JOURNAL OF BACTERIOLOGY, Sept. 1999, p. 5402–5408 Vol. 181, No. 17
0021-9193/99/$04.00+0


# Roles of RuvC and RecG in Phage λ Red-Mediated Recombination

ANTHONY R. POTEETE,* ANITA C. FENTON, AND KENAN C. MURPHY

*Department of Molecular Genetics & Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655*

Received 22 March 1999/Accepted 30 May 1999

**The recombination properties of *Escherichia coli* strains expressing the *red* genes of bacteriophage λ and lacking *recBCD* function either by mutation or by expression of λ *gam* were examined. The substrates for recombination were nonreplicating λ chromosomes, introduced by infection; Red-mediated recombination was initiated by a double-strand break created by the action of a restriction endonuclease in the infected cell. In one type of experiment, two phages marked with restriction site polymorphisms were crossed. Efficient formation of recombinant DNA molecules was observed in *ruvC*$^+$ *recG*$^+$, *ruvC recG*$^+$, *ruvC*$^+$ *recG*, and *ruvC recG* hosts. In a second type of experiment, a 1-kb nonhomology was inserted between the double-strand break and the donor chromosome's restriction site marker. In this case, recombinant formation was found to be partially dependent upon *ruvC* function, especially in a *recG* mutant background. In a third type of experiment, the recombining partners were the host cell chromosome and a 4-kb linear DNA fragment containing the *cat* gene, with flanking *lac* sequences, released from the infecting phage chromosome by restriction enzyme cleavage in the cell; the formation of chloramphenicol-resistant bacterial progeny was measured. Dependence on RuvC varied considerably among the three types of cross. However, in all cases, the frequency of Red-mediated recombination was higher in *recG* than in *recG*$^+$. These observations favor models in which RecG tends to push invading 3′-ended strands back out of recombination intermediates.**

As many as 15 to 20 proteins are thought to be directly involved in homologous recombination in *Escherichia coli* (for reviews, see references 19 and 23). The biochemical activities of most of these proteins have been extensively characterized. Even so, it is difficult to specify the precise roles of most of the recombination proteins. One main reason for the difficulty is that only one of the known proteins, RecA, is strictly required for recombination to take place. Certain recombination events, involving strand annealing rather than strand invasion, can take place in a *recA* null mutant, but these require bacteriophage-encoded recombination functions (36). The nearly complete deficiency of *recA* mutants, blocked at an early step in recombination (5), combined with extensive biochemical characterization of RecA protein have yielded a relatively clear description of RecA's role in recombination: synapsis and strand invasion (for a review, see reference 32.

Several of the recombination proteins of *E. coli* are helicases. Recombinant formation following conjugation or generalized transduction depends upon one of the helicases, the RecBCD protein, which is also a complex nuclease. In the absence of RecBCD, efficient recombination can take place if the *sbcB* gene, as well as the *sbcC* or *sbcD* gene, encoding other nucleases, is mutated. In this setting, recombination depends upon several helicases, including RecG, RecQ, and RuvAB (19). Another helicase, PriA, is important for recombination in otherwise-wild-type cells (18, 33).

One recombination protein, RuvC, is a junction-specific endonuclease that has the properties expected of a Holliday junction resolvase (8). Recombination in wild-type *E. coli* is not highly dependent upon RuvC, though, suggesting either that there is another resolvase or that cutting a Holliday junction is only one of two or more pathways to the production of recombinants. The latter explanation appears to be the case: Lloyd (21) found that recombination in a *ruvC* mutant is dependent on RecG, and biochemical studies of RecG protein have uncovered helicase but not endonuclease activity (24). Rather, Whitby and coworkers (38, 39) have proposed that RecG helicase can process three-stranded junctions in such a way as to generate recombinants without endonucleolytic resolution of a Holliday junction.

The bacteriophage λ homologous recombination system, known as Red, normally promotes highly efficient double-strand break repair and recombination transiently in λ-infected *E. coli*. The Red-induced "hyper-rec" state of *E. coli* can be made permanent if the λ recombination proteins are expressed in the cell in the absence of infecting phage (29, 31). In addition to operating at higher efficiency than other homologous recombination pathways in *E. coli*, Red-mediated recombination is possibly simpler in its early steps (diagrammed in Fig. 1). The λ Gam protein shuts down all the enzymatic activities of RecBCD (16, 28). Double-stranded ends initiate Red-mediated recombination (35, 37). The λ exonuclease (Redα) loads onto double-stranded ends and processively degrades the 5′-ended strand, leaving a 3′ single-stranded tail (20). RecA and λ Redβ protein then cooperate in promoting invasion of the 3′-ended strand into an unbroken homologous duplex (17, 27, 31). (Redβ can promote recombination by strand annealing in the absence of RecA protein, but only if a partner with a double-stranded end at a nonallelic site is provided [36].) In the absence of RecBCD, the only λ proteins required for efficient double-strand break repair and recombination are Redα and Redβ; host proteins carry out all other steps.

An *E. coli* strain containing λ recombination proteins provides an experimental setting in which recombination between nonreplicating λ phages occurs at high frequency at the site of a double-strand break (37). Formation of recombinants between physically marked chromosomes can be monitored di-

* Corresponding author. Mailing address: Dept. of Molecular Genetics & Microbiology, University of Massachusetts Medical School, 55 Lake Ave. North, Worcester, MA 01655. Phone: (508) 856-3708. Fax: (508) 856-5920. E-mail: tpoteete@ummed.edu.

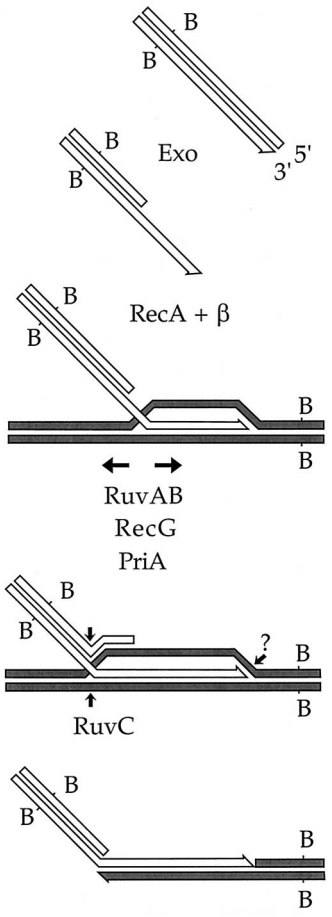


FIG. 1. Possible mechanism of Red-mediated recombination involving strand invasion. The steps, and the roles of the recombination proteins, are described in the text.

rectly by extraction and analysis of DNA from infected cells. It depends upon (i) double-strand breaks, (ii) RecA, (iii) Red proteins, and (iv) inactivation of RecBCD, either by mutation or by λ Gam protein (31).

