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Growth and Cloning of *Tetrahymena pyriformis* on Solid Medium

ENORE GARDONIO, MICHAEL CRERAR, AND RONALD E. PEARLMAN
Department of Biology, York University, Downsview, Ontario, Canada M3J 1P3

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A method of obtaining clones of *Tetrahymena pyriformis* on solid medium has been developed. The medium consists of a basal layer of 1.5% agar topped with 2 ml of 0.3% agar in sterile, plastic petri plates (100 by 15 mm). Both agar layers contain either 2% proteose peptone and 0.1% liver extract (complex medium) or defined medium supplemented with proteose peptone. After drying, 0.5 ml of liquid culture is spread evenly over the top agar, and the plates are then sprinkled lightly and evenly with autoclaved dry Sephadex G-25 (fine). Cell colonies can be observed after 5 days of incubation either by viewing with a microscope or without the aid of a microscope after staining. Plating efficiency is high on either complex or defined medium with a number of strains of *Tetrahymena*, both micronucleate and amicronucleate. Colonies can be picked and transferred to liquid culture for further growth. The existence of clones was demonstrated by plating a mixture of two different drug-resistant mutants. The method should prove useful in selective procedures for the isolation of mutants and for determining survival after treatments such as ultraviolet irradiation.

Tetrahymena pyriformis is a ciliate protozoan that can be readily cultured axenically in liquid culture. *Tetrahymena* can be manipulated for the most part with standard microbiological techniques. *Tetrahymena* has a doubling time of 2.5 h in complex medium and between 5 to 7 h in defined medium. Cells can be grown in large batches with a high yield (6) and can be synchronized by a variety of procedures (3, 10, 12, 14-16, 19-21). For these reasons, this organism has become the subject for many biochemical, morphological, and cytological studies. The biochemistry and physiology of *Tetrahymena* has been reviewed in a recent monograph (9).

Genetic analysis of *Tetrahymena* has not progressed as rapidly as other studies with this organism. Reasons for this are the complexity of gene transfer and expression in *Tetrahymena* and the difficulty in isolating clones of *Tetrahymena* because of the inability of this organism to divide on solid medium without coalescing. Recent progress has been made in the selection of drug-resistant and temperature-sensitive mutants of *Tetrahymena*, all operations being performed in liquid culture (13; C. T. Roberts and E. Orias, Exp. Cell Res., in press).

We have recently been studying the biochemistry of nucleic acid metabolism in *T. pyriformis* (6, 11). We hope to be able to correlate our studies in vitro with function in vivo by isolating mutants blocked in the ability to synthesize deoxyribonucleic acid, ribonucleic acid, or both, and also by isolating mutants with an altered response to agents such as ultraviolet irradiation and drugs such as methyl methane sulfonate and α-amanitin. To facilitate selection of such mutants, we have developed a method for the cloning of *Tetrahymena* on solid medium. This report describes the method. We show that the ability to clone *Tetrahymena* on solid medium can be useful for experiments such as determining survival after ultraviolet irradiation and in selection procedures for the isolation of mutants.

MATERIALS AND METHODS

Strains. The following strains of *T. pyriformis* were used in this study: the amicronucleate strain, GL-C; the micronucleate strains, HSM and B-7, kindly supplied by M. Gorovsky, University of Rochester; a chloramphenicol-resistant mutant of syngen 1, CA-103 (13); and a cycloheximide-resistant mutant of syngen 1, CHX-F3 (C. T. Roberts and E. Orias, in press). Both drug-resistant mutants were isolated and supplied by E. Orias, University of California, Santa Barbara.

Media. Stocks of all strains were maintained in the exponential phase of growth at 28 C, in sterile liquid medium containing 2% proteose peptone (Difco) and

0.1% liver extract L (Nutritional Biochemical Co.) (complex medium). Solid medium was prepared in sterile, plastic petri dishes (100 by 15 mm) and contained complex medium in 1.5% agar topped with 2 to 3 ml of complex medium in 0.3% agar (Difco). Plates also contained 250 μg each of penicillin (Sigma) and streptomycin sulfate (Sigma) per ml. Plates containing the defined medium of Elliott et al. (7), supplemented with 0.04% proteose peptone in agar as above, were also prepared.

Application of Sephadex. Sephadex G-25, fine, was purchased from Pharmacia. Sephadex was applied to the plates from a glass jar closed with a metal cap of diameter 4.5 cm. Small holes, approximately 0.5 mm, were drilled through the cap concentrically from the center of the cap. The capped jar containing the Sephadex was autoclaved prior to each use. The jar was then inverted and clamped at a height to give optimal spreading of the Sephadex on a plate. This height was determined by trial before each experiment. In most of our experiments, the Sephadex jar was clamped 36 cm above the plate. The Sephadex was released by gently tapping the bottom of the jar.

