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Insertion and Deletion Mutations in the *repA4* Region of the IncFII Plasmid NR1 Cause Unstable Inheritance

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Mutants of IncFII plasmid NR1 that have transposons inserted in the *repA4* open reading frame (ORF) are not inherited stably. The *repA4* ORF is located immediately downstream from the replication origin (*ori*). The *repA4* coding region contains inverted-repeat sequences that are homologous to the *terC* inverted repeats located in the replication terminus of the *Escherichia coli* chromosome. The site of initiation of leading-strand synthesis for replication of NR1 is also located in *repA4* near its 3′ end. Transposon insertions between *ori* and the right-hand *terC* repeat resulted in plasmid instability, whereas transposon insertions farther downstream did not. Derivatives that contained a 35-bp frameshift insertion in the *repA4* ORF were all stable, even when the frameshift was located very near the 5′ end of the coding region. This finding indicates that *repA4* does not specify a protein product that is essential for plasmid stability. Examination of mutants having a nest of deletions with endpoints in or near *repA4* indicated that the 3′ end of the *repA4* coding region and the site of leading-strand initiation could be deleted without appreciable effect on plasmid stability. Deletion of the *pemI* and *pemK* genes, located farther downstream from *repA4* and reported to affect plasmid stability, also had no detectable effect. In contrast, mutants from which the right-hand *terC* repeat, or both right- and left-hand repeats, had been deleted were unstable. None of the insertion or deletion mutations in or near *repA4* affected plasmid copy number. Alteration of the *terC* repeats by site-directed mutagenesis had little effect on plasmid stability. Plasmid stability was not affected by a *tus* mutation known to inactivate the termination function. Therefore, it appears that the overall integrity of the *repA4* region is more important for stable maintenance of plasmid NR1 than are any of the individual known features found in this region.

For a bacterial plasmid to be inherited in a stable manner, its replication during the cell division cycle and the partitioning of plasmid replicas to daughter cells during cell division both must be carefully regulated and coordinated. For IncFII plasmid NR1, a 95-kb self-transmissible antibiotic resistance plasmid with a copy number of approximately two per chromosome in *Escherichia coli* (36, 49), those two functions are mediated by the *repA* replicon (25, 34, 48) and the stability (*stb*) locus (25, 27, 28, 41, 42), respectively. Miniplasmids that incorporate *repA* and *stb* are replicated and inherited with characteristics essentially similar to those of the 95-kb parental plasmid (25, 41). Plasmids with mutations that inactivate *stb* are replicated normally but exhibit unstable inheritance, resulting in the accumulation of plasmid-free cells in the population in culture unless continuous antibiotic selection is applied (25, 41).

The essential elements of the *repA* replicon include the replication origin (*ori*) and the *repA1* gene (31, 34, 48), whose protein product binds to the downstream *ori* and is the plasmid-specific replication initiation factor (10, 22, 24, 32). Initiation of plasmid NR1 replication is regulated by controlling the synthesis of the *cis*-acting RepA1 protein (10, 31, 48). The genes encoding the *trans*-acting factors that regulate expression of *repA1* lie upstream from the *repA1* gene (31, 48). Transcription of *repA1* mRNA is regulated by the RepA2 repressor protein (8, 20), and translation of *repA1* mRNA is regulated by RNA-E (19, 47), a 91-base transcript that is complementary to the leader region of *repA1* mRNA (9, 35).

The *repA4* open reading frame (ORF) is located immediately downstream from *ori* (Fig. 1) and is conserved in IncFII plasmids (37, 38). However, there is no known gene product or function associated with the *repA4* ORF, and no transcription promoter has been identified upstream from the gene (1). Although *ori* contains the *cis*-acting DNA sequences required for initiation of replication, the actual site of initiation of leading-strand synthesis for replication of NR1 is located within *repA4* near its 3′ end (4, 23, 29), which is 400 bp downstream from *ori* (Fig. 1). Also contained within *repA4* are two inverted-repeat sequences that are homologous to the core consensus sequence of the inverted repeats that serve as a replication fork trap in the *terC* terminus of the *E. coli* chromosome (14, 17). The binding of Tus protein, encoded by the *tus* gene (15, 17), to the inverted repeats in the chromosome prevents replication forks from proceeding past the *terC* region. The arrest of replication forks by *terC* when bound by Tus is orientation dependent (13, 21, 40). The *terC* inverted repeats in *repA4* also cause termination of replication, at least transiently, that can be detected when they are inserted into other plasmids such as pBR322 (14, 17). The right-hand *terC* repeat in *repA4*, located between bp 2049 and 2062, is oriented such that it would inhibit replication forks entering *repA4* from the left, which is the direction that the NR1 replication fork travels after proceeding unidirectionally around the plasmid from the site of initiation of leading-strand synthesis (4, 23, 29). The left-hand repeat, located between bp 1913 and 1926, would inhibit replication forks entering from the right, perhaps such as those associated with lagging-strand synthesis (29). It is not known whether the *terC* inverted repeats actually participate in termination of NR1 replication. However, similar inverted repeats are present in the replication terminus of plasmid R6K and appear to mediate termination of R6K replication (12, 16, 40). There are no known pheno-

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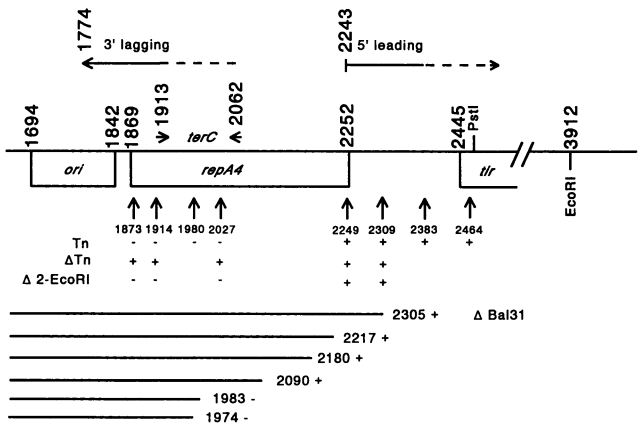

FIG. 1. Map of the *repA4* region of NR1. Numbers refer to the base pair positions within the nucleotide sequence of the *repA* replicon of NR1 (34). This region of plasmid pRR720 lies downstream from the *repA1* gene and includes *ori*, the *repA4* ORF, and the *tir* gene, in which the map is interrupted. Not shown are the *pemI* and *pemK* genes, which are located between *tir* and the *Eco*RI site at bp 3912. All four genes read from left to right in this figure. The *Pst*I site used for mapping insertions is at position 2469. The inverted-repeat sequences that are homologous to the *terC* inverted repeats of the *E. coli* chromosomal replication terminus are located within *repA4* and are indicated by the small horizontal arrows. The 5′ end of the leading strand for replication of NR1 begins at bp 2243 within *repA4*, whereas the 3′ end of the lagging strand is at bp 1774 within *ori*. The sites of insertion of various transposons (Tn) into pRR720 are indicated by the vertical arrows below the map, and the stability phenotype of each insertion mutant is indicated by + or −. Mutants having deleted all but 35 bp of the transposon with use of *Eco*RI are indicated by ΔTn. Other mutants were constructed by deletion of the transposon from its site of insertion as well as the adjacent *Eco*RI fragment to bp 3912 (Δ 2-EcoRI). Additional deletion mutants were constructed by using BAL 31 digestion from position 2309. Positions of the deletion endpoints and the stability phenotypes are indicated.

types associated with inactivation of the termination function of either the *E. coli* chromosome or R6K (7, 17).

