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Comparison of *Bacillus cereus* Bacteriophages CP-51 and CP-53[1]

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Transducing bacteriophages CP-51 and CP-53 were compared. Unlike CP-51, CP-53 appeared to be a lysogenizing phage. CP-51 gave greater frequencies of cotransduction for linked markers than did CP-53. CP-51 was found to be a larger phage which carried more deoxyribonucleic acid (DNA) than CP-53. CP-51 DNA contained about 43% guanine plus cytosine and in addition contained 5-hydroxymethyluracil in place of thymine. CP-53 DNA contained no unusual bases; its guanine plus cytosine content was 37%.

Two generalized transducing bacteriophages have been reported for *Bacillus cereus*. The first, CP-51, was isolated from soil by Thorne who showed that the phage could transduce mutants of *B. cereus* 569 and *B. anthracis* Sterne (26, 27). Further studies by Yelton and Thorne demonstrated that CP-51 could also transduce mutants of *B. cereus* strains 6464, 9139, and T (33). During their studies they discovered a second phage, CP-53, in lysates of CP-51 propagated on strain 6464 and showed that it could transduce auxotrophic mutants of strain 569 to prototrophy. This investigation demonstrates that CP-53 is also a generalized transducing phage for its other host strain, 6464.

In their previous studies Yelton and Thorne showed these phages to be morphologically distinct and to have different sensitivities to ultraviolet (UV) irradiation (33). Studies reported here reveal that the phages also have different transducing abilities. When compared to nonlysogens, cells lysogenized with CP-53 were poor recipients for transduction with the homologous phage; however, transduction by CP-51 was not affected by the presence of CP-53 prophage. CP-51 is shown to be a larger phage than CP-53 and to give greater cotransduction frequencies than CP-53 for linked markers. Physical and chemical methods were used to measure the density, molecular weight, and composition of the phage particles. The phage deoxyribonucleic acid (DNA) species were analyzed to determine their molecular weights and base compositions.

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MATERIALS AND METHODS

Organisms. The organisms used in this investigation were *B. cereus* strains 569 and 6464. The parent strains and mutants derived from them are listed in Table 1. The mutants were isolated by the procedure of Goldberg et al. (9) in which diethyl sulfate is the mutagen, or alternatively, they were derived by treatment of cells with NTG.

Mutagenesis with NTG was accomplished by adding 10 ml of an overnight culture in L broth to 35 ml of fresh L broth containing 25 mg of NTG. The culture was incubated at 37 C on a rotary shaker (250 rev/min) for 6 hr; the cells were pelleted by centrifugation, washed once with PA broth, and resuspended in 20 ml of PA broth. The suspension was incubated on the shaker at 37 C for 48 hr to allow sporulation to occur. The spores were harvested by centrifugation, washed, and heat shocked (65 C, 30 min), and then appropriate dilutions were plated on MlE agar. The plates were incubated at 37 C for 48 hr at which time the minute colonies were picked to selective media to isolate and identify the mutants.

Media and cultural conditions. The media used have all been described in detail previously. NBY medium (26) contained nutrient broth (Difco) and yeast extract (Difco); PA medium (26) contained nutrient broth (Difco) and salts at *p*H 6. L broth (14) consisted of tryptone (Difco), yeast extract (Difco), and NaCl adjusted to *p*H 7; glucose was omitted. The minimal media, Ml0 and MlE, contained a base of salts, glucose, and glutamic acid at *p*H 7. Ml0 (28) also contained alanine, serine, threonine, leucine, isoleucine, and valine. MlE was prepared by adding NBY broth (1% v/v) to the salts-glucose-glutamic acid base. Solid media were prepared by adding 15 g of agar per liter. Difco peptone (1% v/v) was used as a diluent for viable cell counts and phage assays.

Standard saline citrate (SSC) was made by dissolving 8.8 g of NaCl and 4.4 g of sodium citrate in a liter of distilled water. Tris buffer was prepared by dissolving 1.21 g of tris(hydroxymethyl)aminomethane (Tris; Howe and French, Inc.), 5.85 g of NaCl, and

TABLE 1. *Characteristics of the organisms used*[a]

Designation	Tentative genotype	Origin or reference
Bacillus cereus 569	Wild type, prototrophic	NRRL 569
569 UM5	Prototrophic, rough	Spontaneous from 569
569 UM7	*his-1*	DES treatment of 569 UM5
569 UM10	*trp-1*	DES treatment of 569 UM5
569 UM11	*trp-1, str-2*	Spontaneous from 569 UM10
569 UM39	*arg/pro-1*	DES treatment of 569 UM5
569 UM40	*met/cys-5*	DES treatment of 569 UM5
569 UM41	*met-7*	DES treatment of 569 UM5
569 UM42	*met/cys-6*	DES treatment of 569 UM5
569 UM7 (CP-53)	*his-1*, carries CP-53	Infection of 569 UM7
569 UM11 (CP-53)	*trp-1, str-2*, carries CP-53	Infection of 569 UM11
B. cereus 6464 (CP-53)	Wild type, prototrophic, carries CP-53	ATCC 6464
6464 UM4	Prototrophic, cured of CP-53	Altenbern and Stull (1)
6464 UM17 (CP-53)	*met/cys-1*, carries CP-53	DES treatment of ATCC 6464
6464 UM41	*met/cys-3*	DES treatment of 6464 UM4
6464 UM42	*gua-1*	NTG treatment of 6464 UM4
6464 UM45	*met-1*	NTG treatment of 6464 UM4
6464 UM46	*his-1*	NTG treatment of 6464 UM4
6464 UM41 (CP-53)	*met/cys-3*, carries CP-53	Infection of 6464 UM41
6464 UM42 (CP-53)	*gua-1*, carries CP-53	Infection of 6464 UM42

[a] Abbreviations: his, histidine; trp, tryptophan; str, streptomycin resistance; arg, arginine; pro, proline; met, methionine; cys, cysteine; gua, guanine; DES, diethyl sulfate; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Those mutants designated *met/cys* grew on minimal medium containing either methionine or cysteine, and the mutant designated *arg/pro* responded to either proline or arginine.

0.25 g of $MgSO_4 \cdot 7H_2O$ in a liter of distilled water; the *p*H was brought to 7.2 with 1.5 ml of 6 N HCl.

