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ColE1 Copy Number Mutants

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A deletion mutant of the colicin E1-derived plasmid, pDMS6642, exhibited an approximately fourfold increase in copy number. We subsequently isolated hydroxylamine-induced mutants of that plasmid that had a further increase in copy number. Analysis of them suggests that the increased copy number of pDMS6642 is associated with transcriptional readthrough from a Tn*3* transposon into the region of ColE1 containing information that influences plasmid replication. The hydroxylamine mutation in one copy number mutant appeared to increase the plasmid copy number by stimulating readthrough transcription from the Tn*3* transposon into the ColE1 replication control region, whereas the other hydroxylamine mutation acts by another mechanism.

Plasmid ColE1 is stably maintained in *Escherichia coli* strains as a monomeric molecule with a copy number of about 15 per chromosome. Oka et al. (21) have shown that a 420-base-pair (bp) sequence that includes the ColE1 replication origin (the *rep* region) provides all of the information essential for autonomous plasmid replication. It was subsequently shown that a nucleotide sequence between 400 and 600 bp upstream from the replication origin codes for an RNA that influences plasmid copy number (4–6, 18, 25) and incompatibility (RNAI) and also contains a promoter of an RNA primer of ColE1 replication (14).

We previously constructed a deletion mutant called pDMS6642 from a ColE1-derived plasmid. It contains about 860 bp of ColE1 DNA fused to the portion of the Tn*3* transposon that contains the β-lactamase gene and a part of the *tnpR* gene (8, 12; Fig. 1). pDMS6642 has a copy number of about 57 per cell genome. Two hydroxylamine-induced copy number mutant derivatives of pDMS6642 were isolated based on their ability to confer high ampicillin resistance on their hosts. One of those mutants also exhibited a higher production of β-lactamase per plasmid. Further mapping of the mutations and the introduction of insertions that blocked readthrough transcription from the Tn*3* transposon into the ColE1 replication control region led to the conclusion that readthrough transcription from the Tn*3* transposon is the principal cause of the high copy number of both pDMS6642 and the mutant that exhibited the increased production of β-lactamase per plasmid.

MATERIALS AND METHODS

Bacteria, plasmids, and plasmid derivations. The strains of *E. coli* which were used were P678-54 (*thr leu thi rpsT* Sm^r) (5, 6) and Om84 [*tyr*(Am) *trp*(Am) *thy his ilv supD*] (5, 6). Each strain contained appropriate plasmids which were introduced by standard DNA transformation methods (see below). The plasmids used are shown in Table 1, and their construction is shown in Fig. 1. pDMS630 is ColE1 containing a Tn*3* transposon which confers ampicillin resistance (5). pDMS66 (13) is a mutant of pDMS630 in which the end of the Tn*3* transposon associated with the β-lactamase gene is fused to the ColE1 DNA 684 bp upstream from the ColE1 replication origin (20) and 129 bp upstream from the promoter that initiates transcription of the RNA primer of ColE1 replication (10). pDMS6642 was formed by constructing a plasmid that contains only two *Hae*II-generated DNA fragments observed in pDMS66 (Fig. 1). One contains a part of the ColE1 DNA that extends from 185 to 684 bp upstream from the replication origin and is fused to a (approximately 1,200 bp) portion of the Tn*3* transposon that includes the β-lactamase gene and a portion of the *tnpR* gene (8, 12, 13, 20). The other fragment is the 360-bp *Hae*II fragment E that contains the ColE1 replication origin (12, 13, 20). These two fragments were ligated to form pDMS6642. They can only be joined in one way to generate a functional plasmid (11). The location of the *Bam*HI- and *Pst*I-sensitive sites in the Tn*3* portion of the plasmid has previously been identified (8, 10, 13, 24). pDMS1005 (Fig. 1) is a chimera formed by ligating the *Bam*HI-cleaved pDMS6642 and pHSG1, which is a temperature-sensitive DNA replication mutant of pSC101 (5, 7) that was previously used in constructing chimeras with ColE1 plasmid derivatives (5). Such chimeras exhibit temperature-resistant replication so long as the ColE1 component can replicate normally (3, 5, 6). Such chimeras have been shown to exhibit the copy number of the ColE1 plasmid component. No complementation occurs between pHSG1 and ColE1 plasmid derivatives.

pDMS1005 DNA was treated with hydroxylamine (7), and P678-54 transformants exhibiting high ampicillin resistance (4 mg/ml) were selected. Plasmids from two such transformants, pLS53 and pLS102, were studied. The pDMS6642 component of pLS53 and

