Urease Inhibition by EDTA in the Two Varieties of Cryptococcus neoformans

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Cryptococcus neoformans var. neoformans (74 isolates) and C. neoformans var. gattii (44 isolates) were used to test urease activity after growth on both yeast extract-glucose-peptone agar (YEPG) and on YEPG supplemented with 100 μM EDTA. Every isolate grown on YEPG agar for 48 h at 30°C produced a positive reaction within 1 h in a modified rapid urease assay at 37°C. However, isolates grown on YEPG with 100 μM EDTA showed a distinct pattern which corresponded to their varietal status. All but 1 of 74 C. neoformans var. neoformans isolates (98.7%) produced a positive reaction within 1 to 4 h, while none of 44 C. neoformans var. gattii isolates produced a positive reaction within the same period. The urease inhibition results and the casevanine-glycine-bromthymol blue agar test results showed 100% correlation among isolates of C. neoformans var. gattii and 98.7% correlation among isolates of C. neoformans var. neoformans. Two representative isolates of C. neoformans var. gattii (serotypes B and C) were further tested for urease during a prolonged incubation period in urea broth. These isolates failed to show a positive reaction even after 11 h of incubation. The uptake of EDTA was negligible in the two varieties. Extracts of cells grown on YEPG agar showed a high level of urease activity in both varieties. Extracts of cells grown on the agar with 100 μM EDTA showed a marked reduction (86%) of urease activity in one isolate of C. neoformans var. gattii but showed only a 30% reduction in one isolate of C. neoformans var. neoformans. Based on these results, the differential effect of EDTA on the two varieties of C. neoformans appeared to be due to greater inhibition of urease synthesis in C. neoformans var. gattii.

Isolates of Cryptococcus neoformans are divided into two varieties, C. neoformans var. neoformans and C. neoformans var. gattii. These two varieties of C. neoformans are different in morphology in vivo, physiology, serology, ecology, epidemiology, and genetics (1, 4–6). Presently, two diagnostic media, casevanine-glycine-bromthymol blue (CGB) agar and cycloheximide-glycine-phenol red agar, are available to distinguish between these two varieties. These media enable a differentiation of C. neoformans isolates by showing whether the isolates can grow on glycine medium containing either casevanine or cycloheximide. The efficacy of these media was compared by Kwon-Chung et al. (8) in 1982.

Urease activity is one of the most important biochemical properties for members of the genus Cryptococcus and basidiomycetous yeasts in general (3). The characteristics of the enzyme in these yeasts, however, have not been studied. In view of the significant biochemical differences between the two varieties of C. neoformans, the nature of their urease activity might also be different.

Booth and Vishniac (Can. J. Microbiol., in press) recently observed that urease activity, when tested with rapid urea broth of the type isolates of Filobasidiella neoformans var. neoformans (teleomorph of C. neoformans var. neoformans) and Filobasidiella neoformans var. bacillispora (teleomorph of C. neoformans var. gattii), was different after cells were grown on a medium supplemented with EDTA. In this study, we expanded their work, using a modified rapid urea broth, by testing numerous isolates of the two varieties of C. neoformans. After growth on a chelated medium, the urease activity of the isolates of C. neoformans var. gattii was suppressed, while that of isolates of C. neoformans var. neoformans variety was not. This phenomenon is another biological distinction between the two varieties of C. neoformans.

MATERIALS AND METHODS

Isolates and growth conditions. A total of 74 isolates of C. neoformans var. neoformans and 44 isolates of C. neoformans var. gattii were used for the study (Table 1). Of 74 isolates of C. neoformans var. neoformans, 72 isolates were from clinical or natural sources and the remaining 2 isolates (B-3501 and B-3502) were the progeny obtained by crossing a clinical isolate with an isolate from pigeon droppings. All 44 isolates of C. neoformans var. gattii were from clinical sources. Before the study, the isolates were grown on malt extract agar or Sabouraud agar slants for 48 h at 30°C. A loopful of cells obtained from the 48-h slant cultures was streaked on glucose-peptone-yeast extract agar (YEPE) or YEPG with 100 μM EDTA (YEPGE). The cells were then incubated for 48 h at 30°C and tested for urease as described below. The YEPE agar was prepared with yeast extract (3 g), peptone (5 g), glucose (5 g), and agar (18 g) in 1 liter of distilled water. For the YEPGE agar, 100 μM EDTA (0.034 g/liter) was added. The agar medium was dispensed into test tubes (18 by 150 mm; 10 ml per tube), autoclaved, and formed into slants. The pH of this medium varied from 6.5 to 6.7.

