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Characterization of Dimorphism in *Cladosporium werneckii*

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Yeast forms of the dimorphic fungus *Cladosporium werneckii* grow by polar budding and yield a homogeneous yeast phase when cultured at 21 C in an agitated sucrose-salts medium (Czapek-Dox broth). Yeast extract enrichment of such a yeast phase consisting of 10^4 yeasts per ml induces a quantitative conversion of the yeasts to true hyphae. This conversion is not mediated by a transition cell and is often attended by capsule formation. When 10^5 or 10^6 yeasts per ml receive enrichment, a nonquantitative conversion to moniliform hyphae is effected and no capsule formation is observed. Rapid agitation compared to slow agitation or stationary incubation of the nutritionally mediated conversion cultures greatly accelerates the production of lateral hyphal buds or their yeast progenies. These cells appear incapable of undergoing nutritional conversion to hyphae, but instead must grow for several generations in the unenriched sucrose-salts medium to restore conversion competence. Temperature shifts affect directly the morphology and morphogenesis of the yeast in unenriched medium; at 17 C yeasts are smaller and more ovoid than at 21 C, and at 30 C marked conversion of yeasts to moniliform hyphae occurs. A methodology employing the Coulter counter and Coulter channelizer provides evidence that direct correlations do not always exist between the optimum conditions for the growth of *C. werneckii* and the optimum conditions for its yeast-to-mold conversion.

Cladosporium werneckii is a dematiacious black fungus which causes the human skin disease tinea nigra (5, 7, 10, 14, 17). This fungus is known to exhibit both a yeastlike and hyphal morphology although it is not known what bearing this dimorphism has upon the disease. Nielson and Conant reported that totally yeastlike growth is obtained after several transfers using cysteine-starch agar overlaid with cysteine-starch broth (21). Houston et al. (15) after observing growth on a variety of media reported that yeastlike growth is promoted by cysteine enrichment and also by incubation in an atmosphere of carbon dioxide. However, these reports did not cite the precise length of incubation or describe the fungus in detail as to morphology. Carrion (6) has pointed out that several distinct morphologies are characteristically produced by *C. werneckii* in liquid media or in aging surface colonies developing on solidified media. Included among these morphologies are three types of true hyphae and two types of yeastlike growth. Thus, proper characterization of yeast-to-mold conversion in *C. werneckii* must include precise subject definitions and descriptions. Additionally, a defined, homogeneous inoculum should serve as the starting material for all conversion experiments.

In this investigation, we examined the yeast-to-mold conversions of *C. werneckii* by phase-contrast microscopy. In an effort to document the most characteristic aspects of the conversions, only homogeneous populations of yeast-phase cells were used as inocula. Additionally, we established some of the cultural parameters responsible for inducing yeast-to-mold conversions and mold-to-yeast conversions. A quantitative methodology was developed to determine whether yeast-to-mold conversions are directly related to enhanced growth rates.

MATERIALS AND METHODS

Fungus and culture of the yeast phase. *C. werneckii* EPH 4799, a strain isolated from tissue, was a gift from Elizabeth Hodges, Diagnostic Mycology Laboratory, Tulane University Medical School, New Orleans, La. Yeast phase was grown in 100 ml of Czapek-Dox broth (Difco) contained in 250-ml conical flasks stoppered with foam plugs. These cultures were incubated at 21 C with rapid agitation (240 rpm) by using a New Brunswick psycrotherm incubator shaker. The yeast inoculum used for conversion experiments was taken from Czapek-Dox broth (CDB) cultures of 10^6 yeasts per ml in mid-log phase (48 h) of growth. The inoculum cultures were routinely perpet-

uated by allowing populations of yeasts (10^5 yeasts per ml) to grow to approximate concentrations of 5×10^6 yeasts per ml, and then using cells from these cultures to inoculate fresh broth.

