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Isolation and Characterization of Transducing Bacteriophage BP1 for *Bacterium anitratum* (*Achromobacter* sp.)

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A small transducing phage has been isolated against a strain of *Bacterium anitratum*. The particle has a head dimension of 450 A and a tail approximately 200 A long. The latent period is 16 min and the average burst size is 98. The intact particle has an absorption maximum and minimum at 260 and 237 mμ, respectively. The sedimentation coefficient (S_{20}) is 460. The phage contains double-stranded DNA with an $S°_{20,w}$ of 32.8. Moleculcar weight estimates of the deoxyribonucleic acid ranged from 2.33×10^7 to 2.66×10^7 based on sedimentation velocity studies. The percentage guanine plus cytosine compositions of the deoxyribonucleic acid, determined by melting temperature and cesium chloride equilibrium centrifugation, were 40.7 and 42.0, respectively.

This paper concerns studies of a transducing bacteriophage isolated against an organism whose nomenclature is yet to be commonly agreed upon—as the title of this paper suggests. The host bacterium has been known by a variety of names since its initial isolation and description by Schaub and Hauber (23). The following names have appeared most recently: *Acinetobacter anitratus*, *Achromobacter anitratus*, *Herellea vaginicola*, and *Moraxella glucidolytica*. The nomenclature and taxonomy of this and related bacteria have been discussed at length by several authors (3, 22); however, a final decision concerning the nomenclature of this organism is yet to be reached. For operational purposes, the name *Bacterium anitratum* will be used for the host bacterium in this study. The organism is presently listed as *B. anitratum* (strain 15150) by the American Type Culture Collection.

We have examined the genetic relatedness of this organism to other gram-negative diplococci (and cocco-bacilli) of various genera (*Mima*, *Herellea*, *Moraxella*, and *Neisseria*) by deoxyribonucleic acid (DNA) homology studies. Distinct groups and identities have been found among these bacteria by use of DNA-DNA hybridization techniques. Because of these results, it seemed desirable to begin detailed genetic studies on this group of bacteria. Streptomycin resistance has been the only marker transferred between certain members of these bacteria by transformation techniques (5, 11).

To facilitate a detailed study of the genome of these bacteria, we isolated a phage capable of restoring prototrophy to several amino acid auxotrophs derived from the parent bacterium. The details of these experiments will be the subject of a separate report. We present here the characteristics of this bacteriophage.

MATERIALS AND METHODS

Organism and growth. B. anitratum ATCC 15150 was grown routinely in Trypticase Soy Broth (BBL) at 37 C. Phage assays in Trypticase Soy Agar (BBL) were carried out by use of the agar overlay technique (2). The top and bottom layers contained 0.75 and 1.8% agar, respectively.

Phage isolation. Two techniques were used to isolate the phage. In one method, 2 liters of raw sewage freed from particulate matter (45 min at $590 \times g$) were centrifuged for 60 min at $105{,}000 \times g$. The supernatant fluid was decanted, and the pellets were eluted with broth overnight at 4 C. In the second procedure, the particulate-free sewage was filtered through a series of membrane filters (5, 0.8, 0.45, and 0.01-μ pore size; Millipore Corp., Bedford, Mass.). Phage was then eluted as described above from the 0.01-μ filter. In both cases, 1 ml of eluate and 1 ml of log-phase cells (2×10^8 to 5×10^8 bacteria/ml) were added to 30 ml of broth. After overnight incubation, bacteria were removed by low-speed centrifugation, and 10-ml portions of the supernatant

fluid were filtered through sterile 0.45-μ filters. Serial dilutions (10^{-1} to 10^{-5}) of the sterile filtrates were plated and incubated overnight. Single plaques were cored and replated five times. Sterile stocks were then prepared and stored at 4 or -60 C with titers ranging from 10^{10} to 10^{11} plaque-forming units (PFU) per ml.

