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Abstract

Candida albicans is a diploid fungus that undergoes a morphological transition between budding yeast, hyphal, and pseudohyphal forms. The morphological transition is strongly correlated with virulence and is regulated in part by quorum sensing. *Candida albicans* produces and secretes farnesol that regulates the yeast to mycelia morphological transition. Mutants that fail to synthesize or respond to farnesol could be locked in the filamentous mode. To test this hypothesis, a collection of *C. albicans* mutants were isolated that have altered colony morphologies indicative of the presence of hyphal cells under environmental conditions where *C. albicans*

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normally grows only as yeasts. All mutants were characterized for their ability to respond to farnesol. Of these, 95.9% fully or partially reverted to wildtype morphology on yeast malt (YM) agar plates supplemented with farnesol. All mutants that respond to farnesol regained their hyphal morphology when restreaked on YM plates without farnesol. The observation that farnesol remedial mutants are so common (95.9%) relative to mutants that fail to respond to farnesol (4.1%) suggests that farnesol activates and (or) induces a pathway that can override many of the morphogenesis defects in these mutants. Additionally, 9 mutants chosen at random were screened for farnesol production. Two mutants failed to produce detectable levels of farnesol.

Keywords: farnesol-remedial mutants, farnesol-sensing mutants, farnesol-synthesis mutants, quorum sensing, *Candida albicans*, morphological transition.

Résumé

Candida albicans est un champignon diploïde qui subit une transition morphologique entre les levures en herbe, les hyphes et les formes pseudohyphales. La transition morphologique est fortement corrélée à la virulence et est régulée en partie par la détection du quorum. Candida albicans produit et sécrète du farnésol qui régule la transition morphologique levure-mycélium. Les mutants qui ne parviennent pas à synthétiser ou à répondre au farnésol pourraient être verrouillés en mode filamenteux. Pour tester cette hypothèse, une collection de mutants de *C. albicans* a été isolée qui ont modifié les morphologies des colonies, indiquant la présence de cellules hyphales dans des conditions environnementales où *C. albicans* ne pousse normalement que sous forme de levures. Tous les mutants ont été caractérisés pour leur capacité à répondre au farnésol. Parmi ceux-ci, 95,9% sont entièrement ou partiellement revenus à la morphologie de type sauvage sur des plaques de gélose au levure de malt (YM) complétées par du farnésol. Tous les mutants qui répondent au farnésol ont retrouvé leur morphologie hyphale lorsqu'ils ont été recréés sur des plaques YM sans farnésol. L'observation selon laquelle les mutants curatifs du farnésol sont si communs (95,9%) par rapport aux mutants qui ne répondent pas au farnésol (4,1%) suggère que le farnésol s'active et (ou) induit une voie qui peut supplanter bon nombre des défauts de morphogenèse de ces mutants. De plus, 9 mutants choisis au hasard ont été testés pour la production de farnésol. Deux mutants n'ont pas réussi à produire des niveaux détectables de farnésol.

Mots-clés: mutants réparateurs du farnésol, mutants sensibles au farnésol, mutants de synthèse du farnésol, détection du quorum, *Candida albicans*, transition morphologique.

Introduction

The dimorphic fungus *Candida albicans* is the causative agent of vaginal yeast infections, oral thrush, and a series of other fungal maladies. Normally, *C. albicans* is a member of the intestinal tract flora. It becomes a pathogen when conditions permit it to adhere, colonize,

and invade epithelial tissues. Premature infants, surgical patients, and cancer patients receiving immunosuppressive chemotherapy are especially at risk for developing candidiasis. This is a serious disease, as 30%–38% of the patients with candidiasis die (Viudes et al. 2002; Gudlaugsson et al. 2003).

Candida albicans undergoes a morphological transition between a budding yeast form and the hyphal and pseudohyphal forms. The morphological transition is strongly correlated with virulence because yeast- and mycelia-only mutants are non-infective in a mouse model (Lo et al. 1997; Bendel et al. 2003). The mycelial and yeast forms are probably important for colonization and spread, respectively. This morphological transition is regulated by many environmental and chemical signals including temperature and quorum sensing (Hornby et al. 2001). With regard to temperature, *C. albicans* generally grows as yeasts at ≤30 °C and as filaments at 37 °C. With regards to quorum sensing, *C. albicans* produces and secretes an extracellular molecule, farnesol, which regulates the morphological transition (Hornby et al. 2001). During growth at 37 °C, farnesol accumulates, hyphal development is inhibited, and the cells grow instead as budding yeasts. Exogenous farnesol also blocks the yeast to filamentous conversion in response to most, if not all, environmental and chemical inducers (Mosel et al. 2005). Because the morphological transition is critical for pathogenicity, it is important to understand how it is regulated by farnesol. In particular, we ultimately want to know how many farnesol-binding targets exist, and how farnesol binding leads to an altered cell morphology.

