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Characterization of Infectious Deoxyribonucleic Acid from Temperate *Bacillus subtilis* Bacteriophage ϕ105

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Phenol-extracted, infectious deoxyribonucleic acid (DNA) species from ϕ105 phage particles, from ϕ105 lysogenic bacteria, and from induced ϕ105 lysogenic bacteria were sedimented in sucrose gradients. Infectious DNA from ϕ105 particles sedimented like the bulk of mature phage DNA in neutral sucrose. Infectivity of prophage DNA was associated with fast-sedimenting material of heterogenous size. Infectious vegetative phage DNA sedimented somewhat faster than mature phage DNA; it was rapidly converted to a poorly infectious form during the infection.

Deoxyribonucleic acid (DNA) extracted from phage ϕ105 particles, DNA from ϕ105 lysogenic bacteria, and DNA from induced ϕ105 lysogenic bacteria are infectious when assayed in the *Bacillus subtilis* transformation system (8). However, the infectivity of these three DNA species exhibits qualitative and quantitative differences. These differences were suggested to relate to structural differences between the DNA molecules. In the present experiments, we have examined the sedimentation of ϕ105 DNA in sucrose gradients; it will be shown that the different infectious ϕ105 DNA species can be distinguished from each other by their sedimentation properties.

MATERIALS AND METHODS

Bacteria and phage. *B. subtilis* 168 was used as the indicator bacterium. Prophage and vegetative phage DNA were isolated from 168 (ϕ105). *B. subtilis* BR16 (*trp-2, lys-1*) was used to obtain competent cells. Phage ϕ105 has recently been described (2).

Media and growth of bacteria and phage. The media employed and the methods for growing bacteria and phage have been described (7, 8). The procedure of Anagnostopoulos and Spizizen (1) was used to obtain competent bacteria.

Preparation of mature ϕ105 DNA. *B. subtilis* 168 (ϕ105) was grown in 400 ml of T broth in 2,800-ml indentated Fernbach flasks with shaking at 37 C. At a density of 5×10^7 to 10×10^7 bacteria per ml, mitomycin C (MC, Sigma Chemical Co., St. Louis, Mo.) was added at a concentration of 0.5 to 1 μg per ml. After 10 min in the presence of the drug, the bacteria were sedimented and resuspended in 100 ml of fresh T broth and incubated at 37 C with forced aeration. At 50 min after resuspension, 1 mc of thymidine-*methyl*-^3H (specific activity, 6.7 c per mmole; New England Nuclear Corp., Boston, Mass.) was added and the culture was incubated until lysed. The lysates had plaque titers of 10^{10} to 5×10^{10} per ml. To the lysate were added 5 μg of pancreatic deoxyribonuclease per ml (Worthington Biochemical Co., Freehold, N.J.) and 100 μg of lysozyme per ml (Sigma Chemical Co.). After 1 to 2 hr at room temperature, bacterial debris was removed by low-speed centrifugation. The lysate was then centrifuged for 90 min at 19,000 rev/min in the SS-34 rotor in a Sorvall RC-2 centrifuge. The pellet was suspended in 0.2 M NaCl-0.001 M ethylenediaminetetraacetic acid (EDTA), *p*H 8.1. CsCl (Harshaw, optical grade) was added to give a final density of about 1.5. The preparation was then centrifuged for 24 hr at 45,000 rev/min in an SW50 rotor in a Spinco preparative ultracentrifuge. The main phage band was collected through a hole made in the bottom of the tube and dialyzed against two changes of about 1,000 volumes of buffer at 4 C.

DNA was extracted from the phage particles with buffer-saturated phenol (Mallinckrodt, reagent grade), and residual phenol was removed by dialysis as described (8). DNA concentrations were determined by reading the absorbancy at 260 nm, taking 1 unit to correspond to 50 μg.

Preparation of vegetative phage DNA. *B. subtilis* 168 (ϕ105) was grown in 100 ml of T broth with forced aeration at 37 C to a density of 5×10^7 to 10×10^7 bacteria per ml. The prophage was induced with MC as described in the preceding paragraph. Fifty minutes after resuspension in 100 ml of fresh medium, 0.1 mc of thymidine-*methyl*-^3H was added. One minute later, cold thymidine was added to 100 μg per ml. Part of the culture was immediately poured on frozen broth containing 10^{-3} M NaN_3, and the remaining part was similarly treated 9 min later. The bacteria were sedimented, suspended in 0.2 M NaCl-0.001 M EDTA (*p*H 8.1), and lysed with lysozyme and sodium do-

decyl sulfate; DNA was extracted with phenol as recently described (8).

