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Synthesis of Linear Plasmid Multimers in *Escherichia coli* K-12

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Linear plasmid multimers were identified in extracts of *recB21 recC22* strains containing derivatives of the ColE1-type plasmids pACYC184 and pBR322. A mutation in *sbcB* increases the proportion of plasmid DNA as linear multimers. A model to explain this is based on proposed roles of RecBC enzyme and SbcB enzyme (DNA exonuclease I) in preventing two types of rolling-circle DNA synthesis. Support for this hypothesis was obtained by derepressing synthesis of an inhibitor of RecBC enzyme and observing a difference in control of linear multimer synthesis and monomer circle replication. Reinitiation of rolling-circle DNA synthesis was proposed to occur by *recA*$^+$-dependent and *recA*$^+$-independent recombination events involving linear multimers. The presence of linear plasmid multimers in *recB* and *recC* mutants sheds new light on plasmid recombination frequencies in various mutant strains.

DNA exonuclease V (ExoV) is one activity of a multifunctional enzyme (38) called RecBC enzyme because two of its subunits are encoded by the *recB* and *recC* genes (23). Other activities include a DNA helicase (49, 59) and a chi sequence-stimulated DNA endonuclease (47). Genetic analysis of RecBC enzyme began by discovery of *recB21* and *recC22* mutations which reduce both survival after X or UV irradiation and conjugational recombination frequencies (15, 16) and by the discovery of other *recB* and *recC* mutations which cause the same mutant phenotypes (8, 73). Those mutations tested, such as *recB21* and *recC22*, eliminate all known activities of RecBC enzyme measured by assay in vitro (54, 61) and so do not indicate which activities are involved in recombination and recovery from DNA damage. Recently, mutations which appear to affect RecBC enzyme activities differentially have been described (7, 51), and these may prove useful.

Another in vivo function of RecBC enzyme was found to be the degradation of linear DNA. This was inferred from the reduced rate of degradation of unmodified phage DNA attacked in vivo by the *Eco*K restriction system (52). It was also inferred from the indirect suppression of T4 gene 2 mutations by *recB21* or *recC22*, since gene 2 product protects the ends of linear T4 DNA (46). Various phages were also found to encode inhibitors of RecBC enzyme (50, 66, 68), and this was interpreted as indicating an advantage to protection of their DNA. Lambda, for example, carries the *gam* gene whose 16-kilodalton product irreversibly inhibits RecBC enzyme activities in vitro (27). *gam* mutants of lambda showed reduced burst sizes because the transition from monomer circle replication to rolling-circle DNA synthesis did not occur, and the amount of packageable (i.e., multimeric) substrate phage DNA was limited to that formed by recombination (78).

More recently, the in vivo influence of RecBC enzyme on recombination and maintenance of various plasmids has been studied. Plasmid recombinant frequencies were found to be unaffected by *recB* and *recC* mutations (19, 35, 70), unlike conjugational recombinant frequencies which were reduced about 100-fold (8, 71). Plasmid maintenance was, however, affected by *recB* and *recC* mutations alone (21, 51) and in combination with an *sbcB15* mutation (3, 48, 51, 69) or an *sbcA23* mutation (58). The *sbcB15* mutation inactivates DNA exonuclease I (32), and *sbcA23* allows production of DNA exonuclease VIII (2, 62).

To determine how *recB* and *recC* mutations affect maintenance of plasmids related to ColE1, studies of plasmid DNA were carried out. Partially purified plasmid preparations from *recB21 recC22 sbcB15* cells were found to contain a large amount of high-molecular-weight DNA migrating in agarose gel electrophoresis with the mobility of chromosomal DNA fragments (L. W. Ream, Ph.D. thesis, University of California, Berkeley, 1982). Much of this was tentatively identified as plasmid DNA (Ream, Ph.D. thesis), which led to the suggestion that molecular forms other than covalently closed circles are part of the plasmid population in these cells and may contribute to plasmid instability (Ream, Ph.D. thesis). Another study on a *recB21 recC22 sbcA23* strain led to the proposal that a high proportion of plasmid circular multimers causes plasmid instability in this genetic background (58). We continued these studies by determining the molecular nature of plasmid DNA in a *recB21 recC22 sbcB15* strain and by studying the role of the RecBC enzyme in the synthesis of the forms observed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used are listed in Table 1. Plasmid-carrying derivatives were produced by transformation before each experiment, or temporary cultures were kept on petri plates. Permanent stocks were not made. Unless indicated otherwise, cultures were grown in L broth (43) supplemented with the appropriate antibiotics (20 μg of chloramphenicol per ml, 50 μg of kanamycin per ml, 100 μg of ampicillin per ml) at 37°C.

Plasmids. Plasmids used in this study are listed in Table 2 and were kept by storage of DNA at 4°C. pAC400 was derived from pACYC184 by deleting the *Hin*dIII-*Ava*I segment with the appropriate restriction endonucleases, removing the single-strand ends by treatment with DNA polymerase (Klenow fragment), and closing the molecules by treatment with T4 ligase.

