

MUTANT OF LAMBDA BACTERIOPHAGE PRODUCING A THERMOLABILE ENDOLYSIN

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ABSTRACT

CAMPBELL, ALLAN (University of Rochester, Rochester, N.Y.) AND ALICE DEL CAMPILLO-CAMPBELL. Mutant of lambda bacteriophage producing a thermolabile endolysin. *J. Bacteriol.* **85**:1202-1207. 1963.—Endolysin from lambda bacteriophage and a temperature-sensitive mutant thereof was partially purified. The mutant enzyme was distinguishable from the wild type by its greater rate of inactivation by high temperature and by urea. Lysogenic cells carrying the mutant phage did not lyse after induction if kept at 43 C, but, at times around 70 min after induction, rapid lysis occurred following transfer to lower temperatures. This lysis was not inhibited by cyanide or chloramphenicol and therefore probably resulted from enzyme already synthesized at the high temperature. Addition of these inhibitors to the culture at 43 C rapidly destroyed the ability to lyse after a subsequent temperature shift.

It was previously reported (Campbell, 1961a, b) that, in one of a collection of temperature mutants of lambda bacteriophage, lysis did not occur at high temperatures. This was apparently due to a defect in the lytic enzyme endolysin, but the evidence available at that time was indirect. Subsequently, as an adjunct to a study of suppressor-sensitive endolysin mutants, we have made some relevant direct observations.

Recently, Groman (1962) isolated another temperature mutant in which lysis is also affected and called attention to the similarities between his mutant and our own. His mutant accumulates endolysin under conditions where lysis is blocked, from which he reasonably concluded that some step other than endolysin production must be blocked (Groman, 1962; Groman and Suzuki, 1962). The purpose of the present paper is to report the facts concerning our mutant, so that a proper comparison can be made.

MATERIALS AND METHODS

Bacterial and phage stocks. The temperature mutant *hs*₁₂₉ of lambda bacteriophage has been previously described (Campbell, 1961a). It was induced by the ultraviolet restoration technique of Weigle (1953) and picked as one of a group of mutants able to form plaques at 37 C but not at 43 C. As a control, we employed, in some experiments, the wild-type lambda stock from which the mutant was derived. Wild type can form plaques at both 37 and 43 C. We used induced lysogenic bacteria rather than infected bacteria for our experiments. These were prepared by lysogenizing strain C600 of *Escherichia coli* K-12 (Appleyard, 1954) with wild-type lambda and with lambda *hs*₁₂₉.

For the enzyme assays, we used the phage-resistant strain CR63 (Appleyard, McGregor, and Baird, 1956) of *E. coli* K-12.

Medium. Tryptone broth was prepared from the following: Tryptone, 10 g; NaCl, 5 g; thiamine hydrochloride, 1 mg; and deionized water, 1 liter.

Induction. Vegetative phage growth was induced in lysogenic strains by growing cells in Tryptone broth to a density corresponding to a Klett reading of about 40. The cells were centrifuged and resuspended in one-half the growth volume of 0.065 M phosphate buffer (pH 6.8) containing 0.001 M MgSO₄. The cell suspension was then irradiated in a petri dish with a General Electric germicidal lamp, giving a total ultraviolet dose of about 250 ergs/mm². The irradiated suspension was added to an equal volume of broth containing 10⁻³ M MgSO₄ and aerated. Turbidity was followed in a Klett-Summerson photoelectric colorimeter.

Preparation and purification of enzyme. Endolysin of wild-type lambda was isolated from lysates produced by induction, and purified as follows. The protein in the lysates was concentrated by adding powdered (NH₄)₂SO₄, with stirring, to a final concentration of 80% of satura-

tion. In this and all subsequent ammonium sulfate fractionation steps, 70 g of powdered $(\text{NH}_4)_2\text{SO}_4$ were added per 100 ml of fluid (initial volume) for 100% saturation. No corrections were made for changes in volume after the first addition of $(\text{NH}_4)_2\text{SO}_4$.

