JOURNAL OF BACTERIOLOGY, Oct. 1987, p. 4841–4844
0021-9193/87/104841-04$02.00/0

Vol. 169, No. 10

Mutations in the *recD* Gene of *Escherichia coli* That Raise the Copy Number of Certain Plasmids

RALPH SEELKE,[1]* BRUCE KLINE,[2] ROSS ALEFF,[2] RONALD D. PORTER,[3] AND MALCOLM S. SHIELDS[2]

Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin 53201[1]; Section of Microbiology, Department of Cell Biology, Mayo Clinic, Rochester, Minnesota 55905[2]; and Department of Molecular and Cell Biology, Pennsylvania State University, University Park, Pennsylvania 16802[3]

Received 23 March 1987/Accepted 13 July 1987

Chromosomal mutants were isolated in which, for several small plasmids, there was an increased amount of either covalently closed circular plasmid DNA or total plasmid DNA or both. The mutations were mapped to *recD*, which has been shown to affect exonuclease V activity and a variety of plasmid maintenance and replication functions. Our results suggest that rolling-circle plasmid replication can occur in *recD* mutants and that site-specific recombination can resolve the resulting linear multimers into covalently closed circular plasmid forms.

Plasmid copy number, defined as the average number of plasmids per chromosomal equivalent, is a constant in a given strain under a particular set of growth conditions. Plasmids regulate their copy numbers by a variety of mechanisms, some of which have been well characterized (22). However, host DNA replication genes also play a role in plasmid maintenance (3, 21, 26). In addition, mutations in other types of *Escherichia coli* genes have been isolated which have as their primary phenotypes an alteration in the ability of the mutant to maintain properly a resident plasmid (4, 7, 11, 13, 14, 25). We report here that the chromosomal *recD* gene, which is capable of affecting exonuclease V activity, maintenance of some plasmids, and the phenomenon of linear-plasmid multimer production (2, 4, 5), is also capable of affecting levels of either covalently closed circular (CCC) plasmid DNA or total plasmid DNA (i.e., CCC, open circular, and linear forms) or both. We have also identified plasmid regions that seem to determine which of the above mentioned effects occur.

(Portions of this work have been presented previously [R. Aleff, B. C. Kline, and R. Seelke, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H138, p. 131; R. Seelke and S. Lawson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, H131, p. 161].)

The bacterial strains and plasmids used in this study are listed in Table 1. Chromosomally located mutations elevating plasmid copy number (*cop* mutations) were selected by treating strain CSH50(pMF45) bacteria with ethyl methanesulfonate (19) and plating them on AB3 agar (Difco Laboratories, Detroit, Mich.) containing 1 mg of ampicillin per ml. Plasmid pMF45, a mini-F plasmid, normally imparts resistance to about 100 μg of ampicillin per ml when its host is plated at low density. However, cells with putative *cop* mutations form normal-size colonies after overnight growth at 37°C. To prevent selection of sibling mutants, only one putative mutant from each mutagenized culture was retained for analysis.

The mutants thus isolated were confirmed as having chromosomal *cop* mutations by (i) demonstrating that the Cop$^-$ phenotype did not cotransfer to new host cells with plasmid DNA extracted from the Cop$^-$ cells and (ii) curing mini-F from the Cop mutants with acridine orange, reinfecting the cured descendants with mini-F, and subsequently demonstrating that the transformants gave the Cop$^-$ phenotype.

A qualitative assay for the Cop$^-$ phenotype was done by comparing the intensities of plasmid DNA bands in agarose gels. Typically, plasmid DNA was extracted by the alkaline lysis technique (17) from equal cell masses of overnight or late-log-phase cultures grown in antibiotic-supplemented L broth (19). Equal amounts of plasmid-containing extract were then subjected to electrophoresis through 0.8% agarose gels containing 1 μg of ethidium bromide per ml, and the plasmid bands were visualized and photographed by UV transillumination. The intensities of the plasmid bands were on some occasions quantitated by scanning photographic negatives with a Hoeffer GS300 densitometer.

In most cases, quantitative measurements of CCC plasmid DNA were done by the dye-CsCl assay (18). Exponentially growing bacteria were labeled with [^{3}H]thymidine and lysed, and the CCC DNA was separated from other forms by isopycnic centrifugation in an ethidium bromide-CsCl solution. Plasmid copy numbers were determined from the percentage of the total DNA that was found in the CCC DNA form (Table 2, footnote *b*).

