Loss of Lysis Inhibition in Filamentous *Escherichia coli* Infected with Wild-Type Bacteriophage T4

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The plaque enlargement of wild-type T4 bacteriophage observed when assayed in the presence of low concentrations of mitomycin C or after exposure to very low doses of ultraviolet light was studied by using solid as well as liquid culture media. It was found that the filamentous cell formed by the treatment with the agents is responsible for the phenomenon. The filamentous cell was also shown to be characterized not only by the loss of capacity of lysis inhibition but also by a shortening of the latent period. No difference in cellular rigidity could be seen between the filamentous cell and normal cell as far as the analysis from the outside of the cell was concerned, whereas the former cell was shown to be more readily susceptible to phage-induced lysozyme from the inside of the cell. A possible change in the membrane of the filamentous cell and a possible mechanism for lysis inhibition are discussed.

It is well known that, when *Escherichia coli* B infected with wild-type T-even bacteriophage is allowed to undergo successive superinfection, the onset of cell lysis is greatly retarded. This phenomenon of lysis inhibition was first reported by Doermann (4); however, the precise mechanism still remains to be solved.

E. coli B has also been shown to form filaments after treatment with certain types of agents (6, 8, 13, 17) such as ultraviolet (UV) light, mitomycin C, and crystal violet, among others, by the involvement of septum formation (1, 2, 18). Moreover, recent genetic studies on filament formation in *E. coli* have added considerably to an understanding of the cytokinesis mechanism (7, 10, 15, 19).

The present report is concerned with our finding that the plaque of T4r⁺ is expressed as "r" type in *E. coli* B plated on nutrient broth-agar containing a low concentration of mitomycin C or after exposure to low doses of UV light. The study of this phenomenon revealed, through the use of solid as well as liquid media, that the observed plaque enlargement resulted from filament formation of the host cells.

It also became evident that the capacity of lysis inhibition was completely abolished in filamentous cells.

MATERIALS AND METHODS

Bacteria. *E. coli* B and its derivatives, BB, B/r, and B_{s-1} (HCR⁻) (9), and derivatives of *E. coli* K-12, C600 (su-II), CR63 (su-I), and KB, were taken

from our laboratory stock. *E. coli* K-12F⁻ lon⁻ AB1899 (10) and its parent strain F⁻ lon⁺ AB1157 were supplied by P. Howard-Flanders.

Bacteriophages. Bacteriophages T4r⁺ (wild type), T4N93 (tail amber, no. 11, Epstein; reference 5), T4B270 (contracted particle amber, no. 22, Epstein; reference 5), T4eH26 (lysozyme amber, Edgar), and T4eHs90 (lysozyme amber, Streiginger) were used. The first was a laboratory stock. The next three were obtained from M. Sekiguchi, and the last was supplied by A. Tsugita. The high-titer stocks of bacteriophages were prepared on CR63 as a host and were partially purified by repeated differential centrifugations.

Media. The nutrient broth medium consisted of 10 g of polypeptone, peptone, or tryptone (Difco) and 5 g of NaCl per 1,000 ml, pH 7.0.

Glucose synthetic medium consisted of 2.5 g of (NH₄)₂HPO₄, 1.5 g of KH₂PO₄, 3 g of sodium glutamate, 5 g of NaCl, and 10⁻⁴ M MgSO₄ per 1,000 ml, pH 7.0.

Each liquid medium was supplemented with 1.5% agar for hard-solid medium and 0.75% agar for soft-solid medium, 3 ml of which was used as a top layer for plating indicator bacteria.

Agents. Mitomycin C was supplied by Kyowa Hakko Kogyo Co., Tokyo. All of the agents listed in Table 1 were commercially obtained. Egg white lysozyme was purchased from Worthington Biochemical Corp., Freehold, N.J.

Treatment of cells with agents in solid and liquid medium. All *E. coli* strains were used as indicators to observe the change in plaque morphology of T4r⁺ on nutrient-agar plates containing gradient concentrations of the agents. These plates (85-mm diameter) were prepared by placing 20 ml of agent-free agar

over the same volume of nutrient agar containing various concentrations of agents which had been solidified at a slant.

