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Enhanced Production of Farnesol by *Candida albicans* Treated with Four Azoles

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The dimorphic fungus *Candida albicans* excretes farnesol, which is produced enzymatically from the sterol biosynthetic intermediate farnesyl pyrophosphate. Inhibition of *C. albicans* by four azole antifungals, fluconazole, ketoconazole, miconazole, and clotrimazole, caused elevated farnesol production (10- to 45-fold). Furthermore, farnesol production occurs in both laboratory strains and clinical isolates (J. M. Hornby et al., Appl. Environ. Microbiol. 67:2982-2992, 2001) of *C. albicans*.

The inoculum size effect in *Candida albicans* results from the natural production and excretion of an extracellular quorum-sensing molecule (QSM) which our laboratory identified as farnesol (4). Exogenous farnesol, purified from spent media or supplied commercially, prevented mycelial development in both a growth morphology assay and a differentiation assay. The differentiation assay used three chemically distinct triggers for germ tube formation: L-proline, *N*-acetylglucosamine, and serum. In all cases, the presence of farnesol prevented the yeast-to-mycelium conversion, resulting in actively budding yeasts without influencing cellular growth rates (4). We later showed that only the (*E,E*)-isomer of farnesol was biologically active (11) and that *C. albicans* cell extracts contained enzymatic activity to convert [³H]farnesyl pyrophosphate (FPP) to [³H]farnesol (5).

Because FPP is the biosynthetic precursor of both farnesol (5) and ergosterol (10), we reasoned that drugs which block the sterol biosynthetic pathway after FPP might lead to the accumulation of FPP which would, in turn, lead to enhanced farnesol production. This reasoning proved correct; treatment of *C. albicans* with 0.5 μM zaragozic acid B, a potent inhibitor of squalene synthase (2), led to an eightfold increase in the amount of farnesol produced by *C. albicans* (5). At that time our group predicted that all drugs which blocked carbon flow through the sterol pathway would also cause enhanced production of farnesol. The present paper tests that generality by showing that four azole antifungals, fluconazole, clotrimazole, ketoconazole, and miconazole, also boost farnesol production by *C. albicans*. Additionally, because the farnesol-stimulating activity of zaragozic acid B had only been shown with strain A72, we tested the generality of farnesol production in *C. albicans* by analyzing five other strains, including recent clinical isolates.

Growth, sample preparation, and determination of extracellular farnesol by gas chromatography-mass spectroscopy were as previously described (4, 5). Table 1 displays the levels of extracellular farnesol produced by seven strains of *C. albicans*. The four clinical isolates displayed similar levels of farnesol

production as the lab strains A72 and CAI-4. No significant differences were observed. In past work (4), our investigators reported quorum-sensing activity in five other laboratory strains (MEN, SC5314, LGH1095, SG3314, and ATCC10261). Thus, this study has shown a general theme of farnesol production by the majority of *C. albicans* strains used here. At this time we also clarified the distinction between our work, showing that farnesol is the QSM in *C. albicans*, and that of Oh et al. (9), who described the production of farnesoic acid as the QSM in *C. albicans* 10231. *C. albicans* 10231 produced no detectable farnesol (Table 1), thereby revealing a significant metabolic modification in this strain. To date, *C. albicans* 10231 is the only strain tested that does not produce farnesol. Direct comparison of the quorum-sensing activity of farnesoic acid and farnesol showed that farnesoic acid displays only 3.3% of the activity exhibited by (*E,E*)-farnesol (11).

Following our group's work showing increased farnesol production after treatment of *C. albicans* with the squalene synthase inhibitor zaragozic acid B (5), additional sterol inhibitors were studied. Treatment with fluconazole resulted in the dose-response curve shown in Fig. 1. The farnesol excreted increased roughly 13-fold, reaching a maximum of ca. 1.24 mg of farnesol per gram (cell dry weight) at 1 μM fluconazole (Fig. 1 and Table 2). Intracellular farnesol increased in a proportional manner (data not shown). The fluconazole concentration needed for maximal farnesol production (1 μM) corresponded well with the MIC of fluconazole which, for *C. albicans* A72, is ca. 1 μM, although even at 10 μM fluconazole complete inhibition of growth is not achieved. This effect, known as trailing growth, is common in many strains of *C. albicans* (6).