In some cases, the total amount of plasmid DNA per cell mass was determined by using a dot blot technique (23). Known amounts of cells from overnight cultures were immobilized onto nitrocellulose filters, lysed in situ, hybridized with a ^{32}P-labeled mini-F *repE* gene RNA probe, and autoradiographed. The autoradiographs were then scanned in a densitometer.

The results of the dye-CsCl assays with several plasmids in two Cop$^-$ mutants, BK968 (*cop-4* host) and BK969 (*cop-5* host), are shown in Table 2. The plasmids fall mainly into two groups. The copy numbers of plasmids ColE1 and pSC101 were not elevated in either mutant; however, the copy numbers of their Tn*3*-containing derivatives, pSC190 and RSF2124, were elevated. Of the mini-F plasmids tested, pML31 and pMF45 both showed significant increases in CCC plasmid DNA, while pMF21 showed, at most, a twofold increase in the *cop-5* mutant. Surprisingly, a smaller derivative of pMF45, pMF46 (Table 1), showed even higher levels of CCC plasmid DNA than did pMF45. Plasmids pMF45 and pMF46 were also the only ones to show significant plasmid loss when residing in a *cop* mutant host (Table

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
Strains		
CSH50	*ara* Δ(*pro lac*) *thi rpsL*	19
BK968	CSH50 *cop-4*	This study
BK969	CSH50 *cop-5*	This study
Plasmids		
F′ *lac*	*lac*$^+$ *pro*$^+$	1
R386	Tc^r	9
pML31	Mini-F Km^r	16
pMF21	pML31Δ(F40.3–43.1) Km^r	18
pMF45	pML31Δ(F40.3–43.1)ΩTn*3* 46.19 Ap^r Km^r	18
pMF46	pMF45Δ(*Eco*RI *kan*$^+$ fragment) Ap^r Km^s	18
pSC101	Tc^r	6
pSC190	pSC101::Tn*3* Ap^r Tc^r	15
ColE1	Production of or resistance to colicin E1	24
RSF2124	ColE1::Tn*3* Ap^r	24
pCDK2	Ap^r *argA*$^+$; pBR322 replicon	10; S. N. Cohen
pCDK3	Ap^r *thyA*$^+$ *recC*$^+$ *recB*$^+$ *recD*$^+$; pBR325 replicon	10; S. N. Cohen
pZC13	Cm^r *recD*$^+$; pBR325 replicon	4; S. N. Cohen

2, footnote *b*). In addition to the plasmids listed in Table 2, two large IncF1 plasmids, F′*lac* and R386, were also assayed by the dye-CsCl technique; their copy numbers were not elevated in either Cop$^-$ mutant (data not shown).

The concentrations of pML31, pMF21, and pMF45 in strains CSH50 and CSH50 *cop-5* were determined by the dot blot assay. In all three cases, this assay indicated a much larger increase in plasmid DNA in the *cop-5* host than did the dye-CsCl assay. This result showed that the CCC form of the plasmid was a minor fraction of the total plasmid DNA present.

To map the location of the *cop-4* and *cop-5* mutations, we first randomly inserted Tn*10* into the bacterial chromosome (8). We then used P1 lysates of the Tn*10* pools to cotransduce both the tetracycline resistance gene (Tn*10*) and the *cop* allele into a recipient strain containing pMF45. The *cop* Tn*10* recipients were selected on agar with tetracycline and a high level of ampicillin. Next, the *cop* region linked to Tn*10* was transduced into a number of Hfr strains by selecting for tetracycline resistance. Finally, the Tn*10* marker was mapped very near the origin of transfer at 61.5 min in KL16 by standard conjugational techniques (19).

One of the genes located near 61.5 min is *recD*, which has recently been shown to destabilize maintenance of pSC101 and ColE1-type replicons (4). We thus investigated the possibility that the *cop-5* mutation was in *recD*. Using a strain (CR147) possessing an *argA*::Tn*10* mutation as donor, we transduced this mutation into CSH50 *cop-5*(pMF45). Of 100 Tc^r transductants screened, 99 had lost the Cop$^-$ phenotype. This indicated that, as with *recD*, the *cop-5* mutation is tightly linked to *argA*, which maps at 60.5 min. Precise mapping of the *cop-5* mutation to *recD* was accomplished by introducing into BK969 (*cop-5*) both pML31 (Km^r) and one of several compatible plasmids carrying various regions of the chromosome between *thyA* and *argA* (Fig. 1). Plasmids pCDK3 and pZC13 both complemented the Cop$^-$ phenotype, resulting in a dramatic decrease in the copy number of the coresident pML31 plasmid; pCDK2, carrying the *argA* gene, did not (Fig. 1). Although pZC13 also includes a portion of the *recB* gene, the only gene carried in total is *recD*. Similar results were also obtained for BK968 (*cop-4*; data not shown). Additionally, we found that two of the *recD* mutants isolated and characterized by Biek and Cohen (4) show the Cop$^-$ phenotype: When transformed by pMF45, these mutants will grow in the presence of 1 mg of ampicillin per ml and show elevated levels of CCC-form plasmid DNA (S. Lawson and R. Seelke, unpublished results). The *cop-4* and *cop-5* mutations have been designated *recD2201* and *recD2202*, respectively.

