*EVIDENCE FOR CIRCULAR DNA FORMS OF A BACTERIAL PLASMID**

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Colicinogenic factor E_1 (*Col* E_1) is a stable extrachromosomal genetic element present in certain strains of enteric bacteria. This genetic factor determines the production of an extracellular antibiotic protein, termed colicin E_1, which is active against related enteric bacteria. Normally, the production of colicin E_1 occurs spontaneously in only a small proportion of the population of colicinogenic cells, but its synthesis in the majority of the cells in a colicinogenic culture can be induced by agents that are known to interfere with DNA synthesis.[1-3]

An extrachromosomal state for the *Col* E_1 factor has been demonstrated both by studies on the genetic properties of this factor in *Escherichia coli*[4-6] and by studies of the physical and chemical properties of the *Col* E_1 factor in *Proteus mirabilis*.[7] The *Col* E_1 factor has been separated from *Proteus* chromosomal DNA and shown to be double-stranded DNA with a buoyant density of 1.710 gm/cm^3 in a cesium chloride density gradient.[7] Since genetic studies to date have failed to demonstrate an alternative state of integration of the *Col* E_1 factor within the bacterial chromosome, the *Col* E_1 factor is classified as a bacterial plasmid rather than an episome.

A circular configuration has been established for DNA isolated from a number of different sources. The DNA of animal viruses[8-13] and mitochondria[14-18] and the intracellular forms of bacteriophage DNA[19-23] have been shown to exist in a circular, double-stranded form. In addition, genetic[24] and radioautographic studies[25] have indicated a circular configuration for the chromosome of *Escherichia coli*. In the present study, circular forms of DNA are described for a bacterial plasmid, colicinogenic factor E_1, in the organism *Proteus mirabilis*.

Materials and Methods.—Bacterial strain: A streptomycin- and sodium azide-resistant strain of *P. mirabilis* (AC 2505 from A. B. Pardee), requiring nicotinic acid for growth, was made colicinogenic for the *Col* E_1 factor by crossing the *Proteus* strain with an *E. coli* Hfr donor carrying the *Col* E_1 factor. The source of the *Col* E_1 factor is *E. coli* K-30 (isolated by P. Fredericq). The *Proteus Col* E_1+ strain used in these studies produced colicin E_1 with an antibiotic specificity range indistinguishable from the colicin E_1 produced by the *E. coli* K-30 strain. In addition, the colicin produced by *Proteus* E_1+ was immunologically indistinguishable from *E. coli* colicin E_1 by double-diffusion analysis and did not cross-react with antibody specific for *E. coli* colicin E_2.[26]

Spontaneously occurring noncolicinogenic variants of the *Proteus Col* E_1+ strain were isolated at a frequency of 1% after plating a logarithmically growing culture on nutrient agar plates.

Reagents: Lysozyme, trypsin treated with di-isopropyl phosphorofluoridate (DFP), and ribonuclease (3 × crystallized) were purchased from Worthington Biochemical Corporation. Salmon sperm DNA was purchased from Calbiochem.

Growth conditions: The media employed were previously described.[7] The *Proteus Col* E_1+ strain was transferred from a slant and grown overnight in enriched nutrient broth. The culture was diluted and spread on enriched nutrient agar plates, and a single colicinogenic colony was isolated and grown overnight in 10 ml of phosphate-buffered minimal medium as previously described,[7] with the exception of radioisotope and deoxyadenosine. Two liters of the phosphate-buffered minimal medium were inoculated with the overnight culture and the culture was allowed to grow at 37°C with vigorous agitation to a concentration of 8×10^8 cells per ml. The cells were then harvested by centrifugation in the cold.

