Induction of Yeastlike Development in *Aspergillus parasiticus*

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SUMMARY

Vegetative development of *Aspergillus parasiticus* may follow either one of two patterns of morphogenesis (hyphal-yeastlike dimorphism) depending upon the presence or absence of manganese ions in the culture medium. The addition of exogenous amino acids, vitamins and other trace metals had no significant effect upon morphogenetic development or aflatoxin B₁ synthesis. The yeastlike forms are capable of continuous aflatoxin B₁ synthesis under non-proliferating conditions. Varying the Mn²⁺ concentration from 7.3×10^{-4} to 7.3×10^{-3} mM causes a morphological change from yeastlike to hyphal development in a synthetic medium. The yeastlike forms are stable and resist osmotic shock.

INTRODUCTION

Vegetative development in many fungi shows duality of morphogenesis, known as mould-yeast dimorphism, in which the fungus may be in a filamentous form (hyphal-type) or a yeastlike form. Bartnicki-Garcia & Nickerson (1962*a*) established that vegetative growth of *Mucor rouxii* may follow either one of these two patterns. A yeastlike development occurred upon anaerobic incubation under high tensions of CO₂ (Bartnicki-Garcia & Nickerson, 1962*b*). Vegetative morphogenesis was affected by the addition of metal chelating agents. Bartnicki-Garcia & Nickerson (1962*a*) demonstrated that yeastlike development of *M. rouxii* was prevented by EDTA: however, this morphogenetic effect of EDTA could be reversed by transitional group metal ions. Wildman (1966) reported the occurrence of giant cells in small colonies of *Aspergillus flavus* Link grown upon an agar block.

In experiments dealing with the biosynthesis of aflatoxin B₁, we observed the appearance of spherical, yeastlike forms in the synthetic medium. However, these forms probably resemble arthrospores more than a true yeastlike phase since multiplication by budding was not observed (Bartnicki-Garcia & Nickerson, 1962*a*). For convenience we have retained the use of the term 'yeastlike'. This communication presents information about induction of the yeastlike phase in cultures of *Aspergillus parasiticus* and compares the aflatoxin B₁-synthesizing ability of these forms to that of the filamentous form.

METHODS

Organism. Stock cultures of *Aspergillus parasiticus* NRRL 2999 were maintained on potato dextrose agar slants at 30°. Spores were harvested from 5 day slant cultures by covering the culture with sterile deionized water and brushing gently with an inoculation

loop. The spore suspension was placed in sterile 500 ml. centrifuge tubes and centrifuged at 2000 g. The spore pellet was washed with sterile water, filtered over a Millipore* filter, resuspended in sterile deionized water and shaken vigorously to break up clumps of spores. The spores were then counted in a haemocytometer. The suspension was used as the inoculum for liquid medium, care being taken to avoid variation in inoculum size due to sedimentation.

Cultural conditions. Three basal media were used. (1) Yeast extract+sucrose medium: sucrose, 200 g.; yeast extract, 20 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005 g.; FeSO_4 , 0.01 g., and distilled H_2O , 1 l. (2) Bacto yeast nitrogen base media (Difco) supplemented with 5.0 g. glucose and diluted tenfold before use. (3) A synthetic culture medium: glucose, 50 g.; $(\text{NH}_4)_2\text{SO}_4$, 3.0 g.; KH_2PO_4 , 10.0 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 g.; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.5 mg.; $\text{Fe}_2(\text{SO}_4)_3 \cdot 6\text{H}_2\text{O}$, 0.3 mg.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.3 mg.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 15.0 mg.; boric acid, 1.0 mg., and distilled H_2O , 1 l. Various amino acids, vitamins and trace metals were added to the basal media in the concentrations indicated in the text. The various media were dispensed in 50 ml. volumes into 300 ml. Erlenmeyer flasks which were plugged with non-absorbent cotton wool and autoclaved at 121° for 15 min. Each flask was inoculated with 3.2×10^6 spores and incubated at 30° on a New Brunswick rotary shaker at 200 rev./min.

Analytical methods. Mycelia were harvested by filtration through previously dried and weighed Whatman no. 541 filter paper, dried for 2 h. at 110° , cooled and weighed.

Aflatoxin was extracted from the spent culture medium with chloroform. The chloroform extracts were evaporated to dryness in a flash evaporator, and the residue was redissolved in a known volume of CHCl_3 . The various aflatoxins were separated by preparative thin-layer chromatography on silica gel G-HR with 20% acetone in CHCl_3 as developer. The aflatoxin B_1 band was scraped off the plate, and the toxin eluted from the gel with methanol. Its concentration was measured spectrophotometrically (Nabney & Nesbitt, 1964; Holzapfel, Steyn & Purchase, 1966) at 362 nm. with a Beckman-DB spectrophotometer.

