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Genetics

DNA rearrangement mediated by inverted repeats

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ABSTRACT **Inverted repeats of DNA are widespread in the genomes of eukaryotes and prokaryotes and can mediate genome rearrangement. We studied rearrangement mediated by plasmid-borne inverted repeats in *Escherichia coli*. We show that inverted repeats can mediate an efficient and *recA*-independent recombination event. Surprisingly, the product of this recombination is not that of simple inversion between the inverted repeats, but almost exclusively an unusual head-to-head dimer with complex DNA rearrangement. Moreover, this recombination is dramatically reduced by increasing the distance separating the repeats. These results can be readily explained by a model involving reciprocal switching of the leading and lagging strands of DNA replication within the inverted repeats, which leads to the formation of a Holliday junction. Reciprocal strand switching during DNA replication might be a common mechanism for genome rearrangement associated with inverted duplication.**

Repetitive DNA sequences can mediate recombination via various mechanisms (1, 2). In *Escherichia coli*, the *recA*-dependent general recombination proceeds mainly through the RecBCD pathway (1, 2). RecA promotes homologous pairing of DNA molecules and catalyzes the strand exchange reaction leading to the formation of heteroduplex DNA *in vitro* (3). Despite the apparently central role of RecA in homologous recombination, *recA*-independent recombination between tandem direct repeats has been observed in plasmids (4, 5) and the chromosome of *E. coli* (4). This recombination is affected by structural factors such as the distance between the repeats (5–7).

Recombination between direct repeats can lead to deletion of one of the repeats and any intervening sequence, whereas that between inverted repeats can invert the intervening sequence. Relative to recombination between direct repeats, recombination between inverted repeats in *E. coli* has not been extensively studied. The few reported studies of inversion all made use of "genetic switches" of genes. When the promoter of a gene is bracketed by inverted repeats, its orientation can be changed by recombination (inversion) between the repeats, resulting in the reversible alternation of the "on" and "off" states of gene expression. Such a genetic switch was found in phage λ mediated by the inverted insertion sequence elements of Tn*10* and shown to be *recA*-dependent (8). This switching was later shown to occur efficiently via the RecBCD pathway and was suggested to be either intra- or intermolecular (9). Inversion of the *lac* promoter mediated by short inverted repeats in the chromosome of *E. coli* was also shown to be *recA*- and *recBC*-dependent (10). In *Salmonella typhimurium*, inversion between large inverted repeats (>5 kb) separated by large intervals (>60 kb) has been shown to be *recA*- and *recB*-dependent (13).

To further examine recombination between inverted repeats, we created a genetic switch for the *tetA* gene of the plasmid pBR322 by bracketing its promoter with inverted repeats. We show that recombination between inverted repeats in this plasmid system is *recA*- and *recBC*-independent and is dramatically reduced by lengthening the distance separating the repeats. Moreover, instead of the predicted product of simple intramolecular inversion, almost all of the products of this recombination are a special head-to-head dimer. Our results can be readily explained by a replicational model for recombination between inverted repeats.

Table 1. Bacterial strains

Designation	Mutations affecting recombination	Other mutations	Source or ref.
MM294	None	F⁻ *endA1 hsdR17* ($r_k^-m_k^+$)*supE44 thi1 relA1? rfbD? spoT1?*	11
MM294ΔrecA	Δ*recA*	As for MM294	B. Weiss*
RR1	None	F⁻ Δ(*gpt-proA*)*66 leu supE44 ara14 galK2* Δ(*mcrC-mrr*) *lacY1 rpsL20 xyl-5 mtl-1*	12
HB101	*recA13*	As for RR1	11
AB1157	None	F⁻ *thr1 leu6 thi1 lacY1 galK2 ara14 xyl5 mtl1 proA2 his4 argE3 str31 tsx33 supE44 kdgK51*	A. J. Clark†
JC10287	Δ(*srlR-recA*)*304*	As for AB1157	A. J. Clark
JC5519	*recB21 recC22*	As for AB1157	A. J. Clark