The experiments in liquid culture medium were carried out mainly by using *E. coli* B as the host, mitomycin C as the agent, and peptone broth as the medium. *E. coli* was grown in nutrient broth at 37°C. When the optical density (OD) at 660 nm of the culture reached 0.05, agent solutions were added 1:50 (v/v) to obtain the desired final concentrations. Growth was continued until the OD of the untreated culture reached 0.2; the OD of the mitomycin C-treated culture was adjusted to 0.2 by concentration when necessary. This treatment is referred to as a "two-generation" treatment. The cells were then infected with 6×10^9 plaque-forming units per OD per ml two times in 5 min, which corresponded to a multiplicity of infection of 3 to the untreated *E. coli* B.

UV irradiation. The plate was exposed to UV irradiation for 5 sec at 20 min after plating at a distance of 80 cm from a 15-w Hitachi germicidal lamp. When the liquid culture of *E. coli* B reached OD 0.05, it was transferred to a petri dish and irradiated for 5 sec with gentle shaking, as described above.

Assay of the activity of phage-induced lysozyme. Samples were taken from the phage-infected culture and cooled rapidly on crushed ice. Cells harvested by centrifugation were ground with silica sand. The supernatant fluid resulting from centrifugation at $105,000 \times g$ for 90 min was subjected to lysozyme assay according to the method of Sekiguchi and Cohen (16).

Chloroform-treated *E. coli* B cells were used as the substrate. The unit of enzyme activity was expressed as $\Delta OD_{450 \text{ nm}}$ per minute per milligram of protein.

Anti-T4 phage serum. Antiserum was prepared in rabbits. The *K* value of the obtained antiserum was 57.8 in the broth medium.

RESULTS

Change in plaque morphology of T4r⁺ induced by filament-forming agents. During the study on

the ability of low doses of mitomycin C and UV to change *E. coli* cells to filament forms, these agents were found to enlarge the plaque of phage T4r⁺ on *E. coli* B at the same level of concentration. When *E. coli* B was plated on nutrient agar containing a concentration gradient of mitomycin C ranging from 0 to 0.1 $\mu\text{g/ml}$, large plaques formed at a definite level of mitomycin C concentration (about 0.03 to 0.05 $\mu\text{g/ml}$; Fig. 1b). On the non-treated plate, typical small plaques about 1 mm in diameter developed (Fig. 1a). A similar enlargement of plaque size was seen when the upper half of the inoculated plate was irradiated by UV for 5 sec; this contrasted sharply with the small plaques of normal size which developed on the unirradiated area of the plate (Fig. 1c). The morphology of large plaques observed by treatment with UV or mitomycin C was quite similar to that obtained by plating T4r mutants on *E. coli* B. However, when these plaques were isolated and replated on B in the absence of mitomycin C, normal T4r⁺ plaques developed again, indicating clearly that the change in plaque morphology was not due to the mutation of the phage but to the phenotypic effect of the agents.

Microscopic observation of the cell sheets surrounding both types of plaques revealed the dominance of enormously elongated bacteria in the large-plaque area, whereas no such filamentous cells were found in the small-plaque area. This finding led us to investigate the correlation between filament formation of *E. coli* and T4r⁺ plaque enlargement by using various agents and indicator strains. Fourteen agents were found to possess both abilities (Table 1), whereas 48 substances (not listed in the table) were found to be negative in both of these capabilities. In no case did an agent possess only one of these abilities.

FIG. 1. Plaque morphology of T4r⁺ after treatment with mitomycin C and UV. (a) Plaques on nontreated plate; 2×10^8 to 3×10^8 particles of T4r⁺ were plated with *E. coli* B (about 10^8) on a nutrient agar plate. (b) Plaques on a mitomycin C-containing plate in gradient concentration from 0 to 0.1 $\mu\text{g/ml}$. (c) Upper half of the plate was irradiated by UV for 5 sec after plating and incubated at 37°C overnight.

Next, several *E. coli* strains, in which some elongation-negative strains were included, were tested as indicators for correlation between the two phenomena. It was found that *E. coli* BB and B₋₁ were capable of forming filaments and enlarging plaques, whereas *E. coli* B/r, C600, CR63, and KB were not equal in concentration of mitomycin C. A *lon*⁻ mutant of K-12, AB1899, was capable of forming filaments and enlarging plaques. Although its *lon*⁺ parent strain AB1157 showed no ability to form filaments or enlarge plaques at a concentration of less than 0.1 µg of mitomycin C per ml, these abilities appeared coincidentally in the range of 0.1 to 0.2 µg/ml. These results indicate that filament formation and plaque enlargement of wild-type T4 induced by low levels of mitomycin C or UV are closely correlated phenomena.