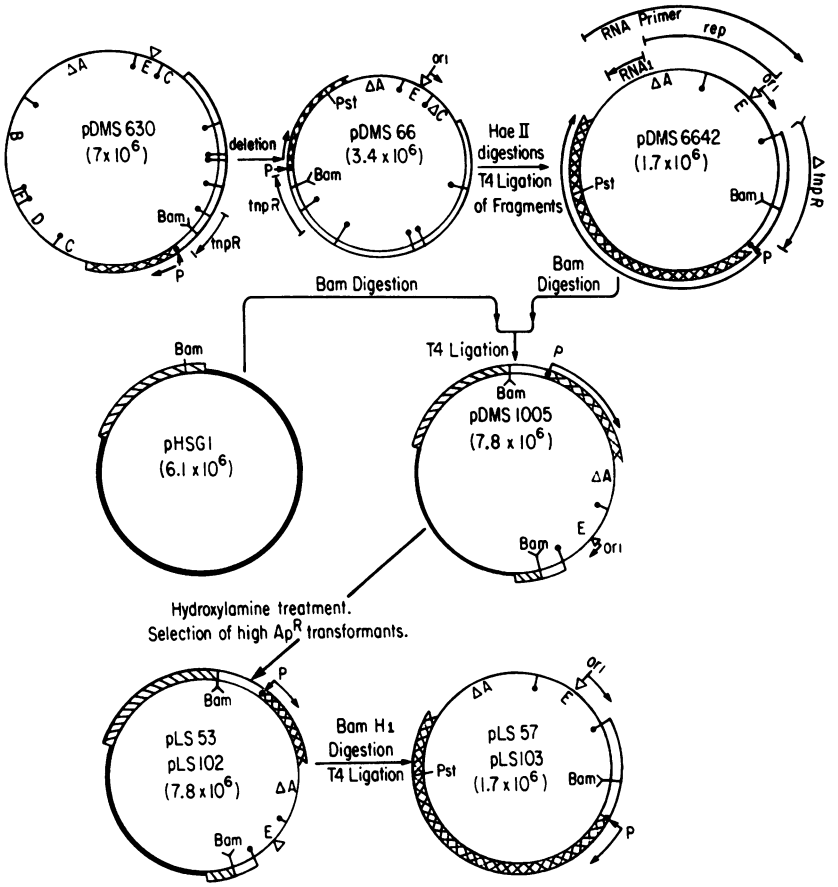

FIG. 1. A diagrammatic representation of the plasmids involved in the formation of pDMS6642, pDMS1005, and the hydroxylamine-induced mutants derived from them. pDMS630 is ColE1 containing a Tn*3* insertion in *Hae*II fragment C of ColE1. Plasmid sizes are given in daltons. Endonuclease-sensitive sites shown are *Bam*HI (Y), *Hae*II (●), and *Pst*I (|). A through F are *Hae*II fragments of ColE1 DNA. The *Hae*II fragments of Tn*3* are not identified. *rep* is the smallest identifiable region containing sufficient information to permit autonomous ColE1 DNA replication. RNAI is the region coding for an RNA transcript that affects ColE1 copy number. RNA primer (→) is the site of initiation and direction of transcription of an RNA primer of ColE1 DNA replication. ori (▽) is the site at which the first deoxyribonucleotide of DNA replication is attached to the RNA primer. The direction of replication is noted (→). ●P→ locates the promoter and direction of transcription of the β-lactamase gene (▩) contained in one end of the Tn*3* transposon (▭). Part of the *tnpR* gene (Δ*tnpR*) is located in the Tn*3* DNA in pDMS6642. ▨ is the tetracycline resistance region in pHSGI, a temperature-sensitive replication mutant of pSC101 containing the gene that confers tetracycline resistance on host cells. pLS53 and pLS102 are hydroxylamine mutants derived from pDMS1005 that confer a high ampicillin resistance phenotype on their hosts. pLS57 and pLS103 are pDMS6642 mutants isolated from pLS53 and pLS102, respectively. Diagrams are not drawn to scale.

pLS102 was recovered by cleaving the DNAs with *Bam*HI, ligating the DNAs, and selecting ampicillin-resistant transformants that contained plasmids that were physically indistinguishable in size and restriction endonuclease digestion patterns from pDMS6642 (Fig. 1). As these plasmids exhibited higher copy numbers and conferred higher levels of ampicillin resistance on their hosts than did pDMS6642, they were designated pLS57 and pLS103.

Plasmid pGA46 was a generous gift of G. An (2; Fig. 3). It contains a promoterless tetracycline resistance gene that can be expressed if linked to a promoter by a *Pst*I-sensitive site (see Fig. 3). It will only confer a tetracycline resistance phenotype on its host if a promoter is inserted in the appropriate orientation at the *Pst*I-sensitive site. The expression of tetracycline resistance after such a ligation leads to the expression of a tetracycline resistance phenotype that is proportional to the promoter activity (2). The plasmid pST29 was a generous gift of J. Tomizawa and T. Som. It is a derivative of RSF1030 and contains a 1,870-bp *Pst*I fragment carrying a chloramphenicol resistance gene that derives from phage P1Cm (1).

Media. LS broth and LS agar are standard nutrient

TABLE 1. Plasmids used

Plasmid	Relevant phenotype[a]	Endonuclease-sensitive sites (no.)[b]	Plasmid size (10^6 daltons)	Origin of plasmid
pDMS630	Ap	*Bam*HI (1) *Eco*RI (1) *Hae*II (10) *Pst*I (4)	7.0	(8, 13, 24)
pDMS66	Ap	*Bam*HI (1) *Hae*II (7) *Pst*I (1)	3.4	(13)
pDMS6642	Ap	*Bam*HI (1) *Hae*II (2) *Pst*I (1)	1.7	(12; Fig. 1)
pDMS1005	Ap	*Bam*HI (2)	7.8	Fig. 1
pHSG1	Tc, Ts rep.	*Bam*HI (1) *Eco*RI (1)	6.1	(5–7)
pST29	Cm	*Pst*I (2)	—	Tomizawa[c] (Fig. 3)
pGA46	Potential Tc	*Pst*I (1)	2.9	(2; Fig. 3)
pLS53	Ap	Same as pDMS1005	7.8	Fig. 1
pLS102	Ap	Same as pDMS1005	7.8	Fig. 1
pLS57	Ap	Same as pDMS6642	1.7	Fig. 1
pLS103	Ap	Same as pDMS6642	1.7	Fig. 1
pLS75	Cm	*Pst*I (2)	2.9	Fig. 3
pLS77	Cm	*Pst*I (2)	2.9	Fig. 3
pLS109	Cm	*Pst*I (2)	2.9	Fig. 3
pLS83	Tc	*Pst*I (2)	4.6	Fig. 3
pLS84	Tc	*Pst*I (2)	4.6	Fig. 3

[a] Drug resistance phenotype conferred by plasmids are ampicillin (Ap), chloramphenicol (Cm), tetracycline (Tc). pHSG1 exhibits temperature-sensitive replication (Ts rep.).

