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Effects of Low Concentrations of Zinc on the Growth and Dimorphism of *Candida albicans*: Evidence for Zinc-Resistant and -Sensitive Pathways for Mycelium Formation

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In this analysis we have examined in detail the effects of low concentrations of zinc on the growth and dimorphism of *Candida albicans*. Evidence is presented that micromolar concentrations of zinc added to growth cultures grown at 25°C (i) cause a twofold increase in the final concentration of spheres at stationary phase, (ii) result in an asynchronous block in the budding cycle at stationary phase, (iii) completely suppress mycelium formation in two independently isolated human strains which produce low but significant levels of mycelia at stationary phase, and (iv) completely suppress mycelium formation in cultures of mutant M10, in which over 60% of the cells form mycelia at stationary phase. In contrast, micromolar concentrations of zinc do not inhibit mycelium formation induced by releasing cells from stationary-phase cultures into fresh medium at 37°C. In addition, if zinc is present in the growth medium of the initial culture at 25°C, the average time of subsequent mycelium formation after release into fresh medium at 37°C is halved. It is demonstrated that the above effects are specific to zinc. The possibility of alternate pathways for mycelium formation is suggested, and the medical implications of this possibility are discussed.

Candida albicans grows either as a budding yeast or as an elongating mycelium. It has been reported that micromolar concentrations of the divalent cation zinc suppress the formation of the mycelial form in vitro (11, 12). In addition, a depression of the in vivo level of zinc has been implicated in *Candida* pathogenesis in individuals suffering from acrodermatitis enteropathica, a disease related to zinc deficiency (2). Therefore, the concentration of the divalent cation zinc in the body fluids of infected individuals may be directly related to the growth, phenotype, and pathogenicity of *C. albicans*. For this reason, we have investigated the specific effects of zinc on the growth and phenotypic conversion of the budding to the mycelial form of *C. albicans* under defined, in vitro conditions.

In this report, we present evidence that micromolar concentrations of the divalent cation zinc specifically cause an increase in the final sphere titer and an asynchronous block in the budding cycle at stationary phase for cultures grown at 25°C. In addition, zinc completely suppresses mycelium formation at 25°C, but it does not suppress mycelium formation in released stationary-phase cultures at 37°C. From these findings, it is proposed that alternate pathways for mycelium formation exist, one sensitive and the other insensitive to zinc. In addition, the possible relationship of these findings to *Candida* pathogenesis is discussed.

MATERIALS AND METHODS

Growth and maintenance of cultures. Stock cultures of strains 3153A, c136, c141, and c157 and of mutant strain M10 were maintained on nutrient agar (9, 10). Strains c136, c141, and c157 were gifts from Helen Buckley of Temple University, Philadelphia, Pa. For experimental purposes, cells from agar cultures were inoculated into 25 ml of the amino acid-containing medium, formulated by Lee et al. (6), in 125-ml Erlenmeyer flasks and rotated at 200 rpm at 25°C. When these initial cultures reached a concentration of approximately 5×10^7 spheres per ml (late log phase), 0.1 ml was transferred to fresh medium, and the new cultures were incubated as above. This second culture and subsequent subcultures were used for experimental purposes. Cultures were serially transferred in liquid medium for no more than 3 weeks to ensure genetic homogeneity. Cells were cloned on agar every 2 months.

Growth in liquid culture was monitored by counting the number of spheres (mother cell or bud, attached or unattached) per milliliter in a hemocytometer or by collecting 5-ml samples of the cell culture on a pretared membrane filter (Millipore Corp.) which was then dried and weighed. The tared weight of the filter was subtracted from the final weight.

Induction of synchronous mycelium formation at 37°C. To induce the synchronous mycelium formation, 1 ml of a 24-h stationary-phase culture, grown

to stationary phase at 25°C, was diluted into 15 ml of fresh medium prewarmed to 37°C in a 50-ml Erlenmeyer flask. The "released" culture was then rotated at 200 rpm at 37°C. The initial sphere concentration in a released culture was approximately 3×10^6 to 5×10^6 per ml. Samples were removed at time intervals and scored for the percentage of spheres possessing evaginations (any detectable outpocketing of the cell surface) and germ tubes (elongate outpocketings with widths no greater than one-third the diameter of their mother cells and with lengths equal to or greater than one-half the diameters of their mother cells). Although we have used the term pseudomycelium in previous publications (7, 9, 10), we are henceforth using the term mycelium to refer to the hyphal growth form of *Candida* which is elongate, tubular, and compartmentalized, with a nucleus in each compartment, visualized by acridine orange staining (10). Germ tubes refer to initial mycelia. Over 100 cells were scored at each time point at ×430 magnification.

