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Isolation and Characterization of a New Bacteriophage, Cp-1, Infecting *Streptococcus pneumoniae*

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Several pneumococcal phages showing a morphology completely different from those of all other previously found pneumococcal bacteriophages have been isolated. Bacteriophage Cp-1, one of the phages isolated, showed an irregular hexagonal structure and a short tail of 20 nm. The virion density was 1.46 g/cm^3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed the presence of nine polypeptides. The polypeptide showing a molecular weight of 39,000 accounted for more than the 90% of the total protein. The nucleic acid of Cp-1 was linear, double-stranded DNA with a mean length of 6.3 μm and a guanine-plus-cytosine content of 41%; its buoyant density was 1.699 and 1.422 g/cm^3 in CsCl and Cs_2SO_4, respectively. Its sedimentation coefficient ($s_{20,w}$) was 19S. Cp-1 DNA showed a remarkable resistance to a large number of restriction endonucleases. A total of 12 fragments, ranging in molecular weight from 1.3×10^6 to 0.09×10^6, were produced by *AluI*, two fragments (molecular weight, 5.5×10^6 and 0.9×10^6) were generated by *Hin*dIII, and two fragments (molecular weight, 6.0×10^6 and 5.7×10^6) were produced by *Hae*III. The easy visualization of the plaques produced by Cp-1, the small size of Cp-1 DNA (12×10^6 daltons), and other biological and physicochemical properties make this phage potentially useful for genetic studies.

Several pneumococcal phages have been isolated (13, 25) and characterized (3, 11, 16) recently. We studied some relatively unusual properties of one of these phages (Dp-1), such as the presence of a lipid (11) and an apparent role of the bacterial murein hydrolase in the liberation of phage progeny (20). Pneumococcal phages also offer possibilities as tools in genetic studies of *Streptococcus pneumoniae*, for which most of the information available has been produced by genetic transformation. Along these lines, we have already described a simple transfection system with phage Dp-4 (21); the physicochemical and biological properties of Dp-4 and its transfecting DNA (6, 10, 19) have been studied in detail and have allowed an investigation of the replication of a phage in a pneumococcus (7). Porter and Guild (17) have described another pneumococcal transfection system using replicating DNA as well as a peculiar form of genetic transfer (18) sensitive to DNase and requiring both competent cells and bacteriophage (pseudotransduction).

In the present paper, we describe the isolation of a new pneumococcal phage (Cp-1) which shows morphological characteristics and physicochemical properties completely different from those previously described for various pneumococcal phages (3, 11, 13, 16, 25). Several characteristics of Cp-1, such as its relative stability and the small size of its DNA, should make it a useful tool for genetic studies.

(A preliminary report of some of the studies described here will be published [R. López, E. García, and C. Ronda, Rev. Infect. Dis., in press].)

MATERIALS AND METHODS

Bacterial strains. Strain R6, a derivative of *S. pneumoniae* strain R36A (Rockefeller University stock) was used mainly as a lawn for the phage described in this paper. A streptomycin-resistant strain, R6st, was constructed by genetic transformation.

Phage. Cp-1 was isolated from throat samples taken from healthy children in Alcalá de Henares, Madrid, by Ramón Dominguez. Details of the isolation technique have been published elsewhere (11). Preparation and purification of the phage are described below.

Cp-1 DNA was isolated by phenol extraction (21) or by phenol extraction after overnight treatment of the phage suspension with 50 μg of proteinase K per ml in a buffer containing 10 mM Tris-chloride (pH 8.0), 10 mM EDTA, 10 mM NaCl, and 0.5% sodium dodecyl sulfate (8).

Determination of the melting temperature. The melting temperature of Cp-1 DNA was determined in standard saline citrate (0.15 M NaCl, 0.015

M sodium citrate [pH 7.0]) by using an automatic apparatus (19830 Tm Analysis System, Beckman Instruments, Inc.) as previously described (6).

Ultracentrifugation techniques. Analytical density gradient centrifugation was performed as described by Szybalsky and Szybalsky (24). The sedimentation coefficient of Cp-1 DNA was determined by the boundary velocity method as described by Bauer and Vinograd (2). Photographs were scanned with a Chromoscan (Joyce-Loebl) recording and integrating densitometer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cp-1 was previously purified by two bandings in CsCl gradients. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out at room temperature with 10% gels as described by García et al. (6).

Titration of phage. In most of the work described here, K-CAT medium (16) was used. Base plates contained K-CAT plus 1.5% agar. Top agar contained K-CAT and 0.8% agar. Catalase (10^3 to 2×10^3 U per plate) was always added to top agar immediately before the agar was poured on the plates. In contrast to the cases of bacteriophages Dp-1 and Dp-4 (21), a single layer of soft agar was sufficient for visualizing Cp-1 plaques.