Materials. All reagent-grade chemicals and chemical reagents, including amino acids, vitamins and trace elements, were purchased from commercial sources.

RESULTS AND DISCUSSION

Induction of yeastlike development. In preliminary experiments on the synthesis of aflatoxin B_1 in a defined medium under shake culture conditions, yeastlike forms of *Aspergillus parasiticus* almost completely replaced the normal hyphal form found in cultures supplemented with yeast extract. In the synthetic medium some yeastlike forms developed within 24 h. of spore germination. After 72 h. most of the culture was in the yeastlike phase. When yeast extract-sucrose (medium 1) was added to this yeast-phase culture, mycelial forms developed from the yeast forms. The importance of a nutrient in yeast extract that was required for induction of the hyphal growth phase was then investigated, as well as other possible deficiencies in the synthetic medium which may have been responsible for the formation of the yeastlike forms.

Effect of vitamins, metal ions and amino acids. In the preliminary experiments three amino acids (histidine, methionine, tryptophan), five metal ions (Zn, Mo, Fe, Cu, Mn)

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

and nine vitamins (biotin, pantothenate, folic acid, inositol, niacin, *para*-amino-benzoic acid, pyridoxine, riboflavin, thiamin) were added separately and in various combinations to the basal synthetic medium and their effect upon morphogenesis was observed at various intervals after inoculation. Yeastlike forms developed only in the absence of added Mn^{2+} ion; Mn^{2+} at 7.3×10^{-1} mM yielded hyphal forms of the organism. The absence of all the metal ions from the basal synthetic medium resulted in either no or scant growth.

Table 1. *Effect of Mn^{2+} concentration upon the morphology of *Aspergillus parasiticus* cultured in synthetic medium*

Mn* (mM)	Morphology	
	Hyphal	Yeastlike
0	—	+
7.3×10^{-5}	—	+
7.3×10^{-4}	—	+
2.0×10^{-3}	+	+
7.3×10^{-3}	+	—
7.3×10^{-2}	+	—
7.3×10^{-1}	+	—

* Mn^{2+} added as $MnCl_2$ to the basal synthetic medium.

Effect of manganese concentration. The responses to various concentrations of Mn^{2+} in the medium are shown in Table 1. The cultures were almost entirely in the yeastlike form in the absence of Mn^{2+} or when Mn^{2+} was added up to 7.3×10^{-4} mM; higher Mn^{2+} concentrations gave the hyphal form. The different concentrations of Mn^{2+} had no significant effect upon total cell yield or aflatoxin B_1 synthesis.

*Morphological development of *Aspergillus parasiticus*.* The first morphological development of the spores was germ-tube formation, which occurred after 20 to 24 h. in the Mn^{2+} deficient synthetic medium. The first signs of morphological change was a protrusion or swelling of the young vegetative hyphae at 48 h. (Pl. 1, fig. 1). These protrusions were found either at hyphal tips or randomly distributed along the length of hyphae (Pl. 1, fig. 2). Subsequently these bodies swelled into large spherical, yeast-like forms between 75 and 90 h. growth (Pl. 1, fig. 3). Further incubation up to 185 h. gave pellets composed almost totally of yeastlike growth. This yeastlike form appeared to be a stable, morphogenetic form of *Aspergillus parasiticus* (Pl. 1, Fig. 4).

The addition of 7.3×10^{-1} mM Mn^{2+} to a synthetic medium containing spores resulted in normal germination and outgrowth throughout development of the culture, producing the hyphal form. No yeastlike forms could be detected under these conditions.

Cultivation of this organism upon agar slants containing the synthetic nutrients minus Mn^{2+} also yielded the yeastlike form of growth.

Manganese appears to be a multifunctional metal in the metabolism of numerous fungal and bacterial systems. It is involved in cell wall synthesis, as well as in DNA, RNA and fatty acid biosynthesis (Hoffman, Scheck & Saffert, 1950; Fox & Weiss, 1964; Jirgena, 1967; Oka, Udagawa & Kinoshita, 1968). Although no evidence is available concerning the effect of Mn^{2+} deficiency upon wall constituents in *Aspergillus parasiticus*, production of yeastlike forms possibly may occur via an incomplete or altered wall-synthesizing mechanism. There appears to have been a lack of protein

synthesis after 85 h. in a Mn^{2+} -deficient medium because transfer of yeastlike forms to fresh synthetic medium (lacking Mn^{2+}) led to no further growth or protein synthesis but growth recommenced on the addition of sufficient Mn^{2+} to such a culture. These observations suggest that in the manganese-deficient medium the organism utilized the available endogenous Mn^{2+} in the spores for germination but that this Mn^{2+} was not sufficient to support hyphal morphogenesis. This Mn^{2+} deficiency might then have inhibited enzyme activities required for wall synthesis and eventually arrested primary metabolism, such as protein, DNA and RNA synthesis systems.