Unlabeled vegetative phage DNA was prepared from bacteria harvested 55 min after resuspension in fresh broth. DNA concentrations were determined as described by Burton (3) with calf thymus DNA as standard.

Preparation of prophage DNA. *B. subtilis* 168 (ϕ105) was grown in T broth to 5×10^7 to 10×10^7 bacteria per ml and "mock-induced." Forty minutes after resuspension in fresh medium, 0.1 mc of thymidine-*methyl*-^3H was added and 20 min later the culture was chilled and DNA was extracted with phenol.

Sucrose gradient centrifugation. Neutral 5 and 20% sucrose solutions were prepared in 0.01 M tris(hydroxymethyl)aminomethane(Tris)-0.001 M EDTA (*p*H 7.4). Alkaline 15 and 30% solutions were prepared in 0.3 N NaOH-0.001 M EDTA (*p*H 12.2 to 12.4). Linear gradients (5 ml) were prepared and 0.1- to 0.2-ml samples, containing not more than 1 μg of DNA were layered on top of the gradients. The samples were centrifuged for 80 min at 40,000 rev/min at 20 C in an SW50 rotor. After a completed run fractions were collected by puncturing the bottom of the tube. A 1-ml amount of Tris-EDTA buffer was added to each fraction. Radioactivity was determined by mixing a 0.5-ml sample and 0.5 ml of 10% trichloroacetic acid. After about 30 min at 4 C, the samples were filtered through HA filters (Millipore Corp., Bedford, Mass.), the filters washed with cold water and air-dried, and the radioactivity on the filters was determined as described (8). Recovery of input radioactivity was better than 80%. The infectivity of each fraction was determined by mixing a proper dilution of the sample with competent BR16 cells. After 30 min for uptake, infectious centers were assayed.

CsCl density equilibrium centrifugation. To 3 ml of sample was added 3.8 g of CsCl (Harshaw, optical grade). The sample was centrifuged for 24 hr at 45,000 rev/min in the SW50 rotor at 20 C. Fractions were collected, and radioactivity and infectivity were determined as described in the preceding paragraph.

RESULTS

Mature phage DNA. DNA extracted from ϕ105 particles sediments as a single molecular species of about 25×10^6 molecular weight in 1 M NaCl (2). ^{3}H-labeled mature DNA was sedimented in neutral sucrose, and the radioactivity and infectivity of each fraction were determined as described. Both activities were found in a single overlapping peak (Fig. 1). No difference was detected in the buoyant density in CsCl of the radioactive and the infectious material (Fig. 2). The infectivity of mature DNA is thus not associated with a material of a shape, size, or density sufficiently different from the bulk of the DNA to be detected in these experiments.

Prophage DNA. Bacterial and prophage genetic markers can reside on the same DNA molecule extracted from lysogenic bacteria (6, 7). When nonlysogenic bacteria are transformed with limiting concentrations of DNA from lysogenic bacteria for markers adjacent to the prophage site (*phe-1*; IlvAl), 2 to 3% lysogenic clones are found among the transformants compared to <0.1% among *trp-2* transformants (*unpublished data*). When DNA from 168 (ϕ105) was sedimented in neutral sucrose, infectivity was associated with heterogenous material sedimenting considerably faster than mature phage DNA (Fig. 3). All of the above findings are consistent with the suggestion that infectious prophage DNA is physically linked to bacterial DNA.

