Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 8818–8821, September 1992
Microbiology

High-efficiency clonal growth of bloodstream- and insect-form *Trypanosoma brucei* on agarose plates

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Communicated by William Trager, June 22, 1992

ABSTRACT **This report describes a method for growing both bloodstream- and procyclic-form *Trypanosoma brucei* as colonies on agarose plates. Procyclic colonies, which took 2 weeks to develop, grew with approximately 17% plating efficiency on SDM-79/0.65% agarose supplemented with 20% (vol/vol) conditioned medium. Bloodstream forms were adapted to *in vitro* growth in liquid HMI-9 medium and then spread on HMI-9/0.65% agarose plates, where they grew to visible colonies in 3–5 days. Plating efficiencies were from 3 to 80%, depending upon the trypanosome variant and experiment. Colonies were proven to be the result of growth from a single cell and contained approximately 10^6 cells at maturity. Colonies were transferred to filters and probed for multicopy and single-copy genes. Potential uses of this method in conjunction with classical and reverse genetic approaches to studying trypanosomes are discussed.**

African trypanosomes are of substantial medical and economic importance because they produce debilitating disease in humans (sleeping sickness) and livestock (nagana) throughout a large portion of sub-Saharan Africa. These parasitic protozoa have also attracted considerable attention from scientists because of their biochemical and genetic characteristics such as multicistronic transcription, trans-splicing of precursor mRNA transcripts, use of trypanothione instead of glutathione, glycosyl-phospholipid anchoring of membrane proteins, an unusual mitochondrial (kinetoplast) DNA structure, and RNA editing (for recent reviews, see refs. 1–5). Additionally, African trypanosomes are famous for their talent for using virtually limitless antigenic variation as a means of surviving the host immune response (6). African trypanosomes have a digenetic life cycle, which includes growth in the bloodstream and tissue spaces of a mammalian host and several distinct developmental stages, including the procyclic form, in an insect host (*Glossina* spp., tsetse fly). Both bloodstream and procyclic forms are highly motile by virtue of a single flagellum, which emerges near the posterior end of the cell and is fused to the cell membrane along its length. Interest in investigating trypanosomes under controlled environmental conditions has spawned several studies describing *in vitro* culture of both forms of the parasite in semi-defined liquid medium (7–15). Procyclics have traditionally been easier to maintain in *in vitro* culture and thus are occasionally referred to as the "culture form." On the other hand, until recently (12–15), bloodstream forms were often difficult to grow in long-term axenic culture.

The ability to grow microorganisms as colonies on solid medium has been enormously useful in genetic and biochemical studies because it provides an expedient way of generating and analyzing clonal populations. Simpson *et al.* (16) and Lee and Van der Ploeg (17) were able to grow procyclics on agarose plates but the methods were limited by low plating efficiencies. Here, we describe a modification of the latter method that produces a substantially higher plating efficiency of procyclics. In addition, we describe the discovery that bloodstream-form trypanosomes are also able to grow rapidly and with high efficiency as colonies on agarose plates and we demonstrate that they can be genetically screened after growth in this manner.

METHODS AND MATERIALS

Trypanosome Stocks. Trypanosomes used in this study were *Trypanosoma brucei* variants MiTat (Molteno Institute Trypanozoon antigen type) 1.2, Mitat 1.4, and MiTat 1.5 (clones 221, 117, and 118, respectively) from strain 427 (18). Also used was a clone designated STIB (Swiss Tropical Institute, Basel) 366E (strain S42), which was isolated in 1966 from a female warthog in Tanzania (19) and was cloned as a metacyclic taken directly from an infected tsetse salivary gland (kindly provided by L. Jenni, Swiss Tropical Institute, Basel). It has since been propagated through a total of eight animals (mice or rats) with intermittent cryopreservation. Prior to use in this study, all of these cell lines were recloned by limiting dilution *in vitro* and checked by immunofluorescence using variant-specific antibodies to ensure expression of the correct variant surface glycoprotein (VSG; except STIB 366E, for which variant-specific antibodies are unavailable).

