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Strain Variation and Morphogenesis of Yeast- and Mycelial-Phase *Candida albicans* in Low-Sulfate, Synthetic Medium

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A low-sulfate synthetic medium was developed in which pure cultures of yeast- and mycelial-phase *Candida albicans* could be cultivated for investigations of the molecular biology of dimorphism. The medium contained ammonium ions, phosphate buffer, salts, glucose, and biotin. Morphogenesis was found to be dependent upon the strain of *C. albicans*. Of six strains tested in the low-sulfate medium at 37°C, three formed mixed cultures of yeasts, true mycelium and pseudomycelium, two formed pure cultures of true mycelium, and one maintained yeast growth. All six strains produced pure cultures of yeasts at 24°C. The buffering capacity of the medium maintained the pH at 6.9 even at high-density cell growth. The low concentration of sulfate and the absence of amino acids in the medium provided conditions in which to radiolabel cellular constituents with [^{35}S]sulfate. For molecular investigations, the use of two strains is suggested, one forming yeasts and one forming true mycelium in low-sulfate medium at 37°C, thus providing controls for both strain variation and for molecular changes induced by environmental change but unrelated to morphogenesis.

Candida albicans, a normally benign member of the human microbial flora, is capable of causing life-threatening disease in patients whose defenses are compromised by a variety of disorders including leukemias, diabetes, and immunodeficiencies (5). The morphogenesis of *C. albicans* from yeast phase to mycelial-phase growth is a hallmark of tissue invasion, yet neither the role of this morphogenesis in the pathogenesis of candidiasis nor the molecular events that initiate and regulate conversion are well understood.

Attempts to identify the environmental stimulus that triggers conversion from the yeast to the mycelial phase have yielded a plethora of often contradictory results. Consequently, a variety of different media have been used to cultivate yeast and mycelial cells for molecular and immunological investigations. Because environmental manipulations such as alterations in carbon or nitrogen sources could potentially induce modifications in cytoplasmic enzymes, or membrane or cell wall components which are unrelated to morphogenesis per se, it is difficult to directly compare the work of different investigators. The problem has been further complicated by the ability of *C. albicans* to form two types of filamentous cells, mycelium and pseudomycelium. These terms have been used incorrectly in the past as synonyms. Recent examination of the ultrastructure of the cell walls and septa of pseudomycelium (i.e., chains of elongated cells with constrictions between cells) and true mycelium (i.e., filaments of uniform diameter without constrictions between cells) by electron microscopy has indicated that the two cell types may be fundamentally different (7, 11).

To facilitate studies of the dimorphism of *C. albicans*, a simple synthetic medium based upon one originally described by Lee et al. (8) has been developed in which morphogenesis is stimulated by a single environmental manipulation (temperature shift). The cultivation of yeast and mycelial cells without concomitant production of pseudomycelium, the strain variation in response to the medium, and the utility of the medium for the incorporation of radiolabel are examined and discussed.

MATERIALS AND METHODS

Cultures. Six strains of *C. albicans* were used. Two strains, designated 550 and 4918, were isolated by Ardell Proctor in 1975 from the skin and blood, respectively, of patients at Duke Hospital. Strain 2252 was originally isolated by Billie Juni in 1972 from a fatal case of candida endocarditis at the University Hospitals, University of Minnesota. The remaining three strains were clinical isolates donated by Rae Ellen Syverson (strain 3153) and by Charlotte Campbell (strains E-136 and E-139). Upon receipt, and semiannually thereafter, each strain was confirmed as *C. albicans* by its ability to form germ tubes in human serum at 37°C and chlamydospores on corn meal agar and by its carbohydrate fermentation and assimilation profile (12).

The strains were maintained at room temperature with biweekly transfers on a medium containing 2% glucose, 1% yeast extract (Difco, Detroit, Mich.), and

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2% agar (GYE). As a control for spontaneous mutation over the course of these studies, multiple cultures of each strain were sealed and stored at −20°C. Before use, the frozen cultures were thawed and immediately streaked onto GYE plates to confirm their purity. Representative colonies were then transferred to fresh GYE slants.

