Effects of Culture Density on the Kinetics of Germ Tube Formation in Candida albicans

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The relationship between culture density or phase of growth at 24.5 °C and the ability of Candida albicans to form germ tubes when shifted to 37 °C was investigated. Evidence is presented demonstrating germ tube production from liquid synthetic medium cultures at all phases of growth. Previous studies reported that only cells from stationary phase cultures were competent to form germ tubes. Comparisons between exponential and stationary phase cultures indicate more rapid and more synchronous germ tube production from cells growing in the exponential phase.

INTRODUCTION

Candida albicans is a dimorphic fungus which exists as normal flora in the gastrointestinal tract of man. Many factors have been reported to affect the yeast to mycelium transformation. The most effective inducers of this morphological change are temperature, pH value of the growth medium, and an alteration in the nature of the nutrient or substrate (Lee et al., 1975; Simonetti et al., 1974; Mitchell & Soll, 1979).

Early-exponential phase cultures shifted from 25 °C to 37 °C were reported to be incapable of germ tube production, whereas 100% of organisms in cultures shifted in late-exponential phase were reported to produce germ tubes (Chaffin & Sogin, 1976). Many investigators have presented additional data which support the concept that only stationary phase cells are capable of mycelial growth (Soll & Bedell, 1978; Mitchell & Soll, 1979; Bell & Chaffin, 1980).

Here we present data showing synchronous, nearly complete transformation of blastospore-forming cells to the hyphal form of growth when cells growing at 24-5 °C in any stage of exponential or stationary phase are diluted into medium at 37 °C. The difference between our experimental system and that used by other investigators is that our 24-5 °C cultures are maintained at low densities in the exponential growth phase (Buckley et al., 1982). The time course of initial germ tube formation differs between exponential and stationary phase cells.

METHODS

Growth and maintenance of stock cultures. Stock cultures of Candida albicans B311 were maintained on Sabouraud's dextrose agar slants at −80 °C. Liquid shake cultures were set up by inoculating a small amount of organism into 50 ml synthetic media developed for Candida (SMC) (Lee et al., 1975). This yielded a culture with no visible turbidity. Medium to be inoculated was stored at 4 °C and brought to room temperature before use. Inoculated flasks were incubated at 24-5 °C in a controlled-environment incubator shaking at 200 r.p.m. (New Brunswick). Warming to ambient temperatures above 24-5 °C was prevented by placing the incubator in a 4 °C cold room.

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Once the initial shake culture exhibited visible turbidity, a drop was used to inoculate a flask containing approximately 50 ml SMC at 24.5 °C. The same dilution was repeated once or twice daily for 48–72 h to keep the cells in the early-exponential phase of growth and at a concentration which never exceeded $1 \times 10^6$ cells ml$^{-1}$. This procedure produced an actively growing culture consisting of budding blastospores, uniform in size and shape, without clumping and free from germ tubes or hyphae. At cell concentrations of less than $3 \times 10^5$ cells ml$^{-1}$ these shake cultures exhibited doubling times of 1.5–2.0 h. Cell growth at 24.5 °C was routinely monitored by measuring absorbance at 675 nm using a Spectronic 710 spectrophotometer (Bausch & Lomb). Cell growth at 37 °C was monitored by measuring \( A_{675} \) and by cell counts using a Neubauer haemocytometer.

Preliminary growth curves and germ tube induction experiments were done in glass flasks containing medium prepared with single-distilled and deionized water. Results were reproducible, and those quoted are typical.

Installation of an ultrapure water-preparation system (Mini-Q system, Millipore) necessitated following the procedure of Bedell & Soll (1979) with modifications. Erlenmeyer flasks (250 ml or 500 ml) made of either polycarbonate or polymethylpentene were machine washed, and rinsed four extra times with Milli-Q water. SMC prepared with single-distilled and deionized water was filter sterilized, stored in polypropylene bottles and supplemented to 0.1 μM with ZnSO$_4$ \( \cdot \) 7H$_2$O before inoculation.

**Temperature shift induction of germ tube formation.** Approximately 18 h before a 37 °C temperature shift experiment, an actively growing culture at 24.5 °C (A$_{675}$ ~ 0-1) was diluted into 50 ml SMC. The amount of inoculum used was calculated to produce the desired cell concentration the next day. All temperature shifts were initiated by diluting or resuspending a culture growing at 24.5 °C to a cell density of 2–3 \( \times \) \( 10^9 \) cells ml$^{-1}$ in SMC prewarmed to 37 °C. This cell density corresponds to an A$_{675}$ of 0.25. To perform temperature shifts on cultures with absorbance values less than or equal to 0.25, the 24.5 °C culture was centrifuged at 500 g for 3 min in a clinical centrifuge before being resuspended in prewarmed SMC. Routinely, the shifts to 37 °C were done in a total volume of 50 ml SMC in 250 ml flasks shaking at 200 r.p.m.

