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Response of Yeast and Mycelial Phases of *Histoplasma capsulatum* to Amphotericin B and Actinomycin D

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The dimorphic fungus *Histoplasma capsulatum* exists in two phases: a unicellular yeast form at 37 C and a mycelium at 25 C. We have found that these two phases have selective drug susceptibilities. The mycelial form of *H. capsulatum* was much more susceptible to the polyene antibiotic amphotericin B than the yeast form; in contrast, the yeast form was more susceptible to the antibiotic actinomycin D. The changes in susceptibility occurred early in the transition between the two phases and permitted the transitions to be blocked by sublethal concentrations of the appropriate drugs.

The dimorphic fungus *Histoplasma capsulatum* exists in two phases: as a unicellular yeast at 37 C and a multicellular mycelia at 25 C (5). We have found differences in amphotericin B (AmB) and actinomycin D (ActD) susceptibilities between these two phases. These differences in susceptibility occurred early in the transition between the two and permitted the temperature-related transition from one phase to the other to be blocked by sublethal concentrations of the drugs.

MATERIALS AND METHODS

Antibiotics and radioactive material. AmB in the form of Fungizone (E.R. Squibb & Sons, Inc., Princeton, N. J.) was dissolved and diluted in distilled water. ActD (Sigma Chemical Co., St. Louis, Mo.) was prepared as an aqueous stock solution (0.5 mg/ml) and stored at −20 C until used. [^{14}C]guanine (8-^{14}C; specific activity, 58 mCi/mmol) was obtained from Schwarz/Mann Radiochemicals, Orangeburg, N.Y.

Organism and growth. *H. capsulatum*, Down's strain, mating type —, a clinical isolate from the culture collection of this laboratory, was maintained in yeast form on a medium containing 2% glucose, 1% yeast extract, and 1.5% agar (GYE). The mycelial form was obtained by germinating and propagating the actively growing yeast cells in Salvin's liquid medium (15) and incubating at room temperature (24 to 26 C) for 2 weeks.

Drug susceptibility assays. Yeast cells from a 3- to 5-day-old plate were suspended in Salvin's medium at a cell number of 2×10^6 cells/ml and incubated without shaking for 24 to 36 h. The cell number was then readjusted to 2×10^6 cells/ml just before the susceptibility assays were performed.

Mycelia were maintained in homogeneous suspension in submerged cultures in Salvin's medium. They were transferred to fresh medium 2 to 3 days before the assay of antibiotic susceptibility. Density of the actively growing mycelia was adjusted with fresh medium so that an equal volume of suspension would give a similar cell mass in dry weight as the yeast cells being tested (dry weight mycelia equal to 2×10^6 yeast cells/ml).

AmB or ActD was added to the assay medium at the concentrations described and the [^{14}C]guanine was added 1 h later at a concentration of 0.1 μCi of medium per ml. One-milliliter samples were taken at 1-,3-, and 6-h intervals. They were precipitated with an equal volume of cold 10% trichloroacetic acid (CCl_3COOH) and were filtered, dried, and counted on a scintillation counter.

Dry weights of the two forms after 3 days of growth in the presence of AmB were measured by mixing 30 to 50 ml of cells with 50% CCl_3COOH to a final concentration of 5%. The cells were collected on preweighed glass fiber filters. The samples were dried in a 150 C oven for 2 h, cooled for 1 h, and weighed on a Metler H20T balance. The total weight minus the weight of the filter paper was defined as the dry weight of the organism. Total ribonucleic acid (RNA) was determined by a modification of the Schmidt-Thannhauser procedure described elsewhere (1, 6). Viability of the organism was determined after 1, 3, 6, and 10 days of growth in the presence of AmB by viable colony counts, or growth of a subculture of 0.1 ml of the undiluted culture on fresh agar medium.