After the stirring was discontinued, the precipitate settled on the surface. It was scooped off and dissolved in 0.065 M phosphate buffer (pH 6.8) containing 0.001 M MgSO_4 . The excess $(\text{NH}_4)_2\text{SO}_4$ was removed by dialysis against 0.02 M phosphate buffer (pH 6.8) for 4 hr.

The first step in the purification consisted of removing nucleic acids with protamine. A solution of protamine sulfate (20 mg/ml) was added, with stirring, to the dialyzed $(\text{NH}_4)_2\text{SO}_4$ fraction. The ratio of protamine sulfate added to protein present was about 1:1. After stirring for 15 min, the precipitate was removed by centrifugation. The supernatant was diluted with 0.065 M phosphate buffer (pH 6.8) containing 0.001 M MgSO_4 to a protein concentration of 1.9 mg per ml. Powdered $(\text{NH}_4)_2\text{SO}_4$ was added with stirring to bring the salt concentration to 50% saturation. After 15 min of stirring, the precipitate was removed by centrifugation. The supernatant was brought to 70% saturation by further addition of $(\text{NH}_4)_2\text{SO}_4$. The precipitate was centrifuged down and dissolved in 0.065 M phosphate buffer (pH 6.8) containing 0.001 M MgSO_4 .

This 50–70 $(\text{NH}_4)_2\text{SO}_4$ fraction was then dialyzed for 4 hr against 0.02 M phosphate buffer (pH 6.8) containing 0.001 M ethylenediaminetetraacetic acid (EDTA). It was then diluted with cold water to a protein concentration of about 5.0 mg per ml, and diethylaminoethyl (DEAE) cellulose was added. The ratio of DEAE to protein was about 20:1. After 20 min of mixing, the precipitate was removed by centrifugation, washed twice with 0.007 M phosphate buffer, and discarded. The washes were combined with the DEAE supernatant, and the mixture was fractionated with $(\text{NH}_4)_2\text{SO}_4$. The 50 to 59% $(\text{NH}_4)_2\text{SO}_4$ fraction contained all the activity and resulted in an over-all purification of 125-fold, compared with the original 80% $(\text{NH}_4)_2\text{SO}_4$ fraction. The over-all yield was 30%.

Mutant enzyme. The isolation of the mutant enzyme presented special problems, because the cells lysed only very slowly at 37 C, and the enzyme turned out to be quite unstable. No detectable activity was found in lysates prepared

in the usual manner for wild-type enzyme. We therefore employed a procedure that was suggested by our finding (see below) that rapid lysis occurs when a culture is shifted from a high temperature such as 37 C down to room temperature (about 24 C for this preparation); 100 ml of C600 (λ $h_{S_{129}}$) were grown up and induced in the usual way. After 65 min of growth at 37 C, they were centrifuged for 5 min, re-suspended in 10 ml of broth plus buffer, and aerated an additional 10 min at 37 C. Next, they were transferred to a bath at 24 C, allowed to stand for 1 hr, and stored in a deep freeze until use.

The mutant enzyme was concentrated with ammonium sulfate and purified 15-fold by steps involving protamine and ammonium sulfate fractionation.

All operations involved in purifying the enzymes after the lysates were obtained were carried out at about 0 C. Centrifugations were made in a Spinco centrifuge at 20,000 $\times g$ for 10 to 15 min.

Enzyme assay. The lytic activity was measured in a Beckman spectrophotometer at 600 $m\mu$ and at room temperature. The experimental cuvette contained phosphate buffer (pH 6.8), 33 μ moles; MgSO_4 , 0.6 μ moles; EDTA-treated cells of strain CR63 prepared according to the method of Jacob and Fuerst (1958); and water to a final volume of 3.0 ml. The initial optical density (OD) was about 0.4. The enzymatic reaction was started by adding between 0.02 and 0.2 ml of the preparation to be assayed. The OD was measured every 15 sec for 2 min. The specific activity was computed as the change in OD between 30 sec and 1 min divided by the weight (in mg) of protein present. The protein concentration of all enzyme preparations was calculated from their light absorption at 260 and 280 $m\mu$ with correction for the nucleic acid content according to Warburg and Christian (1941). It was verified that the activity measured in this way was proportional to the amount of enzyme added.