Isolation of mutant strain M10. A suspension of log-phase cells of strain 3153A, our standard experimental strain, at a concentration of 10^4 cells per ml, was irradiated in an open petri dish with ultraviolet light (260 nm) for 60 s, which resulted in 10% survival. Irradiated cells were immediately plated at very low density (50 to 100 cells per plate) on nutrient agar (9) and incubated at 37°C for 7 days. Wild-type clones grew exclusively in the yeast phase on the agar surface for the first several days and then released mycelia into the supporting agar, forming a cloudy region below the superficial colony. Mutant M10 was initially selected for precocious release of mycelia into the agar.

RESULTS

Growth and induced mycelium formation in strain 3153A. At 25°C, our standard experimental strain, 3153A, grew exclusively in the budding yeast form in defined medium (3, 6, 9). The generation time was approximately 2 h, and cultures reached stationary phase at a final sphere titer of approximately 1.5×10^8 to 3×10^8 per ml (Fig. 1A). At stationary phase, cells accumulated as unbudded singlets at a stage very early in the cell cycle (10). For strain 3153A, virtually no mycelia were observed during the log phase or stationary phase of a growth culture at 25°C.

When stationary-phase cells were inoculated into fresh medium at 25°C, they resumed growth in the budding yeast phase (3, 7, 9); however, when stationary-phase cells were inoculated into fresh medium at 37°C, they formed mycelia synchronously and en masse after an average lag period of approximately 140 min (7, 9). By 140 min, 50% of the released culture had formed small evaginations, and by 165 min, 50% had formed elongated germ tubes the lengths of which were one-half or more the diameters of their mother cells. Each germ tube then grew into a long multinucleate mycelium the length

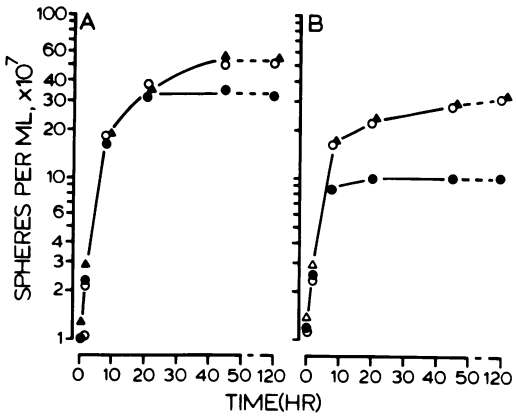

FIG. 1. *Effects of zinc on cell multiplication at 25°C for wild-type strain 3153A (A) and for mutant strain M10 (B). Symbols: ●, no zinc; ○, 2 μM zinc sulfate; ▲, 9 μM zinc sulfate.*

of which was many times the diameter of the mother cell (3, 7).

Effects of zinc on the growth and stationary-phase phenotype of strain 3153A. The addition of micromolar concentrations of zinc sulfate to the growth medium of a culture of strain 3153A had no effect on generation time (Fig. 1A). However, cells grown in the presence of zinc reached a final sphere titer approximately twice that of control cultures lacking zinc (Fig. 1A). This increase in sphere number was not reflected in the dry weights of the stationary-phase cultures. In repeated experiments, the dry weights of 5-ml samples of stationary-phase cultures containing zinc ranged from 1.0 to 1.5 times the respective dry weights of 5-ml samples for parallel stationary-phase cultures lacking zinc. This result was explained in part by the observation that in the presence of zinc, cells did not accumulate primarily as singlets but, rather, appeared to stop growing at several stages of budding, without cell separation. Singlets, which made up more than 65% of the total sphere population of a 48-h stationary-phase culture lacking zinc, made up less than 25% of the total sphere population of a 48-h stationary-phase culture containing zinc. Photomicrographs of untreated and treated cultures are presented in Fig. 2A and B, respectively. All of the above results were also obtained using the chloride rather than the sulfate salt of zinc and using another strain of *C. albicans*, c136, a human isolate of different origin.