[b] Endonuclease-sensitive sites used in ligation. The sites shown in pDMS630, pDMS66, and pDMS6642 represent all sites of those types in those plasmids.

[c] pST29 was obtained from the laboratory of J. Tomizawa. A *Pst*I fragment (1.2×10^6 daltons) (1) was isolated from the plasmid for insertion into other plasmids.

media which have previously been described (5). M9 minimal medium has previously been described (5, 6). It was supplemented with 5 μg of thymine per ml, appropriate amino acid supplements (40 μg/ml), or 0.4% Casamino Acids (Difco Laboratories). It was used for radioactive labeling of plasmid DNA. The concentration of antibiotics in broth or agar was usually 200 μg of ampicillin per ml and 5 μg of tetracycline per ml. Much higher concentrations of drugs were used in testing the maximum allowance concentration (MAC) of drugs. Tetracycline was prepared as needed as a 2-mg/ml solution.

Isolation and purification of plasmid DNA. Plasmid DNA was released from cells by the cleared lysis procedure exactly as described previously (5). The covalently closed circular plasmid DNA was banded in cesium chloride-ethidium bromide density gradients and isolated exactly as previously described (5). The plasmid DNA was dialyzed against an appropriate buffer. Subsequently, the plasmid DNA was either directly examined by agarose gel electrophoresis or first digested with particular endonucleases followed by gel electrophoresis analysis of the fragments generated. The endonuclease digestions were always used to determine whether plasmids formed by ligating specific DNA fragments together had the appropriate structure. Those data are not included in the presentation, although they are referred to in the text.

Transformation of bacteria with DNA. The method used for transformation was exactly as previously described (5, 16). The phenotype of the transformants selected was drug resistance or colicin immunity (5, 6).

Measurement of the MAC. Overnight cultures were diluted so that they contained between 2×10^3 and 1×10^4 bacteria per ml. A 0.1-ml amount of culture was plated on LS agar and on LS agar with various amounts of active ampicillin (200 μg to 22 mg/ml). Ampicillin trihydrate was a gift from Bristol Laboratories. It contains 849 μg of ampicillin per mg. The lowest drug concentration that inhibited the growth of greater than 99% of bacteria was determined. It was almost always noted that a dramatic decline in viable counts of plasmid-containing cells was observed over a 0.5- to 1.0-mg/ml difference in active ampicillin concentration. That drug concentration above which an abrupt decline in viable counts occurred was called the MAC.

The measurement of tetracycline resistance was conducted essentially as described above. Tetracycline and tetracycline-containing agar were always freshly prepared, and the agar was kept in the dark. The inhibition of bacterial growth with increasing amounts of tetracycline was unequivocal and reproducible when measured in increments of 5 μg of tetracycline per ml.

Measurement of β-lactamase activity. β-Lactamase assays were done by a slight modification of the iodometric method described by Sherratt and Collins (23). Bacterial cultures were centrifuged at 4,000 rpm for 10 min. One milliliter of the supernatant was added to duplicate tubes containing 2.5 ml of substrate solution (2.4 mg of ampicillin in 0.1 M phosphate buffer [pH 7.0]) at 30°C and shaken gently in a 30°C water bath for 15 min. A 5-ml amount of 0.016N I_2–0.06 M KI in 2 M sodium acetate buffer (pH 4.2) was then added to each substrate tube. A 1.0-ml amount of the supernatant being tested was added to a control tube. The tubes containing the iodine solution were incubated a further 10 min to allow the iodine to react with the ampicilloic acid generated by the reaction of the β-lactamase with ampicillin. Both the sample and control were centrifuged at 12,000 rpm for 10 min. The absorbance of the supernatant was read at 499 nm in a glass cuvette with a 1-cm light path. The difference in absorbance between the sample and control tubes was corrected to β-lactamase units per milliliter (22, 23) by

multiplying the total volume of liquid (in milliliters) in the total mixture and correcting to 1 ml of sample and 1 h of incubation.

Viable counts on agar plates containing 200 μg of ampicillin per ml were made of all cultures tested, and the β-lactamase units per milliliter were converted to β-lactamase units per 10^8 Ap^r cells in the culture. When a plasmid copy number was determined, the β-lactamase units per 10^8 plasmids was determined by dividing the β-lactamase units per 10^8 cells by the plasmid copy number.

Enzymatic digestion, ligation, and agarose gel electrophoresis of DNA. Enzymatic digestion, ligation, and agarose gel electrophoresis were performed as previously described (6).

Determination of plasmid copy number. Plasmid copy number was calculated from the [^{3}H]thymidine-labeled DNA in the covalently closed circular peak in a cesium chloride-propidium diiodide density gradient exactly as previously described (6). A minimum of three independent determinations was made for all plasmids. The plasmid copy number determined in this way rarely varied by more than ±10%. In all instances, 100% of cells in cultures contained plasmids during the labeling period. It should be noted that plasmid pDMS6642 does not exhibit "relaxation" because all regions of ColE1 that are essential for relaxation are deleted (10). The cesium chloride-propidium diiodide method is therefore quite accurate for determining copy numbers since relaxed circles are not usually produced. This was confirmed by mixing ^{3}H-labeled covalently closed circular DNA with lysates and demonstrating an absence of nicking. Also, to insure that mutants didn't produce significant amounts of unusual relaxed plasmid DNA, we analyzed total lysates by agarose gel electrophoresis. No unusual plasmid bands were identified, thus ruling out that possible source of underestimating plasmid copy number.