Yeast-to-hyphal conversion conditions. To convert yeasts to true hyphae, yeast extract (Difco) was added (1%, wt/vol) to CDB cultures of 10^4 yeasts per ml. These conversion cultures were incubated at 25 C with rotary agitation of 40 rpm. Conversion was equally good at 21 C, but inconveniently protracted; a slow shaking speed was used to retard the accumulation of lateral buds and their yeast progenies. Yeast conversion to moniliform hyphae was accomplished in much the same manner, except that the yeast extract (1%, wt/vol) was added to cultures of at least 10^5 yeasts per ml in CDB and the incubation was conducted at 21 C with rotary shaking at 240 rpm. Nutritionally mediated conversion was also accomplished by the addition of a sterile concentrated solution of yeast extract (250 mg/ml). This solution was prepared by first warming the concentrated solution and then clearing it of particulate matter by successive passage through membrane filter disks of decreasing pore size (8, 5, 3, and 1.2 μm). The sterilization was accomplished by passing the prefiltered solution through a sterile membrane filter disk (0.45 μm). To obtain temperature-mediated conversion, cultures of 10^4 or 10^5 yeasts per ml growing in CDB were shifted from 21 to 25 or 30 C.

Microscopy. Phase-contrast micrographs were obtained by using a Zeiss Universal research microscope and a 40× phase-contrast objective. The micrograph of encapsulated hyphae in India ink was obtained with an American Optical Phase Star microscope and a 40× bright-field objective, Kodak Pan-X film was used for all exposures. When dilute samples were observed the culture samples were concentrated by centrifugation before photography.

Quantitative evaluation of conversion. Cultures were periodically evaluated with regard to their particle number, average particle volume (APV), and relative volume (RV). The particle numbers were obtained by using the Coulter counter (model ZB) fitted with a 70 μm aperture. The APV values were calculated from data collected with the Coulter counter and a Coulter channelizer in the following manner. First, a series of arithmetically increasing size (volume) values was assigned to those channels which registered particle counts. Then the number of particles appearing in each channel was multiplied by the size value assigned to that channel, and the products obtained for all channels were summed. The APV was obtained by dividing this sum by the total number of particles from all the channels. The validity of assigning an arithmetic progression of size values (i.e., 1, 2, 3, ... n) to the channels of the channelizer was demonstrated with the use of a microscope; direct observation established a profile of particle sizes consistent with the data provided by the Coulter channelizer. The APV units thus represent arbitrary units and relate in a proportional way to other APV units; a culture with an APV of 60 units was made up of smaller growth units than one with an APV of 80 units. Translation of these units into an absolute measure of volume was not necessary since yeasts from 21 C control cultures always served as relative standards of volume. The amount of volume present beyond that attributable to the largest yeast doublets reflected yeast-to-mold conversion. The RV was used to estimate the increase or decrease in the rates of growth (measured as volume of biomass per milliliter) of the fungus in experimental cultures relative to yeast-phase controls at any time. For a sampling time, the volume of biomass per milliliter was calculated by obtaining the product of the APV and the particle counts. The product for the control population was then divided into the product for the conversion population to yield the RV. The total volume in the control cultures was expressed as unity (1.0 RV) at each sample time.

RESULTS

Yeast conversion to true hyphae. Logarithmically growing yeasts cultured in CDB consist of more than 95% one- and two-celled units (Fig. 1A). Exhibiting a peanut shape, these cells grow only by apical, unipolar budding. The addition of yeast extract at a final concentration of 1% to CDB cultures containing about 10^4 such yeasts per ml induces a yeast-to-true hyphal conversion. Phase-contrast microscope observations reveal that the first readily discernible morphological change in the converting yeasts becomes apparent about 9 h after the enrichment (Fig. 1B). At this time the yeasts undergo one of two types of morphological changes. Yeasts not having entered a typical budding sequence simply convert directly to hyphae by elongation. In contrast, mother yeast cells having initiated a bud usually undergo little change, whereas the new bud converts to the hypha. For 12 to 24 h, conversion proceeds until the resulting hyphae have a length four or five times that of a yeast singlet (Fig. 1C). A characteristic aspect of the conversion at this time is the pronounced aggregation exhibited by a high percentage of the hyphae. This aggregation is probably related to the capsule which is formed by the fungus when low numbers of yeasts are induced to convert by the addition of yeast extract (Fig. 1D). By 24 h many of the converted mother yeasts are producing lateral buds, while the hyphae are beginning to produce hyphal bud initials (Fig. 1E). The lateral hyphal buds are produced in prolific numbers during subsequent incubation (Fig. 1F). These buds and their yeast progenies continue to increase in number producing a visible culture turbidity by 72 h. Viewed under a microscope, the clusters of mycelium formed between 12 and 36 h are visible as small flocs in the clear medium.