Spores were prepared by inoculating a potato-agar slant (25) and incubating for 5 days at 37 C. Growth from a single slant was collected in 5 ml of sterile distilled water, and the suspension was heated at 65 C for 30 min. This procedure yielded about 10^9 spores per ml.

Propagation and assay of phage. CP-51 was propagated and assayed as described earlier by Thorne (26). CP-53 was propagated and assayed as described previously by Yelton and Thorne (33). CP-51 was stored at 15 C after a 1:10 dilution in NBY broth; CP-53 was stored at 4 C.

Transductions. Recipient cells for transductions were grown by inoculating 25 ml of NBY broth in a 250-ml flask with 5×10^7 spores and incubating for 4 to 5 hr at 37 C on a rotary shaker (250 rev/min). Viable cell counts were made by plating appropriate dilutions on NBY agar. For use in transductions, phage preparations were routinely treated with UV light to inactivate 97% or more of the plaque-forming units (PFU; reference 33). Transductions were performed by adding 0.1 ml of phage suspension and 0.9 ml of cell culture to 18-mm Pyrex tubes and incubating at 37 C for 30 min on a reciprocating shaker in a water bath. After incubation, 0.1-ml samples of the transduction mixture were plated on the appropriate minimal agar plates. Alternatively 1 ml of minimal agar was added to the transduction mixture and the entire volume was overlaid on a minimal agar base. Duplicate samples were plated, and controls to test for spontaneous revertants were always included in each test. Plates were incubated at 37 C and the transductants were scored after 40 hr.

Phage adsorption experiments. The desired numbers of cells and phage particles were mixed in 18-mm tubes and the final volume was brought to 2 ml with NBY broth. After an appropriate incubation time on a reciprocating shaker in a 37 C water bath, each mixture was filtered through an AA membrane (Millipore Corp., Bedford, Mass.) held in a Swinnex apparatus (Millipore Corp.). The filtrate was assayed for phage.

Antiserum. Antisera to phages CP-51 and CP-53 were prepared in rabbits by intravenous injections of phage suspensions having 10^{10} or more PFU/ml (24).

UV irradiation. UV light exposure was carried out with two General Electric germicidal lamps (15 w). A petri dish with 6 ml of the phage suspension was placed 40 cm below the source on a rotating platform (200 rev/min).

DNA extraction. Phage DNA was prepared by a modification of the phenol technique of Mandel and Hershey (17). Purified phage [banded in cesium chloride (CsCl)] was suspended in SSC at *p*H 7 and an equal volume of phenol saturated with SSC at *p*H 8 was added. The solution was gently mixed for 10 min and the phases were separated by centrifugation. The aqueous phase was deproteinized with phenol a second time. After phase separation, phenol was removed from the aqueous phase (10 ml) by dialysis for a total of 24 hr at 4 C against four successive 1-liter volumes of SSC (*p*H 7). The DNA was stored in SSC (*p*H 7) at 4 C. The concentration of DNA was estimated spectro-

photometrically assuming that 1 optical density (OD) unit at 260 nm (1-cm light path) was equal to 50 μg of DNA per ml (32).

Enzymatic hydrolysis of DNA. DNA (1 mg/ml) was hydrolyzed by a two-step enzymatic procedure by using both deoxyribonuclease I and snake venom phosphodiesterase (15). Deoxyribonuclease I at a final concentration of 10 μg/ml was added to the sample of DNA in distilled water, and the mixture was incubated at 37 C for 30 min. It was essential to have the hydrolysate as salt-free as possible to obtain good separation of the nucleotides during chromatography. The *p*H was raised to 8.0 with NaOH after hydrolysis and the solution was heated at 80 C for 20 min to inactivate the enzyme. The precipitate was removed by centrifugation and discarded. The *p*H of the supernatant fluid was raised to 9.3 and 5 μg (final concentration) of phosphodiesterase per ml was added. The mixture was incubated at 37 C, and the *p*H was adjusted to 9.5 at 10-min intervals with NaOH. After 1 hr, the *p*H was stable at 9.5 and the enzyme was inactivated by heating at 80 C for 20 min. The hydrolysate was then chromatographed to separate the nucleotides.

Formic acid hydrolysis of DNA. The method used was that of Wyatt and Cohen (31). Seven hundred micrograms of DNA in 1 ml of distilled water and 0.5 ml of formic acid (88%) were added to a Pyrex tube of 6-mm internal diameter. The tube was sealed in a flame and heated at 175 C for 30 min. After hydrolysis, the tube was opened and the contents were brought to dryness in vacuo at 60 C. The residue was dissolved in 0.1 N HCl and chromatographed to separate the bases.

Chromatography. Separation of nucleotides and bases was obtained by using descending paper chromatography (15). The solvent (30) was isopropanol-12 N HCl-water (65:16:19). The paper was acid-washed Whatman no. 40. The hydrolysates and a mixture containing each of the bases as a control were spotted on the paper, taking care to keep the diameter of the spots small (about 1 cm). The chromatogram was allowed to develop at room temperature for 15 hr at which time it was removed from the tank and air dried. The spots were located by UV light and cut out. The material in each spot was eluted into 0.01 N HCl and examined spectrophotometrically.

Determination of DNA and protein. The DNA content of a sample of phage suspension was determined by the diphenylamine reaction of Burton (4). Calf thymus DNA was used as a standard. Protein was determined by the Lowry assay (16) by using Folin phenol reagent. Bovine serum albumin was used as a standard.

Buoyant density of phage particles. Buoyant densities were determined by isopycnic density gradient centrifugation by using CsCl dissolved in Tris buffer. After equilibrium was reached, 3-drop fractions were collected from the gradient and assayed for PFU. The densities of selected fractions were determined from their refractive indexes. By plotting PFU per milliliter and density against fraction number, it was possible to obtain the buoyant density of the phage particle.

Buoyant density of phage DNA. The procedure described by Mandel et al. (19) was used. *B. subtilis* 168 DNA was included as a marker; its density was assumed to be 1.7034 g/cm^3 (23). Phage DNA (2 μg) and *B. subtilis* DNA (0.5 μg) in 0.38 ml of CsCl solution (density, 1.710 g/cm^3) were centrifuged in the Spinco model E ultracentrifuge at 44,000 rev/min for 24 hr at 25 C. The position of the bands was determined from tracings made by the photoelectric scanning device. The density of the phage DNA was determined from the position of its peak relative to that of the marker (19).