Enlargement of plaque size due to loss of lysis inhibition (LLI) in the *E. coli* B-phage T4r⁺ complex. In the normal condition, T4r⁺ develops small plaques on *E. coli* B, in contrast to the large plaques of T4r mutants. This difference in size of plaques is considered to be due to the lysis inhibition of the T4r⁺-infected complex which is caused primarily by the successive infection of phage (4). If the enlargement of plaque size induced by mitomycin C or UV treatment is due to an interference of the lysis inhibition of the cell-phage complex, these treatments might also cause the liquid culture of *E. coli* B to lyse rapidly, regardless of the successive infection of T4. This expectation was verified by the following experiments. A logarithmically growing culture of *E. coli* B was divided into three parts. The first tube was subjected to 0.1 µg of mitomycin C for 80 min, the second tube was irradiated by UV for 5 sec and incubated for an additional 80 min, and the third part served as an untreated control. Each culture

was then infected with T4r⁺ and the subsequent change of OD was observed. The treated culture began to lyse at 20 min after the first challenge of phage (Fig. 2), whereas the control culture was maintained in a state of lysis inhibition.

Conditions for prevention of lysis inhibition, i.e., duration and concentration of mitomycin C treatment and effect of the media, were sought. The minimum concentration of mitomycin C required for the full appearance of LLI was 0.1 µg/ml when the duration of the treatment was fixed to 80 min, two-generation time experiment (Table 2). Decreasing the concentration of mito-

FIG. 2. LLI of the mitomycin C- or UV-treated culture. *E. coli* B was grown in nutrient broth, treated with mitomycin or UV and, after additional incubation for two generations, inoculated with T4r⁺ at the indicated times (arrow, ∅). The mitomycin C-treated (●) and the UV-treated cultures (▲) began to lyse earlier than the control culture (○).

mycin C under these conditions decreased the extent and rate of the OD drop. This decrease is explained by the presence of a heterogenous popu-

TABLE 1. Agents promoting plaque-type change of T4r⁺ and filament formation of *E. coli* B

Agent	Concn	Agent	Concn
	µg/ml		µg/ml
Crystal-violet	(0 → 10)	3-Amino-6-[2(5-nitro-2-furyl)-vinyl]-1,2,4-triazine	(0 → 0.1)
Dahlia-violet	(0 → 5)	Nitrogen mustard	(0 → 50)
2,6-Diaminopurine	(0 → 1,000)	Bis-β-chloroethylamine	(0 → 500)
Aminopterin	(0 → 100)	Novobiocin	(0 → 500)
Sodium azide	(0 → 50)	Carcinophilline	(0 → 10) ^a
4-Nitroquinoline 1-oxide	(0 → 5)	Neocarzinostatin	(0 → 2.5)
N-methyl-N'-nitro-N-nitroso-guanidine	(0 → 100)	Penicillin	(0 → 100) ^a

^a Units per milliliter. The phage and the indicator bacteria were plated in the same manner as in Fig. 1, on nutrient agar containing a concentration gradient of each test agent indicated in the table. Induction of filament formation by the test agents was determined microscopically on cells taken from the bacterial lawn in the area surrounding large plaques.

TABLE 2. *Conditions of pretreatment for the appearance of LLI^a*

Conditions	Expt 1					Expt 2				
Medium ^b	PP	PP	PP	PP	PP	PP	PP	PP	PP	PP
Mitomycin C ($\mu\text{g/ml}$)	0.10	0.05	0.025	0.012	(-)	0.10	0.10	0.10	0.10	(-)
Generation	2	2	2	2	C	2	1	1/2	1/4	C
Time (min)										
0	.180	.187	.205	.195	.205	.190	.214	.220	.225	.220
20	.205	.215	.220	.220	.230	.175	.192	.225	.235	.224
40	.140	.150	.170	.200	.245	.080	.160	.224	.230	.225
60	.088	.100	.135	.170	.240	.056	.142	.213	.228	.218

