

# Superinfection with Bacteriophage T4: Inverse Relationship Between Genetic Exclusion and Membrane Association of Deoxyribonucleic Acid of Secondary Bacteriophage<sup>1</sup>

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The majority of the deoxyribonucleic acid (DNA) of superinfecting T4 bacteriophage which is injected and not hydrolyzed does not attach to host cell membrane. Low levels of association of secondary phage DNA with membrane appear to be related to temporal genetic exclusion.

The deoxyribonucleic acid (DNA) of superinfecting T4 phage undergoes a sequence of events quite different from that followed by T4 DNA after primary infection. Approximately 50% of the DNA of secondary phage is degraded to acid-soluble form (13) and 80% of that remaining is not injected (11). Electron micrographs by Bayer (Biophys. Soc. Abstr., p. 268a, 1970) suggest that superinfecting phage which fail to inject their DNA are probably not adsorbed to normal phage attachment sites. Genetic markers of the superinfecting phage are, for the most part, excluded from the progeny phage (3). In addition, the per cent attachment of superinfecting parental phage DNA to cell membrane is three- to fourfold lower than that observed in primary infection (4, 5).

That superinfection degradation and exclusion can be independent events was shown conclusively by Fielding and Lunt (7). *Escherichia coli* strains lacking endonuclease I do not solubilize the DNA of superinfecting phage, but temporal exclusion still occurs.

The present work was performed to determine the relationship between exclusion and the percentage of superinfecting phage DNA attached to membrane in cells which lack endonuclease I and from which uninjected T4 DNA has been removed.

## MATERIALS AND METHODS

Procedures for the growth, radioactive labeling, and infection of cells, as well as the magnesium-sarkosyl crystal (M-band) technique for the determination of membrane-bound DNA, were the same as those described previously (4).

<sup>1</sup> Part of this work was presented at the 71st Annual Meeting of the American Society for Microbiology, Minneapolis, Minn., 2-7 May 1971.

**Strains.** *E. coli* ER22, a derivative of *E. coli* B which lacks endonuclease I (6), was obtained from C. C. Richardson. J. R. Walker provided *E. coli* C600 ( $\lambda$ ). T4DrII73 was from the collection of E. B. Goldberg. Other bacterial and phage strains used have been described (4).

**Media, enzymes, and reagents.** Wash fluid used in blender experiments contained, per liter, 0.1 g of gelatin, 0.1 mM CaCl<sub>2</sub>, and 0.1 mM MgSO<sub>4</sub> (10). Tris (hydroxymethyl)aminomethane (Tris)-glucose (TG) medium was that of Burgi (1). Broth, top agar, plates, dilution fluid, and the wash fluid used in phage purification are identical to those used previously (4).

Sarkosyl NL-30 and chloramphenicol were gifts of Geigy Chemical Corp., Ardsley, N.Y., and Parke, Davis & Co., Detroit, Mich., respectively. Egg white lysozyme (grade I, 3× crystallized) was obtained from Sigma Chemical Co., and deoxyribonuclease I (bovine pancreas) was purchased from Worthington Biochemical Corp. All radiochemicals were obtained from New England Nuclear Corp.

**Preparation of <sup>3</sup>H-leu-<sup>32</sup>P-T4D<sub>0</sub>.** *E. coli* BB was grown at 37 C in TG medium, supplemented with 5  $\mu$ g of L-leucine per ml to a cell density of  $5 \times 10^8$  per ml. L-Tryptophan was added (50  $\mu$ g/ml), and the culture was infected with a multiplicity of 5 to 10 T4D<sub>0</sub> per cell. H<sub>3</sub><sup>32</sup>PO<sub>4</sub> (4  $\mu$ Ci/ml) and <sup>3</sup>H-L-leucine (2  $\mu$ Ci/ml) were added 5 and 10 min after infection, respectively. The culture was treated with CHCl<sub>3</sub> 3 hr after infection, and the phage were then purified as reported before (4).