There are three other known genes immediately downstream from *repA4*, referred to as *tir*, *pemI*, and *pemK* (43, 44). These genes are not known to participate in control of replication of NR1. However, the *pem* (plasmid emergency maintenance) genes have been reported to participate in plasmid stable maintenance when cloned into other plasmids such as pBR322 (5, 44). The *tir* gene has been shown to inhibit conjugal transfer of plasmid RP4 (43), which is not otherwise related to IncFII plasmids. Results presented here demonstrate that DNA sequences that include the *terC* repeats within the *repA4* ORF are important for the stable maintenance of NR1, whereas the integrity of the *repA4* ORF itself is not. The *terC* repeats themselves also are not essential, since mutations in them or in *tus* had little effect on stability. Therefore, it appears that the overall integrity of the *repA4* region is more important for stable maintenance of plasmid NR1 than are any of the individual known features found in this region.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* K-12 strains were used in these studies. Strain KP245 (11) was used for plasmid DNA isolation, strain JM105 (46) was used for cloning in M13mp18 and M13mp19 DNA vectors (51), and strains KP435 (11), PK0457 (15), and PK2619 (15) were used for plasmid stability tests. Strain PK2619 is identical to PK0457 except that *tus* is interrupted by insertion of a kanamycin resistance cassette (15). KP245, KP435, PK0457, and PK2619 cells were cultured in 2YT medium (26) containing, per liter, 16 g of tryptone, 10 g of yeast extract (both from Difco Laboratories), and 5 g of NaCl. YT medium (26), containing, per liter, 8 g of tryptone, 5 g of yeast extract, and 5 g of NaCl, was used for culturing JM105 cells. 2YT or YT plates contained 15 g of Bacto Agar (Difco) per liter. Chloramphenicol acetyltransferase (CAT) indicator agar (33) contained 3 mg of crystal violet (Matheson) per liter of 2YT agar. YT top agar contained 6 g of Bacto Agar and 50 μg of 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (Research Organics, Inc.) per liter, plus isopropyl-β-D-thiogalactopyranoside at 0.5 mM. The following antibiotics (Sigma Chemical Co.) were included in the medium where appropriate to select for cells harboring various plasmids: tetracycline hydrochloride (5 mg/liter), kanamycin sulfate (25 mg/liter), streptomycin sulfate (40 mg/liter), sodium ampicillin (50 mg/liter), and chloramphenicol (25 mg/liter). Cells were cultured at 37°C, and growth was monitored by turbidity at 650 nm with a Gilford model 260 spectrophotometer.

Plasmids and isolation of plasmid mutants. Plasmid pRR720 is a stable, low-copy-number 24.6-kb miniplasmid derivative of NR1 that contains the wild-type *repA* replicon, the wild-type *stb* locus, and the *cat* gene for chloramphenicol resistance (41). All mutants listed in Table 1 were derived from pRR720. The mutagenesis of pRR720 by transposon insertion with various derivatives of Tn*1721* (45) has been described elsewhere (41). In brief, donor plasmids that were temperature sensitive for replication were introduced into the same strain with pRR720, followed by curing of the donor plasmids by incubation at 42°C and selection for resistance to chloramphenicol, conferred by pRR720, and tetracycline or kanamycin, conferred by the transposon. Derivatives of pRR720 that conferred resistance to both chloramphenicol and tetracycline or kanamycin were screened for instability on CAT indicator agar (33), on which cells with unstable plasmids form sectored colonies (33, 41). Transposon Tn*1731* is 7.85 kb in length and confers tetracycline resistance, whereas Tn*1733* is 8.9 kb in length and confers kanamycin resistance (45). Both transposons contain one site for *Eco*RI restriction endonuclease near each end, which facilitates mapping their sites of insertion (45). Insertion of these transposons duplicates a 5-bp target sequence (45). pRR720 also contains two sites for *Eco*RI, one located at bp 3912 (Fig. 1) and the second 3.2 kb farther downstream in the *cat* gene (41). To retain chloramphenicol resistance, the two *Eco*RI fragments of pRR720 must be retained in their native orientation in the plasmid (25). Various deletion derivatives of the mutants with transposon insertions were constructed by digestion with *Eco*RI followed by ligation and transformation with selection for chloramphenicol resistance. Plasmids from which the transposon had been deleted were screened for loss of the transposon-encoded resistance. This procedure resulted in deletion derivatives that had lost either the internal transposon *Eco*RI fragment (partial deletion derivatives; designated by suffix "P") or both the transposon fragment and the adjacent downstream fragment from the end of the transposon to the next *Eco*RI site at position 3912 (deletion derivatives; designated by suffix "D"). These plasmids have new *Eco*RI sites created by joining the ends of the transposon or by joining one end of the transposon to the site at position 3912.

Starting with deletion derivative pMRT2309D (Table 1),

TABLE 1. Derivatives of plasmid pRR720[a]

