JOURNAL OF BACTERIOLOGY, Aug. 1975, p. 658-665

Vol. 123, No. 2
Printed in U.S.A.

Class of Small Multicopy Plasmids Originating from the Mutant Antibiotic Resistance Factor R1*drd*-19B2

WERNER GOEBEL[1]* AND RENATE BONEWALD

Gesellschaft für Molekularbiologische Forschung mbH, 33 Stöckheim-Braunschweig, Germany

Received for publication 7 February 1975

The large mutant R-factor R1*drd*-19B2 gives rise to several classes of small, covalently closed circular deoxyribonucleic acids (DNAs), designated as Rsc DNAs, when harbored by the K-12 strain CRT46 which carries a *dna*A mutation. The molecular weights of these DNA molecules range from 3×10^6 to 8.4×10^6. Cells arising from single colonies of CRT46-R1*drd*-19B2 harbor only one to two copies of the large mutant R-factor and in addition 10 to 20 copies of Rsc plasmid of a discrete size class per chromosome. The larger Rsc DNAs carry the ampicillin resistance gene. After transformation the small circular DNAs are present in *Escherichia coli* C in a large number of copies, up to 100 copies per chromosome. Hybridization studies between Rsc plasmids indicate that they possess common DNA sequences.

The depressed antibiotic resistance factor R1*drd*-19 mediates resistance to ampicillin, streptomycin, kanamycin, sulfonamide, and chloramphenicol (10). Cells carrying this plasmid contain one copy per chromosome, which suggests that the replication of this extrachromosomal deoxyribonucleic acid (DNA) is stringently controlled. Recently, Nordström et al. have isolated a mutant of this R-plasmid, factor R1*drd*-19B2, the presence of which results in a severalfold increase in the antibiotic resistances of host cells (13). Strains harboring R1*drd*-19B2 also carry an equally increased number of plasmid copies (13). In an *Escherichia coli* wild-type strain the mutant plasmid is of the same molecular size as the parent R1*drd*-19 factor. Upon transfer into *Proteus mirabilis* it dissociates into a resistance entity and a transfer entity (13), like many other R-factors (4, 12).

In this communication we show that after transfer of R1*drd*-19B2 into the *dna*A mutant CRT46 distinct size classes of small multicopy plasmids are generated which contain the replication genes and, in some cases, the ampicillin resistance gene of the R1*drd*-19B2 parent plasmid.

MATERIALS AND METHODS

Bacterial strains. *E. coli* 1005-R1*drd*-19B2 (13) was obtained from K. Nordström. *E. coli* strains CRT46 and JC411 have been previously described (7). *E. coli* C *met-lac* was obtained from D. Jacob (London).

Media and buffers. Enriched nutrient broth and phosphate-buffered minimal medium (6) were used for the growth of the bacteria. TES buffer contained 0.02 M tris(hydroxymethyl)aminomethane, pH 8.0, 5 mM ethylenediaminetetraacetate, and 0.05 M NaCl. SSC contained 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0. Ampicillin, chloramphenicol, streptomycin, and kanamycin were added to the selection plates in concentrations of 100 μg/ml.

Source of reagents. [*methyl*-^{3}H]thymidine (specific activity, 24.3 Ci/mmol) and carrier-free $H_3{}^{32}PO_4$ were 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). The antibiotics were gifts from Bayer (Leverkusen, Germany).

Bacterial conjugation. The transfer of the R1*drd*-19B2 factor into *E. coli* CRT46 was performed by conjugation on solid plates as described (8). The transconjugants were counterselected by their inability to ferment lactose and to grow at 43 C.

Cell lysis and DNA purification. Cells were grown in phosphate-buffered minimal medium in the presence of [^{3}H]thymidine (5 μCi/ml) and 250 μg of deoxyadenosine per ml. Cells were harvested by centrifugation, washed free of radioactive label, and lysed. The "cleared lysis" procedure used to isolate plasmid DNA has been described (6). The spheroplast-Sarkosyl lysis procedure (1) was used for the determination of the number of plasmid copies. Covalently closed plasmid DNA was isolated from these lysates by cesium chloride-ethidium bromide density gradients and further purified by sucrose gradient centrifugation.

Centrifugation conditions. Cesium chloride-ethidium bromide gradients were prepared by adding

[1] Present address: Institut für Genetik und Mikrobiologie der Universität Würzberg, 87 Würzburg, Röntgenring 11, Germany.

8 ml of a cleared lysate containing 100 μg of ethidium bromide per ml to 7.3 g of CsCl. The solution was centrifuged for 24 h at 44,000 rpm in a Spinco 50Ti rotor at 2 C. Neutral sucrose gradients (5 to 20% in TES buffer) were spun in a Spinco SW50.1 rotor at 45,000 rpm or in a Spinco SW27 rotor at 23,000 rpm at 20 C. All gradients were fractionated by collecting drops from the bottom of the centrifuge tube (6).