**Monitoring germ tube formation.** Immediately following the temperature shift absorbance was measured and a sample was formalin fixed for cell counting. A 300 μl sample of culture was placed in a 1.5 ml Eppendorf centrifuge tube containing 100 μl 37% formaldehyde in water. Samples were then mixed vigorously and refrigerated. Samples were taken and absorbance measured every 45 min for at least 5 h. Samples were counted in a haemocytometer, with blastospores and germ tubes scored as described by Mitchell & Soll (1979). Elongated outgrowths with a width no greater than one third the diameter of their mother cells and with a length equal to or greater than one half the diameter of their mother cells were scored as germ tubes. Spherical outgrowths with a diameter equal to or greater than one half the diameter of their mother cells were scored as blastospores. At least 100 cells were scored, but typically 200–300 cells were scored at 400 × magnification. Percent budding cells was determined for 24.5 °C cultures based on counting at least 200 cells.

**RESULTS**

Cultures of *C. albicans* B311 in exponential phase grew at 24.5 °C with a doubling time of about 2 h, with about 29% of the cells budding (Fig. 1). In cultures with absorbances above 1.3, the doubling time increased and the percentage of budding cells rapidly decreased.

Figure 2 presents the kinetics of germ tube formation in six cultures shifted to 37 °C from points on the growth curve shown in Fig. 1. Shifts to 37 °C from any culture density yielded at least 90% germ tube production. When cultures with absorbances between 0.1 and 1.0 were used in shift experiments, nearly 100% cells produced germ tubes within 3 h after the shift to 37 °C. Cultures with densities in this range also showed almost the same time course of germ tube development. Cultures shifted at lower densities tended to show a slight decrease in the content of germ tubes at various times after the shift (Fig. 2a). This decrease was identical for cultures concentrated by centrifugation to an absorbance of 0.25 or for unconcentrated cultures (data not shown). Cells from exponential phase cultures produced and maintained 100% germ tubes and exhibited a high degree of synchrony in the process. Cells in the stationary phase of growth had a longer lag before the first germ tubes appeared compared to exponential phase cells. When high-density cultures were diluted into spent rather than fresh medium, germ tube formation did not occur (data not shown). Cells shifted to 37 °C from exponential phase cultures at 24.5 °C with absorbances of 0-4 and 0-62 produced greater than 75% germ tubes after 90 min at 37 °C (Fig. 2c, d), whereas a culture with absorbance of 1.1 produced 35% germ tubes after 90 min (Fig. 2e) and a culture with absorbance 3-0 failed to produce germ tubes after 90 min (Fig. 2f). Likewise, the synchrony of germ tube formation decreased in cells shifted from the stationary phase of growth. The time between the first observation of germ tubes and that demonstrating the
Germ tube formation in *C. albicans*

Fig. 1. Growth curve of *C. albicans* at 24.5 °C in synthetic medium. $A_{675}$ (●) was measured using appropriate dilutions at $A_{675}$ above 0.6. The numbers adjacent to the points indicate the percentage of budding cells, this was determined by microscopic observation of fixed cells using techniques described in Methods. The arrows indicate the densities from which portions of culture were taken, adjusted to the same density, and shifted to 37 °C. The letters (a-f) correspond to the individual plots of germ tube formation and cell number and absorbance at 37 °C in Figs 2 and 3.

Fig. 2. Time course of germ tube appearance at 37 °C (●). Portions of culture growing at 24-5 °C were shifted from the densities indicated in Fig. 1. Each portion was adjusted to a density of 2–3 × 10^6 cells ml^{-1} in synthetic medium prewarmed to 37 °C. Samples were fixed in formalin at the times indicated for determination of total cell number and the number of cells bearing germ tubes. The $A_{675}$ values shown in (a)–(f) represent the density of the 24-5 °C culture from which that shift was taken.
Fig. 3. Combined plots of absorbance (●), total cell number (□) and number of germ tubes (■) for each of the cultures shifted to 37 °C as indicated in Fig. 1 and plotted as percentage germ tubes in Fig. 2. The numbers of germ tubes at early times are equivalent to less than 1 in 10⁴ cells.

maximum number of germ tubes was 2.5 h in cells from cultures with absorbances above 2.0 (Fig. 2f). This same interval in cells shifted from the exponential phase varies between 0.7 and 1.5 h (Fig. 2b–e).