Morphogenesis experiments. Yeast cells were germinated to mycelia by incubation in Salvin's medium at 25 C. Mycelia were converted to yeast by incubation in Salvin's medium without addition of reducing agents at 37 C. The cells were observed daily, and photomicrographs of wet mounts of living cells and cells fixed in 5% CCl_3COOH were taken at various intervals of time. The effects of AmB on the yeast-to-mycelial transition (Y→M) and ActD on the mycelia-to-yeast transition (M→Y) were also measured by morphological changes. Because we have found that

AmB lost its potency in Salvin's medium after 24 h, freshly prepared AmB was added to the culture flasks each day to replenish the degraded portion (unpublished data). The amount added was 40% of the original concentration.

RESULTS

Whereas most antibiotics tested were potent against *H. capsulatum* in all phases (unpublished data), AmB and ActD were much more selective and permitted "freezing" of one or another phase during transitional temperature.

Antibiotic susceptibility of yeast, mycelial, and transitional forms. A concentration of 0.005 μg of AmB per ml was able to achieve an 80% inhibition of incorporation of [^{14}C]guanine into RNA of mycelia compared to control cultures (Fig. 1). Ten times more (0.05 μg/ml) AmB was required to achieve the same level of inhibition with yeast cells.

The difference in AmB susceptibility of the yeast and Y→M transitional forms, estimated by radioactive guanine incorporation, was confirmed using dry weight, RNA determination, and viable colony counts (Table 1). For example, 0.005 μg of AmB per ml had no effect on yeast incubated for 3 days at 37 C, but when the cultures were switched to 25 C there was clear inhibition of growth, as measured by these three methods.

When yeast cells had been incubated at room temperature for 24 h, they displayed an AmB dose response pattern similar to that of the mycelial form, although at this time only very small germ tubes were evident (Fig. 1). When the cultures were pulse labeled for 3 h with [^{14}C]guanine, it could be seen that the change in AmB susceptibility of the yeast actually occurred within 4 h after the temperature shift, before any germ tubes were evident (Fig. 2). Figure 2A shows the marked susceptibility of mycelia to 0.005 μg of AmB per ml. Inhibition of RNA synthesis occurred immediately after the addition of AmB. Figure 2B shows the gradual switch to sensitivity to 0.005 μCi of AmB per ml after the yeast was changed to an incubation temperature of 25 C. At 4 h the total [^{14}C]guanine incorporated into RNA was less than 20% of the control cells. Figure 2C shows that the insusceptibility of yeast to 0.005 μg of AmB per ml did not change after the 4 h of incubation at 37 C.

Both forms of *H. capsulatum* were susceptible to ActD, but the yeast form was slightly more susceptible than the mycelial form, as measured by [^{14}C]guanine incorporation (Fig. 3). The difference in susceptibility to ActD between the yeast and mycelial forms was more striking when the cells were first incubated with the drug for 3 days and then subcultured on fresh GYE plates (Fig. 4A-C). A 50-μg/ml amount of ActD did not visibly affect subcultures of mycelia (Fig. 4A), but mycelia incu-

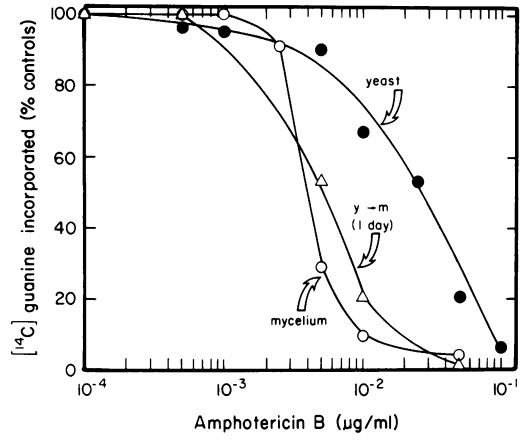

FIG. 1. *Dose response of yeast, mycelial, and yeast to mycelial transitional forms (Y→M) of H. capsulatum to AmB. Yeast cells were incubated at 37 C. Mycelia at 25 C and Y→M represent yeast 24 h after temperature shift to 25 C. The amounts of [^{14}C]guanine incorporated into RNA in the cultures not treated with AmB were: 3,600 counts/min per 53 μg (dry weight) per 3 h in the yeast, 3,870 counts/min per 66 μg (dry weight) per 3 h in the mycelia, and 1,460 counts/min per 53 μg (dry weight) in the Y→M cells.*

