Physiological Study of Cooperative Infection by Restricted Bacteriophage T1

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The ability of certain phages to successfully infect a restricting host at a high multiplicity of infection is known as cooperative infection or cooperation. We have examined the ability of unmodified T1 (T1·0) to participate in cooperative infection in cells possessing the P1 restriction system. We have found that cooperation is dependent upon protein synthesis during the first few minutes after phage infection. However, we have been unable to attribute the necessary protein to a known T1 cistron. Degradation of the restricted T1 genome is approximately equally extensive whether cooperative infection occurs or whether it is blocked by chloramphenicol. It is postulated that an inducible host repair mechanism may be responsible for the phenomenon of cooperative infection.

It has previously been shown that the ability of certain bacteriophages to successfully infect restricting hosts is strongly dependent on the multiplicity of infection (MOI). As the MOI increases, the proportion of infected restricting cells increases at an even greater rate. This has been shown to be true of unmodified λ C (bacteriophage λ grown in *Escherichia coli* C) infecting *E. coli* K (13), for phage ϵ^{18} A (ϵ^{18} grown in *Salmonella anatum*) infecting the restricting strain *Salmonella butantan* (17), for coliphage T*2 infecting *E. coli* B (8), and for coliphage T1·0 (T1 lacking P1 modification) infecting P1 lysogens (6). This phenomenon has been termed "cooperative infection" by Paigen and Weinfeld (13). It is unlikely that a special subpopulation of viruses is responsible for cooperative infection, since an increase in MOI results in a more than proportional increase in the fraction of successfully infected cells.

Whether or not infection by λ or T1 at high MOI will be successful in establishing a replicative cycle in a restricting host is dependent upon the physiological conditions under which the infection is carried out. In the case of λ C infecting *E. coli* K, cooperative infection is severely inhibited by anaerobic conditions, whereas a lack of nutrients in the attachment medium has been shown to destroy cooperative infection of P1 lysogens by T1·0. However, these conditions do not influence the ability of modified λ or T1 to infect the same cells (6, 20).

Weinfeld and Paigen (20) found the cooperative infection of *E. coli* K with λ C sensitive to anaerobiosis for only 3 min after attachment of

the phages. In addition, they discovered that dividing the infecting λ C population into two inputs gave a slightly enhanced level of cooperative infection. Separation of the two portions of infecting λ C by several minutes showed that if aeration was stopped during either period of infection, then the number of successfully infected cells was greatly reduced.

These results imply that the conditions prevailing in the restricting host during the earliest stages of the infectious process are critical in determining the fate of the infecting DNA. In the hope of obtaining an indication of the basis for these effects, we have studied some of the requirements for obtaining a high proportion of successful infections when unmodified T1·0 infects the restricting host B(P1) at high MOI.

MATERIALS AND METHODS

Terminology. To identify modified T1 which has been grown in a P1 lysogen, the designation T1·P is used. T1 grown in a strain which is not P1 lysogenic (i.e., unmodified T1) is termed T1·0.

A bacterium to which one or more T1 has irreversibly attached is referred to as an infected cell. Bacteria which produce any T1 after infection are termed successfully infected cells or yielders, and the ratio of yielders to infected cells is termed the yield ratio. Cells which produce at least one modified T1·P are termed modifiers, and the ratio of modifiers to infected cells is termed the modification ratio.

Phage strains, bacterial strains, and growth media. Most of these have been previously described (3, 4, 5). Strain AB1157, which has an amber suppressor, was from Cynthia Lark, and AB1157(P1) was derived from it in this laboratory. It was used for the propagation of T1am·P stocks. T1⁺·0 was propagated on B and T1⁺·P on B(P1). Propagation and concentration of the stocks were as in Figurski and Chris-

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tensen (5), as were the preparation and concentration of T1 heads and tails.

Preparation of cells for attachment. Log phase *E. coli* B(P1) cells grown in Difco brand nutrient broth (NB) + NaCl media were twice centrifuged, resuspended in NB, and then titered on LB agar (5) plates. Portions of the suspension were pipetted into tubes in an ice bath and aerated for 3 min before the procedures indicated in each figure or table were carried out.