DNA-DNA membrane filter hybridization. The DNA-DNA membrane filter hybridization was performed by the method of Denhardt (5), with some minor modifications which have been described previously (9).

Transformation. Transformation of *E. coli* C by bacterial plasmids was performed by a procedure communicated to us by Jacob. This procedure is essentially a modification of that described by Cohen and Chang (2) but yields almost 10-fold higher transformation rates with *E. coli* C than the latter one. Cells of *E. coli* C logarithmically grown in enriched nutrient broth medium (optical density at 600 nm = 0.7) were chilled on ice and harvested by centrifugation (2 C). The cells were resuspended in 0.5 volume of ice cold 0.1 M $CaCl_2$, kept in ice for 20 min, and pelleted again by centrifugation. The pellet was resuspended in 0.1 volume of cold 0.1 M $CaCl_2$. A 0.1-ml portion of a plasmid DNA solution (2 to 5 μg/ml) was mixed in the cold with 0.2 ml of the cell suspension. The mixture was heated for 40 s at 37 C and then kept in ice for 60 min. Prewarmed (37 C) enriched nutrient broth medium (10-fold excess) was added to the mixture, which was then incubated for 90 min at 37 C. Aliquots of the culture were plated on appropriate selective agar plates.

RESULTS

Characterization of R1drd-19B2 in the dnaA mutants CRT46. The antibiotic resistance factor R1*drd*-19B2 was derived from the parent R1*drd*-19 factor (10) by mutagenesis with ethylmethane sulfonate (13). Both factors have the same molecular weight and carry resistance genes for ampicillin, chloramphenicol, streptomycin, kanamycin, and sulfonamide. However, in contrast to the plasmid R1*drd*-19, which occurs in the cell in one to two copies per chromosome, the mutant R1*drd*-19B2 factor is present in the cell in a 3.5-fold higher number of copies per chromosome (13). The increased number of plasmid copies causes elevated level of antibiotic resistances. It has been claimed that the mutation abolishes the stringent regulation of replication of this plasmid (13). While studying the replication and the integrative suppression of the mutant factor in the *dnaA* mutant CRT46 (unpublished data), we have observed that in the absence of antibiotics in the growth medium a considerable fraction of the supercoiled plasmid DNA synthesized at the permissive temperature (30 C) consists of DNA which is considerably smaller (30*S*) than the original R1*drd*-19B2 factor (supercoiled 76*S*, circular 46 to 48*S*) (Fig. 1B). This small DNA is not present in a CRT46 mutant carrying the parent R1*drd*-19 factor (Fig. 1A). After transferring cells of CRT46 R1*drd*-19B2 several times in antibiotic-free media, the plasmid DNA of this strain consists of more than 50% of this small, covalently closed circular (CCC) DNA. The total fraction of CCC DNA in these cells amounts to about 8% of the total DNA. Taking into consideration the molecular size of the two plasmids (65×10^6 daltons for R1*drd*-19B2 and about 8×10^6 daltons for the small plasmid), one cell of CRT46 R1*drd*-19B2 contains one to two copies of R1*drd*-19B2 and 10 to 15 copies of the small plasmid per chromosome.

Transformation of E. coli C by the large and small plasmid of CRT46 R1drd-19B2. The fractions contributing to peaks I–III of the sucrose gradient (Fig. 1B) were pooled to give three separate DNA solutions. *E. coli* C was transformed with each of these using a modification of the transformation procedure of Cohen and Chang (2), which was communicated to us by Jacob. Table 1 shows that DNA of peaks I and II yields transformants which have the same antibiotic resistance pattern as R1*drd*-19B2 (13). This indicates that these DNA peaks contain the CCC and open circular forms of the original plasmid. In contrast, DNA of peak III yields transformants which are resistant to ampicillin only (Table 1). Plasmid DNA from the (*E. coli* C) transformants obtained with DNAs of peaks I–III of Fig. 1B were isolated by the "cleared lysate" procedure and cesium chloride-ethidium bromide centrifugation (15). Sucrose gradient analysis of the CCC DNA fractions revealed the presence of the large R1*drd*-19B2 plasmid (76*S*) in transformants obtained with DNA from peaks I and II, whereas transformants obtained with DNA from peak III contained only the smaller CCC DNA (31*S*) (Fig. 2A–C).

To determine the amount of CCC DNA present in the cell, the latter transformant was subjected to lysozyme-Sarkosyl lysis (1). About 15% of the total DNA so released can be isolated by dye-buoyant density centrifugation as CCC DNA (Fig. 3A), which consists entirely of small circular 31*S* DNA (Fig. 3B). This amount of covalently closed DNA is higher than in CRT46 and suggests the presence of more than 40 copies of this plasmid (molecular weight, 8.4×10^6) per chromosome. This DNA is designated below as Rsc11 DNA.

