuscript posted online 15 October 2018

Published 21 December 2018

14 α -methylfecosterol instead of 14 α -methylergosta-8,24(28)-dien-3 β ,6 α -diol following azole treatment, which is apparently compatible with continued growth (2). In contrast to the aforementioned resistance mechanisms, inactivation of the *ERG3* gene results in complete azole insensitivity in a single step. Curiously, while azole-resistant *erg3* mutants can be readily selected under *in vitro* conditions (10), they are detected only rarely among azole-resistant clinical isolates (2, 11–13). Furthermore, Miyazaki et al. reported that, while an *erg3* Δ/Δ mutant was apparently azole resistant *in vitro*, it was susceptible in a mouse model of disseminated candidiasis (14). Thus, while loss of Erg3p activity enables *C. albicans* to grow in the presence of the azoles *in vitro*, the contribution of this mechanism to azole resistance in a clinical sense is less certain. Trailing growth is a phenotype observed in a significant proportion of *C. albicans* isolates, and it can be easily mistaken for azole resistance (15). Using the standard CLSI broth microdilution susceptibility testing method, trailing isolates typically appear azole susceptible after 24 h of incubation, but continued growth makes them appear resistant by the 48-h time point. Nonetheless, patients and experimental animals infected with trailing isolates generally respond well to treatment with the azoles (16, 17). The purpose of this study was to determine whether the continued growth of Erg3p-deficient *C. albicans* mutants in the presence of the azoles represents true azole resistance or resembles an exaggerated form of the trailing growth phenotype.

RESULTS

A *C. albicans* *erg3* Δ/Δ mutant exhibits characteristics of both azole resistance and trailing growth. In this study, we used a previously reported *C. albicans* *erg3* Δ/Δ mutant and its isogenic control strain, in which a wild-type (WT) copy of *ERG3* has been reintroduced (18). These strains were compared with the well-characterized azole-resistant clinical isolate TW17, which overexpresses the Mdr1p and Cdr1p efflux pumps, as well as a variant of the target enzyme Erg11p with reduced affinity for azoles (4). Additional reference strains included the azole-susceptible isolate SC5314 and several other *C. albicans* clinical isolates that exhibit either trailing growth or azole resistance. We also constructed a panel of isogenic strains, each possessing a single mechanism known to confer azole resistance, specifically (i) overexpression of the Cdr1p azole efflux pump (strain OCDR), (ii) overexpression of the Mdr1p azole efflux pump (strain OMDR), and (iii) overexpression of the azole target protein, Erg11p (strain OERG), as well as a strain overexpressing all three proteins (strain OTR). Overexpression of *MDR1*, *CDR1*, and *ERG11* was confirmed by quantitative real-time PCR (qRT-PCR) (see Fig. S1A in the supplemental material).

We initially compared the fluconazole susceptibility of each isolate using the standard CLSI broth microdilution protocol (19), and we quantified fungal growth at each drug concentration as the optical density at 600 nm (OD_{600}). As expected, the *erg3* Δ/Δ mutant was apparently insensitive to fluconazole at either the 24-h or 48-h time point (Fig. 1A and B), with a growth profile similar to that of the azole-resistant isolate TW17. One notable difference was that TW17 growth was partly suppressed at very high fluconazole concentrations (64 μ g/ml), whereas growth of the *erg3* Δ/Δ mutant was unaffected. A different response was seen for SC5314 and the JS7, JS17, JS22, and JS23 clinical isolates, each of which exhibited a low fluconazole MIC (≤ 0.5 μ g/ml) but exhibited significant and reproducible levels of residual growth at supra-inhibitory concentrations, at both 24 and 48 h (Fig. 2A and B). Therefore, we classified these as trailing isolates. A third group of isolates (JS28, JS29, and JS40) had intermediate MICs (8 to 32 μ g/ml) and were classified as resistant, according to the CLSI guidelines (19). The OCDR, OMDR, and OERG strains had 4- to 16-fold increases in fluconazole MICs, compared with the isogenic control, while the OTR strain had a MIC approximately 128-fold greater than that of the control strain (Fig. S1B and C). Interestingly, both the OCDR and OTR strains had noticeably more trailing growth at supra-MIC concentrations of fluconazole than did the control strain (Fig. S1B and C), possibly indicating a relationship between elevated *CDR1* expression and trailing growth.

