

Antibiotic Synthesis and Morphological Differentiation of *Cephalosporium acremonium*

C. H. NASH AND F. M. HUBER

Antibiotic Manufacturing and Development Division, Eli Lilly and Company, Indianapolis, Indiana 46206

Received for publication 19 April 1971

In submerged cultures, *Cephalosporium acremonium* exists in four morphological forms: hyphae, arthrospores, conidia, and germings. The phase of hyphal differentiation into arthrospores coincides with the maximum rate of β -lactam antibiotic synthesis. Furthermore, arthrospores, separated by density-gradient centrifugation, possess 40% greater antibiotic-producing activity than any other morphological cell type. In a series of mutants, each with an increased potential to produce β -lactam antibiotics, differentiation into arthrospores was proportional to the increased titer of these antibiotics. Thus, arthrospores exhibit enhanced synthesis of β -lactam antibiotics and appear to be a determining factor in high-yielding mutants. Since a non-antibiotic-producing mutant readily differentiated into arthrospores, antibiotic synthesis and cellular differentiation are not obligately related.

Submerged cultures of *Cephalosporium acremonium* contain various morphological cell types representing stages in the growth cycle. In addition to these morphological changes, this organism synthesizes the β -lactam antibiotics cephalosporin C and penicillin N (1). Cellular differentiation of *C. acremonium* appears in some way to be related to synthesis of these antibiotics. The maximum rate of cephalosporin C production coincides with the conversion of slender hyphal filaments to swollen hyphal fragments, arthrospores (7). In addition, methionine enhances antibiotic synthesis and fragmentation of hyphae (3).

This communication reports the separation by density-gradient centrifugation of morphologically different cells from submerged cultures of *C. acremonium* and the capacity of each cellular form to synthesize β -lactam antibiotics.

MATERIALS AND METHODS

The *C. acremonium* Corda mutant M8650 (4) and its parent strain ATCC 11550 were used in this investigation. A mutant, *ltm-6*, which produces no detectable β -lactam antibiotic, was obtained from Paul A. Lemke of these laboratories.

Cultures were grown at 25°C on a rotary shaker (250 rev/min) in a chemically defined medium (4). This medium was modified by substituting $(\text{NH}_4)_2\text{SO}_4$ (7.5 g/liter) for asparagine, by adding DL-methionine (5 g/liter), and by sterilizing the sugars separately. Inocula were prepared as previously described (2). Eight per cent (v/v) inoculum was added to 100 ml of medium contained in 500-ml wide-mouth Erlenmeyer flasks.

Cells were isolated from 72-hr cultures by centrifugation, washed once with an equal volume of 0.02 M tris (hydroxymethyl) aminomethane-hydrochloride buffer (pH 7.0), and then resuspended in the same buffer.

Morphological forms observed in submerged culture were separated by density-gradient centrifugation. A 5-ml amount of washed cell suspension containing 35 to 45 mg (dry weight) per ml was layered on 75 ml of a 5 to 50% sucrose gradient in the above buffer. The cells were centrifuged through this gradient for 6 min at $206 \times g$. Fractions were obtained by sequentially removing 4-ml samples from the top of the gradient. Cells recovered by centrifugation were immediately suspended in 1 ml of the buffer. Each sample was assayed for cell titer, protein content, and the ability to synthesize β -lactam antibiotics.

Cell titers of gradient fractions were estimated by direct cell count with the aid of a Petroff-Hausser counting chamber. Differential counts were made by determining the frequency of each morphological type among 100 cells selected at random. Cell protein was estimated by the method of Lowry et al. (6).

The capacity of isolated cell types to synthesize penicillin N and cephalosporin C was determined by measuring the incorporation of valine- $U^{14}\text{C}$ into these antibiotics. L-Valine is a recognized precursor of these compounds (1). A $5\text{-}\mu\text{Ci}$ amount of valine- $U^{14}\text{C}$ ($10\ \mu\text{Ci}/\mu\text{mole}$) was added to washed cells, and samples were incubated for 2 hr at 25°C on gyratory water-bath shaker (250 rev/min). After incubation, cells were removed by centrifugation. Ten micro-liters of the supernatant was applied to a diethylaminoethyl cellulose thin-layer plate (J. T. Baker Chemical Co., Phillipsburg, N.J.) and developed with sodium acetate buffer (0.05 M, pH 5.2) at ambient temperature. Penicillin N and cephalosporin C were located by

spraying plates with ninhydrin reagent (0.3 g of ninhydrin, 100 ml of 1-butanol, and 3 ml of acetic acid). In this chromatographic system, both penicillin N and cephalosporin C migrated with an R_F of 0.2,

and valine migrated with an R_F of 0.85. Labeled antibiotic regions were cut out and placed in scintillation vials containing 15 ml of Liquorphor. Radioactivity was determined with a liquid scintillation