Electron microscopy. Phage lysates were purified by two cycles of low-and high-speed centrifugation (20 min at 4,340 $\times$ *g*, 180 min at 78,410 $\times$ *g*). The pellets obtained after high-speed centrifugation were eluted overnight with 0.02 M tris(hydroxymethyl)-aminomethane (Tris), *p*H 7.3, at 4 C. The concentrated phage (10^{11} to 10^{12} PFU/ml) was then layered (0.5 ml) on top of a CsCl gradient consisting of 2 ml at 1.7 g/cc and 2 ml at 1.4 g/cc and was centrifuged for 90 min at 25,000 rev/min in the SW39 rotor with a Spinco L ultracentrifuge. The pure phage band was removed and dialyzed at 4 C against 2% ammonium acetate. The phage was negatively stained with phosphotungstic acid, *p*H 7.2, by use of a pseudo-replica technique or by layering a droplet of the phage-stain mixture on Formvar-carbon coated grids. Micrographs were taken on an RCA EMU-3G electron microscope at a magnification of 75,000 times.

One-step growth experiment. The length of the latent period and the average burst size were determined by one-step growth techniques, as described by Adams (2).

Thermal inactivation. Phage stocks maintained in Trypticase Soy Broth were diluted 10^2- to 10^3-fold in 0.2 M potassium phosphate buffer, *p*H 7.0. The experiment was started by adding 0.50 ml of the diluted phage to 49.5 ml of buffer previously equilibrated to 40 C. The initial phage concentration at time-zero was 3.5 $\times$ 10^5 PFU/ml. A similar flask held at room temperature served as a control. Suitable samples, removed at the time intervals indicated, were immediately cooled in an ice bath. Samples were plated in triplicate.

CsCl buoyant density of phage. Phage lysates were purified by three cycles of low-speed (20 min at 12,000 $\times$ *g*) and high-speed (90 min at 105,000 $\times$ *g*) centrifugation; pellets were resuspended in 0.015 M Tris, *p*H 8.0. The samples of phage, varying in titer from 10^{10} to 5 $\times$ 10^{12} PFU/ml, were adjusted to an initial density of 1.5 g/cc with CsCl. The suspensions were centrifuged for 17 to 22 hr at 22,500 rev/min in the Spinco L (SW39 rotor) ultracentrifuge. Fractions were collected by puncturing the tube and analyzed for infectivity by the agar layer method. The density of cesium chloride in the fractions (seven drops each) was determined from measurements of the refractive index, as described by Ifft et al. (17). A Bausch & Lomb refractometer (abbe-3L) was used to determine the refractive index.

Purification of phage and bacterial DNA. One liter of crude phage lysate (3.5 $\times$ 10^{10} PFU/ml) was centrifuged for 10 min at 10,000 $\times$ *g* to remove cell debris and intact cells. The supernatant fluid was carefully decanted and centrifuged for 60 min at 71,000 $\times$ *g* by use of the 30 rotor in a Spinco L265B ultracentrifuge. The supernatant fluid was discarded, and the pellet was resuspended in 0.85% NaCl, 9.5 $\times$ 10^{-3} M potassium phosphate (*p*H 7.2), and 5 $\times$ 10^{-3} M $MgSO_4$. Deoxyribonuclease (Calbiochem, Los Angeles, Calif.) and ribonuclease (five times crystallized, Sigma Chemical Co., St. Louis, Mo.) were added at concentrations of 40 and 5 μg/ml, respectively, and the mixture was incubated for 60 min at room temperature. Solid CsCl (Varlacoid Chemical Co.) was then added to a density of 1.5 g/cc, and the preparation was centrifuged for 180 min at 35,000 rev/min in an SW65 rotor in the Spinco L265B ultracentrifuge. The pure phage band was removed and dialyzed for 12 hr at 4 C against 100 volumes of buffer (NaCl-$KHPO_4$, *p*H 7.2) with four buffer changes. The pure phage was stored in this same buffer at 4 C over $CHCl_3$. The titer was approximately 10^{13} PFU/ml. DNA was extracted from the pure phage according to the procedure of Abelson and Thomas (1). The pure DNA was stored over $CHCl_3$. DNA concentrations were based upon absorbance at 260 mμ with an extinction coefficient of 20 cm^2/mg. The diphenylamine procedure of Burton (10) for DNA estimation was also used.

Bacterial DNA was isolated according to the technique of Marmur (19).

