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Properties of R1162, a Broad-Host-Range, High-Copy-Number Plasmid

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Regions of plasmid DNA encoding characteristic properties of the IncQ (P-4) group plasmid R1162 were identified by mutagenesis and in vitro cloning. Coding sequences sufficient for expression of incompatibility and efficient conjugal mobilization by plasmid R751 were found to be linked to the origin of DNA replication. In contrast, there was a region remote from the origin, and active in *trans*, that was required for plasmid maintenance. A derivative that was temperature sensitive for stability was isolated. The defect mapped at or near the region required for plasmid maintenance and resulted in far fewer copies of supercoiled plasmid DNA per cell under permissive conditions. A second region required for stability was also identified from the behavior of a deletion derivative of R1162, which did not, however, show an altered number of supercoiled plasmid DNA copies. Finally, a plasmid DNA mutation resulting in a substantially higher copy number was isolated. Plasmid reconstruction experiments suggested that the mutation was linked to the replicative origin.

R1162 is a small (8.7 kilobases [kb]), high-copy-number plasmid conferring resistance to streptomycin (Sm^r) and sulfonamides (Su^r) (4). It is similar or identical to RSF1010 (21) and to other plasmids that have been isolated from diverse backgrounds (4). Together, these plasmids make up the IncQ incompatibility group (IncP-4 in *Pseudomonas*).

The small size and high copy number of IncQ plasmids have made them attractive as molecular cloning vectors. In addition, their host range includes several genera (4, 13, 16), and therefore these plasmids are useful for the transfer of cloned DNA across species barriers. Several investigators have already used R1162 or RSF1010 for this purpose in experiments involving mobilization of DNA between *Escherichia coli* and species of *Pseudomonas* (16, 33). However, relatively little is known about the properties of R1162 or its genetic organization. Heffron et al. (23) isolated derivatives of RSF1010, each containing a transposon inserted at a unique position on the plasmid molecule. A number of insertions inactivated one or both drug resistances. They concluded from the locations of these that the Su^r and Sm^r genes are transcribed as a single unit, with Su^r promoter proximal. DeGraaff et al. (14) examined replicative intermediates of RSF1010 and showed that there is a unique origin of replication 31% of the molecular length from the single *Eco*RI site of the plasmid. Replication may proceed bidirectionally or unidirectionally in either sense. Apart from a partial dependence on DNA polymerase I (20), requirements for the maintenance of IncP-4 plasmids have not been determined.

In this report, we describe our initial efforts to identify and characterize regions of R1162 required for plasmid maintenance, conjugal mobilization, expression of incompatibility, and control of copy number. For this, we have constructed derivatives of R1162, both by mutagenesis of plasmid DNA and by in vitro recombinant techniques. In addition, we have cloned fragments of R1162 and then tested the properties of the resulting recombinant plasmids.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. The *E. coli* K-12 strains used in this study were MV10 (*thr leu thi lacY supE44 tonA ΔtrpE5* λ^-), a derivative of *E. coli* C600 originally from C. Yanofsky; DF1019 (15), a Mu-resistant, nalidixic acid-resistant derivative of C600; CR34 (3), which is C600 *thyA dra*; and GM33 (*dam-3* F^- λ^-), from B. Bachmann. Cells were grown in TYE medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) supplemented as necessary with carbenicillin (Geopen; Pfizer, Inc.) (500 μg/ml) and kanamycin sulfate, streptomycin sulfate, tetracycline hydrochloride, or nalidixic acid (all Sigma Chemical Co.) (25 μg/ml), unless otherwise indicated. For detection of high-level streptomycin resistance and radioactive labeling of DNA, a phosphate-buffered (18) medium was used containing 0.4% glucose, 0.5% Casamino Acids (Difco Laboratories), and 10 μg of thiamine per ml. Table 1 lists the bacterial plasmids used and those constructed during the course of this work.

Mutagenesis of plasmid DNA. In vivo mutagenesis

was carried out with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co.) according to the procedure of Adelberg et al. (1). MV10(R751)(R1162) was treated with 50 μg of the mutagen per ml for a time period sufficient to attain 0.1 to 1% survival (about 20 min at 37°C). Treated cells were then grown in TYE broth for several hours. To segregate plasmid-borne mutations, these cells were mixed at a ratio of 1:1 with DF1019, collected on a nitrocellulose filter, and incubated (filter face up) on a broth plate overnight at 37°C. Transconjugants containing R1162 derivatives conferring a high level of resistance to streptomycin were detected by plating the resuspended mating mixture on phosphate-buffered medium containing nalidixic acid and streptomycin sulfate (500 μg/ml). Transconjugants appeared at a frequency of 10^{-5} to 10^{-6}, relative to the number of colonies obtained when the cells were assayed on plates containing nalixidic acid and streptomycin at the usual concentration of 25 μg/ml.

Plasmid DNA was mutagenized in vitro with hydroxylamine by the procedure of Hashimoto and Sekiguchi (22). Treatment was continued for 24 to 96 h at room temperature, sufficient to cause a 100-fold decrease in transformation efficiency. Conditionally stable derivatives of pMS33 (Table 1) were obtained by transforming MV10 at 30°C to kanamycin resistance (Km^r) with mutagenized DNA. The kanamycin-resistant colonies were replicated with a felt pad onto a broth plate, incubated at 42°C, then retransferred onto a plate containing kanamycin, and again incubated at 30°C. Colonies that did not grow in the presence of kanamycin at 30°C after passage on broth plates at 42°C were purified and saved.

