Enhancement of *Escherichia coli* Plasmid and Chromosomal Recombination by the Ref Function of Bacteriophage P1

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Manuscript received October 10, 1988
Accepted for publication August 10, 1989

ABSTRACT

The Ref activity of phage P1 enhances recombination between two defective *lacZ* genes in the *Escherichia coli* chromosome (*lac*$^-$ × *lac*$^-$ recombination). Plasmid recombination, both *lac*$^-$ × *lac*$^-$ and *tet*$^-$ × *tet*$^-$, was measured by transformation of *recA* strains, and was also assayed by measurement of β-galactosidase. The intracellular presence of recombinant plasmids was verified directly by Southern blotting. Ref stimulated recombination of plasmids in *rec*$^+$ and *rec(BCD)* cells by 3–6-fold, and also the low level plasmid recombination in *recF* cells. RecA-independent plasmid recombination, either very low level (*recA* cells) or high level (*recB recC sbcA recA* cells), was not stimulated. Ref stimulated both intramolecular and intermolecular plasmid recombination. Both normal and Ref-stimulated *lac*$^-$ × *lac*$^-$ chromosomal recombination, expected to be mostly RecBC-dependent in wild-type bacteria, were affected very little by a *recF* mutation. We have previously reported Ref stimulation of *lac*$^-$ × *lac*$^-$ recombination in *recBC sbcB* bacteria, a process known to be RecF-dependent. Chromosomal recombination processes thought to involve activated recombination substrates, *e.g.*, Hfr conjugation, P1 transduction, were not elevated by Ref activity. We hypothesize that Ref acts by unknown mechanisms to activate plasmid and chromosomal DNA for RecA-mediated recombination, and that the structures formed are substrates for both RecF-dependent (plasmid, chromosomal) and Rec(BCD)-dependent (chromosomal) recombination pathways.

WE previously described the discovery and initial characterization of an activity, encoded by bacteriophage P1, that stimulates certain RecA-dependent recombination events in the *Escherichia coli* chromosome (WINDLE and HAYS 1986). Specifically, formation of Lac$^+$ papillae on colonies of the Konrad "hyper-rec-detection" strain KS391 (KONRAD 1977) as a result of recombination between its two defective *lacZ* genes (*lac*$^-$ × *lac*$^-$ recombination) was stimulated as much as a 100-fold by a single (unregulated) copy of this P1 *ref* (*r*ecombination *e*nhancement *f*unction) gene. LU, LU and GOTTESMAN (1989) independently discovered Ref activity on the basis of its stimulation of the RecA-dependent conversion of certain *gal*T: :IS1 bacteria to Gal$^+$.

Expression of the *ref* gene is controlled by an elaborate phage-encoded regulatory mechanism (WINDLE and HAYS 1986; WINDLE, LAUFER and HAYS 1988), but Ref action appears dependent on host recombination functions. Since there is no recombination even in Ref-overexpressing *recA*$^-$ bacteria, Ref appears not to be an independent recombination activity, such as phage lambda Red function (ECHOLS and GINGERY 1968; SIGNER and WEIL 1968). Ref is active not only in bacteria in which most recombination is RecBCD-dependent but also in those in which most recombination is thought to be RecF-dependent (WINDLE and HAYS 1986).

Ref$^-$ phages grow in and lysogenize both wild-type and *rec*$^-$ *E. coli* strains as well as wild-type phages do, so the utility of Ref activity for P1 remains to be determined. A number of RecA-dependent processes in *E. coli*, notably recombination of λ*red*$^-$ phages, are not markedly Ref-stimulated (WINDLE and HAYS 1986).

A better understanding of the mechanism(s) of Ref action would shed light on processes by which chromosomes are activated for homologous recombination, often the rate-limiting factor in determining frequencies (HAYS and BOEHMER 1978; KORBA and HAYS 1980; ZAGURSKY and HAYS 1983), and may illuminate host-virus interactions. Although *lac*$^-$ × *lac*$^-$ chromosomal recombination, observed as the formation of Lac$^+$ papillae on Lac$^-$ colonies (KONRAD 1977), provides a sensitive assay for Ref action, it

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TABLE 1

Bacteria, bacteriophages and plasmids

Number	Recombination genotype	Remainder of genotype/description	Source/reference
A. Bacterial strains			
AB1157	*rec*$^+$	*thr-1 leu-6 thi-1 galK2 proA2 argE3 rpsL-31 tsx-33 supE44 his-4*	A. J. CLARK
BW2033	*rec*$^+$	Hfr Hayes Δ(*argF-lacZ*)U169 *ϕ*80dIIΔ-*lacBK1*	P1(MPh30) × KS391 → Nm^r[Lac$^-$] → PhoA$^+$Nm^s precise excisant
BW2039	*rec*$^+$	Hfr Hayes Δ*lacZMS286*	P1(CG47) × KS391 → Tc^r[Su$^+$Lac$^-$] → Trp$^+$Tc^s precise excisant
C600	*rec*$^+$	*supE thi-1 thr leu lacY*	BACHMANN (1972)
CG47	*rec*$^+$	*supF trp*::Tn*10*	C. GRITZMACHER
CL503	*recB21 recC22*	as N99 but Δ(*argF-lacZ*)U169 *phoA*::Tn*5*	P1(MPh30) × JH314 → Nm^r[Lac$^-$]
CL504	*recB21 recC22*	CL503(λDL10)	This work
CL505	*recB21 recC22*	CL503(λRef$^+$504B)	This work
CL506	*rec*$^+$	MPh30(λDL10)	This work
CL507	*rec*$^+$	MPh30(λRef$^+$504B)	This work
CL508	*rec*$^+$	as C600 but Δ(*argF-lacZ*)U169 *phoA*::Tn*5*	P1(MPh30) × C600 → Nm^r[Lac$^-$]
CL510	*rec*$^+$	CL508(λDL10)	This work
CL511	*rec*$^+$	CL508(λRef$^+$504B)	This work
CL514	*recF143*	as V66 but Δ(*argF-lacZ*)U169 *phoA*::Tn*5*	P1(MPh30) × V66→Nm^r[Lac$^-$]
CL515	*recF143*	CL514(λDL10)	This work
CL516	*recF143*	CL514(λRef$^+$504B)	This work
CL517	*recA56*	DJ1(λDL10)	This work
CL518	*recA56*	DJ1(λRef$^+$504B)	This work
CL520	*recB21 recC22 sbcA23*	as JC8679 but Δ(*argF-lacZ*)U169 *phoA*::Tn*5*	P1(MPh30) × JC8697 → Nm^r[Lac$^-$]
CL522	*recF332*::Tn*3*	CL524(DL11)	This work
CL523	*recF322*::Tn*3*	CL524(λRef$^+$504B)	This work
CL524	*recF332*::Tn*3*	as KS391	P1(JC13285) × KS391 → Ap^r[UV^s; Tc^s]
CL525	*recB21 recC22 sbcA23 recA56*	*srl*::Tn*10* Δ(*argF-lacZ*)U169 *phoA*::Tn*5*	P1(G2) × CL520 → Tc^r[UV^{ss}]
CL526	*rec*(*BCD*)::Tn*5* *recF332*::Tn*3*	as KS391	P1(TSS18) × CL524 → Nm^r[UV^{ss}]
CL527	*rec*(*BCD*)::Tn*5* *recF332*::Tn*3*	CL526(λRef$^+$504B)	This work
CL528	*rec*(*BCD*)::Tn*5* *recF332*::Tn*3*	CL526(λDL11)	This work
CL530	*recB21 recC22 sbcA23*	CL520(λRef$^+$504B)	This work
CL532	*recB21 recC22 sbcA23 recA56*	CL525(λRef$^+$504B)	This work
CL534	*rec*$^+$	as AB1157 but Δ(*argF-lacZ*)U169 *phoA*::Tn*5*	P1(MPh30) × AB1157 → Nm^r[LacZ$^-$]
CL535	*rec*$^+$	CL534(λDL11)	This work
CL536	*rec*$^+$	CL534(λRef$^+$504B)	This work
CL550	*recF143*	as V66 but *pyrA*::Tn*10* Δ(*argF-lacZ*) U169 *phoA*$^+$	EG333 × CL514 → Tc^{r}Sm^r [Nm^{s}Pro$^+$Lac$^-$]
CL551	*recF143*	as CL550 but *rec*(*BCD*)::Tn*5*	P1(TSS18) × CL550 → Nm^r [UV^{ss}]
CL552	*recF143 rec*(*BCD*)::Tn*5*	CL551(λDL10)	This work
CL553	*recF143 rec*(*BCD*)::Tn*5*	CL551(λRef$^+$504B)	This work
CL554	*rec*$^+$	as N99 but Δ(*argF-lacZ*)U169 *phoA*::Tn*5*	P1(MPh30) × N99 → Nm^r[Lac$^-$]
CL555	*rec*$^+$	as CL554 but *pyrA*::Tn*10 phoA*$^+$	EG333 × CL554 → Tc^{r}Sm^r [Nm^{s}Pro$^+$Lac$^-$]
CL556	*rec*(*BCD*)::Tn*5*	as CL555	P1(TSS18) × CL555 → Nm^r[UV^s]
CL557	*rec*$^+$	CL555(λDL10)	This work
CL558	*rec*$^+$	CL555(λRef$^+$504B)	This work
CL559	*rec*(*BCD*)::Tn*5*	CL556(λDL10)	This work
CL560	*rec*(*BCD*)::Tn*5*	CL556(λRef$^+$504B)	This work
DJ1	*recA56*	*srl*::Tn*10* Δ(*argF-lacZ*)U169 *phoA*::Tn*5*	P1(G2) × CL508 → Tc^r[UV^s]
EG333	*rec*$^+$	HfrC, *pyrA*::Tn*10 metB cysG303* Δ(*lac-pro*)X111	E. GOLUB
EL88	*recA56*	*srl*::Tn*10* Δ(*argF-lacZ*)U169 *phoA*::Tn*5*	P1(G2) × MPh30 → Tc^r[UV^s]

