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21. We thank G. Sullivan for advice on the preparation of this manuscript. Supported by grants AI09504 and T32AI07046 from the National Institute of Allergy and Infectious Diseases.

29 May 1985; accepted 27 August 1985

High-Frequency Switching of Colony Morphology in *Candida albicans*

Abstract. *The pathogenic yeast* Candida albicans *switches heritably and at high frequency between at least seven general phenotypes identified by colony morphology on agar. Spontaneous conversion from the original smooth to variant phenotypes (star, ring, irregular wrinkle, hat, stipple, and fuzzy) occurs at a combined frequency of* 1.4×10^{-4}*, but is increased 200 times by a low dose of ultraviolet light that kills less than 10 percent of the cells. After the initial conversion, cells switch spontaneously to other phenotypes at a combined frequency of* 2×10^{-2}*. Switching is therefore heritable, but also reversible at high frequency. The genetic basis of this newly discovered process and its possible role in* Candida *pathogenesis are considered.*

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The dimorphic yeast *Candida albicans* is one of the most pervasive fungal pathogens in man (*1*). Although it has been assumed that its pathogenicity depends to a large extent on its capacity to grow in either a budding or hyphal form, this single developmental characteristic has never seemed to be sufficient to account for its capacity to invade so many diverse body locations, in many instances to evade antibiotic treatment (*2*) and the immune system (*3*), and to opportunistically infect a diverse spectrum of compromised hosts (*4*). In addition, it has been shown that *C. albicans* is a diploid (*5*) with balanced lethals (*6*) and therefore most likely without a sexual phase or meiotic cycle. Where, then, does *C. albicans* obtain the diversity that appears to be basic to its success? The answer to this question may lie in the discovery that *C. albicans* spontaneously and reversibly switches at high frequency between at least seven general phenotypes that can be distinguished by a simple agar assay for colony morphology.

When clonal colonies of a standard strain of *Candida albicans* (3153A) were grown on an amino acid–rich agar (*7*) at 24°C for 7 days, the colony morphology was "smooth" (Fig. 1A). Smooth colonies exhibited an unmottled, or unwrinkled, surface, with no aerial mycelia. Cells removed from the surface of a smooth colony were exclusively in the budding form of growth. On the underside of a mature colony, at the colony-agar interphase, hyphae penetrated the agar. Because of its smooth colony morphology, we will refer to the original parent strain as "original smooth" or "o-smooth."

When large numbers of cells of o-smooth were plated as clones, variant colony morphologies appeared spontaneously at a frequency of 1.4×10^{-4}. In 50,500 colonies examined, we found seven variant colonies: two "star" (Fig. 1B), three "ring" (Fig. 1C), and two "irregular wrinkle" (Fig. 1D). Colonies with the star morphology exhibited a slightly thickened perimeter encompassing a star with between 8 and 12 arms projecting peripherally and equidistant from one another (Fig. 1B). The arms usually did not connect centripetally, and their peripheral tips extended off the colony proper. Colonies with the ring morphology exhibited a very thick perimeter, encompassing up to one-third of their radius (Fig. 1C). The center of a ring colony was relatively thin in comparison, and was usually mottled in texture. Colonies with the irregular wrinkle morphology exhibited deep wrinkling throughout their surface (Fig. 1D). In contrast to o-smooth colonies, cells removed from the surface of star, ring, and irregular wrinkle colonies exhibited both budding and hyphal phenotypes. When original isolates of the three spontaneous variant morphologies were in turn plated as clones, the respective variant phenotype persisted in the majority of offspring. These phenotypes persisted in successive clonal platings.

When cells of o-smooth were plated as clones on agar and immediately treated with low doses of ultraviolet light, resulting in relatively low levels of cell death, variant colony morphologies appeared with even higher frequencies. At an ultraviolet light dose that killed only 8 percent of the cells, 136 out of the 5600 surviving clones exhibited variant colony morphologies, a combined frequency

Table 1. The frequency of variants in an ultraviolet-induced star colony emanating from original smooth and in ring, stipple, fuzzy, and r-smooth colonies emanating from the star.

