JOURNAL OF BACTERIOLOGY, Feb. 1974, p. 379-381

Vol. 117, No. 2
Printed in U.S.A.

Isolation of Germination Mutants of *Dictyostelium discoideum*

RICHARD H. KESSIN[1] AND PETER C. NEWELL
Department of Biochemistry, University of Oxford, Oxford, OX1 3QU, England

Received for publication 5 November 1973

A simple method to separate spores from amoebae of *Dictyostelium discoideum* has been devized and used to isolate spore germination mutants. A subclass of these mutants is temperature sensitive for germination and growth.

The amoebae of the cellular slime mould *Dictyostelium discoideum* aggregate when deprived of food and form spore masses supported by slender stalks. The germination of these spores is a developmental process, as is their formation from individual amoebae. The ability to separate ungerminated spores from amoebae that have germinated successfully permits the isolation of mutants conditionally blocked in spore germination. These mutants are of interest not only because they aid the study of germination, but because defects in spore germination can be attributed to errors in spore formation (6). Because germination mutants may also be temperature sensitive for growth, they may be used in the system of parasexual genetics described by Katz and Sussman (5). Early attempts to enrich for temperature-sensitive germination mutants by using heat shock to kill amoebae were unsuccessful, presumably because the spores lose their heat resistance at an early stage of the germination process. For this reason, the less severe dextrin method of separation described in this paper was tried and found to be successful.

Spores of *D. discoideum* pass into saturated dextrin suspension and sediment during centrifugation, whereas amoebae float. By using this fact, a small number of mutant spores can be recovered from a large population of amoebae that have germinated normally.

MATERIALS AND METHODS

An axenic strain of *D. discoideum*, Ax3, was used and cultured as described by Watts and Ashworth (8). Strain X12 is a recombinant strain carrying chromosomal markers for cycloheximide resistance, brown pigment formation, and the ability to grow in axenic media. Dextrin was prepared by boiling 35 g of dextrin in 100 ml of water with 0.5 ml of chloroform to sterilize the suspension. The cooled suspension was centrifuged at 5,000 × *g* for 30 min to remove sediment.

Mutagenesis was carried out by using *N*-methyl-*N'*-nitro-*N*-nitroso guanidine (NTG) dissolved just before use by shaking at a concentration of 1 mg/ml in salt solution (SS:NaCl, 0.6 g/liter; KCl, 0.7 g/liter; and $CaCl_2$, 0.3 g/liter). Amoebae growing at a density of 2 × 10^6 to 4 × 10^6/ml in axenic medium were harvested by centrifugation at 150 × *g* for 2 min at room temperature. These cells were then resuspended at 10^7/ml in 20 ml of the SS-NTG solution and shaken for 20 min on a rotary shaker at 150 rpm. After mutagenesis the amoebae were washed three times. Axenic cells had a survival rate between 25 and 40% under these conditions as shown by clonal viability tests. The mutagenized amoebae were spread with *Aerobacter aerogenes* on SM agar plates (7) and allowed to devour the bacterial lawn and form fruiting bodies. Spores were harvested onto the lid of a petri dish by hitting the inverted petri dish hard on the bench. The spore suspension was washed once, resuspended in axenic medium at 10^6 to 2 × 10^6 spores/ml, and heat shocked at 45 C for 30 min to destroy amoebae and spore germination inhibitor (4). The spores were shaken for 72 h in a 27 C water bath, during which time almost all observable spores germinated. The germination amoebae and the few remaining ungerminated spores were centrifuged and resuspended in 2 ml of 10 mM Na_2HPO_4-KH_2PO_4, pH 6.4, containing 10 mM ethylenediaminetetraacetic acid. This suspension was layered on 8 ml of saturated dextrin solution (British Drug Houses Ltd., England) in a 10-ml tube and centrifuged at 1,600 × *g* for 20 min at room temperature, after which the dextrin was removed with a Pasteur pipette, the pellet was suspended, and the separation process was repeated. All procedures were done under sterile conditions. Clones derived from individual cells of the second pellet were then grown on lawns of *A. aerogenes* on SM agar plates (7). Spores produced by each clone were then tested for their capacity to germinate by picking one spore mass from each clone into a well of a Linbro 16-mm tissue culture tray (Biocult Laboratories Ltd., Paisley, Scotland PA3 4EP) containing 0.2 ml of axenic medium. The spores were then heat shocked to kill amoebae and to stimulate spore germination (4) by sealing the Linbro tray with tape and floating them in a 45 C bath for 30 min. Many clones could be tested for temperature-sensitive germination by picking into duplicate Linbro trays which were then placed, after heat shocking at 22 or 27 C. Spore germination can be easily observed with an

