Effect of Prophage W on the Propagation of Bacteriophages T2 and T4

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Studies have been undertaken to determine whether the temperate phage ω present in *Escherichia coli* strain W is responsible for the inability of this strain to act as a host for T2 and T4. *E. coli* WS, cured of phage ω , was sensitive to T2 and T4. Lyso-genation of *E. coli* C and WS with phage ω resulted in loss of ability to plate T2 and T4. However, *E. coli* K-12 lysogens still served as hosts for the T-even phage. Two of three WS lysogens studied resembled strain W at the biochemical level. They converted about 30% of infecting T2 deoxyribonucleic acid (DNA) to acid-soluble fragments and limited macromolecular synthesis to a few minutes after infection. The third lysogen did not degrade phage DNA, and nucleic acid and protein synthesis continued for some time, although no phage production occurred. It is concluded that phage ω plays a role in the restriction of virulent phage but that it is not the only factor involved. Since acid solubilization was not observed in all cases of phage ω -mediated restriction of T-even phage, a hypothesis for the restriction has been proposed which is based on an alteration in the cell envelope after lysogenation with phage ω .

During studies of the abortive infection of *Escherichia coli* strain W by T2, it was brought to our attention that this strain is lysogenic (G. Stent, *personal communication*) and that the prophage might play a role in blocking the propagation of virulent phages. We investigated this possibility by characterizing the temperate phage in strain W and by following the course of T2 and T4 infection of bacteria lysogenized by phage ω . We studied in detail a mutant derived from the original *E. coli* strain W which could be infected by the T-even phages. Temperate bacteriophage were not observed in the culture media of this strain; however, when it was again lysogenized with temperate phage obtained from strain W, it could no longer be infected with T2 or T4. The restriction thereby established resembled that observed in the original *E. coli* W strain. These experiments are described in this report.

While performing these experiments, we learned that S. Glover was also investigating the temperate phage ω and its participation in the restriction of phage λ . The results of his experiments have been published (10, 11).

MATERIALS AND METHODS

Bacterial strains. *E. coli* strain W ["Waksman" strain, ATCC 9637 (9)] and *E. coli* strain B were obtained from S. S. Cohen of the University of Penn-

sylvania. By replica plating, G. Stent (University of California) selected a substrain which was sensitive to T2 from strain W. In this paper, this strain has been called WS. Strain WS has a longer division time in minimal media than strain W but shows the same sensitivity to D-serine which is characteristic of strain W (14). *E. coli* strain C (4) was obtained from S. Lederberg of Brown University. *E. coli* K-12 Hfr Hayes $r^- m^-$ (referred to in this paper as K-12) was a gift from S. Glover, Hammersmith Hospital, England (8).

Bacteriophage. T2 and T4 were obtained from S. S. Cohen. The temperate phage from strain W, called ω , was obtained by treating an overnight liquid culture with chloroform at 37°C for approximately 30 min followed by centrifugation at $6,000 \times g$ in a Sorvall RC-2 centrifuge. The supernatant fluid containing the phage was stored at 4°C after the addition of Ca^{++} to give a final concentration of 0.01 M. To obtain high-titer preparations of phage ω from *E. coli* W or from other strains carrying this phage, bacteria were grown in a soft-agar overlay. The phage were extracted from the agar with 0.15 M NaCl and were purified by differential centrifugation.

Bacterial growth conditions and phage methodology. Bacteria were maintained on nutrient agar slants. Cultures were grown with aeration at 37°C in Brain Heart Infusion (BHI) broth or in the synthetic medium previously described (18) supplemented with 0.2% Casamino Acids (Nutritional Biochemicals Corp., Cleveland, Ohio) and 5 g of NaCl per liter. The concentration of bacteria was measured by colony counts on nutrient agar plates and by turbidity read-

ings in a Klett colorimeter. Assays of phage ω were performed as described by Adams (1), with BHI broth solidified with 1.5% agar in a petri plate and with 0.85% nutrient agar containing 0.01 M Ca^{++} as the overlay. T2 and T4 were assayed under these conditions, except that Ca^{++} was omitted and tryptone (Difco) agar plates supplemented with 2 g of sodium citrate per liter and 1.3 g of glucose per liter were used. At 10 min after infection, the surviving bacteria were measured on nutrient agar plates in the presence of phage antiserum. Infected bacteria capable of producing phage ("yielders") were measured with *E. coli* B as the indicator strain after removal of unadsorbed phage with antiserum. Free phage and mature phage present inside infected cells were assayed after treating the culture with chloroform.

