Increased Mutation Rate of *E. coli* K12λ Cultures Maintained in Continuous Logarithmic Growth

JOHN H. NORTHROP

From the Laboratory of The Rockefeller University, the Donner Laboratory of Biophysics and Medical Physics, and the Division of Medical Microbiology and Immunology, School of Public Health, University of California, Berkeley

ABSTRACT Continuous logarithmic growth of *E. coli* K12λ in an automatic culture cell resulted in marked increases in the proportion of several mutants. The P_1 phage-resistant cells increased 10 to 3000 times, the T_2 phage-resistant cells 1 to 1000 times, the neomycin-resistant cells 1 to 10 times, and the virus-producing cells 30 to 70 times. No change occurred in the penicillin-resistant cells. Calculation of the growth curves and direct determination of the mutation rates by the null fraction method showed that the increases in the proportion of mutants were due to increases in the mutation rates.

INTRODUCTION

Antibiotic-resistant cultures usually appear as a result of overgrowth of the culture by a resistant mutant in the presence of the antibiotic. A streptomycin-resistant culture of *B. megatherium*, however, was obtained by prolonged logarithmic growth of a streptomycin-sensitive culture of the organism without exposure to the antibiotic. The proportion of virus-producing cells also increased under these conditions. Analysis of these results showed that the increase in streptomycin-resistant cells in the culture was due partly to an increase in the relative growth rate of the streptomycin-resistant mutants and partly to an increase in their mutation rate. The over-all effect, therefore, was similar to a simultaneous exposure of the culture to a mutagen and to a low concentration of antibiotic (21).

Prolonged logarithmic growth of lysogenic *B. megatherium* in minimal medium led to the appearance of many new phage types (23). At that time this result was thought to be due to adaptation of the organism to the minimal medium. It is probable, however, that the new phage types were also the result of the mutagenic effect of prolonged logarithmic growth.

Increases in the proportion of mutants in logarithmic growth cultures have been observed by Stocker (30), by Novick and Szilard (24), and by the author (17). These increases, however, were the expected changes in a culture

approaching equilibrium conditions, without any change in either mutation or growth rates. In the present experiments the cultures were in equilibrium in the test tube cultures, before the logarithmic growth series was begun. No further change occurred in the test tube cultures.

"Take-over" of a continuous culture by mutants which adhered to the walls of the culture vessel has recently been described by Munson and Bridges (14).

Fox (5) found that the proportion of mutants in continuous logarithmic cultures in complex media increases with increasing growth rate. Drifilm and Tween 80 were used to prevent adherence to the walls of the cell.

Kubitschek and his collaborators (9–12), working with chemostat cultures, found that the proportion of mutants was independent of the growth rate, if growth was limited by lack of tyrosine or by ultraviolet light, but proportionate to growth rate, if growth was limited by aminopurine.

In these experiments the increase in the proportion of mutants is assumed to be a measure of the mutation rate. It is also assumed that growth occurs only in the liquid media and that no cells adhere to the walls of the culture vessel.

The present experiments were designed to rule out these complicating factors. The culture cell used contains a series of rubber rings which wipe the cell walls 500 to 800 times a minute and prevent growth of the organisms on the cell walls without addition of extraneous substances.

The change in mutation rate indicated by the increase in the proportion of some mutants was confirmed by direct determination of the mutation rates by the null fraction method. In addition, it was found to be impossible to calculate the observed increases in the proportion of some mutants without assuming an increased mutation rate.

K12 λ cultures were grown in glucose salt solution at a concentration of 5 to 7 $\times$ 10^8 cells/ml in an automatic cell (19). Control cultures were grown in test tubes and diluted from 1 $\times$ 10^9 to 5 $\times$ 10^6 cells/ml every 24 hr.