Plasmid	Description[b]	Reference or source
Transposon insertion mutants		
pMR1	Unstable; Tn*1732* in *stbA*; *cat kan*	41
pMRT1873	Unstable; Tn*1731* at bp 1873 in *repA4*; *cat tet*	41
pMRT1914	Unstable; Tn*1731* at bp 1914 in *repA4*; *cat tet*	41
pMRT1980	Unstable; Tn*3* at bp 1980 in *repA4*; *cat bla*	41
pMRT2027	Unstable; Tn*1733* at bp 2027 in *repA4*; *cat kan*	41
pMRT2249	Stable; Tn*1733* at bp 2249 in *repA4*; *cat kan*	This study
pMRT2309	Stable; Tn*1733* at bp 2309 near *repA4*; *cat kan*	This study
pMRT2383	Stable; Tn*1733* at bp 2383 near *repA4*; *cat kan*	This study
pMRT2464	Stable; Tn*1731* at bp 2464 in *tir*; *cat tet*	This study
Partial deletion mutants with transposon *Eco*RI fragment removed		
pMR13	Unstable; transposon removed from *stbA* of pMR1; *cat*	28
pMRT1873P	Stable; transposon removed from *repA4* of pMRT1873; *cat*	This study
pMRT1914P	Stable; transposon removed from *repA4* of pMRT1914; *cat*	This study
pMRT2027P	Stable; transposon removed from *repA4* of pMRT2027; *cat*	This study
pMRT2249P	Stable; transposon removed from *repA4* of pMRT2249; *cat*	This study
pMRT2309P	Stable; transposon removed from pMRT2309; *cat*	This study
Deletion mutants with 2 *Eco*RI fragments removed		
pMRT1873D	Unstable; fragments removed from pMRT1873; *cat*	This study
pMRT1914D	Unstable; fragments removed from pMRT1914; *cat*	This study
pMRT2027D	Unstable; fragments removed from pMRT2027; *cat*	This study
pMRT2249D	Stable; fragments removed from pMRT2249; *cat*	This study
pMRT2309D	Stable; fragments removed from pMRT2309; *cat*	This study
pMRT2309B	Stable; pMRT2309D with *Eco*RI site replaced by *Bam*HI linker; *cat*	This study
BAL 31-induced deletion mutants		
pMRB2305	Stable; BAL 31 deletion to bp 2305 of pMRT2309B; *cat*	This study
pMRB2217	Stable; BAL 31 deletion to bp 2217 of pMRT2309B; *cat*	This study
pMRB2180	Stable; BAL 31 deletion to bp 2180 of pMRT2309B; *cat*	This study
pMRB2090	Stable; BAL 31 deletion to bp 2090 of pMRT2309B; *cat*	This study
pMRB1983	Unstable; BAL 31 deletion to bp 1983 of pMRT2309B; *cat*	This study
pMRB1974	Unstable; BAL 31 deletion to bp 1974 of pMRT2309B; *cat*	This study
Site-directed mutants with new *Bgl*II site		
pMRT1873M1	Stable; altered left-hand *terC* site of pMRT1873P; *cat*	This study
pMRT1873M2	Stable; altered right-hand *terC* site of pMRT1873P; *cat*	This study
pMRT1873M3	Stable; altered *repA4* sequence of pMRT1873P; *cat*	This study

[a] pRR720 is a wild-type NR1-derived stable miniplasmid (41).
[b] Abbreviations: *cat*, chloramphenicol resistance; *tet*, tetracycline resistance; *kan*, kanamycin resistance; *bla*, ampicillin resistance.

the new *Eco*RI site was replaced with a *Bam*HI linker to facilitate the creation of deletion mutants with BAL 31 exonuclease. pMRT2309D was partially digested with *Eco*RI, treated with DNA polymerase I Klenow fragment to fill in the single-stranded ends, ligated with 8-bp *Bam*HI linkers (New England Biolabs), digested with *Bam*HI, ligated, and transformed into KP245 with selection for chloramphenicol resistance. In plasmid pMRT2309B, a *Bam*HI site was inserted into the new *Eco*RI site adjacent to position 2309. Plasmids with insertions of the *Bam*HI linker into the *Eco*RI site in the *cat* gene were also obtained from this procedure. It is interesting to note that these plasmids conferred chloramphenicol resistance even though they contained a 12-bp insertion in *cat*.

The new *Bam*HI site in pMRT2309B is the only *Bam*HI site on the plasmid. Mutants containing nested deletions were constructed by digestion of pMRT2309B with *Bam*HI followed by digestion with BAL 31 exonuclease and ligation with a *Bam*HI linker.

Site-directed mutagenesis of *repA4* sequences. The MutaGene M13 *in vitro* Mutagenesis Kit obtained from Bio-Rad Laboratories was used for site-directed mutagenesis of *repA4* DNA. The *Eco*RI fragment from plasmid pMRT1873 from bp 1873 to 3912 (Fig. 1) was cloned in M13mp19. Then the 640-bp *Pst*I fragment from that clone, which contains the *terC* repeats, was cloned in M13mp19. Oligonucleotide primers designed to replace *repA4* sequences with a *Bgl*II restriction site were used to mutagenize the M13 clones according to the protocol provided with the kit, which is based on the method of Kunkel et al. (18). Three 25-base primers were used. Primer 1 (TGTCTGAGTGagatctCTAAAGCGG) spanned the left-hand *terC* repeat from bp 1931 to 1907 (Fig. 2) and replaced the central portion with the agatct *Bgl*II sequence. Primer 2 (GATGAAGTAGagatctCATTCATAA) spanned the right-hand *terC* repeat from bp 2069 to 2045 (Fig. 2) and replaced the central portion with the agatct *Bgl*II sequence. Primer 3 (ACGAGCGTGTagatctAACTTCCAG) was located downstream from the *terC* repeats from bp 2113 to 2089 (Fig. 2) and also introduced a *Bgl*II site into *repA4*. The presence of the mutations was checked by digestion with *Bgl*II. The nucleotide sequence of the 640-bp *Pst*I fragment that contained the mutations was determined to ensure that no other mutations had been introduced. The reverse of the above-described cloning procedure was used to reconstruct *Eco*RI fragments that contained the mutations, which were then inserted into plasmid pMRT1873D to construct plasmids with structures identical to that of plas-

FIG. 2. Nucleotide sequence of the *repA4* region of NR1. The sequence is of the region shown in Fig. 1 with the addition of the 3′ end of *repA1*. Locations of the features from Fig. 1 are indicated. Sites of insertion of transposons are indicated by the upward carets and the abbreviated names of the mutants. The base indicated by the caret is the 5′ end of the 5-bp target sequence duplicated by transposon insertion. Endpoints of the BAL 31-induced deletions are indicated by the leftward carets and the abbreviated names of the mutants. Nucleotides representing the 5′ end of the leading strand and the 3′ end of the lagging strand are in bold type, as is the DnaA box. Nucleotides that are homologous to the *terC* inverted repeats are underlined.

mid pMRT1873P. The mutant plasmids obtained from this procedure are listed in Table 1.

Plasmid stability assay and copy number measurements. The stability of plasmid inheritance was determined by measuring the decrease in the fraction of antibiotic-resistant cells during a period of nonselective growth according to the standard protocol (25). After initial culturing in medium containing chloramphenicol, the cells that harbored various plasmids were repeatedly subcultured by 10^6-fold dilution into drug-free medium and then incubated overnight. At 3-day intervals, appropriate dilutions of the cultures were spread onto drug-free 2YT agar plates, and the antibiotic resistance of at least 100 colonies was tested by replica plating by the toothpick method (26). Representative resistant and sensitive colonies were tested directly for the presence of plasmid DNA by the alkaline minilysate method (39).

The relative copy numbers of plasmids that carry the *cat* gene were estimated from gene dosage effects by measuring CAT specific activity in cell extracts prepared from exponentially growing cultures as described previously (11) except that total protein concentrations were determined with Bio-Rad protein assay kits according to the instructions supplied with the kit. Cells were cultured in medium containing chloramphenicol so that nearly 100% of the cells harbored the test plasmid. Copy numbers were estimated relative to that of plasmid pRR720.

