CIRCULAR DNA FORMS OF A BACTERIAL SEX FACTOR*

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Bacteria are known to possess extrachromosomal genetic elements that can be classified as plasmids or episomes. Bacterial plasmids are extrachromosomal genetic elements that exist solely in the autonomous state,[1] while bacterial episomes are capable of existing either in an autonomous state or stably attached to the bacterial chromosome.[2] Certain bacterial sex factors, or genetic elements that promote the transfer of the bacterial chromosome, exhibit the property of episomes (see Clark and Adelberg[3]). Among the episomal sex factors are certain colicinogenic factors, extrachromosomal genetic elements that determine the production of extracellular, antibiotically active proteins, termed colicins. One of these elements, colicinogenic factor VB (ColVB), has been shown in *Escherichia coli* to stably integrate a portion of its genome into the bacterial chromosome as an alternative to existing in an autonomous state.[4, 5] A derivative of the ColVB factor, the *FColVColBtrycys* factor, has been obtained by Fredericq and shown to carry genetic determinants for bacteriophage T1 sensitivity, the production of colicins V and B, and the biosynthesis of tryptophan and cysteine.[4-6] The *FColVColBtrycys* factor also promotes bacterial conjugation and the transfer both of itself and the bacterial chromosome to female cells and, therefore, is a sex factor.[5, 7, 8]

Theoretical considerations and genetic studies of the interaction of episomes with the bacterial chromosome have led to the prediction that sex factors are circular molecules.[9-12] In the present study the sex factor *FColVColBtrycys* was transferred to a *Proteus mirabilis* strain and identified in this strain as double-stranded DNA with a buoyant density of 1.710 gm/cm³ in a cesium chloride gradient. The physical-chemical properties and the appearance in electron micrographs of the isolated *FColVColBtrycys* factor demonstrate that this sex factor is a circular DNA molecule.

Materials and Methods.—Bacterial strains: The *Proteus mirabilis* strain used in these studies was the nicotinic acid-requiring *Proteus* strain AC 2505 (from A. B. Pardee). A tryptophan- and thymine-requiring mutant of this strain was isolated by treatment with N-methyl-N'-nitro-N-nitrosoguanidine[13] and trimethoprim,[14] respectively. This mutant *Proteus* strain was made colicinogenic for the *FColVColBtrycys* factor by crossing with an *E. coli* strain, YS57, carrying this sex factor. The YS57 *FColVColBtrycys* strain was obtained from C. Yanofsky. The *FColVColBtrycys* factor was isolated by P. Fredericq and shown to carry at least the following sequence of genes: F,cysB,tryA,tryB,tryD,tryC,T1,ColV,ColB.[6] Colicins V and B produced by the *Proteus FColVColBtrycys* strain showed an antibiotic specificity range that was indistinguishable from that of the colicin produced by *E. coli* strains carrying this factor.

The sex factor *FColVColBtrycys* is relatively unstable in the *Proteus* strain. Spontaneously occurring noncolicinogenic variants of the *Proteus FColVColBtrycys* strain were obtained at a frequency of 20% by plating on nutrient agar plates a culture growing logarithmically in the presence of tryptophan. To counterselect noncolicinogenic tryptophan-requiring variants formed by the spontaneous loss of the *FColVColBtrycys* factor, cultures of the colicinogenic *Proteus* strain were grown in a medium lacking tryptophan.

Reagents: Lysozyme, diisopropylfluorophosphate-treated (DFP-treated) trypsin, and ribonuclease (3✕ crystallized) were purchased from Worthington Biochemicals. Salmon sperm

DNA was purchased from Calbiochem. Ethidium bromide was obtained as a gift from Boots Pure Drug Co., Ltd., Nottingham, England.

Media: The enriched nutrient broth and agar employed have previously been described.[15] The tris-buffered minimal medium used was the same as previously described[15] except that 0.2% glycerol was used instead of 0.2% galactose, the thymine concentration was adjusted to 6 µg/ml, and deoxyadenosine was not added. Approximately 1.6 mc of [H³] methyl-thymine was added per liter of medium for the labeling experiments.