For routine titration, 3×10^7 to 5×10^7 cells of strain R6 were mixed with 1 ml of phage suspension in a test tube. After addition of catalase, 5 ml of top agar was added to the tube, mixed, and poured on the plates. Plates were incubated overnight at 30 to 32°C.

Electron microscopy. Purified preparations of bacteriophage in 0.1 M ammonium acetate (pH 7.0) were negatively stained with 2% K-phosphotungstate (pH 7.0) (15), and the grids were examined with a Philips EM300 electron microscope at 80 kV. Purified DNA was processed for electron microscopy as previously described (23). Magnification of photographs was determined with a carbon-grating replica (2,160 lines per mm) from Balzers Union. DNA lengths were measured with a KONTRON MOP-AMO 3 (Messgerate GMBH) apparatus on micrographs enlarged four times.

Cleavage of DNA and electrophoresis of fragments. *Alu*I, *Hin*dIII, *Pst*I, *Xba*I, *Bgl*II, *Bam*HI, *Eco*RI, *Sal*I, *Mbo*I, and *Sma*I were purchased from New England Biolabs, Inc. The conditions of cleavage were those recommended by the suppliers. Samples of DNA (1 to 2 μg) were incubated with the appropriate quantity of enzyme at 37°C for 120 min, heated at 70°C for 10 min, cooled in ice, and mixed with glycerol-containing bromophenol blue as described by Murray and Murray (14). DNA fragments were analyzed by electrophoresis on 1 or 0.7% agarose gels (see below).

RESULTS AND DISCUSSION

Size and morphology of Cp-1. Several phages were isolated (10 different isolations) from throat cultures of children. All these phages showed similar morphology, although differences in size have been detected (Fig. 1). One phage, selected for more detailed study, was designated Cp-1 (for "complutense" phage; complutense is the name given to the people from Alcalá de Henares, Spain, where the bacteriophages were isolated). In negatively stained preparations, the morphology of this bacteriophage turned out to be quite different from those of all other pneumococcal phages described previously (3, 11, 16). The head of Cp-1 (Fig. 2A) had an irregular hexagonal outline of approximately 60 by 45 nm and a tail nearly 20 nm long and 15 nm thick. The head showed a flattened base (Fig. 2C), neck appendages, and head fibers (Fig. 2B). A schematic drawing based on features found by electron microscopy is shown in Fig. 2D. This structure resembles that of *Bacillus subtilis* phage φ29 (4).

Biological properties of Cp-1. Cp-1 formed plaques that were clearer and larger than those previously described in the literature for pneumococcal phages (Fig. 3). This should facilitate the isolation of mutants from Cp-1 and the development of the genetics of this system.

The yield of Cp-1 was rather low, probably due to the low burst size (see below). This problem was eventually circumvented by the following procedure: R6st strain was grown in K-CAT medium (16) at 37°C. At a cell concentration of 2.5×10^7 colony-forming units per ml, the culture was inoculated with Cp-1 at a multiplicity of infection of 2 and incubated at 30°C for 2 h, at which time an equal volume of medium was added, and the incubation was continued at 30°C until the culture lysed (ca. 3 h later). Under these conditions, the titer of the lysates ranged from 4×10^9 to 6×10^9 PFU/ml. After removal of cell debris, NaCl (final concentration, 0.5 M) was added to the supernatant together with polyethylene glycol 6000 (final concentration, 10%) and stored at 4°C overnight. The precipitate was collected by centrifugation ($5.000 \times g$, 15 min), and the pellet was resuspended in 0.1 M NaCl–0.1 M Tris-hydrochloride (pH 7.8)–10 mM $MgCl_2$ before the phage were purified in a preformed CsCl gradient (20, 21).

Cp-1 also differed from the pneumococcal phages Dp-1, Dp-4, ω-3, and ω-4 (13, 16) in its superior stability during storage under different conditions at either low or high phage concentrations (Table 1).

Adsorption and one-step growth. Cp-1 adsorbed to whole cells very poorly; the titration of the supernatants after various times of inoculation is shown in Fig. 4A. It was found that the adsorption rate constant was 2.3×10^{-10} ml/min, a rather low value compared with those of most phages of *Escherichia coli* (1).