[1] Present address: Division of Biological Sciences, Brown University, Providence, R.I. 02912.

inverted microscope and is complete within 24 h in the controls.

RESULTS

The ability of the method to separate spores from amoebae can be seen from the results in Table 1. Amoebae placed on a cushion of dextrin were still floating on it after 20 min of centrifugation at 1,600 × *g*, whereas the spores passed into the dextrin solution or sedimented. Centrifuging at greater speeds resulted in all the spores sedimenting but reduced the viability of the amoebae. Spores which had undergone swelling, one of the early events of spore germination, also passed into dextrin.

Table 2 shows the results of a typical experiment in which spores which were temperature sensitive for germination were selected. The results demonstrate that spores can be separated from a 100-fold excess of amoebae. Approximately 10^6 of the amoebae placed on the first dextrin solution were found in the pellet after centrifugation. These probably ran down the side of the tube when the dextrin was being removed. The number of spores remained relatively constant, although they were difficult to count accurately because of their scarcity. Some of the spores found in the pellet after recentrifugation through fresh dextrin solution were still highly refractile in the phase-contrast microscope after allowing 72 h at 27 C for them to germinate. When all of the cells in the second pellet were plated clonally on lawns of bacteria, very few retained the capacity to form clones and only 41 proved viable. Eight of these proved to be seriously defective in spore germination at 27 C, although a much higher proportion were only slightly defective in germination in this and other experiments. Of 120 viable control spores that had undergone mutagenesis but had not been subject to the dextrin procedure, none showed any inhibition of spore germination.

Table 3 gives the characteristics of several mutants isolated by the dextrin procedure. None germinates as rapidly at 27 C as the AX3 parent strain. All will germinate when brought back to the permissive temperature of 22 C, including strains NP51 and NP53 which remain spores at 27 C for long periods. Two of the temperature-sensitive germinators were also temperature sensitive for growth and did not clear lawns of *A. aerogenes* at 27 C, although they did at 22 C. Because these appear frequently, it is possible that both temperature sensitivities are the result of one mutation, although studies on revertants will be required to eliminate the possibility that two mutations are involved.

To show that the mutants described above were selected rather than isolated by chance, a reconstruction experiment was performed. NP51 spores were mixed with spores of strain X12 at a ratio of $1:1.5 \times 10^5$ and shaken in axenic medium at 27 C for 72 h to allow the X12 spores to germinate. This population of amoebae and a few spores were then centrifuged twice through dextrin solution, and the cells in the final pellet were plated on SM agar. X12 colonies can be distinguished from those of strain NP51 because they form a brown pigment and are cycloheximide resistant. Approximately

TABLE 1. *Centrifugation of spores and amoebae in dextrin solution*[a]

Cells put on dextrin	Cells observed after centrifugation	
	In supernatant (top 2 to 3 ml)	In pellet
10^6 spores	$<10^4$	3×10^5
10^6 amoebae	10^6	$<10^4$

[a] Amoebae or spores were layered on dextrin solution and centrifuged at 1,600 × *g* for 20 min. The top 2 to 3 ml of the dextrin was removed with a Pasteur pipette and examined in a hemocytometer. The remaining dextrin was removed by suction, leaving only 1 ml over the pellet which was resuspended and examined.