This study adds to the list of effects that mutations in the *recD* gene can have (see above). Of particular interest are the results with pML31, pMF45, and pMF21. The first two plasmids showed elevated CCC and total plasmid DNA; pMF21 showed only a slight increase in CCC-form plasmid while showing a large increase in total plasmid DNA. Since these plasmids are nearly isogenic, we have thus shown that plasmid-encoded functions can affect the extent of CCC DNA elevation.

Our results are most simply explained by a modification of the model of Cohen and Clark (5). They showed that mutations or conditions which inhibit the activity of exonuclease V resulted in the production of linear multimers by ColE1-type plasmids. They attribute the presence of linear multimers to the ability of ColE1 in *recB recC* or *recD* mutants to undergo rolling-circle replication, resulting in linear forms that do not readily recircularize. For reasons described below, we believe some plasmids are capable of recircularizing in *recD* mutants. Cohen and Clark (5) did not investigate the effects of the *rec* mutations with other plasmids and did not see an increase in the ColE1 CCC plasmid DNA. In addition, they did not address the effect of an active site-specific recombination system on the fate of the linear multimers.

The resolution of linear multimers into circular monomers

TABLE 2. Amounts of plasmid DNA in Cop$^+$ and Cop$^-$ bacterial hosts

Plasmid	Mass (MDa)[a]	*cop*$^+$ host		*cop-4* host CCC copy no.	*cop-5* host	
		CCC copy no.[b]	Total plasmid DNA[c]		CCC copy no.	Total plasmid DNA
pMF45	12.2	2.0	1×	12.7	12.2	(65 ± 8)×
pML31	10.8	1.4	1×	8.8	7.5	(81 ± 18)×
pMF21	9.4	1.6	1×	ND[d]	2.4–3.2[e]	(68 ± 10)×
pMF46	7.6	2.0		46.0	30.5	
pSC101	6.0	4.0		4.8	6.0	
pSC190	9.0	9.7		52.0	68.0	
ColE1	4.2	10.4		10.0	8.3	
RSF2124	7.4	12.8		25.5	23.7	

[a] MDa, Megadaltons.

[b] Copy number is [(%CCC DNA) × 10^{-2}) (2.5 × 10^9)]/[(mass of plasmid) (fraction of cells retaining plasmid)]. The value 2.5 × 10^9 is the estimated molecular weight of the chromosome. Each value represents the average of two determinations. The individual determinations deviated from the average by 10% or less. Except for the cases in which pMF45 or pMF46 were residing in a *cop-4* or *cop-5* host, the percentage of cells retaining the plasmid was 98% or greater. For the exceptions mentioned, the percentage of cells retaining the plasmid was 56 to 64%.

[c] The relative amount of total plasmid DNA per cell was determined by the dot blot technique on overnight cultures as described in the text. The value for a given plasmid in a *cop-5* host was compared with the value measured in a wild-type host, which was arbitrarily set at 1×.

[d] ND, Not determined.

[e] Copy number was measured in this case only by densitometry of photographic negatives of gels as described in the text. Density of the pMF21 bands isolated from a *cop-5* strain, BK969(pMF21), was 50 to 100% greater than the density of comparable bands isolated from CSH50(pMF21).