Growth conditions: The *Proteus FColVColBtrycys* strain was streaked from a tris-buffered minimal culture onto a tris-buffered minimal agar plate. After incubation at 37°, an individual colony producing both colicin V and colicin B was inoculated into 10 ml of the tris-buffered minimal medium and grown at 37° to late log phase. Two liters of tris-buffered minimal medium containing [H³] methyl-thymine were then inoculated with 2.0 ml of the logarithmically growing colicinogenic *Proteus* culture. The culture was allowed to grow at 37° with vigorous agitation to a concentration of 5×10^8 cells per milliliter and then harvested by centrifugation in the cold. The noncolicinogenic variants of this *Proteus* strain were grown the same way except that 20 µg/ml of tryptophan was added to the tris-buffered minimal medium.

DNA extraction: Cells harvested from 2 liters of media were suspended in 80 ml of cold $2 \times BS$ buffer (0.06 M sodium borate, 0.4 M NaCl, pH 9.0). Cold 1 M sucrose (50 ml), 100 mg of lysozyme (dissolved in 10 ml of BS buffer), and 8 mg of ribonuclease (dissolved in 1.4 ml of BS buffer) were then added to the suspension. After gentle mixing in a 37° water bath for 5 min, the suspension was cooled to 10°, and 10 ml of 0.02 M ethylenediaminetetraacetate (EDTA) was added. After 5 min, 50 ml of a 0.9% sodium lauryl sulfate solution was added and the suspension was rotated on a jar mill at 5° until lysis occurred. The lysate was then mixed with 200 ml of BES (0.03 M sodium borate, 0.005 M EDTA, 0.2 M NaCl, pH 9.0)-saturated phenol (redistilled), and rotated on a jar mill for 30 min at 5°. The buffered phenol layer and the middle protein layer were removed after centrifugation and the extraction of the aqueous phase was repeated. The aqueous solution of DNA was then poured into ZnCl₂-treated dialysis tubing[16] and dialyzed against 6 changes of cold BES during a 15-hr period. Fourteen mg of ribonuclease (pretreated to remove deoxyribonuclease activity by boiling for 10 min in 2.4 ml of 0.15 M NaCl, pH 5.0) was then added, and the solution was incubated at 37° for 120 min with occasional gentle shaking. The solution was again deproteinized by the addition of 200 ml of BES-saturated phenol and rotation on a jar mill at 5° for 30 min. After centrifugation, the aqueous DNA solution was dialyzed in ZnCl₂-treated dialysis tubing against 8 changes of cold TES (0.01 M Tris, 0.01 M EDTA, and 0.2 M NaCl, pH 8.0) during a 20-hr period.

DNA concentration was determined by a diphenylamine colorimetric technique[17] using salmon sperm DNA as the DNA standard.

DNA fractionation: DNA was fractionated on a methylated albumin kieselguhr (MAK) column by the method of Sueoka and Cheng[18] as previously described.[15]

Counting of radioisotope: DNA samples were precipitated on 1×1-in. pieces of Whatman no. 3 filter paper by a modification of the procedure of Bollum.[19] The dried filters were immersed in scintillation vials containing 15 ml of purified toluene-PPO-POPOP and counted in a Beckman liquid scintillation counter.

Density-gradient equilibrium centrifugation: Analytical equilibrium centrifugations in cesium chloride gradients were performed by the method of Meselson, Stahl, and Vinograd[20] as previously described.[15] Densities of DNA's were determined by reference to the peak density of N¹⁵ *Pseudomonas aeruginosa* DNA, which had been previously calculated to have a density of 1.742 gm/cm³.[21]

Alkali denaturation-renaturation: Alkali denaturation-renaturation of DNA samples containing 4–6 µg/ml of DNA were performed by the procedure previously described.[22]

Analytical dye-buoyant-density centrifugation: Analytical dye-buoyant-density centrifugation in a cesium chloride gradient was performed by the procedure of Bauer and Vinograd.[23] A DNA solution containing per milliliter, 15 µg of DNA, 15 µg of ethidium bromide, and 900 mg of CsCl, was centrifuged in a Spinco model E ultracentrifuge according to the procedure described above for density gradient equilibrium centrifugation.

