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Generation of Miniplasmids from Copy Number Mutants of the R Plasmid NR1

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Small, closed circular deoxyribonucleic acid molecules, called miniplasmids, were observed in *Escherichia coli* harboring copy number mutants of the R plasmid NR1 after growth in medium containing tetracycline. The level of tetracycline resistance conferred by the copy mutant plasmids was lower (3 to 6 μg/ml) than that conferred by NR1 (100 μg/ml). The presence of the miniplasmid enhanced the level of tetracycline resistance conferred by the copy mutant. Miniplasmids of molecular weights 4×10^6 to 13×10^6 were found. They carried no antibiotic resistance markers and could be eliminated by growth in the presence of chloramphenicol and/or streptomycin-spectinomycin. Studies with the restriction endonucleases *Eco*RI and *Sal* I indicated that the miniplasmids are derived from the region of the copy mutant plasmids that contains the origin for replication of the resistance transfer factor. There were ~12 copies of the miniplasmid per chromosome, compared with 3 to 6 copies of the copy mutants of NR1. The miniplasmids appeared to be incompatible with the copy mutant plasmids.

NR1 is a conjugative R plasmid that confers resistance to tetracycline (Tc), sulfonamides, streptomycin (Sm)-spectinomycin (Sp), chloramphenicol (Cm) (15, 22), fusidic acid (2), and mercuric ions (25). In most genera of the *Enterobacteriaceae*, NR1 is found as a stable composite R plasmid consisting of the resistance transfer factor that carries the Tc resistance genes (RTF-Tc), and the resistance (r-) determinants component that carries the other drug resistance genes. This is also true in *Proteus mirabilis* when this host strain is cultured in drug-free medium (17, 23). During growth of *P. mirabilis* containing NR1 in medium containing Cm or Sm, there is a dissociation of the R plasmid into the RTF-Tc and r-determinant components. The r-determinant component is selectively amplified and reassociates with the RTF-Tc to form composite R plasmids that contain multiple, tandem (poly-) r-determinants. This phenomenon has been referred to as a "transition," since the R-plasmid deoxyribonucleic acid (DNA) increases in buoyant density from 1.712 to 1.718 g/ml as a result of the increased number of copies of the higher-density r-determinant DNA attached to the RTF-Tc DNA (17, 20, 21, 23). Cells in which a transition has occurred are resistant to increased antibiotic levels as a result of increased gene dosage of the drug resistance genes on the r-determinants (6).

Increased gene dosage resulting in higher levels of antibiotic resistance can also occur in *Escherichia coli* containing NR1. This, however, is usually due to R-plasmid copy number mutants. These mutants undergo more rounds of replication each cell division cycle, resulting in a three- to sixfold increase in the number of copies of the R plasmid per chromosome (1, 14). The poly-r-determinant R-plasmid structures observed during a transition in *P. mirabilis* are not formed in *E. coli* (23). Plasmid copy number mutants of the R plasmid R1 (16) and the cloacinogenic plasmid DF13 (10) have also been described.

Although copy mutants usually increase the level of drug resistance of host cells, the level of resistance to Tc (3 μg/ml) conferred by the copy mutant pRR12 (previously called R12 [13, 14]) is lower than that conferred by NR1 (100 μg/ml) in *E. coli* K-12 and *Salmonella typhimurium* (9; H. Hashimoto, Y. Ike, C. Morris, and R. Rownd, submitted for publication). Several other copy mutants of NR1, isolated more recently in this laboratory, have also been found to confer lower levels of Tc resistance than NR1. In this communication, we show that the level of Tc resistance of cells harboring copy mutants can be increased by generating small circular

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DNA molecules (called miniplasmids) from the copy mutant plasmids. The origin, number of copies per chromosome, and characteristics of miniplasmids generated from several copy mutants of NR1 will be described. A preliminary report of this work has been presented elsewhere (1). Related observations on miniplasmids from the copy mutant pRR12 (13), from a copy mutant of R1 (4), from an ampicillin resistance plasmid R-MS201 (5), and from a ColE1-ϕ80*trp* hybrid plasmid (7) have also been reported recently.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 ML1410 (from H. Hashimoto) is Met^- and nalidixic acid (50 μg/ml) resistant (3). *E. coli* K-12 FL1699 is an F^- derivative of KL16-99 (12; from W. Dove) that was produced by treatment with acriflavin and screening for an $MS2^r$ $f2^r$ $T7^s$ clone (D. Taylor, unpublished data) and is F^- λ^- *thi recA1*. CR34 Thy^- (from W. Dove) is *lac thr leu thi thy* (20 μg/ml; 14). CR34A1 is a spontaneous *nal*^{r} mutant of CR34 Thy^- that also lacks the cryptic plasmid of CR34 (L. Ponton and R. Rownd, unpublished data).

R plasmids. The copy mutants of NR1 used in this study are listed in Table 1. pRR22B was obtained by mutagenizing CR34(pRR22) with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at a concentration of 1 mg/ml, then mating the mutagenized culture with ML1410. The transconjugants were screened and a Cm^s clone was isolated and designated ML1410(pRR22B). This R plasmid is also transfer deficient (Tra^-) (L. Ponton, unpublished data). The Cm^s and *tra* mutations were independently revertible and so are presumably due to two different point mutations (D. Taylor, unpublished data).

