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Localization of Parental Deoxyribonucleic Acid from Superinfecting T4 Bacteriophage in *Escherichia coli*

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High-resolution autoradiography has been employed to localize the nonsolubilized but genetically excluded deoxyribonucleic acid (DNA) of T4 bacteriophage superinfecting endonuclease I-deficient *Escherichia coli*. This DNA was found to be associated with the cell envelope (this term is used here to include all cellular components peripheral to and including the cytoplasmic membrane); in contrast, T4 DNA in primary infected cells, like host DNA in uninfected *E. coli*, was found to be near the cell center. The envelope-associated DNA from superinfecting phage was not located on the outermost surface of the cell since it was insensitive to deoxyribonuclease added to the medium. These results suggest that DNA from superinfecting T-even phage is trapped within the cell envelope.

When *Escherichia coli* previously infected with a T-even phage is superinfected with the same or a similar T-even phage, nearly all of the injected deoxyribonucleic acid (DNA) from the superinfecting phage is degraded to acid-soluble fragments (1, 7). In the mutants of *E. coli* lacking the periplasmic enzyme endonuclease I (endonuclease I^- strains), however, the injected DNA from superinfecting phage is not extensively degraded; less than 5% becomes acid-soluble during the normal course of infection (1, 6). Nevertheless, superinfecting T-even phage are genetically excluded in endonuclease I^- strains (1, 6), and, as measured by complementation, the DNA from such superinfecting phage is not functional (1).

The change which occurs in cells during the first few minutes after adsorption of primary T-even phage and which is responsible for the induction of superinfection breakdown and exclusion has not yet been identified. The accompanying paper (1) demonstrates that the induction of both superinfection breakdown and exclusion requires phage-induced protein synthesis, but no change occurs in endonuclease I activity after infection. It was noted, however, that the injection of DNA from superinfecting phage particles was much less efficient than the injection of DNA from primary phage. These and other observations (10) suggested that the induction of superinfection breakdown and exclusion might result not from an activation of endonuclease I but from a change in the way superinfecting or secondary T-even phage inject their DNA. If, for example, the primary phage induced a change in the cell envelope which resulted in the inefficient passage of DNA from secondary phage through it or entrapment within it, the DNA might become susceptible to attack by endonuclease I, an enzyme thought to reside in the "periplasm" of the cell envelope (reference 3; "cell envelope" is used here to include all cellular components peripheral to and including the cytoplasmic membrane).

In this study, two approaches were used in an attempt to find differences between the intracellular distribution of parental DNA from primary infecting and superinfecting T4 phage. In the first method, it was assumed that the DNA from superinfecting phage would be susceptible to an exogenously added deoxyribonuclease if the infected cells were first converted to spheroplasts. This proved not to be the case. However, a second approach, the direct examination of infected cells by high-resolution autoradiography, indicated that the DNA from superinfecting T4, unlike that from primary phage, is closely associated with the cell envelope. This result supports the hypothesis that the DNA from superinfecting T-even phage becomes trapped in the cell envelope where, in endonuclease I^+ strains, it is rapidly degraded to oligonucleotides.

MATERIALS AND METHODS

Bacteria, phage, media, and most of the procedures employed are described in the accompanying paper (1).

Deoxyribonuclease treatment of T4-infected spheroplasts. Cultures of ER22 were grown to about 6×10^8