**Blending treatment.** Samples were washed twice in cold wash fluid, resuspended in 5.0 ml of the same fluid, and blended with a VirTis "45" homogenizer at setting 45. Control experiments demonstrated that cells can be blended for at least 30 min under these conditions with no loss of viability. Sheared samples were centrifuged for 6 min at 2,800  $\times$  g. The pellet was dissolved in 1.0 ml of 1.0 N NaOH, and 1.0 ml of the supernatant fluid was made 1.0 N NaOH. Hydrolysis was carried out at 70 C for 20 min, after which time

0.2-ml samples were placed directly into a modified Bray's scintillation fluid (16) and counted.

**Degradation of phage DNA.** Superinfection breakdown experiments were performed as described by Fielding and Lunt (7). Methylene blue was used as an indicator during the neutralization step.

**Genetic exclusion studies.** The procedure of Fielding and Lunt (7) was used. *E. coli* C600 ( $\lambda$ ) was the non-permissive host for T4DrII73. Multiplicities of 5 were used for both the initial and secondary infections.

## RESULTS

### Efficiency of injection of superinfecting T4 DNA.

Approximately 40% of the DNA of secondarily infecting T4 is not injected (11). This same low injection efficiency is obtained in host cells lacking endonuclease I, as shown in Fig. 1. Removal of 40% of the secondary phage DNA is accomplished by shearing ER22 cells; 65% of the  $^{32}\text{P}$ -DNA is sheared from B. The apparent difference between ER22 and B cells can be accounted for by the fact that at the time of shearing 30 to 40%

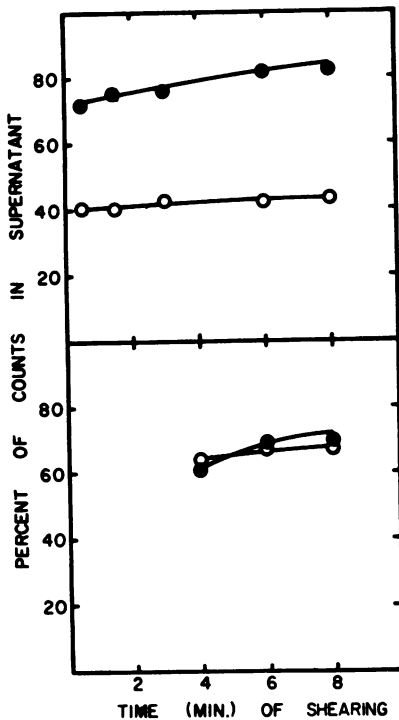


FIG. 1. Removal, by blending, of phage protein and DNA from *E. coli* B and ER22 cells superinfected with  $^3\text{H-leu-}^{32}\text{P-T4D}_0$ . Five minutes after primary infection with a multiplicity of infection (MOI) of 5 phage per cell, cells were superinfected with  $^3\text{H-leu-}^{32}\text{P-T4D}_0$  at an identical MOI. Samples (5 ml) were taken 3 min after superinfection and sheared as described in the text. Results obtained with ER22 cells are presented in the top panel and those with B in the lower panel. Symbols:  $^{32}\text{P}$  label, ○;  $^3\text{H-leu}$  label, ●.

of the superinfecting phage DNA associated with B has already been degraded. The actual amount of  $^{32}\text{P}$ -DNA of superinfecting phage which can be removed by shearing is approximately the same for both hosts. Superinfection breakdown in B also accounts for the equivalent percentages of phage protein and DNA which can be removed. That they are both about 65% is coincidental. Approximately 70% of the protein label and 10% of the parental phage DNA can be removed after 3 min of primary infection of either B or ER22.

**Degradation of phage DNA after infection or superinfection.** Superinfection breakdown does not occur in endonuclease I<sup>-</sup> hosts (7). This observation was confirmed for *E. coli* ER22, as shown in Fig. 2, which compares phage DNA degradation in B and ER22. Less than 18% of the parental DNA label is released into the medium after primary infection of either host. After superinfection, little  $^{32}\text{P}$ -DNA is hydrolyzed by ER22 cells whereas, in B, 50% is solubilized in 15 min. The absence of superinfection breakdown in ER22 cells results in a corresponding increase in sensitivity of secondary phage DNA to deoxyribonuclease. In extracts of superinfected B cells, less than 25% of the DNA of the secondary phage is sensitive to deoxyribonuclease (4). This value increases to 80% in similar experiments performed with ER22 cells.