Switch phenotype	Number of clones	Number of colonies	Total switch colonies	Frequency of individual switch phenotypes						Combined frequency of switching
				Star	Ring	Irregular wrinkle	Stipple	Fuzzy	r-Smooth	
Star	1	25,000	442		7.4×10^{-3}	2.0×10^{-4}	4.1×10^{-3}	6.4×10^{-4}	5.2×10^{-3}	1.8×10^{-2}
Ring	10	57,000	1,398	1.0×10^{-2}		1.0×10^{-3}	4.7×10^{-4}	2.6×10^{-4}	9.8×10^{-3}	2.5×10^{-2}
Stipple	6	36,100	3,933	7.8×10^{-3}	2.9×10^{-3}	7.1×10^{-3}		2.4×10^{-3}	6.1×10^{-3}	1.1×10^{-1}
Fuzzy	4	25,400	665	2.2×10^{-3}	8.3×10^{-3}	5.1×10^{-3}	1.3×10^{-3}		9.4×10^{-3}	2.6×10^{-2}
r-Smooth	5	30,700	444	6.6×10^{-3}	6.3×10^{-3}	1.2×10^{-3}	2.9×10^{-4}			1.4×10^{-2}

of 2.5×10^{-2} for survivors. This represents approximately a 200-fold increase over the frequency of spontaneous switching. In addition to the three spontaneous variant colony phenotypes observed in untreated populations (star, ring, and irregular wrinkle, Fig. 1, B to D), three additional phenotypes were observed: "stipple," "hat," and "fuzzy" (Fig. 1, E to G). Colonies with the stipple morphology were mottled, or bumpy, in appearance (Fig. 1E). Colonies with the hat morphology exhibited a peripheral rim around a domed center (Fig. 1F). A distinct mycelial edge encircling the superficial colony was always evident in the lateral agar. Colonies with the fuzzy morphology exhibited a thickened, irregular rim and thin interior (Fig. 1G). Aerial mycelia composed of hyphal bundles emanated from the entire colony surface. Again, cells removed from the surface of these variant colonies contained hyphae as well as budding cells. When the variants from ultraviolet-treated cell cultures were in turn plated as clones, the variant phenotypes persisted in the majority of offspring.

To measure both the heritability and reversibility of variant phenotypes emanating from o-smooth, we focused initially on the variant star. A single clone of star obtained from an o-smooth culture treated with a low dose of ultraviolet light was grown in liquid nutrient medium to exponential growth phase and then clonally plated. Of 25,000 mature colonies examined, 24,558 (98.2 percent) exhibited the same star morphology as the parent colony, demonstrating the heritability of this switch phenotype (Table 1). However, 442 (1.8 percent) exhibited other switch morphologies, including smooth. This represents a combined frequency of 1.8×10^{-2}. The individual frequencies of ring, irregular wrinkle, stipple, fuzzy, and smooth were 7.4×10^{-3}, 2.0×10^{-4}, 4.1×10^{-3}, 6.4×10^{-4}, and 5.2×10^{-3}, respectively. The smooth colonies emanating from switch phenotypes will be referred to as "revertant smooth," or "r-smooth," because they do not always represent true revertants to o-smooth.

Spontaneous variants from o-smooth were also assessed for switch frequencies. Although this analysis was not as extensive as the preceding one for ultraviolet-induced star, the combined frequency of secondary switching for seven individual clones (three star, two irregular wrinkle, and three ring) was 4.9×10^{-2}, again demonstrating the dramatic increase in spontaneous switching frequency after the initial switch has occurred.