Yeast conversion to moniliform hyphae. Dimorphism in *C. werneckii* is exhibited not

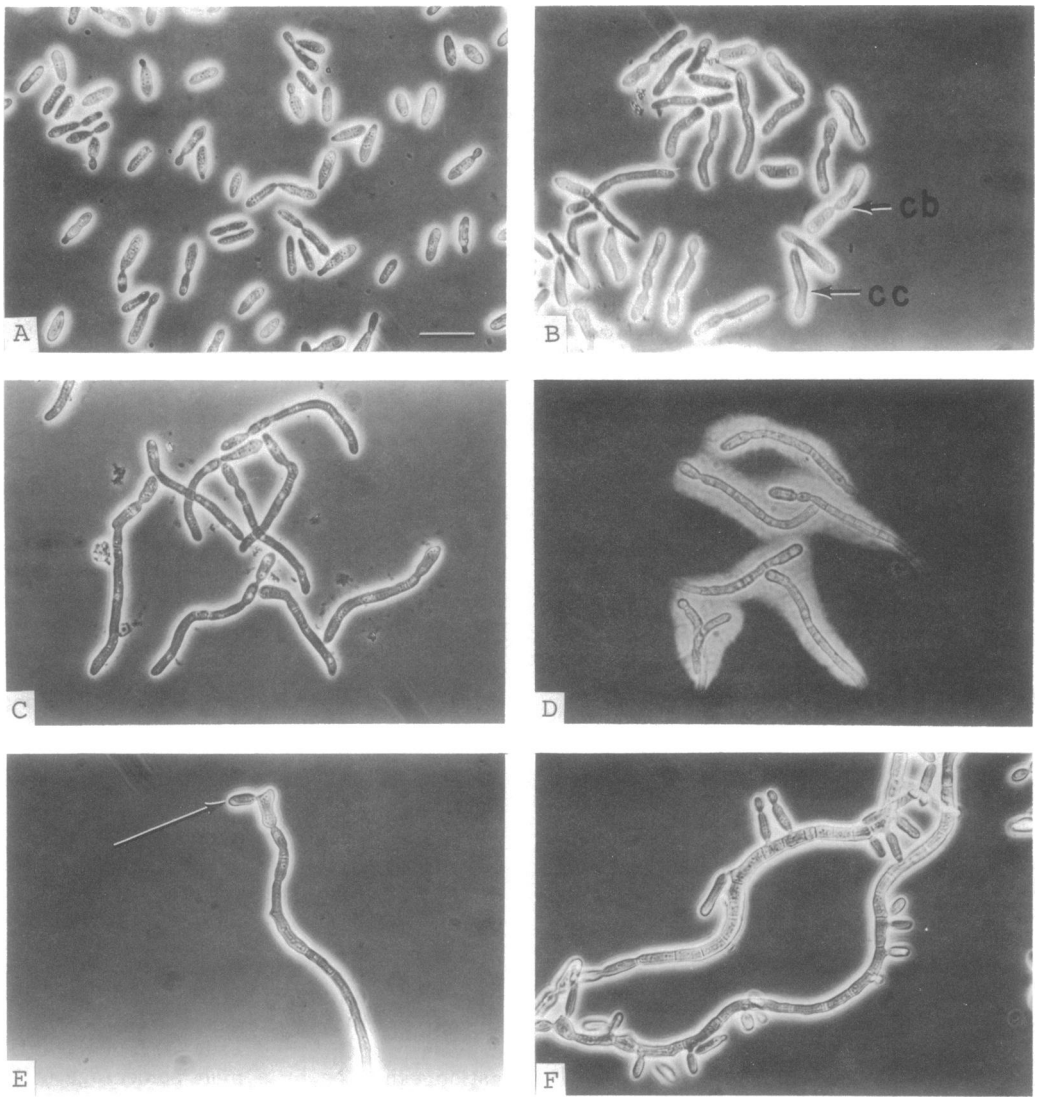

FIG. 1. *Photomicrographs of C. werneckii converting from yeasts (A) to true hyphae (B, C, D, E, F) after enrichment of CDB cultures (10^4 yeasts/ml) with yeast extract (1% wt/vol). Note the polar mode of budding among the yeasts in CDB (A) and the conversions of the mother yeasts (arrow, cc) or buds (arrow, cb) among the yeast 9 h after the enrichment (B). Hyphae from converted yeasts have parallel side walls indicative of true hyphae after 12 h (C) and prominent capsules when observed suspended in India ink (D). After 24 h the converted mother yeasts (E) exhibit daughter buds (arrow), whereas at 36 h the hyphae are producing lateral buds (F). Micrograph D is a light micrograph, whereas the remainder are phase-contrast micrographs. Marker bars represent 10 μm.*