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MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* strains used in this study are listed in Table 1. Plasmid pSVβ was from Clontech. Plasmid pHPH (Fig. 1*B*) was derived from pBR322 (Fig. 1*A*) as follows. The *Sal* I site between the P and H fragments of pBR322 was used for cloning. The P and H fragments were first amplified by PCR with the addition of *Sal* I sites to both ends of them (accomplished by including restriction sites in the PCR primers). After being digested by *Sal* I, both fragments were incubated with pBR322 cut with *Sal* I in a ligation reaction. The product (named pPHPH) with double insertions of P and H in the directions as shown in pHPH was selected. Note that pPHPH has another copy of the P fragment compared with pHPH. This extra P fragment was then deleted to generate pHPH. The pHPβH series of plasmids were derived from pHPH as described in the legend to Fig. 4*A*.

Recombination Tests and Product Analysis. Plasmid recombination was examined as described by Bi and Liu (5). The plasmid substrate was transformed into a strain and the transformants were selected on LB plates containing Ap (100 μg/ml). After ≈20 hr of incubation at 37°C, 10 or more

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Abbreviations: Ap, ampicillin; Tc, tetracycline; R, resistant; S, sensitive; RSS, reciprocal-strand-switching.

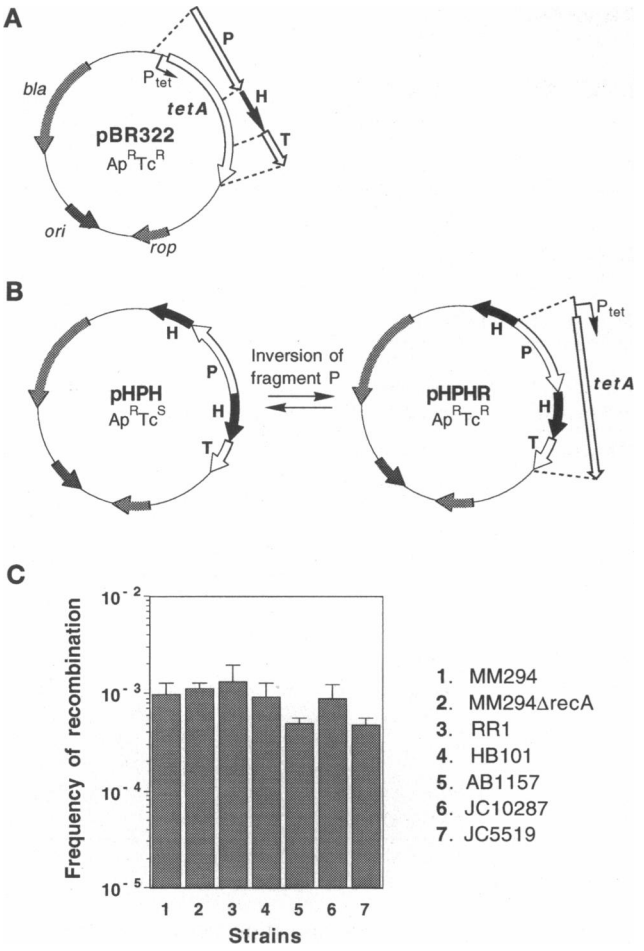

FIG. 1. Recombination between plasmid-borne inverted repeats is efficient and independent of RecA and RecBCD. (*A*) Structure of pBR322. The *tetA* gene (coordinates 86–1276) and its promoter is divided into three parts: P (coordinates 1–651), H (652–1003), and T (1004–1276). (*B*) Genetic switch for *tetA* expression. Shown are pHPH and its predicted product of simple inversion, pHPHR (see text for description). (*C*) Frequency of recombination (in logarithm) of pHPH in various strains (Table 1). Ap, ampicillin; Tc, tetracycline; ^{R}, resistant; ^{S}, sensitive.

colonies were collected and suspended in M9 minimal salts (14). Cells were plated on LB plates containing Tc (20 μg/ml) and Ap (referred to as the Tc plate) to select for cells containing recombined plasmids. Cells were also plated on plates containing only Ap (the Ap plate) to obtain the number of viable Ap^R cells in the suspension. The frequency of recombination was measured as the ratio of the number of colonies on the Tc plate to that on the Ap plate. For each substrate, 3–10 independent experiments were performed and the frequency was calculated as the mean of the data from all experiments. The standard deviation was also calculated. Note that the frequency reported is the overall frequency, not the rate of recombination. For the recombination tests in this work, the rate of recombination as calculated by the method of Luria and Delbrück (15) would be about one order of magnitude lower than the frequency. In addition, in calculation of the frequency, the multicopy number nature of the plasmid substrate is not taken into consideration. Taking the average copy number of pBR322 as 100, the real recombination rate (estimated as the number of events per copy of plasmid per cell per generation) would be about three orders of magnitude lower than the overall frequency.

