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Clustering of Genes Involved in Replication, Copy Number Control, Incompatibility, and Stable Maintenance of the Resistance Plasmid R1*drd-19*

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Plasmid R1*drd-19* is present in a small number of copies per cell of *Escherichia coli*. The plasmid was reduced in size by in vivo as well as in vitro (cloning) techniques, resulting in a series of plasmid derivatives of different molecular weight. All plasmids isolated contain a small region (about 2×10^6 daltons of deoxyribonucleic acid) of the resistance transfer factor part of the plasmid located close to one of the IS*1* sequences that separates the resistance transfer factor part from the resistance determinant. All these derivatives were present at the same copy number, retained the incompatibility properties of plasmid R1*drd-19*, and were stably maintained during cell division. Genes mutated to yield copy mutations also were found to be located in the same region.

Bacterial plasmids have proved to be very useful in studies of DNA replication in bacteria. There are two major reasons for this: (i) plasmid replication relies to a great extent on host functions, and (ii) plasmids are nonessential to the host, thus allowing genetic manipulations without interfering with the growth of bacteria.

We have for some years been engaged in the problem of replication control, and we have chosen the plasmid R1 as a model replicon for this analysis. One important conclusion from our work is that part or all of the genes involved in the regulation of R1 replication are located on the plasmid itself, a conclusion which is primarily based on the isolation and analyses of plasmid copy mutants (22, 23).

The plasmids in the incompatiblity group FII—R1, R6, and R100—are very homologous as judged from heteroduplex analyses (26). Especially in the region supposed to contain the replication genes, no difference between the three plasmids has been observed from these studies. Recently, different publications have presented evidence that the genes required for autonomous replication of the plasmids are located close to one of the two IS*1* sequences on the so-called RTF (resistance transfer factor) part of the molecules (7, 18, 25, 31, 32, 35). It is, therefore, certain that for all these plasmids a functional replication origin must be located here. Furthermore, Timmis and co-workers have shown that also the copy number control genes are located within this region (34, 35). However, the fact that the small plasmids isolated from plasmids R1 and R100 are derived from copy mutants makes a conclusion concerning the location of their copy number contol genes uncertain. This is clearly illustrated by the finding that the copy numbers of these latter small plasmids are higher than those of the parent plasmids (13, 14, 21, 32). Furthermore, a comparison of the data from Kollek et al. (18) and from Timmis et al. (33) indicates that the *inc* genes of the two plasmids R1 and R6-5 are located at different positions in the replication region. Therefore, despite the apparent similarity between the plasmids of the FII group, they may, nevertheless, be organized structurally differently as far as the replication genes are concerned.

We have chosen to reexamine the genetics of the plasmid R1 in order to localize the genes required for plasmid replication including copy number control, incompatibility, and stable maintenance. For this purpose we have used plasmid R1*drd-19*, which has a low copy number (wild type), as the parent plasmid from which gradually smaller plasmid derivatives have been constructed. By analyzing all plasmids constructed from plasmid R1*drd-19* for replication and control properties, it has been possible to demonstrate that, as is the case for plasmid R6-5, plasmid R1*drd-19* has a full complement of all replication genes, including control functions,

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incompatibility, and stable maintenance within a short region of the plasmid molecule.

(Part of the results to be discussed here were presented at the 2nd Tokyo Symposium on Microbial Drug Resistance, Tokyo, 1977 [36].)

MATERIALS AND METHODS

Bacterial strains, media, and growth of cells. The strains used were *Escherichia coli* K-12, except for one case in which *Salmonella typhimurium* LT2 was used. Genotypes and sources of the bacterial strains and plasmids used are listed in Table 1. The bacteria were grown by shaking them in either LB medium (6) or M9 minimal medium (1) supplied with 0.2% glucose and 1% Casamino Acids.

Growth was followed by analyzing optical density in a Klett-Summerson colorimeter or in a Zeiss PMQ3 spectrophotometer.

Preparation of DNA. Plasmid DNA (R1*drd-19* or related plasmids) was prepared from 1- to 3-liter cultures grown to stationary phase in LB medium. Derivatives of the plasmid ColE1 were prepared from 100-ml cultures that had been incubated in the presence of 150 μg of chloramphenicol per ml for 8 to 12 h (8). Cleared lysates were prepared as described by Clewell and Helinski (9). The DNA from the lysates was precipitated with polyethylene glycol by the method of Humphreys et al. (16). The concentrated nucleic acid preparation was eventually run in ethidium bromide-cesium chloride gradients. After harvesting the gradients, we pooled the fractions containing the plasmid DNA, extracted the ethidium bromide with CsCl-saturated isopropanol, and dialyzed the DNA against 3 × 500 ml of TE buffer (10 mM Tris-hydrochloride, 0.5 mM EDTA, pH 9.0).