Preparation of plasmid DNA. Plasmid DNA for use in transformation and standardizing electropherograms was purified by cesium chloride-ethidium bromide (EtBr) density gradient centrifugation of clear lysates (11). We used the lower band. pSB344 DNA from *recA13* host JC2926 (63) was

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TABLE 1. *E. coli* strains

Strain	Genotype					Source or reference
	recB	*recC*	*sbcA*[a]	*sbcB*	Other	
AB1157	+	+	del	+	*b*	1
JC5519	*21*	*22*	del	+	*b*	72
JC7623	*21*	*22*	del	*15*	*c*	25
JC8111	*21*	*22*	del	*15*	*recF143*[c]	25
JC8267	*21*	*22*	del	+	*xonA9*[c]	32
JC8679	*21*	*22*	*23*	+	*d*	20
JC9604	*21*	*22*	*23*	+	*recA56*[e]	20
JC11445	+	+	*23*	+	*d*	A. Templin[f]
JC11451	+	+	del	*15*	*g*	A. Templin[g]
JC11476	+	+	del?	*15*	*thyA264*	A. Templin[h]
JC11834	*21*	*22*	del	*15*	*lexA3*	L. Margossian[i]
JC12122	*21*	*22*	del	*15*	*recJ284*::Tn*10*	S. Lovett[j]
JC15329	*21*	*22*	del	*15*	Δ-306[c,k]	A. Templin[l]
JC15501	+	+	del	*15*	*recD1009*	This work[m]
V218	+	+	del	+	*recD1009*	7

[a] del stands for the absence of Rac prophage in most strains derived from AB1157. *sbcA* and *recE* are both carried by Rac (9) so that most AB1157 deviatives have no potential to express *recE*. The *sbcA23* strains listed are Rac$^+$ derivatives of AB1157 and express the *recE*$^+$ gene.

[b] Other mutations are the following: *thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1*.

[c] In addition to the mutations in footnote *b* these strains have or are likely to have *sbcC201* (40).

[d] JC8679 has the mutations listed in footnote *b* except that it has *his-328* in place of *hisG4*.

[e] JC9604 is *his*$^+$; otherwise it has the mutations in footnote *b*.

[f] JC11445 was derived from JC8679. First a thymine-requiring (Thy$^-$) mutant, JC11437 (*thyA263*), was selected after treatment with trimethoprim (45). JC11437 was exposed to P1 · 3000, and thymine-independent transductants were selected. These were screened by P1 transduction backcrosses to identify *recB*$^+$ *recC*$^+$ transductants (62).

[g] A *sup*$^+$ mutant of JC7689 (34) was selected by the method described by Clark et al. (10). Other mutations are listed in footnote *b*.

[h] Thy$^-$ mutant of JC11451 selected by the trimethoprim method (45).

[i] JC11834 carries *rfa-316* in addition to the mutations listed in footnote *c*. It was isolated as an HK19-resistant mutant of JC9806 (10). JC9806 was isolated exactly as was JC5268 (41) and should be isogenic with it.

[j] Tet^r transductant from JC7623 by P1 · JC12105. JC12105 is described by Lovett and Clark (42).

[k] The full name of this deletion is Δ(*recA-srlR*)306::Tn*10*.

[l] Tet^r transductant from JC7623 by P1 · JC10289. JC10289 is described by Willis et al. (75).

[m] JC15501 was constructed by P1 transduction of JC11476, with phage grown on V218. Thy$^+$ transductants were tested for the *recD1009* mutation by growing P1 on them and transducing JC5422 to Thy$^+$ and testing transductants for the ability to support growth of phage T4 gene 2$^-$ (7). Originally *rec-1009* was thought to affect the *recB* gene (7), but recent results indicate that it affects another gene (*recD*) which encodes a third subunit of the RecBC enzyme (Amundsen et al., in press).

used as an electrophoretic standard for covalently closed circular and relaxed circular monomeric plasmid DNA. pSB344 DNA from *recB21 recC22 sbcA23* host JC7689 contained multimeric circles and was used to mark the electrophoretic mobility of these forms (58). The identity of plasmids in transformants was checked by either of two rapid plasmid DNA preparative techniques (4, 24).

Preparation of total DNA. Total DNA (plasmid and chromosomal) was prepared from cells in the log phase (about 3 × 10^8 cells per ml) by the method described by Gillen et al. (20). This was used for most of the hybridization experiments.

Southern hybridization analysis of molecular forms. To fragment chromosomal DNA, we treated total DNA preparations with a restriction endonuclease that does not cleave the plasmid whose DNA forms are to be monitored. Constant amounts of total DNA (3 μg per lane) were subjected to electrophoresis for 16 h at 20 mA in TBE buffer on a 0.7% agarose gel (44). The DNA was then denatured and transferred onto nitrocellulose filters and hybridized to a ^{32}P-labeled plasmid probe by the Smith and Summers (53) modification of the Southern procedure (55). pAC400 was used as a probe to reveal forms of pSB344. Other plasmids were probed with their own ^{32}P-labeled DNA. ^{32}P labeling was done by the nick translation process (44).

Enzymes. Restriction endonucleases and DNA polymerase were purchased from New England BioLabs, Inc. (Beverly, Mass.) and Boehringer Mannheim Biochemicals (Indianapolis, Ind.), respectively. Enzymatic reactions were performed as directed by the supplier. ExoV (RecBC enzyme) was a generous gift of Andrew F. Taylor and Gerald R. Smith. Digestion with ExoV was performed by the method of Lackey and Linn (37).

Electron microscopy. pSB344 DNA was prepared by cesium chloride-EtBr centrifugation from the JC7623 derivative except that the upper rather than the lower band was harvested. To estimate the fraction of plasmid DNA, we subjected two samples to agarose gel electrophoresis, one untreated and one treated with *Kpn*I and *Sal*I nucleases to fragment the chromosomal DNA without digesting the plasmid DNA. By UV fluorescence of an EtBr-stained gel, we saw one band of DNA in the untreated sample, and this diminished about 50% upon treatment. Similarly, nuclease-treated upper-band DNA was prepared for electron microscopy by the method of Williams (74).

