Sensitivity of Intracellular Bacteriophage λ to Colicin CA42-E2

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Treatment of *Escherichia coli* K-12 infected by λ CI_{ts}857 with colicin CA42-E2 resulted in partial inhibition of the infectious process. Uninfected bacteria were killed by colicin with a probability of about five times that with which similarly treated λ -infected bacteria lose plaque-forming ability. The λ deoxyribonucleic acid (DNA), when present in a bacterial cell either as the replicating DNA of infectious phage or as the nonreplicating DNA of superinfecting phage, was degraded to acid-soluble material after colicin treatment. Analysis of the intermediates of DNA breakdown has revealed that degradation of the DNA to acid-soluble material is preceded by endonucleolytic fragmentation of the chromosome at a limited number of sites. This is the same mechanism of degradation previously observed for *E. coli* DNA after colicin treatment.

Colicins are antibacterial proteins, synthesized by certain strains of the *Enterobacteriaceae*, which kill strains of the same or of closely related species. Study of the mode of action of colicins has revealed that they adsorb to surface receptors and produce a specific lethal biochemical response in sensitive bacteria (15, 20).

Colicins of type E2 specifically inhibit deoxyribonucleic acid (DNA) synthesis and cause breakdown of chromosomal DNA (16, 22; B. L. Reynolds, Ph.D. Thesis, Univ. of Adelaide, 1966). Analysis of the intermediates of DNA breakdown has revealed that DNA degradation to acid-soluble material is preceded by endonucleolytic fragmentation of the chromosome at a limited number of sites (18, 23). This fragmentation of the *Escherichia coli* chromosome takes place in two stages [the scission of single strands of the DNA duplex and the subsequent breakage of the opposite strand nearby (23)] and is the earliest biochemical change observed in sensitive cells after colicin treatment. Therefore, it is thought to be the primary action of this colicin.

The detailed studies on chromosome breakdown reported to date have been limited to the *E. coli* chromosome. The replication of phages T4 and T5 (14) and T2 (B. L. Reynolds, Ph.D. Thesis, Univ. of Adelaide, 1966) was reported to be resistant, although slow degradation of T4 DNA was shown in the presence of chloramphenicol. It is known that colicins of type E2 induce λ lysogens at low multiplicity, but at high colicin multiplicities the proportion of cells releasing λ is reduced (4, 14). It is not known

whether, at the higher multiplicities, λ induction failed to occur, or whether the colicin acted on the induced λ . In this paper, we show that colicin CA42-E2 inhibits the replication of infectious λ and, furthermore, that λ DNA is degraded. Holland and Holland (10) recently reported similar studies with somewhat different results, which are referred to in the Discussion.

MATERIALS AND METHODS

Materials. Nutrient broth (Difco) was prepared double strength plus 0.5% (w/v) sodium chloride, and nutrient agar was Difco blood-agar base prepared as directed without the addition of blood. TG medium was the tris(hydroxymethyl)amino-methane (Tris)-hydrochloride-buffered minimal salts medium described by Nomura et al. (17). Tritiated thymine (TRK-15) and tritiated thymidine (TRA-120) were obtained from the Radiochemical Centre, Amersham, England, and ³²P as orthophosphate (P2B1) was obtained from the Australian Atomic Energy Commission, Lucas Heights, Australia.

Bacterial strains. Bacterial strains are described in Table 1.

Colicin. Colicin CA42-E2 was prepared by the method of Reeves (19) and assayed by spotting loopsful of serial dilutions onto a nutrient agar plate seeded with P512. The highest dilution to give a completely clear zone was taken as the titer in arbitrary units per milliliter (AU/ml; reference 8).

The number of lethal units (LU) per AU, calculated from killing curves (14) with P512, was 9×10^9 LU/AU. This ratio was used to calculate the multiplicity of infection (MOI; reference 14) in all experiments. It should be noted, however, that strain JC2918 and its derivatives are more sensitive to colicin than is P512 (see below); for these strains, there are actually about 1.4×10^{10} LU per indicated AU.

