JOURNAL OF BACTERIOLOGY, June 1974, p. 964-973

Vol. 118, No. 3
Printed in U.S.A.

Plasmids Controlling Synthesis of Hemolysin in *Escherichia coli*: Molecular Properties

WERNER GOEBEL,[1] BRIGITTE ROYER-POKORA, WERNER LINDENMAIER, AND HERMANN BUJARD

Institut für Mikrobiologie und Molekularbiologie der Universität Hohenheim, Hohenheim, Germany, and Institut für Molekulare Genetik der Universität Heidelberg, Heidelberg, Germany

Received for publication 4 February 1974

Covalently closed extrachromosomal deoxyribonucleic acid (DNA) was isolated from alpha-hemolytic wild-type strains of *Escherichia coli*. Most strains examined were able to transfer the hemolytic property with varying frequencies to nonhemolytic recipient strains. Out of eight naturally isolated alpha-hemolytic *E. coli* strains, four contained a set of three different supercoiled DNAs with sedimentation coefficients of 76*S* (plasmid A), 63*S* (plasmid B), and 55S (plasmid C). The sedimentation coefficients and the contour lengths of the isolated molecules correspond to molecular weights of 65×10^6, 41×10^6, and 32×10^6. Three alpha-hemolytic wild-type strains carried only one plasmid with a molecular weight of 41×10^6, and one strain harbored two plasmids with molecular weights of 41×10^6 and 32×10^6. Alpha-hemolytic transconjugants were obtained by conjugation of *E. coli* K-12 with the hemolytic wild-type strains. A detailed examination revealed that plasmids with the same sizes as plasmids B and C of the wild-type strains can be transferred separately or together to the recipients. Both plasmids possess the hemolytic determinant and transfer properties. Plasmid A appears to be, at least in one wild-type strain, an additional transfer factor without a hemolytic determinant. In one case a hemolytic factor was isolated, after conjugation, that is larger in size than plasmid A and appears to be a recombinant of both plasmids B and C.

The extrachromosomal nature of colicin production and multiple drug resistance in *Escherichia coli* is well established (16, 20). These additional properties are governed in most cases by transmissible plasmids. Most naturally isolated R factors and several transmissible colicinogenic (Col) factors show repressed transfer properties. These plasmids code presumably for repressor substances which inhibit their own transfer and also that of other plasmids, such as F factors or mutant R factors (26), which have lost the capability of synthesizing the repressor (15, 17). To date, two basic types of transmissible plasmids have been observed and classified as F-like and I-like (27), since the sex pili formed by cells harboring these plasmids are morphologically and antigenically related to either F or I pili. In some cases the repressor substance determined by these plasmids inhibits its pilus production and transfer of the derepressed factor (fi^+, fertility inhibition); in others it does not (fi^-).

[1] Present address: Lehrstuhl für Biochemie der Techn. Universität Braunschweig, and Gesellschaft für Molekularbiologische Forschung mbH 3301 Stöckheim/Braunschweig, West Germany.

In most of these systems, the genes determining the transfer function, the production of colicin or drug-inactivating enzymes, and the transfer repressor (in repressed systems) reside on a single circular deoxyribonucleic acid (DNA) molecule.

Under certain conditions (e.g., transfer of R factors into *P. mirabilis*) a transmissible R factor can dissociate into an R determinant possessing the genes for inactivation of drugs but not for transfer, and a resistance transfer factor, which exhibits transfer properties but does not determine drug resistance (7, 19).

There are also reports on drug-resistant strains of *Salmonella typhimurium* which seem to harbor two permanently dissociated plasmids, one of which is responsible for determination of the drug resistance without possessing transfer properties, and the other being a transfer factor which promotes the transfer of the first plasmid (1, 2). Whereas the transmissible plasmids like F factors, R factors and some Col factors are rather large supercoiled DNA molecules (6, 7, 9), nontransmissible plasmids like ColE1, ColE2, ColE3 (3), and the R determinants (7) are smaller in size.

The ability of *E. coli* wild-type strains to produce K88 antigen (14, 25), enterotoxin (24), and alpha- and beta-hemolysin (23) seems also to be governed by extrachromosomal elements, since these properties can be transferred at least in some cases to suitable transconjugants.

Since these latter functions are thought to be significant in the pathogenesis of disease caused by these strains (22), it is of considerable interest to learn more about the molecular nature of these systems. Recently we described the isolation of two large supercoiled DNAs from a beta-hemolytic wild-type strain of *E. coli* (12). Upon transferral of the beta-hemolysin determinant to a nonhemolytic *E. coli* strain, both large DNA molecules were found in the transconjugant. To further establish the extrachromosomal nature of the hemolytic systems in *E. coli*, we have continued these studies on alpha-hemolytic wild-type strains and hemolytic *E. coli* K-12 recipients. These studies have revealed that the determinant for alpha-hemolysin production can reside on various transmissible plasmids. They differ in size and in their transfer properties.

MATERIALS AND METHODS

Bacterial strains. The alpha-hemolytic wild-type strains of *E. coli* P224, P157, PM167, PM152, and CM20, the alpha-hemolytic *E. coli* K-12 transconjugants, K-12 (PM152), K-12 (PM167), and K-12(CM20), and the nonhemolytic strain K-12, F^-, lac^-, nal^R used in most instances as recipient strain were kindly provided by H. Williams Smith. The genetic properties of these strains are listed in Table 1. *E. coli* 165β was a gift from D. Kudlaj and CRT46 (18) was given to us by Y. Hirota.

Media. Enriched nutrient broth (ENB) (8) was used for the growth of cultures. Mating was performed on enriched nutrient broth agar. Cells used for DNA extraction were grown in a phosphate-buffered minimal medium with glucose (0.2%) as the carbon source or in tris(hydroxymethyl)aminomethane (Tris)-buffered minimal medium (8) with glycerol as the carbon source. For growth of the alpha-hemolytic wild-type strains, these media were supplemented with thiamine (2 μg/ml). When appropriate, the following antibiotics were used (per milliliter): streptomycin, 100 μg; chloramphenicol, 100 μg; and nalidixic acid, 50 μg. McConkey agar (Difco) was used for distinguishing between lac^+ and lac^- colonies. Blood-agar plates contained 6% washed, ox or sheep erythrocytes in ENB agar.