Restriction enzyme digestion and analysis. DNA in a volume of 10 to 20 μl was mixed with buffer and 1 μl of enzyme solution (1 to 10 U) as described in the recipes delivered by Boehringer Mannheim Corp.

DNA (fragments or closed circles) was visualized on agarose gels (0.7 to 1.4%) run in a vertical gel apparatus designed by D. Blohm. The electrode buffer consisted of Tris (0.036 M), NaH_2PO_4 (0.03 M), and EDTA (0.01 M) with a pH of 7.5. The size of the gels was 200 by 200 by 3 mm, and electrophoresis was performed at 200 V for 3 h at 10°C.

Ligation of DNA and transformation. DNA digested with restriction enzyme was ligated under conditions as previously described (15, 30). Portions of 10 to 50 μl were used for transformation as described by Cohen et al. (10).

Conjugal transfer. Donor and recipient cell cultures were grown exponentially in LB medium. At a density of 2×10^8 cells per ml, 1 ml of each culture was mixed, the mixture was incubated without shaking at 37°C for 30 min, and 0.1 ml of appropriate dilutions was spread on selective plates.

Plasmid copy number determinations. Cultures of cells growing exponentially in 10 ml of M9 minimal medium (see above) containing 250 μg of adenosine per ml were labeled at a density of 5×10^7 cells per ml with 50 μl of [^{3}H]thymidine (20 Ci/mmol, 1 mCi/ml). Growth was continued until the density reached 2×10^8 cells per ml. Amounts of radioactivity in covalently closed circular DNA was analyzed on dye-buoyant density gradients as previously described by Womble et al. (40).

Chemicals and enzymes. All chemicals used were analysis grade. Agarose and antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo. Restriction endonucleases were obtained from Boehringer Mannheim GmbH, and T4 DNA ligase was obtained from Miles Laboratories Ltd., Slough, England. RNase and lysozyme were purchased from Sigma Chemical Co. Radioisotopes were obtained from The Radiochemical Centre, Amersham, Buckinghamshire, England.

RESULTS

Reduction of the size of plasmid R1*drd-19*. Because it is clear that plasmid R1*drd-19* carries information for its own replication control (24), we used a series of approaches (in vivo as well as in vitro) to reduce the size of the

TABLE 1. *Bacteria and plasmids used*

Bacterial strain/plasmid	Parent strain/ plasmid	Genotype[a]	Source	Reference
Bacteria				
E. coli 1100		*endI*	J. Collins	(11)
E. coli SØ824	C600	*thr leu thy dra*	K. Hammer Jespersen	
E. coli W3110 *polA*	W3110	*thy dra polA*	D. Helinski	(17)
E. coli JC2924	AB1157	*thr leu lac xyl mtl pro his rpsL tsx ara arg supE recA56*	A. J. Clark	
E. coli UB1731	J53	*pro met nalA* Tn*802*	P. Bennett	(5)
S. typhimurium LT2M827	LT2	*pro his*	G. Meynell	
Plasmids				
R1*drd-19*		*sul*$^+$ *tra*$^+$ *fin bla*$^+$ *cat*$^+$ *aphA*$^+$ *aadA*$^+$		(20)
R1*drd-19K1*	R1*drd-19*	*aphA*$^+$ *tra*$^+$ *fin*		(4)
pKN102	R1*drd-19*	As R1*drd-19 cop*		(23, 38)
pKN103	R1*drd-19*	As R1*drd-19 cop*		(23, 38)
pSF2124	ColE1	*bla*$^+$ *ColE1*$^+$	J. Collins	(28)
R100 (= NR1)		*cat*$^+$ *aadA*$^+$ *sul*$^+$ *tet*$^+$ *tra*$^+$ *fin*		(12)

[a] Gene symbols are according to the systems of Bachmann et al. (3) and Novick et al. (24).

plasmid without losing the copy number control. The approaches included isolation of the so-called RTF part of the plasmid from *S. typhimurium*, cloning of restriction endonuclease fragments, and isolation of spontaneously arising miniplasmids.

Isolation of the RTF part of the plasmid R1*drd-19*. In *S. typhimurium* the large FII plasmids have been reported to dissociate into the so-called RTF part and resistance determinant (2, 27). Therefore, we transferred plasmid R1*drd-19* by conjugation to an *S. typhimurium* LT2 strain, M827, that was cured of the cryptic plasmid which is normally present in this strain (29). The dissociation of plasmid R1*drd-19* in *S. typhimurium* was demonstrated by analyzing cell lysates on agarose gels or neutral sucrose gradients, where plasmid molecules with lower molecular weight than that of plasmid R1*drd-19* were observed. However, only the RTF parts (40 $\times 10^6$ daltons) or miniplasmids (see below) were detected, but never any plasmid molecule corresponding to the r-determinant (results not shown).