Table 2 illustrates the commonality of increased farnesol production due to sterol inhibition by azole antifungals. Treatment with 1 μM ketoconazole, 1 μM clotrimazole, or 0.5 μM miconazole led to farnesol production increases of 45-, 45-, and 44-fold, respectively. Dose-response curves were not conducted for these three azoles. With the doses chosen, very little growth was observed over 24 h (cell dry weights were ca. 2% of those of the untreated controls). Therefore, the higher levels of farnesol produced with clotrimazole, ketoconazole, and miconazole (Table 2) probably reflected how little growth occurred rather than any mechanistic differences between fluconazole and the other azoles. Significantly, tolnaftate, an inhibitor of

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TABLE 1. Farnesol production by laboratory and clinical isolates of *C. albicans*

Strain of <i>C. albicans</i>	Farnesol produced (mg/g [dry wt]) ^a
A72.....	0.120–0.133
CAI-4.....	0.120–0.133
ATCC 10231.....	<0.005
Clinical isolate 4 ^b	0.125±0.005
Clinical isolate 6.....	0.107±0.012
Clinical isolate 11.....	0.137±0.003
Clinical isolate 12.....	0.131±0.007

^a Cell dry weights were determined for each sample in Tables 1 and 2. Values for A72 and CAI-4 are the average of six or more determinations conducted over a 2-year time span. For *C. albicans* 10231, no farnesol was detected in two independent studies (detection limit of 0.005 mg/g).

^b The four clinical isolates examined were randomly chosen from 12 isolates provided by Thomas Stalder (Lincoln, Nebr.). Values represent the average and variation of duplicate independent measurements.

squalene epoxidation added as a control because it is known to be inactive versus *C. albicans* (1), did not increase farnesol production above wild-type levels (Table 2).

Our investigators previously proposed the general theme that inhibition of the sterol pathway would lead to increased production of farnesol (5). This idea was based on our work with squalene synthase inhibition by zaragozic acid B (5). This theme has now been confirmed for the inhibition of lanosterol 14 α -demethylase by four cytochrome P450-inhibitory azoles (Table 2). For most strains of *C. albicans*, any drug that inhibits

TABLE 2. Enhanced farnesol production by *C. albicans* A72 treated with drugs which target sterol biosynthesis

Antibiotic (concn [μ M])	Farnesol produced (mg/g [dry wt])	Fold increase
None	0.120–0.133 ^a	
Zaragozic acid (0.5)	1.0 ^b	8
Fluconazole ^c (1)	1.24	10
Clotrimazole (1)	5.44	45
Ketoconazole (1)	5.43	45
Miconazole (0.5)	5.31	44
Tolnaftate (1)	0.132 ^d	

^a From Table 1.

^b From reference 5.

^c Fluconazole was kindly provided by Pfizer Pharmaceuticals. Tolnaftate, ketoconazole, clotrimazole, and miconazole were purchased from Sigma. Values for farnesol production are averages of two independent experiments agreeing within $\pm 10\%$.

^d Value in the presence of tolinaftate is an average of two independent experiments agreeing within $\pm 3\%$.

a step in the sterol biosynthetic pathway, thus inhibiting carbon flow to ergosterol, may also increase farnesol production.