RESULTS

Three experiments were carried out. In each case there were marked increases in the various types of cells in the logarithmic cultures compared to the test tube cultures. The virus-producing cells increase 30 to 70 times. The P_1 phage-resistant cells increased 100 times in the first experiment, 3000 times in the second, and 10 in the third. The T_2 phage-resistant cells increased 1000 times in the second experiment, but remained constant in the others. The neomycin-resistant cells increased 10 times in the first experiment, but did not change in the others. The experiments were carried out at about 6 month intervals and variable results are to be expected in this type of experiment, owing to changes in the parent culture.

The resulting changes in the proportion of the types of cells in the second experiment are shown in Fig. 1. The proportion of phage-producing cells in the logarithmic growth cultures increased about 70 times, the T_2 phage-resistant cells increased about 1000 times, and the P_1 phage-resistant cells

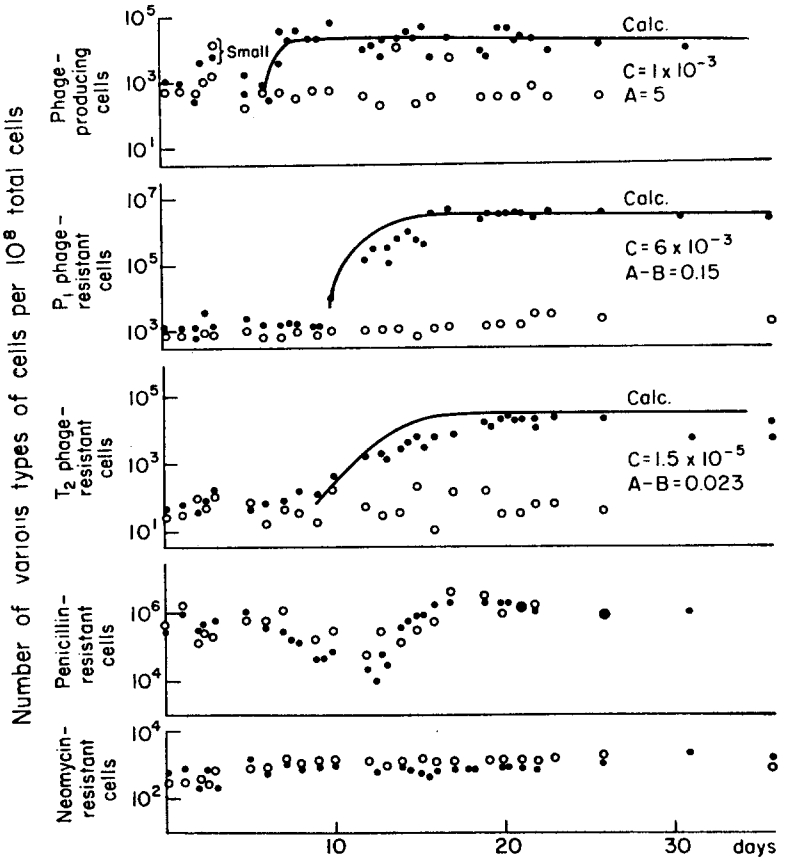

FIGURE 1. The proportion of virus-producing cells, P_1 phage-resistant cells, T_2 phage-resistant cells, neomycin-resistant cells, and penicillin-resistant cells in K12λ cultures growing in glucose salt solution. Open circles, culture grown in test tube and diluted 1/200 every 24 hr. Filled circles, culture in automatic cell with light and stirrer in operation. First 6 days, diluted 1/200 every 24 hr. After that, cell concentration maintained at 5 to 7 × 10^8 B/ml by automatic dilution.

about 3000 times. No significant changes occurred in the neomycin-resistant or penicillin-resistant cells.

The fact that no changes[1] occurred in the first 6 days, before the automatic

[1] A large number of very small plaques appeared on the plates on the 4th day of the experiment, before the automatic dilution was started. The phage titer dropped to the usual value the next day and these small plaques did not appear again.

dilution was started, shows that the change in the cell population is not due to the action of the red light, which activates the automatic dilution, nor to the stirring.