To analyze the products of recombination of a plasmid substrate, 100 or more Tc^R colonies from the recombination tests were used to individually inoculate 2 ml of LB with Ap. After ≈15 hr of growth at 37°C, a quick phenol method (6) was used to isolate plasmid from each culture for analysis by agarose gel electrophoresis.

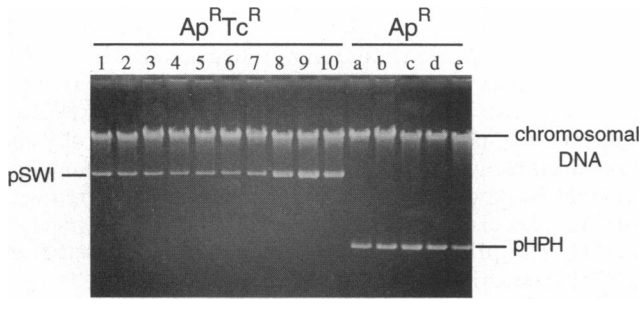

FIG. 2. The major product of *recA*-independent recombination between inverted repeats in pHPH is not that of simple inversion, but a special dimer. Shown are the products of pHPH in HB101. Plasmids were isolated from 100 individual Ap^RTc^R colonies and analyzed by agarose gel electrophoresis. Plasmids from 10 Ap^RTc^R colonies were shown as an example (lanes 1–10). Five samples from Ap^R colonies were also shown as controls (lanes a–e). The structure of the dimeric product (pSWI) is characterized in Fig. 3.

RESULTS

Construction of a Genetic Switch for the *tetA* Gene of pBR322 as a Model System for Studying Recombination Between Inverted Repeats. We constructed a series of plasmids (pHPH and its derivatives described in the legends to Fig. 1*B* and Fig. 4*A*) for studying recombination between inverted repeats in *E. coli*. The basic strategy was to create a genetic switch for the *tetA* gene of pBR322 (Fig. 1*A*) by placing its promoter (P_{tet}) between two inverted repeats. In pHPH (Fig. 1*B*), P_{tet} and part of *tetA* (together designated P) was bracketed with two inverted repeats (H). Note that H is also part of *tetA*. P_{tet} in fragment P of pHPH is in the "wrong" orientation so that *tetA* cannot be expressed. Recombination between the H repeats is expected to cause inversion of P so that the intact *tetA* can be regenerated (in pHPHR, Fig. 1*B*). In order to examine if the distance separating the inverted repeats affects recombination, we also inserted additional DNA fragments in between the H repeats of pHPH (Fig. 4*A*).

Table 2. The major product of recombination between inverted repeats in pHPH is a special dimer (pSWI)

Strain	Products* Special dimer (pSWI), %	Other, %
MM294 (wt)	98†	2‡
MM294ΔRecA	100	—
RR1 (wt)	96†	4‡
HB101 (*recA13*)	100	—
AB1157 (wt)	98†	2‡
JC10287 (Δ*recA*)	100	—
JC5519 (*recBC*)	98†	2‡

wt, Wild type.

*The products of recombination of pHPH were examined as described in the text. Each sample of plasmid was from an individual Ap^RTc^R colony.

†For the $recA^+$ strains MM294, RR1, AB1157, and JC5519, in some of the samples, besides pSWI, the major species, barely detectable amount of plasmid(s) of the size of pHPH is present. The plasmid may be pHPH or its product of simple inversion, pHPHR, or a mixture of them.

‡Samples in which there is a significant amount of a plasmid(s) of the size of the tetramer of pHPH besides pSWI.

