STUDIES ON LYSOGENESIS

II. THE EFFECT OF TEMPERATURE ON THE LYSOGENIZATION OF SHIGELLA DYSENTERIAE WITH PHAGE P1

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Sensitive bacteria, when infected with a temperate phage, either lyse or become lysogenic. With the temperate phage P1 and its host Shigella dysenteriae, the outcome of infection is affected strikingly by temperature. The discovery of this temperature effect has allowed us to study some features of the lysogenization process; these will be described in this paper. The definitions of certain terms and a general discussion of the problems involved in the study of lysogenesis were given in a previous paper (Bertani, 1953a; see also Benzer et al., 1950).

MATERIAL AND METHODS

Bacteria and phages. Strain Sh of S. dysenteriae was used throughout. All platings for phage assay were done on its streptomycin resistant derivative, strain Sh/s (Bertani, 1951). In some experiments another derivative, Sh/s/T6, which is resistant to streptomycin and to phage T6, was used.

Phage P1 was isolated originally from a lysogenic strain of Escherichia coli (Bertani, 1951). It has tadpole shaped particles (electron micrographs by Dr. T. F. Anderson and Mr. A. E. Vatter); it requires Ca++ for adsorption; and on Sh (or Sh/s or Sh/s/T6) it gives turbid plaques 0.3 to 2 mm in diameter (figure 1a).

Lysogenic strains Sh(P1) can be isolated from the bacterial growth visible in the center of plaques formed by P1 on Sh. The amount of free P1 in actively growing cultures of Sh(P1) is of the order of 1 per cent of the number of bacteria present. Phage P1 is adsorbed by cells of Sh(P1) but does not lyse them. While Sh and Sh/s are sensitive to all the T-phages, Sh(P1)

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shows a very low efficiency of plating with phages T1, T3, and T7.

Lysates of P1 occasionally contain virulent mutants, that is, mutants that have lost the ability to form lysogenic complexes with cells of strain Sh. These mutants are found also in the phage produced by Sh(P1). One such isolate, P1 vir, has been used throughout our work. Like P1, the mutant P1 vir has tadpole shaped particles and requires Ca++ for adsorption. On Sh (or Sh/s or Sh/s/T6) it gives clear plaques which are on the average larger than P1 plaques (figure 1b). It is serologically indistinguishable from P1. It does not form plaques when plated on Sh(P1); it is, therefore, a weak virulent mutant (Bertani, 1953b).

Media and procedures. The experimental techniques were those commonly used in work with the well known phages of the T-series (Adams, 1950). LB nutrient medium (Bertani, 1951) was used throughout. Unless otherwise specified, phage assay plates were incubated at 37 C. Phage lysates were prepared with the plate technique of Swanstrom and Adams (1951). P1 neutralizing antiserum was obtained from rabbits which had received repeated injections of P1 lysates. The streptomycin technique (Bertani, 1951) was used to assay free phage produced by cultures of Sh(P1).

Testing of individual colonies for lysogenicity was done by a spot test method. A small amount of broth was inoculated with each colony to be tested and incubated for several hours. During this period, lysogenic isolates produce some phage. Each culture was tested then by spotting a loopful on a streptomycin agar plate covered with Sh/s. The streptomycin sensitive bacteria are killed, while the phage present in lysogenic isolates produces a zone of lysis.

Phage adsorption in nutrient medium was carried out with bacteria from an actively growing
aerated culture (4 × 10^8 to 10^9 cells per ml), spun in the cold, and resuspended to a density of about 2 × 10^6 cells per ml in LB medium with 2.5 to 10 × 10^-2 M CaCl₂ added.

For adsorption in buffer (Benzer, 1952), actively growing cells were spun and resuspended to a density of about 2 × 10^6 cells per ml in buffer (K₂SO₄, 5.0 g; Na₂HPO₄ · 12H₂O, 0.76 g; KH₂PO₄, 0.15 g; NaCl, 4.0 g; dry gelatin, 0.01 g; distilled water, 1 liter) with 2.5 × 10^-3 M CaCl₂ added. The cell suspension was aerated for at least 30 minutes at 37 C. Phage diluted in the same buffer was added and the mixture was incubated for 20 to 30 minutes, after which the mixture was diluted in LB medium. Preliminary experiments showed that the latent period for P1 vir after adsorption in buffer was not significantly different from the latent period after adsorption in nutrient medium and that there was no significant loss of infective centers.