Bacterial mating and transformation. Bacterial matings were routinely carried out by dispensing exponential donor and recipient cells with a syringe onto a 25-mm, 0.45-μm-pore-size filter. The ratio of donor to recipient cells was 1:10, and the total volume of culture used was 1.0 ml. The filter was incubated on a broth plate at 37°C for 90 min; then the cells were suspended in 1.0 ml of fresh broth before plating. Transformation of bacterial cells with DNA was essentially according to the method of Cohen et al. (10).

Isolation and analysis of DNA. For characterization of recombinant DNA, plasmid DNA was isolated from bacteria by the method of Birnboim and Doly (5). Larger quantities of DNA were required for in vitro mutagenesis and plasmid reconstruction experiments, and these were obtained by buoyant density equilibrium centrifugation of Sarkosyl lysates (15) or cleared lysates (26).

Restriction enzymes and T4 polynucleotide ligase were purchased from New England Biolabs and used according to the instructions of the manufacturer, except that dithiothreitol or mercaptoethanol was omitted from the reactions. DNA fragments were analyzed on 0.8% horizontal or vertical agarose gels 2 to 5 mm thick. Electrophoresis was carried out at 5 to 10 V/cm, the DNA was stained with ethidium bromide (approximately 0.1 μg/ml), and the gel was photographed under long-wave UV illumination, using a Tiffen 25A filter.

Estimation of plasmid copy number. Plasmid copy number was estimated essentially as described previously (31). Plasmids were transferred into CR34 by transformation, and the DNA was radioactively la-

TABLE 1. Plasmids used[a]

Plasmid	Relevant properties	Source or construction
Existing		
R1162	IncQ (P-4) Su^r Sm^r Mob^+	G. Jacoby
R751	IncP-1 Tp^r (Tn*402*) Tra^+	24, 32
pBR322	Rep (ColE1) Cb^r Tc^r	7
pCR1	Rep (ColE1) Km^r	12
Constructed for this work		
pMS14	IncQ Su^r Sm^r	*Hae*II-generated deletion of R1162
pMS33	IncQ Su^r Km^r (Tn*903*)	Insertion of 6.7-kb Km^r fragment (30) into *Eco*RI site of R1162
pMS38	IncQ Km^r	Ligation of DNA fragments generated by complete digestion of pMS33 with *Eco*RI and partial digestion with *Hae*II
pMS40	IncQ Km^r	Ligation of DNA fragments generated by complete digestion of pMS38 with *Pvu*II and partial digestion with *Hae*II
pMS63, pMS64, pMS65, pMS92, pMS93, pMS94, pMS95	Cb^r; see text	Replacement of small *Hin*dIII-*Bam*HI fragment of pBR322 with *Hin*dIII-*Mbo*I fragment of pMS40
pMS69	Cb^r	Chimera of pBR322 and pMS40, joined at their *Hin*dIII sites
pMS73	Su^r Sm^r Cop	By in vivo mutagenesis with nitrosoguanidine
pMS88	Su^r Sm^r, Ts (stability)	By in vitro mutagenesis of pMS33 with hydroxylamine

[a] Abbreviations: Inc, expression of incompatibility; Rep, parental plasmid replication system; Tra^+, conjugally self-transmissible; Mob^+, conjugally mobilizable; Cop, present in high copy number, Ts, temperature sensitive.

beled by growing cells at 37°C in phosphate-buffered medium containing thymine (2 μg/ml) and [methyl-^{3}H]thymine (10 μCi/ml). Cells were harvested in log phase and lysed by the Sarkosyl procedure (15), and 0.1 to 0.3 ml of lysate (300,00 to 500,00 cpm) was centrifuged to equilibrium in a CsCl-ethidium bromide gradient. Fractions were then collected and counted as described elsewhere (28).

Estimation of plasmid stability. To obtain an estimate of plasmid stability, cultures were serially cultured in TYE. At least 2×10^3 cells were transferred to fresh medium during the cultivation, after the culture reached the end of log phase. Cells were plated at intervals on broth plates to obtain single colonies, and 50 of these were tested for retention of plasmid-encoded drug resistance by transferring onto plates containing antibiotic.

Rate of loss of plasmid derivatives showing temperature-dependent stability was determined by first growing cultures overnight at 30°C in the presence of kanamycin to ensure retention of the plasmid. Ten milliliters of TYE was inoculated with the overnight culture to an initial concentration of 2.5×10^3 cells/ml and then incubated at 30 or 42°C. Initially and at various times thereafter, the culture was sampled, diluted, and plated on TYE plates. Emerging colonies were scored to determine viable count and replica plated onto medium containing kanamycin to determine loss of the plasmid. At least 150 colonies were screened for every time point sampled.

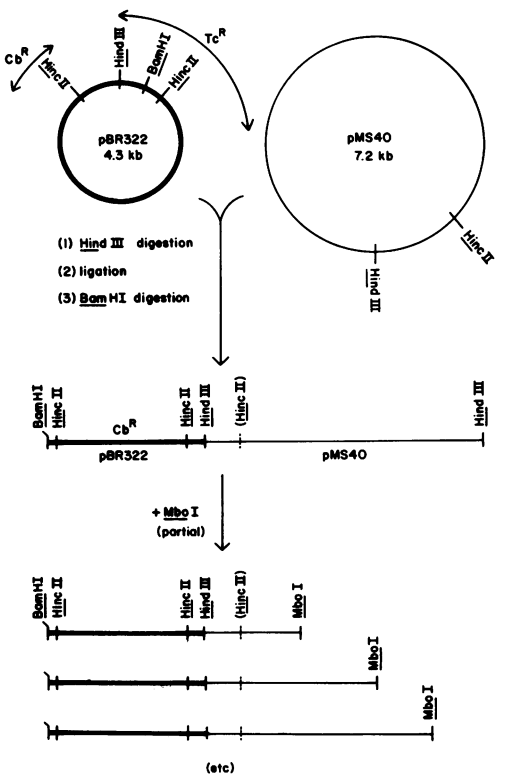

FIG. 1. Construction of pBR322 derivatives containing cloned fragments of pMS40. The heavier line indicates the cloning vehicle DNA. The *Hinc*II site in parentheses may or may not be present in each derivative, depending on the direction from the *Hin*dIII site of pMS40 that has been cloned. Restriction sites for pBR322 are from reference 6.