Sedimentation velocities. Sedimentation coefficients were measured at 20 C in a double-sectored, charcoal-filled Epon centerpiece with a 12-mm path length. The Spinco model E analytical ultracentrifuge fitted with UV optics was used. Scans of the sedimenting material were made at precise intervals with the automatic scanning device. The sedimentation coefficients (*S* values) were calculated from the motion of the 50% concentration point in the boundary region. A plot of the log of the position of this point versus time gave a straight line from whose slope an *S* value was calculated. The *S* values obtained were corrected to zero concentration and water at 20 C. DNA was sedimented at about 24,000 rev/min in SSC. Phage particles were sedimented at about 8,000 rev/min in Tris buffer.

Electron micrographs of DNA. DNA was prepared for shadowing by the microversion technique of Lang and Mitani (13). Two-tenths of a microgram of DNA in SSC was added to 0.9 ml of 0.15 M ammonium acetate at *p*H 6.0. Twenty-five μliters of a 30% Formalin solution (Merck & Co., Rahway, N.J.) was added, and finally 0.1 ml of a cytochrome *c* (Sigma Chemical Co., St. Louis, Mo.) solution (13 μg/ml) was added. After gentle mixing by rotation, approximately 3-mm drops of the mixture were placed on a dental wax surface. Surface evaporation was reduced to a minimum by small wells of distilled water surrounding the DNA drops. At various times, freshly prepared C-coated grids were touched to the droplets for 1 sec. The grids with the protein film attached were defatted by touching to 95% ethanol, washed in distilled water, and then dried face down on Whatman no. 1 filter paper. The grids were rotary-shadowed with Pt-C at an incident angle of 7° in a Bendix-Balzars vacuum plant. Contamination of the grids was kept at a minimum by cooling the vacuum evaporator to −150 C with liquid nitrogen during all evaporation procedures. Grids were examined in a Philips EM 200 electron microscope at an accelerating voltage of 60 kv. Exact magnifications were determined by photographing carbon gratings immediately prior to and after examination of the DNA sample. Hysteresis was eliminated by lens normalization, and specimen contamination was minimized by the use of a liquid nitrogen decontamination device at the level of the specimen. The DNA molecules were photographed and contour lengths were measured with a curvimeter.

RESULTS

Transduction of B. cereus 6464 by CP-53. Transducing phage lysates were prepared by UV

induction of a lysogenic culture of strain 6464. When these lysates were used in transduction experiments, the data in Table 2 were obtained. As these data show, the bacteriophage mediated genetic exchange in this strain. Five different auxotrophic mutants were transduced to prototrophy by CP-53 grown on a prototrophic strain of 6464. The specificity of the transduction was demonstrated by using phage grown on 6464 UM41 as a donor. In this case all of the recipients except 6464 UM41 were transduced to prototrophy. Although not shown in the table, treatment with heat or antiserum, which inactivated the PFU, prevented transduction, whereas treatment of the lysates with deoxyribonuclease had no effect on the recovery of transductants.

There were two Met/Cys requiring mutants used in the experiments reported in Table 2, UM41 and UM17. These mutants were independent isolates and hence were probably not identical. Therefore the colonies obtained in the transduction by using UM17 as a recipient and phage grown on UM41 as the donor represent true transductants and not revertants.

Lysogenizing nature of CP-53. Bott and Strauss (3) have reported that carrier-state cultures of SP-10 in *B. subtilis* contain 10^9 or more PFU/ml. In contrast, we found that cultures of *B. cereus* lysogenized with CP-53 contained only 10^4 PFU/ml 10 hr after inoculation. Although low levels of phage were released spontaneously, CP-53 could be induced by UV irradiation of an infected culture (33). Compared to an unirradiated control, the titer of free phage in the irradiated culture increased 10^4-fold or more in 90 min.

The extent to which strain 6464 was infected with CP-53 was determined by two methods. By plating a known number of spores of strain 6464 in a lawn of sensitive indicator, it was possible to demonstrate that at least 95% of the spores carried the phage. Secondly, all of a large number of isolated colonies derived from single spores of 6464 were infected with CP-53. Hence it seems likely that every cell in a culture carries the phage. Bott and Strauss (3) have shown that carrier-state cultures can be cured of phage infection by growth in the presence of phage-specific antiserum. When antiserum-curing experiments were attempted with cultures of 6464 infected with CP-53, we detected no curing after 24 hr of growth (18 hr followed by a 10% transfer into fresh antiserum broth for 6 hr) in broth containing antiserum even though the antiserum neutralized all of the free phage. Control cultures without antiserum contained about 10^3 PFU/ml.

TABLE 2. *Transduction of Bacillus cereus 6464 by bacteriophage CP-53*[a]

Recipient	Colonies per milliliter		
	Without phage	With phage grown on a prototroph	With phage grown on 6464 UM41 (Met/Cys⁻)
6464 UM41 (Met/Cys⁻)	0	330	0
6464 UM42 (Gua⁻)	15	335	340
6464 UM45 (Met⁻)	40	430	470
6464 UM46 (His⁻)	10	170	160
6464 UM17 (Met/Cys⁻)	0	50	30

[a] Transduction mixtures contained about 2×10^8 colony-forming units and an equal number of plaque-forming units. One-tenth milliliter of the transduction mixture was plated in duplicate on MIE plates.

CP-53 did not form plaques in lawns of infected cells. Clear plaque mutants of the phage arose spontaneously and at a low frequency; however, these mutants were not virulent since they did not form plaques on infected cells. Although a chromosomal location for CP-53 has not been demonstrated, on the basis of the above evidence we presume that the phage is capable of establishing lysogeny.

Transduction of cells lysogenized with CP-53. During the study of transduction of *B. cereus* 6464, it was observed that one of the recipients (6464 UM17) was transduced at a lower frequency than the other mutants (Table 2). Further study showed that this mutant did not allow plaque formation by CP-53 and that it could be induced to yield the phage. The other recipients were all sensitive to CP-53. From these observations it seemed as though the presence of CP-53 as prophage might be inhibiting the transduction of 6464 UM17.

To investigate this possibility, mutants of strains 6464 and 569 were lysogenized with CP-53. This was accomplished by plating CP-53 on the mutant, picking a turbid plaque into PA broth at 37 C, and allowing spores to form. The spores were heat-shocked to inactivate free phage, and dilutions were plated to obtain isolated colonies. One such colony was picked and used as inoculum for growing spores again. These spores were heat-shocked and used as the lysogenic stock of that mutant.