Mutants that fail to synthesize or respond to farnesol should be unable to block the yeast to mycelia conversion and thus would be more likely to grow in the filamentous mode. They may even lose the ability to grow in the yeast form. To test this hypothesis, we isolated a collection of *C. albicans* mutants that had a hyphal or pseudohyphal morphology under environmental conditions (28 °C) where *C. albicans* normally grows as yeasts and characterized the ability of these mutants to respond to farnesol. We found that farnesol-responsive mutants existed and were common. A subset of the mutants was also tested for their ability to synthesize farnesol. Two mutants did not synthesize detectable amounts of farnesol. The analysis of these mutants is an important first step in understanding the role farnesol plays in regulation of the *C. albicans* morphological transition.

Materials and methods

Strains and media

Candida albicans A72 (kindly provided by Patrick A. Sullivan, University of Otago, New Zealand) was originally isolated from a patient by Dr. Antonio Cassone (Rome, Italy). It does not have any auxotrophic markers and it is available from the American Type Culture Collection (Rockville, Md.) as strain MYA2430. *Candida albicans* CAI-4 (*ura3::imm434/ura3::imm434*; Fonzi and Irwin 1993) was obtained from Gerald Fink at the Whitehead Institute for Biomedical Research (Cambridge, Mass.). CAI-4 is derived from SC5314, a clinical isolate, by gene replacement using homologous recombination to replace both copies of its *URA3* gene (Fonzi and Irwin 1993). CAI-4 also has a deletion of the 3′ half of *IRO1* and, thus, is partially defective in iron uptake and utilization (Garcia et al. 2001). A72 and CAI-4 produce spontaneous odd colonies at a frequency of about 1 in 10,000 (0.01%). However, none of these spontaneous odd colonies were stable; they all changed colony morphology within 3–4 transfers. Thus, stability beyond 3–4 transfers was the criterion we used to select stable altered colony morphologies. These cells are either mutants or they have an exceptionally stable colony-type switch. For simplicity in this paper, we use the term "mutants" to describe these cells. A72 and CAI-4 were used in both mutagenesis protocols described below.

Strains were maintained on yeast malt (YM; Difco Laboratories, Sparks, Md.; Tucker, Ga.; Barret et al. 1990) or YEPD (1% *w*/*v* yeast extract, 2% *w*/*v* bacto peptone, 2% *w*/*v* glucose) agar stored at 4 °C. Other media used were yeast nitrogen base (YNB; Difco) supplemented with 2.5 mmol/L *N*-acetylglucosamine, a defined glucose–phosphate–proline medium with 2.5 mmol/L *N*-acetylglucosamine (mGPP), and a modified glucose–salts–biotin (mGSB) medium. The mGPP (Hornby et al. 2001) contained the following on a per liter basis: 20 g glucose, 1.15 g L-proline, 0.55 g *N*–acetylglucosamine, 4 g KH_2PO_4, 3.2 g Na_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 mg $CuSO_4 \cdot 5H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 1 mg $MnCl_2$, 1 mg $FeSO_4$, 20 μg biotin, 200 μg pyridoxine·HCl, 200 mg thiamine·HCl (pH 6.5). The glucose (20% *w*/*v*) and L-proline (100 mmol/L) were autoclaved separately and added aseptically, as were the filter-sterilized vitamins (1000-fold stock prepared in 20% *v*/*v* ethanol). The mGSB (Hornby et al. 2001) medium contained the following on a per

liter basis: 1 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 50 mg $MgSO_4 \cdot 7H_2O$, 50 mg $CaCl_2 \cdot 2H_2O$, and 1 g peptone. After autoclaving, 30 mL of 50% *w*/*v* glucose and 0.4 mL of the mGPP vitamin stock were added aseptically.

Mutagenesis and isolation of mutants

Candida albicans cells with a stable altered colony morphology were selected from cultures treated with 2 mutagenesis protocols. The first protocol consisted of treatment with ethylmethane sulfonate (EMS), to stimulate a high number of transitions at GC sites (Kohalmi and Kunz 1988), followed by ultraviolet (UV) irradiation. *Candida albicans* is diploid and UV irradiation was used to stimulate mitotic recombination, thus making recovery of recessive mutations possible (Kakar et al. 1983). The second protocol consisted of treatment with EMS followed by nitrous acid. Nitrous acid stimulates transitions at and GC sites. In both mutagenesis schemes, A72 and CAI-4 were mutagenized to 50% viability.

