Inhibition of *Histoplasma capsulatum* by *Candida albicans* and Other Yeasts on Sabouraud's Agar Media

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The inhibition of growth of *Histoplasma capsulatum* by *Candida albicans* and other yeasts on Sabouraud's agar was investigated. *Histoplasma* (yeast-phase inoculum) was grown alone and in mixtures with yeasts at 25°C for 4-week periods. As few as 10 colonies of *C. albicans* completely inhibited the growth of approximately 50,000 potential colonies of *Histoplasma*. The pH was determined in cultures of 36 colonies of *Candida* on media containing 1, 2, and 4% glucose by spotting the agar with pH indicators. A drop in the pH became noticeable in all three media about the 3rd day of incubation, and a pH of 3.5 was reached in about 7 days. Subsequently, the pH remained almost stationary in the 4% glucose-agar, rose slowly in the 2% glucose-agar, and rose sharply in the 1% glucose-agar. The growth of *Histoplasma* was inhibited completely at pH 4 and below. When the pH was controlled in mixed cultures, some growth of *Histoplasma* was obtained. Substitution of maltose for glucose delayed the development of acidity and allowed the appearance of numerous mycelial colonies in the presence of *Candida*. This growth was arrested as soon as the medium became acid. Four other species which also acidified the Sabouraud's medium effected similar inhibition. It was thus shown that severe and prolonged acidity produced by some yeasts in the sugar-rich Sabouraud's media is alone sufficient to completely inhibit *Histoplasma* during the standard 4-week incubation of specimens such as sputum.

In diagnostic mycology, *Candida albicans* is frequently encountered in cultures of sputum (1, 2, 6, 13) and it has been found that specimens which are only barely positive on direct examination may contain as many as 10⁶ yeast cells per ml (8). The failure to isolate *Histoplasma capsulatum* from sputum of patients has been commented upon in several publications (3, 5, 12). In a survey of 130 publications dealing with the primary isolation of *H. capsulatum* between 1934 and 1966, it was found that in 57% of these Sabouraud's agar was used as a medium of primary isolation. The inhibition of *H. capsulatum* by *C. albicans* on Sabouraud's agar was reported previously (J. Burns and H. W. Larsh, Bacteriol. Proc., p. 66, 1962) and has also been observed repeatedly in our laboratory.

Since marked acidity is produced by yeasts in glucose-rich media (7, 17, 18), the development of acidity by yeasts in Sabouraud Dextrose Agar was investigated in the present study as a factor in the inhibition of *Histoplasma*, a fungus which requires an initial pH of 6.5 to 8.1 for optimal growth from yeast-phase inoculum (16). Preliminary results of this investigation were reported previously (L. Kapica, C. E. Shaw, and G. W. Bartlett, Bacteriol. Proc., p. 68, 1967).

**Materials and Methods**

**Media.** Commercial media used included Sabouraud Dextrose Agar (Difco), Sabouraud's agar modified (Difco), Mycosel Agar (BBL), Brain Heart Infusion Agar (Difco) to which 5% (v/v) sheep blood was added, and Brain Heart Infusion-blood-agar, as above, to which 1% (w/v) glucose was added. Ingredients per liter of other media prepared were as follows: beef extract-agar (beef extract, 3 g; Neopeptone, 10 g; cerelose, 10 g; sodium chloride, 5 g; agar, 20 g); McGill nutrient blood-agar (beef heart, defatted, infusion from 454 g; proteose peptone, 10 g; sodium chloride, 2.5 g; potassium chloride, 0.2 g; calcium chloride, 0.1 g; agar, 15 g; human blood, 50 ml); Neopeptone (Difco)-maltose- or glucose-agar (Neopeptone, 10 g; maltose or glucose, 40 g; agar, 20 g; autoclaved 50% maltose solution or filter-
sterilized 50% glucose solution added to a separately autoclaved Neopeptone-agar solution).

The final pH of all media except Sabouraud Dextrose Agar (pH 5.6) was pH 7.0 ± 0.5. Sterile media were cooled to 50°C and dispensed in 20-ml volumes into 250-ml Erlenmeyer flasks; they were used within 24 to 48 hr. All flasks were plugged with cotton wool.

