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# Linkage Analysis in *Dictyostelium discoideum* Using Temperature-Sensitive Growth Mutants Selected with Bromodeoxyuridine

RICHARD H. KESSIN,[1] KEITH L. WILLIAMS, AND PETER C. NEWELL
*Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom*

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Amoebae of the cellular slime mold *Dictyostelium discoideum* grown in the presence of bromodeoxyuridine are killed on exposure to near-ultraviolet light. By using this phenomenon, a method was devised by which mutants of *D. discoideum* that are temperature-sensitive for growth can be readily obtained. Three such mutants have been characterized genetically and each was found to be associated with a different linkage group. Two of these linkage groups have not previously been described.

The cellular slime mold *Dictyostelium discoideum* lives as a population of amoebae until the supply of nutrients is depleted. The amoebae then stream together to form aggregates of as many as $10^5$ cells, proceed through a series of developmental stages, and finally produce fruiting bodies consisting of spore masses supported by slender stalks.

As part of a general program to study the genetics underlying this differentiation process, it has become necessary to isolate mutants that are temperature sensitive for growth. Such mutants are useful for the formation of diploids in the parasexual genetic system used in *D. discoideum* (6, 7, 14) and are also valuable chromosomal markers. Satisfactory temperature-sensitive mutants that grow well at 22 C but not at 27 C are rare and are laborious to isolate directly unless very heavy mutagenesis is used. Such treatment generally results in other unrelated mutations, a serious disadvantage if genetic analysis is contemplated. It is therefore desirable to have a selective method for the isolation of temperature-sensitive mutants from lightly mutagenized populations.

Puck and Kao (10) have shown with animal cells that auxotrophic mutants may be recovered from a large population of normal growing cells by using the nucleoside 5'-bromodeoxyuridine (BUdR). This is incorporated into growing cells when it is supplied in the growth medium and renders the cells sensitive to irradiation by near-ultraviolet light, whereas nongrowing cells remain unaffected and survive. The method described in this paper for selecting amoebae that are temperature sensitive for growth is a modification of that of Puck and Kao. We found that the extent of growth in the presence of BUdR and the duration and quality of the irradiation are critical for successful application of the method. A simple system has been devised that is readily reproducible and which incorporates features that should be broadly applicable to the isolation of other kinds of mutants in *D. discoideum*. While this work was in progress, a similar procedure was reported for the myxomycete *Physarum polycephalum* (4).

Recently linkage was established for the first time in *D. discoideum* (6); three linkage groups were found by use of six chromosomal markers. In this study we investigated both the growth characteristics and the chromosomal linkage relationships of three temperature-sensitive mutants isolated after use of BUdR. The mutations were found to be unlinked and two of them were located on hitherto undescribed linkage groups.

## MATERIALS AND METHODS

**Culture of D. discoideum.** Amoebae of strain AX3 and temperature-sensitive strains derived from it were cultured axenically as described by Watts and Ashworth (13). Dihydrostreptomycin sulfate (250 μg/ml), which can be autoclaved, was included in the medium. For growth in liquid culture with the bacterial associate *Aerobacter aerogenes*, the method of Gerisch (3) was used. Growth was at 22 C in 50 to 70 ml of liquid medium in a 250-ml flask in a rotary shaker at 150 cycles/min. The relevant phenotypes and genotypes of the strains used are summarized in Table 1.

**Mutagenesis.** Mutagenesis was always performed on haploid amoebae under the following standardized

[1] Present address: The Biological Laboratories, Harvard University, Cambridge, Mass. 02138.