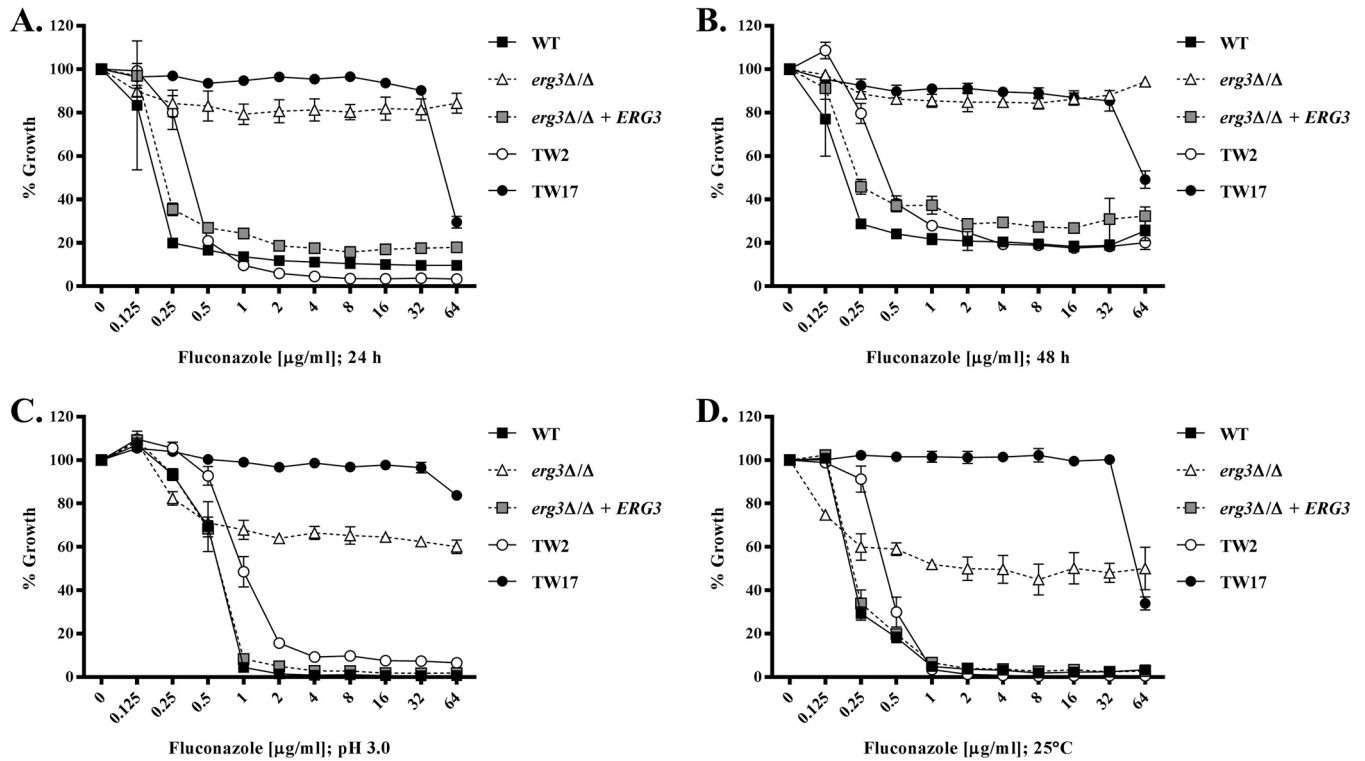


FIG 1 A *Candida albicans* *erg3Δ/Δ* mutant displays features of both azole resistance and trailing growth. (A and B) The fluconazole susceptibilities of a WT strain (GP1), the *erg3Δ/Δ* mutant, *erg3Δ/Δ + ERG3*, and a matched pair of azole-susceptible and azole-resistant clinical isolates (TW2 and TW17) were evaluated using the CLSI broth microdilution protocol; growth was measured as the OD₆₀₀ after 24 h (A) and 48 h (B) of incubation, and results are expressed as a percentage of the growth in the no-drug (DMSO alone) control wells for each strain. (C and D) The same experiment was also conducted with RPMI 1640 medium adjusted to pH 3.0 (C) or with incubation at 25°C instead of 35°C (D), and growth was assessed after 48 h of incubation. Data in all panels are the means ± standard deviations of three biological replicates.

It was shown previously that acidifying the growth medium or reducing the incubation temperature was sufficient to eliminate trailing growth but not true azole resistance (20). Reducing the pH of the RPMI 1640 medium to 3.0 did not dramatically affect the MIC for any of the clinical *C. albicans* isolates but significantly reduced the trailing growth observed with isolates SC5314, JS7, JS17, JS22, and JS23 (Fig. 2C). Similarly, although there was a slight increase (2- to 4-fold) in the MIC for each strain, the relative susceptibilities of the OCDR, OMDR, OERG, and OTR strains were unaltered in the acidified medium (Fig. S1D) but all trailing growth was eliminated. Almost identical results were observed with reduction of the incubation temperature from 35°C to 25°C, in that MICs were not substantially affected but trailing growth was reduced or eliminated (Fig. 1D and 2D; also see Fig. S1E). Interestingly, in acidified medium, fluconazole was able to inhibit the growth of the *erg3Δ/Δ* mutant to a measurable degree at all concentrations $\geq 0.5 \mu\text{g/ml}$, which dramatically reduced the MIC (Fig. 1C). However, the mutant maintained extremely high levels of residual growth at suprainhibitory concentrations of fluconazole, a phenotype resembling the trailing growth phenotype normally observed at pH 7. Similarly, the MIC for the *erg3Δ/Δ* mutant also decreased substantively at 25°C but, in contrast to the trailing isolates, the mutant sustained very high levels of residual growth at suprainhibitory concentrations of fluconazole. Thus, while the *erg3Δ/Δ* mutant appears fully resistant to fluconazole under the conditions of the standard CLSI susceptibility testing protocol, its capacity for continued growth is partly dependent on the specific culture conditions used. Similar results were found for several clinically derived Erg3p-deficient mutants (11), for which growth in the presence of fluconazole is also partly temperature and pH dependent (Fig. 3). In this respect, the continued growth of the *erg3* mutants in the presence of fluconazole shares at least some characteristics with the trailing growth phenomenon.