The difficulties of carrying out one-step-growth experiments with anaerobic (12) or microanaerobic (11) bacterial host cells have been repeatedly pointed out in the literature. For this reason, we performed these experiments as it

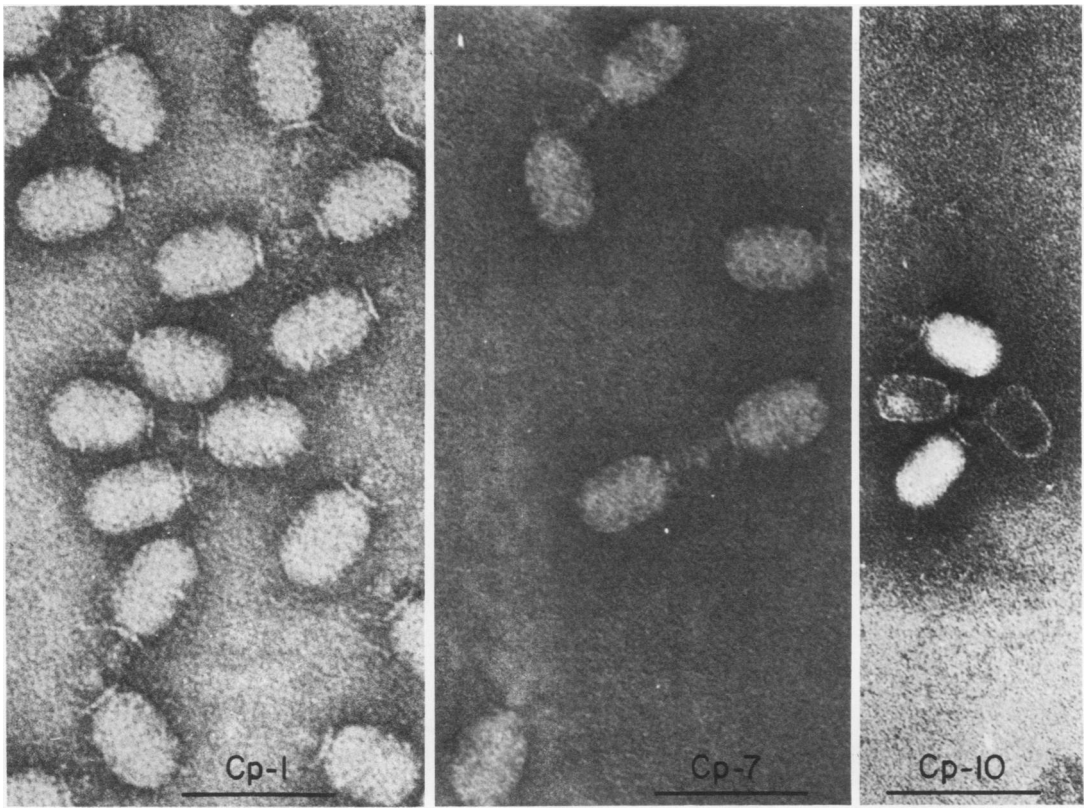

FIG. 1. *Electron micrographs of pneumococcal phages Cp-1, Cp-7, and Cp-10. Preparations were negatively stained with 2% aqueous phosphotungstic acid. Bars, 100 nm.*

was previously suggested for *Clostridium perfringens* (12). In short, R6st strain was grown at 37°C up to a cell concentration of 5×10^7 cells per ml in K-CAT medium. Cp-1 was added (multiplicity of infection, 2) and, after 5 min at 30°C, the culture was centrifuged and resuspended in prewarmed growth medium at the same cell concentration. At various times, samples were withdrawn and assayed for virus. Under these conditions, the latent period for Cp-1 was about 50 min, the rise in phage titer occurred for 20 min, and the apparent burst size was 9 to 11 (Fig. 4B).

Biophysical properties. The buoyant density of purified Cp-1 phage was 1.46 g/cm^3. Purified phage were solubilized in sodium dodecyl sulfate and analyzed by 10% neutral sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The structural proteins were resolved into nine bands; the electropherogram scanned with a spectrophotometer Gilford model 2400 revealed that the area corresponding to the main protein present in the mature particle accounted for more than the 90% of the total protein (Fig. 5). The molecular weights of the polypeptides were 67,000, 53,000, 50,000, 39,000, 34,000, 29,500, 28,000, 20,500, and 13,000.

The melting temperature of Cp-1 DNA in standard saline citrate was 87°C. This value corresponds to a guanine-plus-cytosine content of 42% (9). The buoyant density of double-stranded Cp-1 DNA in neutral CsCl gradients appeared to be identical to that of the host-cell DNA (1.699 g/cm^3). This density corresponds to a guanine-plus-cytosine content of about 40% (9), which is in good agreement with the value determined by the melting temperature. On the other hand, the buoyant density of Cp-1 DNA in Cs_2SO_4 was 1.422 g/cm^3, which suggests that the DNA of this bacteriophage does not contain any odd bases. Heat-denatured Cp-1 DNA showed a single peak in neutral CsCl gradients, with a density of 1.717 g/cm^3. Alkaline (pH 12.0) CsCl gradients of Cp-1 DNA also showed a single peak, with a density of 1.755 g/cm^3. The native DNA of Cp-1 showed a sedimentation coefficient $s_{20,w}$ of 19S, which corresponds to a molecular size of about 12×10^6 daltons (5). The properties of Cp-1 DNA are listed in Table 2.