Colicin had a specific activity of 6 AU/ μ g of pro-

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TABLE 1. *Bacterial strains*

Strain	Genotype ^a
<i>Escherichia coli</i> K-12 JC2918 ^b	<i>thy arg his pro leu thr lac gal ara xyl mtl str^R tsx sup^s λ⁻ λ^S F⁻</i>
<i>E. coli</i> K-12P927	λ ⁺ lysogen of JC2918 ^c
<i>E. coli</i> K-12P512	<i>thy met str^R λ⁻ F⁻</i>
<i>E. coli</i> K-12P855	<i>his λ⁻ cer</i>
<i>E. coli</i> K-12CR34 ^d	<i>thr leu thi thy λ^R (λ CIts-857 ind⁻)</i>

^a Nomenclature used is that recommended by Demerec et al. (3) and Taylor (27). The *cer* locus is colicin E resistance (5, 21; P. Pfaff and E. N. Whitney, Bacteriol. Proc., p. 50, 1971).

^b Strain provided by A. J. Clark.

^c Other markers as in JC2918.

^d Strain provided by J. B. Egan.

tein; protein was determined by the method of Lowry et al. (13).

Radioactive labeling of DNA. ³²P-labeled λ phage was prepared by induction of the λ lysogen CR34 λ *CIts857* and subsequent growth in medium containing ³²P as orthophosphate. The phage were separated from bacterial debris by differential centrifugation and purified further by cesium chloride equilibrium density gradient centrifugation. The purified phage had an average of 0.6 ³²P atoms per phage particle.

The bacterial DNA was uniformly prelabeled as follows. A culture of bacteria grown overnight without aeration in TG medium supplemented with required amino acids at 20 μg/ml and 0.5% (w/v) maltose was diluted 1:50 into fresh medium and aerated for approximately 2 hr. ³H-thymidine, or ³H-thymine for thymine-requiring strains, was added to a final concentration of 0.5 μCi/ml, and incubation with aeration was continued for about 1.5 hr, at which time the bacterial concentration reached 10⁸ to 2 × 10⁸ cells/ml. Bacteria had a division time of about 60 min under these conditions. Thymine-requiring strains were labeled in the presence of 1 μg of thymine per ml. Nonradioactive thymidine or thymine was then added to a final concentration of 50 μg/ml, and incubation was continued for 20 min.

Infection of bacteria with λ *CIts857* phage. Bacterial cultures growing exponentially at 38.5 C in TG medium supplemented with required growth factors and 0.5% (w/v) maltose were washed, suspended at 10⁸ cells/ml in 0.02 M MgSO₄, and infected with λ *CIts857* phage. After 15 min of adsorption at 38.5 C (0.5% of the added phage remained unadsorbed as determined by dilution through broth saturated with chloroform and by plating for plaque formers), the cultures were diluted into TG media supplemented with required growth factors and 0.5% (w/v) maltose at 38.5 C and treated immediately with colicin. Cultures of immune lysogens were superinfected with λ *CIts857* as described above, except at 34.5 C.

DNA degradation. To measure acid-insoluble radioactivity, 0.1-ml samples of the culture were transferred to 2-cm discs of glass-fiber paper (What-

man GF3), which were dropped immediately into cold 5% trichloroacetic acid. The discs were washed with cold 5% trichloroacetic acid and with ethanol and then were dried in a vacuum oven. The samples were counted in a Packard liquid scintillation counter with 5 ml of scintillant (toluene, 1 liter; 2,5-diphenyloxazole, 4 g; 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, 0.5 g) with a 40% efficiency for ³H in DNA.

Sucrose density gradient centrifugation. DNA was extracted from phage-bacterium complexes at various times after colicin treatment by a modification of the method of Smith and Levine (24). Bacterial cultures were prelabeled and superinfected with λ phage as described above. Immediately before colicin addition and at intervals thereafter, 0.2-ml samples were removed for DNA extraction. The samples were added to 0.2 ml of a warmed solution of 100 μg of lysozyme per ml in 0.1 M ethylenediaminetetraacetic acid (EDTA), pH 8. After the samples were incubated at 65 C for 1 min, 20 μliters of 25% (w/v) sodium dodecyl sulfate was added. The resulting lysate was almost clear and was incubated for 10 min with frequent, gentle mixing. The salt concentration was then increased to 1 M by adding 50 μliters of 4 M sodium chloride. After an additional 10-min incubation, the lysed culture was diluted with 0.55 ml of 0.001 M Tris-hydrochloride, 0.015 M sodium chloride, pH 7.4. Mixing was by rolling rather than by shaking. Throughout the procedure great care was taken to avoid degradation by shearing. Pronase, pretreated to remove deoxyribonuclease activity (11), was added at a final concentration of 50 μg/ml, and the lysates were allowed to stand at 30 C for at least 18 hr before sedimentation in sucrose. This procedure completely released the DNA from the bacterial cells. DNA extracts were heated at 50 C for 5 to 10 min immediately before sedimentation in sucrose to dissociate any remaining aggregates.