Media and reagents. The media, antibiotics, and other materials have been described previously (6, 15, 17).

Microbial techniques. Matings and assays for level of antibiotic resistance have been described by Hashimoto and Rownd (6). The protocol for P1 transduction was described by Lennox (11).

Biophysical techniques. Methods for isolating R-plasmid DNA by preparative ethidium bromide-cesium chloride density gradient centrifugation or by nitrocellulose chromatography, for determining the number of plasmid copies per chromosome by ethidium bromide-cesium chloride density gradient centrifugation, for determining sedimentation coefficients by sucrose gradient centrifugation, and for *Eco*RI digestion, agarose gel electrophoresis, and gel photography and analysis have all been described previously (14, 17, 26).

Electron microscopy. The preparation and examination of R-plasmid DNA by electron microscopy and the subsequent analysis of these data have been described previously (17, 26). The length reference standards employed were PM2 (3.53 μm) and ϕX174RF (1.9 μm) DNA. PM2 DNA, when examined by itself, contained no multimers, but ϕX174RF DNA did contain a small fraction of dimers and trimers (R. Warren, personal communication). Consequently, molecules with lengths similar to or multiples of those of the standards were not included as novel DNA species in Table 3.

RESULTS

Initial observations on miniplasmids derived from R-plasmid copy mutants. pRR22B DNA yields 15 fragments after *Eco*RI digestion rather than the 13 fragments observed for NR1 DNA (26). The extra fragments of pRR22B DNA arise from an insertion of molecular weight 3.6×10^6. In several experiments with ML1410 (pRR22B) that had been cloned on plates containing 25 μg of Tc per ml, an additional fragment was observed for pRR22B DNA after digestion with *Eco*RI. The mobility of this additional *Eco*RI fragment (and, hence, its molecular weight) was found to be variable in different experiments, and its intensity was usually greater than expected on the basis of its size relative to the other bands in the gel pattern. In one experiment, this additional band migrated between *Eco*RI fragments D and E (Fig.

TABLE 1. *Characteristics of R plasmids*

Plasmid	Phenotype[a]						Mol wt[b] ($\times 10^6$)	No. of copies per chromosome[c]	Derived from:	Reference
	Tra	Tc	Cm	Sm/Sp	Sa	Rep.				
NR1[d]	+	+	+	+	+	+	58	1		15, 22
pRR12	+	+	+	+	+	Cop.	58	4	NR1	14
pRR21	+	+	+	+	+	Cop.	63	5	NR1-C^+	
pRR22	+	+	+	+	+	Cop.	63	5	NR1-C^+	
pRR22B	-	+	-	+	+	Cop.	63	3	pRR22	L. Ponton, unpublished data

[a] Tra, Transferability; Sa, sulfonamides; rep., replication. +, Wild-type or resistant; -, defective or sensitive; Cop., R-plasmid copy mutant.

[b] Determined by electron microscopy.

[c] Calculated from the percentage of CCC DNA determined in an ethidium bromide-cesium chloride gradient, assuming a molecular weight of 2.5×10^9 for the *E. coli* chromosome.

[d] NR1 has also been designated R100 and R222.

1-1) and, if assumed to be linear DNA, would have a molecular weight of 6.8×10^6. In another experiment with a culture derived from a separate clone, the new fragment migrated slightly faster than *Eco*RI fragment D and, if assumed to be linear DNA, would have a molecular weight of 5.8×10^6 (Fig. 1-2).

pRR22B was transduced to FL1699 by P1*kc*, and transductants were selected on plates containing 25 μg of Tc per ml. *Eco*RI-digested covalently closed circular (CCC) DNA isolated from each of two transductants had an additional fragment of much higher intensity than the other pRR22B fragments (data not shown). If assumed to be linear DNA, these fragments would have molecular weights of 4.2×10^6 and 4.5×10^6, respectively. Sucrose gradient analysis of the undigested DNA of the second sample demonstrated the presence of a 26S species that was presumed to correspond to the 4.5×10^6-dalton fragment (Fig. 2). Examination of these two pRR22B DNA samples by electron microscopy confirmed the existence of a small circular DNA species in the DNA preparations from the two transductants (Fig. 3). The contour lengths of these miniplasmids were determined to be 2.40 and 2.52 μm for the two transductants, respectively. Since these contour lengths agree with the molecular weights determined by agarose gel electrophoresis, these miniplasmids must be migrating as linear DNA on the gels. Therefore, each miniplasmid must contain a single *Eco*RI site.

In other experiments, miniplasmids were observed in *E. coli* strains harboring either pRR22B, pRR21, pRR22, or pRR12 after the host cells had been cloned on plates containing Tc or when Tc (25 to 50 μg/ml) was used in the selective plates in transduction or transformation experiments. The miniplasmids in the cells of individual clones had a unique molecular weight; however, the size of the miniplasmid DNA in different clones was usually not the same, and ranged from 4.5×10^6 to 13×10^6 daltons in different experiments. These initial experiments have shown that it is possible to generate miniplasmids from four different copy mutants after growth of the host cells on medium containing Tc.

