JOURNAL OF BACTERIOLOGY, Apr. 1976, p. 447-453

Vol. 126, No. 1
Printed in U.S.A.

Characterization of a Mini-ColE1 Plasmid

VICKERS HERSHFIELD, HERBERT W. BOYER, L. CHOW,[1] AND DONALD R. HELINSKI*

Department of Biology, University of California at San Diego, La Jolla, California 92037; and Departments of Microbiology and Biochemistry and Biophysics, University of California at San Francisco, San Francisco, California 94112*

Received for publication 28 January 1976

An in vitro constructed plasmid, pVH15, consisting of the entire genome of the plasmid ColE1, the tryptophan operon of *Escherichia coli*, and regions of the bacteriophage φ80pt190, spontaneously gave rise in *E. coli* to a mini-ColE1 plasmid consisting of approximately one-half of the ColE1 genome and a small segment of φ80pt190 DNA. This mini-ColE1 plasmid, designated pVH51, has a molecular weight of approximately 2.1×10^6 and possesses a single *Eco*RI restriction site. Heteroduplex analyses showed that about 90% of the pVH51 plasmid hybridizes to about 50% of the ColE1 plasmid. Phenotypically, pVH51 did not produce colicin E1 but conferred immunity to this colicin. The number of mini-ColE1 plasmid molecules per cell was maintained at a four- to fivefold higher level than normal ColE1. A mini-ColE1 hybrid plasmid, designated pML21 and consisting of pVH51 and the *kan* fragment of plasmid pSC105 inserted at the *Eco*RI restriction site of mini-ColE1, was maintained at a lower copy number level than pVH51. As in the case of normal ColE1, both pVH51 and pML21 continued to replicate in the presence of chloramphenicol. The promotion of conjugal transfer of pVH51 and pML21 by a self-transmissible plasmid was greatly reduced compared with normal ColE1.

The colicinogenic plasmid E1 (ColE1) is a small (4.2×10^6 daltons), extrachromosomal deoxyribonucleic acid (DNA) molecule, stably maintained in *Escherichia coli* (1). Its presence in a cell can be readily detected by the cell's capacity to produce a specific antibiotic protein, colicin E1, and by the immunity of the cell to the lethal action of colicin E1. Insertion of DNA in vitro at the single site in ColE1 sensitive to the *Eco*RI restriction endonuclease produces chimeras of ColE1 that no longer determine the production of active colicin but continue to specify immunity to colicin E1 (11). Logarithmically growing *E. coli* cells harbor 20 to 30 copies of this plasmid per cell (4). Replication of ColE1 has been shown to require an active DNA polymerase I (13) and continues in the presence of chloramphenicol (CM) (2, 4). The insertion of DNA into ColE1 at the *Eco*RI site in the molecule does not alter the number of copies of this plasmid per cell or its ability to continue to replicate in the presence of CM (11).

One of these ColE1 recombinant plasmids, pVH15, has a mass of approximately 14.3×10^6 daltons and consists of the ColE1 genome plus the tryptophan operon of *E. coli* and regions of the genome of the bacteriophage φ80pt190 (11).

[1] Present address: Cold Spring Harbor Laboratory, Long Island, N. Y. 11724.

This study reports some of the properties of a plasmid derived spontaneously from pVH15 in cells growing under selective pressure. This plasmid, designated pVH51, contains one-half of the ColE1 genome and a small portion of the φ80 DNA of pVH15. It has a mass of 2.1×10^6 daltons and possesses a single *Eco*RI cleavage site. The use of this plasmid as a cloning vehicle is also described.

MATERIALS AND METHODS

Organisms. *E. coli* K-12 strain C600 *trp*R$^-$ Δ*trp*E5/pVH15 was the parental strain of C600 *trp*R$^-$ Δ*trp*E5/pVH51. pML21 was constructed in vitro (M. Lovett and D. Helinski, submitted for publication) as a chimera between pVH51 and the *Eco*RI-generated *kan* fragment of pSC105 (6). *Eco*RI cleavage of pVH15 yields three fragments corresponding to linear ColE1 DNA and two *Eco*RI-derived fragments of φ80pt190 DNA of molecular weight 8.5×10^6 and 1.6×10^6, respectively. The larger of the two fragments carries the complete tryptophan operon and the N gene of φ80 (10). pVH51, pML21, and ColE1 were established in C600 by transformation using plasmid DNA purified by dye-buoyant density centrifugation by procedures described elsewhere (7). C600 requires threonine, leucine, and thiamine for growth.