To test whether the variant colony morphologies emanating from the ultraviolet-induced star clone (i) were heritable, (ii) switched to other variant morphologies, and (iii) switched back to star as well as smooth, ten clones of ring, six of stipple, four of fuzzy, and five of r-smooth were individually grown in liquid nutrient medium and plated as clones. Each original ring colony contained cells that had switched to two or more other switch phenotypes. The frequency of variant colonies (other than ring and including r-smooth) ranged between 0.8×10^{-2} and 5.9×10^{-2}. The average frequency for the ten ring clones was 2.5×10^{-2} (Table 1). This was roughly 180 times higher than the frequency of

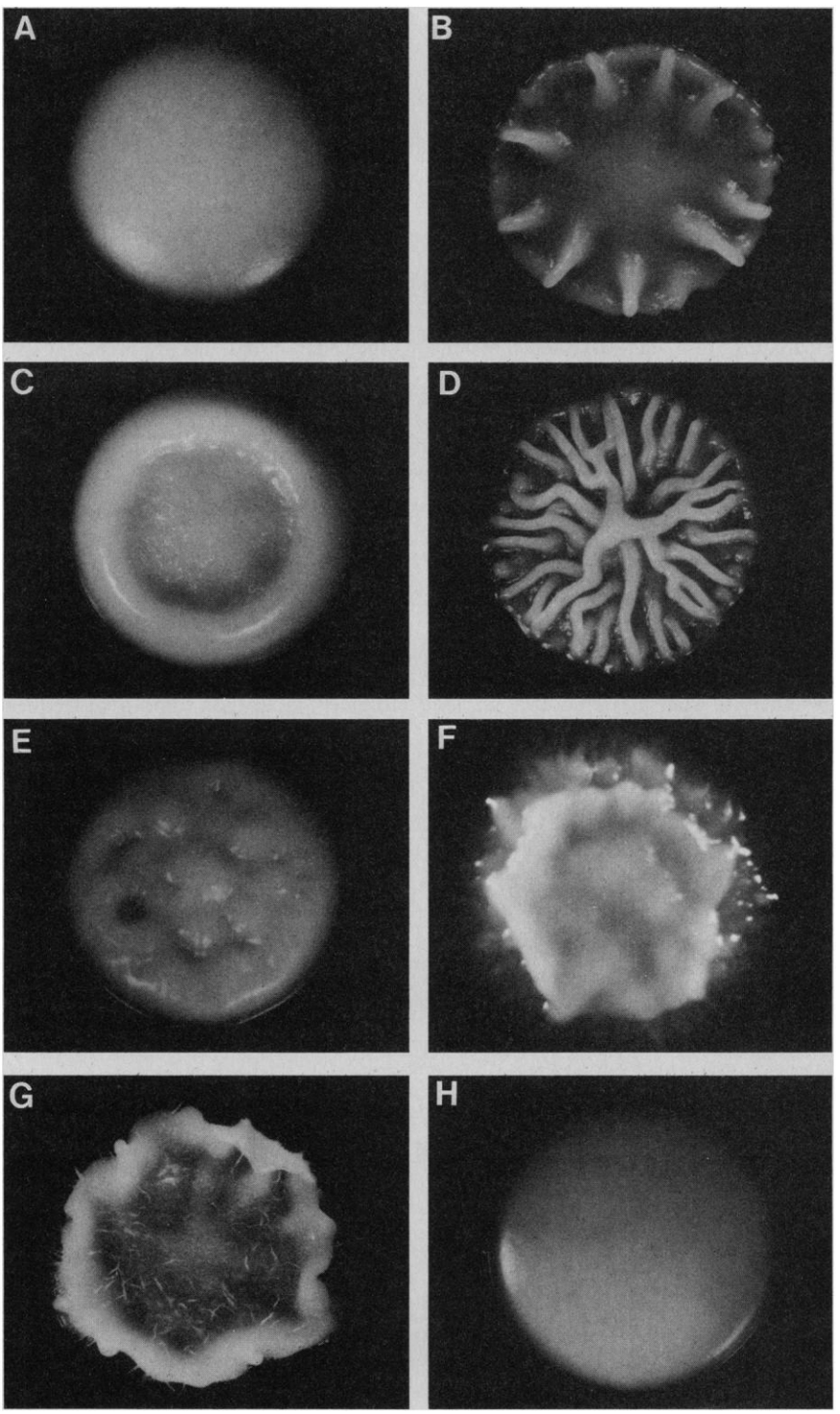

Fig. 1. The variant colony phenotypes of *Candida albicans*. Each panel contains an individual colony representative of each phenotype. Clonal colonies were grown on amino acid–rich agar at 24°C for 7 days (*7*). (A) "o-smooth"; (B) "star"; (C) "ring"; (D) "irregular wrinkle"; (E) "stipple"; (F) "hat"; (G) "fuzzy"; and (H) "r-smooth".

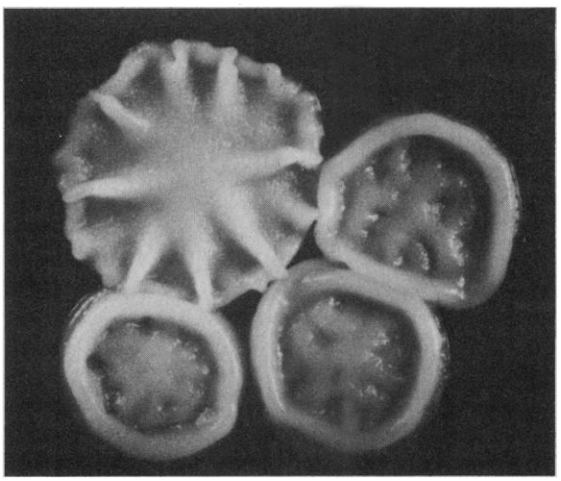

Fig. 2. A switch from ring to star. The agar dish contained 60 colonies, 59 ring and 1 star. This ring clone emanated from an ultraviolet-induced star clone.

variants in o-smooth colonies and roughly the same frequency observed after low ultraviolet treatment of o-smooth. Although all of the tested ring colonies emanating from star exhibited the same original colony phenotype, the frequency of variants was not uniform. It should be noted that the frequency of variants in a colony depends on the time at which the initial switch occurs in the colony