Transduction experiments were performed with these lysogens and their nonlysogenic parents by using both CP-51 and CP-53, grown on prototrophs, as donors. The data in Table 3 show that the frequency of transduction obtained with CP-51 was approximately the same for each

TABLE 3. *Effect of CP-53 prophage on transduction of Bacillus cereus mutants*[a]

Recipient	Transduction with CP-51		Transduction with CP-53	
	Transductants/ml	Frequency	Transductants/ml	Frequency
569 UM7	465	1.6×10^{-6}	560	1.8×10^{-6}
569 UM7 (CP-53)	375	1.3×10^{-6}	50	1.7×10^{-7}
569 UM11	380	1.3×10^{-6}	750	2.5×10^{-6}
569 UM11(CP-53)	390	1.3×10^{-6}	40	1.3×10^{-7}
6464 UM41	240	8.0×10^{-7}	560	1.4×10^{-6}
6464 UM41(CP-53)	210	7.0×10^{-7}	80	1.8×10^{-7}
6464 UM42	345	1.2×10^{-6}	500	1.2×10^{-6}
6464 UM42(CP-53)	380	1.3×10^{-6}	70	1.7×10^{-7}

[a] The multiplicity of infection was about 1 in all cases. One-tenth milliliter of the transduction mixture was plated in duplicate on MlE, except when CP-53 was used to transduce a lysogenic recipient. In that case, 1 ml of minimal agar was added to the transduction mixture and the entire volume was overlaid on the MlE agar base; duplicate tubes were plated. When this procedure was used with the nonlysogenic recipient, no effect on the yield of transductants was observed.

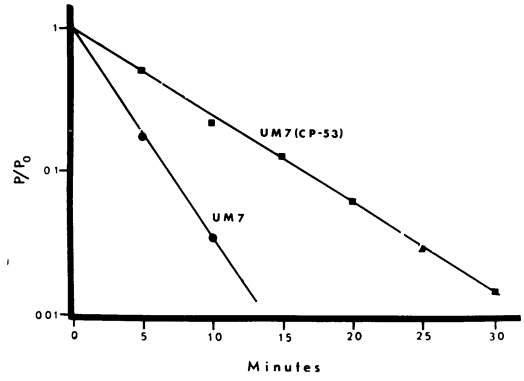

FIG. 1. *Adsorption of CP-53 to lysogenic and nonlysogenic cells of strain 569. Each adsorption mixture contained about 2 × 10⁸ colony-forming units in a total volume of 2 ml, and the multiplicity of infection was about 1. P/P_0 is the fraction of phage remaining unadsorbed.*

recipient. With nonlysogenic recipients, CP-53 gave frequencies equivalent to those obtained with CP-51; however lower frequencies were obtained with CP-53 when lysogenic recipients were used. These data demonstrate that the presence of the prophage interfered with transduction by CP-53; although transduction was not prevented in lysogenic recipients, the frequency was reduced about 10-fold. Since the frequencies of transduction by CP-51 were about the same for lysogenic as for nonlysogenic recipients, integration of DNA transduced by CP-51 was not impaired by the presence of CP-53 prophage.

Adsorption of CP-53 to lysogens. There are examples reported in the literature of lysogenizing phages which alter the surface of the lysogen such that it can no longer adsorb homologous bacteriophage (2, 11). Such bacteriophages are referred to as converting phages. Since CP-53 appears to be a lysogenizing phage, it seemed possible that it might also be a converting phage. If lysogenization with CP-53 reduced the cell's ability to adsorb the phage, it would be possible to explain the low level of transduction obtained when CP-53 was used to transduce lysogens.

The results presented in Fig. 1 show that CP-53 adsorbed more slowly to a lysogen than to a nonlysogen. However, after 30 min, which was the usual period of time for incubating transduction mixtures, more than 98% of the phage had adsorbed to the lysogen.

Cotransduction of linked markers. Three sets of linked markers are known for *B. cereus* 569. They are 569 *his-1* linked to 569 *met/cys-6*, 569 *his-1* to 569 *met/cys-5*, and 569 *arg/pro-1* to 569 *met-7* (T. Bisson, *unpublished data*). Since no double mutants were available, it was not possible to simply select for double transductants on minimal medium. Rather, cells carrying one of the mutations were used as recipients, and transducing phage was grown on cells bearing another of the mutations. The transduction mixtures were plated on minimal medium supplemented with the amino acid required by the donor. The transductant colonies were then picked to selective media to determine whether the requirement of the recipient had been replaced by the requirement of the donor. Such a result would indicate linkage of the markers being examined.

With 569 UM42 (*met/cys-6*) as the recipient and CP-51 grown on 569 UM7 (*his-1*) as the donor, an investigation of the cotransducibility of these loci was undertaken. To demonstrate that the Met^+ transductants did not spontaneously acquire a His^- characteristic, CP-51 grown on wild-type 569 (prototrophic) was also used as a donor. One-tenth milliliter of the transduction mixture was spread in duplicate on M10 agar plates containing 50 μg of L-histidine per ml. The plates were also spread with 0.1 ml of undiluted CP-51 specific antiserum to prevent killing of the transductants. The phage was not irradiated prior to use; it was feared that linkages might be lost during repair of the thymine dimers formed by irradiation. After incubation at 37 C for 3 days, the transductants were picked to M10, M10 supplemented with histidine, and NBY agar plates each spread with 0.1 ml of antiserum to CP-51. The plates were incubated for 2 days at 37 C at which time they were scored for double transductants (those colonies which grew on M10 plus histidine but not on M10 agar plates). With CP-51 grown on 569 UM7, the results showed the markers to be cotransduced at a frequency of 61.5% (123 Met/Cys^+ His^- transductants out of 200 Met/Cys^+ transductants tested). As would be expected, the control with phage grown on a prototroph gave no His^- transductants.

To prove that the *his-1* locus and the *met/cys-6* locus were cotransduced, CP-51 was grown on a suspected double transductant (His^-Met^+) and the phage preparation was used to transduce 569 UM7 (*his-1*) and 569 UM11 (*trp-1*). As a control, CP-51, grown on a prototroph, was used to transduce the same cultures of 569 UM7 and 569 UM11. Both recipients were transducible with phage grown on the prototroph, but the phage grown on the His^- transductant could only transduce the Trp^- mutant to prototrophy. Therefore, the histidine mutation in the transductant was probably identical to that in the original donor.