Ultraviolet irradiation of Tetrahymena. Cells growing exponentially in complex medium (8×10^4 to 10^5 cells/ml) were collected by centrifugation ($2,000 \times g$ for 4 min) and then suspended at a concentration of approximately 2×10^4 cells per ml in inorganic medium (2.75 g of NaCl, 0.25 g of $MgSO_4 \cdot 7H_2O$ made up to 1 liter with 0.005 M potassium phosphate buffer, pH 6.5) (8). A 10-ml amount of cells was irradiated with gentle agitation in uncovered petri dishes (100 by 15 mm). Irradiation was carried out with a General Electric germicidal lamp with maximal output, at 253.7 nm, of intensity 400 ergs per mm^2 per min. After the appropriate dose was administered, 0.1-ml samples of the irradiated cells were removed and added to 10 ml of liquid complex medium. Cells were incubated in the dark for 45 min, and then three samples of 0.5 ml (approximately 100 cells per sample) were plated onto solid complex medium. The plates were incubated in the dark at 28 C for 6 days before colonies were counted as described below.

Observation of colonies. Colonies were observed by viewing the unstained plate with a dissecting microscope. Plates were stained essentially by the method of Chu (5). Methanol (3 ml) was layered on top of the agar for 15 min. The methanol was carefully removed, and 3 ml of stain (2.5 ml of Giemsa stain [Fisher Scientific Co.], 3.0 ml of methanol, and 94.5 ml of water) was gently poured onto the agar. Colonies could be observed within 5 min. After 10 min, the dye was removed and colonies were counted by using a colony counter (American Optical Corp.).

RESULTS

Development of the plating method. Early attempts in our laboratory to clone *Tetrahymena* on solid medium involved the use of 1.5% agar plates topped with soft agar of 0.3 to 0.7%. The *Tetrahymena* were either included in the soft agar and then layered on the hard agar, or they were spread on top of the soft agar in a small volume of medium. In either case, as soon as the soft agar hardened or the liquid in which the *Tetrahymena* were layered on the plate dried, the cells became trapped on top of the soft agar. The trapped *Tetrahymena* became round and did not divide. While the plates were still wet, the *Tetrahymena* swam over a large area of the plate, and some cells may have divided before the liquid dried out. The problem thus became how to keep single cells in an environment where they could remain motile enough to allow division yet be trapped so that they could not migrate across the plate. This was accomplished by sprinkling Sephadex G-25 on top of plates containing medium in 1.5% agar topped with 0.3% soft agar.

The plates, without Sephadex, were dried, after being poured, for 2 days at 37 C or at room temperature for at least a week. *Tetrahymena* were pipetted on top of the agar in a 0.5-ml volume of medium. Cells were distributed uniformly by tilting the plate. Sephadex was sprinkled evenly on top of the plate before the medium was absorbed by the dry agar and before the *Tetrahymena* were trapped on the dry surface of the agar and were unable to swim to the Sephadex beads. The plates were placed in a plastic bag to minimize evaporation and incubated inverted at the appropriate temperature. After incubation for 5 days, colonies could be observed and counted before staining (Fig. 1) or after staining with Giemsa stain (Fig. 2). The

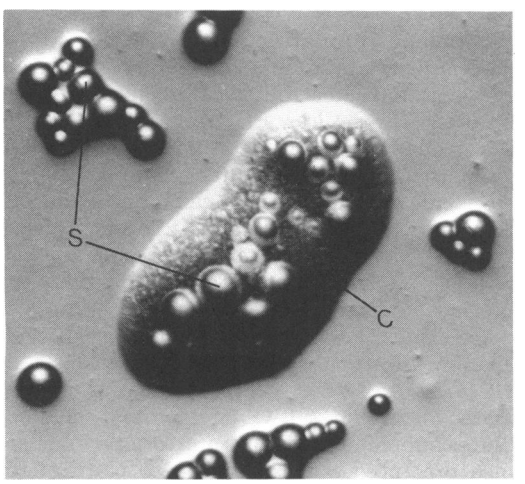

FIG. 1. *Photomicrograph of a colony of T. pyriformis amicronucleate strain GL viewed under a dissecting microscope (SMXXB, Carl Zeiss JENA). Magnification is approximately 200 times. Approximately 100 cells were plated on top of agar in complex medium and incubated for 6 days at 28 C. S, Sephadex beads; C, cells in a colony surrounding Sephadex beads.*