Four linear 5 to 20% (w/v) sucrose gradients were generated simultaneously in 5-ml cellulose nitrate tubes by using a single mixing chamber and a multi-channel roller pump (proportioning pump no. II, Technicon Instruments Corp., Ardsley, N.Y.). A 0.4-ml "cushion" of 60% (w/v) sucrose was put below each gradient. Gradients contained 0.01 M Tris-hydrochloride, 0.001 M EDTA, pH 7.4.

A sample of the DNA extract (50 to 120 μliters) was slowly drawn up into a short length of silicone-rubber tubing (inner diameter, 2.5 mm) connected to a 1-ml Agla micrometer syringe. The sample was then slowly delivered onto the gradient surface. Centrifugation was for 90 min at 35,000 rev/min in the Spinco SW50L rotor at 25 C. The three gradients from each centrifugation were collected simultaneously by pumping the contents of each tube out from the bottom with a multichannel roller pump (proportioning pump no. II, Technicon). Each gradient was pumped onto a strip of glass-fiber paper (1.5 by 26 cm; Whatman GF82), which was moved in 2-cm steps at regular time intervals so that the sucrose gradient was applied to the paper strip discontinuously. The strips were dried, cut into 2-cm segments, and counted directly by liquid scintillation. The spill

over of ^{32}P counts into the ^3H channel was 16%. However, no correction was made for this, as the total number of tritium counts loaded onto each gradient was approximately 20-fold higher than the number of ^{32}P counts. The efficiency of counting of ^3H in DNA in this system varied from 25 to 40% among samples. For this reason, all counts were corrected to absolute activity expressed as disintegrations per minute (dpm; reference 1). The results of each gradient are presented as the absolute radioactivity in each fraction expressed as a percentage of the total dpm recovered from the gradient, plotted against normalized fraction number. The recovery of radioactivity from sucrose gradients was greater than 80%.

The approximate molecular weights indicated in the scale at the top of each sucrose gradient profile were calculated with the equation of Burgi and Hershey (2): $D_1/D_2 = (M_1/M_2)^{0.35}$, where D_1 and D_2 are the distances travelled down a 5 to 20% sucrose gradient by two different native DNA molecules of molecular weights M_1 and M_2 , respectively. DNA from λ phage was used as a reference standard, taking 31 million as its molecular weight.

RESULTS

Effect of colicin on biosynthesis of λ phage. In the studies reported below, the phage λ CIts857 was used. This phage carries the *ts857* mutation and is induced from the lysogenic state at temperatures above 37.5 C (25). Figure 1B shows the effect of colicin on the intracellular infectious development of this phage. At a colicin multiplicity of 6.5, there was no apparent loss in the total number of plaque formers, but the intracellular

infectious development of phage was disrupted in a way that reduced the average burst size. Lysis was complete when the final sample was taken, as chloroform addition did not increase the plaque count.

At higher multiplicities of colicin, there was an initial drop in the total number of plaque formers, indicating that some phage-infected cells are prevented from giving rise to a burst. The proportion of cells that did not give rise to a burst increased with colicin multiplicity but was less than the proportion of uninfected cells killed for any given MOI of colicin.

