Growth of *Histoplasma capsulatum*

VI. Maintenance of the Mycelial Phase

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A medium is described for the maintenance of the mycelial phases of *Histoplasma capsulatum* in their "original" condition of sporulation. A yeast phase medium is reemphasized, not only for conversion and support of the yeast phase, but as an indirect method of maintaining the mycelial characteristics as originally observed.

Stock cultures of the mycelial phase of *Histoplasma capsulatum* rapidly lose the ability to form both macroconidia and microconidia when maintained on Sabouraud's agar or other media commonly employed by the medical mycologist (2, 3, 6, 12). The presence of both types of spores is desired; the microconidium is the primary infectious particle of the mycelial phase (5) and is desired for experimental purposes, whereas the macroconidium serves as the principal diagnostic characteristic of the fungus (3, 6). At present there is no medium which has been completely satisfactory for maintenance of sporulation of this organism (2). The primary purpose of this paper is to report a medium for the maintenance of the mycelial phases in their "original" condition of sporulation; the secondary purpose is to reemphasize the usefulness of the yeast phase medium, not only for conversion and support of the yeast, but also as an indirect method of maintaining the mycelial characteristics as grossly observed.

In 1957 I described a minimal vitamin (MV) medium for maintaining the yeast phase of *Histoplasma capsulatum* (11). Dependent upon the strain of yeast phase used, depleting the medium of one or more of the essential vitamins resulted in either a decreased rate of growth, failure to grow, or, in some instances, a conversion of the yeast phase to the mycelial phase at 37 C. Subsequently, using this minimal medium as a base, the factors required for the conversion of the mycelial phase to the yeast phase were studied (15). The results showed that growth of the yeast phase was greatly stimulated by the presence of citric acid and that the presence of Ca and Mg ions reversed the effect of citric acid, thereby stimulating the growth of the mycelial phase. However, yeast phase was also shown to metabolize the citrate and α-ketoglutarate with a resultant shortening of generation time. Zn ion was also stimulatory to the yeast phase. The combined results of the growth studies were used to modify MV medium to obtain a yeast phase medium which gave excellent growth of the yeast phase and on which viability was maintained for approximately 6 months when stored at 5 C. The yeast phase medium was described by Pine and Drouhet (14), who tested its ability to convert the mycelial phases of *H. capsulatum* and *H. duboisii* to the yeast phase. For maintaining the pure yeast phase, it was superior to other media tested although it did not convert as many strains to the yeast phase as did the Kurong-Yegian inspissated egg medium (8).

The original MV medium was also excellent for maintaining high sporulation in the mycelial phase. During the 17 years of study of the various strains of *Histoplasma*, mycelial phases were maintained on Sabouraud's agar and on MV medium or on the mycelial medium described here. However, the mycelial phases lost their ability to sporulate within several passages on Sabouraud's agar, and cultures preserved in soil or under oil (15) rapidly lost their viability. When first received, the mycelial phases of the organisms were also converted to the yeast phase either by animal passage or by transfer to a conversion medium, and were maintained on blood agar cystine medium of Campbell (4). Subsequent to their formulation, the MV medium or the yeast phase medium was used to maintain the yeast phase. The regeneration of new mycelial stocks from the yeast phases was done by transferring the yeast phase cultures to the mycelial phase medium and incubating them at 25 C for several weeks. Examination of these cultures, comparison of their characteristics with the dead mycelial cultures kept under oil, and correlation of the data of the stock record books and previous publications showed results described in this report.


**TABLE 1. Agar medium for the growth and maintenance of the mycelial phase of Histoplasma capsulatum**

**Part 1. Casein hydrolysate-vitamin-glucose base**

| Steps | Solution (salts, in grams): |
|-------|----------------------------|
| 1.    | Solution I (salts, in grams): |
|       | KH₂PO₄ 8.0                  |
|       | (NH₄)₂SO₄ 8.0              |
|       | MgSO₄.7H₂O 0.86             |
|       | CaCl₂ (anhydrous) 0.08      |
|       | ZnSO₄ 0.05                 |