history as well as the relative rates of cell division of the switch phenotypes. Therefore, the average frequency of variants for a number of independent colonies affords a more accurate representation of switching frequency. All of the ring colonies contained cells that had switched to star and smooth, but not necessarily to irregular wrinkle, stipple, or fuzzy. However, the frequency of these latter morphologies may have been too low to measure for the number of colonies scored. An example of a switch from ring to star in a ring population is presented in Fig. 2.

Each of the six stipple colonies examined exhibited switching to three or more other phenotypes. The frequency of variant colonies (other than stipple and including r-smooth) ranged between 1.6×10^{-2} to 2.7×10^{-1}. The average frequency for the six clones was 1.1×10^{-1} (Table 1). The four fuzzy clones examined exhibited variant colonies (other than fuzzy and including r-smooth) at frequencies between 1.3×10^{-3} and 1×10^{-1}. The average frequency for the four clones was 2.6×10^{-2} (Table 1). Finally, the clones of revertant-smooth exhibited variant colonies (other than smooth) at frequencies between 3.4×10^{-3} and 5.6×10^{-2}. The average frequency for the six clones was 1.4×10^{-2} (Table 1).

Within the r-smooth clones, there appeared to be subphenotypes. One clone, labeled "flat-top," exhibited a flat rather than round surface. Another, labeled "cone," exhibited a peak at the center of the colony. In addition, the limited number of r-smooth clones emanating from star exhibited switch frequencies higher than o-smooth, indicating that they did not represent true revertants to o-smooth.

