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Physical and Biological Properties of Phage ϕ29 Deoxyribonucleic Acid

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Deoxyribonucleic acid (DNA) molecules having a mean length of 5.8 μm were released from purified *Bacillus subtilis* bacteriophage ϕ29 with 2 M sodium perchlorate. Small 0.1 to 0.2-μm molecules were also detected in these DNA preparations. Since intact single chains annealed to form linear duplex molecules, phage ϕ29 DNA was found to be nonpermuted. The molecular weights of single chains of ϕ29 DNA were approximately half that of native DNA, as determined by analytical band sedimentation in CsCl, indicating that ϕ29 DNA is composed of two continuous polynucleotide chains. The molecular weight values of native and annealed ϕ29 DNA from sedimentation agreed with the molecular weight values obtained from electron microscopy. The infectivity of ϕ29 DNA was reduced to a low level by alkaline denaturation and was partially restored by annealing.

Linear deoxyribonucleic acid (DNA) molecules isolated from bacteriophages T2, T4, and P22 are circular permutations of a common sequence, whereas the DNA molecules from bacteriophages T3, T7, and λ have unique nucleotide sequences (12). Thomas and MacHattie (11) have used a denaturation-renaturation test for permutation based on the renaturation into circles of intact, single polynucleotide chains derived from circularly permuted duplex molecules. In the present study, this test demonstrated the nonpermuted nature of *Bacillus subtilis* phage ϕ29 DNA, since intact single chains annealed to form linear duplex molecules. Sedimentation coefficients, molecular weights, and transfection activities of native, denatured, and annealed forms of ϕ29 DNA are reported.

MATERIALS AND METHODS

Phage and host. Bacteriophage ϕ29 was grown on *B. subtilis* strain H and purified as described previously (1).

Extraction of phage DNA. DNA was released from ϕ29 by use of a modification of the sodium perchlorate procedure of Freifelder (4). Concentrated phage ϕ29 in 0.02 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (pH 7.5) containing 0.1 M NaCl and 0.001 M $MgSO_4$ was mixed with an equal volume of 4 M sodium perchlorate in distilled water, and was incubated at 37 C for 45 to 60 min. This method provided quantitative release of ϕ29 DNA molecules.

Denaturation and annealing of ϕ29 DNA. Phage ϕ29 DNA in SSC (0.15 M NaCl-0.015 M sodium citrate) was denatured at concentrations of 100 μg/ml or less in 0.1 M NaOH according to the procedure of Studier (10), taking particular care to avoid shear. After 1 to 2 min at room temperature, the pH of the solution was adjusted to approximately 8 with an appropriate volume of 1.1 M HCl-0.2 M Tris.

DNA in double strength SSC was annealed at concentrations of 100 μg/ml or less by incubation at 65 C for 45 min, according to the method of Ritchie et al. (9).

Sedimentation. The analytical band sedimentation method of Vinograd et al. (13) was used to measure the sedimentation coefficients of native, denatured, and annealed ϕ29 DNA. Sedimentation measurements were made in a Spinco model E ultracentrifuge having a type I 30-mm Kel-F band-forming centerpiece. In general, 0.03 ml of a DNA solution of 20 to 30 μg/ml in SSC was added to the sample well. To the main cell compartment, we added 1.5 ml of a CsCl bulk solution having a density of 1.38 to 1.42 g/cc. Rotor acceleration was suspended at 5,000 rev/min until the DNA had layered on the bulk solution. The rotor was then accelerated to 35,600 rev/min, and ultraviolet absorption photographs were taken at 8- or 16-min intervals with Kodak commercial film. Microdensitometer tracings of the film were made with a Spinco Analytrol film densitometer model RB with an effective slit width of 100 μm.

DNA was sedimented in CsCl solutions of concentrations less than those in which the molecules are buoyant. Under these conditions, contaminating phage ghost proteins floated. Sedimentation coefficients at standard conditions were obtained from observed values in CsCl solutions using the procedures derived by Bruner and Vinograd (3); these investigators found that the product of the observed sedimentation coefficient of the sodium or cesium salts of T7 DNA at infinite dilution (S^0) and the relative viscosity (η_r) of the solution decreased linearly with