Number	Recombination genotype	Remainder of genotype/description	Source/reference
G2	*recA56*	*srl*::Tn*10*	CSONKA and CLARK (1980)
HN356	*recB21*	as N99	H. NASH
JH314	*recB21 recC22*	as N99	SMITH and HAYS (1985)
JH1048	*recA3*	as C600	P1(N100) × C600 → Tc^r[Srl$^-$]
JC8679	*recB21 recC22 sbcA23*	as AB1157	A. J. CLARK (GILLEN, WILLIS and CLARK 1981)
JC13285	*recF322*::Tn*3*	*argE3 his-4 leuB6 proA2 thr-1 rpsL31 lacY1 galK2 mtl-1 xyl-5 tna300*::Tn*10 supE44*	A. J. CLARK (BLANAR *et al.* 1984)
JH1048	*recA3*	as C600	P1(N100) × JH1045 → Srl$^+$[Tc^s, UV^s]
JM101	*rec*$^+$	Δ(*lac-proAB*)(F′*tra*D36*proAB*$^+$) *lacI*^{q} *lacZ* ΔM15	MESSING (1983)
KK1	*rec*$^+$	as KS391, but *leu*::Tn*10*	KORBA and HAYS (1980)
KS391	*rec*$^+$	Hfr Hayes Δ*lacZMS286 ϕ80*dII Δ*lacZBK1*	KONRAD (1977)
KS469	*rec*$^+$	Δ*lacZMS286 ϕ80*dIIΔ*lacZBK1 ara*	B. KONRAD (unpublished)
MPh30	*rec*$^+$	Δ(*argF-lacZ*)U169 *phoA*::Tn*5*	R. WOLF
N99	*rec*$^+$	*galK2 rpsL200*	Meselson strain 28 [Sm^r derivative of W3102 (BACHMANN 1972)]
N100	*recA3*	as N99	Meselson strain 152 (GOTTESMANN and YARMOLINSKY 1968)
TSS18	*rec(BCD)*::Tn*5*	as KS391	This laboratory (T. SCHAEFER, unpublished)
V66	*recF143*	Rac$^-$ *his-4 argA gal*$^-$ *rpeL xyl* (?)*F*$^-$	G. SMITH

Designation	Genotype/remarks	Source/reference
B. Bacteriophage strains		
λDL10	as λDL69, but *lacPO lacZ*$_\alpha$::(multirestriction site insert)	WINDLE (1986)
λDL11	as λDL10, but restriction-site-insert in reverse orientation	WINDLE (1986)
λDL69	BamI°srI(1-2)Δ(*att*$^+$,*int*$^+$) *CI857 nin-5 shn6*° *Hin*dIII, *Bam*HI cloning vector	MIZUSAWA and WARD (1980)
λRef$^+$503H	*ref*$^+$-encoding (H2::Tn*5-503*) fragment from P1::Tn*5-503* inserted into λDL69. High level Ref expression when depressed. Partial insensitivity to P1 regulation. Tn*5* DNA fragment does not encode Nm^r	WINDLE and HAYS (1986)
λRef$^+$504B	*ref*$^+$ *Xba*I-*Sac*I fragment from pUC13-(B8::Tn*5-504*) (encodes neomycin resistance) replacement-cloned into λDL10 at *Xba*I, *Sac*I, sites. Ref phenotype similar to λRef$^+$503H	This work
P1 CmO *c*$^+$	Cm^r	N. STERNBERG
P1 CmO *bof-1*	Derepressed for Ref expression	N. STERNBERG (TOUATI-SCHWARTZ 1979)
P1::Tn*5-503*	Cm^{r}Nm^r. Partially depressed for Ref expression	WINDLE and HAYS (1986)

Designation	Relevant properties/remarks	Source/reference
C. Plasmids		
pACYC184	Cm^{r}Tc^r. Compatible with pBR322	CHANG and COHEN (1978)
pCL102	*bla*$^+$ *lacZ*′$_{2158}$ *cat*$^+$ $_{105}$⟩*lacZ* (Ap^{r}Lac$^+$Cm^r)	This work (Figure 2)
pCL103	*bla*$^+$ *lacZ*$^+$, *bla*$^+$ *lacZ*′$_{2158}$ *cat*$^+$ $_{105}$⟩*lacZ*′$_{2158}$ *cat*$^+$ $_{105}$⟩*lacZ* (Ap^{r}Lac$^+$Cm^r) dimer. Intermolecular recombination product of pCL102	This work (Figure 2)
pCL104	*bla*$^+$ *lac*$^+$, *bla*$^+$ *lacZ*′$_{2158}$ *cat*$^+$ $_{105}$⟩*lacZ* (Ap^{r}Lac$^+$Cm^r) dimer. Product of pMB1044-pCL202 recombination	
pEL101	*bla*$^+$ *lacZ*′$_{2158}$ $_{105}$⟩*lacZ*	This work (Figure 1)
pHN915	*bla*$^+$ *tet*′$_{339}$ $_{23}$⟩*tet* (but Tc^r)	H. NASH
pMB1044	*bla*$^+$ *lacZ*$^+$. Derived from pMLB1031 (pMLB1034 with *Eco*RI site at bp 4305) by insertion of the 246-bp *Hae*III/*Sma*I fragment of pUC9 containing the *lac* operator/promoter region into the *Sma*I site	SILHAVY, BERMAN and ENQUIST (1984)
pTS202	*bla*$^+$ *tet*′$_{339}$ *cat*$^+$ $_{23}$⟩*tet* (Tc^r)	This work (Figure 5)
pTS211	*bla*$^+$ *tet*′$_{970}$ $_{33}$⟩*tet*	This work (Figure 5)
pTS212	*bal*$^+$ *tet*′$_{970}$ *cat*$^+$ $_{33}$⟩*tet*	This work (Figure 5)
pTS221	*bla*$^+$ *tet*′$_{651}$ $_{33}$⟩*tet*	This work (Figure 5)
pTS222	*bla*$^+$ *tet*′$_{651}$ *cat*$^+$ $_{33}$⟩*tet*	This work (Figure 5)

seems inherently unsuitable for mechanistic studies, because of the 2–3-day period required, the imprecision of the Lac$^+$ papillation assay, and the unwieldiness and low copy-number of the bacterial chromosome. Here we describe Ref enhancement of *lac*$^-$ × *lac*$^-$ and *tet*$^-$ × *tet*$^-$ recombination in certain *E. coli* plasmids.

MATERIALS AND METHODS

Bacteria, bacteriophages and plasmids: These are described in Table 1.

Preparation of plasmid DNA: Large-scale plasmid stocks were prepared from 200-ml cultures of appropriately transformed *rec*A bacteria grown overnight at 38° in LB broth (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl). Lysis and plasmid extraction were according to the detergent-lysis procedure described by MANIATIS, FRITSCH and SAMBROOK (1982) except that Triton X-100 (5%) was used instead of sodium dodecyl sulfate, and extraction with phenol-chloroform before (CsCl + ethidium bromide) equilibrium sedimentation was omitted.