Figure 3 shows data from the same experiment but indicating total cell numbers, numbers of cells with germ tubes and A₆₇₅ values for each shift. In these and in many other similar experiments the following differences were observed between cultures shifted from the exponential and stationary phases.

The increase of absorbance at 37 °C followed a pattern dependent on the density of the 24.5 °C culture from which it was taken. The absorbance increased immediately and reached the highest 5 h value when low-density cultures were shifted. Maximum absorbance was 1.8 at 37 °C after 5 h for cultures shifted from an absorbance of 0.08 and 0.15 (Fig. 3a, b) and decreased to a maximum of 1.10 and 0.97 for cultures shifted from absorbances of 0.4 and 0.62, respectively.
Germ tube formation in C. albicans 3005

(Fig. 3c, d). The absorbance at 37 °C decreased for 1-5 h before increasing to a maximum of 0-65 when high-density cultures were shifted, and the absorbance at 37 °C decreased for 2-5 h before increasing to a maximum of 0-48 when stationary phase cultures were shifted.

This trend in absorbance increases seen at 37 °C paralleled the patterns seen with total cell numbers. Cells shifted to 37 °C from low density at 24-5 °C, kept multiplying at an increased rate as germ tubes appeared, and continued multiplying as the maximum percentage of germ tubes was reached (Fig. 3a). Cultures shifted from a slightly higher density kept multiplying but at a decreased rate as germ tubes appeared, and cells stopped multiplying as they completed germ tube formation (Fig. 3b). Cells from cultures with absorbances of about 0-5 stopped multiplying or decreased in number slightly as germ tubes formed (Fig. 3c, d). Cell numbers in cultures shifted from even higher densities decreased markedly while germ tube formation proceeded; this decrease in total cell numbers was also shown in any culture shifted from the stationary phase of growth (Fig. 3e, f). It is likely that the drop in cell numbers and in turbidity is due to cell lysis, since cell ghosts were observed in microscopic preparations.

**DISCUSSION**

Our data show clearly that cells of C. albicans B311 at any density or phase of growth are competent to form germ tubes. The stringent relationship cited by others between phase of growth and competence to form germ tubes was not observed in these studies. Moreover, there appears to be a more complex relationship between phase of growth and the kinetics of germ tube formation and total cell numbers. Whereas growth rate remains fairly constant with increasing cell density up to an absorbance between 1-0 and 1-1 (Fig. 1), the ability to produce germ tubes appears to be greatest in mid-exponential phase cultures. This trend is demonstrated in Fig. 2 where the extent of germ tube formation at 90 min after a shift to 37 °C is greatest in cells taken from 24-5 °C cultures with an absorbance between 0-4 and 0-6. This region of the growth curve represents a stage where growth rate and percentage of budding cells have reached steady state.

Greatest synchrony of germ tube formation occurred when cells from mid-exponential phase cultures were shifted to 37 °C. At these culture densities, cell division ceased as cells prepared to form germ tubes (Fig. 3c, d). Cells shifted from lower culture densities attained less than optimal synchrony and extent of germ tube formation (Fig. 3a, b). Some of these cells continued cell division while others produced germ tubes, yet the majority of the blastospores newly budded at 37 °C also went on to produce germ tubes. At culture densities beyond the exponential phase, cells not only ceased dividing but a large portion of the population was unaccounted for, disappearing presumably through cell lysis. This phenomenon has not been reported in the studies which report that only stationary phase cells are capable of germ tube formation.

The results reported here differ from those of Chaffin & Sogin (1976), and of Soll & Bedell (1978). Both studies concluded that exponential phase cells either failed to form germ tubes or formed only transient germ tubes when shifted from 25 to 37 °C. The chemically defined medium used in their studies is the same as we used in ours. The strain used here, B311, is the same as the 3153A strain used by the other workers.

In our experiments we performed temperature shifts at a constant cell density of 2-3 × 10^6 cells ml⁻¹ (Fig. 3). While germ tube formation occurred at a density as low as 1 × 10^6 cells ml⁻¹, we have had variable conversion at culture densities 10 or 20 times lower. Unfortunately, although Chaffin & Sogin (1976) and Soll & Bedell (1978) specify the culture density at which they performed their experiments, they do not specify the dilution factor used for the temperature shift.

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REFERENCES