TABLE 1. *Description of strains*[a]

| Strain | Parent | Source | Relevant mutant loci[b] | | | | | Relevant phenotype[c] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *acrA* | *cycA* | *tsg* | *bwn* | *whi* | Ac | Cy | G. Res. | Pig-ment | Sp. Col. |
| TS12 | NC4 | E. R. Katz (6) | + | 1 | *D12* | + | *whi* | S | R | NG | NB | W |
| M28 | NC4 | E. R. Katz (6) | + | + | *E13* | *bwn* | + | S | S | NG | B | Y |
| AX3 | NC4 | W. F. Loomis (8) | + | + | + | + | + | S | S | G | NB | Y |
| NP2 | AX3 | This lab | + | + | *A1* | + | + | S | S | NG | NB | Y |
| NP3 | AX3 | This lab | + | + | *B3* | + | + | S | S | NG | NB | Y |
| NP7 | AX3 | This lab | + | + | *C7* | + | + | S | S | NG | NB | Y |
| NP12 | NP3 | This lab | 3 | + | *B3* | + | + | R | S | NG | NB | Y |
| NP14 | TS12 | This lab | 1 | 1 | *D12* | + | *whi* | R | R | NG | NB | W |
| DP2 | TS12/NP2 | This lab | +/+ | 1/+ | *D12/A1* | +/+ | *whi*/+ | S | S | G | NB | Y |
| DP3 | M28/NP2 | This lab | +/+ | +/+ | *E13/A1* | *bwn*/+ | +/+ | S | S | G | NB | Y |
| X2 | DP3 | This lab | + | + | *A1* | *bwn* | + | S | S | NG | B | Y |
| NP56 | X2 | This lab | 6 | + | *A1* | *bwn* | + | R | S | NG | B | Y |
| DP4 | M28/NP14 | This lab | +/1 | +/1 | *E13/D12* | *bwn*/+ | +/*whi* | S | S | G | NB | Y |
| X9 | DP4 | This lab | 1 | 1 | *D12* | *bwn* | *whi* | R | R | NG | B | W |
| DP21 | X9/NP7 | This lab | 1/+ | 1/+ | *D12/C7* | *bwn*/+ | *whi*/+ | S | S | G | NB | Y |
| DP33 | X9/NP12 | This lab | 1/3 | 1/+ | *D12/B3* | *bwn*/+ | *whi*/+ | R | S | G | NB | Y |
| DP44 | TS12/NP56 | This lab | +/6 | 1/+ | *D12/A1* | +/*bwn* | *whi*/+ | S | S | G | NB | Y |

[a] Our nomenclature system is based on that of Demerec et al. (2). Strains produced in this laboratory designated NP are haploid, those deisgnated DP are diploid, and X strains are haploid strains containing reassorted chromosomes from a diploid.

[b] Genes determining temperature sensitivity for growth (27 C) are denoted by *tsg*, for acriflavine resistance (100 μg/ml) by *acr*, for cycloheximide resistance (500 μg/ml) by *cyc*, for brown pigment formation by *bwn*, and for formation of white spores by *whi*. All of these mutations are recessive. + denotes wild-type genotype. The different numbers at mutant loci represent independently isolated mutations.

[c] Ac, Acriflavine; Cy, cycloheximide; G. Res., growth at restrictive temperature (27 C); Sp. Col., spore color; S, sensitive; R, resistant; G, growth; NG, no growth; B, brown; NB, not brown; W, white; Y, yellow.

conditions based on the method of Yanagisawa et al. (16). *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was used as a mutagen and was dissolved by shaking in salt solution (containing, per liter, NaCl [0.6 g], KCl [0.7 g], $CaCl_2$ [0.3 g]) at a concentration of 1 mg/ml. NTG was prepared 20 min before use. Amoebae growing axenically at a density between $2 \times 10^6$ and $4 \times 10^6$/ml were harvested by centrifuging at 150 × *g* for 2 min at room temperature. These amoebae were then resuspended in 20 ml of the salt solution + NTG solution at a concentration of $10^7$ amoebae/ml and placed on a rotary shaker at 150 rpm at 22 C. After 20 min, the mutagenized amoebae were diluted with ice-cold sterile water and washed three times by centrifugation at 150 × *g*. Axenic amoebae had a survival rate between 25 and 40% under these conditions, as shown by clonal viability tests. Amoebae that had not reached a density of $10^6$/ml had a lower survival rate, whereas those above $5 \times 10^6$/ml and approaching stationary phase survived the treatment better. The recovery of axenic amoebae when replaced in axenic medium after mutagenesis was slow and is described in the text.