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cells/ml in M9GC medium supplemented with ^{3}H-thymidine (^{3}H-TdR; 2 μCi/ml, 1.9 Ci/mmole) and isopropyl thiogalactoside (120 μg/ml). The cultures were then centrifuged (in the presence of 63 μg of unlabeled TdR per ml) at room temperature, suspended in fresh M9GC containing 63 μg of unlabeled TdR per ml, and incubated an additional 10 min at 37 C to reduce the pool of acid-soluble ^{3}H-TdR. The cultures were again centrifuged and resuspended in fresh M9GC at 6×10^8 cells/ml. For the superinfection experiment, one culture was infected with unlabeled T4 at a multiplicity of infection (MOI) of 4.4 and 5 min later was superinfected at MOI 4.9 with ^{32}P-labeled T4 of specific activity 4.5×10^{-4} counts per min per plaque-forming unit (PFU). For the primary infection experiment, the order of phage addition was reversed so that the ^{32}P-labeled phage became primary phage. Ten minutes after primary infection, each culture was chilled by threefold dilution with ice-cold M9GC containing 440 μg of bovine serum albumin (BSA) per ml and blended to shear off uninjected phage particles (1). A portion of the blended material was centrifuged at $3,000 \times g$ for 5 min at 4 C, and the pellet was resuspended in a Tris-saline-BSA solution [0.05 M tris(hydroxymethyl)-aminomethane (Tris), *p*H 8; 0.15 M NaCl; BSA, 440 μg/ml]. After an additional centrifugation in the cold, the pellet was suspended in 0.35 ml of 1.5 M sucrose, 0.23 ml of 22% BSA, 0.10 ml of a freshly prepared lysozyme solution (2 mg/ml; Worthington two times crystallized in 0.25 M Tris buffer, *p*H 8.0), 0.06 ml of 4% ethylenediaminetetraacetate (EDTA), and 10 ml of PA-2 medium containing (per liter) Casamino Acids, 10 g; Difco nutrient broth, 10 g; glucose, 10 g; sucrose, 200 g as described by Guthrie and Sinsheimer (8) for the production of spheroplasts. After 10 to 15 min of incubation at room temperature, 0.10 ml of 10% $MgSO_4$ was added to the suspension to complex the EDTA and stop the reaction. Examination by microscope indicated that 40 to 60% of the cells had become spherical; more than 95% of the cells lysed, as measured by the release of preinduced β-galactosidase, upon 10-fold dilution of the suspension into distilled water.

For the measurement of deoxyribonuclease sensitivity, the spheroplast suspension was transferred to 37 C (zero time for the experiment) and 5 min later pancreatic deoxyribonuclease (Sigma Chemical Co., St. Louis, Mo.) was added to 10 μg/ml. Samples removed at intervals were assayed for acid-soluble ^{3}H and ^{32}P label by trichloroacetic acid precipitation as previously described (1). Other samples were taken to monitor cell lysis by the release of preinduced β-galactosidase.

Tritium labeling of T4 phage for autoradiography. High-specific-activity, ^{3}H-TdR-labeled T4 was prepared on a log-phase culture of *E. coli* B. The culture was centrifuged at room temperature and suspended at 4×10^8 cells/ml in fresh M9GC supplemented to 400 μg/ml with deoxyadenosine (AdR) and to 250 μCi/ml with ^{3}H-TdR (24.4 Ci/mmole); it was immediately infected with T4 at MOI 5. After lysis with chloroform at 2.5 hr, purification of the phage was carried out in the presence of unlabeled TdR by differential centrifugation as previously described (1). Approximately 50% of the added ^{3}H-TdR was incorporated into acid-insoluble material; the purified phage contained about 2.2×10^{-4} counts per min per PFU or 1.4 decays per day per phage, assuming 100% plaquing efficiency and 25% counting efficiency. The phage preparation was used within 48 hr.

Tritium labeling of E. coli for autoradiography. Ten milliliters of M9GC containing AdR, 400 μg/ml; uridine, 20 μg/ml; fluorodeoxyuridine (Hoffmann-La Roche, Inc., Nutley, N.J.), 1 μg/ml; unlabeled TdR, 8 μg/ml; and ^{3}H-TdR, 20 μCi/ml (24.4 Ci/mmole) was inoculated with ER22 to 4×10^7 cells/ml. When the culture had attained a density of 3.2×10^8 cells/ml after 2 hr at 37 C, it was harvested in the presence of unlabeled TdR, suspended in fresh M9GC supplemented with 400 μg of unlabeled TdR per ml, and incubated an additional 10 min at 37 C. Approximately 12% of the ^{3}H-TdR label was incorporated, yielding an estimated 16 decays per day per bacterium.

Autoradiography of infected and uninfected cultures. The procedures used to primary infect, superinfect, and blend a culture have been described (1). Blending was shown to be approximately 80% effective in removing phage-coat protein from infected cells.