The average frequencies of switching between star, ring, stipple, fuzzy, and r-smooth are shown in Fig. 3. Switching of irregular wrinkle was not included because of the low frequency of appearance of this phenotype in initial star clones (Table 1). It should also be noted that we found no switches from r-smooth to fuzzy.

To examine in more detail whether variants revert back to true o-smooths, an irregular wrinkle clone (S19) that appeared spontaneously from o-smooth was clonally plated. S19 formed variant colonies at a combined frequency of 5.5×10^{-2}. From among these S19 switch colonies, two r-smooths exhibiting colony morphologies indistinguishable from o-smooth and one star were plated as clones. The switching frequencies of the two r-smooths were 5.1×10^{-4} and $< 1 \times 10^{-4}$; that of the star was 3×10^{-2}. Therefore, the two r-smooths isolated from S19 reverted not only to the colony morphology of o-smooth but also to lower switching frequencies, suggesting true reversion.

In 1968, J. Brown-Thomsen (*8*) examined streaks of 314 strains of *Candida albicans* on malt-extract agar. He discerned 15 different forms as well as variants resulting from parent strains monitored for 6 months. He found differences not only in streak macromorphology but also in cloned colony morphology, fermentation of sugar, assimilation of sugars, reduction of tetrazolium salt, production of chlamydospores (large ovoid cells with thick walls), and the extent of mycelium formation. We found that this extraordinary level of variability does not reflect variation between strains, but rather an inherent capacity for extreme and reversible phenotypic variability within each cell. A standard laboratory strain of *C. albicans* switches spontaneously to a number of variant colony morphologies at an initial frequency of 1.4×10^{-4}. The initial frequency is increased 200-fold by a low dose of ultraviolet light that results in a kill rate of less than 10 percent. After the initial switch, spontaneous switching continues at a frequency of approximately 2×10^{-2}. The high frequency of switching as well as complete reversibility suggests that reversible genetic changes (for instance,

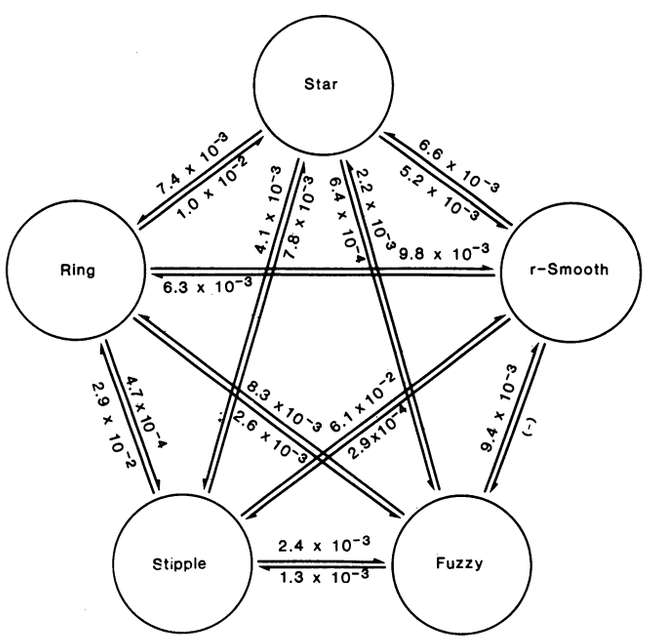

Fig. 3. The frequencies of switching between star, ring, stipple, fuzzy, and r-smooth. These clones emanated from a star clone obtained after low ultraviolet treatment of o-smooth.

changes in the location of mobile genetic elements) (*9*) may be basic to the switching mechanism, although heritable extrachromosomal changes have not been ruled out.

The differences in colony morphology between smooth and variant phenotypes appear to be due to spatial, temporal, quantitative, and qualitative differences in bud and mycelium formation (*10*). However, the molecular or cytological basis for these developmental differences have not been elucidated. All of the seven switch phenotypes described here retain the basic capacity of dimorphism, and are therefore capable of forming buds and mycelia, but the environmental constraints, such as *p*H-regulated dimorphism (*11*), on the transitions between bud and hyphal forms vary markedly between o-smooth and the switch phenotypes (*10*).