TABLE 2. *Separation of amoebae from spores in two centrifugations with dextrin solution*

Cells	Amoebae	Spores (approx)
Initially placed on dextrin solution	7.5×10^7	5×10^5
Observed in pellet	10^6	4×10^5
Observed in pellet after recentrifuging in fresh dextrin solution	$<10^4$	4×10^5

TABLE 3. *Mutants isolated by the dextrin technique*

Mutant	Germination at 22 C		Germination at 27 C		Growth on *A. aerogenes*	
	In 48 h	In 96 h	In 48 h	In 96 h	At 22 C	At 27 C
AX3 (control)	+	+	+	+	+	+
NP 51	−	+	−	−	+	+
NP 52	+	+	−	+	+	+
NP 53	−	+	−	−	+	+
NP 54	+	+	−	+	+	±
NP 55	+	+	−	+	+	−
NP 8	+	+	−	+	+	−

1 in 35 of the recovered cells were cycloheximide sensitive and produced no brown pigment. Thus, there was approximately a 4,000-fold enrichment for the ungerminated spores of strain NP51.

DISCUSSION

Several kinds of mutants might be isolated by the dextrin technique. We may speculate that mutations of certain enzymes might lead to defective spore germination. For example, trehalose is a major energy source for spore germination (3). Defects in trehalase or trehalose-6-phosphate synthetase, both of which are present during the development of *D. discoideum*, might lead to impaired germination. It should also be possible to isolate mutants insensitive to the spore germination inhibitor by using the technique in reverse and recovering amoebae instead of spores. The spore germination inhibitor has recently been identified as dimethylguanosine (1, 2).

An important use of the dextrin technique is the isolation of the class of mutants that are temperature sensitive for growth as well as germination, as these mutants are required for the formation of diploids in the parasexual cycle used for genetic analysis in *D. discoideum* (5).

ACKNOWLEDGMENTS

We thank Dorothy Berry for technical assistance and J. W. Van Sambeek for help with some of the initial experiments.

R. H. K. gratefully acknowledges the support of the Damon Runyon Memorial Fund for Cancer Research.

LITERATURE CITED

1. Bacon, C. W., and A. S. Sussman. 1973. Effects of the self-inhibitor of *Dictyostelium discoideum* on spore metabolism. J. Gen. Microbiol. **76**:331-344.
2. Bacon, C. W., A. S. Sussman, and A. G. Paul. 1973. Identification of a self-inhibitor from spores of *Dictyostelium discoideum*. J. Bacteriol. **113**:1061-1063.
3. Ceccarini, C. 1967. The biochemical relationship between trehalose and trehalase during growth and differentiation in the cellular slime mould *Dictyostelium discoideum*. Biochim. Biophys. Acta **148**:114-124.
4. Cotter, D. A., and K. B. Raper. 1968. Properties of germinating spores of *Dictyostelium discoideum*. J. Bacteriol. **96**:1680-1689.
5. Katz, E. R., and M. Sussman. 1971. Parasexual recombination in *Dictyostelium discoideum*. Proc. Nat. Acad. Sci. U.S.A. **69**:495-498.
6. Newell, P. C. 1971. The development of the cellular slime mould *Dictyostelium discoideum*: a model system for the study of cellular differentiation, p. 87-126. *In* P. N. Campbell and F. Dickens (ed.), Essays in biochemistry, vol 7. Academic Press Inc., London.
7. Sussman, M. 1966. Biochemical and genetic methods in the study of cellular slime mould development, p. 397-409. *In* D. Prescott (ed.), Methods in cell physiology 2. Academic Press Inc., New York.
8. Watts, D. J., and J. M. Ashworth. 1970. Growth of myxamoebae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. Biochem. J. **119**:171-174.