RESULTS

Effect of *recB recC sbcB* genotype on the nature of pSB344 DNA. The physical state of pSB344 DNA in *recB21 recC22 sbcB15* and related strains was investigated by hybridizing total DNA preparations, after electrophoretic fractionation, to a radioactive plasmid probe. pSB344 is a pACYC184 derivative whose *Sal*I site was inactivated by insertion of a 600-base-pair fragment of yeast ribosomal DNA (S. Broido, personal communication). This plasmid was originally used in this study because it is only moderately unstable in the *recB21 recC22 sbcB15* genetic background (data not shown). An autoradiogram of a hybridization experiment is presented in Fig. 1. Most plasmid DNA in *recB*$^+$ *recC*$^+$ *sbcB*$^+$ cells consisted of supercoiled circular monomers, although bands which corresponded to relaxed circular monomers and supercoiled circular dimers were visible (lane 1). An *sbcB15* mutation made little difference in this profile (lane 3). *recB21 recC22* mutations, however, caused 15% of the hybridizable material to appear as a new band, which we have labeled h.m.w. for high-molecular-weight plasmid DNA (lane 2). Addition of *sbcB15* to the *recB* and *recC* mutations led to more plasmid DNA associated with the h.m.w. band. In

TABLE 2. Plasmids

Name	Reference	Native replicon[a]	Derived plasmid	Reference[a]
pAC400	This work[b]	p15	pACYC184	6
pAL231	36	pMB1	pBR322	5
pKC31	R. McMacken[c]	Lambda	None[c]	
pSB344	S. Broido	p15	pACYC184	6
pSF117	22	pMB1	pBR322	5
pSF119	22	pMB1	pBR322	5

[a] The replicons listed are the original plasmids. Except for lambda all are related to ColE1 by similarity.

[b] See Materials and Methods.

[c] Plasmid pKC31 was made by R. N. Rao (Eli Lilly & Co., Indianapolis, Ind.) by cloning the appropriate restriction nuclease fragment of λ *c*I857 *S*am7 (D. Thaler, personal communication). A similar plasmid, pRLM4, has also been made (76).

addition to the h.m.w. band, hybridizable material is present at the origin.

To ascertain the generality of our finding we tested other plasmids. Using pAL231 (36), a derivative of pBR322 (5), as both a test plasmid and a probe, we found h.m.w. DNA in both *sbcB*$^+$ and *sbcB15* strains carrying *recB21 recC22* but not in *recB*$^+$ *recC*$^+$ strains (data not shown). Similarly, pKC31, a λ dv-like derivative of lambda, showed h.m.w. DNA in both *sbcB*$^+$ and *sbcB15 recB21 recC22* strains (data not shown).

Molecular nature of h.m.w. form of pSB344 DNA. The experiment in Fig. 1 was performed with total cell DNA. Plasmid DNA is usually harvested by the cleared lysate method (11) followed by isopycnic centrifugation in the presence of EtBr. We performed such a standard isolation of plasmid DNA from the *recB21 recC22 sbcB15* strain containing pSB344 and examined the two resulting fractions produced by centrifugation. Figure 2A is an autoradiogram of the two fractions analyzed by Southern hybridization with radioactive pAC400 as a probe. The h.m.w. material was exclusively located in the upper (i.e., lower density) fraction thought to consist of noncovalently closed circular DNA and chromosomal DNA fragments (lane 2). The hybridizable DNA at the origin was about equally divided between the two fractions (lanes 1 and 2). Since some nonmigratory material was found in the lower (i.e., more dense) fraction, we suggest that this may consist at least in part of catenated covalently closed circular plasmid DNA.

Rapid plasmid DNA isolation procedures involving DNA denaturation by heat (24) or alkali (4) do not yield h.m.w. plasmid DNA (data not shown). This indicates that h.m.w.

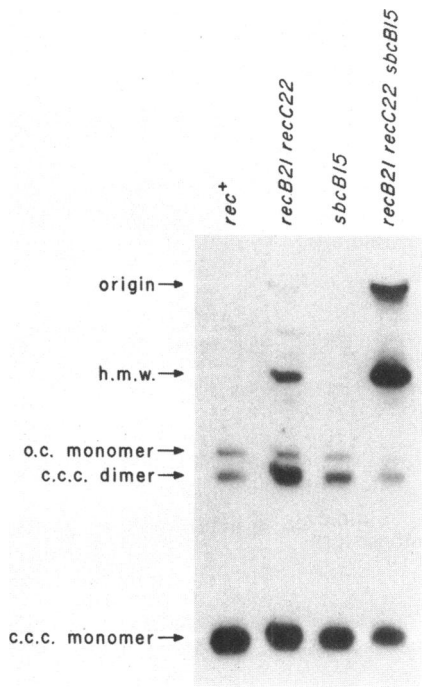

FIG. 1. Southern analysis of pSB344 molecular forms in *E. coli* strains. Total DNA preparations of cells of the designated genotypes harboring pSB344 were electrophoresed and subjected to Southern hybridization (55), using radioactive pAC400 as a probe. The locations of covalently closed circular (c.c.c.) monomers and dimers and of relaxed (o.c.) monomers are indicated. The electrophoretic mobility of high-molecular-weight (h.m.w.) pSB344 derivatives is the same as that of the bulk of nondigested chromosome DNA.

