Copyright © 1997 by the Genetics Society of America

Unraveling a Region-Specific Hyper-Recombination Phenomenon: Genetic Control and Modalities of Terminal Recombination in *Escherichia coli*

Jacqueline Corre, François Cornet,[1] Josette Patte and Jean-Michel Louarn

Laboratoire de Microbiologie et de Génétique moléculaires, Centre National de la Recherche Scientifique, 31062 Toulouse Cedex, France

Manuscript received March 1, 1997
Accepted for publication July 3, 1997

ABSTRACT

The propensity of the terminus of the *Escherichia coli* chromosome for recombination has been further explored, using a test based on the selectable loss of a λ prophage inserted between repeated sequences from Tn*10*. Terminal recombination appears region-specific and unrelated to replication termination in a strain harboring a major chromosomal rearrangement. It requires RecBC(D) activity and must therefore occur between sister chromosomes, to conserve genomic integrity in spite of DNA degradation by RecBCD. Terminal recombination is maximal in the *dif* region and its intensity on either side of this recombination site depends on the orientation of the repeated sequences, probably because of the single χ site present in each repeat. Additional observations support the model that the crossover is initiated by single-strand invasion between sister chromosomes followed by RecBCD action as a consequence of DNA breakage due to the initial invasion event. Crossover location within repeats inserted at *dif* position supports the possibility that sister chromosomes are tightly paired in the centre of the terminal recombination zone. These data reinforce the model that postreplicative reconstruction of nucleoid organization creates a localized synapsis between the termini of sister chromosomes.

TWO distinctive recombination phenomena are associated with the terminus region of the *Escherichia coli* chromosome. One is site-specific recombination at the *dif* locus, which must occur within a short region of the terminus to be effective for exchanges between sister chromosomes, its probable role in the cell cycle (Cornet *et al.* 1996; Kuempel *et al.* 1996; F. Cornet and D. Sherratt, unpublished data). The other, called terminal recombination, is detected by a very high frequency of excisive homologous recombination following insertion of sequence duplications in the region (Louarn *et al.* 1991, 1994). Though the *dif* activity zone (DAZ) is smaller than the terminal recombination zone (TRZ), these regional effects respond similarly to genetic alterations of the region: (i) the DAZ and the TRZ harbor no unique sequence responsible for the physiological proficiency of *dif*-specific recombination (besides *dif*) or for excisive homologous hyperrecombination; and (ii) recombination of both types occurs independently of termination of replication. We attributed both effects to the nature of chromosome organization in the region surrounding the terminus. Postreplicative nucleoid reconstruction was proposed to be a sequence-dependent process creating in the DAZ/TRZ region a transient accumulation of catenation links between sister chromosomes. Catenation may facilitate interchromosomal recombination events of all types (Louarn *et al.* 1994; Cornet *et al.* 1996). This region was in consequence referred to as the nucleoid terminus, to distinguish it from the replication terminus. A similar model of polarized organization of the terminus was also proposed by Kuempel *et al.* (1996).

In this article, we present additional observations on terminal recombination. Further evidence for the uncoupling between termination of replication and terminal recombination is presented. We also report that, in our assay, the orientation within the chromosome of the repeated sequences affects excision frequency. This led us to demonstrate the role of the RecBC system in the phenomenon. Analysis of the recombinants issuing from terminal recombination was also performed. The results suggest that terminal recombination is a two-step process with RecBCD being involved at the late step. The present data indicate that terminal recombination occurs between sister chromosomes that are tightly paired in a small region around *dif* and reinforce our previously proposed model that the synapsis between TRZ/DAZ sister regions is determined by the process of nucleoid reconstruction.

MATERIAL AND METHODS

Bacterial strains, plasmids and bacteriophages: Excision frequency measurements were performed on a set of isogenic strains all derived from strain CB0129 (F^- W1485 *leu thyA deoB* or *C supD*; Bird *et al.* 1972). Strain LN2666, a spontaneous streptomycin-resistant mutant of CB0129, was an intermediate host used in gene substitution experiments. Strain LN850 is also derived from CB0129, in a rather complicated way (Louarn *et al.* 1985). In addition to a large inversion INV(29-78),

Corresponding author: Jean-Michel Louarn, Laboratoire de Microbiologie et de Génétique moléculaires du CNRS, 118 route de Narbonne, 31062 Toulouse Cedex, France. E-mail: louarn@ibcg.biotoul.fr

[1] *Present address:* Microbiology Unit, Department of Biochemistry, University of Oxford, Oxford, United Kingdom.

TABLE 1

Effect of the inactivation of RecBCD on excisive recombination

Tn*10* or *tet* insertion[b]	Position (kb)[c]	Frequency of excision per cell generation[a]		Ratio Rec^+/Rec^-
		In $RecBCD^+$ cells	In $RecBC^-$ cells	
thr::Tn*10*	~0	$4.8 \cdot 10^{-5}$ (LN3458)	$1.4 \cdot 10^{-5}$ (LN3466)	3.4
proAB::Tn*10*	~263	$3.4 \cdot 10^{-5}$ (LN3459)	$1.2 \cdot 10^{-5}$ (LN3467)	2.8
lacZ::Tn*10*	~365	$4.0 \cdot 10^{-6}$ (LN2464)	$1.0 \cdot 10^{-5}$ (LN2950)	4.0
		Within the TRZ		
zda192::tet_{cw}	1437	$1.8 \cdot 10^{-5}$ (LN3473)	$2.3 \cdot 10^{-6}$ (LN3477)	7.8
zda192::tet_{ccw}	1437	$1.0 \cdot 10^{-4}$ (LN3474)	$1.3 \cdot 10^{-5}$ (LN3476)	7.7
zdc310::tet_{cw}	1555	$1.0 \cdot 10^{-4}$ (LN3432)	$1.4 \cdot 10^{-5}$ (LN3439)	7.1
zdc310::tet_{ccw}	1555	$2.0 \cdot 10^{-3}$ (LN3438)	$1.8 \cdot 10^{-5}$ (LN3444)	111
Δ*dif58*::tet_{cw}	1592	$2.8 \cdot 10^{-3}$ (LN2947)	$1.3 \cdot 10^{-5}$ (LN2977)	215
Δ*dif58*::tet_{ccw}	1592	$4.0 \cdot 10^{-3}$ (LN3471)	$2.5 \cdot 10^{-5}$ (LN3479)	160
zdd355::tet_{cw}	1600	$4.0 \cdot 10^{-3}$ (LN3159)	$1.0 \cdot 10^{-4}$ (LN3160)	40
zdd355::tet_{ccw}	1600	$6.0 \cdot 10^{-5}$ (LN3154)	$7.0 \cdot 10^{-6}$ (LN3157)	8.6
zdd370::tet_{cw}	1615	$1.1 \cdot 10^{-3}$ (LN3158)	$6.0 \cdot 10^{-6}$ (LN3174)	180
zdd370::tet_{ccw}	1615	$1.0 \cdot 10^{-4}$ (LN3507)	$1.0 \cdot 10^{-5}$ (LN3520)	10

[a] Averages of measurements made on at least five independent clones. Clone-to-clone variations higher than a factor of 2 are frequently observed. Strain numbers in our collection are indicated within parentheses.

[b] Numbers associated with *zxy* designations of *tet* insertions refer to their positions on the physical map of the terminus region established by BOUCHE (1982).

[c] Map positions are given according to the physical map of BERLYN *et al.* (1996).

