The “Finger,” a Unique Multicellular Morphology of *Candida albicans* Induced by CO₂ and Dependent upon the Ras1-Cyclic AMP Pathway

Karla J. Daniels, Claude Pujol, Thyagarajan Srikantha, and David R. Soll
Developmental Studies Hybridoma Bank, Department of Biology, The University of Iowa, Iowa City, Iowa, USA

Most experiments exploring the basic biology of pathogenic microbes are performed *in vitro* under conditions that do not usually mimic those of their host niche. Hence, developmental programs initiated by specific host cues may be missed *in vitro*. We have tested the effects of growing low-density agar cultures of the yeast pathogen *Candida albicans* in concentrations of CO₂ found in the gastrointestinal tract. It is demonstrated that in physiological concentrations of CO₂ at 37°C, yeast cells form a heretofore undescribed multicellular “finger” morphology distinct from a previously described stalk-like structure induced by high doses of UV irradiation that kills more than 99.99% of cells. The finger extends axially, is uniform in diameter, and is visible to the naked eye, attaining lengths of 3 mm. It is composed of a basal yeast cell monolayer adhering to a semispherical crater formed in the agar and connected to a basal bulb of yeast cells at a fragile interface. The bulb extends into the long shaft. We propose that a single, centrally located hypha extending the length of the shaft forms buds at compartment junctions that serve as the source of the yeast cells in the shaft. A mutational analysis reveals finger formation is dependent upon the pathway Ras1—Cdc35—cyclic AMP (cAMP) (PDE2→)→Tpk2→Tec1. Because of the mechanically fragile interface and the compactness of bulb and shaft, we suggest that the finger may function as a multicellular dispersal mechanism produced in host niches containing high levels of CO₂.

In most experiments exploring the basic biology of microbial pathogens, the *in vitro* conditions employed are far from those the microbe encounters in the host. This is true for the nutrient, ionic, and gaseous composition of the supporting media, released host metabolites, fluid flow, and a variety of host substrates (21, 22, 39, 55). Since most pathogens have evolved in response to host conditions, the employment of *in vitro* conditions that do not accurately mimic *in vivo* conditions may be insufficient for induction of the full repertoire of developmental programs the pathogen is able to express. In the case of the yeast pathogen *Candida albicans*, most experiments are performed in air, which contains 0.04% CO₂. However, CO₂ in the interstitial fluids of tissues can attain concentrations higher than 5%, and that of the gastrointestinal tract can attain concentrations higher than 20% (1, 29). The CO₂ content of the gastrointestinal tract, therefore, reaches concentrations up to 500 times that of air. Indeed, high physiological levels of CO₂ have dramatic effects on major developmental programs of *C. albicans*, most noteworthy the formation of hyphae (24, 38) and the phenotypic switch from the white to opaque phenotype (19, 32), a prerequisite for mating (34, 37).

Given the impact that physiological concentrations of CO₂ had been demonstrated to have on already established developmental programs, we wondered whether simply growing *C. albicans* in an environment containing host levels of CO₂ might reveal additional developmental programs heretofore unidentified. We show here that plating cells at low density on a defined agar medium and incubating the cultures in 20% CO₂ at 37°C induces a complex multicellular morphology in a majority of a/a strains, but not in MTL-homozygous strains. We show that this new morphology is distinct from the “stalk-like” structures formed by survivors of high doses of UV (11, 49). We have named this complex morphology the “finger” because it resembles the transient multicellular finger morphology formed by the social amoeba *Dictyostelium discoideum* in the generation of a sporangium (41, 52). The *C. albicans* finger, which is visible to the naked eye, reaches lengths in excess of 3 mm and is composed primarily of densely packed, viable yeast-phase cells with apparently a single central hypha traversing the length of the shaft, and it has a base composed of a yeast cell bulb that sits atop a hemispherical, highly coherent basal monolayer of yeast cells. The monolayer adheres tightly to the surface of a hemispherical agar cavity. We propose that a single core hypha in the shaft releases yeast cells at compartmental junctions, resulting in a “fountain effect” in which one hypha extends vertically, acting as the source of budding yeast cells that multiply, pack, and support the thick shaft. The shaft is of uniform diameter along its entire length, contains no spaces or pockets of dead cells, and is encapsulated, making it difficult to disperse the yeast cells it harbors without strong mechanical disruption. The basal bulb, contiguous with the shaft, is connected to the basal monolayer at a fragile interface. The ready and precise release through mechanical perturbation after dehydration of the bulb and main shaft from the basal monolayer and the encapsulation of the bulb and shaft suggest that the finger may function as a multicellular vehicle for dispersal. Mutant analysis reveals that finger development is regulated by the Ras1-cyclic AMP (cAMP) pathway, targeting the transcription factor Tec1, the same pathway regulating hypha formation (11, 30, 47, 51). Given the observation that physiological levels of CO₂ induce formation of the complex finger morphology at physiological temperature, one wonders if other developmental programs and morphological states have been missed in *C. albicans*, or for that matter in other microbial pathogens, because physiological conditions have not been accurately mimicked *in vitro*.
Materials and methods