**Treatment of amoebae with BUdR.** Filter-sterilized BUdR was added to amoebae growing in axenic medium. The concentration of BUdR was 150 μg/ml (0.49 mM). After growth in BUdR, amoebae were harvested, washed once by centrifugation, and resuspended in salt solution at a concentration of $5 \times 10^4$/ml. Five milliliters of the amoebae suspension was placed in a petri dish (50 by 12 mm) and covered with the plastic lid. Controls were covered with aluminium foil. The petri dish was placed in a tray on a layer of wet towelling (for cooling purposes) 10 cm beneath a 50-W standard ultraviolet germicidal lamp (Hanovia Lamps Ltd., Slough, U.K.). The plastic lid of the

petri dish filters out all light below 280 nm while allowing a proportion of the near-ultraviolet light through. Petri dishes from three different sources behaved similarly. The same result may be achieved with an OX9A filter (Chance-Pilkington Bros. Ltd., St. Asaph, Flintshire, U.K.) instead of the plastic petri dish lid. By using a thermopile calibrated by the British National Physical Laboratory, we determined that approximately $1.2 \times 10^3$ ergs/s/cm$^2$ of light energy between 310 and 390 nm penetrate the lid of a petri dish situated 10 cm from the light source. This is about 10% of the energy received by the thermopile in the absence of the plastic filter. The majority of this 10% is at the lower and more useful end of the range. For inactivation of BUdR-containing bacteriophage T4, light of a wavelength around 313 nm is optimal (11). The amount of energy received by the thermopile is not greatly reduced by adding 5 ml of salt solution to the lid to simulate the condition of the amoebae being exposed.

After varying periods of exposure to near-ultraviolet light, amoebae were removed from the plastic petri dishes and tested for viability. The amoebae stick to the plastic petri dishes and care must be taken to dislodge them by drawing the suspension of amoebae several times through a pipette. The irradiated amoebae, or the controls covered with foil, were plated clonally on SM agar (12) with *A. aerogenes* as a food source. Clones appeared after 4 to 5 days. The BUdR-treated amoebae formed plaques more slowly than the controls.

**Screening for growth-temperature-sensitive mutants.** Control colonies, or those surviving BUdR treatment at the restrictive temperature, were transferred with toothpicks to duplicate SM agar plates spread with *A. aerogenes* a few minutes previously. One sample was placed at 27 C (26.8 ± 0.3 C) and the other plate was kept as a control at 22 C. When colonies were widely distributed or an existing array of colonies had to be retested, a replica plating technique was used. For this technique we used a florist's flower-stem holder containing many small spikes on a round base the size of a petri dish. As many as six impressions can be made from one master plate. When sampling with toothpicks or replica plating, the petri dish to be incubated at 27 C was always touched first to bias against temperature-sensitive growers. The plate to be incubated at 22 C was then touched, without returning to the suspect colony. Another method which has the advantage of speed was sometimes used. Amoebae were allowed to form small colonies at the permissive temperature of 22 C. The size of these colonies was marked on the back of the plates which were then placed at 27 C, the restrictive temperature. Clones that did not spread beyond the original boundaries were retested.

A mutant was considered temperature sensitive if it failed to grow at 27 C after being tested twice. Mutants to be used in genetic analysis were more stringently analyzed and used only if an initial inoculum of $2 \times 10^5$ amoebae/plate failed to clear a lawn of *A. aerogenes* at 27 C after 4 days, while sister amoebae at 22 C had devoured the lawn and formed fruiting bodies. Temperature-sensitive mutants were stored on silica gel (9) after two clonal passages.

**Formation of diploids.** Diploids that were temperature resistant for growth at 27 C were selected by the methods described previously (14) at a frequency of approximately one in $10^5$ from two temperature-sensitive haploid strains.

**Assignment of mutations to linkage groups.** The methods used were those previously used for *Aspergillus nidulans* (5) and for *D. discoideum* (6, 15). About $5 \times 10^4$ diploid amoebae heterozygous for cycloheximide resistance were plated in association with *A. aerogenes* on agar-solidified growth medium containing cycloheximide (500 μg/ml). After 4 to 5 days, cycloheximide-resistant clones appeared at a frequency of about one in $10^3$ amoebae plated. These comprised both haploids and homozygous diploids that were distinguished on the basis of spore size, the diploid spores being twice the volume of haploid spores. Only haploids were used to assign genetic markers to linkage groups.

