Growth of Myxamoebae of the Cellular Slime Mould *Dictyostelium discoideum* in Axenic Culture

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(Received 14 April 1970)

1. A simple axenic medium suitable for the growth of the myxamoebae of a strain of the cellular slime mould *Dictyostelium discoideum* is described.
2. Procedures suitable for the growth of this strain in liquid and on solid media are described.
3. Conditions suitable for initiating the cell differentiation of myxamoebae grown axenically are described.

The life cycle of the cellular slime mould *Dictyostelium discoideum* is divided into two mutually exclusive phases. In the first phase (feeding) the single-celled myxamoebae multiply by binary fission, using as food soil bacteria. In the second phase (differentiation) these hitherto solitary myxamoebae associate on a solid surface to form multicellular and macroscopic aggregates in which, after a period of time, the component cells become either the stalk or the spore cells of the mature fruiting body (Bonner, 1967). This cell differentiation of the myxamoebae can be induced at will by withdrawal of food, and thus this organism is an attractive model system for studying the biochemical events associated with cell differentiation. Such studies have been hampered, however, by the necessity of growing the myxamoebae on bacteria. Sussman & Sussman (1967) described a complex axenic medium in which *D. discoideum* myxamoebae can grow. We report the isolation of a strain of *D. discoideum* that will grow on a simpler axenic medium and describe procedures for its cultivation in liquid and on solid media.

RESULTS AND DISCUSSION

Isolation of *D. discoideum* Ax-2. Sussman & Sussman (1967) described a complex axenic medium for *D. discoideum* that contains liver extract and foetal calf serum in addition to inorganic salts, glucose, peptone and yeast extract. Dr Sussman kindly sent us his strain, which was capable of growth in this medium. Attempts were then made to eliminate the most complex component, namely foetal calf serum, from the medium. Subculture of the cells into a medium that contained only half

the recommended concentration led to poor growth at first, but after repeated subculture the doubling time and growth yield became similar to those reported for cells grown with the higher concentration of serum. The concentration of serum was then halved again and the cells were subcultured until the growth yield and rate had recovered to their previous values, and the procedure was repeated until the cells eventually had no requirement for the serum. Similarly it was possible to omit the liver extract from the medium after the cells had been adapted to growth in such a medium by repeated subculture into media containing steadily decreasing concentrations of liver extract.

This procedure probably results in the selection of a mutant strain adapted for growth in the poorer medium (Ashworth & Watts, 1970), and we term our strain selected in this fashion *D. discoideum* Ax-2.

Conditions for growth of *D. discoideum* Ax-2. Strain Ax-2 was selected for its ability to grow in a medium containing in 1 litre: Oxoid bacteriological peptone (14.3g), Oxoid yeast extract (7.15g), D-glucose (15.4g), Na₂HPO₄·12H₂O (1.28g) and KH₂PO₄ (0.486g), final pH 6.7. Growth occurs in this medium with the same doubling time of 8-9h and growth yield if the glucose is autoclaved separately and then added to the other sterile components of the medium or if the medium is autoclaved with all the components present. Schwalb & Roth (1970) have also selected a strain that will grow in a similar medium.

Glucose is not an essential component of the medium, since the myxamoebae will grow with almost the same growth rate in a medium lacking added glucose (Fig. 1). This medium is not, however, entirely free of carbohydrate, and a very small uptake of the carbohydrate can be detected during

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Fig. 1. Growth kinetics of *D. discoideum* Ax-2 myxamoebae in medium lacking added carbohydrate (O) and in glucose-containing medium (Δ). The 700 ml cultures in 2-litre Erlenmeyer flasks were shaken at 22°C in a rotary shaker and the increases in cell number were determined by haemocytometer counts.

growth of the cells (Ashworth & Watts, 1970). Addition of fructose, mannose or maltose to the glucose-free medium will increase the rate of growth of the myxamoebae and the cell yield, but other compounds that might have been expected to promote growth appear to have no effect or are toxic (Table 1). The toxicity is probably an effect of the compound added rather than a salt or osmotic effect, since addition of sodium chloride (0.1 M final concentration) has no effect on growth.