only by conversion of yeasts to true hyphae, but also by conversion under appropriate conditions to moniliform hyphae. The development of moniliform hyphae results from either the use of large inocula in the presence of yeast extract (Fig. 2A) or from shifting unsupplemented cultures from 21 to 30 C (Fig. 2B). Moniliform hyphae resulting from enrichment usually remain short, growing no longer than 100 μm, and produce large numbers of lateral hyphal buds. Moniliform hyphae induced by a temperature shift usually grow longer, appear somewhat narrower in diameter, and produce fewer lateral hyphal buds. Both the nutritionally induced moniliform hyphae and the temperature-induced moniliform hyphae, when observed in

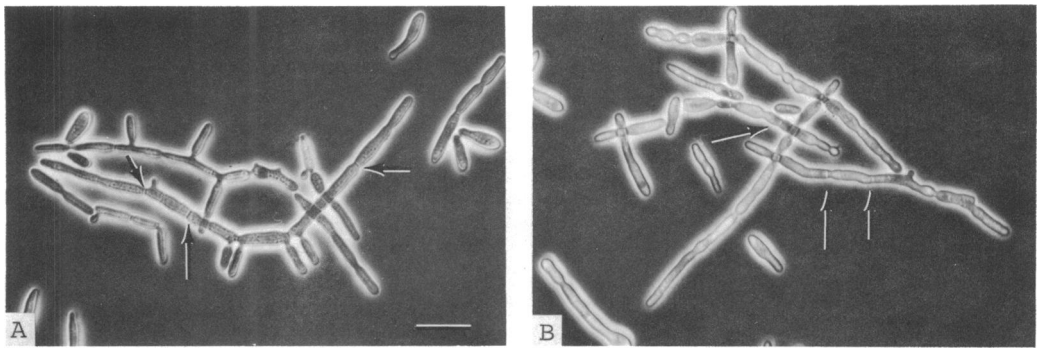

FIG. 2. *Phase-contrast micrographs of moniliform hyphae formed in response to enrichment (A) of CDB yeast cultures (10^6 yeasts/ml) with yeast extract (1%, wt/vol) or in response to a temperature shift (B) from 21 to 30 C of CDB yeast cultures (10^4 or 10^6 yeasts/ml). Note the simple septa-like partitions (arrows) among the hyphae produced in response to either condition. Marker bars represent 10 μm.*

thin section with the electron microscope, exhibit simple septa having a septal pore and associated Woronin bodies (R. Hardcastle and R. Gustafson, unpublished results). The presence of such simple septa in these hyphae documents their moniliform nature.

Effect of agitation on converted cultures. The nature of cultures resulting from nutritionally mediated conversion differs significantly according to the amount of agitation. When a culture receives no agitation (i.e., stationary incubation) it retains a mold appearance even though yeastlike lateral buds and their progenies become substantial. However, when a similar culture receives vigorous agitation the production of lateral hyphal buds and their progenies appears to accelerate, the hyphae clump and stop their rapid elongation, and the culture itself exhibits a turbidity characteristic of a homogeneous population of yeasts. The lateral hyphal buds and their yeast progenies produced in nutritionally mediated conversion cultures exhibit no structural or cytoplasmic differences when viewed with the phase-contrast microscope and compared with yeasts in CDB cultures. However, differences are readily apparent upon culture. Yeasts from CDB are completely conversion competent, whereas yeasts having arisen in enriched conversion cultures are not. When yeasts of the latter type are inoculated into enriched medium at a level of 10^5 cells per ml, the result is simply a perpetuation of conversion-incompetent yeast population. When the inoculum rate is 10^4 cells per ml an abortive attempt at conversion occurs, resulting in the production of pleomorphic budding units of growth. Such units are short, have an aberrant appearance, and produce numerous buds. Only after conversion-incompetent yeast cells have gone through a number of cell division cycles in minimal medium is nutritional conversion competence restored. This seems to suggest the necessity of exhausting an intracellular supply of some factor, either present in yeast extract or induced by the presence of yeast extract, which inhibits the conversion of yeast-like cells to hyphae.