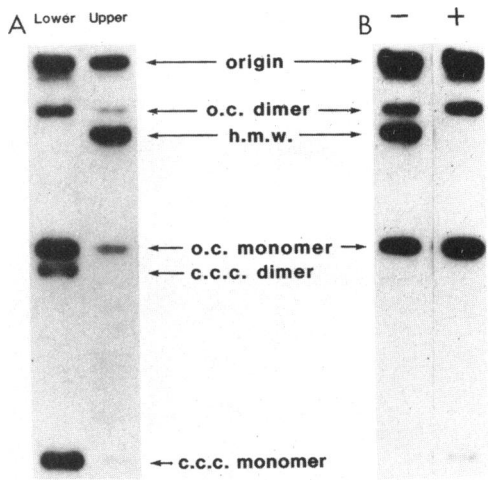

FIG. 2. Cesium chloride-EtBr density gradient fractionation of pSB344 DNA and digestion of h.m.w. DNA by ExoV. (A) Clear lysate of JC7623 cells harboring pSB344 was fractionated by CsCl-EtBr density gradient centrifugation. The band marked lower contained DNA of higher density than the band marked upper. These were withdrawn with a syringe, and their DNA was purified and subjected to Southern hybridization analysis. See the legend to Fig. 1 for further information. (B) DNA from the upper band of a CsCl-EtBr gradient, such as that shown in panel A, was treated with ExoV. Untreated DNA was added to lane −, and treated DNA was added to lane +. Southern analysis was performed as stated in the legend of Fig. 1.

DNA might consist of linear molecules, circles with single-strand gaps, or nicked circles. Since h.m.w. DNA did not migrate as any of the nicked or covalently closed circular multimers, we favor the hypothesis of linear molecules or gapped circles.

Both of these forms would be digestible by RecBC enzyme (61). To test for the presence of these forms, we treated with RecBC enzyme an upper fraction of plasmid DNA, isolated in a similar fashion to that shown in Fig. 2A. The h.m.w. band disappeared after digestion, while the other plasmid DNA components of the fraction appeared to be untouched (Fig. 2B). Because of the substrate specificity of RecBC enzyme (61), this result rules out the identity of h.m.w. DNA as some form of covalently closed or nicked circular DNA and makes likely its identity as linear multimers or gapped circles. Since S1 nuclease digestion did not change the mobility of h.m.w. DNA (data not shown), we favor the hypothesis of linear multimers. The plasmid DNA at the origin was not digested by RecBC enzyme; it may be catenated nicked circular or very highly branched DNA.

To examine h.m.w. DNA by electron microscopy, we attempted unsuccessfully to isolate it from agarose gels by several techniques. Finally, we examined DNA from an upper fraction of a CsCl-EtBr gradient (see Materials and Methods). The preparation contained many short (<6 kilobases) linear molecules, probably restriction fragments of the chromosome, and larger molecules in the range from 14 to 30 kilobases, i.e., plasmid trimer to heptamer length. Twenty-five of the long molecules were photographed. All were linear. No circular molecules were seen. Twenty-four of the molecules were clearly unbranched, and one had a possible branch.

Restriction endonuclease digestion of the upper fraction of plasmid DNA yielded DNA with the electrophoretic mobility of linear monomers and faster-migrating fragments of

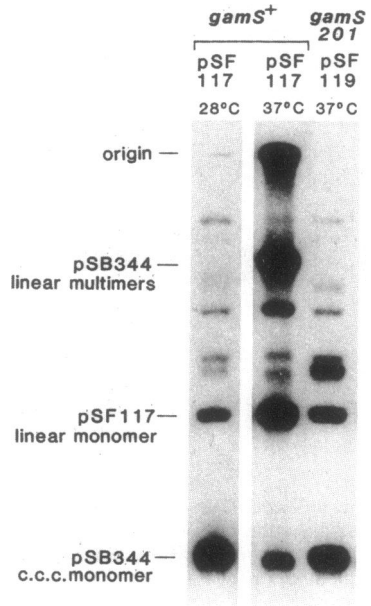

FIG. 3. Derepression of *gamS*$^+$ leads to accumulation of plasmid linear multimers. Cultures of JC11451 (*sbcB15*) cells harboring pSB344 and pSF117 or pSF119 were grown overnight at the indicated temperature. Total DNA preparations were treated with *Sal*I to convert pSF117 and pSF119 into linear monomers. Subsequent analysis was performed as described in the legend to Fig. 1.

plasmid DNA which were visible as a smear (data not shown). These fragments were absent from a digest of the lower fraction and were eliminated from the upper fraction if, before digestion with restriction endonuclease, the sample was digested with ExoV (data not shown). Such fragments would be produced from linear plasmid DNA with circularly permuted ends.

We think that all these results indicate that the h.m.w. band consists of linear multimers with circularly permuted ends. We cannot rigorously exclude the possibility that there are also circular multimers with single-strand gaps or rolling circular molecules in h.m.w. DNA. Our electron microscopy experiment, however, makes it plausible that these are not a major fraction of the molecules. Consequently, we will henceforth speak of h.m.w. DNA as linear multimers.

Migration of the linear multimers as a single band would seem to indicate that they are reasonably homogeneous in length. Agarose gels of 0.7% cannot, however, resolve molecules above 20 kilobases in length (44), and we saw a considerable range of length with the electron microscope. Since the linear multimers comigrated with fragments of the chromosome, we think it possible that they are formed during isolation by breakage of longer linear multimers.

Formation of linear plasmid multimers by induction of *gam* activity. RecBC enzyme can be inhibited in vivo and in vitro by the product of the *gam* gene of lambda (66, 67). Conditional expression of *gam* might be expected, therefore, to lead to conditional formation of linear plasmid multimers. To test this, we used pSF117, a plasmid carrying a shortened form of *gam* (*gamS*$^+$), and the *c*I857 gene (22). Thus, *gamS* on pSF117 is transcribable from the p_R promoter of lambda, which is under control of the thermally labile *c*I857 product. *gamS* transcription can be derepressed in a thermally regulatable way (22). Derepressing *gamS*$^+$, in cells carrying pSF117, converts *recB*$^+$ *recC*$^+$ cells to partial *recB recC* phenocopies in that ExoV activity cannot be detected in vitro (22) and UV resistance decreases but not to *recC* mutant levels (S. A. Friedman and J. B. Hays, personal communication). Presumably, partial inactivation of RecBC enzyme occurs in vivo because the smaller *gamS* rather than the larger *gamL* product is encoded by pSF117.