Ultraviolet spectrum of phage and phage DNA. Pure phage was diluted in NaCl-$KHPO_4$-$MgSO_4$ buffer to 4 $\times$ 10^{11} PFU/ml. Phage DNA was diluted in 1 $\times$ SSC (0.15 M NaCl plus 0.015 M sodium citrate). The absorption spectra were determined by use of a model 2000 recording spectrophotometer (Gilford Instruments, Oberlin, Ohio).

Sedimentation velocity studies of phage DNA and phage. In these experiments, sedimentation measurements were made at 20 C in a Spinco E analytical ultracentrifuge with ultraviolet light at 265 mμ to follow the boundary by use of Kodak commercial film.

Phage DNA was analyzed at 42,040 rev/min with photographs taken at 8-min intervals. A 12-mm Kel-F centerpiece was used in these studies. In some instances, the DNA solution was slowly added to a partially assembled cell by use of a plastic pipette with a 1.5-mm bore. In other experiments, an appropriate amount of stock DNA was added as described, and the remainder of solvent (1 $\times$ SSC) was added through the filling hole after cell assembly. DNA concentrations ranged from 17 to 112 μg/ml. The absorption photographs were scanned with a Joyce-Loebl microdensitometer. The DNA sedimentation constant was corrected to $S^\circ_{20,w}$ according to the equation of Svedberg and Peterson (26). The partial specific volume for sodium-DNA was taken to be 0.556 (14, 25).

Sedimentation analysis of pure phage was conducted at 15,220 rev/min in 0.15 M NaCl-0.02 M $KHPO_4$ (*p*H 7.2) and 5 $\times$ 10^{-3} M $MgSO_4$. Photographs were taken at 2-min intervals. The phage concentrations varied from an A_{260} value 0.15 to 11.0. To calculate the $S^\circ_{20,w}$, the partial specific volume value for *Escherichia coli* phage T-7 was used (0.65). A 12-mm aluminum centerpiece was used in this study.

Thermal denaturation of phage DNA and bacterial DNA. The melting temperature (T_m) of the nucleic acid was determined from the denaturation profile ob-

tained from experiments carried out with a Gilford 2000 spectrophotometer equipped with a thermister probe for direct temperature recording. The solvent used was 1 × SSC. The equation of Marmur and Doty (20) was used to calculate the GC base composition of the DNAs from the T_m.

Equilibrium centrifugation of phage DNA in CsCl. The procedure of Meselson et al. (21) was used to determine the buoyant density of phage DNA in a cesium chloride gradient. *Clostridium perfringens DNA* (ρ = 1.691 g/cc) and *Micrococcus lysodeikticus DNA* (ρ = 1.731 g/cc) were included as internal markers. DNA in 0.05 M Tris (*p*H 8.5) and 10^{-3} M ethylenediaminetetraacetate was brought to an initial density of 1.72 g/cc by the addition of solid cesium chloride and was centrifuged for 22 hr at 44,770 rev/min at 25 C in a Spinco E analytical ultracentrifuge. Ultraviolet absorption photographs were scanned with a Joyce-Loebl microdensitometer. Calculations of DNA guanine plus cytosine (GC) base composition were based on the method of Schildkraut, Marmur, and Doty (24).

Zone centrifugation of phage DNA in sucrose gradients. ^{32}P-labeled T7 phage DNA was prepared by growing *E. coli* B in the glycerol medium of Fraser and Jerrel (13) with the exception that 0.64 mM potassium phosphate was used instead of the usual phosphate concentration. After reaching a cell density of 10^8 to 2 × 10^8/ml, 2 mc of carrier-free ^{32}P-phosphoric acid (Tracerlab, Waltham, Mass.) was added to a concentration of 5.0 μc/ml. Bacteriophage T7 was added 30 min later at a multiplicity of 0.5. After lysis of the culture, cell debris was removed by a 10-min centrifugation at 6,000 × *g*. The phage was pelleted from the supernatant fluid by centrifugation at 31,000 × *g* for 90 min. The phage pellet was resuspended in T-phage buffer (2) and banded by layering 1 ml onto a CsCl gradient consisting of densities 1.7, 1.5, and 1.3 g/cc (1.3 ml each). This was centrifuged for 60 min at 40,000 rev/min (SW65 rotor) in a Spinco L265B centrifuge. The pure phage band was removed and dialyzed against four changes of T buffer (100 volumes each change). The specific activity of the T7 bacteriophage suspension was 3.55 × 10^5 counts per min per absorbance unit (1.0) at 260 mμ.