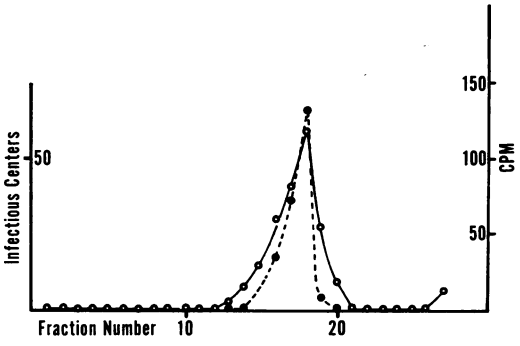

FIG. 1. *^{3}H-labeled DNA from ϕ105 particles was sedimented in neutral sucrose. Twelve-drop fractions were collected and assayed for radioactivity* (○) *and infectivity* (●). *Sedimentation is from right to left in this and the following figures.*

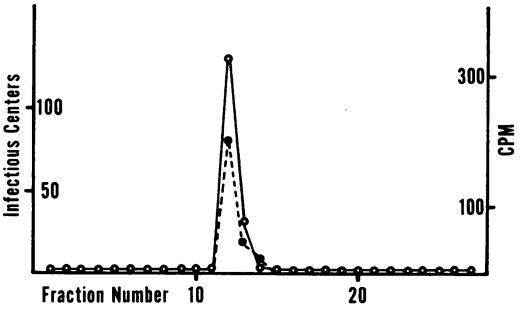

FIG. 2. *^{3}H-labeled DNA from ϕ105 particles was banded in CsCl. Fractions were collected and assayed for radioactivity* (○) *and infectivity* (●).

Vegetative phage DNA. About 40 min after resuspension of an MC-induced culture of 168 (ϕ105) in fresh broth, the rate of DNA synthesis increased; this was accompanied by a parallel increase in the infectivity of DNA extracted from the culture (8). Similar results are observed in lytically infected bacteria (*unpublished data*). The infectivity of vegetative DNA was proportional to DNA concentration, indicating that one DNA

molecule is sufficient to establish an infection (Fig. 4).

The increase in infectivity of DNA from induced bacteria stops well before total phage DNA synthesis stops and before the major fraction of mature phage is formed (8; *unpublished data*). This could be interpreted to mean that infectious vegetative DNA is derived from a pool of limited size which is turned over during the course of the infection.

Unlabeled DNA from MC-induced 168 (ϕ105) was extracted together with ^{3}H-labeled DNA from mature phage. The DNA was sedimented in neutral sucrose. The infectivity was associated with material sedimenting about 13 times faster than radioactive material (Fig. 5). No difference in buoyant density in CsCl was found between the radioactive and infectious material (Fig. 6).

DNA was next extracted from MC-induced 168 (ϕ105) after a ^{3}H-thymidine pulse and chase as described. The samples were sedimented in neutral sucrose and the fractions obtained were assayed for infectivity and radioactivity (Fig. 7a, b). In the pulsed sample, the radioactivity showed a broad distribution with two peaks; the infectivity was associated with the faster sedimenting material.

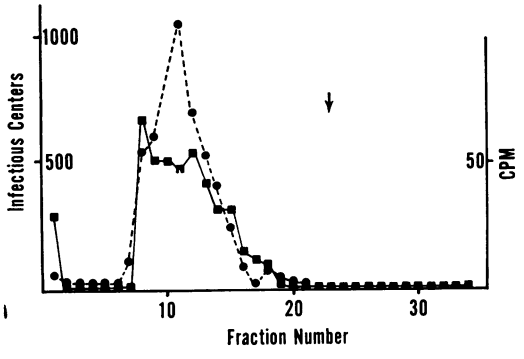

FIG. 3. *DNA was extracted from "mock-induced" 168 (ϕ105) as described and sedimented in neutral sucrose. Nine-drop fractions were collected and assayed for radioactivity (■) and infectivity (●). The arrow marks the position of ^{3}H-labeled mature DNA sedimented in another tube in the same run.*

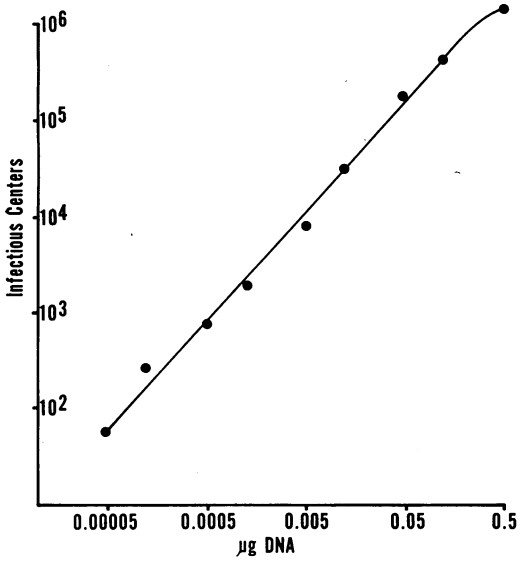

FIG. 4. *Dilutions of DNA extracted from 168 (ϕ105) 55 min after MC induction were assayed for infectivity.*