Measurement of MOI and the modification or yield ratios. Subsequent to the performance of the procedures indicated in each table or figure, the infected cell suspension was diluted with an equal volume of NB + NaCl at 0 C and immediately centrifuged for 5 min at $4,000 \times g$. The number of unattached phage was then titered on LB agar plates, using the amber permissive strain KB3 as a plating culture. This determination allows calculation of the MOI and the number of infected cells, assuming a Poisson distribution of phage on cells. To determine the modification and yield ratios, the pellet of infected cells was then resuspended, serially diluted, and titered on LB agar plates using KB3 or AB1157(P1) as a plating culture. Each plaque formed on strain AB1157(P1) resulted from a modifier cell, whereas each plaque formed on KB3 resulted from a yielder cell. AB1157(P1) was used for plating modifiers instead of KB3(P1) because its restriction property is more stable.

Titration of T1 tails. T1 tails were titered by mixing with a large (20- to 100-fold) excess of T1 heads at a concentration of 5.0×10^8 heads per ml. After mixing tails with heads, 30 min at 27 C were allowed for assembly into whole particles. The assembled particles were titered on KB3, and the resulting titer was compared with the plaque-forming ability of the head or tail preparations alone. From unpublished studies of the kinetics of the assembly process, we have concluded that at least 90% of the tails were detected by this procedure.

Preparation of T1 ghosts. Ghosts were prepared by a method similar to that of Kaiser (10). T1 was heated for 20 min at 70 C. At the conclusion of this treatment, 99% of the phage were inactivated. This preparation was negatively stained and examined under the electron microscope. The electron micrographs (not presented) revealed that almost all heat-treated phage heads were filled with the negative stain and had apparently ejected their DNA.

Preparation of radioactive phage. Radioactive T1·0 was prepared by infecting log phase *E. coli* KB3 in the presence of 1.2 mM of adenosine per ml and 16 μ Ci of [methyl-³H]thymidine per ml. Two hours were then allowed for lysis, the lysate was made 0.005 M in MgCl₂, and 50 μ g of deoxyribonuclease I per ml (Worthington) were then added. This reaction mixture was incubated for 30 min at 37 C, during which time 40 to 60% of the cold trichloroacetic acid precipitable counts were made acid soluble. The lysate was then centrifuged twice for 90 min at $105,000 \times g$, the supernate was discarded, and the pellet was resuspended each time in NB. The amount of acid precipitable non-phage radioactivity that would not attach to *E. coli* B was then determined (never >

18%). This figure was then subtracted from the total radioactivity in the preparation, and the remaining radioactivity was considered to be in phage.

DNA solubilization. The solubilization of T1 DNA in the restricting host was followed by removing 0.5-ml portions of the infected cells at various times and placing them in a test tube containing an equal volume of 10% trichloroacetic acid at 0 C. This mixture was allowed to sit on ice for 30 min, and the precipitate was collected on a glass fiber filter (Whatman GF/C). The test tube which had contained the sample was then washed three times with 10 ml of 5% trichloroacetic acid at 0 C, and the washes were poured over the filter. The filter was then dried, added to 10 ml of liquid scintillation counting solution in low K glass vials, and counted in a liquid scintillation counter. The counting solution was 4 g of New England Nuclear Omnifluor (98% 1,5-diphenyloxazole, 2% *p*-bis[o-methylstyryl]benzene) in 1.0 liter of Eastman Kodak scintillation grade toluene. The counting efficiency was always between 48 and 50%.

RESULTS

The effect of divided input. We found that administering the same T1·0 phage input in two separate portions did not decrease the efficiency of cooperation (Table 1); if anything, there was a slight increase. This is the same result reported by Weinfeld and Paigen (20) for the infection of *E. coli* K by λ C. This double input type of protocol was used in several of the experiments below.

The effect of attachment of phage tails or phage ghosts. One way of looking at the above result is to assume that the first portion of infecting phage initiated a change in the cell such that the second portion of phage had a better chance of establishing a successful infection. Since there is some indication (14) that the restriction enzymes are located near the surface

TABLE 1. The effect of divided phage input on cooperative infection^a

First T1·0 administration (min)	Second T1·0 administration (min)	Total MOI	Modification ratio ($\times 10^2$)
0		9.0	1.7
0	0	17.5	7.0
0	2	18.0	11.0
0	4	18.0	9.8
0	6	18.5	9.5

^a B(P1) cells were prepared for attachment as described. They were then placed in tubes and transferred under constant aeration to a 37 C water bath at T = -3 min. Three minutes later (T = 0 min), the cells were infected with the first portion of T1·0. The second portion containing the same number of phages was added at the time indicated. The MOI and modification ratio were then determined as described.

of the cell, a surface alteration brought about by the attachment of the first portion of phage could conceivably alter the ability of surface enzymes to act on subsequently invading DNA molecules.