Media. L agar (pH 7.4) contained (per liter) 10 g of tryptone (Difco), 5 g of yeast extract (Difco), 5 g of NaCl, and 13.5 g of agar. N-salts medium contained

0.047 M Na_2HPO_4, 0.022 M K_2PO_4, 0.018 M $(NH_4)_2SO_4$ and 2×10^{-6} M $FeCl_3$ (pH 7.1) (14). N-salts medium was supplemented with 0.4% vitamin-free Casamino Acids (Difco), 5 μg of thiamine per ml, and 0.2% glucose or glycerol. Deoxyadenosine at 200 μg/ml was added to enhance the uptake of radioactive thymine.

Growth, labeling conditions, and preparation of lysates. All cultures were grown at 37 C. Measurement of cell mass was performed on a Klett-Summerson colorimeter using filter no. 54 (green). In experiments in which the level of plasmid DNA was measured, the cells were incubated while being shaken in N-salts-Casamino Acids-glucose medium containing deoxyadenosine and [^{3}H]thymine (18 Ci/mmol; New England Nuclear Corp.) to a cell density of 2×10^8 to 4×10^8 cells per ml, at which time a sample was taken. Growth and incorporation of [^{3}H]thymine were stopped by addition of sodium azide (5×10^{-2} M, final concentration) and chilling the culture. When cells were further incubated in the presence of CM, this inhibitor was added at a final concentration of 170 μg/ml. Samples were lysed, and a portion of the lysate was centrifuged to equilibrium in a cesium chloride-ethidium bromide gradient as described previously (4).

To determine the presence of a plasmid DNA-protein relaxation complex, 30-ml cultures were grown to 2×10^8 to 4×10^8 cells/ml in supplemented N-salts-Casamino Acids-glycerol medium in the presence of deoxyadenosine and [^{3}H]thymine. After addition of sodium azide and lowering of the temperature, cells were sedimented and washed with TES buffer (0.05 M tris(hydroxymethyl)aminomethane(Tris) [pH 8.0], 0.005 M ethylenediaminetetraacetic acid [EDTA; pH 8.0], and 0.05 M NaCl) and resuspended in 1 ml of 25% sucrose (0.05 M Tris, pH 8). Samples were then lysed as previously described (12) with 0.025% Triton X-100, and the lysates were cleared at 40,000 × *g* for 20 min.

Sucrose density gradients. Sucrose gradient (5 to 20%) centrifugation, collection of fractions, and counting of radioactivity were carried out as described (3). Cleared lysates were run on gradients containing 0.5 M NaCl, 0.05 M Tris (pH 8), and 0.005 M EDTA (pH 8.0) and were centrifuged for 120 min at 45,000 rpm in a Beckman SW50.1 rotor.

Agarose gel electrophoresis. *Eco*RI endonuclease cleavage of DNA was carried out as described (11), and samples were analyzed on 0.7% agarose gels in Tris-borate buffer (pH 8.2) at 70 V for 5.5 h. The DNA bands were stained with 0.5 μg of ethidium bromide per ml in buffer and photographed with a shortwave ultraviolet transilluminator.