Isolation of Rsc DNA from single colonies

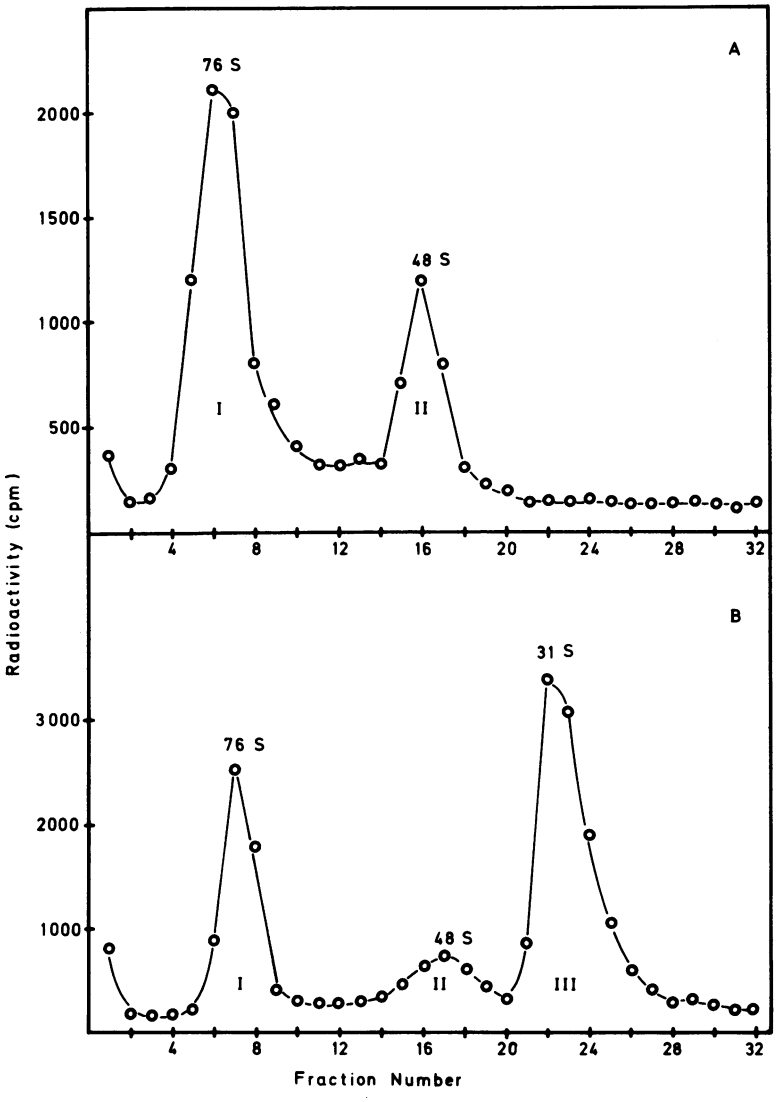

FIG. 1. *Neutral sucrose gradient centrifugation of plasmid DNA from E. coli CRT46 R1drd-19 and E. coli CRT46 R1drd-19B2. Plasmid DNA was isolated from the cleared lysates of the corresponding strains by cesium chloride-ethidium bromide centrifugation. The fractions containing plasmid DNA were dialyzed against TES buffer. Two-tenths milliliter of plasmid DNA prepared in this way was mixed with 5 μl of a solution of ^{32}P-labeled Col E1 DNA (23S, indicated by the arrow), and the mixture was centrifuged in a Spinco SW50.1 rotor at 45,000 rpm and 20 C for 60 min. Ten-drop fractions were collected from the bottom of the tube. (A) Plasmid DNA of CRT46 R1drd-19; (B) plasmid DNA of CRT46 R1drd-19B2.*

of CRT46 R1drd-19B2. Comparing the band width of the 31*S* peak of CRT46 R1*drd*-19B2 (Fig. 1B) to that of Rsc DNA of *E. coli* C transformed by this DNA (Fig. 2C and 3B), it is obvious that the former is somewhat broader and hence may represent a collection of heterogeneous DNA molecules. To test this possibility, cultures of CRT46-R1*drd*-19B2 were raised from single colonies and labeled with [^{3}H]thymidine. Plasmid DNA was isolated by dye-buoyant centrifugation of the cleared lysates. ^{3}H-labeled CCC DNA was mixed with covalently closed ^{32}P-labeled Col E1 DNA (23*S*) as internal marker and analyzed on neutral sucrose gradients.