The curves were calculated by means of the following equation (22):

$$\frac{M}{W_0} = \frac{C(e^{(B-A)t} - 1)}{B - A} + \frac{M_0(e^{(B-A)t})}{W_0} \tag{1}$$

in which

M = mutants/ml.

W_o = wild cells/ml — constant — $\gg M$

B = growth rate of mutant $= \frac{1}{t} \ln \frac{M}{M_o}$ t = days

A = growth rate of wild $= \frac{1}{t} \ln \frac{W}{W_0}$

C = mutation time rate constant; i.e., fraction of wild cells changing to mutants per day without any assumption about cell division. At equilibrium $\frac{C}{A - B} = \frac{M}{W}$. When $C \ll (A - B)$, $C = 2\lambda A$, where λ is the usual mutation frequency rate constant ("mutation rate"), as determined by the null fraction method (8). C is used rather than λ, since it requires one less assumption and since the appearance of the virus-producing cells cannot be calculated by the assumption that their appearance follows cell division (cf. Northrop, reference 18).

The change from virus-free to virus-producing cells is calculated in the same way as for the other types of cells, since this is the simplest assumption capable of explaining the facts and since the effect of mutagens on this change is similar to their effect on recognized mutants in the culture (20). The objection that the change to virus-producing cells cannot be a mutation, because the proportion of virus-producing cells is too high, does not apply here. The proportion of virus-producing cells is lower than that of either the P_1 phage-resistant, penicillin-resistant, or T_2 phage-resistant cells.

It may be noted that there is a lag of about 10 days (50 generations) before the final proportion of P_1 phage-resistant or T_2 phage-resistant cells is reached. This lag is due to the difference in the growth rates of the wild and of the mutant cells $(A - B)$, as predicted by the equation, and does not require any special assumption about mutagenic or phenotypic lag. The curves are similar in every respect to those obtained after exposure of the culture to mutagenic agents (20).

The values of the constants used to calculate the results are shown on the

figure and in Table I. The values for C, the mutation time rate constant used to calculate the results, are as close as can be expected to the values obtained by direct experiment, using the null fraction method (8). This agreement is direct experimental evidence that the increase in the proportion of mutants is due to an increase in their mutation rates. The greatest discrepancies are in the values for the mutation rate of the test tube cultures determined by the null fraction method or calculated from the time curves. The null fraction determinations of the mutation rates must be carried out with very dilute suspensions (1×10^2 to 1×10^4 cells/ml), while the experimental cultures contain 1×10^7 to 1×10^9 cells/ml, and hence, some differences in the

TABLE I

VALUES OF CONSTANTS BY DIFFERENT METHODS

Value of $(A-B)$ (difference in growth rates of wild and mutant cells) and C (mutation time rate constant) determined by different methods for virus-producing cells, P_1 phage-resistant, and T_2 phage-resistant cells in AC (automatic cell) or TT (test tube) cultures

Type of cell	Virus-producing		P_1 Phage-resistant		T_2 Phage-resistant	
Culture	TT	AC	TT	AC	TT	AC
Constant $(A-B)$ determined by direct means	5	5	0.1	0.1	0.5	
From equation (1)		5		0.15		0.023
λ by null fraction	5×10^{-6}	3×10^{-5}	1×10^{-6}	1×10^{-4}	1×10^{-7}	1×10^{-6}
$C = 2\lambda A$	5×10^{-5}	3×10^{-4}	10×10^{-6}	1×10^{-3}	10×10^{-7}	1×10^{-5}
C from $\frac{C}{A-B} = \frac{M_e}{W_e}$	3.5×10^{-5}	10×10^{-4}	1.5×10^{-6}	4×10^{-3}	5×10^{-7}	1.5×10^{-5}

mutation rates are to be expected. The results cannot be calculated by assuming changes in growth rate, with constant mutation rate. In order to calculate the results on the assumption that the mutation rate in the continuous culture is the same as that in the test tube culture, it is necessary to assume that $(A - B)$, the difference in the growth rates of P_1 phage-resistant cells and the normal cells in the test tube cultures, is 450 per day. This is an impossible value since A is 5 per day by direct measurement and B is positive.

