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Circular Deoxyribonucleic Acid from *Shigella dysenteriae* Y6R

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Circular deoxyribonucleic acid was isolated from *Shigella dysenteriae* Y6R and was found to consist of six species having molecular weights of 10^6, 1.3×10^6, 2.6×10^6, 3.8×10^6, 20×10^6, and 24×10^6 daltons. These size classes were partially resolved by sucrose density gradient centrifugation. The minicircles (10^6 and 1.3×10^6) were found to have a buoyant density in CsCl of 1.710 g/ml. The 3.8×10^6 dalton class had a density of 1.707 g/ml. The two largest species had a density of 1.702 g/ml. Two other strains, *S. sonnei* II and *S. dysenteriae* 60, also contained circular deoxyribonucleic acid.

Covalently closed circular duplex deoxyribonucleic acid (DNA) is more resistant to irreversible denaturation than native DNA in any other topological configuration (15; M. Rush and R. C. Warner, *submitted for publication*). It is therefore possible to treat a cellular lysate with alkali and then neutralize it, with the result that, of all of the DNA molecules present, only those having closed strands will remain intact. The separation of native, circular, duplex DNA from alkali-denatured, single-stranded DNA is accomplished by nitrocellulose chromatography, since single-stranded material binds to nitrocellulose whereas native DNA does not. We report here the isolation by this technique of six different species of circular DNA present in *Shigella dysenteriae* Y6R.

MATERIALS AND METHODS

S. dysenteriae Y6R was obtained from I. Tessman; *S. dysenteriae* 60 and *S. sonnei* II, from W. L. Barksdale. Ethidium bromide was obtained from Boots Pure Drug Co. Ltd.; lysozyme, from Worthington Biochemical Corp., Freehold, N.J.; and ^{32}P-orthophosphoric acid, from E. R. Squibb & Sons, New York, N.Y.

The methods employed for preparative and analytical ultracentrifugation (13, 14) and for electron microscopy (12, 13) were described previously.

Preparation of circular DNA. *S. dysenteriae* Y6R was grown at 37 C to stationary phase in 1 liter of modified 3XD medium (3), washed with four 100-ml portions of 0.15 M ethylenediaminetetraacetate, *p*H 8.0, and frozen in 100 ml of this buffer. Cells were lysed by thawing in the presence of 10 μg of lysozyme per ml, and circular DNA was isolated as previously described for the penicillinase plasmid of *Staphylococcus aureus* (12). An average of 100 μg of pure form I (covalently closed, circular duplex DNA) was obtained per liter of culture. ^{32}P-labeled circular DNA was prepared by adding 20 mc of carrier-free orthophosphoric acid to cells in X medium (8) 1 hr before harvesting. A specific activity of 4,000 counts per min per μg of DNA was obtained.

RESULTS

Basic properties of S. dysenteriae Y6R circular DNA. As indicated in the ultracentrifuge scans

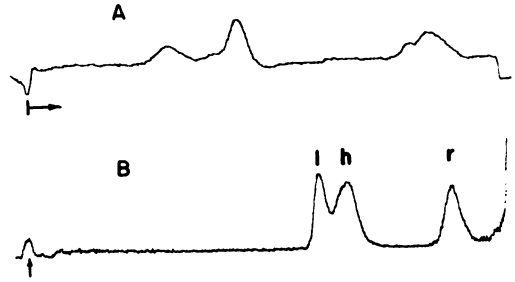

FIG. 1. *Analytical ultracentrifugation of isolated circular DNA. (A) Ultraviolet scan of bands present after 41 min of zonal centrifugation through 3 M CsCl, 0.15 M NaCl, 0.015 M Na citrate at 40,000 rev/min and 20 C. (B) Ultraviolet scan of bands present after 36 hr of centrifugation in CsCl at 48,000 rev/min and 25 C. The lighter sharp band (l) has a buoyant density of 1.702; the heavier broad band (h) has an average density of 1.711. The very heavy band (r) is denatured DNA of Micrococcus luteus, a marker with a density of 1.742. A total of about 1 μg of circular DNA was present in each experiment. An arrow indicates the meniscus. By estimating the fraction of total DNA representing minicircular molecules (1.3×10^6 and 1×10^6 daltons) from the scan in A, and by assuming a 50% preparative yield, it can be calculated that each cell contains about 10 molecules of this size.*