Strain maintenance and growth. The genotypes and sources of the control and mutant strains of C. albicans used in this study are listed in Table S1 in the supplemental material. Strains were maintained in 20% glycerol at −80°C. For routine growth, supplemented Lee’s medium (5, 27) was used unless otherwise stated. For experimental purposes, cells from four 5-day colonies were grown at 25°C in suspension to stationary phase prior to plating. Agar cultures were plated at a density of 40 to 60 CFU per 10-cm plate at 37°C in 20% CO2 or air, as noted. Four different agar cultures were used: (i) supplemented Lee’s medium in 2% agar (5, 27), (ii) Spider medium in 2% agar (31), (iii) yeast extract-peptone-dextrose (YPD) in 2% agar (17, 18), and (iv) YPD in 4% agar (11). Colonies were grown for a minimum of 5 days and a maximum of 10 days.

SEM. Agar cultures were fixed by placing osmium tetroxide crystals in the agar petri dish, replacing the lid, and placing the dish in a fume hood overnight. Following osmium vapor fixation, the samples were postfixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 24 h. The specimens were removed, washed in 0.1 M cacodylate buffer (pH 7.2), and subjected to an alcohol dehydration series (50%, 70%, 85%, 95%, and 100%). After the second change of 100% ethanol, a 50/50 mixture of 100% ethanol and hexamethyldisilizane (HMDS) was placed in the dish for 30 min, followed by 2 changes of pure HMDS. The plates were then allowed to air dry in a hood. Once dried, a scalpel was used to cut out areas of fingers and mount the specimens on Cambridge-style scanning electron microscope (SEM) specimen mounts. The mounts were sputter coated with 60/40 gold-palladium and imaged with a Hitachi S-4800 field emission scanning electron microscope (Hitachi Corp., San Diego, CA) located in the Central Electron Microscopy Research Facility (CMRF) at the University of Iowa.

Stalk-like structure formation. To induce stalk-like structures (11), 2 × 106 cells were plated on air-dried 4% agar plates containing YPD medium. UV irradiation followed the protocol of Morrow et al. (40), resulting in approximately 99.99% cell death. The plates were then incubated at 29°C in air for 5 days.

Results

CO2 induces finger formation in two natural a/α strains. Yeast cells of the natural, unrelated a/α strains P37037 and P37039 (35, 56, 57) were plated at low density (40 to 60 CFU per 10-cm plate) on 2% agar containing supplemented Lee’s medium (5, 27) and incubated at 37°C for 7 days either in air, which contains 0.04% CO2, or in a mixture of 80% air and 20% CO2 (20% CO2). After 10 days in air, over 99% of the colonies of both strains were uniformly smooth and round (Fig. 1A and B). After 10 days in 20% CO2, however, the colonies of both strains exhibited the following four distinct phenotypes (Fig. 1C and D): (i) the uniformly “smooth” round colony (Fig. 1E); (ii) a highly “myceliated” colony composed of a small, relatively round, smooth center and a peripheral halo of mycelia penetrating the surrounding agar (Fig. 1F); (iii) a “medusa” colony, composed of a roughly round colony with thick tapered “tentacles” emanating outwardly on the surface of the agar (Fig. 1G); and (iv) the “finger” extending aerially (Fig. 1H).