All isolates were streaked from malt extract agar or Sabouraud agar onto CGB agar plates and also were tested for serotypes by the previously described agglutination test (5).

Urease test. The rapid urea broth (RUH broth) developed by Roberts (11) was modified for this study. The modified RUH was prepared in 2× concentration with urea (4.0 g), yeast extract (0.02 g), phenol red (2.0 mg), KH2PO4 (0.273
TABLE 1. Urease test on isolates of C. neoformans grown in the presence of 100 \( \mu \)M EDTA (agar medium)

<table>
<thead>
<tr>
<th>C. neoformans isolates</th>
<th>No. of isolates positive at different incubation times (h)</th>
<th>% Positive:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4</td>
<td>At 4 h in RUH broth</td>
</tr>
<tr>
<td>var. neoformans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serotype A</td>
<td>18/61 56/61 61/61 61/61</td>
<td>100.0</td>
</tr>
<tr>
<td>Serotype D</td>
<td>4/13 10/13 12/13 12/13</td>
<td>92.3</td>
</tr>
<tr>
<td>var. gattii</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serotype B</td>
<td>0/30 0/30 0/30 0/30</td>
<td>0</td>
</tr>
<tr>
<td>Serotype C</td>
<td>0/14 0/14 0/14 0/14</td>
<td>0</td>
</tr>
</tbody>
</table>

* Test contained at least 10^6 cells per ml of modified RUH broth.

and Na\(_2\)HPO\(_4\) (0.285 g). The ingredients were dissolved in 100 ml of distilled water and then filter sterilized. The pH of this RUH broth was 6.8. To test the urease activity, a loopful of yeast cells grown for 48 h at 30°C on YEPG or YEPGE agar was suspended in 2 ml of sterile distilled water. The \( A_{500} \) of the cell suspension measured with a spectrophotometer (Ultraspec; LKB Instruments, Inc., Gaithersburg, Md.) was 0.8 to 1.0; the cell count varied from 1 \( \times \) 10^8 to 2 \( \times \) 10^9/ml. The cell suspension was vortexed, and 1 ml of the suspension was added to 1 ml of 2\( \times \) RUH broth. The RUH broth and the cell suspension were kept in an ice bath until they were combined. The RUH broth culture was then placed in a 37°C shaking water bath (40 oscillations per min). The tube was read every hour for 4 h. Magneta red color was considered a positive reaction, and orangish yellow color was considered a negative reaction. An unclear or borderline reaction resulting in orangish color was read with a spectrophotometer by the procedure described below. Cells were centrifuged for 10 min at 1,500 \( \times \) g. The supernatant (1 ml) was then removed with a Pasteur pipette and was mixed with 1 ml of distilled water in a cuvette. The \( A_{500} \) of the mixture was read against a blank cuvette containing RUH broth (1 ml of 1\( \times \) RUH broth mixed with 1 ml of distilled water). All readings over 0.3 manifesting a color of magenta red or orange-pink were considered positive reactions.

**Effect of EDTA on growth rate.** YEPG broth containing various concentrations of EDTA was used to determine the growth rate of one isolate from each variety of C. neoformans (B-3501 and NIH 191). Seed cultures were grown overnight in YEPG broth, and 0.25 ml of the cultures was transferred into Coleman spectrophotometer tubes containing 4.75 ml of fresh YEPG broth with or without EDTA. The tubes were incubated at 30°C on a roller drum. The initial \( A_{500} \) of the cultures varied from 0.03 to 0.05 as measured with a Coleman spectrophotometer (model 6120). Absorbancy of the culture was measured every 60 min.