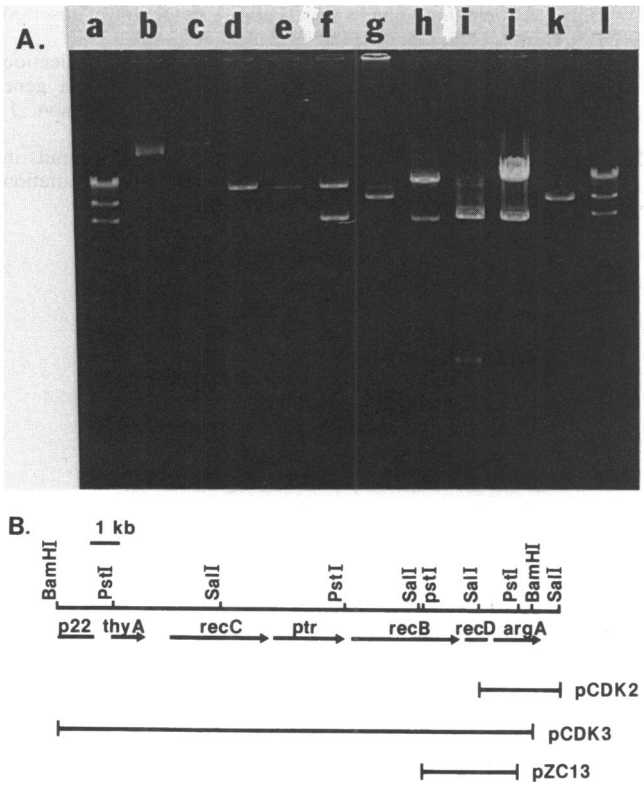

FIG. 1. Comparison of plasmid copy numbers of pML31 in Cop^+ and Cop^- strains of *E. coli* and complementation of the Cop^- phenotype by the $recD^+$ gene. (A) Lysates from ca. 1.1×10^8 cells (0.51 A_{550} units) from late-log-phase cultures of each strain (lanes b to h) or ca. 0.22 A_{550} units from overnight cultures (lanes i to k) were electrophoresed as described in the text for 120 min at 100 V (4 V/cm). Lanes b and c contain uncut plasmid DNA; lanes d to k contain DNA that has been digested with *Bam*HI. Lanes: a and l, λ DNA cut with *Hin*dIII for linear DNA size standards; b, pML31 from CSH50 *cop-5*(pML31); c, pML31 from CSH50(pML31); d, pML31 from CSH50 *cop-5*(pML31); e, pML31 from CSH50(pML31); f, pML31 and pCDK2 from CSH50 *cop-5*(pML31, pCDK2); g, pML31 and pZC13 from CSH50 *cop-5*(pML31, pZC13); h, pML31 and pCDK3 from CSH50 *cop-5*(pML31, pCDK3); i, pCDK2; j, pCDK3; k, pZC13. Sizes of λ DNA fragments, in kilobase pairs, from top to bottom: 23.13, 9.41, 6.56, 4.36, 2.32, 2.03, and 0.56. (B) Genetic map of the chromosomal regions carried by pCDK2, pCDK3, and pZC13. This figure is taken from Fig. 8 of Biek and Cohen (4) and Fig. 1 of Dykstra et al. (10).

by site-specific recombination could explain those situations in which the CCC plasmid DNA was elevated. Site-specific recombination could be provided by either Tn*3* (12) or *oriV1* (20). For example, pMF21 shows at most a twofold increase in CCC plasmid DNA in BK969; when pMF21 acquires either *oriV1* (pML31) or Tn*3* (pMF45), that increase becomes about sixfold. The same explanation could also hold for the increase in CCC plasmid seen when pSC101 and ColE1 acquire Tn*3* and are placed in a *recD* host. We are currently examining the plasmids listed in Table 2 for evidence of rolling-circle replication.

In summary, the simplest explanation for our data is that rolling-circle plasmid replication in *recD* mutants occurs in a number of plasmids and that the combination of this form of replication with an active site-specific recombination system results in elevated levels of CCC plasmid DNA as well.

The expert technical assistance of Sobomabo Lawson and Lisa Musmanno is gratefully acknowledged. We thank Donald Biek and Stanley Cohen for providing plasmid-containing strains and *recD1901*- and *recD1903*-containing strains.

This work was supported by the Mayo Foundation; grant GM25604 from the U.S. Public Health Service, National Institutes of Health (to B.C.K.); the University of Wisconsin-Milwaukee Graduate School Research Committee; and the Shaw Fund of the Milwaukee Foundation.