Bacteriophage BP1 DNA was labeled with ^{3}H by growing the host cells in a medium composed of: 0.5% sodium succinate, 0.1% sodium acetate, 0.05% yeast extract, 0.05% Casamino Acids, 0.1% NH_4Cl, 0.02% $MgSO_4$, 0.001% CaCl, 0.003% $MnCl_2$, 10^{-5}% $FeSO_4$, and 6.4 × 10^{-4} M potassium phosphate. When the cell density reached 10^8 per ml, 5 mc of ^{3}H-adenine (21 mc/mmole; Tracerlab) was added. The culture was infected immediately with BP1 at a multiplicity of 0.5. Purification of the phage after cell lysis was carried out as described for phage T7. The specific activity of the pure BP1 phage suspension was 3.18 × 10^5 counts per min per absorbance unit (1.0) at 260 mμ. Sedimentation experiments were carried out as soon as possible after phage purification. The procedure of Ableson and Thomas (1) was used to examine the sedimentation of single polynucleotide chains from BP1 and T7 DNA in alkaline sucrose gradients. Linear 5 to 20% sucrose (w/v) gradients were prepared at 20 C in 0.9 M NaCl and 0.1 M NaOH. DNA was released from the pure phage by adjusting a suspension of phage to 0.10 M Na_3PO_4 and allowing the mixture to stand for 10 min at 20 C. A 0.2-ml sample was slowly layered on top of a 4.8-ml gradient by use of a 1-ml plastic pipette (Falcon Plastics, Los Angeles, Calif.). A controlled rate of flow (0.6 ml/min) was maintained by using a model 180 density gradient fractionator (Instrumentation Specialities Co., Inc., Lincoln, Neb.). The tubes were centrifuged for 2.25 hr at 35,000 rev/min at 20 C in a Spinco L265B ultracentrifuge. The rotor was allowed to decelerate at the end of the run without breaking. Each tube was then fractionated with the model 180 density gradient fractionator. Fractions of four drops each were collected in vials containing 10 ml of Bray's solution (9), and radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Phage and plaque morphology. Examination of the electron micrograph (Fig. 1) of BP1 stained with phosphotungstic acid reveals a particle possessing a hexagonal head and short tail. Measurements across the hexagonal faces ranged from 410 to 484 A, with the average being approximately 450 A. The tail length was approximately 200 A. More detail of the head and tail can be seen in the inset (Fig. 1A). The capsomeric arrangement suggests a regular icosahedron structure.

The center of the plaque (2 to 3 mm diameter) formed by BP1 after 24 hr at 37 C frequently was up to 50 microcolonies growing within the circle, which in turn is bounded by a 5-mm halo. BP1 will not form plaques on other related bacterial strains from which other phages have been isolated (4) and described.

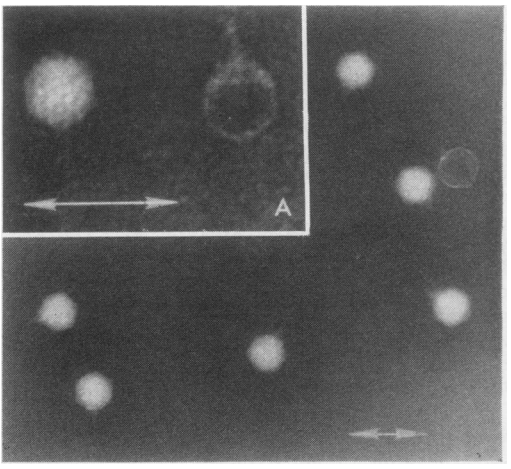

FIG. 1. *Electron micrograph of a negatively stained preparation of BP1. Tail features and capsid structure are more evident in the inset (A). One phage ghost is visible in this photograph. The scale represents 100 mμ.*

One-step growth experiment. The average burst was 98 per cell, and the end of the latent period occurred at 16 min. These data are based upon six experiments.