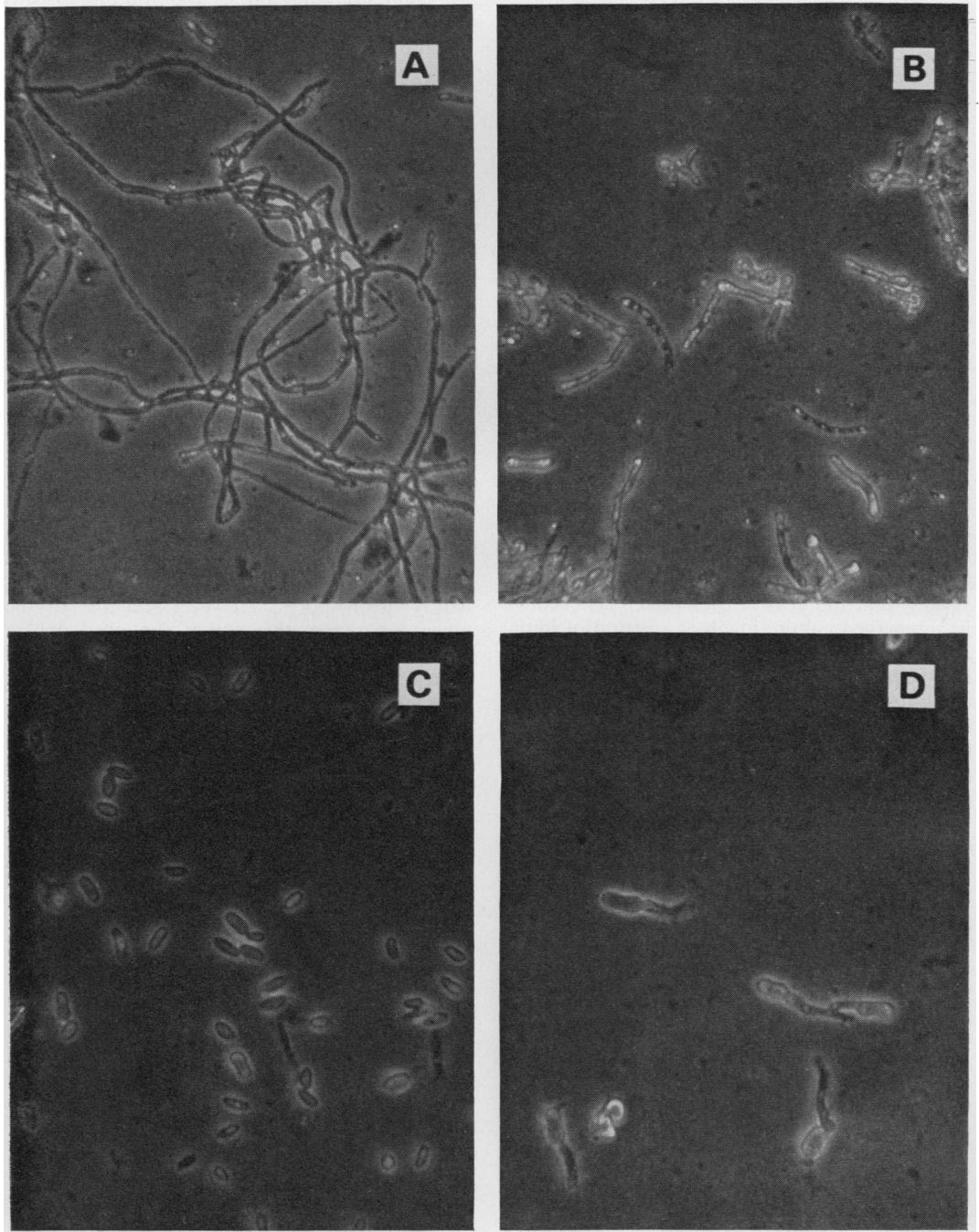


FIG. 1. Various morphological forms observed in submerged cultures of *Cephalosporium acremonium*. (A) Hyphae, (B) arthrospores, (C) conidia, (D) germinating conidia.