Density-gradient preparative centrifugation: Density-gradient preparative centrifugation of DNA in a cesium chloride gradient was performed in a fixed-angle type-50 rotor in a Spinco model L ultracentrifuge using the conditions described by Flamm *et al.*[24] A 1-ml solution con-

taining 9.3 μg of DNA and 832 mg of cesium chloride in TE buffer (0.01 M Tris, 0.001 M EDTA, pH 8.4) was centrifuged at 33,000 rpm for 60 hr at 25°. Fractions (0.05-ml) were collected and aliquots of these fractions were counted as described above.

Electron microscopy: The procedures for the preparation of the DNA for electron microscopy have been previously described.[22] Essentially, the technique of Kleinschmidt and Zahn,[25] subsequently modified by Stoeckenius,[26] was employed. The DNA specimens were rotary-shadowed with uranium and examined in a Phillips 200 electron microscope. Contour lengths were determined as previously described.[22]

Results.—Buoyant density characterization of DNA from Proteus FColVColBtrycys: DNA, prepared from the *Proteus FColVColBtrycys* strain by the phenol procedure, was examined by equilibrium centrifugation in a cesium chloride gradient. As shown in Figure 1a, the *Proteus FColVColBtrycys* strain possessed two detectable DNA species. The major band of DNA, corresponding to *Proteus* chromosomal DNA, had a buoyant density of 1.700 gm/cm³ in the CsCl gradient. The minor band of DNA, present to the extent of 4.9 per cent of the total DNA, exhibited a buoyant density of 1.710 gm/cm³. This "satellite" DNA was not present in the parental noncolicinogenic *Proteus* strain (Fig. 1b) or in a *Proteus* strain that had spontaneously lost the ability both to grow in the absence of tryptophan and produce colicins V and B.

The satellite DNA from the *Proteus FColVColBtrycys* strain could be enriched by fractionation of the DNA from the colicinogenic *Proteus* strain on an MAK column. The early DNA-containing fractions of the MAK column contained essentially all of the DNA of a buoyant density of 1.710 gm/cm³. The initial one or two fractions of the DNA peak generally consisted of a mixture that was approximately 50 per cent DNA of a buoyant density of 1.710 gm/cm³ and 50 per cent *Proteus* chromosomal DNA. The amount of "satellite" DNA recovered from the MAK column was determined from microdensitometer tracings of density gradient equilibrium centrifugation analysis of the individual "satellite" DNA-containing fractions. Approximately 5.1 per cent of the DNA eluted from the column had a buoyant density of 1.710 gm/cm³. This "satellite" DNA was not present in comparable fractions obtained from MAK column chromatography of the DNA from the noncolicinogenic *Proteus* parent, or spontaneously occurring noncolicinogenic *Proteus* variants.

Reversible alkali denaturation of FColVColBtrycys DNA: The acquisition by a *Proteus* cell of this "satellite" DNA when the cell acquires the ability both to produce colicins V and B and grow in the absence of tryptophan, and the loss of this DNA when the cell loses these properties, indicates that this DNA species with a buoyant density of 1.710 gm/cm³ represents the *FColVColBtrycys* factor in the colicinogenic *Proteus* strain. The *FColVColBtrycys* DNA was examined for its ability to resist denaturation by alkali treatment. Unfractionated DNA from the *Proteus FColVColBtrycys* strain was treated with 0.1 N NaOH, neutralized, and analyzed by centrifugation in a CsCl density gradient. As shown in Figure 1c, approximately 35 per cent of the *FColVColBtrycys* DNA failed to be denatured by conditions that completely denatured *Proteus* chromosomal DNA (as indicated by a shift in buoyant density of approximately 0.015 gm/cm³). These conditions also completely denatured the DNA from the noncolicinogenic parental *Proteus* strain (Fig. 1d) and *E. coli* chromosomal DNA which has a buoyant density of 1.710 gm/cm³ in the cesium chloride gradient.