Colonies with combinations of these traits were also prevalent. Examples of mature finger morphologies are presented in Fig. 1H through P. In Fig. 1H, O, and P, colonies have formed both fingers and tentacles, and in Fig. 1P, a finger has bent over and contacted the substratum. Similar results were obtained when cells were incubated in 10% CO2 (data not shown) and 20% CO2 at 29°C (data not shown).

Cell composition of a finger. Fingers formed by strains P37039 and P37037 were remarkably uniform in length, reaching 2 to 3 mm within 10 days (Fig. 1H through P). They were also remarkably uniform in diameter along the entire shaft (Fig. 1H through P). The ratio of diameter to length averaged 1:7 (n = 15). For most fingers (Fig. 1H through O), the diameter remained uniform along their lengths. In many cases, fingers fell to the substratum, but even then, they maintained the finger form (Fig. 1I, N, and P). The yeast cells in the shaft usually did not immediately resume cell division when the shaft contacted the nutrient agar substratum. This suggested either that an encapsulating pellicle formed at the surface of a shaft or that the cells in a shaft were nonviable. To distinguish between these alternatives, cells from mechanically disrupted fingers were plated at low density on agar. The level of viability was >80% in three repeat experiments, supporting the
alternative suggestion that the shaft of a finger was encapsulated. In a minority of cases, the tip of a bent finger contacting the agar grew peripherally on the surface of the agar (Fig. 1P).

When the shaft of a finger was picked from a culture with a dissection needle or loop, strong mechanical disruption was necessary to disperse the cells. Compression of a finger under a coverslip on a glass slide caused the finger to erupt, releasing cells. The great majority (\(>99.9\%\)) of the cells were in the yeast phase, while a minority (\(<0.1\%\)) were in the hyphal phase (Fig. 2A through D).

A minority of the yeast cells were budded. When cell cultures were further diluted, the unbudded phenotype of the great majority of yeast cells was evident, as were small clusters of budding cells that formed at the distal end of each elongate hyphal cell compartment (Fig. 3E and F).

In all cultures forming fingers, the medusa, with tentacles along the agar surface, represented a prominent colony phenotype (Fig. 1C and D). In contrast to fingers, the shape of the tentacles along the substratum was tapered, rather than tubular. When cells from tentacles were dispersed and examined in a manner similar to that used to examine the cells of fingers, the same cell phenotypes were observed. The majority of cells (\(>99\%\)) were in the un budded yeast form, a minority in all stages of the budding process, and a small minority in the hyphal form (data not shown). The tapered shape suggested that unlike fingers, yeast cells along the length of a tentacle continued to multiply during extension, presumably because of the availability of a direct food source in the underlying agar. The tapered morphology of a tentacle reflected decreasing age distally and hence the amount of time cells had undergone multiplication.

**Unique architecture at the finger base.** The shafts of fingers frequently fractured when fixed for scanning electron microscopy (SEM) (Fig. 3A). An analysis of the cells lining the walls of the fractures reinforced the conclusion that the great majority of cells in the shaft of a finger were in the yeast form (Fig. 3B). Cracks examined along the entire length of the shafts revealed no large spaces or regions of swollen cells. Fixation for SEM frequently resulted in the release of fingers and the basal bulb from the agar substratum. When the agar substratum was examined after detachment, hemispherical craters were visible (Fig. 3C and D). A crater presumably represented the location in the agar from which the shaft and bulb had detached. Agar surfaces upon which cells had not been cultured were free of craters (data not shown), indicating that formation of the basal bulb caused the crater. When analyzed at higher magnification by SEM, a tightly packed monolayer of unbudded yeast cells lining each crater was observed (Fig. 3E and F). Clean separation of the proximal bulb and the monolayer indicated that a fragile interface existed between them.