Crude small-scale plasmid stocks (MANIATIS, FRITSCH and SAMBROOK 1982) were extracted from 1.5-ml stationary-phase cultures grown in LB-broth plus ampicillin (40 μg per ml) at 32°, by the boiling method of HOLMES and QUIGLEY (1981), with Triton X-100 (5%) substituted for sodium dodecyl sulfate. Extracts were treated successively with equal volumes of phenol and chloroform, and precipitated with ethanol.

DNA procedures: Restriction enzymes (from Bethesda Research Laboratories or New England Biolabs) were used as specified by the suppliers. For joining of DNA fragments, phage T4 DNA ligase was used overnight at 16° under conditions specified by the supplier (New England Biolabs). To fill in restriction-fragment ends with 5′ overhangs, phage T4 DNA polymerase was employed in the presence of deoxynucleotide triphosphates as described by MANIATIS, FRITSCH and SAMBROOK (1982). *Xho*I linkers (p-CCTCGAGG, from New England Biolabs) were joined to restriction fragments with filled-in ends by treatment of a 100:1 linker: fragment mixture with T4 DNA ligase (400 units per 0.5-μg fragment). For transformation with plasmid DNA, bacteria were grown in 20-ml LB broth cultures to early exponential phase and treated with $CaCl_2$ essentially as described by MANDEL and HIGA (1974).

Measurement of recombination: *β-galactosidase assay*: Bacteria were transformed with plasmids pEL101 or pCL102, with selection on LB-plates (LB broth plus 1.5% agar) containing ampicillin (40 μg/ml). After 48 hr at 32°, multiple colonies (50–100 colonies washed from plates with 2 ml LB broth) were used to inoculate 10 ml of LB broth plus ampicillin (40 μg/ml) for overnight growth at 32°. For β-galactosidase assays, aliquots of these cultures were diluted 100-fold with minimal "A" medium (MILLER 1972) and grown to early exponential phase. Permeabilization with chloroform plus sodium dodecyl sulfate, and measurement of hydrolysis of orthonitrophenyl-β-galactoside (ONPG), were as described by MILLER (1972). Miller units represent ONPG hydrolysis rates normalized for turbidities of cell cultures, *i.e.*, they correspond to specific activities.

Plasmid-transformation assay: Overnight cultures, grown from multicolony inocula as described above for β-galactosidase assays, were used for preparation of crude small-scale plasmid stocks as described above. *recA* bacteria (strain DJ1) were transformed with these plasmid stocks. For assaying *lac*$^-$ × *lac*$^-$ recombination, transformants were spread on McConkey-lactose plates (4% McConkey base agar, 1% lactose) containing ampicillin (40 μg per ml), and both total Ap^r transformants and Lac$^+$Ap^r bacteria counted.[4] For assaying *tet*$^-$ × *tet*$^-$ recombination, transformants were spread on LB plates containing ampicillin, and on LB plates containing both ampicillin and tetracycline (12.5 μg per ml).

Southern-blot assay: Crude small-scale plasmid preparations were treated with restriction enzymes and subjected to electrophoresis in 0.8% agarose (in 80 mM Tris-phosphate buffer, pH 8.0); DNA was transferred to nitrocellulose filters as described by MANIATIS, FRITSCH and SAMBROOK (1982). Prior treatment of the filters for 3–4 hr at 41°, mixing with 10^7 cpm of radioactive probe [linear pEL101 DNA ^{32}P-labeled by the random-oligo-primer method of FEINBERG and VOGELSTEIN (1983)], hybridization overnight at 41°C, washing of filters, and exposure to KODAK X-OMAT AR film were as described by SILHAVY, BERMAN and ENQUIST (1984).

Determination of relative transformation efficiencies: Stocks of plasmid pMB1044 (6.5-kb Lac$^+$ Cm^s monomer) and pCL103 (17.9-kb Lac$^+$ Cm^r dimer) (see Figure 2) were used to transform strain DJ1 at a series of decreasing plasmid:cell ratios. In the range where transformation efficiencies were directly proportional to amount of plasmid, average transformation efficiencies were: pMB1044, 4.5×10^{12} CFU per μmole plasmid and pCL103, 1.0×10^{12} CFU per μmole plasmid.

RESULTS

Ref-stimulated plasmid recombination: In order to determine whether Ref activity could stimulate recombination in which one partner was not the bacterial chromosome, we deleted the *lacZMS286* gene from the *lac*$^-$ *lac*$^-$ strain KS391 (creating strain BW2033) and introduced plasmid pMB1034 (not shown), in which *lacPO* and a small N-terminal portion of *lacZ* are missing (SILHAVY, BERMAN and ENQUIST 1984). Formation of Lac$^+$ papillae by recombination between the plasmid and the chromosomal Φ80dIIΔ*lacBK1* gene, in BW2033 (pMB1034) bacteria, was virtually undetectable in the absence of Ref activity: no papillae in individual colonies after 72-hr incubation, and only an occasional papilla in heavy streaks. However, BW2033 (pMB1034) (λRef$^+$503B) lysogens showed about ten papillae per colony after only 48 hr.

In order to test *lac*$^-$ × *lac*$^-$ recombination in easily manipulable substrates, we constructed plasmid EL101 (Figure 1). This plasmid contains a tandem partial duplication of the *lacZ* gene–a 1.1-kb[4] N-terminal segment followed by a 2.0-kb C-terminal segment–and thus can become *lac*$^+$ only by break-and-join "splices" (STAHL 1979) within the 1.0-kb (*Cla*I-*Sac*I) overlap region (′*lacZ*′) (Figure 1). Recombination of pEL101 was assayed in two ways: transforma-

[4] Abbreviations used: Ap^r, Ap^s; Cm^r, Cm^s; Nm^r, Nm^s; Sm^r, Sm^s; Tc^r, Tc^s: resistant, sensitive, to ampicillin, chloramphenicol, neomycin, streptomycin, tetracycline, respectively. CFU: colony-forming units. kb: kilobase pairs. *lac*′, *tet*′; ′*lac*, ′*tet*: *lac*, *tet* genes deleted for 5′, 3′ portions, respectively (subscripts indicate deletion endpoints). ′*lac*′: gene missing both 5′ and 3′ portions. $(lac^-)^2$, $(tet^-)^2$: genomes incorporating two (not necessarily identical) defective copies of indicated genes. *tet*::Xho_{23}: *tet* gene with *Xho*I linker inserted at base-pair 23. UV^s, ultraviolet-light sensitive; UV^{ss}, ultraviolet-light hypersensitive.