Analytical dye-buoyant-density analysis of Proteus FColVColBtrycys DNA:
Bauer and Vinograd have shown that the buoyant density of a closed circular
DNA-ethidium bromide complex at saturation is greater by approximately 0.04
gm/cm³ than a linear or open circular DNA-ethidium bromide complex.[23] Since
a substantial amount of the *FColVColBtrycys* DNA reacted to alkali treatment as
closed circular double-stranded DNA, the effect of ethidium bromide on the
buoyant density of this DNA was examined.   Unfractionated DNA from the
Proteus FColVColBtrycys strain and from the noncolicinogenic parental *Proteus*
strain were centrifuged to equilibrium in a cesium chloride gradient containing
ethidium bromide.   As shown in Figure 2a, the DNA from the noncolicinogenic
Proteus parent banded as a single DNA component, while the DNA from the
Proteus FColVColBtrycys strain (Fig. 2b) exhibited two DNA components with the
minor component possessing a higher buoyant density than the bulk of the DNA.
Preliminary experiments have revealed that this heavier minor component when
isolated from the dye-cesium chloride mixture exhibits a buoyant density of 1.710
gm/cm³.   As shown in Figure 2b, the main DNA band of *Proteus FColVColBtrycys*
DNA usually possessed a shoulder on the heavy side of the gradient.   This shoulder
presumably represents linear and open circular *FColVColBtrycys* DNA.

Isolation of FColVColBtrycys DNA: Unfractionated DNA from the *Proteus
FColVColBtrycys* strain was banded in a preparative cesium chloride density
gradient in four tubes in a type-50 fixed-angle rotor.   The density profile from
one of the tubes is shown in Figure 3.   Fractions 33–35 were pooled with com-
parable fractions from the other three tubes.   This pooled DNA was examined
for its content of *FColVColBtrycys* DNA by equilibrium centrifugation in a cesium
chloride gradient before and after treatment with alkali.   The pooled fractions
contained essentially pure *FColVColBtrycys* DNA.   The results of treatment with
alkali followed by rapid neutralization indicate that 5–10 per cent of sex factor
DNA remained in the form of a covalently intact circle after isolation by this
procedure.

Examination of FColVColBtrycys DNA by electron microscopy: The *FColVCol-
Btrycys* DNA from the pooled fractions of the preparative equilibrium centrifuga-
tion was also examined by electron microscopy.   The majority of the DNA fila-
ments observed had the appearance of open circular double-stranded DNA (Fig.
4a).   In addition to this configuration a very small percentage of the DNA mole-
cules appeared in a supercoiled form (Fig. 4b).   The contour length of the open
circular molecules was calculated for 18 randomly chosen strands.   An average
length of 54.5 μ was found with a standard deviation of ±1.7 μ (Fig. 5).   In
addition to the supercoiled and fully extended circular molecules, linear DNA
molecules were observed with lengths that were approximately equal to or smaller
than the circular DNA molecules.

Discussion.—The colicinogenic factor *FColVColBtrycys* examined in these experi-
ments is a sex factor.   Studies in *E. coli* have shown that this factor promotes
bacterial conjugation, chromosomal recombination, and its own transfer to female
cells.[5, 7, 8] Also, cells carrying this factor are susceptible to the male-specific
bacteriophage, μ.[8] In addition to the genetic determinants for colicin V and
colicin B production and fertility, this factor carries genes of the tryptophan and
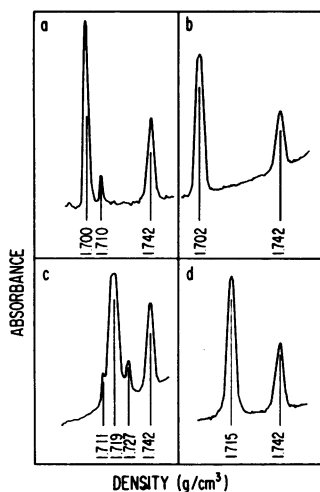cysteine loci and genetic determinants for T1 bacteriophage susceptibility.[4–6]