Buffered Sabouraud Dextrose Agar and Sabouraud's agar modified were prepared by using 0.1 M phosphate buffer as a solvent or by adding a separately autoclaved suspension of CaCO₃ to the media just before distribution. Final concentration of the CaCO₃ was 0.5% and the pH was 7.3. The pH of the Neopeptone-glucose-agar was adjusted to several acid pH levels by adding precalculated volumes of 3.5 M HCl to sterile samples of the autoclaved medium cooled to 50°C. The medium was then stirred thoroughly and dispensed at once.

Strains tested. A strain of H. capsulatum, no. 649, isolated from a case of pulmonary histoplasmosis at the Royal Victoria Hospital in Montreal in 1965, was used throughout the experiments. Four strains isolated from recent cases of histoplasmosis at different hospitals in Montreal, one strain obtained from the American Type Culture Collection, and a mixture of six strains were used for comparative studies. Stock cultures were maintained in yeast phase at 37°C on sealed slopes of McGill nutrient blood-agar. The strain of C. albicans used throughout the study was isolated from a culture of a specimen of bronchial aspirations in which it was observed to be inhibitory to H. capsulatum. For comparative studies, six other strains of C. albicans isolated from clinical specimens and seven other species of yeast including C. parapsilosis, C. tropicalis, C. curvata, C. brumptii, C. cattenuata, Cryptococcus neoformans, and Saccharomyces cerevisiae were used. Stock cultures were maintained on Sabouraud's agar modified at 4°C.

Inoculum and inoculating procedure. Yeast-phase H. capsulatum was grown on sealed slopes of McGill nutrient blood-agar at 37°C for 2 days, harvested with a loop into 10 ml of saline, and filtered through glass wool to remove large aggregates of cells. The density of this stock suspension was read on a model 7 Coleman photo-nephelometer (Standard 21) and was adjusted to give a reading between 25 and 50. One ml of a 10⁻³ dilution of stock suspension was mixed with 1 ml of 0.7% agar at 45 to 50°C; 1 ml of this agar suspension was used to inoculate one flask. A thinly confluent growth was obtained in which individual colonies could no longer be discerned. These dilutions were estimated by serial platings on Sabouraud's agar modified to produce 5,000 to 7,000 colonies per flask. Microscopic examinations of the stock suspensions showed that the majority of the infective units were in the form of small clumps of two to three cells with a good proportion of single cells; larger clumps were occasionally encountered. C. albicans and other yeasts were grown on Sabouraud's agar modified for 2 days; inoculum was prepared in the same manner as that of H. capsulatum. A 0.5-ml amount of a 10⁻³ dilution produced the desired number of colonies, about 35 per flask. Careful readjustment in the preparation of the dilutions resulted on most occasions in closely reproducible numbers; however, extra units were usually prepared to allow for elimination of those carrying unsuitable numbers of colonies. Microscopic examinations of the stock suspensions showed that single and budding cells only were present.

Mixed populations were prepared by combining equal volumes of double-strength saline suspensions of the two organisms just prior to mixing with the agar. All tests were set up in triplicate. After 1 to 2 hr on the bench, the flasks were placed in a 25°C incubator for a standard 4-week incubation. The cultures were examined once a week.

In some experiments, mycelial-phase inoculum of H. capsulatum was required. This was prepared by placing sterile analytical filter paper rings (outside diameter, 2 cm; inside diameter, 1 cm), manufactured by Carl Schleicher & Schuell Co., Keene, N.H., on plates of Neopeptone-glucose-agar. The rings adhered well to the surface moisture of the agar. The plates were then flooded with a double volume of standard yeast-phase inoculum and were incubated for 3 days at 25°C. The rings were then transferred with sterile forceps, without damage, to the test media and placed with the surface bearing inoculum in an upright position.

Determination of pH in agar media. Since agar pH cannot be measured with electrodes, bromocresol purple (pH 6.8 to 5.2), bromocresol green (pH 5.4 to 3.8), and bromophenol blue (pH 4.5 to 3.0) were used. All three were nontoxic for Candida, but only bromocresol purple was nontoxic for Histoplasma. pH determinations were made by incorporating indicator in the medium at a final concentration of 0.002% or by spotting the agar with drops of 0.04% solutions of indicator. The color of the spots was read against similarly made spots on standards. (Standards were prepared by acidifying lots of Neopeptone-glucose-agar to 12 pH levels between pH 3.0 and 6.5 and distributing the agar in 20-ml portions to flasks in the routine manner.) Standards were used because colors obtained at a given pH did not correspond precisely with those quoted by the Merck Index, probably because of the organic nature of the medium. Shades of colors read consistently by the same person differed sufficiently to allow, in most cases, the determination of pH differences of as little as 0.25 of a unit.