In this way we isolated the RTF part from plasmid R1*drd-19* (pKN904) and from two copy mutants, plasmids pKN102 (pKN905) and pKN103 (pKN906) (23, 38). These plasmids have all been transferred by conjugation to *E. coli*, where they were analyzed further.

Cloning of *Eco*RI fragments from plasmid R1. In analogy with the experiments of Timmis et al. (35), we have isolated small plasmids from R1*drd-19* consisting of one restriction enzyme fragment carrying replication functions and one fragment carrying antibiotic resistance genes. In addition, we have cloned several DNA fragments from plasmid R1*drd-19* onto the cloning vector pSF2124. In these experiments we used the restriction endonuclease *Eco*RI, which cleaves the plasmid R1*drd-19* into 17 linear fragments ranging in size from 0.1×10^6 to 12×10^6 daltons (7). Figure 1 is a reconstruction of the physical map of R1*drd-19* from the data of Blohm and Goebel (7), which is included to illustrate the position of the different fragments on the plasmids. Figure 2A shows the fragment pattern of *Eco*RI-digested R1*drd-19* DNA analyzed on an agarose gel; 12 bands are clearly visible. The upper band representing the largest DNA fragments contains two fragments of almost identical size. The smallest fragments are not seen in this gel. The plasmids obtained from the ligation experiments (with or without plasmid pSF2124) are listed with their respective phenotypes in Tables 2 and 3.

To analyze for the presence of replication functions on the fragments cloned on pSF2124,

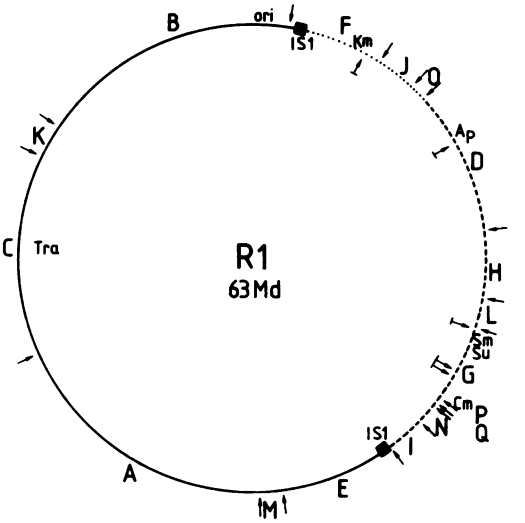

FIG. 1. *Physical map of plasmid R1drd-19, reconstructed from Blohm and Goebel (7). The arrows mark the position of sites sensitive to restriction endonucleases EcoRI (outside arrows) and BamHI (inside arrows). (A to Q) The respective EcoRI fragments; (····) the part of the r-determinant that is deleted spontaneously from R1drd-19 plasmids with high frequency; (-----) the deletion in plasmid R1drd-19K1.*

we tried to transform DNA of all hybrid plasmids into the strain W3110 *polA*. Only plasmid pKN191 could be established in this strain, whereas all other plasmids failed to give rise to plasmid-carrying clones (Table 2). It is known that plasmid ColE1 (pSF2124) requires a high concentration of DNA polymerase I for its replication and that plasmid R1*drd-19* does not (17). Therefore, we took this result as an indication of the localization of the replication functions from R1*drd-19* on plasmid pKN191.

Plasmids containing only *Eco*RI fragments from R1*drd-19* were isolated after selection for either ampicillin (pKN501) or kanamycin (pKN500) resistance (Table 3). These plasmids were found to contain one large *Eco*RI fragment (about 12×10^6 daltons) and, in the case of pKN501, an additional fragment of 6.5×10^6 daltons, whereas the plasmid pKN500 contained, in addition to the 12×10^6-dalton fragment, a 4.5×10^6-dalton fragment (Fig. 2A). To find out whether the large fragment in both cases was the same, the plasmid DNAs were digested with restriction endonuclease *Sma*I. Plasmids pKN501 and pKN500 are very similar with respect to the *Sma*I fragments obtained (Fig. 2B); the differences may easily be ascribed to the difference between the small *Eco*RI fragments. Plasmid pKN191 contains the same large

TABLE 2. *Properties of plasmids constructed by cloning EcoRI fragments of plasmid R1drd-19 onto the vector pSF2124 (ColE1)*

Plasmid	Mol wt (× 10^6)	*Eco*RI fragment from R1[a]	Antibiotic resistance[b]	Incompatibility towards R1	Replication in *polA*[c]
pKN184	19	A		–	–
pKN185	17	C		–	–
pKN186	10	H		–	–
pKN188	9	J		–	–
pKN189	12	F	Km	–	–
pKN191	19	B		+	+
pKN192	11	G	Sm, Su	–	–
pKN194	8.5	K		–	–
pKN195	9.5	I		–	–
pKN196	8.2	M		–	–

[a] See Blohm and Goebel (7) and Fig. 1.