Candida albicans A72 cells from a 5- to 7-day-old YM agar plate were transferred to 50 mL mGSB medium and grown at 30 °C for 30 h to stationary phase with shaking at 200 r/min. The cells were recovered by centrifugation for 10 min at 4350*g* (6000 rpm in a Beckman JA-20 rotor) and washed 3 times with 10 mL of 0.05 mol/L potassium phosphate buffer (pH 6.5). The washed cells were resuspended in 10 mL of this buffer. A "time 0" cell titer was determined by removing a 0.1 mL sample that was diluted and plated onto YM agar to give 150–200 colonies per plate. Two hundred fifty microliters of EMS was added to the remaining 9.9 mL cell suspension. This mixture was incubated for 40 min at room temperature with gentle mixing every 5 min. EMS was then neutralized by adding 10 mL of 6% *w*/*v* sodium thiosulfate and incubating for 20 min at room temperature. The cells were washed once with 10 mL of 6% *w*/*v* sodium thiosulfate and twice with 10 mL of 0.05 mol/L potassium phosphate buffer (pH 6.5). The cells were resuspended in 10 mL of 0.05 mol/L potassium phosphate buffer (pH 6.5), diluted (by a factor of 10^{-4} and 10^{-5}), and plated on YM agar. The plates were subjected to 50 J of ultraviolet light C (UV-C) (2.5 J/s for 20 s) and incubated at 30 °C until colonies were visible.

Nitrous acid mutagenesis was basically identical to that used by Kakar et al. (1983). Cells were grown and collected via centrifugation as described for EMS mutagenesis. However, the cells were washed and

resuspended in 10 mL of 0.1 mol/L sodium acetate buffer (pH 4.5) instead of in the phosphate buffer used for EMS. A time 0 titer was obtained in an identical fashion as for EMS mutagenesis. The remaining 9.9 mL of cell suspension was centrifuged and resuspended in 9.9 mL of filter-sterilized 0.1 mol/L sodium acetate buffer (pH 4.5) containing 0.06 mol/L sodium nitrite and incubated at room temperature. After 15–25 min, mutagenesis was stopped by the addition of 5 mL of 0.5 mol/L potassium phosphate buffer (pH 7.5). Mutated cells were diluted using 0.05 mol/L phosphate buffer (pH 7.5) to obtain 150–200 colonies when 0.1 mL was spread on a YM agar plate. The YM agar plates were incubated at 30 °C until colonies were visible. The normal colony morphology on YM agar plates at 30 °C is a smooth, circular colony.

The mutagenized colonies were examined for odd colony morphologies. Cells from odd morphology colonies were transferred to both fresh YM agar and mGPP agar plates. The mGPP plates selected against auxotrophs. Isolates that formed colonies of similar size to the wild-type, grew in mGPP, and maintained their odd colony morphology for at least 4 transfers on YM agar plates were saved for future study. The protocol for *C. albicans* CAI-4 was the same as for A72 except that all media were supplemented with 40 mg/L uridine.

Phase-contrast microscopy

Cells from the isolate of interest were inoculated onto a YM agar plate and incubated at 28 °C for 5 d for A72 mutants or 7 d for CAI-4 mutants. A longer incubation period was required for CAI-4 to compensate for its slower growth rate. For smooth colonies, all visible cellular material from a 2.5 mm colony or a 2.5 × 2.5 mm section from a line inoculation was suspended in 1.0 mL of sterile 0.05 mol/L potassium phosphate buffer (pH 6.5) and vortexed for 1 min to separate the cells. If the isolate of interest came from a colony whose texture was hard, indicating filaments only, or rubbery, indicating a high percentage of filaments, a 5-times larger area of cells was removed and homogenized using a sterile Pyrex hand homogenizer with 5 mL of phosphate buffer. Homogenization was necessary because in these cases vortexing did not result in individual cells. Cells were observed using a 40× phase-contrast lens on an Olympus BH2-RFCA microscope equipped with an Olympus C-2000 zoom digital camera (Olympus America Inc., Melville, N.Y.).

Farnesol-response assays

YM agar plates were supplemented with 10, 50, or 100 μmol/L mixed isomer farnesol (Sigma, St. Louis, Mo.). A frozen stock of 100 mmol/L farnesol in ethanol was brought to room temperature and aliquoted into empty petri dishes. The liquid medium (55 °C) was added and the plates were swirled to mix. Line inoculations were used and plates were incubated at 28 °C for 5 or 7 d for A72 and CAI-4, respectively. For each isolate, the morphology of the streak was compared with a control plate with no added farnesol. Isolates were examined for any reduction in length or density of hairs and (or) reduction in wrinkles.

Analysis of farnesol-production assays by gas chromatography and mass spectrometry (GC/MS)

Cells were grown in mGPP at 30 °C. Extracellular farnesol from cell-free supernatants was analyzed by GC/MS as described previously (Hornby et al. 2001; Hornby and Nickerson 2004).