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**FIG 2** Cells prepared from fingers are predominately in the yeast form, with rare hyphae (“H” arrowheads). Finger shafts were picked with a needle and then pressed on a microscope slide by applying pressure to a coverslip in order to release cells. (A to D) Examples of cells from fingers pressed under microscope coverslips. (E and F) Diluted samples containing examples of buds forming at the distal ends of compartments along hyphae.
Visualization of core hyphae in tentacles. Tentacles, like fingers, also fractured during SEM fixation (Fig. 4A). Because of the stability afforded by attachment to the agar and their flat contour (Fig. 4A), we were able to view the location of hyphae in the crevices of fractures in tentacles by SEM. The great majority of cells lining crevices were in the yeast form (Fig. 4B through F), consistent with the results obtained by analyzing dispersed cells. However, in almost every deep crevice examined (six of eight), we observed a single core hypha (Fig. 4B through F). In sequential fractures along a tentacle, a single core hypha could be visualized in each (Fig. 4C through E). Because of finger detachment, the subsequent dilution of fingers resulting from the addition of fixative and the tubular rather than flat morphology of a finger, it was more difficult to find and scrutinize deep crevices for a core hypha, as was possible with tentacles. Given that cell preparations generated from fingers and tentacles had the same low concentration of long hyphal profiles with buds formed at the apical ends of compartments (<10^−3), we suggest that as is the case in tentacles, a single core hypha penetrates the length of a finger.

Medium and strain specificity. Three media were tested for finger formation: supplemented Lee’s medium (5, 27), Spider medium (31), and YPD medium (17, 18). All media contained 2% agar. Tests were done in 20% CO₂ or in air at 37°C. For the two major α/α test strains, P37039 and P37037, fingers and tentacles formed on agar containing supplemented Lee’s medium, and only in 20% CO₂ (Fig. 5A and D). Supplemented Lee’s medium is a defined medium, containing glucose as its carbon source (5, 27). It is supplemental with a limiting concentration of zinc (5, 53). The two test strains P37039 and P37037 did not form fingers or tentacles on Spider medium (31) in 20% CO₂ or in air (Fig. 5D). Spider medium is a poor medium containing mannitol as the carbon source (31). On Spider medium, all colonies uniformly formed intertwined aerial hyphae and hyphae in the agar (Fig. 5B). The two test strains P37039 and P37037 also did not form fingers or tentacles on YPD medium, either in 20% CO₂ or in air (Fig. 5D). YPD medium is a rich, undefined medium with glucose as the carbon source (17, 18). All colonies formed on YPD medium exhibited a uniformly large colony dome with hyphae penetrating the agar in 20% CO₂ or in air (Fig. 5C). Fingers also did not form on supplemented Lee’s medium in 20% CO₂ at 37°C, when the colony density was above 100 per 10-cm plate, a condition that severely restricts colony growth (data not shown).

To test strain specificity, we first tested 11 unrelated α/α strains on agar containing each of the three media, in 20% CO₂ or air at 37°C. The 11 strains represented three of the five major clades (I, II, and SA) first discriminated by Pujol et al. (45) (Fig. 5D). Seven of the 11 strains (63%) formed fingers and tentacles, and only on supplemented Lee’s medium (Fig. 5D). The seven included mem-

FIG 3 Scanning electron micrographs of incipient fingers and craters. Cell preparations were incubated for 5 to 7 days in 20% CO₂ at 37°C. (A) An incipient finger with a fracture arising from fixation. (B) Magnified fracture revealing yeast cells compacted in the wall of a fracture. (C and D) A crater at different magnifications. (E and F) Examples of the compacted yeast cell monolayer lining craters.
bers of all three major clades (Fig. 5D). The strains that formed fingers and tentacles included SC5314, a member of clade I, which is the most common laboratory strain used in genetic and molecular studies of C. albicans. Six MTL-homozygous strains were next tested. They included two natural a/α strains (P37005 and 12C), two natural α/α strains (WO-1 and P57072), and two spontaneous derivatives of the α/α strain P37039 (P37039 α/α and P37039 a/a) (35). The six MTL-homozygous strains represented two of the major clades of C. albicans (I and II) (Fig. 5D). None of the four natural MTL-homozygous strains or the spontaneous derivatives P37039 α/α or P37039 a/a formed fingers or tentacles on supplemented Lee’s medium, Spider medium, or YPD medium, in high CO₂ or air (Fig. 5D). On supplemented Lee’s medium, the four α/α strains that did not form fingers or tentacles and all six MTL-homozygous strains instead formed colonies with halos of hyphae penetrating the surrounding agar. On Spider medium, the colonies of all 17 tested strains formed dense aerial hyphae and hyphae penetrating the agar, but no fingers or tentacles along the surface of the agar (Fig. 5B and D). Finally, on YPD medium, all 17 tested strains formed uniformly smooth, large colonies with hyphae penetrating the agar, but no fingers or tentacles (Fig. 5C and D). Hence, a majority of α/α strains, but no a/a or α/α strains, formed fingers and tentacles, and only in high CO₂ on supplemented Lee’s medium at 37°C.