RESULTS

Lower limits of inhibition. Sabouraud Dextrose Agar was inoculated with mixed inocula containing the standard density of Histoplasma and decreasing densities of Candida (Table 1). As few as 10 colonies of Candida scattered over the agar surface completely inhibited the growth of Histoplasma. Fewer colonies allowed irregular pockets of atypical colonies to appear where the space between the yeast colonies was somewhat larger than that on the rest of the agar. It was also observed that the fewer the yeast colonies per flask the larger they grew, and it was calculated that scattered colonies of C. albicans were
TABLE 1. Lower limits of inhibition of Histoplasma capsulatum by Candida albicans

<table>
<thead>
<tr>
<th>No. of colonies/ flask</th>
<th>Colony diameter</th>
<th>Area occupied</th>
<th>Growth of H. capsulatum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>5.5</td>
<td>24</td>
<td>No growth</td>
</tr>
<tr>
<td>26</td>
<td>8.5</td>
<td>16</td>
<td>No growth</td>
</tr>
<tr>
<td>10</td>
<td>8.0</td>
<td>14</td>
<td>Poor, nonaerial</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>14</td>
<td>Poor, nonaerial</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>14</td>
<td>Abundant, typical</td>
</tr>
<tr>
<td>0</td>
<td>16</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

* Sabouraud Dextrose Agar, initial pH, 5.6; 28 days, 25 C.

completely inhibitory to Histoplasma when they occupied as little as 15 to 20% of the agar surface.

When increasingly heavy inocula of Histoplasma were tested against the lowest inhibitory numbers of C. albicans on Sabouraud Dextrose Agar, it was found that 10 scattered colonies of the yeast were completely inhibitory to about 50,000 (heaviest inoculum tested) potential colonies of Histoplasma.

It was observed that the nature of the inhibition was fungistatic rather than fungicidal. When agar blocks, apparently free of growth, were lifted after 2 weeks of incubation from among the Candida colonies and squashed on fresh slopes of Sabouraud Dextrose Agar, growth of Histoplasma appeared within a few days.

**pH changes in cultures of C. albicans on Neopeptone-glucose-agar.** In preliminary tests, Sabouraud Dextrose Agar, in which bromocresol purple indicator had been incorporated, was inoculated with pure and mixed populations of the two organisms. Since the pH of this medium was 5.6, the initial color of the medium was green. In all Candida-carrying units, yellow zones appeared around each colony of yeast 2 to 3 days after inoculation. These zones enlarged visibly at 5 days, and at 7 days the whole medium was yellow. In pure cultures of Histoplasma, the medium turned purple when growth appeared in about 10 to 14 days, indicating a rise in the pH.

Acid production by the yeasts was demonstrated by the dissolution of CaCO₃ (Fig. 1; 11). The buffering action of the carbonate visibly restricted the spread of acidity to narrow zones around the colonies. Whenever groups of colonies occurred, the zones coalesced to form transparent pools.

**pH changes produced by C. albicans over a 4-week period in Neopeptone-glucose-agar medium are presented in Fig. 2.** The greatest drop in the pH was observed in the 4% glucose medium (Sabouraud Dextrose Agar). By the 5th day of incubation, the pH had dropped to 3.75 to 4.0 and, by the 14th day, to 3.0 to 3.25. After that, a very slow rise occurred to pH 3.5. This medium thus remained at a pH below 4 for about 24 days of the 4-week period. In the 2% glucose medium (Sabouraud's agar modified), the pH curve dropped to its lowest point (pH 3.5 to 3.75) about the 12th day. It then began to rise and reached pH 4.75 at the end of the 4th week. In the 1% glucose medium, the first pH reading varied considerably according to the distance...
from the nearest colony (Fig. 2, broken line). The lowest point of this curve (pH 3.5) occurred at about the 10th day of incubation. After that, the curve rose sharply to the original level of pH 6.5.