Measurements of plasmid stability. A plasmid-containing colony was picked from an appropriate selective agar and suspended in LS broth, and the cultures were incubated at either 30 or 42°C. These cultures were diluted about 1/1,000 every 12 h and grown for up to 40 generations. The bacterial viable counts of each culture were determined by plating the cultures on both LS agar and LS agar containing ampicillin and incubating them at 30°C. If a constant difference in plating efficiency was noted on agar with and without ampicillin, 100 colonies were picked from the LS agar and tested by a streak test, as previously described (5, 6), to determine the numbers of ampicillin-sensitive colonies present.

Determination of DNA nucleotide sequence. The method of Maxam and Gilbert (17) was used for determining DNA nucleotide sequence.

RESULTS

Isolation and characterization of hydroxylamine-induced plasmid mutants showing increased copy number. The chimeric plasmid pDMS1005 (Table 1, Fig. 1; see above) was originally isolated to create a vehicle for identifying mutations which affect the replication of ColE1. The chimera was produced by ligating plasmid pHSG1 (5), which exhibits temperature-sensitive replication, to a ColE1 deletion mutant pDMS6642 (see above) via their *Bam*HI-sensitive sites (Fig. 1). pDMS1005 replication is temperature resistant, indicating that it replicates via the ColE1 replication process (see above; 3, 5, 6). Insertion of DNA into the *Bam*HI-sensitive site of pHSG1 inhibits the expression of the plasmid-conferred tetracycline resistance phenotype (5), whereas insertion of DNA into the *Bam*HI-sensitive site in the Tn*3* transposon nucleotide sequence of pDMS6642 did not inhibit β-lactamase expression. pDMS1005, therefore, confers an ampicillin resistance and tetracycline sensitivity phenotype on its host. It has previously been shown that when the constitutively expressed β-lactamase gene is carried on a plasmid, the level of ampicillin resistance manifested by a host cell is proportional to the plasmid copy number (9, 19). This was reconfirmed for derivatives of pDMS630, a ColE1 derivative used in these experiments (Table 1, Fig. 1) that were ligated to pHSG1 (5, 6). Since cells carrying pDMS1005 were maximally resistant to 1.5 mg of ampicillin per ml, it was assumed that mutants which confer a significantly higher level of ampicillin resistance on their hosts would probably exhibit an increased plasmid copy number. Other causes of increased β-lactamase activity in a cell must, however, be considered.

pDMS1005 was mutagenized in vitro with hydroxylamine, as described previously (7, 12), and that DNA was used to transform *E. coli* P678-54. Transformants were selected which were resistant to at least 4 mg of ampicillin per ml. The MAC of cells, the MAC per plasmid, and the β-lactamase production per plasmid of two independently isolated highly ampicillin-resistant mutants, pLS53 and pLS102, are shown in Table 2. Although pLS53 exhibited a small but consistent increase in copy number compared with pDMS1005, pLS102 exhibited an almost fourfold increase in copy number (Table 2). In the case of pLS102, the increased plasmid copy number and both increased MACs and β-lactamase production per cell were consistent with the interpretation that the increased copy number is responsible for the increased drug resistance. In the case of pLS53, the increased copy number cannot be the sole cause of increased drug resistance.

The localization of the mutation to the pDMS6642 component in each mutant chimera was undertaken. The pDMS6642 components of pLS53 and pLS102 were isolated from the respective chimeras, as described in the legend to Fig. 1 and above. These plasmids, which were identical to pDMS6642 in size and endonuclease cleavage pattern (data not shown), conferred high levels of ampicillin resistance on their host.

TABLE 2. Measurements of copy number, ampicillin resistance, and β-lactamase activity

Plasmid	Stability[a]		Copy no.	MAC of ampicillin (mg/ml)	MAC/ plasmid[b]	β-Lactamase/ 10^8 plasmids[c]
	30°C	42°C				
pDMS1005	S	S	10 ± 1.4	1.5	0.15 ± 0.02	0.19 ± 0.04
pLS53	S	U	15 ± 1.4	12.0	0.80 ± 0.08	0.74 ± 0.08
pLS102	S	U	38 ± 4.0	6.0	0.16 ± 0.02	0.24 ± 0.03
pDMS6642	S	S	57 ± 2.1	2.0	0.04 ± 0.002	0.05 ± 0.01
pLS57	U	U	84 ± 3.6	15.0	0.19 ± 0.01	0.12 ± 0.02
pLS103	U	U	140 ± 7.6	6.0	0.04 ± 0.002	0.06 ± 0.02
pDMS630	S	S	15 ± 1.8	1.5	0.10 ± 0.01	0.21 ± 0.02

[a] S, Stable; U, unstable.
[b] MAC per plasmid is the MAC divided by the plasmid copy number.
[c] β-Lactamase per 10^8 plasmids is the β-lactamase level per 10^8 cells in the culture divided by the plasmid copy number. Each β-lactamase assay was performed a minimum of four times.

The pDMS6642-type plasmids isolated from pLS53 and pLS102 were named pLS57 and pLS103, respectively (Fig. 1 and Table 2).

The copy number (Table 2) of pDMS6642 was significantly greater than that of pDMS630, and both pLS57 and pLS103 had copy numbers significantly greater than that of pDMS6642.

