# Appearance of Virulent Bacteriophages in Lysogenic *E. coli* Cultures after Prolonged Growth in the Presence of Triethylenemelamine

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ABSTRACT Continuous culture of *coli* 12λ, P22, 600–434, 600–21, and 600–299 in the presence of triethylenemelamine (TEM) results in the appearance of a new virulent virus which attacks the parent culture. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) is effective with 600–21 and ultraviolet light with 12λ and 600–21. The cultures which produce the virulent virus continue to do so indefinitely in the absence of the mutagen, but are not lysogenic for the virus. Most of the cells in such cultures are resistant to the virus and do not produce any, but there are a few mutant cells sensitive to the virus and the virus multiplies by infection of these sensitive mutants.

## INTRODUCTION

Mutagens, as a rule, increase the proportion of all mutants present to approximately the same extent, rather than cause the appearance of new mutants (Muller, 1954). K12λ cultures, for example, exposed to ultraviolet light, hydrogen peroxide, or triethylenemelamine (TEM) produce 2 to 20 times as many phage-producing cells, neomycin-resistant cells, penicillin-resistant cells, $P_1$ phage-resistant cells, and $T_2$ phage-resistant cells, as do the control cultures (Northrop, 1966). In those experiments the cultures were exposed to the mutagens for a short time only. If *coli* 12λ or P22 cultures are grown continuously in the presence of TEM, an entirely different result is obtained. Under the latter conditions a new virulent virus appears which cannot be found in the untreated cultures and which attacks the parent culture itself (Northrop, 1965). These virulent viruses cause more or less

complete lysis of the untreated lysogenic cultures or of *coli* K12S. The present article reports an extension of these studies to other strains of *coli*.

## MATERIALS AND METHODS

*Culture Medium* Tryptone broth (Sinsheimer, 1959), or nutrient broth (NB), or yeast extract peptone (YEP) (Northrop, 1939).

*Plating Method* Sinsheimer (1959).

*Stock Cultures* Transfer by loop in NB daily.

*Test for Virulent Phage* Toluene was added to sample to prevent resistant cells from overgrowing the plaques. One drop was placed on YEP plate made with stock culture of strain used, or diluted and assayed as above.

*Test for Phage-Resistant Colonies* $10^6$ to $10^7$ cells were added to 1 ml high titer phage ($10^8$ to $10^9$ phage/ml), which had been sterilized by shaking with toluene, 3 to 6 hr at 25°C, 0.2 ml agar added, and the suspension spread on YEP plate.

*Cultures* The writer is indebted to Dr. F. Jacob for the following lysogenic *coli* cultures and for information concerning them:

*P22* Defective prophage related to λ, noninducible by ultraviolet light; 600–434 prophage and 600–21 prophage related to λ, inducible by ultraviolet light; 600–299 prophage not related to λ, noninducible. The numbers are those of Dr. Jacob's laboratory.

The TEM was kindly furnished by Dr. Ruegsegger of Lederle Laboratories.

## RESULTS

The results obtained with *coli* 600–434, 600-21, and 600-299 are summarized in Table I. All the cultures produce a virulent phage after 5–20 days' growth in the presence of TEM. Cultures of K12λ, P22, and 600-434 do not produce any virulent phage in the absence of mutagens, but 600-21 and 600-299 do, and mutagens have no striking effect on the appearance of the virulent phage in the latter culture.

*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) had some effect on 600-21 and 600-299, but not on 600-434. Hydrogen peroxide had some effect on 600-299, but not on K12λ, P22, or 600-434. Ultraviolet light gave positive results with K12λ and 600-21, but not with P22 or 600-434. Butadiene dioxide gave positive results with 600-434. Hydroxylamine, urethan, maleic hydrazide, and versene did not produce the virulent virus and, if anything, decreased its incidence in 600-299. Cultures of *coli* K12S, *megatherium* 899, and *megatherium* KM gave negative results with all the mutagens.

TEM evidently is thus almost specific in causing the appearance of the

TABLE I

APPEARANCE OF VIRULENT PHAGES IN *E. coli* CULTURES GROWN CONTINUOUSLY IN THE PRESENCE OF MUTAGENS

Experimental Procedure Four tubes, containing 5 ml tryptone, were inoculated from one single colony each. Four tubes, containing the mutagen, inoculated from these four tubes. All tubes were incubated for 24 hr at 37 °C and 0.3 transferred to fresh 5 ml culture medium. Transfer was repeated daily and each tube was assayed for a virulent virus. Each 5 ml culture contains approximately $5 \times 2 \times 10^9 = 10^{10}$ cells. The incidence of the virus, therefore, is

$$\frac{\text{No. of positive cultures}}{\text{Total number of cultures tested} \times 10^{10}}$$

In the case of 600-434, for instance, 620 control cultures were tested and all were negative. The incidence of the virulent virus in the controls is therefore $\frac{<1}{6 \times 10^{12}}$. In the TEM series there was 1 positive out of about 20 cultures, corresponding to an incidence of $\frac{1}{2 \times 10^{11}}$, or >30 times as high as in the control series.