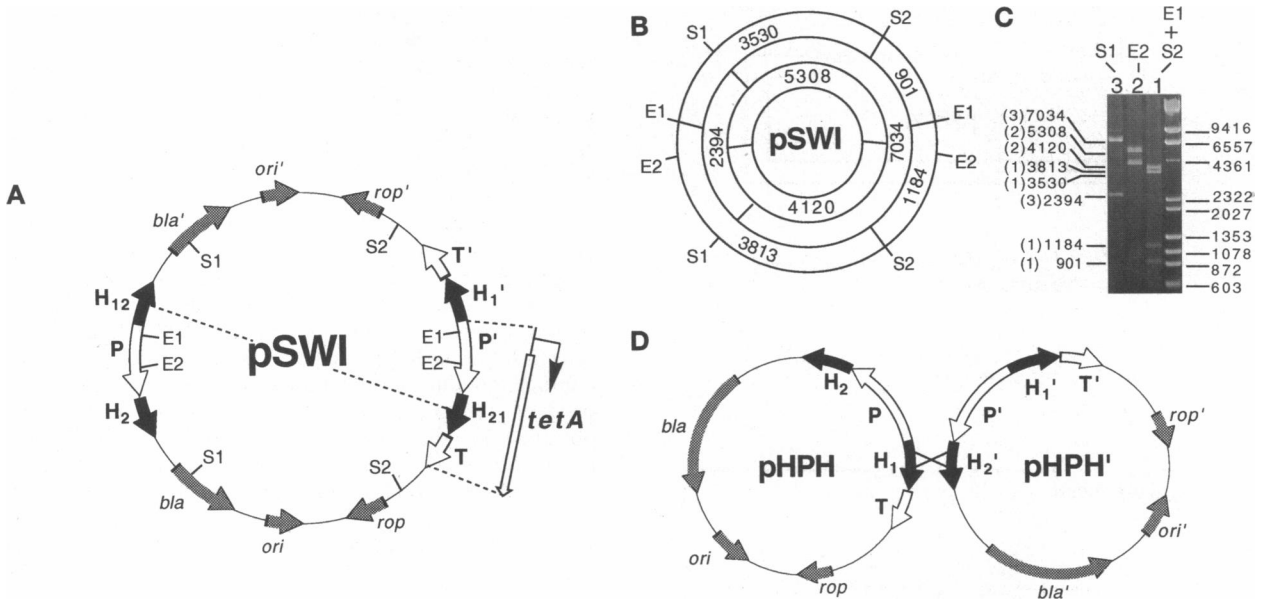

FIG. 3. The major product of *recA*-independent recombination between plasmid-borne inverted repeats is an unusual dimer containing inverted duplications. (*A*) Structure of the product (pSWI) of *recA*-independent recombination of pHPH deduced from restriction analyses (see *B* and *C*.) Note that pSWI contains large inverted duplications separated by the P fragments which are flanked by the inverted H repeats. An intact *tetA* is present in pSWI (compare pSWI with pBR322 in Fig. 1*A*). The dashed line separates the two monomeric components (pHPH) of pSWI. The four H repeats in pSWI were designated H_{12}, H_{21}, H_1' and H_2, respectively. E1, E2, S1, and S2 are the sites for the restriction enzymes *Eco*RV, *Eco*NI, *Sca* I, and *Sty* I, respectively. (*B*) Predicted fragments generated from various restriction digestions of pSWI. Outer circle, fragments generated by E1 plus S2 digestion. Middle and inner circles, fragments generated by S1 and E2 digestion, respectively. (*C*) Restriction analyses of the product of pHPH. Results from three sets of digestions of the product are shown. Lanes 1, 2, and 3, product digested with E1 plus S2, E2, and S1, respectively. (*D*) Theoretical scheme for pSWI formation. pSWI can be produced by an intermolecular reciprocal crossover between two H repeats (H_1 and H_2') of two pHPH molecules. H_{12} and H_{21} in pSWI (see *A*) would be the repeats involved in this crossover. However, this is unlikely the mechanism for pSWI formation (see *Discussion*) but is drawn to help explain the structure of pSWI.