Media. Four synthetic media were investigated. The formulations of these media, which are modifications of the synthetic medium (SM) described by Lee et al. (8), are presented in Table 1. Both SM and buffered synthetic medium (bSM) contain the same eight amino acids, glucose, and biotin, but differ in their salt composition. Two additional media, basic salts (BS) and low-sulfate medium (LSM), consist of salts, glucose, and biotin. The media were prepared by separately autoclaving a 50% glucose stock solution and salt solutions, with (SM and bSM) or without (BS and LSM) the amino acids. A biotin stock solution was prepared at 1,000-fold concentration (1 mg/ml) and sterilized by filtration. To prepare the complete media, each 200-ml solution of basic salts and amino acids (if present) was supplemented with the aseptic addition of 5.0 ml of glucose and 0.2 ml of biotin stock solutions. All chemicals were reagent grade.

Cultivation of yeast- and mycelial-phase cells. Rigorous attention to detail in the standardization of growth conditions was necessary to achieve maximal growth and reproducible morphology of the yeast and hyphal growth forms. Preliminary experiments determined that the following protocol yielded uniform results. Cells of *C. albicans* from a 48-h GYE slant were suspended in sterile deionized water and centrifuged at 1,000 × *g* for 3 min. The pellet was resuspended at a concentration of approximately 10^9 yeast cells per ml in sterile deionized water, and this suspension was used to initiate cultures in the medium to be tested (SM, bSM, BS, or LSM) at a cell density of 10^6 to 10^7 yeast cells per ml, as estimated by counting the cells in a hemacytometer chamber. The cells were then incubated overnight in a rotary shaker (model G76, New Brunswick Scientific Co., New Brunswick, N.J.) at 150 rpm at room temperature until early stationary phase was reached, and a final census of 2 × 10^8 to 3 × 10^8 yeast cells per ml, consisting of single yeast cells (<5% budding forms), was obtained. The cells were then centrifuged, washed once, and resuspended in fresh medium at 10^7 to 5 × 10^7 yeast cells per ml as determined by hemacytometer chamber count. Cultures were established from this suspension of cells and incubated in rotary water bath shakers at 150 rpm at two temperatures. Most experiments employed 200-ml culture volumes in 500-ml Erlenmeyer flasks. For preliminary experiments, tubes containing 1 ml of culture medium were often inoculated directly with the washed cells from the GYE slant, eliminating the overnight culture.

Evaluation of morphology. During the first 6 h, at least 200 cells of each strain were scored directly in hemacytometer chamber. Only true germ tubes were scored as mycelial cells (i.e., those outgrowths half the diameter of the yeast cell and lacking a constriction at the base). Typically, an incipient germ tube reached 5 μm in length before it was unequivocally identifiable as a true hypha and not an initial pseudohypha. The yeast cell at the base of the germ tube was not scored separately, and the length of the growing filament was not reflected in the quantitation. Yeast cells were denoted as single (without bud), double (with one bud) or clump (three or more cells attached together). Whereas monomorphical growth was easily quantified, it was very difficult to enumerate mixed populations of yeast and mycelial cells because the cells tended to clump and become entangled. Therefore, the relative percentage of mycelial cells in a mixture of cell types must be considered approximate, even though the overall morphological response of such strains was quite predictable. Because of their variable morphology, pseudomycelial cells often closely resembled yeast cells and could not be separately quantitated. Pseudomycelial formation was usually associated with clumping, and it was noted whether pseudomycelial growth comprised more or less than half of the cells in these clumps.

Photomicrography. The morphological changes were documented with photomicrographs. At different times after incubation, loopfuls of each culture were mixed with lactophenol-cotton blue on glass slides, overlaid with cover slips, and photographed through bright-field optics with a 35-mm Leitz camera mounted on a Leitz Ortholux microscope.