Effects of zinc on the growth and stationary-phase phenotype of strains producing low and high levels of mycelia at stationary phase at 25°C. Cultures of strains c141 and c157 exhibited growth characteristics similar to

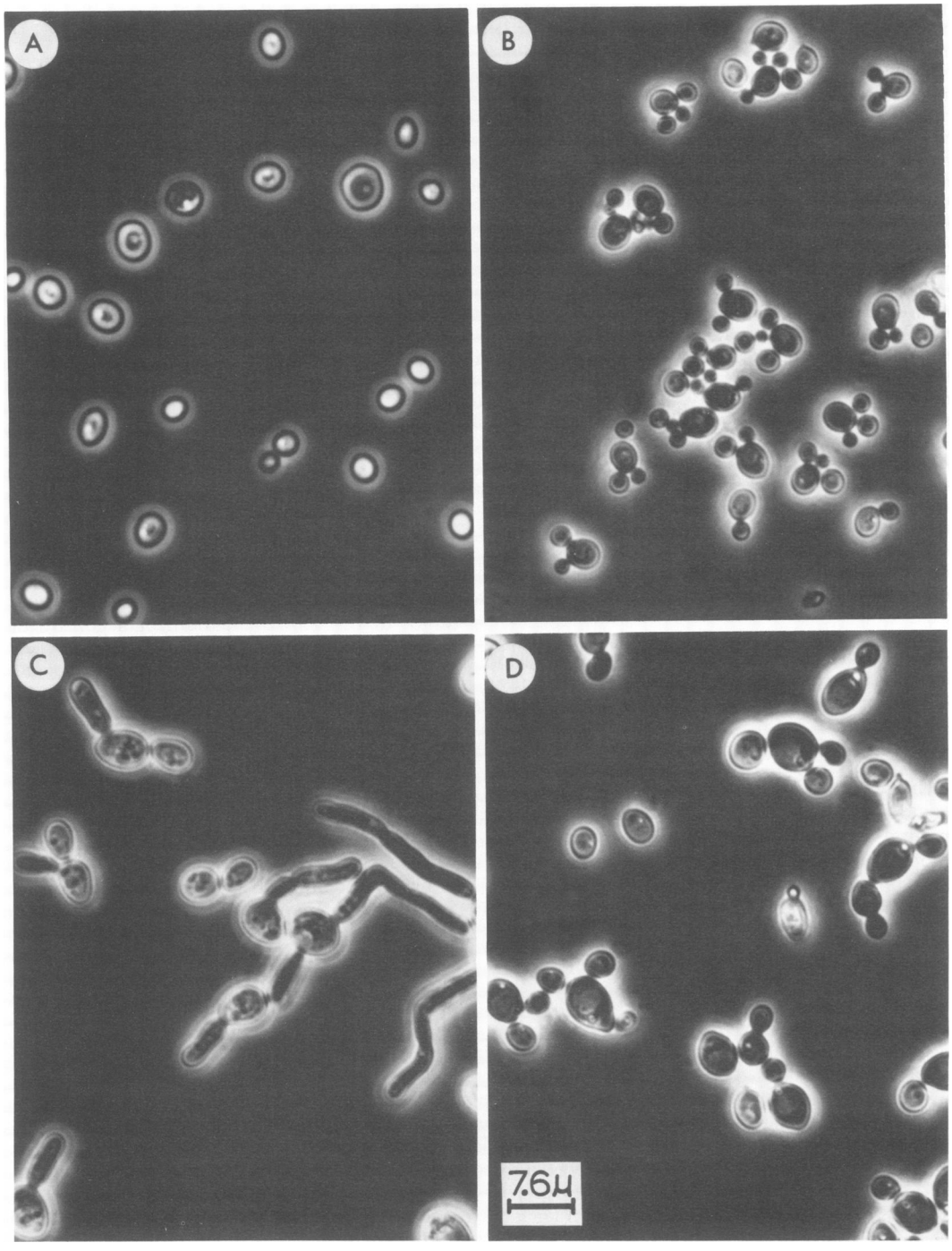

FIG. 2. *Photomicrographs of stationary-phase cultures grown at 25°C in the absence and presence of zinc. (A) Strain 3153A in the absence of zinc; (B) strain 3153A in the presence of 2 μM zinc sulfate; (C) mutant strain M10 in the absence of zinc; (D) strain M10 in the presence of 2 μM zinc sulfate.*

