Isolation and Characterization of Supercoiled Circular Deoxyribonucleic Acid from Beta-Hemolytic Strains of *Escherichia coli*

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Covalently closed circular deoxyribonucleic acid (DNA) molecules were isolated by cesium chloride centrifugation in the presence of ethidium bromide from a naturally occurring beta-hemolytic *Escherichia coli* strain (SC52). The open circular forms have contour lengths of 2.25 ± 0.1 μm, 24.0 ± 0.3 μm, and 29.5 ± 0.5 μm. The beta-hemolytic character of *E. coli* SC52 can be transferred by conjugation to a nonhemolytic recipient strain. Analysis of the supercoiled DNA of the hemolytic recipient demonstrated that the two large supercoiled DNA molecules of *E. coli* SC52 are transferred during this event, too. A beta-hemolytic laboratory *E. coli* strain and several of its derivatives have been shown to contain at least one circular DNA molecule, slightly larger in size than those isolated from *E. coli* SC52 and its conjugant. The possible significance of these DNA molecules for hemolysin production and transfer is discussed.

An increasing number of bacterial functions have been shown to be controlled by extrachromosomal genetic elements. Studies on colicinogeny (1, 9) and multiple drug resistance (6, 15) have clearly established that these characters are mediated by autonomously replicating deoxyribonucleic acid (DNA) molecules which are functionally and physically distinct from the bacterial chromosome. The extrachromosomal DNA studied to date could be isolated as supercoiled circular molecules with widely differing molecular weights. Whereas the transmissible episomes represent rather large DNA molecules [F and F′ factors (8), R factors (6), Col I, Col V (Helinski, personal communication)], the nontransmissible plasmids are normally considerably smaller in size [Col E1, E2, E4 (1), R-determinants (6)]. In addition to colicinogeny and drug resistance, three other properties, namely, the ability to produce K88 antigen (19), enterotoxin (17), and alpha and beta hemolysins (18), have been shown by genetic experiments to be governed in some strains of *Escherichia coli* by transmissible factors. These functions are commonly thought to be significant in the pathogenesis of disease caused by these strains (16), yet we are aware of no information on the molecular nature of these transmissible factors. We have therefore investigated the DNA of a naturally isolated beta-hemolytic *E. coli* strain and several beta-hemolytic laboratory strains. In this report, the isolation and characterization of circular DNA molecules related to the production of beta hemolysin in *E. coli* are described.

**MATERIALS AND METHODS**

**Bacterial strains.** *E. coli* SC52 was isolated from ox intestines and kindly provided by P. Hummel. Strain SC52 was shown to be beta-hemolytic on sheep and ox blood-agar. *E. coli* Hfr CR34 dna43, a temperature-sensitive mutant deficient in DNA replication, was provided by H. Schaller. MRW44 is a conjugant of *E. coli* Hfr CR34 dna43, which was generously given to us by G. Buttin. *E. coli* Hfr H (Col E1) and YS40 were a gift from D. R. Helinski.

**Media.** Enriched nutrient broth (7) was used for growing overnight cultures. Mating was also performed in this medium. Phosphate-buffered minimal medium was previously described (10).

**Growth and labeling conditions.** A 1-ml inoculum of the strain to be studied was added to 20 ml of phosphate-buffered minimal medium and grown to the log phase (cell density, 5 × 10^9 cells/ml). The medium was supplemented with 3H-thymine (5 μCi/ml) and deoxyadenosine (250 μg/ml) or with 3H-thymidine (5 to 10 μCi/ml).

**Transfer of the HlyB factor.** The donor strain SC52 and the recipient strain Hfr H (Col E1) azif were grown to log phase in enriched nutrient broth (ENB). Both strains were mated in a ratio of 1:10 on hard agar and incubated overnight at 37 C. A loopful of bacteria taken from these plates was suspended in ENB medium (10 ml). Portions (0.1 ml) of several dilutions of this suspension were plated on hard agar containing 100 μg of sodium azide per ml. The resulting colonies were tested for beta hemolysin production on ox blood-agar.

**Elimination conditions.** *E. coli* SC52 was grown to
the log phase; the culture was diluted 1:50 and further
incubated for 2 hr at 30 C. A sample of this culture (1
ml) was suspended in 10 ml of ENB medium con-
taining 20, 30, 40, or 100 f.m. of acridine orange per ml.
Elimination conditions in the presence of ethidium
bromide were as described by Bouanicha et al. (2).