If the proportion of λ -infected cells prevented by colicin from giving rise to a burst is used to calculate the number of LU adsorbed in the same way that the proportion of cells killed can be used to calculate the true number of LU adsorbed (14), then about five times as much colicin as is required to kill an uninfected cell must be adsorbed to prevent an infected cell from giving rise to a burst. However, when the replication of λ is blocked by carrying out phage adsorption and subsequent colicin treatment in the presence of 0.005 M KCN, the uninfected cells are killed by colicin with a probability about two times that with which similarly treated λ -infected bacteria lose plaque-forming ability. The sensitivity of λ -infected cells in this case is equivalent to that observed by Nomura after colicin induction of λ from a lysogen (14).

Analysis of the distribution of total phage yields among single bursts indicates that the fraction that survives the action of colicin (when phage multiplication does occur) produces a greatly reduced burst (Fig. 2). Thus, when phage-infected bacteria were treated with colicin (MOI, 70), only 30% of the complexes produced a burst. The range in burst size for colicin-treated (MOI, 70) complexes was 3 to 11 phages released per cell, with an average burst size of 6, whereas the range for untreated complexes was 16 to 123, with an average burst of 67. If a correction is made for tubes containing more than one phage-infected bacterium, the average burst size for uninfected complexes was 57.

Degradation of infectious λ DNA. The initial studies attempting to demonstrate colicin-induced degradation of λ DNA were carried out under conditions in which the intracellular development of infectious phage was blocked with KCN. However, no degradation of phage or bacterial DNA of phage-infected bacteria (MOI, 5) occurred under these conditions after colicin treatment. Similarly, bacterial cultures carried through the same procedure but not infected with phage showed no DNA degradation after colicin treatment, indicating that the inhibition of degradation

FIG. 1. Effect of colicin at various multiplicities on the replication of infectious phage λ CIts857 in P512. P512 was infected with λ CIts857 (multiplicity of infection, 0.2). After 15 min of adsorption at 38.5 C, the culture was diluted 100 times into supplemented TG medium at 38.5 C, and colicin was added at the multiplicities indicated. Colony formers (A) and total plaque formers (B) were determined at the indicated times after colicin addition.

FIG. 2. Distribution of total phage yields among single bursts after colicin treatment of replicating λ CIIts857. P512 was infected with λ CIIts857 (multiplicity of infection, 0.4) in the presence of 0.005 M KCN. After adsorption, the culture was diluted 1,000 times into supplemented TG medium at 38.5 C and treated with colicin at a final concentration of 0.008 arbitrary units/ml (multiplicity of infection, 70). After 10 min, cultures were diluted 2.5×10^{-6} into fresh media, and 79 0.5-ml samples were incubated at 38.5 C for 2 hr before plating each sample for total plaque formers.

results from the conditions used and not from infection by λ .

Degradation of both λ DNA and bacterial DNA was observed when λ -infected cultures (MOI, 6) undergoing phage replication were treated with colicin, regardless of whether KCN was present during phage adsorption (Fig. 3). Although initial rates of degradation of λ DNA and bacterial DNA were similar, the breakdown of λ DNA was less extensive. In this instance with P512 used as host, the rate of degradation of bacterial DNA in infected cells was slower than that in uninfected cells, suggesting that λ -infected cells are "partially tolerant" to the action of colicin.

We have observed that, after colicin treatment of P512, DNA degradation occurred at a slower rate and breakdown was less extensive than with some other auxotrophic strains of *E. coli* K-12 treated under equivalent conditions. For this reason, the above experiment was repeated with JC2918 as host (Fig. 4). In λ -infected JC2918 (MOI, 5) treated with colicin, degradation of bacterial DNA and λ DNA occurred more rapidly and was more extensive than that observed with P512 as host. Similarly there was greater reduction in the average burst size in λ -infected JC2918 treated with colicin. In contrast to the observation with P512 (Fig. 3), the degradation of bacterial DNA occurred at the same rate in infected and uninfected cells.

Degradation of superinfecting λ DNA. It is known from the report of Wolf and Meselson (28) that superinfecting λ DNA does not replicate in an immune lysogen. Most of the superinfecting λ DNA is converted to two circular DNA species:

FIG. 3. Degradation of ^3H -thymine-labeled bacterial DNA and ^{32}P -labeled λ DNA after colicin treatment of λ CIIts857-infected P512. Half of a prelabeled bacterial culture was infected with ^{32}P λ CIIts857 (multiplicity of infection, 6) in the presence of 0.005 M KCN. The other half was carried through the same procedure but was not infected with phage. After adsorption, the cultures were diluted 10 times into supplemented TG media at 38.5 C, and colicin was added to a final concentration of 4 arbitrary units/ml (multiplicity of infection, 360). The average phage burst determined 120 min after colicin addition was 1.5 for colicin-treated cultures and 37 for untreated cultures.