DNA isolation and manipulation. Plasmid DNA isolation, restriction endonuclease digestion, gel electrophoresis, ligation of restriction fragments, and transformation of *E. coli* cells with plasmid DNA were performed as described elsewhere (25, 39). All enzymes were used as recommended by the suppliers. Restriction endonuclease fragments were purified from agarose gels with DEAE-membranes or from 0.8 or 1.4% low-melting-point agarose gels (39). The alkaline minilysate method (39) was used for screening of plasmid sizes and restriction endonuclease analysis of plasmid DNA. M13 phage DNA was isolated and manipulated as described in the instructions obtained with the New England Biolabs M13 Cloning and Sequencing System.

Nucleotide sequence analysis. Kits for sequencing single-strand M13 phage DNA were purchased from New England Biolabs. Dideoxy sequencing was performed according to the instructions supplied with the kit, using [α-^{35}S]dATP. Phage DNA that contained inserted DNA fragments from the *repA4* region was prepared as described in the sequencing kit instructions. The orientations of the inserted *Eco*RI fragments were determined by digestion of double-stranded phage DNA with *Pst*I, which has sites at position 2469 of the inserted fragment (Fig. 1) and also in the phage polylinker. Orientation B is defined as having the *repA4* ORF in the direction opposite that of *lacZ* of the phage, such that the *repA4* sequence determined from the primed single-stranded phage DNA would read from 5′ to 3′ (left to right) as presented in Fig. 1. M13 clones with inserted DNA fragments that contained the *terC* repeats were obtained only in orientation A. Kits for sequencing double-stranded phage DNA were purchased from United States Biochemical (Sequenase version 2.0), and sequencing was performed according to the instructions supplied with the kit. Standard sequencing primers and reverse sequencing primers were used. For determining the sequence of the partial deletion mutants from which the transposons had been deleted, synthetic oligonucleotide primers that allowed sequencing across the sites of deletion in both directions were obtained.

Similar methods were used to confirm the presence of site-directed mutations within *repA4*.

Northern (RNA) blot analysis of *repA4* transcription. RNA samples were subjected to Northern blot analysis according to published methods (3, 39), with slight modifications essentially as described previously (27). Intact RNA samples were obtained from cultures of KP245 cells containing either no plasmid, wild-type plasmid pRR720, or copy mutant plasmid pRR942 (25, 50), using CsCl block gradient centrifugation (27). RNA samples were prepared for electrophoresis by mixing 5 μl of RNA sample with 15 μl of deionized formamide, 5 μl of formaldehyde, and 3 μl of 10× morpholine propanesulfonic acid (MOPS) running buffer (3), heating the mixture at 65°C for 10 min, and then chilling it on ice. The denatured RNA samples were then electrophoresed in 1.2% agarose-formaldehyde gels at 3 V/cm. The sizes of the RNA transcripts were estimated by comparison with the mobilities of the RNA Ladder molecular weight standards (Bethesda Research Laboratories) that were electrophoresed in the same gels. The locations of the standard bands were determined by staining with ethidium bromide prior to electrophoresis and photographing the gel with UV light. The RNA samples to be transferred to nitrocellulose were not stained with ethidium bromide. The RNA was transferred from the gels to supported nitrocellulose membranes (Optibind; 0.45-μm pore size; Schleicher & Schuell) and hybridized to ^{32}P-labeled DNA probes. The hybridization products were detected by autoradiography.

DNA restriction fragments from *repA4* or *repA1* were used to prepare the probes. From *repA4*, an *Eco*RI-*Rsa*I fragment (bp 1873 to 2103) contained the 5′ half of *repA4*, including the *terC* repeats, and an *Rsa*I-*Fok*I fragment (bp 2103 to 2258) contained the 3′ half of *repA4*. From *repA1*, a 288-bp internal *Sma*I fragment (bp 1001 to 1289) was used. The DNA fragments were labeled by nick translation with [α-^{32}P]dCTP (Amersham). Nick translation reagents and enzymes were obtained as a kit from Amersham.

RESULTS

Isolation and characterization of unstable plasmid mutants with transposon insertions in *repA4*. Plasmid pRR720 is a 25-kb miniplasmid derived from NR1 (95 kb) that contains the *repA* replicon, the *stb* locus, and the *cat* gene for chloramphenicol resistance (41). It is stably maintained at a low copy number of one to two per chromosome in an *E. coli* host (41). pRR720 was used previously for the characterization of the *stb* locus (28, 41), and its replication and stability properties have been extensively studied. During mutagenesis of pRR720 by insertion of derivatives of transposon Tn*1721* and subsequent screening for unstable mutants, it was discovered that not all unstable mutants had insertions in *stb* (28, 41). Transposon Tn*1721* has recognition sites for *Eco*RI near each of its ends, which facilitates mapping the sites of insertion (45). Mapping with restriction endonucleases *Eco*RI and *Pst*I indicated that some unstable mutants had transposon insertions in the *repA4* ORF (designated Tn in Fig. 1). A number of other mutants that remained stable but had transposon insertions near *repA4* were also characterized (Fig. 1). The stability of each representative mutant is shown in Fig. 3 in comparison with that of stable parental plasmid pRR720. The numbers in the names of the mutant plasmids indicate the sites of transposon insertion in the DNA sequence. The unstable mutant pMRT1914 is an example of a plasmid with a transposon insertion near the 5′ end of *repA4*, whereas the stable plasmid pMRT2464 is an example with a transposon insertion downstream from *repA4* in the *tir* gene.

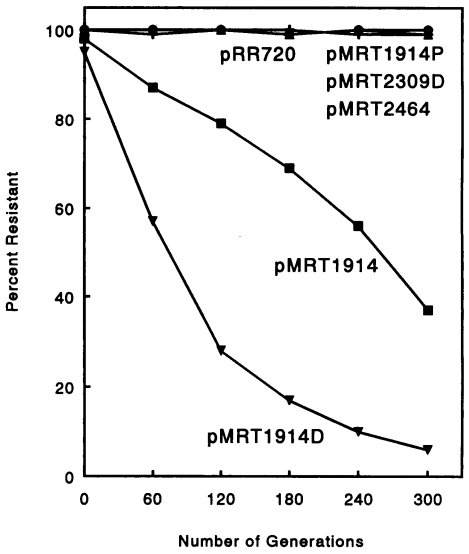

FIG. 3. Stability tests of pRR720 and several insertion or deletion mutants. The percentage of PK0457 cells that were resistant to chloramphenicol after various periods of nonselective growth in drug-free medium was determined by replica plating. The test plasmids were wild-type pRR720 (●), insertion mutant pMRT1914 (■), in which the transposon was inserted in *repA4*, insertion mutant pMRT2464 (◆), in which the transposon was inserted in *tir*, deletion mutant pMRT1914P (▲), in which the transposon *Eco*RI fragment was deleted from pMRT1914, deletion mutant pMRT1914D (▼), in which both the transposon and adjacent *Eco*RI fragments were deleted from pMRT1914, and deletion mutant pMRT2309D (+), in which both the transposon and adjacent *Eco*RI fragments were deleted from pMRT2309.