**Uptake of radiolabeled ETA.** Ethylenediaminetetra(2\(^{-}\)14\textsuperscript{C})acetic acid, sodium salt (specific activity, 48 mCi/mmole; Amersham Corp., Arlington Heights, Ill.) was used for the uptake test. One isolate of C. neoformans var. neoformans (B-3501) and one isolate of C. neoformans var. gattii (NIH 191) were grown overnight in 100 ml of YEPG broth. A 5-ml sample of each culture was transferred into 1,000 ml of fresh broth and incubated on a 30°C shaker (160 rpm) for 4 h. Cells in the exponential growth phase were harvested by centrifugation, washed three times with glucose-ammonium broth (2), and resuspended in glucose-ammonium broth (10^6 cells per ml). Samples of 2 ml were dispensed into five 25-ml flasks. To one sample, Formalin (1%) was added and stirred for 1 h at 25°C. Cells were washed three times with 15 ml of distilled water before being suspended into the original volume in a glucose-ammonium broth. This sample was used as one of the controls. The other control was a sample which was incubated on ice throughout the test. The Formalin-treated control and the three remaining flasks were placed in a 30°C shaking water bath (10 oscillations per min), and 1 \( \mu \)Ci of radiolabeled ETA was added to all five flasks. Pulsed cells were incubated for 1, 3, and 5 min before the uptake was terminated by the addition of cold glucose-ammonium broth. The cells were spun for 10 min at 1,500 \( \times \) g (4°C) and were washed two times with cold glucose-ammonium broth and suspended in 0.5 ml of saline with azide (0.1%). A 0.5-ml portion of each sample was added to 20 ml of Aquosol (New England Nuclear Corp., Boston, Mass.) and counted with a scintillation counter (Packard 2000; Packard Instrument Co., Inc., Downers Grove, Ill.).

**Cell extracts and ammonia assay.** The cells of B-3501 and NIH 191 grown on YEPG or YEPGE agar media for 48 h at 30°C were harvested, washed with buffered saline, and divided into two aliquots. One portion was vortexed with glass beads to fracture the cells and obtain cell extracts as described previously (10). The other portion was suspended in 1 liter of RUH broth (\( A_{500} \) = 0.8) and incubated overnight. The cells were collected and fractured by the glass bead method in the same way as the other sample. After the breakage, the supernatant was collected by centrifugation at 3,000 \( \times \) g for 10 min to remove the beads and large particles. The supernatant was further centrifuged at 16,600 \( \times \) g at 4°C for 15 min to obtain cell wall-free extracts. Protein concentration in the extracts was estimated by the use of BCA (protein assay reagent; Pierce Chemical Co., Rockford, Ill.) with a bovine serum albumin standard.

The ammonia assay was performed by a Conway diffusion dish method (7). The protein concentration of cell extracts was adjusted to 400 \( \mu \)g/ml with distilled water; the concentration of aqueous urea used was 1 M. Falcon tubes (5 ml) containing 0.5 ml of cell extract, 0.1 ml of urea solution, and 1.4 ml of distilled water were incubated for 4 h in a 37°C water bath. Control tubes were prepared the same way but excluding the cell extract. The reaction mixture was transferred to Conway dishes. Ammonia was released into the gaseous phase by K\(_2\)CO\(_3\) and trapped in standard HCl solutions. NH\(_3\) was determined by back titration with NaOH and methyl red indicator (13). Urease activity was expressed as micromolar NH\(_3\) per hour per milligram of protein.