RESULTS

Isolation of mini-ColE1 plasmid. The ColE1-*trp* plasmid, pVH15, is lost at high frequency in a host strain carrying a *trp*R$^-$ mutation when grown in the presence of tryptophan (11). Under these growth conditions, a smaller plasmid element, detected by sucrose gradient analysis, was observed in a culture of a previously pure clone of C600 *trp*R$^-$Δ*trp*E5/pVH15. Cells carrying only the smaller plasmid were subsequently obtained after growing C600 *trp*R$^-$Δ*trp*E5/pVH15 on L agar overnight. Cultures of these strains showed no detectable colicin production, but when streaked against purified colicins they were found to have retained colicin E1 immunity. CsCl-ethidium bromide centrifugation of a cleared lysate of a culture of one of these strains labeled with [^{3}H]thymine yielded a supercoiled DNA peak that sedimented in a neutral sucrose gradient as a 2.1×10^6-dalton molecule. This mini-ColE1 plasmid was designated pVH51. pVH51 was established in C600 by transformation with purified pVH51 supercoiled DNA. A neutral sucrose gradient analysis of this plasmid from the C600/pVH51 strain is shown in Fig. 1A. All subsequent experiments with pVH51 were carried out with the C600/pVH51 strain.

Analysis of pVH51 using the *Eco*RI restriction endonuclease showed that this plasmid possessed a single *Eco*RI restriction site. A non-self-replicating fragment of DNA was inserted in vitro into pVH51 at this site, and the hybrid plasmid was established by transformation (7) initially in C600 *trp*R$^-$Δ*trp*E5*recA* and then in C600 for all subsequent analyses. This fragment of DNA, generated by *Eco*RI cleavage of pSC105 (6) plasmid DNA, had a molecular weight of 4.5×10^6 and contained the genetic determinant of kanamycin resistance (*kan* fragment). Sucrose gradient analysis of the mini-ColE1-*kan* hybrid, designated pML21, indicated that the main DNA peak sedimented as a 30*S* molecule, corresponding to a molecular weight of approximately 6.6×10^6 (Fig. 1B). The faster sedimenting peak of DNA in this gradient exhibited a sedimentation value expected for a dimer of supercoiled pML21. The sedimentation value of the slowest sedimenting DNA species in this gradient corresponds to that expected for the open, circular forms of monomer pML21.

Structure of pVH51 and pML21. The analysis of *Eco*RI digests of the pVH51 and pML21 plasmids by agarose gel electrophoresis is shown in Fig. 2. pVH51 was cleaved to a single molecular species of 2.1×10^6 daltons (Fig. 2B), whereas pML21 generated two fragments corresponding to the *kan* fragment of pSC105 and the parental pVH51 plasmid DNA (Fig. 2).

A representative heteroduplex between *Eco*RI-cleaved pVH51 DNA and ColE1 DNA is shown in Fig. 3. The heteroduplex shows a double-stranded region of precise homology of about 2.0×10^6 daltons, corresponding to 48% of one end of the linear ColE1 DNA molecule. A

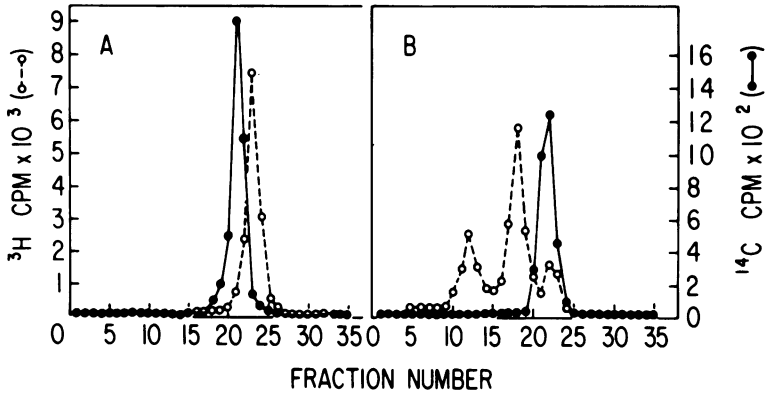

FIG. 1. *Sedimentation analysis of purified supercoiled DNAs. Supercoiled plasmid DNAs were isolated after CsCl-ethidium bromide centrifugation of cleared lysates from C600 strains grown in the presence of glucose as previously described (12). Approximately 20,000 counts/min of [^{3}H]thymine-labeled pVH51 (A) and pML21 (B) DNA were centrifuged in the presence of [^{14}C]thymine-labeled ColE1 marker DNA (23S) on a 5 to 20% sucrose gradient. The gradients were centrifuged for 120 min at 45,000 rpm in a Spinco SW50.1 rotor. Fractions were collected onto Whatman no. 1 filter paper, and the radioisotope was counted as described in Materials and Methods.*