Rifampin was used at a concentration of 0.01
up/ml in ENB medium as previously described (11).
Elimination with sodium dodecyl sulfate (SDS) was
performed essentially as described by Tomoda et al.
(20). Final SDS concentration was 10%. Incubation
times at 30 C were 48, 24, and 72 hr.

Test for hemolysis production. Single colonies were
stabbet on blood-agar plates containing 6% defibri-
nated ox blood or sheep blood in ENB agar. Beta-
hemolytic colonies formed clear zones around the
resulting colony after incubation for 15 hr at 37 C.

Test for colicinogeny. Single colonies were tested for
coliogeny by stabbing them on the sensitive E. coli
strain YS40 as described by Kahn and Helinski (12).

Cell lysis and isolation of supercoiled DNA. Cell lysis of the beta-hemolytic E. coli strain was performed by
the lysozyme-Brij 58 technique as described by Clewell
and Helinski (5) with some minor modifications. The
viscous lysates were clarified by centrifugation at 8,000
x g for 15 min at 4 C with the use of a G-50 K refrig-
erate centrifuge (WKF, Germany). The pellet which
contains most of the chromosomal DNA was dis-
carded. The cleared supernatant fluid was centrifuged
in a cesium chloride-ethidium bromide gradient (14). A
mixture of 2.0 ml of cleared supernatant, 1.5 ml of
TES buffer (0.03 M tris(hydroxymethyl)aminomethane
(Tris), 0.005 M ethylenediaminetetraacetic acid (EDTA)
and 0.05 M NaCl, pH 8.0), 0.5 ml of ethidium bromide
solution (1 mg/ml), and 3.8 g of CsCl were added to a
centrifuge tube of a type 50 fixed-angle rotor.

Dye-buoyant density centrifugation was carried out
for 18 hr at 0 C and 44,000 rev/min in an L50 ultra-
centrifuge with a type 50 fixed-angle rotor.

For electron microscopy studies, DNA was extracted
from a 1-liter culture by the phenol technique and fur-
ther purified by centrifugation in a cesium chloride-
ethidium bromide gradient.

Sucrose gradient centrifugation and counting of ra-
dioisotope. Sucrose gradient centrifugation was per-
formed by using an SW50 rotor or an SW65 rotor and
an L50 or L2-65B ultracentrifuge. Portions of DNA
samples (0.2 ml in dilute SSC (0.015 M NaCl, 0.0015 M
sodium citrate, pH 7.0)) were layered on 5 ml of 5 to
20% linear sucrose gradients containing 0.1 M NaCl,
0.001 M EDTA, and 0.01 M Tris (pH 8.0). Alkaline
sucre gradients were performed on 5 ml of 5 to 20%
linear sucrose gradients containing 0.2 M NaOH, 0.001
M EDTA, and 0.7 M NaCl. The samples were cen-
trifuged at 45,000 rev/min for the indicated times at
20 C. Ten-drop fractions were collected from the bottom of the
tube either directly on filter papers or in small vials
from which samples were spotted on small filter discs
(1 by 1 cm). The filter discs were treated with 10% tri-
chloroacetic acid and then washed twice with alcohol
and finally with ether. The dried filter papers were
placed in scintillation vials containing 10 ml of a PPO-
POPOP [2,5-diphenyloxazole-1,4-bis-2-(5-phenylox-
azoxy)] scintillation liquid, and counted in an SL40
liquid scintillation counter (Inter technique, France).

Electron microscopy. Grids for electron microscopy
were prepared by the method of Kleinschmidt and
Zahn (13) and were examined with a Siemens I elec-
tron microscope. Photographs of the circular DNA
molecules were enlarged, and their contour lengths
were measured.