the density of the solvent (ρ) over a wide range of density. In CsCl, the $S^0\eta_r$ versus ρ lines at $S = O$ passed through the buoyant densities. Extrapolation of these lines to the density of water ($\rho = 1.0$) yielded $(S^0\eta_r)_w$ values in NaCl which agreed with literature values for the standard sedimentation coefficients ($S^0_{20,w}$). Thus, the $(S^0\eta_r)_w$ values were considered standard sedimentation coefficients. In the present study, uncorrected sedimentation coefficients were calculated from the motion of the maximum of the concentration profile. The plots of the logarithm of the distance from the sedimenting peak to the axis of rotation (r) versus time (t) were usually linear over the length of the cell. $S^0\eta_r$ values were calculated by use of the relative viscosities of aqueous CsCl solutions from the data of Bruner and Vinograd. One $S^0\eta_r$ value and the buoyant density ($S^0\eta_r = O$) were plotted versus the densities of the CsCl solutions to yield straight lines which were extrapolated to $(S^0\eta_r)_w$, the standard sedimentation coefficients. For alkaline denatured DNA, two $S^0\eta_r$ values corresponding to two different CsCl densities were used rather than a buoyant density value.

Concentration dependence of the sedimentation coefficient was not detected at the DNA concentrations used; however skewing of the sedimenting band was observed in a 12-mm cell with higher DNA concentrations (200 μg/ml).

Transfection assay. Assay for infectious ϕ29 DNA was conducted according to the procedure of Reilly and Spizizen (8), except that streptomycin-resistant *B. subtilis* strain H was used as the indicator bacterium.

Electron microscopy. DNA molecules were prepared for electron microscopy by use of a procedure modified from the protein film technique of Kleinschmidt et al. (5). DNA at a concentration of 2 μg/ml in double strength SSC was mixed with an equal volume of 0.04% cytochrome *c* in 4 M NaCl; 0.2 ml of the mixture was spread onto an 80 cm² surface of 0.15 M ammonium acetate. Electron micrographs of DNA molecules were taken at a magnification of 10,000 ×. Procedures for measuring molecular lengths were described previously (1).

All micrographs were taken with a Siemens Elmiskop IA electron microscope.

RESULTS

Nonpermuted nature of ϕ29 DNA. DNA molecules were released from purified phage ϕ29 upon treatment with 2 M sodium perchlorate (Fig. 1A). Fifty molecules had a mean length of 5.80 ± 0.12 μm and ranged in length from about 5.4 to 6.1 μm (Fig. 2A). We also observed very short, 0.1- to 0.2-μm DNA molecules that constituted about 1% of the total DNA by weight. The relative numbers of short and long molecules shown in Fig. 2A may not accurately reflect the ratio of these molecules in a larger population of perchlorate-released DNA molecules. Additional measurements are necessary to definitively establish the relative numbers of short and full-length molecules.

Denaturation of ϕ29 DNA in 0.1 M NaOH at a concentration of 50 μg/ml or less, followed by neutralization, resulted in preparations containing less than 1% duplex molecules as estimated by electron microscopy. The bulk of the DNA was "puddled," indicating conversion of single chains to a compact form.

Incubation of neutral denatured ϕ29 DNA in double strength SSC at 65 C for 45 min resulted in efficient reformation of linear duplex molecules (Fig. 1B). A distribution of molecular lengths of annealed ϕ29 DNA is shown in Fig. 2B. The bulk of the annealed DNA resembled native DNA in length, though a few fragments of varying length were observed. The mean length of annealed molecules longer than 5 μm was 5.67 ± 0.21 μm. Therefore, ϕ29 DNA is nonpermuted, since a circularly permuted collection of intact, complementary single chains should anneal to produce circles.

Strand dissociation of ϕ29 DNA. The denaturation-renaturation test for permutation is useful for DNA molecules having continuous polynucleotide chains and probably cannot be effectively applied to molecules that have interruptions in either strand. Analytical band sedimentation of denatured ϕ29 DNA was performed to determine continuity of the single polynucleotide chains. Sedimentation patterns of native, alkaline denatured, neutral denatured, and annealed forms of ϕ29 DNA are shown in Fig. 3. The standard sedimentation coefficients and molecular weight values for native, alkaline denatured, neutral denatured, and annealed forms of ϕ29 DNA are given in Table 1. The mean lengths of the molecules longer than 5 μm in the native and annealed preparations shown in Fig. 2 were 5.80 and 5.67 μm, respectively. Assuming ϕ29 DNA is a double helix sodium salt in the B crystallographic form and that the base pairs are 340 pm apart, the molecular weights of the native and annealed DNA molecules would be 11.1×10^6 and 10.9×10^6, respectively. Thus, the molecular weight values obtained from sedimentation agree well with values calculated from electron microscopic length measurements. The molecular weights of the single chains of ϕ29 DNA are approximately half that of the native DNA, indicating that ϕ29 DNA is composed of two continuous polynucleotide chains.