Adaptation of Bloodstream Forms to Liquid Culture. Trypanosomes were grown in either mice or rats to subsaturation density (1–5 × 10^8 cells per ml of blood) and adapted to grow in HMI-9 medium essentially as described (12). Briefly, 1 ml of infected blood [collected with 0.2 vol of citrate glucose anticoagulant (0.1 M sodium citrate/0.04 M glucose, pH 7.7)] was diluted in 9 ml of HMI-9 medium and then centrifuged at 200 × *g* for 5 min at room temperature. The supernatant (containing trypanosomes) was transferred to a T-25 culture flask and incubated upright at 37°C for 2 hr to allow the majority of the remaining blood cells to settle. Trypanosomes in the supernatant were then used to initiate 5-ml cultures in HMI-9 at a density of 1.2 × 10^6 cells per ml. Cultures were fed daily by adding 2.5 ml of fresh medium after removing an equal volume. In the initial stages, many cells died. Once the trypanosomes began to divide rapidly (when densities recovered to 10^6 cells per ml), cultures were routinely diluted 1:10 to 1:20 daily to maintain densities in the range of 10^5–10^6 cells per ml.

Preparation of Agarose Plates and Growth of Colonies. Agarose plates were prepared using 0.65% agarose as suggested by Lee and Van der Ploeg (17). To achieve the desired final concentration of ingredients, it was necessary to prepare and then mix 2× solutions of medium and agarose. 2× HMI-9 medium was prepared by dissolving 5.31 g of powdered Iscove's modified Dulbecco's medium (IMDM, GIBCO/BRL) and 0.907 g of sodium bicarbonate in 92 ml of low-conductivity water followed by the addition of 40 ml of

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Abbreviation: VSG, variant surface glycoprotein.

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heat-inactivated fetal bovine serum, 40 ml of Serum Plus (both from JRH Biosciences, Lexanarks), and 4 ml of each of the following 100× solutions: 100 mM (13.6 mg/ml, in 0.1 M NaOH) hypoxanthine, 5 mM (2.82 mg/ml) bathocuproine sulfonic acid (disodium salt), 150 mM (18.2 mg/ml) cysteine, 20 mM (1.4 μl/ml) 2-mercaptoethanol, 100 mM (11.0 mg/ml) pyruvate, 16 mM (3.9 mg/ml) thymidine, and penicillin (10,000 units/ml)/streptomycin (10 mg/ml) solution. This mixture was then equilibrated to 50°C in a water bath. The 2× SDM-79 medium (containing 40% conditioned medium) was prepared by dissolving 7.33 g of powdered SDM-79 (JRH Biosciences) in 83 ml of H_2O followed by addition of 32 ml of fetal bovine serum, 1.15 ml of hemin (2.5 mg/ml in 50 mM NaOH), and 80 ml of conditioned SDM-79 medium (conditioned by growth of procyclics to late logarithmic phase; about 10^7 cells per ml). After adjusting the pH to 7.4 with NaOH, the medium was filter-sterilized and warmed to 50°C in a water bath. A 2× agarose solution was prepared by suspending 2.6 g of agarose (type VII low melting temperature, Sigma) in 200 ml of distilled water. After autoclaving, the molten agarose was cooled to 50°C and then mixed with either the 2× HMI-9 medium or 2× SDM-79 medium, and 20-ml portions were transferred to 100 × 15 mm Petri dishes. The agarose was allowed to set for 30 min. Plates were used immediately or stored at 4°C for up to 1.5 months. Prior to use, plates were dried with the lids off in a laminar-flow hood for 1 hr (1.4 g of H_2O evaporated). Trypanosomes were diluted in HMI-9 or SDM-79 to desired densities and 100 μl was gently spread over each plate with a glass spreader (flame-sterilized after immersion in 70% ethanol). Plates were further dried for 15 min and incubated either in a 37°C humid CO_2 incubator (bloodstream forms) or at 27°C without CO_2 (procyclics) until colonies appeared.

We have recently devised a simplified procedure, not used in this study, for preparing the agarose plates: mix 10 parts of 1× medium, warmed to 50°C, with 1 part of 6.5% agarose, also equilibrated to 50°C. Although this results in a medium that is 10% diluted, approximately 7% of the moisture evaporates during drying, thus bringing the osmolarity back to near physiological level. Plates prepared in this manner yield plating efficiencies equivalent to plates prepared with 2× solutions.