For premature lysis of phage infected cells, Doerrmann’s (1952) technique was employed. Samples of infected cells were diluted to a density of about 4 × 10^8 bacteria per ml in LB medium containing 10^-2 M NaCN and phage T6 (about 10^8 per ml). The lysing mixture was left 30 minutes at 37 C, diluted, and plated on Sh/s/T6. Control experiments with higher bacterial concentrations showed that the lysing mixture employed produces visible lysis.

RESULTS

Effect of temperature on multiplication of P1 vir. Several one step growth experiments were done with P1 vir on strain Sh at 37 C (figure 2a). The length of the latent period varied from 43 to 53 minutes, averaging 47 minutes. The burst size was between 400 and 500.

When the bacteria were similarly infected in buffer at 37 C, diluted in LB medium at 37 C, and 5 minutes later brought to 20 C or 24 C, the latent period of P1 vir was greatly prolonged. Plaque counts started to increase between 3 and 3½ hours at 24 C and between 4 and 7 hours at 20 C. The apparent burst size of P1 vir at 24 C was about 10; at 20 C it was 2 or 3.

Possibly, at low temperature only a very small proportion of the infected cells are lysed. This view is confirmed by other observations. Assay plates of P1 vir incubated at 20 C do not show any plaques although the bacterial layer is well developed. In one step growth experiments at 20 C one observes a gradual, irreversible loss of infective centers during the latent period: if
It seems, therefore, that at 20 C the lytic process of P1 \( \text{vir} \) is blocked in a majority of the infected cells. We wondered at which point in the multiplication process of P1 \( \text{vir} \) the block occurred. Doermann (1952) showed with several phages that mature phage is present inside infected cells some time before they lyse. Doermann's experiment was repeated with P1 \( \text{vir} \) both at 37 C and at 20 C. In two experiments with infected cells, incubated at 37 C and prematurely lysed, intracellular mature phage began to appear 13 and 16 minutes, respectively, before the end of the latent period (figure 2b). At 20 C no intracellular phage was detected as late as 5 hours after infection.

**Effect of temperature on multiplication of P1.** Several one step growth experiments with P1 at 37 C gave values between 43 and 52 minutes (average 47) for the latent period. The burst size varied between 150 and 350.

The effect of low temperatures parallels that on P1 \( \text{vir} \). At 24 C and 20 C the latent period was prolonged and the apparent burst size reduced. No plaques were obtained when P1 assay plates were incubated at 20 C.

There is an important difference, however, in the reactions of P1 and P1 \( \text{vir} \) to low temperature. While the irreversible loss of P1 \( \text{vir} \) yielders at 20 C was very gradual, with P1 the loss occurs rather abruptly. After a two hour exposure at 20 C, the number of infected cells that liberate P1 if plated at 37 C is reduced by a factor 5 to 10 and stays at that level or decreases very slowly thereafter if the treatment is prolonged (table 1).

**Lysogenization with phage P1.** In platings of P1 infected cells treated for two hours at 20 C, several tiny plaques, each centered by a small colony, were observed. Such centered plaques were occasionally observed also with infected cells kept at 37 C all the time, but in much smaller numbers. Since centered plaques are produced often by lysogenic bacteria plated together with indicator bacteria, the possibility was considered that those P1 infected cells that are lost as infective centers after exposure at 20 C survive and become lysogenic.

Experiments designed to test these points (table 2) showed that the P1 infected cells that fail to appear as phage yielders after treatment at 20 C are accounted for by new lysogenic complexes. In the experiment of table 2 a multiplicity of infection of 4.2 was used and 87.5

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*Figure 2.* One step growth curves for P1 \( \text{vir} \) on Sh at 37 C under conditions of spontaneous and premature lysis. Adsorption in buffer. Sh input (colony counts) = \( 2.4 \times 10^9 \) per ml. P1 \( \text{vir} \) input = \( 7.3 \times 10^9 \) per ml. Unadsorbed phage measured by centrifugation at time 0 (= time of dilution into nutrient medium) = 23 per cent of input. In the spontaneous lysis experiment (curve a) the unadsorbed phage was neutralized with antiserum before the end of the latent period. In the premature lysis experiment (curve b) the data were corrected by subtracting the unadsorbed phage, represented by the line c.
per cent of the (infected) bacteria were found to be lysogenic after the low temperature treatment. Equally high frequencies of lysogenization were obtained when multiplicities of infection as low as 0.05 were used. The low temperature treatment, therefore, is an efficient method of inducing lysogenization for phage P1.