These analyses revealed the presence of at least four size classes of Rsc DNAs, with sedimentation coefficients of 31*S*, 27*S*, 23 to 24*S*,

TABLE 1. *Transformation of E. coli C by DNAs of peaks I, II, and III*[a]

DNA	No. of transformants isolated on ampicillin plates	Antibiotic resistances of the transformants			
		Amp	CM	Sulf	Strep
Peak I	97	+	+	+	+
Peak II	15	+	+	+	+
Peak III	2,500	+	−	−	−

[a] *E. coli* was transformed as described in Materials and Methods with DNA of peaks I, II, and III (Fig. 1B). DNA solutions contained 5 to 10 μg/ml. Transformant colonies were selected (by spreading 0.1 ml of the transformation mixture) on enriched nutrient broth agar plates containing 100 μg of ampicillin per ml. Amp, Ampicillin; CM, chloramphenicol; Sulf, sulfonamide; Strep, streptomycin. All antibiotics were applied in concentrations of 100 μg/ml.

and 21*S* (Fig. 4A–E). Out of 40 single colonies of CRT46 R1*drd*-19B2 analyzed by this procedure, 20 belonged to class I (Rsc11; 31*S*), 9 to class II (Rsc12; 27*S*), 7 to class III (Rsc13;, 23 to 24*S*), and 3 to class IV (Rsc14; 21*S*). One colony had a Rsc DNA of 34 to 35*S*, which may represent an additional class V (Rsc10). All colonies of CRT46 R1*drd*-19B2 analyzed contained, in addition to the Rsc plasmids, one to two copies of the large factor R1*drd*-19B2. The CCC form of this plasmid sediments to the bottom in the gradients (Fig. 4A–E). The open circular form sediments at 46 to 48*S*.

Transformation of E. coli C by Rsc DNAs. To test whether all Rsc DNAs are independently replicating plasmids carrying the ampicillin resistance gene, *E. coli* C was transformed with isolated CCC Rsc DNAs belonging to the different size classes. DNAs of classes I to III, but not class IV, gave rise to ampicillin-resistant transformants with high frequencies (Table 2). All ampicillin-resistant transformants obtained with the isolated Rsc DNAs contained high levels of CCC DNA (15 to 20% of the total cellular DNA) with homogeneous DNA species identical in size to the original transforming Rsc DNA. This indicates that, depending on the molecular size of the Rsc plasmids, 50 (for Rsc11) to 100 (for Rsc14) plasmid copies per chromosome are present in the cell. Rsc10 DNA (34 to 35*S*; class V) also yielded ampicillin-resistant transformants. However, these contained CCC DNA of 31*S*. None of the ampicillin-resistant transformants were able to transfer its Rsc DNA during mating with an appropriate recipient strain, indicating that Rsc DNAs do not carry the transfer genes of the R1*drd*-19B2 factor.

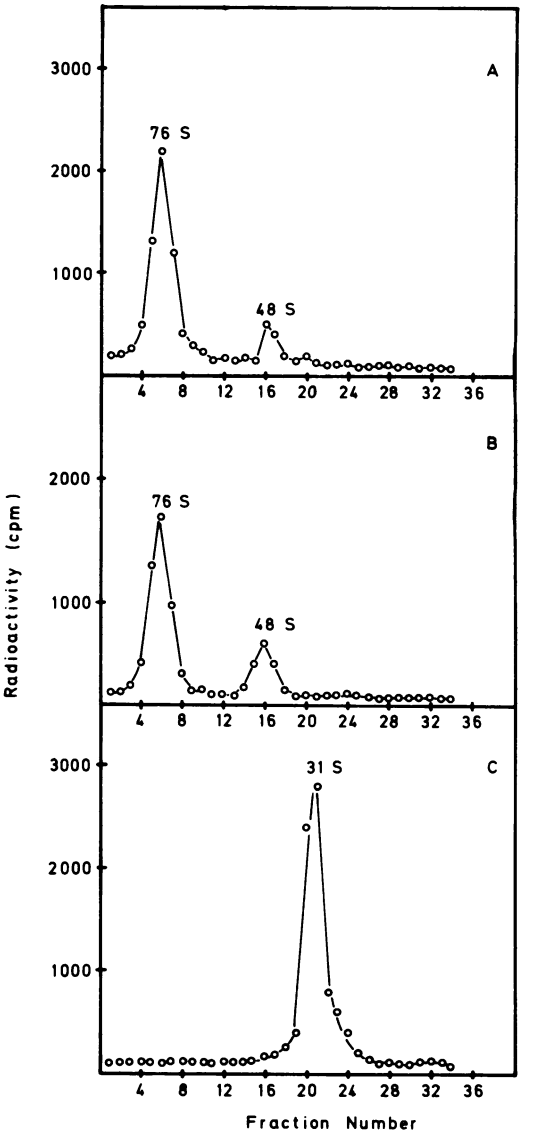

FIG. 2. *Sucrose gradient analysis of plasmid DNAs from E. coli C cells transformed with DNAs of peaks I, II, and III of Fig. 1B. Fractions composing peaks I, II, and III in Fig. 1B were separately pooled, and E. coli C was transformed, using them as described in the text. Plasmid DNA was isolated from cleared lysates of the transformant strains by cesium chloride-ethidium bromide gradient centrifugation. Fractions containing covalently closed DNA were further analyzed on neutral 5 to 20% sucrose gradients (1 h, 20 C, 45,000 rpm, SW50.1 rotor). (A) Plasmid DNA from E. coli C transformed by DNA of peak I; (B) plasmid DNA from E. coli transformed by DNA of peak II; (C) plasmid DNA from E. coli C transformed by DNA of peak III. S values are related to covalently closed Col E1 DNA (23S).*