Cloning procedures. Table 1 shows the construction of smaller derivatives of R1162. Plasmid pMS14, a *Hae*II-generated deletion of R1162, was constructed in the laboratory of J. A. Shapiro. For the generation of the recombinant plasmids pMS38 and pMS40, arising from the ligation of *Hae*II-digested DNA, pMS33 (Table 1) DNA was used as the starting material. This plasmid was chosen because it is known that the gene(s) for inactivation of kanamycin lies on a single *Hae*II fragment (25). Selection for sulfonamide resistance is undesirable because this often results in a large background. Selection for streptomycin resistance is also to be avoided because the coding sequence of this resistance is at the distal part of a larger transcriptional unit (23), and so there are likely to be *Hae*II sites between this sequence and its promoter. The *Hae*II digestions were incomplete. To avoid transformation by molecules cut once with *Hae*II, DNA was also digested to completion with another restriction enzyme before ligation (Table 1). Molecules cleaved with this second enzyme, and also cleaved only once with *Hae*II, would have heterologous ends and would therefore transform recipient cells very inefficiently.

Fragments of pMS40 DNA were cloned (Fig. 1) by first extracting plasmid DNA from *E. coli* strain GM33, which is deficient in N^6-deoxyadenosine methylase. The DNA was mixed with fully methylated pBR322 DNA, the mixture was then digested with *Hin*dIII, and the fragments were ligated. The resulting digest was then partially cleaved with *Mbo*I, an enzyme with a recognition site of low specificity, which is fully active only on undermethylated DNA (17). The mixture was finally digested to completion with *Bam*HI, an enzyme which generates cohesive ends homologous to those generated by *Mbo*I. The net result of these procedures is to generate, among other molecular products, a set of derivatives in which the small *Hin*dIII-*Bam*HI fragment of pBR322 is replaced by a *Hin*dIII-*Mbo*I fragment of pMS40 (Fig. 1). These molecules may be isolated by transforming cells to carbenicillin resistance (Cb^r) and then screening for loss of resistance to tetracycline (Tc^r). In this way, two nested sets of fragments may be cloned, extending in either direction a variable distance from the *Hin*dIII site of pMS40.

To characterize these fragments, we needed to know both the size of the insertion in each pBR322 derivative and the direction from the *Hin*dIII site of pMS40 cloned in each case. This was readily accomplished by analysis of *Hinc*II-generated DNA fragments on agarose gels. There were two *Hinc*II sites in pBR322 (6) and one in pMS40, near the *Hin*dIII site (Fig. 1). Therefore, the presence or absence of a third *Hinc*II site in the hybrid plasmid indicated the direction from the *Hin*dIII site of pMS40 that had been cloned. Smaller fragments not large enough to include the *Hinc*II site of pMS40 could not be identified in this way. However, such small fragments did not include any R1162 DNA (Fig. 2), and derivatives containing them were not studied. The size of the inserted DNA was calculated from the size of the unique *Hinc*II