counter. Labeled substrates and scintillation fluid were obtained from Nuclear-Chicago Corp., Chicago, Ill. In some experiments, antibiotic activity was estimated by a standard microbiological plate method (4) with *Salmonella gallinarum* as the test organism.

Residual dry weight was determined by washing packed cells derived from 10 ml of whole broth with an equal volume of 0.1 N HCl, and suspending the cellular residue with 5 ml of water. The material was dried at 100 C for 24 hr and weighed.

RESULTS

Four cell types of *C. acremonium* were observed in submerged cultures (Fig. 1): hyphae (fine filaments with scattered septa), arthrospores (thick, short, highly septate forms), conidia, and germlings.

The distribution of these morphological forms and the concentrations of the β -lactam antibiotics during the fermentation are shown in Table 1. Antibiotic synthesis started at about 38 hr of incubation, and the rate of synthesis (per unit weight of mycelium) reached a maximum at 68 hr. Before initiation of antibiotic synthesis, mycelial growth consisted of 58 to 65% hyphae. Between 26 and 48 hr, considerable differentiation of hyphae was observed, as demonstrated by an increase in the percentage of arthrospores and the appearance of conidia. These results suggest that the growth cycle of *C. acremonium* in submerged culture involves a period of initial hyphal development followed by extensive fragmentation into arthrospores and formation of conidia. Concurrent with this differentiation was maximum antibiotic synthesis, which implicates arthrospores as the morphological cell type primarily responsible for antibiotic synthesis.

TABLE 1. Frequency of cell types and accretion of cephalosporin C during fermentation

| Time (hr) | Rate of antibiotic production ^a | Percentage | | | |
|-----------|--|------------|--------------|---------|------------|
| | | Hyphae | Arthrospores | Conidia | Germ-lings |
| 0 | 0.0 | 60 | 30 | 0 | 10 |
| 15 | 0.0 | 58 | 32 | 0 | 10 |
| 23 | 0.0 | 66 | 31 | 0 | 3 |
| 38 | 0.1 | 30 | 38 | 19 | 12 |
| 46 | 0.8 | 23 | 45 | 20 | 12 |
| 60 | 2.1 | 20 | 50 | 20 | 10 |
| 68 | 2.2 | 19 | 53 | 18 | 14 |
| 82 | 1.2 | 22 | 62 | 18 | 0 |
| 90 | 0.7 | 16 | 59 | 15 | 12 |

^a Rate of total β -lactam antibiotic synthesis (micrograms of total bioactivity per minute per milligram of cell protein).

The high degree of heterogeneity in the cell population of this fermentation prevented direct confirmation that the arthrospore was the morphological form possessing the greatest capacity to synthesize antibiotics. The morphologically different cell types were therefore separated in a sucrose gradient, and their antibiotic synthetic capacity was determined. In this system, the incorporation of the label paralleled the appearance of total β -lactam antibiotic activity detected by bioassay (Fig. 2). Furthermore, the labeled material was inactivated by β -lactamase (*Bacillus cereus*) treatment and had a maximum ultraviolet absorption at 265 nm. Levels of antibiotic below 10 $\mu\text{g/ml}$ (limit of bioassay) can be quantitated with this method, which makes it exceedingly attractive when using low cell concentrations.