Ras1-cAMP pathway regulates finger formation. Because we found that both fingers and tentacles contained rare hyphae and that in tentacles the core hypha clearly penetrated the entire length of the tentacle, we hypothesized that in both structures, a core hypha may serve as the original source of yeast cells in the stalk of a finger and hence may be the basic driving force for extension (see Discussion). Therefore, we tested whether the Ras1-cAMP pathway, which plays a role in the regulation of hypha formation (12, 26, 31, 43, 47, 51), also plays a role in the regulation of finger and tentacle formation. In hypha formation, this signal transduction pathway and targeted transcription factors include the following: Ras1→Cdc35→cAMP (Pde2— )→Tpk2 (Tpk1)→Efg1→Tec1. Ras1 is a GTPase that activates adenylate cyclase (12), Cdc35 is an adenylate cyclase that catalyzes cAMP formation (36), Pde2 is a negative regulator that hydrolyzes cAMP (23), Tpk2 is an isoform of the catalytic domain of cAMP-dependent protein kinases (7, 9, 55a), Efg1 is a DNA-binding protein (28), and Tec1 is a transcription factor that regulates genes involved in hypha formation (50). We first tested whether deletion of genes encoding key proteins in the Ras1-cAMP pathway blocked CO₂ induction of finger and tentacle formation. The Δras1/Δras1, Δtpk2/Δtpk2, and Δtec1/tec1 deletion mutants were generated in the α/α laboratory strain SC5314 (58) (see Table S1 in the supplemental material). The Δcdc35/Δcdc35 (31) and Δefg1/Δefg1 (47) deletion mutants were generated in the auxotrophic derivatives of SC5314 (see Table S1). CO₂ induced incipient finger and tentacle formation in the parent strain, SC5314 (Fig. 6A). CO₂ did not induce finger or tentacle formation.
formation in the Δras1/Δras1, Δcdc35/Δcdc35, and Δtpk2/Δtpk2 mutants (Fig. 6B, C, and D, respectively). One thousand colonies were analyzed for each strain, with uniform results. In addition, CO2 did not induce finger or tentacle formation in the Δefg1/Δefg1 and Δtec1/Δtec1 deletion mutants (Fig. 6E and F, respectively). Microscopic analysis of 4 × 10⁴ cells in the fingers and tentacles formed by parental strain SC5314 revealed that the great majority (>99.9%) were in the unbudded yeast form and that a small minority (<0.1%) were in the hyphal form (Fig. 6G and H), proportions similar to those observed for strains P37039 and P37037 (Fig. 2). In marked contrast, no hyphae were observed in preparations of cells from the colonies formed by the Δras1/Δras1, Δcdc35/Δcdc35, Δtpk2/Δtpk2, Δefg1/Δefg1, and Δtec1/Δtec1 mutants (data not shown). For each mutant, over 4 × 10⁴ cells were examined. Finger formation in high CO2, therefore, is regulated by the same Ras1-CAMP pathway (Ras1¡Cdc35¡Tpk2¡Efg1) and the same targeted transcription factor (Tec1) that regulate hypha formation (2, 24, 46, 50).