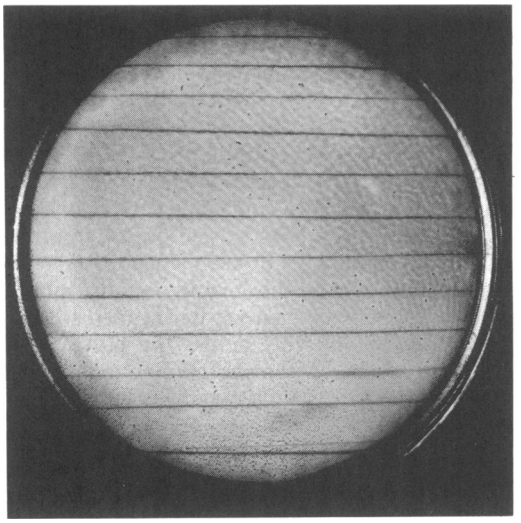

FIG. 2. *Colonies of T. pyriformis strain GL viewed after being stained with Giemsa as described in Materials and Methods. Approximately 116 cells of T. pyriformis GL were plated onto agar in complex medium, incubated for 5 days, and then stained with Giemsa. The small grains (lighter in the middle and gradually darker towards the outside of the plate) are Sephadex beads. Colonies can be seen as mostly irregular-shaped spots, larger and darker than the Sephadex beads. Parallel lines were drawn in pencil on the back of the plate to aid in counting.*

stained colonies were well separated and could be unambiguously counted with a colony counter.

Efficiency of plating. Plating efficiency was determined by adding a known number of cells (determined electronically with a Coulter Counter B, Coulter Electronics Co.) to the plates and then counting colonies after 5 days of growth either before or after staining. Because electronic determination of cell number is accurate only to approximately ±2% and because cultures were diluted after determining cell number electronically but before plating, the plating efficiencies we calculated are approximate values. Table 1 shows the plating efficiencies of wild-type *T. pyriformis* strains GL-C, HSM, and B-7 on complex medium plates. Data for strain GL-C on minimal medium plates supplemented with proteose peptone are also presented. Determination of plating efficiency does not depend on whether colonies are counted before or after staining.

Viability of colonies. Colonies can be picked from unstained plates with a wire loop, wire probe, or sterile toothpick and transferred to liquid medium for growth. Data for colonies picked at random with a wire loop from plates of a number of strains of *Tetrahymena* are presented in Table 1. Care must be taken in picking colonies for transfer to liquid medium since operation is performed while viewing the plate with a microscope. Unless a micromanipulator is used some "mis-picking" can occur. Mis-picking rather than the existence of nonviable colonies probably is the reason for low apparent viability in some experiments.

Colonies are clones. To prove that the colonies we observe on plates are clones derived from single cells, we plated a mixture of drug-resistant syngen 1 mutants. The mutants used were strain CA-103, resistant to 250 μg of chloramphenicol per ml, and strain CHX-F3, resistant to 10 μg of cycloheximide per ml. Plates containing only one of the above mutants were included in the experiment as controls. After incubation for 5 days, colonies were picked at random from the plates before being stained and incubated in 2 ml of liquid medium. Samples from tubes that showed growth after 48 h were transferred to liquid medium containing either chloramphenicol (250 μg/ml) or cycloheximide (10 μg/ml). After incubation for 2 days, the drug-containing media were scored for growth. Table 2 shows the results of one such experiment. Of 30 colonies that grew in drug-free liquid medium, only one grew in both cycloheximide- and chloramphenicol-containing medium. The other 29 colonies grew in either medium plus chloramphenicol or in medium plus cycloheximide, but not in both. This suggests that these colonies are clones arising from single, mutant cells. The reason for the preponderance of cycloheximide-resistant colonies picked may simply be attributable to random picking or may be because of the slow growth rate of CA-103 (13). Colonies picked from plates containing a single mutant cell type and not a mixture of the two mutants grew only in medium containing the drug to which they were resistant.