Chemicals. [*Methyl*-^{3}H]thymidine (specific activity, 24.3 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, England), Brij 58 was obtained from Merck (Darmstadt, Germany), ethidium bromide was from Calbiochem (Los Angeles, Calif.), and lysozyme was from Serva (Heidelberg, Germany).

Test for alpha-hemolysin production. Hemolysin production was tested by stabbing single colonies on blood agar. Alpha-hemolytic colonies formed large, clear zones around the growing colony after incubation for 15 h at 37 C. When low frequencies of hemolytic colonies were expected, ENB plates containing up to 10^4 colonies were replica-plated on blood-agar plates. After incubation for 5 h at 37 C, even a very small number of hemolysin-positive colonies could be visualized by this procedure.

Transfer of hemolytic factors. Donor and recipient cells were grown separately in enriched nutrient broth to a cell density of approximately 5×10^8 cells per ml. One part (0.05 ml) of the donor cultures was mixed with five parts of the recipient cultures and spread on the surface of ENB agar plates. The plates were incubated at 37 C for 15 to 20 h. A loopful of the mated culture was resuspended in 5 ml of enriched nutrient broth. Alternatively, donor and recipient at a ratio of 1:10 were mated in broth for 1 h at 37 C. Dilutions were prepared to obtain individual colonies on appropriate selective agar plates. When the first procedure was used, the frequency of transfer is expressed as the percentage of recipient colonies that obtained the extrachromosomal marker. With the second procedure, the frequency of transfer is given as the ratio of hemolytic recipient colonies to the number of donor cells.

Growth and labeling conditions. A 1-ml inocu-

TABLE 1. *Hemolytic bacterial strains*

Strain	Genetic markers	Type	Origin
P224	tra^-,[a] hly^+	Wild	H. W. Smith
PM152	tra^+, hly^+	Wild	H. W. Smith
PM167a	tra^+, hly^+	Wild	H. W. Smith
PM167b	tra^+, hly^+	Variant of PM167a	
PM157a	tra^+, hly^+	Wild	H. W. Smith
PM157b	tra^-, hly^+	Wild	H. W. Smith
CM20	tra^+, hly^+	Wild	H. W. Smith
165β	tra^+, hly^+	Wild	D. Kudlaj
K-12(PM152-1)	tra^+, hly^+, nal^R, lac^-	Transconjugant of *E. coli* K-12 with PM152	H. W. Smith
K-12(CM20)	tra^+, hly^+, nal^R, lac^-	Transconjugant of *E. coli* K-12 with CM20	H. W. Smith
K-12(PM167-1)	tra^+, hly^+, nal^R, lac^-	Transconjugant of *E. coli* K-12 with PM167a	H. W. Smith

[a] The symbols tra^+ and tra^- indicate that the corresponding hemolytic strain has the ability to transfer the hemolytic determinant or lacks it, respectively. hly^+ indicates that the strain produces hemolysin. nal^R, nalidixic acid resistance.

lum of the strain to be studied was added to 30 ml of phosphate-buffered minimal medium and grown to the log phase (cell density 2×10^8 to 3×10^8 cells per ml). The medium was then supplemented with deoxyadenosine (250 μg/ml) and labeled with [*methyl*-^{3}H]thymidine (5 to 10 μCi/ml) for two to three generations at 37 C.

Isolation of revertants of a dnaA mutant in which the mutation is suppressed by the integration of hemolytic factor. Fractions of a logarithmically growing culture of the *dnaA* mutant CRT46 (18) harboring hemolytic factor(s) were spread on broth-agar plates and incubated at 43 C for 2 days. The resultant colonies were tested for integrative suppression by their sensitivity to acridine orange, as described by Nishimura et al. (18).

Preparation of extrachromosomal DNA. Radioactively labeled extrachromosomal DNA was prepared by procedure (5, 10) that yields cleared lysates containing more than 50% of the total extrachromosomal DNA and less than 5% of the total chromosomal DNA. A mixture consisting of 2.0 ml of the cleared lysate, 1.5 ml of Tris-ethylenediaminetetraacetic acid (EDTA)-NaCl buffer (0.05 M Tris-hydrochloride, 0.05 NaCl, 0.005 M EDTA, pH 8.0), 0.5 ml of an ethidium bromide (2,7 - diamine - 10 - ethyl - 9 - phenylphenanthridinium bromide) solution (1 mg/ml), and 3.8 g of CsCl was added to a centrifuge tube and spun in a type 50 fixed-angle rotor (21). For larger-scale DNA extractions, the same procedure was used to obtain cleared lysates from 100-ml cultures of cells, and the cesium chloride-ethidium bromide gradients were centrifuged in a Ti 60 rotor (11).

Sucrose gradient centrifugation and counting of radioisotopes. Extrachromosomal DNA isolated by cesium chloride-ethidium bromide centrifugation was analyzed further on neutral 5 to 20% linear sucrose gradients containing 0.1 M NaCl, 0.001 M EDTA, and 0.01 M Tris-hydrochloride (pH 8.0) for appropriate times (20 C, 45,000 rpm) in an SW65 rotor on a Beckman model L2-50 or L2-65B preparative ultracentrifuge. Alkaline sucrose gradient determinations were performed on 5 to 20% linear sucrose gradients containing 0.2 M NaOH and 0.7 M NaCl. Where indicated, ^{32}P-labeled ColE1 DNA (3) or R1*drd*19 DNA (W. Goebel, Eur. J. Biochem., in press) were used as internal markers. Fractions were collected either directly on filter paper squares or in small vials. In the latter case, small volumes of each fraction were spotted on filter paper squares which were then treated with 10% trichloroacetic acid and washed twice with alcohol and finally once with ether. The dried filter papers were placed in scintillation vials containing 10 ml of scintillation fluid {2,5-diphenyloxazole; 1,4-bis-[2]-(5-phenyloxazolyl)benzene} and counted in an SL30 or SL40 liquid scintillation counter (Intertechnique, France).