In this study, we examined the properties of Red-expressing *E. coli* bearing mutations in *ruvC* and *recG*, singly and in combination. We found that the dependence of Red-mediated recombination on RuvC was variable among the different types of crosses tested. Red-mediated recombination is more efficient in a *recG* mutant than in a *recG*$^+$ strain, in contrast to recombination via other pathways (RecBCD, RecF, or RecE), which is reduced in *recG* mutant (22). However, as in wild-type *E. coli*, this Red-mediated recombination was, in most cases, highly dependent upon RuvC in the *recG* mutant. These observations distinguish between models that have been proposed for the roles of RuvC and RecG in recombination (38, 39).

## MATERIALS AND METHODS

**Bacteria.** Except as noted, all bacterial strains used in this study were derivatives of *E. coli* AB1157 (*argE3 his-4 leuB6 proA2 thr-1 ara-14 galK2 lacY1 mtl-1 xyl-5 thi-1 rpsL31 tsx-33 supE44*); it and JC15329 [Δ(*srlR-recA*)*306*::Tn*10*] were obtained from A. J. Clark. Strains CS85 (*eda-51*::Tn*10 ruvC53*) (34) and N2731 (*recG258*::*kan*) (22) were obtained from F. W. Stahl. Strain CS85 was reconstructed by transduction of AB1157 with a P1 lysate grown on strain CS85, with selection for tetracycline resistance. A tetracycline-sensitive derivative, TP438, was subsequently selected on the basis of fusaric acid resistance (26). TP440 (*ruvC53 recG258*::*kan*) was constructed by transduction of TP438 with a P1 lysate grown on N2731, with selection for kanamycin resistance. Phage recombination functions and the EcoRI restriction-modification system were introduced into these strains by simultaneous transformation with plasmids pTP223 and pMB4, as described previously (31).

Strain KM22, Δ(*recC ptr recB recD*)::$P_{tac}$-*bet-exo kan*, has been described previously (29). KM32, Δ(*recC ptr recB recD*)::$P_{tac}$-*gam-bet-exo cat*, was constructed similarly.

Strain TP507, in which the *recC*, *ptr*, *recB*, and *recD* genes are replaced by a cassette consisting of $P_{tac}$-*gam-bet-exo*, the *Pae*R7 restriction-modification system, and $P_{lac}$-*c*I, was constructed by crossing KM22 with a linear DNA fragment as described previously (29). Recombinants were selected on the basis of their immunity to λ infection. The linear fragment was generated by digestion with *Nde*I and *Bam*HI of plasmid pTP822, which was constructed from a series of intermediates as follows. (i) Plasmid pTP800 was made by ligation of a polycloning site, consisting of the partially complementary oligonucleotides AATTGGG CCCAGATCTCCATGGCCGCGGTCTAGAGCTC and AATTGAGCTCTA GACCGCGGCCATGGAGATCTGGGCCC, into the *Eco*RI site of pKM125 (29). One isolate, in which the orientation of the inserted sequence was *thy*A-*Apa*I-*Bgl*II-*Nco*I-*Sac*II-*Xba*I-*Sac*I-*argA* (see Fig. 4), was selected. (ii) The source of the $P_{tac}$-*gam-bet-exo* sequence was plasmid pTP234 (*Ptac-gam-bet-exo* operon fusion inserted into the *Eco*RI site of pBR322, containing λ sequences from the *Sal*I site immediately upstream from *gam* to the *Acc*I site immediately downstream from *exo*), which served as a template for PCR with oligonucleotides GACATAAGATCTCCGACATCATAACGGTTCTGGCAA and GACATAA GATCTTTGCGCCTACCCGGATATTATCGT. The resulting 2.1-kb fragment was digested with *Bgl*II and ligated into the *Bgl*II site of pTP800. One isolate, in which the orientation of the inserted sequence is such that the λ *red* genes are transcribed in the direction *thyA-argA*, was selected and designated pTP806. (iii) The source of the *Pae*R7 restriction-modification system (*pae*) was plasmid pAORM3.8 (12), which served as a template for PCR with oligonucleotides ACAATTCCATGGGCCGATGATTTAGTGAGGTCGTCA and ACAATTTC TAGACCTCCAAGCCCGAATGATCGAGAA. The resulting 2.5-kb fragment was digested with *Nco*I and *Xba*I and ligated between the corresponding sites in pTP806, generating plasmid pTP808. (iv) The source of $P_{lac}$-*c*I was plasmid pKB280 (4), which served as a template for PCR with oligonucleotides AGTT GCTCTAGAACTCATTAGGCACCCCAGGCTTTA and AGTTGCTCTAGA TTATCAGCTATGCGCCGACCAGAA. The resulting 0.9-kb fragment was digested with *Xba*I and ligated into the *Xba*I site of pTP808. One isolate, in which the orientation of the inserted sequence is such that the λ *c*I gene is transcribed in the direction *thyA-argA*, was selected and designated pTP810. (v) An *Apa*I-*Sac*I fragment from pTP810 containing $P_{tac}$-*gam-bet-exo pae* $P_{lac}$-*c*I was inserted between the *Apa*I and *Sac*I sites in pKM145-6 to generate pTP822. pKM145-6 is similar in structure to pKM125 but has no antibiotic resistance determinant between the *recBCD*-flanking sequences (29a).

The chromosomal $P_{tac}$-*gam-bet-exo pae* $P_{lac}$-*c*I operon of strain TP507 represses expression of *lacZ* from the $P_L$ promoter of an infecting λ phage at least 1,000-fold and restricts the efficiency of plaque formation by *Pae*R7-unmodified λ *imm P22 h80* approximately $10^4$- to $10^5$-fold (data not shown). Although the λ recombination and repressor genes in the substitution are nominally under the control of the wild-type *lac*I gene of this strain, effective expression is seen even in the absence of inducer. Derivatives of TP507 bearing *rec* and *ruv* mutations were constructed by P1 transduction, with selection for antibiotic resistance. Donor strains were as listed above.

**Phages.** λ wild type and λ *nin5* were obtained from F. W. Stahl. λ *sR1° RFLP381 sR3° sR4° sR5°* and *sR1° RFLP382 sR3° sR4° sR5°*, referred to below as λ RFLP381 and λ RFLP382, have been described previously (31). Additional RFLP substitutions were crossed into the λ chromosome as described previously (31); recombinants were selected as described or on the basis of plaque formation on a phage P2 lysogen (Spi$^-$ phenotype) and were given the numerical designations of their plasmid parents (e.g., λ RFLP835 was made by crossing λ wild type with plasmid pTP835). Phages were propagated in a Δ(*recC ptr recB recD*)::$P_{tac}$-*bet-exo cat* derivative of W3110 *strA594 lac gal*, which was constructed by P1 transduction with KM32 as the donor and selection for chloramphenicol resistance.