**Attachment of the DNA of superinfecting phage to membrane.** After superinfection of *E. coli* B, little of the secondary T4 DNA is found associated with membrane (5). The poor injection efficiency and the superinfection breakdown which occur make this result difficult to interpret, however. Sheared samples of superinfected ER22 cells eliminate both of these complications and permit examination of injected DNA of the secondary phage.

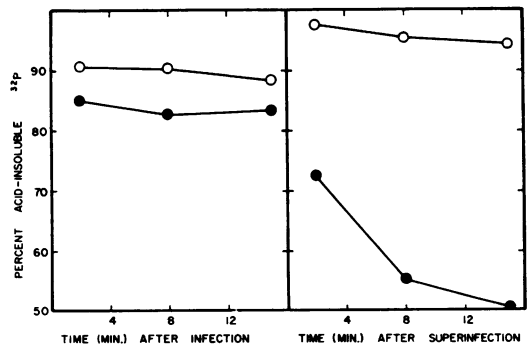


FIG. 2. Breakdown of  $^{32}\text{P-T4D}_0$  DNA after infection (left) or superinfection (right) of *E. coli* B and ER22. Multiplicities of 5 were used in each infection. Purification of the  $^{32}\text{P-T4D}_0$  phage employed involved a terminal dialysis step (7) in addition to the normal purification procedure. Symbols: ○, infection of ER22 cells; ●, infection of B cells.

As shown in Table 1, the percentage of superinfecting phage DNA attached to membrane in sheared preparations of ER22 cells is low. If it is assumed that only DNA which is bound to membrane is capable of being replicated, the above result suggests a basis for exclusion in T4. Table 1 also shows that approximately the same low percentage (25 to 30%) of secondary phage DNA is membrane-bound in all four cases, despite the fact that differences (described below) of up to 10-fold in recovery of  $^{32}\text{P}$ -DNA were observed. As has been described, shearing removes 40% of the phage DNA initially associated with cells after secondary infection, and superinfection breakdown renders acid-soluble an additional 50%. Thus the predicted recoveries of  $^{32}\text{P}$  phage DNA are as follows: nonsheared ER22 cells, 100%; sheared ER22 cells, 60%; nonsheared B cells, 50%; and sheared B cells, 10%. The actual recoveries, based on the results of five experiments, are  $100 \pm 16\%$ ,  $54 \pm 3\%$ ,  $55 \pm 12\%$ , and  $11 \pm 4\%$ , respectively. The amount of cell membrane and DNA recovered is unaffected by shearing or superinfection breakdown; the determinations of actual  $^{32}\text{P}$ -DNA recovery were calculated from double-label experiments by using recovery of one or the other of these two cell constituents as an internal standard.

The 25 to 30% figure for attachment after superinfection results in part from some adventitious attachment of phage to M-band. After secondary infection with  $^3\text{H}$ -leu- $^{32}\text{P}$ -T4, approximately 20% of the leucine label appears in the M-band prepared from both sheared and nonsheared samples.

**Effect of chloramphenicol on attachment of superinfecting T4 DNA to membrane.** Genetic exclusion occurs in both B and ER22 (7).

TABLE 1. *Percentage of macromolecules in M-band after superinfection<sup>a</sup>*

Component	Macromolecules (%) in			
	Sheared cells		Unsheared cells	
	B	ER22	B	ER22
Membrane.....	45	37	43	48
T4 DNA.....	30	27	25	26
Host DNA.....	67	65	65	69

<sup>a</sup> Multiplicities of infection of 5 were used for both primary and secondary infection. Cells were superinfected at 5 min, and samples were taken for analysis by the  $\text{Mg}^{2+}$ -sarkosyl crystal technique 2 min later. Cell membrane and host DNA were labeled with glycerol-2- $^3\text{H}$  and thymidine-methyl- $^3\text{H}$ , respectively (5).