Electron microscopy. Electron microscopy was preformed as described by Lang and Mitani (13). A Siemens Elmiskop I was used and photographs were taken at magnifications of $\times$5,000 or $\times$10,000. The photographs were evaluated as described previously (4); DNA form II of bacteriophage PM2 was used as an internal standard. The contour length of this DNA has been determined to be 3.22 ± 0.07 μm, resulting in a molecular weight of $(6.70 \pm 0.20) \times 10^6$.

RESULTS

Isolation and characterization of supercoiled DNA from alpha-hemolytic wild-type strains. Smith and Halls (24) observed previously that some hemolytic wild-type strains of *E. coli* are able to transfer the hemolysin determinant by conjugation to appropriate recipients. This suggests that this property may be determined by extrachromosomal transmissible plasmids.

To examine the molecular nature of such hemolytic factors, we have tried to isolate supercoiled DNA from several alpha-hemolytic wild-type strains of *E. coli*, none of which has been found after analysis to carry R factors or Col factors. Cleared lysates of these strains were prepared (5, 10) and centrifuged to equilibrium in CsCl gradients containing an excess of the dye ethidium bromide (21). The radioactivity profiles of the gradients from all the hemolytic wild-type strains were similar. In addition to a major band (at a buoyant density of $\rho = 1.59$ g/cm^3), a second band with higher buoyant density, characteristic of closed circular DNA, was evident. This indicates that all the strains possess extrachromosomal, supercoiled DNA.

To further characterize this DNA, fractions of the heavier band were pooled and centrifuged on neutral sucrose density gradients. ^{32}P-labeled, supercoiled ColE1 DNA (23*S*) or supercoiled R1*drd*19 DNA (75*S*) were added as internal markers. Three types of sedimentation patterns were observed (Fig. 1A–C). The DNA pattern of type I (Fig. 1A) shows distinct peaks at 76, 63, and 55*S* and a broader peak at 36 to 42*S*. The DNAs sedimenting at 76 and 63*S* are homogeneous, supercoiled DNA species, as shown by their high sedimentation rates during alkaline sucrose gradient centrifugation, by their high buoyant densities after renewed banding in CsCl-ethidium bromide gradients, and by electron microscopy (Fig. 2, Fig. 3). From the sedimentation coefficients of the supercoiled DNAs and the contour length measurements of the relaxed molecules (Table 2), the molecular weights of these two species are calculated to be 65×10^6 and 41×10^6. We will refer to these DNAs as plasmid A (65×10^6 molecular weight) and plasmid B (41×10^6 molecular weight). The fractions sedimenting at 55*S* contain a mixture of an additional supercoiled DNA and relaxed DNA of plasmid A. The supercoiled DNA can be purified by renewed dye buoyant density centrifugation. This DNA,

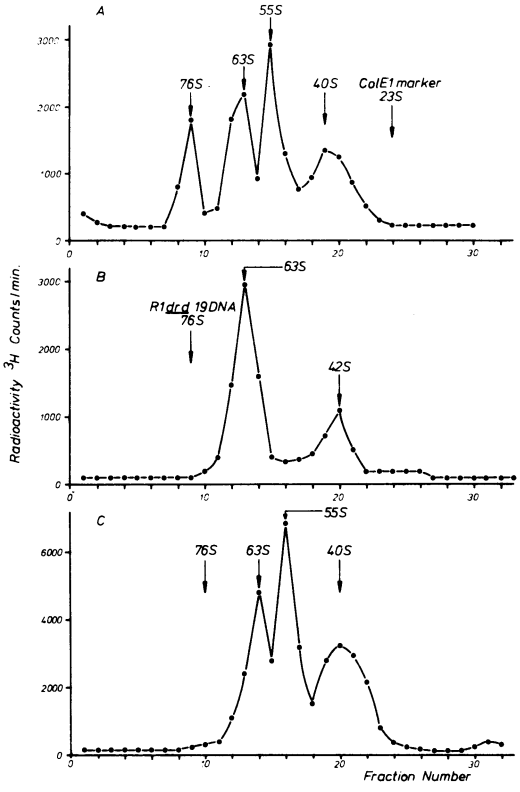

FIG. 1. *Sucrose gradient analyses of the supercoiled DNA of three hemolytic Escherichia coli wild-type strains purified by cesium chloride-ethidium bromide equilibrium centrifugation. The fractions of the heavy satellite band were pooled and dialyzed. Portions (0.2 ml) of these fractions were layered on neutral 5 to 20% sucrose gradients and centrifuged for 60 min at 45,000 rpm in a Spinco SW65 rotor at 20 C. Ten-drop fractions were collected from the bottom of the tube directly on filter papers, which were then assayed for ^3H radioactivity. The indicated S values were obtained by use of an internal 76S ^3H-labeled supercoiled R1drd19 DNA standard. (A) E. coli PM152, (B) E. coli P224, (C) E. coli P167b.*

termed plasmid C, has a molecular weight of 32 $\times 10^6$ as determined by its sedimentation coefficient and contour length (Fig. 2). The broad band at 36 to 42*S* in the neutral sucrose gradient contains a mixture of open circular DNA of plasmids B and C. Four hemolytic *E. coli* wild-type strains, PM167a, PM152, CM20, and P157a (Table 1). showed this pattern with three supercoiled plasmid DNA species. (The original strain P157 was found to be a mixture of two hemolytic strains, designated as P157a and P157b.)