The experiment of table 2 showed also that some lysogenization may occur at 37°C. This raises the problem of the accuracy of the plating technique with incubation at 37°C for assaying the titer of preparations of P1. Clearly, particles that upon plating lysogenize the first cell on which they are adsorbed are lost as plaque formers. Since some lysogenization occurs at 37°C (see table 2), phage assays at 37°C do not give the full titer of P1 preparations. There is evidence, however, that the absolute titer cannot be much higher than the plaque count at 37°C. If P1 infected cells are exposed to 40 or 42°C, for various time intervals during the latent period, or if P1 assay plates are incubated at 40°C, there is an increase in the number of lytic centers, which, however, never exceeds the number of lytic centers at 37°C by more than a factor of 1.3.

A comparison was attempted of the amount of phage antigens in a preparation of P1, which was used in several lysogenization experiments, and in 3 preparations of P1 vir, using the antibody blocking test (DeMars et al., 1953). The assumption was made that plaque count assays are satisfactory for titration of the virulent phage (see a discussion in Luria et al., 1951) and that P1 particles have the same antibody blocking ability as P1 vir particles. The rather preliminary tests indicated that the total amount of phage material in the P1 preparation could not exceed by more than a factor 3 the amount corresponding to the plaque count at 37°C and probably was much below that limit.

The lysogenic colonies obtained after treatment of infected cells at low temperature are indistinguishable from Sh(P1) strains, obtained from the secondary growth of P1 plaques, both in the amount of P1 they liberate (at 37°C) and in their resistance pattern towards the T-phages.

After lysogenization, the cells divide at the same rate as the parental strain before infection. The lysogenization process, though, delays somewhat the first cell division after infection (figure 4).

Determination of the temperature sensitive
sensitive period. Exposure of Sh cells infected with either P1 or P1 vir to 20°C for 2 hours produces, as said before, a loss of phage yielders which is much greater for P1 than for P1 vir. One step growth experiments, in which infected cells are returned to 37°C after the 2 hours at 20°C, show that the latent period is only slightly affected by the treatment as though the process leading to lysis had been stopped completely. Instead, the average burst size is usually doubled (figure 5). This indicates that, while the phage development is blocked, materials are synthesized inside the cell, which can later be utilized by phage and thus permit a larger burst size. It also suggests that the phage development is blocked by low temperature at a specific stage.

Attempts were made to determine the time in the latent period at which the temperature sensitive stage occurs by exposing P1 infected cells at various times during the latent period (figure 6). The results indicate that the temperature sensitive period occurs after the first 20 minutes of the latent period as measured at 37°C.

This conclusion was confirmed for P1 vir. Infected cells were kept for 20 and 30 minutes, respectively, at 37°C and then transferred to 20°C. In the first culture, phage liberation did not start before 4 hours; in the second culture, liberation began at 80 minutes.

All the temperature experiments described above were done with Sh cells grown previously at 37°C. The same temperature effect can be observed if both cell growth and phage adsorption are carried out at 20°C. This shows that the block in the phage development produced by low temperature is not mediated by a process of adaptation to growth at 20°C in cells grown at 37°C.

**Effect of temperature on lysogenic strain Sh(P1).**

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**TABLE 1**

The effect of low temperature on the multiplication of phage P1

Cells of strain Sh were infected with P1 in buffer (low multiplicity). After adsorption (time 0), the mixture was diluted into LB medium containing antiphage serum at 37°C. Samples were placed at 20°C for various periods of time and plated for plaque count; the plates were incubated at 37°C. The plaque counts do not include centered plaques (see text).