The different morphological forms were concentrated into three broad overlapping zones in the sucrose gradient (Fig. 3): conidia in fractions 1 through 4, arthrospores in the intermediate fractions, and hyphae in fractions 11 to 16. Germinating conidia were distributed in the conidial fraction and the hyphal fraction according to the relative length of the germ tube. The greatest antibiotic synthetic activity was found in fractions 8 through 10. These fractions corresponded with the zone containing predominantly arthrospores. However, relative to the other two zones, this region contained only a small proportion of the total cell count and protein. The capacity of washed cell suspensions to synthesize the antibiotics was essentially un-

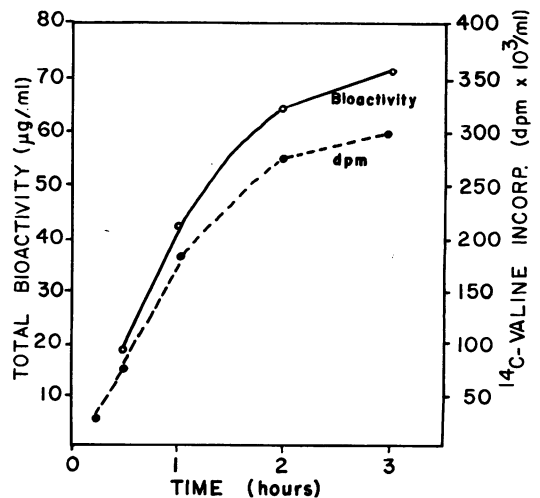


FIG. 2. Incorporation of valine- U - ^{14}C into β -lactam antibiotic (\bullet) and appearance of bioactivity (\circ) in washed cell suspensions of *Cephalosporium acremonium*.

altered by prior incubation in sucrose solutions ranging in concentration from 5 to 40%. These results demonstrate that arthrospores, as a major cell type, have an enhanced capacity to synthesize the antibiotics.

Since arthrospores possessed the greatest synthetic capacity for cephalosporin C and penicillin N, strains with enhanced antibiotic synthesis would be expected to differentiate into arthrospores more readily. This concept was tested by comparing the percentage of arthrospores and antibiotic titer in 72-hr cultures of strains with enhanced capacities to produce cephalosporin C and penicillin N. A direct relationship was found between the formation of arthrospores and the synthesis of β -lactam antibiotic (Fig. 4). Strain M8650-3 synthesized more than twice the quantity of antibiotic as strain M8650-1 and formed arthrospores at about twice the frequency. These data suggest that increased differentiation into arthrospores is a determinant in strains with enhanced synthetic capacities.

If an obligate relationship exists between

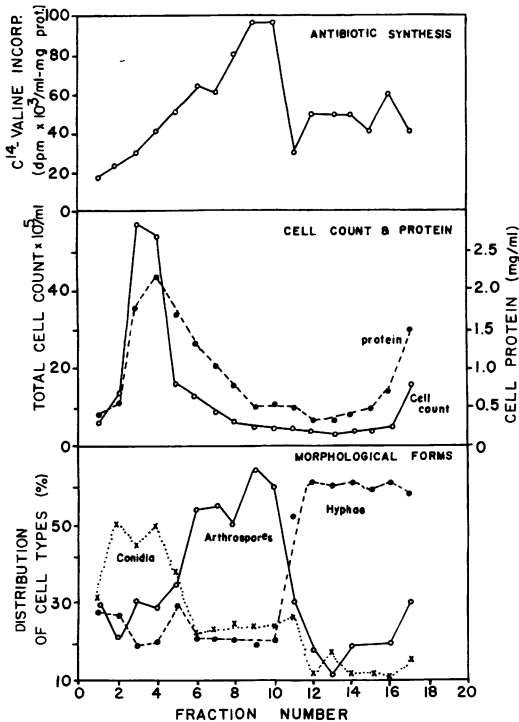


FIG. 3. Frequency of morphological types, total cell count and protein, and antibiotic synthetic activity of various fractions obtained from 72-hr culture by density-gradient centrifugation. Density increased from 5 to 50% sucrose from left to right.