In the case of the virus-producing cells, B is 0, since these cells do not continue to grow and direct measurement shows little or no difference in the growth rate of the two cultures. The increase in virus-producing cells, therefore, must be due to an increase in the mutation rate.

A sample from the automatic cell after 2 weeks' continuous logarithmic growth, transferred in test tubes, retained a high proportion of P_1 phage-resistant and T_2 phage-resistant cells for about 10 days (50 generations). The virus-producing cells, however, returned to the original proportion in about 20 generations (Fig. 2).

The time required for a culture to reach equilibrium condition, after a change in either mutation rate or growth rate, is inversely proportional to the difference in the growth rates of the wild and the mutant cells. The half-time to equilibrium (22) is $t_{50} = \frac{\ln 2}{(A - B)}$. The value for $(A - B)$ for the virus-producing cells is 5, while for the P_1 phage-resistant cells it is 0.15 and for the

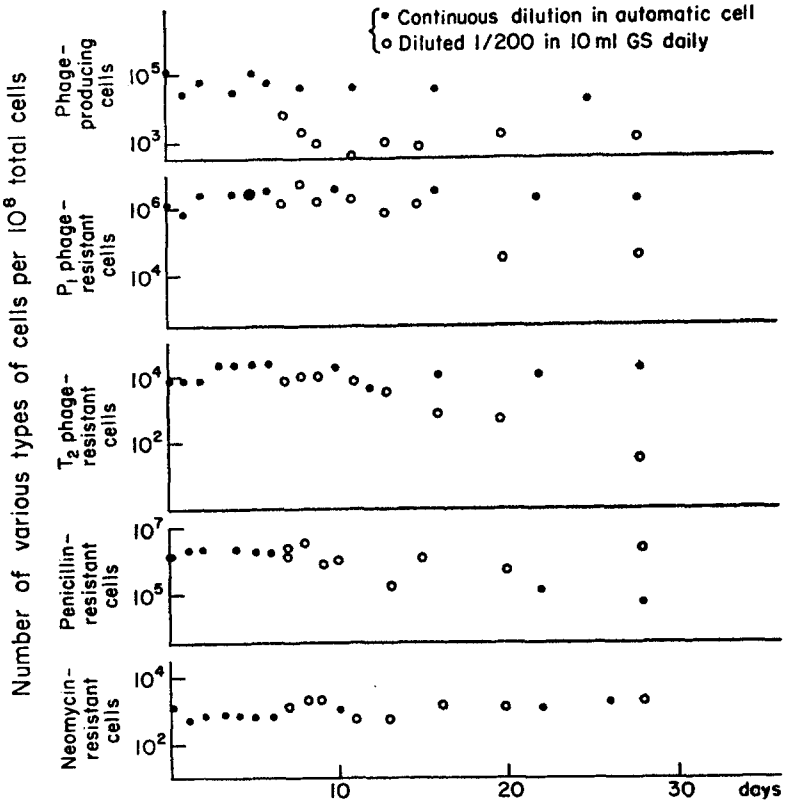

FIGURE 2. Proportion of various types of cells in K12λ cultures grown in test tubes after 2 wks' continuous logarithmic growth in automatic culture cell.

T_2 phage-resistant cells, 0.08. The virus-producing cells, therefore, reach equilibrium much more rapidly than the T_2 phage-resistant or P_1 phage-resistant cells, after either an increase or decrease in the mutation rate.

The over-all result is the same as that in the case of *B. megatherium* cultures kept in continuous logarithmic growth.

The same mechanism probably accounts for the remarkable increase in virulence of pneumococcus cultures kept in continuous logarithmic growth (4). It is possible that the increase in virulence of pathogenic bacteria during epidemics is also due to continued rapid growth.