The ice-cold blended material from ^{3}H-TdR-labeled, T4-infected cultures and the chilled cell suspension from an uninfected, ^{3}H-TdR-labeled culture were centrifuged ($3,000 \times g$, 5 min, 5 C) and suspended in fresh cold medium to remove unbound label. One-tenth volume of gluteraldehyde-picric fixative [0.26 M gluteraldehyde, 0.1 M sodium cacodylate, 0.026 M picric acid, 0.003 M $Ca(NO_3)_2$, 0.002 M $MgCl_2$; *p*H 7.4] was added. The cells were then centrifuged ($2,000 \times g$, 5 min, room temperature), suspended in one volume of the gluteraldehyde-picric fixative, and left overnight at room temperature. They were washed twice with *p*H 7.2 cacodylate rinsing buffer [0.15 M sodium cacodylate, 0.187 M sucrose, 0.003 M $Ca(NO_3)_2$, and 0.002 M $MgCl_2$], fixed for 1 to 2 hr in 1% osmium tetroxide (in cacodylate buffer), washed twice with 0.1 M sodium acetate (*p*H 7.3), and then stained with 0.25% uranyl acetate. After two additional washings with acetate buffer, the final pellet was dehydrated through 100% acetone and propylene oxide and embedded in Epon. Thin sections were cut with a Porter-Blum MT 2 microtome and picked up on uncoated, 300-mesh grids. The sections were stained with uranyl acetate followed by lead citrate and then were carbon-coated on one side. This side of each grid was coated with a monolayer of Ilford 4 emulsion by using a stripping film technique (12). The grids were dried and stored over silica gel for approximately 2 months. The exposed grids were developed with paraphenylene diamine and examined in the electron microscope. Suitable fields were photographed at the same magnification (ca. 40,000-fold on final 8 by 10 inch print), and measurements were made of the distance from the center of each silver grain to the outer edge of the nearest cell wall.

RESULTS

Susceptibility of superinfecting DNA to exogenous deoxyribonuclease. Superinfecting DNA is not unusually susceptible to exogenous deoxy-

ribonuclease. The results of the accompanying study of the induction of superinfection breakdown and exclusion suggested that the injected DNA from superinfecting phage, unlike the DNA from primary phage, might not penetrate the cytoplasmic membrane but instead might be trapped in the cellular envelope. If this were so, the superinfecting phage DNA might be sensitive to added extracellular deoxyribonuclease, especially if the cell wall were first partially degraded by digestion in hypertonic medium (spheroplasting).

Two comparable cultures of the endonuclease I⁻ strain ER22 were induced for β-galactosidase production and labeled for about two generations with ^{3}H-TdR as described above. The prelabeled cells were then primary infected or superinfected at high multiplicity with ^{32}P-labeled T4, blended to remove uninjected phage DNA, and converted to spheroplasts by lysozyme-EDTA treatment. The spheroplast suspension was incubated at 37 C in the presence of Mg^{2+} and pancreatic deoxyribonuclease, and samples were removed to determine the amount of ^{32}P-labeled phage DNA and ^{3}H-labeled cellular DNA that had been solubilized. Cell lysis was monitored by the release of preinduced β-galactosidase.

As can be seen in Fig. 1a, there was no detectable difference in deoxyribonuclease susceptibility between superinfecting T4 DNA and *E. coli* DNA. Prior to addition of deoxyribonuclease, 19% of the ^{3}H-label was acid-soluble, presumably because of incomplete removal of the ^{3}H-TdR pool before infection. Immediately after addition of the pancreatic deoxyribonuclease to the superinfected spheroplasts, 20% of the phage DNA and 15% of the bacterial DNA were rapidly solubilized, a result which was not surprising since 25% of the spheroplast suspension had lysed before the deoxyribonuclease was added. The significant result, however, was that, throughout the subsequent course of the incubation, the ratio of per cent ^{32}P-counts to per cent ^{3}H-counts released by the enzyme did not vary significantly from 1.0. At the time the culture was chilled for blending (10 min after primary infection), only a small amount of the host DNA should have been degraded to acid-soluble products owing to the action of phage-induced deoxyribonucleases (2). If the superinfecting T4 DNA had been exposed to the extracellular deoxyribonuclease because of its presumed location at or near the surface of the cell, one would expect that the ratio given in Fig. 1a would increase rapidly (to a value of 4 to 5 in the experiment described in Fig. 1a) after the addition of deoxyribonuclease. At later times this ratio would be expected to decrease slowly as the spheroplasts lysed. In the control culture (primary infection, Fig. 1b), the ratio remained close to 1.0 throughout the period of incubation as had been expected.