Results

Isolation of C. albicans heritable colony morphological variants

Candida albicans cells with a stable altered colony morphology were isolated following mutagenesis of 2 parent strains, A72 and CAI-4. These parental strains were chosen because they are well-characterized farnesol-producing and farnesol-responsive strains that have been previously used in in vitro studies (Hornby et al. 2001, 2003; Hornby and Nickerson 2004; Shchepin et al. 2003).

EMS–UV mutagenesis

For our first collection, cells were treated with EMS, followed by UV irradiation. One hundred thirty-four (0.1%) of the ca. 90 500 A72 survivors and 606 (0.6%) of the ca. 95 000 CAI-4 survivors had an odd colony morphology that was maintained following at least 4 transfers on YM or YEPD agar at 30 °C (**Table 1**). Under these conditions, unmutagenized A72 and CAI-4 cells form smooth colonies with yeast cells

Table 1. Categories of colony morphology mutants obtained from *C. albicans* A72 and CAI-4.

	A72		*CAI-4*	
Category	*EMS–UV*	*EMS – nitrous acid*	*EMS–UV*	*EMS – nitrous acid*
Hairy	129	158	167	49
Stippled	1	6	0	0
Hairy stippled	1	25	0	12
Wrinkled	0	5	1	3
Hairy wrinkled	2	23	424	90
Mountain-like	0	0	14	0
Shaggy dog	1	0	0	0
Total	134	217	606	154

Note: *Candida albicans* cells were mutagenized by either EMS followed by UV irradiation or by EMS followed by nitrous acid application. Colonies with a stable altered morphology were selected on YM agar at 30 °C.

only (**Figs. 1 and 2**). The mutants fell into 7 distinct categories based on colony type: hairy, stippled, hairy stippled, wrinkled, hairy wrinkled, mountain-like, and shaggy dog. Representative mutants of each type are shown in Fig. 1. The majority of A72 mutants (96.3%) were in

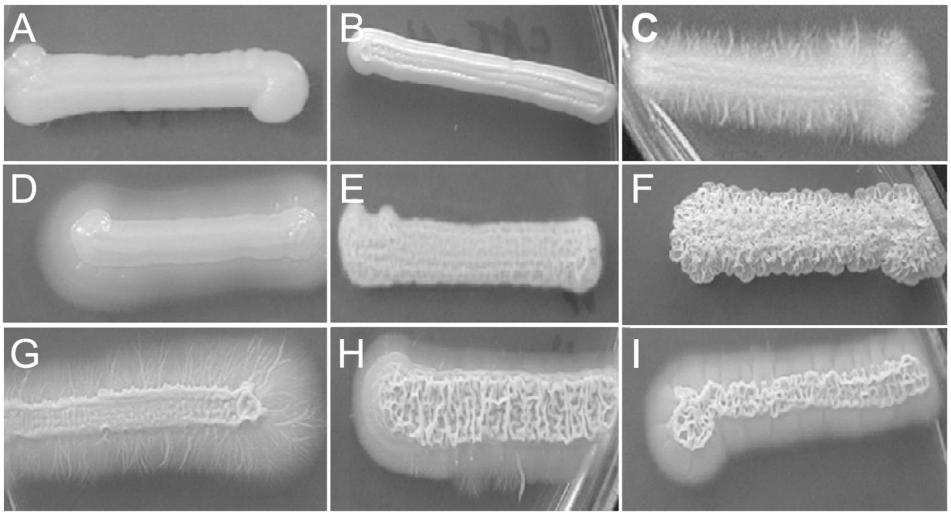

Fig. 1. Representative examples of colony morphologies of parental strains and mutants obtained in the screen. *Candida albicans* cells were plated on YM agar and incubated for 5 d at 28 °C. Representative colony morphologies for (A) parental strain A72, (B) parental strain CAI-4, and mutants (C) shaggy dog, (D) hairy, (E) stippled, (F) wrinkled, (G) hairy stippled, (H) hairy wrinkled, and (I) mountain-like. The shaggy dog and mountain-like morphologies were unique for mutants of A72 and CAI-4, respectively. Mutants with the other mutant colony morphology types were recovered from both A72 and CAI-4.