FIG. 4. Degradation of ^3H -thymidine-labeled bacterial DNA and ^{32}P -labeled λ DNA after colicin treatment of λ CIIts857-infected JC2918. Prelabeled bacterial cultures, at 2.5×10^8 cells/ml, were infected with ^{32}P -labeled λ CIIts857 (multiplicity of infection, 5) or left uninfected. After adsorption, the cultures were diluted 10 times into supplemented TG media and treated with colicin at a final concentration of 2.6 arbitrary units/ml (multiplicity of infection, 90). The average phage burst size determined 60 min after colicin addition was 0.02 for colicin-treated cultures and 30 for untreated cultures.

component I (supercoils, both DNA strands covalently closed) and component II (circles, one strand not covalently closed), both of which are thought to be intermediates in vegetative λ DNA replication (29). The presence of these nonreplicating species of λ DNA after superinfection of an immune lysogen made it possible to test the sensitivity of nonreplicating λ DNA to colicin (Fig. 5). The breakdown of superinfecting λ DNA to acid-soluble material, as in the breakdown of infectious λ DNA, occurred at about the same rate as the breakdown of host DNA but was less extensive. However, the absolute rates of λ DNA degradation in the two experiments cannot be compared directly, as it was necessary to carry out the experiments at different temperatures (34.5 C for superinfecting DNA and 38.5 C for infectious λ DNA).

To characterize the mechanism of λ DNA degradation, DNA was extracted from P927 superinfected by λ CIts857 at various times after colicin addition and sedimented in neutral sucrose density gradients (Fig. 6). Both λ DNA and *E. coli* were progressively fragmented by double-strand breakage. At time 0, the bulk of the λ DNA sedimented as complete linear molecules of 3×10^7 molecular weight, with a small part sedimenting faster as circles. By 5 min, there was some fragmentation of λ DNA, and by 10 min only 50% of the λ DNA remained as whole molecules, al-

though less than 5% had been completely degraded to acid-soluble material by this stage. By 20 min, both types of DNA had been fragmented to about 5×10^6 to 10×10^6 molecular weight. Unfortunately, under the conditions used, smaller fragments which may be present at 60 min merged in the gradient with the acid-soluble material.

DISCUSSION

This study was carried out as part of an investigation into the mechanism by which colicins of type E2 induce breakdown of cellular DNA. Most previous studies have investigated the breakdown of the *E. coli* chromosome. In this report, we show that the DNA of λ phage is also sensitive to the action of this colicin.

The results presented above show that, after colicin CA42-E2 treatment of λ -infected *E. coli* cultures, the intracellular development of infectious λ CIts857 is inhibited. The λ DNA is degraded to acid-soluble material as is the host DNA, suggesting that the adsorption of colicin to a sensitive bacterial cell produces the same biochemical response in these two species of DNA. Furthermore, after colicin treatment of an immune lysogen superinfected with λ , the λ DNA, that does not undergo replication is similarly degraded to acid-soluble material. In this case, it was further shown that the breakdown of λ DNA involves endonucleolytic double-strand breakage

FIG. 5. Degradation of ^3H -thymidine-labeled bacterial DNA and ^{32}P -labeled λ DNA after colicin treatment of P927 superinfected with λ CIts857. Prelabeled bacterial cultures at 34.5 C were left uninfected or were superinfected with ^{32}P -labeled λ CIts857 at various multiplicities. After adsorption, the cultures were diluted 10 times with supplemented TG media at 34.5 C and treated with colicin at a final concentration of 1.3 arbitrary units/ml (multiplicity of infection, 90).