[b] Resistance in addition to the Ap resistance already present on pSF2124. Abbreviations: Ap, Ampicillin; Sm, streptomycin; Su, sulfonamides; Km, kanamycin.

[c] Tested as establishment of the plasmids in W3110*polA*.

TABLE 3. *Properties of plasmids derived from plasmid R1drd-19*

Plasmid	Parent plasmid	Mol wt (× 10^6)	Antibiotic resistances[a]	Level of Ap resistance[b] (μg/ml)	Copy no.[c]	Incompatibility towards R1
R1*drd-19*		63	Km, Ap, Sm, Su, Cm	100	1	+
R1*drd-19K1*	R1*drd-19*	50	Km		1	+
pKN904 (RTF)	R1*drd-19*	40	None		1.7	+
pKN501	R1*drd-19*	18	Ap	75	1	+
pKN500	R1*drd-19*	16	Km		1	+
pKN799	R1*drd-19K1*	53	Km, Ap (Tn*802*)	2,400[d]	1	+
pKN801	pKN799	14	Ap	2,400	1–2	+
pKN800	pKN799	8.0	Km, Ap	2,400	1–2	+
pKN1562	R1*drd-19K1*::Tn*802*	6.5	Km		1–2	+
pKN1562Ap	R1*drd-19K1*::Tn*802*	13	Km, Ap	75	ND	ND
pKN102	R1*drd-19*	58	Ap, Sm, Su, Cm	400	3–4	+
pKN905 (RTF)	pKN102	40	None		3–4	+
pKN520	pKN102	18	Ap	400	3–4	+
pKN103	R1*drd-19*	63	Km, Ap, Sm, Su, Cm	200	2	+
pKN906 (RTF)	pKN103	40	None		2.5	+
pKN402	pKN400[e]	4.6	None		30°C: 20–30 40°C: uncontrolled	+ (at 30°C)

[a] Abbreviations: Ap, Ampicillin; Cm, chloramphenicol; Sm, streptomycin; Su, sulfonamides; Km, kanamycin; ND, not determined.

[b] Single cell resistance (38).

[c] Measured on ethidium bromide-cesium chloride gradient, and expressed relative to copy number of plasmid R1*drd-19*, which is set to 1.

[d] The single cell resistance of strain UB1731 (Tn*802*) was found to be 200 to 300 μg/ml.

[e] Runaway replication mutant of plasmid R1*drd-19* (39).

*Eco*RI fragment as the two self-cloned plasmids (Fig. 2B). The antibiotic resistance genes contained in these plasmids (kanamycin resistance in pKN500, ampicillin resistance in pKN501) have been shown to be located on the two small *Eco*RI fragments F and D (7), respectively. We conclude that all functions required for autonomous plasmid replication are located on one of the two largest *Eco*RI fragments of plasmid R1*drd-19*.

The final identification of the *Eco*RI fragment carrying the replication functions was done by comparing the *Sma*I fragment pattern of pKN500, pKN501, and pKN191 with that published by Goebel and co-workers (14) for miniplasmids derived from a copy mutant of R1*drd-19*. These plasmids have been claimed to contain part of the so-called *Eco*RI B fragment—the second largest of the *Eco*RI fragments—and they also show the same characteristic large number of very small fragments. Thus, the large *Eco*RI fragment common to all the described plasmids is the B fragment.

Isolation of plasmids carrying only a small part of the replication fragment. The plasmid R1*drd-19K1* is a deletion mutant of