Systematic characterization of miniplasmids. To examine the generation and behavior of miniplasmids in a more systematic manner, several copy mutants were transferred to the same host strain under conditions that were known not to result in the formation of miniplasmids. In all of our initial experiments, miniplasmids were only observed in cells that had been cultured in medium containing Tc. ML1410 was chosen as the host because a clone contain-

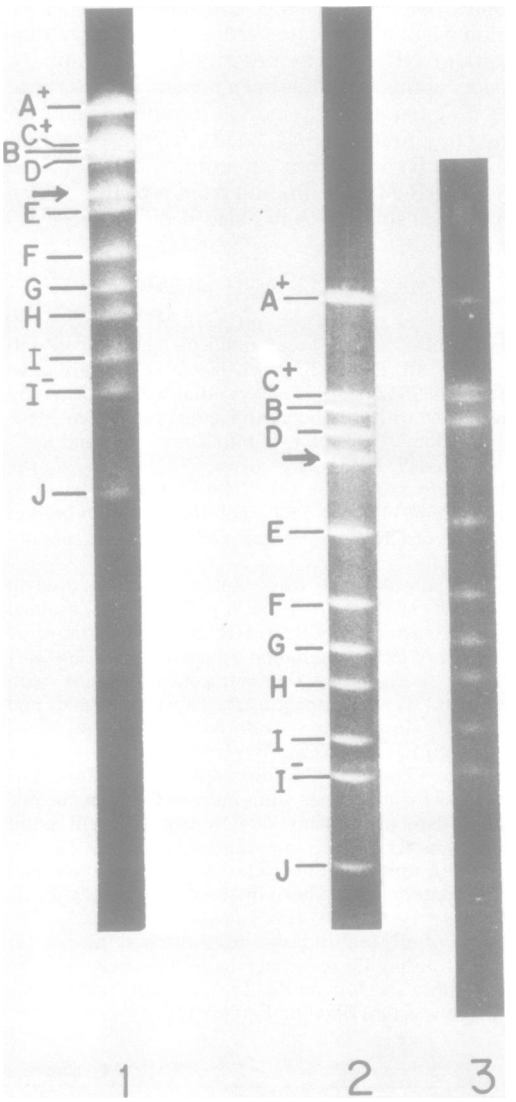

FIG. 1. *Agarose-ethidium bromide gel electrophoresis of EcoRI restriction endonuclease fragments of copy mutant plasmid DNA and miniplasmid DNA. The miniplasmid DNA fragments are indicated by arrows in the various gels. The DNA was isolated as CCC DNA by nitrocellulose column chromatography and purified by neutral cesium chloride density gradient centrifugation. The EcoRI patterns are: (1) pRR22B DNA with miniplasmid DNA having a molecular weight of 6.8×10^6, analyzed on a 1% agarose gel; (2) pRR22B DNA with miniplasmid DNA having a molecular weight of 5.8×10^6, analyzed on a 0.6% agarose gel; and (3) pRR22B DNA without miniplasmid DNA, analyzed on a 0.6% agarose gel.*

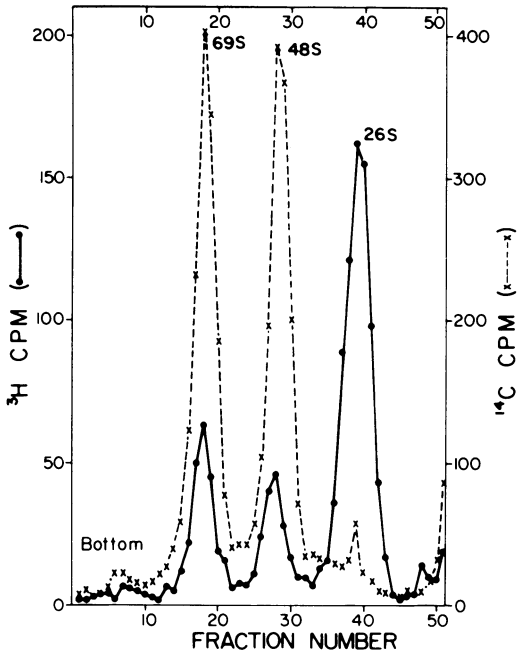

FIG. 2. *Neutral sucrose gradient profiles of pRR22B DNA and miniplasmid DNA. NR1 marker [^{14}C]DNA (×) was sedimented with [^{3}H]DNA (●) isolated from E. coli harboring pRR22 as described in the text. The DNAs were sedimented in 5 to 20% (wt/wt) sucrose gradients.*

ing pRR22B, a *tra* Cm^s derivative of pRR22, was available that lacked miniplasmids. pRR21 and pRR22 were transferred to ML1410 by bacterial mating, and the transconjugants were selected on plates containing Cm (25 μg/ml) and Sp (100 μg/ml). ML1410(pRR22B) and ML1410(NR1) were also cloned on these plates so that these cells would have received similar treatment.

R-plasmid DNA isolated from each of these strains was digested with *Eco*RI and analyzed by electrophoresis on 0.6% agarose gels. Analysis of the patterns indicated that all of the expected *Eco*RI fragments of the copy mutant plasmids were present in stoichiometric amounts; no novel or intensely fluorescing bands were observed (Fig. 4-1). When undigested DNA was examined, only one band was observed in the gel, which corresponded to the CCC copy mutant DNA (data not shown).