Plasmid Recombination Between Inverted Repeats Is Efficient and Independent of RecA and RecBCD. Recombination between inverted repeats in pHPH (Fig. 1*B*) was examined in various strains of *E. coli* (Table 1). Surprisingly, recombination as scored by Tc^R is very efficient (with a frequency of about $0.5–1.0 \times 10^{-3}$) and is *recA*-independent (Fig. 1*C*, compare 1, 3, and 5 with 2, 4, and 6, respectively). Moreover, it is also *recBC*-independent (Fig. 1*C*, compare 5 with 7). Similar results were also obtained using another pBR322-based substrate in which P_{tet} was bracketed with inverted repeats of 559 bp in length (data not shown). These results indicate that there is a mechanism(s) other than conventional *recA*-dependent recombination (1, 2) for inverted repeats-mediated recombination.

The Product of *recA*-Independent Plasmid Recombination Between Inverted Repeats Is an Unusual Head-to-Head Dimer. The expected product of recombination between inverted repeats is that of an simple intramolecular inversion.

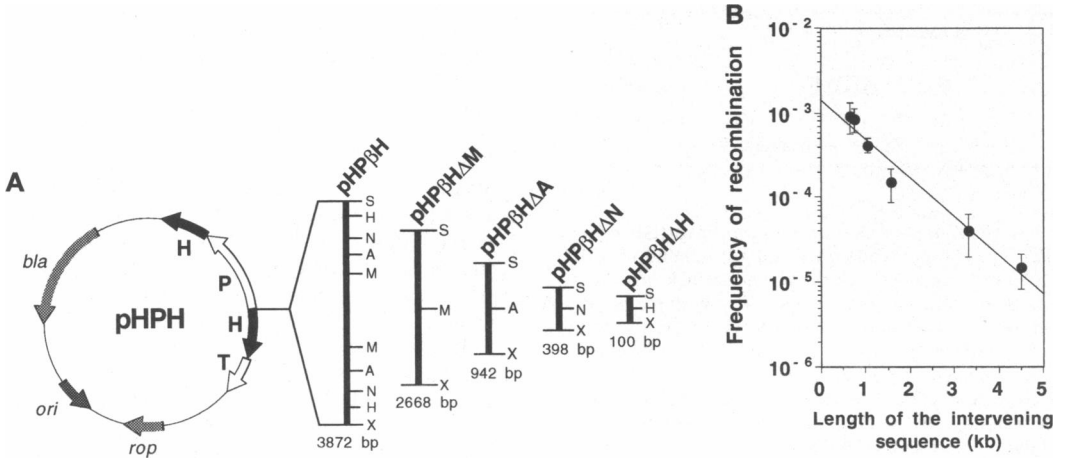

FIG. 4. *recA*-independent plasmid recombination between inverted repeats is greatly reduced by lengthening the intervening sequence. (*A*) pHPβH series of plasmids: pHPH derivatives with various intervening sequences between the H repeats. The *Sal* I–*Xho* I fragment (3872 bp) of pSVβ was inserted into pHPH at the *Sal* I site between the P fragment and the H fragment adjacent to fragment T, generating pHPβH. Then, the *Mlu* I fragment, the *Apo* I fragment, the *Not* I fragment, and the *Hpa* I fragment were deleted from pHPβH, generating the plasmids pHPβHΔM, pHPβHΔA, pHPβHΔN, pHPβHΔH, respectively. S, H, N, A, M, and X, *Sal* I, *Hpa* I, *Not* I, *Apo* I, *Mlu* I, and *Xho* I sites, respectively. (*B*) Increasing the distance separating the inverted repeats inhibits *recA*-independent recombination between them. Recombination tests of pHPH and the pHPβH series of plasmids were performed in HB101. The logarithm of the frequency of recombination was plotted as a function of the length of the intervening sequence.