Anderson (Ph.D. Thesis, Washington University, 1970) found that protein synthesis is required for the induction of genetic exclusion after T4 infection. A similar requirement for protein synthesis exists if  $\phi\text{X174}$ - or S13-infected cells are to establish superinfection exclusion (12, 18). In our hands, 200  $\mu\text{g}$  of chloramphenicol per ml added 30 sec after primary infection completely prevented exclusion. The results shown in Table 2 demonstrate that inhibition of protein synthesis shortly before or immediately after primary infection results in the attachment of 85% of the DNA of superinfecting phage to membrane. This percentage is identical to that found in a normal primary infection or in a primary infection which takes place in the presence of chloramphenicol. The degradation of host DNA which occurs after T4 infection accounts for the reduced percentage of host DNA attached to membrane in the control culture.

## DISCUSSION

The results presented indicate that an inverse correlation exists between the attachment of T4 DNA to membrane and temporal genetic exclusion. Exclusion occurs in both *E. coli* B and ER22, and only 25% of the secondary phage DNA appears in the M-band. Genetic exclusion does not occur if protein synthesis is inhibited after the initial infection, in which case 85% of superinfecting DNA is membrane-bound.

It is difficult to state precisely the actual amount of superinfecting DNA which is membrane-bound. Recovery of the secondary DNA is 10 times higher when nonsheared ER22 cells are used as hosts than when sheared preparations of B are used, yet in both cases 25% of the DNA is present in the M-band. The extent of exclusion is approximately 90 to 95% in both cases. Because exclusion is not complete, it is possible that a constant amount of superinfecting DNA attaches to mem-

TABLE 2. *Effect of chloramphenicol on membrane attachment of DNA after superinfection<sup>a</sup>*

Addition of chloramphenicol	DNA in M-band (%)	
	Host DNA	DNA of superinfecting phage
None.....	51	26
2 min preinfection.....	96	87
0.5 min postinfection..	97	88

<sup>a</sup> Cells were superinfected at 5 min, and samples were taken at 8 min after primary infection. Chloramphenicol (4 mg/ml in 50% ethanol) was added to a final concentration of 200  $\mu\text{g}$ /ml at the times indicated.

brane in all cases and that this DNA is responsible for progeny phage which bear markers of the superinfecting phage. If it is assumed that all of DNA present in M-bands prepared from sheared *E. coli* B cells is membrane bound, then 2.5 to 3% of the DNA of superinfecting phage which is initially associated with host cells is attached to membrane. This value is in good agreement with the amount of superinfecting phage DNA which is transferred to progeny phage. When cells are superinfected at 5 min, 2% of the superinfecting phage DNA appears in progeny phage (8).

It is probable that most of the secondary phage DNA never penetrates the cell membrane. The rapid release of fragments of secondary phage DNA into the medium and the location of endonuclease I in the cell periplasm (14) suggest that the injected DNA may be trapped in this region. Work employing autoradiographic techniques also supports this hypothesis; the DNA of superinfecting phage is associated with cell wall, whereas parental DNA in a primary infection is found in the cell cytoplasm (Anderson, Ph.D. Thesis, Washington University, 1970).

After T4 infection, cells develop not only the ability to exclude superinfecting phage but also an increasing resistance to lysis from without (19) and a tolerance to infection with ghosts (2). These observations combined with results which indicate that changes in host permeability (17) and phospholipid synthesis (9, 15) occur after infection are generally attributed to alterations which take place in host membrane. The absence of genetic exclusion in the presence of chloramphenicol and the high percentage of attachment of secondary T4 DNA to membrane in this case suggest that these membrane alterations require protein synthesis. This requirement may also be reflected in the recent demonstration that development of tolerance to ghosts is prevented by chloramphenicol or sodium azide (2).

The fact that a relatively low percentage of DNA from superinfecting T4 is attached to cell membrane may also serve as an additional criterion by which methods for the determination of membrane-bound DNA can be tested.

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