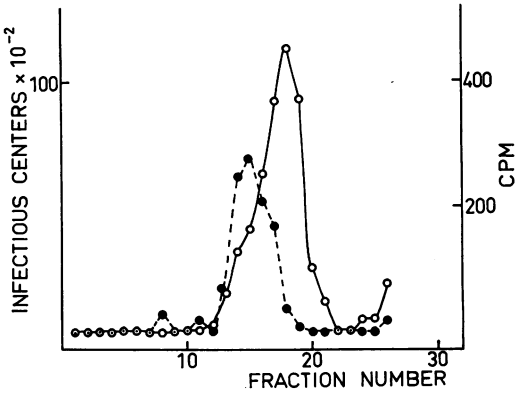

FIG. 5. *DNA extracted from induced 168 (ϕ105) together with ^{3}H-labeled DNA from ϕ105 phage particles was sedimented in neutral sucrose. Twelve-drop fractions were collected and assayed for radioactivity (○) and infectivity (●).*

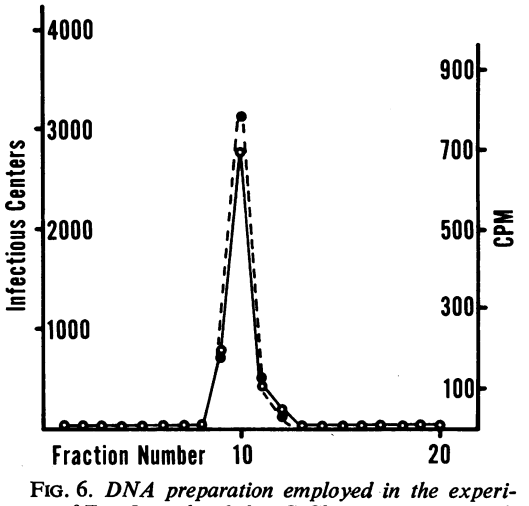

FIG. 6. *DNA preparation employed in the experiment of Fig. 5 was banded in CsCl. Fractions were collected and assayed for radioactivity (○) and infectivity (●).*

In the chased sample, most of the radioactivity was found at a position corresponding to the slower sedimenting labeled material in the pulsed sample. The infectious material sedimented faster than the radioactive material; i.e., labeled material moved from the infectious material during the chase. This is also shown by the fact that the ratio of acid-precipitable radioactivity to infectivity increased with increasing time of incubation of the induced cells with ^{3}H-labeled thymidine.

Pulsed and chased vegetative DNA and ^{3}H-labeled mature DNA were sedimented in alkaline sucrose (Figs. 8a to c). One peak of radioactive material, at identical positions, was found in each sample. We did not observe any fast-sedimenting material in these experiments, indicating that covalently closed circular DNA molecules are not abundant in our preparations.