The satellite DNA of the second type is separated into two sharp peaks at 63 and 42*S* which represent one homogeneous DNA species in the supercoiled (63*S*) and relaxed (42*S*) configuration (Fig. 1B). The size of this plasmid is equivalent to that of plasmid B of type I. Three of the hemolytic *E. coli* wild-type strains examined (P157b, P224, and 195β) showed this pattern.

Satellite DNA of type III (Fig. 1C) consists of two supercoiled DNAs sedimenting at 63 and 55*S*. The corresponding open circular forms sediment at 36 to 42*S*. The only hemolytic wild-type strain which showed this pattern was PM167b, a variant of PM167a. This strain has obviously lost plasmid A without showing an altered behavior in the hemolysin production.

Transfer of the hemolytic determinant and characterization of the plasmid DNA in the hemolytic transconjugants. To test the possible involvement of plasmids A, B, and C in the genetic determination of hemolysin, we mated *E. coli* K-12 and B with some of the hemolytic wild-type strains and selected for hemolytic transconjugant colonies. Most hemolytic wild-type strains tested were able to transfer the hemolytic property with varying frequencies into *E. coli* K-12 and with lower efficiencies into *E. coli* B (Table 3). There were two nontransmissible wild-type strains, P224 and P157b. These contain a plasmid identical in size to plasmid B.

With most hemolytic wild-type strains of *E. coli*, high transfer frequencies were observed upon mating of the donor and the recipient on solid agar, whereas mating of the strains in broth yielded only low numbers of hemolytic transconjugants (Table 3). This indicates that these hemolytic wild-type strains have repressed transfer properties.

Single colonies of the hemolytic transconjugants were selected, and the plasmid DNA transferred during the conjugation was analyzed. As expected, the wild-type strain 165β, which carries only plasmid B, transferred this plasmid during conjugation to the recipient cell. This indicates clearly that plasmid B determines hemolysin production and transfer in this strain. Hemolytic transconjugants containing plasmid B can also be obtained upon mating of *E. coli* K-12 with the wild-type strains P224 and P157b when the transfer is promoted by a suitable transfer factor.

Hemolytic transconjugants obtained by conjugation of *E. coli* K-12 with wild-type strains of type I, however, showed a more complex behavior. With *E. coli* PM152 as donor, most of the transconjugants (five single hemolytic transconjugant colonies were analyzed) carry the plasmids B and C (Fig. 4A), whereas one carries a plasmid which is larger in size than plasmid A

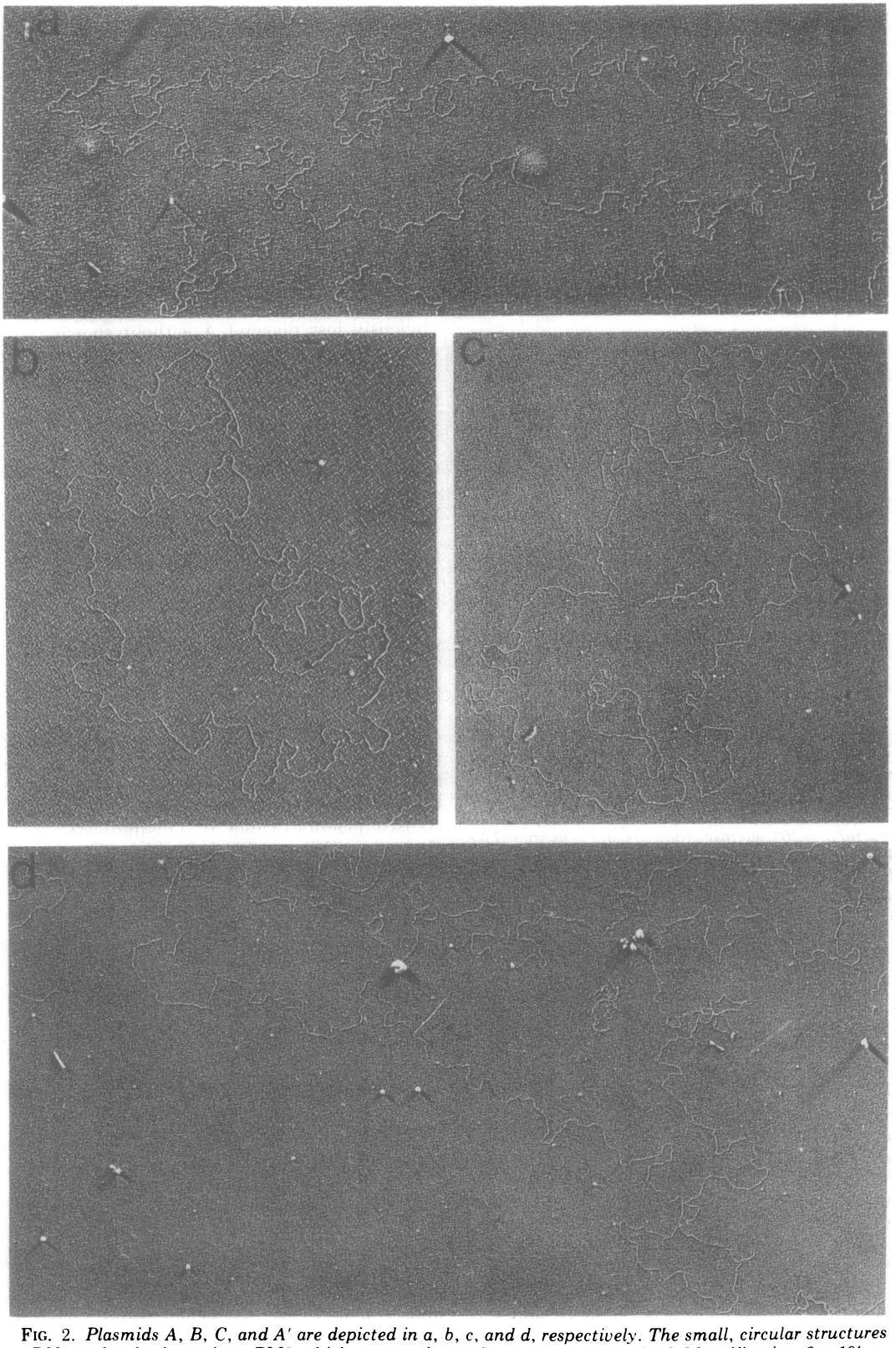

FIG. 2. *Plasmids A, B, C, and A' are depicted in a, b, c, and d, respectively. The small, circular structures are DNA molecules from phage PM2, which were used as an internal length standard. Magnification:* 2×10^4.