Comparison of the ability of CP-51 and CP-53 to cotransduce linked markers. The demonstration that cotransduction of linked genes could be observed when singly marked recipients were used made it possible to compare the abilities of CP-51 and CP-53 to cotransduce pairs of genetic markers. Two sets of linked markers were used (569 *his-1* and 569 *met/cys-5*, 569 *arg/pro-1* and 569 *met-7*), and each mutant was used as a donor and a recipient with each phage. The data obtained from these experiments are presented in Table 4. They show that CP-51 gave greater frequencies of cotransduction than did CP-53.

Physical and chemical characteristics of CP-51 and CP-53 phage particles. The bacteriophage particles were analyzed to determine their chemical composition, buoyant density, and molecular weight. The size of the phage particles was determined from electron micrographs. The data from these experiments are given in Table 5 and show CP-51 to be a larger phage with a greater density and molecular weight than CP-53.

Thermal denaturation of bacteriophage DNA. Phage DNA dissolved in SSC was thermally denatured by the procedure described by Mandel and Marmur (18). *B. subtilis* 168 DNA was melted simultaneously as a control and gave a T_m of 87.5 C. De Ley reported a value of 87.6 C for this DNA (6). Melting profiles for the DNA species are shown in Fig. 2. Both phage DNA species gave sharp transitions with a hyperchromic increase of about 39%. CP-51 DNA melted at 77.7 C which corresponds to a guanine plus cytosine (GC) content of approximately 20%, and CP-53 DNA melted at 84.1 C giving a GC content of about 36%. The low T_m value for CP-51 DNA suggested the possibility of an unusual base in the DNA of this phage.

Buoyant density of phage DNA. It has been shown that the buoyant density of a DNA molecule can be correlated with its GC content. To further substantiate the results obtained by thermal denaturation studies, the buoyant density of each phage DNA was determined, and the corresponding GC content of the phage DNA was calculated (19). Figures 3 and 4 show examples of scans made of CP-51 and CP-53 DNA, respectively. Based on the average of two or more determinations by this procedure, CP-51 DNA had a density of 1.7457 g/cc implying a GC content of 87.5%, and CP-53 DNA had a density of 1.6973 g/cc which corresponds to a GC content of 38.2%. The high value obtained with CP-51 DNA, which conflicted with the value obtained by melting experiments, indicated the presence of an unusual base. The value of 38% GC obtained with CP-53 DNA was in close agreement with the 36% GC value obtained by melting. Hence, it is unlikely that CP-53 DNA contains any unusual bases.

Base composition of phage DNA. To determine the bases present in the phage DNA species, they were subjected to both formic acid and enzymatic hydrolysis. The hydrolysates were chromatographed to separate the individual bases, and the bases were identified from their R_F values and their spectral properties. Pure samples of various bases were used as controls and the values obtained were compared to literature values (Table 6). Based on these data, the DNA of CP-51 contained adenine, guanine, cytosine,

TABLE 4. *Cotransduction of linked markers by bacteriophages CP-51 and CP-53*[a]

Donor	Recipient	Per cent cotransduction with	
		CP-51	CP-53
569 UM7 (*his-1*)	569 UM40 (*met/cys-6*)	79.0 (158/200)[b]	30.5 (40/131)
569 UM40	569 UM7	85.5 (77/90)	32.2 (54/168)
569 UM39 (*arg/pro-1*)	569 UM41 (*met-7*)	20.5 (41/200)	0 (0/200)
569 UM41	569 UM39	17.0 (51/300)	0 (0/300)

[a] The multiplicity of infection was about 1 in each case. Transductants were selected on M10 agar plates supplemented with the amino acid required by the donor. The transductant phenotype was determined by picking to M10, M10 supplemented with the amino acid required by the donor, and NBY agar plates. Those transductants which did not grow on M10 but did grow on supplemented M10 and NBY were scored as doubles.

[b] The numerator represents the number of doubles; the denominator represents the number of transductants that were tested.

TABLE 5. *Properties of bacteriophages CP-51 and CP-53 and their deoxyribonucleic acids (DNA)*

Phage or DNA	Size in nanometers		Weight per cent DNA	Per cent guanine-cytosine	Molecular weight (daltons)	Density (g/cm³)	Unusual base
	Head	Tail					
CP-51 phage	89.7	20.0 × 159.9	56.7		102×10^6[a]	1.527 ± 0.004	
CP-53 phage	66.2	12.5 × 275.9	51.9		28.8×10^6[a]	1.486 ± 0.004	
CP-51 DNA				43	57.9×10^6[b]	1.7457 ± 0.0002	HMU[c]
CP-53 DNA				37	16.5×10^6[d]	1.6973 ± 0.0006	None

[a] Calculated from the $S_{20,w}$ value by the equation of Pitout et al. (20).

[b] Average of molecular weights obtained from *S* value, band-width, and per cent of phage that was DNA.

[c] 5-Hydroxymethyluracil.

[d] Average of molecular weights obtained from band-width, contour length, and per cent of phage that was DNA.

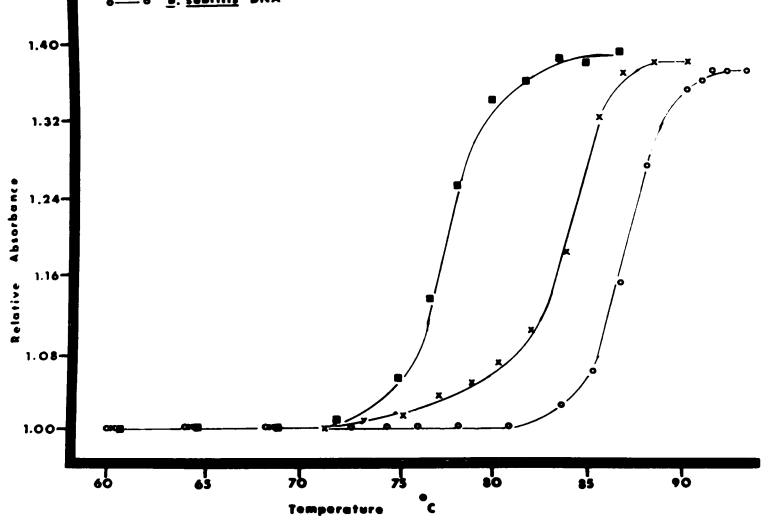

FIG. 2. *Melting profiles of DNA preparations. DNA (20 μg/ml) was dissolved in saline citrate, pH 7.0.*