[d] The Δ(*recBC*)::Ap^r deletion was crossed into the various lysogens by conjugation with Hfr2926.

it carries a *dnaA46* mutation, a ϕ80*imm*λind⁻ prophage, and a *manA*::Mu insertion. Strain LN3412 derives from LN850 in two steps: (i) crossing in a Δ*tus*::Ap^r mutation (with concommitant loss of the linked *manA*::Mu insertion), and (ii) elimination of the ϕ80*imm*λind^- prophage, which hindered application of the excision test. These modifications involved several transductional and conjugational crosses. All Tn*10* insertions have been previously described (LOUARN *et al.* 1991, 1994). Constructions of Δ(*dif2600*)::Ap^r, Δ(*dif58*)::tet_{cw} and tet_{ccw} deletions-insertions (subscript cw and ccw designations indicate the orientation, clockwise and counterclockwise, respectively, of the *tet* insertion as in Figure 1D), of *zdc310*::tet_{cw} and tet_{ccw}, *zdd355*::tet_{cw} and tet_{ccw}, and *zdd370*::tet_{cw} insertions have been previously reported (LOUARN *et al.* 1994; CORNET *et al.* 1996). Other *tet* insertions and the Δ(*recBC*)::Ap^r tagged deletion were constructed for the present work (see below). All map positions in Table 1 are given according to the chromosome map Ecomap9 compiled by BERLYN *et al.* (1996). Strain LN2463, an Hfr that promotes an early transfer of a *lamB* (λ^r) allele, is described in LOUARN *et al.* (1991). Integration-excision vectors pFC13, pFC20 and pLN135 are described in CORNET *et al.* (1994, 1996). Bacteriophage λTSK is described in REBOLLO *et al.* (1988).

Media and general methods: Luria-Bertani (LB)-rich medium is described in MILLER (1992). Antibiotic concentrations used were as follows: ampicillin (Ap): 25 μg/ml; chloramphenicol (Cm): 25 μg/ml; kanamycin (Kn): 25 μg/ml; streptomycin (Sm): 20 μg/ml for the Sp/Sm interposon and 200 μg/ml for *rpsL* alleles; tetracyclin (Tc): 15 μg/ml. All *in vivo* or *in vitro* genetic experiments were performed according to standard procedures (SAMBROOK *et al.* 1989; MILLER 1992).

Construction of chromosomal *tet* insertions: A chromosomal fragment chosen among our collection of cloned segments of the region (BEJAR and BOUCHE 1983) is first subcloned on an integration-excision vector such as pLN135. The 2.9-kb *Bgl*II segment from Tn*10*, called here *tet*, which harbors the *tetAR* genes and confers tetracyclin resistance (Tc^r), is then cloned into the fragment. The recombinant plasmid is eventually introduced into a *rpsL* (Sm^r, recessive) mutant strain. The vector harboring a temperature-sensitive replication machinery, a chloramphenicol resistance determinant (Cm^r) and a *rpsL*⁺ (Sm^S, dominant) gene, substitution of the chromosomal wild-type sequence by the modified one is performed in two steps: (i) selection at 42° for Cm^r Tc^r that are also Sm^S; (ii) subsequent selection of Tc^r Sm^r that are also Cm^S.

Excision frequency measurements: Bacteria harboring the Tn*10* or *tet* insertion of interest were infected by λTSK and lysogens were sought for by selection of kanamycin-resistant (Kn^r) derivatives at 30°. Clones retained for further study were streptomycin-resistant (Sm^r), tetracyclin-sensitive (Tc^S) and temperature-sensitive (Ts), had also to be phage producers and to give rise to Tc^r derivatives (excisants) when incubated at 42°. These criteria could only be fulfilled if the prophage is integrated into the chromosome as shown Figure 1A. A *lamB* (λ^r) allele was then crossed in by conjugation with Hfr strain LN2463. The use of λ^r lysogens made null the risk of reinfection and killing of cured bacteria by phages produced after thermal induction. Average frequency of cured bacteria is the mean of frequencies of temperature-resistant (Tr) derivatives measured on at least five independent clones (clone-to-clone variations by a factor of ≥2 are not uncommon). Excision frequencies per cell generation were calculated by dividing the frequency of cured bacteria by the estimated age of the clones (25–30 generations). This calculation is valid because we observed no selective advantage at 30° for the cured bacteria (LOUARN *et al.* 1991).

Construction of a tagged *recBC* deletion: The *Eco*RI-flanked chromosomal fragment of bacteriophage λ460 from the ordered hybrid phage collection of KOHARA *et al.* (1987), which carries the *recB* and *recC* genes, was cloned into integration-excision vector pFC20 and then the fragment between *Hin*dIII sites at ordinates 2960.0 and 2967.9 kb on Ecomap9 was replaced by the ΩAp^r interposon (FELLAY *et al.* 1987); these

*Hin*dIII sites fall within *recB* and *recC*, respectively. Substitution of wild-type *recB recC* genes with this tagged deletion was performed as described above in a spontaneous Sm^r resistant mutant of Hfr PK191, which transfers early the *recBCD* region, to yield strain LN2926. Phenotypes associated with the Ap^r character were an increased UV sensitivity and a strongly decreased recipient ability in transductional crosses that are typical of $RecBC^-$ mutants.

RESULTS

The orientation of the repeats flanking the excisable DNA may control excision frequency: To assay terminal recombination, we measured the frequency of excision of a prophage λTSK that was inserted by recombination with a series of chromosomal *tet* sequences (Figure 1, A and B; LOUARN *et al.* 1991, 1994). The *tet* segment (alone or as part of Tn*10* transposon) has been inserted at 14 positions in the terminus region; at five sites both orientations of the *tet* sequence were available. The results are shown in Figure 2. Excisive recombination was maximal at the *dif* locus (position 5 in Fig. 2; in this insertion the *tet* segment tags a 58-bp deletion eliminating *dif*), and both orientations at this site yielded similar high frequencies. To the left of *dif*, excisive recombination was up to 50 times more frequent for the tet_{ccw} orientation, whereas to the right of *dif* the tet_{cw} orientation gave more frequent excisions. These results indicate that terminal recombination occurs in a polarized fashion on both sides of the *dif* region. The indifference of recombination at *dif* to the orientation of *tet* might result either from inactivity of the polarizing phenomenon in the region surrounding *dif* or because of the Dif^- phenotype itself, which is associated with some induction of the SOS system (KUEMPEL *et al.* 1991). This point is still under investigation. Figure 2 is drawn according to the first model.

Regional specificity of terminal recombination is not determined by the replicative process: Independence of terminal recombination from termination of replication has been previously supported by the finding that the TRZ remained invariant when the region where replication forks meet was displaced more than 200 kb away from its normal zone, the *psrA* (*terC*) region (LOUARN *et al.* 1994). We report here that a much more dramatic alteration of chromosome organization does not change the localization of the TRZ.

We have previously isolated a strain (LN850; LOUARN *et al.* 1985) having undergone a large chromosomal inversion due to recombination between two inverted copies of IS*5*, IS*5*F at 29 min (1398 kb on Ecomap9), and IS*5*T at 78 min (3662 kb). This inversion puts the *dif* site only 11 map minutes away from *oriC* (Figure 3). In spite of the new location of the site, the strain remains Dif^+ (CORNET *et al.* 1996). This strongly suggests that *dif* activity is controlled by intrinsic properties of the terminus region, not by distance from the origin. We report here a similar observation for the TRZ. The

