

# Increased Fragility of *Escherichia coli* After Infection with Bacteriophage M13

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Male strains of *Escherichia coli* infected with filamentous phage M13 released the progeny phage particles from intact cells. At the same time, the cells continued to grow and multiply at a slightly lower rate than the uninfected cells. Concomitant with the phage release, lipopolysaccharide from the cell wall of the infected cells was also released. The buoyant density of *E. coli* HfrC in diaginol, 1.25 g/cc, did not change as a result of infection. Detergents like sodium dodecyl sulfate and Sarkosyl specifically lysed the infected cells. The infected cells showed enhanced fragility as indicated by inactivation by various stresses, namely heat, osmotic shock, and freezing and thawing. It is concluded that the infection with M13 causes certain alterations in the surface structure of *E. coli*, thus making the cells more fragile.

M13 belongs to the group of recently discovered filamentous phages containing single-stranded circular deoxyribonucleic acid (DNA) and specific for male strains of *Escherichia coli* (9, 10, 14). The progeny phages have the unique property of "oozing" out of the intact host cells while the latter continue to grow and multiply (11). Salivar, Tzagaloff, and Pratt (25) and Brown and Dowell (3) showed that the M13-infected cells grow at only a slightly slower rate than the healthy cells. Schwartz and Zinder (27) showed that spheroplasts of the healthy and the M13-infected cells are equally stable.

Although these studies showed no apparent change in the cellular structure of *E. coli* after infection with M13, Falaschi and Kornberg (6) showed that, along with the progeny M13 phage, lipopolysaccharide (LPS) was released into the medium. We considered it likely that the release of progeny phage particles from intact cells was associated with the damage to the surface structure of the latter as a result of which LPS was released into the medium. Therefore, it was expected that the infected bacteria would be more fragile than the healthy ones. The present studies describe the results of some experiments designed to show that this is indeed the case. On the other hand, the buoyant density of whole cells, which is a measure of their gross chemical composition, did not change as a result of infection.

## MATERIALS AND METHODS

**Culture conditions.** M13 bacteriophage (wild type) and its host, *E. coli* HfrC, were obtained from P. H. Hofschneider, Max Planck Institute, Munich, Ger-

many. The bacteria were grown in a modified M9 medium (25) at 35 to 37 C on a rotary shaker with good aeration to a density of  $10^8$  to  $2 \times 10^8$  cells/ml, and then the phage was added at a multiplicity of 10 to 20. The high multiplicity of infection was necessary because of poor absorption of the phage to the host (22). At specified times after infection, the infected and the control cultures grown under identical conditions were chilled, and the cells were harvested in the cold by centrifugation.

The viable counts of the bacteria were determined by their colony-forming ability after dilutions in saline-phosphate medium (25), plating on nutrient agar, and incubation at 37 C overnight. The phage was assayed by the usual double-layer technique by the method of Adams (1).

**Estimation of LPS.** LPS is macromolecular and is sedimentable by ultracentrifugation at  $100,000 \times g$  for 120 min (6, 15). 2-Keto,3-deoxy octonate and colitose are the unique constituents of LPS in *E. coli* (7, 8), and they can be estimated with thiobarbituric acid reagents (5). The LPS released in the bacterial culture filtrate was estimated as follows. Thirty-milliliter amounts of the healthy and the infected cultures were centrifuged at low speed ( $10,000 \times g$  for 10 min) to remove the cells and then were subjected to ultracentrifugation in a Spinco ultracentrifuge (model L in rotor no. 30) at  $105,000 \times g$  for 120 min. The pellets containing LPS were suspended in saline-phosphate, and then a sample was used for chemical estimation of colitose. Since the absolute content of colitose in LPS of the particular *E. coli* strain used was unknown, the amount of LPS was expressed as extinction at 532 nm of the specific color produced.

**Spectrophotometry.** The cell density of a bacterial culture was determined at 660 nm in a Beckman DU or Hilger-Watts spectrophotometer and sometimes in a Klett-Summerson colorimeter (no. 66 filter). The

release of ultraviolet-absorbing materials from cells in the medium was followed by measuring the absorbance of the cells at 260 nm in the spectrophotometer.