[^{35}S]sulfate incorporation. Proteins were radioactively labeled with ^{35}S by growing cells in LSM with $Na_2{}^{35}SO_4$ (see Table 1). The radionuclide was obtained

TABLE 1. *Modifications of SM of Lee et al. (8)*[a]

Medium	Salts						Amino acids								Glucose	Biotin
	$(NH_4)_2SO_4$	NH_4Cl	$MgSO_4 \cdot 7H_2O$	K_2HPO_4	KH_2PO_4	NaCl	L-Ala	L-Leu	L-Lys	L-Met	L-Orn	L-Phe	L-Pro	L-Thr		
SM	5.0[b]		0.2	2.5		5.0	0.5	1.3	1.0	0.1	0.0714	0.5	0.5	0.5	12.5	0.001
bSM	5.0		0.2	14.0	6.0	5.0	0.5	1.3	1.0	0.1	0.0714	0.5	0.5	0.5	12.5	0.001
BS	5.0		0.2	14.0	6.0	5.0									12.5	0.001
LSM		1.33	0.001	14.0	6.0	5.0									12.5	0.001

[a] Preparation of media: Salts and amino acids were dissolved in deionized water, dispensed in 200-ml aliquots into 500-ml Erlenmeyer flasks, covered with parchment paper caps, and autoclaved. To each 200 ml of presterilized medium, 5.0 ml of glucose stock and 0.2 ml of biotin stock were added aseptically. Glucose stock, 40× (0.5 g/ml): 50 g of glucose was dissolved in 100 ml of deionized water and autoclaved. Biotin stock, 1,000× (1 mg/ml): 0.1 g of *d*-biotin was added to 50 ml of deionized water, dissolved by the dropwise addition of 1 N NaOH, and adjusted to pH 7.0 with 1 N HCl, and the volume was brought to 100 ml with deionized water. The solution was sterilized by filtration and stored at 4°C.

[b] All amounts are grams per liter.

from New England Nuclear Corp. (Boston, Mass.) with a specific activity close to 1 Ci/mmol. Each 200-ml culture of cells at an initial density of 10^7 yeast cells per ml was incubated in LSM with 0.5 mCi of [^{35}S]-sulfate (2.5-μCi/ml final ^{35}S concentration) for 20 h. Triplicate samples of 0.5 ml were pelleted, washed twice with BS, precipitated with 3.0 ml of ice-cold trichloroacetic acid, filtered, washed with ice-cold trichloroacetic acid and 50% ethanol, dried, and counted in a liquid scintillation counter (model LS-233, Beckman Instruments, Inc., Fullerton, Calif.) with counting efficiency of 55%.

RESULTS

Inoculum. Preparations of single yeast cells (i.e., without buds) inoculated into SM and incubated at 37°C germinated more rapidly, in synchrony, and with fewer pseudomycelial cells than preparations of actively budding yeasts. To maximize yield, cultures were initiated with a high inoculum (10^7 to 5×10^7 cells per ml) of early stationary-phase cells.

Strain variation. In the SM medium, all six strains of *C. albicans* grew satisfactorily in the yeast phase at 24°C without forming filaments. However, the response to temperature elevation varied. The percentage of filamentation reached by each strain in SM is shown in Fig. 1. Strains 4918 and E-139 formed true germ tubes which continued to elongate and branch, producing tangled mycelial mats by 18 h. Macroscopically, mycelial cultures appeared very flocculent. After 18 h, reversion began to occur as yeast cells started budding from the hyphal tips and septa. Strains 550, E-136, and to some extent 3153 reproducibly formed mixed cultures of yeast cells, mycelium, and pseudomycelium. Typically with these three strains, the pseudomycelial form was predominant during the first 6 h, after which reversion to yeast-phase growth occurred within 6 to 9 h. Reversion was evinced by the appearance of yeast cells budding from mycelial or pseudomycelial cells. Strain 2252 maintained yeast-phase growth at both 24 and 37°C. The reversion of strain 4918 is shown in Fig. 2.