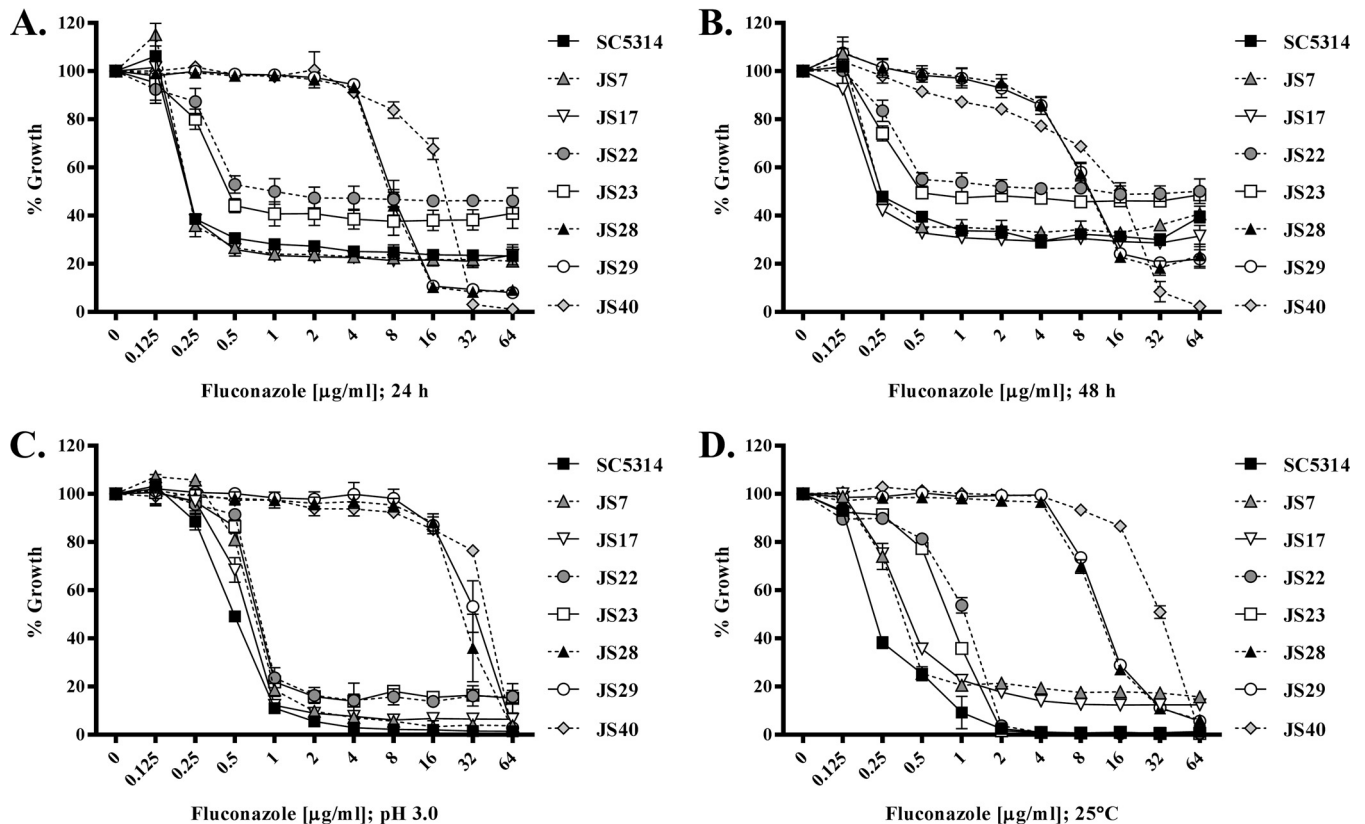


FIG 2 Trailing growth but not azole resistance is eliminated by adjusting the incubation temperature or the pH of the medium. (A and B) The fluconazole susceptibilities of the WT isolate SC5314 and a set of clinical isolates (JS strains) were evaluated using the CLSI broth microdilution protocol; growth was measured as the OD_{600} after 24 h (A) and 48 h (B) of incubation, and results are expressed as a percentage of the growth in the no-drug (DMSO alone) control wells for each strain. (C and D) The same experiment was also conducted with RPMI 1640 medium adjusted to pH 3.0 (C) or with incubation at 25°C (D), and growth was assessed after 48 h of incubation. The means \pm standard deviations of three biological replicates are indicated for all panels.

However, the phenotype of the *erg3* Δ/Δ mutant is distinct from that of the trailing growth isolates, as the growth observed in the presence of fluconazole is substantially more robust under all conditions tested. The *erg3* phenotype also contrasts with that of the truly resistant isolates TW17, OTR, JS28, JS29, and JS40, for which growth in the presence of fluconazole is largely independent of the culture conditions used.

Finally, the azole antifungals are fungistatic rather than fungicidal, with *C. albicans* survival in the presence of growth inhibitory concentrations of fluconazole depending on signaling through the calcineurin phosphatase (21–23). Inhibition of the calcineurin pathway with cyclosporine A thus renders the azoles fungicidal and eliminates trailing growth (21–23). Therefore, we determined whether the capacity of the *erg3* Δ/Δ mutant to grow in the presence of fluconazole was affected by cyclosporine A. While cyclosporine A did not significantly alter the MIC for any *C. albicans* strain, it eradicated the trailing growth observed previously for SC5314, JS7, JS17, JS22, and JS23 (Fig. 4B; also see Fig. S2B). These data confirm that, while trailing growth is dependent on calcineurin signaling, true azole resistance is not. Cyclosporin A treatment was not sufficient to restore fluconazole sensitivity or to reduce the growth of the *erg3* Δ/Δ mutant in the presence of fluconazole (Fig. 4A; also see Fig. S2A). This capacity to survive and to proliferate in the absence of calcineurin signaling is consistent with true azole resistance, rather than an exaggerated form of the trailing growth phenotype. Thus, while the *erg3* Δ/Δ mutant shares at least some characteristics with the trailing growth phenomenon, other characteristics more closely resemble true azole resistance.