Dissolve the above in 500 ml and make to 1 liter with distilled water; store at 5 C; add 250 ml/liter of medium.

| Steps | Solution II (minor elements, in grams): |
|-------|-----------------------------------------|
| 2.    | FeSO₄.7H₂O 5.7                           |
|       | MnCl₂.6H₂O 0.8                           |
|       | NaMoO₄.2H₂O 0.15                         |

Store at 5 C; good indefinitely or until red precipitate forms; add 10 ml/liter.

| Steps | Solution III: casein hydrolysate* 10% acid hydrolyzed-vitamin-free solution; add 40 ml/liter. |
|-------|-------------------------------------------------------------------------------------------------|
| 3.    | Inositol 200                                                                                  |
|       | Thiamine hydrochloride 200                                                                      |
|       | Calcium pantothenate 200                                                                      |
|       | Riboflavin 200                                                                                 |
|       | Nicotinamide 100                                                                               |
|       | Biotin 10                                                                                     |

Suspend and make to 1 liter in distilled water; store at 5 C; suspension is good for 6 months to 1 year; add 10 ml/liter.

| Steps | Solution V (hemin): suspend 200 mg of hemin in 10 ml of distilled water and bring into solution by the addition of a few drops of concentrated ammonium hydroxide; final volume is made to 100 ml; store at 5 C; add 1 ml/liter. |
|-------|-------------------------------------------------------------------------------------------------|

| Steps | Solution VI (thiolic acid): dissolve 10 mg of DL-Thiolic acid in 10 ml of 95% alcohol; store in deep freeze; add 0.1 ml/liter. |
|-------|---------------------------------------------------------------------------------------------------------------------------------|
| 6.    | Solution VII (coenzyme A): dissolve 10 mg of coenzyme A in 10 ml of distilled water; add two drops of 0.05% Na₂S·5H₂O solution made in freshly boiled distilled water; store in deep freeze; add 0.1 ml/liter. |

| Steps | Solution VIII (oleic acid): suspend 100 mg of oleic acid® in 50 ml of distilled water and neutralize (NaOH) to pH 7.0; adjust final volume to 100 ml. |
|-------|---------------------------------------------------------------------------------------------------------------------------------|
| 10.   | Suspend 2 g of nonsoluble potato starch (Baker's) in 50 ml of distilled water and pour into 450 ml of boiling water; add 10 mg of oleic acid solution (10 ml of solution VIII); add 15 g of agar; autoclave for sterilization at 121 C for 20 min; while the agar starch base is still hot, add the 500 ml of casein hydrolysate-vitamin-glucose base; mix thoroughly and heat nearly to boiling with constant mixing; tube aseptically and slant.

| Steps | Solution IX (organic additions): |
|-------|---------------------------------|
| 11.   | Organic additions (in grams):   |
|       | Glucose 10.0                    |
|       | a-Ketoglutaric acid 1.0         |
|       | L-Cysteine-HCl 1.0              |
|       | Glutathione-reduced 0.5         |
|       | L-Asparagine 0.1                |
|       | L-Tryptophan 0.02               |

9. Neutralize carefully to pH 6.5 with 20% KOH; make to 500 ml and sterilize by filtration through a 0.45-μm membrane filter (Millipore) to give a 2× concentrated basal solution.

**Part 2. Agar starch base**

10. Solution VIII (oleic acid): suspend 100 mg of oleic acid® in 50 ml of distilled water and neutralize (NaOH) to pH 7.0; adjust final volume to 100 ml.

11. Suspend 2 g of nonsoluble potato starch (Baker's) in 50 ml of distilled water and pour into 450 ml of boiling water; add 10 mg of oleic acid solution (10 ml of solution VIII); add 15 g of agar; autoclave for sterilization at 121 C for 20 min; while the agar starch base is still hot, add the 500 ml of casein hydrolysate-vitamin-glucose base; mix thoroughly and heat nearly to boiling with constant mixing; tube aseptically and slant.