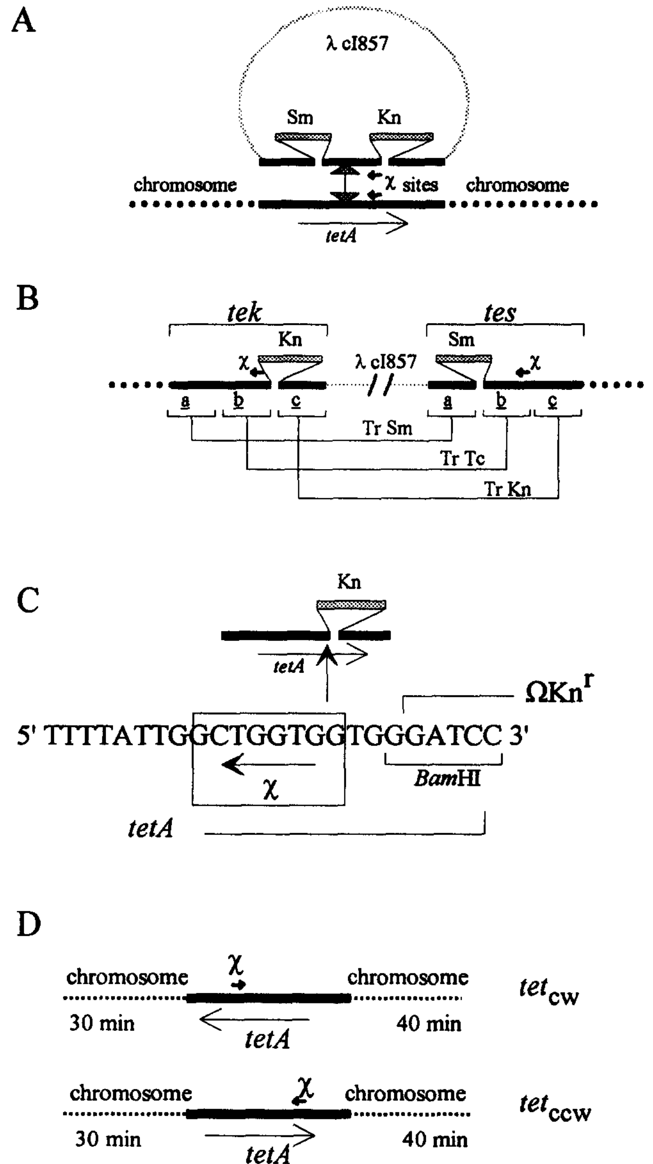


FIGURE 1.—Assay for excision frequency determinations. (A) Integration of λTSK by homologous recombination within a resident *tet* sequence leads to a Ts Tc^S strain when integrative recombination, symbolized by a double arrow, proceeds between the Sm^r and the Kn^r insertions. (B) Prophage excision by homologous recombination in a Ts Tc^S lysogen results in a cured bacterium that is Tr and either Sm^r, Tc^r or Kn^r dependent upon the *tet* segments (a, b or c) where the exchange takes place. *Tes* and *tek* designations refer to *tet* sequences harboring a Sm^r or a Kn^r insertion, respectively. (C) A χ site exists on the *tet* sequence at a few nucleotides from the *Bam*HI site, which limits internally the Kn^r insertion. As it is oriented, it favors the shift from Exonuclease V activity to recombinase activity of a RecBCD complex travelling on DNA from right to left, in the direction opposite that of *tetA* transcription. No other χ site is found in λTSK. (D) Our convention for *tet* insertion orientation in the chromosome, with reference to the *tet* χ site orientation.

strain used for this study, LN3412, is a derivative of inversion strain LN850 rendered Tus^- and cured of a resident $\phi 80 imm\lambda$ prophage (see MATERIAL AND METHODS). As a consequence of the *tus* mutation, replication

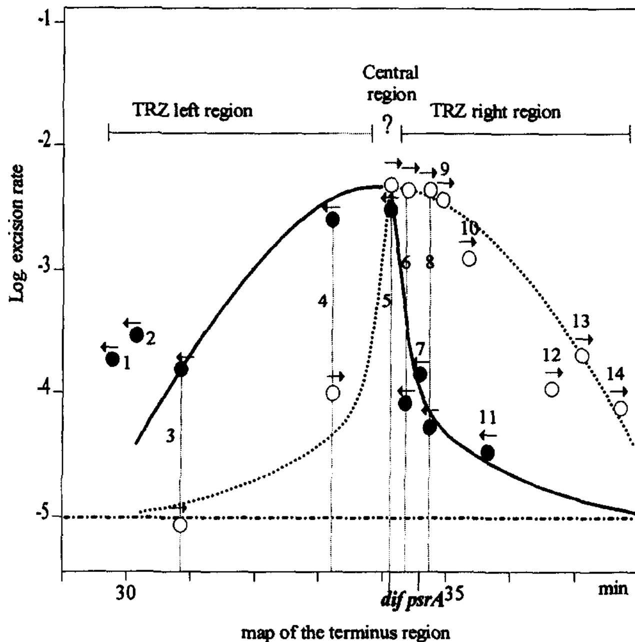


FIGURE 2.—Semi-log plot of prophage excision frequencies per cell generation *vs.* map position in the terminus region of the resident *tet* sequences used for prophage integration. Map positions in minutes. Only two chromosomal markers are indicated: recombination site *dif* and replication pause site *psrA/terC*. O, tet_{cw}; ●, tet_{ccw}. Arrows above circles recall *tet* χ site orientations, according to the convention of Figure 1C. Values reported for Tn*10* insertions are from LOUARN *et al.* (1991, 1994). The curve joining the tet_{ccw} positions is drawn symmetric to the curve joining the tet_{cw} positions with respect to a vertical axis at *dif*. The dashed horizontal line refers to the average excision frequency observed outside the terminus region. List of insertions: 1: *zci119*::Tn10_{ccw}; 2: *zcj152*::Tn10_{ccw}; 3: *zda192*::*tet* (cw and ccw); 4: *zdc310*::*tet* (cw and ccw); 5: *dif58*::*tet* (cw and ccw); 6: *zdd355*::*tet* (cw and ccw); 7: *zdd365*::tet_{ccw}; 8: *zdd370*::*tet* (cw and ccw); 9: *zde381*::Tn10_{cw}; 10: *zde395*::Tn10_{cw}; 11: *zde406*::Tn10_{ccw}; 12: *zdf237*::Tn10_{cw}; 13: *zdg232*::Tn10_{cw}; 14: *zdh57*::Tn10_{cw}.

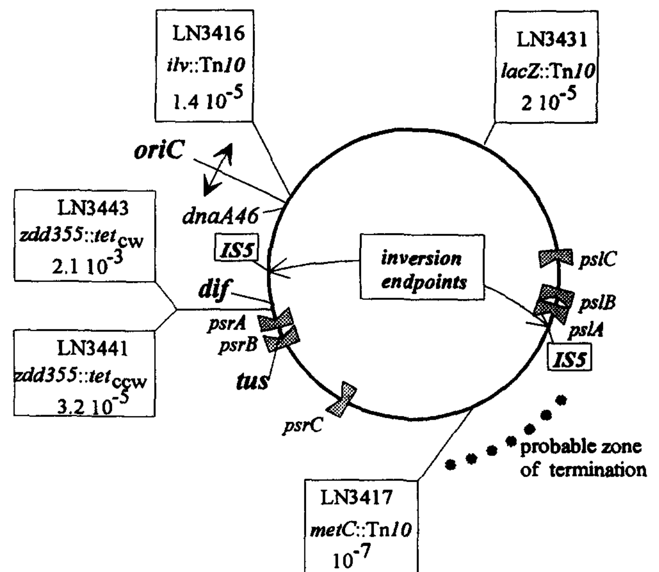


FIGURE 3.—Indigenous recombination in INV29-78 Tus$^-$ strains. The locations, on a circular map of the rearranged chromosome, of the different *tet* or Tn*10* insertions assayed for Tc^r recombinant formation are shown. Designations of the insertions, frequencies of Tc^r recombinants per cell generation and strain names are given within frames. Orientations at *zdd355* are indicated by the same symbols as on the normal chromosome. Shown also are the positions of sites and genes of interest: *oriC*, origin of replication; *dnaA46*, gene controlling initiation at *oriC*,, Ts allele; *dif*, site for chromosome dimer resolution; *psrA*, *B*, and *C*, pause sites inhibiting clockwise moving forks on the normal chromosome; *pslA*, *B*, and *C*, pause sites inhibiting counterclockwise moving forks; *tus*, the gene controlling synthesis of the protein inhibiting fork movement at pause sites; IS*5*, insertion sequence involved in the generation of the large inversion. As inferred from unpublished marker frequency data, the probable zone of replication termination in Tus$^-$ conditions is indicated by a curved dotted line (termination in Tus$^+$ conditions takes place in the vicinity of *psrC*).