Alkaline denaturation of ϕ29 DNA in single strength SSC at high DNA concentrations (about 200 μg/ml), followed by neutralization, apparently resulted in incomplete denaturation, since analytical band sedimentation in a 12-mm cell detected a peak at the position expected for native DNA (Fig. 4). Moreover, these neutral denatured samples contained a third, rapidly

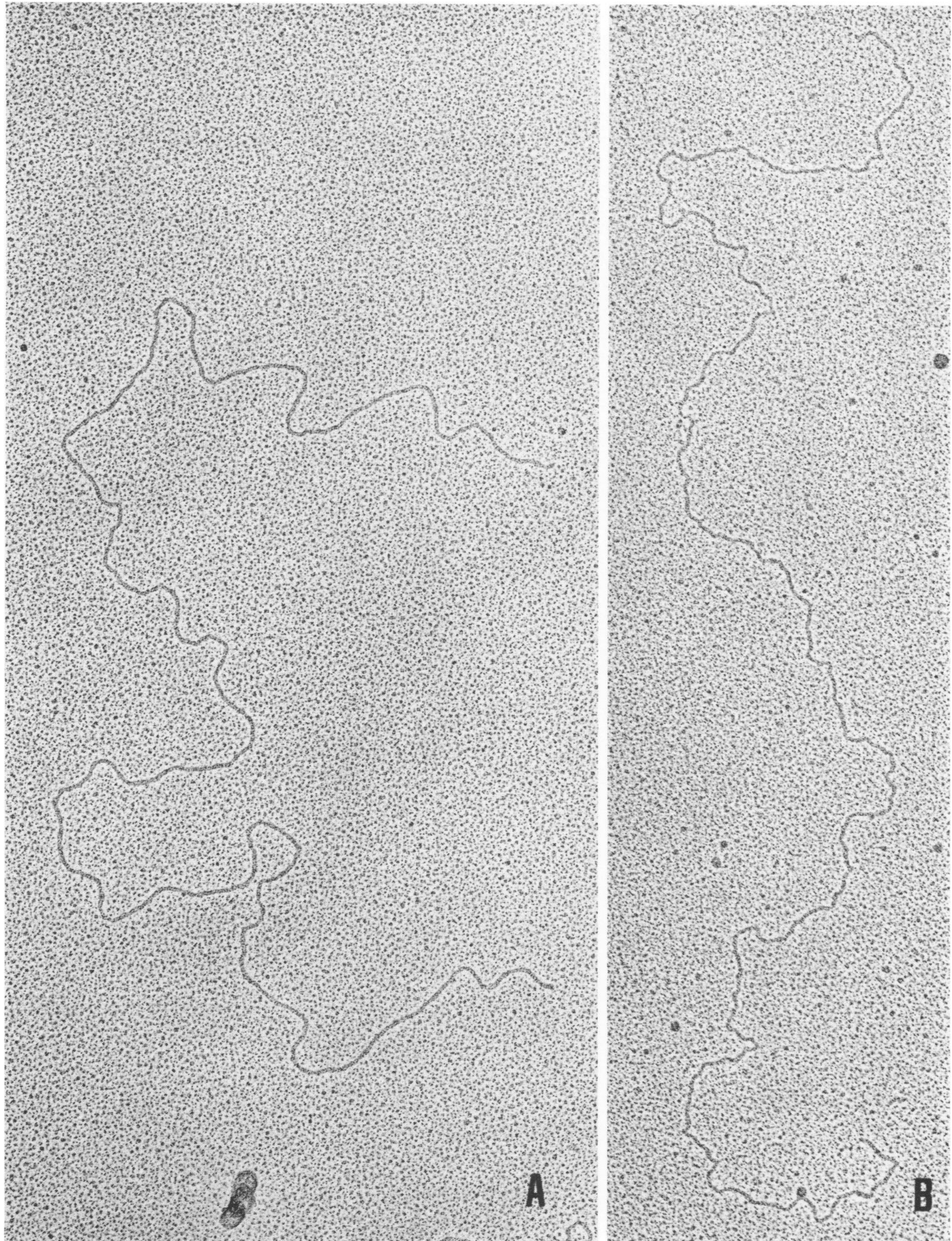

FIG. 1. (*A*) *Native ϕ29 DNA released by sodium perchlorate.* $\times$ *54,000.* (*B*) *Phage ϕ29 DNA annealed in double strength SSC at 65 C.* $\times$ *51,000.*

sedimenting band having a standard sedimentation coefficient approximately double that of the neutral denatured peak. The presence of this band might be explained by incomplete strand separation during alkali treatment, followed by transition of single-stranded regions to a compact form upon neutralization.