The exact sites of insertion in the mutants with a transposon in or near *repA4* were determined by nucleotide sequence analysis (Fig. 2) except for mutant pMRT1980, which resulted from insertion of Tn*3* and was located by restriction mapping alone. In the four unstable mutants, the sites of insertion were between *ori* and the right-hand *terC* repeat, whereas in the four stable plasmids, the sites of insertion were downstream from the site of initiation of leading-strand synthesis (Fig. 1 and 2). No mutants with insertions between the right-hand *terC* repeat and the site of leading-strand synthesis were obtained. These results indicated that transposon insertion in the 5′ half of *repA4* resulted in plasmid instability, whereas insertion downstream from *repA4*, including in the *tir* gene, did not.

Analysis of *repA4* mRNA transcription. To determine whether the *repA4* ORF was transcribed, Northern blot analysis was used to probe for RNA transcripts. RNA was prepared from host strain KP245 without plasmids, from KP245 harboring pRR720, or from KP245 harboring pRR942. pRR942 contains the *cat* and *repA* regions, like pRR720, but has a copy number about five to six times higher than that of pRR720, which makes detection of rare transcripts more sensitive (50). RNA was probed with labeled DNA from the left half of *repA4* (including *terC* sequences) or the right half of *repA4* (without *terC*). A control DNA probe consisting of part of the upstream *repA1* gene, which is transcribed at a low level (8, 50), was also included in these experiments. The results indicated that RNA isolated from host cells without a plasmid contained a

transcript of about 1,400 bases that hybridized to the left-hand *repA4* (*terC*) probe but not to the right-hand *repA4* probe (data not shown). This most likely represents a transcript that includes one of the *E. coli terC* repeats. No additional transcripts with homology to left- and right-hand *repA4* probes were observed for RNA prepared from KP245 harboring either pRR720 or pRR942. For the control (*repA1*) probe, transcripts of about 1,400 and 1,100 bases were observed in the RNA from KP245 harboring either pRR720 or pRR942 (data not shown). These transcripts were absent from the KP245 host RNA sample. The *repA1* signal was about five times stronger for the pRR942 RNA samples than for those from pRR720, as expected on the basis of their copy numbers. These results indicate that *repA4* is not transcribed at a level detectable by these methods and therefore may not produce a gene product, at least under the conditions of these experiments.

Isolation and characterization of mutants with deletions in *repA4*. To determine which parts of the *repA4* region were responsible for the unstable phenotypes of the mutants with transposon insertions in *repA4*, deletion mutants were constructed by using the *Eco*RI sites at the ends of the transposons. Two types of deletion were obtained. In the first type, partial digestion was used to delete only the internal 8-kb *Eco*RI fragment of the transposon. In these plasmid derivatives, all but 30 bp of the transposon were deleted. Those remaining 30 bp plus the 5-bp target duplication result in a 35-bp frameshift insertion within the *repA4* coding region. The presence of the 35-bp insertions in these partial deletion derivatives was confirmed by nucleotide sequence analysis. For the second type of deletion, both the 8-kb transposon *Eco*RI fragment and the adjacent downstream *Eco*RI fragment ending at position 3912 (Fig. 1) were deleted. These deletion derivatives are missing all of the NR1 DNA between the site of transposon insertion and the downstream *Eco*RI site at position 3912, including the *tir*, *pemI*, and *pemK* genes.

Partial deletion of the internal *Eco*RI fragment of the transposon restored stability to every mutant examined (designated ΔTn in Fig. 1), as illustrated for pMRT1914P (Fig. 3), in which the insertion occurred at bp 1914 near the beginning of the *repA4* coding region. In mutants pMRT1873P and pMRT1914P, the shifted translational reading frame terminates prematurely at a UGA codon at position 1956; in pMRT2027P, it terminates at a UAA codon at position 2113 (Fig. 2). Since these mutants are unlikely to produce a functional RepA4 protein, it seems unlikely that a *repA4* gene product is required for plasmid stability. The restoration of stability to these partial *Eco*RI deletion derivatives also indicated that these plasmids still contained a functional *stb* locus.

In contrast to the results for the partial deletion derivatives, the stability phenotypes of the derivatives from which the two *Eco*RI fragments were deleted, such as pMRT1914D (Fig. 3), depended on the site of transposon insertion (designated Δ 2-EcoRI in Fig. 1). Deletion derivatives from unstable insertion mutants produced unstable plasmids, whereas those derived from stable insertion mutants produced stable plasmids (Fig. 1). Because the plasmids from which two *Eco*RI fragments had been deleted between positions 2249 and 3912 (Fig. 1) were stable (Fig. 3; Table 1), the absence of the *tir*, *pemI*, and *pemK* genes from these plasmids had little effect on their stability.

Copy numbers of pRR720 and various mutants. If the transposon insertions in *repA4* and the deletions derived from them interfered with plasmid replication, then plasmids with such mutations might have a reduced copy number in the cell. Since NR1 and its miniplasmid derivative pRR720 are low-copy-number plasmids, any reduction in copy number could result in unstable inheritance. The copy numbers of the various unstable mutants were estimated and compared with that of parental plasmid pRR720 (Table 2). The mutants with transposons inserted in the 5′ end of *repA4* (pMRT1873 and pMRT1914) and the partial and double *Eco*RI fragment deletion mutants derived from them all had copy numbers indistinguishable from that of pRR720, even though all of these mutants were unstable. For comparison, the copy number of a stable mutant with a transposon inserted downstream from *repA4*, pMRT2309, was also found to be unaffected by the insertion (Table 2). Therefore, none of the insertion or deletion mutations in *repA4* had measurable effects on plasmid copy number that could account for the observed instability of the plasmids.

TABLE 2. Copy numbers of pRR720 and various mutants

Plasmid	Description	Relative copy number[a] (mean ± SD)
pRR720	Wild type	1.00 ± 0.19
pMRT1873	Transposon in *repA4*	0.99 ± 0.23
pMRT1873P	Delete transposon	1.07 ± 0.04
pMRT1873D	Delete 2 fragments	0.97 ± 0.24
pMRT1914	Transposon in *repA4*	1.01 ± 0.16
pMRT1914P	Delete transposon	0.95 ± 0.27
pMRT1914D	Delete 2 fragments	0.94 ± 0.20
pMRT2309	Transposon near *repA4*	0.97 ± 0.06

[a] Estimated by CAT activity relative to that of pRR720.