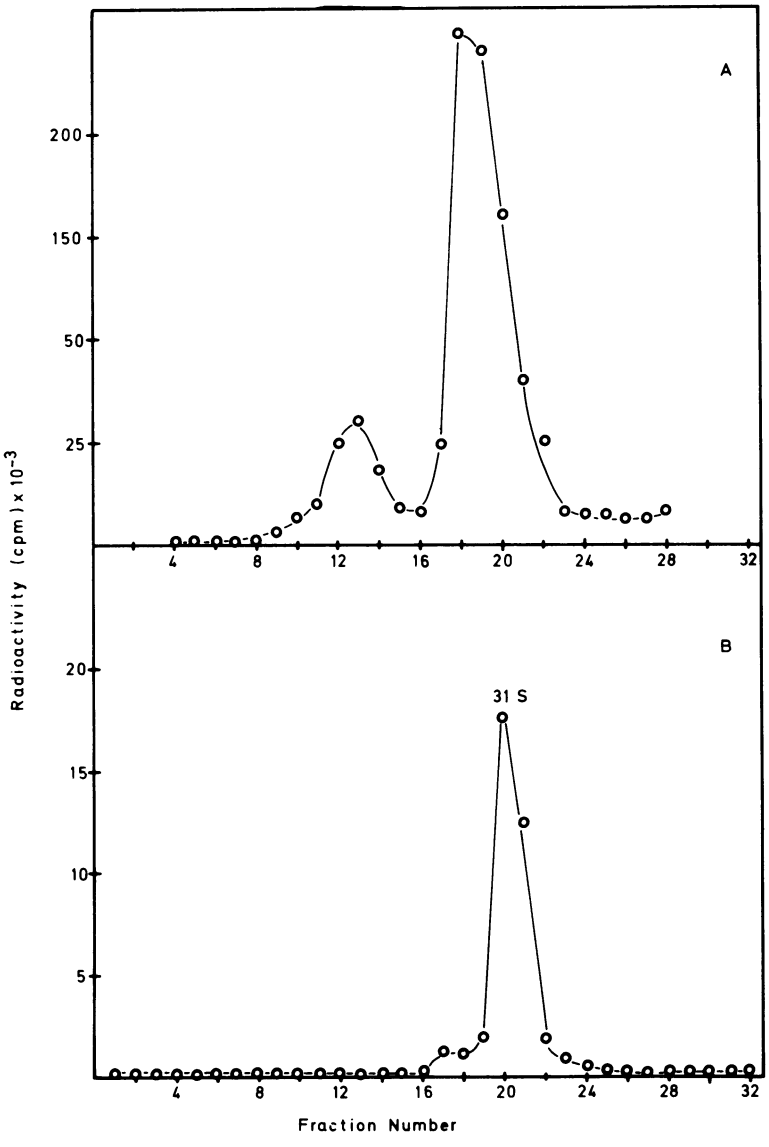

FIG. 3. *Plasmid DNA isolated from a Sarkosyl lysate of E. coli C Rsc11. Cells of E. coli C Rsc11 were lysed with Sarkosyl as described (1). The viscous lysate was sheared by pipetting it through the hole of a 1-ml pipette, mixed with appropriate amounts of cesium chloride and ethidium bromide, and centrifuged to equilibrium in a Spinco Ti50 rotor (24 h, 44,000 rpm, 2 C). Fractions (15 drops) were collected from the bottom of the tube into small vials from which 10 μl was taken for determination of the radioactivity (A). The fractions containing the covalently closed plasmid DNA (fractions 12 to 14) were dialyzed against TES buffer and centrifuged through a neutral 5 to 20% sucrose gradient as described in Fig. 1B.*

DNA-DNA hybridization between Rsc DNAs. To test the relationship between the various size classes of Rsc DNA molecules, DNA-DNA hybridization was performed with the isolated Rsc DNAs. Purified Rsc DNAs of the size classes I–IV (21*S*, 24*S*, 27*S*, and 31*S*) were fixed in increasing amounts to nitrocellulose filters which were annealed to ^{32}P-labeled Rsc11 DNA (31*S*) by the method of Denhardt (5). Before fixation to the nitrocellulose filters, supercoiled Rsc DNAs were opened and denatured by heating the DNA solution for 15 min at 100 C in the presence of 0.1 NaOH. This treatment converts practically all of the supercoiled DNA to open circular or linear DNA, as demonstrated by sucrose gradient analysis. After neu-