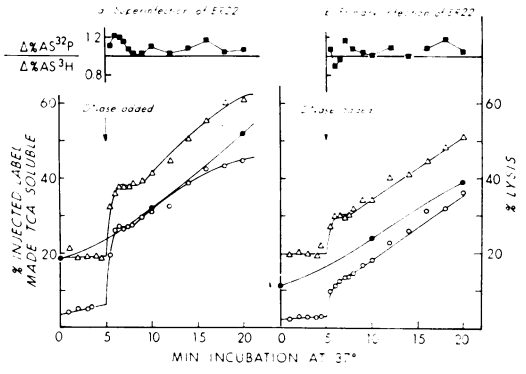

FIG. 1. *Sensitivity of superinfecting T4 DNA to extracellular deoxyribonuclease. The process of infection and spheroplast production is described in Methods. (a) Superinfection by ^{32}P-labeled T4: adsorption, 87%; injection (of adsorbed phage DNA), 56%. (b) Primary infection by ^{32}P-labeled T4: adsorption 98%; injection, 75%. Symbols: (○) per cent acid-soluble ^{32}P label (phage DNA), (△) per cent acid-soluble ^{3}H label (bacterial DNA), (●) per cent supernatant β-galactosidase (measure of cell lysis), and (■) ratio of phage DNA (△%AS^{32}P) to bacterial DNA (△%AS^{3}H) solubilized by the added pancreatic deoxyribonuclease.*

Location by autoradiography of primary and secondary phage DNA. The location of intracellular components in relation to the cell envelope can sometimes be determined directly by high-resolution autoradiography (11). By this procedure it was determined that DNA of primary phage is located near the cell center and DNA of secondary phage is located near the cell envelope. To determine the intracellular distribution of parental T4 DNA, cultures of ER22 were either primary infected or superinfected with high-specific-activity, ^{3}H-TdR-labeled T4. The infected cultures were blended to remove uninjected phage DNA and then fixed and embedded. Thin sections of the infected cells were prepared for autoradiographic studies as described above. Representative examples of the relationship of grains to bacteria are shown in Fig. 2.

A histogram of the primary infected cells harvested 5 min after infection is shown in Fig. 3a. Chloramphenicol (200 μg/ml) had been added 5 min before infection to increase injection efficiency and to prevent exclusion of late adsorbing phage particles (1). Chloramphenicol also prevents phage development, including the formation of a fast sedimenting complex containing parental phage DNA (9), but it does not prevent