strains 3153A and c136 in all respects but one. Both strains produced low but significant levels of mycelia at stationary phase at 25°C. After 40 h in stationary phase at 25°C, between 0 and 0.2% of cells of strains 3153A and c136 produced mycelia; in contrast, between 4 and 8% of cells of strains c141 and c157 produced mycelia. When zinc sulfate was added to a final concentration

of 9 μM in growth cultures of c141 and c157 at 25°C, the same effects described for strains 3153A and c136 were obtained. The final sphere titer at stationary phase increased twofold, and cells accumulated predominantly in a budded rather than in a singlet form. In addition, the presence of zinc completely suppressed the formation of mycelia at stationary phase. In both cultures, no mycelia were found in 5,000 scored cells after 50 h in stationary phase in the presence of zinc.

To examine further zinc suppression of mycelium formation at 25°C, we isolated an ultraviolet-induced mutant strain of 3153A, M10, in which a majority of cells formed mycelia at stationary phase at 25°C. M10 grew in the budding yeast form during the early and midlog phases of growth, with an average generation time of approximately 2 h (Fig. 1B). As the population entered stationary phase, spheres began forming elongate mycelia (Fig. 3). After 20 h in stationary phase, over 50% of the sphere population possessed one or more mycelia (Fig. 3 and 2C).

The addition of micromolar concentrations of zinc to a growth culture of M10 at 25°C produced effects similar to those for strains c141 and c157: the generation time remained unaffected (Fig. 1B), the final concentration of spheres at stationary phase was approximately twofold that of untreated cultures (Fig. 1B), cells were asynchronously blocked in the budding cycle, and the formation of mycelia at stationary phase was completely suppressed (Fig. 3). Photomicrographs of untreated and treated cultures are presented in Fig. 2C and D, respectively.

Specificity of the suppression of mycelium formation by zinc at 25°C. At concentrations of 0.1 μM or less, zinc sulfate had no effect on mycelium formation at stationary phase in cultures of M10 grown at 25°C (Fig. 4). At a concentration of 0.2 μM, zinc sulfate suppressed mycelium formation by more than 90% (Fig. 4). At concentrations of 2 μM or above, it suppressed mycelium formation by 100% (Fig. 4). Suppression was not due to a general divalent cation effect but was, rather, due specifically to the divalent cation zinc. When the divalent cations Ca^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, and Co^{2+} were individually added to growth cultures of M10 cells at 25°C at concentrations of 9 and 90 μM, neither an increase in final sphere concentration nor suppression of mycelium formation was observed at stationary phase (Table 1). The divalent cation Co^{2+} did depress the growth rate and did cause the production of blunt-ended, shortened mycelia. The effects of cobalt are now being investigated in more detail. The divalent cation Mg^{2+} was not tested since it was already a normal component of the growth medium at millimolar concentrations (6). Both the chloride and sulfate salts of zinc gave identical results. Together, these data indicate that the divalent cation zinc is a specific and potent inhibitor of mycelium formation in stationary-phase cultures of the mutant strain M10 grown at 25°C.