RESULTS

To see whether there was a structural difference between the purified endolysin derived from the mutant and that obtained from the wild type, their sensitivity to thermal inactivation and to some chemicals was compared.

Thermal inactivation. If the two enzymes were

incubated for 30 min at 37 C and then assayed, the wild-type enzyme retained all its activity, whereas that of the mutant enzyme completely disappeared. At 31 C, the wild-type enzyme was stable, but the rate of inactivation of the mutant enzyme was slow enough to measure conveniently. Figure 1 shows the kinetics of inactivation of the mutant enzyme at 31 C. A mixture of wild-type and mutant enzyme was included to eliminate the possibility that the thermal instability was due to something in the lysate other than the enzyme itself. The approximate additivity seen makes this possibility unlikely.

Chemical inactivation by urea. Working with *Neurospora tyrosinase*, Sussman (1961) showed that urea inactivated heat-sensitive mutant enzymes to a greater extent than it did the wild-type enzyme. We obtained similar results with our enzymes. Incubation for 15 min with 2 M urea provided a satisfactory differentiation between the two. In these experiments, equal volumes of the enzyme and 4 M urea were mixed and then incubated for 15 min at room temperature (23 to 24 C). Samples were immediately removed and assayed for enzymatic activity.

The activity of the mutant enzyme was found to be completely destroyed by 2 M urea, whereas

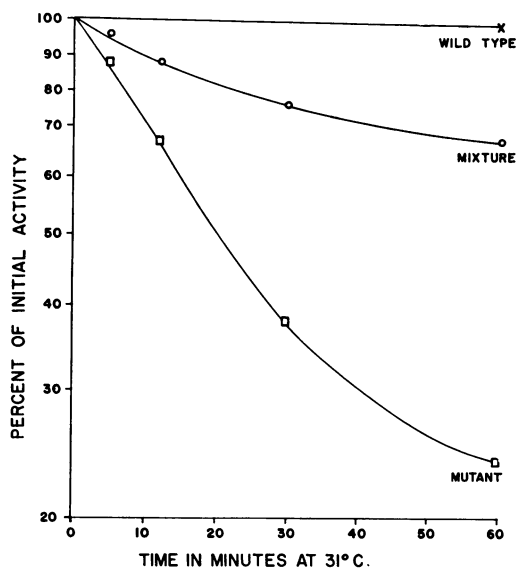


FIG. 1. Thermal inactivation of mutant and wild-type enzymes. Purified preparations of mutant and wild-type enzyme and a 1:1 mixture (on an activity basis) were incubated at 31 C. Samples were removed at various times and assayed immediately.

the wild-type enzyme remained active. The exact result with the wild type varied from preparation to preparation, and seemed to depend somewhat on the purity of the enzyme. The activity of the crude 80% ammonium sulfate fraction was not affected by 2 M urea. Under the same conditions, the activity of the crude 80% ammonium sulfate mutant enzyme was completely destroyed. A 10× purified preparation of the wild-type enzyme was inactivated 28% by 2 M urea, whereas a 125× purified preparation was inactivated 35%.

Control experiments showed that urea added to the assay mixture in the same final concentration as with the sample pretreated with urea (i.e., 0.2 M) had no effect on the activity of the mutant enzyme not previously exposed to urea, and that the mutant enzyme was only inactivated about 25% by 15 min of exposure to room temperature in the absence of urea.

Inhibition by polyamines. Brown (1960) reported that spermine was an effective inhibitor of the autolysis of cell walls prepared from a marine bacterium. This autolysis is presumably due to some lytic enzyme in the preparation. It seemed of interest to test the effect of spermine and other polyamines on lambda endolysin. When spermine was added to the assay mixture at a concentration of 2×10^{-4} M, a 48% inhibition of wild-type enzyme was seen. Mutant enzyme under the same conditions was inhibited 45%. Spermidine and putrescine at the same concentration had very little inhibitory activity on either enzyme. The results therefore do not distinguish between the wild type and the mutant enzyme.