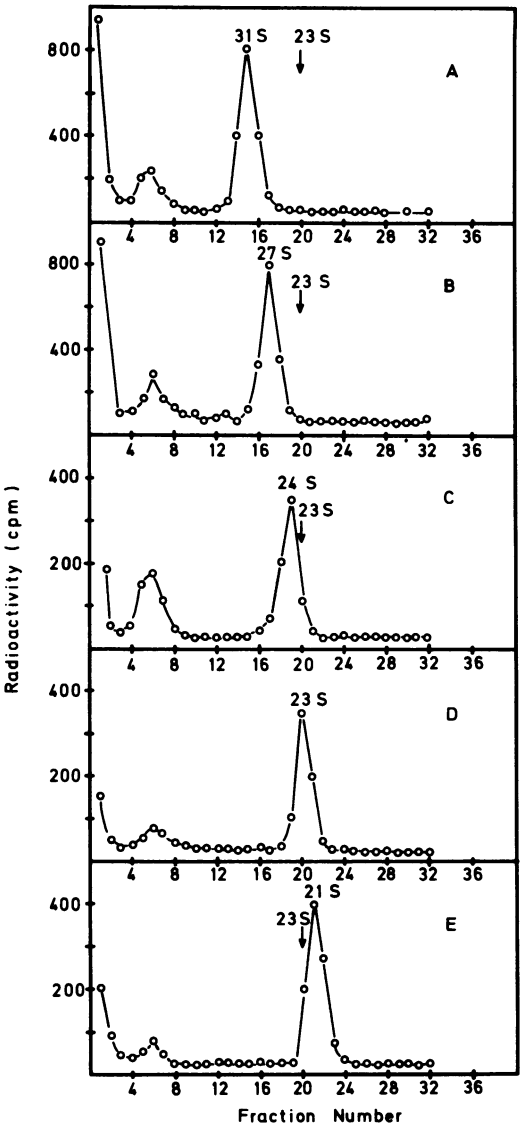

FIG. 4. *Sucrose gradient analyses of plasmid DNA of CRT46 R1drd-19B2 cultures originating from single colonies. Cultures of E. coli CRT46 R1drd-19B2 were raised from single cell clones and labeled with [^3H]thymidine (5 μCi/ml), and cleared lysates of the cells were prepared. Plasmid DNA was isolated by cesium chloride-ethidium bromide centrifugation. Fractions containing the covalently closed plasmid DNA were further analyzed on neutral 5 to 20% sucrose gradients (SW50.1, 2 h, 45,000 rpm, 20 C). (A-E) Plasmid DNA from culture of CRT46 R1drd-19B2 raised from five single colonies.*

tralization with 0.1 N HCl to pH 7.0, increasing amounts of denatured ^3H-labeled Rsc DNAs were fixed on filters, which were then incubated with constant amounts of heat-denatured ^{32}P-labeled Rsc11 (31*S*) DNA. The latter was degraded by sonic treatment to DNA fragments sedimenting at about 8*S*. Denaturation of the fragmented Rsc11 DNA by alkali with subsequent neutralization yielded the same results.

Figure 5A shows the hybridization saturation curves obtained. At saturation 45% of the ^{32}P-labeled Rsc11 DNA (31*S*) was bound to ^3H-labeled Rsc11 DNA (31*S*) present on the membrane filter. This value was taken as 100% homology, and hybridizations with the other Rsc DNAs were normalized to this value. Related to the corrected 100% efficiency of hybridization with homologous Rsc11 DNA, 80, 55, and 35% hybridization is obtained with Rsc12 DNA (27*S*), Rsc13 DNA (24*S*), and Rsc14 DNA (21*S*), respectively, Taking into consideration the molecular weights of the various Rsc DNAs (8.4×10^6 for Rsc11 DNA, 6.8×10^6 for Rsc12 DNA, 5×10^6 for Rsc13 DNA, and 3×10^6 for Rsc14 DNA [1]), the data obtained are in good agreement with the assumption that all Rsc DNAs contain common nucleotide sequences.

As expected, Rsc11 DNA hybridizes completely to R1*drd*-19B2 DNA, whereas only 40% hybridization is obtained between Rsc11 DNA and R1*drd*-16 DNA. The latter was derived from R1*drd*-19 DNA by a deletion mutation and carries only the kanamycin resistance gene (10) (Fig. 5B).

DISCUSSION

The replication of the antibiotic resistance factor R1*drd*-19 is stringently controlled in the *E. coli* cell; i.e., this plasmid occurs in the cell in one to two copies per chromosome (10, 13). Nordström et al. (13) have recently isolated a copy mutant of this plasmid, R1*drd*-19B2, which in contrast to the parent plasmid is present in the cell in a 3.5-fold higher amount.