Effect of zinc on synchronous mycelium formation in released stationary-phase cultures at 37°C. When cells from a stationary-phase culture of strain 3153A grown at 25°C in the absence of zinc were diluted into fresh medium prewarmed to 37°C and lacking zinc, they

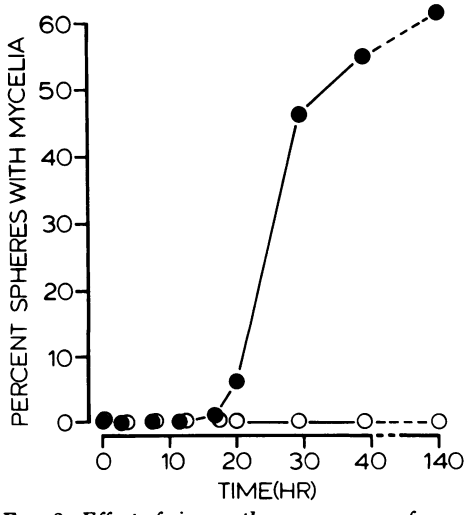

FIG. 3. *Effect of zinc on the appearance of mycelia at stationary phase in cultures of strain M10 grown at 25°C. Symbols: ●, in the absence of zinc; ○, in the presence of 2 μM zinc sulfate.*

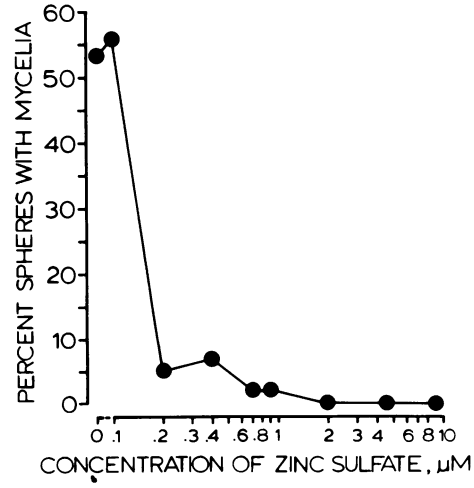

FIG. 4. *Effects of various concentrations of zinc sulfate on the final proportion of cells of strain M10 possessing mycelia at stationary phase at 25°C. Cells were scored for mycelia approximately 40 h after cultures entered stationary phase.*

TABLE 1. *Specificity of zinc suppression of mycelium formation at stationary phase for mutant M10 at 25°C*[a]

Ion	Ion concn (μM)	Generation time during log phase (h)	Approximate time culture entered stationary phase (h)	Sphere concn at stationary phase (spheres per ml)	% Spheres with mycelia at stationary phase (40 h)
None		2.5	20	5×10^7	55
Zn^{2+}	9	2.5	20	1.5×10^8	0
Zn^{2+}	90	2.5	20	2×10^8	0
Ca^{2+}	9	2.5	20–25	7×10^7	53
Ca^{2+}	90	2.5	20–25	7×10^7	32
Cu^{2+}	9	2.5	20–25	6×10^7	52
Cu^{2+}	90	2.5	20–25	6×10^7	59
Fe^{2+}	9	2.0	20–25	9×10^7	55
Fe^{2+}	90	2.0	20–25	9×10^7	47
Mn^{2+}	9	2.25	20	6×10^7	49
Mn^{2+}	90	2.25	20	6×10^7	38
Co^{2+}	9	4	30–40	4×10^7	30
Co^{2+}	90	6	40–50	2×10^7	22[b]

[a] Stationary-phase measurements were taken 40 h after the initiation of growth at time zero. Specific ions were added to growth medium as $ZnSO_4$, $CaCl_2$, $CuSO_4$, $FeSO_4$, $MnCl_2$, and $CoCl_2$.

[b] Since cells in 90 μM cobalt entered stationary phase late, the percentage of cells with mycelia is presented for 100-h cells.

synchronously formed mycelia rather than buds after an average lag period of approximately 165 min (Fig. 5A) (9). When stationary-phase cells were diluted into fresh medium prewarmed to 37°C and containing 9 μM zinc sulfate, they also formed mycelia synchronously after an average lag period of 165 min (Fig. 5A). Concentrations of zinc sulfate as high as 900 μM in the release medium had no effect on either the proportion of cells forming mycelia or the average time of mycelium formation; millimolar concentrations of zinc sulfate delayed but did not suppress mycelium formation. Similar results were obtained for strain c136 and for the majority of cells, which have not formed mycelia at 25°C, of strains c141 and c157.