Drug sensitivity selection on plates. To demonstrate that this technique for plating and cloning can be used in selective procedures for the isolation of mutants, we observed the growth of various mutant strains of *Tetrahymena* on selective medium. The mutant strains used had been selected in liquid culture (13; C. T. Roberts and E. Orias, in press). Strain CA-103, resistant to 250 μg of chloramphenicol per ml, was plated on solid complex medium containing 250 μg of chloramphenicol per ml and on plates containing 10 μg of cycloheximide per ml. Drugs were generally included in the agar and were added to the agar by filtration through sterile membrane filters (Sartorius, 0.2 μm). Table 3

TABLE 1. *Efficiency of plating Tetrahymena pyriformis on solid media containing Sephadex*

Strain	Temp (C)	Agar medium	Dissecting microscope				Giemsa staining				No. of colonies picked from plates	No. of colonies which grew in liquid complex medium
			Colonies/plate	Mean	Standard deviation	Plating efficiency (%)	Colonies/plate	Mean	Standard deviation	Plating efficiency (%)		
GL	28	Complex	88 65 57 90 105 79 41 120	81	±25	70	95 56 51 81 92 113	81	±24	70	10	9
GL	28	Defined plus proteose peptone (0.04%)	45 38 36	40	±5	29	45 37 35	39	±5	29	10	6
HSM	28	Complex	32 70 46 53 37 53	49	±14	53	27 62 37 45 32 45	41	±12	44	10	10
B-7[a]	32	Complex	64 55 61 80 87	69	±14	70	62 56 61 78 82	68	±11	69	10	9

[a] This strain shows some tendency for cells to migrate from colonies surrounding Sephadex beads. Some colonies have been observed not surrounding beads. To prevent this apparent migration, extra care in drying the plates must be used. Also, Sephadex should be spread very sparsely so that the distance between beads is greater than with the other strains.

TABLE 2. *Demonstration that colonies on plates arise from single cells (experiments done with complex medium at 28 C)*

Strain	No. of colonies picked	No. of colonies that grew in drug-free liquid complex medium	No. of colonies that grew in liquid complex medium containing cycloheximide (10 μg/ml)	No. of colonies that grew in liquid complex medium containing chloramphenicol (250 μg/ml)	No. of colonies that grew in liquid complex medium containing cycloheximide (10 μg/ml) and in liquid complex medium containing chloramphenicol (250 μg/ml)
CA-103 plus CHX-F3[a]	40[b]	30	21	8	1
CHX-F3	5[c]	4	4	0	0
CA-103	5[d]	5	0	5	0

[a] Approximately 46 CHX-F3 and 41 CA-103 were plated on each of five plates.
[b] Colonies were picked at random from three different plates.
[c] Colonies were picked at random from plates onto which approximately 93 cells per plate had been added.
[d] Colonies were picked at random from plates onto which approximately 103 cells per plate had been added.

shows that strain CA-103 grows on plates containing chloramphenicol but not on plates containing cycloheximide. A similar experiment done with strain CHX-F3, a mutant resistant to 10 μg of cycloheximide per ml, shows that growth occurs on plates containing cycloheximide and not on plates containing chloramphenicol (Table 3). Wild-type cells do not grow on plates in the presence of either chloramphenicol (250 μg/ml) or cycloheximide (10 μg/ml). This experiment demonstrates that strains carrying genetic markers involved with drug resistance behave the same when grown on solid or liquid medium. We conclude that isolating clones on solid medium containing selective agents can be used in selection procedures for the isolation of mutant strains.

Sensitivity to ultraviolet irradiation. *Tetrahymena* is an organism much used in radiation biology studies (18). It is quite resistant to ultraviolet irradiation (2), but exact inactivation kinetics have been difficult to obtain, in part because of the necessity to use indirect methods (1, 4). Figure 3 shows a dose-response curve for *T. pyriformis* GL-C obtained by plating irradiated cells on solid complex medium. The mean lethal dose is 350 ergs/mm². These data are in qualitative agreement with data obtained using indirect measurements (1, 2, 4). It is difficult to compare the mean lethal dose values quantitatively because of differences in strains used and in the stage of the cell cycle analyzed (2). This technique can be expanded for use with other inactivation techniques (e.g., X ray, methyl methane sulfonate) and can also be very valuable in selection, screening, and comparison of mutants with an altered response to ultraviolet irradiation.