The *C. albicans* *erg3* Δ/Δ mutant sustains significant membrane damage upon fluconazole treatment. We next compared the amount of membrane damage sustained upon azole treatment. A cytoplasmic version of NanoLuc luciferase (Nluc)

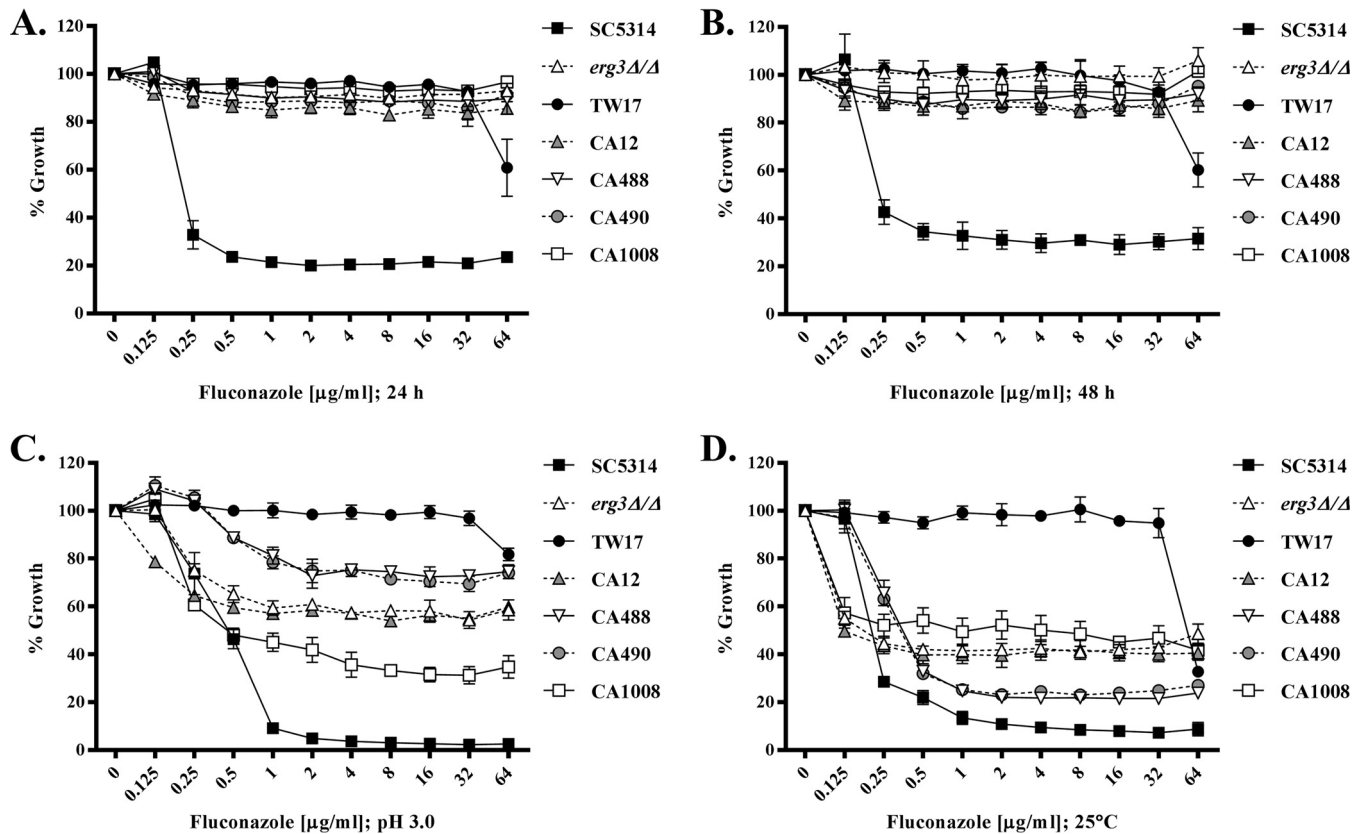


FIG 3 The growth of *Candida albicans* Erg3p-deficient clinical isolates in the presence of fluconazole is partly temperature and pH dependent. (A and B) The fluconazole susceptibilities of *C. albicans* SC5314, the *erg3Δ/Δ* mutant, the azole-resistant isolate TW17, and the previously reported Erg3p-deficient clinical isolates CA12, CA1008, CA488, and CA490 were compared using the CLSI broth microdilution protocol; growth was quantified as the OD₆₀₀ after 24 h (A) and 48 h (B) of growth, and results are expressed as a percentage of the no-drug (DMSO alone) control wells. (C and D) The same experiment was conducted with RPMI 1640 medium adjusted to pH 3.0 (C) or with incubation at 25°C (D), and growth was assessed after 48 h. The means ± standard deviations of three biological replicates are shown for each panel.

