Effect of Relative Humidity on Survival of Candida albicans and Other Yeasts

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Individual blastospores of Candida albicans were deposited on the surface of 50-mm membranes (Millipore Corp.) and placed within sealed glass chambers at various relative humidities (RH). After 48 hr, virtually all cells maintained at 100 and 10% RH had survived, but 84% of the cells maintained at 60% RH failed to develop into colonies when transferred to Sabouraud medium. No morphological abnormalities could be observed in cells surviving low RH values, but their initial rate of multiplication after transfer to Sabouraud medium was greatly reduced, compared to that demonstrated by cells maintained at 100% RH. At 60% RH, the exposure time required to kill 50% of the blastospores was 2 to 3.5 days. The inimical effect of 60% RH was confirmed in a total of 21 isolates of C. albicans. No deleterious effect was noted when 12 other species of yeasts were subjected to 10, 60, and 100% RH. The single isolate of Candida brumptii and 1 out of the 20 isolates of Cryptococcus neoformans tested also failed to grow after blastospores had been exposed to 60% RH for 4 days.

Fungi causing disease in man have been studied extensively in vitro, but their relationships to relative humidity (RH) have received scant attention. Marples (8) has suggested that blastospores of Candida albicans are susceptible to desiccation. In her study, a suspension of cells in a yeast extract broth was allowed to evaporate to dryness. She observed that the number of viable yeast cells detected by plate count dropped rapidly, and, within 3 hr, a 1,000-fold decrease in colony counts was obtained. With the particular model used, however, an alternative explanation for the loss of viability could have been that cellular proteins were denatured by increasingly hypertonic conditions in the culture medium as it dried out. The present study was undertaken in an attempt to determine whether a decrease in RH, in the absence of other cultural variables, did exert any effect on the viability of blastospores of C. albicans. It was initiated as part of a wider study on the existence of strain differences of pathogenic fungi. The original intention was to determine whether different strains of C. albicans and other pathogenic fungi were affected equally by low levels of RH.

MATERIALS AND METHODS

Organisms. Twenty-one isolates of C. albicans were tested. They were obtained from several mycological centers in the United States and the United Kingdom. Their selection for this study was based on variation in anatomical and geographical sites of origin, serotype (6), laboratory age, and phenotypic variation. All isolates were streak purified and identified by formation of chlamydospores on corn meal agar, filamentation in serum after 3 hr at 37 C, and by fermentation of glucose, galactose, and maltose.

Isolates were maintained on Sabouraud agar slants at 4 C and subcultured twice yearly. Their laboratory age ranged from 1 to 13 years. For quantitative studies, subcultures of the standard lab strain (CA 148) were prepared on 2.5%, malt extract-agar (Difco). Cells from slants maintained at 37 C for 48 hr were harvested, washed three times in sterile distilled water, resuspended in sterile water, and adjusted by counting chamber to give a final concentration of ca. 200 cells per ml. The proportion of single cells in the test suspension based on counts of 500 cells ranged from 90 to 99%, i.e., in the steady state of growth. Single cells were more readily obtained at 37 C than at 25 C. Since the accuracy of the method was likely to be compromised by the presence of budding cells, the inoculum was prepared from cells grown at 37 C. For nonquantitative studies, isolates were grown overnight at 25 C on Sabouraud agar slants. This yielded a cell suspension where virtually all the cells were budding, i.e., in the logarithmic phase of growth. After three washes in sterile distilled water, suspensions were standardized visually to approximately equal optical densities (about 10² cells per ml).

Fourteen other species of yeasts were studied. These were Cryptococcus neoformans (20 isolates), C. diffuens (2 isolates), C. albicus (1 isolate), C. laurentii (1 isolate), Candida tropicalis (12 isolates), C. parapsilosis (4 isolates), C. guilliermondii (2 isolates), C.
krusei (2 isolates), C. utilis (1 isolate), C. brumptii (1 isolate), Saccharomyces cerevisiae (3 isolates), Torulopsis glabrata (5 isolates), Rhodotorula mucilaginosa (1 isolate), R. rubra (1 isolate).

Test material consisted of washed blastospores in logarithmic phase of growth. They were suspended in sterile distilled water and adjusted by visual comparison to give approximately comparable opacities.

Relative humidity chambers. These were prepared from 9-cm glass petri dishes. The required RH was obtained by adding appropriate mixtures of glycerin and distilled water (2). RH values in initial experiments were obtained with graded aqueous solutions of potassium hydroxide or sulfuric acid (11), but, although results were apparently unrelated to the choice of RH system, all data reported below were obtained in atmospheres whose RH was determined by glycerin-water mixtures (RH solutions).