Standard methods were used to obtain cultures lysogenic for phage ω . Phage preparations were plated on the bacteria and those bacteria growing in the center of the resulting turbid plaques were picked and purified by streaking on agar plates. Cultures from isolated colonies were tested for their susceptibility to infection and for their ability to produce phage ω .

Enzyme assays. Deoxycytidylate hydroxymethylase and phage lysozyme were assayed by published procedures (17, 20). To prepare extracts of infected cells, the culture, at a concentration of 5×10^8 cells/ml, was infected with an input multiplicity of 5 phage per bacteria. At the desired times, 50-ml samples of cells were chilled, centrifuged, and resuspended in 2 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.5) containing 0.01 M dithiothreitol. After freezing and thawing, the cells were disrupted by brief sonic treatment and the debris was removed by centrifugation at $30,000 \times g$ for 15 min.

Isotope incorporation. The incorporation of ^3H -uracil and ^{14}C -isoleucine into acid-precipitable material was determined by the filter-paper disc method of Bollum (5). A mixture of the isotopically labeled compounds containing 4 μC of uracil (40 $\mu\text{C}/\mu\text{m}$) and 2 μC of isoleucine (250 $\mu\text{C}/\mu\text{m}$) was added to 4 ml of the infected culture. The Casamino Acids in the medium provided carrier for the radioactive isoleucine.

Acid solubilization of parental phage deoxyribonucleic acid (DNA). Phage labeled with ^{32}P were prepared by infecting cells in low phosphate medium (6) containing 20 μg of phosphate per ml and $^{32}\text{P}_i$ (specific activity, 0.4 $\mu\text{C}/\mu\text{g}$). After lysis of the cells with chloroform and treatment with deoxyribonuclease and ribonuclease, the phage were purified by a cycle of low- and high-speed centrifugation followed by a second centrifugation at low speed. Bacteria at a concentration of 5×10^8 cells/ml were infected with ^{32}P -labeled phage at the desired multiplicity. To determine the extent of acid solubilization of parental phage DNA, 1-ml samples were removed, at different times after infection, into 0.1 ml of 50% trichloroacetic acid. An 0.1-ml amount of a solution containing 10 mg of bovine serum albumin per ml was then added. After thorough chilling, the precipitate was removed by centrifugation and 0.5 ml of the supernatant fluid was dried in a stainless-steel planchet and counted in a windowless gas flow counter. To correct for phage that failed to adsorb to the bacteria, the cells were

centrifuged from a sample of the infected culture and the acid-precipitable radioactivity in the supernatant fluid was determined. The amount of ^{32}P -labeled material adsorbed to the cells was calculated by subtracting the radioactivity resulting from unadsorbed phage from the total radioactivity. The amount of acid-soluble material produced was expressed as the percentage of ^{32}P -labeled material adsorbed to the cells that became acid soluble.

Radioactivity determinations. Isotopically labeled compounds were purchased from the New England Nuclear Corp., Boston, Mass., and from the Volk Chemical Co., Skokie, Ill. ^{32}P was obtained from the Squibb Institute for Medical Research, New Brunswick, N.J. To determine the amount of ^{14}C and ^3H in the same sample, a Tri-Carb liquid scintillation spectrophotometer (Packard Instrument Co., Inc., Downers Grove, Ill.) was used. A Nuclear-Chicago gas flow counter was used for the determination of ^{32}P and for the deoxycytidylate hydroxymethylase assays.

Preparation for electron microscopy. Phage preparations were dialyzed against 1% ammonium acetate for 90 min and were mixed with twice their volume of 2% ammonium molybdate (pH 6.8) or potassium phosphotungstate (15). Specimens were placed on carbon-coated grids with micropipettes and were examined in a Siemens Elmiskop electron microscope having an accelerating voltage of 80 kv and a magnification of 40,000 to 60,000 times.