**Isoptic centrifugation of bacteria in diagnol.** Cahn and Fox (4) used equilibrium ultracentrifugation in Renografin gradients to separate transformable from nontransformable cells of *Bacillus subtilis*. Diagnol (sodium acetate), an X-ray-opaque compound (a May and Baker product) similar to Renografin, was used in the present studies for equilibrium ultracentrifugation of *E. coli* cells. A standard plot of density of diagnol in saline-phosphate versus refractive index at 25°C in visible light was obtained by using a Carl Zeiss 1245 Abbe refractometer.

**Chemicals.** Sarkosyl NL 97 and Brij 58 were gifts of Geigy Chemical Corp. and Atlas Chemical Industries, respectively. Trypsin and lysozyme were purchased from Sigma Chemical Co. Tween 80 was a Koch-light product. Other chemicals used were of reagent grade.

## RESULTS

**Growth rate of healthy and M13-infected bacteria.** There was a small but significant increase in the generation time of *E. coli* HfrC after infection with M13, regardless of whether viable count or turbidity of the culture was measured (Fig. 1). The extracellular phage count shows that phage particles were secreted by the growing bacteria at an approximately exponential rate between 30 min and 4 hr after infection. The rate of phage production decreased when the bacterial culture reached the late log phase. These results confirm the earlier observations of Salivar, Tzagaloff, and Pratt (25) and Brown and Dowell (3) obtained with different host strains.

The length of the healthy and of the infected cells was determined on the log-phase cells after fixing and staining with crystal violet and then measuring by means of an ocular micrometer at a magnification of 1,500. The average length of *E. coli* HfrC varied with the age of culture from 2.0 to 2.4  $\mu\text{m}$ . In one case, when the healthy and the infected cultures were examined under identical conditions, the average lengths of the healthy and of the infected bacteria were approximately 2.4 and 2.5  $\mu\text{m}$ , respectively. This result supported the earlier observation of Brown and Dowell (3) that the average volume of the bacteria does not change significantly after infection with M13.

**Release of LPS from infected cells.** Figure 2 shows the kinetics of release of LPS as measured by the estimation of colitose and of M13 phage from the infected culture. It appears that the release of LPS continued almost linearly when the rate of phage production decreased to a very low level. It is likely that the degradation of the envelope structure continued in the infected cells even when the cells almost ceased to excrete

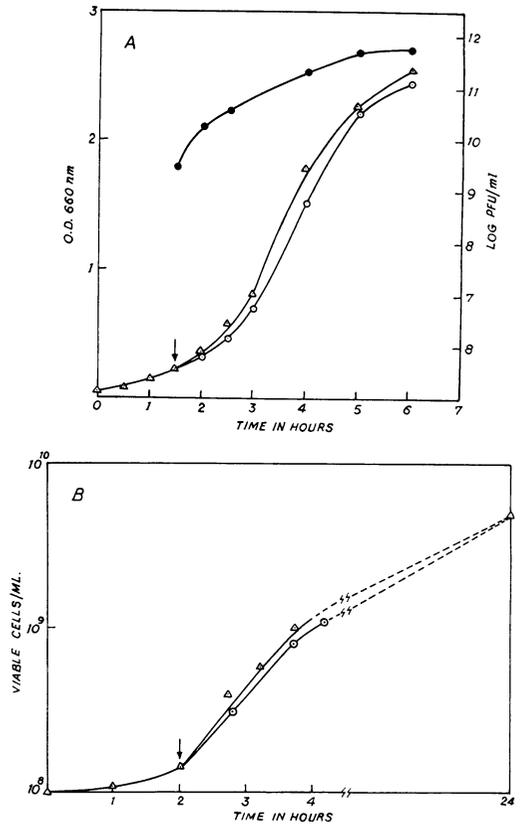


FIG. 1. Growth curves of healthy and M13-infected *E. coli* HfrC. (A) Growth as measured by absorbance at 660 nm. Symbols:  $\Delta$ , healthy cells;  $\circ$ , infected cells;  $\bullet$ , titer of extracellular phage. (B) Growth as measured by viable count. Symbols:  $\Delta$ , healthy cells;  $\circ$ , infected cells. The arrow indicates the time of infection.