Colony Lifts and Hybridizations. Approximately 100 bloodstream-form trypanosomes were spread on each agarose plate. Plates were incubated at 37°C in a humid CO_2 incubator for 4–4.5 days to allow growth of colonies. Plates were then chilled at 4°C for 2 hr after marking the position of each colony on the bottom. Duralose membranes (Stratagene) were briefly wetted on a blank HMI-9/agarose plate and then carefully overlaid onto the colonies. After adsorption for 1 min, a needle was used to punch holes through the membrane and agarose for orientation. Each filter was then rapidly peeled from the agarose and immediately placed on one layer of Whatman 3MM paper, soaked in denaturation solution (0.5 M NaOH/1.5 M NaCl), for 5 min, followed by transfer to another piece of Whatman paper, soaked in 3 M NaOAc (pH 5.2), for 5 min. After drying at room temperature for 30 min, DNA was cross-linked to the filters by exposure to 120 mJ of UV radiation. Probes used for hybridization included (*i*) a 644-base-pair *Acc* I–*Sac* II fragment of the *T. brucei* β/α tubulin intergenic region excised from plasmid pEB1NBA (P. Hevezi and G.A.M.C., unpublished data), (*ii*) a 803-base-pair *Hin*dIII fragment including the 5′ variable region of the 221 VSG expression linked copy gene extricated from pTgB221.1 (kindly provided by P. Borst, Netherlands Cancer Institute, Amsterdam) (20), and (*iii*) a 698-base-pair *Fsp* I–*Aat* II fragment from pGEM-4 (Promega) used as a control. Each fragment was gel-purified and recovered using a Prep-A-Gene kit (Bio-Rad). Probes were random-primer-labeled using [α-^{32}P]dCTP, purified from unincorporated nucleotides, and hybridized to filters using a Prime-It gold kit, NucTrap push columns, and QuikHyb solution, respectively, according to the manufacturer's instructions (Stratagene). Hybridization was carried out at 68°C for 2 hr followed by two 15-min washes at 20°C in 2× standard saline citrate (SSC)/0.1% SDS and one 30-min wash at 60°C in 0.1× SSC/0.1% SDS.

RESULTS AND DISCUSSION

Growth of Bloodstream-Form Colonies. Three variants from strain 427 (117, 118, and 221) and one clone from strain S42 (366E) were adapted to axenic culture conditions in HMI-9 medium. After initiating primary cultures, a period of adaptation was observed, characterized by slow growth for 7–16 days (depending on the variant). After this lag, the cultured trypanosomes exhibited population doubling times of 8–10, 7–10, 6–7, and 7–9 hr for variants 117, 118, 221, and 366E, respectively. As revealed by an indirect immunofluorescence assay using variant-specific antibodies, variants did not switch antigenic types during the adaptation period. However, consistent with the results of others (21, 22), over the course of several months, switching occurred at low levels in cloned populations.

Since the trypanosomes were growing well in liquid culture, we decided to attempt to grow them on semi-solid agarose plates prepared with HMI-9 medium. In the seminal experiment, 100 variant 221 cells were spread on several HMI-9/0.65% agarose plates and incubated at 37°C. After 3.5 days, small opaque colonies were visible with the unaided eye. After a further 1-day incubation, these developed into mostly spherical but sometimes irregularly shaped white colonies 1–2 mm in diameter (Fig. 1*A*). Microscopic examination (Fig. 1 *B–D*) showed that each colony consisted of two regions: a saturation-density core region and a peripheral region of tapering density in which individual cells could be distinguished. Cells in the colony are in constant movement and the core region consists of a swarming mass of cells. Despite the high level of motility, individual cells were rarely seen migrating further than 100–200 μm away from the edge of the colony. Furthermore, satellite colonies generated from straying cells were rarely observed. The first colonies grown of variant 366E were mainly "loose" colonies intermixed with a few moderately tight colonies. However, after transferring cells from one of the moderately tight colonies to a fresh plate, only tight colonies resulted. This suggests that the ability to form high-density tight colonies is a selectable trait.

