ROLE OF CARBON DIOXIDE IN THE DIMORPHISM OF *COCCIDIOIDES IMMITIS*[1]

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Converse (J. Bacteriol., **74**, 106, 1957) described a procedure for the conversion of arthrospores and mycelial fragments of strain M-11 of *Coccidioides immitis* to the spherule form. He used a defined medium containing Tamol "N," and reported maintenance of the spherule form through three serial transfers. As a result of the need for a continuing supply of spherules for studies in this laboratory, the method and medium of Converse were adapted to prolonged serial culture.

Two-hundred-ml quantities of the glucose-ammonium acetate medium of Converse containing 0.05 per cent of Tamol "N" in 1-L Erlenmeyer flasks closed with cotton plugs were inoculated with spherules at a concentration of 2.0 mg dry weight per ml. Initially the spherules were grown from arthrospores in the same medium. The cultures were incubated for 16 hr on a rotary shaker at an amplitude of 1 inch and 112 cycles per min at 34 to 35 C. Under these conditions, the density of the culture generally increased to 4.0 to 4.5 mg per ml as determined by the dry weight of material retained by an HA Millipore membrane. Transfers were made to fresh medium after the cultures were centrifuged and the supernatant medium discarded. These cultures have been maintained for as many as 160 transfers with little development of mycelium.

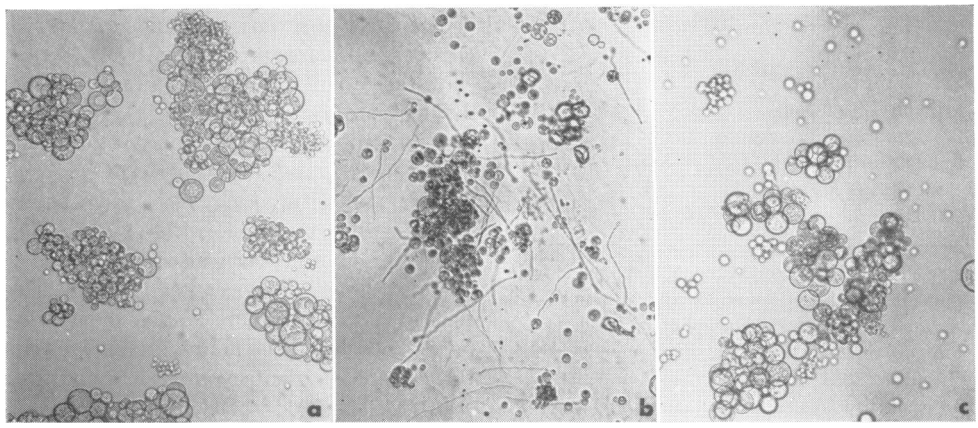

Figure 1. Effect of size of inoculum and CO_2 on morphology of *Coccidioides immitis*: *a*, 2.0 mg per ml inoculum, incubated in air; *b*, 0.2 mg per ml inoculum, incubated in air; *c*, 0.2 mg per ml inoculum, incubated in 90 per cent air-10 per cent CO_2. Magnification 153×.

It was observed that growth of the spherule form with minimal development of mycelium was favored by a large inoculum. Moreover, the development of mycelium in cultures prepared at low cell densities was suppressed by incubation in a mixture of 90 per cent air and 10 per cent CO_2. The effect of size of inoculum and added CO_2 are shown in figure 1. Tamol "N" medium was inoculated with spherules from a well established culture (120 transfers) at levels of 2.0 and 0.2 mg per ml. The cultures were distributed in 5-ml volumes in 25-ml flasks which were incubated on the shaker in air or in 220-ml sealed jars in which the gas phase was adjusted to 90 per cent air and 10 per cent CO_2 by the method of partial pressures. After 16 hr, wet mounts were prepared and

[1] Strain M-11 of *Coccidioides imitis* was supplied by Mr. John L. Converse, U. S. Army Chemical Corps, Fort Detrick, Frederick, Maryland.

TABLE 1

Effect of 10 per cent CO_2 on spherule formation by Coccidioides immitis

Spore Suspension	Age of Spores*	(No. of Spherules per ml) × 10^{-4}	
		Air	90% air, 10% CO_2
	weeks		
1	2	5.1 ± 2.7†	14.5 ± 6.9†
2	12	<0.25	32.9 ± 3.8
3	48	1.27 ± .36	10.4 ± 1.7

* Stored at 4 C.

† Confidence intervals are twice the standard error of the means.

photographed. Little or no mycelium appeared in cultures in which the inoculum was 2.0 mg per ml and incubated in air (figure 1*a*) or 0.2 mg per ml and incubated in 10 per cent CO_2 (figure 1*c*). In contrast, considerable mycelium appeared in the culture inoculated at 0.2 mg per ml and incubated in air (figure 1*b*). It seems likely that the favorable effect of the larger inoculum was due to a greater concentration of metabolic CO_2.

A similar effect of CO_2 was observed on the conversion of arthrospores and mycelial fragments to spherules in Tamol "N" medium. In table 1 are shown the number of spherules over 10 μ in diameter produced in 40 hr by three different arthrospore suspensions incubated in air and in 90 per cent air-10 per cent CO_2. Direct counts were made in a hemocytometer and 8 or more square mm were counted for each culture. Although the response of the three suspensions to CO_2 differed, the superiority of the CO_2-grown cultures is evident. These observations are in contrast to those of Converse and Besemer (J. Bacteriol., **78,** 231, 1959) who found no improvement in spherule yield in 10 per cent CO_2. The explanation may lie in differences in the size and metabolic activity of the inoculum and the volume of the cultures.

CONCENTRATION AND ISOLATION OF AUXOTROPHIC MUTANTS OF SPOREFORMING BACTERIA

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Unlike many other classes of mutants, the efficient isolation of auxotrophic mutants has necessitated the development of techniques such as the penicillin method (Lederberg and Zinder, J. Am. Chem. Soc., **70,** 4267, 1948; Davis, J. Am. Chem. Soc., **70,** 4267, 1948) and the filtration technique (Fries, Nature, **159,** 199, 1947) which have proved useful in the isolation of auxotrophs of certain microbial species. Though these mutants with specific nutritional deficiencies have proved to be valuable in genetic and metabolic studies, such studies have been restricted mainly to species whose auxotrophs can be isolated by the above methods. The need for extending them to other less well studied species and developing methods more suitable than those used so far, need hardly be stressed. Isolation of mutants of sporeforming bacteria is reported here.

Attempts to isolate auxotrophic mutants of *Bacillus subtilis* in this laboratory using the penicillin method have not proved as effective as with *Escherichia coli*. A method for concentrating auxotrophs taking advantage of the difference in thermolability of spores and vegetative cells has been developed and has proved to be more efficient than the penicillin method. A spore suspension of *B. subtilis* was irradiated with ultraviolet light, washed, and inoculated into a minimal medium of the following composition in grams per liter of distilled water (Demain, J. Bacteriol., **75,** 517, 1958): K_2HPO_4, 30; KH_2PO_4, 10; NH_4Cl, 5; NH_4NO_3, 1; Na_2SO_4, 1; $MgSO_4$, $7H_2O$, 0.1; $MnSO_4 \cdot 4H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2$, 0.005; L-alanine, 0.89; L-glutamic acid, 1.47; L-asparagine, 1.32; glucose, 10; pH 6.8 to 7.0. The glucose and the amino acids were autoclaved separately and then added. After incubating at 28 C until maximal germination occurred, the culture was placed in a boiling water bath for varying periods, depending on the strain used. Heating time was determined by