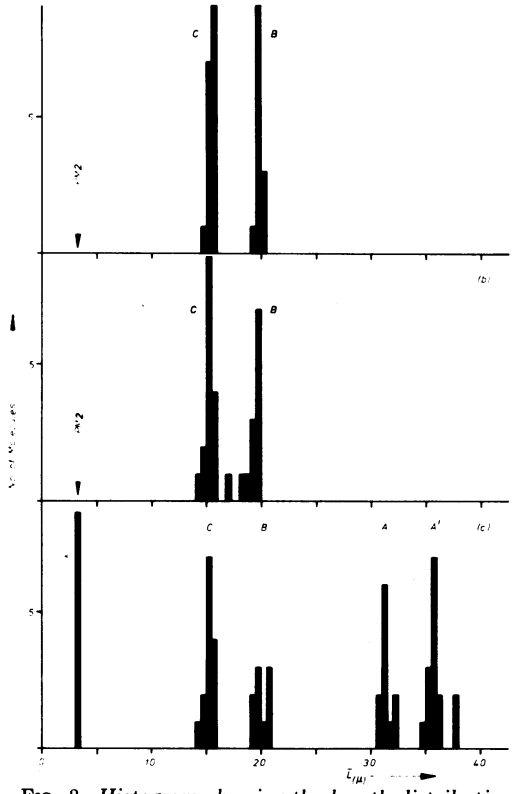

FIG. 3. *Histogram showing the length distribution of plasmids A, B, C, A', and PM2 DNA. In all preparations the length was calculated with respect to PM2 DNA, which was set as 3.20 μm. (a and b) Plasmids B and C of two different transconjugants of E. coli K-12 with PM152, K-12(PM152-2), and K-12(PM152-3). (c) Plasmids A, B, and C of PM152 and plasmid A' of the transconjugant K-12(PM152-1). L, Length in micrometers.*

of the wild strain (80 to 82*S*; 75 × 10^6 molecular weight) (Fig. 4B). The latter strain has been designated as plasmid A' (Fig. 2). Its molecular weight appears to be the sum of the molecular weights of plasmids B and C of *E. coli* PM152 (Table 2). With PM167 as donor we have isolated hemolytic transconjugants of *E. coli* K-12 which contain either plasmid B, plasmid C, a combination of plasmids A and B, or

TABLE 2. *Contour lengths and molecular weights of plasmids A, B, C, and A'*[a]

Plasmid	Contour length of the relaxed molecule L (μm)	Mol wt (sodium salt) × 10^{-6}	Rate of sedimentation of form I DNA
A'	36.0 ± 0.8	74.9 ± 2.2	80–82*S*
A	31.4 ± 0.5	65.4 ± 2.0	76*S*
B	20.0 ± 0.6	41.6 ± 1.4	63*S*
C	15.2 ± 0.5	31.6 ± 1.0	55*S*

[a] For electron microscopy, DNA was diluted into 0.15 M ammonium acetate. At this ionic strength, the linear density of double-stranded DNA is (2.08 ± 0.06) × 10^6 daltons/μm.

TABLE 3. *Transfer frequencies of the hemolytic determinant by hemolytic wild-type strains and transconjugants of E. coli K-12*[a]

Donor strain	Transfer frequencies during conjugation on solid ENB agar		Transfer frequencies during conjugation in ENB broth[b]	Plasmids present in the donor strain
	E. coli B[c]	*E. coli* K-12[c]		
PM167a	10^{-1}	9.2×10^{-1}	4×10^{-5}	A, B, C
PM152	10^{-2}	3.1×10^{-1}	3.5×10^{-5}	A, B, C
P157a	1.5×10^{-2}	3.9×10^{-1}	3.8×10^{-5}	A, B, C
P157b	[d]	[d]	[d]	B
CM20	1.6×10^{-2}	1.5×10^{-1}	8×10^{-5}	A, B, C
P224	[d]	[d]	[d]	B
K-12(PM152-1)	4×10^{-1}	9.8×10^{-1}	6×10^{-5}	A'
K-12(PM152-2)	1.8×10^{-1}	3×10^{-1}	2×10^{-5}	B, C
K-12(PM152-3)	2.1×10^{-1}	3.2×10^{-1}	1.8×10^{-5}	B, C
K-12(PM167-1)	2.5×10^{-2}	5.3×10^{-1}	6×10^{-5}	B
K-12(PM167-2)	1.8×10^{-2}	1.3×10^{-1}	3.6×10^{-5}	A, B
K-12(PM167-3)	ND[e]	1.1×10^{-1}	2.8×10^{-5}	A, B, C
K-12(PM167-4)	ND	2.7×10^{-3}	2.6×10^{-5}	C
K-12(CM20)	ND	6×10^{-4}	2×10^{-5}	B

[a] Transfer frequencies were determined as described in Materials and Methods.
[b] Recipient was *E. coli* K-12.
[c] Recipient.
[d] Transfer frequencies were less than 10^{-6}.
[e] Not determined.