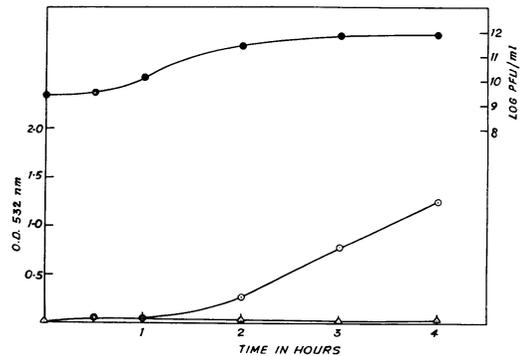


FIG. 2. Kinetics of release of colitose in lipopolysaccharide from healthy and M13-infected *E. coli* HfrC. The amount of colitose is expressed as the absorbance of the thiobarbituric acid reaction product. Symbols:  $\Delta$ , colitose released from healthy cells;  $\circ$ , colitose released from infected cells;  $\bullet$ , the titer of extracellular phage.

progeny phage particles. Whether the loss of LPS from the infected cells was compensated for by its increased synthesis is, however, not clear.

**Buoyant density of cells in diiginol.** The buoyant density of whole bacteria is, at best, a crude measure of the content of different cellular constituents, namely, protein, ribonucleic acid (RNA), DNA, envelope materials, and minerals. Nevertheless, we investigated whether the buoyant density of the infected cells was significantly different from that of the healthy cells. There was only one opalescent band of bacteria in a discontinuous gradient of diiginol, 20 to 50% (w/v) after ultracentrifugation at  $32,000 \times g$  for 30 min in an SW39 swinging bucket rotor in a Spinco ultracentrifuge (model L). The density of the band was  $1.25 \text{ g/cm}^3$ , whether the healthy and the infected cells were banded in a mixture or separately. This indicates that the bacterial density was not altered as a result of infection. Therefore, we may conclude that the overall composition of the cell was not grossly altered as a result of infection, in spite of the release of LPS and synthesis of phage-specific components.

**Susceptibility of infected cells to detergents.** The susceptibility of the infected cells to various stresses and detergents was compared to that of the healthy cells. Firstly, the efficiency of various detergents and enzymes in lysing intact cells was studied turbidimetrically. D. Pratt (*personal communication*) observed that sodium dodecyl sulfate (SDS) at certain concentration specifically lyses the infected cells. In the present studies, the healthy and the infected cells (2 hr after infection) suspended in growth medium in the log phase were treated with sodium deoxycholate (DOC), SDS, Sarkosyl, Brij 58, Tween 80, and also with trypsin and lysozyme. Among these, Brij 58 (1%, w/v), Tween 80 (0.1%, v/v), trypsin (10  $\mu\text{g/ml}$ ), and lysozyme (10  $\mu\text{g/ml}$ ) did not lyse either the healthy or the infected cells. Trypsin at 100  $\mu\text{g/ml}$  partially lysed both types of cells. DOC at a concentration of 0.1% (w/v) completely lysed both the types of cells. However, SDS and Sarkosyl distinguished strikingly the healthy from the infected cells. Figure 3 shows that the healthy cells were not only not lysed in the presence of 0.1% (w/v) SDS or 0.5% (w/v) Sarkosyl but continued to grow at the same rate as the control in the absence of the detergents. On the other hand, about 80% of the infected cells were rapidly lysed in the presence of these detergents. Thus, the detergent lysis could be used for selectively destroying the M13-infected bacteria.

The healthy and the M13-infected cells were equally unsusceptible to lysozyme. This may be surprising since lysozyme sensitivity of *E. coli*

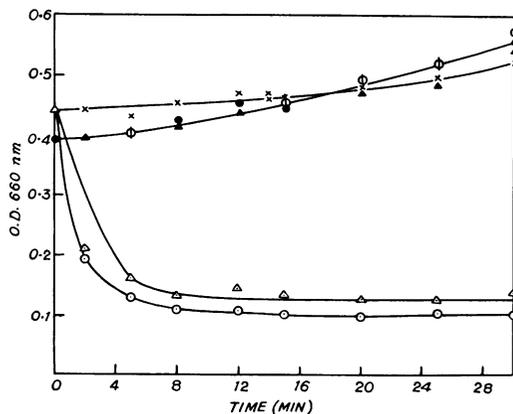


FIG. 3. Lysis of M13-infected *E. coli* HfrC in the presence of SDS (0.1%, w/v) and Sarkosyl (0.5%, w/v) as measured by the decrease of absorbance at 660 nm. Symbols: ●, untreated healthy cells; ▲, healthy cells in the presence of SDS; ◊, healthy cells in the presence of Sarkosyl; ×, untreated infected cells; ○, infected cells in the presence of SDS; △, infected cells in the presence of Sarkosyl.

is brought about by ethylenediamine tetraacetate (EDTA; 23). EDTA treatment releases LPS from *E. coli* (13) as infection with M13 did. Thus, either in the quality and quantity of LPS released or in causing other changes in the surface structure, the effect of EDTA treatment is different from that of infection with M13.