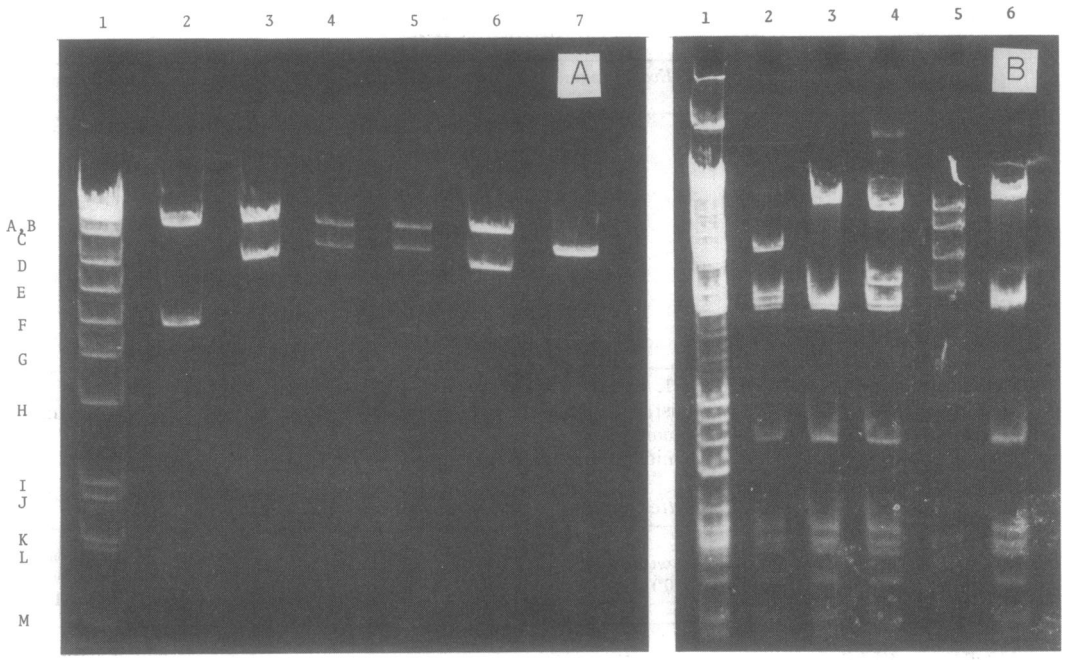

FIG. 2. *Restriction enzyme fragments from plasmids carrying the cloned large EcoRI fragments. (A) Fragments generated by EcoRI digestion. (lane 1) R1drd-19; (lane 2) pKN500; (lane 3) pKN501; (lane 4) pKN191; (lane 5) pKN184; (lane 6) pKN520; (lane 7) pSF2124. (B) Fragments generated by SmaI digestion. (lane 1) R1drd-19; (lane 2) pKN500; (lane 3) pKN501; (lane 4) pKN191; (lane 5) pKN184; (lane 6) pKN520. Enzyme digestions and agarose gel electrophoresis were performed as described in the text. The gels contained 1% agarose. After the electrophoresis, the gels were placed in water containing 0.5 μg of ethidium bromide per ml for 1 h. The stained gels were illuminated with short-wavelength UV light, and photographs were taken through a red filter (Kodak, Wratten M25) with Ilford HP-5 film.*

R1*drd-19*, which only mediates resistance to kanamycin (4). From the *Eco*RI digest pattern it was shown that many fragments have been deleted, and on comparing the pattern with the map of plasmid R1*drd-19* (7) it is clear that the deletion covers all of the r-determinant except for the fragments F (the kanamycin resistance fragment) and J (Fig. 1). This plasmid has only one site susceptible to the restriction endonuclease *Bam*HI, and this site is located close to one of the *Eco*RI sites of the F fragment (7; Fig. 1). The rationale of the following experiments was first to introduce random new *Bam*HI sites into R1*drd-19K1*, aiming in particular at different positions within the *Eco*RI fragment B of plasmid R1*drd-19*. This was done by insertion of the transposon Tn*A* (Tn*802*) (5) carrying the β-lactamase gene responsible for ampicillin resistance and also carrying one site recognized by restriction endonuclease *Bam*HI. From such plasmids, *Bam*HI fragments containing the replication functions of R1*drd-19* were isolated after ligation and transformation into a plasmid-free *E. coli* strain selecting for either kanamycin or ampicillin resistance. The size of such fragments should, of course, depend on the location of Tn*802* in the R1*drd-19K1* plasmid. The actual resistance pattern should in each case depend upon the orientation of the insertion.

Plasmid R1*drd-19K1* was transferred by conjugation to the strain UB1731, which harbors Tn*802* in the chromosome. The resulting transconjugant strain was mated with strain 1100, and selection was made on minimal plates containing ampicillin and kanamycin. Because strain UB1731 is an F^- strain, the only way to mobilize Tn*802* into strain 1100 is by translocation of the transposon from the chromosome of UB1731 onto the plasmid R1*drd-19K1*.

In such a conjugation experiment a translocation frequency of approximately 10^{-3} was found. A preliminary screening of the resulting plasmids showed that Tn*802* was inserted at several locations as judged from the *Eco*RI fragment patterns (not shown).

We isolated 500 clones of bacteria carrying R1*drd-19K1*::Tn*802* plasmids. All were found to be transmissible by conjugation, indicating that in all cases Tn*802* was inserted outside the *tra* genes. Because of the limited resolution of frag-

ments larger than 10×10^6 daltons, insertions of Tn*802* into the *Eco*RI A and B fragments should not result in any observable change in the *Eco*RI fragment pattern. Therefore, one plasmid, pKN799, which had gained the transposon without a change in the *Eco*RI pattern, was analyzed further (Fig. 3). The plasmid DNA (pKN799) was digested with *Eco*RI, ligated, and transformed to competent *E. coli* cells. Clones resistant to ampicillin were found to contain a plasmid, pKN801, of 14×10^6 daltons. The *Sma*I fragment pattern of this plasmid was not much different from that found for the plasmids containing the *Eco*RI B fragment (not shown). It is, therefore, clear that Tn*802* must be inserted into the B fragment and that this fragment alone is capable of autonomous replication. Next, construction of a plasmid derivative of pKN799 extending from the *Bam*HI site of the F fragment to the *Bam*HI site in Tn*802* was attempted. Plasmid pKN799 was cut with *Bam*HI, ligated, and transformed to *E. coli* 1100 selecting for kanamycin or ampicillin resistance. It turned out that all clones independent of the selection were resistant to both antibiotics. Furthermore, all resistant clones contained a plasmid (pKN800) of size 8.0×10^6 daltons (Fig. 4A).