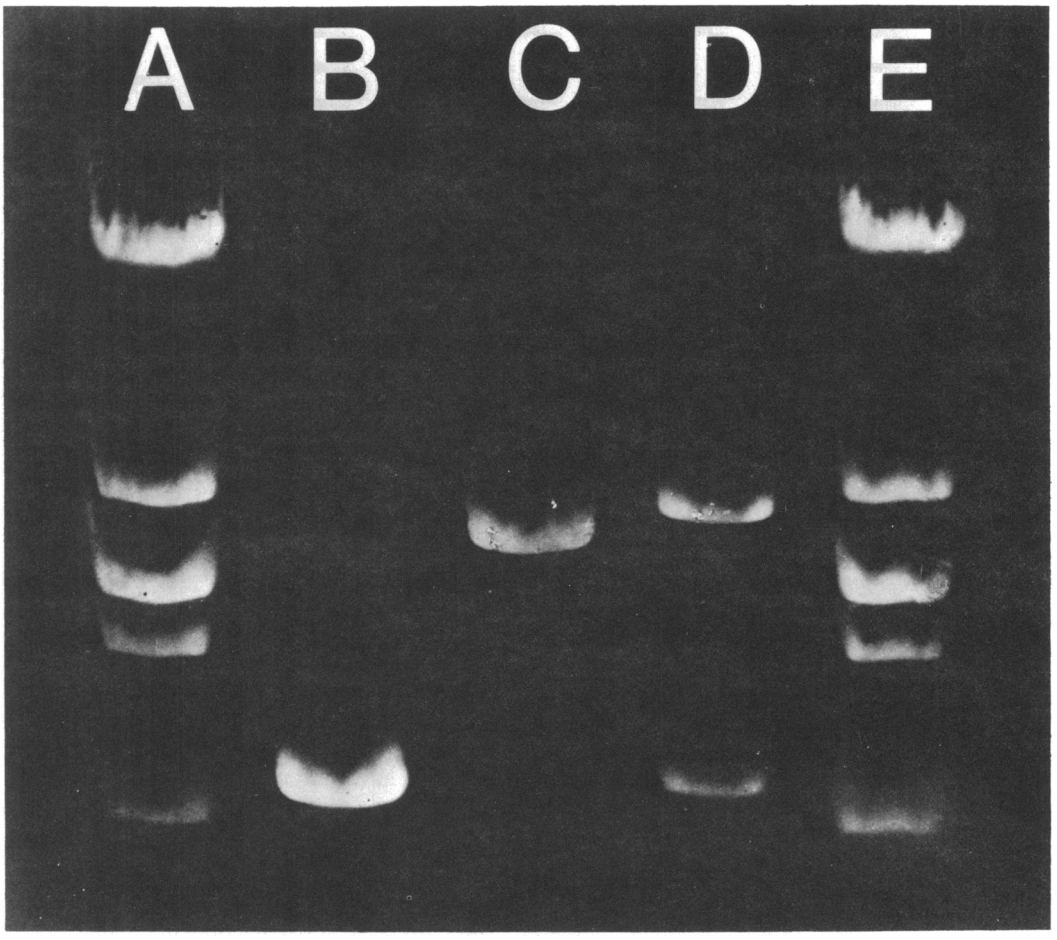

FIG. 2. *Agarose slab gel electrophoresis of plasmid DNA cleaved with EcoRI endonuclease. The digested DNA (0.4 to 0.6 μg) was applied to the sample slots in 50-μl volumes containing 10% sucrose. Agarose gel electrophoresis was carried out as described in Materials and Methods. Molecular weight estimates are based on λ fragments generated by EcoRI. (A) and (E) λ fragments with molecular weights of 13.70 × 10^6, 4.68 × 10^6, 3.7 × 10^6, 3.56 × 10^6, 3.03 × 10^6 and 2.09 × 10^6, respectively (the 3.7 × 10^6 and 3.56 × 10^6 bands are not separated). (B) pVH51, molecular weight of 2.1 × 10^6. (C) ColE1, molecular weight of 4.2 × 10^6. (D) pML21 fragments, molecular weights of 4.5 × 10^6 and 2.1 × 10^6, respectively.*