New substitution sequences were assembled by making a variety of derivatives of plasmid pTP368 (31). This plasmid contains λ sequences from 21,226 to 23,130 and 33,498 to 34,499; sequences cloned between the λ segments, on recombination with phage, replace λ sequences normally between them. The intermediate plasmids were made as follows. For pTP812, the *cat* gene from pCDK3 (9) was amplified by PCR with oligonucleotides ATCATCGCTAGCATGAGAACGTT GATCGGCACGTAAG and ATCATCGGTACCGGGCCCGACCGGGTCGA ATTTGCTTTCGAA. The resulting 0.9-kb fragment was digested with *Kpn*I and *Nhe*I and ligated between the *Kpn*I and *Nhe*I sites in pTP368. pTP813 was constructed by conversion of the *Xho*I site of pTP368 to an *Apa*I site by insertion of an oligonucleotide, TCGATCGGGCCCGA. For pTP815, the *Xho*I site in pTP812 was eliminated by digestion with *Xho*I, filling in the ends with DNA polymerase and deoxynucleoside triphosphates, and ligating. pTP816 was constructed by conversion of the *Afl*II site in pTP815 to a *Pae*R7 (*Xho*I) site by substitution of a corresponding segment, bounded by *Nhe*I and *Sac*I sites, from pTP817. pTP817 was constructed by conversion of the *Afl*II site in pTP813 to a *Pae*R7 (*Xho*I) site by insertion of an oligonucleotide, TTAACGCCTCGAGG CG. pTP835 and pTP836 were constructed by conversion of the *Apa*I sites of

pTP816 and pTP817 to *Bgl*II sites by insertion of an oligonucleotide, GCAGA TCTGCGGCC. For pTP863 and pTP864, the *Pae*R7 (*Xho*I) sites of pTP835 and pTP836, respectively, were eliminated by digestion with *Xho*I, filling in the ends with DNA polymerase and deoxynucleoside triphosphates, and ligating with a *Hin*dIII linker (CAAGCTTG).

Derivatives of λ designed to monitor recombination events with the cell chromosome were constructed as described above. Two plasmid intermediates were involved. pTP818 was constructed by conversion of the *Afl*II site in pTP368 to a *Pae*R7 (*Xho*I) site by insertion of an oligonucleotide, TTAACGCCTCGAGGCG. For pTP819, sequences between the two *Xho*I sites of pTP818 were replaced by a segment in which the *cat* gene is flanked by *lac* sequences, assembled from three PCR-generated parts: (i) *lacZ* sequences amplified from *E. coli* AB1157 DNA with primers GACGCACTCGAGGCGTTAACCGTCACGA and CTCCAGTGGC CGGCGATGATGCAGCGGCGTCAGCAGTTGTT; (ii) *cat* sequences amplified from DNA from an *E. coli* strain bearing transposon Tn*9* with primers ATCATCGCCGGCCACTGGAGCACCTCAAAAACACCA and TCATCAG GGCCCGACCGGGTCGAATTTGCTTTCGAA; and (iii) *lacZ* and *lacY* sequences amplified from *E. coli* AB1157 DNA with primers ACCCGGTCGGG CCCTGATGACCCGTGCACCGCTGGATAACG and GACGCACTCGAGG CACACAGCGCCCAG. Parts i and ii and parts ii and iii were joined in pairs by PCR, making use of the overlaps of their primer sequences, and subcloned in intermediate plasmids via their *Xho*I and *Ngo*M1 and their *Apa*I and *Xho*I ends, respectively. The two subassemblies were recombined in vitro by cutting and joining at the *Nco*I site in *cat*. This construction makes use of two sites in the *E. coli lac* operon that differ by 1 bp from *Pae*R7 sites, in such a way that when the *lac*::*cat819* segment is cut from the phage that bears it, the ends of the resulting DNA fragment match the cell chromosome exactly.

**Crosses.** Bacterial host strains were cultured and infected with parent phages at a multiplicity of seven each as described previously (31). When plasmid-bearing strains were used as hosts, retention of the plasmid pMB4 bearing the *Eco*RI restriction-modification system was measured as described previously (31); in cases in which less than 85% of the cells retained pMB4, the experiment was discontinued.

Phage DNA was extracted from 10-ml samples of λ RFLP-infected cell cultures by the use of lysozyme and a phenol-chloroform-isoamyl alcohol mixture as described previously (31); in later experiments, phenol was substituted for the phenol-chloroform-isoamyl alcohol mixture. DNA samples (10% of the total) were digested overnight at 37°C with 15 U of *Bgl*II and 2.5 U of RNase A and then subjected to electrophoresis in 0.7% agarose gels and transferred by capillary flow to Zeta-Probe (Bio-Rad) membranes. In experiments with λ RFLP381 and λ RFLP382, blots were probed with $^{32}$P-labeled RNA as described previously (31). In experiments with other λ RFLP phages, the prehybridization mixture was composed of 1 mM EDTA, 0.5 M sodium phosphate buffer [pH 7.2], 7% sodium dodecyl sulfate, and 200 μg of yeast RNA (baker's yeast RNA, type III; Sigma). Following 30 min of prehybridization at 60°C, $^{32}$P-labeled *cat* gene probe ($10^7$ cpm) was added. Following incubation at 60°C overnight, the filters were washed and processed as described previously (31).

The *cat* gene probe was generated as follows. Plasmid pTP801 (made by cloning the *cat* gene, in the form of a DNA fragment generated by PCR with oligonucleotide primers CGGGATCCCGTGAGACGTTGATCGGCACGTA AGA and CGGGATCCCGGACCGGGTCGAATTTGCTTTCGAA, into the *Bam*HI site of pUC19) was cut with *Bam*HI to generate a 0.9-kb fragment, which was purified by electrophoresis in a 0.7% agarose gel. The 0.9-kb band was removed, and the DNA was extracted by centrifugation of the gel slice through an Easy Clean agarose gel DNA extraction filter (Primm Labs). Approximately 25 ng of the purified DNA was denatured by boiling it for 10 min and then placed on ice and added to a random priming reaction mixture containing 3 μl of a mixture of 0.167 mM (each) dATP, dGTP, and TTP (50 μCi of [α$^{32}$P]dCTP; 3,000 Ci/mmol), 5 U of Klenow polymerase, and 2 μl of Boehringer Mannheim hexanucleotide mix (10× concentration) in a total volume of 20 μl. After incubation at 37°C for 30 min, the reaction mixture was extracted twice with phenol and then once with ether, and DNA was precipitated twice with ethanol and then redissolved in 100 μl of water and boiled for 10 min before being added to the membranes.

Autoradiographs were scanned in a Molecular Dynamics Personal Densitometer SI. Quantitation of bands was done by the use of ImageQuant software on an Apple computer.