Table 2 shows the results when T1 tails or T1 ghosts replace the first portion in a divided input experiment. It is clear that these particles had no effect whatsoever. The infectious centers obtained were equal to those obtained from the second input alone. If a surface change is important in the establishment of successful cooperative infection, neither T1 tails nor T1 ghosts effect that change.

The effect of the inhibition of protein synthesis. To test whether infection of restricting cells by T1·0 might result in the synthesis of a protein which could alter the outcome of the infection, the following experiments were undertaken. Chloramphenicol (50 $\mu\text{g}/\text{ml}$) was added prior to T1·0 infection in a cooperative experiment and had the following effect: cooperative infection was eliminated. The small fraction of infected cells yielding phage was approximately equal to that obtained in single-phage infections (data not shown). Apparently only the "exceptional cells", which are unable to restrict (11, 13), were successfully infected. The results presented in Fig. 1 show that the period during which the infection was susceptible to inhibition lasted only about 4 min; after this time most of the successfully infected cells had become insensitive to chloramphenicol. We also showed that chloramphenicol had no effect on the infection of B(P1) by T1·P, and that chloramphenicol greatly reduced yield ratio as well as modification ratio when T1·0 infected B(P1) at high MOI (data not presented). This

last observation rules out the possibility that chloramphenicol was blocking modification of the progeny without blocking successful infection of the cells.

We then turned to the divided input type of

FIG. 1. The effect of the addition of chloramphenicol at different times on cooperative infection. B(P1) cells were prepared for attachment as described. The cells were then transferred to a 37 C bath at $T = -3$ min. T1·0 was added to all tubes at $T = 0$. Chloramphenicol was added to each tube at the time indicated on the abscissa. Chloramphenicol was washed out between $T = 10$ min and $T = 30$ min. The modification ratio was determined as described.

TABLE 2. The effect of T1 tails and T1 ghosts on cooperative infection of *E. coli* B(P1)^a

Expt no.	Tube	Addition time		MOI	Multiplicity of tails attached or of ghosts added	Modification ratio ($\times 10^3$)
		0 min	4 min			
1	1	T1·0	T1·0	19.0		17
	2	NB	T1·0	11.5		6.0
	3	T1 tails	T1·0	10.0	10.5	6.5
	4	T1·0	T1 tails	11.5	5.5	6.0
	5	T1 tails	NB		11.5	<0.003
2	1	T1·0	T1·0	15.5		4.7
	2	T1 Ghosts	T1·0	8.2	8.4	0.22
	3	NB	T1·0	7.8		0.30

^a B(P1) cells were prepared as described. They were infected at the times indicated with phage, ghosts, or tails. At 8 min, the tubes were all chilled in an ice bath and diluted with an equal volume of chilled NB + NaCl. In the case of tails, the MOI was determined by correcting for tails that remained free in the supernatant fluid after infection; in the case of ghosts, no assay for efficiency of attachment was made. Tail assay and modification ratios were determined as described.

experiment to see whether protein synthesized during infection by the first portion of phage would be sufficient for the second phage input to make its contribution to overcoming the restriction system. As can be seen from the results in Table 3, this was not the case: the presence of chloramphenicol during the second phage input prevented that infection from increasing the number of successfully infected cells. We did not attempt to test the effect of chloramphenicol during the first infection only, since it is not certain how rapidly any residual effect of chloramphenicol treatment might be reversed by washout. However, it is difficult to see how protein synthesized during the second infection would be able to help the phage added first, since the reverse is not true. We conclude that protein synthesis during each of the two infections is necessary to increase the number of cooperatively infected cells.

The effect of amber mutations. A series of *T1am* mutants were isolated and mapped by Michalke (12). Work in this laboratory (5) has established the phenotype of these amber mutants, which have been placed in 18 genes. Mutants in gene 1 and gene 2 synthesize no phage DNA and are presumably blocked in some early step in the T1 infectious cycle. These were thought to be the best candidates for a role in controlling the protein required for cooperative infection, but mutants in all of the other known genes were also tested.

Testing of the mutants for their ability to synthesize the protein required for cooperation was accomplished by means of a divided input experiment. The suppressorless strain B(P1) was first infected with an amber mutant (*T1amX*:0) and subsequently with wild-type phage (*T1*⁺:0). It was reasoned that if a phage-coded protein were required for success-

ful cooperative infection, then T1 with an amber mutation in a gene required for the expression of that protein should be unable to participate in establishing cooperative infection in the suppressorless strain. As a result of the mutant's inability to cooperate, the level of successful infection would be that corresponding to the input of T1⁺ only. On the other hand, if *T1amX* could synthesize the necessary protein, then the level of successful infection would reflect the entire MOI. As seen in the examples in Table 4, the *T1am* mutants in genes 1 and 2 were able to participate in establishing successful infections. Similar results were found with *am* mutants representing all of the known T1 genes (data not shown). It can also be seen in Table 4 that the order of addition of *T1am* and T1⁺ did not influence the results.