RESULTS

Nature of the prophage in *E. coli* strain W. A typical overnight culture of *E. coli* strain W, treated with chloroform to kill the bacteria, contained from 10^5 to 10^6 infectious particles per ml. These particles produced turbid plaques which varied in size and shape on *E. coli* strains C and K-12. The same variation in plaque morphology was found when the virus present in individual plaques was plated. The virus present in the clear plaque observed occasionally (approximately $1:10^5$) always gave rise to clear plaques which

TABLE 1. Relative efficiency of plating of phage ω

Phage ^a	Bacterial host	
	<i>E. coli</i> C	<i>E. coli</i> K-12
ω -W.....	1 ^b	1
ω -C.....	1	10^{-5}
ω -K-12.....	1	10^3
ω -WS.....	1	1

^a The origin of phage ω has been indicated after the convention of Arber and Dussoix (3); the phage designation ω is followed by the bacterial host from which the preparation was made; e.g., phage from *E. coli* C = ω -C.

^b The plating efficiencies were calculated relative to *E. coli* C which was taken as 1.

appeared to be virulent mutants of the temperate phage. The number of plaque-forming units present in the supernatant fluid from strain W was essentially the same whether *E. coli* K-12 or C was used as the indicator (Table 1). Growth on *E. coli* strains C or K-12 altered the plating efficiency of phage ω so that it showed a preference for the strain on which it grew last (Table 1).

The phage present in the supernatant fluid from a culture of strain W failed to produce plaques on strain WS. By using this phage to infect strain C and the progeny of this infection (ω ·C) to infect strain K-12, a preparation of ω ·K-12 was obtained which contained 5×10^7 phage per ml. This preparation gave plaques on strain WS with an efficiency of 10^{-6} . Bacteria growing in the center of these plaques were isolated and tested for lysogeny. The three lysogenic strains studied in detail were resistant to phage ω infection and produced phage ω . Phage from two of the lysogens, strains WS(ω)1C and WS(ω)2B, were readily detected in the supernatant fluid from broth cultures. The third lysogen, strain WS(ω)0, produced far fewer phage than strains W, WS(ω)1C, and WS(ω)2B; the soft-agar overlay technique was required to detect the phage produced by this strain.

The low efficiency of plating of phage ω on strain WS was independent of the source of the phage; it was equally low with phage ω derived from a lysogenic strain of WS or from the other strains of bacteria. The low efficiency of plating on strain WS was therefore not due to a host-induced modification of the type exhibited by ω ·K-12 and ω ·C (Table 1; 10); it is a property inherent to strain WS.

Electron microscopic examination of a high-titer preparation made by infection of *E. coli* strain C with phage from strain W showed a bacteriophage-like particle (Fig. 1). In all preparations examined, collapsed heads and contracted sheaths were observed. The bacteriophage resembled P2 in size and shape (2) but did not possess the immunity properties of P2.

Effect of lysogenization with phage ω on the biological characteristics of T2 and T4 infection. To determine whether the presence of phage ω as a prophage affected the propagation of T2 and T4, lysogenic strains of *E. coli* C, K-12, and WS were isolated from plaques made with a high-titer preparation of phage ω .

Clones were designated as lysogenic if they produced phage ω and were insensitive to infection by phage ω carrying the correct host range specificity. Subsequently, the lysogenic strains able to act as a host for T2 and T4 were compared with the nonlysogenic parental strains. The five strains of *E. coli* K-12 (ω) tested plated T2 and

T4 with the same efficiency as *E. coli* K-12. The five strains of *E. coli* C (ω) tested plated T2 with an efficiency of less than 10^{-2} ; T4 failed to produce plaques on either *E. coli* C or *E. coli* C (ω). The properties of these lysogenic cultures were not investigated further.