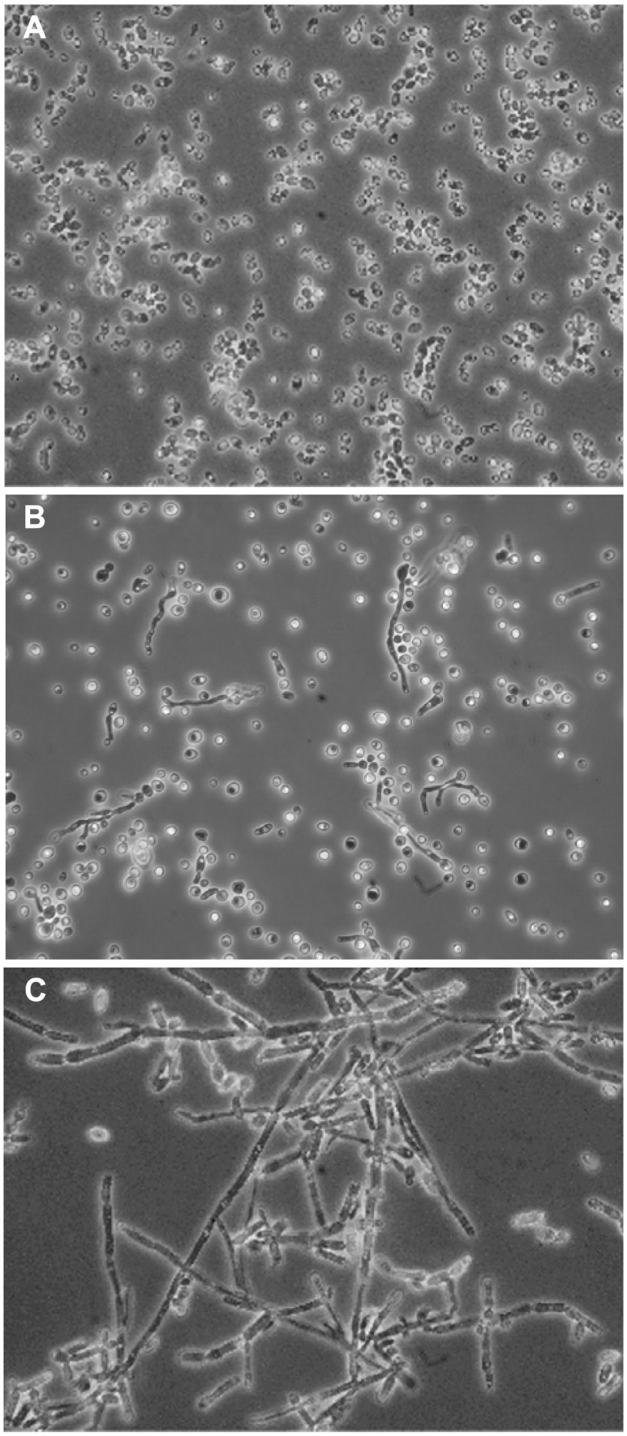

Fig. 2. Examples of cell morphologies of parental strains and mutants obtained in the screen. (A) Parental strain A72 (average cell size 4 μm × 4.8 μm), (B) a hairy mutant (average mycelia width 2.5 μm) and (C) a shaggy dog mutant (average mycelia width 3.5 μm).

the hairy category, whereas the majority of CAI-4 mutants (70%) were in the hairy wrinkled category. The second-highest number of CAI-4 mutants (26.6%) was in the hairy category (Table 1). Each of these mutants was stable for ≥4 transfers and, for each mutant, a survey of a minimum of 1000 colonies showed uniform colony morphology.

A clear relationship between *C. albicans* colony morphology and cell morphology has been well established (Radford et al. 1994). To confirm that colony morphology correlated with individual cell morphology, we examined a subset of the mutants grown at 28 °C by microscopy. This subset consisted of all 740 of the mutants generated by EMS–UV. Unmutagenized A72 and CAI-4 strains grew as yeasts only. Of the 740 EMS–UV mutants described in Table 1, 1 A72 mutant in the shaggy dog category and 14 CAI-4 mutants in the mountain-like category grew as filaments only. The other 725 EMS/UV mutants all grew as a mixture of yeasts and filaments. Representative micrographs of the A72 parent strain and mutants are shown in Fig. 2. The variety of altered colony types is similar to those observed for phenotypic colony-type switching by Radford et al. (1994) and Perez-Martin et al. (1999).

EMS – nitrous acid mutagenesis

Low doses of UV irradiation (>90% survival) cause an increase in the frequency of colony-type switching in *C. albicans* (Slutsky et al. 1985; Soll 1997). To reduce the possibility that some of the mutants we recovered in our screen were not actually mutants, but might instead have resulted from exceptionally stable colony-type switching, we mutagenized A72 and CAI-4 with EMS, followed by nitrous acid. Currently, there is no reference for either EMS or nitrous acid inducing phenotypic switching. In this screen, we found that 217 (0.3%) of the ca. 80 420 A72 survivors and 154 (0.2%) of ca. 75 000 CAI-4 survivors maintained odd colony morphology following ≥4 transfers on YM agar (Table 1). These mutants fell into 6 distinct categories based on colony morphology: hairy, stippled, hairy stippled, wrinkled, hairy wrinkled, and mountain-like. No shaggy dog mutants were isolated. Again, the majority of A72 mutants (72.8%) were in the hairy category, whereas the majority of CAI-4 mutants (58.4%) were in the hairy wrinkled category with the second highest number (31.8%) in the hairy category. Thus, similar results were obtained when EMS was used in combination with either UV or nitrous acid.