It can be seen from Table 4, however, that the plaque morphology mutant T1Ge, which was previously noted (6) to be associated with erratic results in cooperative infection, was unable to enhance the level of successful infection in these experiments, regardless of whether it was added first or second. This mutant maps close to *am* 2 and *am* 46 (12), which places it in the tail region of the T1 map (5). It makes an alkaline plaque on pH indicator media. The functional alteration in this mutant is unknown.

The degradation of the DNA from restricted T1. In other phage-host systems, a majority of the DNA from restricted phage is degraded to acid-soluble fragments by the host (1, 8, 17). The later stages of this degradation depends in part on recBC nuclease acting on DNA which is sensitized by the restriction endonuclease (15). It has been shown that phages T4 and λ code for proteins that interfere with the action of the recBC nuclease (16, 18).

TABLE 3. *The effect of chloramphenicol on cooperative infection*^a

Tube no.	Addition time					MOI	Modification ratio ($\times 10^2$)
	-2 min	0 min	+2 min	+4 min	+7 min		
1		T1:0		NB	NB	9.1	1.1
2		T1:0		T1:0	NB	16.4	4.3
3		T1:0		T1:0	Chloramphenicol	16.8	3.7
4		T1:0	Chloramphenicol	T1:0		17.3	1.1
5	Chloramphenicol	T1:0		T1:0		16.4	0.11

^a Log phase B(P1) cells were prepared for attachment as described, and 3.4 ml of cells were placed in each tube. The tubes were then transferred under constant aeration to a 37 C water bath at -3 min. Then, 0.20-ml portions of T1:0, chloramphenicol, or NB were added at the times indicated in the table. Each 0.20-ml portion of T1:0 contained 10 phage per cell, and each portion of chloramphenicol consisted of a total of 200 μ g of chloramphenicol, resulting in a final concentration of 50 μ g/ml. At 8 min, all tubes were transferred to an ice bath and diluted with an equal volume of chilled NB + NaCl. The MOI and modification ratios were determined as described.

TABLE 4. Tests on T1 mutants for their ability to participate in cooperative infection^a

Expt no.	Tube no.	Phage added at		Total MOI	Modification ratio ($\times 10^3$)
		0 min	4 min		
1	1	NB	T1 ⁺ ·0	9.6	0.36
	2	T1 ⁺ ·0	T1 ⁺ ·0	19.5	2.5
	3	T1am5·0 (gene 2)	T1 ⁺ ·0	18.0	3.0
	4	T1am16·0 (gene 1)	T1 ⁺ ·0	17.5	2.5
	5	T1am15·0 (gene 5)	T1 ⁺ ·0	20.0	3.9
2	1	NB	T1 ⁺ ·0	7.5	0.42
	2	T1 ⁺ ·0	T1 ⁺ ·0	15.0	3.7
	3	T1am16·0	T1 ⁺ ·0	15.0	3.6
	4	T1 ⁺ ·0	T1am16·0	15.0	2.6
	5	T1Ge·0	T1 ⁺ ·0	15.0	0.39
	6	T1 ⁺ ·0	T1Ge·0	14.7	0.80

^a Log phase B(P1) cells were prepared as described, and 3.6 ml were dispersed to each tube. At -3 min, the tubes were moved to the 37 C water bath, and the procedures shown in the table were performed with constant aeration. All phage preparations were diluted to the same concentration and added in 0.20-ml portions when indicated in the table. At 8 min, all tubes were transferred to an ice bath and diluted with an equal volume of chilled NB + NaCl. The MOI and modification ratio were determined as described.

We reasoned from these observations that chloramphenicol might be blocking cooperative infection by preventing the synthesis of a T1 protein that would inhibit degradation of DNA.

To test this hypothesis, the degradation of restricted DNA to acid solubility was followed in the presence and absence of chloramphenicol. As can be seen in Fig. 2, chloramphenicol had very little effect on T1·0 degradation. This result does not rule out large differences in the molecular weight of the remaining DNA, which was not degraded to the degree required for acid solubility. The small increase in degradation seen in the presence of chloramphenicol (3 to 7%) is repeatable and is probably related to the observation that chloramphenicol slightly improves T1 attachment (see Table 3 and Fig. 1 which show that early chloramphenicol addition increases the number of attached phage or MOI).