DNA extraction: Cells harvested from 2 l of media were washed one time with 100 ml of cold TS (0.03 *M* tris, 0.05 *M* NaCl, pH 8.0) and suspended in 75 ml of cold TS. Cells were lysed by the addition of 40 mg lysozyme (dissolved in 4 ml TS) and 4 mg of ribonuclease (dissolved in 1 ml TS) followed by gentle shaking on a rotary shaker for 10 min at 37°C. The suspension was then cooled to 10°C and 20 ml of 0.1 *M* ethylenediaminetetraacetate (EDTA) was added. After 5 min at this temperature, 25 ml of 2.5% sodium lauryl sulfate was added and the suspension incubated at 37°C to 45°C until lysis occurred. Twenty ml of 5 *M* sodium perchlorate, 120 ml of TES (0.05 *M* NaCl, 0.005 *M* EDTA and 0.03 *M* tris, pH 8.0) saturated phenol (redistilled), and 60 ml of chloroform were then added to the lysate and the mixture was rotated on a jar mill at room temperature for 30 min. The organic phase and the middle protein layer were removed after centrifugation and the extraction of the aqueous phase was repeated. Two vol of absolute ethanol were layered on top of the aqueous solution of DNA and the DNA precipitate was collected on a glass rod by gentle stirring. The DNA precipitate was dissolved in 50 ml of cold dilute saline-sodium citrate (0.015 *M* NaCl, 0.0015 *M* sodium citrate, pH 7.0). Generally 1 day was allowed for complete solution of the DNA precipitate. The salt concentration was adjusted to 0.15 *M* NaCl and 0.015 *M* sodium citrate, pH 7.0, 14 mg of ribonuclease (pretreated to remove deoxyribonuclease by heating at 80°C for 10 min in 2.4 ml of 0.15 *M* NaCl, pH 5) was added and the solution incubated at 37°C for 120 min with gentle rotation. The solution was deproteinized by the addition of phenol and chloroform, rotation on the jar mill, and centrifugation two successive times as described before. The DNA was again collected on a stirring rod following the addition of 2 vol of absolute ethanol. The precipitate was then dissolved in 50 ml of dilute saline-sodium citrate.

DNA concentration was determined by a diphenylamine colorimetric assay.[27] Salmon sperm DNA was used as the DNA standard for this assay.

DNA fractionation: DNA was fractionated on a methylated albumin kieselguhr (MAK) column by the method of Sueoka and Cheng[28] as previously described.[7]

Density-gradient equilibrium centrifugation: Analytical equilibrium centrifugation in a cesium chloride gradient was performed by the method of Meselson, Stahl, and Vinograd[29] as previously described.[7] N^{15} *Pseudomonas aeruginosa* DNA (kindly supplied by F. Hickson) was employed as reference DNA. Densities of DNA were determined by reference to the peak density of the N^{15} *Ps. aeruginosa* DNA which was taken to be 1.742 gm/cm³.[30]

Alkali denaturation-renaturation: MAK column fractions of DNA, which contained 2 to 4 μg/ml of DNA in 0.7 *M* NaCl and 0.05 *M* sodium phosphate, pH 7.0, were adjusted to 0.1 *N* NaOH by the addition of 1 *N* NaOH and chilled. The pH of the DNA solution was approximately 12.2. After 5 min at 0°C the DNA solution was adjusted to pH 8.5 by the addition of a mixture of 0.3 *N* HCl and 0.7 *M* tris.

Electron microscopy: DNA, taken directly from the MAK column, was gently mixed with a 0.2% solution of DFP-treated trypsin in 0.1 *M* ammonium acetate so that the final concentration of DNA was 1–4 μg/ml. After the slow drawing of the DNA into a motor-driven large-bore pipette, the solution was delivered down a wet glass rod onto the surface of an aqueous solution of 0.1 *M* ammonium acetate. The resultant film was picked up and shadowed essentially as described by Kleinschmidt and Zahn,[31] subsequently modified by Stoeckenius.[9] Micrographs were taken on glass plates in a Philips 200 electron microscope in which the magnification was carefully calibrated at least once in every 12 plates with a carbon grating replica of 2,160 lines per mm.

To determine the length of the DNA molecules, the negative was projected, the DNA contour traced, and string carefully laid along the contour. From the known magnification the lengths were then calculated (Figs. 5, 6).