When cells from a stationary-phase culture of strain 3153A were grown to stationary phase in the presence of 9 μM zinc sulfate and then released into fresh medium prewarmed to 37°C and either lacking or containing 9 μM zinc sulfate, mycelia formed relatively synchronously on the majority of spheres, and they did so in approximately one-half the time it took for a released cell culture initially grown to stationary phase in the absence of zinc (Fig. 5A). Similar results were obtained for strain c136 and for the majority of cells, which have not formed mycelia at 25°C, of strains c141 and c157.

We were unable to test the effects of zinc on mycelium formation at 37°C in released cultures of stationary-phase cells of mutant strain M10 initially grown to stationary phase at 25°C in the absence of zinc because of the high proportion of mycelia already present in these cultures. However, we could test the effect of zinc at 37°C on cultures grown at 25°C in the presence of zinc. M10 cells grown to stationary phase at 25°C in the presence of zinc were released into fresh medium prewarmed to 37°C which either contained or lacked zinc. In both cases, they formed mycelia synchronously in half the time it took cells of strain 3153A grown to stationary phase in the absence of zinc (Fig. 5B).

DISCUSSION

In this report, the effects of micromolar concentrations of zinc on the growth and dimorphism of *C. albicans* have been analyzed in detail under defined in vitro conditions. Evidence has been presented which demonstrates that the addition of zinc to the medium of a growth culture at 25°C has four discrete effects on stationary-phase cells: (i) a twofold increase in the final sphere concentration, (ii) an asynchronous block in the budding cycle, (iii) suppression of mycelium formation, and (iv) a decrease in the time of synchronous mycelium formation after release into fresh medium prewarmed to 37°C. Evidence has also been presented that the addition of zinc to a released culture of stationary-phase cells at 37°C does not suppress the formation of mycelia, demonstrating that zinc does not suppress mycelium formation under all conditions.

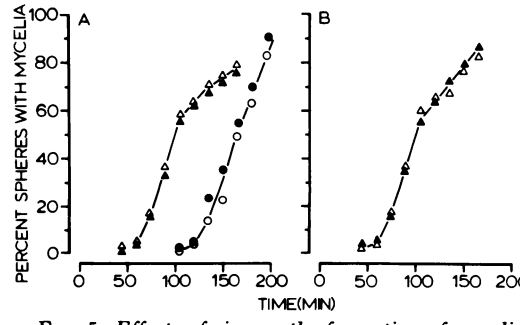

FIG. 5. *Effects of zinc on the formation of mycelia after release from stationary phase by dilution into fresh medium at 37°C for wild-type strain 3153 (A) and mutant strain M10 (B). Symbols: ●, no zinc during growth at 25°C and during release at 37°C; ○, no zinc during growth at 25°C, 9 μM zinc sulfate during release at 37°C; ▲, 9 μM zinc during growth at 25°C, no zinc during release at 37°C; △, 9 μM zinc sulfate during growth at 25°C, 9 μM zinc sulfate during release at 37°C.*

Possibility of a zinc chelator at stationary phase. It was previously demonstrated that the final sphere concentration at stationary phase in *Candida* cultures grown at 25°C is not directly regulated by the availability of nutrients in the medium (8, 9). Cell-free medium of stationary-phase cultures will support new rounds of growth, indicating that the cessation of cell multiplication may be due to the accumulation of a labile or volatile growth inhibitor in cultures at high cell density (9). The twofold increase in the final concentration of spheres and the slight increase in dry weight at stationary phase caused by the addition of micromolar concentrations of zinc indicates that the effect of the putative growth inhibitor could be partially reversed by zinc. This possibility is given some credence by recent observations on the effects of chelators on the growth of *Saccharomyces cerevisiae* (5). The chelating agents *O*-phenanthroline and 8-hydroxyquinoline when added in microgram quantities to cultures of *Saccharomyces* inhibited growth, blocking cells as unbudded singlets in a fashion similar to the proposed inhibitor in *Candida* cultures (9). In the *Saccharomyces* investigation (5), it was also found that the specific addition of zinc reversed the inhibitory effects of the chelators. These similarities suggest that *Candida* cells may produce an extracellular chelator which is labile, functions as a growth inhibitor, effects the acquisition of mycelium inducibility at 37°C, blocks cells in G1, and is partially inactivated by the addition of zinc. The possibility is now under investigation.