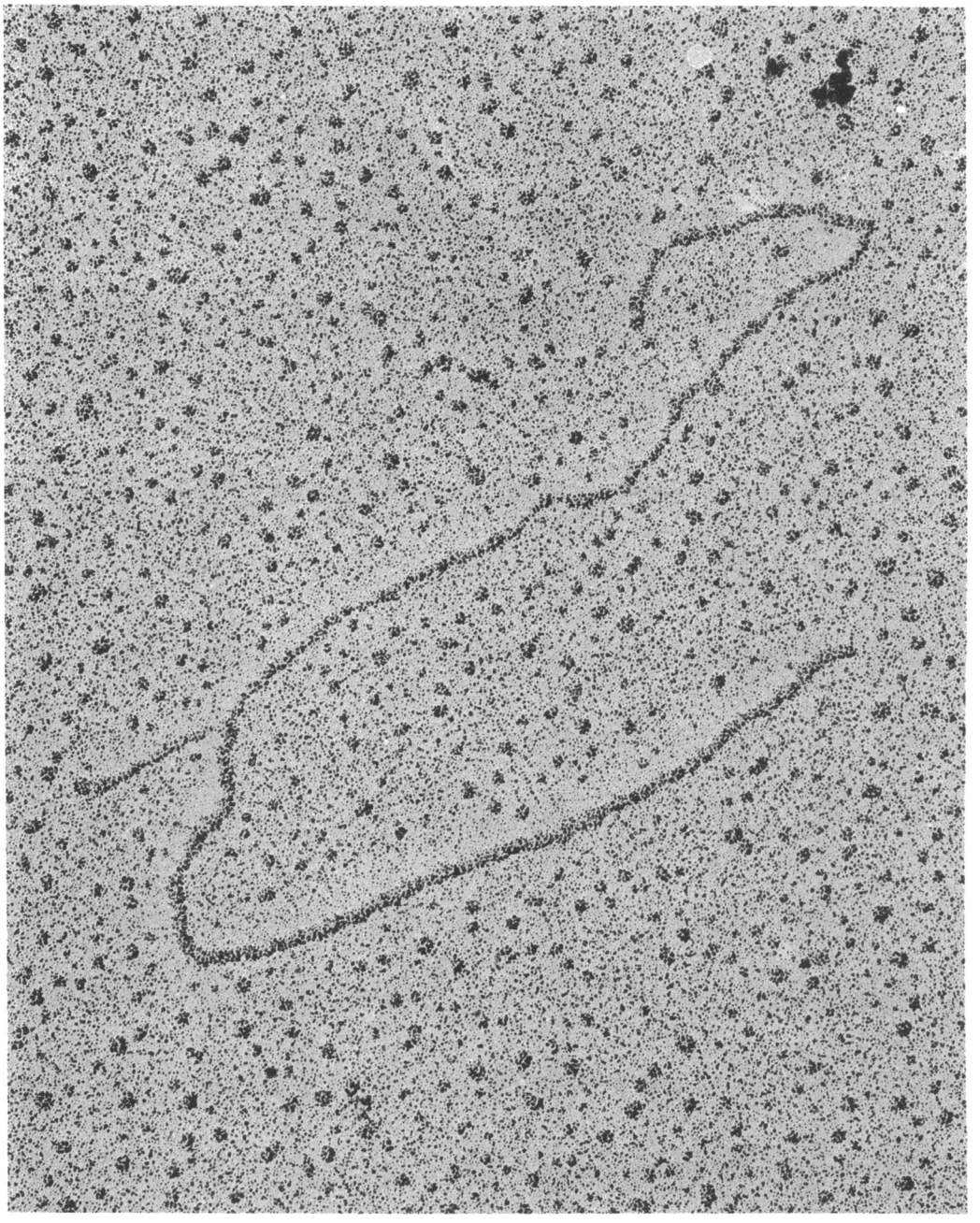

FIG. 3. *Approximately 10 μg each of pVH51 and ColE1 were cleaved to linear molecules by digestion with EcoRI endonuclease. After extraction with phenol and then ether, the samples were dialyzed against 10 mM Tris-hydrochloride (pH 8.5) and 1 mM EDTA. Methods for denaturation and neutralization of the mixed DNA samples and formamide spreading techniques for electron microscopy have been described (5).*

short single-stranded tail, or region of nonhomology between pVH51 and ColE1, corresponds to about 10% of the pVH51 molecule. These results indicate that all of pVH51 is derived from ColE1 except the short region that most likely represents a portion of φ80pt190 DNA incorporated into pVH15.