forks do not pause at terminator sites; we have checked that in the inversion strain the displaced 29–40-min region is replicated early without any indication of fork pausing at *psrA* or *psrB* (data not presented). Strain LN3412 received, *via* phage P1-mediated transduction, Tn*10* or *tet* insertions at the chromosomal positions indicated in Figure 3. These derivatives were lysogenized by λTSK and identified by their Tc^S phenotype. Prophage excision frequency was not directly measured due to the presence of the temperature-sensitive *dnaA46* mutation (conserved because this mutation associated with the inversion causes a rich medium sensitivity that helps verify the presence of the rather unstable inversion; LOUARN *et al.* 1985). Instead, we determined the frequencies of reconstitution of active *tetA* genes, by selecting for Tc^r derivatives. In noninverted chromosomes, a Tc^r recombinant is formed every two to three prophage excision events (Table 2), and conversely most Tc^r recombinants selected at 30° are also cured of the prophage (not shown). The data, reported in Figure 3, indicate clearly that in the inversion strain the zone around *dif* is also a zone of hyperfrequent recombination between direct repeats; furthermore, at each point tested the frequencies of recombination seen in the inversion strain are in the same range of values as seen without the inversion. In addition, the effect of the orientation of *tet* at position *zdd355* is conserved in the inverted strain. Thus, the combined effects of a major chromosomal inversion plus inactivation of the replication pause system do not alter either the positioning of the TRZ on the chromosome, which in strain LN3412 is now located close to *oriC* and far from the termination region, nor the orientation dependence. Clearly, the TRZ location is determined by intrinsic features of the zone and not by features of the replication process.

Excisions at high frequency in the TRZ depend on RecBCD: The orientation effect implies the presence in the *tet* sequence of elements able to orient the action of recombination enzymes. One such element could be

the 5′GCTGGTGG3′ octamer, χ (SMITH *et al.* 1981). RecBCD loaded on double-stranded DNA at a free end degrades DNA from this end until it encounters the 3′ end of a χ site, as written above; it then creates recombinogenic single-stranded material that favors recombination on the 5′ side of χ (KOWALCZYKOWSKI *et al.* 1994). The sequences involved in the excision assay harbor a χ site within each *tet* repeat (Figure 1C). It was thus reasonable to examine the role of the RecBCD complex in the orientation effect.

Analysis of the role of RecBCD in terminal recombination has been made easier by the construction of a tagged deletion Δ(*recBC*)::Ap^r (see MATERIALS AND METHODS). Table 1 shows the effect of the *recBC* deletion on prophage excision frequencies as a function of the prophage chromosomal location and orientation. At all positions tested outside the TRZ, excision frequencies, already low, were decreased only three- to fourfold in RecBC$^-$ conditions. This indicates that along most of the chromosome, excisive recombination does not depend strictly on RecBCD, as already pointed out by MAHAN and ROTH (1989). In contrast, within the TRZ, introduction of the Δ(*recBC*)::Ap^r mutation into lysogens displaying hyperexcisive recombination resulted in a dramatic decrease of the excision frequency down to the level observed at positions outside the TRZ (Table 1). These data demonstrate that the RecBC enzyme is responsible for the high excision frequencies characteristic of terminal recombination and support the possibility that the orientation of the *tet* sequence controls the efficiency of terminal recombination because it harbors a unique χ site.

Distribution of resistance characters among cured bacteria in terminal recombination: If usage of the *tet* χ sites is responsible for the orientation effect on excision frequency, the crossover point within the duplicated *tet* sequences and hence the antibiotic resistance of the excisants recovered should be determined by the χ site location. The situation is illustrated in Figure 4, which shows that the *tet* χ site correctly oriented to favor excisional exchanges is the one located on *tek.* This site would activate RecBCD recombinase only when the enzyme travels in the prophage-to-chromosome direction and after passing through the Kn^r determinant. In consequence, RecBCD recombinase should tend to exclude the Kn^r determinant from the Tr recombinants, and this effect was expected to be strong at least at these positions of the TRZ where excisive recombination is both RecBC- and orientation-dependent. To test this possibility, we have determined the relative frequency of the three types of excisants at various positions, by testing colonies of cured Tr bacteria for conserved antibiotic resistances. Results are reported in Table 2.

Outside the TRZ: Whatever the location of the chromosomal *tet* insertion outside the TRZ and in RecBCD$^+$ conditions, Kn^r excisants were always found at a lower frequency than Sm^r or Tc^r excisants. They constituted

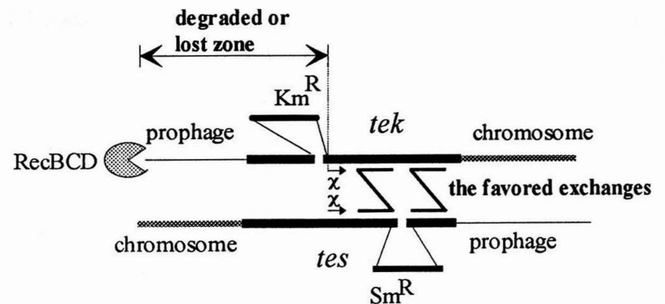


FIGURE 4.—Prophage curing by RecBCD-catalyzed recombination between the repeats flanking the prophage. The paired *tes* and *tek* sequences are drawn irrespective of their belonging or not to sister chromosomes. When the *tet* χ site controls the unequal exchange and considering the mode of action of RecBCD (KOWALCZKOWSKY *et al.* 1994), excisive recombination is observed when RecBCD, loaded at a DSB located to the left of *tek*, degrades successively prophage material, the c̲ part of the *tet* segment (with reference to Figure 1B) and the Kn^r insertion before it reaches the correctly oriented *tet* χ site, which belongs to *tek.* Recombination must eventually proceed between the b̲ regions (ending with Tr Tc^r excisants) or the a̲ regions (ending with Tr Sm^r excisants) of *tek* and *tes* partners. The zig-zags indicate the recombinant molecules linked during the process.

on the average ~10% of excisants. This low Kn^r frequency was only slightly increased in RecBC$^-$ conditions (data not shown) and therefore is a characteristic of the system largely independent of RecBCD usage.

In the TRZ flanking regions: At *zda192, zdc310, zdd355* and *zdd370* positions, Kn^r excisants were also found, in proportions quite comparable to those observed at positions outside the TRZ and irrespective of *tet* orientation. Again, Kn^r excisant frequencies were not much increased in RecBC$^-$ conditions (not shown). This is at variance with our prediction.

In the center of the TRZ: At Δ(*dif58*)::*tet* position, where both orientations are equally active, the situation was quite different: Kn^r recombinants became very rare (less than 1% of excisants at both situations analyzed), and the involvement of RecBC in this deficit was demonstrated by an ordinary frequency of Kn^r excisants when these strains were made RecBC$^-$ (Table 2). The prediction that the action of RecBCD should hinder the generation of Kn^r excisants seems therefore to be valid at this position only. The lack of Kn^r recombinants is not due solely to the inactivation of *dif.* when the prophage was inserted at positions *zda310*::tet_{ccw} or *zdd370*::tet_{cw} (both displaying high excision frequency; Figure 2), the presence of Δ(*dif2600*::Ap^r) had no effect either on excision frequency (not shown) or on the proportion of Kn^r excisants (Table 2; compare LN3438 to LN3472, and LN3158 to LN3537).