Increased farnesol could also be used to screen drugs that target the sterol biosynthetic pathway and to examine the sensitivity of clinical isolates to known sterol-inhibitory drugs. While the hypothesis that azoles promote farnesol production via inhibition of the ergosterol pathway is attractive, at this time we cannot exclude the possibility that pleiotropic effects

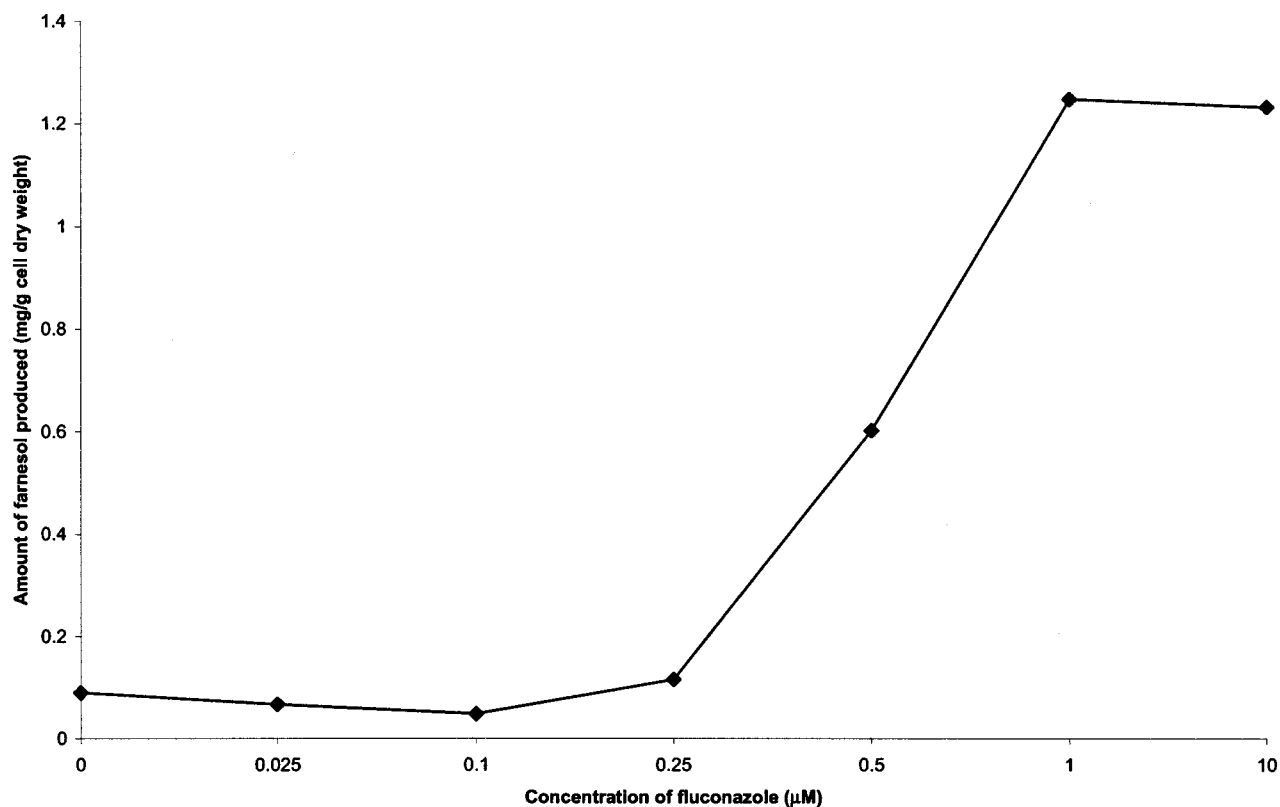


FIG. 1. Increased farnesol production by fluconazole-treated cells. Each point is the average of at least two separate experiments agreeing within $\pm 10\%$.

of the azole family affect farnesol independently of sterol biosynthesis.

Elevated production of farnesol at sublethal levels of fluconazole (Fig. 1), coupled with the known ability of farnesol to cause a shift from mycelia to yeast in *C. albicans* (4), provides an attractive explanation for past work which noted that sub-inhibitory concentrations of azole drugs inhibited hyphal development and maintained the cells in the yeast morphology. Interestingly, this inhibitory effect was independent of any inhibition on growth of the fungus (3, 8). Odds (7) also mentioned in his comprehensive review of *Candida* that “most azoles are able to prevent or greatly perturb hyphal growth of *C. albicans*. The drugs retard or annul the initial outgrowth of germ tubes and entirely prevent hyphal branching, thus leading to cultures of largely or entirely yeast-form cells, even on media that normally support development of long hyphae.”

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