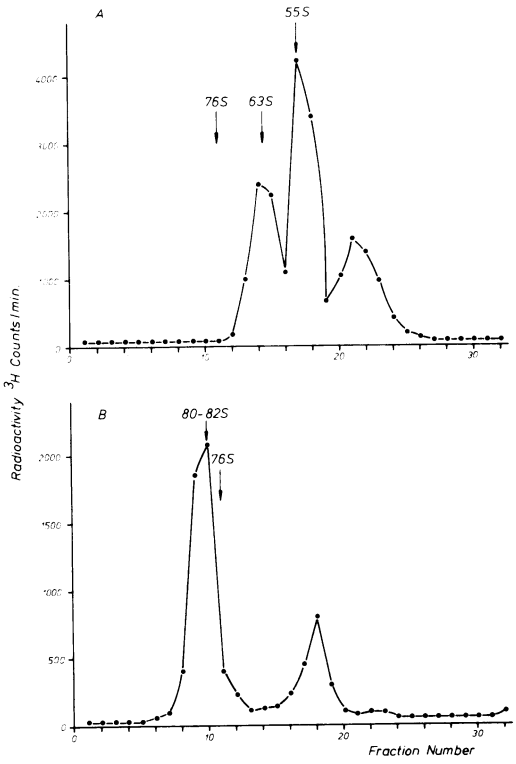

FIG. 4. *Sucrose gradient analyses of the supercoiled DNA of K-12 transconjugants obtained by mating with PM152. Supercoiled DNA from both K-12 transconjugants K-12(PM152-1) and K-12(PM152-2) was isolated by cesium chloride-ethidium bromide centrifugation. Neutral sucrose gradients were performed under the same conditions as described in Fig. 1. (A) K-12(PM152-2), (B) K-12(PM152-1).*

plasmids A, B, and C (Fig. 5). A single transconjugant colony was isolated by chance which had received during conjugation with PM167 only one plasmid having the same size as plasmid A of the donor strain. However, this strain was nonhemolytic.

The transfer properties of various hemolytic K-12 transconjugants obtained with the hemolytic *E. coli* strains PM152 and PM167 as donors were tested. K-12(PM152) transconjugants carrying plasmids B and C showed a rather high transfer frequency when mated with *E. coli* B or K-12 on solid agar (Table 3). An even higher transfer frequency was observed with the K-12(PM152) transconjugant harboring the large plasmid A′ when the mating was performed on solid agar but, as with the former K-12(PM152) transconjugants carrying plasmids B and C, rather low transfer frequencies (5.5×10^{-5}) were obtained upon mating in broth.

A K-12(PM167) transconjugant carrying only plasmid B showed a rather high transfer frequency on solid agar (Table 3), whereas the transfer frequency observed for a transconjugant carrying only plasmid C was quite low. In this case only transfer to *E. coli* K-12 was tested. The frequencies of transfer by the K-12(PM167) transconjugant strains harboring plasmids A and B or A, B, and C were intermediate in value.

One hemolytic K-12 transconjugant obtained by mating with the wild-type strain CM20 carried only plasmid B and showed an extremely low transfer frequency (Table 3) even when mated on solid agar. From the results described above, it appears that plasmids B and C of PM167, or at least plasmids of the same size as B and C of PM167, determine independently both hemolysin production and transfer

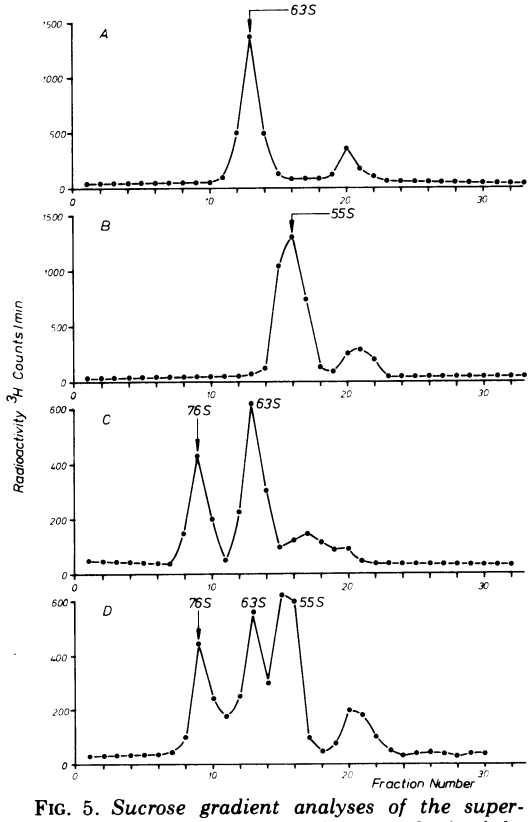

FIG. 5. *Sucrose gradient analyses of the supercoiled DNA of K-12 transconjugants obtained by mating with PM167a. Supercoiled DNA from four different K-12 transconjugants was isolated by cesium chloride-ethidium bromide centrifugation and analyzed further on neutral 5 to 20% sucrose gradients as described in Fig. 1. (A) K-12(PM167-1), (B) K-12(PM167-4), (C) K-12(PM167-2), (D), K-12(PM167-3).*

properties. Plasmid A of PM167 may be an additional transfer factor without the hemolytic determinant. No such conclusions can be drawn for the plasmids of PM152 since no transconjugants could be obtained which harbored separately plasmid B or C. The large plasmid in one of the transconjugants does not seem to be identical with plasmid A of the donor strain.

Integrative suppression of the dnaA mutation T46 by plasmids of E. coli PM152. It has been shown previously that transmissible F-like plasmids can integrate into specific sites of the chromosome in *dnaA* mutants (18), causing a "reversion" of the temperature-sensitive initiation defect of the mutant at the elevated temperature. This phenomenon has been termed integrative suppression (18). Assuming that plasmids B and C of PM152 are both independent transfer factors carrying the hemolytic determinant, it was believed that only one of the two plasmids would integrate during integrative suppression of a *dnaA* mutant, leaving the other in an extrachromosomal state. The *dnaA* mutant CRT46 was therefore mated with PM152. Hemolytic colonies of CRT46 were selected. Out of three transconjugant colonies tested, all were still temperature sensitive and had again received plasmids B and C during conjugation (Fig. 6A). Integratively suppressed, temperature-resistant strains of these hemolytic CRT46 strains were obtained with a frequency of 4×10^{-5} and tested for sensitivity to acridine orange, as described by Nishimura et al. (18). Analysis of the extrachromosomal DNA of several of these temperature-resistant strains showed that indeed either plasmid B or C was in the extrachromosomal state (Fig. 6B and C), with the other plasmid obviously integrated into the chromosome, since these strains had received Hfr properties, and transferred the chromosome in the clockwise direction. The temperature-sensitive control culture CRT46 (PM152) did not transfer chromosomal markers to any appreciable extent. By transfer of the extrachromosomal plasmid of the integratively suppressed strains into new *E. coli* K-12 recipients, it could be demonstrated that both plasmids B and C of PM152 can function as independent transmissible hemolytic factors.