Each chamber consisted of two bottom portions of glass petri dishes, one inverted on top of the other. They were rendered air- and water-tight by sealing the junctions with Chemfluor Lab-type (Chemplast Inc.) or Parafilm “M” (America Can Co.). A portion of glass tubing (3 cm broad by 1.5 cm high) placed within the chamber served as a support for the test material and prevented contact with the 15 ml of RH solution in the base of the chamber. A disc of heavy filter paper placed within the lids of chambers maintained at 100% RH effectively minimized disruption of test layers of cells by preventing the formation and fall of water drops from the lower surfaces of the lids. The range of RH values studied was 10 to 100%, at 10% intervals.

Chambers were maintained at 25 C. Fresh RH solutions were prepared before each trial and discarded immediately after use. All glassware was clean and sterile prior to use. RH solutions were not sterilized.

Cell morphology and viability. The morphology of individual cells was observed by spreading a cell suspension containing about 5 x 10⁶ cells per ml on the top surfaces of clean, sterile microscope slides, and placing these within chambers at appropriate RH values. After exposure, a drop of 0.2% methylene blue in 0.2 M KH₂PO₄ and a cover slip were placed over the layer of cells, and the slide was examined microscopically.

Viability studies: quantitative. The primary objective of these studies was to determine the effect of varying RH on the viability of individual blastospores of C. albicans. Pretreatment cultural conditions were manipulated to yield cells in the steady state of growth, where budding would be absent and where depletion of endogenous food reserves would accordingly be minimal. Additional advantages in using single rather than budding cells included ease and accuracy of cell counts and the avoidance of spurious cell population increases caused by the abridgment of daughter cells during manipulation.

By careful counts and appropriate dilutions with sterile distilled water, cell densities of about 100 to 150/ml were prepared. After being shaken to ensure uniform distribution, sufficient cell suspension was pipetted into a sterile membrane filter assembly (Millipore Corp.) to cover the 50-mm membrane completely and to permit the eventual deposition of ca. 200 individual yeast cells on its surface after negative pressure filtration.

After filtration, the membrane was removed from the assembly with sterile forceps, transferred to an RH chamber, and maintained at 25 C for 48 hr. It was then transferred to the surface of a Sabouraud agar plate and incubated at 37 C for 2 or 3 days. Viable cells developed into visible colonies which were counted and recorded. As controls, three membranes were removed from the membrane filtration assembly and placed directly onto Sabouraud plates. Three membranes were used for each RH value. All experiments were duplicated. In later experiments, membranes were maintained in the chambers for 2, 4, and 7 days prior to removal and subsequent evaluation of colony counts. Two strains of C. albicans were used, namely, CA 148 and CA 251, isolated from the esophagus of a pig and human sputum, respectively.

Viability studies: qualitative. When it became clear that for cells of C. albicans there was a relationship between RH and viability, studies were extended to include other isolates of C. albicans and other species of yeasts. In this phase of the work, suspensions of cells in sterile distilled water were pipetted onto individual sterile filter-paper discs (Whatman no. 7), 0.6 cm in diameter. Two discs were inoculated with each test isolate. These were placed on sterile microscope slides and introduced into chambers at 10, 60, or 100% RH. At 2, 4, and 7 days, discs were removed, placed on the surface of Sabouraud plates, and incubated at 25 C. Growth was recorded as present or absent.

RESULTS

Quantitative. Results of the initial quantitative study are shown in Table 1.

After the demonstration that fewer cells survived at 60 to 70% RH than at other levels tested, attempts were made to establish the relationship between time of exposure and loss of viability. This was done by preparing multiple RH chambers, each containing single membranes (Millipore Corp.) and each bearing a standardized number of single blastospores on its surface. Since the greatest loss of viability occurred at 60% RH, the test chambers were maintained at 10, 60, and 100% RH, with glycerin-water mixtures. At daily intervals, three membranes were removed from chambers at these RH values, transferred to Sabouraud plates, and incubated at 37 C; counts were made on the number of colonies developing. Results are shown in Figure 1. This experiment was done three times with similar results. It should be noted that, whereas loss of viability at 100% RH is negligible at 9 days, there was no growth from cells maintained at 60% RH for 7 days or longer. The average exposure required to kill 50% of the blastospores in the three trials was noted to be 2, 3.5, and 2.5
TABLE 1. Effect of 48 hr of exposure to different relative humidities (RH) on survival of Candida albicans

| RH   | No. of colonies developing on Sabouraud agar plates | Per cent survival a |
|------|-----------------------------------------------------|---------------------|
| 10   | 266                                                 | 106                 |
| 20   | 191                                                 | 76.4                |
| 30   | 181                                                 | 72.4                |
| 40   | 119                                                 | 47.6                |
| 50   | 103                                                 | 41.2                |
| 60   | 42                                                  | 16.8                |
| 70   | 71                                                  | 28.4                |
| 80   | 192                                                 | 76.8                |
| 90   | 250                                                 | 100                 |
| 100  | 251                                                 | 100                 |

a Average of three tests.
b Compared to cells maintained at 100% RH.

days, respectively. Cells maintained at 10% RH had little or no loss of viability within the first 72 hr but then showed an increasing mortality rate thereafter. A considerable proportion of cells (about 25 to 30%) remained viable, even after 11 days, and some survivors were noted (5%) at the termination of the experiment on the 16th day.