TABLE 1. *Growth of the yeast cells of H. capsulatum incubated at 37 C and Y → M cells incubated at 25 C in the presence of AmB*[a]

Yeast	AmB (μg/ml)	% Control		
		Increase in dry wt	Increase in total RNA	Viability
37 C	0.0	100.0	100.0	100.0
	0.005	86.3	66.0	100.0
	0.01	75.4	84.8	73.7
	0.05	0.0	4.4	1.90
25 C	0.0	100.0	100.0	100.0
	0.005	10.4	23.0	29.1
	0.01	4.55	21.0	0.04
	0.05	3.20	2.86	0.0

[a] Growth was measured by increase in dry weight and RNA after 3 days of incubation with the antibiotic. The increase in dry weight was 1.80 mg, and in RNA 141 μg, in 50 ml of Y → M cells not treated with AmB. Viability was determined by serial dilution. There were 114 × 10^4 colonies/ml in the yeast culture at 3 days and 125 × 10^4 colonies/ml in the Y → M cell cultures.

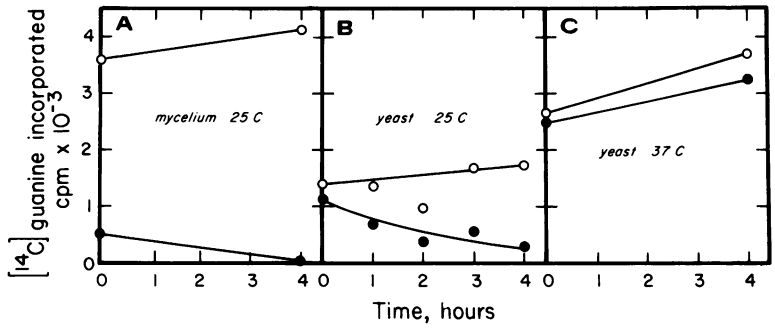

FIG. 2. *Changes in AmB susceptibility after the incubation temperature of yeast cells of H. capsulatum was switched from 37 to 25 C. AmB (0.005 μg/ml) was added at time 0, and the cells were pulse labeled for 3 h with [14C]guanine at the times indicated. Symbols: (○) control; (●) cells incubated with 0.005 μg of AmB per ml.*

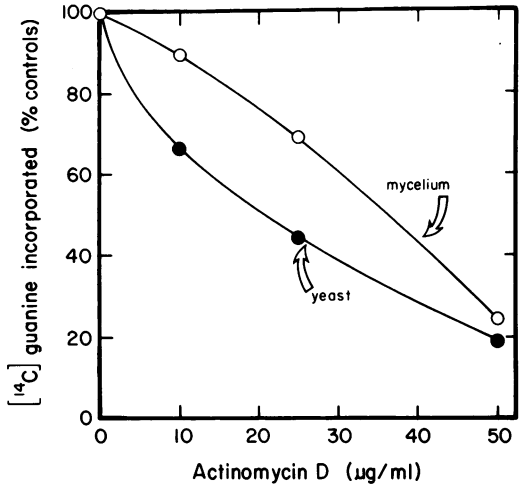

FIG. 3. *Dose response of mycelial and yeast forms of H. capsulatum to ActD. Mycelia were incubated at 25 C and yeast at 37 C. The amounts of [14C]guanine incorporated into the RNA of cultures not treated with AmB were 3,500 counts/min per 45 μg (dry weight) per 6 h for yeasts and 1,800 counts/min per 20 μg (dry weight) per 6 h for mycelia.*

bated at 37 C for 3 days became more susceptible to ActD (Fig. 4B); yeast cells at 37 C were clearly even more susceptible (Fig. 4C).