Fig. 1. Growth and aflatoxin B_1 synthesis by *Aspergillus parasiticus* growing in a synthetic medium. Results are expressed as mg. dry wt/culture flask and μ g. aflatoxin B_1 produced. ●—●, Mass (mg. dry wt) yield; ○—○, aflatoxin B_1 synthesis.

Comparison of aflatoxin B_1 synthesis by hyphal and yeastlike forms. The synthesis of aflatoxin B_1 , a potent hepatocarcinogen (Wogan, 1969), by hyphal and yeastlike forms of *Aspergillus parasiticus* in various media was investigated. The yeastlike form in Mn^{2+} -deficient synthetic medium began to synthesize just before the growth rate began to decrease (Fig. 1); this stage of culture is termed the secondary phase or idiophase of growth (Bu'Lock *et al.* 1965). Toxin synthesis continued to increase until the pH reached 2.5 to 2.3.

The addition of Mn^{2+} before spore germination resulted in development of the hyphal form only and a slight delay in aflatoxin synthesis. The presence of Mn^{2+} ion at concentrations sufficient to support growth of the hyphal form had no appreciable effect upon total toxin synthesis by either the yeast or hyphal forms.

When the yeastlike forms were transferred to fresh synthetic media, they were incapable of further replication or increase in total cell mass. However, aflatoxin synthesis recommenced until again limited by the drop in pH of the medium. These yeastlike forms could be transferred every 24 to 48 h. to fresh synthetic medium for continuous production of aflatoxin B_1 . Cells were still capable of toxin synthesis after being transferred over a period of 30 days, at which time the experiment was ended.

Growth of the organism in yeast extract + sucrose medium resulted in high yields of

the hyphal form and of aflatoxin (Fig. 2). In this medium slowing of vegetative growth was followed by the onset of toxin synthesis. Growth of *Aspergillus parasiticus* was sparse on Bacto yeast nitrogen base media, yielding only 110 mg. of mycelial dry weight in the hyphal form. Not only did aflatoxin synthesis fail to occur, but also many of the spores failed to germinate. This medium contained all the necessary vitamins, amino acids and trace metals usually required for growth of many fungi.

Fig. 2. Growth and aflatoxin B₁ synthesis by *Aspergillus parasiticus* growing in a yeast extract-sucrose medium. Results are expressed as mg. dry wt/culture flask and μg. aflatoxin B₁ produced. ●—●, Mass (mg. dry wt) yield; ○---○, aflatoxin B₁ synthesis.

Characteristics of yeastlike forms. The yeastlike form of *Aspergillus parasiticus* were resistant to lytic enzymes and osmotic shock. If the yeastlike form arose through either the incomplete synthesis of hyphal wall or through an altered wall synthesis, it would possibly be more susceptible to osmotic shock or enzymic lysis. This hypothesis was tested by incubating the yeastlike forms in 0.75 M sorbitol and 0.5 M-NaCl for a 20 h. incubation period followed by suspension in distilled water. The yeastlike forms were harvested and observed microscopically for breakage. The majority were intact with little evidence of cellular destruction. Since cell walls of most fungi contain lipid, polysaccharide and cellulose or chitin, the yeastlike forms were treated with chitinase, lipase, lipoxygenase and cellulase enzymes but no hydrolysis resulted.

Upon transfer of yeastlike forms to a synthetic medium containing Mn²⁺ further growth produced filamentous, hyphal forms, which indicated again the need for Mn²⁺ for formation of hyphal mycelial.

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EXPLANATION OF PLATE

Fig. 1. Swelling of young hyphae of *Aspergillus parasiticus* in submerged culture, growing in synthetic medium (minus Mn^{2+}). $\times 800$.

Fig. 2. Swelling and protrusion of the hyphal tips of *A. parasiticus* in submerged culture, growing in synthetic medium (minus Mn^{2+}). $\times 800$.

Fig. 3. Development of complete spherical yeastlike bodies of *A. parasiticus* in submerged culture, growing in synthetic medium (minus Mn^{2+}). $\times 800$.

Fig. 4. Mature yeastlike forms of *A. parasiticus* in submerged culture, growing in synthetic medium (minus Mn^{2+}). $\times 800$.