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Exonucleases I, III, and V Are Required for Stability of ColE1-Related Plasmids in *Escherichia coli*

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The stability of two ColE1-related plasmids (pRSF2124 and pMB9) was examined in strains of *Escherichia coli* multiply deficient in exonucleases I (*sbcB*), III (*xthA*), or V (*recB recC*). Any combination of exonuclease I, III, and V deficiency resulted in dramatically decreased stability of both pRSF2124 and pMB9. Inactivation of the RecF pathway by introducing either *recF* or *recJ* mutations to the *recB recC sbcB* background resulted in nearly wild-type levels of stability for both plasmids. In contrast, the introduction of *uvrD3 uvr-257*, *uvrE100*, or *recL152* into the *recB21 recC22 sbcB15* strain did not affect plasmid stability. Furthermore, the amount of plasmid DNA recovered from pRSF2124 or pMB9 transformants of a *xthA1 sbcB15* strain was strikingly reduced relative to that of a wild-type control. Taken together, these results suggest that some aspect of DNA repair is required for stable maintenance of ColE1-related plasmids in *E. coli*.

Although transformation of *Escherichia coli* K-12 with ColE1 and related plasmids has been well documented, surprisingly little is known about the molecular mechanism of the process. Entry of DNA into the cell is Ca^{2+} dependent, facilitated by mild heat shock, and appears to be unaffected by the conformation of the plasmid DNA (5, 6, 19). Kretschmer et al. (14) analyzed the kinetics of plasmid uptake and suggested that a single molecule was sufficient for successful transformation, although more than one plasmid molecule could be taken up by a competent cell (5). Weston et al. (28) observed that plasmid DNA became tightly bound to the outside of *E. coli* cells in the presence of Ca^{2+} and other divalent cations. After heat shock, only 0.1 to 1% of the plasmid DNA became DNase resistant when saturating amounts of plasmid were used. As yet, the host functions involved in the mechanisms of DNA entry have not been identified.

Maintenance of plasmid molecules after cell entry appears to depend on both replication of plasmid molecules and segregation of plasmid progeny during host cell division. For example, Kingsbury and Helinski (13) demonstrated that replication of ColE1 required host DNA polymerase I. When ColE1 synthesis was inhibited in a temperature-sensitive *polA12* strain, loss of plasmid molecules was observed after a short lag period. On the other hand, Meacock and Cohen (20) showed that certain deletion derivatives of pSC101 were considerably less stable than the parent plasmid. The kinetics of plasmid loss were not consistent with those predicted for random segregation, suggesting that the deleted regions were required for efficient partitioning.

Plasmid instability may also be related to other aspects of DNA metabolism. Barbour et al. (J. Supramol. Struct., Suppl. 2, p. 63, 1978) reported that F′ *lac* plasmids were unstable in *recB recC*-deficient (exonuclease V^-) hosts and that induction of β-galactosidase enhanced plasmid loss. Introduction of either *recA* or *recF* mutations into the exonuclease V-deficient host partially restored stability. It was suggested that inefficient repair of plasmid molecules damaged during transcription resulted in the observed instability. Others have shown that ColE1-related plasmids are not stably maintained in *recB recC sbcB*-deficient (exonuclease V^-, exonuclease I^-) hosts (10, 22, 27). As with F′ *lac* plasmids, stability was restored upon introduction of either *recA* or *recF* mutations into the *recB21 recC22 sbcB15* background (22).

In the present study, the stability of two ColE1-related plasmids was examined in a variety of hosts deficient in enzymes associated with genetic recombination and DNA repair. Stability of pRSF2124 and pMB9 was shown to be dramatically reduced in strains deficient in any combination of exonucleases I, III, and V. Furthermore, introduction of a *recJ* mutation to a strain deficient in both exonucleases I and V restored stability of the three plasmids tested to the same extent as a *recF* mutation.