Copy number and conjugal transfer of pVH51 and pML21. Strains carrying ColE1 normally have approximately 2 to 3% of their

DNA that can be isolated as supercoiled ColE1. This is about 15 copies per chromosome. In logarithmically growing cultures, approximately 5% of the total DNA in cells harboring pVH51 exists as supercoiled DNA, an average of about 57 copies per chromosome or 114 copies per cell, assuming two genome equivalents per cell (8). Unexpectedly, only 4 to 4.5% of the total DNA of the pML21-containing strain of *E. coli* was present as the supercoiled DNA form of this plasmid. This level of plasmid DNA corresponds to about 15 copies per chromosome.

Both pVH51 and pML21 continued to replicate in the presence of CM to approximately the same final level as ColE1. As shown in Fig. 4, all three plasmids attained a level of about 46% of their total DNA as supercoiled DNA after overnight incubation of the cells in the presence

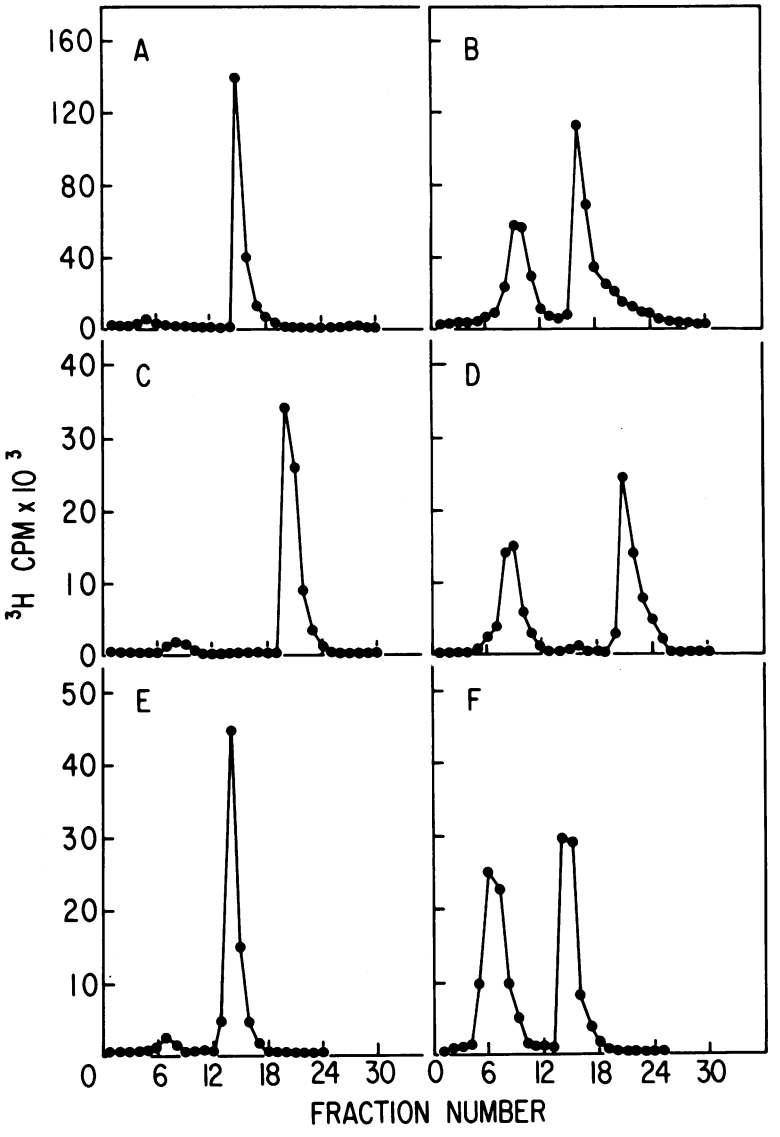

FIG. 4. *Dye-buoyant density centrifugation of Sarkosyl lysates of cells before and after incubation in the presence of CM. A, C, and E represent lysates of midlog-phase cultures of C600 cells containing ColE1, pVH51, and pML21, respectively, at the time of addition of CM. B, D, and F are lysates prepared from the same cultures (ColE1, pVH51, and pML21, respectively) 20 h after 170 μg of CM per ml was added. The cultures exhibited a generation time of 50 min in the N-salts medium containing Casamino Acids before CM was added. Fractions were collected and counted as described in Materials and Methods.*

of CM in N-salts medium containing Casamino Acids. This amount of plasmid DNA corresponds to 935, 350, and 500 copies per chromosome for pVH51, pML21, and ColE1, respectively.

As in the case of ColE1, pVH51 and pML21 are non-self-transmissible but mobilizable by a self-transmissible plasmid. However, the promotion of transfer of pVH51 and pML21 by the self-transmissible plasmids R100drd and R64drd11 was considerably lower than found for ColE1. Under mating conditions where these R plasmids promote the transfer of ColE1 to 25% of the recipient cells, only 0.005% of the recipients received and maintained pVH51 or pML21.

Examination of strains for plasmid relaxation complexes. It has been observed that ColE1 DNA can be purified from gently prepared lysates as a relaxation complex consisting of supercoiled ColE1 DNA plus protein material (3). Treatment of this complex with certain agents that alter protein structure, such as sodium dodecyl sulfate, results in a conversion of the supercoiled DNA in the complex to the open, circular DNA form (3). A high proportion of ColE1 DNA can be obtained in the form of relaxation complex by growing the cells in N-salts-Casamino Acids medium containing glycerol as a carbon source.