Results.—Isolation of Col E_1 from Proteus Col E_1+: *Proteus mirabilis Col E_1+* possesses chromosomal DNA with a buoyant density in a cesium chloride gradient of 1.700 gm/cm³, and a relatively small amount of satellite DNA with a buoyant density of 1.710 gm/cm³.[7] This satellite DNA, corresponding to the *Col* E_1 factor, is present in the amount of 0.2 to 0.3 per cent of the total DNA. Fractionation on an MAK column of *Proteus Col* E_1+ DNA, prepared by the Marmur procedure, results in an enrichment of *Col* E_1 DNA in the initial column fractions that contained DNA.[7] If the DNA is prepared by a phenol procedure, *Col* E_1 DNA can be ob-

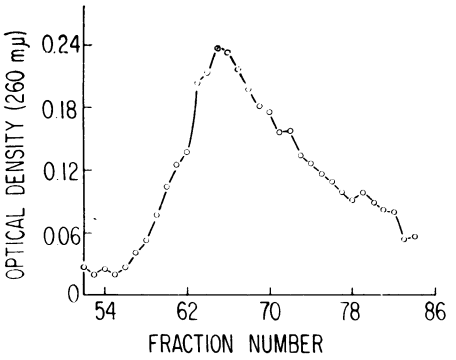

FIG. 1.—DNA elution profile of *Proteus* E_1^+ DNA on a methylated albumin column. Approximately 2 mg of DNA was added to a 3.0 cm × 4.0 cm column of MAK. Linear gradient elution was employed, using 110 ml of initial buffer (0.6 *M* NaCl, 0.05 *M* sodium phosphate, pH 6.7, and 110 ml of final buffer (0.9 *M* NaCl, 0.05 *M* sodium phosphate, pH 6.7). Fractions of approximately 2 ml were collected.

tained in essentially a pure form in the first or second column fractions that contained DNA. Presumably, this is due to a greater size of the average chromosomal fragment isolated by the phenol procedure and the preferential retention of larger DNA molecules by the MAK column under these chromatographic conditions. The results of an MAK column fractionation of DNA from *Proteus Col* E_1^+ prepared by the phenol procedure are shown in Figure 1. Figure 2 represents microdensitometer tracings of the initial three fractions of the MAK column that contain DNA. As shown in Figure 2, the first two fractions consist of essentially pure *Col* E_1 DNA.

Reversible alkali denaturation of Col E_1 DNA: Alkali denaturation and rapid neutralization of linear DNA or open circular double-stranded DNA (containing at least one single-strand break) increase its buoyant density in CsCl by approximately 0.015 gm/cm^3.[32, 33] Treatment of covalently intact, circular duplex DNA with alkali followed by neutralization does not result in denaturation or a change in buoyant density.[9] Purified satellite DNA, obtained by fractionation of the DNA

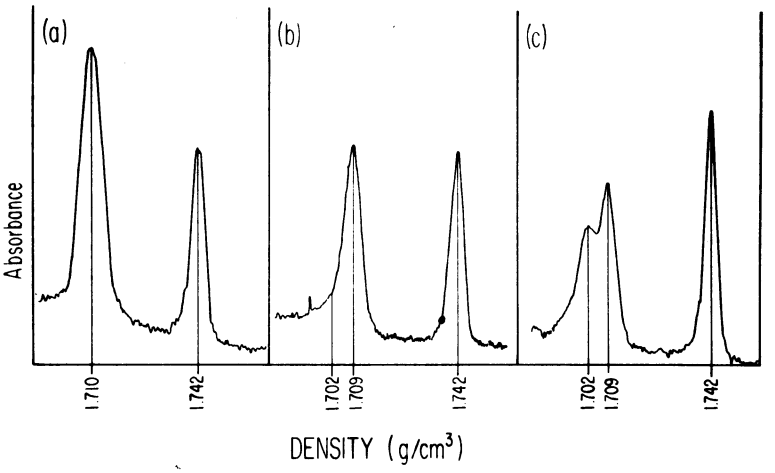

FIG. 2.—Microdensitometer tracings of the results of cesium chloride equilibrium centrifugation of the DNA of the first 3 DNA-containing fractions of the column described in Fig. 1. (*a*) Fraction 59. (*b*) Fraction 60. (*c*) Fraction 61.