(Promega Corp.) (24) was expressed in the *erg3Δ/Δ* mutant and reference strains, so that Nluc release into the culture supernatant could be used to detect loss of plasma membrane integrity (Fig. 5). Substantial amounts of Nluc activity were detected in the culture supernatant of the WT reference strain SC5314 after treatment with 5 µg/ml

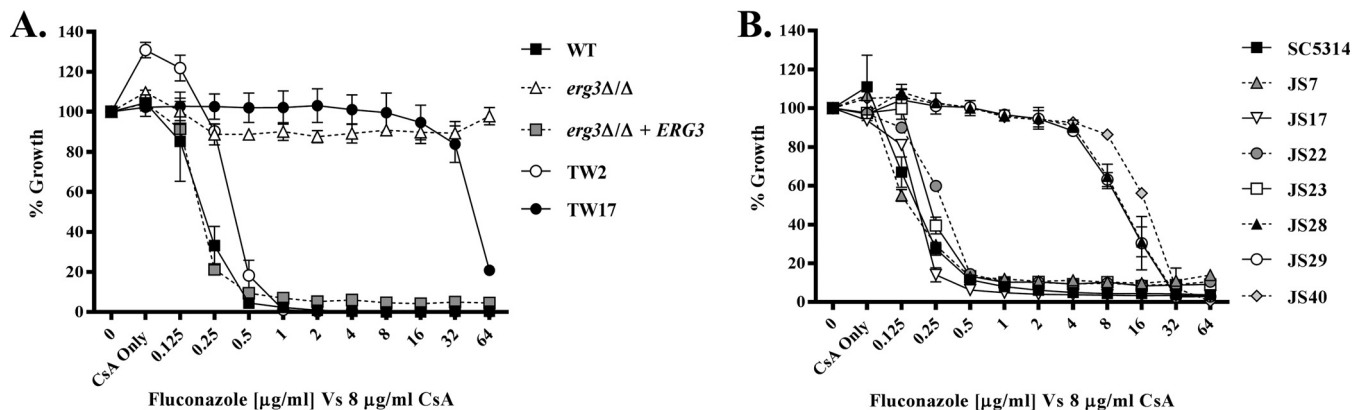


FIG 4 Growth of the *Candida albicans* *erg3Δ/Δ* mutant in the presence of fluconazole is not dependent on calcineurin signaling. (A) The growth of the WT strain (GP1), the *erg3Δ/Δ* mutant, and a pair of matched azole-susceptible and azole-resistant clinical isolates (TW2 and TW17) was compared in fluconazole dose-response experiments in the absence or presence of 8 µg/ml cyclosporine A (CsA), according to the CLSI broth microdilution protocol. The growth of each strain was measured as the OD₆₀₀ after 48 h, and results are expressed as a percentage of growth in the no-drug (DMSO) control wells. (B) The same experiment was performed with strain SC5314 and a set of clinical isolates (JS strains). The means ± standard deviations of three biological replicates are shown in each panel.

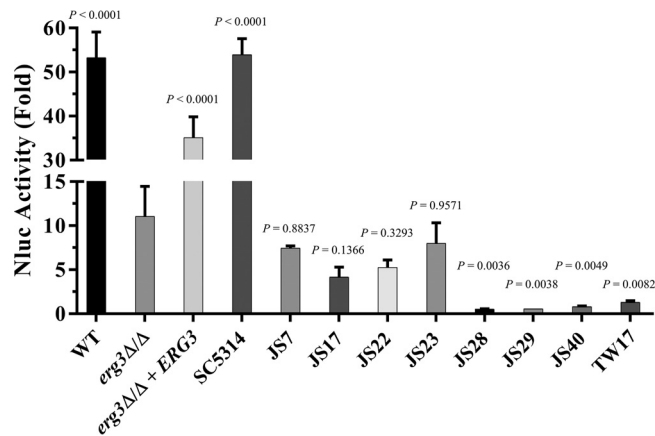


FIG 5 The *Candida albicans* *erg3Δ/Δ* mutant sustains membrane damage following fluconazole treatment. The *Candida albicans* WT strain (GP1), the *erg3Δ/Δ* mutant, *erg3Δ/Δ + ERG3*, a group of trailing isolates (SC5314, JS7, JS17, JS22, and JS23), and a set of azole-resistant isolates (JS28, JS29, JS40, and TW17) were transformed with plasmid pDUPNluc. The resulting strains were then grown in 96-well plates in YNB medium with 5 μ g/ml fluconazole or 0.5% DMSO (no-drug control). After 24 h of incubation at 35°C, culture supernatant was removed from each well and Nluc activity was measured using a luminescent substrate. Nluc activity was normalized with respect to growth (OD_{600}), and results are expressed relative to the no-drug controls for each strain. The means \pm standard deviations for three independent transformants of each strain are shown. The means for the experimental groups were compared using one-way analysis of variance and Tukey's multiple-comparison test. The *P* values for each strain, compared to the *erg3Δ/Δ* mutant, are indicated.

fluconazole for 24 h at 35°C (~50- to 60-fold increases over untreated cells) (Fig. 5), indicating loss of plasma membrane integrity. More modest amounts of Nluc were released from the trailing isolates JS7, JS17, JS22, and JS23 (~5- to 10-fold increases over untreated control cells), while no increase in Nluc release was detected for the resistant isolates JS28, JS29, JS40, and TW17. Interestingly, upon fluconazole exposure, the *erg3Δ/Δ* mutant released significantly more Nluc than the resistant isolates and in fact released levels similar to those of the trailing isolates. Nonetheless, the amount of Nluc released was substantially less than those for the isogenic *ERG3*⁺ revertant strain, the WT reference strain (GP1), and the corresponding clinical isolate from which all of these laboratory-derived strains were ultimately derived (SC5314). Thus, while the *erg3Δ/Δ* mutant tolerates fluconazole treatment better than the WT strain, it is not unaffected. In conclusion, these data confirm that the ability of the *erg3Δ/Δ* mutant to endure the consequences of azole exposure is qualitatively and quantitatively distinct from true azole resistance conferred by enhanced drug efflux or changes in the target enzyme Erg11p.