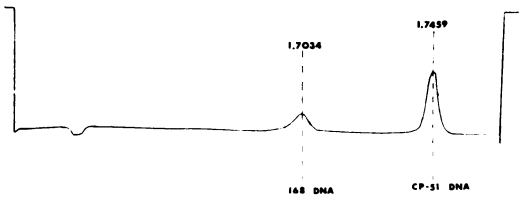

FIG. 3. *Buoyant density of CP-51 DNA. CP-51 DNA (2 μg) and B. subtilis DNA (0.5 μg) as a marker DNA of known density were centrifuged in CsCl at 44,000 rev/min for 24 hr at 25 C. A tracing of the cell made by the photoelectric scanning device is shown.*

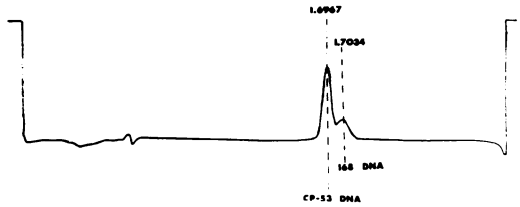

FIG. 4. *Buoyant density of CP-53 DNA. The conditions were the same as those described in the legend to Fig. 3.*

TABLE 6. *Identification of the bases present in CP-51 and CP-53 deoxyribonucleic acids (DNA)*[a]

Source	R_F	250/260	280/260	Identity of base
Guanine standard	0.25	1.29	0.81	
Spot no. 1 CP-51	0.25	1.24	0.76	Guanine
Spot no. 1 CP-53	0.28	1.36	0.80	Guanine
Literature	0.25	1.37	0.84	
Adenine standard	0.40	0.78	0.37	
Spot no. 2 CP-51	0.39	0.77	0.39	Adenine
Spot no. 2 CP-53	0.40	0.78	0.37	Adenine
Literature	0.36	0.76	0.38	
Cytosine standard	0.52	0.46	1.46	
Spot no. 3 CP-51	0.47	0.41	1.52	Cytosine
Spot no. 3 CP-53	0.49	0.48	1.53	Cytosine
Literature	0.47	0.48	1.53	
HMU standard	0.68	0.78	0.33	
Spot no. 4 CP-51	0.69	0.78	0.33	HMU[b]
Literature	0.65	0.77	0.32	
Thymine standard	0.81	0.65	0.54	
Spot no. 4 CP-53	0.78	0.71	0.55	Thymine
Literature	0.77	0.67	0.53	

[a] Literature values for the R_F were obtained from *Methods in Virology* (15); the other literature values were obtained from the *Handbook of Biochemistry* (22). The experimental data were obtained by formic acid hydrolysis of the DNA species. As standards, pure samples of the bases obtained from Sigma Chemical Company were used. The bases were eluted into 0.01 N HCl after chromatographic separation and used for spectrophotometric analysis. The R_F values were obtained by paper chromatography by using isopropanol-HCl-water (65:16:19) as the solvent system.

[b] 5-Hydroxymethyluracil.

TABLE 7. *Base composition of the bacteriophage deoxyribonucleic acids*[a]

Phage	Base	Mole per cent as determined by: Enzymic hydrolysis	Mole per cent as determined by: Formic acid hydrolysis
CP-51	Adenine	27.9	29.1
	Guanine	21.9	20.5
	Cytosine	22.0	22.1
	5-HMU[b]	28.0	29.1[c]
CP-53	Adenine	29.5	31.3
	Guanine	18.3	18.3
	Cytosine	19.9	18.2
	Thymine	32.2	31.8

[a] The base composition was determined by eluting spots from a paper chromatogram into 0.01 N HCl and determining the optical density (OD) of the eluate at its lambda maximum. The mole composition was calculated from the formula: micromoles/liter = OD at λ max/extinction coefficient at λ max.

[b] 5-Hydroxymethyluracil.

[c] Since hydrolysis with formic acid partially degrades 5-HMU, it was assumed that the amount of HMU in the DNA hydrolysate would equal the amount of adenine present.

and 5-hydroxymethyluracil (HMU). CP-53 DNA was shown to contain adenine, guanine, cytosine, and thymine. The demonstration of 5-HMU in the DNA of CP-51 was consistent with the low T_m and high buoyant density obtained with this DNA.

The base compositions of the phage nucleic acids were then calculated from the amount of each base present in the hydrolysates. The results obtained are found in Table 7. With CP-51 DNA, a GC content of 43.9% was obtained by enzymatic hydrolysis; hydrolysis with formic acid gave a value of 42.6% GC. Enzymatic hydrolysis of CP-53 DNA gave a GC content of 38.2%, whereas formic acid hydrolysis gave a value of 36.5% GC.

DNA molecular weight from band-width at equilibrium. When DNA is banded in a CsCl gradient, the width of the band is determined by diffusion of the molecules away from their equilibrium position. Since the amount of diffusion varies inversely as the weight of the molecule, a measurement of the band's width is an indirect measure of the molecule's weight (5). CP-51 and CP-53 DNA species were banded in CsCl solution in the model E analytical ultracentrifuge at 20 C, and tracings were made of the DNA bands with an automatic scanning device (Table 8). When equilibrium had been reached, the width

TABLE 8. *Molecular weights of CP-51 and CP-53 deoxyribonucleic acids (DNA) as determined by the band-width method*[a]

DNA	σ (cm)	ω (radians/sec)	ρ_0 (g/cm^3)	r (cm)	β	Molecular weight (daltons)
CP-51	6.45×10^{-3}	4613	1.7067	7.00	1.199×10^9	54.3×10^6
CP-53	1.18×10^{-2}	4625	1.6855	6.76	1.190×10^9	17.0×10^6

[a] The DNA species were banded in CsCl solution in the model E analytical ultracentrifuge at 20 C. Tracings were made of the DNA bands by using ultraviolet light (265 nm) and the automatic scanning device. The molecular weight was calculated by using the equation (5): $MW = RT\rho_0\beta/\sigma^2\omega^4r^2$.