**RESULTS**

**Urease activity.** When the isolates were tested in the modified RUH broth after growth on YEPG agar at 30°C for 48 h, they showed urease activity within 1 h regardless of varietal status. However, the isolates grown on YEPGE agar showed a distinct pattern which corresponded to varietal status. Table 1 shows that all but 1 of 74 C. neoformans var. neoformans isolates produced positive urease activity within a 1- to 3-h incubation period at 37°C. However, none of 44 C. neoformans var. gattii isolates produced a positive urease reaction during the same period. In addition, two type cultures of F. neoformans var. bacillispora (anamorph, C. neoformans var. gattii NIH 191 [serotype C, \( \alpha \) mating type]) and NIH 444 [serotype B, \( \alpha \) mating type], tested under the same conditions remained negative even at 11 h (test discontinued after 11 h). A positive reaction of isolates in the urea broth was manifested by color changes from orangish yellow to red or magenta. Spectrophotometric determination of the
TABLE 2. Inhibition of urease activity in the cultures grown on an agar medium with EDTA and the recovery of the activity after 17-h incubation in RUH broth

<table>
<thead>
<tr>
<th>Strain</th>
<th>Urease activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before RUH broth</td>
</tr>
<tr>
<td>C. neoformans var. neoformans</td>
<td></td>
</tr>
<tr>
<td>(B-3501)</td>
<td></td>
</tr>
<tr>
<td>No EDTA</td>
<td>115</td>
</tr>
<tr>
<td>EDTA (100 µM)</td>
<td>80</td>
</tr>
<tr>
<td>C. neoformans var. gattii</td>
<td></td>
</tr>
<tr>
<td>(NIH 191)</td>
<td></td>
</tr>
<tr>
<td>No EDTA</td>
<td>87</td>
</tr>
<tr>
<td>EDTA (100 µM)</td>
<td>12</td>
</tr>
</tbody>
</table>

*a Cell extracts were incubated with urea for 4 h (see Materials and Methods).

** Methods.

positive reaction had an A956 ≥ 0.3. Absorbancy of the urea broth supernatant collected from C. neoformans var. neoformans cultures after a 4-h incubation ranged from 0.4 to 0.9, except for one isolate (0.03) that gave a negative reaction. Absorbancy of the urea broth supernatant collected from C. neoformans var. gattii cultures ranged from 0.02 to 0.2. Results of the urease test and the CGB reaction showed remarkable correlation: 100% of the isolates of C. neoformans var. gattii, and 98.7% of the isolates of C. neoformans var. neoformans. The one isolate of C. neoformans var. neoformans which did not conform to the test was neither a clinical nor a natural isolate but the result of a genetic cross in the laboratory.

Growth rate of B-3501 and NIH 191 in the presence of EDTA. Representative isolates of the two varieties of C. neoformans grown in YEPEG broth at 30°C showed that resistance toward EDTA was different in both isolates. The isolate B-3501 (C. neoformans var. neoformans) grew well in the presence of 100 µM EDTA, showing only a 6-min prolongation of generation time as compared with the control. In the presence of 600 µM EDTA, the isolate still grew well but showed a 34-min-longer generation time than the control. The isolate NIH 191 (C. neoformans var. gattii) was more sensitive than B-3501 to EDTA. In the presence of 100 and 600 µM EDTA, the generation time of isolate NIH 191 increased by 25 min and 2.5 h, respectively. The growth rate of the two isolates on YEPEG slants with 100 µM EDTA, however, was not noticeably different.

Uptake of radiolabeled EDTA. The radiolabeled EDTA that accumulated in the cells after a 5-min incubation was negligible in both B-3501 and NIH 191 (data not shown). The count showed that less than 100 pM EDTA accumulated in 10^6 cells of NIH 191 in 5 min and that less than 5 pM accumulated in a similar number of B-3501 cells.

Urease activity of cell extracts. Urease activity of the cell extracts obtained from both B-3501 and NIH 191 cultures grown on YEPEG agar showed no effect when EDTA was added to the reaction mixture (data not shown). The addition of 100 µM EDTA to YEPEG agar inhibited enzyme activity in cell extracts of both isolates but to different degrees. The urease activity of B-3501 grown in the presence of EDTA showed 30% inhibition compared with that of the control. The enzyme activity of NIH 191, however, showed 86% inhibition compared with the control (Table 2). The enzyme activity of the cell extracts obtained after the cells were removed from YEPEG agar and incubated in the RUH broth for 17 h showed complete recovery in B-3501 but only 50% recovery in NIH 191 (Table 2).