The level of Tc resistance of ML1410 harboring the copy mutant pasmids was determined by the plate assay described by Hashimoto and Rownd (6). NR1 confers resistance to Tc at a level of 100 μg/ml. ML1410 containing pRR21 and pRR22 was resistant to 6 μg of Tc per ml and ML1410(pRR22B) was resistant to only 3 μg of Tc per ml.

The ML1410 transconjugants harboring the copy mutant plasmids that had been selected

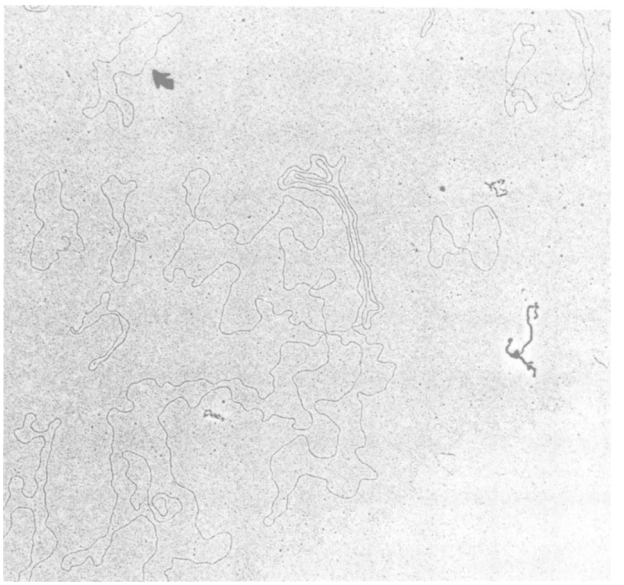

FIG. 3. *Electron micrographs of pRR22B and miniplasmid DNA molecules. The DNA used in the experiment shown in Fig. 2 was spread with PM2 DNA (arrow) as a reference length standard (3.53 μm). The other small circular DNA molecules are miniplasmids with a contour length of 2.54 ± 0.05 μm. The larger circular molecules are pRR22B DNA with a contour length of 35.7 ± 0.5 μm.*