FIG. 6. Degradation of ^3H -thymidine-labeled bacterial DNA and ^{32}P -labeled λ DNA after colicin treatment of P927 superinfected with λ CIIs857. Prelabeled bacteria at 4×10^9 cells/ml were superinfected with ^{32}P -labeled λ CIIs857 (multiplicity of infection, 1.75). After adsorption, the cultures were diluted 10 times into supplemented TG media at 34.5°C and treated with colicin at a final concentration of 6.4 arbitrary units/ml (multiplicity of infection 140) at 34.5°C . At the indicated times during degradation, samples were removed and analyzed for acid-insoluble radioactivity (A) or the DNA was extracted and sedimented on sucrose density gradients (B). Gradients were spun for 90 min at 35,000 rev/min at 25°C . Symbols: solid line, ^{32}P ; dashed line, ^3H .

of the DNA before degradation to acid-soluble material (exonuclease action). This is the mechanism of breakdown observed for *E. coli* chromosomal DNA (18, 23), in which it also has been shown that single-strand breakage precedes double-strand breakage (23).

It was first observed by Endo, Kamiya, and Ishizawa (4) that colicin E2 induces the develop-

ment of λ in lysogenic *E. coli* cells. Maximal induction (30% of initial lysogenic bacteria) occurred at a colicin MOI of 1, and Nomura (14) showed that nearly all killed cells could be accounted for by the plaque formers. At higher multiplicities, the number of plaque formers decreased exponentially at a rate about one-half of that of the loss of colony-forming ability. How-

ever, it is not possible to conclude from this experiment whether colicin at high multiplicities fails to induce λ , damages the capacity of host cells to support λ replication, or directly affects induced λ .

In our experiments with infectious λ , colicin still destroyed plaque-forming ability and, in this case, must act either on the host's capacity to support λ replication or directly on the vegetative λ . Compared with its ability to kill uninfected cells, colicin destroyed the plaque-forming ability of infected cells with an efficiency of 50% when λ had been preadsorbed in the presence of KCN to stop replication; when λ replication had started before colicin addition, the efficiency was 20%. Colicin appears to act directly on λ as the λ DNA is degraded. However, it is possible that colicin also has some effect on the host's capacity to support λ replication.

Nomura (14) has shown that colicin can cause some degradation of T4 DNA if phage replication is inhibited by chloramphenicol. In our experiments λ DNA is degraded rapidly (Fig. 4), without the addition of chloramphenicol.

Thus λ DNA can be degraded under the same conditions under which host DNA is degraded, although degradation of λ DNA is less extensive. It was also observed with P512, in which the rate of degradation was generally low, that host DNA was degraded less rapidly in λ -infected cells than in uninfected cells. The reason for this is not known.

Holland and Holland (10) have reported that λ DNA is not degraded after colicin treatment. They used relatively low multiplicities of colicin, giving 1 to 10% survival of uninfected cells and about 30% breakdown of bacterial DNA (Holland, *personal communication*). This corresponds to an MOI of about 2 to 5, which is much lower than the MOI we used and probably accounts for the apparent discrepancy in our results.

Thus, at high multiplicities, colicin produces efficient degradation of host DNA, less efficient but extensive degradation of λ DNA, and only inefficient degradation of T4 DNA. This variation in efficiency, may result from differences in base composition of these species of DNA. For example, in contrast to *E. coli* and λ , T4 DNA contains 5-hydroxymethyl cytosine (glucosylated) rather than cytosine.

However, the endonuclease involved in colicin-induced degradation may act at specific sites on the DNA, and the observed differences in degradation may result from differences in the distribution of such sites. These sites must occur at intervals of approximately 10^6 molecular weight along the *E. coli* chromosome (23). Cytosine-rich clusters occur at intervals of 10^6 to 2×10^6

molecular weight in the DNA of *E. coli* and λ phage but are far less frequent in T4 DNA (26) and thus may be the specific site for the endonuclease.

It is of interest that *E. coli* endonuclease II, in conjunction with other nucleases, fragments *E. coli* DNA to pieces of about 10^6 molecular weight and that, by analogy with the very similar T4 endonuclease II, the specificity may be for cytosine-rich clusters (7, 12). The possibility that endonuclease II may be involved in colicin-induced DNA degradation is very interesting as this enzyme has been implicated in both recombination (6) and replication (9). This possibility is compatible with the hypothesis (20) that colicins induce lethal modifications of changes that occur normally during recombination.

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