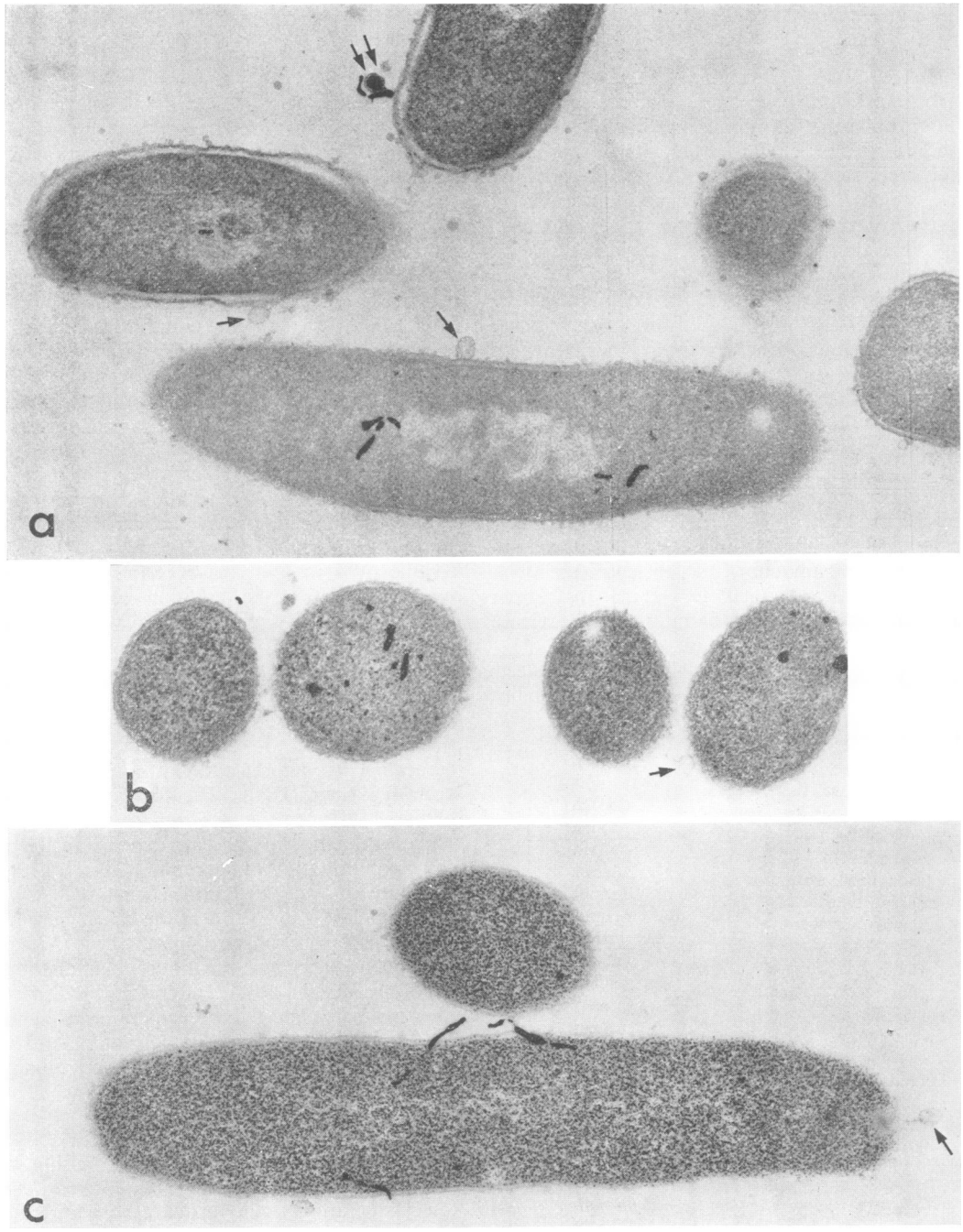

FIG. 2. *Electron micrographs of thin-section autoradiograms of T4-infected ER22. Representative micrographs from the experiments illustrated in Fig. 3. (a) Primary infection by ^{3}H-TdR-labeled T4 in the presence of chloramphenicol (CAP). (b) Primary infection by labeled T4, no CAP. (c) Superinfection by labeled T4. It is evident that grains tend to be centrally located after primary infection with T4, with (a) or without (b) CAP, whereas they tend to be localized near the cell surface after superinfection (c). Phage ghosts (single arrow) are evident in all the micrographs, and an intact phage with an associated grain (double arrow) is present in (a).* × *32,700.*