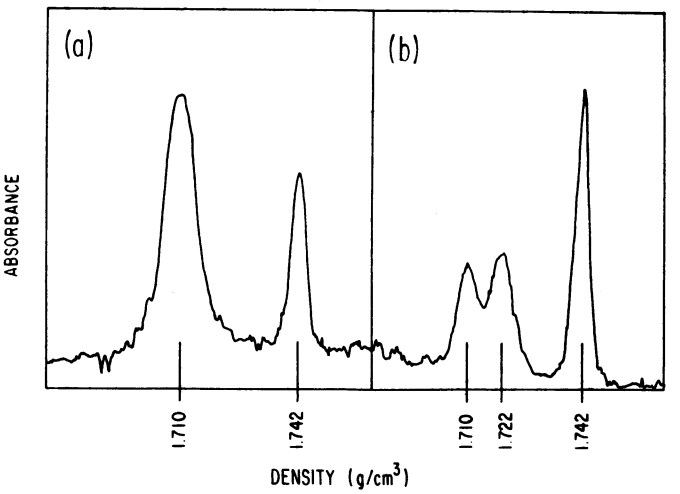

FIG. 3.—Microdensitometer tracings of the results of cesium chloride equilibrium centrifugation of *Col* E_1 DNA obtained by chromatography on an MAK column. (*a*) Untreated *Col* E_1 DNA. (*b*) *Col* E_1 DNA treated with alkali and neutralized as described in *Materials and Methods*.

of *Proteus Col* E_1+ on an MAK column, was denatured with 0.1 *N* alkali, neutralized, and analyzed by centrifugation in a CsCl density gradient. As shown in Figure 3, approximately 50 per cent of an MAK column preparation of essentially pure satellite DNA failed to be denatured by this treatment. In some MAK column preparations approximately 90 to 100 per cent of the satellite DNA resisted denaturation by this treatment. Under these conditions preparations of *P. mirabilis* chromosomal DNA or *E. coli* DNA, either unfractionated or fractionated on an MAK column, were completely denatured, as indicated by a displacement of these DNA preparations to a higher buoyant density.

Examination of Col E_1 DNA by electron microscopy: MAK column fractions containing pure *Col* E_1 DNA were also examined by electron microscopy. The DNA filaments observed had the typical appearance of double-stranded DNA largely in the form of supercoiled circles (Fig. 4a). In addition to this configuration, approximately 10 to 20 per cent of the circular molecules obtained from the initial fractions of the MAK column appeared in an open circular form (Figs. 4a and *b*). The contour length of these open and twisted circular molecules was calculated for 47 randomly chosen strands. An average length of 2.33 μ was determined with a standard deviation of ± 0.06 μ (Fig. 5).

In addition to the 2.3-μ length class of circular *Col* E_1 DNA, open and supercoiled circular forms of approximately twice this contour length were observed. These 4.7-μ molecules are illustrated in Figures 4*c*, *d*, and *e*. Open circular forms of this larger size class have been observed without any overlapping or twisting of the strands. The length distribution for 29 randomly chosen strands of this class of *Col* E_1 DNA gives an average contour length of 4.73 ± 0.10 μ (Fig. 6). The proportion of 2.3-μ and 4.7-μ forms of *Col* E_1 in a bacterial population could not be determined accurately from these data, since only the initial one or two fractions of the

total eluate from the MAK column were examined. Since the MAK column fractionates on the basis of size in addition to the guanine-cytosine content of the DNA,[28]

FIG. 4.—Electron micrographs taken of *Col* E_1 DNA purified by MAK column chromatography. In every case except that shown in (*e*) the DNA was extraced by the phenol procedure described in *Materials and Methods.*

(*a*)Open and supercoiled circular DNA of the 2.3-μ class. × 49,000.