To isolate a number of different but analogous plasmids, we made a mixed culture of 50 different clones containing R1*drd-19K1*::Tn*802* plasmids from which we prepared plasmid DNA. The DNA was cut with *Bam*HI, subjected to ligation conditions, and transformed to *E. coli*. We isolated several kanamycin-resistant clones that were found to carry plasmids of various sizes. One of these, pKN1562, is discussed in more detail in this communication. It has a molecular weight of 6.5×10^6 (Fig. 4A). The plasmid carries only kanamycin resistance. Plasmid DNA was digested with *Pst*I (Fig. 4B), and restriction enzyme maps were constructed (Fig. 5). These maps are very similar to those recently published by Kollek et al. (18) with respect to the region needed for plasmid replication. Because only DNA to the left of the *Eco*RI site is required for plasmid replication, it is seen that 2.3×10^6 or less daltons of the *Eco*RI B fragment is necessary to establish plasmid replication. (Most likely part of the inserted Tn*802* is deleted

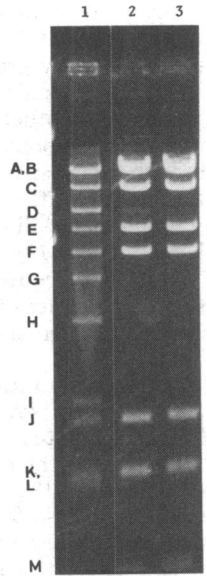

FIG. 3. *EcoRI fragments from plasmids R1drd-19, R1drd-19K1, and pKN799. (lane 1) R1drd-19; (lane 2) R1drd-19K1; (lane 3) pKN799. The electrophoresis and subsequent analysis were performed as described in the legend to Fig. 2.*

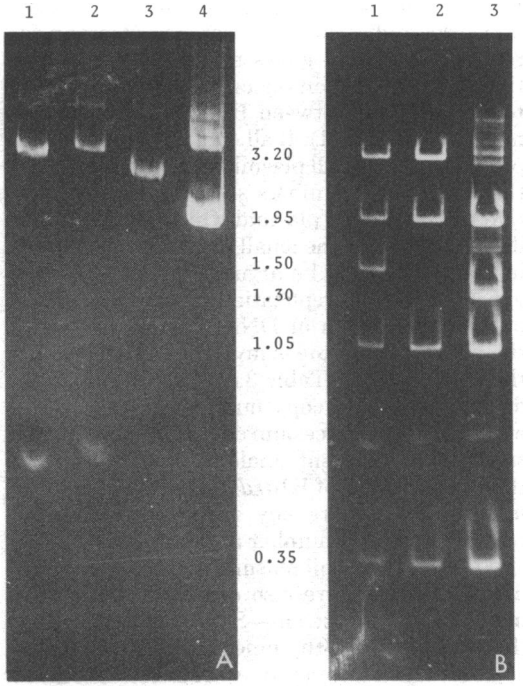

FIG. 4. *Agarose gel analysis of miniplasmids derived from R1drd-19 and pKN400. (A) Covalently closed DNA molecules of plasmids (lane 1) pSF2124 (as a marker), (lane 2) pKN800, (lane 3) pKN1562, and (lane 4) pKN402 (the upper bands represent open circular and linear DNA molecules of the plasmid). (B) Fragments generated by digestion with PstI of (lane 1) pKN800, (lane 2) pKN1562, and (lane 3) pKN402 (partial digest). The numbers at the bands indicate the molecular weight ($\times 10^6$) of the DNA fragments determined by comparison with an EcoRI digest of R1drd-19 DNA (not shown). The concentration of agarose was 1.4%. The electrophoresis and subsequent analysis were performed as described in the legend to Fig. 2.*

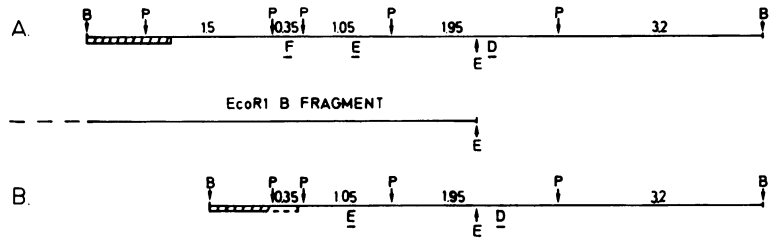

FIG. 5. *Restriction enzyme maps of plasmids (A) pKN800 and (B) pKN1562. Abbreviations: $E^{\uparrow}$, EcoRI site; $B_{\downarrow}$, BamHI site; $P_{\downarrow}$, PstI site; E, E, and D, PstI fragment nomenclature for R1 as described by Kollek et al. (18). Numbers express the molecular weight (×10^6) of the PstI-fragments. Hatched areas indicate the position of Tn802 in the plasmids.*

in plasmid pKN1562, but the exact extension of this deletion is not known.)