To investigate the stability of the culture-adapted phenotype, culture-adapted 118 trypanosomes were grown in mice for 3 days until subsaturation parasitemias were attained. They were then used to initiate liquid cultures and spread on plates for growth of colonies. The trypanosomes continued to grow rapidly in liquid culture without a lag period and formed colonies with high plating efficiency. Thus these results suggest that the trypanosomes were able to retain their culture-adapted phenotype after growth in at least one animal. Ramifications of this include the possibility of monitoring genotypic or phenotypic changes in the parasite throughout the course of infection by screening colonies (see below).

High plating efficiencies (>50%) were obtained in the initial plating experiments. This provided some evidence that a colony could be generated from a single cell. This was later proven when single cells of culture-adapted 221 trypanosomes were spotted on a plate and their growth was monitored quantitatively over time (Fig. 2). Interestingly, no lag period was detected; cells began to divide immediately at the same doubling time as in liquid culture (≈6 hr). They continued to divide rapidly until they reached just over 10^6 cells per colony on day 5. After a very short stationary phase, viability began to decline swiftly to a point, on day 8, when quantities of viable cells fell below the level of detection.

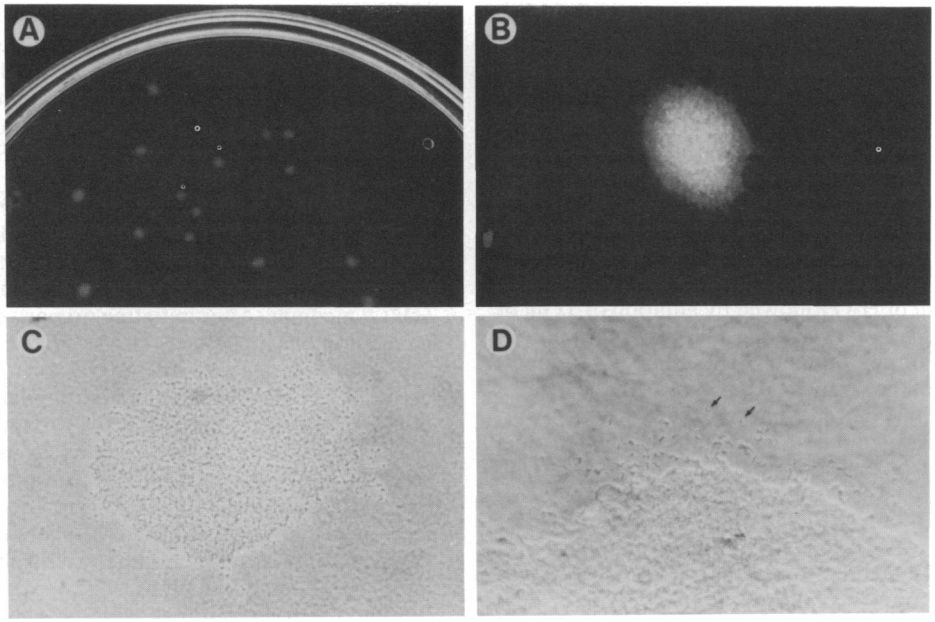

FIG. 1. Colonies of bloodstream-form *T. brucei*. (*A*) Unmagnified colonies. (*B–D*) Magnified individual colonies. Arrows in *D* indicate individual cells. (*B–D*, ×10, ×30, and ×80, respectively.)

Although it is unclear what caused the rapid loss of viability, a critical nutrient in the medium may have been depleted.

Plating efficiency measurements for S427 and S42 variants are shown in Table 1. The plating efficiencies are within the same range observed for other trypanosomatids grown on agar or agarose: *Leishmania donovani*, 58% (23); *Leishmania tropica*, 34% (23); *Leishmania tarentolae*, 30–100% (24); *Trypanosoma cruzi*, 11% (25); *Herpetomonas megaseliae* and *Crithidia harmosa*, 80–90% (26); *Crithidia fasciculata*, 30–50% (27); and *Leptomonas seymouri*, 60% (V. Bellofatto, personal communication). It is not clear why 100% plating efficiency was not achieved. However, this may be an intrinsic property of the parasite since cloning efficiency in liquid medium is also less than 100% (≈70% for 221 trypanosomes; unpublished data). Regardless, the observed plating efficiencies on HMI-9/agarose medium are sufficient for most applications.