Physiological observations on the mutant phage. Figure 2 shows the optical density curves of induced lysogens carrying lambda hs_{129} or wild type lambda incubated at 34 or 43 C. Whereas wild type lysed rapidly at 43 C, the mutant showed little lysis in some experiments and none in others. Even at 34 C, lysis was considerably delayed in the mutant. In Fig. 3 and 4, we see the effect on the mutant of starting growth at one of these temperatures and switching to the other at various times after induction. It is seen that a shift from 34 to 43 C prevented lysis completely, and, if lysis had already commenced before the shift, it stopped almost immediately. A shift from 43 to 34 C, on the other hand, allowed lysis to occur, provided it was not done too late.

All these observations are understandable from the results of the *in vitro* enzyme assays. The enzyme is very thermolabile, and therefore lysis does not occur at elevated temperatures. After a shift down to 34 C, new enzyme may be synthesized, provided the cell has not passed

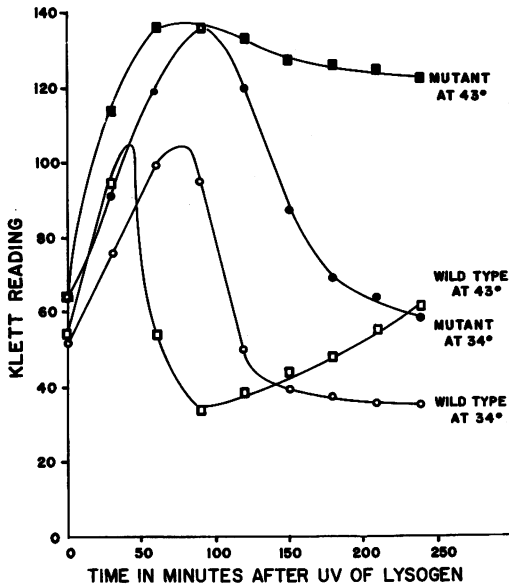


FIG. 2. Optical density curves for lysogens of wild-type and mutant phage, incubated after induction at 34 and 43 C.

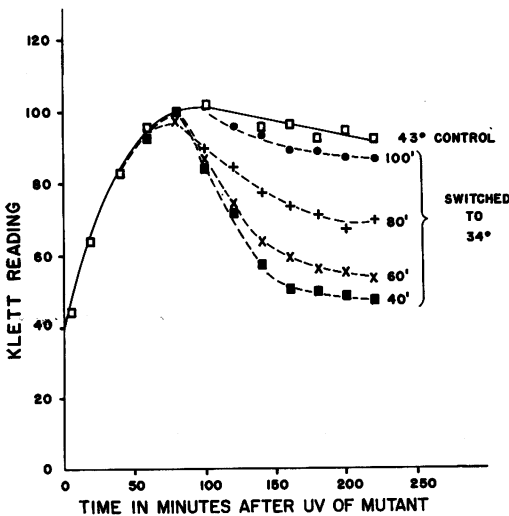


FIG. 3. Effect on lysis of shift down from 43 to 34 C at various times after induction of the mutant lysogen.

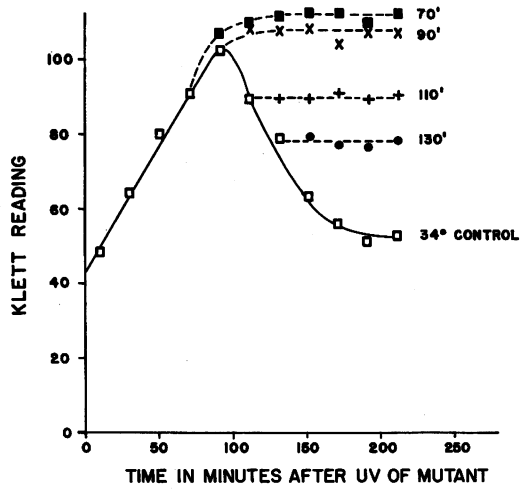


FIG. 4. Effect on lysis of shift up from 34 to 43 C at various times after induction of the mutant lysogen.