DISCUSSION

Urease is an enzyme that commonly occurs in the basidiomycetous yeasts (3). The enzyme activity is readily detected by various methods performed routinely in clinical laboratories for diagnostic purposes (10). Booth and Vishniac (in press) observed that if the isolate was first grown on chelated agar medium and then tested with RUH broth, the urease activity of the type isolate of F. neoformans var. neoformans was substantially higher than that of F. neoformans var. bacillispora. The present study with 74 isolates of C. neoformans var. neoformans and 44 isolates of C. neoformans var. gattii revealed that the urease activity of the two varieties, after growth on chelating medium, was significantly different. This biochemical phenomenon is another example of the biological distinctiveness of the two varieties.

Booth and Vishniac found that the cation Ni^{2+} (but not others), when incorporated into the YEPEG agar, eliminated the inhibitory effect of EDTA in the type isolates of F. neoformans var. bacillispora. Our observation supported their results (data not shown). This suggests that either urease activity was strengthened by the inclusion of nickel or that nickel blocked the EDTA effect by chelation. Association of nickel with urease has been known in some plants. The urease from jack bean was found to contain bound nickel (9). Booth and Vishniac also found that the Ni^{2+} transport system was different in both varieties of the C. neoformans isolate (J. L. Booth and H. S. Vishniac, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, K88, p. 217). The type isolate of F. neoformans var. neoformans exhibited a significantly greater affinity for Ni^{2+} than did the type isolate of F. neoformans var. bacillispora. Additional isolates of the two varieties need to be studied before the phenomenon can be attributed to a varietal difference.

In the present study with the cell extract, it was clear that EDTA had a different effect on the urease of the two varieties of C. neoformans. Cell extracts from one culture of C. neoformans var. gattii (NIH 191) grown on YEPEG agar with 100 µM EDTA showed an 86% reduction in urease activity. However, it appears that the urease in isolates of C. neoformans var. neoformans was more resistant to EDTA since only a 30% reduction was seen in the activity of B-3501. Complete restoration of urease activity was seen in B-3501 during incubation of cells in RUH broth, while only a 50% restoration was seen in NIH 191. Altering the incubation temperature to 30°C did not make any difference (data not shown).

The urease inhibition phenomenon can be used as another diagnostic criterion for varietal identification of C. neoformans isolates. Normally, CGB reaction is sufficient for the separation of the two varieties. However, since L-canavanine may not be readily obtained in certain countries, there may be a need to employ alternate biochemical methods for the varietal diagnosis.

The method is easy and inexpensive to perform. RUH broth used in this technique can be stored in a refrigerator for up to a month without losing efficacy. When we tested the original RUH broth used by Booth and Vishniac, we found that it produced 10 to 20% false-positives from isolates of C. neoformans var. gattii. The modified RUH broth used in our study contained 2.01 x 10^{-2} M phosphate instead of 1.34 x 10^{-2} M. This change eliminated false-positives and resulted in specific reactions in 1 to 3 h. Cell concentration in
the RUH broth was another important factor in obtaining a specific reaction in the shortest amount of time. When the RUH test system was initiated with $A_{600} \leq 0.5$, a positive result was not observed in many isolates until 4 h, whereas it took only 1 h when the $A_{600}$ was 0.8 to 1.0. When the RUH broth system was converted to the agar system, results were ambiguous and differentiation of the two varieties was not possible. The results with most isolates were visually clear, so spectrophotometric reading was unnecessary. Only 5% of isolates produced borderline results (orange, orange-pink) and required spectrophotometric reading. Since borderline results can be produced by having insufficient cells in the RUH broth, it is recommended that the tests be repeated with a loopful of cells. It is important that the supernatant collected from RUH broth culture for spectrophotometric testing is free from cells. The turbidity resulting from the particles gives erroneous readings.

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LITERATURE CITED