Kinetics of conversion. Using 10^4 yeasts per ml as inoculum, conversion mediated nutritionally is essentially quantitative; with 10^5 or 10^6 yeasts per ml conversion is less than quantitative and is designated moniliform. Although conversion at the higher inoculum levels is less than quantitative, advantage can be taken of the moniliform conversions for other purposes. This results from the fact that no aggregation of the yeasts or hyphae is associated with these conversions. Thus, studies of the growth kinetics of yeast-to-moniliform hyphal conversion (both temperature mediated and nutritionally mediated) were conducted by using the Coulter counter and Coulter channelizer. The utility of these instruments lies in the fact that cultures need not be sacrificed in order to measure the relationships between total growth and particle counts, such measurements constituting the essence of conversion kinetics.

Exponential-phase yeasts (10^5 or 10^6 yeasts per ml) inoculated into CDB and incubated at 21 C increase in APV reaching maximum values after about 24 h, at which time the APV begins to decline (Fig. 3A and B). Yeast cultures exhibiting these kinetics and grown identically served as controls for all conversion experiments. APV values above the maximum (i.e., an APV of approximately 80) were considered to represent some degree of yeast-to-hyphal conversion. The addition of yeast extract either 24 h after inoculation (Fig. 3B) or initially (Fig. 3A) effects a dramatic increase in the APV for

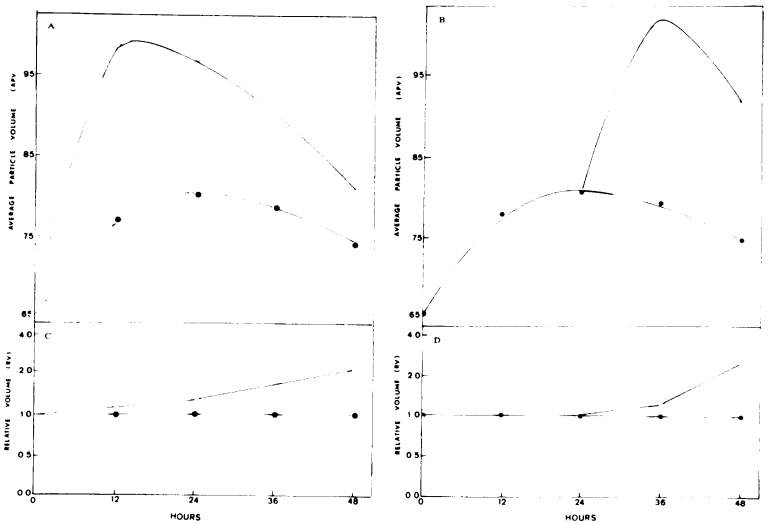

FIG. 3. *Kinetics of nutritionally induced conversion to moniliform hyphae. Increases in APV and RV with enrichment at zero time (A, C) and enrichment after 24 h (B, D). Symbols: ●, yeast phase in unsupplemented CDB; ○, conversion units after supplementation with 1% yeast extract.*

about 12 h after which the APV declines. Samples from the control cultures observed with a microscope exhibit predominantly three classes of fungal units: singlets, singlets having initiated a bud, and doublets. In contrast, samples from converted cultures contain moniliform hyphae of varying lengths, and yeasts in various phases of bud formation. Prolific numbers of lateral hyphal buds are being produced by the moniliform hyphae 12 h after the addition of yeast extract. This apparently accounts for the subsequent decrease in APV. Based on relative volume estimates (Fig. 3C and D) it is apparent that an overall increase in growth rate of *C. werneckii* occurs when yeast-to-moniliform hyphal conversion is induced nutritionally.

Varying the temperature of incubation affects directly the APV of yeast-phase cells growing in CDB. Decreases in temperature 24 h after inoculation (Fig. 4B) or initially (Fig. 4A) effect decreases in APV, whereas increases in temper-