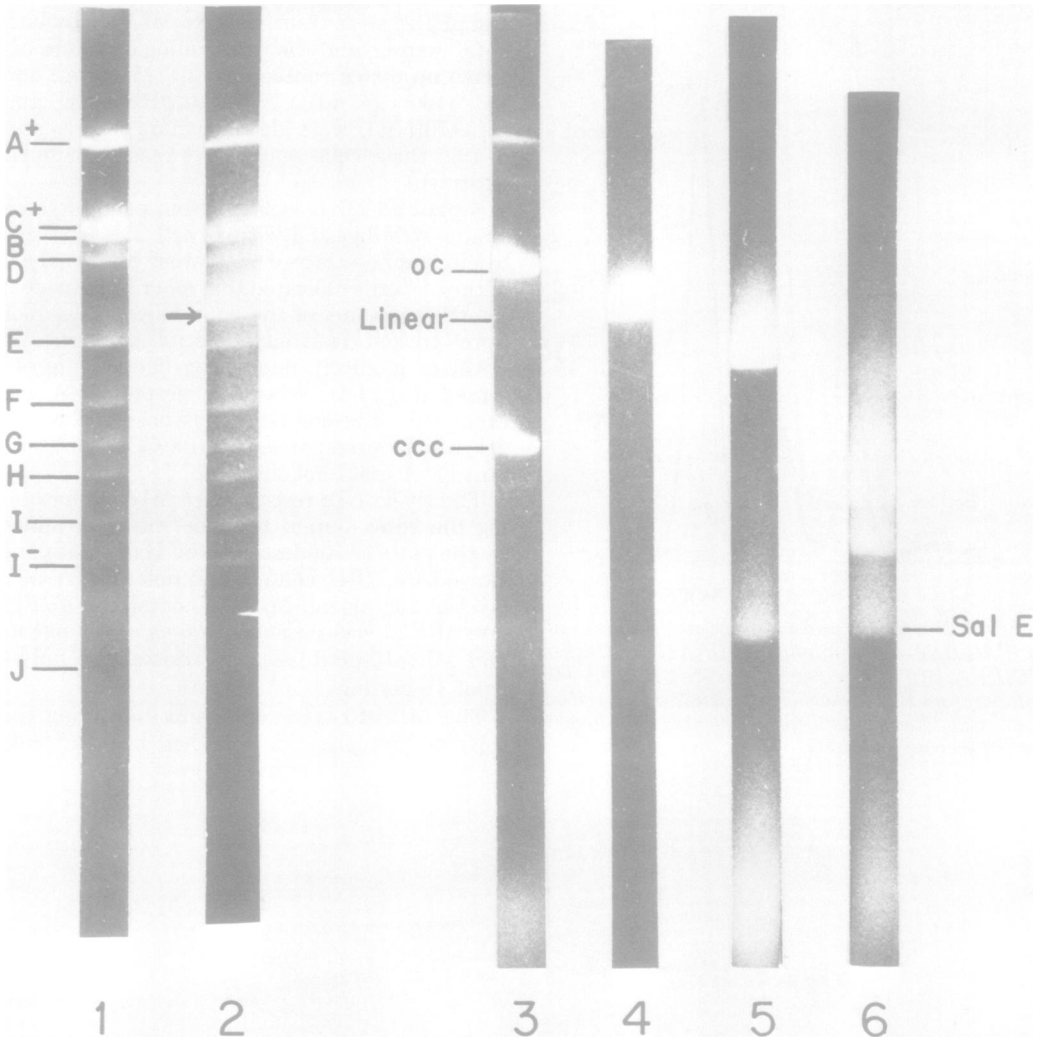

FIG. 4. *Photographs of miniplasmid and copy mutant plasmid DNAs subjected to electrophoresis on 0.6% agarose gels. The CCC DNAs were purified from crude lysates by preparative ethidium bromide-cesium chloride density gradient centrifugation. The patterns are of: (1) EcoRI-digested pRR21 DNA; (2) EcoRI-digested pRR21 DNA and miniplasmid DNA with a molecular weight of 5.7 × 10⁶ (arrow); (3) undigested 5.7 × 10⁶-dalton miniplasmid DNA from drug-sensitive cells harboring only miniplasmids (shown in order of decreasing mobilities are the CCC, open circular (OC), and concatamer forms); (4) EcoRI-digested miniplasmid DNA; (5) Sal I-digested miniplasmid DNA; and (6) Sal I and EcoRI-digested miniplasmid DNA.*

on plates containing Sp were then streaked on plates containing Tc at either 25 or 50 μg/ml About 10^{-2} to 10^{-3} of the cells formed colonies on these plates. Single clones with enhanced Tc resistance were then used to inoculate cultures of the cells in drug-free Penassay broth for isolation of DNA. The results obtained for clones of ML1410 that harbored the different copy mutant plasmids were similar, so we will discuss only the results for ML1410(pRR21). Only about 50% of the clones that grew on plates containing 25 or 50 μg of Tc per ml were found to contain

miniplasmids, and our discussion will deal only with those.

CCC R-plasmid DNA, isolated from a culture that had been inoculated with a clone of ML1410(pRR21) resistant to 50 μg of Tc per ml, was digested with *Eco*RI and analyzed by electrophoresis. In addition to the *Eco*RI fragments of pRR21 DNA, an intensely fluorescing extra band was observed to migrate slightly slower than *Eco*RI fragment E of pRR21 (Fig. 4-2). If linear in conformation, this band corresponds to a miniplasmid with a molecular weight of 5.7×10^6.

After extensive growth of this culture, which contained both pRR21 and 5.7×10^6-dalton miniplasmids in drug-free Penassay broth, antibiotic-sensitive clones could be isolated. Approximately 5% of the cells were drug-sensitive after 20 generations of growth in drug-free medium. These drug-sensitive clones contained CCC DNA, which was isolated and analyzed on gels. The undigested DNA separated into three bands on an agarose gel, corresponding, in order of decreasing electrophoretic mobility, to the CCC, nicked or open circular, and what are thought to be concatenated forms of the miniplasmid DNA (Fig. 4-3). After *Eco*RI digestion of the miniplasmid DNA, only a single band of molecular weight 5.7×10^6 was observed (Fig. 4-4). This band was not present in the gel pattern of the undigested miniplasmid DNA (Fig. 4-3), suggesting that it corresponds to the linear form of the DNA. These observations indicate that the miniplasmid has a single *Eco*RI site.

Digestion of the 5.7×10^6-dalton miniplasmid DNA with the restriction endonuclease *Sal* I produced two fragments that had molecular weights of 4.5×10^6 and 1.2×10^6 (Fig. 4-5). The smaller *Sal* I fragment corresponds in size to *Sal* I fragment E of NR1 or pRR21 DNA (C. Barton, unpublished data). Double digestion of miniplasmid DNA with *Eco*RI and *Sal* I yielded fragments that had molecular weights of 2.5×10^6, 2.0×10^6, and 1.2×10^6 (*Sal* I E) (Fig. 4-6). These results are compatible with the miniplasmid containing the region of the pRR21 plasmid indicated in Fig. 5. The miniplasmid appears to include the part of *Eco*RI fragment H that comes from the RTF-Tc component (referred to as ΔH_2 [26]), possibly the IS*1* element on *Eco*RI fragment H of NR1 DNA, and part of *Eco*RI fragment B (including all of *Sal* I fragment E [1.2×10^6] but not all of *Sal* I fragment D [4.5×10^6]).

Other miniplasmids of molecular weight less than 8×10^6 isolated in this laboratory have also been found to have single *Eco*RI sites and to contain *Sal* I fragment E and a second *Sal* I fragment whose size depends on the molecular weight of the miniplasmid, which indicates that all the miniplasmids examined originate from the same region on the parent plasmid. None of these miniplasmids confers resistance to any of the antibiotics to which NR1 (or its copy mutants) confers resistance, including Tc. This is consistent with the locations of the Cm, Sm-Sp, sulfonamide, and Tc drug resistance genes on the R plasmid (Fig. 5).