DISCUSSION

The most noteworthy finding reported here is that protein synthesis is required immediately following infection by restricted T1 for cooperative infection to occur. Furthermore, in a divided input experiment, the protein synthesized following the first infection does not create conditions so that the second input of phage can overcome P1 restriction without further protein synthesis. This result resembles the effects of anaerobiosis in the case of λ C infecting *E. coli* K (20).

This result contrasts sharply with the recent report by Heip et al. (9), who have shown that chloramphenicol does not block the ability of

FIG. 2. The effect of chloramphenicol on the degradation of T1·0 in B(P1). B(P1) cells were prepared for attachment and placed in two aeration tubes at 0 C. At $T = -6$ min, either NB or chloramphenicol were added to the tubes (final chloramphenicol concentration was 50 μ g/ml). At $T = -5$ min, [^{3} H]thymidine-labeled T1⁺·0 was added to the cells (no infection or degradation of DNA occurs at 0 C). At $T = -1$ min, the total number of acid precipitable counts present (17,000 counts/min) was determined (this is the 100% figure on the graph). At $T = 0$, the tubes were transferred to a 37 C water bath and samples were removed at the times indicated. Acid-precipitable radioactivity was determined as described. Symbols: ▲, no chloramphenicol; ●, with chloramphenicol.

unmodified λ to successfully infect restricting *E. coli* K strains at high MOI. The experiments of Weinfeld (19) had already indicated that λ C can, at high MOI, overcome strain K restric-

tion, but not the restriction specified by P1. Also, enhanced recombination accompanies cooperative infection with T1 (6), but not with λ (20).

It thus appears that these two phenomena, despite their superficial similarity, may be mechanistically very different. We prefer to continue to use the term cooperative infection to refer to any case in which several phage particles interact to successfully infect a restricting host. As more information is gained about the nature of the process in any one case, a more specific term, related to the mechanism, can be applied, such as the term "abolition of restriction" used by Heip et al. (9).

Since it appears that, in the case of T1 infecting B(P1), protein synthesis is required during both phage inputs, it seems likely that the induction of the relevant protein is inefficient. A cell infected with only a few phage may have a low probability of synthesizing enough of this protein for the establishment of a successful infection. Additional phage, added under conditions that permit protein synthesis, will convert an additional proportion of the cells into yielding cells.

The requirement for protein synthesis argues against a simple saturation model of cooperative infection. The saturation model envisions the cooperative infection of a restricting cell as the result of saturating the restriction enzymes with unmodified DNA, such that these enzymes cannot act on viral DNA which is injected subsequently (2, 20).

The question remains whether the protein is of viral, cellular, or P1 prophage origin. The only T1 mutant which was unable to participate in cooperative infection as judged by the double input experiment was T1Ge which maps in the tail region. Under the conditions of the double input experiment (low salt), this mutant attaches, immediately shuts off *E. coli* B DNA synthesis, and gives no phage DNA synthesis and no cell lysis (D. Figurski, personal communication). The fact that T1Ge-0 DNA is degraded upon infection of B(P1) under the same conditions (data not presented) indicates that its DNA is injected. Since infection by T1Ge under these conditions is so physiologically abnormal, the failure of the mutant to stimulate cooperative infection may not indicate that the mutant is specifically deficient in the protein required for cooperation.

The results of Freshman et al. (6) indicated that extensive recombination accompanies cooperative infection in the case of T1. We have made a more extensive study of this recombination and found additional features that distin-

guish it from the recombination seen in ordinary T1 crosses (Potts and Christensen, manuscript in preparation).

M. Radman (*In Molecular and Environmental Aspects of Mutagenesis*, in press) has recently described an inducible *E. coli* UV repair system, and Ganesan and Smith (7) have shown a requirement for protein synthesis in Rec-dependent repair of UV and X-ray damage. Some of the X-ray damage (nicks) may be similar to some of the damage caused by restriction enzymes.

We propose the hypothesis that the phenomena of cooperative infection of B(P1) by T1-0 can best be understood in terms of an inducible, recombination-dependent repair system which can act on the fraction of restriction-damage DNA, which escapes degradation, and which has the potential to generate intact, replicating genomes. This repair system may have elements in common with other inducible or recombination-dependent repair systems.

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