The effect of *gam* activity on the distribution of pSB344 molecular forms in an *sbcB* mutant (JC11451) is presented in Fig. 3. *sbcB15* cells harboring pSB344 and pSF117 were grown at 28 and 37°C. Total DNA preparations were treated with *Sal*I endonuclease, which has one site on pSF117 and none on pSB344, and were subjected to agarose gel electrophoresis. The fragments were then hybridized to pAC400 by the Southern method (55). pSB344 multimers were not observed when *gamS*$^+$ was repressed (at 28°C) but were observed when it was derepressed by growth at 37°C. DNA prepared from a culture harboring pSB344 and pSF119, a *gamS201* derivative of pSF117, served as a control, since the *gam* allele which is derepressed in this case encodes an inactive protein (22). No linear pSB344 multimers were observed in the control DNA.

Kinetics of linear multimer synthesis. The kinetics of linear plasmid multimer accumulation after derepression of *gamS*$^+$ was investigated in *sbcB15* cells (JC11451) harboring pSF117 (Fig. 4). In this system pSF117 served both as the carrier of *gamS*$^+$ and as the test plasmid. Cells in the logarithmic phase of growth at 28°C were transferred to 37°C for the designated period of time. Total DNA was prepared, and equal amounts (3 μg) were digested with *Kpn*I (which does

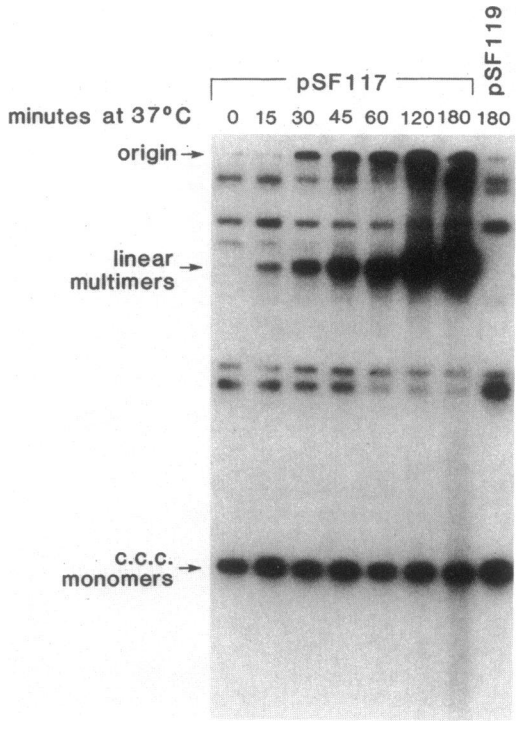

FIG. 4. Kinetics of accumulation of linear multimers of pSF117 after derepression of *gamS*$^+$ in *sbcB15* cells. Samples of an exponential-phase culture of JC11451 harboring pSF117 or pSF119 were transferred from 28 to 37°C for the indicated period of time. Total DNA was prepared from the cells. Equal amounts of DNA (3 μg) were treated with *Kpn*I to fragment the chromosome but not the plasmid and were subjected to Southern hybridization analysis with radioactive pSF117 as a probe. c.c.c., Covalently closed circular.

not cleave pSF117) and subjected to Southern hybridization with radioactive pSF117 as a probe. An autoradiogram of the Southern blot (Fig. 4) indicates that no linear multimers were present when the cells were grown at 28°C. Fifteen minutes after transfer from 28 to 37°C, linear multimers were visible, and they increased in amount with time at 37°C. After 45 min linear multimers represented most of the hybridizable material in the cell. No linear multimers were observed when a similar strain carrying pSF119 in place of pSF117 was incubated at 37°C.

The substantial increase in hybridizable material at the linear multimer band was not accompanied by a corresponding decrease in the hybridizable material in the supercoiled circular monomer bands, suggesting an increase in the total amount of plasmid DNA after derepression of *gamS*$^+$. To determine the relative amount of plasmid DNA in the preparations presented in Fig. 4, the same amounts of total DNA preparations were digested with *Eco*RI, which has a unique site on pSF117, electrophoresed in the presence of EtBr, and photographed (Fig. 5, insert). The results (Fig. 5) show that the ratio of plasmid to chromosome DNA increased about 25-fold so that after 3 h about 50% of the total DNA was plasmid DNA. The kinetics of accumulation approximated an exponential function until about 60 min and thereafter appeared to be linear. During the entire period there was little change in the amount of DNA present as plasmid circular supercoiled monomers relative to chromosomal DNA (Fig. 5). This means that pSF117 DNA must have accumulated in the other forms we noted, i.e., linear multimers and nonmigratory DNA. No accumulation of pSF119 DNA occurred, indicating that a functional *gamS*$^+$ gene was required.