Elimination of miniplasmids by selection with Cm or Sp. The culture of ML1410(pRR21) described in the preceding section, containing both miniplasmids (5.7×10^6) and the pRR21 copy mutant plasmid, was cultured in Penassay broth containing 25 μg of Cm and 50 μg of Sp per ml for 20 generations. The *Eco*RI pattern of the CCC DNA isolated from this culture was the same as the *Eco*RI pattern of the CCC pRR21 DNA from a culture without miniplasmids. Extra fragments corresponding to miniplasmid DNA were not observed. Miniplasmids could also be eliminated by cloning cells that harbor both a miniplasmid and the copy mutant plasmid on plates containing Cm at 25 to 50 μg/ml and/or Sm at 25 μg/ml. In these experiments, a clone from the Cm + Sp plates was used to inoculate cultures in drug-free Penassay broth to prepare cells for isolation of CCC R-plasmid DNA.

Number of copies of pRR21 and miniplasmids. To estimate the number of copies of pRR21 in cells without miniplasmids, a clone of ML1410(pRR21) was selected from a plate containing Sm and used to inoculate a culture of the cells in drug-free Penassay broth. DNA was isolated from the culture and centrifuged in an ethidium bromide-cesium chloride density gradient. The percentage of CCC pRR21 DNA was found to be 13.0% of the chromosome DNA, which corresponds to 5.1 copies of the copy mutant R plasmid per chromosome. In a similar experiment, the percentage of CCC DNA in a culture of drug-sensitive cells containing only a miniplasmid (5.7×10^6 daltons) derived from pRR21 was 2.7%, which corresponds to 12 copies of the miniplasmid per chromosome (Table 2). Thus, both the copy mutant plasmid and the miniplasmid derived from it have an increased number of plasmid copies per chromosome in comparison with the R plasmid NR1, which has only about one copy per chromosome in *E. coli* (14). The percentage of CCC DNA in cells harboring both pRR21 and miniplasmids was found to be intermediate between the values observed for cells harboring pRR21 or miniplasmids alone.

Observation of other small circular

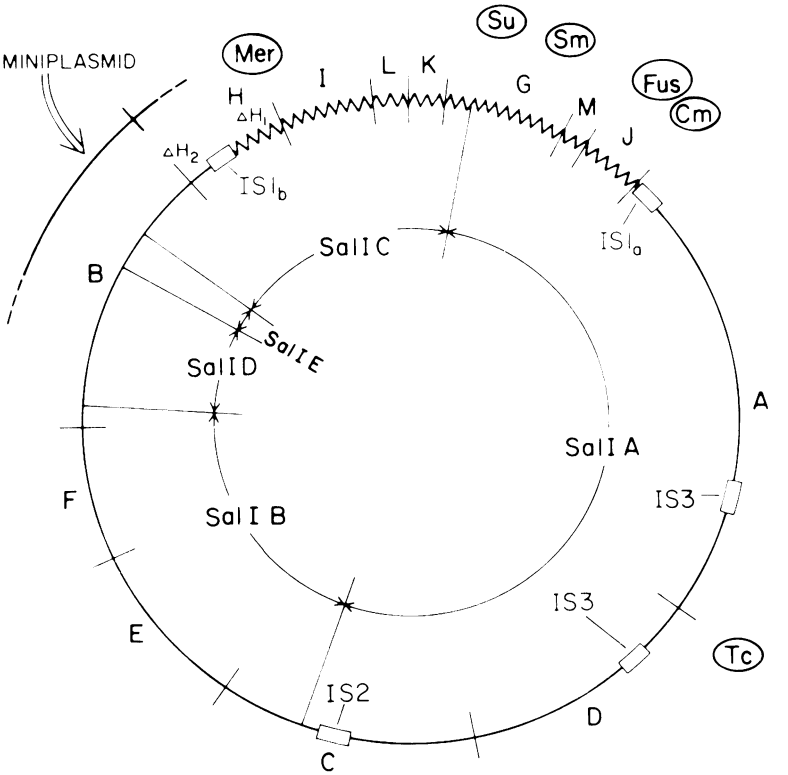

FIG. 5. *Origin of miniplasmids on the EcoRI and Sal I restriction endonuclease maps of the R plasmid NR1. The proposed origin of the miniplasmids characterized in this study is designated on the EcoRI (26) and Sal I (C. Barton, R. Warren, A. Easton, and R. Round, submitted for publication) restriction endonuclease maps of NR1. ——, The RTF-Tc component; ∿∿, the r-determinants component of the composite R plasmid. As described in the text, one extremity of the miniplasmid is proposed to be located at the IS1 element on EcoRI fragment H, and the miniplasmid DNA includes subfragment ΔH_2 (0.8×10^6 daltons), the segment between the EcoRI B-H cleavage site and the Sal I C-E cleavage site (2.0×10^6 daltons), Sal I fragment E (1.2×10^6 daltons), and a variable-sized segment proceeding clockwise on the physical map to the other extremity of the miniplasmid. The dashed ends of the miniplasmid indicate that it has not been definitely established that all of the miniplasmids contain the IS1 element and that the position of the other end is variable. Mer, Mercuric ions; Su, sulfonamides; Fus, Fusidic acid.*

DNAs in R⁺ *E. coli*. During electron microscopic examination of R-plasmid DNA preparations for determination of contour lengths, a number of other small circular DNA species were observed that differed in contour length from the miniplasmids (Table 3). The proportion of these other small plasmids was considerably lower than that of the copy mutant plasmids or miniplasmids in the CCC DNA preparations. Their origin is presently unknown.