Table 2. Farnesol reversal of "mutant" colony phenotype.

Category	*A72*	*CAI-4*
Complete response	191	292
Partial response	135	448
No response	25	20
Total	351	760

Note: The effect of exogenous farnesol was measured by examining colony phenotype on YM agar containing 0 and 100 μmol/L farnesol at 28 °C. Mutants were categorized as "complete response" if they reverted to a wild-type phenotype (smooth and entire colonies) in the presence of 100 μmol/L farnesol. Mutants were categorized as "partial response" if the presence of 100 μmol/L farnesol caused a decrease in hair length, hair density, and (or) a decrease in wrinkling. Mutants were categorized as "no response" if farnesol caused no visible changes in colony morphology.

Farnesol-responsive mutants

All of the A72 and CAI-4 mutants (1111) were screened for their responsiveness to farnesol. They were tested on YM medium containing 0, 10, 50, and 100 μmol/L farnesol at 28 °C. It should be noted that for wild-type cells, 1– 2 μmol/L farnesol is sufficient to block the yeast to mycelial conversion (Mosel et al. 2005). A very high percentage of these mutants responded to farnesol addition by changing their colony morphology. On YM plates with farnesol, 191 (54.4%) of 351 A72 mutants and 292 (38.4%) of 760 CAI-4 mutants fully reverted to wild-type morphology (**Table 2**) as judged by the formation of smooth and entire colonies (**Fig. 3**). Another group of mutants partially reverted in the presence of farnesol. In this group, which included 135 (38.5%) of 351 A72 mutants and 448 (58.9%) of 760 CAI-4 mutants, farnesol reduced the wrinkled appearance in the center of the colony and shortened the hairs on the periphery, while simultaneously making the hairs less dense (Fig. 3; Table 2). Thus, 326 (92.9%) of the A72 mutants and 740 (97.4%) of the CAI-4 mutants responded to farnesol. These mutants were designated "farnesol responders". Significantly, all farnesol responders exhibited a temporary or reversible response to farnesol. That is, they reverted towards wild-type colony morphology on YM plus farnesol, but then regained their altered colony morphology when restreaked on YM without farnesol. Microscopic examination of the responding mutants confirmed that colony morphology changes were accompanied by decreased filaments and an increased percentage of budding yeasts (data not shown).

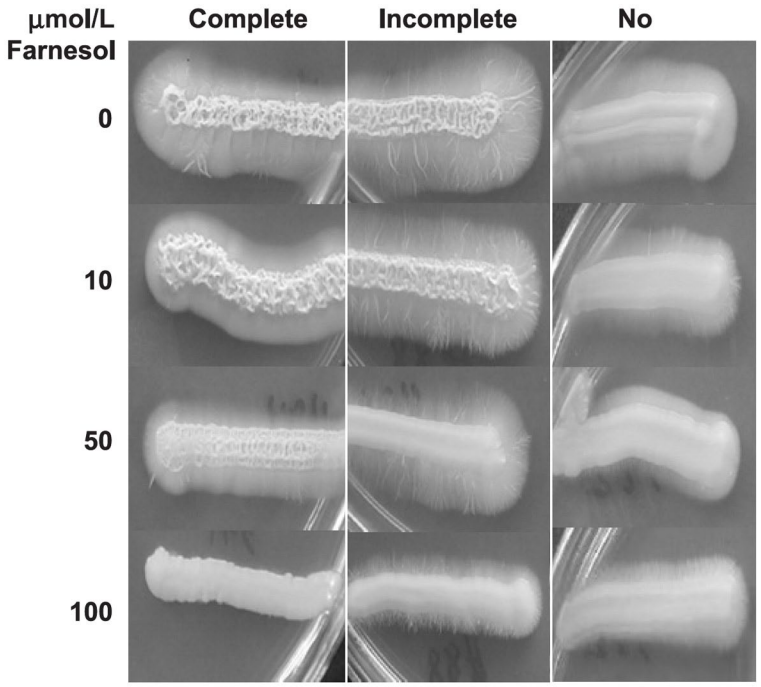

Fig. 3. Representative examples of mutants that revert to wild type (complete response), partially revert (incomplete response), or show no change (no response) in the presence of farnesol. *Candida albicans* cells were plated on YM agar containing 0, 10, 50, or 100 μmol/L farnesol and incubated for 5 d at 28 °C.