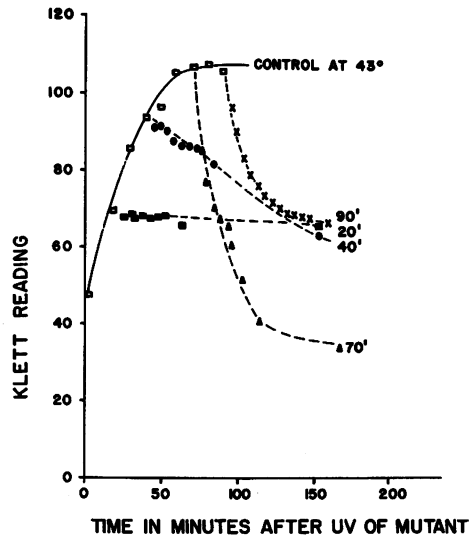


FIG. 5. Effect on lysis of placing the culture of the mutant lysogen at 24 C without aeration at various times after induction and growth at 43 C.

some "point of no return," as indicated by the very poor lysis seen after a shift at 100 min.

Less expected were the results shown in Fig. 5, where induced lysogens were switched at various times to room temperature (24 C) and kept there without aeration. From the several families of such curves we have run, the following conclusions can be drawn. (i) A shift down to room temperature before 30 min does not result in

lysis. (ii) The most rapid complete lysis is seen if the shift down is made at about 70 min. (iii) At times later than 70 min, the initial rate of lysis after a shift down is rapid, but the final amount of lysis becomes progressively less with longer times, as though a progressively larger fraction of cells were becoming "nonlysable" as time proceeds. (iv) The rate of lysis is maximal if the shift-down temperature is about 23 C.

These findings were unexpected because it seemed unlikely under these conditions that such rapid lysis could be caused by enzyme synthesized after the shift down, and almost all of that synthesized before the shift should have been thermally inactivated. To distinguish these alternatives, we added at the moment of the shift KCN (to a final concentration of 0.005 M) or chloramphenicol (final concentration, 25 $\mu\text{g}/\text{ml}$) or both. Either inhibitor should prevent the synthesis of new enzyme after the shift, but neither had a detectable effect on the rate of lysis. We therefore concluded that no enzyme synthesis need occur at the lower temperature in order to see lysis after a shift down.

From the *in vitro* experiments, we know that the mutant enzyme had a half-life of 21 min at

31 C and 3.9 min at 37 C. We calculate that the half-life at 43 C should be 0.7 min. So most of the activity seen in a shift down from 43 C should be owing to enzyme formed during the last minute before the shift. If we should inhibit enzyme synthesis for several minutes before the shift, we should prevent lysis. In our first experiment, we tried the effect of cyanide and chloramphenicol, alone and in combination, on lysis when they were (i) added at 50 or 70 min after induction, simultaneously with shift down; (ii) added at 50 min after induction, prior to a shift down at 70 min. Normal lysis was seen after the shift down in the first case, but not in the second.

To pinpoint the time more closely, several experiments of the type shown in Fig. 6 were carried out, in which chloramphenicol was added at 0, 1, 3, and 5 min before a shift down. One would like to do all the shifts simultaneously, but this is technically impossible. What was done instead was to bracket the experiment with the controls. The two curves without chloramphenicol thus came from shifts at 66 and 75 min, those with chloramphenicol simultaneously with the shift, at 67 and 74 min. For the 5-min curve, chloramphenicol was added at 68 min, and the shift down was at 73 min, etc. The similarity of the bracketing curves, together with the fact that the control rate of lysis reached a maximum at about 70 min, makes this a valid procedure and allows one to use the same induced culture for both the experimental and control curves.