As shown before (W. Goebel, Eur. J. Biochem., in press), the large plasmid of K-12(PM152), designated as plasmid A', is also able to cause integrative suppression of CRT46. However, only some temperature-resistant variants that are all hemolytic have integrated the whole plasmid. Most of them have a plasmid of the same size as plasmid B or plasmid C in an extrachromosomal state. This suggests that

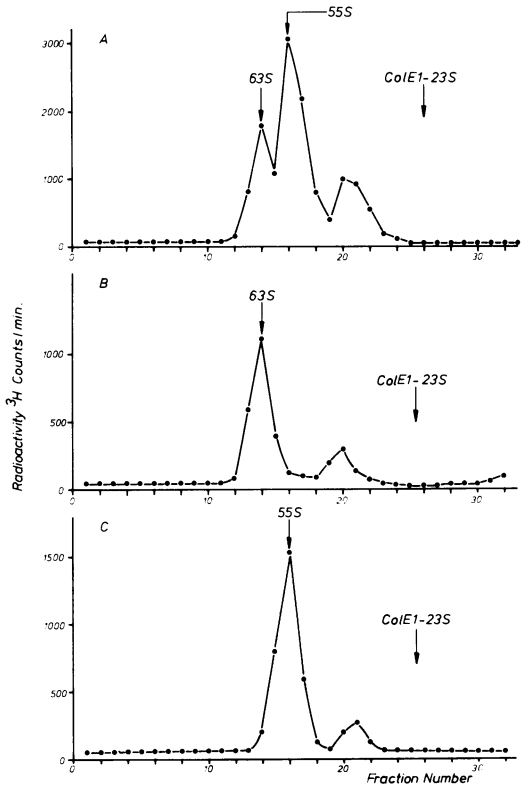

FIG. 6. *Sucrose gradient analyses of the supercoiled DNA of temperature-insensitive hemolytic revertants of CRT46 suppressed by the integration of the hemolytic plasmids of PM152. Supercoiled DNA was isolated by cesium chloride-ethidium bromide centrifugation and analyzed further on neutral 5 to 20% sucrose gradients as described in Fig. 1. (A) Hemolytic, temperature-sensitive mutant CRT46-(PM152); (B and C) temperature-resistant strains of CRT46 (PM152) suppressed by the integration of one hly plasmid.*

plasmid A', which has a molecular weight equal to the sum of those of plasmids B and C, is a stable recombinant of plasmids B and C. Further evidence for this assumption is derived from hybridization studies which will be published elsewhere.

DISCUSSION

Closed circular plasmid DNAs have been isolated from alpha-hemolytic wild-type strains of *E. coli* that appeared to be related to the synthesis and transfer of the hemolytic property. They clearly differ in size with molecular weights of 65×10^6, 41×10^6, and 32×10^6 for plasmids A, B, and C, respectively, and can therefore be readily distinguished. In those wild-type strains which carry only plasmid B, there is clear-cut evidence that the genes for

hemolysin and for transfer reside on this plasmid. Most of the hemolytic wild-type strains, however, carry all three plasmids, and the question arises whether one or more of these plasmids are concerned with hemolysin production and/or transfer. It can be ruled out that the three plasmids arise from a heterogeneous population of three different types of cells, each having a different plasmid, since the cultures analyzed for plasmid DNA were raised from single colonies. In addition, the amount of plasmid DNA isolated from these strains equals roughly one copy of each plasmid per cell. Two lines of evidence suggest that plasmids B and C, or at least plasmids of the same size as plasmids B and C of the original wild-type strains, are independent transmissible hemolytic plasmids. First, *E. coli* K-12 transconjugants that contained only plasmid B or plasmid C could be isolated after mating with one such wild-type strain. In both cases this plasmid proved to be a transmissible factor carrying the hemolytic determinant. Secondly, transconjugants of a *dnaA* mutant carrying plasmids B and C from another wild-type strain gave rise to integratively suppressed strains that had one of the two plasmids integrated into the host chromosome whereas the other remained in an extrachromosomal state. Further conjugation with new recipient strains led to hemolytic transconjugants having only one of the two plasmids.

Plasmid A may well be an independent transfer factor that does not carry the hemolysin determinant, since the only transconjugant which possessed a separated plasmid A was not hemolytic. The large plasmid A′ with hemolysin and transfer genes does not seem to be identical with plasmid A of the wild-type strain. It appears to be larger in size (75×10^6 molecular weight) and may be a recombined DNA molecule consisting of plasmids B and C. Its size equals the sum of these two smaller plasmids. In addition, it obviously dissociates during integrative suppression of a *dnaA* mutant into plasmids B and C, one being integrated into the chromosome and the other remaining in an extrachromosomal state.

Most hemolytic wild-type strains of *E. coli* tested were capable of transferring the alpha-hemolytic property to appropriate recipients. The transfer frequency for the hemolytic determinant of the wild-type strains and their transconjugants was low, resembling that of repressed R factors. However, plasmid B seems to promote its transfer more efficiently than plasmid C.