* This medium is essentially the yeast phase medium described by Pine and Drouhet (14). To convert the mycelial phase maintenance medium to the yeast phase medium, 10 g of citric acid is added to the casein hydrolysate-vitamin-glucose base before neutralization and adjustment to 500 ml. Only 12.5 g of agar is used per liter of medium. The minimal vitamin medium originally described (11), which is also recommended for mycelial phase maintenance, is identical to the MPM medium except ZnSO₄ and χ-ketoglutaric acid are deleted. To make liquid media for either yeast phase or mycelial phase, delete agar, reduce the concentration of starch to 0.5 g per liter of final medium and reduce the concentration of oleic acid to 1 mg per liter of final medium.

* From Nutritional Biochemicals Corp.

**MATERIALS AND METHODS**

Cultures. Strains 6617, 6621, 6622, 6623, 6624, and F851 and other strains of *H. capsulatum* were obtained from C. W. Emmons, National Institutes of Health, Bethesda, Md., and have been maintained since 1952. Their cultural characteristics, as observed after several passages on Sabouraud's medium, were described by Pine and Peacock (15). Strain 28 was obtained from the Mycology Laboratory, National Communicable Disease Center (NCDC), Atlanta, Ga. Strain A811 was obtained from the Microbiological Reagents Unit, Biological Reagents Section, NCDC. Strain 2591 of *H. duboisii* was obtained from the Department of Microbiology, Duke University Medical Center,
Fig. 1. Sporulation characteristics of Histoplasma capsulatum strain 6617. (a) Mycelial phase culture grown in 1961 on the minimal vitamin (MV) medium and stored under oil to date. Medium dark phase optics. 645 X. Arrows denote masses of microconidia and single macroconidium. (b) Mycelial phase culture grown in 1963 on the mycelial phase maintenance (MPM) medium. Dark field. 300 X. (c) Mycelial phase culture from yeast phase inoculum grown on the MPM medium in 1969. Dark field. 150 X. (d) As (c), the field depicted is that contained within the square of (c). Medium dark phase. 645 X. (e) As (d) medium dark phase. 1455 X. Microconidia are those indicated by arrow in (d).

Durham, N.C.; the remaining strains of H. duboisi were obtained from Edouard Drouhet, Institut Pasteur, Paris, France (14). Transfers of all the strains were made every 4 to 6 months; cultures were incubated at 25 C for 2 to 4 weeks and were then stored at 5 C. Comparative characteristics of transfers of a given strain over several years were based on culture and transfer records and on comparative morphological aspects observed microscopically in wet mounts and recorded by photography.

Microscopic observation and photography. Mycelial fragments were taken from the surface of cultures and mounted in a 10% gelatin solution with 0.01% merthiolate. Observations and photographs were made with a medium, dark phase microscope and a Polaroid Land camera using type 50 film. Negatives were processed, and prints having the desired characteristics of contrast were made.

Media. The mycelial phase maintenance (MPM) medium and the yeast phase medium are described in Table 1. Sabouraud's medium (1) and Brain Heart Infusion (BHI) medium were also used.
FIG. 2. Sporulation characteristics of Histoplasma capsulatum, strain 6623. (a) Mycelial phase culture grown in 1961 on the MV medium and stored under oil to date. Medium dark phase. 285 ×. (b) Mycelial phase culture grown in 1968 on the MPM medium. White light. 285 ×. Note mass of macroconidia. (c) As (b). 600 ×. Macroconidia. (d) Mycelial phase culture grown in 1968 on Brain Heart Infusion agar. White light. 600 ×. (e) Mycelial phase culture from yeast phase inoculum grown on the MPM medium in 1969. White light. 600 ×. Note mass of macroconidia similar to those of (b) above. (f) As (e) above, showing individual tuberculate macroconidia.