Chemicals and enzymes. Reagents were obtained from the following sources: CsCl (high purity), Penn Rare Metals; Sarkosyl NL-97, Geigy; "Ultra pure" $(NH_4)_2SO_4$, Schwarz/Mann; ethidium bromide, Calbiochem-Behring; tetracycline and amino acids, Sigma Biochemical Co.; ampicillin (Omnipen), Wyeth Pharmaceutical Co.; tryptone, yeast extract, and agar, Difco Laboratories; and agarose, SeaKem.

Lysozyme (40,000 U/mg) was obtained from Worthington Diagnostics; RNase A (60 Kunitz U/mg) was purchased from Sigma; pronase (89,000 proteolytic units/g) was obtained from Calbiochem-Behring; *Eco*RI (10 U/μl) was purchased from Bethesda Research Laboratories.

Media and buffers. Luria broth, LC top agar, and M56 buffer were used as previously described (15). Luria broth agar plates were made by the addition of 20 g of agar to each liter of Luria broth. Ampicillin (Ap) or tetracycline (Tc) were added to final concentrations of 20 μg/ml. TA buffer (pH 8.05) contained 40 mM Tris, 2 mM EDTA, 20 mM sodium acetate, and 10 mM NaCl.

Bacterial strains and plasmids. The strains used in this study were all derivatives of *E. coli* K-12 (Table 1). Inheritance of *rec* mutations was determined by sensitivity to UV light, using the replica plating techniques of Clark and Margulies (4). *xthA* mutations were analyzed by sensitivity to 0.08% methylmethane sulfonate in Luria broth agar plates. The plasmids used were the generous gift of D.

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TABLE 1. *E. coli* K-12 strains[a]

Strain	Genotype	Reference/source
AB1157	*arg pro thr leu his*	17
JC411	*thyA* ColE1	W. D. Rupp
JC5519	*arg pro thr leu his recB21 recC2*	17
JC7623	*arg pro thr leu his recB21 recC22 scB15*	17
JC7689	*arg pro thr leu sbcB15*	17
JC7694	*endA sbcB15*	17
JC8111	*recF143 arg pro thr leu his recB21 recC22 sbcB15*	9
JC8161	*recJ148 arg his pro thr leu recB21 recC22 sbcB15*	9
JC9253	*xthA1 arg his pro thr leu thy sbcB15*	A. J. Clark
JC9255	*xthA1 arg pro thr leu recB21 recB22 sbcB15*	A. J. Clark
SK1280	*uvrE100 arg his pro thr leu recB21 recC22 sbcB15*	S. R. Kushner
SK1298	*recL152 arg his pro thr leu recB21 recC22 sbcB15*	S. R. Kushner
SK1314	*xthA1 arg his*	29
SK1733	*xthA1 arg his pro leu thr recB21 recC22*	SK1717 × P1 (JC5519), Thy^+ transductant
SK1801	*uvrD3 uvr-257 arg his pro leu thr recB21 recC22 sbcB15*	S. R. Kushner
W3110	*thyA* ColE2	J. Inselburg

[a] Nomenclature conforms to that of Bachmann (1).

Vapnek. pRSF2124 (24) is ColE1 containing a Tn*3* insertion, and pMB9 is a derivative of a ColE1-like plasmid (pMB1). The details of its construction appear elsewhere (3).

Bacterial strains were transformed by the method of either Cohen et al. (6) or Kushner (16). pRSF2124 transformants were selected on plates containing Luria broth plus Ap; pMB9 transformants were selected on plates containing Luria broth plus Tc.

Plasmid stability was determined by growing the transformants in the absence of selection on Luria broth agar plates for approximately 20 generations. Antibiotic resistance and expression of other plasmid-encoded markers as well as markers pertinent to the host strain were then tested by replica plating.

Colicin E1 activity was detected by an overlay assay as described by Ozeki et al. (21). Patches surrounded by cleared zones were scored as cea^+ (colicin E1 activity).