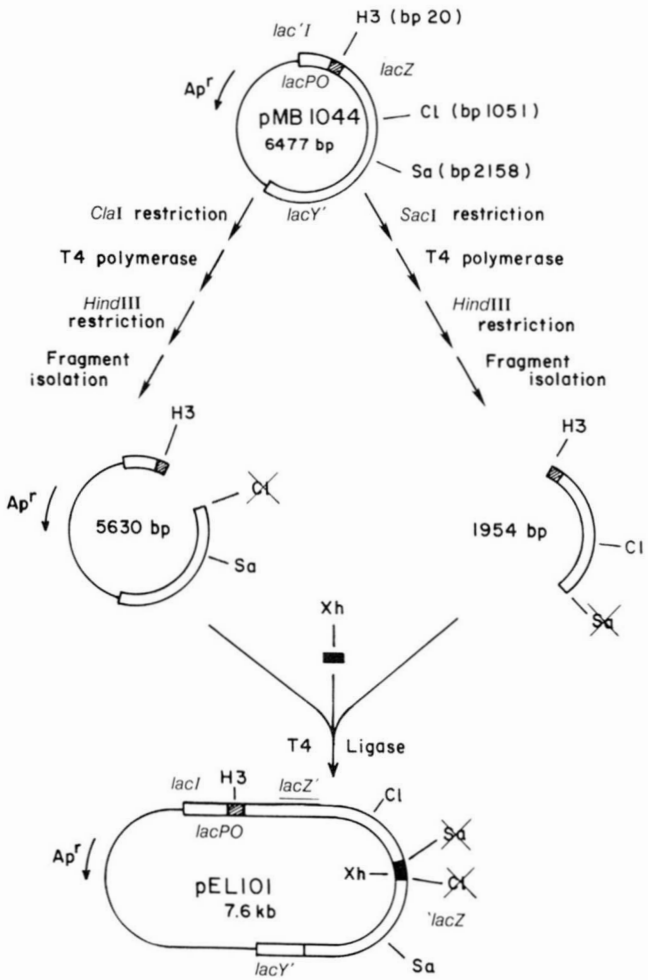

FIGURE 1.—Construction of the *lacZ'*`*lacZ* plasmid pEL101. Details of procedures are given in MATERIALS AND METHODS section: *Sac*I or *Cla*I restriction of plasmid pMB1044; treatment with phage T4 DNA polymerase in the presence of deoxynucleotide triphosphates to produce blunt ends; *Hin*dIII restriction; isolation of large *Hin*dIII-blunt (*Cla*I) and small *Hin*dIII-blunt (*Sac*I) fragments by agarose-gel-electrophoreses and electroelution; ligation of a mixture of the isolated fragments and *Xho*I linkers (phosphorylated by treatment with phage T4 polynucoleotide kinase in the presence of ATP); transformation of *recA* bacteria (strain JH1048), pooling of Ap^r transformants, small-scale extraction of plasmid DNA, transformation of Δ*lacZ recA56* bacteria (EL88); screening of small-scale plasmid preparations from cultures grown from individual colonies by *Xho*I and *Eco*RI-plus-*Hin*dIII restriction and agarose-gel electrophoresis. (□), *lac* DNA (*lacZ'*, bp 1-2158; '*lacZ*, bp 1051–bp 3350). (▨), 204-bp multirestriction site insert (*Hin*dIII, *Pst*I, *Sal*I, *Bam*HI, *Sma*I, *Bam*HI) replacing codons 5 through 8 of the *lacZ* structural gene, in frame (SILHAVY, BERMAN and ENQUIST 1984). (■–Xh), *Xho*I linker, CCTCGAGG. Restriction-enzyme sites: H3, *Hin*dIII; Cl, *Cla*I; Sa, *Sac*I. Ap^r, ampicillin-resistance determinant.

tion of *recA* Δ*lacZ* bacteria with small-scale plasmid preparations made from liquid cultures grown from colonies washed off plates, and direct measurement of β-galactosidase in similarly obtained log-phase cultures (Table 2). By both assays, Ref enhanced *lac*$^-$ × *lac*$^-$ plasmid recombination about fivefold. In *recB recC* cells, *lac*$^-$ × *lac*$^-$ plasmid recombination, Ref-stimulated or not, was about the same as in *rec*$^+$

TABLE 2

Recombination of plasmid pEL102

Strain	Pertinent phenotype	β-Galactosidase specific activity (Miller units)	Frequency of Lac$^+$-plasmid transformants among Ap^r (×10^2)
CL506	Rec$^+$ Ref^0	12 ± 1	0.16
CL507	Rec$^+$Ref$^+$	71 ± 9	0.85
CL504	Rec(BCD)$^-$ Ref^0	12 ± 3	0.12
CL505	Rec(BCD)$^-$ Ref$^+$	85 ± 5	0.63

Large-scale preparation of purified stocks of plasmid pEL101 from *recA* bacteria (strain DJ1), transformation of the indicated bacterial strains to ampicillin resistance and plate-growth as indicated, β-galactosidase assays on liquid cultures grown 4 hr from cells washed off plates, small-scale plasmid preparations from same cultures after overnight growth, transformation of *recA* Δ*lacZ* bacteria (strain DJ1), and spreading on XGal-ampicillin plates were as described under MATERIALS AND METHODS. β-Galactosidase specific activities (Miller units) are averages of values for two different original transformation plates for each strain (range indicated). Lac$^+$ plasmid frequencies are mong 4800 (CL506), 1400 (CL507), 1700 (CL504), and 3700 (CL505) total ampicillin-resistant (Ap^r) colonies scored.

bacteria, unlike *lac*$^-$ × *lac*$^-$ chromosomal recombination, which appears mostly RecBCD-dependent (WINDLE and HAYS 1986).

Analysis of the structures of recombinant plasmids: Plasmid pEL101 (Figure 1) can yield a Lac$^+$ product by either intramolecular or intermolecular recombination. The respective products, *lac*$^+$ monomers and dimeric plasmids encoding *lac*$^+$ and *lacZ'*`*lacZ'*`*lacZ* genes (*lac*$^+$, *lacZ'*`*lacZ'*`*lacZ* dimers), are readily distinguished by physical analysis. We used plasmids extracted from Rec$^+$Ref$^+$ cultures (strain CL507) to transform *recA* Δ*lacZ* bacteria (strain DJ1). The structures of plasmids obtained from a few Lac$^+$ transformants selected at random were analyzed by *Hin*dIII and *Xho*I restriction and agarose gel electrophoresis. Of five plasmids analyzed, four were *lac*$^+$ monomers and one was a *lac*$^+$, *lacZ'*`*lacZ'*`*lacZ* dimer (data not shown).

To facilitate further product analysis, we constructed plasmid pCL102 by inserting a fragment encoding *cat* (chloramphenicol resistance) into pEL101 (Figure 2). The product, pCL102, yielded both Lac$^+$Cm^s products, presumably by intramolecular recombination, and Lac$^+$Cm^r products (*e.g.*, pCL103), presumably by intermolecular recombination, with a small bias towards the latter (Table 3). A number of colonies, obtained by transformation of *recA* mutants with plasmids originally extracted from Ref$^+$ cultures, were used to prepare plasmids for analysis by *Hin*dIII and *Sst*I-plus-*Cla*I restriction and agarose gel electrophoresis (Figure 3). All (4/4) of the Cm^{s}Lac$^+$ plasmids analyzed yielded the restriction pattern expected for an intramolecular recombinant (pMB1044). Analysis of two Cm^{s}Lac$^+$ plasmids is shown in Figure 3: *Hin*dIII restriction yielded a 6.5-kb linear molecule (lanes E, F), in contrast to the 9.0-

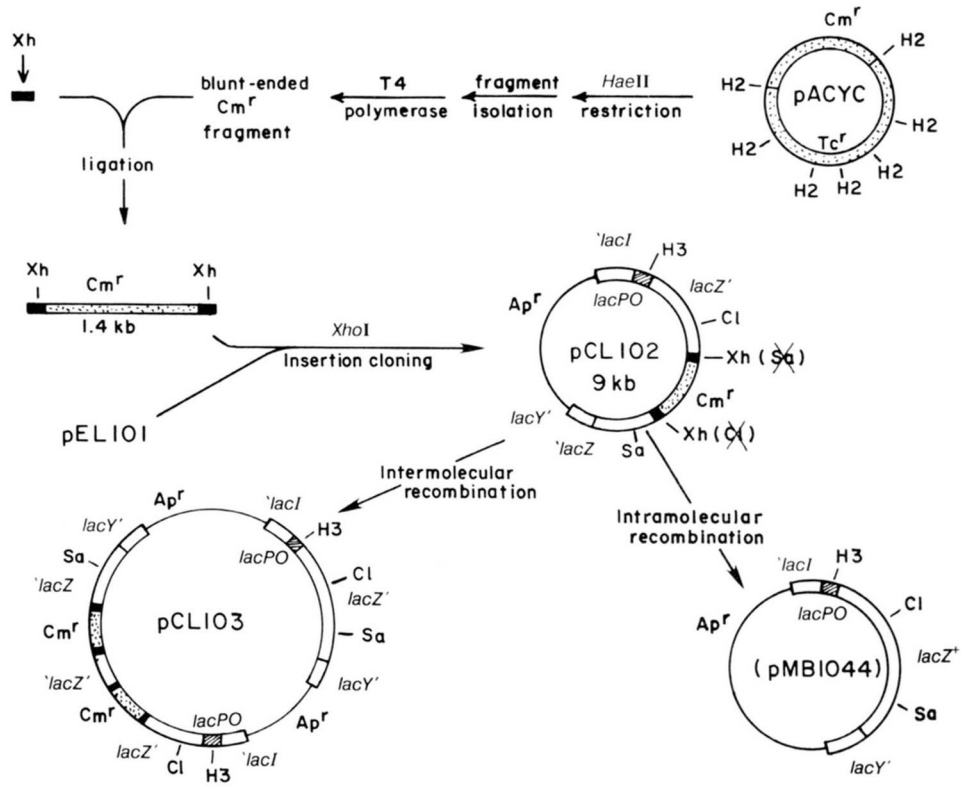

FIGURE 2.—Construction and recombination of plasmid pCL102. Details of construction procedures are given in MATERIALS AND METHODS section: *Hae*II restriction of plasmid pACYC184 and isolation of 1.4-kb Cm^r-encoding fragment by agarose gel electrophoresis and electroelution; treatment with phage T4 DNA polymerase to create blunt ends; ligation to *Xho*I linkers (CCTCGAGG); *Xho*I digestion of Cm^r fragment and ligation with *Xho*I-digested pCL101; transformation of JM101 bacteria and selection of Ap^{r}Cm^r colonies; screening of small-scale plasmid preparations by *Xho*I restriction and agarose-gel electrophoresis. pCL101 and pMB1044 are products expected for intermolecular and intramolecular recombination within *lacZ*, respectively. (□), *lac* DNA (*lacZ'*, bp 1-2158; '*lacZ'*, bp 1051–2158; '*lacZ*, bp 1051–3350). (▨), in-frame multi-restriction-site insertion in *lacZ* (see legend to Fig. 1). (■–Xh), *Xho*I linker, CCTCGAGG. (▤, Cm^r-encoding 1.4-kb fragment from pACYC. Restriction enzyme sites: H2, *Hae*II; Xh, *Xho*I; Cl, *Cla*I; Sa, *Sac*I; ⊠, ⊠, sites destroyed by DNA polymerase fill-in; H3, *Hin*dIII.