RESULTS

The results obtained in maintaining the various strains of *H. capsulatum* are best described by the histories of several specific mycelial cultures. Certain strains of these cultures were chosen for description because they are recognized by defined mycelial characteristics; others are of interest because of their history and present usage.
Strain 6617. I initially used strain 6617 for a period of several years to determine certain growth requirements of the yeast phase (9, 10, 18). This strain, isolated by C. W. Emmons from a Georgia skunk, was characterized by its very numerous production of masses of microconidia and by the virtual absence of macroconidia. Its yeast phase was originally isolated by mouse passage, but was easily obtained by conversion of the mycelium on the blood-agar-cystine medium. This strain was classified as a 1,4 serotype by Kaufman and Blumer (7). Soil cultures of the mycelium were prepared in 1953 by using mycelial fragments and yeast phase cells as inocula. Stock cultures made on Sabouraud’s agar rapidly lost sporulating ability and viability (15). Mycelial stock cultures were made in 1957 on the MV medium from soil stocks initially inoculated with yeast phase. These cultures were then maintained on the MV medium and the MPM medium and transferred every 6 months. MV cultures placed under oil in 1961 rapidly lost viability but were saved at 5 C until the present time. Figure 1a shows a mass of microconidia imbedded in the dense mycelium of this oil culture. These masses of spores were also formed in subsequent transfers as shown in a dark field photograph (Fig. 1b) of a MPM medium stock culture prepared in 1963. In 1969, stock cultures of mycelium were also initiated from yeast phase cultures first started in 1952. In Fig. 1c, the masses of microconidia distributed throughout the mycelium are readily observed under dark field and with medium dark phase (Fig. 1d). These are not ungerminated yeast phase cells of inoculum as shown in Fig. 1e, where characteristic morphology and the mycelial points of attachment of the microconidia are apparent. Only two macroconidia were observed in a single microscopic mount of this strain. Thus the mycelial characteristics of the culture were maintained by mycelial passage on MPM medium from 1957 to at least 1963 with the evidence presently on hand. Furthermore, the yeast phase, maintained from 1952 to the present time, converted on MPM medium to form mycelial phase which also showed the original characteristics of sporulation. Several microscopic mounts from transfers to both Sabouraud’s and BHI slants were devoid of spores; others showed very few spores, although mycelial growth was more abundant on these two media.

Attempts were made to induce sporulation of strains 6570, 6576, 6617 and 6621 by mouse inoculation of the sterile mycelial cultures from Sabouraud’s slants. Strains 6617 and 6570 failed to grow; strains 6621 and 6576 were isolated on Sabouraud’s medium in their original form and could not be induced to sporulate by transfer to the MV medium.

Strain 6623. The sequence of transfers and
FIG. 4. Characteristics of Histoplasma capsulatum strains 6624 and A811. (a) Mycelial phase of strain 6624 grown from yeast phase inoculum on the MPM medium in 1969. 645 X. Note numerous macroconidia. (b) Mycelial phase of strain A811 grown from yeast phase inoculum on MPM medium in 1969. Note single macroconidium in essentially sterile mycelium. (c) Yeast phase cells of strain 6624. Medium dark phase. 645 X. (d) Yeast phase cells of strain A811 medium dark phase. 645 X.
preparation of cultures of this strain (15) were identical to those for strain 6617. This culture was remarkable in its profuse production of macroconidia on MV (see reference 12 on p. 46; Fig. 4D). By observation of the culture preserved under oil in 1961 (Fig. 2a), it is seen that the culture still retained this characteristic sporulation after transfer on MPM medium for 7 years (Fig. 2b). Tubercules of the macroconidia are shown in Fig. 2c. The great reduction in sporulation observed after a single passage of the mycelial phase on BHI agar is shown in Fig. 2d. Strain 6623 lost its ability to sporulate within three transfers on Sabouraud's medium. Conversion of the yeast phase to mycelium on either the MPM or Sabouraud's medium gave a dark brown mycelial mat solid in many areas with macroconidia (Fig. 2e and 2f). The growth obtained on BHI slants, however, was virtually sterile by comparison.

**Strain 6621.** The maintenance and transfer schedule of strain 6621 was identical to that of strain 6617. Originally isolated on Sabouraud's agar from a case of histoplasmosis in man, it was obtained in 1952 and described as having many micro- and macroconidia. This culture was of the 1,4 serotype (7) and was maintained on the MV medium in both yeast and mycelial phases. On Sabouraud's agar, the mycelial phase was described in 1958 (15) as forming no macroconidia and few microconidia. Figure 3 shows that the mycelial phase obtained on the MPM medium by conversion of the yeast phase produced abundant micro- and macroconidia, and has, in general, the morphological characteristics of the original culture.