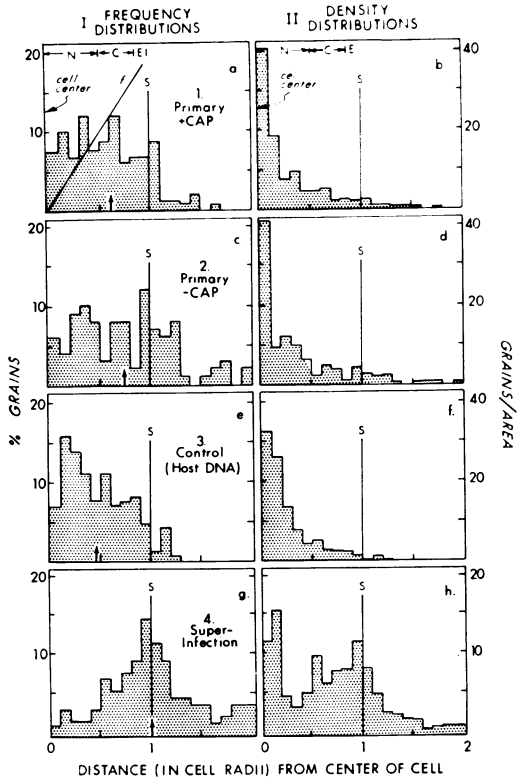

FIG. 3. *Histograms of frequency and density of grain distributions as function of distance from center of cell. Micrographs such as those in Fig. 2 were analyzed. The distance from each grain to the outer edge of the nearest cell wall was determined for all grains within one cell radius of a cell wall. Column I gives the frequency distributions; column II the density distributions calculated as described in Results. Grain counts have been normalized to 100 (actual numbers counted are given in Table 1). The frequency distribution that would result from a uniform density distribution is indicated by line f in panel a. In each panel, the center of the cell is at the left (0 radius), and the cell surface is at the line marked S (one radius). At the tops of panels a and b, approximate locations of the nuclear region (N), the cytoplasmic region (C), and the envelope region (E) are indicated. In this experiment actual values measured for 30 randomly selected cells were: cell radius, 358 ± 22 nm; radius of nuclear region, 185 ± 32 nm; and width of cell envelope, 40 ± 10 nm. The experiments were as follows. Panels a and b: primary infection with chloramphenicol (CAP); ER22 at 3.2 × 10^8 cells/ml infected at MOI 9.5 with ^{3}H-TdR-labeled T4 5 min after CAP added to 200 μg/ml; 80% of T4 added adsorbed, 84% of adsorbed T4 injected. Culture chilled 5 min after infection, blended, and prepared for autoradiography as described in Methods. Panels c and d: primary infection without CAP; as in panels a and b, but no CAP added; adsorption, 82%; injection, 70% of adsorbed T4. Mean cell radius was 364 ± 27 nm. Panels e and f: uninfected control cells; ER22 labeled with ^{3}H-TdR. Mean cell radius was 356 ± 25 nm. Panels g and h: superinfection; ER22 at 3.3 × 10^8 cells/ml infected with unlabeled T4 at MOI 4.7 and 5 min later by ^{3}H-TdR-labeled T4 at MOI 12.4; adsorption of labeled T4, 80%; injection, 44% of adsorbed T4. Culture chilled 5 min after secondary infection. Mean cell radius was 350 ± 28 nm.*

attachment of phage DNA to the cell membrane as measured by the sarcosyl "M-band" technique (5). If the primary phage DNA were uniformly distributed throughout the cell cytoplasm, one would expect grain frequency to increase linearly as a function of distance from the cell center (Fig 3a line f), and then to decrease abruptly at the cell wall. The grain distribution across the chloramphenicol-treated, primary infected cells was, however, relatively uniform; only a few grains were further than 0.1 cell radius outside the cell wall. Thus, a disproportionate amount of the phage DNA was located in the "nuclear" region near the center of the cell.

An approximate mass-density distribution was constructed from the data shown in Fig. 3a. This calculation assumed the bacterial cell to be a cylinder of unit cross-sectional radius, and this cross section was divided into concentric circles with increments in radius of 0.1. When the grain frequency in each annular area between two of the circles was divided by this area, the resulting histogram gave the probability of finding a segment of phage DNA per unit of area (or volume) as a function of distance from the long axis of a cell (Fig. 3b). This histogram illustrated more clearly than the original that the parental T4 DNA in primary-infected, chloramphenicol-treated cells had a high probability of residing near the cell center. There were no indications in this or in the subsequent experiments that the parental T4 DNA was preferentially located at the ends of the cell. In this experiment, most cell sections exhibited a well defined nuclear region and a well defined, though often plasmolysed, cell envelope.

The histograms of parental T4 DNA 5 min after infection in cells not pretreated with chloramphenicol (Fig. 3c and d) were similar to those in the pretreated cells, and both resembled the histograms of bacterial DNA in an uninfected culture (Fig. 3e and f). In the primary infection experiment without chloramphenicol, the nuclear regions of most cells had disintegrated; nevertheless, the parental phage DNA was preferentially located in that region.

The histograms of parental T4 DNA from superinfecting phage were very different (Fig. 3g and h). In this case, a large fraction of the grains were associated with the cell envelope. Forty-

three per cent of the grains were found within ±0.2 cell radii of the cell wall, whereas the peak in the density distribution (Fig. 3h) near the cell center results from only five grains and is not statistically significant.

