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Kinetics of Fungal Growth and Phosphatase Formation in *Aspergillus nidulans*

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ABSTRACT

DORN, G. (Albert Einstein College of Medicine, New York, N.Y.), AND W. RIVERA. Kinetics of fungal growth and phosphatase formation in *Aspergillus nidulans*. J. Bacteriol. **92**:1618–1622. 1966.—A liquid culture system is described for growing *Aspergillus nidulans* in a manner amenable to kinetic studies. Changes in dry weight, protein, deoxyribonucleic acid (DNA), inorganic phosphate, alkaline phosphatase, and acid phosphatase activity were followed over a 40-hr interval. In both limiting- and high-phosphate media, the doubling time of DNA during the logarithmic phase was 3.5 ± 0.5 hr. Of the four phosphatases which have been found in this fungus, three were markedly derepressed by low levels of inorganic phosphate.

Up to this time, attempts to characterize the kinetics of growth in fungi have been hampered by the inability to obtain homogeneous cultures. Specifically, aggregation of germinating spores, hyphal intertwining, and the presence of various developmental stages within a single mycelial mat contribute to the heterogeneity of culture samples.

In 1963, Mackintosh and Pritchard (9) observed the formation of microcolonies when *Aspergillus nidulans* was grown on solid agar medium in the presence of surface-active compounds such as sodium dodecyl sulfate or sodium deoxycholate. These agents, although restricting radial growth of this fungus, did not appear to affect its viability or normal differentiation.

The present work was undertaken to test the feasibility of using surface-active substances to obtain individual colonies of homogeneous composition in liquid cultures of *A. nidulans*, thereby facilitating enzyme kinetic analysis. The acid and alkaline phosphatases of this organism, previously investigated by genetic and electrophoretic methods (5, 6), were employed here as a model to assess the potentials of any new liquid culture system for the study of enzyme regulation in fungi. The effects of high-and limiting-phosphate conditions on a number of culture parameters are discussed.

MATERIALS AND METHODS

Organism. A biotin-requiring strain *bi*1 of *A. nidulans* was obtained from the stock maintained in the Department of Genetics, Glasgow University, Glasgow, Scotland. The details of the strains, media, symbols, and routine methods employed in the genetics of *A. nidulans* have been described elsewhere (11, 12).

Liquid culture media. High-phosphate deoxycholate medium was made up as follows: $NaNO_3$, 6.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 1.5 g; D-glucose, 10.0 g; Casamino Acids (Difco), 0.6 g; sodium deoxycholate, 0.4 g; D-biotin (Calbiochem), USP, 15.0 μg; trace elements (copper, iron, manganese, and zinc); and double-distilled water, 1.0 liter. The *p*H was adjusted to 6.5 with 4 N NaOH before sterilization, and the medium was autoclaved at 120 C for 15 min.

Limiting-phosphate deoxycholate medium is identical to the high-phosphate medium with the exception that the KH_2PO_4 is omitted. Unless specified to the contrary, all ingredients are of analytical reagent standard.

Method of cultivation. A New Brunswick Microferm Unit fitted with a 14-liter chamber was used. The chamber was filled with 10 liters of medium; the unit was operated at 37 C with an air flow of 0.1 liter per minute. The impellers were set to rotate at 300 rev/min. At time zero, 10^8 *bi*1 spores were inoculated into the chamber; 25-ml samples were collected over a 40-hr period. Samples of the cultures were assayed for dry weight, protein, deoxyribonucleic acid (DNA), inorganic phosphorus, acid phosphatase, and alkaline phosphatase activity.

Dry weight. Mycelia obtained from 10-ml samples were placed on preweighted filter papers and washed twice with 10 ml of distilled water. The papers were then dried in an oven at 90 C for 24 hr and were weighed.

Total protein. Protein was measured by the Lowry et al. (8) modification of the phenol method. A 2-ml portion of each sample was washed twice with barbital

buffer (*p*H 8.25), and the mycelium was disrupted in a Biosonik sonic extractor for 1 min. The mycelial suspension was assayed for total protein. Crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was used as a standard.

DNA. The mycelia from 10-ml samples were washed twice with distilled water. After the addition of 1 ml of 0.5 N perchloric acid, the mycelial suspension was assayed for DNA according to Burton's (2) modification of Dische's (4) diphenylamine method. Highly polymerized DNA type VI (Sigma Chemical Co.) was used as a standard.