**Strain 6624.** Strain 6624 was isolated from a human case of histoplasmosis and was obtained in 1954. It produced large amounts of both macroconidia and microconidia. Both yeast and mycelial phases were maintained in the same manner as described for strain 6617. The mycelial phase of strain 6624 was sent to the NCDC, where it was converted to the yeast phase on BHI agar and was given the culture number A811. It was evaluated as a yeast phase antigen in the Complement Fixation Test (19) and became standard yeast phase antigen prepared by the Biological Reagents Section. Comparison of the characteristics of the yeast phase of strain 6624 with those of strain A811 in 1965 showed a marked morphological difference between them; whereas strain 6624 was composed of typical budding yeasts (Fig. 4c), strain A811 was a monilial variant in which the yeasts were linked as in a string of beads and which contained varying amounts of mycelium (Fig. 4d). No antigenic differences were observed between them (13, 16). The mycelial phase of strain 6624 still showed abundant sporulation in 1963, whereas sporulation was exceedingly limited, if not absent, in A811. Conversion of the yeast phase to the mycelium on the MPM medium in 1969 gave cultures which reflected these same characteristics of the two strains (Fig. 4a, b). The results indicated that a mutation of the mycelial phase which had occurred on BHI or Sabouraud's agar and which had resulted in a loss of sporulation was carried genotypically by the yeast phase for approximately 12 years.

**Results in general.** Two media, the MV or the MPM medium, have been used successfully to maintain the sporulation of 17 strains. However, certain strains which were received as asporogenous cultures or strains of limited sporulation were not stimulated to produce spores (strains *H. duboisii*, 936, 1085, RV 10097; *H. capsulatum* A811 and 107). Other strains, *H. duboisii* 2591 and *H. capsulatum* 28 which were sterile on Sabouraud's medium, showed abundant sporulation on the MPM medium. No major differences between the MV medium and the MPM medium were noted, although the MV medium would be preferred theoretically, since it lacks zinc ion and α-ketoglutaric acid, both of which stimulate yeast phase growth. The use of the yeast phase medium as a medium for growth of the mycelial phase at 25°C is definitely not recommended, since slow growth of glabrous mycelium is often obtained in which many atypical yeast-like forms appear. Indeed, certain strains (strains 6619 and 6622) showed a slow aberrant growth of the yeast phase at room temperature.

**DISCUSSION**

In the years they have been studied, the yeast phase strains have been characterized by certain nutritional, amino acid, and vitamin requirements (9–11). The mycelial phases have not, however, shown these requirements (12); thus strain 6623 was known by its very characteristic formation of macroconidia and 6617 by its abundant production of microconidia without macroconidia. Maintenance of these characteristics when strains were grown on the MPM medium was evident for 17 years, as were the less characteristic sporulating abilities of some 17 additional strains, which have included serotypes 1,4 and 1,2,3 of Kaufman and Blumer, and strains of *H. duboisii*.

Artis and Baum (3) studied the effects of variation in media on the formation of the tuberculate spores and found that the addition of phosphate to Sabouraud's agar stimulated nine strains to produce tuberculated or smooth macroconidia. Two passages of the yeast phase through hamsters
successfully induced the production of macroconidia on Sabouraud’s agar by several strains. Anderson and Marcus (2) found that macroconidia formation was best supported on several synthetic media containing asparagine and ammonium sulfate as sources of nitrogen; the medium which best supported macroconidia formation was Sabouraud’s with phosphate (3).

The MV medium was initially described as a yeast phase maintenance medium, but the maintenance medium for the yeast phase was further modified to include ZnSO₄, α-ketoglutaric acid, and citric acid. With few exceptions, each of the factors present in the media have been found to exert a beneficial effect for either mycelial or yeast phase growth or for conversion of one phase to the other; it is recommended they be retained. “Vitamin Free” casein hydrolysate-enzymatic (Nutritional Biochemicals Corp. Cleveland, Ohio) cannot be substituted for casein hydrolysate-acid, since it inhibits yeast phase growth.

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