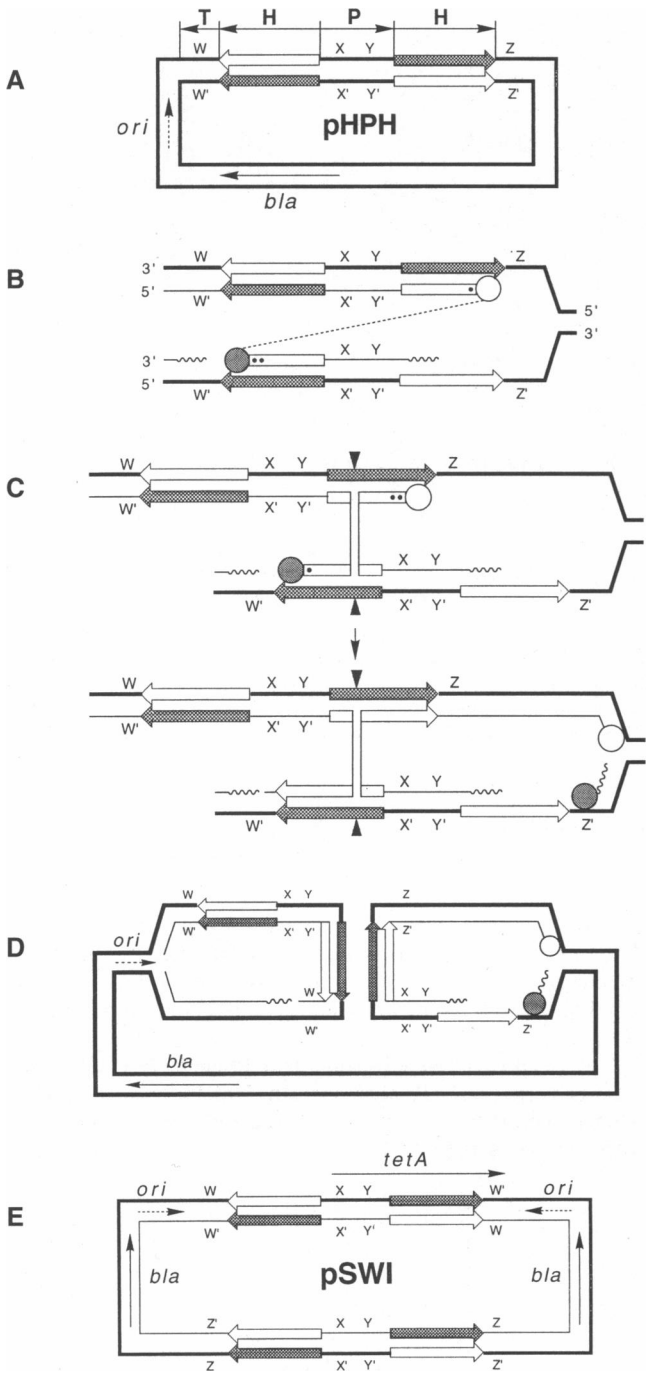

FIG. 5. RSS model for recombination between inverted repeats. (*A*) Plasmid pHPH. The H repeats and the P and T fragments (Fig. 1*B*) are shown. The two strands of DNA are shown as thick lines. Each strand of a repeat is shown as an arrow. Open arrows correspond to the 5′ → 3′ orientation of DNA and shaded arrows correspond to the 3′ → 5′ orientation. Markers W, X, Y, and Z are arbitrary. The origin of replication (*ori*) is shown as a dashed arrow. The *bla* gene is shown as an arrow. (*B*) Inverted repeats being synthesized at a replication fork. Open circle, leading strand DNA polymerase activity; shaded circle, lagging strand polymerase. Thin lines, nascent strands of DNA. The 3′ end of the leading strand is indicated by a dot, and the 3′ end of the lagging strand is indicated by two dots. Wavy lines represent the RNA primers for lagging strand synthesis. The dashed line indicates that the two polymerase activities may be actually associated in a complex (the DNA polymerase III holoenzyme) (19, 20). (*C*) Reciprocal switching of the leading and lagging strands within the inverted repeats. The 3′ end of the nascent leading strand dissociates from the leading strand polymerase and template and switches to the lagging strand polymerase and template, and vice versa. Replication continues after the switching. Sooner or later, the junction generated in *C* will be resolved by DNA endonuclease and ligase. The arrowheads indicate the positions of endonuclease digestion. (*D*) Structure of the replicating plasmid after the junction in *C* is resolved in the manner as shown. (*E*) Completion of replication results in a special dimer (pSWI) as is also illustrated in Fig. 3*A*.