The MAC per plasmid and β-lactamase per plasmid were very similar for pDMS6642 and pLS103 as they were for pDMS1005 and pLS102, from which they were derived. The MAC per plasmid and β-lactamase production per plasmid were, however, significantly higher for pLS57 than for pDMS6642. These findings are consistent with the interpretation that the mutation in pLS57 may directly cause an increased β-lactamase production, which secondarily causes the observed increase in plasmid copy number. They are also consistent with the interpretation that the mutation in pLS103 affects the control of the plasmid copy number without altering the β-lactamase production of the pDMS6642 plasmid. Since mutations in pLS57 and pLS103 induce changes in copy number and β-lactamase activity similar to those exhibited by pLS53 and pLS102, it is reasonable to assume that the mutation affecting each chimera resides in its pDMS6642 component.

While making these measurements, we determined the stability of all of the plasmids at 30 and 42°C (see above), since if this is not done, an undetected large plasmid loss during growth could affect the interpretation of measurements. The results in Fig. 2 show that both pLS53 and pLS102 were stable, whereas pLS57 and pLS103 exhibited a small but detectable instability at 30°C. When cells were grown without ampicillin for 40 generations, a maximum of 5% exhibited a loss of plasmids. All of the mutants were unstable at 42°C (Fig. 2). The plasmid instability is, therefore, not responsible for the increased MAC per plasmid of β-lactamase per plasmid ratio exhibited by pLS53 and pLS57.

Localization of the mutations affecting pLS57 and pLS103. If an appropriate *Bam*HI-*Pst*I-generated fragment (Table 3) is derived from either pLS57 or pLS103 and ligated to an appropriate DNA fragment derived from pDMS6642, the hybrid plasmid which is formed should express the mutant plasmid phenotype. pDMS6642, pLS57, and pLS103 DNAs were each cleaved with both *Bam*HI and *Pst*I. The fragments (A and B; Table 3) from each digest were carefully purified twice by gel electrophoresis. It was essential to reduce cross-contamination of fragments and to eliminate undigested DNA which could obscure the results. Each fragment from a mutant was then ligated to the other fragment from pDMS6642, and the newly formed hybrid

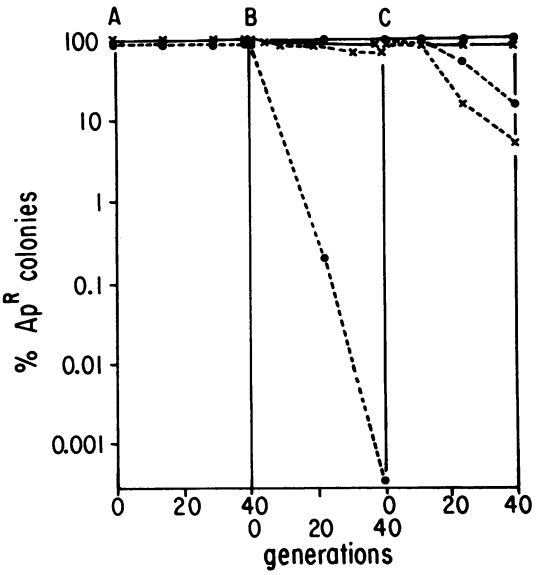

FIG. 2. Plasmid stability in cells grown in LS broth at 30°C (——) or at 42°C (– – – –). (A) pDMS1005 (●), pDMS6642 (X); (B) pLS53 (●), pLS57 (X); (C) pLS102 (●), pLS103 (X).

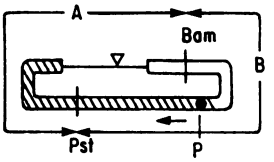

TABLE 3. Results of transformation by plasmids reconstituted from fragments derived from mutants and pDMS6642

Source of fragments[a]		Transformants[b]		MAC of transformants (mg of ampicillin per ml)[c]
Mutant	pDMS6642	200 μg/ml	4 mg/ml	
pLS57A	B	405	2	2
pLS57B	A	299	155	15
pLS57A	—	2	0	NT
pLS57B	—	10	2	NT
—	A	0	0	—
—	B	0	0	—
pLS103A	B	138	158	5
pLS103B	A	196	0	2
pLS103A	—	1	0	NT
pLS103B	—	0	0	—
—	A	0	0	—
—	B	0	0	—

[a] *Bam*HI-*Pst*I digests of the pDMS6642, pLS57, and pLS103 plasmids produce an A and B fragment. These were independently purified, mixed, and ligated. ▭ and ▥ are the portions of the Tn*3* transposon not containing and containing, respectively, the β-lactamase gene. ——— is the ColE1 DNA. ▽ is the replication origin. ●P → is the site of the promoter of the β-lactamase gene and its direction of transcription. —, None.

[b] The number of transformants in 0.01 ml of transformation mixture plated on L agar containing either 200 μg or 4 mg of ampicillin per ml. The amount of A and B DNA used in controls for ligation and transformation was identical to that used in the experimental samples.

[c] The MAC of transformants of each type was taken from the 4-mg/ml plate when possible. NT, Not tested.

plasmids were introduced into P678-54 by transformation. The ability of the transformants to grow on 4 mg of ampicillin per ml was initially taken to indicate that the plasmids had acquired the mutant phenotype. The plasmid copy number of the transformants was also measured, as was the MAC of the cells after introduction of the plasmids into Om84. The results (Table 3) show that when pLS57 fragment B was ligated to pDMS6642 fragment A, the new plasmid exhibited the information which led to increased plasmid-mediated β-lactamase production which was identical to that exhibited by pLS57. When the pLS103A fragment was ligated to pDMS6642 fragment B, a new plasmid was also formed that led to increased plasmid-mediated β-lactamase production identical to that exhibited by pLS103. The newly formed plasmids had copy numbers which were the same as pLS57 and pLS103 (data not shown).

The fragment 103A contains the nucleotide sequence for the ColE1 RNA1 product in which mutations have been shown to cause an increased-copy-number phenotype (4, 18). The negative results of the reverse ligation and the negligible background transformation of individual fragments eliminated the possibility that the transformants were produced by reconstituted or contaminating original mutant DNA (Table 3).