Isolation and characterization of mutants with deletions in *repA4* induced by digestion with BAL 31 exonuclease. Although deletion of two *Eco*RI fragments from plasmids with transposons in the upstream region of *repA4* resulted in instability, it was not possible to determine from the available mutants whether deletion of the *terC* repeats, deletion of the site of initiation of leading-strand synthesis, or deletion of some other unidentified function within *repA4* was responsible for the phenotype, since the entire region was deleted (Fig. 1). To examine this question, mutants having a nest of deletions induced by digestion with BAL 31 exonuclease from position 2309 were constructed, starting with stable derivative pMRT2309D. Such mutants are designated by the letter B and the base pair position of the deletion endpoint (Table 1). The extent of the deletion in each mutant was determined by nucleotide sequence analysis (Fig. 2). Plasmids with deletions into the 3′ coding region of *repA4*, such as mutant pMRB2217 (Fig. 1 and 2), remained stable. This finding confirmed that the *repA4* ORF per se was not required for stability. Results for mutants like pMRB2217 also indicated that deletion of the site initiation of leading-strand synthesis had little effect on stability. However, mutants with deletions that removed the right-hand *terC* repeat, such as pMRB1983, were unstable (Fig. 1). The instability of BAL 31 deletion mutant pMRB1983 was comparable to that of pMRT2027D, constructed by deletion of two *Eco*RI fragments as described above (data not shown). Therefore, mutants from which the *terC* repeats were deleted, either with *Eco*RI or with BAL 31, were unstable, and those that retained the *terC* repeats were stable.

Effects of *tus* and *terC* mutations on plasmid stability. To determine whether termination of replication at the *terC* repeats was important for stable maintenance of NR1, the termination function was inactivated either by mutation of

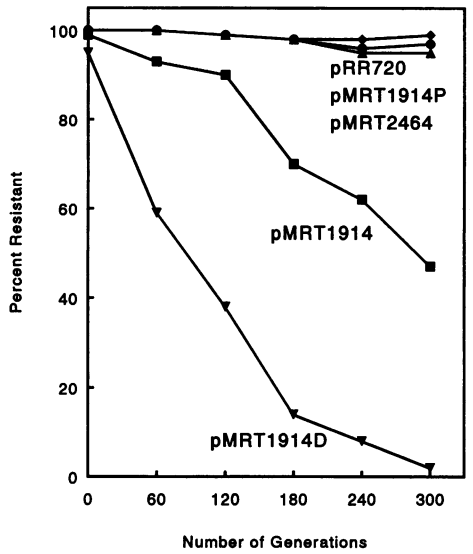

FIG. 4. Effects of the *tus* mutation on the stability of pRR720 and its derivatives. The percentage of PK2619 (*tus*) cells that were resistant to chloramphenicol after various periods of nonselective growth in drug-free medium was determined by replica plating. The test plasmids were wild-type pRR720 (●), insertion mutant pMRT1914 (■), insertion mutant pMRT2464 (◆), deletion mutant pMRT1914P (▲), and deletion mutant pMRT1914D (▼).

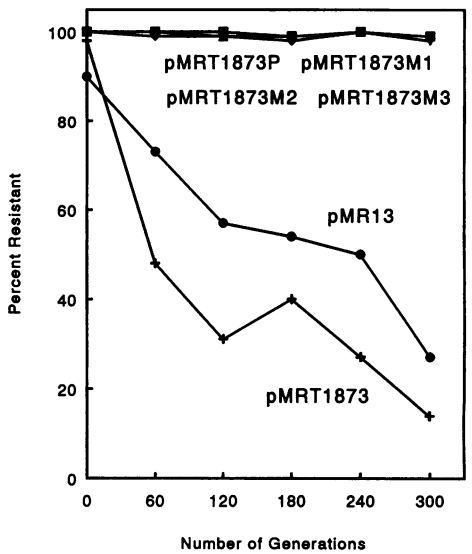

FIG. 5. Effects of *terC* mutations on the stability of pRR720 derivatives. The percentage of KP435 cells that were resistant to chloramphenicol after various periods of nonselective growth in drug-free medium was determined by replica plating. The test plasmids were insertion mutant pMRT1873 (+), deletion mutant pMRT1873P (■), site-directed mutants pMRT1873M1 (▲), pMRT1873M2 (▼), and pMRT1873M3 (◆), and deletion mutant pMR13 (●).

the *tus* gene or by site-directed mutagenesis of the *terC* repeats. The same set of plasmids examined in *tus*$^+$ host PK0457 was introduced into the isogenic *tus* mutant PK2619. In PK2619, the *tus* gene is interrupted by insertion of a kanamycin resistance cassette such that no active Tus protein is produced (15). The stability of the plasmids in the *tus* mutant host (Fig. 4) was indistinguishable from that in the *tus*$^+$ host (Fig. 3). Plasmids pRR720, pMRT1914P, and pMRT2464 were stable in both *tus*$^+$ and *tus* mutant hosts, indicating that Tus protein is not required for stable maintenance of these plasmids. Likewise, plasmids pMRT1914 and pMRT1914D were unstable in both hosts, indicating that Tus protein alone was not responsible the unstable phenotypes of these mutant plasmids.

Plasmids pMRT1873M1, pMRT1873M2, and pMRT1873-M3 were constructed from stable plasmid pMRT1873P, using site-directed mutagenesis to introduce *Bgl*II sites in the left-hand *terC* repeat, in the right-hand *terC* repeat, and in *repA4* downstream from the *terC* repeats, respectively (Materials and Methods; Table 1). Similar mutations in the central core sequences of the *terC*-like repeats of plasmid R6K inactivated the termination function, since the repeats were no longer able to bind Tus protein (40). The stability of the three site-directed mutants was indistinguishable from that of stable plasmid pMRT1873P (Fig. 5). For comparison, the results for unstable control plasmids pMRT1873 (transposon in *repA4*) and pMR13 (frameshift in *stbA*) are also presented (Fig. 5). These results indicate that neither of the *terC* repeats in *rep4A* is essential for stable maintenance of these plasmids. Combined with the results for the *tus* mutant host presented above, these findings suggest that termination of replication at the *terC*-like repeats is not essential for stability of NR1.

DISCUSSION

The process of DNA replication includes initiation, elongation, and termination. Termination is the final stage of replication prior to segregation of the completed replica molecules to the daughter cells at cell division. Therefore, interference with termination might have consequences for separating the nascent replicas, which might impede their segregation at division. The segregation process, called partitioning for plasmids (2, 30), is essential for stable plasmid inheritance, since interference with the process results in the segregation of plasmidless cells (2, 30). Most plasmids that have been examined are found to encode functions required for their partition (2, 30), such as the *stb* locus of NR1 (25, 41). The *stb* function requires that there exist at least two individual plasmid molecules for partition to occur at division (25, 41). It is important to note that the unstable mutants described here contain a functional *stb* locus, so there must be some other element that is also essential for stable inheritance of these plasmids that is altered by insertion or deletion in the *repA4* region. It seems unlikely that a *repA4* gene product is involved in plasmid stability, since frameshift mutations in *repA4* did not cause instability, nor was there any evidence for transcription of a *repA4* mRNA. Therefore, some structural component within the *repA4* DNA sequence might be important.