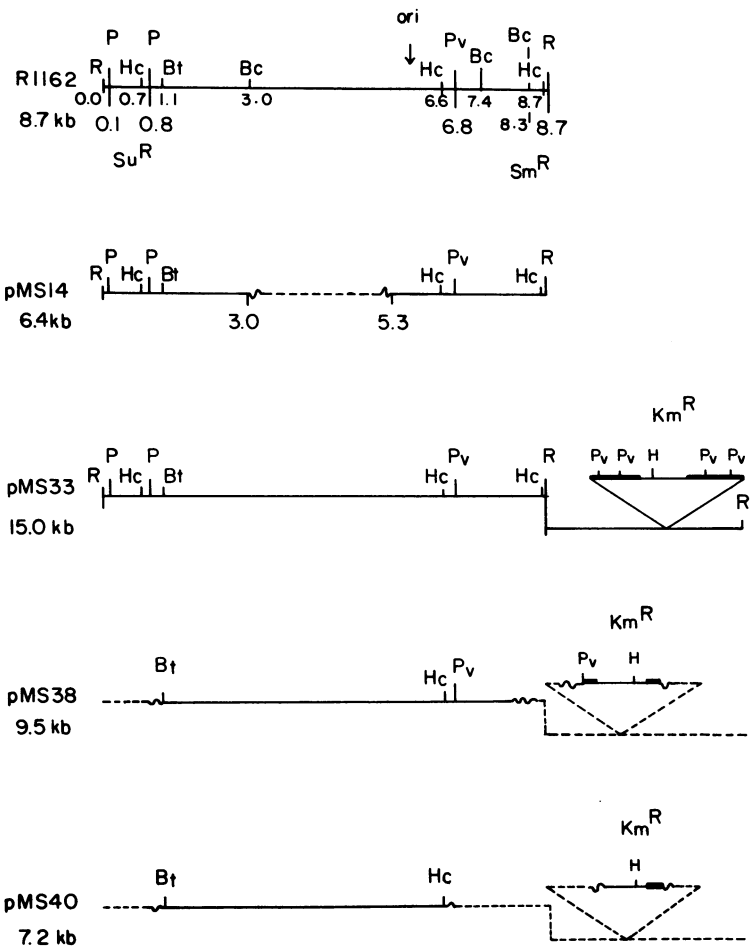

FIG. 2. Map of R1162 and derivatives. The Km fragment cloned in pMS33 is drawn at a lower level than the R1162 DNA, to indicate its independent origin. Within the Km fragment is Tn*903*; this is drawn above the remaining DNA. The thicker portions of the transposon indicate inverted repeat DNA. Dashed lines designate deleted DNA, with the short wavy lines showing uncertainty for their endpoint. Certain restriction enzyme cleavage sites (R. Meyer, M. Hinds, and M. Brasch, unpublished data; 2, 9, 16, 19, 35) are shown for *Eco*RI (R), *Pst*I (P), *Hin*cII (Hc), *Bst*EII (Bt), *Bcl*I (Bc), *Pvu*II (Pv), and *Hin*dIII (H).

fragment for each derivative, along with a knowledge of the distances between the *Hin*cII-*Hin*dIII and *Hin*cII-*Bam*HI cleavage sites of pBR322 (6) and, when present, the size of the small *Hin*cII-*Hin*dIII fragment of pMS40 (0.9 kb) (Fig. 2).

RESULTS

Isolation of smaller derivatives of R1162 and cloned fragments of pMS40. Smaller derivatives of R1162 were generated by partial digestion of plasmid DNA with *Hae*II (see Materials and Methods). We obtained plasmid pMS14 (Fig. 2), which was deleted between approximately 3.0 and 5.3 kb coordinates on the R1162 map. Deletion derivatives were also obtained by *Hae*II digestion of pMS33, followed by ligation of the resulting fragments. The plasmid pMS33 consists of R1162 with a fragment of DNA, encoding Km^r on the transposon Tn*903* (30, 34), inserted into the *Eco*RI cleavage site. Despite numerous attempts, no deletion of R1162 DNA was obtained beyond the *Bst*EII site at 1.1 kb or before the *Hin*cII site at 6.6 kb (Fig. 2). The smallest plasmid obtained, pMS40 (Fig. 2), was saved for further characterization. Together with the deletion in pMS14, these results suggest that essential plasmid genes are encoded within 1.1 to 3.0 and 5.3 to 6.6 kb on the R1162 map. This conclusion is consistent with the observation of DeGraaff et al. (14), who, on the basis of electron microscopy, placed the origin or replication within the 5.3- to 6.6-kb segment. However, there are no other functions known to be encoded within these two regions.

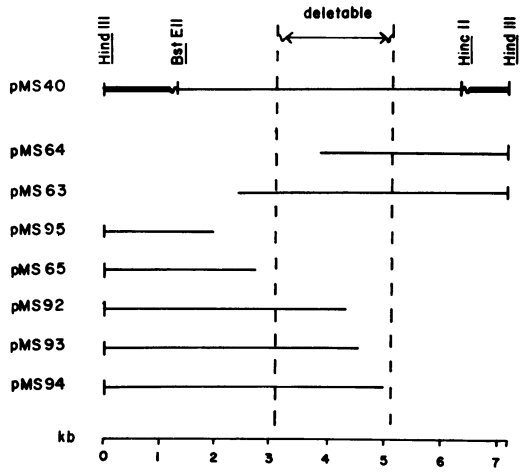

FIG. 3. Fragments of pMS40 cloned into pBR322. A map of pMS40 is drawn at the top; the heavy lines indicate DNA derived from the Km^r fragment, and the wavy lines regions of uncertain origin. DNA corresponding to that deleted in pMS14 is shown by the vertical dashed lines. Below the map, horizontal lines indicate regions of pMS40 DNA cloned into pBR322 to generate the plasmid named at the left.

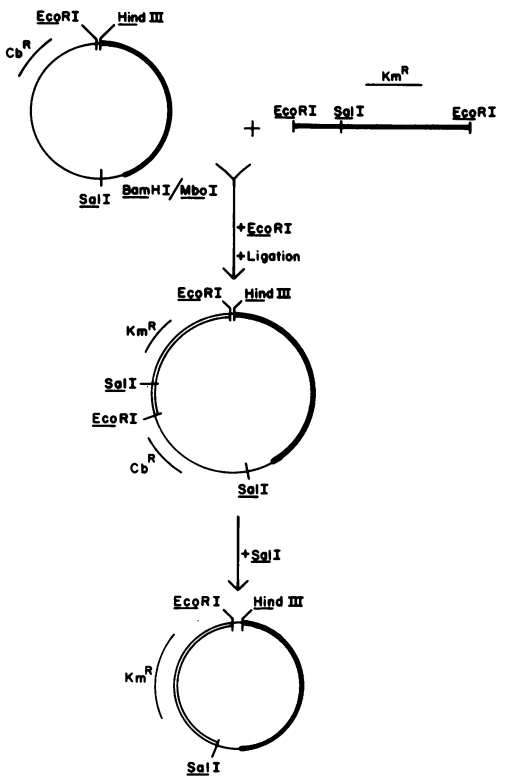

FIG. 4. Method for generating kanamycin-resistant satellite plasmids. A pBR322 derivative containing cloned pMS40 DNA (see text) is indicated in the upper left, with the heavier line representing the pMS40 DNA. To the right is the Km^r DNA fragment used in the cloning.