DISCUSSION

The present data confirm the absence of direct relationship between terminal recombination and termina-

TABLE 2

Distribution of antibiotic resistance markers among cured bacteria

Strain	Tn*10* or *tet* location/ orientation	Rec functions (relevant genotype)	Frequency among excisants of[a]		
			Sm^r	Tc^r	Kn^r
	Outside the TRZ				
LN2464	*lacZ*	+	58	25	17
LN3458	*thr*	+	75	22	3
LN3459	*proAB*	+	58	32	10
	In the left region of the TRZ				
LN3473	*zda192*/cw	+	46	36	18
LN3474	*zda192*/ccw	+	52	45	3
LN3432	*zdc310*/cw	+	54	39	7
LN3438	*zdc310*/ccw	+	32	49	19
LN3472	*zdc310*/ccw	Δ*dif2600*	26	37	37
	In the central region of the TRZ				
LN2947	*dif*/cw	Δ*dif58*	70	30	<1
LN2977	*dif*/cw	Δ*dif58* Δ*recBC*	37	43	20
LN3471	*dif*/ccw	Δ*dif58*	70	30	<1
LN3479	*dif*/ccw	Δ*dif58* Δ*recBC*	34	44	22
	In the right region of the TRZ				
LN3159	*zdd355*/cw	+	53	38	9
LN3154	*zdd355*/ccw	+	44	41	15
LN3158	*zdd370*/cw	+	46	45	9
LN3537	*zdd370*/cw	Δ*dif2600*	59	36	5
LN3507	*zdd370*/ccw	+	37	58	5

[a] Values are percentages; Kn^r values are average of at least three determinations performed on independent clones, involving a total number of cured clones over 100 for each strain. Important clone-to-clone variations (more than a factor of 2) could be observed in the frequencies of a given recombinant type.

tion of replication and leave no doubt about the central role of the RecBC complex in the frequent excision events characteristic of terminal recombination. These findings help to clarify our understanding of events occuring in the terminus region, but it must be kept in mind that the artificial system used in our analysis may interfere with and modify the course of events occurring on normal chromosomes (*i.e.*, harboring no duplicated sequences in the region).

Our results show that elevated recombination occurs in the TRZ even when that region is displaced to a position close to *oriC*. This demonstrates the lack of a link to site-specific pausing or termination of replication. This absence of a relationship with termination of replication does not support the previously proposed possibility that events occuring at stalled replication forks are inducers of the strong recombinogenic activity of the terminus (LOUARN *et al.* 1991; HORIUCHI *et al.* 1994; KUZMINOV 1995).

The invariance of the TRZ activity fits in with our current view of how the regional control may operate (LOUARN *et al.* 1994; CORNET *et al.* 1996). The major contention is that a sequence-dependent structure is assembled on each side of the nucleoid terminus and must be reconstructed after each replication cycle. The reconstruction may proceed through a strictly defined (but unknown) series of events, so that it ends near *dif*. Catenation links between the two sister chromosomes after replication may be propelled toward the nucleoid terminus by the reconstruction process. Accumulation of catenation links in the nucleoid terminus may create a synapsis between the sister chromosomes in this region, and cuts introduced by topoisomerases/decatenases may initiate terminal recombination events.

Though largely speculative, this picture of postreplicative events in the terminus region is consistent with existing results and provides a convenient framework for interpreting not only terminal recombination, but also the confinement of *dif* activity to the same zone (CORNET *et al.* 1996; KUEMPEL *et al.* 1996). It also helps understanding of the observation that many segments of the region are refractory to genetic inversion (REBOLLO *et al.* 1988). In the chromosome harboring the large inversion, at least one of the structural units (on the *zdd355* side of *dif*) should remain unaffected.

A first question is raised by the involvement of RecBC in terminal recombination. The RecBCD complex, once loaded on DNA at a free end, first acts as a nuclease until it finds a correctly oriented χ site. How does a cell undergoing terminal recombination cope with this extensive chromosome degradation? When RecBC-dependent terminal recombination occurs on normal chromosomes (*i.e.*, carrying no duplicated sequences), the repair of the double-strand break (DSB) required for RecBCD loading must involve sequences from a second chromosome present in the affected cell. A second copy is needed to repair the loss of material due to exonuclease V activity. Being RecBC-dependent, terminal recombination is most probably an interchromosomal event, as is *dif*-specific recombination. However, in our assay, intrachromosomal RecBC-dependent recombination is not *a priori* excluded: when a DSB occurs within the prophage, the RecBCD complex that travels towards *tek* will encounter a χ site correctly oriented for promoting the excision exchanges depicted in Figure 4. Could terminal recombination be induced by a special behavior of the λ prophage when present in the terminus region? This seems quite unlikely because this prophage is noninducible and, moreover, the effect of flanking sequence orientation on terminal recombination cannot be accounted for by the generation of DSBs at a fixed position within the prophage. The RecBC dependence indicates strongly that terminal recombination occurs between two complete sister chromosomes, since the TRZ is normally the last part of the chromosome to be replicated.

A second question is raised by the involvement of RecBCD. How is the free end (DSB) generated, which is required for RecBCD loading? Though the effects of inactivation of the *tet* χ site have not yet been tested, the orientation effect and the requirement for RecBC are consistent with a key role of this site in controlling the frequency of excisive recombination. The active orientation (*i.e.*, giving rise to high excision frequency; Figure 2) makes this site trigger the recombinase activity of RecBCD complexes only when they travel along the chromosome in the *dif*-to-*oriC* direction on either side of *dif*. The simplest explanation is that a DSB is generated in the central region of the TRZ of one of the sister chromosomes (cutting of the two sister chromosomes simultaneously and at the same location must be lethal); this cutting would provide a fixed region for RecBCD loading on the chromosome. This possibility (to which we refer as the terminal *DSB* model) is illustrated Figure 5. Figure 5A shows how prophage excision is generated, according to this model, when the *tet* sequences are in the active orientation. It demands that *tek'*, distal from *dif* on the linearized chromosome, recombines with *tes*, proximal to *dif* on the uncut chromosome. But repair events initiated at the *tes'* χ site, which lies between the postulated DSB and the *tek'* χ site, should result in equal exchanges, not in excision exchanges, and these equal exchanges should be in the majority. The occurence of DSB in the region should be extremely high if this model were correct.

The model of a systematic DSB in the center of the TRZ has several problems. One is that the region can be deleted without affecting terminal recombination (LOUARN *et al.* 1994). Thus, the region carries no essential cut site. A second negative aspect of the model is shown in Figure 5B. The repair of a terminal DSB should preferentially generate a dimeric chromosome, because RecBCD creates preferentially 3'-recombinogenic ends (KOWALCZYKOWSKI *et al.* 1994) and because resolution cuts might be preferentially placed in noncrossover strands (as shown for RuvC *in vitro*, BENNETT and WEST 1995). Such dimers should require *dif*-specific recombination for resolution. If this were the preferential pathway for excisive terminal recombination, we would expect high excision frequencies to depend on Dif$^+$ phenotype. No such dependence has been observed (LOUARN *et al.* 1994). A third line of argument comes from the survey of the *E. coli* chromosome sequence for χ sites along ~90 kb on either side of *dif*. Figure 6 shows that (i) along more than 40 kb centered on *dif* only one χ site is found, facing *dif* [the orientation opposite that of the *tet* χ site when prophage excision is high (Figure 2)], (ii) further away on either side of *dif*, there is a strong preference (two thirds of the sites) for χ sites in the active orientation (facing *oriC*). When a DSB is generated near *dif*, this χ site distribution would allow RecBCD to degrade ~1% of the chromosome before carrying out repair, and this repair should be initiated at one of the *oriC*-facing χ sites found at ~20kb from *dif* on either side. Consequently, a RecBCD complex loaded at a DSB close to *dif* should have much less chance to reach, for instance, *zdc310*::*tet* or *zdd370*::*tet* (several active χ sites between *dif* and