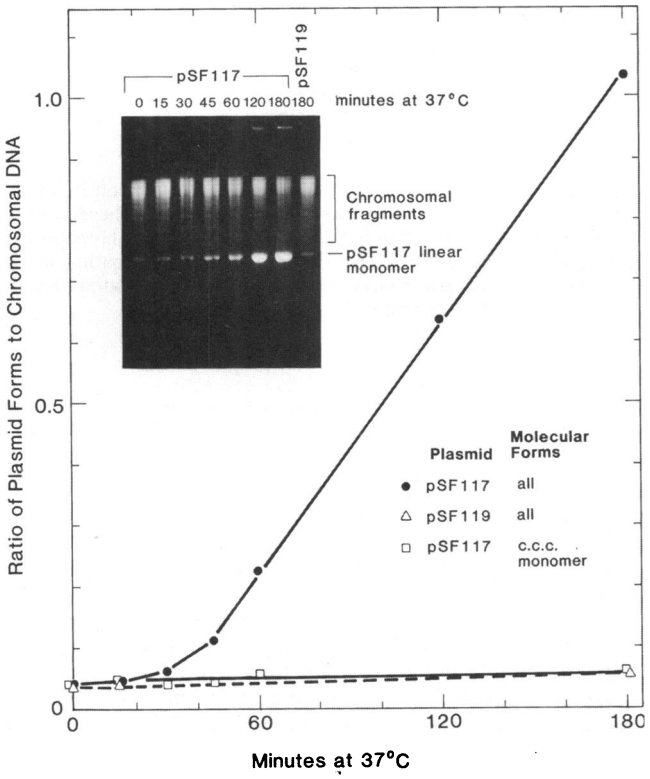

FIG. 5. Synthesis of pSF117 DNA after derepression of *gamS*$^+$ in *sbcB15* cells. Cells were obtained as described in the legend to Fig. 4. Equal amounts (3 μg) of total cell DNA were prepared and digested with *Eco*RI to fragment the chromosome and monomerize plasmid DNA and were subjected to agarose gel electrophoresis in the presence of EtBr (44). The electropherogram was photographed under UV illumination (insert), and the negative was scanned with a microdensitometer. The ratio of linear plasmid monomer DNA to chromosomal DNA fragments longer than the linear plasmid was determined for each sample as described in Table 3, footnote *c*. Ratios of circular plasmid monomers to total plasmid DNA were determined at each time point by cutting out the nitrocellulose bands obtained by hybridization with ^{32}P-labeled probe (Fig. 4) and counting them in a Beckman scintillation counter. These ratios were multiplied by corresponding ratios obtained from the insert to yield the ratios plotted. c.c.c., Covalently closed circular.

TABLE 3. Effect of mutations on occurrence of plasmid linear multimers

	Genetic background[a]	*rec*, *sbc*, and *xon* mutations[b]	% Linear multimers[c]
A.	AB1157	None	<1.0
	JC5519	*recB21 recC22*	14
	JC11451	*sbcB15*	<1.0
	JC7623	*recB21 recC22 sbcB15*	74
B.	JC15501	*recD1009 sbcB15*	63
	JC8267	*recB21 recC22 xonA6*	52
C.	JC15329	*recB21 recC22 sbcB15* Δ(*recA-srlR*)*306*::Tn*10*	2.0
	JC11834	*recB21 recC22 sbcB15 lexA3*	53
	JC8111	*recB21 recC22 sbcB15 recF143*	38
	JC12122	*recB21 recC22 sbcB15 recJ284*::Tn*10*	47
D.	JC11445	*sbcA23*	<1.0
	JC8679	*recB21 recC22 sbcA23*	60
	JC9604	*recB21 recC22 sbcA23 recA56*	61

[a] All strains carried pSB344. The names of plasmid-free immediate ancestors are given.
[b] For additional mutations see Table 1.
[c] Determined by microdensitometer tracing, cutting out the peaks corresponding to the plasmid forms from a photocopy of the tracing, and weighing the papers. The numbers are averages of values from three to eight experiments.

Effect of various mutations on amount of linear plasmid multimers. To learn some of the factors influencing the amount of linear plasmid multimers in exponentially growing cultures, we tested several genotypes. Table 3 presents the percentage of total plasmid DNA found as linear multimers as determined by microdensitometer scanning of autoradiograms of Southern hybridization experiments similar to the ones presented in Fig. 1. Results for the standard genotypes with which we were dealing are presented in Table 3A.

In Table 3B we present the effect of substituting *recD1009* for *recB21 recC22*. This substitution had no appreciable effect on the amount of linear multimers. In vivo *recD1009* affects phenomena attributable to the ExoV activity of RecBC enzyme (e.g., T4 gene 2 mutations are suppressed), but the cells are recombination proficient and UV resistant (7; S. K. Amundsen, A. F. Taylor, A. M. Chaudhury, and G. R. Smith, Proc. Natl. Acad. Sci. USA, in press). This would appear to indicate a differential effect of *recD1009* on the in vivo activities of RecBC enzyme (7; Amundsen et al., in press). If that appearance is confirmed experimentally, we will conclude that it is the ExoV activity of RecBC enzyme, and not the other activities, that inhibits formation of linear multimers. It is, however, possible that the differential effect in vivo reflects a threshhold phenomenon and is caused by an equal decrease in all activities of RecBC enzyme. If that