AmB and ActD blockage of phase transition. When yeast cells were incubated at 25 C in the presence of 0.005 μg of AmB per ml less than 5% of yeast cells germinated at 3, 6, and 12 days, whereas 30 to 50% of the cells in the control flask started to germinate at 1 day and were completely germinated by 3 days (Fig. 5). This lack of germination on the AmB-treated cultures was not explainable as a result of killing, because 100% of the yeast cells were viable after 1 day, 30% at 3 days, and 10% at 6 and 12 days.

When the addition of AmB was delayed for 4 h after the yeast had been incubated at 25 C, it was less effective in preventing germination, with 20% of the cells showing germ tubes after 3 days.

The transition of mycelia to yeast phase at 37 C was arrested in a similar way by ActD. Four days after incubation at 37 C began, hyphal tip constriction could be seen in the mycelia without ActD, whereas no gross morphological changes were observed in the cells

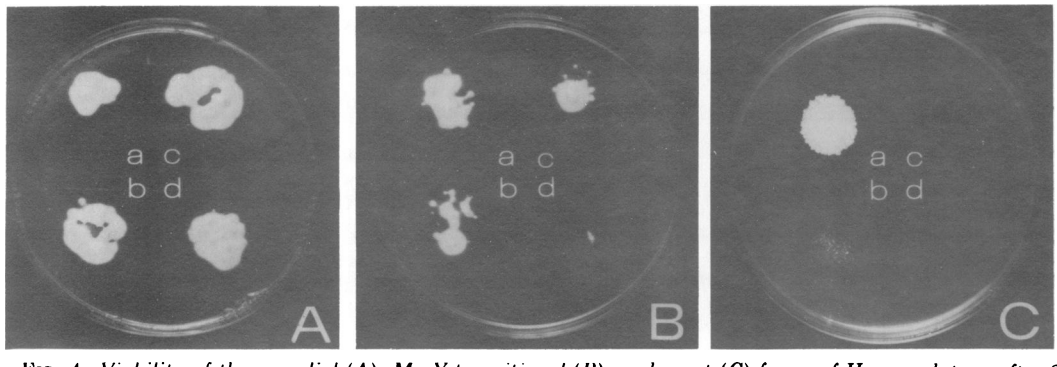

FIG. 4. *Viability of the mycelial (A), M→Y transitional (B), and yeast (C) forms of H. capsulatum after 3 days of incubation with 0 (a), 1) (b), 25 (c), and 50 (d) μg of ActD per ml. Samples (0.1 ml) of the cultures were plated on glucose-yeast extract agar. The mycelial and transitional forms were incubated at 25 C and yeast cells were incubated at 37 C.*