The effect of T2 and T4 infection on lysogens of *E. coli* WS was studied in greater detail. T2 adsorbed to and killed strain WS and its lysogens but the lysogens failed to produce phage (Table 2). A similar experiment showed that, although T4 infected strains WS and B with the same efficiency, T4 did not readily adsorb to the lysogenic strains derived from strain WS (Fig. 2). The kinetics of adsorption show that T4 rapidly attached to strain WS and after the eclipse period the number of plaque-forming units increased. About 60 min after infection, between 60 and 100 phage per cell were produced. The strains lysogenic for phage ω failed to adsorb more than 50% of the T4 added and did not produce progeny phage. After infection of the WS lysogens with T4, the viable count and turbidity of the culture increased. Similar results were obtained whether the experiments were performed in BHI broth or synthetic media.

Nucleic acid and protein synthesis after infection of strain WS and its lysogenic derivatives. The biological aspects of T2 infection of WS (ω) strains resemble the abortive infection of *E. coli* strain W (7; C. Fowler, Ph.D. Thesis, Univ. of Pennsylvania, Philadelphia, 1951). Therefore, we investigated whether the similarities extend to the biochemical level. Pizer and Cohen showed that phage ribonucleic acid and the viral enzyme deoxycytidylate hydroxymethylase were synthesized after *E. coli* W was infected with T2 (16). Subsequent work has demonstrated that the synthesis of these phage-directed polymers comes to an abrupt stop early after infection (H. S. Smith and L. I. Pizer, Bacteriol. Proc., 1966, p. 126). The extent of phage synthesis in the lysogenic strains was determined. After infection by T2, ^{14}C -isoleucine and ^3H -uracil were incorporated into acid-precipitable material (Fig. 3). Strain WS and the lysogen WS(ω)0 incorporated isotope for at least 40 min. Strains WS(ω)1C and WS(ω)2B stopped incorporating isotope by 5 min. Therefore, the capacity to synthesize nucleic acid and protein after infection was severely limited in two of the three lysogens examined.

By assaying cell-free extracts of T2-infected lysogens for deoxycytidylate hydroxymethylase and lysozyme activity, evidence was obtained that the isoleucine incorporation (Fig. 3) measured phage protein synthesis. Extracts of WS(ω)1C and WS(ω)2B had approximately 10% of the

FIG. 1. Electron micrograph of phage ω examined by negative contrast. Marker represents 100 nm.

TABLE 2. Infection of *E. coli* WS and its lysogens by T2^a

Bacterial strain	EOP ^b	Bacteria/ml	T2/ml	Bacteria surviving infection (%)	Bacteria yielding T2 (%)	Average burst ^c at	
						30 min	60 min
WS.....	1	3.2×10^8	1.2×10^9	5.5	116	75	275
WS(ω)1C.....	$< 10^{-5}$	7.8×10^8	5.5×10^9	1.6	0.010	0	0
WS(ω)2B.....	$< 10^{-5}$	6.4×10^8	5.5×10^9	1.5	0.006	0	0
WS(ω)0.....	$< 10^{-5}$	9.1×10^8	5.5×10^9	0.41	0.500	0	0

^a Bacteria were grown to a Klett reading of between 100 and 120 in mineral medium and then were infected. At 10 min, 2% of the input phage was unadsorbed.

^b Efficiency of plating relative to *E. coli* strain B.

^c The average burst size was determined on chloroform-treated samples of the culture.

hydroxymethylase activity found in extracts of WS and WS(ω)0 and lacked lysozyme activity (Fig. 4). Strain WS(ω)0 differed from the other two lysogens at the biochemical level in that after infection isotope incorporation continued longer

and at least one late protein, lysozyme, was synthesized. Since strain WS(ω)0 also incorporated labeled thymidine into DNA, the lack of phage production cannot be explained by a limitation of phage synthesis to early functions.

Acid solubilization of T2 DNA. A characteristic of infection of strain W is that about 30% of the infecting T2 DNA is converted to acid-soluble fragments (H. S. Smith and L. I. Pizer, *Bacteriol. Proc.*, 1966, p. 126; H. S. Smith, Ph.D. Thesis, Univ. of Pennsylvania, Philadelphia, 1967). Strains WS(ω)1C and WS(ω)2B resembled strain

FIG. 2. Adsorption of T4 to strain WS and its lysogens. Lysogenic cultures were grown in synthetic media to a concentration of about 7×10^8 per ml and then were infected with T4 at an input multiplicity of about 3 phage per cell. Strain WS was infected at 3×10^8 cells per ml with a multiplicity of 9 phage per cell.