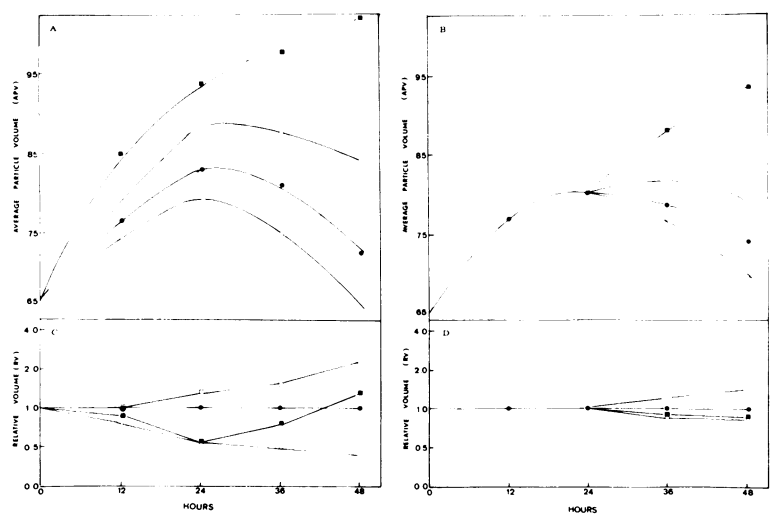

FIG. 4. *Kinetics of temperature-induced conversion to moniliform hyphae. Increases in APV and RV with temperature shifts at zero time (A, C) and temperature shifts after 24 h (B, D). Symbols: yeast phase in cultures incubated at 21 C (●) and 17 C (○); conversion units in cultures at 25 C (□) and 30 C (■).*

ature 24 h after incubation (Fig. 4B) or initially (Fig. 4A) result in increases in APV. When differential temperatures of incubation are imposed initially there is a proportional and wider divergence in APV by 48 h than observed when the shifts are imposed at 24 h. Microscope examination of the growth reveals that cultures at 17 C contain yeast singlets, yeasts having initiated bud formation, and doublets characteristic of control cultures. In addition, a subpopulation of yeasts can be identified which are smaller and more spherical. At 25 C numerous hyphal units having lengths three and four times that of large yeast singlets are observed among the yeast singlets and doublets. Greatly extended hyphal units are observed after conversion at 30 C. Microscope study of these cultures also show significant numbers of the three- and four-cell moniliform hyphal units common to cultures incubated or shifted to 25 C.

Because an APV of greater than 80 is considered to represent some degree of yeast-to-hyphal conversion, it is apparent that relatively little conversion is effected by imposing a temperature of 25 C on a population of control yeast phase, whereas pronounced conversion is effected upon the yeasts by a temperature of 30 C (Fig. 4A). In the 30 C cultures (Fig. 4A) which exhibit the most pronounced conversion either microscopically or in terms of APV, the overall growth rate based on relative volume is depressed for about 36 h before rising to levels slightly higher than exhibited by controls. In contrast, cultures growing at 25 C show a consistently higher growth rate than controls, indicating that 25 C is the optimal temperature of those employed for the growth of *C. werneckii*. Not unexpectedly, yeasts grown at 17 C show a slower rate of growth than yeasts in control cultures. When temperature shifts are made after 24 h (Fig. 4B, D), growth rates based on relative volume reflect similar comparative responses to cultures incubated initially at the corresponding temperatures (Fig. 4A and C).

DISCUSSION

For most fungi whose dimorphism is nutritionally mediated, a low oxidation-reduction potential and/or elevated carbon dioxide content are implicit on the conditions required to maintain yeast growth and/or effect mold-to-yeast conversion. Salvin (25) found that sulfide ions or —SH groups are essential for the growth of the yeast phase of *Histoplasma capsulatum*, and Pine (23) obtained the yeast phase of this fungus with a synthetic medium containing cysteine. Scherr (26) reported that an incubation temperature of 37 C is less critical than sufficient concentrations of —SH groups for maintaining yeast growth of *H. capsulatum*. It was also his observation that an optimal oxidation-reduction potential maintains yeast growth in lieu of exogenously supplied —SH groups. Conversion of *H. capsulatum* mycelium to yeast has been accomplished by using several highly reduced, complex media, including blood agar slants sealed with paraffin (8), congealed egg-potato flour medium (18), and cysteine-blood medium (4). Nickerson and Mankowski (20) reported that yeastlike cultures of *Candida albicans* are prevented from reverting to the mold phase with the addition of cysteine. *Sporothrix schenkii* shows a requirement for increased carbon dioxide tensions in order to convert from mold to yeast (9), as does *Histoplasma farciminosum* (2). Yeastlike development in *Mucor rouxii* was shown to depend on the presence of carbon dioxide and the absence of oxygen (1).