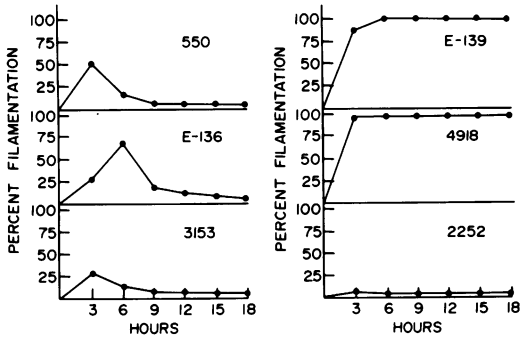

FIG. 1. *Variation of six strains of C. albicans in response to synthetic medium (SM) at 37°C. Percent filamentation was enumerated from hemacytometer chambers as described in Materials and Methods. Each point represents mean of three separate experiments. All strains produced <1% filamentation in SM at 24°C.*

Despite the varied morphological growth response of the six strains in SM, each strain was capable of forming true mycelia (hyphae). They all produced germ tubes in human serum within 90 min at 37°C and subsurface filaments in GYE slants at 24°C (Fig. 3).

pH. The growth of this large number of cells at both 24 and 37°C caused the pH of SM to drop precipitously within 3 h from pH 6.9 to 3.0. The addition of 120 mM potassium phosphate buffer (bSM, Table 1) stabilized the pH of the medium at pH 6.9. The morphological response of each strain to bSM at 37°C was similar to its response in SM at 37°C (Fig. 1). The varied responses of these strains (e.g., the early reversion of E-136, 550, and 3153) were not affected by the increasing acidity of SM, and, indeed, appeared to be independent of pH shifts from 7.0 to 3.0. Maintenance of the pH of SM at neutrality, however, minimizes the possibility of pH-induced macromolecular changes in yeast-phase or mycelial-phase cells and permits the use of bSM for investigations involving molecular inhibitors.

Amino acid and sulfate ion concentration. Because of the varied strain response to SM, it was important to determine which, if any, of the eight amino acids in the SM were necessary for conversion from yeast to filamentous growth. Moreover, deletion of some or all of the amino acids with a concomitant decrease in the sulfate ion concentration of the medium would facilitate incorporation of radiolabel into cellular macromolecules (e.g., [^{35}S]sulfate into cytoplasmic proteins). Consequently, two media were developed: BS (Table 1), lacking all eight amino acids, and LSM, lacking the amino acids and substituting ammonium chloride for ammonium sulfate. Both were tested for their ability to support temperature-induced filamentation. All six strains responded to BS and LSM as they had to SM and bSM, and results were similar to those shown in Fig. 1. It is apparent that the eight amino acids and high sulfate content of SM are not essential for either growth or morphological conversion of these cells. On the basis of these experiments, LSM was concluded to be a satisfactory medium for molecular investigations.

Growth kinetics. The growth rates of strains 4918 and 2252 in LSM at 24 and 37°C were determined by dry weight analyses (Fig. 4). The mean doubling times calculated from those data are as follows: 4918 at 24°C, 3.7 ± 0.2 h; 4918 at 37°C, 1.8 ± 0.2 h; 2252 at 24°C, 3.6 ± 0.3 h; and

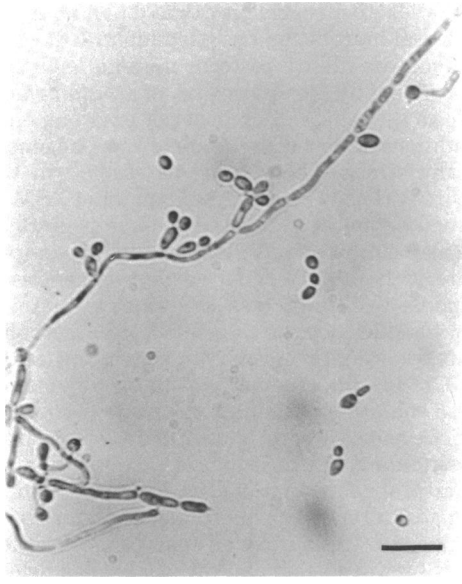

FIG. 2. *Reversion of strain 4918 in SM. The culture was inoculated at low density (10^5 yeast cells per ml) and incubated at 37°C for 36 h. Bar equals 25 μm.*