Termination of bidirectional replication at the termini of plasmid R6K and the *E. coli* chromosome involves the binding of Tus protein to the *terC* inverted repeats (13, 15, 21, 40). When bound by Tus, the *terC* repeats impede the progress of a replication fork (13, 40). The impediment is orientation dependent (17, 21), and the inverted *terC* repeats are situated so as to trap replication forks between them, resulting in termination of replication when the forks converge (14, 17). Deletion of the *tus* gene inactivates termination mediated by the *terC* repeats but has no other observ-

able phenotype for *E. coli* (17). Deletion of the *terC*-like repeats from plasmid R6K also prevents termination at the normal location but again has no other observable effect on the plasmid (7).

The mutants with the transposons inserted between *ori* and the right-hand *terC* repeat, such as pMRT1914, were quite unstable (Fig. 3). These plasmids still contain the *terC* repeats and other sequences in the *repA4* region, but the insertion of the transposon has displaced them by 8 kb. The site of leading-strand initiation is also displaced in these mutants, but apparently new sites can substitute for them in initiation of replication (29) without obvious effects on plasmid copy number (Table 2). However, displacement of the *terC* repeats by 8 kb would cause the replication fork to encounter them in an uncustomary location, which might contribute to the overall instability of the plasmids. Although the insertions had no measurable effect on plasmid copy number, it is possible that small effects that could have contributed to the instability occurred.

Two genes located downstream from *repA4* have been reported to contribute to plasmid stability (5, 44). Referred to as *pem* on NR1 (44) or *parD* on related IncFII plasmid R1 (5), these genes appear to encode a poison-and-antidote system that under certain circumstances can kill plasmid-free segregants (6, 44). However, results presented here and earlier (41) indicate that the *pem* genes contribute little to the stable maintenance of NR1 derivative pRR720. When either the *stb* locus (41) or the *repA4* region is inactivated by mutation, the plasmids are unstable, even though they contain the intact *pem* genes. Plasmids that retain intact *stb* and *repA4* loci, such as pMRT2309D, remain stable even if the *pem* genes are deleted. Therefore, any effects of the *pem* genes on the stability of pRR720 must be of much lower magnitude than those of the *stb* and *repA4* loci.

Unlike the case for plasmid R6K (7), deletion of the region containing the *terC* repeats from mutants such as pMRT1914D and pMRB1983 (Fig. 1) caused the NR1-derived plasmids to be unstable. However, inactivation of the termination function by either a *tus* host mutation or site-directed mutation of the *terC* repeats had little effect on plasmid stability. These findings suggest that as for *pem*, termination of replication at *terC* is of little importance by itself. However, in the case of the unstable deletion mutants, the *terC* repeats were deleted in addition to the site of initiation of leading-strand synthesis, the *pem* genes, and regions containing potential unknown functions. It may be that each of these sites or functions (known or unknown) by itself contributes only minimally to overall plasmid stable maintenance, but in combination, the effects become significant. It should also be noted that although the effects of a given mutation may be minimal under the experimental conditions used in the laboratory, even small contributions to plasmid stable maintenance might be significant to the overall fitness and survival of a plasmid in nature.

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REFERENCES

1. **Armstrong, K. A., H. Ohtsubo, W. R. Bauer, Y. Yoshioka, C. Miyazaki, Y. Maeda, and E. Ohtsubo.** 1986. Characterization of the gene products produced in minicells by pSM1, a derivative of R100. Mol. Gen. Genet. **205**:56–65.
2. **Austin, S. J.** 1988. Plasmid partition. Plasmid **20**:1–9.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, New York.
4. **Bernander, R., M. Krabbe, and K. Nordstrom.** 1992. Mapping of the *in vivo* start site for leading strand DNA synthesis in plasmid R1. EMBO J. **11**:4481–4487.
5. **Bravo, A., G. de Torrontegui, and R. Diaz.** 1987. Identification of components of a new stability system of plasmid R1, ParD, that is close to the origin of replication of the plasmid. Mol. Gen. Genet. **210**:101–110.
6. **Bravo, A., S. Ortega, G. de Torrontegui, and R. Diaz.** 1988. Killing of *Escherichia coli* cells modulated by components of the stability system ParD of plasmid R1. Mol. Gen. Genet. **215**:146–151.
7. **Crosa, J. H., L. K. Luttropp, and S. Falkow.** 1978. Molecular cloning of replication and incompatibility regions from the R-plasmid R6K. J. Mol. Biol. **124**:443–468.
8. **Dong, X., D. D. Womble, V. A. Luckow, and R. H. Rownd.** 1985. Regulation of transcription of the *repA1* gene in the replication control region of IncFII plasmid NR1 by gene dosage of the *repA2* transcription repressor protein. J. Bacteriol. **161**:544–551.
9. **Dong, X., D. D. Womble, and R. H. Rownd.** 1987. Transcriptional pausing in a region important for plasmid NR1 replication control. J. Bacteriol. **169**:5353–5363.
10. **Dong, X., D. D. Womble, and R. H. Rownd.** 1988. *In vivo* studies of the *cis*-acting replication initiator protein of IncFII plasmid NR1. J. Mol. Biol. **202**:495–509.
11. **Easton, A. M., and R. H. Rownd.** 1982. The incompatibility product of IncFII plasmid NR1 controls gene expression in the plasmid replication region. J. Bacteriol. **152**:829–839.
12. **Hidaka, M., M. Akiyama, and T. Horiuchi.** 1988. A consensus sequence of three DNA replication termination sites on the *E. coli* chromosome is highly homologous to the *terR* sites of the R6K plasmid. Cell **55**:467–475.
13. **Hill, T. M., and K. J. Marians.** 1990. *Escherichia coli* Tus protein acts to arrest the progression of DNA replication forks *in vitro*. Proc. Natl. Acad. Sci. USA **87**:2481–2485.
14. **Hill, T. M., A. J. Pelletier, M. L. Tecklenburg, and P. L. Kuempel.** 1988. Identification of the DNA sequence from the *E. coli* terminus region that halts replication forks. Cell **55**:459–466.
15. **Hill, T. M., M. L. Tecklenburg, A. J. Pelletier, and P. L. Kuempel.** 1989. *tus*, the *trans*-acting gene required for termination of DNA replication in *Escherichia coli*, encodes a DNA-binding protein. Proc. Natl. Acad. Sci. USA **86**:1593–1597.
16. **Horiuchi, T., and M. Hidaka.** 1988. Core sequence of two separable terminus sites of the R6K plasmid that exhibit polar inhibition of replication is a 20 bp inverted repeat. Cell **54**:515–523.
17. **Kuempel, P. L., A. J. Pelletier, and T. M. Hill.** 1990. *tus* and the terminators: inhibition of replication in *Escherichia coli*, p. 297–306. *In* K. Drlica and M. Riley (ed.), The bacterial chromosome. American Society for Microbiology, Washington, D.C.
18. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-directed mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.
19. **Light, J., and S. Molin.** 1983. Post-transcriptional control of expression of the *repA* gene of plasmid R1 mediated by a small RNA molecule. EMBO J. **2**:93–98.
20. **Light, J., E. Riise, and S. Molin.** 1985. Transcription and its regulation in the basic replicon of plasmid R1. Mol. Gen. Genet. **198**:503–508.
21. **MacAllister, T., G. S. Khatri, and D. Bastia.** 1990. Sequence-specific and polarized replication termination *in vitro*: complementation of extracts of *tus*$^-$ *Escherichia coli* by purified Ter protein and analysis of termination intermediates. Proc. Natl. Acad. Sci. USA **87**:2828–2832.
22. **Masai, H., and K. Arai.** 1987. RepA and DnaA proteins are required for initiation of R1 plasmid replication *in vitro* and interact with the *oriR* sequence. Proc. Natl. Acad. Sci. USA **84**:4781–4785.
23. **Masai, H., and K. Arai.** 1989. Leading strand synthesis of R1