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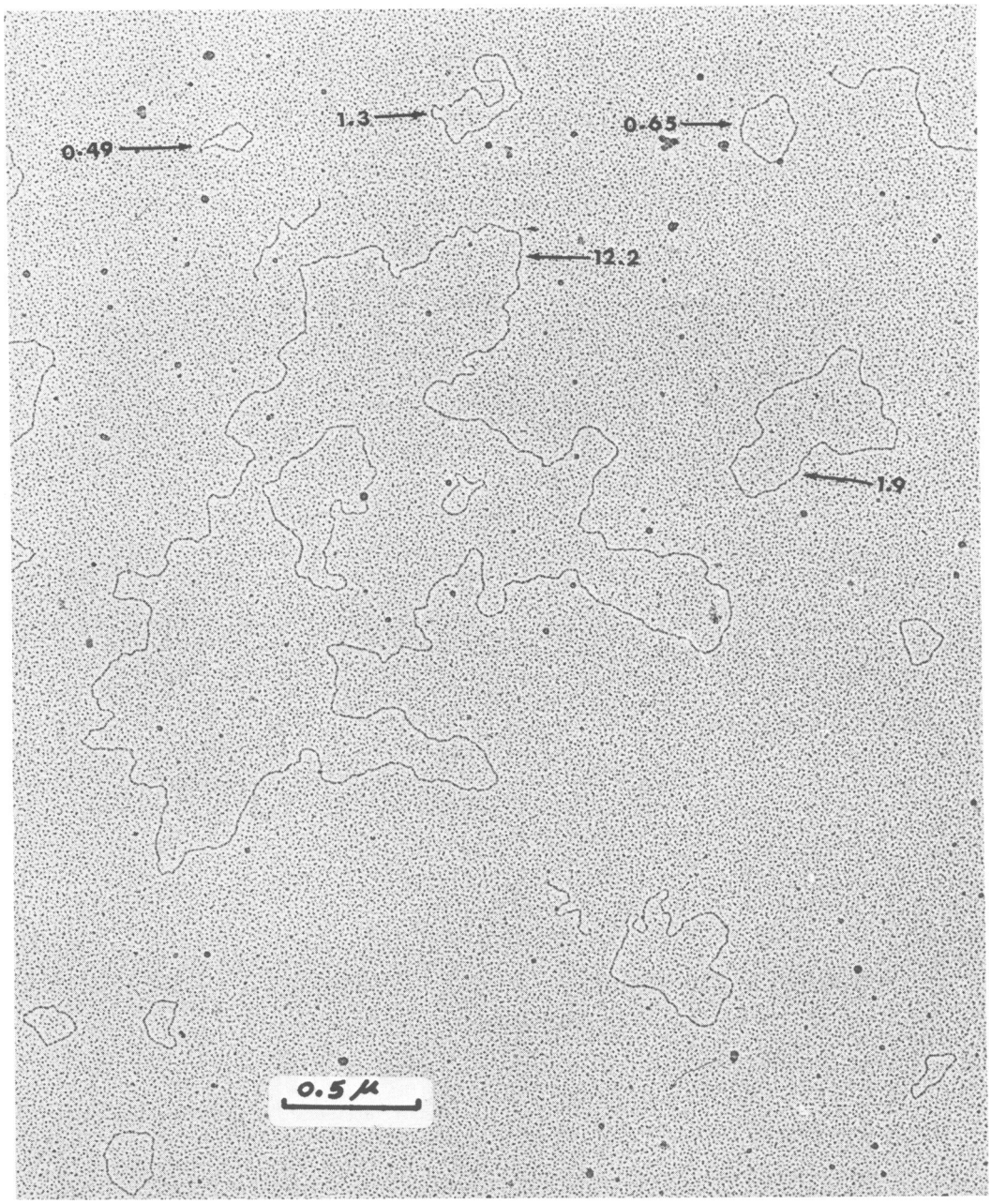

FIG. 2. *Electron micrograph of unresolved circular DNA of S. dysenteriae Y6R, showing members of five of the six size classes present in this strain. Mean contour lengths are indicated by the arrows. Not shown is a molecule representing the class with a contour length of 10.2 μm.*

shown in Fig. 1, and confirmed by the electron micrograph in Fig. 2, form I DNA isolated from *S. dysenteriae* Y6R contained several molecular species. The contour lengths and estimated molecular weights of the six distinct circular molecules present are listed in Table 1. The five peaks identifiable in a tracing of a sedimentation run (Fig. 1A) were therefore assigned to unresolved minicircles [a general term (9) referring to molecules with molecular weights in the range of 10^6 daltons] and to resolved species with molecular weights of 2.6×10^6, 3.8×10^6, 20×10^6, and 24×10^6 daltons. Since the separation of bands in buoyant CsCl, as shown in