In crosses between λ *lac*::*cat819* and bacterial hosts, the bacteria were cultured and infected with phage as described previously (31). The multiplicity of infection was 5, except as noted. After 1 h at 37°C, the infected cells were titered on Luria-Bertani agar plates and on Luria-Bertani agar plates supplemented with chloramphenicol (20 μg/ml), isopropylthiogalactopyranoside (250 μg/ml), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (133 μg/ml) for detection of recombinants.

## RESULTS

**Red-mediated recombination independent of RuvC and RecG.** Genetic and biochemical studies of recombination in *E. coli*, summarized in the introduction, suggest that a *recG ruvC* double mutant, which is highly defective in recombination, is specifically blocked at the resolution step. A prediction that follows from this idea is that unresolved recombination intermediates might accumulate in replication-blocked crosses between λ phages in a *recG ruvC* double mutant. To test this, we constructed single- and double-mutant strains bearing plasmids that express *red* genes, λ *c*I repressor, and the *Eco*RI restriction-modification system. The somewhat surprising result of phage crosses in these strains (Fig. 2) was that the frequency of recombination relative to that in the wild type was increased by mutation of *recG* and only slightly affected by mutation of *ruvC*, either alone or in combination with *recG*. In seeking to understand this lack of dependence on either RuvC or RecG, we considered and tested a number of possible explanations.

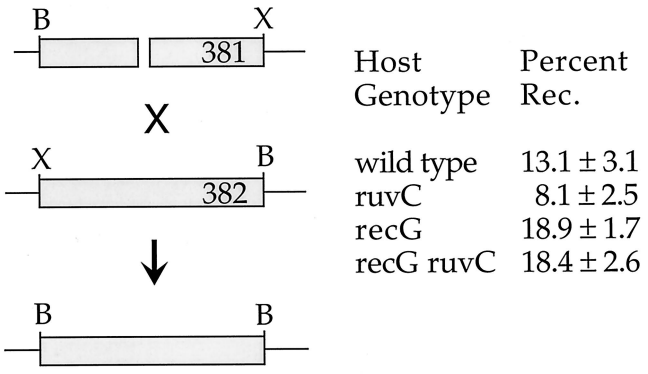


FIG. 2. Recombination (Rec.) between nonreplicating λ chromosomes, one of which has a double-strand break. The phages, λ RFLP381 (381) and λ RFLP382 (382), bear a substitution that replaces λ genes between *orf194* and *ssb* with unrelated sequences; the ends of the substitution are marked with different restriction sites: B, *Bgl*II, and X, *Xba*I. The host cells bear two plasmids: pTP223, which expresses λ genes *c*I, *gam*, *red*β, and *red*α, and pMB4, which encodes the *Eco*RI restriction-modification system. The phages each have a single *Eco*RI restriction site in the substitution sequence; in the case of λ RFLP382, it was made uncuttable by prior passage through an *Eco*RI-modifying host. DNA was extracted from infected cells 60 min after infection, digested with *Bgl*II, and analyzed on Southern gels. Parental and recombinant bands from three experiments were quantitated; the means and standard errors are indicated.

One explanation for the recombination proficiency of the *ruvC recG* double mutant might be that the strain had acquired a suppressing mutation. The *rusA* mutation described by Mahdi et al. (25), in particular, activates a RuvC-like resolvase encoded by a cryptic prophage. However, tests (data not shown) of the *ruvC recG* strain showed that it is as recombination-deficient in conjugative crosses and transduction, and as UV sensitive, as the original strain described by Lloyd (21).

Another explanation for the high frequency of Red-mediated recombination might be that the phages themselves express a resolving function. The most likely candidate for such a function would be the *rap* gene in the λ *nin* region (15), which Mahdi et al. (25) have proposed to be a Holliday junction resolvase. This explanation is unlikely for two reasons: (i) the crosses are carried out in host cells that overexpress λ repressor from a plasmid, so few λ genes (none from the *nin* region) should be transcribed, and (ii) in redesigning the tester phages (see below), we have deleted the *nin* region and still detected efficient recombination in the *ruvC recG* mutant host.

The explanation we consider most likely is that the process observed in the λ crosses does not necessarily involve simple break-join recombination but rather can proceed via branch migration and heteroduplex formation, as diagrammed in Fig. 3a. A control experiment showed that mismatch repair would necessarily be involved in this alternative pathway. We con-

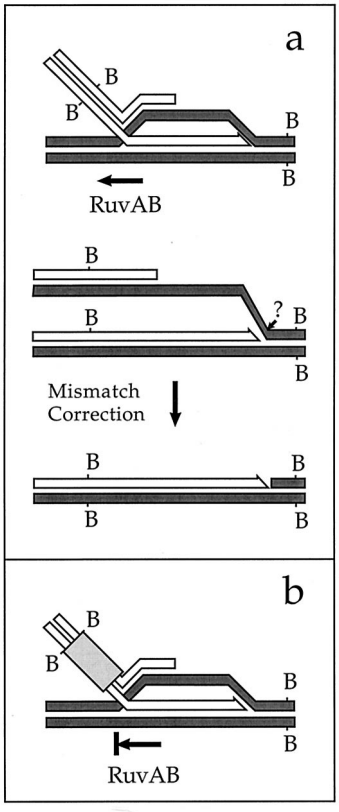


FIG. 3. (a) Possible mechanism of recombinant formation via the Red pathway, independent of RuvC and RecG. The first steps (not shown) would be the same as in Fig. 1. Branch migration, possibly directionally biased due to the absence of RecG, leads to heteroduplex formation at the site of marker B. Mismatch repair then converts the heteroduplex to a homoduplex, bypassing the need for endonucleolytic processing of a Holliday junction. On the right side, endonucleolytic cleavage and trimming of the displaced strand or, possibly, extension of the invading 3′ end would produce a structure that would be counted as a recombinant following extraction, digestion with *Bgl*II, and analysis on a Southern gel. (b) A heterologous sequence inserted between the invading end and the marker B is predicted to block the pathway shown in panel a, making recombination more dependent upon RuvC and/or RecG.

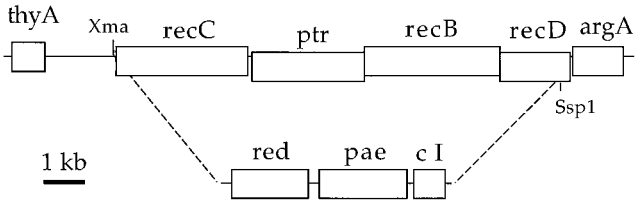


FIG. 4. Map of the Δ(*recC-recD*)::*red-pae-c*I substitution in strain TP507 and derivatives. Sequences between the *Xma*I site upstream from *recC* and the *Ssp*I site near the C-terminus-encoding end of *recD* are replaced. The block of sequence designated "red" includes (from left to right) the promoter $P_{tac}$ and λ genes *gam*, *red*β, and *red*α. pae includes the restriction endonuclease and methylase genes of *Pseudomonas aeruginosa* R7, transcribed rightward. The λ *c*I gene is fused to the promoter $P_{lac}$*UV5* and is also transcribed rightward.

structed mismatched heteroduplexes with the structure that would be produced in an event of the type pictured above and found that they could not be digested with the restriction enzyme (*Bgl*II) used to characterize phage chromosomes extracted from the infected cells (data not shown); therefore, branch migration alone cannot account for the apparent recombination. We then attempted to test the role of mismatch repair genetically. A *ruvC recG mutS* triple mutant was constructed and transformed with Red, *c*I, and *Eco*RI-expressing plasmids. However, the resulting strain grew poorly, particularly in liquid culture, and the phage crosses could not be carried out.