It was noted that the rates of growth of colonies developing on the membranes after transfer from the RH chambers to the surfaces of Sabouraud plates varied according to the prior level of main-
tained relative humidity. Those from membranes maintained at 100% RH were large and could be readily counted at 24 hr; those from 10 to 50% RH were minute and required incubation for 2 to 3 days before attaining the same size as 24-hr-old colonies from cells kept at 100% RH. Although there was a clear and seemingly linear correlation between the rate of colony growth and the degree of saturation of the chamber atmosphere, this bore no relationship to the numbers of cells surviving at different RH values. The numbers of viable blastospores maintained at 100 and 10% RH were almost identical, but the rates of growth of colonies derived from these cells differed greatly. Apparently, cells exposed to the lowest RH values tested remained viable but were subjected to an appreciable physiological stress; removal of conditions causing the stress did not restore cells immediately to their previous

TABLE 2. Survival of Candida albicans after relative humidity tests

| Duration of exposure (day) | No. of isolates surviving a at relative humidity |
|----------------------------|-----------------------------------------------|
|                            | 10% | 60% | 100% |
| 2                          | 20  | 20  | 20   |
| 4                          | 20  | 15  | 20   |
| 7                          | 20  | 6   | 20   |

a Twenty isolates were tested.

TABLE 3. Species of yeasts tested for susceptibility to relative humidity (RH)

| Species                  | No. of isolates tested | No. of isolates failing to survive a | Conditions of lethality for susceptible isolates |
|--------------------------|------------------------|-------------------------------------|-----------------------------------------------|
| Cryptococcus neoformans  | 20                     | 1                                  | 60% RH for 4 days                             |
| C. diffluens             | 2                      | 0                                  |                                               |
| C. albidos              | 1                      | 0                                  |                                               |
| C. laurentii            | 1                      | 0                                  |                                               |
| Candida tropicalis       | 11                     | 0                                  |                                               |
| C. parapsilosis          | 4                      | 0                                  |                                               |
| C. guilliermondii        | 2                      | 0                                  |                                               |
| C. krusei               | 2                      | 0                                  |                                               |
| C. utilis               | 1                      | 0                                  |                                               |
| C. brumptii             | 1                      | 1                                  | 60% RH for 2 days                             |
| Saccharomyces cerevisiae | 3                      | 0                                  |                                               |
| Torulopsis glabrata      | 5                      | 0                                  |                                               |
| Rhodotorula mucilaginosa | 1                      | 0                                  |                                               |
| R. rubra                | 1                      | 0                                  |                                               |

a At 10, 60, or 100% RH for 2, 4, or 7 days.
physiological status and this delayed the resumption of cell multiplication.

Trials with the second isolate (CA 251) of C. albicans showed a similar, if less striking reduction in the numbers of cells surviving the middle ranges of RH.

**Qualitative.** In these studies, survival was recorded only as present or absent. Results obtained with 20 additional isolates of C. albicans are shown in Table 2.

These data are consistent with those presented in the quantitative study (Table 1). Only 6 out of 20 isolates survived exposure to 60% RH for 7 days: in all but one of these, survival was represented by development of a single colony on one of the two duplicate test discs. It, therefore, seems evident that susceptibility of blastospores to 60% RH is common to all isolates of C. albicans tested.

Results obtained with other yeasts are presented in Table 3.

**DISCUSSION**

Most terrestrial fungi grow in damp habitats. A high water content in the substrate is almost a prerequisite for establishment of fungal growth, and, as a rule, dispersion spores are not produced unless the surrounding atmosphere has a relative humidity approaching 100%. Studies on the relationship between fungi and relative humidity have focused largely on the control of spore production and germination, usually by plant pathogenic species (3, 7). Few specific investigations have been made on the effects of RH on the vegetative stages of fungal growth, and these have concentrated almost exclusively on molds associated with deterioration in storage of economically important materials (5, 10). Fungi causing systemic infections in man are almost always acquired from an exogenous source and, although likely to experience wide ranges of humidity in their natural environments, few laboratory studies have been made on their survival at reduced RH. It has, however, been shown that the mycelial phase of Histoplasma capsulatum is incapable of growth below 98% RH, in contrast to Sporothrix (Sporotrichum) schenckii, which can grow at 93% RH (9). In another study (4), arthrospores of Coccioidoides immitis maintained at 50% RH showed little reduction in viability although a marked loss was noted at low RH and elevated temperatures (37 C).