LITERATURE CITED

1. **Achtman, M., N. Willetts, and A. J. Clark.** 1972. Conjugational complementation analysis of transfer-deficient mutants of F*lac* in *Escherichia coli*. J. Bacteriol. **110**:831–842.
2. **Amundsen, S. K., A. F. Taylor, A. M. Chaudhury, and G. R. Smith.** 1986. *recD*: The gene for an essential third subunit of exonuclease V. Proc. Natl. Acad. Sci. USA **83**:5558–5562.
3. **Arai, T., and R. Clowes.** 1975. Replication of stringent and relaxed plasmids, p. 141–155. *In* D. Schlessinger (ed.), *Microbiology—1974*. American Society for Microbiology, Washington, D.C.
4. **Biek, D., and S. N. Cohen.** 1986. Identification and characterization of *recD*, a gene affecting plasmid maintenance and recombination in *Escherichia coli*. J. Bacteriol. **167**:594–603.
5. **Cohen, A., and A. J. Clark.** 1986. Synthesis of linear plasmid multimers in *Escherichia coli* K-12. J. Bacteriol. **167**:327–335.
6. **Cohen, S. N., and A. C. Y. Chang.** 1977. Revised interpretation of the origin of the pSC101 plasmid. J. Bacteriol. **132**:734–737.
7. **Cress, D. E., and B. C. Kline.** 1976. Isolation and characterization of *Escherichia coli* chromosomal mutants affecting plasmid copy number. J. Bacteriol. **125**:635–642.
8. **Davis, R. W., D. Botstein, and J. R. Roth (ed.).** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Dennison, S.** 1972. Naturally occurring R factor, derepressed for pilus synthesis, belonging to the same compatibility group as the sex factor F of *Escherichia coli* K-12. J. Bacteriol. **109**:416–422.
10. **Dykstra, C. C., D. Prasher, and S. R. Kushner.** 1984. Physical and biochemical analysis of the cloned *recB* and *recC* genes of *Escherichia coli* K-12. J. Bacteriol. **157**:21–27.
11. **Ely, S., and W. Staudenbauer.** 1981. Regulation of plasmid DNA synthesis: isolation and characterization of copy number mutants of mini-R65 and mini-F plasmids. Mol. Gen. Genet. **181**:29–35.
12. **Heffron, F.** 1983. Tn*3* and its relatives, p. 223–260. *In* J. A. Shapiro (ed.), Mobile genetic elements. Academic Press, Inc., New York.
13. **Jamieson, A., and P. Berquist.** 1976. Genetic mapping of chromosomal mutations affecting the replication of the F-factor of *Escherichia coli*. Mol. Gen. Genet. **148**:221–223.
14. **Kingsbury, D., and D. Helinski.** 1973. Temperature sensitive mutants for the replication of plasmids of *Escherichia coli*. I. Isolation and specificity of host and plasmid mutations. Genetics **74**:17–31.
15. **Kopecko, D., J. Brevel, and S. N. Cohen.** 1976. Involvement of multiple translocating DNA segments and recombinational hot spots in the structural evolution of bacterial plasmids. J. Mol. Biol. **108**:333–360.
16. **Lovett, M. A., and D. R. Helinski.** 1976. Method for the isolation of the replication region of a bacterial replicon: construction of a mini-F′*km* plasmid. J. Bacteriol. **127**:982–987.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Manis, J., and B. Kline.** 1978. F plasmid incompatibility and copy number genes: their map locations and interactions. Plasmid **1**:492–507.
19. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **O'Connor, M. R., and M. H. Malamy.** 1984. Site-specific recombination in the *oriV1* region of the F factor. Cold Spring Harbor Symp. Quant. Biol. **49**:421–434.
21. **Otten, M. R., M. Wlodarcyzk, B. C. Kline, and R. Seelke.** 1980.

Control of plasmid R6K copy numbers in isogenic *rep*$^+$ and *rep* *Escherichia coli* strains. Mol. Gen. Genet. **177**:493–499.
22. **Scott, J. R.** 1984. Regulation of plasmid replication. Microbiol. Rev. **48**:1–23.
23. **Shields, M., B. C. Kline, and J. E. Tam.** 1986. A rapid method for the quantitative measurement of gene dosage: mini-F plasmid concentration as a function of cell growth rate. J. Microbiol. Methods **6**:33–46.
24. **So, M., R. Gill, and S. Falkow.** 1975. The generation of a ColE1:Ap^r cloning vehicle which allows detection of inserted DNA. Mol. Gen. Genet. **142**:239–249.
25. **Wada, C., and T. Yura.** 1984. Control of F plasmid replication by a host gene: evidence for interaction of the *mafA* gene product of *Escherichia coli* with the mini-F *incC* region. J. Bacteriol. **160**:1130–1136.
26. **Womble, D., and R. Rownd.** 1979. Replication of plasmids in mutants of *Escherichia coli* temperature-sensitive for initiation of deoxyribonucleic acid synthesis. Plasmid **2**:95–108.