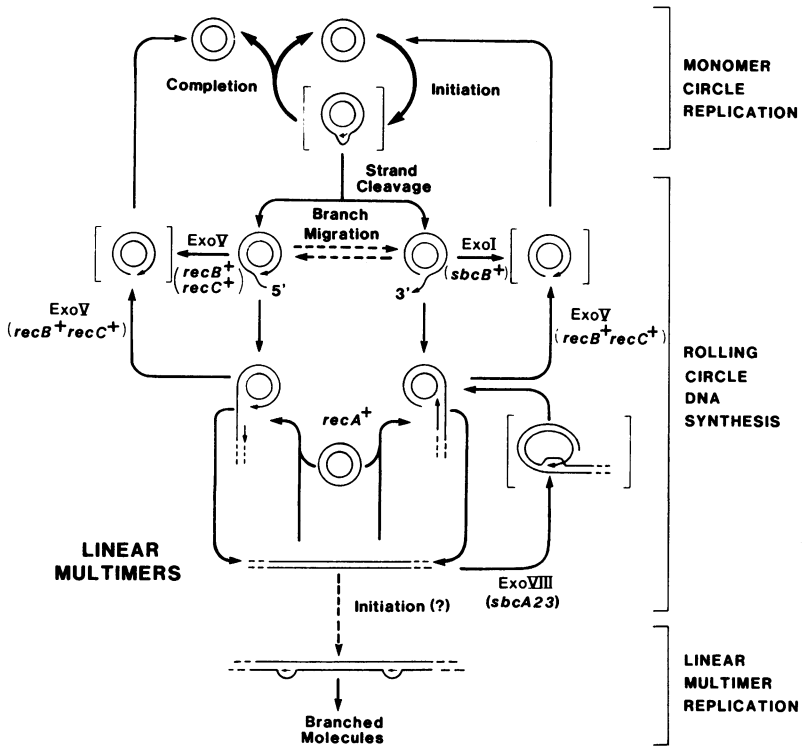

FIG. 6. Hypothetical roles of ExoV (RecBC enzyme) and exonuclease I (SbcB enzyme) in plasmid DNA metabolism. Concentric circles represent duplex circular plasmid DNA, and parallel lines represent duplex linear plasmid DNA. An arrowhead associated with these symbols represents a 3′ terminus of a DNA strand. Dashed lines associated with these symbols represent an indefinite extent of DNA. Hypothetical intermediates that are not critical to the argument are in brackets and are included for illustration. Arrows represent reaction paths and not necessarily single enzyme reactions. Dashed arrows represent possible reaction paths. Names of enzymes and genes are indicated near the reaction path arrows with which they are associated by hypothesis. See text for further description. Exo, Exonuclease.

is confirmed in vitro, we will not be able to conclude which activity is responsible. In Table 3B we also present the effect of substituting *xonA6* for *sbcB15*. *xonA* is the same gene as *sbcB*. Although *xonA6* suppresses *recB21 recC22* less completely than *sbcB15* (32), it acts similarly to increase the percentage of linear plasmid multimers.

Table 3C shows the effects on linear multimer accumulation of adding various mutations to the *recB21 recC22 sbcB15* background. Deletion of *recA* led to a 35-fold reduction in the amount of linear plasmid multimers. To test the possibility that $recA^+$ is required to derepress the *lexA* regulon, we used *lexA3* which encodes a noncleavable repressor (39). The high percentage of linear multimers was not affected. To test the possibility that the requirement for $recA^+$ implied a requirement for all the genes of the RecF pathway of conjugational recombination, we tested *recF143* and recJ284::Tn*10*. These mutations blocked conjugational recombination in the *recB21 recC22 sbcB15* background as did *recA13* (25, 42), but they only caused a small reduction in the amount of linear plasmid multimers.

Table 3D shows the effects of *sbcA23* used as a substitute for *sbcB15*. By itself *sbcA23* did not lead to the accumulation of linear multimers, but like *sbcB15* it led to greater accumulation when *recB* and *recC* were mutant. In the *recB21 recC22 sbcA23* background *recA56* had no apparent effect on accumulation of linear multimers. This contrasts with the strong effect of the deletion of *recA* in the *recB21 recC22 sbcB15* background. We think this means that there is a difference in *recA* dependence of accumulation in the two genetic backgrounds, but we can be sure of this only when we test the same *recA* mutation in both backgrounds.

DISCUSSION

Plasmids normally replicate in wild-type *Escherichia coli* cells to yield circular monomers (30). In this paper we demonstrate, for plasmids of the ColE1 type, that RecBC enzyme inhibits a process whose major product is linear multimers. For this demonstration we used both strains carrying mutations inactivating RecBC enzyme and strains carrying a plasmid encoding a mutant form of lambda *gam* protein which nonetheless inhibited RecBC enzyme. By examining *sbcB15* and *sbcA23* mutants we found that both mutations increased the amount of plasmid linear multimers. We also found that the linear multimer accumulation in the *sbcB15* mutant differs from that in the *sbcA23* mutant in its $recA^+$ dependence. Finally, we found that, unlike monomer circle replication, production of plasmid linear multimers is not effectively controlled by the mechanism which controls monomer circle copy number.

To rationalize these findings, we present in Fig. 6 a plausible outline of the steps which may be involved. We hypothesize that an intermediate in theta-type monomer circle replication is sometimes degraded to an open circular by-product with a redundant single strand. The redundant strand can be removed by exonuclease or RecBC enzyme depending on whether a 3′ or 5′ terminus is available. Alternatively, the by-products can act as substrates to

initiate either of two forms of rolling-circle DNA synthesis. RecBC enzyme, however, would normally degrade the linear product of this synthesis, preventing its accumulation. In the absence of RecBC enzyme, rolling-circle DNA synthesis would lead to a net increase in linear DNA. The increase would be stimulated by the absence of exonuclease I because more by-product molecules would initiate rolling-circle DNA synthesis. In the presence of exonuclease I more initiation could occur by *recA*-dependent recombination between linear end products and monomer circles or by *recA*-independent exonuclease VIII-catalyzed intramolecular recombination of the linear multimer products. To explain the presence of nonmigratory plasmid DNA, we included the possibility that multiple initiations of DNA synthesis on a linear multimer molecule could produce a highly branched molecule which would be expected not to migrate under the electrophoresis conditions we employed.