The findings presented in this report indicate that vegetative cells of C. albicans are adversely affected by levels of relative humidity below 100%. In this respect, the findings are consistent with the conclusion reached by Marples (8) although relationships between decreasing RH and cell death are evidently more complex than previously reported.

In this study, death of the cells was equated with their inability to develop colonies when transferred to the surface of a nutrient medium. The qualitative studies, involving cell suspensions containing about 10⁴ to 10⁶ times the number of cells used in the quantitative membrane filter experiments, also showed that, whereas extremes of RH (10 and 100%) were relatively innocuous to blastospores of C. albicans, mid-ranges of humidity were lethal. This effect was noted not only when the RH was controlled with glycerin but also when potassium hydroxide solutions were used (11). It is therefore considered possible that the observed lethality could be a function of the RH itself, rather than an antimicrobial effect of glycerin, such as that which has been reported against Serratia marcescens and E. coli at intermediate levels of RH (12).

Microscopic examination of cells maintained for 2 to 3 days at 60% RH showed some morphological changes when compared to cells kept at 100% RH. Individual cell outlines were somewhat irregular, and the cell contents were poorly differentiated. Vacuoles were absent and cytoplasm was divided into irregular, coarsely reticular masses. When examined at 24 or 48 hr, some cells could be observed resembling those in the original suspension or those maintained at 100% RH.

It is of interest that cells of C. albicans can survive better at 10% RH than at 60% RH. The ability of C. albicans to form chlamydospores did suggest a mechanism which could account for this finding, but chlamydospores were not seen in cells maintained at any RH. In examining blastospores exposed to 10% RH for 3 days on glass slides, no morphological differences could be recognized when compared to those maintained in a saturated atmosphere. With the passage of time, no change in the cytomorphology of surviving cells could be observed. The possible existence of a physiological equivalent of the chlamydospore can be proposed but not substantiated although it would provide an attractive explanation for the findings. In this context, the marked delay between transfer of cells maintained at low RH values to the surface of culture media and appearance of colonies is noteworthy and may be attributable to metabolic readjustments of the cells to rehydration. If the rate of severity of desiccation stress, rather than its duration, is the major factor promoting deployment of endogenous safety mechanisms, this might account for the failure of cells maintained at 60% RH to switch to protection-oriented
metabolism. This concept is speculative, however, and is unsupported by any other reported data, or by microscopic observations of the cells in the current studies.

The reported experiments were devised without any major consideration of the rate at which equilibrium of the atmospheric water vapor was achieved within the RH chamber nor was attention paid to the rate of hydration of cells after removal from the chamber. It cannot be denied that either or both of these factors could influence the survival of C. albicans. It is, nevertheless, of interest that these same factors apparently played a less significant role in determining the susceptibility of yeasts other than C. albicans.

The demonstration that cell death occurred in 14 of 20 isolates of C. albicans maintained at 60% RH for 7 days shows that the phenomenon is common within this species. Only the single isolate of C. brumpti tested and 1 out of 20 isolates of Cryptococcus neoformans reacted in the same manner. It should be noted that this strain of C. neoformans also differed from other test isolates by its very slow growth although it is not known whether this observation is correlated with its susceptibility to desiccation. The present studies were concerned solely with the survival of individual yeast cells under conditions of reduced atmospheric moisture, and were initiated to substantiate and extend the earlier observations by Marples (8). It is of interest and perhaps of significance that cells of C. albicans are killed by relatively short exposure to levels of moderate humidity, whereas those of C. neoformans are not. In nature, cells of C. albicans exist endogenously within vertebrate hosts at anatomical sites which are at or near total saturation. In contrast, cells of C. neoformans, in common with the other species of Candida and Cryptococcus tested, often occur saprophytically, where it could be anticipated that they would be subjected to conditions of varying RH. The ability to withstand fluctuating humidity might be more readily associated with organisms liable to experience such conditions than with organisms adapted to environments where the RH approaches 100%.

In view of the close phylogenetic relationships presumed to exist between C. albicans and C. tropicalis, it is noteworthy that, whereas all 20 isolates of the former species are affected by mid-ranges of RH, none of the 11 isolates of C. tropicalis was killed. It is not anticipated that an RH-susceptibility test could afford a ready means of differentiating between these two species but there may be some value in exploring the relationships between RH or survival of filamentous strains of C. albicans and C. tropicalis, which have been considered by some authors as co-variants of the same species (1).

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