The finger is distinct from the stalk-like morphology. Engelberg et al. (11) found that the high doses of UV irradiation that killed 99.95% of plated Saccharomyces cerevisiae cells induced the formation of stalk-like structures in air on predried 4% agar containing YPD medium. The stalk-like structures grew to over 10 mm in length. They contained large acellular spaces under the tip and along the stalk between the outer cell layer and an inner cell core (49). Engelberg et al. (11) also found that comparable levels of UV irradiation of C. albicans resulted in the formation of similar stalk-like structures, but they did not subsequently analyze the internal architecture, as they did for S. cerevisiae (49). To compare fingers and stalk-like structures, we repeated the protocol of Engelberg et al. (11) and obtained rare stalk-like structures (Fig. 7A and B), which were much longer than fingers (Fig. 7C). Stalk-like structures formed at a frequency of 1 in a half-million irradiated cells. The frequency of finger formation among survivors was 1 in 50. Both the midregion and tip region of the C. albicans stalk-like structure were composed of compacted yeast cells (Fig. 7D and E). Budding cells represented a minority of cells and were more abundant in the tip region (Fig. 7E, red arrowheads). In contrast to fingers, we found no hyphae in cell preparations of either the midregion or the tip region, in a combined analysis of over 10⁶ cells. Staining with Sytox green, a DNA dye that penetrates only dead cells, revealed that over 90% of the yeast cells in the C. albicans stalk-like structure were viable (Fig. 7D). Plating experiments verified a similar high level of viability (>80%). No bulb structure similar to that at the base of a finger was evident in a stalk-like structure. Stalk-like structures were inhibited from forming when UV-irradiated cells were incubated in 20% CO2, the inductive

FIG 5 Medium and strain specificity of finger induction by 20% CO2 at 37°C. (A) Finger and medusa colonies formed by strain P37039 on agar containing supplemented Lee’s medium. (B) Colonies of strain P37039 with aerial hyphae and hyphae penetrating agar containing Spider medium. (C) Smooth colonies of strain P37039 with hyphae penetrating agar containing YPD medium. (D) Finger and tentacle formation by 17 strains in 20% CO2 or air (0.04% CO2) at 37°C on agar containing the three test media. +, finger and tentacle formation; −, no finger or tentacle formation. All experiments were repeated at 29°C with similar results.
signal for finger formation. Formation of stalk-like structures required air-dried 4% agar and formed on YPD agar, as reported by Engelberg et al. (11). In contrast, finger formation occurred on 2% agar that did not have to be air dried, and finger formation was inhibited by YPD medium. The differences between CO2-induced fingers and UV-induced stalk-like structures, listed in Fig. 7F, are, therefore, numerous and fundamental, indicating that the formation and regulation of the two morphologies are developmentally distinct.

DISCUSSION
Finger architecture. The four definable architectural features of a finger are the basal yeast cell monolayer at the substratum, the fragile interface, the proximal bulb, and the contiguous 3-mm shaft. The bulb and shaft are composed primarily of compacted yeast cells, a majority of which are unbudded and a minority of which are budded. The rare hyphae observed in dispersed cell preparations (<0.1%) were usually in the process of budding. The observation that up to 5% of yeast cells in a dispersed finger cell preparation possessed buds at all stages of development suggested that yeast cells, formed at the distal end of each hyphal compartment, are released and continue to undergo limited cell division. Staining and plating experiments revealed that the majority of yeast cells in a finger are viable. However, division of yeast cells in the shaft must be highly regulated and restricted, given the uniformity of the shaft diameter along its length. Similarly, hyphal branching and formation must be repressed, thus limiting hyphal growth to the core hypha.

The phenotypic domains of a finger, therefore, are complex and constant. The basal monolayer of unbudded yeast cells lining the crater is extraordinarily compacted, suggesting that the cells are highly cohesive. This basal layer differs from the yeast cell layer at the base of a C. albicans biofilm in several respects. First, the basal layer of a finger is a monolayer, whereas that of a biofilm is a
poly layer (16, 25). Second, the yeast cells in the basal layer of a biofilm give rise to a distal carpet of vertically oriented hyphae embedded in a dense supporting matrix (4, 6, 8), whereas the upper portion of a finger consists almost exclusively of cohesive yeast cells. Third, biofilms do not form a definable interface between the basal layer and upper portion, as fingers do between basal layer and bulb.