RESULTS

Isolation and characterization of supercoiled
DNA of the naturally occurring beta-hemolytic E.
coli strain SC52. Cesium chloride density centri-
fugation in the presence of the dye ethidium bro-
mide is the commonly used method to decide
whether a DNA preparation contains supercoiled
Circular DNA molecules. The beta-hemolytic E.
coli strain SC52 was therefore gently lysed by the
lysozyme-Brij 58 procedure. The bulk of the
chromosomal DNA of this lysate was removed by
low-speed centrifugation to avoid the precipi-
tation of large circular DNA molecules. The cleared
lysate was immediately subjected to dye-
buoyant density centrifugation. Fractionation of the
resulting gradient yields the profile shown in
Fig. 1. It is evident from this gradient that, in
addition to the light density band containing
linear duplex DNA, a more dense band is
present which is at a position characteristic for
supercoiled DNA. The DNA present in the
heavy band represents about 3% of total DNA. To

FIG. 1. Fractionation of the DNA of a cleared lysate of E. coli SC52, grown in a 30-ml culture, after cesium chloride density centrifugation in the presence of ethidium bromide. The cleared lysate was prepared by the lysozyme-Brij 58 procedure. After centrifugation in a cesium chloride gradient in the presence of ethidium bromide, fractions (15 drops) were collected from the bottom of the tube in small vials. Portions of each fraction (0.02 ml) were spotted on filter discs and assayed for 3H-radioactivity as described in Materials and Methods.
Fig. 2. Sucrose gradient analysis of the supercoiled DNA of E. coli SC52 purified by cesium chloride-ethidium bromide equilibrium centrifugation. The fractions of the heavy satellite band were pooled and dialyzed against diluted SSC. A portion (0.2 ml) of this fraction was layered on a neutral 5 to 20% sucrose gradient and centrifuged for 60 min at 45,000 rev/min in a Spinco SW 65 rotor at 20°C by using a Beckman L2-65B centrifuge. Ten-drop fractions were collected from the bottom of the tube directly on filter papers, which were assayed for 3H-radioactivity as described in Materials and Methods. The indicated S_{w,20} values were obtained by use of an external 23S 3H-labeled supercoiled Col E1 DNA standard.

Further analyze the supercoiled DNA of this strain, the pooled fractions of the more dense band were run through neutral and alkaline sucrose gradients. Figure 2 demonstrates that several DNA species sedimenting at different velocities are present in the heavy band of the dye-buoyant density gradient. Above pH 12.5, the sedimentation coefficient of supercoiled DNA molecules increases considerably (21), whereas linear or open circular molecules are denatured and sediment more slowly. When the sucrose density gradient centrifugation of the isolated DNA was performed under alkaline conditions, two very fast-sedimenting peaks are observed, whereas the rest of the DNA sediments in one broad band at around 46S (Fig. 3). Based on the data reported by Clayton and Vinograd (4), the results indicate that the two large DNA molecules which sediment at 72S and 66S under neutral conditions are present in a supercoiled conformation, whereas the DNA sedimenting at around 45S represents the corresponding open circular DNA molecules which remain unresolved under the conditions described. The DNA sedimenting under neutral conditions at 23S is also in a supercoiled conformation, since its sedimentation coefficient is increased to around 50S in alkaline gradient under our conditions. All sedimentation coefficients are related to the supercoiled form of Col E1 DNA (1), which always cosedimented as a marker.

Electron microscopy of the circular DNA molecules of E. coli SC52. To confirm the physical data of the DNA present in the heavy band of the dye-buoyant density gradient, electron micrographs were taken of phenol-extracted DNA samples from E. coli SC52. The supercoiled DNA was separated from the chromosomal material by centrifugation in cesium chloride gradients containing ethidium bromide. Circular DNA of three different contour lengths are observed in supercoiled and open circular conformation (Fig. 4). The contour lengths of these molecules determined from enlarged pictures are shown in Table 1. No molecules with intermediate sizes are observed. The molecular weights of the DNA molecules determined from the contour lengths agree quite well with those calculated from the sedimentation coefficients (Table 2).

Test for drug resistance and colicinogeny. Since it is known from genetic data that hemolysin production is governed, at least in some E. coli
FIG. 4. Electron micrographs of resolved circular DNA of beta-hemolytic E. coli SC52, showing members of the three classes with mean contour lengths of 2.25 μm (A), 24.0 μm (B), and 29.5 μm (C).

TABLE 1. Contour-length measurements and molecular weights of the circular DNA molecules extracted by dye-buoyant density centrifugation of E. coli SC52

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of molecules measured</th>
<th>Contour lengths of open circular molecules (μm)</th>
<th>Molecular wt*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC52</td>
<td>15</td>
<td>2.25 ± 0.1</td>
<td>4.4 x 10^6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>24.0 ± 0.3</td>
<td>47 x 10^6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>29.0 ± 0.5</td>
<td>58 x 10^6</td>
</tr>
</tbody>
</table>

*Calculated from contour lengths on assumption that 1 μ of DNA = 1.96 x 10^6 daltons (6).