Cp-1 DNA isolated by phenol treatment of

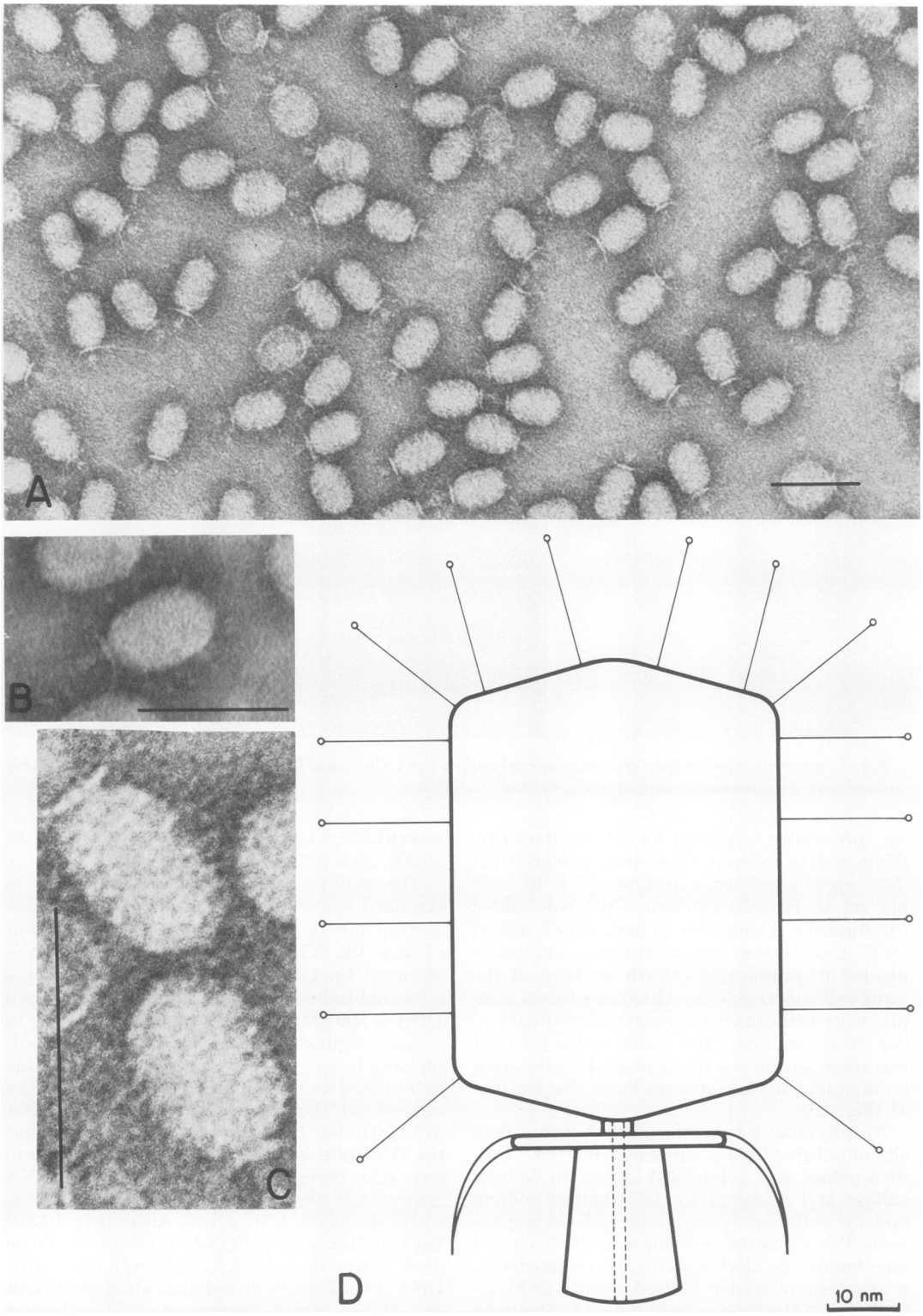

FIG. 2. *(A) Electron micrograph of a negatively stained preparation of purified Cp-1. (B and C) Phage particles showing details of the head projections and the base plate. (D) Schematic drawing of Cp-1. Bars in A, B, and C, 100 nm.*