Because two temperature-sensitive strains were used to construct diploids, some way of distinguishing the temperature-sensitive mutations in haploid segregants was necessary. In the experiments where temperature-sensitive mutations were to be assigned to linkage groups, one strain contained the unassigned temperature-sensitive mutation and the other always contained the mutation *tsgD12*, which has previously been assigned to linkage group II (6,15). The mutation *tsgD12* could thus be identified by its segregation with the white spore marker and *acrA* (see Table 3). Since the incidence of mitotic crossing-over is very low, chromosomes assort randomly without significant genetic exchange between homologues. Genetic markers may therefore be assigned to linkage groups by analyzing cosegregation of markers. Thus *tsgD12* could be identified because it cosegregated with *whi* (see Table 3), and the unknown temperature-sensitive mutation could be located by analyzing haploid segregants lacking *tsgD12* (i.e., not white). In this way extensive back-crossing studies to rigorously identify the temperature-sensitive mutation were not needed routinely, although such studies always confirmed the validity of the method based on the cosegregation of markers.

**Materials.** *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was obtained from Ralph Emanuel, Ltd., Wembley, England. Yeast extract and proteose peptone were obtained from Oxoid. BUdR (grade A) was purchased from Sigma or Calbiochem. Petri dishes were purchased from Dyos Plastics, Surbiton, Surrey, U.K.; Falcon Plastics, Oxnard, Calif.; and A/S Nunc, Denmark (tissue culture grade).

## RESULTS

**Effects of growth in axenic medium containing BUdR.** Amoebae that had grown in the presence or absence of BUdR were irradiated with filtered ultraviolet light as described in Materials and Methods. Only those amoebae that had been grown in the presence of BUdR

were killed by the filtered ultraviolet light (Fig. 1). Non-irradiated amoebae remained viable under these conditions for at least 24 h. The effectiveness of the ultraviolet filtering system is indicated by the finding that exposure of amoebae, grown with or without BUdR, to ultraviolet irradiation without the plastic filter resulted in almost complete loss of viability in a few seconds.

In other organisms (1) deoxyadenosine increases the effectiveness of BUdR treatment,

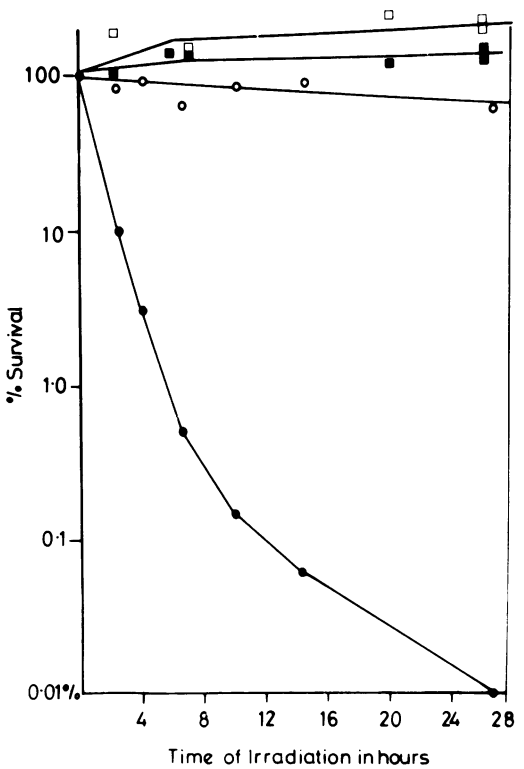


FIG. 1. *Survival of amoebae after exposure to BUdR. Amoebae of strain AX3 were grown for three doublings in axenic medium containing 150 μg of BUdR per ml, after which they were harvested and washed once by centrifugation at 150 × g. Control and BUdR-treated amoebae were resuspended in salt solution at a density of 5 × 10⁴/ml. Five milliliters of this suspension of amoebae was placed in a sterile petri dish (60 by 15 mm) and irradiated through the lid as described in the Materials and Methods. Control and BUdR-treated amoebae in petri dishes covered with foil were also analyzed. The temperature under the light did not rise above 23 C. Symbols:* □, *no BUdR, no near-ultaviolet light (covered by foil);* ■, *no BUdR, exposed to near-ultraviolet light;* ○, *150 μg of BUdR per ml (0.49 mM), no near-ultraviolet light (covered by foil);* ●, *150 μg of BUdR per ml, exposed to near-ultraviolet light.*