We cloned fragments of pMS40 DNA, extending from the *Hin*dIII site a variable distance in either direction to *Mbo*I sites (Materials and Methods; Fig. 1), by replacing the small *Hin*dIII-*Bam*HI fragment of pBR322. Our objective was to identify and map properties encoded by the fragments. Seven derivatives were selected for further study. Figure 3 shows the DNA from pMS40 that was included in each derivative.

Identification of R1162 DNA required in *trans* for plasmid maintenance. Our experience in generating smaller derivatives of R1162 in vitro suggests that DNA between 1.1 and 3.0 kb is essential for maintenance. We show here that this is true, and that the required function(s) encoded by this region may be provided in *trans*. The plasmids pMS63 and pMS64 lacked a complete 1.1- to 3.0-kb segment, which extends from the *Bst*EII site to the edge of the deletable region, but should contain the origin of replication of R1162 and adjacent genes (cf. Fig. 2 and

TABLE 2. Transformation of MV10 and MV10(R1162) with *Sal*I-digested Km^r plasmid DNA

Incoming DNA	Recipient	Transformation frequency[a]	Fraction of transformants that are Km^r Cb^{sb}
pMS63-Km^r	MV10	$<10^{-7}$	0/2
	MV10(R1162)	6×10^{-7}	7/10, 8/10, 10/10, 6/10
pMS64-Km^r	MV10	2.2×10^{-7}	0/10, 0/4, 0/4, 0/3
	MV10(R1162)	1.6×10^{-6}	4/10, 3/10, 7/10, 6/10
pMS69-Km^r	MV10	5.3×10^{-6}	10/10, 10/10, 9/10, 10/10
	MV10(R1162)	1.7×10^{-5}	7/10, 8/10, 10/10, 8/10
pBR322-Km^r	MV10	3.5×10^{-7}	0/6, 0/10, 3/8,[c] 1/8[c]
	MV10(R1162)	9.8×10^{-7}	0/10, 0/10, 0/10, 0/10

[a] Transformants per 0.5 μg (approximately) of DNA.
[b] After exposure to DNA, cells were divided into four tubes for expression. Results are for each tube giving transformants after plating.
[c] Deletion derivatives of pBR322-Km^r in which part of the Cb^r gene has been lost.

TABLE 3. Transformation by plasmid DNA isolated from Km^r transformants on MV10(R1162)

Plasmid in MV10 recipient	No. of colonies with screened drug resistance[a]			
	pMS63-derived DNA		pMS64-derived DNA	
	Km^r [b] Sm^r [c]	Sm^r [b] Km^r [c]	Km^r [b] Sm^r [c]	Sm^r [b] Km^r [c]
1. None	20	0	20	0
2. pMS69	0		0	
3. pMS95	20	0	20	0
4. pMS65	20	0	20	0
5. pMS92	3	0	15	0
6. pMS93	3	0	8	0
7. pMS94	3	0	8	0
8. pBR322	20	0	20	0

[a] Twenty colonies tested in each case.
[b] Characteristic selected for.
[c] Characteristic screened.

3). To test whether this remaining R1162 DNA constitutes a stable replicon, we cloned an *Eco*RI fragment encoding Km^r (30) into the *Eco*RI site of pMS63 and pMS64. This fragment was the same as that used in the construction of pMS33 (Fig. 2), but for clarity we have not indicated the presence of Tn*903*. Recombinant molecules were saved that had the Km^r fragment inserted in the orientation shown in Fig. 4. This orientation permits subsequent excision by *Sal*I digestion of a single fragment containing the Km^r gene(s), all of the previously cloned pMS40 DNA, and a small amount (275 base pairs) of nonessential pBR322 DNA. To construct positive and negative controls, we also inserted the Km^r fragment into pBR322 and into pMS69, a chimera between pBR322 and the complete pMS40, joined at their *Hin*dIII sites. We then cleaved the DNA of these plasmids with *Sal*I and transformed MV10 and MV10(R1162) with the digests. Colonies were selected for kanamycin resistance and subsequently screened for susceptibility to carbenicillin (Cb^s) (Table 2). Evidently the *Sal*I-generated Km^r fragment of pMS63 and pMS64 could be rescued only if R1162 was present in the recipient cell. Such a result could occur in two ways: (i) by recombination between homologous regions of R1162 and the incoming DNA or (ii) by persistence of the *Sal*I fragment as a satellite plasmid, dependent on R1162-specified gene products. In the first case, the streptomycin and kanamycin resistances are likely to have become linked. To test this, plasmid DNA was isolated from a kanamycin-resistant colony of MV10(R1162), resulting from the transformation with *Sal*I-generated DNA, and used to transform MV10 and MV10(pMS69) (Table 3, lines 1 and 2). When kanamycin resistance was selected in the MV10 background, the colonies were also streptomycin resistant. However, the kanamycin-resistant transformants of MV10(pMS69) were susceptible to streptomycin (Sm^s). The streptomycin-resistant transformants of MV10 were kanamycin susceptible in every case. These results are explained if the *Sal*I fragments derived from pMS63-Km^r and pMS64-Km^r replicate only as a satellite plasmid, dependent on R1162-specific products encoded by the pBR322-pMS40 chimera, pMS69. The DNA essential for replication must be located in the 1.1- to 3.0-kb segment of R1162, since the 3.0- to 5.3-kb region is deleted in pMS14 (Fig. 2) and therefore is not essential, and the remaining DNA is present in pMS63 and pMS64. This was confirmed by the observation that pMS92, pMS93, and pMS94 (Fig. 3), which contained the 1.0- to 3.0-kb region cloned in pBR322, were able to maintain the *Sal*I-generated kanamycin-resistant DNA as a satellite plasmid (Table 3, lines 5 through 7). The essential genes must lie fairly close (less than 0.5 kb) to the 3.0-kb endpoint, because neither pMS65 nor pMS95 could support satellite plasmid replication (cf. Fig. 3 and Table 3, lines 3 and 4).