Inorganic phosphorus. The amount of inorganic phosphorus in the culture medium was determined by the method of Dryer, Tammes, and Routh (7).

Phosphatase activity. Phosphatase activity was measured by a modification of the methods of Ohmori (10) and of Bessey, Lowry, and Brock (1). The *p*-nitrophenol liberated from *p*-nitrophenylphosphate (Sigma 104) at 30 C was measured at 410 mμ in a Coleman spectrophotometer. The substrate *p*-nitrophenylphosphate (5 mg/4 ml) was dissolved in either 0.6 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 9.5 (alkaline phosphatases), or in 0.6 M acetate buffer, *p*H 4.8 (acid phosphatases). To 0.4 ml of the appropriate reaction mixture was added a 0.1-ml sample of the culture material. The reaction was stopped, and the reaction product was stabilized with 1 ml of 1 N NaOH. Enzyme activity was expressed as micromoles of *p*-nitrophenol released per hour per milliliter of culture material.

Enzyme extraction from the mycelium. Approximately 250 ml of culture material was collected at specific time intervals. The mycelium was harvested on a Büchner funnel, washed with distilled water, resuspended in 25 ml of 0.025 M Veronal buffer (*p*H 8.2), and disrupted in a high-speed homogenizer at 0 C. The debris was separated by centrifugation at 20,000 × *g* for 30 min. A mixture of 4 parts of supernatant fluid to 1 part of chloroform was shaken for 15 min, and the precipitated material was removed by centrifugation at 2,500 × *g* for 10 min. This latter treatment does not affect the phosphatases, but does remove the deoxycholate and extraneous lipid material which interfere with electrophoresis. For electrophoresis, the crude extract was concentrated to approximately 1 ml by collodion membrane ultrafiltration.

Electrophoresis. Poulik's (13) modification of Smithies' (14) method for horizontal starch-gel electrophoresis was used to identify the four phosphatases. Each sample was soaked onto a strip of Whatman 3 MM chromatography paper and inserted into the gel. Electrophoresis was conducted at room temperature with a stabilized d-c potential of 6 v/cm for 5 hr. After this, the gel (initial *p*H, 8.5) was flooded with 0.4 M acetate buffer (*p*H 4.8) which contains the substrate α-naphthylphosphate and a diazonium coupling salt (fast garnet B). In the presence of a phosphatase, α-napthol was released and coupled with the diazonium salt to form an insoluble and colored complex. The flooding of the gel with the acetate buffer resulted in the gradual lowering of *p*H, and thus permitted the detection of phosphatase components with *p*H optima in the range between *p*H 4.8 and 8.5.

RESULTS

Effects of deoxycholate on growth. Preliminary experiments were performed to test the effect of various concentrations of deoxycholate on the growth of *A. nidulans* in liquid culture. In the absence of deoxycholate, the growing centers coalesced and became entangled around the impeller. On the other hand, the addition of between 0.04 and 0.08% deoxycholate to the culture produced two very desirable effects, namely, (i) restriction of radial growth accompanied by inhibition of aggregation between individual growing centers, and (ii) homogeneity of colony size (average diameter = 1 ± 0.2 mm after 25 hr of growth). Samples could be taken from a *single* culture as frequently as desired, provided that the total volume of the culture was not significantly altered. A 25-ml sample provided sufficient material for the accurate determination of all the variables studied here, namely, dry weight, amounts of protein, DNA, inorganic phosphate, and phosphatase activity.

Growth of Aspergillus in high-phosphate medium. Figure 1 shows the changes in various growth parameters of *Aspergillus* grown in high-phosphate deoxycholate medium. Figures 1A and B illustrate both the smooth type of growth curves obtainable under these conditions, and the parallel increases in dry weight and protein content. The low level of protein relative to dry weight (approximately 8%) appears to be a characteristic feature of fungi (3).

The DNA curve (Fig. 1C) suggests three distinct phases of growth: a lag phase (0 to 13 hr), a logarithmic phase (13 to 25 hr), and a complex terminal phase. Direct estimates of cell doubling are not feasible in a multicellular, coenocytic fungus such as *Aspergillus*. However, the doubling time of DNA production during the "logarithmic phase" gives an indirect estimate of nuclear doubling; on high-phosphate deoxycholate medium, this is 3.5 ± 0.5 hr.