Growth of Histoplasma on Neopeptone-glucose-agar at acid pH. Since the drop of pH in Neopeptone-glucose-agar had a lag phase of about 3 days or a period sufficiently long to allow a reversion of the yeast phase to mycelial phase, the effect of pH on a 3-day-old mycelial phase was determined. Flasks of Neopeptone-glucose-agar, adjusted to seven pH levels from 3.0 to 6.5, were inoculated with rings of 3-day-old mycelial-phase inoculum in one series and with the standard yeast-phase inoculum in another series. Table 2 shows the detrimental effect of pH 4.5 on growth from yeast-phase inoculum; at pH 4 and below, no growth occurred. The 3-day-old mycelial-phase inoculum exhibited some tolerance to acid pH. The growth in this series appeared first on the rings in the form of aerial tufts of mycelium (about 50 to 60 colonies per ring were counted under best conditions) which, when the pH was suitable, spread rapidly to the agar medium. At pH 4.5, growth, although still fairly vigorous on the rings, spread slowly to the agar. At pH 4, only a few tufts of mycelium appeared on the rings late during the incubation, and the hyphae did not grow onto the acid agar.

Effect of glucose concentration on inhibition. Flasks of Neopeptone-glucose-agar were prepared with five glucose concentrations between 0.1 and 4% and were inoculated with pure and mixed populations (Table 3). Whereas both organisms in pure population grew better at higher glucose concentrations, the degree of inhibition of Histoplasma in mixed populations was directly related to the glucose concentration. As the glucose concentration went down to 1% and less, colonies of Histoplasma began to appear depending on the distance from the nearest Candida colonies. In the last two series, growth of Histoplasma, although meager, appeared all over, in spite of the presence of Candida; tufts of aerial mycelium were actually observed on top of Candida colonies. In the two last series, growth of H. capsulatum in pure and mixed cultures in the absence of glucose showed that the quantity of growth did not appear to differ in the two situations, but the growth in mixed populations was somewhat stunted as compared to that in pure populations.

Effect of buffering on inhibition. Sabouraud Dextrose Agar and Sabouraud's agar modified were buffered with 0.1 M phosphate buffer at pH 7 and calcium carbonate at pH 7.3. The cultures were inoculated with the standard mixed and pure populations of the two species. (A rather high population of 77 colonies per flask of Candida was obtained in this experiment.) Bromocresol purple was incorporated in all units. The buffers reduced the duration of the acid phase in all cultures carrying the yeast, i.e., the media turned yellow later than under unbuffered conditions and became alkaline before the end of the experimental 4-week incubation, sooner in Sabouraud's agar modified than in Sabouraud Dextrose Agar. The carbonate buffer controlled the acidity more effectively than the phosphates; the opacity in these media disappeared almost completely in Sabouraud Dextrose Agar and in about 25% in Sabouraud's agar modified.

In mixed cultures, the buffers were only partially successful in counteracting the inhibition, the effect varying with the buffers and the media used. The phosphates failed completely in Sabouraud Dextrose Agar, i.e., no growth of Histoplasma appeared; in Sabouraud's agar modified, some growth which was atypical and nonaerial
appeared at the end of the 3rd week of incubation. Both carbonate-buffered media produced growth of Histoplasma, a little more abundantly in Sabouraud’s agar modified than in Sabouraud Dextrose Agar; this growth was also atypical. In pure cultures, both buffers affected adversely the growth of Histoplasma in quantity, quality, and time of appearance. The carbonate buffer did not affect Candida, but the phosphates did; the colonies appeared late and remained very small for about one week. This growth improved during the 3rd week and was normal at the end of the 4th week.

Inhibition on Neopeptone-maltose-agar. In pure populations, typical growth of Histoplasma appeared 3 days earlier and was more vigorous than on Sabouraud Dextrose Agar. Colonies of Candida were slow to appear both in pure and mixed populations. In mixed populations, pinpoint filamentous colonies of Histoplasma comparable to those in pure populations appeared in as little as 6 to 7 days over the entire agar, regardless of the presence of Candida. However, this growth failed to develop further and, at 28 days, close examination was still required to detect its presence on the medium. A further incubation period of 3 weeks showed no change.

When a pH curve analogous to those in Fig. 2 was constructed, it was found that, in this medium, C. albicans produced a drop in pH which was delayed by 2 to 3 days, as compared to that produced in Sabouraud Dextrose Agar. After 7 days of incubation, however, the maltose medium became and remained as acid as the 4% glucose medium.