The alternative, branch migration-heteroduplex formation-mismatch repair pathway to recombinant formation could in principle be blocked by insertion of a heterologous sequence between the double-strand break and the marker used to score recombination (a restriction site, in this case), as pictured in Fig. 3b. Recombination might then be channeled through a resolvase-requiring pathway.

**Redesign of phages and hosts for detection of resolvase-dependent recombination events.** For reasons described above, we constructed new variants of the λ RFLP phages (see Fig. 5 [top]). The λ sequences replaced by the new RFLP substitutions are the same as in the previous versions, but three main changes were made. (i) In the new phages, the leftmost 1 kb of sequence in the substitution is either the same as before or has been replaced by a heterologous sequence, the *cat* gene of transposon Tn*9*. (ii) A unique *Pae*R7 restriction site has been introduced near the middle of the 5-kb substitution sequence (near the *Eco*RI site of the previous versions). This change was introduced because other studies had demonstrated the greater efficiency of the *Pae*R7 restriction enzyme in promoting recombination events in vivo, relative to the *Eco*RI system employed previously (36). *Pae*R7-uncuttable partners were constructed by insertion of *Hin*dIII linkers into the *Pae*R7 sites. (iii) The *nin* region was eliminated by crossing in the deletion *nin5*.

The extraordinary recombination proficiency of *E. coli* strains bearing λ *red* genes in their chromosomes in place of *recBCD* (29) led us to place the other functions needed for λ RFLP crosses—*c*I repressor and the *Pae*R7 restriction-modification system—in the chromosome as well (Fig. 4). This approach eliminates the need for plasmids in the host strains, a significant advantage. Some bacterial strains lacking recombination functions grow relatively poorly when they bear plasmids. The plasmids' replication, moreover, is affected by mutations of the host recombination genes, as well as expression of phage recombination functions. For example, in bacteria in which RecBCD is inactivated, much plasmid DNA is found in the form of linear multimers, apparently as the result of rolling-circle replication (7).

As detailed in Materials and Methods, we constructed a cassette bearing the λ genes *gam*, *bet*, and *exo*, the *Pae*R7 restriction-modification system, and λ *c*I repressor and recombined it into the chromosome of *E. coli* AB1157 in place of *recBCD* (Fig. 4). The resulting strain, TP507, is recombination proficient in general and hyper-rec with respect to recombination with short linear DNA fragments, as described previously (29). Mutant alleles of *recG* and *ruvC* were introduced into the TP507 background by P1 transduction.

**Resolvase-dependent Red-mediated recombination.** Crosses between the new λ RFLP phages were predicted to have different outcomes, depending upon which partner was cut, as diagrammed in Fig. 5 (top). Cutting the non-*cat*-bearing phage should result in the production of recombinants independent of resolution functions, as seen previously. However, according to the scheme outlined in Fig. 3, a double-strand break in the *cat*-bearing phage should lead to the production of a recombination intermediate that can be processed into a recombinant only by the action of a resolvase.

The involvement of resolution functions in recombination in the new system was tested in the experiment shown in Fig. 5

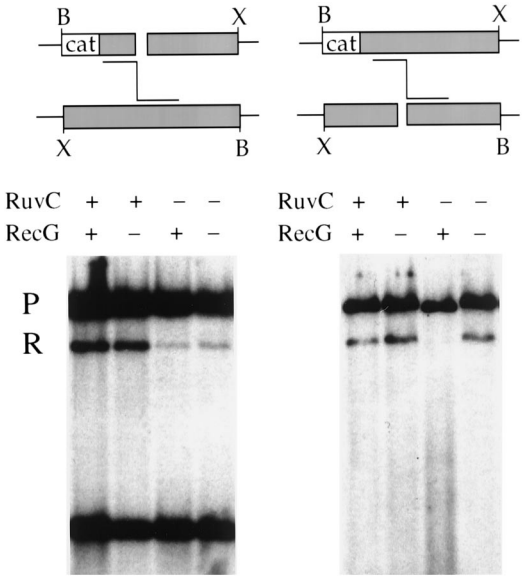


FIG. 5. (Top) Recombination between λ RFLP phages bearing a heterologous sequence. As indicated, production of a recombinant follows different potential pathways, depending upon which phage is cut. On the left, the invading partner has homology (1 kb) at the end but has a heterologous sequence (the *cat* gene; 1 kb) between the end and the restriction site marker B (*Bgl*II). Formation of recombinants by the mechanism diagrammed in Fig. 3 is blocked. On the right, the invading strand encounters no substantial heterology, only the restriction site polymorphism. Formation of recombinants by the mechanism diagrammed in Fig. 3 is permitted. The cross on the left involves phages λ *RFLP835 nin5* (above) and λ *RFLP864 nin5* (below); that on the right involves λ *RFLP863 nin5* (above) and λ *RFLP836 nin5* (below). (Bottom) Autoradiographs of Southern gels of *Bgl*II-digested DNA extracted from host cells 60 min after infection and probed with *cat* sequences. The positions of parental (P) and recombinant (R) bands are indicated. The lowest band results from cutting with *Pae*R7 in vivo and with *Bgl*II in vitro. The bacterial strains were all Δ(*recC-recD*)::*red-pae-c*I. +, present; −, absent.

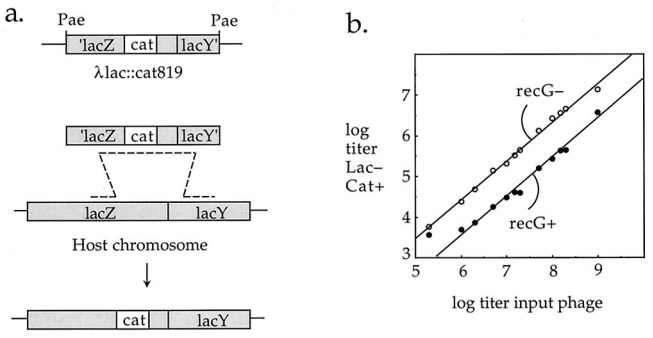


FIG. 6. (a) Recombination between λ *lac*::*cat819 nin5* and the bacterial chromosome. Phage repressor in the infected cell prevents transcription of phage genes. *Pae*R7 restriction endonuclease releases a DNA fragment that recombines with the chromosome, generating a chloramphenicol-resistant, Lac⁻ recombinant. (b) Production of recombinants following infection with λ *lac*::*cat819 nin5*. Log-phase cells (approximately $10^8$/ml) were infected at various multiplicities and, after 1 h of further aeration, were plated for total and recombinant (*cat*⁺ *lac*) titers. Open circles, Δ(*recC-recD*)::*red-pae-c*I *recG258*; solid circles, Δ(*recC-recD*)::*red-pae-c*I. Total cell counts increased approximately fourfold during the experiment.