Immunity to colicin E1 was determined by replica plating the transformants onto Luria broth agar plates previously spread with 0.2 ml of partially purified colicin E1. Resistance to colicin E2 was also examined to eliminate interference of spontaneous chromosomal resistance to colicin E1. The dilutions chosen for the immunity assays were derived from colicin titers determined in a separate experiment.

Partial purification of colicins E1 and E2. Colicin E1 and colicin E2 were partially purified by a modification of the procedure of Herschman and Helinski (8).

Extraction of plasmid and total DNA. pRSF2124 and pMB9 transformants of JC9253 were picked from selective plates and grown overnight at 37°C in Luria broth [JC9253(pMB9)] or Luria broth supplemented with 20 μg of ampicillin per ml [JC9253(pRSF2124)]. Plasmid DNA subsequently was extracted by the alkaline rapid screen method of Ish-Horowicz and Burke (11).

Total cellular DNA was prepared by the method of Davis and Vapnek (7). Overnight standing cultures of JC9253 transformants were grown in Luria broth (pMB9) or Luria broth plus Ap (pRSF2124) and used to inoculate flasks containing 1 liter of unsupplemented L broth. The cultures were grown with shaking at 37°C to a density of $\sim 5 \times 10^8$ cells per ml. Detection of plasmid DNAs was accomplished by Southern blot hybridization (25). ^{32}P-labeled plasmid DNAs were prepared according to Rigby et al. (23).

Plasmid stability in exonuclease-deficient strains. Fresh transformants of exonuclease-deficient strains were picked from selective medium and tested for plasmid stability. After a defined period of growth (~20 generations) in the absence of selection, transformants were tested for the expression of at least two plasmid-encoded genes. AB1157 (wild type) and strains deficient in either exonuclease I (JC7689), III (SK1314), or V (JC5519) showed no differences in their abilities to maintain either pMB9 or pRSF2124 (Table 2).

In contrast, neither pMB9 nor pRSF2124 transformants of SK1733 (*recB21 recB22 xthA1*) maintained drug resistance or other plasmid-encoded markers after growth in the absence of selection (Table 2). Similarly, none of the pMB9 transformants of JC9255 (*recB21 recC22 sbcB15 xthA1*) retained immunity to colicin E1 or resistance to Tc. Furthermore, no Ap^r pRSF2124 transformants of JC9255 were ever obtained with either transformation procedure.

pRSF2124 transformants of JC9253 (*sbcB15 xthA1*) retained resistance to Ap after growth in the absence of selection, but none of these strains produced active colicin. In addition, pMB9 transformants of JC9253 were immune to colicin E1, but showed levels of Tc resistance intermediate between those of Tc^r and Tc^s controls patched on the same plates. When these transformants were grown overnight in L broth supplemented with 20 μg of Tc per ml, no growth was observed.

The number of pMB9 transformants obtained with JC7623 (*recB21 recC22 sbcB15*) was greatly reduced compared with wild-type controls. Subsequent testing showed that all Tc^r transformants obtained were sensitive to UV light, in contrast to the expected UV^r phenotype (17). On the other hand, pRSF2124 transformants retained their UV^r phenotype but

TABLE 2. Effect of exonuclease I, V, or III deficiencies on plasmid stability[a]

Strain	Genotype	pRSE2124 Ap^r cea^+/total tested	pMB9 Tc^r ice^+/total tested
AB1157	Wild type	116/116	60/60
JC7689	*sbcB15*	74/74	109/109
SK1314	*xthA1*	20/20	80/80
JC5519	*recB21 recC22*	80/80	80/80
JC9253	*sbcB15 xthA1*	0/93[b]	0/114[c]
SK1733	*recB21 recC22 xthA1*	1/100	11/49
JC7623	*recB21 recC22 sbcB15*	0/29	ND[e]
JC9255[d]	*xthA1*	ND[e]	0/22
SK1280[d]	*uvrE100*	12/100	0/1
SK1801[d]	*uvrD3 uvr-257*	33/138	0/5
SK1298[d]	*recL152*	17/55	0/10
JC8161[d]	*recJ148*	20/27	52/58
JC8111[d]	*recF143*	84/85	62/80

[a] Stability of plasmids was determined by examining retention of plasmid markers in transformants grown in the absence of selection. The markers were analyzed as described in the text.