As soon as the virulent virus appears, the tube is dropped from the series. Thus a ratio of 4/80 shows that all four replicate cultures developed the virus by the time 80 cultures had been transferred and assayed. 0/160 shows that no virus appeared in any of the 4 replicate tubes after 40 transfers each.

| Culture | | K12 λ | Coli P22 | 600-434 | 600-21 | 600-299 | K12S |
|---|---|---|---|---|---|---|---|
| Mutagen | Series No. | Positive/Total tests | | | | | |
| 0 (Control cultures) | 1 | 0/150 | 0/120 | 0/160 | 4/160 | 4/90 | 0/200 |
| | 2 | | | 0/120 | | 1/50 | |
| | 3 | | | 0/160 | | 4/130 | |
| | 4 | | | 0/180 | | | |
| Triethylenemelamine (TEM) (0.1 mg/ml) | 1 | 4/25 | 4/36 | 4/75 | 4/40 | 4/120 | 0/200 |
| | 2 | | | 4/42 | | 4/45 | |
| | 3 | | | 4/80 | | 4/6 | |
| | 4 | | | | | 3/43 | |
| *N*-Methyl-*N'*-nitro-nitroso-guanidine (MNNG), 0.05-0.1 mg/ml | 1 | | | 0/50 | 4/24 | 4/42 | |
| | 2 | | | | | 0/20 | |
| | 3 | | | | | 3/46 | |
| $H_2O_2$, $10^{-3}$ M | 1 | 0/120 | 0/120 | 0/160 | | 4/60 | |
| | 2 | | | | | 2/20 | |
| | 3 | | | | | 4/15 | |
| U.V., 5 min at 15 cm | 1 | 4/38 | 0/120 | 0/160 | 2/40 | 0/30 | |
| | 2 | | | | | 1/20 | |
| | 3 | | | | | 2/60 | |
| Butadiene dioxide, 0.05 mg/ml | 4 | | | 2/102 | | 2/63 | |
| Hydroxylamine, 0.02 mg/ml | 4 | | | 0/162 | | 1/146 | |
| Urethan, 10 mg/ml | 4 | | | 0/166 | | 0/146 | |
| Maleic hydrazide, 2 mg/ml | 4 | | | 0/166 | | 0/146 | |
| Versene, 0.2 mg/ml | 4 | | | | | 0/146 | |

virulent viruses. This mutagen reacts with N-7 of guanine (Wheeler, 1967), as does MNNG (Singer and Fraenkel-Conrat, 1967).

The appearance of the virulent virus is the result of prolonged growth in the presence of the mutagen, and is not due simply to the fact that a large number of cultures were tested. If the same number of cultures are grown for 1 day only in the presence of the mutagen, no virulent phage is found. This result is summarized in Table II. In this experiment 3 sets of 20 tubes each were inoculated from a stock culture of 600-434. Set A contained tryptone broth, B and C contained tryptone + 0.1 mg TEM/ml. The A cultures were transferred every day 0.3/5 ml into tryptone. The B tubes were inoculated 0.3/5 ml each day from the corresponding A tubes into tryptone + TEM. The C set was transferred daily into tryptone + TEM. This C set,

TABLE II

DAYS' GROWTH REQUIRED TO PRODUCE VIRULENT PHAGE WITH 600-434 GROWING IN TRYPTONE ± TEM

| Transfer No. (≈ 1 day's growth) | A 20 tubes, 5 ml each, transfer 0.3/5 daily in tryptone | B 0.05 mg TEM/ml in tryptone. Inoculate 0.3/5 daily from A cultures | C Tryptone + 0.05 mg TEM/ml transfer 0.3/5 daily from preceding C cultures |
|---|---|---|---|
| | Ratio positive/total tubes | | |
| 1-5 | 0/100 | 0/100 | 0/100 |
| 6 | 0/20 | 0/20 | 8/20 |
| 7 | 0/20 | 0/20 | 12/20 |
| 8 | 0/20 | 0/20 | 19/20 |
| | No virulent phage in tryptone or in tryptone + TEM for 1 day. All tubes develop virulent phage after 5 to 10 daily transfers in tryptone + TEM. | | |

therefore, grew in the presence of TEM throughout the experiment, while the same number of B cultures grew 1 day each in the presence of TEM.