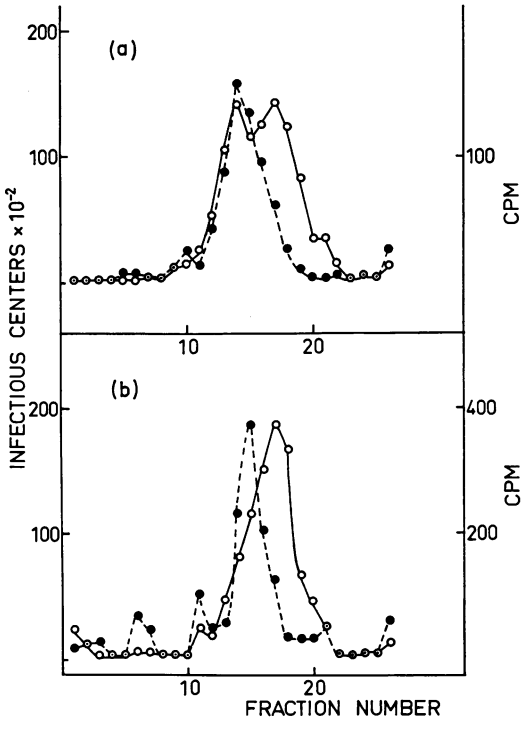

FIG. 7. *Pulsed and chased DNA from MC-induced 168 (ϕ105) was sedimented in neutral sucrose. Twelve-drop fractions were collected and assayed for radioactivity (○) and infectivity (●). (a) Pulsed DNA; (b) chased DNA. The peaks of radioactivity and infectivity were also assayed for rescue of wild-type alleles of ts-marker N9 (see reference 8). In Fig. 7a, the fast peak gave 3.6 × 10^4 and the slow peak gave 3.3 × 10^4 infectious centers in the rescue experiment; in Fig. 7b, the infectivity peak gave 3.3 × 10^4 and the radioactive peak gave 5.6 × 10^4 infectious centers in the rescue experiment.*

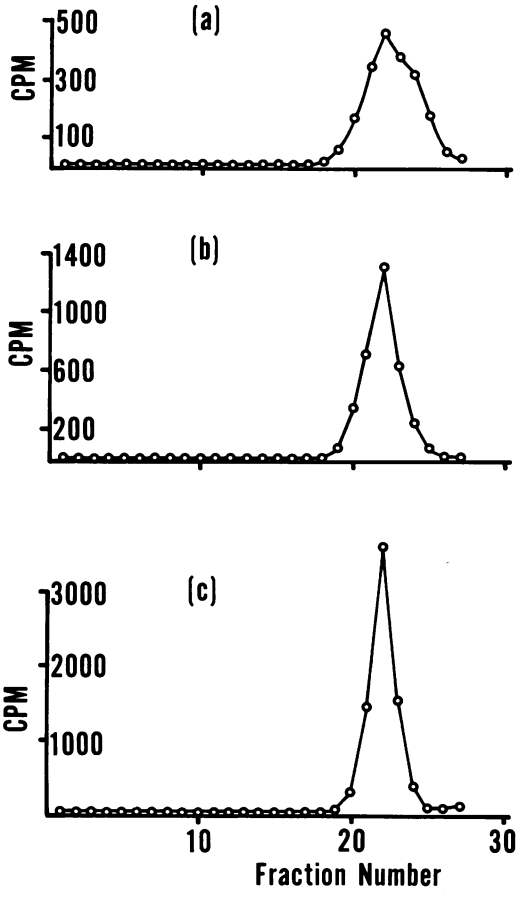

FIG. 8. *(a) Pulsed DNA from induced 168 (ϕ105), (b) chased DNA from induced 168 (ϕ105), and (c) ^{3}H-labeled mature DNA were sedimented in alkaline sucrose, and the total radioactivity of each sample was determined.*

DISCUSSION

After phage DNA is taken up by competent *B. subtilis*, it is rapidly degraded by bacterial nucleases (4, 8). We have suggested that the infectivity of ϕ105 DNA is dependent on its ability to escape degradation by being removed from an internal binding site in the competent cell (8). Removal from this binding site was thought to occur mainly by recombination (prophage integration or marker rescue) or by replication, and the efficiency of removal from the binding site was suggested to depend on the structure of the DNA.

The infectivity of mature ϕ105 DNA was considered to depend on some rare event, which allows the DNA to escape degradation and initiate a productive infection, rather than on the pres-

ence of some minor species of DNA structurally different from the bulk of the DNA. The present experiments have failed to demonstrate any difference between infectious mature DNA and the bulk of mature DNA. This supports, but does not prove, our previous suggestion.

Prophage DNA is thought to owe its infectivity to bacterial DNA covalently linked to prophage DNA. In accordance with this suggestion, infectious prophage DNA sediments as a heterogenous species and considerably faster than mature DNA.

Infectious vegetative DNA sediments 1.2 to 1.5 times faster than mature DNA in neutral sucrose. It is converted to a poorly infectious, more slowly sedimenting form during the infection. The infectious vegetative DNA most likely is a replicative intermediate of ϕ105 DNA. The distribution of radioactivity of pulsed DNA in alkaline sucrose indicates that this intermediate contains single strands both shorter and longer than those [of homogenous size (2)] found in mature DNA. The data do not permit any conclusions as to the exact structure of infectious vegetative DNA; it might be of a type similar to that suggested for replicating coliphage λ DNA by Kiger and Sinsheimer (5).

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