**Effect of freezing and thawing.** It is known that freezing alters the permeability of *E. coli* cells (2, 28). The infected cells were found to be far more susceptible to inactivation than the healthy cells after repeated freezing and thawing treatment. Two hours after infection, the infected cells along with the control healthy cells in the log phase were harvested by centrifugation in the cold in sterile tubes. The cells were then washed three times with cold saline-phosphate buffer and resuspended in the same buffer. The suspended cells were then subjected to repeated cycles of freezing the tubes in a salt-ice freezing mixture for 5 min with shaking and then thawing by holding at room temperature. Samples were assayed for viable cells after the treatment. Table 1 shows that, although the numbers of bacteria after harvesting were comparable, a much larger fraction of infected cells was inactivated by repeated freezing.

It has been known that 10% glycerol protects *E. coli* cells from inactivation during freezing (2, 20). Hence the freezing and thawing treatment was carried out in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.0) containing 10% glycerol. Table

1 shows that the infected cells were five times more susceptible to the freezing treatment than the healthy cells.

The loss of turbidity of a bacterial suspension is a measure of lysis during freezing and thawing. We found that the infected cells lost 20% turbidity (660 nm) as compared to 24% for the healthy cells after three cycles of freezing and thawing in saline-phosphate. Thus, lysis might account for only a fraction of total inactivation and could not explain the increased susceptibility of the infected cells to freezing.

Ultraviolet-absorbing materials are released from bacteria either after lysing or after certain treatments like Tris-EDTA washing (18) or after freezing and thawing (2). This material is presumably comprised of soluble nucleotides and RNA degradation products (18). Hence we compared the amount of 260 nm-absorbing materials released from the same amount of the healthy and the M13-infected cells after freezing and thawing treatment. Table 2 shows that, although there was some difference in optical density (OD) at 260 nm of the saline-phosphate supernatants in different steps, the overall amount of 260-nm absorbing materials released from the healthy and the infected cells was comparable. Thus, we may conclude that there was no difference in the permeability of the healthy and the infected cells as a result of freezing and thawing.

**Effect of osmotic shock.** Although it appears unlikely that the lethal effect of freezing is due to osmotic shock (20), Neu and Heppel (19) have shown that osmotic shock also damages the rigid cell wall and cytoplasmic membrane. The periplasmic enzymes and ultraviolet-absorbing materials are released from the cells into the medium after osmotic shock. In the present

studies, log-phase infected cells, 2 hr after infection (200 Klett units), and healthy cells (230 Klett units) were centrifuged, resuspended in 0.01 M Tris-hydrochloride (pH 8.0) containing 20% sucrose, and incubated for 30 min at 0 C.

TABLE 1. Effect of freezing and thawing treatment on the viability of healthy and M13-infected *E. coli* HfrC

| Step   | Viable count (cells/ml of suspension) |                   |
|--|---------------------------------------|-------------------|
|  | Healthy                               | Infected          |
| Bacteria suspended in saline phosphate buffer  |                                       |                   |
| Time of infection  | $1.4 \times 10^8$                     | $1.4 \times 10^8$ |
| Time of withdrawal of culture  | $5 \times 10^8$                       | $5 \times 10^8$   |
| Concentration by centrifugation and resuspension   | $1.4 \times 10^{10}$                  | $8 \times 10^9$   |
| After third washing  | $1.4 \times 10^{10}$                  | $7 \times 10^9$   |
| After freezing and thawing (one cycle)   | $9 \times 10^9$                       | $5 \times 10^9$   |
| After freezing and thawing (three cycles)  | $7 \times 10^9$                       | $2 \times 10^8$   |
| Survival, last 4 steps above (%)   | 50                                    | 2.5               |
| Bacteria suspended in 10% (v/v) glycerol containing 0.01 M Tris (pH 8)   |                                       |                   |
| Time of withdrawal of culture  | $5 \times 10^9$                       | $2 \times 10^9$   |
| Cell suspension in glycerol in the same volume as the culture after freezing and thawing (cells kept frozen for 42 hr at -15 C and then slowly thawed) | $3 \times 10^9$                       | $6 \times 10^8$   |
| Cell suspension (above) in glycerol, frozen and thawed in salt-ice mixture (one cycle)   | $2 \times 10^9$                       | $1.6 \times 10^8$ |
| Survival expressed relative to the original culture (%)  | 40                                    | 8                 |