Properties of small derivatives and cloned fragments of R1162. Ability of the pBR322 derivatives containing cloned fragments of pMS40 to eliminate R1162 from the cell was tested by transforming MV10(R1162) to carbenicillin resistance and screening carbenicillin-resistant colonies for streptomycin resistance (Table 4). A comparison of these results with Fig. 3 indicates that the determinants for group Q (P-4) incompatibility must lie near the origin of replication, in the 5.3- to 6.6-kb region of R1162.

R1162 was non-self-transmissible, but was efficiently mobilized by plasmid R751 (24, 32) (Table 5, line 1). The derivatives pMS14 and pMS40 were also mobilizable, so that the determinants for this capability must lie in the 1.1- to 3.0-kb or 5.2- to 6.6-kb regions of R1162. Because pBR322 was only very poorly mobilized by R751 (Table 5), but the pBR322-pMS40 chimera (pMS69) was efficiently transferred, the derivatives containing fragments of pMS40 DNA could be tested to locate the R1162 coding

TABLE 4. Transformation of MV10(R1162) with pBR322 derivatives containing cloned fragments of pMS40

Incoming plasmid	No. of transformants remaining Sm^{r}[a]
pMS63	0
pMS64	0
pMS95	50
pMS65	50
pMS92	50
pMS93	50
pMS94	50
pMS69	2
pBR322	50

[a] Fifty tested in each case.

sequences required for mobilization. The results (Table 5) indicated that the determinants for mobilization, like those for incompatibility, were linked to the origin in the 5.3- to 6.6-kb region of R1162.

The stability of pMS14 and pMS40 was tested by serially culturing strains containing these plasmids in medium without antibiotics and then at various times streaking the culture on broth plates and testing isolated colonies for drug resistance (Table 6). Plasmid pMS14 was unstable, whereas pMS40 was just as stable as the parental R1162. Estimates of plasmid copy number (Table 7) indicated that the instability of pMS14 was not due to a substantially lower copy number for this plasmid. DNA required for stable maintenance must therefore be located wholly or in part within the 3.0- to 5.3-kb region of R1162, deleted in pMS14.

Derivatives of R1162 with altered properties. Derivatives of pMS33 (Fig. 2) temperature sensitive for maintenance were isolated by mutagenesis of plasmid DNA with hydroxylamine (Materials and Methods). One temperature-sensitive plasmid, pMS88, was selected for study. Figure 5 shows the rate of loss of this plasmid from log-phase cells grown in broth at 30 and 42°C. In contrast, pMS33 was stable in cells grown under identical conditions (greater than 80% retention of kanamycin resistance).

TABLE 5. Conjugal mobilization of R1162 derivatives by R751 into the recipient DF1019

Test plasmid in donor	Transconjugants/ donor cell
1. R1162	1.0×10^{-1}
2. pMS14	1.1×10^{-1}
3. pMS40	5.0×10^{-2}
4. pBR322	$<1 \times 10^{-6}$
5. pMS69	2.2×10^{-1}
6. pMS64	6.3×10^{-3}
7. pMS63	1.6×10^{-1}
8. pMS95	$<1 \times 10^{-6}$
9. pMS65	$<1 \times 10^{-6}$
10. pMS92	$<1 \times 10^{-6}$
11. pMS93	$<1 \times 10^{-6}$
12. pMS94	6.8×10^{-5}

TABLE 6. Stability of R1162 derivatives in MV10

Plasmid	No. of colonies drug resistant[a]					
	0[b]	13	39	52	75	88
R1162	50	50	50	50	50	50
pMS14	29	17	0	0	0	0
pMS40	50	50	50	50	50	50

[a] Fifty tested in each case.
[b] Number of generations in liquid broth.

The temperature-dependent instability of pMS88 could be complemented in *trans* by pMS92 (Fig. 3). MV10(pMS88) was exposed to pMS92 DNA, and 12 transformed colonies (selected at 30°C for carbenicillin resistance) were purified by streaking on the plates containing carbenicillin and incubated at 42°C. We then tested two isolated colonies from each streak for retention of pMS88. The results (Table 8, line 1) showed that stability of pMS88 at 42°C was substantially restored. In contrast, pMS65 (Fig. 3) failed to complement pMS88 (Table 8, line 2). Although the R1162 derivative pMS14 was also maintained unstably in MV10 (Table 6), this defect was not complemented (at 37°C) by pMS92 (Table 8, line 4). There was a further difference between pMS14 and pMS88: whereas pMS14 was maintained at a normal number of supercoiled copies per cell, there were far fewer copies of pMS88 under permissive conditions than of the parental pMS33 plasmid (Table 7).