Switching has been shown to occur not only in our standard laboratory strain but also in strains of *C. albicans* isolated from the mouths of healthy individuals (*12*) and in the related yeast *C. tropicalis* (*13*). It therefore seems reasonable to suggest that switching may provide *C. albicans* and related infectious yeasts with the diversity that is expected of such pervasive and successful pathogens. Switching may provide an organism with the capacity to (i) invade diverse body locations, (ii) evade the immune system in a fashion analogous to *Salmonella* (*14*) and *trypanosomes* (*15*), or (iii) change antibiotic resistance. It may also account for a significant portion of the 90 or more *Candida* "species" that have been described (*16*).

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17. The authors are indebted to M. Staebell, P. Jennings, and Z. Sunleaf for technical assistance and to C. Newlon and J. Yarger for valuable discussions. Supported in part by NSF grant PCM-8202380, NIH grant GM25832, and a grant from the University of Iowa Research Foundation.

21 May 1985; accepted 12 September 1985

Psoriatic Fibroblasts Induce Hyperproliferation of Normal Keratinocytes in a Skin Equivalent Model in Vitro

Abstract. *A skin equivalent model has been used to fabricate tissues with psoriatic and normal cells. Psoriatic fibroblasts can induce hyperproliferative activity in normal keratinocytes. The psoriatic epidermis from lesions continues to proliferate at high rates for at least 15 days in this model, and normal fibroblasts are unable to suppress this hyperproliferation. The primary defect in psoriatic skin may reside in the dermal fibroblast.*

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Psoriasis is a common skin disease characterized clinically by the appearance of sharply circumscribed scaly red plaques. Histopathological changes in established psoriatic plaques are essentially epidermal, with keratinocyte hyperproliferation in the basal layers and incomplete keratinization. The main changes in the dermis consist of papillary edema, dilatation of the papillary capillaries, and the appearance of a moderate infiltrate of inflammatory cells. The role of dermal fibroblasts in inducing plaque formation is the subject of this report.

There is strong evidence that the disease may not be of systemic origin but that the defect may originate within the skin (*1*). When full-thickness psoriatic skin is grafted to the nude mouse, the epidermis from lesions [involved psoriatic (PP) skin] maintains most of its pathological features for at least 6 weeks and the epidermis from apparently normal areas [uninvolved psoriatic (PN) skin] can develop certain features of the psoriatic lesion (*1*). However, the rates of proliferation and the patterns of differentiation of keratinocytes from PP or PN skin monocultured in vitro do not differ from each other or from the rates and patterns of normal cultured keratinocytes (*2*).

Keratinocytes cultivated without a living dermal component may be an incomplete model for studying interrelations within the skin that could explain the mechanism of plaque formation. To overcome this handicap we have adapted a model system (*3*), called a skin equivalent, made from matrix materials with dermal and epidermal cells to study this aspect of the disease. The dermal component, called a dermal equivalent, originates from the contraction of a collagen gel by dermal fibroblasts (*4*) that have first been grown as conventional monolayer cultures on plastic. In the original experiments, the epidermis was applied to the dermal equivalent as a cell suspension (*3*); in the experiments reported here a new procedure, the insertion of a small full-thickness punch biopsy into the dermal equivalent, was used. The multilayered epidermis that develops in vitro by a process of epiboly (Fig. 1, A through C) consists of tightly associated, well-differentiated keratinocytes emanating from an organized basal layer of cells (*5*). Using the skin equivalent model, we have studied the outgrowth of keratinocytes from punch biopsies of PP and PN skin harvested from the forearms of untreated patients afflicted with chronic plaques of psoriasis and from punch biopsies of normal skin (NN) from age- and sex-matched healthy volun-