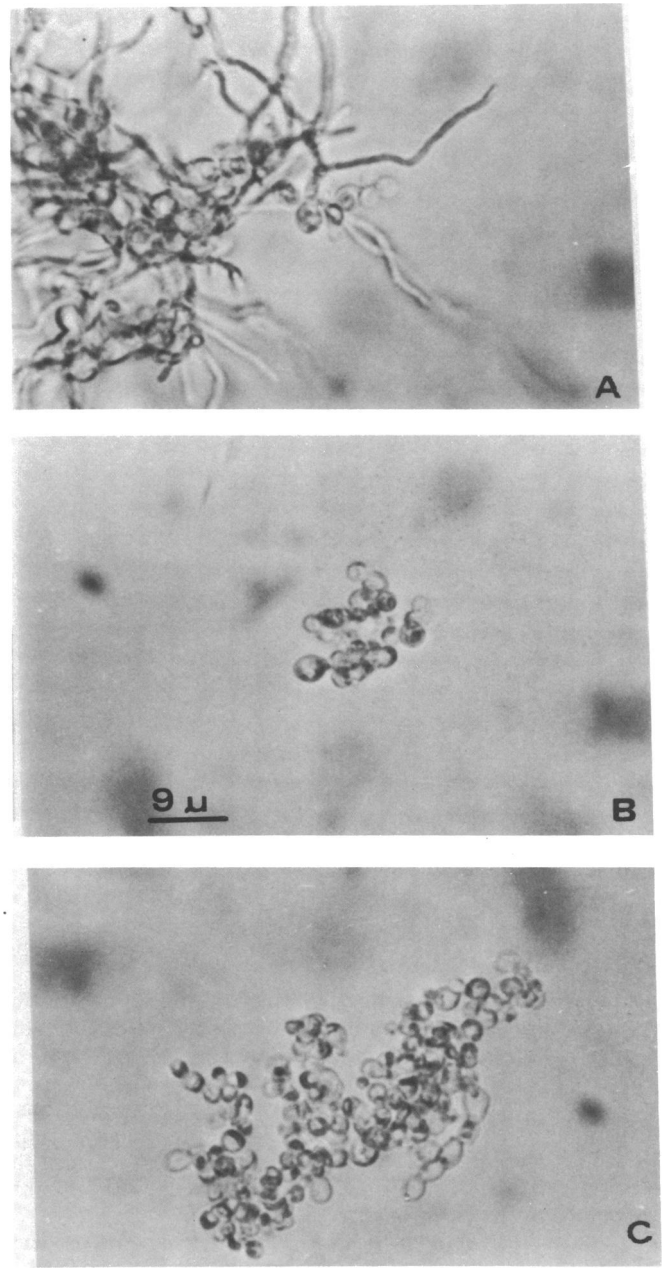

FIG. 5. *Inhibition of Y→M transition by AmB. Yeast cells of H. capsulatum were incubated for 3 days at 25 C. The concentrations of AmB in the cultures were (A) 0 μg/ml, (B) 0.005 μg/ml, and (C) 0.01 μg/ml.*

with ActD. At 7 days clusters of yeast cells were seen among the mycelial masses without ActD (Fig. 6A). In the cells incubated for 7 days with 10 μg of ActD per ml, only occasional hyphal tip constrictions were seen, but no unicellular yeast could be found (Fig. 6B). Most of the hyphal tips examined were filled with cytoplasm, indicating that all the cells were still viable. In the cells incubated with 25 μg of ActD per ml, slightly constricted and swollen hyphae were observed, and 50% of the hyphae were vacuolated (Fig. 6C). In the cells incubated with 50 μg of ActD per ml, all but a few hyphae were vacuolated and empty (Fig. 6D). Nevertheless,