[b] All transformants were Ap^r but *cea*.

[c] All transformants were ice^+ but only weakly tetracycline resistant.

[d] Strain is also *recB21 recC22 sbcB15*.

[e] ND, Not determined, because no transformants were obtained.

rapidly lost their ability to produce colicin E1 and their resistance to Ap after growth on nonselective medium. Freshly picked pRSF2124 transformants of JC7623 did not grow when restreaked directly onto plates containing Luria broth plus Ap.

Addition of other alleles involved in DNA repair or recombination to the *recB21 recC22 sbcB15* genetic background affected plasmid stability to various degrees (Table 2). Stability of both plasmids was restored to nearly wild-type levels by either the *recJ148* or *recF143* allele. *recB21 recC22 sbcB15* transformants carrying either *uvrD3 uvr-257*, *uvrE100*, or *recL152* showed intermediate levels of stability with pRSF2124, but none of these three alleles affected pMB9 stability in the *recB21 recC22 sbcB15* background. Strains carrying *uvrD3 uvr-257*, *uvrE100*, *recJ148*, *recF143*, or *recL152* alone maintained both plasmids (data not shown).

Analysis of pRSF2124 and pMB9 plasmid DNA in JC9253. Transformants of strain JC9253 (*sbcB15 xthA1*) expressed Ap^r (pRSF2124) and were ice^+ (pMB9), but failed to produce active colicin (pRSF2124) and expressed Tc^r weakly (pMB9). Since marker analysis of plasmid stability gave conflicting results, plasmid DNA from five independent pRSF2124 and pMB9 transformants of JC9253 was isolated by the alkaline rapid screen technique of Ish-Horowicz and Burke (11). Plasmid DNA could not be detected from these transformants, although both purified plasmid DNA and plasmid DNA from a wild-type strain were clearly visible (data not shown). To verify this result, total cellular DNA isolated from two independent pRSF2124 transformants of JC9253 was digested with *Eco*RI and subjected to Southern blot analysis, using ^{32}P-labeled pRSF2124 plasmid DNA as a hybridization probe (Fig. 1). The results indicate that plasmid DNA was present in greatly reduced amounts in the *sbcB15 xthA1* strain, compared with the wild-type control strain. Similar results were obtained with pMB9 transformants (data not shown).

The results of the experiments presented here demonstrate that some combination of functionally active exonuclease I, exonuclease III, and exonuclease V is required for the maintenance of ColE1-related plasmids in *E. coli*. Strains lacking any two of these enzymes either could not maintain the tester plasmids, had dramatically reduced levels of plasmid DNA, or could not be transformed with these plasmids. Although it has been shown previously that exonuclease I (*sbcB*)-exonuclease V (*recB recC*) double mutants did not maintain ColE1 plasmids (10, 22, 27), it was not expected that the *sbcB xthA* and *recB xthA* strains would exhibit similar behavior.

It is unlikely that the absence of these exonucleases directly affects plasmid replication, since there is no evidence to suggest that they are individually required for ColE1 replication (26). Indeed, there is no phenotype associated with *sbcB* (exonuclease I) single mutants. The only common link between the nucleases is that they are associated with a variety of DNA repair pathways in vivo. Accordingly, reduced repair efficiency—whether it be for UV-induced photoproducts (*recB*, *recC*, and *sbcB*) or apurinic sites (*xthA*)—may lead to plasmid instability. Barbour et al. (J. Supromol. Struct., Suppl. 2, p. 63, 1978) have suggested a similar link between F plasmid instability and faulty repair of damage which might accumulate during transcription. Thus, the reduced amount of plasmid DNA isolated from *xthA1 sbcB15* strain JC9253 could reflect faulty or inefficient repair of plasmid DNA, indirectly resulting in reduced replication, increased degradation, or abnormal partitioning during segregation.