TABLE 1. *Mean contour lengths and estimated molecular weights of the six circular DNA species present in S. dysenteriae Y6R*[a]

Species	No. of molecules measured	Mean length ± SD (μm)	Estimated molecular wt (daltons)
1	134	0.487 ± 0.014	0.97 × 10^6
2	35	0.652 ± 0.017	1.3 × 10^6
3	30	1.30 ± 0.03	2.6 × 10^6
4	30	1.91 ± 0.03	3.8 × 10^6
5	16	10.2 ± 0.10	20 × 10^6
6	23	12.2 ± 0.12	24 × 10^6

[a] Molecular weights were calculated by assuming a value of 2 × 10^6 daltons/μm for the sodium salt of DNA.

Fig. 1B, depends upon base composition and not size, it was necessary to resolve the mixture before assigning a buoyant density to each size class.

Isolation of individual molecular species of S. dysenteriae Y6R circular DNA. After a preliminary ethidium bromide-CsCl gradient clarification (Fig. 3) to remove the small amount (1 to 2%) of contaminating linear host cell DNA present in the preparation, ^{32}P-form I DNA was partially resolved into its component fractions by means of successive sucrose gradient centrifugations (Fig. 4). Thus, fractions 1 to 6 of the gradient represented in Fig. 4A contained a mixture of large rings (20 × 10^6 and 24 × 10^6 daltons), and fractions 19 to 28 of the same gradient contained an unresolved mixture of the remaining components. As can be seen in Fig. 4B, these components were partially resolved after centrifugation through a new gradient. By means of this procedure, pure samples of minicircular DNA (fractions 20 to 23 in Fig. 4B) and of 3.8 × 10^6 daltons DNA (fractions 8 to 12 in Fig. 4B) were obtained. The 2.6 × 10^6 dalton species was only partially resolved in this gradient.

Properties of individual molecular species of S. dysenteriae Y6R circular DNA. An electron micrograph of a preparation of minicircular molecules is shown in Fig. 5. This preparation contained two distinct classes of DNA, as is evident from the contour length histogram presented in Fig. 6, and formed a single broad peak in buoyant CsCl (48,000 rev/min) with a density of 1.710. Resolved 3.8 × 10^6 dalton molecules had a density of 1.707, and a mixture of the large rings (20 × 10^6 and 24 × 10^6 daltons) formed a single sharp band with a density of 1.702.

Isolation of circular DNA from two other Shigella strains. To learn whether the presence of any or all of these six circular DNA species is typical of the genus *Shigella*, we attempted to isolate covalently closed molecules from two other strains, *S. sonnei* II and *S. dysenteriae* 60. Both strains did contain such forms. Although they were apparently not the same size as those present in Y6R, the measurements were not sufficiently precise to establish clearly that they are different. *S. sonnei* II contained three species with molecular weights of 1.4 × 10^6, 3.4 × 10^6, and 4.8 × 10^6 daltons, whereas *S. dysenteriae* 60 contained only one species with a molecular weight of 2 × 10^6 daltons.

DISCUSSION

Both form I and form II of circular duplex DNA have been isolated from a wide spectrum of bacterial sources. In some cases, these molecules have been identified as episomes (2, 4) or as plasmids (6, 10), allowing for detailed correlations among their genetic, biochemical, and physical characteristics. In others, the functional significance of the isolated molecules has remained obscure (1, 5, 11); the circular DNA

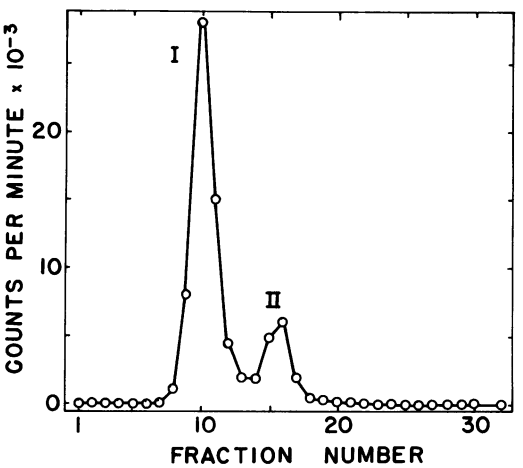

FIG. 3. *Sedimentation equilibrium of purified ^{32}P-labeled circular DNA in CsCl containing ethidium bromide. The solution contained 700 μg of ethidium bromide, 75 μg of DNA (1.3 × 10^3 counts per min per μg), and 0.07 mmoles of tris(hydroxymethyl)amino methane-hydrochloride pH 7.5, in 7.0 ml of CsCl, density 1.59 by refractometry. Fractions were collected by means of a microsiphon after 44 hr of centrifugation in a type 65 angle head rotor at 50,000 rev/min and 20 C, and were assayed by scintillation counting. The density increases from right to left. Although some of the species in the circular DNA preparation differ slightly in buoyant density (Fig. 1B), only a single heavy band (I) was formed during preparative ethidium bromide-CsCl gradient centrifugation. Fractions 6 to 12 were combined, dialyzed against 0.015 M NaCl, 0.0015 M Na citrate, and concentrated by flash evaporation to a volume of 100 μliters.*