To test the presence of pVH51 and pML21 as relaxation complex, cleared lysates of cells containing these plasmids were treated with 0.4% sodium dodecyl sulfate. Treated and untreated samples were then centrifuged in a 5 to 20% neutral sucrose gradient. The sodium dodecyl sulfate treatment failed to convert any significant amount (<10%) of pVH51 or pML21 to the open, circular DNA form under conditions where about 60% of normal ColE1 DNA was found in the form of relaxation complex. These results indicate that pVH51 and pML21 either are not present as relaxation complex, present to a very low extent as complex, or, unlike normal ColE1, extraction of these plasmid DNA molecules from the cell under the conditions employed results in dissociation of the relaxation proteins from the plasmid DNA.

DISCUSSION

The mechanism responsible for the generation of the mini-ColE1 plasmid, pVH51, from the ColE1-*trp* plasmid pVH15 is unknown. On the basis of the structure of the heteroduplex between pVH51 and normal ColE1, it is possible to conjecture that mini-ColE1 arose by a recombination event between the midpoint of the ColE1 portion of pVH15 and a region of the ϕ80pt190 segment of this plasmid.

Several properties of the parental plasmid, pVH15, have been retained in mini-ColE1. In addition to the failure to produce colicin E1, the plasmid continues to specify immunity to colicin E1 despite the loss of approximately one-half of the ColE1 DNA. Furthermore, as in the case of the parental plasmid, mini-ColE1 is present as multiple copies per cell. However, the deletion of a substantial amount of ColE1 DNA has apparently affected the regulation of the number of copies of this plasmid per cell, since a considerably higher level (estimated at 114 copies per cell) was observed. Finally, pVH51 has retained the ability to replicate in the presence of CM. The final yield of pVH51 after continued replication in the presence of CM is approximately 45% of the total DNA, a level similar to that obtained in the presence of CM for normal ColE1 and the ColE1 chimeras pVH15 (ColE1-*trp*) and pML2 (ColE1-*kan*) (11). It is clear that the final yield of ColE1 and ColE1 hybrid plasmids in the presence of CM is related to the total mass of plasmid DNA rather than the number of copies of the plasmid element.