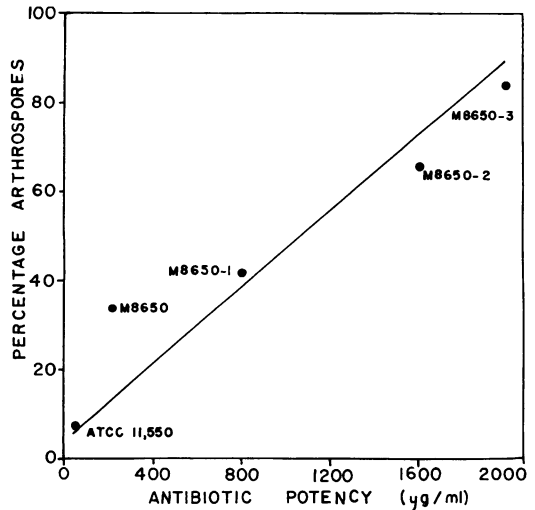


FIG. 4. Formation of arthrospores by strains of *Cephalosporium acremonium* having increasing potential to produce β -lactam antibiotic.

cellular differentiation and antibiotic synthesis, then a mutant which synthesizes no detectable β -lactam antibiotic would not be expected to form appreciable quantities of arthrospores. Such a mutant, *ltm-6*, was examined after 74 hr of incubation and 80% of the population was observed to be arthrospores. Therefore, cellular differentiation was not obligately related to antibiotic synthesis.

DISCUSSION

The synthesis of β -lactam antibiotics by *C. acremonium* was studied in relation to morphogenesis. In submerged culture, four morphological forms, i.e., hyphae, arthrospores, conidia, and germlings, are present and represent different stages of growth (7; W. Gams, *Cephalosporium-artige Schimmelpilze* (hyphomycetes), *in press*). Study of this growth cycle during submerged fermentation indicated a temporal relationship between accretion of antibiotic and the conversion of hyphae into arthrospores.

To ascertain if any specific morphological form possessed enhanced capacity to synthesize antibiotic, individual cell types were isolated by density-gradient centrifugation and compared for their potential to synthesize cephalosporin C and penicillin N. Arthrospores synthesized 40% more of the antibiotics than any other morphological type.

If differentiation into arthrospores is a determining factor in antibiotic synthesis, then environmental conditions and mutational events which enhance β -lactam antibiotic synthesis

should also stimulate the formation of arthrospores. Examination of several mutant strains of *C. acremonium*, each with increased potential to produce antibiotic, revealed that antibiotic synthesis was proportional to an increased titer of arthrospores. Furthermore, supplementation of growth media with methionine enhances both the synthesis of antibiotic and the formation of arthrospores (3).

Differentiation of a nonproducing mutant, however, clearly eliminates an obligate relationship between morphogenesis and antibiotic synthesis. Therefore, the superior synthetic potential of arthrospores must be related to some metabolic characteristic which permits arthrospores to be superior to other morphological forms in the synthesis of β -lactam antibiotics but not in differentiation per se. The yeast phase of the dimorphic fungus, *Histoplasma capsulatum*, possesses a more active sulfate permease than does the hyphal phase (5). An analogous phenomenon could explain the enhanced antibiotic synthetic capacity of arthrospores.

ACKNOWLEDGMENTS

We are grateful to P. A. Lemke for providing strain *lm-6* and to Richard Pieper for excellent technical assistance.

LITERATURE CITED

1. Abraham, E. P. and G. G. F. Newton. 1967. Penicillins and cephalosporins, p. 1-16. In D. Gottlieb and P. D. Shaw (ed.), Antibiotics, vol. 2. Springer-Verlag, New York.
2. Caltrider, P. G., and H. F. Niss. 1966. Role of methionine in cephalosporin synthesis. *Appl. Microbiol.* 14:746-753.
3. Caltrider, P. G., F. M. Huber, and L. E. Day. 1968. Effect of methionine and sulfate on the metabolism of *Cephalosporium acremonium*. *Appl. Microbiol.* 16:1913-1918.
4. Dennen, D. W., and D. D. Carver. 1969. Sulfatase regulation and antibiotic synthesis in *Cephalosporium acremonium*. *Can. J. Microbiol.* 15:175-181.
5. Garrison, R. G., H. T. Dodd, and J. W. Hamilton. 1970. The uptake of low molecular weight sulfur-containing compounds by *Histoplasma capsulatum* and related dimorphic fungi. *Mycopathol. Mycol. Appl.* 4:171-180.
6. Lowry, D. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
7. Smith, B., S. C. Warren, G. G. F. Newton, and E. P. Abraham. 1967. Biosynthesis of penicillin N and cephalosporin C: antibiotic production and other features of metabolism of a *Cephalosporium* sp. *Biochem. J.* 103:877-890.