DISCUSSION

A number of R-plasmid copy mutants have been isolated that confer a higher level of drug resistance to Cm, Sm, and Sp to host cells, owing to gene dosage effects (14, 16). However, copy mutants derived from NR1 confer a considerably lower level of resistance to Tc (3 to 6 μg/ml) than NR1 (100 μg/ml). The reason for this is presently unknown.

When *E. coli* or *S. typhimurium* cells that harbor copy mutant plasmids are cloned on plates containing a high concentration of Tc, variant clones that have levels of Tc resistance comparable to that conferred by NR1 (Hashimoto et al., submitted for publication) are observed at a comparatively high frequency (10^{-2} to 10^{-3}). A substantial fraction of these cells with enhanced Tc resistance have been found to contain miniplasmids in addition to the copy mutant plasmid. The miniplasmids in the cells of individual clones have a unique molecular weight. However, the size of the miniplasmid DNA in different clones is usually not the same, and has ranged from 4.5×10^6 to 13×10^6 daltons in our experiments.

If cells harboring both miniplasmids and copy mutant plasmids are cultured in medium containing either Cm, Sm, or Sp (sulfonamides have not been examined), the miniplasmids are lost from the cells and only CCC copy mutant plasmid DNA is observed in the cells after a sufficient period of growth. On the other hand, drug-sensitive cells harboring only miniplasmids are formed during growth of cells harboring both miniplasmids and copy mutant plasmids in drug-free medium. These observations suggest that the copy mutant plasmid and the miniplasmid are incompatible. This is presumably because the miniplasmid is derived from the copy mutant plasmid and is able to replace the copy mutant plasmids in the multicopy pool of R plasmids in the cells. If miniplasmids are present in the cells to enhance their level of Tc resistance, then there would be fewer copies of the copy mutant plasmids. Cells harboring a high proportion of miniplasmids would occasionally produce daughter cells that lacked the copy mutant plasmid. These cells would be drug sensitive, since the miniplasmid does not carry drug resistance genes. Growth of cells harboring both miniplasmids and copy mutant plasmids in medium containing either Cm, Sm, or Sp would be selective for cells harboring only copy mutant plasmids, since these cells would have the highest level of drug resistance. These considerations also explain why the percentage of CCC plasmid DNA in cells harboring both miniplasmids and copy mutant plasmids is intermediate between the percentages observed for cells harboring either only miniplasmids or only copy mutant plasmids.

TABLE 2. *Number of plasmid copies per chromosome*

Plasmid[a]	Mol wt ($\times 10^6$)	% CCC DNA[b]	No. of copies/chromosome[c]
NR1	58	2.4	1.4
pRR21	63	13.0	5.1
Miniplasmid	5.7[d]	2.7	12.0

[a] Plasmid harbored by ML1410.

[b] Average of percent CCC DNA from three ethidium bromide-cesium chloride density gradients of DNA from exponential cultures.

[c] Calculated assuming *E. coli* chromosome molecular weight of 2.5×10^9.

[d] Determined by electron microscopy and mobility of *Eco*RI fragments on agarose gels.

The above interpretation can also explain why the level of Tc resistance is increased in cells harboring a mixture of miniplasmids and copy mutant plasmids. Since the decreased resistance to Tc conferred by copy mutants appears to be due to the increased number of copies of the R plasmid, miniplasmids would increase the level of resistance to Tc by decreasing the number of copies of the copy mutant plasmids in the host cells. Since both the miniplasmid and the copy mutant plasmid must be present for enhanced Tc resistance, the level of Tc resistance may depend on the relative number of copies of the miniplasmid and the copy mutant within individual cells.

The fragments produced by treatment of the DNA of several miniplasmids with *Eco*RI and

TABLE 3. *Molecular weights and frequencies of circular DNA species in E. coli*

Host	R plasmid	R plasmid mol wt ($\times 10^6$)[a]	Miniplasmid mol wt ($\times 10^6$)[a]	Other plasmids mol wt ($\times 10^6$)[a]
$CR34A_1$	NR1[b]	57.5 ± 0.78 (45)[c]		12.50 ± 1.4 (5)
FL1699	pRR21[d]	62.1 ± 1.33 (39)	5.92 ± 0.23 (78) 11.6 (1)	4.30 ± 0.21 (12) 7.37 ± 0.37 (3)
CR34	pRR22	64.1 ± 0.62 (47)		9.20 ± 0.74 (5) 13.6 ± 0.26 (31) 48.1 (1) 73.4 (1)
ML1410	pRR22B	62.4 ± 0.64 (62)		4.45 (1)
FL1699	pRR22B-1	63.4 ± 0.80 (43)	4.26 ± 0.09 (217)	13.3 (1)
FL1699	pRR22B-6	62.8 ± 0.73 (50)	4.47 ± 0.09 (77) 8.92 ± 0.11 (2)	26.5 (1) 77.5 (1)

[a] Contour lengths determined by using PM2 as cospread length standard; molecular weights were calculated from contour lengths by using lambda DNA (30×10^6; 17.06 μm) as a standard.