We analyzed the products of recombination of pHPH under various genetic backgrounds. One hundred or more Ap^RTc^R colonies from recombination tests of pHPH in each strain tested were analyzed. Unexpectedly, for all *recA*$^-$ strains tested, all of the Ap^RTc^R colonies examined contained a single species of plasmid of the size of dimeric pHPH as revealed by ethidium bromide staining (Fig. 2 and Table 2). Restriction analysis (Fig. 3*C*) revealed that this plasmid is an unusual dimer (designated pSWI) consisting of inverted duplications (Fig. 3*A*). Plasmid pSWI is certainly not the product of a simple intramolecular inversion between the H repeats of pHPH because such a product (pHPHR in Fig. 1*B*) has the size of pHPH. For the *rec*$^+$ strains AB1157, MM294, and RR1, and the *recBC* strain JC5519, each of the 100 Ap^RTc^R colonies also contained mainly or only the special dimer pSWI instead of the product of simple inversion (Table 2). Similar results were also obtained for the other pBR322-based substrate mentioned above (data not shown). These results indicate that *recA*-independent recombination between inverted repeats is not simple inversion.

Plasmid Recombination Between Inverted Repeats Is Highly Sensitive to the Distance Separating the Repeats. To examine if the distance separating the inverted repeats can affect recombination, we derived from pHPH the pHPβH series of plasmids (Fig. 4*A*) in which the H repeats were separated by intervening sequences of 751, 1049, 1593, 3319, and 4523 bp, respectively. Using these plasmids and pHPH, recombination between inverted repeats as a function of the length of the intervening sequence was examined. As the distance between the H repeats increased, the frequency of *recA*-independent recombination decreased exponentially (Fig. 4*B*). However, the pHPβH series of plasmids still yielded special dimers similar to pSWI (data not shown) indicating that lengthening the intervening sequence did not affect the type of recombination involved.

DISCUSSION

We have demonstrated that plasmid-borne inverted repeats can mediate an efficient and *recA*- and *recBC*-independent recombination which produces almost exclusively an unusual head-to-head dimer with complex DNA rearrangement. In theory, pSWI (Fig. 3*A*), the unusual dimeric product of pHPH, can be formed by an intermolecular reciprocal exchange between two pHPH monomers (Fig. 3*D*). However, this is unlikely to be the case for the following reasons. (*i*) Intermolecular recombination is rare in *recA*$^-$ strains. In *recA* mutants there is virtually no intermolecular conjugational recombination (16). Oligomer formation from monomeric plasmid is *recA*-dependent (17). It has been shown that recombination between two compatible plasmids is greatly reduced in a *recA*$^-$ strain, with a frequency of $\approx 4 \times 10^{-6}$ (18), >100-fold lower than the frequency of pSWI formation (Fig. 1*C*). (*ii*) If the special dimer is formed by intermolecular recombination, increasing the distance between the inverted repeats should not affect its formation. However, its formation is greatly reduced as the intervening sequence increases (Fig. 4*B*).

It is striking that, even in *recA*$^+$ strains, very few, if any, products of pHPH detected are that of simple inversion between the H repeats (Table 2). One possible explanation is that the simple inversion product (pHPHR, Fig. 1*B*) is either toxic to, or cannot be stably maintained in, host cells. This was ruled out by the fact that pHPHR (constructed *in vitro*) was

able to transform cells and be stably maintained on medium containing only Ap or medium containing both Tc and Ap (data not shown). Why simple intramolecular inversion is not the predominant recombination is unclear.

DNA replication has been invoked to explain *recA*-independent deletion between direct repeats and accompanying rearrangements (4–7). However, these models cannot explain simple inversion between inverted repeats or the formation of pSWI from pHPH. Here we propose a model for *recA*-independent recombination between inverted repeats, which involves reciprocal switching of the leading and lagging strands when the repeats are being replicated.