TABLE 2. Release of ultraviolet-absorbing materials from healthy and M13-infected cells after freezing and thawing treatment<sup>a</sup>

| Determination  | Healthy           |                   |                       | Infected          |                   |                       |
|--|-------------------|-------------------|-----------------------|-------------------|-------------------|-----------------------|
|  | OD <sub>260</sub> | OD <sub>280</sub> | OD <sub>260/280</sub> | OD <sub>260</sub> | OD <sub>280</sub> | OD <sub>260/280</sub> |
| First wash supernatant                                 | 0.71              | 0.50              | 1.46                  | 0.64              | 0.44              | 1.46                  |
| Second wash supernatant                                | 0.34              | 0.21              | 1.60                  | 0.64              | 0.36              | 1.80                  |
| Third wash supernatant                                 | 0.53              | 0.30              | 1.74                  | 0.50              | 0.26              | 1.84                  |
| Supernatant after three cycles of freezing and thawing | 2.58              | 1.18              | 2.26                  | 1.91              | 0.91              | 2.16                  |
| Total  | 4.16              | 2.19              | 1.90                  | 3.69              | 1.97              | 1.88                  |

<sup>a</sup> A log-phase *E. coli* culture was infected with M13 90 min after infection; 40 ml each of the healthy and the infected cultures was centrifuged in the cold ( $10,000 \times g$  for 10 min). The cell pellets were washed three times with 5 ml of saline-phosphate and were finally suspended in the same volume of saline-phosphate. The cell suspensions were subjected to three cycles of freezing and thawing, and the absorbance of the supernatants at 260 and 280 nm was determined and expressed per  $10^9$  cells.

The suspensions were again centrifuged in the cold and the bacterial pellets were resuspended in the same volume of 0.01 M Tris-hydrochloride, pH 8.0. Table 3 shows the viability of the shocked cells. It is evident that, whereas the healthy cells were completely resistant to the osmotic shock, the infected cells were largely inactivated. When EDTA was added, most of both the healthy and infected cells were killed. The turbidity of the healthy cells after osmotic shock dropped from 0.645 to 0.635 OD<sub>660 nm</sub> units and that of the infected cells dropped from 0.53 to 0.43. There was no difference in the release of periplasmic enzymes, 5'-nucleotidase and endonuclease I (19), and ultraviolet-absorbing materials from the healthy and the infected cells after osmotic shock.

**Kinetics of heat inactivation.** Russell and Harries (24) have shown that heat treatment of *E. coli* cells in suspension causes damage to the cytoplasmic membrane of the cells and subsequent release of RNA-degradation products into the medium. We compared the kinetics of killing of the healthy and the infected cells by heat. The cells were harvested 2 hr after infection (OD at 660 nm was 0.86 for the healthy and 0.77 for the infected cells) and suspended in saline-phosphate. The cell suspensions under identical conditions were immersed in a water bath at 50 C, and samples were assayed for colony-forming survivors at various times. The infected cells were inactivated about twice as fast as the healthy cells (Fig. 4). The ultraviolet-absorbing materials released were measured to be 1.49 and 0.83 OD<sub>260 nm</sub> units per 10<sup>9</sup> cells of the infected and the healthy bacteria, respectively, after 19 min of heating at 50 C. Thus, both cellular inactivation and the release of ultraviolet-absorbing materials indicated a greater susceptibility of infected cells to heat.

TABLE 3. Effect of osmotic shock on the viability of healthy and M13-infected *E. coli* HfrC

| Determination   | Viable count (cells/ml of suspension) |                       |
|---|---------------------------------------|-----------------------|
|   | Healthy                               | Infected              |
| Time of infection.....                                      | 1.6 × 10 <sup>8</sup>                 | 1.6 × 10 <sup>8</sup> |
| After concentration by centrifugation and resuspension..... | 5 × 10 <sup>9</sup>                   | 2 × 10 <sup>9</sup>   |
| After osmotic shock.....                                    | 5 × 10 <sup>9</sup>                   | 2.5 × 10 <sup>8</sup> |
| Survival (%).....   | 100                                   | 12.5                  |