Conditions	Expt 3						Expt 4			
Medium	PP	PP	PP	PP	PP	PP	PP	BP	BT	GS
Mitomycin C ($\mu\text{g/ml}$)	.10	.20	.40	.80	1.6	(-)	.10	.10	.10	.10
Generation	2	1	1/2	1/4	1/8	C	2	2	2	2
Time (min)										
0	.205	.205	.210	.205	.205	.190	.200	.195	.210	.220
20	.185	.190	.190	.193	.188	.184	.220	.210	.232	.220
40	.110	.148	.185	.198	.200	.200	.113	.123	.220	.230
60	.020	.025	.110	.135	.160	.165	.068	.075	.150	.260

^a The following factors were examined: concentrations of mitomycin C required in the two-generation treatment (experiment 1), periods of treatment at a mitomycin C concentration of 0.1 $\mu\text{g/ml}$ (experiment 2), effects of both in reciprocal changes (experiment 3), and dependency on the medium (experiment 4). OD was measured at 20-min intervals.

^b PP, polypeptone; P, peptone; T, tryptone broth; GS, glucose synthetic medium.

lation with regard to the effect of mitomycin C; i.e., some of the cells were capable of undergoing lysis inhibition and some were not. Microscopic observation of the culture revealed the presence of various proportions of filamentous cells which decreased proportionately to the decrease of mitomycin C concentration. A notable fact was that the onset of OD drop was always at 20 min after the first infection, regardless of mitomycin C concentration. The presence or absence of mitomycin C after phage infection never affected the appearance of LLI. When the cells were pretreated for various periods with fixed concentrations of mitomycin C at 0.1 $\mu\text{g/ml}$, the minimal effective treatment was found to be two-generation (Table 2, experiment 2). One-generation treatment decreased the extent of OD drop. When the pattern of OD was surveyed at various concentrations and duration of treatment, such that the product of the two factors was kept constant, the minimal positive condition was one-generation treatment at a concentration of 0.2 $\mu\text{g/ml}$.

Treatment at higher concentrations of mitomycin C for shorter periods of time did not develop LLI (Table 2, experiment 3). For this effect to be positive, UV treatment required long post-incubation after the irradiation. Morphological observation revealed development of filamentous cells during this rather long period of mitomycin

C treatment at lower doses and a long postincubation period after UV irradiation. Thus it is concluded that, for the full appearance of LLI, treatment of cells for more than one generation with a relatively low concentration of mitomycin C is adequate.

During the course of treatment with 0.1 $\mu\text{g/ml}$, OD increase and synthesis of net deoxyribonucleic acid, ribonucleic acid, and protein were found to proceed at almost the same rate as those of the control for more than 80 min. After two-generation treatment, the cell number ratio of the treated culture to the untreated culture which indicated the synchronization coefficient of filament induction was 1:3, and the survival of the treated culture was 2% of the initial cell number.

It is known that filament induction is decreased in nutritionally poor medium compared with rich medium. The effect of several media on the appearance of LLI was tested (Table 2, experiment 4). Various broth media examined so far have the capacity to support filament formation and LLI, whereas salt medium failed to show either of these effects.

To determine how phage progeny are produced in mitomycin C-treated cells (0.1 μg of mitomycin C, two generation), a one-step growth experiment was carried out. The control cell began to lyse at 25 min after infection (Fig. 3), thus being pre-

vented from successive infection. The mitomycin C-treated cell started to lyse and to release progeny in 20 min; in this case, the latent period was the same as that of the concentrated culture (Fig. 2), in which the successive infections were inevitable.

Increase of the step size in the mitomycin C-treated cell is explained as a reflection of the volume increase of the mitomycin C-treated cell. Thus, it can be said that the filamentous cell is characterized not only by LLI but also by the shortening of the latent period. The LLI is different from the "lysis from without" (3), for the LLI has a definite latent period and is phage-productive.

Effect of mitomycin C treatment on cell wall rigidity. To account for the LLI observed in the mitomycin C-treated *E. coli*-T4r⁺ complex, several experiments were performed. To clarify possible changes in fragility of the cell wall of the mitomycin C-treated culture, the treated and untreated cells were collected, and the rate of digestion of the cells was followed by measuring OD change during treatment with ethylenediaminetetraacetate (EDTA), egg white lysozyme, or EDTA plus lysozyme (11, 14) from outside the cells. However, no differences could be found between the cells (Fig. 4), even when phage-induced lysozyme was used instead of egg white lysozyme.