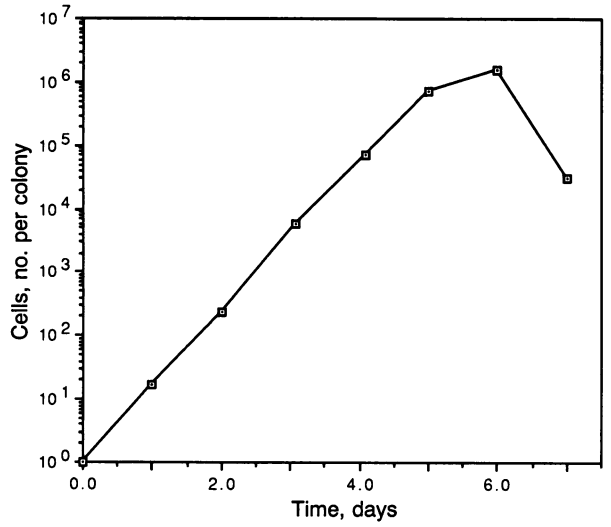

FIG. 2. Measurement of cell growth in colonies. Individual cells were spotted on different regions of a plate and the cell number was determined daily either by directly counting the cells under the microscope (days 0–2) or by transferring the colony (using a Pasteur pipette) to a small amount of liquid medium followed by quantitation in a Neubauer counting chamber (days 3–7). At day 8, number of cells was below the level of detection ($<10^3$ cells per colony). Each value is the average of three colonies.

We have also grown *T. brucei* bloodstream forms of GUTat 3.1 and ILTat 1.4 on HMI-9/agarose plates with plating efficiencies of 75 ± 10% (n = 3) and 49 ± 8% (n = 3), respectively. Furthermore, on HMI-93 (28)/agarose plates, we have generated *Trypanosoma congolense* IL3000 and IL1180 bloodstream-form colonies; however, initial plating efficiencies were low (≤5%).

Factors Affecting Colony Growth. To prevent cells from straying on the plate, Lee and Van der Ploeg (17) found it necessary to remove moisture from the surface of the plate by drying in a laminar flow hood for 1 hr prior to, and 0.5 hr after, spreading the cells. We also found that 1 hr was an optimal prespreading drying time but, in our hands, drying for 15 min was sufficient after plating. HMI-9 medium contains $NaHCO_3$ and the pH is buffered in the presence of CO_2. However, during storage and drying in air, the pH of the medium increased as indicated by a change in color of the phenol red. Nevertheless, this did not have a noticeable effect on growth rate or plating efficiency. In fact, equilibrating the plates in a humid CO_2 incubator just prior to spreading the cells had a marked negative effect on plating efficiency, possibly by rehydrating the surface. Orientation of the plates (upright or inverted) was unimportant. However, the density of the donor culture used to inoculate the plates was critical for optimal results. Highest plating efficiencies were obtained

Table 1. Plating efficiency of bloodstream-form trypanosomes grown on HMI-9/agarose plates

Variant	Strain	Plating efficiency A	Plating efficiency B
117	S427	24 ± 14	32 ± 16
118	S427	50 ± 18	78 ± 15
221	S427	43 ± 12	81 ± 12
366E	S42	8 ± 3	3 ± 2

Plating efficiency was calculated as (number of colonies ÷ number of cells plated) × 100. A and B indicate results from two experiments in which 100 cells (calculated) were spread per plate. Each value is the mean ± SD of four plates.

when cells were taken from early to midlogarithmic phase cultures, $<2 \times 10^6$ cells per ml (data not shown).

Colony Screens. The ability to probe or assay a large number of clonal populations for specific genetic or biochemical markers is a powerful experimental tool. This can be expediently performed by transferring colonies onto a membrane filter. To examine the feasibility of screening trypanosome colonies, variant 221 colonies were lifted onto nitrocellulose filters and hybridized with ^{32}P-labeled DNA probes for multicopy (tubulin, 20 copies per cell) and single copy (221 VSG) genes (Fig. 3). In both cases a signal was detectable after overnight exposure to x-ray film. Similar results were obtained when variant 118 colonies were probed with the 221 VSG gene (data not shown), thus eliminating the possibility that the 221 VSG probe was hybridizing to the 221 VSG mRNA in the initial experiment. No hybridization was detected on a control filter probed with a fragment from pGEM-4. At least two lifts could be performed from the same plate; this may allow screening for two different markers on replica filters and viable cells could be recovered after the procedure. Detection of proteins using antibody probes has not yet been attempted, but we assume it is technically possible. It may also be possible to screen colonies on filters for mutants defective in biochemical pathways (29, 30).