TABLE 2. *Transformation of E. coli C by isolated Rsc DNAs*[a]

DNA	Transformation frequency for ampicillin resistance	Sedimentation coefficient of CCC DNA in transformants (*S*)
Rsc10	10^{-4}	31
Rsc11	5×10^{-4}	31
Rsc12	6×10^{-4}	27
Rsc13	6.5×10^{-4}	24
Rsc14	0	

[a] Transformation of *E. coli* C by Rsc DNAs isolated from cultures of CRT46 R1*drd*-19B2 as described in the text was performed as in Table 1. Concentration of the transforming DNA solutions was 10 μg/ml.

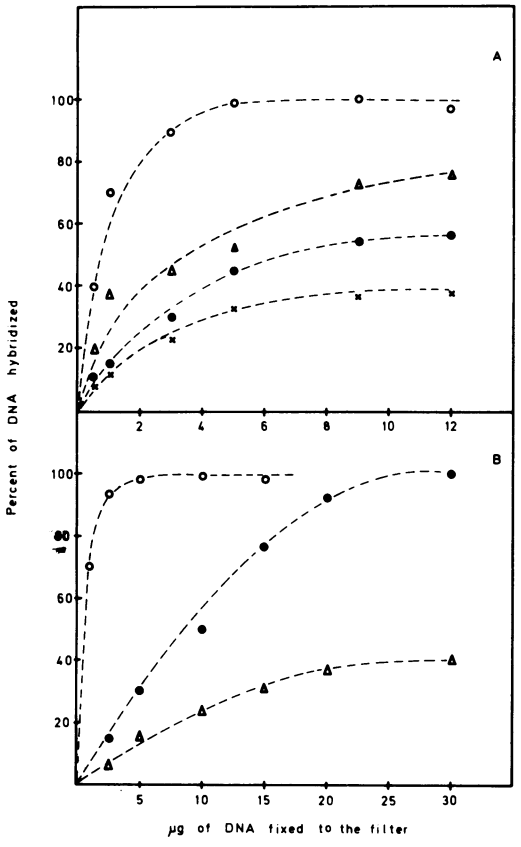

FIG. 5. *DNA-DNA hybridization of ^{32}P-labeled Rsc11 DNA of E. coli C-Rsc11 with (A) various fixed amounts of ^{3}H-labeled Rsc11 DNA* (○), *Rsc12 DNA* (▲), *Rsc13 DNA* (●), *and Rsc14 DNA* (×) *and (B) with increasing fixed amounts of ^{3}H-labeled R1drd-19B2 DNA* (●) *and R1drd-16 DNA* (△). *Annealing was performed as described in Materials and Methods in a total volume of 1.2 ml containing approximately 0.05 µg of ^{32}P-labeled Rsc11 DNA 10^{5} counts/min of DNA per µg. The percent values are normalized as described in the text.*

Both plasmids have, however, the same size and exhibit the same antibiotic resistances. The level of resistance against the various antibiotics is higher with R1*drd*-19B2 in parallel to the increased number of plasmid copies in the cell. The reason for the relaxed control of the mutant plasmid DNA replication may be the loss of a negatively acting control element for the initiation of replication (13, 14). When transferred into the *dna*A mutant CRT46, which has a temperature-sensitive lesion in the initiation of chromosomal DNA (11), the generation of small CCC DNA molecules (Rsc DNA) is observed at the permissive temperature. Low quantities of these small Rsc plasmids can also be observed in other temperature-sensitive DNA replication mutants at 30 C and even in a wild-type K-12 strain when the strains are kept for longer periods of time in antibiotic-free media or in media with ampicillin as the only antibiotic. However, the amount of Rsc plasmid in the *dna*A mutant is considerably higher and may be over 50% of the total supercoiled DNA of this particular strain. Individual cells of the *dna*A mutant, carrying the mutant factor R1*drd*-19B2, always contain one or two copies of this large plasmid and in addition 10 to 20 copies of Rsc plasmid of a discrete size class per chromosome. These small DNA molecules can be divided into at least four discrete size classes ranging from 3×10^{6} to 8.4×10^{6} daltons. The Rsc plasmids are autonomously replicating units independent of the large R1*drd*-19B2 factor. After transformation of *E. coli* C by Rsc DNAs, ampicillin-resistant transformants are obtained, except with the DNA of the smallest size class (Rsc14). All Rsc DNAs occur in the transformant cell in a large number of copies (between 50 and 100 copies per chromosome). This may indicate that the Rsc DNAs still contain the mutation responsible for the multicopy phenotype of the parent plasmid R1*drd*-19B2, although one can not exclude the possibility that the small Rsc DNAs replicate under an entirely different control system than the parent plasmid, thus yielding the high number of plasmid copies.