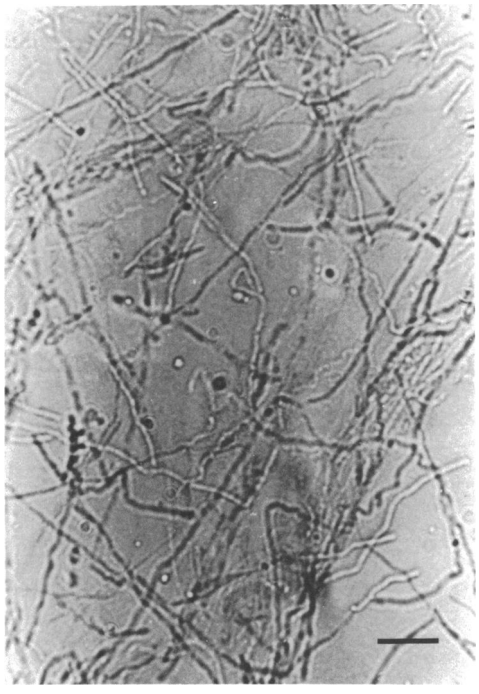

FIG. 3. *Hyphae produced by strain 2252 and penetrating the agar of a GYE slant (tease mount of an agar section). Bar equals 25 μm.*

2252 at 37°C, 1.9 ± 0.15 h. The growth rate of both strains at 37°C (1.8 h) is twice the rate at 24°C (3.6 h). With an inoculum of 2×10^7 to 3×10^7 cells per ml, stationary phase is reached in two to three doublings (7 to 10 h at 24°C, and 4 to 6 h at 37°C). Therefore, the maximum yield of actively growing cells was obtained by harvesting 37°C cultures at 3 h and 24°C cultures at 6 h.

The incorporation of [^{35}S]sulfate by strains 4918 and 2252 in LSM at 24 and 37°C is shown in Fig. 5. These studies confirm the growth kinetic data obtained from measurement of the total cell mass (Fig. 4). As before, at 3 and 6 h respectively, 37 and 24°C cultures have incorporated similar amounts of the radionuclide and have nearly reached maximal incorporation.

Microscopic examination revealed actively budding yeasts in 6-h cultures of 4918 and 2252 in LSM at 24°C and in 3-h cultures of 2252 at 37°C. At 3 h of growth at 37°C, 100% of cells of strain 4918 had formed germ tubes mostly 20 to 40 μm in length.

DISCUSSION

The LSM described in this paper has been shown to be an effective medium for the cultivation of both yeast- and mycelial-phase *C. albicans*. LSM is the simplest medium designed for this purpose described to date. The advantages of LSM are that the pH of the medium is maintained near neutrality despite high cell density, that cellular macromolecules may be readily radiolabeled, and that morphogenesis is stimulated by a simple temperature shift from 24 to 37°C. Furthermore, with the protocol described herein, it is possible to obtain pure cultures of

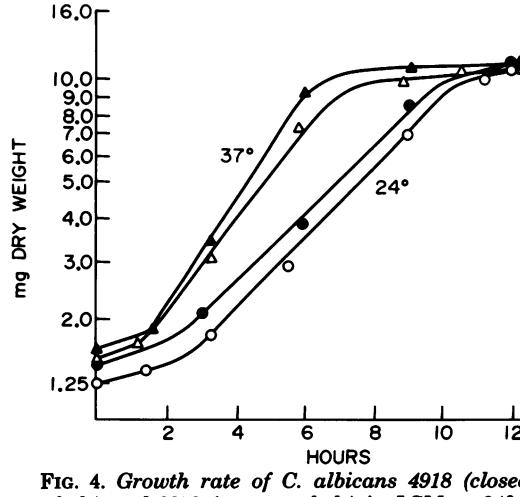

FIG. 4. *Growth rate of C. albicans 4918 (closed symbols) and 2252 (open symbols) in LSM at 24°C (circles) and 37°C (triangles). Cultures were initiated at a density of 10^6 yeast cells per ml. Samples (5 ml) of each culture were filtered through preweighed Millipore filters (0.45 μm porosity); the filters were dried overnight and reweighed. Each point represents the mean of triplicate determinations.*