TABLE 3

Analysis of products of recombination plasmid pCL102

Strain	Phenotype	Total	Lac$^+$	Lac$^+$/total ($\times 10^2$)	Lac$^+$Cm^r	Corrected ratio Lac$^+$Cm^r/Lac$^+$ ($\times 10^2$)
CL510	Rec$^+$Ref^0	1.8×10^4	17	0.09	9	20
CL511	Rec$^+$Ref$^+$	6.6×10^3	50	0.76	31	26

Large-scale preparation of a purified stock of plasmid pCL103 from *recA* bacteria (strain DJ1), transformation of indicated bacteria to ampicillin resistance, growth on plates at 32° for 48 hr, multi-colony inoculation into broth, overnight growth, transfer and growth for 4 hr in fresh broth, small-scale plasmid extraction, transformation of *recA* Δ*lacZ* bacteria (strain DJ1) to ampicillin resistance on McConkey-lactose plates and scoring of Lac$^+$ colonies were as described under MATERIALS AND METHODS. Colonies were tested for chloramphenicol resistance (Cm^r) by toothpicking onto LB plates containing the drug (30 μg/ml). Ratio of Lac$^+$ Cm^r among total Lac$^+$ transformants was corrected for the relative transformation efficiencies of pMB1044 (Lac$^+$Cm^s) and pCL103 (Lac$^+$Cm^r): 4.5×10^{12} CFU per μmole plasmid and 1.0×10^{12} CFU per μmol plasmid respectively (see MATERIALS AND METHODS).

kb *Hin*dIII-restricted parental pCL102 (lanes I, J), and *Sst*I(*Sac*I isoschizomer) + *Cla*I restriction (lanes G, H) yielded the 1.0-kb fragment interior to *lac*$^+$ (designated '*lacZ'*) plus the 5.5-kb fragment (common to all plasmids) that encodes the vector backbone and the N- and C-terminal *lac* portions that flank '*lacZ'*, rather than the 3.5-kb '*lacZ'cat*$^+$'*lacZ'* fragment from pCL102 (lanes K, L).

Of four apparent intermolecular recombinant (Cm^{r}Lac$^+$) plasmids, three appeared similar to pCL103, and one yielded the restriction pattern expected for a double recombinant. A sample of each was analyzed (Figure 3): the pCL103-type molecule was cut by *Hin*dIII nuclease into 6.5-kb and 11.4-kb fragments (lane A) (corresponding to the size of two 9.0-kb pCL102 monomers), and *Sst*I + *Cla*I restriction (lane C) yielded the 1.0-kb 'lacZ' fragment, two copies of the 5.5-kb backbone fragment, and a 5.9-kb fragment that encodes '*lacZ'cat*$^+$'*lacZ'cat*$^+$'*lacZ'*; the apparent double recombinant, designated pCL104, was cut by *Hin*dIII into 6.5 and 9.0-kb fragments (lane B) that correspond to complete copies of pCL102 (lanes I, J) and pMB1044 (lanes E, F), and *Sst*I + *Cla*I restriction yielded the 1.0-, 3.5- and 5.5-kb (double) bands (lane D) expected for a pCL102-pMB1044 cointegrate (compare with lanes G, H and K, L), or for an intramolecular recombinant of pCL103. Thus all Lac$^+$ plasmids appeared to be the

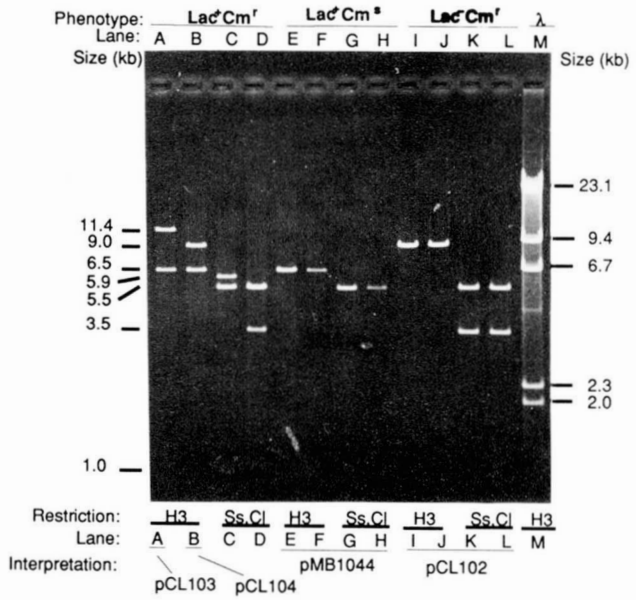

FIGURE 3.—Restriction analysis of pCL102 recombination. Strain DJ1 (RecA⁻) was transformed with pCL102 plasmids propagated in strain CL511 (Rec⁺Ref⁺), as described in the legend to Table 3. Four colonies displaying each indicated phenotype were randomly selected for extraction of plasmids and analysis by *Hin*dIII (H3) and *Sst*I + *Cla*I (Ss, Cl) restriction, electrophoresis in 1% agarose and ethidium bromide staining. Representatives of each of the three recombinants identified (pMB1044, pCL103, pCL104; see text), as well as pCL102 parental plasmids, were re-analyzed simultaneously, along with *Hin*dIII-digested phage λ DNA.

result of one of two well-defined homologous recombination events.

Ref-stimulated plasmid and chromosomal recombination in *rec* mutants: Most homologous recombination processes in *E. coli* are completely RecA-dependent or nearly so, but some, *e.g.*, Hfr conjugational recombination (CLARK 1973), are highly RecBCD-dependent, whereas others, *e.g.*, some plasmid recombination processes (JAMES, MORRISON and KOLODNER 1982), are highly RecF-dependent. We tested the ability of Ref activity to stimulate recombination of plasmid pCL102 in various mutant bacteria (Table 4). As expected, plasmid recombination was RecBCD-independent and mostly RecF-dependent. Ref not only stimulated plasmid recombination about fivefold in Rec⁺ and Rec(BCD)⁻ bacteria, but also stimulated the residual recombination in RecF⁻ cells. We do not know whether this last result reflects leakiness of the *recF143* mutation or stimulation of some RecF-independent plasmid recombination process. The low recombination in *recA* cells was not increased by Ref activity. Similar results were obtained when pEL101 plasmids were propagated in Ref⁰ and Ref⁺ bacteria and transformed into DJ1 (*recA* Δ*lac*) for detection of Lac⁺ plasmids: the Lac⁺ frequencies among plasmids propagated in *rec*⁺ (CL510) and *recB recC* (CL504) Ref⁰ control bacteria were about the same, and were elevated four- to sixfold in the corresponding Ref⁺

TABLE 4

Recombination of plasmid pCL102 in *rec* mutants

Control strain[a]	Ref⁺ strain[b]	Recombination phenotype	Relative plasmid recombination[c]	Ref stimulation factor[d]
CL557	CL558	Rec⁺	(1)	6 ± 2
CL559	CL560	Rec(BCD)⁻	1 ± 0.5	4 ± 1
CL515	CL516	RecF⁻	0.1 ± 0.1	4 ± 1
CL517	CL518	RecA⁻	0.05 ± 0.1	0.8 ± 0.2
CL520	CL530	Rec(BCD)⁻, SbcA	5 ± 2	2 ± 0.5
CL525	CL532	Rec(BCD)⁻, SbcA RecA⁻	3 ± 2	0.9 ± 0.2

Plasmid pCL102 purified from *recA* bacteria (DJ1) was used to transform the indicated strains to ampicillin resistance. Colonies were grown on rich plates at 32° for 48 hr. From 50 to 200 transformant colonies were mixed together in LB broth and used to inoculate LB broth supplemented with ampicillin for overnight growth at 32°. This overnight culture was used to inoculate minimal medium A for growth at 32° for 4 hr. Aliquots of these cultures were used in β-galactosidase assays as described under MATERIALS AND METHODS. Data correspond to averages for three to four trials. Standard deviations are indicated.