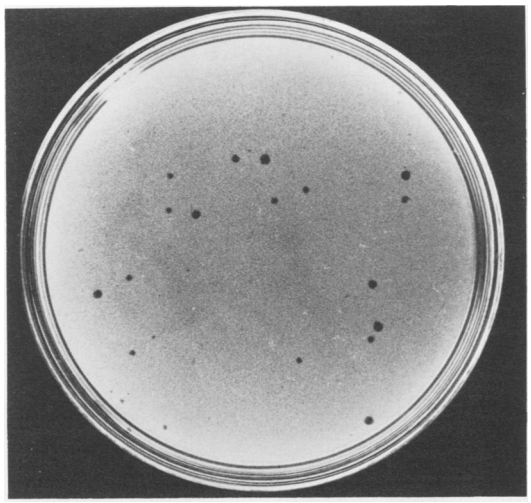

FIG. 3. *Plaques produced by Cp-1 on agar plates containing K-CAT medium.*

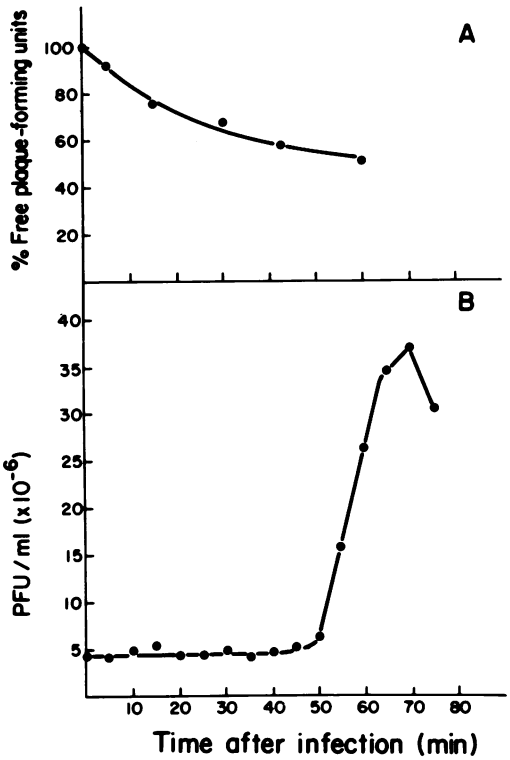

FIG. 4. *(A) Adsorption of Cp-1 to whole R6 cells. To 5 × 10⁷ cells per ml in K-CAT medium, enough purified Cp-1 was added to give a multiplicity of infection of 2. At the indicated times, samples were withdrawn and centrifuged at 10,000 × g for 10 min, and the titers of the phage remaining in the supernatant were determined on the lawns of R6 cells. (B) One-step-growth curve of the Cp-1 (for details, see text).*

purified phage (Fig. 6) appeared as linear, double-stranded molecules when examined by electron microscopy. The mean length was 6.3 ± 0.3 μm, corresponding to a molecular size of 11.9 × 10^6 ± 0.6 × 10^6 daltons (23).

TABLE 1. *Stability of Cp-1 under various storage conditions*

Addition	Phage titer (PFU/ml) after 1 month at:[a]			
	4°C		−70°C	
	Crude lysate	Purified phage	Crude lysate	Purified phage
None	10^9	10^{11}	ND[b]	3×10^{10}
1 M NaCl	3×10^8	4×10^{10}	ND	10^{10}
10% Glycerol	1.5×10^9	1.2×10^{11}	2.5×10^9	2×10^{11}
1 M NaCl + 10% glycerol	5×10^8	7×10^{10}	10^9	8.5×10^{10}

[a] Crude lysates in K-CAT medium and purified phage in 0.1 M Tris-hydrochloride (pH 7.8)–0.5 M NaCl–10 mM $MgCl_2$ were used. The titer of the crude lysate at the beginning of the experiment was 3×10^9 PFU/ml, and the titer of purified phage at the beginning of the experiment was 2.5×10^{11} PFU/ml.

[b] ND, Not determined.