plasmid replication *in vitro* is primed by primase alone at a specific site downstream of *oriR*. J. Biol. Chem. **264**:8082–8090.
24. **Masai, H., Y. Kaziro, and K. Arai.** 1983. Definition of *oriR*, the minimum DNA segment essential for initiation of R1 plasmid replication *in vitro*. Proc. Natl. Acad. Sci. USA **80**:6814–6818.
25. **Miki, T., A. M. Easton, and R. H. Rownd.** 1980. Cloning of replication, incompatibility and stability functions of R plasmid NR1. J. Bacteriol. **141**:87–99.
26. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **Min, Y., A. Tabuchi, D. D. Womble, and R. H. Rownd.** 1991. Transcription of the stability operon of IncFII plasmid NR1. J. Bacteriol. **173**:2378–2384.
28. **Min, Y. N., A. Tabuchi, Y. L. Fan, D. D. Womble, and R. H. Rownd.** 1988. Complementation of mutants of the stability locus of IncFII plasmid NR1. Essential functions of the *trans*-acting *stbA* and *stbB* gene products. J. Mol. Biol. **204**:345–356.
29. **Miyazaki, C., Y. Kawai, H. Ohtsubo, and E. Ohtsubo.** 1988. Unidirectional replication of plasmid R100. J. Mol. Biol. **204**: 331–343.
30. **Nordstrom, K., and S. J. Austin.** 1989. Mechanisms that contribute to the stable segregation of plasmids. Annu. Rev. Genet. **23**:37–69.
31. **Nordstrom, K., S. Molin, and J. Light.** 1984. Control of replication of bacterial plasmids: genetics, molecular biology, and physiology of the plasmid R1 system. Plasmid **12**:71–90.
32. **Ohtsubo, H., T. B. Ryder, Y. Maeda, K. Armstrong, and E. Ohtsubo.** 1986. DNA replication of the resistance plasmid R100 and its control. Adv. Biophys. **21**:115–133.
33. **Proctor, G. N., and R. H. Rownd.** 1982. Rosanilins: indicator dyes for chloramphenicol-resistant enterobacteria containing chloramphenicol acetyltransferase. J. Bacteriol. **150**:1375–1382.
34. **Rosen, J., T. Ryder, H. Inokuchi, H. Ohtsubo, and E. Ohtsubo.** 1980. Genes and sites involved in replication and incompatibility of an R100 plasmid derivative based on nucleotide sequence analysis. Mol. Gen. Genet. **179**:527–537.
35. **Rosen, J., T. Ryder, H. Ohtsubo, and E. Ohtsubo.** 1981. Roles of RNA transcripts in replication incompatibility and copy number control in antibiotic resistance plasmid derivatives. Nature (London) **290**:794–797.
36. **Rownd, R. H., D. D. Womble, X. Dong, V. A. Luckow, and R. P. Wu.** 1985. Incompatibility and IncFII plasmid replication control, p. 335–354. *In* D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (ed.), Plasmids in bacteria. Plenum Press, New York.
37. **Ryder, T., J. Rosen, K. Armstrong, D. Davison, E. Ohtsubo, and H. Ohtsubo.** 1981. Dissection of the replication region controlling incompatibility, copy number, and initiation of DNA synthesis in the resistance plasmids, R100 and R1, p. 91–111. *In* D. S. Ray (ed.), The initiation of DNA replication. Academic Press, Inc., New York.
38. **Ryder, T. B., D. B. Davison, J. I. Rosen, E. Ohtsubo, and H. Ohtsubo.** 1982. Analysis of plasmid genome evolution based of nucleotide-sequence comparison of two related plasmids of Escherichia coli. Gene **17**:299–310.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Sista, P. R., S. Mukherjee, P. Patel, G. S. Khatri, and D. Bastia.** 1989. A host-encoded DNA-binding protein promotes termination of plasmid replication at a sequence-specific replication terminus. Proc. Natl. Acad. Sci. USA **86**:3026–3030.
41. **Tabuchi, A., Y. N. Min, C. K. Kim, Y. L. Fan, D. D. Womble, and R. H. Rownd.** 1988. Genetic organization and nucleotide sequence of the stability locus of IncFII plasmid NR1. J. Mol. Biol. **202**:511–525.
42. **Tabuchi, A., D. D. Womble, and R. H. Rownd.** 1992. Autoregulation of the stability operon of IncFII plasmid NR1. J. Bacteriol. **174**:7629–7634.
43. **Tanimoto, K., T. Iino, H. Ohtsubo, and E. Ohtsubo.** 1985. Identification of a gene, *tir* of R100, functionally homologous to the F3 gene of F in the inhibition of RP4 transfer. Mol. Gen. Genet. **198**:356–357.
44. **Tsuchimoto, S., H. Ohtsubo, and E. Ohtsubo.** 1988. Two genes, *pemK* and *pemI*, responsible for stable maintenance of resistance plasmid R100. J. Bacteriol. **170**:1461–1466.
45. **Ubben, D., and R. Schmitt.** 1986. Tn*1721* derivatives for transposon mutagenesis, restriction mapping and nucleotide sequence analysis. Gene **41**:145–152.
46. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
47. **Womble, D. D., X. Dong, R. P. Wu, V. A. Luckow, A. F. Martinez, and R. H. Rownd.** 1984. IncFII plasmid incompatibility product and its target are both RNA transcripts. J. Bacteriol. **160**:28–35.
48. **Womble, D. D., and R. H. Rownd.** 1986. Regulation of IncFII plasmid DNA replication. A quantitative model for control of plasmid NR1 replication in the bacterial cell division cycle. J. Mol. Biol. **192**:529–548.
49. **Womble, D. D., and R. H. Rownd.** 1988. Genetic and physical map of plasmid NR1: comparison with other IncFII antibiotic resistance plasmids. Microbiol. Rev. **52**:433–451.
50. **Womble, D. D., P. Sampathkumar, A. M. Easton, V. A. Luckow, and R. H. Rownd.** 1985. Transcription of the replication control region of the IncFII R-plasmid NR1 *in vitro* and *in vivo*. J. Mol. Biol. **181**:395–410.
51. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.