[a] Lysogenic for λDL10.

[b] Lysogenic for λRef⁺504B.

[c] Ratio of specific β-galactosidase activity to that of wild-type strain.

[d] Ratio of specific β-galactosidase activity of Ref⁺ strain to that of Ref⁰ control.

derivatives (CL511, CL505); the low residual recombination in a *recF* mutant (CL515) was elevated fourfold in the Ref⁺ derivative (CL516), but propagation in the corresponding *recA* pair (CL517, CL518) resulted in very low recombination and no Ref stimulation.

In order to verify that β-galactosidase measurements and Lac⁺-plasmid transformation frequencies corresponded at least qualitatively to intracellular plasmid distributions, populations extracted directly from *rec*⁻ (pCL102) and *rec*⁺ (pCL102) bacteria were analyzed *en masse*, by *Cla*I-plus-*Sst*I restriction, gel electrophoresis and "Southern" blotting with a pCL102 probe (Figure 4). Fragments corresponding to the *lacZ'*`*lacZ* parent pCL102 (5.5 and 3.5 kb), and unique fragments corresponding to pMB1044-type intramolecular recombinants (1.0-kb: Figure 4, lane K; see also Figure 3, lane G) and pCL103-type intermolecular recombinants (1.0, 5.9 kb: Figure 4, lane A; see also Figure 3, lane C) were readily distinguished from one another. By this technique we detected no recombinant bands among plasmids from *recA* and *recF rec(BCD)* bacteria (Figure 4, lanes J, H, I) and faint recombinant bands among RecF⁻ plasmids (lanes F, G). (This is more obvious in autoradiograms exposed for longer times.) In plasmid populations from *rec*⁺ (lanes D, E) and *rec(BCD)* (lanes B, C) bacteria, the recombinant bands were relatively prominent. Increases in recombinant bands in the presence of Ref activity could be detected for *rec*⁺ bacteria, where the bands were not too dark (lane B *vs.* C, 5.9 kb; lane D *vs.* E, 1.1 kb), and for *recF* bacteria in more heavily

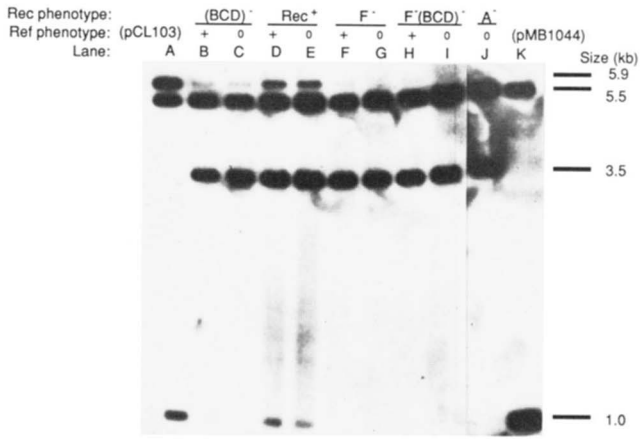

FIGURE 4.—Southern blot analysis of pCL102 recombination in *rec* mutants. Propagation of various strains containing pCL102 and lysogenic for λDL11 (or λDL10) or for λRef⁺504B, extraction of plasmids, *Hin*dIII + *Sst*I restriction, electrophoresis, transfer of bands to filter paper and hybridization with a pEL101 probe were as described under MATERIALS AND METHODS. Lanes correspond to the following strains: B, CL560; C, CL559; D, CL558; E, CL557; F, CL516; G, CL515; H, CL533; I, CL552; J, DJ1 (no λ prophage). Lanes A and K contained pCL104 and pMB1044 DNA, respectively.

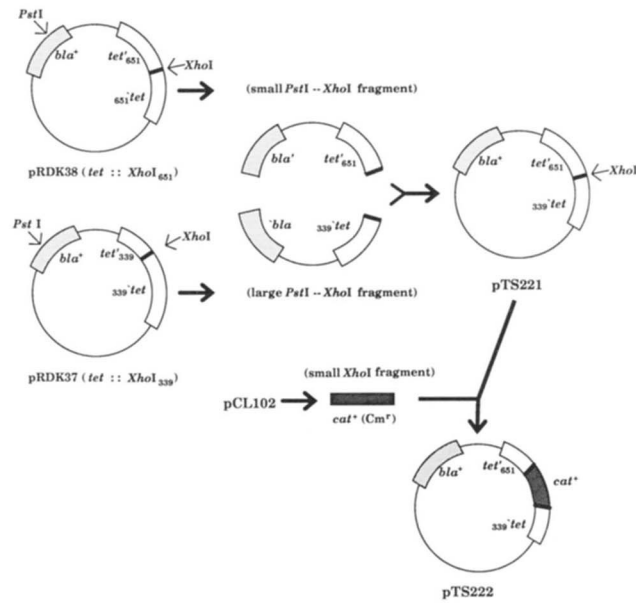

FIGURE 5.—Construction of *tet'* `*tet* plasmids. Details of procedures are given in MATERIALS AND METHODS section. Plasmids pTS221 and pTS222: *Pst*I + *Xho*I restriction of pRDK38 and pRDK37 and electrophoresis-electroelution isolation of small (*bla'tet'*$_{651}$) and large (*'bla*$_{339}$`*tet*) fragments, respectively; ligation, transformation of *recA* bacteria and isolation of Apʳ Tcˢ transformants; verification of pTS221 structure (*bla*⁺*tet'*$_{651\ 339}$`*tet*) by restriction analysis, including presence of *Xho*I site; isolation of small *Xho*I fragment (encoding *cat*⁺) from pCL102 and insertion into *Xho*I site of pTS221; transformation of *recA* bacteria and selection for ApʳCmʳTcˢ transformants; verification of structure of plasmid pTS222 (*bla*⁺ *tet*$_{651}$ *cat*⁺ $_{339}$`*tet*) by restriction analysis. Plasmids pTS211 and pTS212: *Nru*I restriction of pBR322 and brief *Bal*31 digestion (estimated end-point at bp 970); digestion with S1 endonuclease and fill-in using *E. coli* DNA polymerase Klenow fragment; attachment of Xho linkers (CCTCGAGG), ligation and transformation of strain C600; identification of ApʳTcˢ transformants and demonstration of *Xho*I sensitivity of corresponding plasmid (pTS110); *Pst*I + *Xho*I treatment of pTS110 and isolation of *`bla tet'*$_{970}$ fragment; ligation of *`bla tet'*$_{970}$ to *bla'* $_{339}$`*tet* fragment from pRDK37 (see above); isolation of *bla*⁺ *tet'*$_{970\ 339}$`*tet* plasmid pTS211 as for pTS221 above; insertion of *cat*⁺ *Xho*I fragment from pCL102 into pTS211 to produce *bla*⁺ *tet'*$_{970}$ *cat*⁺ $_{339}$`*tet* plasmid pTS212, as for pTS222 above. Construction of pTS202: insertion of *Xho*I *cat*⁺ fragment from pCL102 into pHN915 to produce *bla*⁺ *tet*$_{339}$ *cat*⁺ $_{2}$`*tet* plasmid pTS202, as for pTS212 above.

exposed autoradiograms (not shown). However, this technique is neither as sensitive nor as quantitatively accurate as the β-galactosidase or plasmid-transformation assays. Recombinant bands were also detected in plasmids extracted from *recA recB recC sbcA* and *recB recC sbcA* bacteria (data not shown).

We previously observed that both Ref-independent and Ref-stimulated *lac*⁻ × *lac*⁻ chromosomal recombination (Lac⁺ papillation) mostly required RecBCD function, but that the residual recombination in *recBC* mutants was Ref-enhanceable (WINDLE and HAYS 1986). Here we tested the effect of Ref activity on *lac*⁻ × *lac*⁻ chromosomal recombination in *recF*, and *recF rec(BCD)* mutants (data not shown). The *recF* bacteria (CL522, CL523) behaved similarly to *rec*⁺ bacteria with respect to both unstimulated and Ref-stimulated (about 50-fold) levels of Lac⁺ papillation. The corresponding *recF rec(BCD)* derivatives CL527 and CL528, showed greatly reduced, but Ref-enhanceable, recombination (no Lac⁺ papillae and about one-half papillae per colony, respectively). Thus Ref stimulates recombination in the absence of RecBCD functions, in the absence of RecF functions, and in the apparent absence of both. In the last case we have not ruled leakiness of the *rec* mutations.