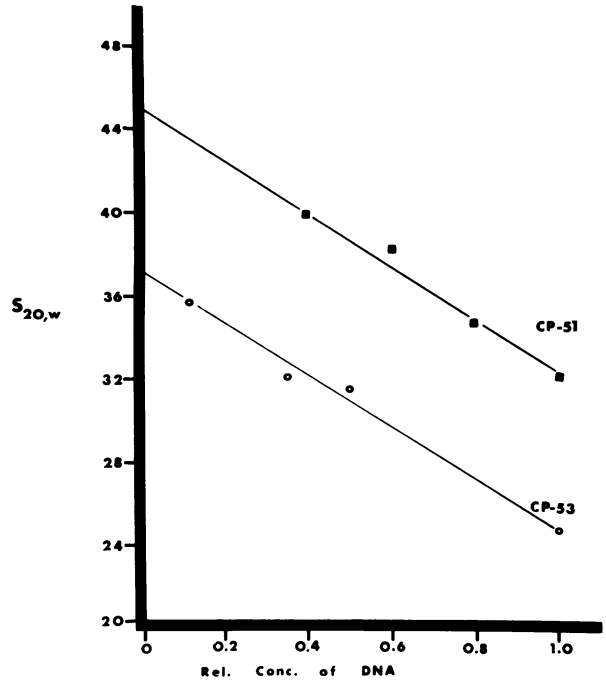

FIG. 5. *Relationship between $S_{20,w}$ and the concentration of CP-51 and CP-53 DNA. DNA was sedimented at 24,000 rev/min in saline citrate.*

of the band formed by each DNA was measured and a molecular weight was calculated by the equation (5): $MW = RT\rho_0\beta/\sigma^2\omega^4r^2$. By this procedure CP-51 DNA gave a molecular weight of 54.3×10^6 daltons and CP-53 DNA gave a molecular weight of 17.0×10^6 daltons. Because of the large size of the CP-51 DNA molecules, great care had to be taken in loading the DNA into the centrifuge cell to avoid shearing the molecules. The molecular weights obtained for the DNA species by this method compared favorably with the values calculated by multiplying the weight of the phage particle by the per cent of the phage that was DNA; this procedure gave a value of 57.8×10^6 daltons for CP-51 DNA and 15.0×10^6 daltons for CP-53 DNA.

DNA sedimentation velocity studies. Sedimentation velocity studies were also done to determine the molecular weight of each phage DNA. *S* values were calculated at several concentrations of DNA, corrected to $S_{20,w}$, and then these values were plotted against DNA concentration to obtain $S_{20,w}$ values by extrapolation to zero concentration (Fig. 5). CP-51 DNA was found to have an $S_{20,w}$ value of 45.2, whereas CP-53 DNA had an $S_{20,w}$ value of 37.3. Computation of a molecular weight from these values with Freifelder's equation (8) gave 61.6×10^6 daltons for CP-51 DNA and 35.5×10^6 daltons for CP-53 DNA. The value for CP-51 DNA was in good agreement with those obtained by alternate methods. However, the value for CP-53 DNA was

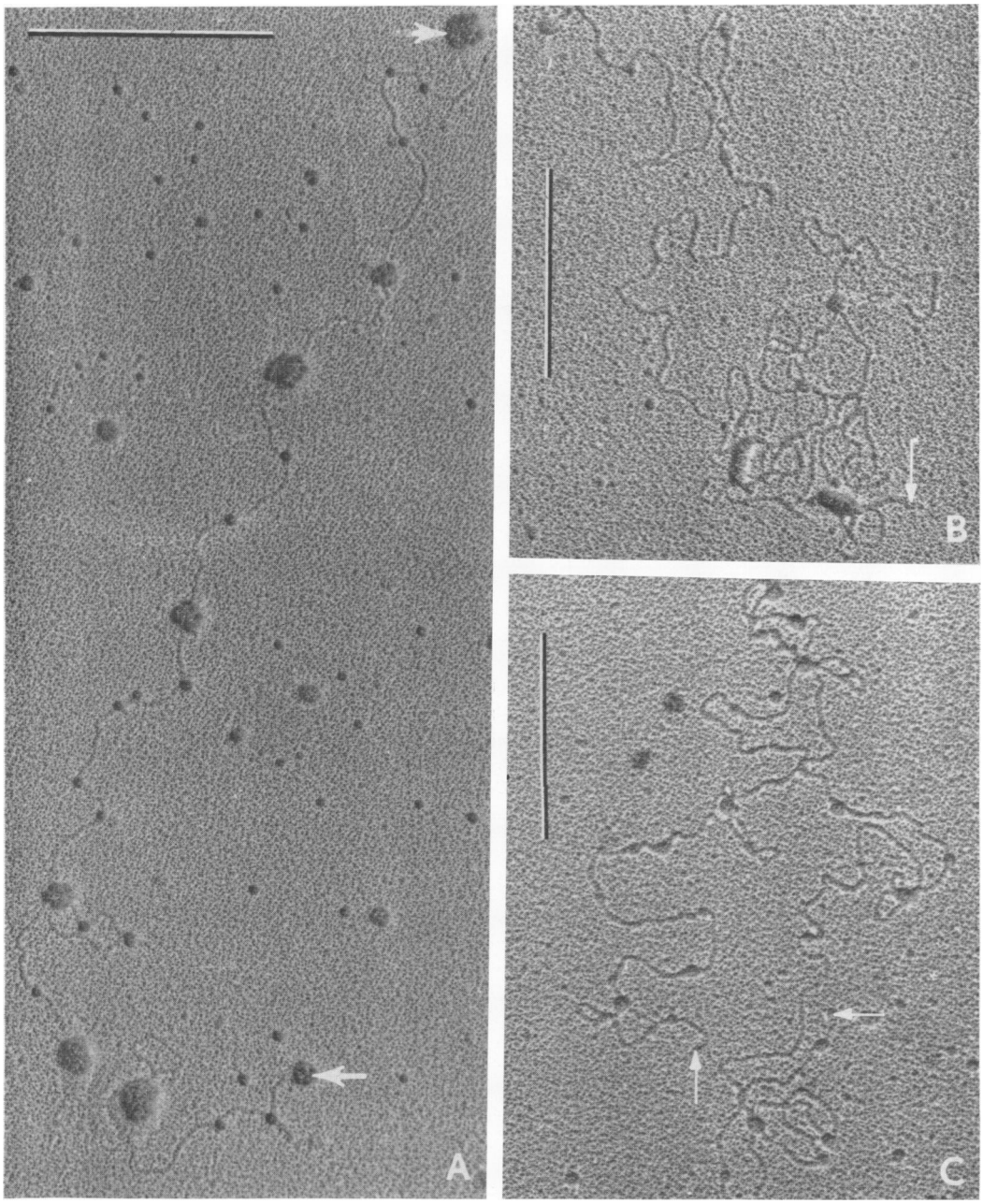

FIG. 6. *Electron micrographs of CP-53 DNA. Arrows indicate the ends of the DNA molecules. (A) × 68,700; (B) × 78,200; (C) × 59,900. Bar = 0.5 μm.*

approximately twice that from previous experiments.