but it did not do so with *D. discoideum* amoebae under our conditions. Fluorodeoxyuridine has a slight enhancing effect for killing with BUdR, but it was not used routinely.

Figure 2 shows the effect of BUdR on the growth rate in strain AX3. Growth was only slightly affected by BUdR (150 μg/ml) before four to five doublings had occurred when there was a fairly sudden cessation of growth. After one, two, three, and four generations of growth in axenic medium containing BUdR, amoebae were tested for the ability to withstand exposure to near-ultraviolet light. The number of survivors decreased by roughly an order of magnitude with each doubling (Fig. 2). The viability of the non-BUdR-treated amoebae when exposed to near-ultraviolet light after one, two, three, and four generations was 97, 87, 94, and 90%, respectively. A BUdR-resistant strain, selected as described by Loomis (8), showed no sensitivity to near-ultraviolet light after growth in BUdR.

**Selection of mutants temperature-sensitive for growth.** Mutants temperature-sensitive for growth were isolated by shifting growing mutagenized amoebae from the normal temperature

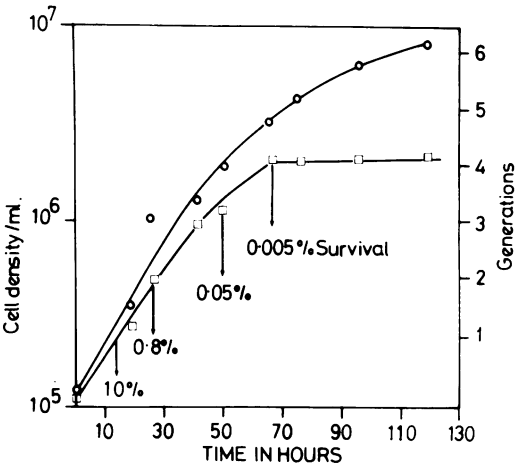


FIG. 2. *Growth and survival after varying times of exposure to BUdR. Amoebae of strain AX3 were grown for one, two, three, and four generations in the presence and absence of 150 μg of BUdR per ml in axenic medium and then prepared for irradiation as described in Materials and Methods. Each sample was irradiated for 18 h. Control and BUdR samples were taken for each point. The controls all showed high viability (see text). Survival of the BUdR-treated samples is given in the figure and is calculated as the ratio of irradiated survivors to non-irradiated survivors. Killing after one doubling was analyzed in a separate experiment. Symbols:* ○, *control, no BUdR;* □, *BUdR treated.*

of 22 C to 27 C, the restrictive temperature. After 24 h, to allow time for temperature-sensitive growth mutants to cease deoxyribonucleic acid replication, BUdR was added and was (presumably) only incorporated into the deoxyribonucleic acid of growing amoebae. On exposure to near-ultraviolet light, only those capable of growth at 27 C should be killed, whereas those that had for one reason or another not been able to grow at 27 C in axenic medium should survive to a greater extent. Several other classes of mutants will inevitably be coselected, including those that had not yet begun to grow after mutagenesis, BUdR-resistant mutants (4), and those amoebae that are incapable of growth on axenic medium but will grow on bacteria. The last class includes potential auxotrophs which may be amenable to isolation when a defined medium is available.