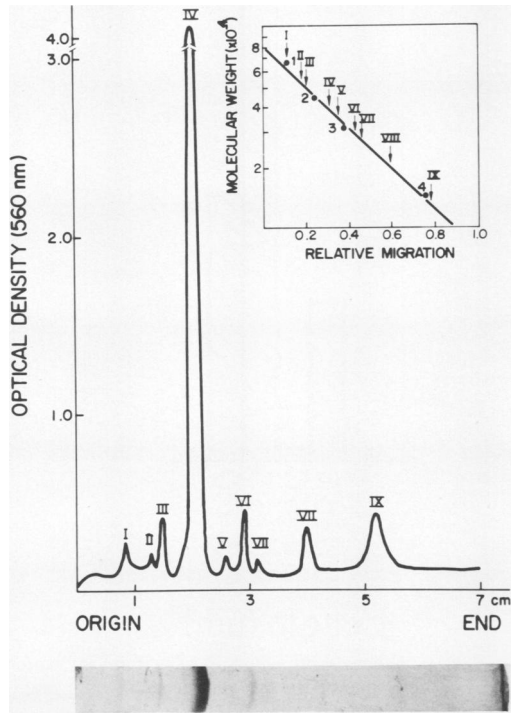

FIG. 5. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified Cp-1. The insert shows the migration of the polypeptides and their estimated sizes relative to the following molecular weight markers: 1, bovine serum albumin (67,000); 2, ovalbumin (45,000); 3, deoxyribonuclease I (31,000); 4, lysozyme (14,400).*

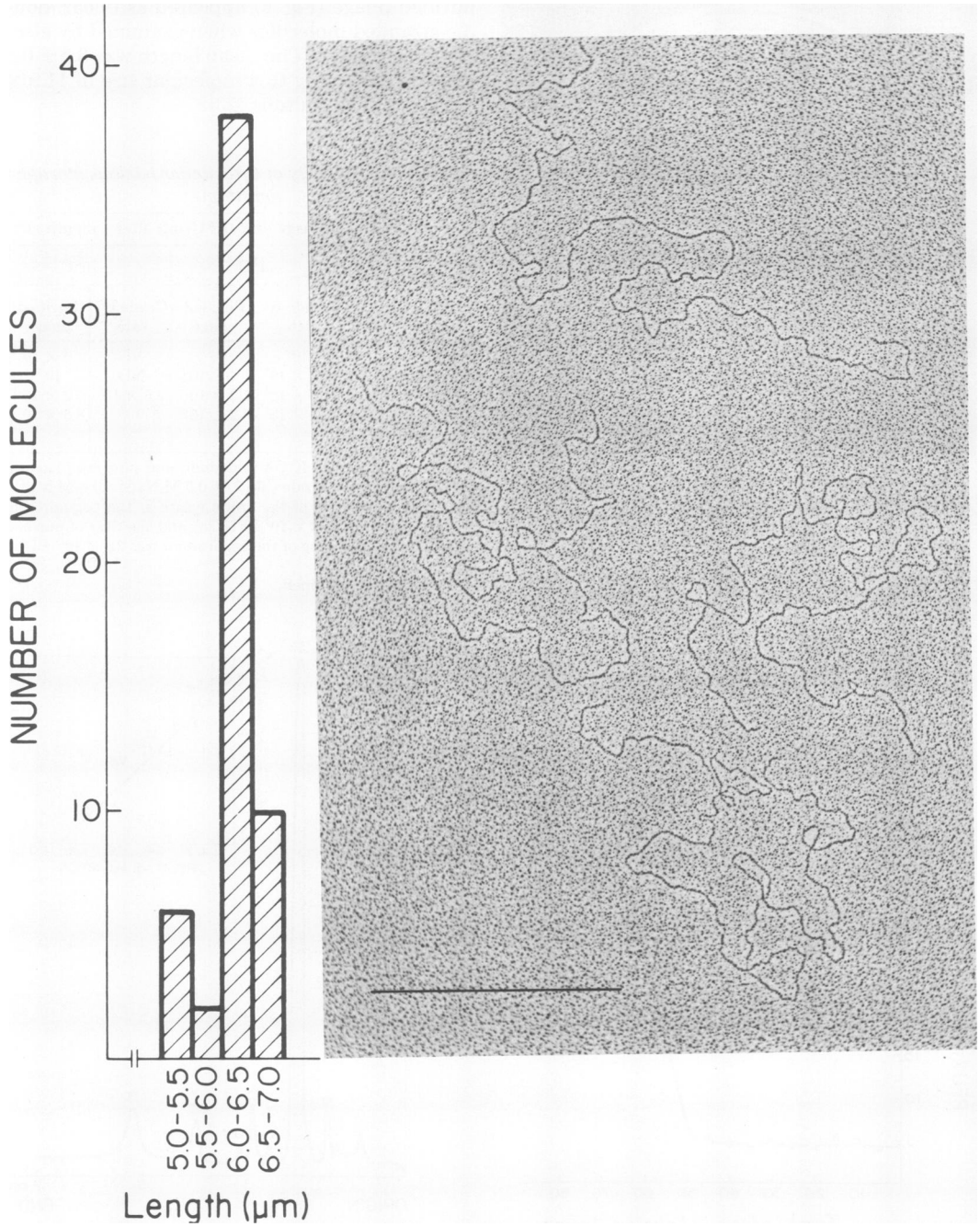

FIG. 6. *Electron micrograph of four linear molecules of Cp-1 DNA. The size distribution of 56 molecules of Cp-1 DNA is shown on the histogram. Bar, 1 μm.*