Copy number of the R1*drd-19* fragment plasmids. The data presented above show that a short region of the RTF part of the plasmid R1*drd-19* allows stable replication of the plasmid, thus indicating the presence of origin for replication and all genes necessary for replication. This replication region is located very close to the junction between RTF and the r-determinant (7, cf. Fig. 1). If all control genes are also present on the small plasmids it is to be expected that their copy numbers should be identical to that of the parent plasmid. On the other hand, if more copies of the small plasmids are present in the cell, it may be argued that one or more genes involved in replication control are located outside this region of DNA or that the size of the plasmid molecule is involved in determining the copy number. Table 3 contains results from determinations of copy numbers (measured on ethidium bromide-cesium chloride gradients) for some of the different small plasmids relative to the copy number of R1*drd-19*. It is obvious that in no case is there any significant difference between the copy number of the large R1*drd-19* and that of the small plasmid. The copy number determinations were also carried out in a thymine auxotrophic strain—SØ824—after labeling the cells with [^{3}H]thymine, but in no case was any difference compared to strain 1100 observed (not shown). Table 3 also contains results from determinations of the level of ampicillin resistance which has been found to be proportional to the gene dosage, i.e., copy number (38). Plasmid pKN1562 was digested with *Eco*RI and fused to the *Eco*RI fragment carrying the β-lactamase gene (the 6.5 × 10^6-dalton fragment from pKN501), and the resulting plasmid, pKN1562-Ap, was included in the analysis of the other ampicillin-resistant plasmids. The data support the conclusion that no gross alteration of the copy number is observed for the small plasmid derivatives. This type of measurement is an important alternative to determinations on CsCl gradients because it is to be suspected that recoveries of covalently closed circular molecules from cell extracts to some degree are influenced by the size of the plasmid molecule. Determinations of single cell resistance to ampicillin, on the other hand, should be completely independent of the molecular weight of the replicon, and exclusively be a measure of gene dosage (copy number) as described by Uhlin and Nordström (38). A comparison of the copy numbers obtained by the two methods shows a general tendency to yield slightly higher numbers for the small plasmids when using CsCl gradients, whereas the resistance level to ampicillin is unchanged or may be even slightly reduced compared to the R1*drd-19* plasmid. The single cell resistance to ampicillin of plasmid pKN799 and its small derivatives was found to be approximately 20 times higher than that of R1*drd-19*, although the copy number of this plasmid measured on dye-buoyant density gradients is low. Similar high-resistance plasmids were found having Tn*802* inserted in other parts of the plasmid (not shown). It is possible that transcription readthrough from a strong outside promoter may be the reason for this.

The low copy numbers for all small plasmids isolated from plasmid R1*drd-19* strongly suggest that all genes involved in control of plasmid replication must be placed close to the replication origin on the *Eco*RI fragment B of plasmid R1*drd-19*.

Miniplasmids from copy mutants of plasmid R1*drd-19*. Previously, several copy mutants of R1*drd-19* were isolated ranging in copy numbers from 1 to 10 times the copy number of R1*drd-19* (37, 38). Many of these mutant plasmids show a tendency to dissociate into smaller plasmids carrying only a few or none of the resistance genes in addition to a replicative origin (13; our unpublished data). We have isolated a large number of such miniplasmids from several copy mutants, including mutants expressing a conditional copy number phenotype. In addition, we have constructed one plasmid in vitro

consisting of the *Eco*RI fragments B and D from the copy mutant plasmid pKN102. This plasmid is pKN520, the copy number of which was found to be identical to that of pKN102 (Table 3). Because the copy numbers of miniplasmids derived from copy mutants are often higher than those of their parents (13, 32), we also analyzed a copy mutant with a very distinct phenotype correlated to the copy number control: plasmid pKN402, which is derived from plasmid pKN400 (39). Plasmid pKN400 has a copy number of 2 to 3 at 30°C and uncontrolled replication at temperatures above 37°C. Plasmid pKN402 was isolated from cells sensitive to all antibiotics to which plasmid R1*drd-19* (pKN400) mediates resistance. The size of the plasmid is 4.6×10^6 daltons (Fig. 4A), and it was found to contain the *Pst*I fragments known to be involved in replication of plasmid R1*drd-19* (Fig. 4B). The copy number of the small plasmid was found to be 25 to 30 times that of plasmid R1*drd-19* at 30°C (Table 3). When cells carrying plasmid pKN402 are grown in LB medium at temperatures above 37°C, the plasmid replication is uncontrolled (the copy number may be increased up to 5,000) and the cells die after five to six doublings of the cell number—a phenotype similar to that of pKN400 (39). These results directly show that the copy mutation(s) resulting in the temperature-dependent copy number phenotype of pKN400 is present on plasmid pKN402, and thus located in the vicinity of the replication origin.