The remaining 25 (7.1%) of 351 A72 mutants and 20 (2.6%) of 760 CAI-4 mutants did not respond to farnesol (Table 2) as judged by an unchanged colony morphology on YM plates with 100 μmol/L farnesol. These mutants were designated "farnesol non-responders". The non-responding mutants did not exhibit decreased filamentation (data not shown).

Farnesol production in 9 of the C. albicans colony morphology variants

An attractive model suggests that mutants locked into the filamentous mode either failed to synthesize farnesol or failed to respond to farnesol. To test this hypothesis, 4 mountain-like mutants (filamentous only) and 5 hairy wrinkled mutants (yeast–filament mixtures) were analyzed for farnesol production by GC/MS (**Table 3**). These mutants, chosen at random, were all derived from CAI-4. Farnesol production in the CAI-4 parent was 0.11 ± 0.02 mg/g cells. All but 2 of the

Table 3. Extracellular farnesol produced by parent strains and selected mutants derived from the CAI-4 parent.

Strain	*Colony morphology*	*Category Farnesol response*	*Farnesol concentration (mg/g cell dry mass)*
CAI-4	Parent N/A	0.11 (±0.02)*	
H125	Mountain-like	Complete	Not detectable†
H1	Mountain-like	Partial	0.10
M3	Mountain-like	Partial	0.64
H383	Mountain-like	Complete	0.29
H152	Hairy wrinkled	Complete	0.09
H68	Hairy wrinkled	Complete	0.14
H253	Hairy winkled	Complete	0.62
H88	Hairy wrinkled	Complete	0.46
H121	Hairy wrinkled	Complete	Not detectable†

Note: Mountain-like and hairy wrinkled mutants were randomly selected for analysis.

* Twenty replicates were used to measure farnesol production in the CAI-4 parent strain. This value (0.11 mg/g cell dry mass) agrees nicely with that of (0.12 mg/g cell dry mass) previously reported based on 6 determinations (Hornby and Nickerson 2004). The amount of farnesol for the other samples is an average of duplicate samples that agree ±10%.

† No farnesol was detected in 2 independent studies (detection limit of 0.005 mg/g).

mutants produced extracellular farnesol at levels that were similar to or greater than the CAI-4 parent. H125, a mountain-like mutant, and H121, a hairy wrinkled mutant, did not produce detectable farnesol.

Discussion

We have characterized a collection of *C. albicans* mutants that are altered in their colony morphology. One thousand one hundred eleven stable mutants with altered colony morphology were obtained and then tested for their response to farnesol. We found that farnesol-responsive mutants existed and were common. The majority of our morphological mutants (92.9% of the A72 mutants and 97.4% of the CAI-4 mutants) responded reversibly to farnesol. These mutants represent a novel collection of conditional morphological mutants. Presumably, the differences in farnesol responsiveness could result from partial loss-of-function mutations in 1 gene, mutations in several different genes, or activation and (or) induction of a second farnesol-response pathway that overrides the morphogenesis defect in the mutants. Because farnesol-responsive mutants are reversible and because they

are so common relative to mutants that fail to respond to farnesol, we think it is likely that farnesol activates and (or) induces a pathway that can override the morphogenesis defects in many of the farnesol-responsive mutants. *Candida albicans* is a diploid fungus. In this regard, the significance of farnesol remedial mutants does not depend on whether the mutants in our collection are: (*i*) altered in both copies of a gene, as was intended from the double mutagenesis protocols used (Kakar et al. 1983); (*ii*) single-copy haplo-insufficient mutants (Uhl et al. 2003); or (*iii*) exceptionally stable phenotypic switches. In each case, the reversal of the colony morphology phenotype by farnesol should be of interest.

We expect many of the mutants in our collection to result from haplo-insufficiency. Uhl et al. (2003) used transposon mutagenesis to conduct a large-scale loss-of-function genetic screen for haplo-insufficient mutations that affect the switch between yeast and filamentous growth in *C. albicans*. They identified mutations in 146 genes from a library of 18 000 strains. This study was significant because they demonstrated the utility of haplo-insufficiency for identifying mutations in genes affecting the morphogenic switch in *C. albicans*. We believe that many of our mutants may also be haplo-insufficient for the following reasons: (*i*) as Uhl et al. (2003) demonstrated, haplo-insufficient mutations affecting morphogenesis are common; (*ii*) we recovered morphological mutants at frequencies of 0.1% for A72 and 0.6% for CAI-4, values similar to the frequency of morphological mutants in the Uhl et al. (2003) screen (0.08%); and (*iii*) we recovered the 7 categories of morphological mutants at similar frequencies after treatment with different combinations of mutagens (Table 1).