FIG. 3. Kinetics of isotope incorporation into acid-precipitable material. Bacteria at about 5×10^8 cells per ml were infected with T2. Multiplicities of 6.4 and 4.5 phage per cell were used for strains WS and WS(ω)0, respectively. For strains WS(ω)1C and WS(ω)2B, the multiplicity was 20. Survivors were less than 0.1% in all cases. The isotopically labeled compounds were added to the cultures immediately before infection, and 0.1-ml samples of the cultures were removed to paper discs at the times indicated.

FIG. 4. Kinetics of phage enzyme production. The bacterial cultures are represented by the same symbols that were used in Figs. 2 and 3. The solid lines show the kinetics of deoxycytidylate (dCMP) hydroxymethylase production. Lysozyme production is shown by the dashed line. Each point represents enzyme assays on a separate sample of an infected culture.

FIG. 5. Production of acid-soluble fragments from T2 DNA. The bacteria were infected with ^{32}P -labeled T2 at the multiplicities of infection (moi) shown on the curves.

W in that they rapidly solubilized T2 DNA (Fig. 5). This degradation of viral DNA was distinguishable from the superinfection breakdown phenomenon described by Leslie et al. (13), since it occurred at both high and low multiplicities of infection and was essentially completed by 10 min after addition of T2 to the bacteria.

Acid solubilization of T2 DNA was not extensive when strain WS(ω)0 or strain WS was the host (Fig. 5). The fragmentation of the DNA of the infecting phage correlates with the early cessation of viral synthesis but does not appear to be a requirement for restriction.

DISCUSSION

The object of this investigation was to determine whether the presence of temperate phage in

E. coli strain W is responsible for the inability of this strain to act as a host for T2 and T4. On the basis of the similarities between T2 and T4 infection of strain W and the strains of WS made lysogenic for phage ω , it was possible to conclude that phage ω plays a role in the restriction of the virulent phage. However, since lysogens of K-12 could be infected with T2 or T4, the presence of the temperate phage is not the only factor involved in limiting infection.

Another aspect of phage ω restriction is that not all of the WS lysogens behaved in a manner similar to strain W at the biochemical level. The two lysogens that produced large quantities of phage ω converted about 30% of the infecting T2 DNA to acid-soluble fragments and limited macromolecular synthesis to a few minutes after infection. This behavior was similar to that found with strain W. The third lysogen, although showing similar biological characteristics, failed to degrade phage DNA or bring the synthesis of nucleic acid and protein to an abrupt halt early after infection. From these results, it appears that, although the acid solubilization of T2 DNA may contribute to the disruption of the infection, it is not essential for the establishment of restriction. Possibly related to this is the observation that with strain W at least 50% of the DNA from the infecting phage was still intact; i.e., indistinguishable from DNA present in mature phage by zone sedimentation through neutral or alkaline sucrose gradients at times after infection when acid solubilization was completed (H. S. Smith, Ph.D. Thesis, Univ. of Pennsylvania, Philadelphia, 1967).

In view of our inability to ascribe all of the aspects of restriction to nuclease activity, we have considered a second hypothesis; according to this hypothesis, restriction is the result of an alteration in the cell envelope after lysogenization with phage ω . As a consequence of this change, the adsorption of T4 would be reduced and the sealing of the cell membrane after T2 infection impaired. The incomplete resealing would lead to continued leakage of cell constituents and to the disruption of polymer synthesis. Excessive leakage has been reported (19) to accompany the restriction of rII mutants in K-12 (λ). The differences in behavior after infection shown by different bacterial strains lysogenic for the same prophage might arise from differences in cell envelopes and hence the degree of alteration produced by the establishment of lysogeny. This hypothesis accounts for most of the experimental observations and should be testable with the strains described in this paper.