In proposing this, we purposefully followed the outlines of a proposal for the influence of RecBC enzyme on lambda phage replication (17). The similarity between ColE1-type plasmids and lambda is supported by our showing that a plasmid derived from λ dv produces linear multimers in a *recB21 recC22 sbcB15* strain. We also showed that a short form of the lambda *gam* gene, whose wild-type product is necessary to allow production of linear phage multimers, has a similar effect on ColE1-type plasmids. Furthermore, we showed that the presence of exonuclease VIII, which is isofunctional to the lambda *redX* exonuclease (26, 33), stimulates formation of linear multimers just as does the *redX* product in the λ replication system (17, 55). We note, however, one important difference. Rolling-circle DNA synthesis enhances the viability of lambda because the linear multimer product is a substrate for virion production (18, 56), while it is likely to decrease the viability of cells harboring plasmids because it is not subject to synthesis initiation control by the control product RNA-I (see below).

In Fig. 6 we interpreted the *recA* dependence of linear multimer synthesis in the *recB21 recC22 sbcB15* background as reinitiation of rolling-circle synthesis owing to intermolecular recombination between linear end products and monomer circles. The small effects of *recF* and *recJ* mutations on linear multimer accumulation might argue against a major role for *recA*-dependent recombination in the reaction, but it is possible that *recF*- and *recJ*-dependent steps are superfluous in the linear-by-circular recombination shown in Fig. 6 (see below). We cannot rule out a *recA recF*- and *recJ*-dependent pathway for initiating rolling-circle replication by recombination of two circular monomers. By testing the *lexA3* mutation we ruled out the possibility that *recA* is necessary to derepress the *lexA* regulon. We did not, however, rule out another regulatory role for *recA*, nor did we rule out the possibilities that *recA* protein stabilizes a replication intermediate, as proposed for stable DNA replication (29), or protects the linear final products from degradation by the remaining DNA exonucleases, e.g., exonuclease III and exonuclease VII.

The negligible effect of substituting *sbcA23* for *sbcB15* we also interpreted as reinitiation of rolling-circle replication by intramolecular recombination of the linear multimers. The *recA* independence of this reinitiation process may be due to the dispensibility of *recA* product for intramolecular recombination (19, 35). Alternatively, dispensibility may be due to a partially active *recA*-product-like protein produced as a result of the *sbcA* mutation. Such a product might be an analog of the *redB* (beta) product of lambda (28).

We showed that it is possible to study the kinetics of formation of linear multimers by derepressing a gene whose product inhibits RecBC enzyme. After derepression of *gamS*$^+$, linear plasmid multimers could be detected in less than half a generation time and accumulated thereafter. During the period of accumulation, no major change in the ratio of plasmid circular monomers to chromosomal DNA was apparent. This is consistent with our hypothesis because rolling-circle DNA synthesis should not be subject to normal replication control. Such control is exerted by a small antisense RNA (RNA-I) which combines with the preprimer transcript and inhibits primer formation (64, 65). Continuation of rolling-circle DNA synthesis is expected to be independent of such priming events. The increasing copy number of the RNA-I structural gene might be expected to cause a decrease in the frequency of monomer circles, since new rounds of theta-type replication would be blocked. Linear plasmids make poor templates for RNA-I transcription, however (77), so that the concentration of RNA-I would increase more slowly than its gene copy number. Theta-type replication would therefore continue unabated. A limit is expected when the low transcription rate on linear multimers begins to increase the RNA-I concentration. Subsequent loss of monomer circles through segregation may lead to segregation of plasmid-free cells. By correlating changes in plasmid DNA forms with the segregation of plasmid-free cells we hope to shed light on the causes of plasmid instability.

Our finding that ColE1-type plasmids can synthesize linear multimers in the absence of RecBC enzyme provides an explanation for several recent findings. For example, linear plasmid multimers occur in transducing particles of Mu and T4 phages (31, 60). Since infection by both phages leads to inhibition of RecBC enzyme (50, 68), it is logical to propose that linear plasmid multimer synthesis is activated during infection. The dramatic increase in the net amount of plasmid DNA after inactivation of RecBC enzyme may also explain the observation that colicin production in cells carrying the ColE1 derivative pRSF2124 is enhanced under these conditions (C. L. Bassett and S. R. Kushner, personal communication). Since *cea* is controlled by the *lexA* repressor (13, 14), an increase in plasmid copy number should titrate the repressor and derepress colicin synthesis. Under these conditions one might also expect to observe derepression of the entire *lexA* regulon including *lexA* itself.

Finally, we note that our discovery of linear plasmid multimers allows a new interpretation of the finding that *recB* and *recC* mutations stimulate *recF*$^+$-independent plasmid recombination (12, 19). We now infer that this type of recombination uses linear plasmid multimers as a substrate. Further, we infer that the *recF*$^+$-dependent plasmid recombination pathway occurs between and within circular molecules. We can also now reinterpret the finding of *sbcA* mutant stimulation of plasmid recombination in the *recB21 recC22* and not in the *recB*$^+$ *recC*$^+$ background (12, 19, 35). *sbcA* mutations allow synthesis of exonuclease VIII which can act on linear but not circular molecules (26), and linear molecules are present as recombination substrates only in the *recB recC* mutant strains. The use of linear molecules as recombination substrates in a *recB21 recC22 sbcA23* genetic background was recently demonstrated by Symington et al. (L. S. Symington, P. Morrison, and R. Kolodner, J. Mol. Biol., in press).

In this paper we demonstrated that ColE1-type plasmids can undergo a type of DNA synthesis which yields linear multimers and that this type of synthesis is prevented or inhibited by RecBC enzyme. The question whether other

bacterial replicons, such as F, R1, and *oriC* plasmids, can undergo a similar type of replication in *recB recC* mutants is under investigation.

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