(*b*) An open 2.3-μ circular molecule juxtaposed to its supercoiled allomorph. × 56,000.

(*c*) Supercoiled forms (2.3 μ and 4.7 μ). × 56,000.

(*d*) Open circular forms (2.3 μ and 4.7 μ). × 56,000.

(*e*) Supercoiled circular form of *Col* E_1 DNA, 4.7-μ, extracted by the Marmur procedure.[7] Super-coiled forms thus extracted appeared more tightly twisted. × 56,000.

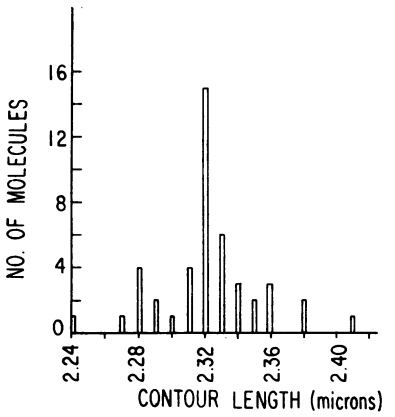

FIG. 5.—Contour lengths of 47 strands of the 2.3-μ size class.

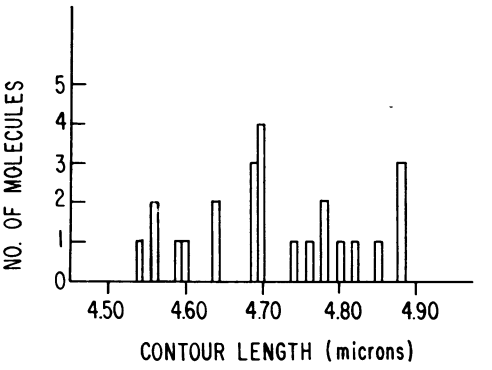

FIG. 6.—Contour lengths of 29 strands of the 4.7-μ size class.

the samples examined by electron microscopy probably were enriched for the 2.3-μ class of circular *Col* E_1 DNA. Studies presently being carried out on the total population of molecules of *Col* E_1 DNA indicate that the ratio of 2.3-μ to 4.7-μ supercoiled molecules varies from 2:1 to 4:1 in different *Col* E_1 DNA preparations.[34] Circular molecules of a length other than the 2.3-μ and 4.7-μ categories were not observed in the electron-microscopic preparations. In addition to the coiled and fully extended cyclic molecules, linear molecules of a variety of lengths were observed in various preparations (Fig. 4*a*). Most of the rod-shaped molecules were shorter than or equal in length to the circular forms and probably represent fragmented circular *Col* E_1 DNA and some contaminating chromosomal DNA. However, these studies do not rule out the possibility of naturally occurring linear forms of the *Col* E_1 DNA.

Circular forms of DNA molecules were not observed in several identically prepared preparations of DNA from the noncolicinogenic *P. mirabilis* parent strain, or in DNA preparations of several independently isolated noncolicinogenic spontaneous variants of the *P. mirabilis Col* E_1+ strain. As shown previously,[7] these noncolicinogenic strains do not contain the satellite DNA of a buoyant density of 1.710 gm/cm^3 that is characteristic of the *Col* E_1 DNA.

Discussion.—A previous study on colicinogenic factor E_1 established that this bacterial plasmid in *P. mirabilis* consists of double-stranded DNA with a buoyant density of 1.710 gm/cm^3 in a cesium chloride gradient.[7] It was shown that when a *Proteus* cell acquires the ability to produce colicin E_1 it also acquires this species of DNA, and when the cell spontaneously loses the ability to produce colicin E_1, it loses this "satellite" DNA. Finally, *Col* E_1 DNA, purified from a mixture of *P. mirabilis Col* E_1+ cells labeled with H^3-thymine and *P. mirabilis Col* E_1− cells labeled with C^{14} thymine was shown to contain only H^3 thymine.[7]