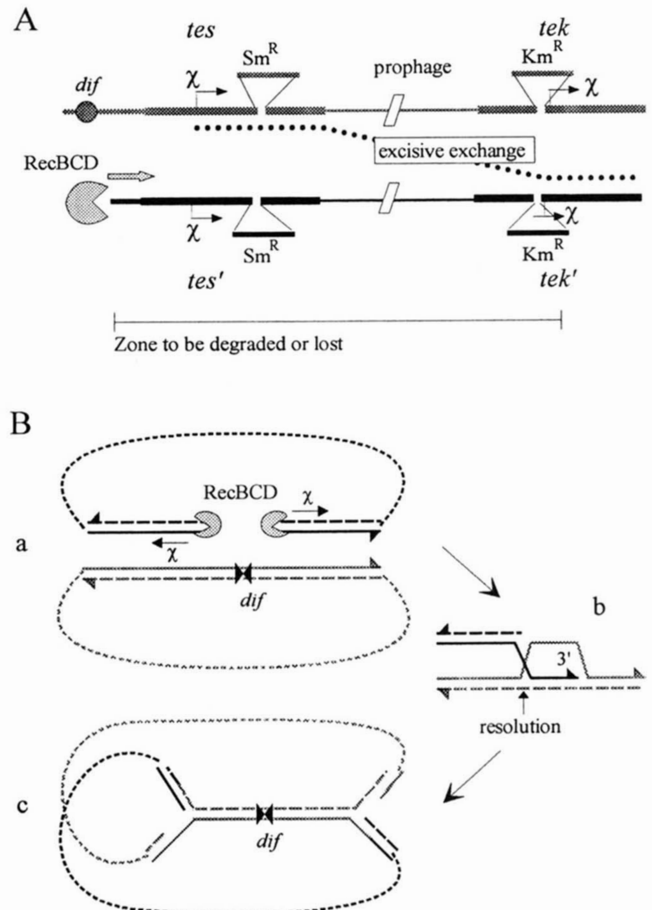


FIGURE 5.—The terminal double-strand break (DSB) model. (A) Events required for prophage excision after generation of a DSB in the *dif* region on one of sister chromosomes. The *tet* sequences are drawn in the active orientation. The required recombination between *tek'* and *tes* can occur only after RecBCD had travelled along the cut chromosome from the point of entry near *dif* to *tek'*. The complex must find the *tes'* χ site on its way and may catalyze an equal exchange with the sister chromosome. RecBCD should seldom reach *tek'*. (B) Possible sequence of events for terminal DSB repair. (Ba) RecBCD complexes loaded onto each DNA end degrade DNA till they meet a correctly oriented χ site and create 3' recombinogenic ends. Like strands are shown by continuous or dashed lines. (Bb) One heteroduplex joint after invasion by the 3' tail. The noncrossover strand is shown after the resolution cut at the junction, but prior to the presumed migration of this junction driven by RecG (see LLOYD and LOW 1996 for further insights). (Bc) A later stage after joining of the 3' end generated by the resolvase on the invaded chromosome to the 5' end generated by RecBCD on the invading one: the recombination junctions resemble replication forks and may be converted into them by recruitment of replication enzymes. Eventually, these forks replicate the intervening portion of the chromosome, with formation a chromosome dimer whose resolution may involve site-specific recombination at *dif*.

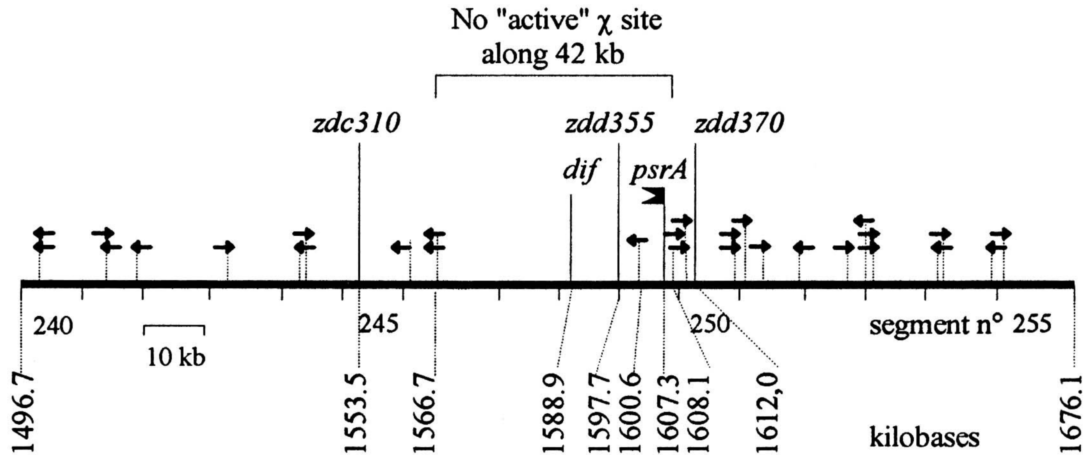


FIGURE 6.—Distribution of χ sites in the TRZ. Ordinates refer to segments of the *E. coli* genome sequence established by F. R. BLATTNER and collaborators: GenBank accession no. U00096, segments EA000240–EA000255. The χ sites (approximate positions) are represented by arrows as in Figure 1C; in the "active" orientation (with reference to the excision test) the arrowhead faces *oriC*. The location (in kb) on this map of several other loci of interest is also given.

these insertions) than *zdd355*::*tet* (within the χ-free region; Figure 6). In spite of this, at these three positions excision efficiencies remain within the same order of magnitude and display strong orientation dependence (Figure 2).

The alternative possibility is that the events of terminal recombination that we are able to detect are two-step processes in which RecBCD acts at a late phase. The model, here referred to as the terminal strand invasion model, assumes that the initial event is a nick that allows a first step of single-strand exchange between intercatenated sister chromosomes. Catenation links might facilitate the alignment of partner molecules, which in turn could favor invasion of the nicked duplex by a strand from the sister chromosome. The nick might arise from the decatenation process itself (DIGATE and MARIANS 1989; KATO *et al.* 1990; ADAMS *et al.* 1992). The DSB responsible for the RecBCD-dependent second step would be created at resolution of strand invasion, when the resolvase cuts the noncrossover strands. Formally, the recombinant structure formed during the first step (Figure 7) resembles that postulated for integration of a donor single-stranded DNA into the recipient chromosome during transformation in *Streptococcus pneumoniae* (MARTIN *et al.* 1996).

In this model, the prophage deletion event may be generated in two ways: (i) when strand invasion occurs between prophage sequences and (ii) when unequal strand invasion occurs between *tes* on one chromosome and *tek* on the second chromosome. In the first mode, shown in Figure 8, the deletion can only be generated during the RecBCD-dependent DSB repair, entailing a systematic loss of the Kn^r marker (see also Figure 4). In the second mode, strand invasion itself may generate the deletion, as shown in Figure 9, so that the Kn^r marker may remain associated with the deletion. The observation that Kn^r excisants were quite scarce when the prophage was inserted at the *dif* position suggests that the first mode was predominant at this position, whereas in the flanking regions the higher frequency of Kn^r recombinants might indicate a higher contribution of the second mode. The ratio between the two modes is probably controlled by various parameters. One of them may be the tightness of pairing between sister chromosomes. It may be that the sliding of the intercatenated chromosomes along each other, which is required for the unequal pairing characteristic of the second mode, is reduced in the *dif* region. This suggests the possibility that the sister chromosomes might form a synaptic structure in the *dif* region.

The terminal strand invasion model fits in with most

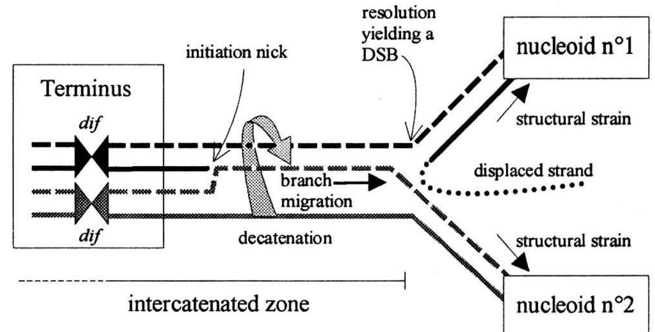


FIGURE 7.—The terminal strand invasion model: intercatenation and structural strain. We postulate that a nick on one of the intercatenated chromosomes allows a nonreciprocal replacement of the nicked strand by the identical strand from the second chromosome. Such nicks might be due to the activity of decatenases. An additional postulate is that the tensions imposed on the exchanging molecules by the process of nucleoid reorganization will orient in the *dif*-to-*oriC* direction strand displacement and subsequent strand invasion/branch migration. If resolution occurs on a noncrossover strand, as indicated, a free end with single-strand overhang is generated. Only when this free end is made blunt can RecBCD act. For the sake of clarity, catenation links are not shown.