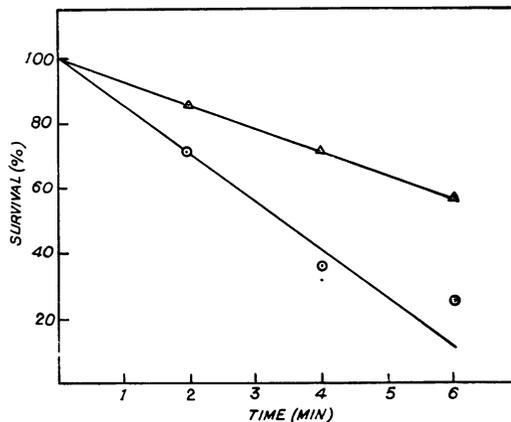


FIG. 4. Kinetics of inactivation of *E. coli* HfrC at 50 C. Symbols: Δ, healthy cells; ○, infected cells.

DISCUSSION

We have confirmed the observation of previous workers (3, 25) that M13-infected *E. coli* cells multiply at only a slightly lower rate than healthy cells. The size of the cells also did not change as a result of infection. Since the progeny phage particles were released from the intact and growing bacteria, the surface structure of the latter must have undergone subtle changes. The release of LPS specifically from the infected cells supports this possibility. However, the nature of the lesions in the cell envelope as a result of the infection is not clear. It has been found in the present studies that the rate of LPS production increased, whereas the rate of phage production decreased considerably in the late stage of infection. This may indicate that persistent infection in the absence of active phage synthesis would continue to alter the surface structure of the host.

The quantitative studies of the release of LPS in relation to the total bacterial mass have not been carried out. That the buoyant density of bacteria did not change as a result of infection is consistent either with the fact that the amount of LPS lost per cell was insignificant or that the lost LPS was being replaced in the infected cell. The loss of LPS from the infected cells is reminiscent of the LPS release from healthy *E. coli* cells after EDTA treatment found by Leive (13). The infected cells, like the EDTA-treated cells, but unlike the healthy cells, were susceptible to actinomycin D (12; Roy and Mitra, *in press*). On the other hand, the infected cells did not exhibit other features of EDTA treatment, namely susceptibility to lysozyme (23) and release of ultraviolet-absorbing materials (18).

LPS is believed to contribute to the structural rigidity of the cell envelope (26). However, after loss of about half of their LPS, cells do not become osmotically fragile (12, 13). Nevertheless, the fact that the infected cells after repeated subculture partially lysed (Roy and Mitra, *unpublished data*) suggests that the cell envelope underwent alterations other than the loss of LPS, resulting in the weakening of its structure. A comprehensive understanding of this phenomenon would perhaps lead to the elucidation of the mechanism of release of progeny M13 phage from the intact cells.

In any case, it has been shown in the present studies that the infected cells were preferentially susceptible to certain detergents, osmotic shock, freezing, and heat. It is likely that different components of the macromolecular and highly complex surface structure were targets of these different treatments. Only SDS and Sarkosyl specifically lysed the M13-infected cells whereas other surface-active agents like DOC, Brij 58, and Tween 80 could not distinguish the healthy from the infected cells. This indicated that SDS and Sarkosyl might be acting at the same site in the bacterial envelope, whereas, among the latter, DOC lysed indiscriminately and was different in action from Brij 58 and Tween 80.

All of the different mechanical and thermal stresses preferentially inactivated the infected cells, but, here again, these stresses were not identical in action. Thus, osmotic shock and freezing did not show any difference between the healthy and the infected cells as regards the release of soluble ultraviolet-absorbing materials, whereas heat treatment produced greater release of the ultraviolet-absorbing materials from the infected cells. In all cases, however, the applied stresses caused damage to the envelope structure of the cells (2, 19, 20, 24, 28). Although the healthy cells could efficiently repair the damage and survive, for the infected cells the damage was irreversible because of the already weakened surface structure.

M13 is a small phage with a single-stranded ring DNA of about  $2 \times 10^6$  daltons molecular weight (16, 17). It has only eight cistrons, most of which have been identified as either coding for the phage coat proteins or proteins (enzymes) related to phage DNA synthesis (21). Whether an M13 cistron codes for an enzyme which damages the bacterial envelope structure or whether some phage specific-protein(s) inhibits the synthesis of structural component(s) of cell envelope remains to be elucidated.

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