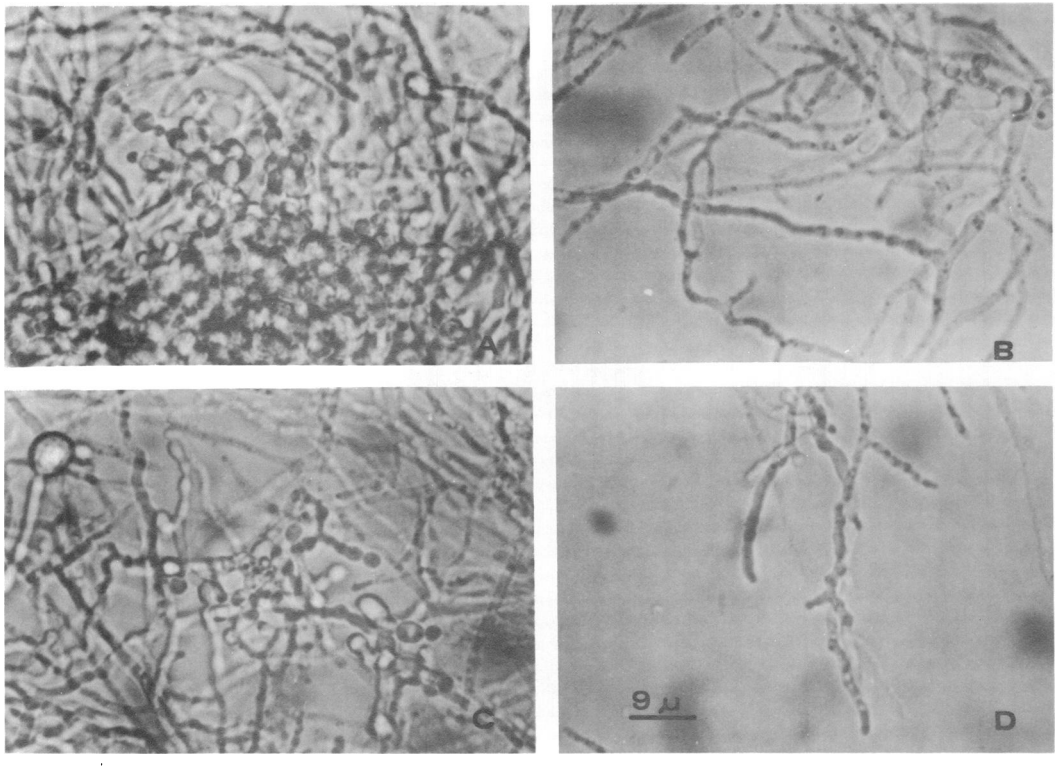

FIG. 6. *Inhibition of M→Y transition by ActD. Mycelia of H. Capsulatum were incubated for 7 days at 37 C. The concentrations of ActD in the cultures were (A) 0 μg/ml, (B) 10 μg/ml, (C) 25 μg/ml, and (D) 50 μg/ml.*

subcultures of these mycelia, even after incubation with ActD for 7 days, were viable and grew out as yeast at 37 C and as mycelia at 25 C.

DISCUSSION

Phase transitions in microorganisms, particularly in sporulating *Bacillus subtilis*, can be arrested at specific stages by appropriate mutational lesions, and spores traditionally can be classified into several stages by changes in the envelope that lead to progressive resistance to several substances (chloroform, octanol, etc.) (17).

The case of *H. capsulatum* described here is analogous in that differential drug susceptibilities exist for the cells in different growth states; however, here the transition is from one growing state to another, rather than from a growing cell to a storage form. Very likely many other growth phase-specific inhibitors exist, perhaps with even greater selectivity.

The inhibition of the transition by AmB and ActD probably does not occur by nonspecific killing. If that alternative were true, all of the cells eventually would have been killed by these agents. Since AmB was fungistatic for as long as 12 days after the Y→M shift began, and the ActD-inhibited M→Y transition produced little killing even at 7 days, this mechanism is not likely.

The observation that delay of addition of AmB for 4 h after the temperature shift eliminated the inhibition of the transition argues that a critical event occurs within this 4-h period, and the AmB has to be added within this time period to achieve its effect. The subsequent cell death in the yeast incubated in AmB at 25 C would probably then be a secondary effect.

The mechanism by which AmB and ActD arrested the Y→M and M→Y transition is unknown. AmB likely caused leakage of essential elements from the cell (2–4, 11). However, it might act by a very different mechanism; for example, it might have prevented early changes in the cell envelope essential for the transition by binding to the cell membrane (9–13, 16).

Since ActD blocks RNA transcription at many sites, the difference in susceptibility of the two morphological forms of *H. capsulatum*

is probably based on differences in membrane permeability, as in other cases of ActD resistance (8). Yeast cells would then be more permeable to ActD than mycelia, and therefore more susceptible.

The time in the Y→M transition when the yeast cells assumed the mycelial susceptibility to AmB, 4 h after transfer to 25 C, is consistent with the notion of a change in the cell envelope. At about that time, electron microscopic observations have shown an involuted membrane system at a region near the cell wall (7). The data are also consistent with observations on *Aspergillus fumigatus*, for which a 15-fold increase in AmB methyl ester susceptibility occurred during the early stage of conidial germination, before appearance of germ tubes (14).

The arrested transition produced by the differential susceptibility of the yeast and mycelial forms to AmB and ActD may be useful in elucidating features of morphogenesis. For example, the arrested transition produced by the differential susceptibility of the yeast and mycelial forms might aid in selection of nontransforming mutants or synchronization of cultures. Initial trials with yeast incubated at 25 C in the presence of AmB for 1 to 3 days and then washed free of antibiotic have shown yeast germinating uniformly, with good synchrony (unpublished data).

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