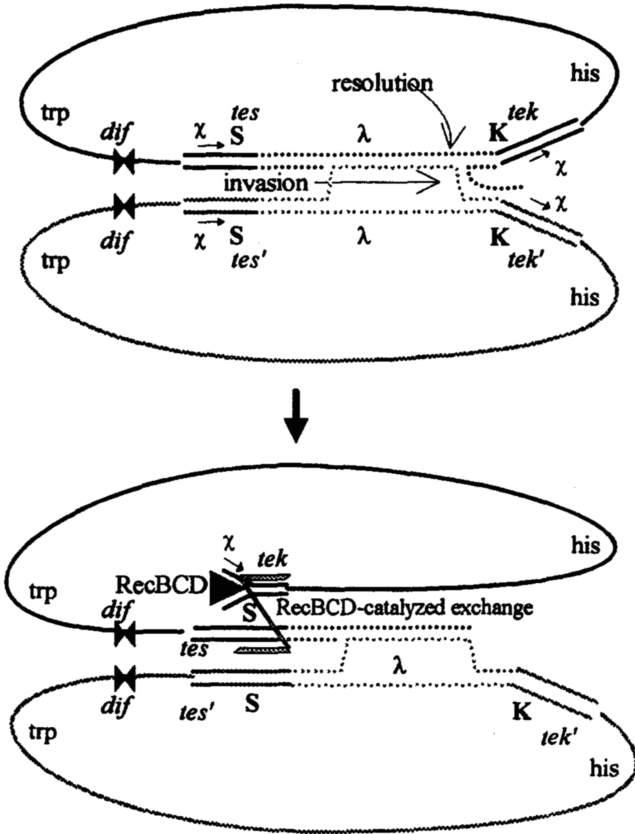
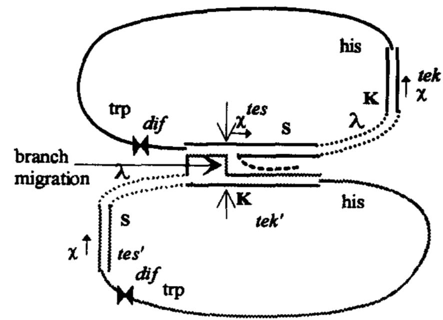
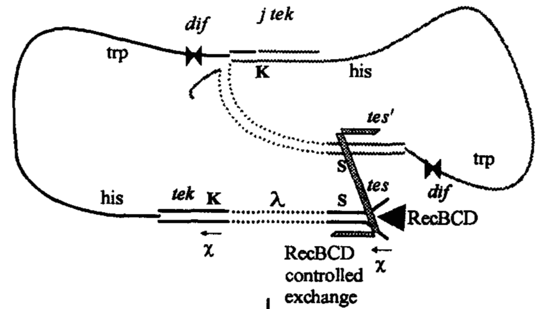


FIGURE 8.—A possible model for two-step terminal recombination resulting from equal strand invasion. (A) Equal strand invasion, as proposed in Figure 7, has occured between prophage DNAs. Invasion has already proceeded in the *dif*-to-*oriC* direction, and resolution will create a free end located within prophage DNA. (B) Subsequent evolution under RecBCD control. RecBCD (black triangle), once converted into a helicase at the *tek* χ site (after having degraded the Kn^r determinant), may catalyze recombination between *tek* and *tes* (indicated by the zigzag). The net result is the immediate creation of two monomeric chromosomes, one cured and the second still lysogenic, with no need for subsequent *dif*-specific recombination. The chromosome orientation is deduced relative to the positions of the *trp* (28 min) and *his* (45 min) operons. Other conventions as in Figure 5.

A Ist step RecBCD independent



B 2nd step RecBCD dependent



C dimer resolution at *dif*

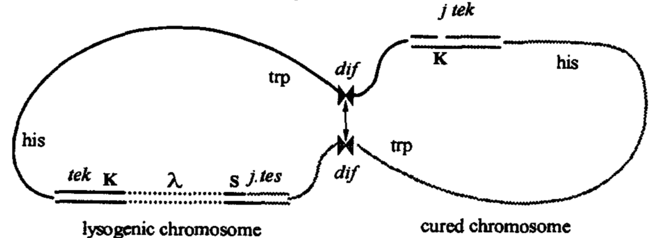


FIGURE 9.—A possible model for two-step terminal recombination resulting from unequal strand invasion. (A) First step. Unequal strand invasion had occurred between the c regions (with respect to Figure 1B) of *tek'* and *tes*. Prophage excision will result from resolution cuts on noncrossover strands (indicated by vertical arrowheads). The drawing shows the generation of a junction *tek* (*j.tek*) sequence between the sister chromosomes. (B) Second-step. RecBCD (black triangle), loaded at the free end resulting from resolution, immediately meets the *tes* χ site and catalyses recombination (symbolized by a zigzag) between *tes* and *tes'* or between λ sequences, with creation of a chromosome dimer. Prophage material still attached to the *j.tek* sequence will be eventually lost. (C) Chromosome monomerization. XerC/XerD-mediated recombination between *dif* sites (symbolized by a double arrow) will yield one cured chromosome harboring a *tek* sequence and one lysogenic one. Other conventions as in Figure 8.

of the other observations. For instance, the scheme drawn in Figure 8 shows how excisive recombination may occur without generation of a chromosome dimer and subsequent requirement for resolution at *dif*. It does however leave a difficulty in that, by itself, it generates no polarity of excisive exchanges. What could be responsible for the directionality of terminal recombination, which diverges on either side of the *dif* region? Some directionality may result from the tensions exerted on the DNA of this region by the reconstruction of nucleoid structure after replication. We suspect that these tensions drive branch migration preferentially in one direction. The rule should be that the recombination branch migrates against the flux of catenation links (Figure 7), that is in the *dif*-to-*oriC* direction. This prediction comes from considerations of events required to generate the expected deletion when the *tet* sequences are in the active orientation (see Figures 8 and 9). In consequence, resolution of terminal strand invasions should occur at the branch distal from *dif*.

It is safe to postulate that terminal strand invasion

events can generate DSBs in the TRZ even in the absence of repeated sequences. When their RecBCD-controlled repair results in circular dimer formation as shown in Figure 5B, such dimers may be preferentially resolved by *dif*-specific recombination. It could well be that $RecBC^{-}$ bacteria display a low viability (CAPALDO-KIMBALL and BARBOUR 1971; RYDER *et al.* 1996; in our hands only 40% of Δ(*recBC*) bacteria give rise to a colony) mainly because they cannot repair the DSBs issuing from terminal strand invasion events and that Dif^{-} mutants display a poor viability [only 60–70% of the bacteria are able to give rise to a colony (BLAKELY *et al.* 1991; KUEMPEL *et al.* 1991; CORNET *et al.* 1996)] because they cannot resolve the dimers due to RecBCD fixing the DSBs sometimes associated with terminal strand invasions. The observation that the double deficiency Δ(*dif*) Δ(*recBC*) is no more detrimental than either single deficiency (data not shown) is consistent with this view.

Finally, it may appear surprising that a large region around *dif* is poor in χ sites, considering its propensity for recombination. This is true also for χ-like sites: no GCTAGTGG and ACTGGTGG sequences, which display some χ activity (CHENG and SMITH 1987), are present in a 50-kb segment around *dif*. This could be related to the second function that has been recently attributed to χ sites: initiation of binding and pairing of DNA by RecA (TRACY and KOWALCZYKOWSKI 1996). We suspect that the absence of χ sites protects the cell against situations (nicks, etc.) that might otherwise evolve toward recombination between sister chromosomes. Too many recombination figures might entangle the sister chromosomes rather than free them.