Relationship of the zinc effect to the cell cycle. In the absence of zinc, the majority of cells in most strains of *Candida* which we have tested accumulate as unbudded singlets at a stage early in the budding cycle at stationary phase at 25°C (9). In the presence of zinc, cells appear to be blocked at various stages of the budding cycle, based on the presence and size of buds. However, we have not measured the average deoxyribonucleic acid content of nuclei so we cannot be sure if nuclear deoxyribonucleic acid is unreplicated, replicated, or both in a stationary-phase culture of zinc-treated cells. It is important that one not assume the state of the nucleus based on the presence or size of a bud since budding and nuclear events have been separated on different dependent pathways (4, 10).

If the time of synchronous mycelium formation at 37°C depends upon a cell cycle parameter of the blocked stationary-phase population, then the decrease in time observed for zinc-treated cells may be due to a change in that parameter caused by zinc. For instance, in the absence of zinc, cells are blocked in G1, but in the presence of zinc they may be blocked at a later stage. This would imply that the time necessary for cells to progress from G1 to the stage in which zinc-treated cells are blocked may represent a portion of the preevagination period. Alternatively, the cell cycle may have nothing to do with the timing of evagination. Rather, it may be that the stationary-phase phenotype is altered by zinc so that a rate-limiting process which is essential for evagination is precociously stimulated by the presence of zinc, thus decreasing the preparative time for evagination after release from stationary phase at 37°C. All of these possibilities must be directly tested.

Possibility of alternate pathways for mycelium formation. The most interesting and potentially important result we have obtained in this investigation is that alternate pathways may exist for mycelium formation in *Candida*. We have demonstrated that mycelium formation in cultures of strains c141 and c157 and of mutant strain M10 at stationary phase at low pH and low temperature is completely suppressed by less than a 2-μM concentration of zinc but that synchronous mycelium formation in stationary-phase cultures of all tested strains released into fresh medium at high pH (>5.5) and high temperature (37°C) is completely resistant to zinc at even millimolar concentrations. We previously presented evidence that synchronous mycelium formation in released stationary-phase cultures exhibits a strict requirement for both high pH and high temperature (7, 9). These two conditions are not required for mycelium formation at stationary phase at 25°C in strains c141 and c157 and in mutant strain M10 (Bedell and Soll, unpublished observations).

The mycelia formed under the two sets of conditions, or along the two pathways, are superficially indistinguishable when compared under the compound microscope with phase optics. However, one or more molecular aspects of the mycelia formed along the two pathways must be different to account for the radical differences in environmental requirements and zinc sensitivity we have demonstrated in this investigation.

Medical significance of the zinc effect. The possibility that mycelia can form by different pathways has special significance for both the antibiotic treatment of *Candida* and for immunological investigations of *Candida* antigens. In the former case, antibiotics must be screened for their effects on the two different pathways of mycelium formation in a single strain since differences similar to zinc sensitivity may exist. In the latter case, antigenic differ-

ences between mycelia formed by the different pathways may exist. This may cause confusion in studies in which antibodies against the antigens of mycelia formed by one pathway are used against antigens of mycelia formed by the second pathway. It is therefore extremely important to discover which pathway, zinc sensitive or zinc resistant, predominates during different pathogenic infections.

Because of the large number of key roles played by the divalent zinc ion in the defense mechanisms of individuals suffering from infectious diseases (1), it is difficult to assess the direct effects of zinc on the growth and phenotypic conversion of dimorphic yeasts in vivo. For instance, in patients suffering from the hereditary disorder acrodermatitis enteropathica, normal zinc absorption through the small intestine is depressed (2). Individuals suffering from this disease usually are infected with *Candida*, but an increase of zinc in the diet causes a remission of the symptoms of the defect, including a reduction in *Candida* infection (2). The reduction of *Candida* infection may be due to a direct effect of increased zinc at the site of infection or to a stimulation of the host's defense mechanisms. We have directly examined the effects of micromolar concentrations of zinc on growth and dimorphism of *C. albicans*, and we have observed that zinc neither depresses growth nor suppresses the formation of the invasive mycelium under all conditions.

ACKNOWLEDGMENTS

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