Thermal stability in phosphate buffer. The thermal inactivation of BP1 in buffer at 40 C was biphasic in nature, as shown in Fig. 2. Each point represents an average of five experiments. The inactivation rates k_1 and k_2 for the primary and secondary rates of inactivation were 0.104 min^{-1} and 0.032 min^{-1}, respectively. The fraction of phage surviving for 60 min under these conditions was 0.07. A similar family of curves was observed even at phage concentrations 10^4-fold greater than those reported here and at higher temperatures with phage suspended in buffer or broth. In the latter case, temperatures between 50 and 60 C must be used for any appreciable inactivation to be observed. The data do not mean that the phage population consists of two fractions differing in thermal stability, since an extrapolation of the curves (to the ordinate) obtained at various temperatures always resulted in different intercepts. In addition, the same type of curve was observed if phage were eluted from a single plaque (representing survivors after 60-min exposure) and were again exposed at various temperatures. Similar thermal inactivation results have been reported by Kaplan (18) with vaccinia virus and by Welker (28) with a *Bacillus stearothermophilus* bacteriophage. Other numerous examples of this type of inactivation kinetics were discussed by Hiatt (16) in a recent review.

Buoyant density in cesium chloride. The buoyant density of the phage in CsCl was calculated to be 1.516 g/cc.

Absorption spectrum of BP1 and BP1 DNA. The intact phage had an absorption maximum at 260 mμ and minimum at 237 mμ. The 260:230 ratio was 1.26 and 260:280 ratio was 1.61. The absorption maximum and minimum for BP1 DNA were 258 and 231 mμ, respectively. The 260:230 ratio was 2.49 and the 260:280 ratio was 1.93.

Sedimentation studies of phage and DNA. The sedimentation coefficient of phage BP1 was determined at 20 C with four concentrations of phage ranging from absorbancy (260 mμ) of 0.15 to 11.0. From a plot of $1/S$ versus A_{260} extrapolated back to the ordinate, a sedimentation coefficient (S_{20}) of 460 was calculated. If one uses the value of 0.65 for the partial specific volume (that of coliphage T7) to calculate an $S^\circ_{20,w}$, then the coefficient will be 480. This probably represents an upper limit, since phage T7 is slightly larger than BP1.

The sedimentation coefficient of BP1 DNA determined in 1 × SSC at 25 C was calculated from plotting the reciprocal of the sedimentation coefficient corrected to water at 20 C versus concentration (Fig. 3). The $S^\circ_{20,w}$ of BP1 DNA was calculated to be 32.8. The molecular weight of the DNA was 2.33 × 10^7 if determined by the equation of Eigner and Doty (12) and 2.66 × 10^7 if calculated by the equation of Studier (25).

Melting temperature of phage and bacterial DNA. Thermal denaturation profiles of BP1 DNA and host-cell DNA were carried out at various DNA concentrations in 1 × SSC. The increase of relative absorbance in a typical experiment is shown in Fig. 4. As might be expected, BP1 DNA had a narrower melting range (approximately 2 to 3 C less) than host-cell DNA. The melting temperatures for BP1 and host DNA

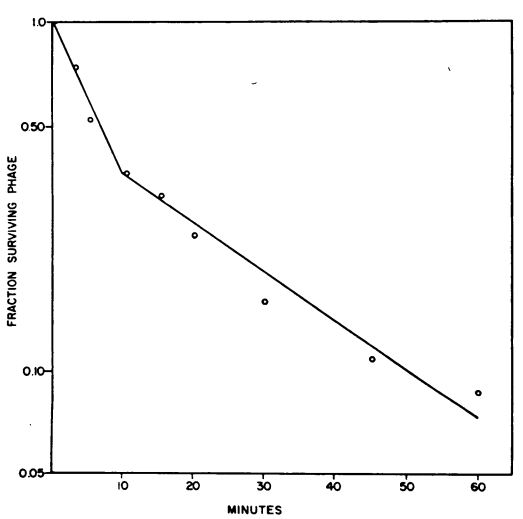

FIG. 2. *Thermal inactivation of BP1 in 0.02 M potassium phosphate, pH 7.0. Inactivation rates k_1 and k_2 were derived from slopes of the upper and lower parts of the curve, respectively. Each point represents an average of five experiments.*