DISCUSSION

As various research groups and different fields of microbiology use the terms drug "resistance" and "tolerance" inconsistently, or even interchangeably, we will use the following definitions based on the underlying molecular mechanisms. On a molecular level, resistance implies that a drug is unable to engage or to modulate sufficiently the activity of its target protein and thus is ineffective in inhibiting microbial growth. In contrast, drug tolerance implies that a microbe is able to endure the consequences of a drug acting upon its target, and thus it can survive and/or proliferate in the presence of that drug. According to these definitions, drug efflux mechanisms (which prevent the azoles from accessing their intracellular target), elevated target protein expression, and mutations that reduce azole binding or inhibition of Erg11p all represent mechanisms of drug resistance. However, the residual growth observed with trailing isolates should be considered a form of azole tolerance, as the isolates are able to survive and to proliferate in the presence of the fungistatic azoles despite Erg11p inhibition (15, 25, 26). In this context, loss of Erg3p activity should also be considered a form of azole tolerance, as the azoles engage and inhibit Erg11p in both the WT strain and *erg3* null mutants, altering the sterol composition of both. The implications of these distinctions

become apparent when the physiological consequences are considered. As reported by others, we find that changing the temperature or pH under which the CLSI assay is conducted is sufficient to eliminate the trailing growth observed with the azoles. Thus, while trailing isolates are to some degree able to proliferate in the presence of the azoles under standard conditions, the physiological consequences of ergosterol depletion and the accumulation of abnormal sterols can be more severe when the culture conditions are changed. Trailing growth also depends on the induction of stress responses that are activated by calcineurin (23). In contrast, azole resistance conferred by increased efflux pump or target protein expression is independent of the culture conditions and calcineurin signaling. The results of this study underscore the fact that, while Erg3p-deficient mutants appear completely insensitive to the azoles in standard MIC assays, there are significant physiological consequences that compromise membrane integrity and function. Specifically, following azole-mediated inhibition of Erg11p activity, *erg3* null mutants accumulate 14 α -methylfecosterol instead of 14 α -methylergosta-8,24(28)-dien-3 β ,6 α -diol, which accumulates in the WT strain (2, 11, 27). While 14 α -methylfecosterol can apparently provide adequate function to support full growth under the conditions of the standard CLSI assay (19), the level of dysfunction is sufficient to impinge upon growth rates when the conditions (e.g., pH or temperature) are altered. Unlike many of the trailing isolates, however, *erg3* null mutants are able to persist and to proliferate to a significant cell density in the presence of fluconazole under all conditions tested. Also, in contrast to trailing isolates, the capacity of *erg3* mutants to proliferate in the presence of the azoles does not depend upon calcineurin signaling. Finally, the Nluc release assay revealed that *C. albicans erg3* null mutants are not invulnerable to azole treatment but sustain substantively less membrane damage than the WT strain. We reported previously that azole exposure leads to fragmentation of the fungal vacuole, a large acidified intracellular organelle (25). Azole treatment also induces vacuolar fragmentation in the *C. albicans erg3 Δ/Δ mutant, but to a much lesser extent than for the *ERG3*⁺ control strains (Fig. S3). These observations confirm that the level of membrane dysfunction resulting from azole-induced shifts in the cellular sterol content is less severe in *erg3* null mutants than *ERG3*⁺ strains. In summary, *C. albicans erg3* null mutants do not completely conform to the characteristics of either trailing growth or true azole resistance, possessing some qualities of both phenotypes.*

Of course, from the perspective of clinicians, the only pertinent point is whether a patient will respond to treatment with an azole antifungal. When interpreted in this light, the results of this study may be revealing. It has been well established that elevated expression of drug efflux pumps and Erg11p contributes to azole resistance and treatment failure in clinical settings (4). In contrast, most *C. albicans* isolates that exhibit trailing growth have been associated with favorable outcomes following treatment with azoles (28). In a mouse model of disseminated infection, *C. albicans* trailing isolates were reported to be susceptible to fluconazole treatment (17, 26). In addition, we recently reported that a *C. albicans* endosomal trafficking mutant that exhibited significant levels of trailing growth *in vitro* was susceptible to fluconazole treatment in a mouse model of vaginal candidiasis (16).

However, a recent study suggested that the trailing phenotype results from the slow growth of subpopulations of cells that are able to endure the deleterious effects of drug treatment, which may have implications in clinical settings (29). Clinical isolates from patients with persistent candidemia were also found to have higher levels of trailing than those readily cleared by azole treatment. The repeated isolation of trailing isolates from patients with oropharyngeal candidiasis following treatment with azoles could also indicate that such isolates are more difficult to eradicate from infected patients than are nontrailing isolates (28).