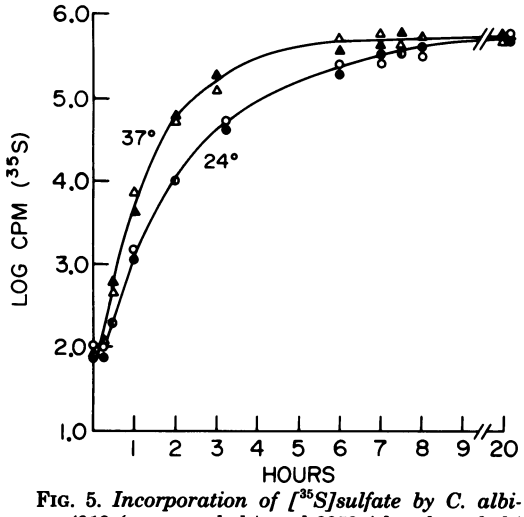

FIG. 5. *Incorporation of [^{35}S]sulfate by C. albicans 4918 (open symbols) and 2252 (closed symbols) at 24 and 37°C in LSM. Curves indicate identical rates of incorporation by both strains at 24 and 37°C. Cells at an initial density of 10^7 yeast cells per ml were incubated in LSM with 2.5 μCi of [^{35}S]sulfate per ml for 20 h. Each point represents the mean of triplicate samples. See text for experimental details. Symbols:* ○, *4918-24Y;* ●, *2252-24Y;* △, *4918-37M;* ▲, *2252-37Y.*

yeasts and pure cultures of true mycelium in which the cells have converted nearly synchronously. The ease with which yeast- and mycelial-phase cells may be manipulated in LSM makes it an ideal medium for investigations of the molecular biology of *C. albicans*.

Six strains of *C. albicans* were tested for their ability to form filaments in LSM at 37°C. Two (4918 and E-139) produced 100% germ tubes; three (E-136, 550, and 3153) produced mixed populations of yeasts, pseudomycelium, and true mycelium; and one (2252) maintained yeast growth. All six strains produced only yeasts in LSM at 24°C. This striking variation in the response of each strain to identical environmental conditions suggests several very interesting properties of the morphogenesis of *C. albicans* in synthetic media.

By analogy to the other dimorphic fungal pathogens, it has been presumed that the morphogenesis of *C. albicans* can be triggered by a single environmental factor or set of factors (e.g., temperature, CO_2 levels, or specific carbon source). Attempts to identify this "morphogen" (9) in serum or in defined synthetic media have produced a bewildering array of often contradictory observations. Considering the strain variation noted in this study and by others (4, 6, 10), it now seems clear that many purported morphogens only stimulate certain strains to convert from yeast to mycelial growth and that no single, universal morphogen has been identified.

Since the ability to form mycelial cells is a property of all typical strains of *C. albicans*, it follows that there is some set of environmental conditions which will stimulate morphogenesis in any strain. The six strains studied in this report variously produced filaments in LSM or human serum at 37°C, and hyphae penetrated the agar surface of a GYE slant at room temperature. Others have reported morphogenesis in response to such diverse conditions as biotin or zinc insufficiency, or as a function of carbohydrate, phosphate, or amino acid concentrations (2, 3, 8, 13–16). The mechanism by which all of these conditions stimulate the same morphogenetic response is perplexing. Furthermore, strain variation and the potential of such varied environmental manipulations to stimulate cellular changes unrelated to morphogenesis make the significance of purported biochemical differences between yeast and mycelial cells unclear, particularly if only one strain or medium was investigated. Indeed, certain molecular changes that accompany morphogenesis have been attributed by other investigators to the strain or medium utilized in those studies (1, 3). We propose that strain- and medium-dependent cellular changes may be controlled simultaneously, by using both a strain that produces only yeasts at 37°C in LSM (e.g., 2252) and one which produces 100% germ tubes in LSM at 37°C (e.g., 4918) and examining molecular parameters in yeast cells of each strain at 24°C (to locate strain differences) and yeast and mycelial cells at 37°C (to identify changes in the yeast cells stimulated by the temperature shift but unrelated to or insufficient for morphogenesis, in addition to morphology-dependent changes). By this method, we have identified changes in cytoplasmic proteins that are apparently related to morphogenesis as well as to strain and temperature (M. Manning, Ph.D. thesis, Duke University, Durham, N.C., 1979).

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