When CO2 induces finger formation, it also induces tentacle formation. We have demonstrated that a single hypha is at the core of a tentacle. From that observation and the observation that very long, rare hyphae are present in cell preparations of the shaft of fingers at the same low concentration found in tentacles, we infer that a core hypha also traverses the length of each finger. We have not been able to identify a basal monolayer or fragile interface at the origin of a tentacle, because a tentacle contacts the nutrient agar substrate along its entire length, precluding mechanical dissociation upon SEM fixation, which mediated identification of these structures at the base of the finger. A tentacle also differs from a finger in that it is flat and tapered rather than tubiform, presumably because of continued lateral growth due to the direct availability of an underlying food source. A finger exhibits uniform width along its length, possibly because of the restricted food source at the basal monolayer. Because tentacles are always formed when fingers are formed and not formed when fingers are not formed, we have tentatively concluded that they reflect a similar developmental process, one aerial and the other along the surface of nutrient agar.

**Ras1-cAMP pathway.** Because high levels of CO2 induce hypha formation (3, 23) and hypha formation in turn appears to play a central role in the morphogenesis of fingers and tentacles, we tested and found that the Ras1-cAMP pathway, which regulates hypha formation (12, 30, 46, 47, 51) (Fig. 8A), also regulates finger and tentacle formation (Fig. 8B). This pathway plays a role not only in hypha and finger formation, but also in the stimulation of the white-to-opaque phenotypic transition by CO2, N-acetylglucosamine, and glucose (19, 20) (Fig. 8C). Finally, this pathway has been shown to play a key role in regulating a/α biofilm formation (Fig. 8D) (56), but not a/a or α/α biofilm formation (48, 58,
The latter is regulated by the mitogen-activated protein (MAP) kinase pathway, although the targeted transcription factor in this case again is Tec1 (Fig. 8E) not Cph1, the target in the pheromone response pathway for the opaque cell mating response (Fig. 8F) (48, 58, 59). We have tentatively concluded that the Ras1-cAMP pathway plays an essential role in finger formation, presumably by regulating extension of the core hypha. Our results, however, do not discriminate between an exclusive role in core hypha extension and a more general one that would include other aspects of finger formation, such as the formation of the yeast cell monolayer, the fragile interface, the basal bulb, the agar crater, and the morphogenetic processes regulating restricted multiplication of yeast cells along the shaft.

**Distinction between the finger and stalk-like structure.** Engelberg et al. (11) previously had shown that high doses of UV irradiation that killed approximately 99.95% of S. cerevisiae or C. albicans cells induced formation of stalk-like structures among the few surviving cells of either species. Here we have reproduced that observation. We found that these long, tapered stalk-like structures do not form in response to CO₂ at 37°C. They are in fact inhibited from forming if irradiated cells are incubated in 20% CO₂. Moreover, stalk-like structures form on YPD, which we found inhibited finger formation. There is also no morphological indication that a bulb forms at the base of a stalk-like structure or that a hypha penetrates the core, two features of fingers. Tentacle formation is also not associated with the formation of stalk-like structures. Finally, we found that stalk-like structures disassociate into single-cell suspensions upon the most incidental contact, whereas fingers are highly resistant to mechanical disruption, suggesting the latter are encapsulated in a protective pellicle. We did, however, find that in contrast to stalk-like structures formed by UV-irradiated S. cerevisiae (11), those formed by C. albicans contain viable yeast cells lacking vacuoles, as was the case for the cells contained in CO₂-induced fingers. We also found no spaces or regions of dead cells, as is the case in S. cerevisiae stalks (49). The numerous differences between the C. albicans stalk-like structures and fingers indicate that they are phenotypically distinct. Since stalk-like structures form only after 99.95% cell death caused by UV irradiation, it seems highly unlikely that they arise through mutation and play no role in the life history of C. albicans. Fingers, on the other hand, are formed by up to 50% of cell populations of clinical strains in response to 10 to 20% CO₂, a condition encountered in the host gut. Therefore, we believe that fingers formed in response to high CO₂ on agar reflect a pathologically relevant phenotype in the developmental repertoire of C. albicans.