(bottom). In this experiment, DNA was extracted from cells infected with RFLP phages, digested with *Bgl*II endonuclease, run in a Southern gel, and hybridized with a *cat*-specific probe. In crosses in which the *cat*-bearing phage was cut in vivo, three bands were seen: parental, recombinant, and a 1,990-bp fragment generated by *Pae*R7 cutting in vivo and *Bgl*II cutting in vitro. The last band was not present in DNA extracted from crosses in which the non-*cat*-bearing phage was cut. The three bands correspond to ones seen previously (31).

The fraction of label in recombinant bands in Fig. 5 (bottom) and similar autoradiograms was quantitated; the results are shown in Table 1. As seen previously, the two phages recombine efficiently in the *red*-expressing host that lacks RecBCD but is otherwise wild type for recombination functions. In contrast to previous results, however, recombination was reduced more than 15-fold by mutation of *ruvC*. The RuvC dependence of recombination was seen regardless of which parent was cut. Recombination was increased by mutation of *recG*; this effect, too, was independent of which parent was cut. In a *recG* mutant background, however, loss of *ruvC* function resulted in a 16-fold decrease in recombination when the *cat*-bearing chromosome was cut but only a slight (possibly insignificant) decrease when the non-*cat*-bearing chromosome was cut.

TABLE 1. Effects of *ruvC* and *recG* mutations on λ Red-mediated recombination between phage chromosomes

| Bacterial strain[b] | Percent recombinant[a] | |
|---|---|---|
| | *cat*-bearing phage cut | Non-*cat*-bearing phage cut |
| Wild type | 8.4 ± 5.4 | 13 ± 4.0 |
| *ruvC* | 0.6 ± 0.4 | 0.3 ± 0.3 |
| *recG* | 21 ± 9.3 | 28 ± 1.3 |
| *ruvC recG* | 1.3 ± 0.2 | 23 ± 2.6 |

[a] The percent of total *cat* gene sequences found in recombinant DNA molecules in the crosses shown in Fig. 5 and two similar experiments were measured as described in the text. The means and standard errors are shown.
[b] All strains bear the Δ(*recC-recD*)::*red-pae-c*I substitution.

**Biological-recombinant formation.** We sought to address the question of whether the formation of recombinant DNA molecules in λ RFLP crosses correlates with the production of biological recombinants. We constructed a variant of the RFLP phages, λ *lac*::*cat819*, which, upon infecting an immune host with the *Pae*R7 restriction-modification system, is cut by the *Pae*R7 nuclease, releasing a 4-kb fragment with *lacZ* and *lacY* sequences at its ends and the *cat* gene in the middle (Fig. 6a). Red-mediated recombination results in replacement of the chromosomal *lacZ* gene with a mutant allele that is defective for *lacZ* function but confers chloramphenicol resistance on the recombinant. According to the rationale diagrammed in Fig. 3, production of chloramphenicol-resistant recombinants would be expected to require the same functions as production of recombinant DNA molecules in λ RFLP crosses in which the *cat*-bearing chromosome is cut (Fig. 5, top left).

Infection of a Δ*recBCD*::*red-pae-c*I *recG* host with λ *lac*::*cat819 nin5* at low multiplicity produced Lac⁻ chloramphenicol-resistant recombinants at the rate of approximately 0.02 per infecting phage. This frequency was independent of multiplicity over a range of 0.001 to 1, indicating that infection with a single phage is sufficient to produce a recombinant (Fig. 6b). When this strain was infected with λ *lac*::*cat819 nin5* at high multiplicity, as many as 18% of the infected cells gave rise to chloramphenicol-resistant recombinants (data not shown).

The abilities of (Δ*recBCD*::*red-pae-c*I) *ruvC*, *recG*, and *recG ruvC* mutants to recombine with λ *lac*::*cat819* and λ *lac*::*cat819 nin5* are compared in Table 2. The two phages gave comparable frequencies of recombination in all hosts, indicating that λ genes in the *nin* region did not significantly affect recombinant

TABLE 2. Effects of *ruvC* and *recG* mutations on gene replacement frequency

| Bacterial strain[a] | Relative frequency of Lac$^-$ Cm$^r$ recombinants[b] | |
|---|---|---|
| | λ *lac*::*cat819* | λ *lac*::*cat819 nin5* |
| Wild type | 1.0 | 1.0 |
| *ruvC* | 0.32 ± 0.11 | 0.49 ± 0.07 |
| *recG* | 4.8 ± 1.7 | 7.8 ± 4.1 |
| *ruvC recG* | 0.20 ± 0.12 | 0.20 ± 0.13 |
| *recA* | NT | 0.011 ± 0.004 |

[a] All strains bear the Δ(*recC-recD*)::*red-pae-c*I substitution.
[b] Wild-type values ranged from 0.19 to 0.57%. The means and standard errors from two to four experiments are given. NT, not tested.

formation. Loss of *ruvC* function reduced the recombination frequency two- to threefold, while loss of *recG* function increased it five- to eightfold. The effect of the *ruvC* mutation was much greater in a *recG* mutant background, resulting in a 20- to 40-fold decrease in recombination. A *recA* mutation, by comparison, reduced recombination 90-fold.

An interesting and as yet unexplained aspect of recombination between λ *lac*::*cat819* and *E. coli* strains bearing the Δ*recBCG*::*red-pae-c*I substitution is that up to one-third of the chloramphenicol-resistant recombinants in some crosses were LacZ$^+$. Production of these LacZ$^+$ recombinants appears to depend on the same functions as the LacZ$^-$ recombinants—in particular, recA and, in a *recG* background, *ruvC* (data not shown). Their retention of *lacZ* function implies that the recombination event that generated them was not simple gene replacement. The LacZ$^+$ recombinants may contain duplications of the *lac* region. This idea is favored by the observation that many of them gave rise to spontaneous LacZ$^-$ colonies on restreaking (unpublished observations).

## DISCUSSION

The results of experiments examining Red-mediated recombination between linear and circular partners suggest two main interpretations concerning the roles of RecG and RuvC. (i) the main pathway to recombinant formation involves RuvC and (ii) RecG impedes this main recombination pathway. However, RecG may also stimulate recombination via a RuvC-independent pathway.