Mean values of the grain distributions and their standard deviations are given in Table 1. With this data the grain distributions were compared by *t* tests (Table 2). The results suggest that, even in the two primary infection experiments, the difference in the distribution of grains, although small, is significant ($P < 0.025$). On the other hand, the distributions from the uninfected cells and the secondary infected cells show much more pronounced differences, both from each other ($P < 0.001$) and from the primary infected cells ($P < 0.005$ or <0.001).

DISCUSSION

In an endonuclease I⁻ mutant of *E. coli* B that genetically excludes but does not solubilize superinfecting T4 DNA, high-resolution autoradiography has shown that this DNA was mainly associated with the cell envelope. In contrast, DNA from primary phage is localized near the center of the cell, with a distribution similar to, but not identical to, that of bacterial DNA prior to infection.

TABLE 1. *Means and standard deviations of grain distributions*

Expt	No. of grains	Mean distance from cell center (in cell radii)	Standard deviation (in cell radii)
Primary-infection + CAP[a]	148	0.602	±0.379
Primary-infection − CAP	100	0.746	±0.480
Uninfected control	145	0.476	±0.313
Secondary infection	130	1.003	±0.427

[a] Chloramphenicol.

TABLE 2. *Comparison of grain distributions by t test*

Comparison	*t*	Degrees of freedom	Probability
Secondary infection, primary infection + CAP[a]	8.388	276	<0.001
Secondary infection, primary infection − CAP	4.367	228	<0.001
Secondary infection, uninfected control	11.868	273	<0.001
Uninfected control, primary infection + CAP	3.043	291	<0.005
Uninfected control, primary infection − CAP	9.137	243	<0.001
Primary infection − CAP, primary infection + CAP	2.925	246	<0.025

[a] Chloramphenicol.

The resolution of the autoradiographic technique, however, is not sufficient to indicate the precise relationship of the DNA from superinfecting phage particles to the cell envelope. Thus, it is not possible to ascertain directly from these experiments whether such DNA is trapped within the layers of the cell wall-membrane complex or whether it is bound in some way to one or both surfaces of the cell envelope.

If naked DNA from superinfecting phage particles were bound to the outer surface of cells, one would expect it to be sensitive to added extracellular deoxyribonuclease; however, such sensitivity was not found. DNA from superinfecting phage particles might also be bound to the outer cell surface in the form of uninjected phage particles insensitive to deoxyribonuclease. This possibility can be ruled out since the blending procedure removed the vast majority of uninjected particles. The efficiency of this treatment was confirmed by the finding that phage particles were observed only very rarely in autoradiographs of blended preparations.

The DNA from superinfecting phage particles might also be bound to the cytoplasmic side of the plasma membrane. However, when superinfected cells are gently lysed and sedimented through neutral glycerol gradients, very little of the DNA from the superinfecting T4 is associated with the "fast sedimenting complex" believed to represent membrane-bound DNA. Rather, superinfecting DNA is found as free DNA of less than unit length (1). In contrast, primary DNA, the bulk of which was found to be near the center of the cell by autoradiographic analysis, sediments mainly with the fast sedimenting complex.

A possible explanation of the results reported above is that in endonuclease I⁻ strains, where the DNA from superinfecting phage is not extensively degraded, such DNA becomes trapped in the cell envelope. Such trapped DNA might be insensitive to deoxyribonuclease treatment of spheroplasts for several reasons. Spheroplasting does not appear to remove large amounts of cell wall material or to alter significantly the structure of the cell envelope as seen in electron micrographs (5); thus, entrapped DNA may not be sufficiently exposed by spheroplasting to render it deoxyribonuclease sensitive. Alternatively, the spheroplasting procedure itself may allow entrapped DNA to pass through the plasma membrane and into the cytoplasm.

The results are therefore consistent with the "failure-to-penetrate" model of the T-even phage exclusion phenomenon outlined in the accompanying paper (1). Experiments involving ^{32}P-labeled T2 and *E. coli* B which were performed in 1955 by Puck and Lee (10) are also compatible with this view of superinfection and led them to conclusions similar to those presented here.

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