Our reciprocal-strand-switching (RSS) model is detailed in Fig. 5 using pHPH as an example. Fig. 5*A* indicates the substrate pHPH. At the replication fork, the leading strand DNA polymerase (19, 20) is copying the second repeat (proximal to the fork), while the lagging strand polymerase is copying the first repeat (Fig. 5*B*). Fig. 5*C* indicates reciprocal switching of the leading and lagging strands within the inverted repeats and continuation of replication. Note that the junction created by strand switching is formally a Holliday junction (21). It can be resolved by endonuclease and ligase activities. If the nascent strands (thin lines in the junction) are cut and religated after exchange, the original fork structure is regenerated as if nothing has happened. However, if the template strands are cut and religated after exchange as illustrated in Fig. 5*C*, a dumbbell-shaped replicating plasmid would be formed (Fig. 5*D*). Completion of replication would result in an unusual complex dimer (pSWI) consisting of inverted duplications (Fig. 5*E*). Plasmid pHPH has the unidirectional origin of replication (*ori*) of pBR322 (22). However, it is obvious that plasmid with a bidirectional *ori* will also generate the structure in Fig. 5*E*. Note that the sequence of the markers W, X, Y, and Z in pHPH is WXYZ (Fig. 5*A*). In the product (pSWI), two new joints, ZX and YW, are created, and the intact *tetA* (corresponding to the sequence of markers XYW) is regenerated. The rest of pSWI consists of two large inverted duplications (also see Fig. 3*A*). In conclusion, we propose that RSS within the inverted repeats of pHPH during replication leads to the formation of the special dimer pSWI.

None of the processes of the RSS model involves RecA or RecBCD and thus the proposed recombination between inverted repeats is *recA*- and *recBC*-independent. The distance effect on recombination between inverted repeats (Fig. 4*B*) can also be explained by the RSS model. Since, in general, synthesis of the leading and lagging strands is coordinated spatially (19, 20), reciprocal strand switching might occur efficiently only when the two repeats are separated by a relatively short distance, perhaps in the range of an Okazaki fragment (1–2 kb in length; refs. 19 and 20).

Simple strand switching in replication has been first proposed to explain certain deletions in phage λ (23) and phage Mu excision by aborted transposition (24). It was also used to explain the generation of inverted duplications in certain gene amplification events in mammalian cells (refs. 25 and 26; reviewed in ref. 27). These models (23–26) all invoke that replication switches strands (templates) and proceeds around the replication fork and can be referred to as "single-strand-switching" models. The RSS model is different from these models as follows. (*i*) In the RSS model, *reciprocal* switching of *both* nascent strands of replication has been proposed and a Holliday junction is formed after the switching. (*ii*) In the RSS model, strand switching occurs within *preexisting inverted repeats* at the replication fork. Although both the single-strand-switching model and the RSS model can explain the formation of inverted duplications, only the RSS model can explain the inverted repeats-mediated complex rearrangement observed in this study.

It is not known if the complex rearrangement mediated by inverted repeats in plasmids also occurs in the chromosomes of cells. If it occurs in the *E. coli* chromosome, the product would be a head-to-head dimeric chromosome (structurally similar to pSWI), which might be lethal to the cell. This might be the reason why this kind of rearrangement has not been detected in *E. coli*. If the rearrangement occurs in a linear chromosome, the products would be two palindromic DNA molecules (as can be extrapolated from Fig. 5). Interestingly, palindromic chromosomes have been found in mammalian cells (for an example, see ref. 28). If two such events occur in the same chromosome, three products would be generated: two palindromic chromosomes and an extrachromosomal circle. The circle would contain mostly an inverted duplication separated by two unique DNA segments. Interestingly, this circle is structurally similar to the H circle in the protozoan parasite *Leishmania* (29, 30) and certain λ*dv* plasmids derived from phage λ (23), as well as certain circular amplicons in mammalian cells (31). It is noteworthy that the H circle is originated from the H locus which is flanked by *two pairs of inverted repeats* in the *Leishmania* chromosome (29, 30). In light of our RSS model, it is possible that the H circle is formed via RSS *at both pairs of inverted repeats* bracketing the H locus during bidirectional DNA replication. It will be interesting to investigate whether the RSS model underlies the mechanism of certain gene amplification and genome rearrangement events.

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