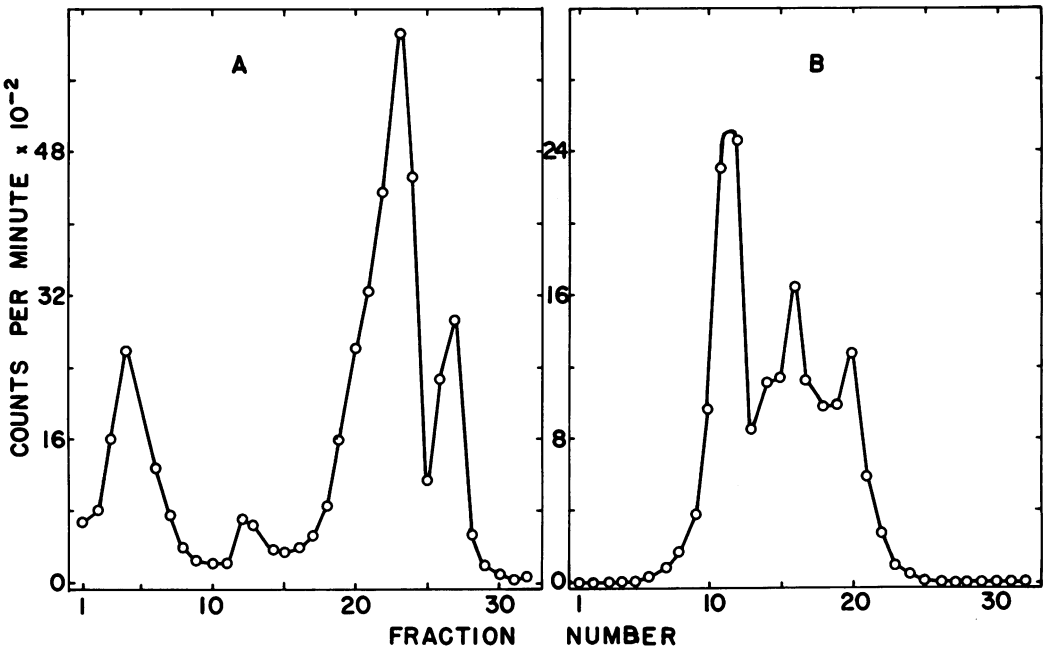

FIG. 4. *Sucrose gradient sedimentation of ^{32}P-labeled circular DNA. Linear gradients (13 ml) prepared from 5 to 23% (w/v) sucrose in 0.15 M NaCl, 0.015 M citrate were centrifuged at 40,000 rev/min in a Spinco SW 41 rotor. (A) DNA (60 μg) from the heavy ethidium peak represented in Fig. 3 after 5 hr of centrifugation at 4 C. Fractions 19 to 28 were pooled, dialyzed, and concentrated to a volume of 200 μliters. (B) Combined fractions 19 to 28 from A, and another gradient similar to A, after 8.5 hr of centrifugation at 5 C through another gradient. The ordinates are the total counts per minute in each fraction.*