Incompatibility and stability. The phenomenon of incompatibility has been claimed to be part of the copy number control mechanism (37). Because our results strongly suggest that all genes involved in copy number control of plasmid R1*drd-19* are located close to the replication origin, we also analyzed the small plasmid derivatives for incompatibility properties.

The different plasmids were transformed to the *E. coli* K-12 *recA* strain JC2924. Then plasmid R1*drd-19* or R100 was transformed by conjugation, and clones were purified. After growth in medium without any antibiotics for more than 20 generations, single cell colonies were tested for the presence of either plasmid (test for resistance pattern). All plasmids carrying the replication region of plasmid R1*drd-19* were incompatible with R1*drd-19* (Table 3). Similar experiments have been done with miniplasmids from copy mutants, and in all cases they have been found to be incompatible with plasmid R1*drd-19*, although the rates of segregation of these in many cases were found to be quite low (data not shown). Thus, the incompatibility functions are also located genetically in the region around the replication functions of R1*drd-19*.

The loss of plasmid R1*drd-19* from cells growing without any selection pressure to maintain the plasmid is normally negligible. However, it was found that all of the small plasmid derivatives from R1*drd-19* were slightly unstable, being lost from the cells with a rate of 0.1 to 0.5% per generation, but this segregation rate is significantly below that which can be calculated for a random partitioning of the plasmids at cell division. Assuming a copy number of one per chromosome, equivalent random partitioning should result in 3 to 5% plasmid-free segregants per cell generation.

DISCUSSION

The aim of the present work was to define the part of the plasmid R1*drd-19* genome which is responsible for replication, stable maintenance, incompatibility, and copy number control of the plasmid. By in vivo deletion of the r-determinant, it was possible to demonstrate that all replication control functions are located on the RTF part of the plasmid. The RTF part is a replicon, although there was no evidence that the r-determinant can replicate autonomously in *E. coli* or in *S. typhimurium*. Furthermore, all control functions are present on one fragment (B) obtained by cleavage of the plasmid with the restriction endonuclease *Eco*RI. This fragment can function as a replicon, and its copy number is indistinguishable from that of the full-size plasmid R1*drd-19*. Hence, *Eco*RI fragment B carries the origin of vegetative replication (7), the genetic information for all plasmid-mediated replication functions as well as all control functions. Furthermore, by reducing the size of the plasmid even more, it became clear that the genetic information needed for replication, as well as for normal copy number control, is clustered in a small region adjacent to the IS*1b* sequence that marks the border between the RTF part of plasmid R1*drd-19* and the r-determinant. The small, low-copy-number plasmids generated from plasmid R1*drd-19* have essentially the same maintenance stability as the full-size plasmid, indicating that the genetic information for partitioning of the plasmids between daughter cells at division is also located in this small region. The exact size of the smallest part of plasmid R1*drd-19* (mini-R1) that allows wild-type control of replication is not known at present, but Goebel and co-workers (14) recently showed that in the case of copy mutant pKN102 the two *Pst*I fragments E and F (Fig. 5) are sufficient for autonomous plasmid replication. We have isolated analogous plasmids from R1*drd-19*, and even these seem to retain a low copy number, but they are not stably maintained during cell growth (to be published).

Our data are in full agreement with those published for plasmid R6-5 by Timmis et al. (33). Similar results have also been published for plasmid F (19). However, the analyses of the small plasmids generated from copy mutant plasmids show that the reduction in size results in increased copy numbers (13, 32). The reason for this is at present unknown. This means that miniplasmids isolated from copy mutant plasmids cannot directly be used for analyses of copy number control functions. Furthermore, because it has been shown previously that there is a connection between the *inc* and the *cop* functions (37), the *inc* properties of such copy mutant miniplasmids may also be difficult to analyze and interpret. Thus, the apparent differences between the location of the *inc* gene on plasmid R1*drd-19* and R6-5 as indicated from the data of Kollek et al. (18) and Timmis et al. (33) may reflect such problems.

In conclusion, the data presented here, together with previously published data on FII plasmids (18, 26, 31, 33), show that most likely the three major plasmids of this group—R1, R6, and R100—have a small DNA region in common in which all genes necessary for replication, control of replication, and partitioning of the plasmids are located. These functions are expressed "normally" only if the plasmids analyzed carry the wild-type alleles of the control gene.

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