Cell lysis caused by infection of T-even phage is known to require the synthesis of phage-induced lysozyme. When challenged by lysozyme amber mutants T4eHs90 and T4eH26, the cultures did not lyse during a 3-hr observation period, although they accumulated phage progeny in the cell (Fig. 5). When the same experiment was performed using T4B270 and T4N93, which were capable of producing lysozyme but incapable of

forming progeny in *E. coli* B, the OD patterns of the mitomycin C-treated and the untreated cultures were found to be quite similar to those of the culture infected with wild-type T4r⁺. These results indicate that the appearance of LLI requires the phage-induced lysozyme, as does normal lysis, and does not depend on the formation of phage particles inside the cells. A possibility that the formation of phage-induced lysozyme may be delayed under the state of lysis inhibition was

FIG. 4. Fragility of the treated cell. The treated cells (mitomycin C at 0.1 $\mu\text{g/ml}$, two-generation) were collected, washed twice, suspended in tris(hydroxymethyl) aminomethane-hydrochloride buffer, 100 mM, pH 7.8, at a density of OD 0.43, and incubated at 37 C. The suspension was added with $10^{-3}M$ EDTA ($\circ$), 100 μg of lysozyme per ml (Δ), or EDTA plus 100 μg of $10^{-3}M$ lysozyme per ml (∇).

FIG. 3. Progeny production of the mitomycin C-treated cells in one-step growth. The cells were infected with T4r⁺ (multiplicity of infection, 3) for 5 min, diluted 10 times into antiserum at 5 min, further diluted $10^4 \times$ into broth at 10 min, and incubated. Infective centers were $1.2 \times 10^6/\text{ml}$ of the treated culture and $3.2 \times 10^6/\text{ml}$ of the untreated culture.

FIG. 5. Lysis curves of lysozyme amber phage T4eHs90 (∇), amber phage T4B270 (Δ), and wild-type T4r⁺ ($\circ$). Procedures were the same as those in Fig. 2.

tested by measuring the activity of lysozyme formed in the mitomycin C-treated and the untreated cells in the course of infection. Lysozyme activity started to rise at 15 min and was synthesized to almost the same extent in both cultures (Fig. 6). Therefore, lysis inhibition represents the state of the cell in which lysis is repressed even in the presence of lysozyme.

DISCUSSION

The experiments presented here indicate that the enlargement of T4r⁺ plaques on mitomycin C- or UV-treated *E. coli* is a property of filamentous cells induced by these treatments for the following reasons. (i) Both effects were obtained by the treatment of relatively low doses of mitomycin C or UV for more than one generation of treatment; (ii) the enlargement of plaques was always associated with the filamentous bacterial lawn; (iii) both effects were positive only in *E. coli* strains capable of forming filaments; (iv) both effects were positive only in rich media; and (v) all filament-forming agents so far tested could develop plaque enlargement.

One-step growth and other experiments showed that the latent period in the filamentous cell was apparently shortened and was not affected by successive infections. The filamentous cell was, however, demonstrated to possess the same rigidity as normal cells when treated with lysozyme, EDTA, or EDTA plus lysozyme from outside the cell, thus suggesting the intactness of the outer and the middle layers (rigid layer; reference 12). The experiment with amber phage also showed that the treated cell was extremely susceptible to phage-induced lysozyme.

FIG. 6. Activity of lysozyme formed in mitomycin C-treated (○) and untreated (●) cells in the course of infection.

From these results, we suggest two possibilities about the mechanism of LLI and lysis inhibition. (i) The cytoplasmic membrane is involved in LLI, but we postulate that there is a timing mechanism involved which makes the membrane susceptible to the passage of lysozyme at a specific time after infection. At this time, the normal lysis-inhibited cell is capable of making the necessary repairs to prevent contact of lysozyme with its substrate, whereas the mitomycin C- or UV-treated cell is not. (ii) Regarding the establishment of lysis inhibition, Doermann (4) described the necessity of successive infection, although its function is still obscure. Our data showed that the effect of superinfecting phage was not expressed in the treated cell. Therefore, it seems reasonable to assume that the target responsible for the effect of successively infecting phage is a cytoplasmic membrane. Moreover, by the successive adsorption of phage to a normal cell-phage complex, some metabolic or mechanical process involving the modification of the membrane may be initiated (so that the phage-induced lysozyme does not reach the substrate through the membrane), whereas the membrane of the MC- or UV-treated cell-phage complex shows no such initiation.

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