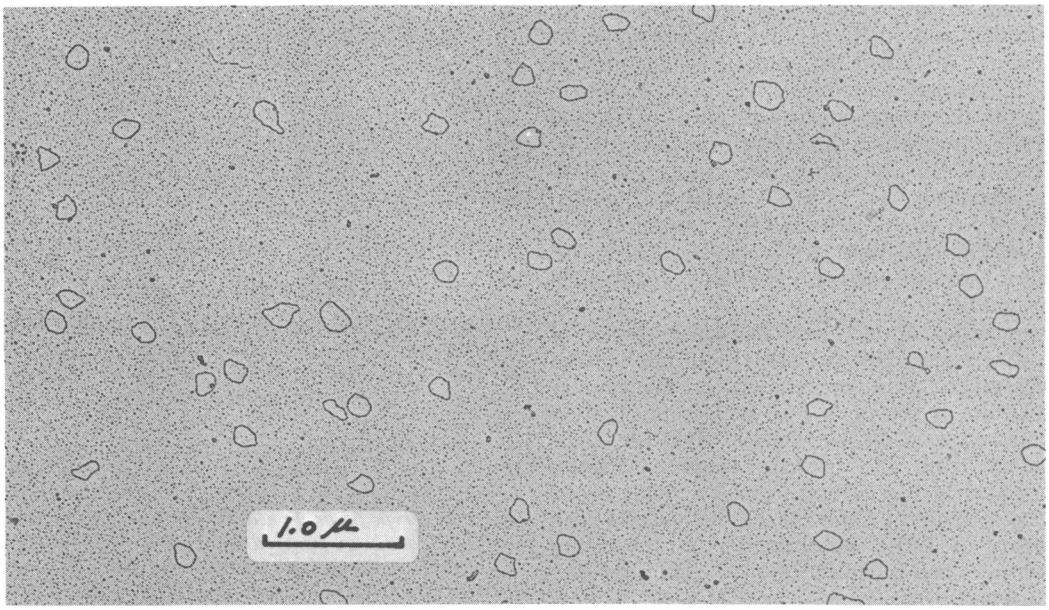

FIG. 5. *Electron micrograph of resolved minicircular DNA, showing members of the two size classes with mean contour lengths of 0.49 and 0.65 μm.*

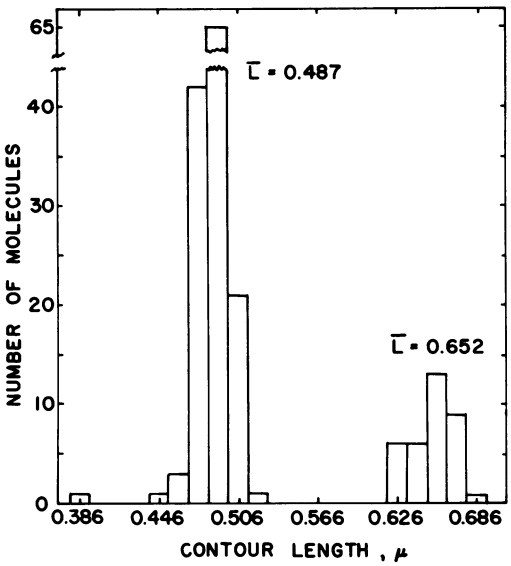

FIG. 6. *Contour length distribution of minicircular DNA. Of the 169 molecules randomly chosen, 134 were of the smaller class (mean length: 0.487 ± 0.014 μm), and 35 were of the larger class (mean length: 0.652 ± 0.017 μm).*

isolated from *S. dysenteriae* Y6R, and described in this paper, falls into the latter class. Nevertheless, it is tempting to speculate on the possible functions of such species. In light of present knowledge regarding the types of genetic characteristics determined by extrachromosomal elements (6), resistance to antimicrobial agents such as antibiotics, heavy metals, and other metabolic poisons would appear to be the best candidates. If these were the characteristics determined, one might even suspect that, because of its size, the 20 × 10^6 dalton species is a sex factor, the 3.8 × 10^6 dalton species is a nontransferable plasmid, and the 24 × 10^6 dalton molecule is a recombinant of the two. It is also possible that one or more of the species is due to a phage, present either as an autonomous prophage or as an integrated one in which occasional induced cells yield progeny phage DNA. However, preliminary investigations pertaining to such properties in *S. dysenteriae* Y6R were unrevealing. Circular DNA was isolated from two other strains of *Shigella* in an attempt to see whether any of these six DNA molecules is characteristic of *Shigella*. However, as previously noted, the circular DNA molecules isolated from these strains appear to be unrelated to those from Y6R.

Because of their unusually small size, the 10^6 and the 1.3 × 10^6 dalton species (minicircular DNA) present in Y6R are of special interest. Such molecules can code for only a limited number of proteins or specific ribonucleic acids, and it is not apparent why a cell possessing more than 10 copies of such a molecule would have an evolutionary advantage over one in which this information is present in only one copy. Nevertheless, minicircular DNA has been identified both in bacterial (1, 5) and in animal (16) cells.

The possibility of preparing pure samples of the various size classes of circular DNA from Y6R and other *Shigella* strains should allow detailed biochemical investigations of the nature, origin, and replication of these molecules.

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ADDENDUM IN PROOF

Circular DNA has also been isolated from *Shigella* by H. S. Jansz, J. Zandberg, J. H. vander Pol, and E. F. J. van Bruggen (Eur. J. Biochem. 9:156, 1969).

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