Inhibition by other strains of C. albicans and other species of yeasts. Six other strains of C. albicans were paired against six other strains of H. capsulatum on Sabouraud Dextrose Agar, and total inhibition was observed in every case. Bromocresol purple included in the medium indicated a pH below 5.2 over the 4-week incubation period. Four other species of yeasts, C. tropicalis, C. parapsilosis, S. cerevisiae, and Cryptococcus neoformans, tested against H. capsulatum 649 on Sabouraud Dextrose Agar, also produced total inhibition. The yeast population varied from 21 to 62 colonies per flask. Bromocresol purple, included in the medium, indicated a pH below 5.2 in all cases except in cultures of C. parapsilosis, which became alkaline in the last days of incubation. Some yeasts were found (C. curvata, C. brumptii, and C. catenulata) which allowed stunted and atypical growth of Histoplasma to appear on Neopeptone-glucose (2%)-agar. Bromocresol purple indicator, incorporated in the medium, showed that the pH drop produced by these species was less severe than that produced by species which caused complete inhibition. Although the agar turned yellow in cultures of C. brumptii and C. catenulata, purple patches were left at the end of the 4th week; in the case of C. curvata, the agar did not turn yellow but remained green to yellow-green.

Growth of Histoplasma in the presence of C. albicans on other media. Sabouraud Dextrose Agar and Sabouraud’s agar modified served as control media and exhibited the usual inhibition. (These cultures were kept for another 4 weeks at 25 C. Toward the end of that period, i.e., after 8 weeks of incubation, a small amount of growth of Histoplasma appeared among the Candida colonies on Sabouraud’s agar modified but not on Sabouraud Dextrose Agar.) Mycosel Agar and beef extract-agar produced partially inhibited growth of Histoplasma which appeared late and was obviously reduced in quantity. On about the 10th day, the three blood-containing media produced a heavy but atypical growth of Histoplasma with very poorly developed aerial mycelium. Characteristically, the colonies appeared over the entire agar surface without any evidence of inhibition, and even grew underneath Candida colonies.

Discussion

The difficulty in obtaining growth of H. capsulatum in the presence of C. albicans and other yeasts on the two commercial brands of Sabouraud’s agar appears to be centered around the production by the yeast of an acid pH in the medium. The acid pH alone is strongly inhibitory to Histoplasma. This would agree with results by Garrison (4), who found that the oxidation of glucose by the yeast phase of H. capsulatum had a definite pH optimum of 7.0, whereas hydrogen ion concentrations below pH 4 were inhibitory for both endogenous respiration and glucose oxidation. Since the severity of the inhibition is directly related to the glucose concentration of the medium, detoxification of the medium presumably takes place once the sugar supply of the medium becomes exhausted, and subsequent utilization of organic acids begins. Thus, media containing low concentrations of glucose (0.1 to 1.0%) allowed some growth of Histoplasma within the 4-week incubation period, whereas media containing higher concentrations of glucose allowed growth only after 8 weeks of incubation (Sabouraud’s agar modified) or allowed no growth at all (Sabouraud Dextrose Agar). Although undoubtedly there are other factors which play a role in inhibition, it is conceivable that the prevailing low pH inactivates proteolytic enzymes of Histoplasma and, consequently, no visible growth occurs under conditions of nitrogen
stavation. That this supposition is probably correct was shown by the fact that the inhibitory state of the medium is fungistatic rather than fungicidal.

Since control of pH in Sabouraud's medium was only partially successful in reducing the inhibition, and since clinical specimens would probably contain fewer infective units of Histoplasma than the inoculum used in this study, it could be shown that chances of isolation of Histoplasma from yeast-carrying specimens would be small even if alterations were made in these media. Although prolonged incubation of cultures for 2 to 3 months may result in positive cultures, this technique is both unreliable and impractical. Existing naturally media, such as brain heart infusion-glucose-blood-agar (9, 10, 14, 15), grow Histoplasma in the presence of yeasts without apparent reduction in the number of colonies; therefore, it is strongly recommended that these media be used, rather than Sabouraud's, for the primary isolation of Histoplasma from specimens of the upper respiratory tract. The growth of Histoplasma on such media, although atypical, will become easily recognizable with experience. However, since other fungi may assume similar atypical morphology on these media, the suspected fungus must be transferred for positive identification to a medium on which Histoplasma, in pure culture, will exhibit typical growth and sporulation; for this purpose, the Sabouraud's media are most appropriate. In cases where colonies of Histoplasma are growing in close proximity to yeasts, purification of the fungus of yeasts will be necessary before transferring to Sabouraud's agar. This may be accomplished by intraperitoneal passage through mice or, in case of C. albicans, by the use of the biotin-free medium devised by Burns and Larsh (Bacteriol. Proc., p. 66, 1962).

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