Fragment B from pLS57 (illustration, Table 3) contained only Tn*3* DNA. The *Bam*HI- and *Pst*I-sensitive sites, which are not in the ColE1 DNA of pDMS6642, have been sequenced in the Tn*3* transposon (8, 24). Included in the Tn*3* sequence are the β-lactamase promoter region and a portion of the *tnpR* gene which does not include its promoter (Fig. 1; 8, 24). The *Hae*II-sensitive site in Tn*3* closest to the β-lactamase gene splits the *tnpR* gene (8). Since the MAC of cells carrying pLS57 is increased, it was thought that pLS57 contained an up-promoter mutation in the previously identified β-lactamase promoter. A Sau3A-1 fragment was identified, which contains the β-lactamase promoter region of the gene in pDMS6642 (24; Dyann Worth, personal communication). The region surrounding the β-lactamase promoter of pLS57 and pDMS6642 was sequenced by the Maxam-Gilbert method (17). The sequence, which extended for 50 nucleotides above and 100 nucleotides below the Pribnow box located at the beginning of the β-lactamase sequence (24; data not shown), was identical to that reported by Sutcliff

(24). The mutation in pLS57 is, therefore, not in the immediate region identified with the promoter sequence of the β-lactamase gene, but toward or in the *tnpR* gene sequence. There are about 650 nucleotides between the β-lactamase promoter and the point at which the *tnpR* gene has been cut (8; Fig. 1).

Role of transcription from the Tn*3* sequence of pDMS6642 on plasmid copy number. The effect of the mutation located in the pLS57 *Bam-Pst* fragment B of both increasing β-lactamase production and plasmid copy number suggested the hypothesis that the high copy number of pDMS6642 and its mutants may be influenced by readthrough transcription from the Tn*3* portion of the plasmid into the ColE1 DNA adjacent to it. The ColE1 DNA which is adjacent to the Tn*3* transposon contains the replication control information of the plasmid which is expressed at the transcriptional level (25). Endonuclease fragment mapping of pDMS66 and pDMS6642 (data not shown) indicates that both the β-lactamase gene and the part of the *tnpR* gene that is present are oriented so that the normal transcription direction of those Tn*3* sequences is towards the ColE1 replication control region (8). The readthrough transcription hypothesis was tested by examining the effect on plasmid copy number of introducing insertions into the *Pst*I site of pDMS6642 and its mutants.

A 1,900-bp *Pst*I-generated fragment from pST29 (which codes for chloramphenicol resistance) was inserted into the *Pst*I site of pDMS6642, pLS57, and pLS103 in both orientations (Table 1 and Fig. 3). The sequence of the inserted fragment contains at least one transcription termination site associated with the chloramphenicol resistance gene, as well as other potential genetic information (1). The copy numbers of plasmids pLS77-1 and 2 (containing the insertion into pMS6642 in each orientation) and pLS75-1 and 2 (containing the insertion into

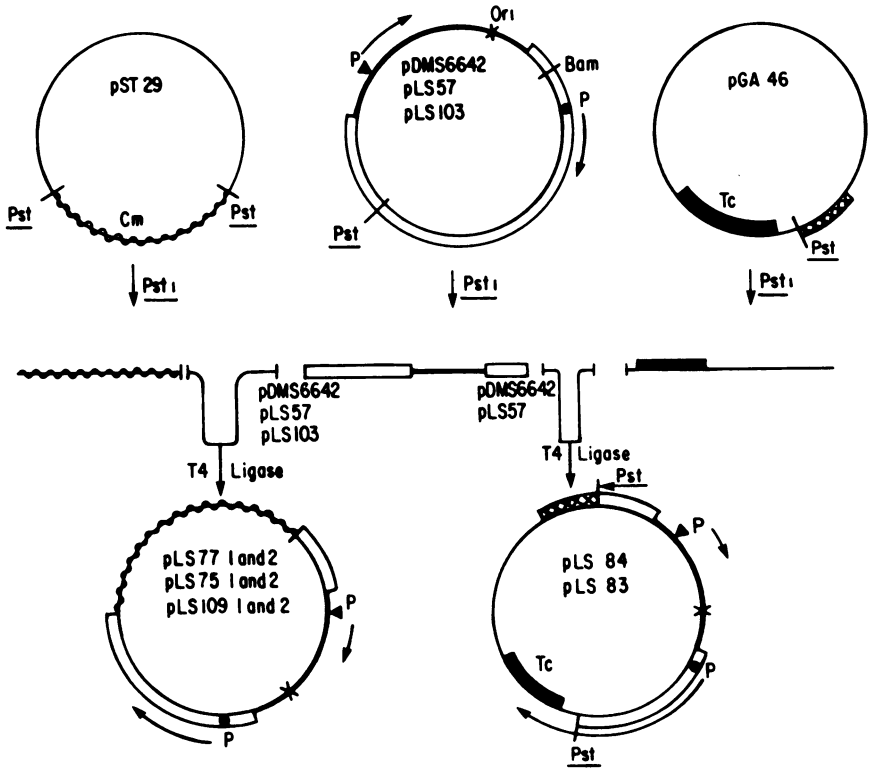

FIG. 3. Diagrammatic representation of the formation of insertions into the *Pst*I-sensitive site of pDMS6642, pLS57, and PLS103. Insertion derivatives of pDMS6642 are pLS77 and pLS84. Insertion derivatives of pLS57 are pLS75 and pLS83. pLS109 is an insertion derivative of pLS103. The two possible orientations (1 and 2) of the *Pst*I fragment (——) derived from pST29 are not distinguished in the diagram. That fragment contains information conferring chloramphenicol resistance (Cm). ● P is the location of the β-lactamase promoter and ▼ P is the location of the promoter of the RNA primer of ColE1 DNA replication. → indicates the direction of transcription. X Ori is the origin of DNA replication. ▭ is part of the Tn*3* transposon. ▬ is part of the pGA46 conferring tetracycline (Tc) resistance when fused with a promoter-containing sequence. ⊠ is part of the end of the β-lactamase gene with one end defined by the *Pst*I-sensitive site.