Amoebae mutagenized as described in Materials and Methods require 2 to 3 days at 22 C to recover and to show net increases in cell number. When these mutagenized amoebae are plated clonally in association with *A. aerogenes* they are 25 to 40% viable, but the colonies show variable growth rates and 2 to 5% carry heritable morphological defects. To make procedures as standard as possible, mutagenized amoebae were allowed to grow from $10^5$ to $10^6$/ml before being subjected to BUdR selection. Thus, in any one experiment sibling temperature-sensitive mutants may be isolated. This can easily be overcome by subdividing the population of mutagenized amoebae before they begin to grow. When experiments were done with nonmutagenized amoebae, no temperature-sensitive mutants were isolated, so the frequency of temperature-sensitive growth mutants in a nonmutagenized population is extremely low.

**Evidence of selection with BUdR.** To show that the BUdR selection does not kill nondividing amoebae, several reconstruction experiments were performed (Table 2). Temperature-sensitive mutants were incubated at 22 and 27 C for the same time. If truly temperature sensitive for growth and originally selected by this technique, they should survive in the presence of BUdR at 27 C but not at 22 C. Strain AX3 is killed at both temperatures, whereas strain X2 carrying the temperature-sensitive mutation *tsgA1* and strain NP3 carrying *tsgB3* are not killed to the same extent at 27 C as at 22 C. Similarly, strain M28, which is not capable of growth in axenic medium at any temperature, survives BUdR treatment under conditions where only 0.2% of strain X2 amoebae survive. We therefore believe that BUdR does not kill nongrowing amoebae of *D. discoideum* to the same extent as growing amoebae and that these temperature-sensitive growth mutants were selected by the method described.

**Growth characteristics of selected mutants.** Several of the growth-temperature-sensitive mutants selected using BUdR were further tested for their ability to grow in liquid axenic medium and also non-nutrient liquid medium (10 mM potassium phosphate, pH 6.5) with added *A. aerogenes* as a food source. The results (Fig. 3 and 4) show that the mutants differed in their ability to grow. For example, strain X2, carrying the growth-temperature-sensitive mutation which is designated *tsgA1*, was completely temperature sensitive in both axenic medium and in association with bacteria. When grown on agar-solidified medium in association with *A. aerogenes*, it did not show any tendency to revert (reversion frequency less than $10^{-7}$). The absence of reversion may be due to lethality after a short period at the restrictive temperature. Strain NP7 carrying the mutation *tsgC7* was also completely temperature sensitive in axenic medium (although it might be noted that the growth rate at 22 C in axenic culture was considerably less than the control AX3

TABLE 2. *Survival of temperature-sensitive mutants in the presence of BUdR*[a]

| Strain | Temperature-sensitive mutation | Growth at 22 C | | | Growth at 27 C | | |
|---|---|---|---|---|---|---|---|
| | | Initial density (per ml) | Final density (per ml) | Survival (%) | Initial density (per ml) | Final density (per ml) | Survival (%) |
| AX3 | | $10^5$ | $6 \times 10^6$ | 0.05 | $10^5$ | $1.2 \times 10^6$ | 0.04 |
| X2 | *tsgA1* | $2.5 \times 10^5$ | $3.2 \times 10^6$ | 0.2 | $10^5$ | $10^5$ | 18 |
| NP3 | *tsgB3* | $5 \times 10^5$ | $3.4 \times 10^6$ | 1-2 | $2.6 \times 10^5$ | $4 \times 10^5$ | 28 |
| M28[b] | *tsgE13* | $10^5$ | $10^5$ | 16.3 | | | |

[a] Mutant or strain AX3 amoebae, previously grown at 22 C in axenic medium, were inoculated in 10 ml of axenic medium in a 125-ml flask and incubated at 22 or 27 C as described in the legend to Fig. 3. After 24 h, BUdR was added to a final concentration of 150 μg/ml and the amoebae were incubated further. After several doublings of the 22 C sample had taken place, both sets of amoebae were harvested and irradiated for 18 h.

[b] Does not grow in axenic medium.