We obtained R1162 derivatives conferring high-level streptomycin resistance by nitrosoguanidine mutagenesis of a donor strain, followed by conjugal mobilization of the plasmid and selection of transconjugants able to grow on

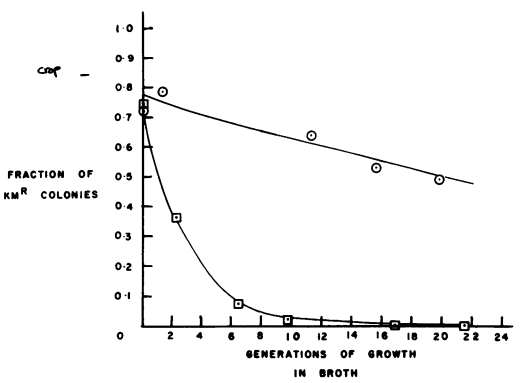

FIG. 5. Rate of loss of pMS88 from MV10 grown in broth without antibiotics at 30°C (⊙) and at 42°C (⊡).

TABLE 7. Estimates of plasmid copy number[a] in MV10

Plasmid	Copy no.
R1162	57
pMS14	41
pMS40	81
pMS73	251
pMS88[b]	5
pMS33[b]	81

[a] Number of copies of supercoiled plasmid DNA per chromosome (molecular weight, 2.5×10^9; 8).
[b] Plasmid isolated from cells grown at 30°C.

streptomycin (500 μg/ml) (Materials and Methods). The majority of colonies isolated contained mutations in the plasmid DNA. When the plasmid DNA was extracted from these strains, it efficiently transformed MV10 to streptomycin (125 μg/ml) (Table 9). In contrast, DNA obtained from a transconjugant (isolate 6) in a control experiment, where initial selection was for streptomycin resistance on plates containing 25 μg of the antibiotic per ml, did not give a similar number of highly streptomycin-resistant transformants. We examined the plasmid from isolate 1 in more detail, designating it pMS73. A copy number estimate of the plasmid showed that it was present in a substantially greater number of copies per cell than R1162 (Table 7). Thus, pMS73 had a mutation in a gene (*cop*) involved in copy number control.

We took advantage of the location of the *BclI* cleavage sites in R1162 (Fig. 2) to partially map the *cop* mutation. Both pMS73 and R1162 were digested with *BclI*, and the fragments were electrophoresed on an agarose gel. The two large fragments were separately recovered from the gel (41), and various combinations of these were ligated to reconstruct the replicon. The small *BclI* fragment was not included, since it did not contain any essential DNA (Fig. 2). We then transformed MV10 to sulfonamide resistance with the various DNA mixtures, and screened the sulfonamide-resistant colonies for plasmid content. (We were unable to use streptomycin resistance in these experiments because this drug resistance is lost with the small *BclI* fragment). Unfortunately, sulfonamide resistance does not show a clear gene-dosage effect, so we screened for copy number directly. MV10(pCR1) was transformed with the reconstructed plasmids, and the plasmid DNAs of these strains were then isolated, linearized by digestion with *Eco*RI, and run on an agarose gel. By examination of the gel, we estimated relative molecular yields of the plasmids. This method worked well: pMS73 could be readily distinguished from R1162 by this method (Fig. 6). To provide suitable plasmids for comparison in the experiment, positive controls were included where all of the *BclI* fragments in the plasmid reconstruction were derived from either R1162 or pMS73 (Table 10). The *cop* mutation evidently lay within the 3.0- to 7.4-kb fragment. Regardless of mechanism for control of copy number, deletion of the *cop* region should substantially alter the plasmid copy number. However, neither pMS14 nor pMS40 showed copy numbers greatly different from those of R1162 (Table 7). We therefore think it likely that the *cop* sequence is within the 5.3- to 6.6-kb segment of R1162.

TABLE 8. Complementation of stability defects by pMS92

Test plasmid	Incoming plasmid	Purified colony pairs expressing test plasmid drug resistance[a]		
		RR	RS	SS
1. pMS88	pMS92	11	0	1
2. pMS88	pMS65	0	0	12
3. pMS88	pBR322	0	0	12
4. pMS14	pMS92	0	3	9
5. pMS14	pBR322	0	1	11

[a] Twelve pairs tested. RR, both resistant; RS, one resistant; SS, neither resistant. Colonies were purified at 37°C (pMS14) or 42°C (pMS88).

DISCUSSION

Coding sequences required for plasmid replication and maintenance are frequently located in a small region of the molecule (29, 30, 36, 38). This feature has permitted the isolation of miniplasmid derivatives of many different naturally occurring plasmids. Although these plasmids are frequently less stable then the parental replicon, they retain many of its characteristic properties. Perhaps the extreme in this regard is the isolation of ColE1 derivatives consisting of little more than the origin of replication (*ori*) and sufficient adjacent DNA to encode an RNA primer (36). Because R1162 is like ColE1 in

TABLE 9. Transformation of MV10 with DNA from high-level streptomycin-resistant transconjugants

Isolate no.	Streptomycin concn used for selection of transconjugant (μg/ml)	Transformants/ml on plates containing streptomycin at:	
		25 μg/ml:	125 μg/ml:
1	500	7.5×10^3	7.2×10^3
2	500	5.2×10^5	5.4×10^5
3	500	6.3×10^4	5.1×10^4
4	500	7.8×10^4	6.0×10^4
5	500	9.5×10^4	190
6	25	9.8×10^4	<50