pLS57 in each orientation) were reduced to about 20 copies per chromosome. The copy numbers of pLS109-1 and -2 (containing the insertion into pLS103 in each orientation) exhibited an absolute reduction in copy number similar to that shown by pLS77-1 and -2, but the final base-line copy number was much higher than that of pLS77-1 and -2 or pLS75-1 and -2 (Table 4). The effects on copy number appeared to be independent of the direction of insertion (Table 4). The insertion had the predicted effect of reducing the copy number of all three plasmids, supporting the hypothesis that the high copy number of pDMS6642 depends on a continuity between the region of the Tn*3* transposon upstream from the *Pst*I site and the ColE1 genetic information involved with the plasmid's replication control. The fact that the effect was to reduce the copy number of pLS57 and pDMS6642 to the same base line while reducing the copy number of pLS103 to a higher base line cannot be considered insignificant. The mutation in pLS57 maps upstream, in terms of transcription (8), from the *Pst*I site, and affects β-lactamase synthesis, whereas the mutation in pLS103 maps downstream from the *Pst*I site and doesn't effect β-lactamase synthesis. The facts that in the inserted sequence (i) there is at least one nucleotide sequence identifiable as a transcription termination site, (ii) numerous translational termination sites exist that possibly could affect transcription, and (iii) other genetic information is probably coded for provide a variety of possible explanations of the effect of the insertion on copy number (1).

TABLE 4. Effect on copy number of inserting a DNA fragment into the *Pst*I-sensitive site of pDMS6642 and its derivatives

Parental plasmid[a]	Plasmid + insertion[b]	Copy no.
pDMS6642 (57)	pLS77-1[c]	23
	pLS77-2	21
pLS57 (84)	pLS75-1[c]	24
	pLS75-2	21
pLS103 (140)	pLS109-1[c]	99
	pLS109-2	122
pDMS6642	pLS84[d]	22
pLS57	pLS83[d]	20

[a] The parental plasmid into which the insertion was introduced. The number in parentheses is that plasmid's copy number.

[b] No. 1 and 2 refer to an arbitrary designation of the orientation of the inserted fragment (Fig. 3) as determined by endonuclease cleavage of the plasmids and subsequent electrophoretic analysis of the DNA (data not shown). The orientations are identical in all plasmids designated 1 and in all designated 2.

[c] The inserted DNA is a *Pst*I-cleaved 1,900-bp fragment derived from pST29 containing a chloramphenicol resistance gene.

[d] The inserted DNA is a *Pst*I-cleaved pAG46.

The effect on plasmid copy number of inserting another large DNA fragment with more than one site for termination of transcription, pGA46 (4,600 bp), was tested by inserting it into the *Pst*I-sensitive site of pDMS6642 to form the plasmid pLS84 and into the *Pst*I-sensitive site of pLS57 to form the plasmid pLS83 (Fig. 3). One further advantage of using pGA46 plasmid DNA in this test is that when cleaved with *Pst*I, a promoterless nucleotide sequence that can potentially express tetracycline resistance is exposed. The level of tetracycline resistance conferred by attaching a particular promoter to the pGA46 sequence is reported to be proportional to the transcriptional activity stimulated by it (2). Therefore, as a result of inserting pGA46 into these plasmids, two different effects should be seen if the interpretation of the previous results is to be supported. First, the insertion should reduce the copy number of both pDMS6642 and pLS57 to the same extent as previously observed with the 1,900-bp fragment. Second, the promoter(s) that affect pLS57 copy number and β-lactamase production should cause a higher level of expression of tetracycline resistance by pGA46 than the promoter(s) of pDMS6642.

The result of inserting pGA46 into pDMS6642 and pLS57 was to reduce the copy number of the chimeras pLS84 and pLS83 to 22 and 20 copies, respectively (Table 4). Both chimeras expressed tetracycline resistance. pLS84-containing cells grew on agar containing 5, but not 10, μg of tetracycline per ml, whereas pLS83-containing colonies grew on agar containing 15, but not 20, μg per ml. The higher tetracycline resistance conferred by pLS83 is, therefore, not related to plasmid copy number. The result suggests that transcriptional activity is higher in the Tn*3* region of pLS57 than in the same region of pDMS6642. It also shows that the insertion reduces the copy number of both plasmids to the same level, which was identical to the level caused by inserting the *Pst*I fragment carrying the chloramphenicol resistance gene into the same site. It should be noted that for the chimeras pLS83 and pLS84 to produce a tetracycline resistance phenotype, the plasmids necessarily had to be inserted in one particular orientation (Fig. 3; 2). If they had been inserted in the opposite orientation, a chimera that would confer a tetracycline sensitivity-ampicillin resistance phenotype would have been produced because the *Pst*I-sensitive site in pGA46 is at one end of the terminal part of a β-lactamase gene. The inverted insertion would have matched the first part of the β-lactamase gene of pDMS6642 with the last part of the β-lactamase gene, which

is all of that gene that exists in pGA46 (Fig. 3). This was not observed in either pLS83 or pLS84. The result, therefore, further supports a model of readthrough transcription affecting copy number and directly supports the interpretation that β-lactamase production per pLS57 plasmid is increased.