Similarly, the relative importance of *ERG3*-inactivating mutations in azole resistance in clinical settings has been difficult to determine. It is clear that, while *erg3* mutants can be readily selected *in vitro* following exposure to azoles (10, 30, 31), relatively few have been reported among azole-resistant clinical isolates. Loss of Erg3p activity itself blocks ergosterol biosynthesis, resulting in membrane abnormalities, hypersensitivity to some

physiological stresses, defective hyphal growth, and reduced virulence in a mouse model of disseminated infection (13, 14, 18, 32). The resulting reduction in pathogenic fitness may result in the rapid elimination of *erg3* mutants from infected patients, effectively imposing a counter-selective pressure against *ERG3* inactivation. In a recent study, we reported that, despite severe defects in virulence (i.e. an inability to cause lethality), an *erg3Δ/Δ* deletion mutant was relatively resistant to fluconazole treatment in a mouse model of disseminated infection, as treatment did not significantly reduce levels of *erg3Δ/Δ* colonization of kidney tissue. Strikingly, in a mouse model of vaginal candidiasis, the *erg3Δ/Δ* mutant not only was able to readily colonize and to induce pathology but also was resistant to fluconazole treatment (18). These findings clearly confirm that loss of Erg3p activity does result in azole resistance within the mammalian host, although it also results in niche or context-specific defects in pathogenicity. Importantly, our previous study used immunocompetent mice for the model of disseminated infection, and it is possible that the virulence of the *erg3Δ/Δ* mutant would be greater in immunosuppressed mice, i.e., in the absence of stress imposed by the immune defenses of the mammalian host. If this proved to be the case, then it would imply that *erg3* mutants not only are azole resistant but also are able to cause productive infections in patients at greatest risk of IFIs, namely, those with profound immune dysfunction. To date, just one study has used immunosuppressed mice to test the virulence of *erg3* mutants (13). The study revealed that such mutants appeared to have attenuated virulence. However, other genetic factors unrelated to Erg3p dysfunction could not be ruled out as the cause of the virulence defects, since isogenic strains complemented with a WT *ERG3* allele were not available for comparison. Moreover, the azole susceptibility of those strains was not tested in that model of infection (13). Either way, our previous *in vivo* data support the idea that loss of Erg3p activity is sufficient to confer resistance to the azole antifungals within the mammalian host and, by inference, patients (18).

The low frequency with which *erg3* azole-resistant mutants have been reported to date likely reflects several factors. First, diminished virulence of *erg3* null mutants has been observed (see above). Second, while loss of Erg3p function seems to afford some protection against the antifungal activity of azoles, it is less than that conferred by enhanced drug efflux and/or changes in the target protein. Specifically, *erg3* null mutants are not invulnerable to the physiological consequences of azole exposure and in fact sustain significant membrane damage upon treatment. Third, the frequency of Erg3p-deficient clinical isolates may be underestimated, as a detailed biochemical analysis of cellular sterol contents is needed to confirm deficiencies in C-5 desaturase activity, and this is not routinely performed. Furthermore, most reports to date have focused exclusively on the analysis of clinical isolates with mutations that completely inactivate the *ERG3* gene. However, relatively small adjustments in Erg3p activity may be sufficient to confer azole resistance (18), and such subtle changes are difficult to detect, even through analysis of cellular sterol contents.

In summary, our results establish that, while loss of C-5 sterol desaturase activity enables *C. albicans* to grow in the presence of the azole antifungals, this phenotype is both qualitatively and quantitatively distinct from azole resistance conferred by enhanced drug efflux or changes in the target protein, Erg11p. Our results also clearly distinguish the *erg3* mutant phenotype from the trailing growth phenomenon.

MATERIALS AND METHODS

Growth conditions. *C. albicans* was routinely grown at 30°C on YPD medium (1% yeast extract, 2% peptone, 2% dextrose), supplemented with uridine (50 μg/ml) when necessary. Transformant selection was carried out on minimal YNB medium (6.75 g/liter yeast nitrogen base without amino acids, 2% dextrose, 2% Bacto agar), supplemented with the appropriate auxotrophic requirements described for *S. cerevisiae* (33) or 50 μg/ml uridine.

Plasmid construction. All oligonucleotides used in this study are listed in Table S1 in the supplemental material. Plasmids pKE1 (34), pKE1Nluc (35), pKE4 (16), pKE4CDR1 (16), pAR8 (36), pAR8-ERG11 (36), pKE1:GFP-YPT72 (25), and pDUP3 (37) were described previously.

pGHT3 was produced by amplifying the *TEF1-MCS-ADH1* 3'UTR (where UTR stands for "untranslated region" and MCS stands for "multiple cloning site") cassette from pKE4 using the primer pair TEF1prF-

BamHI and ADH1-3'UTRR2-Apal and was cloned into the BamHI and Apal restriction sites of pGEMHIS1. For construction of the plasmid pGHT-MDR1, the *MDR1* open reading frame (ORF) was amplified from SC5314 genomic DNA with HiFi Platinum *Taq* (Invitrogen) and the primer pair MDR1ORFF-EagI and MDR1ORFR-MluI and then was cloned between the EagI and MluI restriction sites of pGHT3. Plasmid pDUPNluc was produced by amplifying the *ACT1* promoter, the *NLUC* ORF, and the *ADH1* 3' UTR from pKE1Nluc using the primer pair ACT1prom-Clal-F and ADH1term-Spel-R and was cloned into pDUP3 using the Clal and Spel restriction sites.

C. albicans strains. All strains used in this study are listed in Table S2. The *erg3Δ/Δ* mutant was constructed in a previous study (18). Transformation of *C. albicans* with DNA constructs was performed using the lithium acetate method (38). Gene deletion strains were constructed with the PCR-based approach described by Wilson et al. (39), using the *ura3Δ/Δ his1Δ/Δ arg4Δ/Δ* strain BWP17 (kindly provided by Aaron Mitchell, Carnegie Mellon University). Strains TW2 and TW17 (4) were kindly provided by Theodore C. White (University of Missouri-Kansas City). Strains CA12, CA488, CA490, and CA1008 were kindly provided by Steven Kelly (Swansea University, UK). Strains JS7, JS17, JS22, JS23, JS28, JS29, and JS40 were kindly provided by Jack Sobel (Wayne State University).