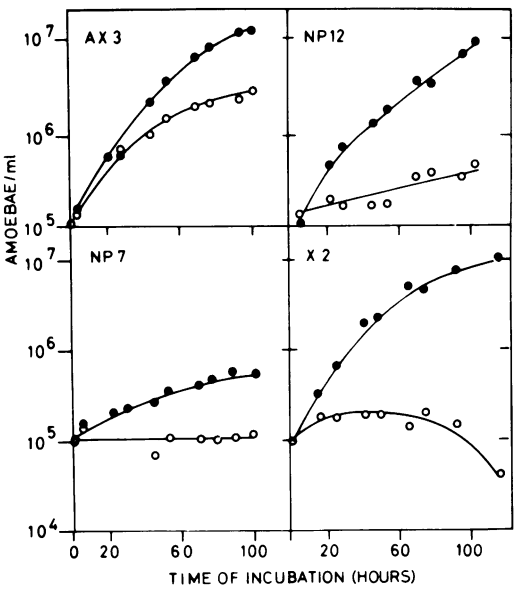


FIG. 3. *Growth of temperature-sensitive mutants at 22 and 27 C in liquid axenic culture. Each mutant, previously grown for at least one passage in axenic medium at 22 C, was inoculated into 50 ml of axenic medium in a 250-ml flask. Those at 22 C were shaken on a rotary shaker at 150 rpm and those at 27 C were shaken in a reciprocal action water bath at 120 cycles/min. Symbols:* ●, *22 C;* ○, *27 C.*

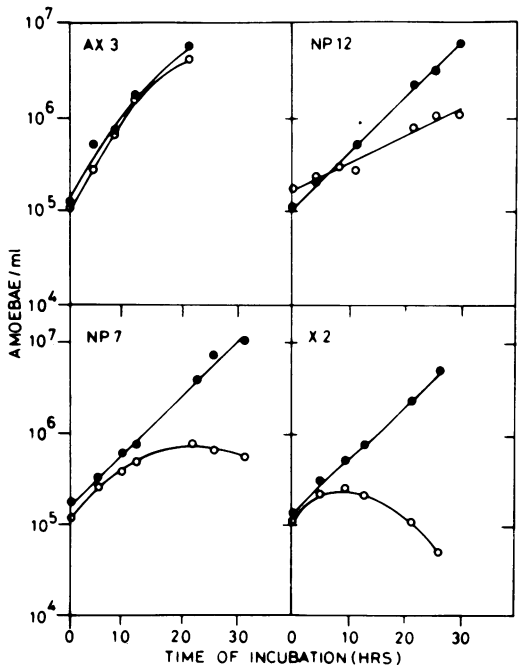


FIG. 4. *Growth of temperature-sensitive mutants at 22 and 27 C in liquid culture with bacteria as food source. The amoebae used in these growth tests were taken from bacterially grown liquid cultures at 22 C in logarithmic phase. Incubation conditions were identical to those described in Fig. 3, except that pregrown A. aerogenes in 10 mM potassium phosphate buffer (pH 6.5) replaced the axenic medium. Symbols:* ●, *22 C;* ○, *27 C.*

strain), but in association with bacteria it was capable of three to four generations of growth at 27 C before finally stopping; this was noticed consistently.

Strain NP12 carrying the mutation *tsgB3* is capable of slow growth in axenic medium at 27 C and a limited amount (three to five generations) of slow growth in association with bacteria. With bacteria on solid medium, single amoebae will produce tiny colonies just visible to the naked eye after 5 days of growth. This strain reverts to normal growth on solid medium at a frequency of about one in $2 \times 10^5$ amoebae plated.

**Linkage group analysis of the mutants.** In previous studies in this laboratory (15), acriflavine resistance mutations (*acrA*) have been characterized and located on linkage group II. By using these mutations and those previously established by Katz and Sussman (6), the linkage relationships of the temperature-sensitive mutations *tsgA1*, *tsgB3*, and *tsgC7* were established. Representative experiments are shown in Table 3. All three temperature-sensitive mutations were recessive since the diploids DP2, DP21, DP33, and DP44 (Table 1) all grew at the restrictive temperature. One mutation, *tsgB3*, is located on a previously established linkage group (linkage group IV) carrying the brown pigment marker (6, 15). The other two mutations did not segregate with any of the then known genetic markers and were therefore assigned to new linkage groups (III and V). Linkage group III has since been shown to bear one of the genes for the ability of strain AX3 to grow in axenic medium (15).