<table>
<thead>
<tr>
<th>Treatment of Infected Cells Before Plating</th>
<th>Plaque Counts (Plaque Yielders)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>20°C</td>
</tr>
<tr>
<td>0 through 30 min (Control)</td>
<td>853</td>
</tr>
<tr>
<td>0 through 5 min</td>
<td></td>
</tr>
<tr>
<td>5 through 95 min</td>
<td>177</td>
</tr>
<tr>
<td>5 through 110 min</td>
<td>99</td>
</tr>
<tr>
<td>5 through 125 min</td>
<td>85</td>
</tr>
<tr>
<td>5 through 140 min</td>
<td>80</td>
</tr>
<tr>
<td>0 through 10 min</td>
<td></td>
</tr>
<tr>
<td>10 through 100 min</td>
<td>156</td>
</tr>
<tr>
<td>10 through 115 min</td>
<td>121</td>
</tr>
<tr>
<td>10 through 130 min</td>
<td>80</td>
</tr>
<tr>
<td>10 through 145 min</td>
<td>70</td>
</tr>
</tbody>
</table>

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**TABLE 2**

The effect of temperature on the proportion of P1 infected cells that become lysogenic

Cells of strain Sh were infected with P1 at 37°C (adsorption in nutrient medium). After 5 minutes a sample was diluted into LB medium containing antiphage serum at 37°C to neutralize the unadsorbed phage. Another sample was diluted into chilled LB medium and centrifuged to measure the unadsorbed phage. At 15 minutes bacterial assays were made from the serum tube, by diluting and spreading on agar plates, which had been cooled previously at 20°C or warmed at 37°C. The precooled plates were kept at 20°C for 2 hours following spreading and then incubated at 37°C; the prewarmed plates were incubated immediately at 37°C. One hundred colonies from each series were spot tested for lysogenicity. A phage assay for phage yielders was made at 30 minutes from the serum tube.

<table>
<thead>
<tr>
<th></th>
<th>Phage Yielders (at 37°C)</th>
<th>Bacterial input</th>
<th>Phage input</th>
<th>Unadsorbed Phage</th>
<th>Multiplicity of Infection</th>
<th>Infected Bacteria (Expected)</th>
<th>Phage Yielders (at 37°C)</th>
<th>Bacteria Surviving Infection (Colony Formers)</th>
<th>Frequency of Lysogenic Colonies Among Survivors</th>
<th>Proportion of Infected Bacteria Lysogenized at 37°C</th>
<th>Proportion of Infected Bacteria Lysogenized at 20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.6 X 10^9/ml</td>
<td>1.2 X 10^9/ml</td>
<td>5.3 X 10^9/ml</td>
<td>4.2</td>
<td>1.6 X 10^9/ml</td>
<td>1.4 X 10^9/ml</td>
<td>3.8 X 10^7/ml</td>
<td>94/100</td>
<td>22.5 per cent</td>
<td>87.5 per cent</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>at 37°C by expected 7% of survivors</td>
<td>97/100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Phage production in cultures of Sh(Pl), as in all other lysogenic bacteria investigated in this respect, occurs by lysis of a small fraction of cells at each generation. Single bursts experiments showed that in cultures of Sh(Pl) at 37 C in the exponential phase of growth the fraction of cells lysed per generation is of the order of 10^-4 per generation. The average burst size is not smaller than the estimate obtained from one step growth experiments with Sh cells infected with Pl at 37 C.

It was natural to ask whether Sh(Pl) could produce phage when growing at 20 C. As shown in figure 7, phage production stops (or is greatly reduced) soon after transfer of Sh(Pl) cells from 37 C to 20 C. In cultures passaged several times at 20 C, little or no free phage can be detected. The same result is obtained when Na citrate (ca 0.5 per cent) is added to the medium to block the Ca++ present and thus reduce the rate of adsorption of Pl. The absence of free phage in cultures of Sh(Pl) growing at 20 C, therefore, is not caused by readsoption of the phage onto the lysogenic cells. Lack of phage production by Sh(Pl) at 20 C is not due to loss of lysogenicity; all of 48 colonies produced from Sh(Pl) cells grown at 20 C were able to produce phage when tested at 37 C. Low temperature, therefore, does not interfere with the multiplication and transmission of the prophage but only with the lytic process leading to production of infective phage.