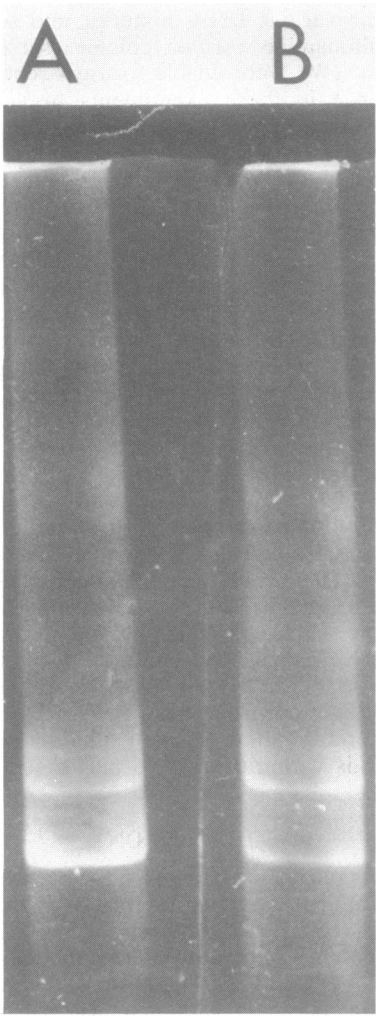

FIG. 6. Agarose gel electrophoresis of *Eco*RI-cleaved plasmid DNA derived from (A) isolate 1 and (B) isolate 6 (Table 9). The plasmid PCR1 (12) (upper band) was coextracted from the cells.

being a small, high-copy-number plasmid and in having a requirement for DNA polymerase I (although poorly characterized for R1162) (20, 27), we thought that it might be possible to isolate drastically smaller derivatives for this plasmid as well. However, our investigations indicate that the requirements for maintenance of R1162 are more complex. In particular, there is a coding sequence, remote from the origin, which specifies at least one product active in *trans* and necessary for plasmid maintenance. In this regard, R1162 resembles the much larger, broad-host-range IncP-1 plasmids (32, 37). R1162 and other IncP-4 plasmids apparently have a larger host range than initially appreciated (13). Perhaps the nonclustering of essential functions represents the recent acquisition of additional replication maintenance determinants that increase plasmid independence from host cytoplasm.

Efficient conjugal mobilization of ColE1 requires a *cis*-acting site (*bom*) near *ori* and a second, less well-defined region (*mob*), distant from *ori* and specifying, at least in part, products active in *trans* (40). These products probably make up the plasmid relaxation complex, which introduces a nick essential for conjugal transfer into the DNA at *bom*. Mob$^-$ derivatives, in addition to being poorly mobilized, are almost always deficient in activity of the relaxation complex (40).

Coding sequences sufficient for mobilization of R1162 are linked to the origin of replication. In agreement with this, Nordheim et al. (35) have shown that the site of the relaxation complex nick for RSF1010, a plasmid possibly identical to R1162, is very close to *ori*. Because pMS14 and pMS40 are mobilized by R751 as well as pMS63 and pMS64, all functions necessary for mobilization of R1162 are encoded within the 5.3- to 6.6-kb region of the plasmid. In contrast, ColE1 must retain a large fraction of its genome, in addition to the *bom* site, for efficient mobilization by R751 (39). Possibly R1162 requires fewer relaxation complex components for mobilization or is more successful in parasitizing the components of the R751 vector. Such adap-

TABLE 10. Plasmid reconstruction experiments to locate *cop* mutation

Source of *Bcl*I fragments		Transformants/ml	Plasmid phenotype[a]	
8.3–3.0 kb	3.0–7.4 kb		High copy no.	Wild type
pMS73	R1162	2.5×10^4	0	4
R1162	pMS73	1.6×10^4	3	1
pMS73	pMS73	3.4×10^4	4	0
R1162	R1162	2.3×10^4	0	4
	pMS73, R1162	9.2×10^2	4	3
pMS73, R1162		2.4×10^3	1	3

[a] After allowing for uptake of DNA, cells were divided into four parts for expression. One transformant arising from each part was then tested to avoid examining sibs. Wild type indicates having parental copy number.

tations might be expected in a plasmid of broad host range.

R1162 encodes two distinct functions required for plasmid stability. One of these is missing in the deletion derivative pMS14, which although unstable replicates with a nearly normal copy number. The defect is not complemented by pMS92 (Fig. 3). A second stability function is defined by the temperature-sensitive mutation in pMS88. The defect is complemented by pMS92, but not by pMS65. Because pMS92 but not pMS65 contains the entire essential region for replication of the R1162 satellite plasmids, the temperative-sensitive mutation must be adjacent to, or within, the region essential for plasmid maintenance.

Under permissive conditions and in the presence of selection, there are far fewer copies of supercoiled pMS88 DNA per cell than for the parental pMS33 plasmid. A mutation (*cop*) resulting in a higher number of copies of R1162 is located near *ori*, in a region of the plasmid different from that containing the temperature-sensitive mutation of pMS88. One interpretation of these observations is that the copy number of R1162 is normally controlled by *cop*, but that a second, positively acting product, encoded within the essential 1.1- to 3.0-kb region of the plasmid, is also required for replication. This product would be defective in the case of pMS88 and would become rate limiting for replication even at a permissive temperature, resulting in fewer copies of the plasmid per cell. However, other explanations are also possible. For example, the mutation in pMS88 might bring about an altered relaxation complex which is adventitiously nicking the DNA, causing a decline in the number of supercoiled molecules and, perhaps as a consequence, faulty replication or maintenance. Mutations of this type were probably isolated by Collins et al. (11) in ColE1 DNA. We think such an explanation unlikely here because, as discussed above, all of the R1162-specified components of the relaxation complex are probably encoded within the 5.3- to 6.6-kb region of the plasmid. Further experiments are now being conducted to elucidate the role of the temperature-sensitive gene product.

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