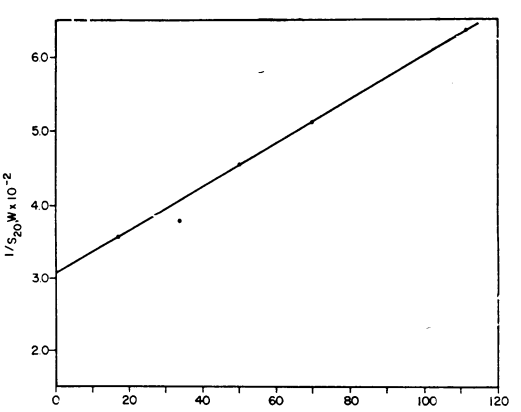

FIG. 3. *Sedimentation coefficient of BP1 DNA as a function of concentration.*

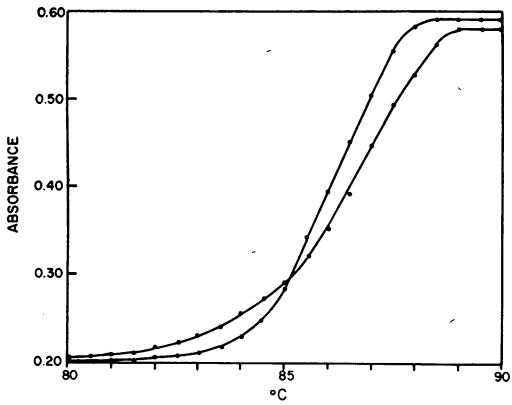

FIG. 4. *Thermal denaturation profile of BP1 DNA (○) and host-cell DNA (●) carried out in 1 × SSC.*

were 86.0 and 86.5 C, respectively. The data suggest that the BP1 nucleic acid is in fact double-stranded DNA. The pure nucleic acid reacted characteristically with diphenylamine. In addition, a hyperchromic increase of absorbance at 260 mμ was observed when 5 μg of deoxyribonuclease/ml was added to BP1 nucleic acid. No effect was seen when ribonuclease was added to 10 μg/ml levels.

Buoyant density of BP1 DNA in CsCl. The density of BP1 DNA after equilibrium centrifugation was calculated to be 1.702 g/cc. This calculation is based upon the position in the gradient of BP1 DNA relative to the DNA of *C. perfringens* and *E. coli* (Fig. 5), which were included as internal standards. DNA of this density has a % GC content of 42.8. This is in close agreement with the composition determined from the melting temperature, suggesting that no unusual bases are present in the DNA, or at least not in large amounts.

BP1 DNA sedimentation in alkaline sucrose gradients. Cosedimentation of alkali-denatured BP1 DNA and T7 DNA is shown in Fig. 6. A single peak represents DNA of both phages. Virtually identical profiles were obtained when each phage DNA preparation was run separately, and in all cases the profiles were highly reproducible. Displacement of the profile to the right (toward the bottom of the tube) by longer periods of centrifugation did not result in the elimination of peak coincidence. Similar profiles and peak coincidence were also seen in experiments where the ratio of BP1 to T7 DNA was varied. The molecular weight of T7 DNA is $2.5 \pm 0.1 \times 10^7$ (12, 25), which means that this is the upper limit for the weight of BP1 DNA.

The properties of intact BP1 and BP1 DNA are summarized in Tables 1 and 2, respectively.

DISCUSSION

The bacteriophage described in this work is morphologically similar to coliphages T3 and T7 and to other small phages having "short tails" (6–8). It is morphologically distinct from the phages previously isolated and described (4) from related strains of bacteria. In comparison with these phages, phage BP1 is markedly less stable to heat and the latent period is 10 to 15 min shorter.