No virulent phage appeared in either the A or B set during the 8 days, nor in the C set for the first 5 days. In the next three transfers all but one tube in the C set produced the virulent phage.

*Changes in Cultures of coli 600-434 in the Presence of TEM*

The results of an experiment in which cultures of 600-434 were transferred daily in tryptone ± 0.1 mg TEM are shown in Fig. 1. The cultures were transferred for 3 days with no TEM present and during that time no significant changes occurred. The 4th to 18th transfers were in tryptone containing 0.1 mg TEM/ml. The cell concentration decreased when grown in the TEM medium, but no change occurred in virus production until the 10th transfer. At that time $10^3$ plaques/ml appeared on 600-434 plates. The next day there were $10^6$ to $10^7$ plaques per ml and that titer remained nearly

constant throughout the experiment. The 18th transfer was made into tryptone alone and also into tryptone + TEM. Both the cultures continued to produce about the same concentration of the virulent phage. The plaque count on K12S plates remained about the same throughout the experiment,

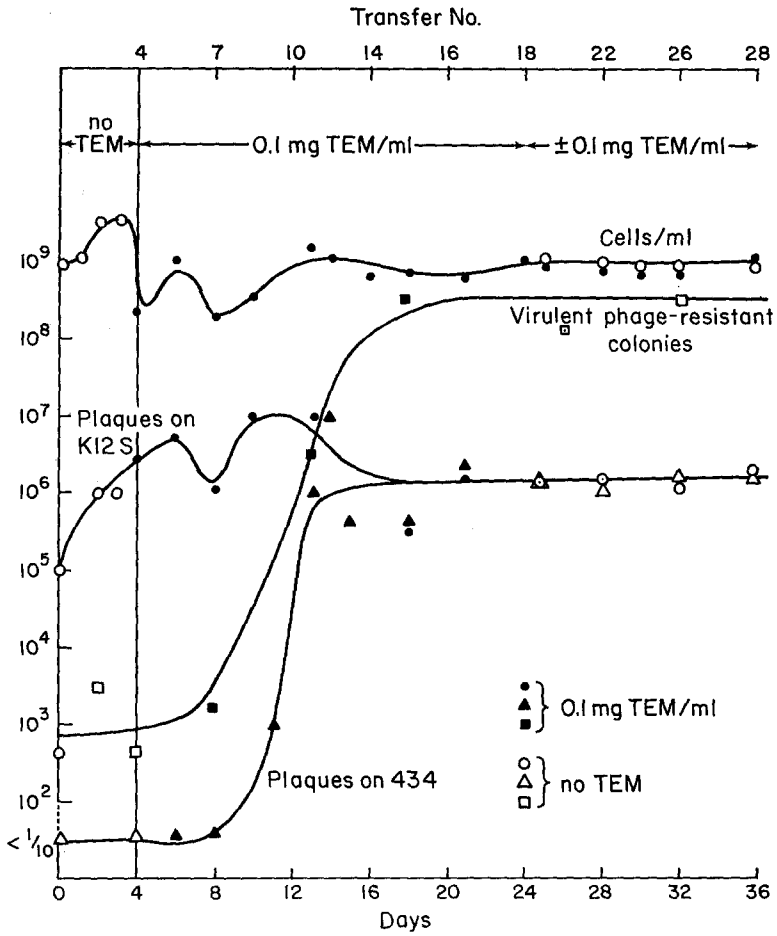


FIGURE 1. Changes in 600-434 cultures grown in the presence of 0.1 mg TEM/ml. Cultures transferred 1/day 0.3/10 ml in tryptone medium ±0.1 mg TEM/ml, 37°C, 24 hr. Assayed for plaques on 600-434 and K12S and for 600-434 phage-resistant colonies, and cell concentration by optical density.

but for the first nine transfers there were no corresponding plaques on 434 plates. After the virulent phage appeared, the plaque count on K12S or 434 was about equal, probably because the virulent phage infects both the K12S and 434 cultures. The plaques formed by the virulent phage were clearer than those of the lysogenic phage, but the difference was not sufficient to allow differential counts to be made.

The proportion of colonies resistant to this virulent phage increases rapidly as soon as the phage appears and soon practically all cells in the culture are resistant to the virus.