Some of our mutants may also result from colony-type switching. *Candida albicans* can spontaneously and reversibly switch between at least 7 general phenotypes that can be seen by changes in colony morphology, a form of phenotypic switching (Slutsky et al. 1985). Spontaneous colony variants of *C. albicans* 3153A occur at a frequency of 1.4×10^{-4} (0.01%). The phenotype of the colony variants is heritable, because the phenotypes persist through several clonal platings, with the caveat that these variants themselves have a high rate of secondary spontaneous switching. For example, colony variants of 3153A have secondary switching frequencies of 4.9×10^{-2} (4.9%). Low doses of UV light (>90% survival) increase phenotypic switching in *C. albicans* 3153A by a factor of approximately 200 (Slutsky et al. 1985). Again,

the UV-treated 3153A colony variants have a high secondary- switching frequency (2.5×10^{-2} or 2.5%). Our screen also recovered high-frequency switchers, but we did not keep them. Instead, the mutants described herein were selected to give a low secondary-switching frequency and were distinct from high-frequency switchers in 3 ways: (*i*) many of our mutants were originally isolated in 2001 and their morphological phenotype has been maintained through greater than 10 transfers; (*ii*) for each mutant, a survey of a minimum of 1000 colonies showed uniform colony morphology; and (*iii*) all of our farnesol responders were reversible; they returned to the altered colony morphology when farnesol was removed. Thus, although some of our mutants might have resulted from a colony-type switch, that switch must be exceptionally stable.

Here we have shown that farnesol-responsive mutants are common. *Candida albicans* is normally able to respond to 1–2 μmol/L farnesol. These farnesol-responsive mutants may require exogenous farnesol either because their response to farnesol is attenuated such that they do not respond to the concentrations of farnesol normally encountered in colonies or because high concentrations of farnesol hyperactivates a second, dominant mechanism that inhibits filamentation by overriding the morphogenesis defect of the mutants. Another example in which farnesol exhibited an overriding control on cellular physiology is provided by Granshaw et al. (2003). In *Neurospora crassa*, circadian rhythms can be seen as a series of bands or conidiation regions on the surface of agar media. Mutations in 3 different genes (*frq*, *wc-1*, and *wc-2*) led to loss of circadian rhythm. However, addition of farnesol or geraniol (10–100 μmol/L) restored rhythmic banding to strains lacking a circadian rhythm owing to mutations in any 1 of these 3 genes. Granshaw et al. (2003) interpreted their data in terms of a two-oscillator model in which farnesol activated or amplified the remaining oscillator.

Mutants that fail to make detectable farnesol could have a defect in farnesol synthesis or they could hyperactivate a farnesol-degradation or -conversion pathway. Further analysis will be necessary to distinguish between these possibilities. Farnesol is synthesized from farnesyl pyrophosphate, an intermediate in the highly conserved sterol biosynthetic pathway (Hornby et al. 2003). An in vitro assay has been developed for farnesol synthesis from farnesyl pyrophosphate (Hornby et al. 2003) and we are currently using it to purify and identify the

proteins responsible for farnesol biosynthesis. Mutants that fail to make farnesol will be useful in complementation studies, i.e., do the genes identified by the reverse biochemical approach actually complement the mutations? This will be an important confirmation that the correct gene or genes have been cloned and a test for whether or not we have identified all of the genes for farnesol biosynthesis.

Mutants that do not respond to farnesol may be lacking a critical farnesol receptor, a signal transduction pathway activated by farnesol, or have mutations in a morphogenic pathway that overrides the farnesol response. For instance, some of these mutants could be defective in *CHK1* function (Kruppa et al. 2004). Chk1p is a histidine kinase that functions in a two-component signal transduction pathway and *C. albicans* strains lacking both copies of *CHK1* do not respond to farnesol (Kruppa et al. 2004). It may be possible to identify the missing proteins in the mutants by comparing the pattern of proteins from wild-type and mutant *C. albicans* on two-dimensional gels.

We observed differences in the frequencies of mutants in distinct morphological categories for A72 versus CAI-4. The majority of A72 mutants (96.3%) were in the hairy category, whereas there were 2 major categories for the CAI-4 mutants (70% were hairy wrinkled and 26.6% were hairy; Table 1). These differences may reflect genetic differences between the strains. For instance, CAI-4 might be heterozygous for a recessive mutation that results in formation of hairy wrinkled colonies, whereas A72 is wild type for both alleles. If so, mutants with a hairy wrinkled phenotype would be recovered more frequently from CAI-4. Similarly, differences in the genetic backgrounds of A72 and CAI-4 might explain the apparent dichotomy leading to shaggy dog colonies in mutants of A72 and mountain-like colonies in mutants of CAI-4. Alternatively, this colony difference could be a further manifestation of the *iro-1* defect in CAI-4 (Garcia et al. 2001).

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