## DISCUSSION

We chose to apply the BUdR selection technique to the isolation of mutants that are temperature sensitive for growth because these mutants are useful for the parasexual system of genetic analysis (6, 14). In this system diploid amoeba, formed by fusion of haploid amoebae bearing nonallelic temperature-sensitive growth mutations, are selected by their ability to grow at the restrictive temperature. These mutants also serve as excellent chromosomal markers for segregation and chromosomal recombination studies.

We did not find temperature-sensitive growth mutants occurring without selection at the

TABLE 3. *Assignment of temperature-sensitive alleles to linkage groups by analyzing haploid segregants of suitably marked diploids*[a]

| Allele[b] | Linkage group | | | | | No. |
|---|---|---|---|---|---|---|
| | I | II | III | IV | V | |
| *tsgA* | | | | | | |
| Diploid TS12 / DP44 NP56 | *cycA1* / + | + *whi* *tsgD12* / *acrA6* + + | + / *tsgA1* | + / *bwn* | | |
| Haploid segregants | *cycA1* | *acrA6* + + | + | + | | 18 |
| | *cycA1* | *acrA6* + + | + | *bwn* | | 21 |
| | *cycA1* | *acrA6* + + | *tsgA1* | + | | 6 |
| | *cycA1* | *acrA6* + + | *tsgA1* | *bwn* | | 13 |
| *tsgB* | | | | | | |
| Diploid X9 / DP33 NP12 | *cycA1* / + | *acrA1* *whi* *tsgD12* / *acrA3* + + | | *bwn* + / + *tsgB3* | | |
| Haploid segregants | *cycA1* | *acrA1* *whi* *tsgD12* | | *bwn* [c] | | 16 |
| | *cycA1* | *acrA1* *whi* *tsgD12* | | + [c] | | 12 |
| | *cycA1* | *acrA3* + + | | *bwn* + | | 14 |
| | *cycA1* | *acrA3* + + | | + *tsgB3* | | 12 |
| *tsgC* | | | | | | |
| Diploid X9 / DP21 NP7 | *cycA1* / + | *acrA1* *whi* *tsgD12* / + + + | | *bwn* / + | + / *tsgC7* | |
| Haploid segregants | *cycA1* | *acrA1* *whi* *tsgD12* | | *bwn* | [c] | 9 |
| | *cycA1* | *acrA1* *whi* *tsgD12* | | + | [c] | 10 |
| | *cycA1* | + + + | | *bwn* | + | 6 |
| | *cycA1* | + + + | | *bwn* | *tsgC7* | 10 |
| | *cycA1* | + + + | | + | + | 6 |
| | *cycA1* | + + + | | + | *tsgC7* | 8 |

[a] Haploids were obtained as described in Materials and Methods. The temperature-sensitive muations *tsgA* and *tsgC* are unlinked since *tsgA* is linked to one of the genes involved in growth in axenic medium whereas *tsgC* is not (15). The relative positions of the chromosomal markers have not yet been established.

[b] For strain DP44, secondary selection on acriflavine was used to obtain haploids not containing *tsgD12*.

[c] In these cases the genotype was not established.

frequency described by Loomis (7). A selective method of obtaining such mutants was therefore important. The mutant selection method using BUdR should also be useful, however, for the isolation of other kinds of mutants such as auxotrophs when a defined medium or medium defined for certain metabolites has been developed.

The temperature-sensitive growth mutants that were isolated show distinctly different growth abilities. This suggests that they bear mutations at different sites on the genome, and this is confirmed by genetic analysis which showed that they were located on different linkage groups. Most of the temperature-sensitive mutants that have been characterized, including two of the three described here, show some slow growth at the restrictive temperature, using either axenic or bacterial culture or both. Haugli and Dove (4) found that all the temperature-sensitive growth mutants that they isolated from the myxomycete *Physarum polycephalum* had this "leaky" characteristic. In contrast, one of the three mutations described here (*tsgA1*) grows for no more than one generation when switched to the restrictive temperature and shows no tendency for further leaky growth. Besides making this a very valuable mutation for genetic work, it is of intrinsic interest since it may code for a temperature-sensitive mutant protein connected with the cell or nuclear replication system.

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