At present we use the following conditions for culturing the cells: (a) 10 ml of medium in a 100 ml Erlenmeyer flask (not shaken); (b) 60 ml of medium in a 250 ml Erlenmeyer flask (not shaken); (c) 700 ml of medium in a 2-litre Erlenmeyer flask (shaken on a rotary shaker with an eccentric rotation of 3 cm at 150–170 rev./min). Procedure (a) is useful for various experimental purposes, (b) for maintaining stocks and (c) for obtaining large yields of cells. The growth rates and yields in the 10 ml and 60 ml cultures may be increased, if required, by shaking at 100–150 rev./min. A rotary shaker has been found most satisfactory for shaking the cultures, although strain Ax-2 (but not some other axenic strains) will also grow well if shaken on a reciprocal shaker. It has also been possible to grow the

Table 1. Growth of myxamoebae with various carbon sources

The myxamoebae were grown in a medium containing yeast extract and peptone (for details see the text). Sugars were added to give a final concentration of 18 mg/ml except for cellobiose (9.8 mg/ml) and galactose (15 mg/ml). The sodium salts of the acids were added as solutions at pH 6.4–6.7 to give final concentrations of 0.1 M. Myxamoebae growing exponentially in the yeast extract-peptone medium in the absence of added sugar were added to the media to give an initial cell density of 3×10^4 cells/ml. The cultures of final volume 10.8 ml in 100 ml Erlenmeyer flasks were incubated without shaking at 22°C.

Additional carbon source	Cell density (cells/ml)	
	After 124 h	After 172 h
Glucose	2.6×10^6	3.4×10^6
Mannose	2.7×10^6	4.0×10^6
Maltose	7.7×10^6	4.7×10^6
None	2.1×10^6	2.3×10^6
Ribose	2.3×10^6	2.9×10^6
Lactose	1.3×10^6	1.6×10^6
Cellobiose	9.6×10^5	1.9×10^6
Galactose	9.6×10^5	1.6×10^6
Sucrose	9×10^5	1.5×10^6
Aspartate	4.8×10^5	7.1×10^5
Glutamate	2.7×10^5	7.8×10^5
Gluconate	2.4×10^5	3.8×10^5
Lactate	1.5×10^5	3.3×10^5
Succinate	1×10^5	1.4×10^5
Acetate	2×10^4	6×10^4

myxamoebae in a New Brunswick 5-litre continuous culture apparatus if the rates of aeration and agitation are kept to the minima permitted by the apparatus.

Care must be taken to ensure that the cells are kept at close to the optimum temperature for growth (22–23°C), since the cells only grow well in a narrow range of temperatures. Temperatures below 20°C or above 25°C can cause cessation of growth and eventually death of the cells.

Growth on solid media. Considerable difficulty was experienced in finding conditions that would allow the myxamoebae to grow on solid media, although they grow readily in a lawn of bacteria on 2% nutrient agar (Sussman, 1966). The concentration of the agar proved to be critical, and we have found that best growth occurs on media solidified with 0.5% (w/v) Ionagar (Oxoid), growth rates decreasing progressively on concentrations above 0.5%. Below this concentration the agar is so soft that it becomes difficult to handle. For convenience we pour a bottom layer of solid medium containing 2% (w/v) Ionagar, allow this to set and then pour over it a thinner layer of 0.5% Ionagar.

EXPLANATION OF PLATE I

Growth of *D. discoideum* Ax-2 myxamoebae on the surface of glucose containing medium solidified with agar (b and d) and embedded in such a medium (a and c). (a) and (b) $0.64\times$ actual size; (c) and (d) individual colonies magnified $\times 64$. Cells were stained with Nitro Blue Tetrazolium dye.

Myxamoebae (or spores) of strain Ax-2 are then spread on the surface with a sterile glass spreader. Each cell gives rise to a colony that is difficult to see, since the cells form a layer that is only one cell thick and tend to migrate from the centre (Plate 1d). The colonies can be seen more easily if a solution of 0.1% (w/v) Nitro Blue Tetrazolium dye is gently layered on top, since the colonies then stain deep purple after a few hours incubation at 22°C (Plate 1b). The colonies rarely form fruiting bodies, even if left for several weeks, but the cells will readily form fruiting bodies if they are washed off the surface of the agar with sterile water and deposited on Millipore filter-grade supports (cat. no. AABP 047 00) as described by Sussman (1966).

Owing to the tendency of the myxamoebae to migrate, it is not possible to plate them on agar surfaces at high densities and it is difficult to show that growth is truly clonal. Fortunately spores of strain Ax-2 can be suspended in molten 0.5% agar medium at 42°C and can then be poured quickly over a layer of cold solid 2% agar medium. The resulting colonies (Plate 1c) are formed in the agar layer and are much more compact than those formed during growth on the surface. Growth is rather slower in the embedded colonies, but the colonies can be seen without prior staining. Myxamoebae do not survive suspension in molten agar at 42°C and, if the same procedure is used with them, plating efficiencies are highly variable. However, mixtures of agar and gelatin (which lowers the setting temperature) or use of carrageenan (Parker, Bernstein & Green, 1968) in place of agar has enabled us to show that myxamoebae can also give rise to definite and discrete embedded colonies similar to those shown in Plate 1(c). The surface colonies can be successfully replica-plated with sterile velvet pads in the manner originally developed for bacterial colonies.