It has to be mentioned, however, that the small size of a plasmid per se does not lead automatically to an increased number of copies in the cell. For instance, cells of *E. coli* C transformed by the small plasmid pSC101 (2), which originates from the larger antibiotic resistance factor R6-5, harbor only a few plasmid copies per chromosome.

Hybridization studies of the various Rsc DNAs indicate that they contain common nucleotide sequences. The data obtained exclude the possibility that the Rsc DNAs are tandemly arranged repeats of a small basic unit. There are also two main arguments against the generation of Rsc DNAs by dissociation of the original R1*drd*-19B2 DNA into transfer factor and resistance determinants as observed with many R-factors under a variety of conditions (4, 12). (i) The large DNA found in the *dna*A mutant CRT46 R1*drd*-19B2 still contains the Rsc DNA sequences and is identical in size to the R1*drd*-19B2 factor as it exists in the original host. No intermediate DNA which could correspond to the resistance transfer factor part (12) was ever observed in CRT46 R1*drd*-19B2 (ii) Rsc DNAs (except Rsc14) carry only the ampicillin resistance gene but not the other

antibiotic resistances and hence cannot represent the whole resistance determinant.

There is no clear evidence as to how these small Rsc DNAs are generated, and every model proposed at the moment would be entirely speculative. It also remains to be seen whether its increased generation in CRT46 is due to the *dnaA* mutation or to other differences in the genetic background of this strain.

All Rsc DNAs carrying the ampicillin resistance gene have single cleavage sites for the restriction enzymes EcoRI (P. J. Greene, M. C. Betlach, H. M. Goodman, and H. W. Boyer, *In* R. B. Wickner, ed., *Methods in Molecular Biology. DNA Replication and Biosynthesis*, in press) and HindIII (16) (H. Maier, G. Luibrand, and W. Goebel, manuscript in preparation), making these small multicopy plasmids suitable as vectors for in vitro recombinations (3). Such recombinations with EcoRI-cleaved Rsc11 DNA and other suitable plasmid DNAs (Col E1 and pSC101) have already been successfully carried out by us and will be described elsewhere (H. Maier, R. Kollek, and W. Goebel, manuscript in preparation).

LITERATURE CITED

1. Bazaral, M., and D. R. Helinski. 1968. Characterization of multiple circular DNA forms of colicinogenic factor E1 from *Proteus mirabilis*. Biochemistry **7**:3513-3519.
2. Cohen, S. N., and A. C. Y. Chang. 1973. Recircularization and autonomous replication of a sheared R-factor DNA segment in *Escherichia coli* transformants. Proc. Natl. Acad. Sci. U.S.A. **70**:1293-1297.
3. Cohen, S. N., A. C. Y. Chang, H. W. Boyer, and R. B. Helling. 1973. Construction of biologically functional bacterial plasmids *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **70**:3240-3244.
4. 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.
5. Denhardt, D. T. 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641-646.
6. Goebel, W. 1970. Studies on extrachromosomal elements. Replication of the colicinogenic factor Col E1 in two temperature sensitive mutants of *Escherichia coli* defective in DNA replication. Eur. J. Biochem. **15**:311-320.
7. Goebel, W. 1974. Studies on the initiation of plasmid DNA replication. Eur. J. Biochem. **41**:51-58.
8. Goebel, W., B. Royer-Pokora, W. Lindenmaier, and H. Bujard. 1974. Plasmids controlling synthesis of hemolysis in *Escherichia coli*: molecular properties. J. Bacteriol. **118**:964-973.
9. Goebel, W., and H. Schrempf. 1972. Isolation of minicircular DNA molecules from wild strains of *Escherichia coli* and their relationship to other plasmids. J. Bacteriol. **111**:696-704.
10. Meynell, E., and N. Datta. 1967. Mutant drug-resistant factor of high transmissibility. Nature (London) **214**:885-887.
11. 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.
12. Nisioka, T., M. Mitani, and R. Clowes. 1969. Composite circular forms of R-factor deoxyribonucleic acid molecules. J. Bacteriol. **97**:376-385.
13. Nordström, K., L. C. Ingram, and A. Lundbäck. 1972. Mutations in R factors of *Escherichia coli* causing an increased number of R-factor copies per chromosome. J. Bacteriol. **110**:562-569.
14. Pritchard, R. H., P. T. Barth, and J. Collins. 1969. Control of DNA synthesis in bacteria. Symp. Soc. Gen. Microbiol. **19**:263-297.
15. 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. Natl. Acad. Sci. U.S.A. **57**:1514-1521.
16. Smith, H. O., and D. Nathans. 1973. A suggested nomenclature for bacterial host modification and restriction systems and their enzymes. J. Mol. Biol. **81**:419-423.