The simple, nonreduced sucrose-salts medium (CDB) which supports yeast growth of *C. werneckii* strain EPH 4799 is markedly different from the complex, highly reduced media described in the reports mentioned above for the yeast growth of *H. capsulatum* and *C. albicans*. Moreover, incubation temperature and degree of agitation affect the homogeneity of yeastlike growth of *C. werneckii* in CDB. (In this context, homogeneity refers to the extent to which there is a presence of one and two-celled yeast units and an absence of pleomorphic units and short moniliform hyphae.) Homogeneity of yeast growth is greater at 21 C than at 25 C, and greater with rapid instead of slow agitation or stationary incubation. Possibly the improvements in the quality of yeast growth at 21 C and with more rapid agitation are linked to an increased availability of oxygen for the cells, since the solubility of oxygen in aqueous solutions varies inversely with the temperature (12) and directly with the amount of agitation the solution receives (11). This is in direct contrast to the effect which oxygen or agitation has on the yeast growth of *Mucor rouxii* (1), i.e., causing yeast cells to grow as filaments.

Nielson and Conant (21) reported that successive transfers of *C. werneckii* on cysteine-starch agar overlaid with cysteine-starch broth are necessary for inducing yeastlike growth. This is interpreted, relative to our findings with strain 4799, as being similar to the growth of lateral hyphal buds and their progeny (Fig. 5) after conversion in enriched medium. In contrast, Houston et al. (15) obtained yeastlike growth with *C. werneckii* in unsupplemented

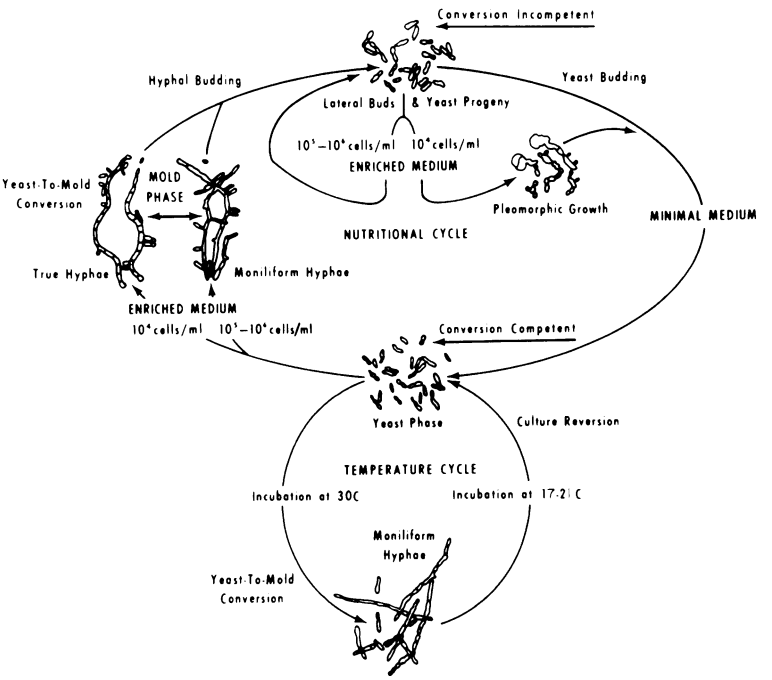

FIG. 5. *Proposed asexual life cycle depicting the morphological responses made by C. werneckii strain 4799 to changes in nutrition, cell number, and temperature.*

CDB incubated broth at 22 and 37 C. With strain 4799 we obtained homogeneous yeast growth in CDB cultures incubated at 17 to 21 C, but found total growth inhibition at 37 C on this medium and a number of other standard laboratory media. Studies are now under way to determine if the inability of strain 4799 to grow at 37 C is the exception or the rule relative to several other strains of *C. werneckii*. Houston et al. (15) also found that *C. werneckii* grows in thioglycolate broth as a form "intermediate" between yeasts and hyphae. Although the intermediate form was not described it seems possible that it represents what we define as moniliform hyphae; the complex, highly reduced nature of thioglycolate broth is consonant with the requirements for nutritional conversion to moniliform hyphae in strain 4799.