It thus appears that all transferable hemolytic wild-type strains, as well as the hemolytic transconjugants obtained by mating with them, harbor plasmids of two different sizes (plasmids B and C) with the hemolysin determinant and the transfer genes residing on the same plasmid. There may be an additional fertility factor (plasmid A) present in some wild-type strains, but it is not necessarily related to the synthesis and the transfer of the hemolytic property. There is no evidence for a permanent dissociation of the hemolytic factor into a determinant and a transfer factor as observed for some R factors in *Proteus mirabilis* (7) and *Salmonella typhimurium*, (1, 2) and as claimed for the factor determining the K88 antigen in *E. coli* (14).

ACKNOWLEDGMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

F. Lingens is thanked for his support; we are obliged to H. William-Smith and D. Kudlaj for giving us most of the hemolytic strains. R. Marsh is thanked for reading of the manuscript.

LITERATURE CITED

1. Anderson, E. S., and M. J. Lewis. 1965. Drug resistance and its transfer in *Salmonella typhimurium*. Nature **206**:579-583.
2. Anderson, E. S., and M. J. Lewis. 1965. Characterization of a transfer factor associated with drug resistance in *Salmonella typhimurium*. Nature (London) **208**:843-849.
3. Bazaral, M., and D. R. Helinski. 1968. Circular DNA forms of colicinogenic factors E1, E2 and E3 from *Escherichia coli*. J. Mol. Biol. **36**:185-194.
4. Bujard, H. 1970. Electron microscopy of single-stranded DNA. J. Mol. Biol. **49**:125-137.
5. Clewell, D. B., and D. R. Helinski. 1969. Supercoiled circular DNA-protein complex in E. coli: purification and induced conversion to an open circular DNA form. Proc. Nat. Acad. Sci. U.S.A. **62**:1159-1166.
6. Clewell, D., and D. R. Helinski. 1970. Existence of the colicinogenic factor-sex factor *Col* I-P9 as a supercoiled circular DNA-protein relaxation complex. Biochem. Biophys. Res. Commun. **41**:150-162.
7. Cohen, S. N., and C. A. Miller. 1970. Non-chromosomal antibiotic resistance in bacteria. II. Molecular nature of R-factors isolated from *Proteus mirabilis* and *Escherichia coli*. J. Mol. Biol. **50**:671-687.
8. De Witt, W., and D. R. Helinski. 1965. Characterization of colicinogenic factor E1 from a non-induced and a mitomycin C-induced *Proteus* strain. J. Mol. Biol. **13**:692-703.
9. Freifelder, D. 1968. Studies with *Escherichia coli* sex factors. Cold Spring Harbor Symp. Quant. Biol. **33**:425-434.
10. Goebel, W. 1970. Studies on extrachromosomal elements. Replication of the colicinogenic factor ColE1 in two temperature sensitive mutants of *Escherichia coli* defective in DNA replication. Eur. J. Biochem. **15**:311-320.
11. Goebel, W., and D. R. Helinski. 1970. Nicking activity of an endonuclease I-transfer ribonucleic acid complex of *Escherichia coli*. Biochemistry **9**:4793-4801.
12. Goebel, W., and H. Schrempf. 1971. Isolation and characterization of supercoiled circular deoxyribonucleic acid

from beta-hemolytic strains of *Escherichia coli*. J. Bacteriol. **106**:311-317.
13. Lang, D., and M. Mitani. 1970. Simplified quantitative electron microscopy of biopolymers. Biopolymers **9**:373-379.
14. Leth Bak, A., G. Christiansen, C. Christiansen, and A. Stenderup. 1972. Circular DNA molecules controlling synthesis and transfer of the surface antigen (K88) in *Escherichia coli*. J. Gen. Microbiol. **73**:373-385.
15. Meynell, E., and N. Datta. 1967. Mutant drug resistant factors of high transmissibility. Nature (London) **214**:885-894.
16. Meynell, E., G. G. Meynell, and N. Datta. 1968. Phylogenetic relationships of drug-resistance factors and other transmissible bacterial plasmids. Bacteriol. Rev. **32**:55-83.
17. Meynell, G. G., and A. M. Lawn. 1968. Filamentous phages specific for the I sex factor. Nature (London) **217**:1184-1186.
18. Nishimura, Y., L. Caro, C. M. Berg, and Y. Hirota. 1971. Chromosome replication in *Escherichia coli*. IV. Control of chromosome replication and cell division by an integrated episome. J. Mol. Biol. **55**:441-456.
19. Nisioka, T., M. Mitané, and R. Clowes. 1969. Composite circular forms of R factor deoxyribonucleic acid molecules. J. Bacteriol. **97**:376-385.
20. Novick, R. P. 1969. Extrachromosomal inheritance in bacteria. Bacteriol. Rev. **33**:210-235.
21. Radloff, R., W. Bauer, and J. Vinograd. 1967. A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Nat. Acad. Sci. U.S.A. **57**:1514-1521.
22. Smith, H. W. 1969. Veterinary implications of transfer activity, p. 213-226. *In* Bacterial episomes and plasmids. J. and A. Churchill Ltd., London.
23. Smith, H. W., and S. Halls. 1967. The transmissible nature of the genetic factor in *Escherichia coli* that controls haemolysin production. J. Gen. Microbiol. **47**:153-161.
24. Smith, H. W., and S. Halls. 1968. The transmissible nature of the genetic factor in *Escherichia coli* that controls enterotoxin production. J. Gen. Microbiol. **52**:319-334.
25. Stirm, S., F. Ørskov, I. Ørskov, and A. Birch-Anderson. 1967. Episome-carried surface antigen K88 of *Escherichia coli*. III. Morphology. J. Bacteriol. **93**:740-748.
26. Watanabe, T., . and T. Fukasawa. 1962. Episome-mediated transfer of drug resistance in enterobacteriaceae. IV. Interactions between resistance transfer factor and F-factor in *Escherichia coli* K-12. J. Bacteriol. **83**:727-735.
27. Watanabe, T., H. Nishida, C. Ogata, T. Arai, and S. Sato. 1964. Episome-mediated transfer of drug resistance in enterobacteriaceae. VII. Two types of naturally occurring R factors. J. Bacteriol. **88**:716-726.