TABLE 2. Molecular weights of the circular DNA molecules of E. coli SC52 calculated from S_w,20 values

<table>
<thead>
<tr>
<th>S_w,20 of supercoiled molecules</th>
<th>Molecular wt*</th>
</tr>
</thead>
<tbody>
<tr>
<td>23S</td>
<td>4.2 - 4.3 x 10^6</td>
</tr>
<tr>
<td>66S</td>
<td>46 x 10^6</td>
</tr>
<tr>
<td>72S</td>
<td>58 x 10^6</td>
</tr>
</tbody>
</table>

*Calculated from S_w,20 values from the data of Clayton and Vinograd (4).

strains, by a transmissible episome (17), it was quite likely that one or more of the observed circular DNA molecules may represent the hemolytic factor. In addition, we tested E. coli SC52 for drug resistance and colicinogenicity. E. coli SC52 was not resistant to any of the antibiotics tested (penicillin, chloramphenicol, streptomycin, sulfonamid, kanamycin, tetracycline), but it proved to be colicinogenic on the sensitive strain YS40. It was also colicinogenic on strain C600 (Col E1, V; Table 3).

Elimination experiments. Several methods have been described (2, 11, 20) for eliminating extrachromosomal DNA elements. From those methods tested (acridine orange, ethidium bromide, sodium dodecyl sulfate, and rifampin), none succeeded in eliminating beta-hemolysin production in E. coli SC52. In all cases about 10^6 treated E. coli colonies were tested. Colicinogenicity
could not be eliminated either from *E. coli* SC52 during the various treatments.

**Transfer of the beta-hemolytic character of *E. coli* SC52 to a nonhemolytic recipient.** Conjugation experiments were carried out between the beta-hemolytic *E. coli* strain SC52 and the colicinogenic strain Hfr *H* (Col E) to see which DNA forms are transferred to a resulting beta-hemolytic recipient. Of several hundred Hfr *H* (Col E) colonies tested, we were able to isolate one with the ability to produce beta-hemolysin. This *E. coli* Hfr *H* (Col E, Hly) colony can be distinguished from an *E. coli* SC52 (Hly, Col E) colony by the following criteria. (i) The recipient colony is azide-resistant like *E. coli* Hfr *H* (Col E),. (ii) The recipient strain synthesizes colicin E, and not the colicin produced by *E. coli* SC52 as shown in Table 2.

**Characterization of the supercoiled DNA of the beta-hemolytic recipient.** The extrachromosomal DNA of the conjugant *E. coli* Hfr *H* (Col E, Hly) was analyzed by dye-buoyant density centrifugation and sucrose density centrifugation as described. Whereas strain *E. coli* Hfr *H* (Col E) does not contain any fast-sedimenting DNA molecules but only the supercoiled Col E DNA, the beta-hemolytic *E. coli* Hfr *H* (Col E, Hly) possesses, in addition to the Col E DNA, the two large DNA molecules already observed in the donor strain *E. coli* SC52 (Fig. 5). Since the amount of DNA sedimenting at 23S does not increase in *E. coli* Hfr *H* (Col E, Hly) as compared to that of *E. coli* Hfr *H* (Col E), it is unlikely that the small supercoiled DNA form of *E. coli* SC52 is also transferred during conjugation.

**Characterization of the extrachromosomal DNA of beta-hemolytic *E. coli* Hfr CR34 dna43 and its conjugants.** While testing our laboratory strains for their possible ability for hemolysin production, we found that the temperature-sensitive mutant deficient in DNA replication, *E. coli* Hfr CR34 dna43, was able to form clear zones when plated on ox blood-agar, which is characteristic for beta-hemolysin production. In addition, we observed that a derivative of *E. coli* Hfr CR34 dna43, namely the rec- strain MRW44 which has received the temperature-sensitive replication marker by conjugation from *E. coli* Hfr CR34 dna43 (3), is also beta-hemolytic. It is quite possible that the hemolytic factor is transferred to the latter strain during conjugation. As shown in Fig. 6A and 6B, both beta-hemolytic strains contain one large DNA molecule, sedimenting in the supercoiled conformation at around 78S and in its open circular form at 47S, related to the supercoiled Col E DNA.