Digestion with restriction enzymes. We have previously found that Dp-4 DNA has unusual physical properties (6). In addition, mature Dp-4 DNA resists a large number of restriction nucleases assayed so far (unpublished data). Cp-1 DNA also showed a remarkable resistance to most of the restriction nucleases tested (i.e., *Pst*I, *Xba*I, *Bgl*II, *Bam*HI, *Eco*RI, *Sal*I, *Mbo*I, and *Sma*I). Cp-1 DNA was digested with restriction endonuclease *Alu*I, and the cleavage prod-

ucts were separated by electrophoresis on 1% agarose slab gels (Fig. 7). Table 3 shows the molecular weights obtained for the Cp-1 restriction fragments. The molecular weights of Cp-1 *Alu*I fragments ranged from 1.3×10^6 to 0.09×10^6, and the sum of the molecular weights of these fragments was 5.15×10^6. The discrepancies between this value and those obtained by analytical ultracentrifugation and by electron microscopy (see above) could be resolved if we assume that some bands (i.e., C, E, and H of Fig. 7) in the *Alu*I digest of Cp-1 DNA actually consisted of several fragments of similar sizes, as was apparent from the restriction pattern as well as from the densitometer tracing of the fragment intensities (data not shown). On the other hand, Cp-1 DNA was cleaved by *Hin*dIII and yielded four bands (10.8×10^6, 6.5×10^6, 5.5×10^6, and 0.9×10^6 daltons) by electrophoresis on 0.7% agarose slab gels (Fig. 8, lane f); nevertheless, when the incubation of the DNA with *Hin*dIII was continued for a longer period, the cleavage products were resolved only in two bands (Fig. 9, lane a) corresponding to bands C and D. Comparisons of the complete and the partial digests of Cp-1 DNA with *Hin*dIII (Fig. 8 and 9) suggest that this enzyme cut the DNA as it is shown in Fig. 9. Partial cleavage produced four bands, a cut at I produced bands A and D, and a cut at II produced bands B and C, whereas complete digestion produced only bands C and D. Band C consisted of two fragments of similar size (5.5×10^6).

Finally, *Hae*III cut Cp-1 DNA once; the two fragments obtained (Table 3) gave molecular weights of 6.0×10^6 and 5.7×10^6 (T.A. Trautner,

TABLE 2. *Biophysical characteristics of Cp-1 DNA*

Characteristic	Measurement
Buoyant density (g/cm^3)	
Neutral CsCl (native DNA)	1.699
Neutral CsCl (thermally denatured DNA)	1.711
Alkaline CsCl	1.755
Cs_2SO_4	1.422
Sedimentation coefficient ($s_{20,w}$)	19S
Melting temp (°C)	87
Type	Linear duplex
Contour length (μm)	6.3 ± 0.3
Mol wt (×10^6)	12
Guanine-plus-cytosine content (%)	~41

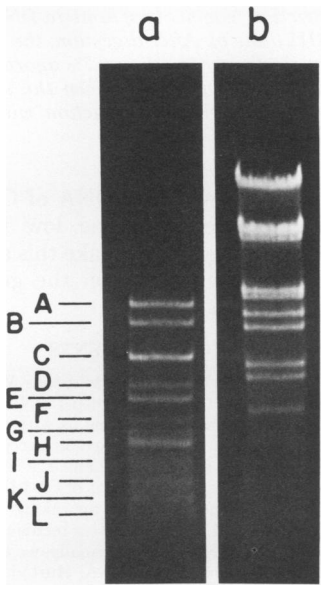

FIG. 7. *Analysis of Cp-1 DNA digested with restriction endonuclease AluI. After digestion with the enzyme, the DNA was subjected to electrophoresis on a 1% agarose gel. Cleavage conditions of DNA, gel composition, and electrophoresis conditions are described in the text. Fragments were visualized by ethidium bromide staining. An EcoRI- HindIII digest of lambda DNA (lane b) was included as the molecular weight standard.*

TABLE 3. *Molecular weight of Cp-1 DNA restriction fragments produced by digestion with AluI, HindIII, and HaeIII*

Fragment	Mol wt (×10^6)
*Alu*I fragment[a]	
A	1.30
B	1.05
C	0.70
D	0.46
E	0.39
F	0.29
G	0.25
H	0.22
I	0.17
J	0.13
K	0.10
L	0.09
*Hin*dIII fragment[b]	
C	5.50
D	0.90
*Hae*III fragment[b]	
A	6.00
B	5.70

[a] Molecular weights were estimated from the relative mobility of each fragment compared with the mobilities of fragments of an *Eco*RI-*Hin*dIII double digest of lambda DNA (14).