Growth of Aspergillus in low-phosphate medium. Figure 2 represents the time course of changes in experimental parameters in low-phosphate liquid deoxycholate medium. The rapid increase in growth seen between 20 and 30 hr in Fig. 1A and B is notably absent from Fig. 2A and B.

Figures 2B and C suggest that, by 22 hr, inorganic phosphate had become growth-limiting, with a consequent decrease in the rate of protein and DNA synthesis. Just before the inorganic phosphate in the medium reached its lowest level (2 μg/ml), there was a rapid and substantial increase in alkaline and acid phosphatase activity

(Fig. 2D). In contrast to the high-phosphate cultures, there was a three- to fourfold increase in alkaline and acid phosphatase activity in the limiting-phosphate cultures. The rapid rise in phosphatase activity was coupled with an increase of inorganic phosphorus.

Electrophoresis. It has been demonstrated previously (5, 6) that, on limiting-phosphate medium, four major electrophoretically distinct phosphatases are produced in the *bi*1 strain of *A. nidulans*. The two slowest components have maximal activity at alkaline *p*H, whereas the activity of the other two components is maximal at acid *p*H. For reference purposes, the phosphatases have been numbered 1 through 4, proceeding from the slowest to the fastest migrating component. To determine the time of appearance of the four phosphatases during growth on low-phosphate medium, large samples were collected at various time intervals, and the mycelial extracts were subjected to starch-gel electrophoresis (Fig. 3, samples A, B, C, D, E, and F). Phosphatase 2 appeared to be present from the onset of growth and increased at a rate proportional to the growth of the culture. Phosphatases 1 and 3 appeared rapidly, shortly before the inorganic phosphate in the medium reached the growth-limiting level (2 μg/mg). Phosphatase 4 appeared between 23 and 25 hr of growth; by this time the other three phosphatases were already present in substantial amounts. In high-phosphate liquid medium, large amounts of phosphatase 2 and a trace of phosphatase 4 were formed (Fig. 3, sample G).

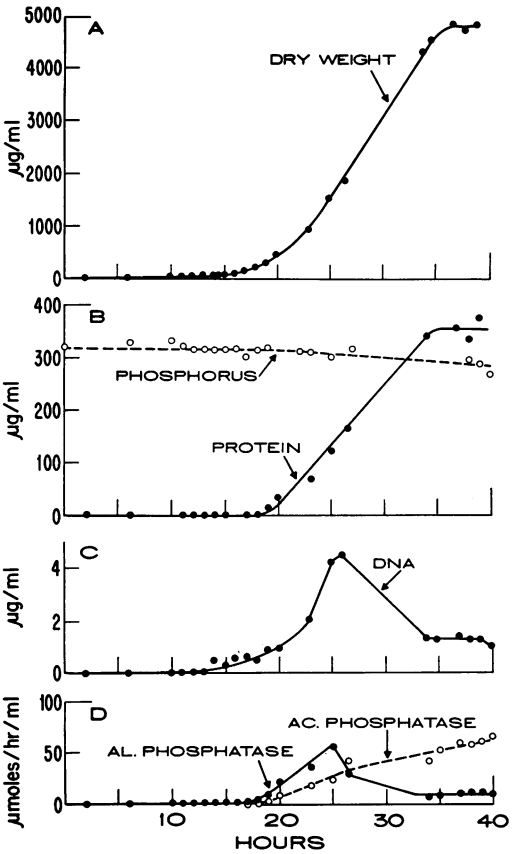

FIG. 1. *Changes in dry weight, protein, inorganic phosphorus, DNA, and phosphatase activity in high-phosphate culture. (A) Variation in dry weight; (B) variation in protein and inorganic phosphorus; (C) variation in DNA; and (D) variation in alkaline (pH 9.5) and acid (pH 4.8) phosphatase activity. Each point represents the average of four samples taken from four independent cultures.*