Although Ref activity did not stimulate the residual plasmid recombination in *recA* bacteria (Table 4, line 4), it seemed possible that more efficient RecA-independent processes might be enhanceable. Plasmid recombination in *sbcA* bacteria (*rec*⁺ or *recA*) is thought to proceed via a RecA-independent RecE-dependent process (FISHEL, JAMES and KOLODNER 1981). We found *lac*⁻ × *lac*⁻ plasmid recombination to be more efficient in *recB recC sbcA* than *rec*⁺ bacteria. Ref did not enhance recombination in *recA recB recC sbcA* cells and only slightly stimulated that in *recB recC sbcA* mutants (Table 4). Similar results were obtained using pEL101 plasmids (data not shown). In these experiments the observed Ref stimulation of recombination in the control strain (CL536) (data not shown) ruled out any requirement for the cryptic Rac prophage (LOW 1973), present in many *E. coli* K-12 strains but absent from AB1157 and its derivatives (KAISER and MURRAY 1979).

The UV-sensitivity of *recB recC* mutants is reversed by *sbcA* and *sbcB* mutations, concomitant with restoration of recombination proficiency (BARBOUR *et al.* 1970; KUSHNER *et al.* 1971). Presumably some recombination-dependent daughter-strand-gap repair process that deals with the consequences of replication of

TABLE 5

Recombination of *tet′ ‵tet* plasmids

Plasmid	Relevant genotype	Homologous overlap (bp)	Average recombination frequency [($Ap^rTc^r/Ap^r \times 10^2$)] Ref^0	Ref$^+$	Average Ref stimulation factor
Experiment 1					
pTS211	tet'_{970} $_{339}$‵tet	611	0.87 ± 0.65	2.0 ± 1.5	2.4 ± 0.8
pTS212	$tet'_{970}cat^+$ $_{339}$‵tet	611	0.48 ± 0.20	1.1 ± 0.5	2.6 ± 1.2
pTS221	tet'_{651} $_{339}$‵tet	312	0.10 ± 0.01	0.15 ± 0.01	1.5 ± 0.1
pTS222	tet'_{651} cat^+ $_{339}$‵tet	312	0.13 ± 0.07	0.69 ± 0.45	5.0 ± 1.2
pCL102	$lac'_{2158}cat^+$ $_{105}$‵lac	1007	0.29 ± 0.06 (0.23)	0.99 ± 0.18 (0.77)	3.2 ± 0.8 (3.3)
Experiment 2					
pTS212	tet'_{950} cat^+ $_{339}$‵tet	611	0.11 ± 0.04	0.47 ± 0.07	5.2 ± 1.2
pTS222	tet'_{651} cat^+ $_{339}$‵tet	312	0.07 ± 0.02	0.47 ± 0.09	7.2 ± 1.7

Plasmids were prepared from *recA* bacteria (JH1048) and transformed into C600 for selection by growth for 16 hr on LB plates containing ampicillin (50 μg/ml). Colonies (about 500) were washed off plates with broth. Resuspended cultures were used for preparation of crude small-scale plasmid stocks; these were used to transform JH1048 cells. Transformants were spread on plates containing ampicillin and ampicillin plus tetracycline (12.5 μg/ml) for determination of the recombination frequency, defined as the fraction of tetracycline-resistant bacteria (Tc^r) among ampicillin-resistant (Ap^r). Data correspond to average from four (experiment 1) or three (experiment 2) *recA* transformations with each crude plasmid preparation, standard deviations are indicated. For pCL102, data in parentheses correspond to frequencies calculated from sum total Ap^r and Ap^rTc^r bacteria for all trials.

damaged DNA (RUPP *et al.* 1971) is abolished. We observed no significant change in the UV-sensitivity of *recF*, *recB recC*, or *recF recB recC* mutants in the presence of a λRef$^+$ prophage, in contrast to the Ref effects on *lac*$^-$ × *lac*$^-$ recombination described above (data not shown). Thus in this respect Ref activity does not replace RecBCD function or compensate for its absence.

Ref stimulation of $(tet^-)^2$ plasmid recombination: In order to determine whether Ref stimulation was specific for the *lac* sequences involved in the plasmid and chromosomal recombination processes described above, we constructed $(tet^-)^2$ plasmids analogous to pEL101 and pCL102. In this case the availability of plasmids with unique *Xho*I linkers inserted into the *tet* gene at several different *Taq*I sites (DOHERTY, MORRISON and KOLODNER 1983) made it possible to construct substrates with different lengths of homologous overlap and with different homologous-overlap sequences of the same length. The N-terminal *tet*-fragments used are designated as tet'_{23}, tet'_{339}, . . . , depending on the site of the *Xho*I linker, and the C-terminal fragments similarly as $_{23}$‵tet, $_{339}$‵tet. The construction of the tet'_{651} $_{339}$‵tet plasmid pTS221 (analogous to pEL101), and the tet'_{651} cat^+ $_{399}$‵tet plasmid pTS222 (analogous to pCL102) is diagrammed in Figure 5. The results of experiments in which $(tet^-)^2$ plasmids were propagated on *rec*$^+$ bacteria in the absence and presence of Ref activity, and the frequency of *tet*$^+$ plasmids was assayed by extraction of plasmids, transformation of *recA* bacteria, and selective plating, appear in Table 5. Ref activity stimulated recombination of *tet′cat*$^+$‵*tet* plasmids about as well as it stimulated pCL102 recombination. Thus it appears that Ref stimulation is not markedly sequence-specific. Ref stimulation may increase with decreasing length of homology when the recombining sequences are relatively far apart (compare lines 2 and 6 with lines 4 and 7 in Table 5). [Note that in the case of pTS211 and pTS221 (Table 5, lines 1, 3), the decrease in homology was accompanied by a 50% reduction in the spacing between the *tet* homologies, and the recombination frequency decreased significantly. In both pTS212 and pTS222 (lines 2, 4) the homology-homology separation is relatively large, because of the intervening *cat*$^+$ segment.]

The tet'_{339} $_{23}$‵tet plasmid pHN915 confers tetracycline resistance, most likely because tet'_{339} encodes $_{ptet}$ (but not a complete *tet* gene) and $_{23}$‵tet encodes translation signals and the entire *tet* coding sequence (but not a promoter). Insertion of the *cat*$^+$ fragment, forming pTS202, nearly eliminates tetracycline resistance, presumably because p_{tet}-initiated transcription is terminated. Ref increased the apparent recombination frequency of pTS202 about fourfold. However, there was a background of many very small colonies when cells transformed with pTS202 were spread on tetracycline plates. Since a little p_{tet} transcription may leak through the Cm^r fragment, and there may be a high background of nonrecombinational events that increase the leakage, the apparent Ref stimulation factor for pTS202 may be too low. However, this plasmid does provide a third sequence, distinct from those in pTS221 and pCL102, whose recombination is Ref-stimulated.

In plasmid pRDK41, the *tet*::*Xho*I$_{23}$ plasmid pRDK35 is joined in tandem to the *tet*::*Xho*I$_{1267}$ plasmid pRDK39 (DOHERTY, MORRISON and KOLODNER 1983). Ref stimulated formation of Tet$^+$ plasmids three- to fourfold during propagation of *rec*$^+$

(pRDK41) bacteria on solid and liquid media (data not shown). Here the various Tet$^+$ recombinant products that can be formed (DOHERTY, MORRISON and KOLODNER 1983)–monomers; *tet*$^+$, *tet*::*Xho*I$_{23}$:*Xho*I$_{1207}$ dimers; gene-conversion dimers such as *tet*$^+$, *tet*::*Xho*I$_{23}$; higher-order oligomers–were not distinguished.