## PROPAGATION OF THE VIRULENT VIRUS

Cultures of 600-434 or 600-21 which have produced the virulent virus after growth in the presence of TEM continue to do so indefinitely with or without the presence of the mutagen. Colony analysis of such cultures, however, shows that they are not lysogenic but that the virus is propagated by infecting a few sensitive mutants which arise from the virus-resistant cells. The results of such an experiment are shown in Table III.

A culture of 600-21 which had produced a virulent phage when grown in the presence of MNNG was diluted in steps of 10. 1 ml of each dilution was

TABLE III

CONDITIONS FOR CONTINUOUS PRODUCTION OF VIRULENT VIRUS

600-21 transferred 22 times in NB + MNNG. Diluted as below. Add 1 ml of each dilution to 10 ml NB, grow up, and assay for B, plaques on K12S and on 600-21. Numbers in parentheses indicate calculated values.

| Dilution | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ |
|---|---|---|---|---|---|---|---|---|
| Cells/ml | $10^7$ | ($10^6$) | ($10^5$) | ($10^4$) | ($10^3$) | ($10^2$) | ($10^1$) | (1) |
| Plaques/ml on 600-21 | ($10^5$) | ($10^4$) | ($10^3$) | $10^2$ | (10) | <10 | <10 | <10 |
| 1 ml added to 10 ml NB—grown up 37°C—assayed | | | | | | | | |
| Cells/ml | ← all more than $10^8$ → | | | | | | | 0 |
| P/ml on 600-21 | + | + | + | − | − | − | − | − |
| P/ml on K12S | + | + | + | + | + | + | + | − |

added to 10 ml of nutrient broth, grown up at 37°C and assayed for phage on K12S and on 600-434. Those tubes which were inoculated with $>10$ cells all produced phage for K12S. Those cultures which were inoculated with $>10^2$ virulent phage particles (equivalent to $>10^4$ cells) all produced more of them, but those that received $<10^2$ virulent phage particles did not produce any.

These results indicate that these latter virulent virus-producing cultures are not lysogenic, since the virulent virus is not carried by the bacterial cells. The continual production is accomplished by infection of a few sensitive bacterial mutants which arise during the growth of the culture. The titer of such cultures is usually about $10^5$ virus particles per $10^9$ cells. If an infective cell is assumed to produce 100 virus particles, then there is about 1 sensitive cell per $10^6$ cells. This is of the order of magnitude to be expected for the incidence of a mutant.

The correctness of this explanation is confirmed by the fact that growth of the culture in media containing versene, which prevents infection of sensi-

tive cells, results in the loss of virulent virus, although the lysogenic virus continues to be produced. Transfer of the culture by loop also results in loss of the virulent virus, since, in this case, too few virulent virus particles are in the inoculum.

The results with 600-299 are similar except that the incidence of the virulent virus in the control tubes is higher than with 600-21 or 600-434. Also single colonies from old virus-producing cultures are often sensitive to the virus and are lysed by the virus. Such sensitive cells are able to exist in old cultures in the presence of the virus since infection cannot occur in a culture which has approached its maximum cell concentration.

The virulent viruses obtained in this way all produce small clear plaques on the parent culture or on K12S plates. They all cause more or less complete lysis of K12S or of the parent culture.

Each lysogenic culture plates on all the other lysogenic cultures, and those cultures which produce the virulent virus plate on all the others, including their own parent strain.

The virulent virus obtained in this way may be related to the virulent phage mutants described by Jacob and Wollman (1954), Lederberg and Lederberg (1953), Bertani (1965), and Kaiser and Jacob (1957).

## DISCUSSION

The fact that in some cultures (P22 or K12λ) very high λ phage is produced before the virulent phage appears, suggests that the virulent virus is the result of a mutation of the phage itself. Repeated attempts, however, to obtain a virulent mutant by treatment of λ phage were unsuccessful.

The fact that prolonged growth in the presence of the mutagen is required suggests that some mutant gradually overgrows the culture in the presence of the mutagen and that the virulent phage is then produced by this mutant. There is a marked increase in all mutants tested in the presence of TEM, but no correlation could be found between the increase in various mutants and the production of the virulent virus. The cultures do not become resistant to TEM.

The production of the virulent phage from lysogenic cultures may explain the many early reports of the production of phages "*de novo*" (reviewed by Raettig). At that time the widespread occurrence of lysogenic cultures was not realized and it is quite probable that the cultures which produced the phage were lysogenic. The results may also be related to the occurrence of virulent animal viruses described by Gross (1958), Duran-Reynals (1952), and Yamafuji (1964).

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