Growth of Procyclic Colonies. Although growth of procyclic colonies has been accomplished previously (17), initial plating efficiency was in the order of 0.01%, which is too low for most applications. Brun and Schonenberger (9) supplemented their medium with conditioned medium for clonal growth of procyclics in liquid culture. We altered the Lee and Van der Ploeg method (17) to include conditioned medium and this resulted in a marked increase in plating efficiency. Addition of 20% conditioned medium increased plating efficiency from ≤0.5 to ≈17% (Table 2). However, supplementation with greater amounts of conditioned medium (40 and 60%) did not result in a further significant increase. These results suggest that, unlike bloodstream forms, procyclics require conditioned medium for efficient growth from a single isolated cell.

Potential Uses of the Plating Technique. Both bloodstream-form and procyclic *T. brucei* can be cloned in liquid medium (9, 31). However, growth of this parasite as colonies on agarose plates offers several important advantages over cloning in liquid. (*i*) Large numbers of clones can be generated and easily quantitated on a single plate. (*ii*) Relative growth rates can be determined by direct visual observation of colony size. (*iii*) Most importantly, clones can be conveniently transferred *en masse* to a solid support (e.g., nitrocellulose membranes) for rapid screening. As a result of these attributes, the plating technique may find its greatest power when used in conjunction with stable transfection of trypanosomes and isolation of mutants. Indeed, we recently demonstrated that clones of bloodstream-form stable transfectants can be easily selected on HMI-9/agarose plates (unpublished data). Furthermore, gene expression in individual colonies may be studied by introducing an enzyme that can be detected colorimetrically *in situ* (e.g., β-galactosidase). Although mutants have yielded tremendous insight toward understanding biological processes in other organisms, few mutant trypanosomes have been described. The ability to easily grow trypanosomes as colonies will stimulate exploration of this approach.

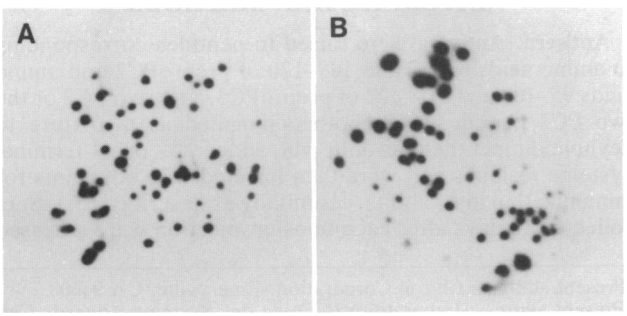

FIG. 3. Bloodstream-form colony screens. Different filters were hybridized with ^{32}P-labeled tubulin probe (*A*) or 221 VSG probe (*B*) and exposed to x-ray film at −70°C for 16 hr and 23 hr, respectively.

Table 2. Effect of conditioned medium on plating efficiency of procyclic trypanosomes grown on SDM-79/agarose plates

Conditioned medium, %	Plating efficiency
0	<0.5*
20	17 ± 6.4
40	13 ± 2.6
60	22 ± 10

Plating efficiency was calculated as (number of colonies ÷ number of cells spread) × 100. Values are mean ± SD of three plates.
*No colonies were generated from 200 cells (calculated) spread on each plate.

We thank Hiro and Kazuko Hirumi for help with growing *T. congolense* and the additional *T. brucei* strains (GUTat 3.1 and ILTat 1.4); Garry Takle for technical advice; Pradeep Patnaik, Michael Duszenko, Peter Overath, and Sam Black for helpful discussions; and Anant Menon and Vivian Bellofatto for critical reading of the manuscript. This work was supported by National Institutes of Health Grant AI21729.

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