Although several of the above properties of the parental plasmid were retained in mini-ColE1, it was observed that the transfer of this plasmid, promoted by the self-transmissible plasmids R100drd and R64-11, was reduced to a very low level. In addition, whereas a substantial portion of the DNA of the parental plasmid pVH15 can be isolated in the form of relaxation complex (unpublished observation), similar extraction conditions did not yield a significant level of pVH51 in the form of relaxation complex.

Mini-ColE1 has a single *Eco*RI cleavage site, it is smaller than normal ColE1, and it has a more elevated copy level—these properties make this plasmid a potentially more suitable cloning vehicle than normal ColE1. In addition, the greatly reduced transmissibility of mini-ColE1 provides more effective containment of the plasmid to the host cell. However, in the one instance of insertion of the *kan* fragment of DNA into mini-ColE1, the number of copies per cell of the resulting plasmid chimera was lowered considerably. It remains to be seen whether or not the insertion of DNA into mini-ColE1 will consistently result in a reduction in the copy level. Finally, as in the case of normal ColE1, the insertion of DNA into mini-ColE1 does not affect the ability of this plasmid to continue to replicate in the presence of CM.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grants AI-07194 and GM-14378 from the National Institute of Allergy and Infectious Diseases and the Na-

tional Institute of General Medical Sciences, respectively, and National Science Foundation Research grant GB-29492. V. Hershfield was supported by Public Health Service Postdoctoral Fellowship GM54149.

LITERATURE CITED

1. **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.
2. **Clewell, D. B.** 1972. Nature of ColE1 plasmid replication in the presence of chloramphenicol. J. Bacteriol. 110:667-676.
3. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. U.S.A. 62:1159-1169.
4. **Clewell, D. B., and D. R. Helinski.** 1972. Effect of growth conditions on the formation of the relaxation complex of supercoiled ColE1 deoxyribonucleic acid and protein in *Escherichia coli*. J. Bacteriol. 110:1135-1146.
5. **Chow, L. T., H. W. Boyer, E. Tischer, and H. M. Goodman.** 1974. Electron microscopic mapping of the attachment sites on SV40 DNA during lytic infection. Cold Spring Harbor Symp. Quant. Biol. 39:109-117.
6. **Cohen, S. N., A. C. Chang, H. W. Boyer, and R. Helling.** 1973. Construction of biologically functional plasmids *in vitro*. Proc. Natl. Acad. Sci. U.S.A. 70:3240-3244.
7. **Cohen, S. N., A. C. Y. Chang, and C. L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. 69:2110-2114.
8. **Cooper, S., and C. E. Helmstetter.** 1968. Chromosomal replication and the division cycle of *Escherichia coli* B/r. J. Mol. Biol. 31:519-540.
9. **Fredericq, P.** 1954. Transduction genetique des propertes colicinogenes chez *Escherichia coli* et *Shigella sonnei*. C. R. Soc. Biol. 148:399-402.
10. **Helling, R. B., H. M. Goodman, and H. W. Boyer.** 1974. Analysis of endonuclease R *Eco*RI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. J. Virol. 14:1235-1244.
11. **Hershfield, V., H. W. Boyer, C. Yanofsky, M. Lovett, and D. R. Helinski.** 1974. Plasmid ColE1 as a molecular vehicle for cloning and amplification of DNA. Proc. Natl. Acad. Sci. U.S.A. 71:3455-3459.
12. **Katz, L., D. T. Kingsbury, and D. R. Helinski.** 1973. Stimulation by cyclic adenosine monophosphate of plasmid deoxyribonucleic acid replication and catabolite repression of the plasmid deoxyribonucleic acid-protein relaxation complex. J. Bacteriol. 114:577-591.
13. **Kingsbury, D., and D. R. Helinski.** 1970. DNA polymerase as a requirement for the maintenance of the plasmid colicinogenic factor E1. Biochem. Biophys. Res. Commun. 41:1538-1544.
14. **Nomura, M., and C. Witten.** 1967. Interaction of colicins with bacterial cells. III. Colicin tolerant mutations in *Escherichia coli*. J. Bacteriol. 94:1093-1111.