DISCUSSION

The copy number of pDMS6642 was reduced from 57 to about 20 per chromosome after either a 1,900- or 4,600-bp fragment was inserted into the *Pst*I-sensitive site of the Tn*3* β-lactamase gene carried by the plasmid. As has been noted before (5, 6) and as was illustrated here, the copy numbers of the ColE1 plasmid derivatives are not simply a reflection of plasmid size. If they were, very different effects of the two insertions should have been seen. The cause of the reduction in plasmid copy number by these insertions is of interest because the insertions are at a point between at least one well-characterized constitutively active promoter of β-lactamase and a well-characterized deletion (20) carried in pDMS6642 that fused the end of the β-lactamase gene to the ColE1 nucleotide sequence about 129 bp upstream of the promoter for the RNA primer of ColE1 DNA replication. As both insertions have transcription termination signals (1, 2), it seems reasonable to think that the reduction of the copy number of pDMS6642 to the same level by both insertions was due to a block in transcription readthrough from the Tn*3* region and was not due to several independent but identical effects of transcription that was initiated within the different insertions. It should also be considered that if readthrough transcription affects ColE1 copy number, then a large insertion of 1900 bp could very well have an effect simply on the basis that it may reduce the efffciency of readthrough, without any specific termination site being present. Nonspecific transcription termination events have often been observed when RNAs have been studied. One such example is the RNA transcripts produced from the RNA primer promoter of DNA replication of ColE1 examined in vitro (14, 25). Some of those transcripts appear to spontaneously terminate at a variety of sites (14, 25). These findings, therefore, suggest that the increased copy number of pDMS6642 is caused by readthrough transcription from the Tn*3* region of the plasmid into the ColE1 replication control region.

The hydroxylamine-induced mutation in pLS57 is located in the Tn*3* component of pDMS6642. The mutation causes an increase in its host's resistance to ampicillin, increased β-lactamase production per plasmid, and in addition, an increased plasmid copy number. It was argued that the increased β-lactamase transcription in pLS57 further increased the transcriptional readthrough into the ColE1 replication control region and thus caused the increase in copy number (about 30 copies) over that seen in pDMS6642. This was supported by the observation that the insertion of the 1,900- and 4,600-bp fragments into the *Pst*I site of the β-lactamase gene reduced the copy number of pLS57 to 20. The insertions not only blocked the effect of the mutation in pDMS6642 on the plasmid copy number, but also blocked the additional effect of the hydroxylamine mutation on the copy number. The simultaneous reversal of the copy number effect caused by the deletion in pDMS6642 and the hydroxylamine mutation suggested that the mechanism by which both caused the increased copy number is the same, namely, readthrough transcription. The higher production of β-lactamase per plasmid by pLS57 is most likely caused by a mutation that has generated a new promoter upstream from the normal β-lactamase promoter, which was found to have an unchanged nucleotide sequence. The effect of an increased promoter activity influencing β-lactamase production in pLS57 was further substantiated by demonstrating an increased expression of a tetracycline resistance gene that relies on a promoter of the β-lactamase gene for expression.

The hydroxylamine-induced mutation in pLS103 increased the copy number of pDMS6642 without altering the production of β-lactamase per plasmid. The mutation is located in the DNA A fragment shown in the illustration in Table 3. That DNA fragment contains the end of the β-lactamase gene, the replication control region of ColE1, including the replication origin, and about 300 to 350 bases (8) that reside between the *Hae*II-sensitive site that marks a ColE1-Tn*3* junction (Fig. 1, pLS57 and pLS103) and the *Bam*HI site in the Tn*3* DNA. Since the mutation did not alter the β-lactamase production of pLS103, it is assumed that it does not alter the plasmid copy number by significantly altering transcription passing through the β-lactamase gene. Mutations in the RNA1 gene of ColE1, which are in the ColE1 replication region, are known to increase the plasmid copy number (4, 18, 25). If the increased copy number of pDMS6642 is caused both by readthrough transcription from a promoter in Tn*3* in the direction of β-lactamase transcription and by a mutation in the DNA A fragment that does not rely on such readthrough transcription, then it was reasoned that insertions placed at the *Pst*I-sensitive site in pLS103 could dissect the two processes, causing the increased copy number of that plasmid. The insertion should lower the copy number of the pLS103 plasmid by about 35

copies per chromosome, the amount attributed to readthrough transcription from Tn*3*. If the effect on copy number is unaffected by the mutation in the DNA A fragment by such an insertion, the new plasmid (pLS109) will have a higher base-line copy number than pDMS6642 carrying the same insertions (e.g., pLS77). The results in Table 4 show that the base-line copy number of those plasmids (pLS109) was higher (between 99 and 122) than for pDMS6642, and the average drop in copy number was about 30. The result is taken to further support the interpretation that readthrough transcription in pDMS6642 and its derivatives has a positive effect on pDMS6642 replication.

The striking consistency of the results obtained from quite different kinds of measurements of the three plasmids, pDMS6642, pLS57, and pLS103 and their derivatives is taken to strongly support the model of readthrough transcription affecting the copy number in pDMS6642.

An increased transcriptional readthrough from the Tn*3* DNA of pDMS6642 or pLS57 could cause an increased plasmid copy number by affecting the transcription of either the RNA primer of DNA replication or of the RNAI gene product. These observations suggest that transcriptional control of ColE1 replication in vivo can be profitably examined by using pDMS6642 and DNA fragments that contain specific modulatable promoters.

This study also points up some problems and potential advantages of studying the control of replication of a plasmid from which, as frequently occurs in engineered plasmids, much of the genetic information has been removed that otherwise buffers the control system from unusual transcriptional activity.

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