In conclusion, a comparison should be made between the abortive infection of strain W and

Shigella (P2). Phage ω resembles P2 morphologically, and both of these temperate phages interfere with T2 infection only when they are present in certain bacterial strains (12). Furthermore, viral synthesis stops early after T2 infection of both strain W and *Shigella* (P2) (K. Fields, Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, 1968; Pizer, Smith, and Lederberg, *unpublished data*). However, unlike strain W, viral DNA is not degraded in *Shigella* [(P2) H. S. Smith, Ph.D. Thesis, Univ. of Pennsylvania, Philadelphia, 1967]. Therefore, the suppression of T2 infection in *Shigella* (P2) is an example of an early restriction that does not involve destruction of the viral template.

It appears that a number of factors are involved in the restriction of T-even phage infection by P2-like phage, and the interaction of these factors leads to a variety of consequences which makes it difficult to assign cause and effect relationships. Although the most striking effect with strain W and the two WS lysogens was acid solubilization of the infecting DNA, this was not always observed, and a hypothesis consistent with most of the data available is one based on an altered cell envelope.

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LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
2. Anderson, T. F. 1960. On the fine structures of the temperate bacteriophages P1, P2 and P22. Proc. European Regional Conf. Electron Microscopy, Delft, 1960, p. 1008-1011.
3. Arber, W., and D. Dussoix. 1962. Host controlled modification of bacteriophage λ . J. Mol. Biol. 5:18-36.
4. Bertani, G., and J. J. Weigle. 1953. Host controlled variation in bacterial viruses. J. Bacteriol. 65:113-121.
5. Bollum, F. J. 1959. Thermal conversion of non-priming DNA to primer. J. Biol. Chem. 234: 2733-2734.
6. Burgi, E. 1963. Changes in molecular weight of DNA accompanying mutations in phage. Proc. Natl. Acad. Sci. U.S. 49:151-155.
7. Cohen, S. S. 1953. Studies on controlling mechanisms in the metabolism of virus infected bacteria. Cold Spring Harbor Symp. Quant. Biol. 18:221-235.
8. Colson, C., S. W. Glover, N. Symonds, and K. A. Stacey. 1965. The location of the genes for host

- controlled modification and restriction in *Escherichia coli* K12. *Genetics* **52**:1043-1050.
9. Davis, B. D., and E. S. Mingioli. 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B₁₂. *J. Bacteriol.* **60**:17-28.
 10. Glover, S. W., and G. Kerszman. 1967. The properties of a temperate bacteriophage W ϕ isolated from *Escherichia coli* strain W. *Genetics Res.* **9**:135-139.
 11. Kerszman, G., S. W. Glover, and J. Aronovitch. 1967. The restriction of bacteriophage λ in *Escherichia coli* strain W. *J. Gen. Virology* **1**:333-347.
 12. Lederberg, S. 1957. Suppression of the multiplication of heterologous bacteriophages in lysogenic bacteria. *Virology* **3**:496-513.
 13. Leslie, S. M., R. C. French, A. F. Graham, and C. E. van Roogen. 1951. The breakdown of T 2r⁺ bacteriophage upon infection of its host *Escherichia coli*. *Can. J. Med. Sci.* **29**:128-143.
 14. Maas, W. K., and B. D. Davis. 1950. Pantothenate studies. I. Interference by D-serine and L-aspartic acid with pantothenate synthesis in *Escherichia coli*. *J. Bacteriol.* **60**:733-745.
 15. Nagington, J., A. A. Newton, and R. W. Horne. 1964. The structure of Orf virus. *Virology* **23**:461.
 16. Pizer, L. I., and S. S. Cohen. 1961. The synthesis of an enzyme and other polymers in abortive infection of *Escherichia coli* strain W. *Biochem. Biophys. Acta* **53**:409-411.
 17. Pizer, L. I., and S. S. Cohen. 1962. Purification and properties of the deoxycytidylate hydroxymethylase and studies of its origin. *J. Biol. Chem.* **273**:1251-1259.
 18. Pizer, L. I., and M. L. Potochny. 1964. Nutritional and regulatory aspects of serine metabolism in *Escherichia coli*. *J. Bacteriol.* **88**:611-619.
 19. Sekiguchi, M. 1966. Studies on the physiological defect in rII mutants of bacteriophage T4. *J. Mol. Biol.* **16**:503-522.
 20. Sekiguchi, M., and S. S. Cohen. 1964. The synthesis of messenger RNA without protein synthesis. *J. Mol. Biol.* **8**:638-659.