[b] Molecular weights were estimated from the relative mobility of each fragment compared with the mobilities of fragments of a lambda *Hin*dIII digest (14).

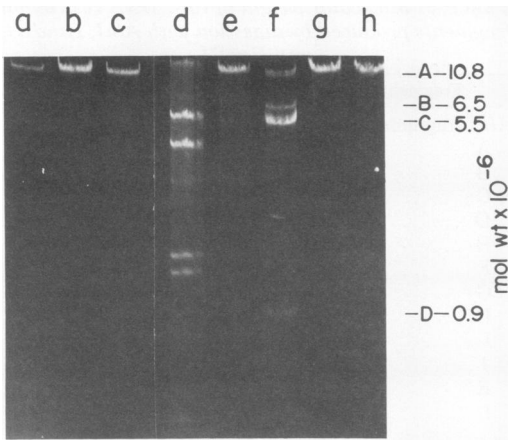

FIG. 8. *Analysis of Cp-1 DNA treated with restriction enzymes EcoRI, HindIII, BamHI, and SmaI (lanes e through h, respectively) and of lambda DNA digested with HindIII (lane d). Native φ29 DNA (lane a), native φ29 and native Dp-1 together (lane b), and native Dp-1 (lane c) were also subjected to electrophoresis on a 0.7% agarose slab gel. Conditions were described in the text.*

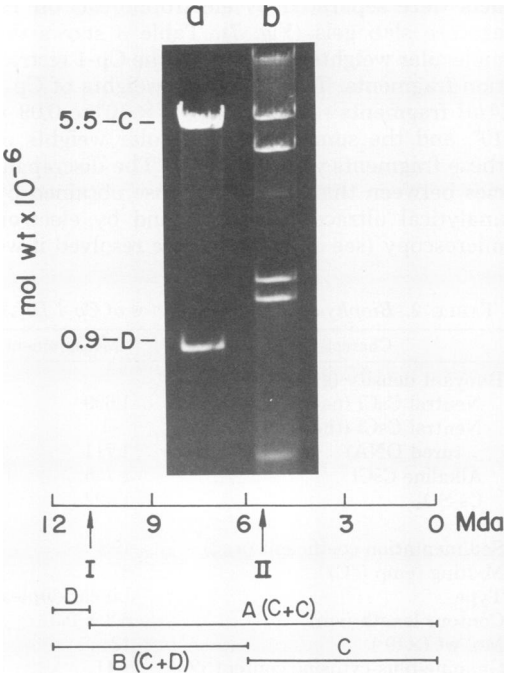

FIG. 9. *Restriction fragments of Cp-1 DNA completely digested with restriction enzyme HindIII (lane a) and restriction fragments of lambda DNA digested with HindIII (lane b). After digestion, the DNA fragments were electrophoresed on 0.7% agarose gels. At the bottom of the figure are shown the sites of the cleavage of Cp-1 DNA by restriction endonuclease HindIII (see text).*

personal communication). Fig. 8 also shows the positions of intact φ29 DNA (lane a); Cp-1 DNA (lane c) and φ29 plus Cp-1 DNA (lane b), as well as the positions of Cp-1 DNA treated with *Eco*RI, *Bam*HI, and *Sma*I (lanes e, g, and h, respectively).

On the other hand, Cp-1 phage attacked both *Dpn*I- and *Dpn*II-containing strains (S. Lacks, personal communication), which may indicate that its DNA is resistant to both enzymes. In, support of this possibility is the fact that Cp-1 DNA was resistant to *Mbo*I, an isoschizomer of *Dpn*II. Although no discrepancies in the guanine-plus-cytosine content have been found for Cp-1 DNA, as determined by the melting temperature and by the buoyant density analysis (see above), the relative insensitivity of Cp-1 DNA to the restriction endonucleases suggests that this DNA may contain either abnormal or substituted bases, as previously suggested for Dp-4 DNA (6). Alternatively Cp-1 DNA may contain the four normal bases and may have lost the restriction sites for many endonucleases, as was recently pointed out for T7 (22).

Another interesting feature of Cp-1 is that the DNA obtained by the proteinase K method had no biological activity in transfection. However, if phage DNA was prepared in the absence of proteases, the material from the interphase of the phenol-treated DNA appeared to have transfecting activity (unpublished data). This observation, along with other physicochemical data recently obtained in our laboratory (i.e., the phenol-treated DNA does not penetrate into agarose gels), suggests the presence of a protein(s) associated with the DNA of Cp-1. This possibility, together with the low molecular weight of the DNA, should make this system an interesting one for studies on the genetics of pneumococcal phages.

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