DISCUSSION

The results reported here demonstrate that low temperatures block the lytic development
of phage P1. This occurs both with lysogenic cells and with sensitive cells infected with P1 or P1 vir. It is simplest to assume that one and the same reaction is responsible for the block in all three cases. This reaction, needed for the production of infective phage, does not occur within the first 20 minutes of the latent period at 37°C in the lytic cycle; in the lysogenic cycle it must follow the prophage stage (Bertani, 1953a). How completely the reaction is blocked at 20°C is not exactly known; information on this point is limited by the precision of the available techniques.

![Figure 6. Determination of the temperature sensitive period. Cells of strain Sk were infected in buffer with P1 (multiplicity ca 0.25). At time 0 they were diluted into LB medium at 37°C. At various times, phage assays were made by plating on precooled petri dishes. These were incubated at 20°C for 2½ hours and then transferred at 37°C. Control assays were made just before the end of the latent period; these plates were incubated at 37°C all the time. The control assays are represented by the horizontal broken line in the diagram. The plaque counts are plotted as a function of the time of plating on precooled agar.](image-url)

**Figure 7.** Effect of low temperature on the production of phage by lysogenic Sk(P1) cells. A culture of Sk(P1) growing in LB medium at 37°C was suddenly diluted into medium at 20°C. Bacterial and free phage titers (corrected for the dilution) are plotted against time.

The effect of temperature seems to be specific for phage P1. It has not been observed with the phages of the T1–T7 group nor with the temperate phage P2.

With P1 infected cells, low temperature not only blocks the lytic process but also increases the proportion of cells that become lysogenic. This may simply be a consequence of the fact that the lytic pathway is blocked if one assumes that each particle of P1 has the double potency of either lysing or lysogenizing Sk cells upon infection and that no irreversible reaction intervenes between the stage at which the choice between lysis and lysogenization is made and the stage at which phage development is blocked by low temperature.

The finding that a temperature change can change the proportion of cells that become lysogenic instead of being lysed upon infection with P1 makes improbable the existence of genetic differences between the two classes of cells before infection.

Temperature effects on phage production by lysogenic bacteria were noticed in the past (Gildemeister and Herzberg, 1924; Bordet and Bordet, 1946) but were not analyzed in connection with the problem of lysogenization. Lieb (1953) observed an effect of temperature on the
lysogenization process with phage $\lambda$ of *E. coli*. Here temperature did not seem to affect the "lysis vs lysogenization" decision in the infected cells but rather the time at which the infected cells that were not lysed (or their progeny) established permanent lysogenicity. Lieb gave evidence for such a two step picture of the process of lysogenization with phage $\lambda$. We did not test its validity for phage *P1*. The two systems show a common feature: the delay in growth of the lysogenized clones. The cause of this delay is not known.

Lieb (1953) with phage $\lambda$ and Boyd (1951) with phage *A1* of *Salmonella typhi-murium* demonstrated that more infected cells become lysogenic at higher multiplicities of infection. In a few experiments to test this point with phage *P1*, we failed to observe any effect of multiplicity of infection on lysogenization. Additional experiments are needed to explain this difference.

Cytological studies of the *P1* system (Murray, 1953) showed that the initial morphological changes in the cell nuclei following infection of *Sh* with *P1* are identical in those cells destined for lysis and for lysogenization. In the latter the changes are completely reversible since *Sh(P1)* cells are cytologically indistinguishable from *Sh* cells.

ACKNOWLEDGMENT

We wish to acknowledge the collaboration of Dr. R. G. E. Murray, University of Western Ontario, in the cytological observations reported here and the kind hospitality extended by him to one of us.

SUMMARY

Exposure to 20 C blocks the lytic development which would occur at 37 C when phage *P1* or its virulent mutant *P1 vir* infects its host *Shigella dysenteriae*, strain *Sh*. The temperature sensitive stage occurs after the first 20 minutes of the latent period (at 37 C) of these phages but before any mature phage is formed intracellularly. Low temperature also blocks production of phage by lysogenic cells *Sh(P1)*. Cells of strain *Sh* infected with the temperate phage *P1* become more frequently lysogenic when exposed at 20 C; thus, the lysogenic path is favored at low temperature over the lytic path of infection.

REFERENCES


Luria, S. E., Williams, R. C., and Backus, R. C. 1951 Electron micrographic counts of bacteriophage particles. J. Bact., 61, 179-188.