Biological properties of denatured and annealed

ϕ29 DNA. The infectivity of native, neutral denatured, and annealed ϕ29 DNA for competent *B. subtilis* was determined by use of a standard transfection assay. Neutral denatured DNA retained less than 4% of the transfecting activity of native DNA (Table 2). Reformation of linear duplexes by annealing, however, restored biological activity to as much as 30% of the native DNA. Hence, only a fraction of linear molecules reformed from single polynucleotide chains were biologically active.

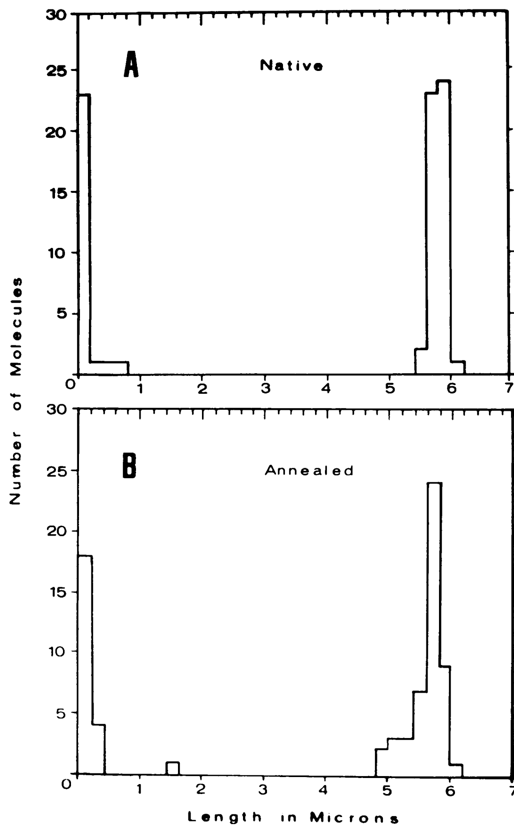

FIG. 2. *Distribution of lengths of native (A) and annealed (B) ϕ29 DNA molecules.*

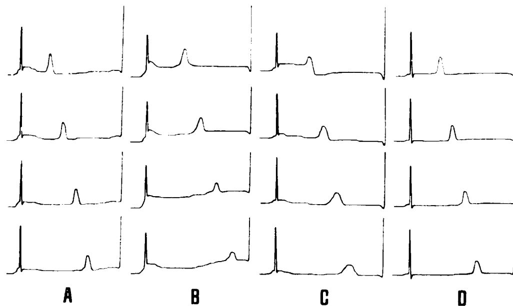

FIG. 3. *Analytical band sedimentation patterns of ϕ29 DNA. Sedimentation is from left to right. Intervals between frames were 16 min for native, alkaline denatured, and annealed DNA molecules, and 8 min for neutral denatured DNA. (A) native DNA; (B) alkaline denatured DNA; (C) neutral denatured DNA (pH 8); (D) annealed DNA.*

TABLE 1. *Molecular weight of ϕ29 DNA (sodium salt) from sedimentation and electron microscopy*

Form of ϕ29 DNA	$s^0_{20,w}$	Mol wt from sedimentation[a]	Mol wt from electron microscopy[b]
Native	24.5	11.5×10^6	11.1×10^6
Alkaline denatured	26.4	5.6×10^6	
Neutral denatured	50.4	5.1×10^6	
Annealed	23.5	10.2×10^6	10.9×10^6

[a] Calculated from the equations of Studier (10).

[b] Calculations were based on the measurements shown in Fig. 2 and an assumed value of 192 daltons/100 pm.

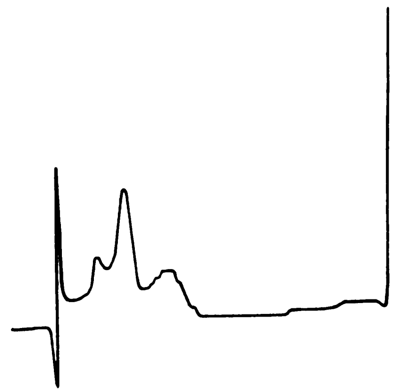

FIG. 4. *Analytical band sedimentation pattern of ϕ29 DNA denatured at 200 μg/ml, neutralized, and sedimented.*

TABLE 2. *Infectivity of native, denatured, and annealed ϕ29 DNA*

Expt	Infectious centers/ml[a]		
	Native	Neutral denatured	Annealed
1	5.3×10^5	4.5×10^3 (1%)[b]	3.8×10^4 (7%)
2	8.4×10^5	2.2×10^4 (3%)	1.4×10^5 (17%)
3	1.2×10^6	4.7×10^4 (4%)	2.3×10^5 (19%)
4	4.9×10^5	2.0×10^4 (4%)	8.1×10^4 (17%)
5	1.5×10^5	2.9×10^3 (2%)	4.5×10^4 (30%)

[a] Competent cells (3×10^8/ml) were incubated with 2.5 μg of ϕ29 DNA per ml in experiments 1 and 4, 5 μg of DNA per ml in experiments 2 and 3, and 1 μg of DNA per ml in experiment 5. Infectious centers were assayed after incubation of the mixtures for 45 min at 37 C.