In the present study the alkali denaturation properties and electron-microscopic analysis of the *Col* E_1 factor from noninduced cells indicate that the DNA of the noninduced *Col* E_1 factor is of a circular configuration. Moreover, two types of circular forms with respect to size are observed, circular forms of approximately 2.3-μ and 4.7-μ contour lengths. Both extended open and coiled circular forms are

observed for each type. The conclusion that both types represent the *Col* E_1 factor is based upon the buoyant density of 1.710 gm/cm^3 for both types and the observation that a cell acquires simultaneously the 2.3-μ and 4.7-μ circular classes upon acquisition of the ability to produce colicin E_1. Furthermore, a *Proteus Col* E_1^+ cell that loses the ability to produce colicin E_1 also simultaneously loses both the 2.3μ- and 4.7-μ circular forms. Studies are presently being carried out to determine if the 4.7-μ form represents a duplication of the genetic information possessed by the 2.3-μ molecule, or if it contains additional genetic information.

The response of *Col* E_1 DNA to treatment with alkali, followed by rapid neutralization, and the appearance of *Col* E_1 DNA upon electron microscopic examination indicate that the circular forms of the *Col* E_1 DNA consist of two covalently closed circular strands that are in a supercoiled configuration similar to that initially described for polyoma DNA.[11] Studies are in progress to obtain additional physical chemical evidence for the supercoiled configuration of the *Col* E_1 factor. Supercoiled double-stranded circular forms have also been shown for the DNA of SV40,[11] the replicative form of ϕX174,[19, 20, 35] the intracellular forms of bacteriophage λ,[21, 22] M13,[23] and mitochondrial DNA from several sources.[14, 15] As in the case of *Col* E_1 DNA, the cyclic DNA from the animal and bacterial viruses is predominantly in the supercoiled configuration with a much smaller proportion of open circular forms.

The contour lengths of 2.3 μ and 4.7 μ correspond to molecular weights of 4.5×10^6 and 9.2×10^6, respectively, assuming that the mass per unit length in these electron-microscopy preparations is similar to the β form of the sodium salt of double-stranded DNA.[36] The size of the *Col* E_1 factor is considerably less than the size estimated by a DNA agar technique for the bacterial episome, the F factor or derivatives of the F factor.[37] The *Col* E_1 factor is also considerably smaller than the circular chromosome of the related bacterium *E. coli*, which has a molecular weight of approximately 2.3×10^9.[25]

It is not known whether the 2.3-μ and 4.7-μ cyclic forms exist simultaneously in the same cell or are present separately in different cells in a bacterial culture. It is clear, however, that in every case tested, the progeny of single cell isolates of a *Proteus Col* E_1^+ culture are capable of giving rise to both cyclic forms. Essentially two possibilities are being considered for the relationship between these two cyclic forms. The 4.7-μ cyclic form may represent a stable intermediate in the duplication of the 2.3-μ form or a by-product of duplication as a result of a recombinational event. The other possibility is the formation of the 4.7-μ cyclic form as a result of the opening of the circular form of two 2.3-μ molecules followed by closure with each other, rather than self-closure. It is also possible that single and double sizes of circular genetic elements are peculiar to transferable extrachromosomal genetic elements in bacteria and is related to the potential for transfer of these elements by conjugation. Experiments are presently being carried out to test these various possibilities. Molecules of twice the usual size of the replicative form of the bacteriophage ϕX174 have also been observed.[38] More recently Radloff, Bauer, and Vinograd[39] reported circular forms of DNA of a variety of size classes in extracts of HeLa cells.

Summary.—*Col* E_1 factor DNA isolated from *P. mirabilis Col* E_1^+ cells resists denaturation by exposure to high pH followed by rapid neutralization. Electron-

microscopic analysis of *Col* E_1 DNA revealed circular double-stranded molecules that were predominantly in a tightly twisted configuration. Approximately 10 to 20 per cent of the molecules were of the open circular type. The supercoiled and open circular DNA forms of *Col* E_1 were of two size classes having contour lengths of 2.33 μ and 4.73 μ.

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