**Hypothesis for the extension of a finger.** The shaft of a finger can grow aerially to a length of 3 mm in 7 days, representing a minimum rate of extension of approximately 430 μm per day, assuming the rate of extension is constant. A plausible hypothesis for extension can be formulated in which a single hypha extending apically releases yeast cells from the distal end of each cellular compartment (Fig. 9), a budding characteristic of true hyphae (42). The yeast cells formed by hyphae would then undergo limited cell division, perhaps controlled in part by the lack of nutrients after release. The core hypha would continue to grow apically and continue to release yeast cells. Subsequent hypha formation by yeast cells and branching of the core hypha would be severely restricted, even though CO₂ represents an inducer of the yeast-hypha transition (10, 43). Extension of the core hypha and the formation of yeast cells might be fueled by nutrients that pass up the hypha from the nutrient agar substratum. The cytoplasm of hyphal compartments is contiguous (14, 54) and thus may function in a fashion similar to that of phloem cells in plants, transporting nutrients distally (Fig. 9). Transportation through compartments could be mediated through septal pores, which have been described in C. albicans (13, 15). As yeast cells multiply around the core hypha, they in turn would support the thick shaft, facilitating vertical extension of the very thin, fragile core hypha (Fig. 9). Presumably the bulb in the crater, attached to the basal yeast cell monolayer, would anchor the shaft (Fig. 9). The rare stalk-like structure that is induced by UV, however, attains heights five times that of a finger, but contains no hyphae. Scherz et al.
loosely packed and lack cohesion compared to the densely packed may explain why the yeast cells in a stalk-like structure are so plication at the tip and do so independently of a core hypha. This thereof, nutrients can flow up the stalk to support yeast cell multi-
(49) hypothesized that growth of stalk-like structures in S. cerevisiae was through the multiplication of yeast cells at the base and that cells multiplying basally pushed distal cells apically. They ar-
gued that distal cells in the S. cerevisiae stalk-like structure died for lack of nutrients and provided evidence that was the case. How-
ever, we found that the cells along the stalk-like structure of C. albicans remain viable, even at the tip, which is at odds with the hypothesis of Scherz et al. (49) as applied to C. albicans. Our ob-
servations that the stalk-like structure of C. albicans can grow apically without a core hypha, that cells in the stalk-like structure are viable, and that budding continues in the tip region provide an alternative mechanism to the one suggested by Scherz et al. (49) for S. cerevisiae stalk-like structures and the one we have suggested for C. albicans fingers (Fig. 9). We suggest that in stalk-like struc-
tures, nutrients can flow up the stalk to support yeast cell multi-
FIG 9 A model of the finger morphology and a hypothesis for the extension of the shaft. In the proposed hypothesis, a single core hypha extends vertically from the bulb, releasing yeast cells from the distal end of each hyphal compart-
ment. These yeast cells in turn undergo restricted cell division, which termi-
nates when the diameter of the shaft reaches approximately 0.4 mm. This would explain the uniform diameter along the length of the shaft. We assume in the model that the hypha traverses the length of the finger at its core and that a pellicle encapsulates the shaft.

The possible role of the finger. The complex architecture and compartmentalization of morphological domains in the course of finger formation suggest that the process involves a development-
tal program that functions over time and space. Although we have found no detailed description in vitro or in vivo of the finger mor-
phology in the literature, it is possible that fingers have been ob-
served by medical mycologists over the years in stored agar cul-
tures in which gas exchange is limited, leading to a buildup of CO2. The apparent complexity of the morphogenetic program and spec-
ific induction by the same high levels of CO2 that are attained in the gastrointestinal tract and interstitial regions of select host tis-
ues suggest that the finger may have evolved as a result of host colonization and the associated challenges of high CO2 in colo-
nized host niches. Moreover, the apparent fragility of the interface between the basal monolayer and bulb suggests that one possible role the finger may play is as a multicellular vehicle for dispersal. This of course represents a hypothesis rooted in in vitro observa-
tions that must be verified in vivo. What is clear is that analysis of the phenotypic potential of C. albicans in high CO2, a physiologi-
cal condition not often used in vitro, has revealed a new develop-
mental program, the formation of the finger. One must, therefore, wonder how many other programs and phenotypes remain un-
identified in the developmental repertoire of C. albicans or, for that matter, other microbial pathogens.

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