The close agreement in estimating the GC composition of the DNA from melting experiments and cesium chloride equilibrium centrifugation suggests that the DNA base composition is not unusual. However, chemical analysis would be needed to detect small quantities of unusual bases. It is concluded that the phage contains double-stranded DNA, since one observes an increase in absorbance of the nucleic acid with heating, a similar increase with the addition of deoxyribonuclease, a strong diphenylamine reaction, and sedimentation in neutral and alkaline sucrose gradients characteristic of double-stranded DNA. The molecular weight estimates made by sedimentation velocity and sucrose density gradient centrifugation are in close agree-

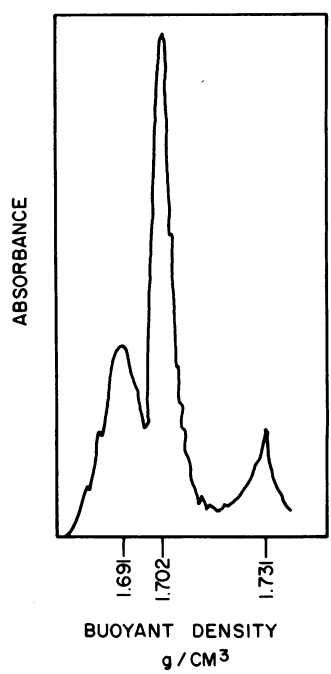

FIG. 5. *Cesium chloride equilibrium centrifugation of BP1 DNA. The DNA of Clostridium perfringens (ρ = 1.691) and Micrococcus lysodeikticus (ρ = 1.731) serve as internal markers.*

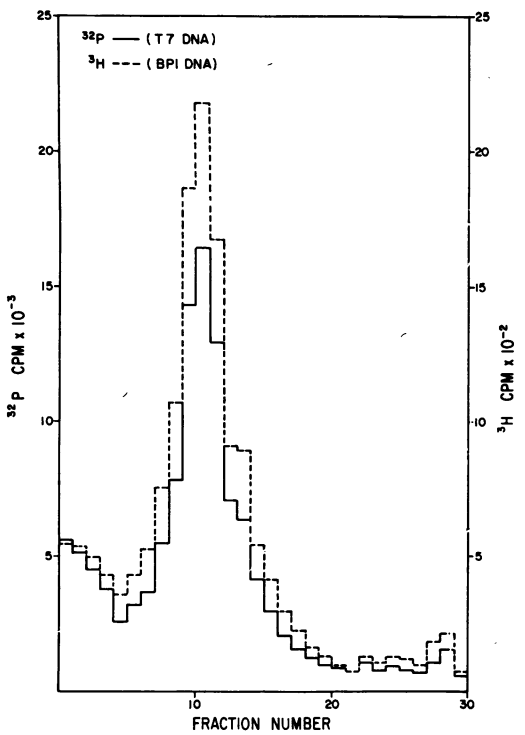

FIG. 6. *Sedimentation profile of alkali-denatured BP1 DNA and T7 DNA in alkaline sucrose gradient. The bottom of the gradient is to the right.*

TABLE 1. *Properties of bacteriophage BP1*

Head size	450 A
Tail length	200 A
Plaque morphology	2–3 mm center, 5-mm halo
Latent period	16 min
Burst size	98
Thermal inactivation (40 C)	Biphasic, k_1 = 0.104 min^{-1} k_2 = 0.032 min^{-1}
Buoyant density (CsCl)	1.516 g/cc
S_{20}	460
λ max	260
λ min	237

ment. Since alkali-denatured BP1 DNA sediments in a single symmetrical peak in alkaline sucrose gradients, we can conclude that no interruptions are present in the single polynucleotide strands. The weight of the BP1 DNA is close to that of coliphage T7 DNA, as might be expected since the two phages have similar dimensions.

Using this phage, we have been able to demonstrate transduction of several amino acid auxotrophs derived from the parent strain. Therefore, it should be possible to construct a genetic map for this group of bacteria. This phage is presently being used to establish linkage of the loci by use of arginine and histidine auxotrophs. The results of these and related transduction studies will be the subject of a separate communication.

TABLE 2. *Properties of bacteriophage BP1 DNA*

Melting temperature	86.0 C
Density in CsCl	1.702 g/cc
% GC	40.7 (T_m); 42.8 (*p*CsCl)
$S^{\circ}_{20,w}$	32.8
Molecular weight	2.33×10^7
λ max	258 mμ
λ min	231 mμ

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