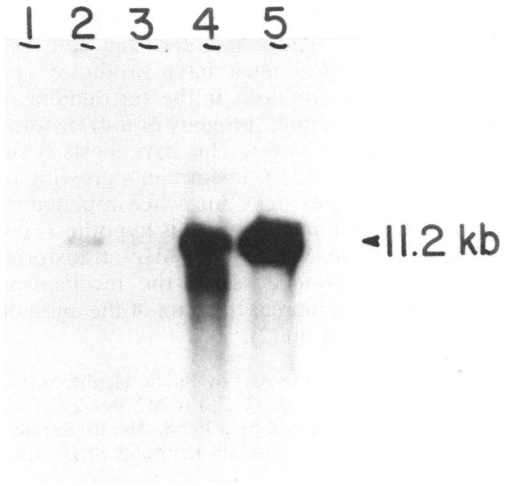

FIG. 1. Analysis of plasmid DNA from pRSF2124 transformants of JC9253. Total DNA was extracted by the method of Davis and Vapnek (7), digested with *Eco*RI, electrophoresed on a 0.8% agarose gel, and transferred to nitrocellulose paper as described by Southern (25). The filter was hybridized with ^{32}P-labeled (23) pRSF2124 plasmid DNA. Equivalent amounts of DNA were added in lanes 1 to 4. Lane 1, total DNA from JC9253; lanes 2 and 3, total DNA from two independent transformants of JC9253(pRSF2124); lane 4, total DNA from a wild-type control [AB1157(pRSF2124)]; lane 5, 0.4 μg of purified pRSF2124 plasmid DNA.

Plasmid instability in the *recB21 recC22 sbcB15* strain may arise through a different mechanism. Recent observations that the level of *recA* protein is increased in *recB sbcB* double mutants (12) suggest that genes under the control of the *lexA* repressor may be involved with ColE1 plasmid instability. These findings indicate that in *recB recC sbcB* strains, induction of the RecF pathway (normally repressed by *lexA*) could lead to the formation of plasmid intermediates which accumulate in the absence of exonucleases I and V and which cannot be stably maintained by the cell. Inactivation of this pathway by introducing a *recA*, *recF*, or *recJ* mutation (Table 2) prevents the formation of these plasmid intermediates, thereby increasing plasmid stability in *recB21 recC22 sbcB15* strains. Once more information is available regarding the molecular events of the RecF pathway, it should be possible to better understand the observations reported above.

Although exonuclease I-exonuclease V double mutants are phenotypically UV^r (17), all of the pMB9 transformants of JC7623 (*recB21 recC22 sbcB15*) were UV^s (see also reference 27). The most likely explanation for this phenomenon was the isolation of spontaneous mutations which inactivated the RecF pathway, since the *recF143* and *recJ148* alleles also stabilized pMB9 transformants of this strain (Table 2). In fact, when one of the UV^s transformants was analyzed genetically by a P1 transduction rescue experiment, it was shown to remain genotypically *recB21* and *sbcB15* (S. R. Kushner, unpublished data).

The fact that UV^r pRSF2124 transformants of JC7623 were obtained is most likely a consequence of the nature of ampicillin resistance. The mechanism of Ap^r involves pro-

duction of β-lactamase, a cell-surface enzyme which degrades the antibiotic before it enters the cell. pRSF2124 transformants of JC7623 must have produced enough β-lactamase to degrade antibiotic in the surrounding medium, thus allowing either "cured" progeny or untransformed cells to survive on selective plates. This hypothesis is supported by the fact that pRSF2124 transformants growing on selective medium failed to express Ap^r when replated on plates containing Luria broth plus Ap. If this hypothesis is correct, it could also explain why UV^r pMB9 transformants of JC7623 were not obtained, since the mechanism of Tc resistance involves an increased efflux of the antibiotic from cells that have taken it up (2).

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