In the presence of RecG function, the degree of dependence of Red-mediated recombination on RuvC varied substantially among the three types of crosses described in the preceding section. The dependence was greatest in crosses between nonreplicating phage chromosomes in non-plasmid-bearing cells (Fig. 5). In the other cases, recombination appeared to be nearly independent of RuvC.

The contribution of RecG to Red-mediated recombination was less variable among experiments: in all cases, elimination of RecG function increased the frequency of recombination. In both types of crosses that were carried out in non-plasmid-bearing cells—between nonreplicating λ chromosomes and between short linear DNA fragments and the bacterial chromosome—the resulting higher-frequency recombination was also more dependent on RuvC (except when recombinant formation could in theory proceed via branch migration, heteroduplex formation, and mismatch repair, as pictured in Fig. 3). The simplest interpretation of these observations is that RecG opposes the main pathway of Red-mediated recombination, which is RuvC dependent. The data in Table 2 suggest that RecG may also promote an alternative, RuvC-independent recombination pathway: recombination frequencies in the *ruvC* mutant were slightly higher than in the *ruvC recG* double mutant.

An alternative interpretation of the effects on Red-mediated recombination of the *recG258* allele is that they do not result from inactivation of *recG* but rather from a function expressed by the mini-Tn*10* used to generate the allele (22). This interpretation is ruled out by the observation that a simple deletion allele of *recG* exhibits the same stimulation of Red-mediated recombination as *recG258* (unpublished observations).

Our description of the roles of RecG and RuvC is similar to a model proposed by Whitby and coworkers (39), in which RecG actually aborted genetic exchanges resulting from RecA-mediated strand invasion but then allowed RecBCD to catalyze exchanges at the ends of the incoming DNA by an unspecified mechanism. Although these researchers found biochemical evidence for RecG's ability to act in this way, the fact that a *recG* mutant is recombination deficient relative to wild type apparently led them to favor other interpretations of RecG's activity in recombination (1, 38).

The generation of late-arising revertants of *lacZ* mutants under conditions of selection is another cellular activity that apparently is impeded by RecG (10, 13). This activity, sometimes called "adaptive mutability," is dependent upon recombination functions (6, 13). Its dependence upon recombination functions may be a consequence of the involvement of gene amplification in the process (3). Harris et al. (13) proposed a model for recombination associated with adaptive mutation, in which RecG aborts 3′-end invasion and promotes 5′-end invasion.

The initial steps of Red-mediated recombination are perhaps less complex than those of RecBCD-mediated recombination or of RecBCD-dependent adaptive mutation. The λ exonuclease specifically and processively degrades the 5′-ended strand of double-stranded DNA (20), leaving exclusively 3′-ended single strands for synapsis and strand invasion. Production of these 3′-ended single-strand tails in λ-infected cells has been observed directly (14). The observation that RecG inhibits Red-mediated recombination that is constrained to proceed via strand invasion favors models in which RecG tends to push out invading 3′ ends.

The model diagrammed in Fig. 1 and 3 accounts reasonably well for recombination between linear and circular partners in a *red*-expressing cell lacking *recBCD* and *recG* functions: double-stranded ends are channeled nearly exclusively through a pathway that involves the creation of 3′-ended single-strand tails, which invade an unbroken homologous duplex. If and only if branch migration is impeded by a significant nonhomology, recombinant formation is dependent upon nucleolytic resolution of the resulting Holliday junction by RuvC. In the absence of RuvC, presumably, a recombination intermediate something like the structure diagrammed in Fig. 3b accumulates. We have not observed such an intermediate, but the methods employed in the extraction and restriction enzyme digestion of DNA from λ-infected cells might be expected to favor its dissociation by spontaneous branch migration.

The model diagrammed in Fig. 1 and 3 does not account so well, by itself, for what happens in a *recG*$^+$ cell. A more complete model would account for three questions raised by the data in Fig. 2 and Tables 1 and 2. (i) Why was recombination between nonreplicating λ chromosomes, with no substantial nonhomologies, dependent upon RuvC in one set of crosses (Table 1, non-*cat*-bearing phage cut) and independent in another (Fig. 2)? One possible explanation is that the interacting DNA sequences in question were not identical in the two experiments and branch migration could proceed all the

way past the restriction site polymorphism in the face of opposition by RecG in one case but not in the other. (ii) The crosses diagrammed in Fig. 5 (left side) and Fig. 6 both involve recombination of a linear *cat*-bearing chromosome with a circular chromosome. Why was recombinant formation dependent upon RuvC in the former case and independent in the latter? A key difference between the two crosses is that replication of both partners is blocked in the λ cross (Fig. 5) whereas replication of only the linear partner is blocked in the linear-by-host chromosome cross (Fig. 6). The passage of a replication fork may potentiate alternative pathways for the resolution of recombination intermediates. (iii) How does RecG promote, rather than impede, RecBCD-mediated recombination? One possibility is that RecBCD-mediated recombination proceeds primarily via 5′-ended strand invasion, but extensive studies of the activities of RecBCD, both in vitro (2) and in vivo (11), favor the view that RecBCD generates invading 3′-ended strands. Together, these observations are consistent with the idea that RecBCD may function in the processing or resolution of recombination intermediates that it participates in generating (5, 30). Perhaps the normal role of RecG is to facilitate this function of RecBCD.

## ACKNOWLEDGMENTS

We thank Rik Myers and Susan Rosenberg for helpful discussions and Michael Volkert for critical reading of the manuscript.

This work was supported by Public Health Service grant GM51609 from the National Institutes of Health.