In our previous experiments with chloramphenicol at 50 min and a shift down at 70 min, we had noticed some decrease in optical density during the 20 min at 43 C. In the present experiment, it was impossible to take any readings during the period between chloramphenicol addition and shift down, but there was an initial drop before the first reading which far exceeded the subsequently observed rate in those cultures where lysis was slow. We do not know the origin of this drop; the slight dilution caused by the addition of the chloramphenicol solution explains only a small fraction of it. At any rate, subsequent to this drop, there was almost complete inhibition of lysis if chloramphenicol was added 5 min prior to shift down, and even the addition at 1 min before shift down substantially decreased the rate of lysis. Addition at the time of the shift down did not affect the rate, although the final amount of lysis was decreased by the presence of chloramphenicol.

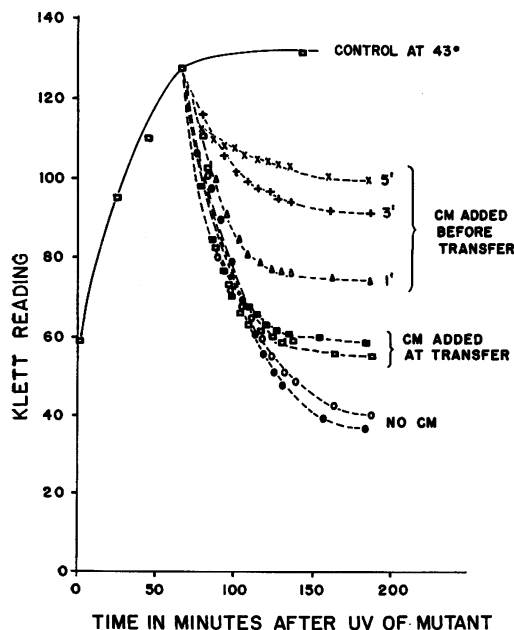


FIG. 6. Effect on lysis of addition of chloramphenicol (CM) simultaneously with and shortly before transferring culture to 24 C without aeration. Details in text.

This shows that essentially all of the enzyme responsible for the rapid lysis after the shift is synthesized during a few minutes close to the time of shifting. This agrees with the observed high rate of thermal inactivation *in vitro* and suggests that, in such a blocked mutant, the enzyme is manufactured in enormous excess. It does not completely prove that enzyme synthesis occurs immediately before rather than immediately after the shift, since there could be some lag before the chloramphenicol stopped protein synthesis in every cell.

DISCUSSION

It seems clear from the *in vitro* data that the *hs*₁₂₉ mutation has caused a structural alteration in the endolysin, making it more sensitive to denaturation by heat and by urea. It is obvious from the results of Groman (1962) and Groman and Suzuki (1962) that the endolysin from their mutant is much more stable than ours. The mutants are similar to one another in that (i) even if lysis is in progress at the time of a shift up in temperature to 43 or 44 C, it ceases very quickly at those temperatures; (ii) a shift down in temperature causes rapid lysis.

The lysis after shift down creates a new problem. The rapid onset of the lysis, together with the results of the chloramphenicol experiments, suggest that this lysis is due to enzyme synthesized at the high temperature immediately before shifting. However, if this is so, why don't the cells lyse at the high temperature? They must be maintaining a high steady-state concentration of enzyme for at least one-half hour, and yet the optical density remains constant. Either it takes some time for newly formed enzyme to find its substrate, during which time it is inactivated, or there is a reversible as well as an irreversible effect of temperature on lysis by *hs*₁₂₉.

The second possibility cannot be tested directly, because the assay by cellular lysis does not work well at high temperatures. We cannot even say whether the enzyme is more or less active at 43 than at 25 C. It is possible that the endolysin of Groman's mutant is reversibly, but not irreversibly, inactivated by heat. Alterna-

tively, the similar behavior of his mutant and ours might be a common property of all mutants affecting the lytic process, even if they affect different steps of it.

ACKNOWLEDGMENTS

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