Preservation and maintenance of stocks. Sussman (1966) has recommended that spores of *D. discoideum* should be freeze-dried and be stored in the same way as freeze-dried bacteria, although this procedure in our hands often results in considerable loss of viability. It also requires expensive equipment and a phial of cells should only be used once. However, we found that spores of *D. discoideum* Ax-2 can be stored on dry sterile silica-gel crystals. This procedure has been used to store other fungal spores (Perkins, 1962; Barrat, Johnson & Ogata, 1965), and we have found, in agreement with Reinhardt (1966), that slime mould spores may be stored in this way for at least 2 years without any significant loss in viability. We use a procedure identical with that described by Perkins (1962).

It is often difficult to keep sterile complex nutrient medium such as that required for growing *D. discoideum*. Contamination of cultures by

bacteria or yeasts may be checked by plating out samples of the media at 37°C, since myxamoebae are rapidly killed at this temperature. If frequent contamination is a serious problem, streptomycin (250 µg/ml) can be added to the media without any serious effects on either the growth rates or yields.

Cell differentiation of axenically grown cells. Since cells of strain Ax-2 may be grown both axenically and on a large scale, they have obvious advantages for biochemical and genetic studies over the cells of the non-axenic strain. However, if these cells are to be used in preference to those of the non-axenic strain, it is also essential that they should differentiate and form fruiting bodies in the same way as cells grown on bacteria. Some difficulties were experienced with early axenic strains since they tended to become either phenotypically or genotypically unable to aggregate, but no such difficulties have been found with strain Ax-2.

The cells may be harvested, washed with water at 0°C and then placed as aqueous suspensions on Millipore filter supports and allowed to form fruiting bodies as described by Sussman (1966). The pattern of differentiation then seems to depend on the conditions in which the cells have previously been grown and on the number of cells on the Millipore supports (0.8 µm pore size, 47 mm diameter, cat. no. AABP 047 00).

(a) At a density of 10^8 cells/Millipore support, all cells grown axenically in the complex medium containing fructose or glucose will form fruiting bodies. The differentiation tends, however, to be asynchronous and many of the fruiting bodies have an abnormal appearance.

(b) Cells grown axenically in the complex medium containing no added sugar rarely form fruiting bodies when at a density of 10^8 /Millipore support, but, after the formation of the first finger and slug stages, tend to regress into undifferentiated clumps of cells or to form what appear to be the stalks of fruiting bodies having no spore masses.

(c) Fruiting bodies are formed from all the cells if only 2.5×10^7 cells are placed on the Millipore supports even if the cells have previously been grown in the absence of glucose or fructose. The synchrony of the differentiation is improved at this cell density for myxamoebae grown on glucose or fructose and few aberrant fruiting bodies are detectable. It would thus appear that it is preferable to use for the axenic strain of cells a lower cell density on the Millipore supports than is usually used for cells grown on bacteria (10^8 cells/Millipore support).

The time-course for the appearance of the various stages involved in the formation of the fruiting bodies is slightly different for the strain Ax-2 from that for *D. discoideum* NC-4. The first 'fingers' are formed 12–13 h after the myxamoebae

have been placed on the Millipore supports and the 'slugs' appear at 15h. These further differentiate into second 'fingers' by 18h and the fruiting bodies are almost complete at 20h. Thus the fruiting bodies are formed about 3-4h earlier than in *D. discoideum* NC-4.

(d) The axenic strain of cells will also grow on bacteria, although not as rapidly as *D. discoideum* NC-4, and the synchrony of the differentiation of such cells is satisfactory when 10^8 cells are placed on each Millipore support. When the cells are first grown on bacteria, the fruiting bodies formed from them have stalks that are clearly much shorter than those of the non-axenic strain, but the fruiting bodies can become more like those formed from the non-axenic strain of cells if they are formed from cells that have been grown on bacteria for many generations. However, the cells have been found to retain their ability to grow axenically even when they have previously been grown for many months in association with *Aerobacter aerogenes*.

Any tendency for myxamoebae of the axenic strain not to differentiate well may usually be overcome by growing the cells clonally on bacteria and starting new axenic cultures from the spores of a

clone of cells that forms good fruiting bodies. This procedure should, however, only be necessary at rare intervals for *D. discoideum* Ax-2.

We thank Mrs Janet Quance and Miss Kathryn Warrington for technical assistance and the Science Research Council for financial support.

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