Transcription and *lac*$^-$ × *lac*$^-$ recombination: KOBYASHI and IKEDA (1977) reported that rifampicin inhibited recombination between λ bacteriophages in the presence of chloramphenicol, and inferred that transcription was stimulating recombination. Thus putative differences in transcription activity might explain the differences between the extent of Ref stimulation of (*lac*$^-$)^2 plasmids and (*lac*$^-$)^2 bacterial chromosomes. However, we found that during growth in TBY broth or glycerol-minimal medium, addition of the *lac* inducer IPTG had no effect on the frequency of *lac*$^-$ × *lac*$^-$ chromosomal recombination in KS391 bacteria, as determined by subsequent plating on McConkey-lactose plates (Lac$^+$ cell frequency was about 10^{-5} after growth without *lac* induction or after growth in the presence of three different concentrations of IPTG). We also tested the effect of Ref activity on *lacZ*$^+$ expression, both repressed and IPTG-induced, with negative results: β-galactosidase synthesis during growth in the absence of Ref (strain N99) in glycerol-minimal medium was 2.3 ± 0.1 without IPTG and (1.9 ± 0.2) × 10^3 (average for two cultures) in 0.1 mM IPTG; in the presence of Ref activity [N99(λRef$^+$504B)] the corresponding values were 1.3 ± 0.1 and (2.5 ± 0.1) × 10^3 (averages for four cultures). Thus transcription of genes and Ref stimulation of recombination within them appear uncoupled.

Recombination processes not markedly stimulated by Ref expression: We have previously reported that several RecA-dependent recombination processes–*e.g.*, excision of a phage λ tandem duplication, reassortment of genetic markers by IS1 × IS1 recombination in a P7-P1 hybrid phage, precise excision of a *lacZ*::Tn*10* insertion–were stimulated less than twofold by Ref activity (WINDLE and HAYS 1986). We sought further information about possible specificity of Ref stimulation by testing a variety of additional recombination processes, focusing on relatively efficient processes in which the DNA structure of at least one recombining partner was in other than closed duplex form, and thus might be presumed to be already activated for recombination.

Two extensively studied recombination processes affecting the *E. coli* chromosome, those associated with Hfr conjugation and with P1 transduction, involve DNA structures thought to be recombinagenic: temporarily single-stranded regions in Hfr DNA and linear duplex DNA, respectively. We compared linkage of *pyrA*::Tn*10* to other donor alleles, at both nearby and more distant loci, in conjugal crosses between the Hfr donor EG333 and strain AB1157 lysogenic for either λDL11 or λRef$^+$504B. The respective frequencies of *pyrA*::*Tn*10 transconjugants carrying donor alleles in the Ref^0 and Ref$^+$ crosses were: *thr*$^+$, 96% and 95%; *leu*$^+$, 93% and 87%; *met*$^-$, 94% and 99%; *lac*$^-$, 74% and 64%. Linkages between pairs of auxotrophic markers appeared normal in both cases, and yields of total tetracycline-resistant transconjugants differed by less than a factor of two. In the case of P1-mediated transductions, using phages grown on strain KK1, both the yield of *leu*::Tn*10* transductants and the linkage between the *leu* and *ara* loci were measured in recipients lacking Ref activity [strain KS469 and KS469 (DL10)] and in those producing high levels of Ref activity [KS469 (λRef$^+$503H)]. The number of *leu*::Tn*10* transductants varied less than twofold among the three recipients, and the respective frequencies of co-transduction with *ara*$^+$ were 68%, 60% and 74%, respectively. In another experiment, we used P1 phages grown on Δ*lac*ZMS286 *phoA*::Tn*5* or Δ*lac*ZBK1 *trp*::Tn*10* bacteria for transduction of KS391 derivatives. Here the exchanges involve the same chromosomal *lacZ*$^-$ genes whose recombination with one another is stimulated two orders of magnitude by Ref activity. Nevertheless, the presence of Ref in the recipients did not affect transduction efficiencies (data not shown). We conclude that Ref activity does not significantly increase either conjugational or transductional recombination frequencies.

Although Ref does not appear essential for normal lytic growth or lysogeny, in *E. coli* at least, Ref-stimulated recombination could play a role in resistance of P1 to DNA damage, as RecA-dependent daughter-strand-gap repair does in *E. coli* (RUPP *et al.* 1971). However, the yield from infections with UV-irradiated phages did not vary markedly (twofold at most) as a function of multiplicity (0.2–10 phages per cell) ["multiplicity reactivation" (HUSKEY 1969)], and *ref*$^+$ phages showed at most a twofold increase over *ref*$^-$ phages in survival at higher multiplicities.

DISCUSSION

Ref activity significantly stimulated recombination of several different well-defined plasmid substrates. In the case of (*lac*$^-$)^2 plasmids, substantially similar results were obtained using two different recombination assays–segregation of plasmids by extraction from cells and transformation of *recA* cells, and measurement of β-galactosidase–and the intracellular presence of appropriate plasmids was verified qualitatively by Southern blotting of plasmid populations. Measurement of β-galactosidase is much more rapid than segregation of plasmids by transformation and is more sensitive and quantitatively reliable than Southern blotting (as well as somewhat simpler). It thus appears the method of choice.

The key observations are the following: (1) Ref stimulated plasmid recombination in strains other than KS391, the strain in which the Ref effect was discovered (WINDLE and HAYS 1986). (2) Ref stimulated both *lac*$^-$ × *lac*$^-$ and *tet*$^-$ × *tet*$^-$ plasmid recombination. (3) Ref stimulated intramolecular and intermolecular processes. (4) Ref stimulated RecF-dependent plasmid recombination. It also stimulated chromosomal recombination in *recF* bacteria, a process thought to depend on RecBCD function (ZIEG and KUSHNER 1977). (5) Four processes thought to involve DNA already activated for recombination–conjugal recombination, P1 transduction, multiplicity reactivation of UV-irradiated phages, and RecE-dependent plasmid recombination (in *recB recC sbcA* mutants)–were not affected by Ref activity. (6) Ref stimulated *lac*$^-$ × *lac*$^-$ plasmid recombination three to six-fold, but stimulated *lac*$^-$ × *lac*$^-$ chromosomal recombination 20–100-fold (WINDLE and HAYS 1986), even though the propagation times and conditions for the two measurements were about the same.

At what stage(s) in recombination processes does Ref act? Several observations argue that Ref mediates an initial activation step that increases substantially the amount of recombinagenic DNA that is a substrate for "recombinases" (products of genes implicated directly in recombination). First, if RecF and RecBCD functions may be presumed to act after activation [except perhaps in the case of RecBCD and Chi sites (TAYLOR *et al.* 1985)], because they are each implicated in several different recombination processes (presumably involving different activated substrates), then the fact that Ref stimulates both RecF-dependent and RecBCD-dependent processes suggests a role for Ref in activation. Second, Ref fails to significantly stimulate recombination processes for which activated substrates are thought to be already present–conjugation (single-stranded Hfr DNA), P1 transduction (linear duplexes), multiplicity reactivation (daughter-strand gaps in replicated UV-irradiated DNA), plasmid recombination in *recB recC sbcA* bacteria (3′-OH single-stranded ends?). Third, Ref stimulates both intramolecular and intermolecular processes.

None of the above considerations rule out involvement of Ref in some rate-limiting postactivation step common to RecF- and RecBCD-dependent processes, heteroduplex repair, for example. What is the evidence that activation is in fact rate-limiting in *E. coli* recombination? First, metabolically inert λ phage DNA is a poor recombination substrate (HAYS and BOEHMER 1978). Second, mutants hyper-rec for chromosome recombination have invariably proved to be defective in DNA metabolism rather than being recombinase overproducers (KONRAD 1977; ZIEG, MAPLES and KUSHNER 1978). Third, massive overproduction of known recombinases increases λ phage recombination relatively little (KORBA and HAYS 1980; ZAGURSKY and HAYS 1983). (Similar studies have not been performed for plasmid recombination.) Thus the activation stage is a likely target for recombination-enhancing activities.

Why does plasmid recombination seem so much less Ref-sensitive than chromosomal recombination? This may not actually be the case. The frequency of Ref-dependent chromosomal recombination (Lac$^+$ cells) during overnight colony growth and subsequent overnight growth in broth is about 10^{-4} (WINDLE and HAYS 1986), whereas the frequency of recombinant plasmids in Ref$^+$ cells propagated under roughly similar conditions is about 10^{-2} (Tables 4 and 5). A judgement as to whether Ref stimulates plasmid or chromosomal recombinations more efficiently depends on whether stimulation factors or absolute Ref-dependent frequencies are compared. If Ref activates DNA for recombination, as we argue above, then it appears that it activates plasmids more efficiently than it activates bacterial chromosomes. Although there are other efficient activation mechanisms for plasmids, there appear to be none such for chromosomes. Thus Ref-activated plasmid recombination is a physiologically significant process whose "signal" relative to Ref-independent "noise" is low but acceptable. Since plasmids offer many experimental advantages, they seem the best choice for further study of Ref recombination-stimulation mechanisms.

This work was supported by National Institutes of Health grants GM 22585 and GM 33236 to J.B.H. This is technical report No. 8678 from the Oregon Agricultural Experiment Station.

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