The WT strain (GP1), the *erg3Δ/Δ* mutant, *erg3Δ/Δ* + *ERG3*, and the TW17, JS7, JS17, JS22, JS23, JS28, JS29, and JS40 strains were transformed with NgoMIV-linearized pDUP3 or pDUPNluc and selected on YPD plates containing 200 μg/ml nourseothricin. Strains overexpressing *Cdr1p* (OCDR strain), *Mdr1p* (OMDR strain), *Erg11p* (OERG strain), or all three proteins (OTR strain) were constructed by sequentially transforming BWP17 with plasmids carrying the *URA3*, *HIS1*, or *ARG4* markers, as follows. The OCDR strain was made by sequential transformation with pKE4CDR1 (linearized with NheI), pGHT3 (linearized with NruI), and pAR8 (linearized with Clal). The OMDR strain was made by serial transformation with pKE4 (linearized with NheI), pGHT-MDR1 (linearized with NruI), and pAR8 (linearized with Clal). The OERG strain was made by sequential transformation with pKE4 (linearized with NheI), pGHT3 (linearized with NruI), and pAR8-ERG11 (linearized with Clal). The OTR strain was made by sequential transformation with pKE4CDR1 (linearized with NheI), pGHT-MDR1 (linearized with NruI), and pAR8-ERG11 (linearized with Clal). A WT control strain (XCTRL) carrying the vectors only was made by successive transformation with pKE4 (linearized with NheI), pGHT3 (linearized with NruI), and pAR8 (linearized with Clal). Correct integration of pKE4/pKE4CDR1, pAR8/pAR8-ERG11, and pGHT3/pGHT-MDR1 was confirmed by PCR analysis using the primer pairs LUXINTDEF/LUXINTDETR, ARG4DEF7/ARG4DETR7, and HIS1DEF9/HIS1DETR5, respectively.

RNA extraction. Each *C. albicans* strain was grown overnight at 30°C in YPD medium, subcultured to an OD₆₀₀ of 0.2, and incubated for 6 h at 30°C, with shaking. Cells were collected by centrifugation at 3,500 rpm for 5 min. The supernatant was poured off, and the cell pellets were stored at -80°C. RNA was extracted from the cell pellets using the hot phenol method described by Schmitt et al. (40). RNA pellets were eluted in nuclease-free water, and the quantity and purity were determined spectrophotometrically by measuring the absorbance at 260 nm and 280 nm.

Quantitative RT-PCR. cDNA was synthesized using the Verso cDNA synthesis kit (Thermo Scientific) with random hexamers, according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed with primer pairs that bind within the *ACT1* (*ACT1-FWD-qRT-PCR* and *ACT1-RVS-qRT-PCR*), *CDR1* (*CaCDR1-F-qPCR* and *CaCDR1-R-qPCR*), *MDR1* (*BMR1-F* and *BMR1-R*), or *ERG11* (*CaERG11-RVS-qRT-PCR* and *CaERG11-FWD-qRT-PCR*) (Table S1) coding sequences, using the Maxima SYBR Green/ROX qPCR master mix (2×; Thermo Scientific), as indicated by the manufacturer. Reactions were completed in a CFX96 real-time system (Bio-Rad). Expression levels of *CDR1*, *MDR1*, and *ERG11* among the strains were compared to those of *ACT1* (normalizing gene) using the 2^{-ΔΔCT} method (41).

Antifungal susceptibility testing. Antifungal susceptibility testing of all the strains included in this study was performed using the broth microdilution method described in CLSI document M27-A3 (19), in a 96-well plate format. All drugs for susceptibility testing used in this study were diluted 2-fold in dimethyl sulfoxide (DMSO) at 200 times the final concentration. RPMI 1640 medium (Sigma-Aldrich) was prepared according to the CLSI protocol; the medium was buffered with morpholinepropanesulfonic acid (MOPS), and the pH was adjusted using NaOH and HCl. Plates were incubated without shaking for 24 or 48 h at 25°C or 35°C. The content of each well was carefully resuspended with pipetting up and down before the OD₆₀₀ was measured using a BioTek Cytation 5 plate reader.

Luciferase-based membrane integrity assay. *C. albicans* strains expressing Nluc were cultured overnight at 30°C in YPD medium. Cells were then washed and resuspended in distilled water, and cell density was adjusted to 1 × 10⁷ cells/ml in YNB broth. One hundred microliters of each cell suspension was added to wells of a round-bottom, 96-well plate containing 100 μl of YNB medium plus 5 μg/ml fluconazole or 0.5% DMSO. After incubation for 24 h in a 35°C standing incubator, the cells in each well were resuspended by mixing using a multichannel pipette. Plates were then centrifuged at 1,800 rpm for 5 min to pellet the cells. Twenty-five microliters of supernatant was transferred to a white flat-bottomed 96-well plate, and Nluc activity was determined using the Nano-Glo luciferase assay reagent (Promega Corp.), according to the manufacturer's instructions. Plates were further centrifuged at 1,500 rpm for 2 min, and luminescence was read using a Cytation 5 plate reader (BioTek Instruments). Cell growth for each strain was determined as the OD₆₀₀ of 1:10 dilutions of the cell cultures. Luminescence readings were normalized to the corresponding OD₆₀₀ for each sample.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01337-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

ACKNOWLEDGMENTS

Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases (grant R01AI099080). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

We thank Promega Corporation for permission to produce and to utilize the *C. albicans* adapted NanoLuc coding sequence. We also thank Jack Sobel (Wayne State University) for providing strains used in this study.

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