FIG. 1.—Microdensitometer tracings of results of cesium chloride equilibrium centrifugation of (a) *Proteus FColVColBtrycys* DNA, (b) *Proteus* DNA, (c) *Proteus FColVColBtrycys* DNA treated with alkali and neutralized as described in *Materials and Methods*. (d) *Proteus* DNA treated with alkali and neutralized.
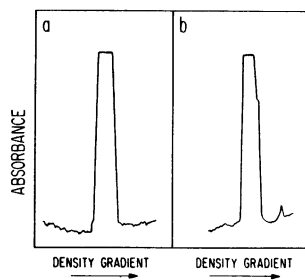

FIG. 2.—Microdensitometer tracing of results of dye-buoyant density centrifugation of (a) *Proteus* DNA and (b) *Proteus FColVColBtrycys* DNA.
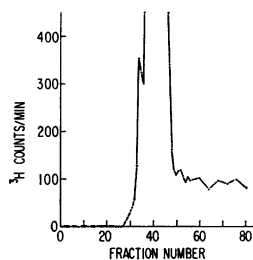

FIG. 3.—Preparative cesium chloride equilibrium centrifugation of *Proteus FColVCol-Btrycys* DNA.

When present in the *Proteus* strain, the *FColVColBtrycys* factor can be characterized as a double-stranded DNA molecule with a buoyant density of 1.710 gm/cm³. This characterization is based upon the acquirement of this DNA species by the *Proteus* strain when this strain acquires the ability both to produce colicins V and B and to grow in the absence of tryptophan, and the loss of this "satellite" DNA when the strain spontaneously loses these properties.

The *FColVColBtrycys* factor has been transferred from the *Proteus* strain back to an *E. coli* strain, where it exhibits the same properties as found in the original *E. coli* donor strain. Thus, genetic linkage and other properties of this factor are not apparently altered by its transfer to and maintenance in the *Proteus* strain.

The fact that a substantial percentage of the isolated *FColVColBtrycys* DNA resists denaturation by treatment with alkali followed by neutralization indicates that a significant amount of this DNA exists in the form of covalently closed circular molecules. This conclusion is supported by the electron microscopy studies that have revealed both fully extended open and supercoiled circular DNA forms of this factor. The displacement of the *FColVColBtrycys* DNA from the bulk of the DNA by equilibrium centrifugation in cesium chloride in the presence of ethidium bromide also indicates that a significant amount of this DNA exists in a supercoiled configuration. Supercoiled double-stranded circular forms have
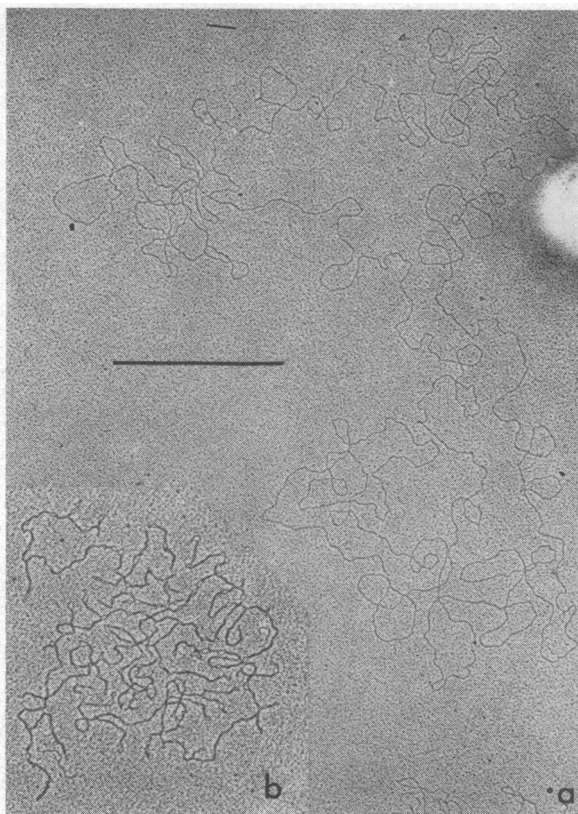
Fig. 4.—Electron micrographs taken of *FColVColBtrycys*
DNA purified by preparative cesium chloride equilibrium
centrifugation. (*a*) Typical open circular molecule and
(*b*) tightly coiled molecule.
The scale line represents 1.0 μ.