## REFERENCES

1. **Al-Deib, A. A., A. A. Mahdi, and R. G. Lloyd.** 1996. Modulation of recombination and DNA repair by the RecG and PriA helicases of *Escherichia coli* K-12. J. Bacteriol. **178:**6782–6789.
2. **Anderson, D. G., and S. C. Kowalczykowski.** 1997. The translocating RecBCD enzyme stimulates recombination by directing RecA protein onto ssDNA in a χ-regulated manner. Cell **90:**77–86.
3. **Anderson, D. I., E. S. Slechta, and J. R. Roth.** 1998. Evidence that gene amplification underlies adaptive mutability of the bacterial *lac* operon. Science **282:**1133–1135.
4. **Backman, K., and M. Ptashne.** 1978. Maximizing gene expression on a plasmid using recombination in vitro. Cell **13:**65–71.
5. **Birge, E. A., and K. B. Low.** 1974. Detection of transcribable recombination products following conjugation in Rec+, RecB−, and RecC− strains of *Escherichia coli* K12. J. Mol. Biol. **83:**447–457.
6. **Cairns, J., and P. L. Foster.** 1991. Adaptive reversion of a frameshift mutation in *Escherichia coli*. Genetics **128:**695–701.
7. **Cohen, A., and A. J. Clark.** 1986. Synthesis of linear plasmid multimers in *Escherichia coli* K-12. J. Bacteriol. **167:**327–335.
8. **Dunderdale, H. J., F. E. Benson, C. A. Parsons, G. J. Sharples, R. G. Lloyd, and S. C. West.** 1991. Formation and resolution of recombination intermediates by *E. coli* RecA and RuvC proteins. Nature **354:**506–510.
9. **Dykstra, C. C., D. Prasher, and S. R. Kushner.** 1984. Physical and biochemical analysis of the cloned *recB* and *recC* genes of *Escherichia coli* K-12. J. Bacteriol. **157:**21–27.
10. **Foster, P. L., J. M. Trimarchi, and R. A. Maurer.** 1996. Two enzymes, both of which process recombination intermediates, have opposite effects on adaptive mutation in *Escherichia coli*. Genetics **142:**25–37.
11. **Friedman-Ohana, R., and A. Cohen.** 1998. Heteroduplex joint formation in *Escherichia coli* recombination is initiated by pairing of a 3′-ending strand. Proc. Natl. Acad. Sci. USA **95:**6909–6914.
12. **Gingeras, T. R., and J. E. Brooks.** 1983. Cloned restriction/modification system from *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **80:**402–406.
13. **Harris, R. S., K. J. Ross, and S. M. Rosenberg.** 1996. Opposing roles of the Holliday junction processing systems of *Escherichia coli* in recombination-dependent adaptive mutation. Genetics **142:**681–691.
14. **Hill, S. A., M. M. Stahl, and F. W. Stahl.** 1997. Single-strand DNA intermediates in phage λ's Red recombination pathway. Proc. Natl. Acad. Sci. USA **94:**2951–2956.
15. **Hollifield, W., E. Kaplan, and H. Huang.** 1987. Efficient RecABC-dependent, homologous recombination between coliphage lambda and plasmids requires a phage *ninR* region gene. Mol. Gen. Genet. **210:**248–255.
16. **Karu, A. E., Y. Sakaki, H. Echols, and S. Linn.** 1975. The γ protein specified by bacteriophage λ. J. Biol. Chem. **250:**7377–7387.
17. **Kmiec, E., and W. K. Holloman.** 1981. β protein of bacteriophage λ promotes renaturation of DNA. J. Biol. Chem. **256:**12636–12639.
18. **Kogoma, T., G. W. Cadwell, K. G. Barnard, and T. Asai.** 1996. The DNA replication priming protein, PriA, is required for homologous recombination and double-strand break repair. J. Bacteriol. **178:**1258–1264.
19. **Kowalczykowski, S. C., D. A. Dixon, A. K. Eggleston, S. D. Lauder, and W. M. Rehrauer.** 1994. Biochemistry of homologous recombination in *Escherichia coli*. Microbiol. Rev. **58:**401–465.
20. **Little, J. W.** 1967. An exonuclease induced by bacteriophage λ. II. Nature of the enzymic reaction. J. Biol. Chem. **242:**679–686.
21. **Lloyd, R. G.** 1991. Conjugational recombination in resolvase-deficient *ruvC* mutants of *Escherichia coli* K-12 depends on *recG*. J. Bacteriol. **173:**5414–5418.
22. **Lloyd, R. G., and C. Buckman.** 1991. Genetic analysis of the *recG* locus of *Escherichia coli* K-12 and of its role in recombination and DNA repair. J. Bacteriol. **173:**1004–1011.
23. **Lloyd, R. G., and K. B. Low.** 1996. Homologous recombination, p. 2236–2255. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, D.C.
24. **Lloyd, R. G., and G. J. Sharples.** 1993. Dissociation of synthetic Holliday junctions by *E. coli* RecG protein. EMBO J. **12:**17–22.
25. **Mahdi, A. A., G. J. Sharples, T. N. Mandal, and R. G. Lloyd.** 1996. Holliday junction resolvases encoded by homologous *rusA* genes in *Escherichia coli* K-12 and phage 82. J. Mol. Biol. **257:**561–573.
26. **Maloy, S. R., and W. D. Nunn.** 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. J. Bacteriol. **145:**1110–1112.
27. **Muniyappa, K., and C. M. Radding.** 1986. The homologous recombination system of phage λ: pairing activities of β protein. J. Biol. Chem. **261:**7472–7478.
28. **Murphy, K. C.** 1991. λ gam protein inhibits the helicase and χ-stimulated recombination activities of *Escherichia coli* RecBCD enzyme. J. Bacteriol. **173:**5808–5821.
29. **Murphy, K. C.** 1998. Use of bacteriophage λ recombination functions to promote gene replacement in *Escherichia coli*. J. Bacteriol. **180:**2063–2071.

29a. **Murphy, K. C.** Unpublished data.

30. **Myers, R. S., and F. W. Stahl.** 1994. Chi and the RecBCD enzyme of *Escherichia coli*. Annu. Rev. Genet. **28:**49–70.
31. **Poteete, A. R., and A. C. Fenton.** 1993. Efficient double-strand break-stimulated recombination promoted by the general recombination systems of phages λ and P22. Genetics **134:**1013–1021.
32. **Roca, A. I., and M. M. Cox.** 1997. RecA protein: structure, function, and role in recombinational DNA repair. Prog. Nucleic Acid Res. Mol. Biol. **56:**129–223.
33. **Sandler, S. J., H. S. Samra, and A. J. Clark.** 1996. Differential suppression of *priA2::kan* phenotypes in *Escherichia coli* K-12 by mutations in *priA*, *lexA*, and *dnaC*. Genetics **143:**5–13.
34. **Sharples, G. J., F. E. Benson, G. T. Illing, and R. G. Lloyd.** 1990. Molecular and functional analysis of the *ruv* region of *Escherichia coli* K-12 reveals three genes involved in DNA repair and recombination. Mol. Gen. Genet. **221:**219–226.
35. **Stahl, F. W., I. Kobayashi, and M. M. Stahl.** 1985. In phage λ, *cos* is a recombinator in the Red pathway. J. Mol. Biol. **181:**199–209.
36. **Stahl, M. M., L. Thomason, A. R. Poteete, T. Tarkowski, A. Kuzminov, and F. W. Stahl.** 1997. Annealing vs. invasion in phage λ recombination. Genetics **147:**961–977.
37. **Thaler, D. S., M. M. Stahl, and F. W. Stahl.** 1987. Double-chain-cut sites are recombination hotspots in the Red pathway of phage λ. J. Mol. Biol. **195:**75–87.
38. **Whitby, M. C., and R. G. Lloyd.** 1995. Branch migration of three-strand recombination intermediates by RecG, a possible pathway for securing exchanges initiated by 3′-tailed duplex DNA. EMBO J. **14:**3302–3310.
39. **Whitby, M. C., L. Ryder, and R. G. Lloyd.** 1993. Reverse branch migration of Holliday junctions by RecG protein: a new mechanism for resolution of intermediates in recombination and DNA repair. Cell **75:**341–350.