EXPERIMENTAL METHODS

Culture medium, glucose salt solution (6).

Plating agar for phage determinations (29).

The automatic cell was that described previously (16, 19), except that a heating coil was placed around the culture media inlet tube. This was necessary with *E. coli* in order to prevent the organism from growing in the inlet tube itself. The inlet tube enters a dropping column and is not connected directly to the culture. The growing culture, therefore, is not heated. This apparatus maintains constant cell concentration by turbidimetric control of the addition of culture medium. It differs in principle, therefore, from the chemostat, in which the growth rate is limited by the supply of some necessary constituent of the medium (13, 24).

Theoretically, conditions are much simpler than in the chemostat (15), but experimentally they are more complicated. In the present apparatus, the walls of the vessel are continuously cleaned by rapid (500 to 800/min) strokes of rubber rings. In case cells still adhere to the walls or wipers, the cell may be cleaned by operating the wipers at 2000 to 3000 strokes/min for a short time. The organisms removed after cleaning the cell in this way contained the same proportion of mutants as before.

P_1 phage-resistant and T_2 phage-resistant colonies were determined by adding 0.1 ml culture to 1 ml high titer P_1 or T_2 phage. Then 0.2 ml agar was added and the suspension spread on 15 ml plating agar.

Neomycin-resistant and penicillin-resistant colonies were determined by spreading 0.1 ml suspension on 15 ml yeast extract peptone agar containing 20γ penicillin/ml or 1γ neomycin/ml. The penicillin-resistant colonies varied, probably because there is no definite plateau in penicillin-resistant cells, and hence slight variations in the concentration of penicillin cause marked differences in the number of surviving colonies.

Cross-plating experiments showed that the mutants were individually distinct, except that some neomycin-resistant colonies were also penicillin-resistant, and some P_1 phage-resistant colonies were also T_2 phage-resistant.

Lambda phage determination, 0.5 ml toluene added to 5 ml of culture. Sample removed from below toluene after ½ hr and diluted in peptone. 1×10^7 K12S cells/ml added and suspension plated by Gratia's double layer technique as used by Adams (1).

The burst size[2] of the K12λ in glucose salt solution is about 20 and the number of phage-producing cells, therefore, is calculated as 1/20 of the phage titer. No significant difference was found between the burst size of the logarithmic and test tube cultures.

Mutation rate by null fraction method, 10 to 20 small samples of the culture containing no mutants were grown for 1 to 4 hr and the number of mutants and the increase in the number of cells determined by plating.

The mutation frequency rate constant, λ, =

[2] It appears that this term is a misnomer, since recent electron microscope photographs (7, 27) show that some phages, at any rate, are released from the intact cell. These results confirm those from this laboratory which showed that phage release may precede lysis (26).

$$\frac{\ln \dfrac{\text{Total No. of samples}}{\text{No. of mutant samples}}}{\text{Increase in No. of cells per sample}}$$

C, the mutation time rate constant, $= 2\lambda A$, where A is the growth rate of the culture. The figures in Table I are the means of 5 to 10 determinations.

It is possible to determine the mutation rate in this way, since the high rate persists for several days, after removal from the continuous culture cell (Fig. 2).

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[3] Figs. 1 and 2 of this paper are reversed. Fig. 1 corresponds to the title of Fig. 2 and Fig. 2 to the title of Fig. 1.

[4] Similar equations have been derived by Deskowitz and Shapiro (3), Shapiro (28), Delbrück (2), and Novick and Szilard (25). These derivations, however, did not take into account the fact that when a microbial organism divides, the number of new cells increases by 2, but the total population increases by 1. If the proportion of mutants is very small, as Novick and Szilard assumed, this peculiarity affects only the numerical value of the mutation frequency rate constant, but if the mutation rate is significant compared to 1, failure to correct for the 2 new cells predicts completely incorrect results. Otherwise, these equations differ only in notation from those of Novick and Szilard.