also been shown for colicinogenic factor E_1[22] and the DNA of animal viruses,[27, 28] bacteriophage,[29–34] and mitochondria.[35, 36]

Electron microscopic preparations of *FColVColBtrycys* DNA contain molecules that are mostly in an open circular form with a considerably smaller amount in the tightly twisted configuration. Assuming that a single break in one of the two DNA strands of this sex factor DNA can result in an opening of the supercoiled configuration, it is reasonable to expect that a considerable reduction in the percentage of supercoiled forms has resulted from breaks in the DNA molecule that occur during the DNA extraction, purification, and preparation for electron microscopy.

The molecular weight estimated for the *FColVColBtrycys* factor is 107×10^6, assuming a linear density of 196 daltons/angstrom. This size is considerably

larger than that shown for the circular DNA form of colicinogenic factor
$E_1(ColE_1)$.[22] The smaller form of the $ColE_1$ DNA has a molecular weight of 4.5
$\times 10^6$. This is consistent with the fact that
the $ColE_1$ factor apparently does not carry the
genetic determinants of fertility or genes associ-
ated with any of the known chromosomal genetic
loci in contrast to the $FColVColBtrycys$ factor.
The $FColVColBtrycys$ factor is also larger than
the sex factor F'-13L which carries the *lac* locus
in addition to F genes. The size of the F'-13L
factor, estimated by a DNA agar technique, is
equivalent to a molecular weight of approximately
60×10^6.[37] The molecular weight of the F region
of the F'-13L factor has been estimated to be
approximately 40×10^6.[37] The $FColVColBtrycys$

FIG. 5.—Frequency distribution
of lengths of 18 circular $FColVCol-$
$Btrycys$ DNA molecules.

factor is approximately 5 per cent of the size of the circular chromosome of the
related bacterium *Escherichia coli* which has a molecular weight of approximately
2.3×10^9.[38]

The amount of $FColVColBtrycys$ DNA recovered from the colicinogenic *Proteus*
strain is approximately 5 per cent of the total DNA. Assuming that the molecular
weight of the chromosome of *P. mirabilis* is similar to that of *E. coli*, these recovery
data indicate the presence of one copy of the $FColVColBtrycys$ factor per copy of
chromosome in the bacterial cells. A similar conclusion has been made with
regard to the estimation of the number of *Flac* factors per chromosome in exponen-
tially growing *E. coli* cells.[10]

The demonstration of circular DNA forms for the $FColVColBtrycys$ factor
supports recent models proposed for the interaction of sex factors with the bacterial
chromosome.[9, 10, 12] These models are based upon a pairing of a circular sex
factor with a circular chromosome followed by breakage and a crossing-over
involving the two circular molecules. Genetic studies involving the bacterial sex
factor F14 have also provided some evidence for the circularity of sex factors.[12]

Presently, experiments are being carried out to examine other physical-chemical
properties of the $FColVColBtrycys$ factor and to determine the effect on the struc-
ture of this sex factor of conditions that lead to the induced production of colicin B.

Summary.—The bacterial sex factor, $FColVColBtrycys$, was isolated from a
tryptophan- and thymine-requiring *Proteus mirabilis* strain. This sex factor was
characterized as double-stranded DNA with a buoyant density of 1.710 gm/cm³
in a-cesium chloride gradient. Electron microscopic analysis of the $FColVCol-$
$Btrycys$ DNA revealed o en circular and supercoiled molecules. The average
contour length of the open circular DNA form was 54.5 μ ±1.7 μ. Analyses of
$FColVColBtrycys$ DNA by equilibrium centrifugation in a cesium chloride gradient
in the presence of ethidium bromide, and after treatment with alkali followed by
rapid neutralization, indicate that approximately 35 per cent of the isolated
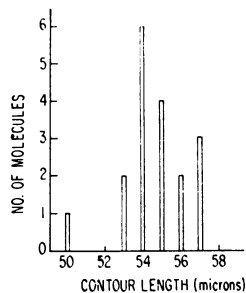
FColVColBtrycys DNA was present as covalently intact and supercoiled circular DNA molecules.

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