[b] Percentage values in parentheses refer to infectivity of denatured and annealed DNA as compared to the infectivity of the native DNA (100%) in each experiment.

DISCUSSION

Two classes of ϕ29 DNA molecules were observed in perchlorate-treated preparations, "whole" lengths and very short 0.1- to 0.2-μm lengths. The presence of the very short molecules is unexplained. Since the ϕ29 DNA was released from phage freshly purified on CsCl, it is unlikely that the small molecules originated from degraded DNA which was an external contaminant of the phage suspension. We hesitate to refer to the short pieces of DNA as "fragments," since they are not accompanied by a spectrum of longer breakage products. However, breaks in single chains near the ends of whole molecules might render these regions perferentially shear-fragile. Moreover, the action of contaminating deoxyribonuclease cannot be ruled out since the DNA was released in the presence of magnesium. Freifelder (4) reported, however, that deoxyribonuclease is rendered ineffective by 5 M sodium perchlorate during the release of phage T7 DNA in the presence of magnesium. Finally, the short molecules might be regarded as a second chromosome, were it not for their variability in length. Since the small molecules were only about 1 to 3.5% the length of whole molecules and the composite error in length measurements was estimated at about 3%, evidence for the origin of small pieces from ends of whole molecules was not obtained. Whether there are equal numbers of 0.1- to 0.2-μm molecules and whole molecules is not yet known. Length distributions of phage T3 DNA published by Lang et al. (6) showed relatively large numbers of small DNA fragments, but the T3 data differed from the present length distributions of ϕ29 DNA in that the T3 preparations contained a spectrum of DNA fragments intermediate in length between very short pieces and whole lengths.

It was previously shown that phenol-extracted ϕ29 DNA gives skewed distributions of lengths, with observed modal class values ranging from 5.7 to 5.9 μm. These values agree with the 5.8-μm length of ϕ29 DNA released with sodium perchlorate. Phenol- and perchlorate-extracted ϕ29 DNA molecules differ, however, in that a spectrum of fragments of varying length are found in phenol preparations but not in perchlorate samples.

Values for the molecular weight of ϕ29 DNA obtained from analytic band sedimentation agree well with molecular weight values obtained from electron microscopy. The length of ϕ29 DNA has also been confirmed by Bott (2).

The denatured forms of ϕ29 DNA are apparently the single strands of the native duplex molecule since the molecular weight of denatured ϕ29 DNA in both neutral and alkaline CsCl is about half that of the native form as determined by Studier's (10) empirically derived relationships between S and M. Since strand separation was efficient when ϕ29 DNA was denatured at a concentration of 50 μg/ml or less in 0.1 M NaOH, the full-length linear duplex molecules formed by annealing resulted from random association of single chains with a unique sequence. Denaturation of a collection of molecules with cyclic permutations of the same sequence would yield single polynucleotide chains of different permutation. These single chains, as in the case of phage T2 DNA (7), would anneal to form overlapped structures and then circular molecules. Therefore, phage ϕ29 DNA resembles T3 and T7 DNA in that reformed molecules are full-length, linear duplexes.

Circular molecules were not obtained by merely annealing perchlorate-extracted ϕ29 DNA in five times concentrated SSC at 60 C, but were readily prepared by annealing phenol-extracted ϕ29 DNA under similar conditions (Charles A. Thomas, Jr., *personal communication;* D. L. Anderson, *unpublished data*). Therefore, the cohesive ends of ϕ29 DNA are apparently preserved by phenol extraction, but one or both of the ends are altered or lost during the perchlorate treatment. If the small 0.1- to 0.2-μm molecules found in perchlorate preparations are indeed ends of whole molecules, then a cohesive end would be attached to these fragments. Alternatively, cohesive ends may have been eliminated by nuclease activity during isolation of the DNA.

The partial rather than complete restoration of the transfecting activity of ϕ29 DNA upon annealing is unexplained, although slight imperfections were noted in some of the annealed linear duplex molecules. Unrenatured short regions of otherwise duplex molecules were frequently observed by electron microscopy.

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