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**Table 3. Colicinogenic of *E. coli* SC52, Hfr *H* (Col E) and the conjugant Hfr *H* (Col E, Hly)**

<table>
<thead>
<tr>
<th><em>E. coli</em> strains</th>
<th>Colicinogenic on test strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YS 40</td>
</tr>
<tr>
<td>SC52</td>
<td>+</td>
</tr>
<tr>
<td>Hfr <em>H</em> (Col E)</td>
<td>+</td>
</tr>
<tr>
<td>Hfr <em>H</em> (Col E, Hly)</td>
<td>+</td>
</tr>
</tbody>
</table>

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**Fig. 5. Sucrose gradient analyses of the supercoiled DNA of *E. coli* Hfr *H* (Col E) and *E. coli* Hfr *H* (Col E, Hly).** Supercoiled DNA from both strains was isolated by cesium chloride-ethidium bromide centrifugation of the corresponding cleared lysates. Neutral sucrose gradients as described in Fig. 2 were used. Centrifugation was performed for 60 min at 45,000 rev/min in a Spinco SW50 rotor at 20 C by using a Beckman L50 centrifuge. Fractionation was carried out as described in Fig. 2. A, *E. coli* Hfr *H* (Col E); B, *E. coli* Hfr *H* (Col E, Hly).
The isolation of three species of circular supercoiled DNA from a naturally isolated beta-hemolytic E. coli strain raises the question: Which DNA molecule represents the actual genome carrying the gene for beta-hemolysin production? It has been previously shown that, in several hemolysin-positive strains, hemolysin production is governed by a transmissible episome (18). Studies on the physical nature of transmissible factors which consist of a transfer factor and another determinant often covalently linked together always revealed large DNA molecules, for example, R factors, Col V, Col B.

Two DNA molecules isolated from the beta-hemolytic strain E. coli SC52 have molecular weights approaching those of the above-mentioned transmissible episomes, whereas the third DNA form is considerably smaller. Upon transferring the ability of hemolysin production to a nonhemolytic E. coli strain, both large DNA molecules are cotransferred to the recipient strain. This result suggests that at least one, possibly the largest of these DNA molecules, is the hemolytic factor. The other DNA molecule could then represent the dissociated transfer factor of the hemolytic factor or another transfer factor not linked to a determinant or at least not to a determinant one can identify. Upon transferring a transmissible factor like the hemolytic factor, another transfer factor which is also present in the donor cell has a good chance of going over as well. The colicinogenic character of this E. coli strain is not transferred together with the hemolytic property to the recipient strain. This suggests that the gene for colicin production is not linked with either of the large DNA forms but resides probably on a nontransmissible plasmid. The small supercoiled DNA molecule isolated from E. coli SC52 could account for this character. It is interesting to notice that the extrachromosomal DNA molecules encoding the production of colicin E₈, E₉, and E₁₀ have similar molecular weights (1). These latter extrachromosomal elements are also nontransmissible plasmids (12).

The extrachromosomal DNA isolated from the beta-hemolytic E. coli Hfr CR34 dna43 and its derivatives is reproducibly slightly larger than the largest DNA molecule isolated from the beta-hemolytic E. coli strain SC52. This could mean that the beta-hemolytic determinant is linked to another transfer factor or that other determinants which cannot be identified are linked to the same transfer factor in addition to the beta-hemolytic determinant.

When the beta-hemolytic E. coli Hfr CR34 dna43 is mated with a nonhemolytic recipient, the transmission of the beta-hemolytic character is associated with the transfer of a single supercoiled DNA molecule similar in size to the largest DNA form in E. coli SC52. Considering

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**Fig. 6.** Sucrose gradient analyses of the beta-hemolytic E. coli strain Hfr CR34 dna43 and its beta-hemolytic derivatives. Neutral sucrose gradients, as described in Fig. 2, were used. Centrifugation was performed for 90 min at 45,000 rev/min in an SW65 rotor at 20°C with a Beckman L2-65B centrifuge. Fractions were carried out as described in Fig. 2. A. E. coli Hfr CR34 dna43; B-MRW44; C, nonhemolytic E. coli 1100 (○) and beta-hemolytic E. coli 1100 (Hy) (●).
that hemolysin production in E. coli has been shown in several cases to be governed by an extrachromosomal element (18), it is not unlikely that this large supercoiled DNA molecule represents the hemolytic factor of this strain.

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LITERATURE CITED