Contour length of CP-53 DNA. Because of the discrepancy in the experimental values for the molecular weight of CP-53 DNA, a third method was used to measure this molecular weight. Electron micrographs of the DNA molecules were taken and the contour lengths of the molecules were measured. The contour length of a DNA molecule is related to its molecular weight (207×10^4 daltons/μm) as reported by Lang (12). Some sample molecules are shown in Fig. 6. Ten molecules were measured and their average molecular weight was calculated to be 16.5×10^6 daltons ±

0.5×10^6 daltons. This value is consistent with that obtained by the band-width method. As a control, DNA from bacteriophage PM2, a known circular DNA molecule with a molecular weight of from 5×10^6 to 6×10^6 daltons (7), was also photographed and its contour length was measured. The photographs of PM2 DNA showed supercoiled and open-circular molecules with a contour length of 2.56 μm (5.3×10^6 daltons). Hence, the results obtained by electron microscopy with CP-53 DNA appear to be accurate.

Shape of the phage DNA molecules. The sharp melting transitions of the phage DNA species indicated that the DNA species were double-stranded molecules. They left unresolved the question of whether the DNA species were linear or circular. If they were circular, they would have to be nicked circles so that the strands could separate on heating. To resolve this question the DNA species were subjected to band sedimentation in alkaline CsCl (*p*H 12.5). At this *p*H, DNA is denatured. Since linear molecules separate into two equivalent strands, only one band is seen; however, circular molecules with a single-strand nick separate into a linear and a circular strand and thus give two bands during sedimentation. When such experiments were done with CP-51 and CP-53 DNA species, only one band was formed in each case indicating that the molecules were linear. That CP-53 DNA is a linear molecule is further supported by the electron micrographs of this DNA.

Table 5 summarizes some of the properties of the phage DNA species. CP-51 DNA had a richer GC content than CP-53 DNA, was more dense than CP-53 DNA in large part due to the substitution of HMU for thymine, and weighed about 3.5 times as much as CP-53 DNA. Both DNA species were linear, double-stranded molecules.

DISCUSSION

The results presented here extend published data showing that CP-53 is a generalized transducing phage. Mutants representing five different auxotrophic markers have been tested, and each was transduced to prototrophy by the phage. Transducing frequencies as high as 2×10^{-6} were obtained. Other markers have not yet been tested in transduction experiments.

Cells lysogenized with CP-53 were not transduced at a high frequency by the homologous phage even though transduction by CP-51 was not impaired. CP-53 adsorbed somewhat slower to lysogens than to nonlysogens, but the reduced rate of adsorption was probably not great enough to account for the lower yield of transductants.

A similar effect of prophage on the frequency of transduction has been reported in the P22-*Salmonella* system (34). Transduction frequencies were reduced 5- to 20-fold in a lysogenic recipient.

Rao has shown that *S. typhimurium* lysogenized with P22 excludes superinfecting homologous phage (21). Although P22 adsorbed to and injected its DNA into lysogens, the superinfecting genome was lost somehow. The loss of the superinfecting genome was not due to repression since the cells were induced prior to superinfection. The route of entry was important since introduction of the superinfecting phage via conjugation did not lead to exclusion. Although Rao did not explore the possibility, it seems possible that transducing particles could be similarly excluded.

Since transduction is the only reported method of genetic exchange in *B. cereus*, genetic mapping of this organism depends upon cotransduction of linked markers. The usefulness of a phage for mapping studies can be correlated to the size of the piece of DNA it can carry. Phages which transfer large segments of the host chromosome are desirable if one is ordering markers over the entire genome. In their studies on cotransduction in *B. subtilis*, Tyeryar et al. (29) concluded that cotransduction frequencies were directly related to the size of the piece of DNA carried by a phage. By using phages SP-15, PBS-1, and SP-10, they showed that the phage with the largest genome gave the greatest frequency of cotransduction, and the smallest phage gave the lowest frequency. In these studies with CP-51 and CP-53, we found that CP-51 gave greater cotransduction frequencies than CP-53. By analogy to the *B. subtilis* phages it appeared as though CP-51 had a larger genome than CP-53. Proof of this was obtained subsequently by physical techniques.

Although no mapping of the *C. cereus* chromosome has been attempted, the similarity of the two sets of linked markers in this organism to a pair of linkage groups in *B. subtilis* is striking. The *his-1*, *met/cys-5* linkage group might correspond to the *hisB*, *metB* region of the *B. subtilis* map, whereas the *met-7*, *arg/pro-1* group could be representative of the *metA*, *argC* area. If this proves to be the case, the chromosome map of *B. cereus* may be very similar to that of *B. subtilis*.

Physical and chemical studies on the bacteriophages showed them to be dissimilar. They were morphologically different, had different densities and molecular weights, and the base compositions of their DNA species were distinct. CP-51 had more guanine and cytosine in its DNA than did CP-53 and in addition it contained 5-HMU in place of thymine. The finding of HMU in the

DNA of CP-51 was of interest since this base has also been reported in the DNA of many *B. subtilis* phages (22). It is not clear why this substitution should occur, but pyrimidine substitutions appear to be a widespread phenomenon among phages. In several coliphages 5-hydroxymethylcytosine replaces cytosine (22).

When the molecular weight of CP-53 DNA was determined by sedimentation velocity, an *S* value was obtained which gave a weight about twice that measured in other ways. Although we have no explanation for this discrepancy, we have ruled out the possibility of circular molecules on the basis of the data from the alkaline sedimentation and electron microscopy. Perhaps CP-53 DNA dimerized in a manner similar to that reported by Hershey et al. for lambda phage DNA (10).

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