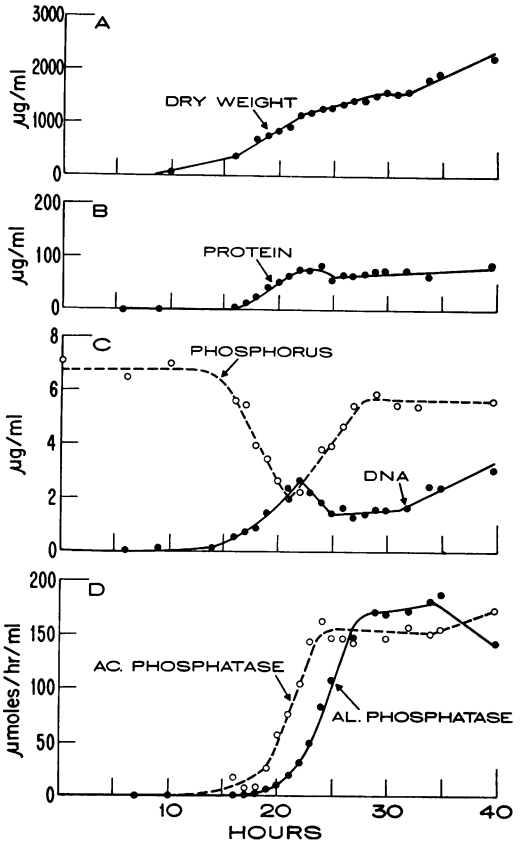

FIG. 2. *Change in dry weight, protein, DNA, inorganic phosphorus, and phosphatase activity in limiting-phosphate culture. (A) Variation in dry weight; (B) variation in protein; (C) variation in DNA and inorganic phosphorus; and (D) variation in alkaline (pH 9.5) and acid (pH 4.8) phosphatase activity. Each point represents the average of four samples taken from four independent cultures.*

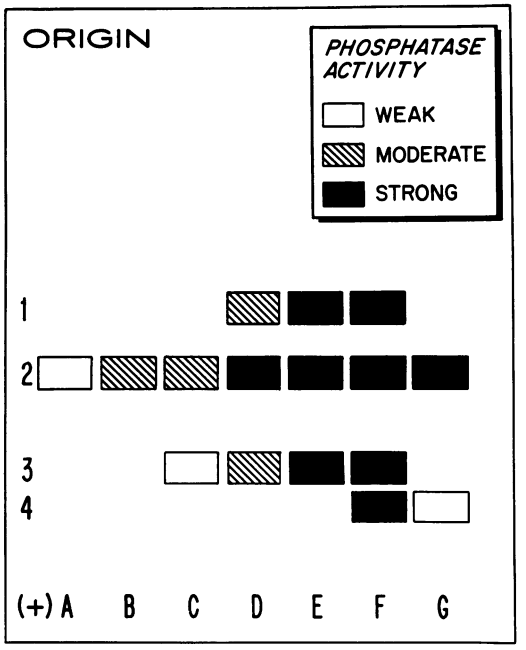

FIG. 3. *Electrophoretic pattern of the alkaline (1 and 2) and acid (3 and 4) phosphatases in crude extracts of the bi1 strain grown on limiting- or high-phosphate medium for various time intervals. Limiting phosphate: (A) 16 hr, (B) 19 hr, (C) 20 hr, (D) 21 hr, (E) 23 hr, and (F) 25 hr. High phosphate: (G) 40 hr.*

DISCUSSION

The results reported here demonstrate that sodium deoxycholate induces *A. nidulans* to grow in liquid culture in a manner which makes it amenable to kinetic studies. Although, in this instance, changes in amounts of protein, DNA, dry weight, inorganic phosphorus, alkaline phosphatase, and acid phosphatase activity were measured at hourly intervals over a 40-hr period, the method can conceivably be extended to include more parameters and shorter sampling intervals. Tatum, Barratt, and Cutter (15) reported that sodium deoxycholate induces colonial growth of *Neurospora* and *Syncephalastrum* on agar medium; hence, our liquid culture system may also be applicable to these fungi.

As in *Escherichia coli* (16), conditions for the formation of the alkaline and acid phosphatases differed strikingly from one another. Phosphatases 1 and 3 were completely absent in high-phosphate cultures (Fig. 3G), but they were rapidly formed when inorganic phosphate was lowered to a level limiting to growth. Phospathase 2 appeared to be a constitutive enzyme; in both limiting- and high-phosphate cultures it was present from the onset of growth, and increased at a rate proportional to cell growth. Phosphatase 4 apparently required both a low level of inorganic phosphate and another unknown factor, since only trace amounts of this enzyme were found in high-phosphate cultures; it appeared in limiting-phosphatase cultures only after the other three phosphatases had appeared.

Under conditions of limiting phosphate, the derepression of the alkaline and acid phosphatases was undoubtedly important to the life of the organism. At least one of these enzymes probably recycled the small amount of phosphate present and thereby enabled growth to continue.

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