[b] $\phi X174$RF DNA included as second length standard.

[c] Numbers in parentheses represent the number of molecules measured of each designated size class.

[d] $\phi X174$RF DNA cospread instead of PM2 DNA.

Sal I restriction endonucleases indicate that the miniplasmids are derived from a unique region of the copy mutant plasmid. In the case of the 5.7×10^6-dalton miniplasmid derived from pRR12, the 1.2×10^6-dalton fragment of a *Sal* I digest and of an *Eco*RI-*Sal* I double digest should correspond to *Sal* I fragment E, which is located within *Eco*RI fragment B (Fig. 5). The 2.0×10^6-dalton fragment of the *Eco*RI-*Sal* I double digest should correspond to the DNA segment between the *Eco*RI site between *Eco*RI fragments B and H and the *Sal* I site between *Sal* I fragments E and C. Since the insertion element IS1 has been implicated in the formation of deletions in the galactose operon of *E. coli* (19) and the dissociation of the r-determinants component of composite R plasmids (8, 18, 26; J. Miller and R. Rownd, manuscript in preparation), it is likely that the IS*1* element located on *Eco*RI fragment H (26) at the RTF-Tc::r-determinants junction is also included on the miniplasmid. According to this interpretation, the DNA segment from the B-H *Eco*RI site up to and possibly including the IS*1* element (0.8×10^6 daltons, referred to as ΔH_2 in Fig. 5) would be joined to the 4.9×10^6 segment of *Eco*RI fragment B, which is adjacent to the B-H *Eco*RI site proceeding counterclockwise on the physical map (Fig. 5), to form the 5.7×10^6-dalton miniplasmid. The DNA of this miniplasmid would produce all of the fragments observed by treatment of the DNA with *Eco*RI, *Sal* I, and *Eco*RI plus *Sal* I. The other miniplasmids would be formed by a similar mechanism. The size of a miniplasmid would be determined by the location of the second recombination site counterclockwise from the IS*1* element.

Recent studies on replicating molecules of pRR12 DNA isolated from *P. mirabilis* have shown that there is an origin of replication on *Sal* I fragment E, which is located within *Eco*RI fragment B (28; R. Warren, D. Womble, C. Barton, A. Easton, and R. Rownd, submitted for publication). Thus, at least one of the origins of replication of pRR12 (and presumably of NR1 and the other copy mutants) is located on all of the miniplasmids. *Eco*RI fragment B of the closely related R plasmid R6-5 (27) and of NR1 and pRR12 (Y. Miki, A. Easton, and R. Rownd, unpublished data) have been cloned and are capable of autonomous replication. Our smallest miniplasmids (4.2×10^6 daltons) would contain less than one-half of *Eco*RI fragment B (the region between the *Sal* I D-E cleavage site and the *Eco*RI B-H cleavage site). Since the segment of the miniplasmid that consists of a part of *Eco*RI fragment H (ΔH_2; 0.8×10^6 [26]) is not necessary for its autonomous replication, the size of the replicator region of the copy mutant plasmids (and presumably of NR1) must be less than 3.4×10^6 daltons.

The number of copies of miniplasmids per chromosome is two- to threefold higher than the number of copies per chromosome of the copy mutant R plasmid from which it was derived. Hershfield et al. (7) also observed that a mini-ColE1 plasmid had a higher copy number than ColE1. It is presently not known whether the two- to threefold-higher number of copies of the miniplasmids is due to lower degree of breakage (nicking) of the smaller miniplasmid DNA during isolation and handling of the CCC DNA, or whether there is a difference in the control of the replication of the miniplasmids in comparison with the copy mutant plasmids. In any case, these preliminary experiments suggest that the miniplasmids retain the copy mutant phenotype and that the mutation which results in an increased number of copies of the R plasmid per cell resides on the miniplasmid. If this interpretation is correct, at least one gene that regulates the number of rounds of replication of the R plasmid NR1 is located within the segments of NR1 from which the smallest miniplasmids are derived.

Mickel and Bauer (13) have also reported the presence of miniplasmids in *E. coli* cells harboring pRR12 (called R12 in their experiments) which enhance the level of resistance of the cells to Tc. Our results are similar to theirs, except that they observed a much broader range of sizes for the miniplasmids that they isolated (from 3.7×10^6 to 55.3×10^6 daltons). They have also postulated that the miniplasmids contain a segment of pRR12 DNA that includes the IS*1* element on *Eco*RI fragment H and a variable segment of the R plasmid, proceeding counterclockwise on the R plasmid physical map (Fig. 5). The number of copies of the miniplasmids per chromosome was not examined in their experiments.

In a number of experiments on the characterization of the DNA of copy mutants in *E. coli*, a number of small circular molecules of variable but nonrandom size has been observed in different experiments. These species are usually present in a very low weight percentage relative to the total plasmid DNA, and their origin is unknown at the present time. Just as in the case of miniplasmids, these plasmids could contribute to the reservoir of plasmids of unknown origin and function (cryptic plasmids) that have been observed in a wide variety of bacterial genera.

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