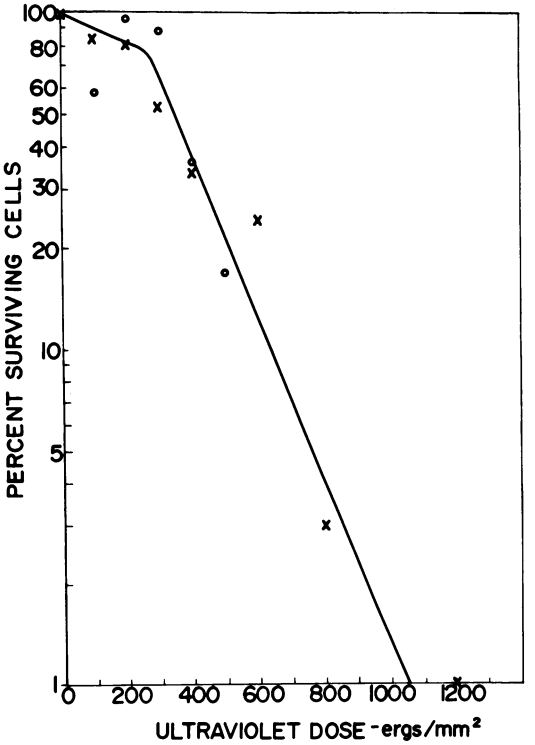

FIG. 3. *Ability of T. pyriformis amicronucleate strain GL to form colonies after treatment with varying doses of ultraviolet irradiation. Irradiation, plating, and growth of cells were as described in Materials and Methods. Percent survival represents the percentage of cells which form colonies on solid complex medium after 5 days of incubation in the dark at 28 C. Each symbol (×, O) represents a separate experiment. At least three plates were counted after staining with Giemsa to obtain the data for each dose in each experiment.*

TABLE 3. *Growth on plates containing cycloheximide or chloramphenicol*

Strain	Medium	Temp (C)	No. of colonies on plates containing cycloheximide (10 μg/ml)	Mean	No. of colonies on plates containing chloramphenicol (250 μg/ml)	Mean
CHX-F3[a]	Complex	28	95	79 ± 11	0	
			73		0	
			69		0	
			80		0	
CA-103[b]	Complex	28	0		70	78 ± 11
			0		93	
			0		75	
			0		73	
			0		80	

[a] Approximately 105 *Tetrahymena* (cell number electronically determined) added to each plate.
[b] Approximately 100 *Tetrahymena* (cell number electronically determined) added to each plate.

DISCUSSION

West et al. (17) have described an agar layer method for determining the activity of diverse materials against selected protozoa. The problem of restricting the organisms adequately without inhibiting growth was not completely solved for *T. pyriformis* by these workers. We have found that on a wet agar plate colonies can be observed. These colonies arise, however, from many cells which divide and swim to coalesce around a small number of cells. The colonies do not arise from division of a single cell.

We chose to sprinkle Sephadex beads on the plates to aid in restricting the movement of cells while still allowing them to remain motile and divide. The reasons for this choice were as follows. Dry Sephadex beads take up liquid and produce a liquid environment around each bead. If the Sephadex is sprinkled uniformly and if the agar is dry before adding the cells, most of the liquid in which the cells are added will be taken up by the agar and by the Sephadex, and interbead spaces should remain dry, thus preventing the *Tetrahymena* from swimming to adjacent beads. Thus, single cells are localized around beads in a liquid environment with sufficient nutrients to allow for motility, growth, and division. The cell suspension added to the agar must contain few enough cells so that colonies are sufficiently far apart and do not coalesce when they grow. We have found that plating between 100 and 150 cells per plate results in maximal plating efficiency.

The Sephadex beads are heavy enough to make a slight indentation on the surface of the 0.3% agar. This allows for a larger volume of liquid to surround each bead than would be observed if the beads were added directly to 1.5% agar where no indentation on the surface occurs. This, in turn, allows the colonies to grow to a large size.

Sephadex must be applied to the plate before the liquid in which the *Tetrahymena* are added to the plate becomes completely absorbed by the agar. Trapped cells cannot swim to the beads. The Sephadex beads would essentially have to hit the *Tetrahymena* for the cells to reach the liquid area around the beads. A wet plate allows the cells to swim to a neighboring bead as the liquid becomes absorbed by the agar. The absorption of liquid by the beads almost certainly helps the *Tetrahymena* to reach a bead. Plating efficiency is probably determined mainly by the dryness of the plate when the Sephadex is added and by the uniformity of spreading of the Sephadex.

The method we have described enables a number of strains of *T. pyriformis*, both micronucleate and amicronucleate, to be grown and manipulated on solid medium in petri plates. Clones can be isolated and selections can be performed. We have been unable so far to replica plate colonies. Colonies must be transferred from plates to liquid medium in microtest plates or in tubes for further manipulation. The method makes possible the use of the standard techniques of the microbial geneticist with *T. pyriformis*. We are at present using this method to aid in the selection of mutants of *Tetrahymena* altered in deoxyribonucleic acid and ribonucleic acid metabolism.

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