Nickerson and Mankowski (20) described yeast-to-mold conversion as an interruption of the budding process without a concomitant interruption of growth. However, because of the inherent technical difficulties associated with mold growth evaluation (3), little attention has been directed toward determining whether conversions are attended by significant growth rate changes. In consideration of this, we evaluated on the basis of volume differences the changes in growth rates accompanying the yeast-to-mold conversions in *C. werneckii*. Theoretically, where conversion from yeasts to hyphae is quantitative, it is possible to calculate the differential growth rates of one yeast cell and one hypha. However, where conversion is less than quantitative, as in the case of *C. werneckii* converting to moniliform hyphae, it is more appropriate to compare the differential growth rates of entire cultures, not individual cells. This allows one to say that conversion is or is not attended by an increase in the total relative volume of a nonquantitative conversion culture. Based on relative volume, we have demonstrated that nutritionally mediated conversion is attended by an increased growth rate (Fig. 3C and D). In contrast, the pronounced temperature-mediated conversion occurring at 30 C is attended by a decrease in growth rate (Fig. 4C and D) well through the conversion period.

The increase in APV of control cultures at 24 h (Fig. 3A and B; 4A and B) reflects the general effect which inoculum size has on the morphogenesis of *C. werneckii*. These cultures, inoculated with 10^5 yeasts per ml, show substantially less of an APV increase at 24 h than cultures inoculated with 10^4 yeasts per ml, and substantially more of an increase then cultures inoculated with 10^6 yeasts cells per ml (R. Hardcastle and P. Szaniszlo, unpublished data). Such increases in APV in minimal media appear to be the result of elongation of yeast

cells while the yeasts come to equilibrium with new cultural conditions. After 24 h, equilibrium is being approached and APV declines.

In nutritionally mediated conversion to moniliform hyphae (Fig. 3A and B) the APV of conversion cultures shows a decline after the peak APV is reached. This decline is apparently caused by the rapid onset of yeastlike growth resulting from the increase in growth rate of unconverted yeast cells and the production of lateral hyphal buds and their progenies. That nutritionally induced moniliform hyphae produce copious numbers of lateral buds is shown in Fig. 2A. The sustained gradual increase in APV during temperature-induced conversion to moniliform hyphae at 30 C (Fig. 4A and B) reflects the more quantitative nature of this system's conversion and its low level of production of lateral hyphal buds (Fig. 2B).

The polar pattern of budding exhibited by the yeastlike cells of *C. werneckii* is, to our knowledge, unique among dimorphic, pathogenic fungi. Of the four types of yeast growth patterns described by Streblova (27), the apiculate budding pattern as illustrated by *Saccharomycodes ludwigii* appears most applicable to *C. werneckii*. The yeast cells of the other dimorphic pathogens grow in a fashion similar to that of *Saccharomyces cerevisiae*, a pattern of growth described as the budding pattern. The unusual physiology of yeast growth in *C. werneckii*, compared to the other pathogenic, dimorphic fungi may be related to the fact that *C. werneckii* is the only one of the group which grows by the apiculate budding method. The absence of a transition cell between the converting yeast and the resulting hypha of *C. werneckii* is also relatively unique among the pathogenic dimorphic fungi. With *C. werneckii* elongation proceeds directly from converted yeasts or buds. This facility of conversion may be characteristic of a more highly developed dimorphic system than those of organisms reported to develop transition cells, such as *Paracoccidiodes brasiliensis* (5) and *Phialophora dermatitidis* (22).

Howard (16) described three mechanisms by which hyphae of dimorphic pathogenic fungi convert to the yeast phase. In this regard *C. werneckii* resembles *C. albicans* (13) and *P. dermitidis* (22) in that the hyphal phases produce lateral buds which in turn give rise to the yeast phase by budding. As shown in this study, the yeast-phase cells of *C. werneckii* can in turn not only convert back to the hyphal phase, but can also convert to one or more of several growth forms intermediate between true yeast and true hyphae. Most characteristic among these intermediate forms are the moniliform hyphae and the aberrant pleomorphic forms. This trait of the organism can be envisioned, at least on a superficial level, as representing a phylogenetic recapitulation of sorts, although any speculation as to the order of such hypothetical events would be unfounded at this time. It further appears reasonable to speculate that other fungi, also characterized as vegetatively polymorphic, are culturally trapped between total yeast-like growth and total hyphal growth and that under the proper conditions could be made to grow totally in either form. A diagram is presented (Fig. 5) which depicts the nutritional and temperature-dependent responses of *C. werneckii* discussed herein.

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