We thank Drs. JEAN-PIERRE CLAVERYS and DAVID LANE for their critical reading of the manuscript and many helpful discussions. We are grateful to A. KUZMINOV and F. STAHL for their interest and comments. This work was supported in part by a research contract from the Association pour la Recherche sur le Cancer (ARC). F.C. was supported by an ARC fellowship.

LITERATURE CITED

ADAMS, D. E., E. M. SHEKHTMAN, E. L. ZECHIEDRICH, M. B. SCHMID and N. R. COZZARELLI, 1992 The role of topoisomerase IV in partitioning bacterial replicons and the structure of catenated intermediates in DNA replication. Cell **71:** 277–288.

BEJAR, S., and J. P. BOUCHE, 1983 Molecular cloning of the region of the terminus of *Escherichia coli* K12 DNA replication. J. Bacteriol. **153:** 604–609.

BENNETT, R. J., and S. C. WEST, 1995 RuvC protein resolves Holliday junctions via cleavage of the continuous (noncrossover) strands. Proc. Natl. Acad. Sci. USA **92:** 5635–5639.

BERLYN, M. K. B., K. B. LOW, K. E. RUDD and M. SINGER, 1996 Linkage map of *Escherichia coli* K12, Ed. 9, pp. 1715–1902 in *Escherichia coli and Salmonella Cellular and Molecular Biology*, Ed. 2, edited by F. C. NEIDHARDT, R. CURTISS III., J. L. INGRAHAM, E. C. C. LIN, K. B. LOW *et al.* American Society for Microbiology, Washington, DC.

BIRD, R. E., J. LOUARN, J. MARTUSCELLI and L. CARO, 1972 Origin and sequence of chromosome replication in *Escherichia coli*. J. Mol. Biol. **70:** 549–566.

BLAKELY, G., S. COLLOMS, G. MAY, M. BURKE and D. SHERRATT, 1991 *Escherichia coli* XerC recombinase is required for chromosomal segregation at cell division. New Biol. **3:** 789–798.

BOUCHE, J. P., 1982 Physical map of a 470 × 10^3 base-pair region flanking the terminus of DNA replication in the *Escherichia coli* K12 genome. J. Mol. Biol. **154:** 1–20.

CAPALDO-KIMBALL, F., and S. D. BARBOUR, 1971 Involvement of recombination genes in growth and viability of *Escherichia coli* K12. J. Bacteriol. **106:** 204–212.

CHENG, K. C., and G. R. SMITH, 1987 Cutting of chi-like sequences by the RecBCD enzyme of *Escherichia coli*. J. Mol. Biol. **194:** 747–750.

CORNET, F., I. MORTIER, J. PATTE and J.-M. LOUARN, 1994 Plasmid pSC101 harbors a recombination site, *psi*, which is able to resolve plasmid multimers and to substitute for the analogous chromosomal *Escherichia coli* site *dif*. J. Bacteriol. **176:** 3188–3195.

CORNET, F., J. LOUARN, J. PATTE and J.-M. LOUARN, 1996 Restriction of the activity of the recombination site *dif* to a small zone of the *Escherichia coli* chromosome. Genes Dev. **10:** 1152–1161.

DIGATE, R. G., and K. J. MARIANS, 1989 Molecular cloning and DNA sequence analysis of *Escherichia coli topB*, the gene encoding topoisomerase III. J. Biol. Chem. **264:** 17924–17930.

FELLAY, R., J. FREY and H. K. KRISCH, 1987 Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of Gram-negative bacteria. Gene **52:** 147–154.

HORIUCHI, T., Y. FUJIMURA, H. NISHITANI, T. KOBAYASHI and M. HIDAKA, 1994 The DNA replication fork blocked at the *ter* site may be an entrance for the RecBCD enzyme into duplex DNA. J. Bacteriol. **176:** 4656–4663.

KATO, J. L., Y. NISHIMURA, R. IMAMURA, H. NIKI, S. HIRAGA *et al.*, 1990 New topoisomerase essential for chromosome segregation in *Escherichia coli*. Cell **63:** 393–404.

KOHARA, Y., K. AKIYAMA and K. ISONO, 1987 The physical map of the whole *Escherichia coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50:** 495–508.

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.

KUEMPEL, P. L., J. M. HENSON, L. DIRCKS, M. TECKLENBURG and D. F. LIM, 1991 *dif*, a *recA*-independent recombination site in the terminus region of *Escherichia coli*. New Biol. **3:** 799–811.

KUEMPEL, P. L., A. HOGAARD, M. NIELSEN, O. NAGAPAN and M. TECKLENBURG, 1996 Use of a transposon Tn*dif* to obtain suppressing and non-suppressing insertions of the *dif* resolvase site of *Escherichia coli*. Genes Dev. **10:** 1162–1171.

KUZMINOV, A., 1995 Breakage of arrested replication forks in *E. coli*. BioEssays **17:** 733–741.

LLOYD, R. G., and K. B. LOW, 1996 Homologous recombination, pp. 2236–2255 in *Escherichia coli and Salmonella Cellular and Molecular Biology*, Ed. 2, edited by F. C. NEIDHARDT, R. CURTISS III., J. L. INGRAHAM, E. C. C. LIN, K. B. LOW *et al.* American Society for Microbiology, Washington, DC.

LOUARN, J., F. CORNET, V. FRANCOIS, J. PATTE, and J.-M. LOUARN, 1994 Hyperrecombination in the terminus region of the *Escherichia coli* chromosome: possible relation to nucleoid organization. J. Bacteriol. **176:** 7524–7531.

LOUARN, J.-M., J. P. BOUCHE, F. LEGENDRE, J. LOUARN and J. PATTE, 1985 Characterization and properties of very large inversions of the *E. coli* chromosome along the origin-to-terminus axis. Mol. Gen. Genet. **201:** 467–476.

LOUARN, J.-M., J. LOUARN, V. FRANCOIS and J. PATTE, 1991 Analysis and possible role of hyperrecombination in the termination region of the *Escherichia coli* chromosome. J. Bacteriol. **173:** 5097–5104.

MAHAN, M. J., and J. R. ROTH, 1989 Role of *recBC* function in formation of chromosomal rearrangements: a two-step model for recombination. Genetics **121:** 433–443.

MARTIN, B., G. J. SHARPLES, O. HUMBERT, R. G. LLOYD and J. P. CLAVERYS, 1996 The *mmsA* locus of *Streptococcus pneumoniae* encodes a RecG-like protein involved in DNA repair and in three-strand recombination. Mol. Microbiol. **19:** 1035–1045.

MILLER, J. H., 1992 *A Short Course in Bacterial Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

REBOLLO, J. E., V. FRANCOIS and J.-M. LOUARN, 1988 Detection and

possible role of two large nondivisible zones on the *Escherichia coli* chromosome. Proc. Natl. Acad. Sci. USA **85:** 9391–9395.

RYDER, L., G. J. SHARPLES and R. G. LLOYD, 1996 Recombination-dependent growth in exonuclease-depleted *recBC sbcBC* strains of *Escherichia coli* K12. Genetics **143:** 1101–1114.

SAMBROOK, J., E. F. FRITSCH and T. MANIATIS, 1989 *Molecular Cloning: A Laboratory Manual,* Ed. 2, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SMITH, G. R., S. M. KUNES, D. W. SCHULTZ, A. TAYLOR and K. L. TRIMAN, 1981 Structure of chi hotspots of generalized recombination. Cell **24:** 429–436.

TRACY, R. B., and S. C. KOWALCZYKOWSKI, 1996 In vitro selection of preferred DNA pairing sequences by the *Escherichia coli* RecA protein. Genes Dev. **10:** 1890–1903.

Communicating editor: N. L. CRAIG