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Type III Restriction Is Alleviated by Bacteriophage (RecE) Homologous Recombination Function but Enhanced by Bacterial (RecBCD) Function

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Previous works have demonstrated that DNA breaks generated by restriction enzymes stimulate, and are repaired by, homologous recombination with an intact, homologous DNA region through the function of lambdoid bacteriophages lambda and Rac. In the present work, we examined the effect of bacteriophage functions, expressed in bacterial cells, on restriction of an infecting tester phage in a simple plaque formation assay. The efficiency of plaque formation on an *Escherichia coli* host carrying EcoRI, a type II restriction system, is not increased by the presence of Rac prophage—presumably because, under the single-infection conditions of the plaque assay, a broken phage DNA cannot find a homologue with which to recombine. To our surprise, however, we found that the efficiency of plaque formation in the presence of a type III restriction system, EcoP1 or EcoP15, is increased by the bacteriophage-mediated homologous recombination functions *recE* and *recT* of Rac prophage. This type III restriction alleviation does not depend on *lar* on Rac, unlike type I restriction alleviation. On the other hand, bacterial RecBCD-homologous recombination function enhances type III restriction. These results led us to hypothesize that the action of type III restriction enzymes takes place on replicated or replicating DNA in vivo and leaves daughter DNAs with breaks at nonallelic sites, that bacteriophage-mediated homologous recombination reconstitutes an intact DNA from them, and that RecBCD exonuclease blocks this repair by degradation from the restriction breaks.

A restriction endonuclease recognizes a specific DNA sequence and introduces a double-strand break (DSB). A cognate modification enzyme methylates the same sequence and thereby protects it from cleavage. Together, these two enzyme activities form a restriction-modification (RM) system. The genes coding these enzymes are often tightly linked and can be termed an RM gene complex. The RM systems have been classified into type I, type II, and type III (59).

Type II restriction enzymes, such as EcoRI found on a plasmid of *Escherichia coli*, cut DNA at or near their recognition sequence (53). Typical type III restriction enzymes, such as EcoP1 encoded by bacteriophage/plasmid P1, and EcoP15 encoded by plasmid p15B, consist of two polypeptides (4, 55). One subunit encoded by the *mod* gene is responsible for target recognition and modification, and another encoded by the *res* gene is responsible for restriction. EcoP1 recognizes 5′AGACC, while EcoP15 recognizes 5′CAGCAG. The EcoP1 and EcoP15 RM systems are homologous except for the target sequence recognition region in the *mod* gene (25).

Unlike type II modification, type III modification methylates only one DNA strand at its recognition sequence due to missing adenine residues in the complementary strand (2). Type III restriction requires two unmodified recognition sites that must be in inverse orientation (42). All unmodified recognition sequences are in the same orientation on the newly replicated DNAs and would not allow restriction (42). A type III restriction enzyme, an ATP-dependent helicase, bound at its unmodified recognition sequence pulls duplex DNA from one direction (43; see also reference 41). When two enzyme molecules bound at two recognition sequences of opposite orientation meet, they introduce a DSB into DNA 25 to 27 bp inward from each recognition site. Type I restriction enzymes, such as EcoKI coded by the *hsd* locus of *Escherichia coli*, show similar reactions except that the enzyme pulls DNA from both directions and that the DNA between the two recognition sequences will be cleaved upon encountering two enzyme molecules (45).

Restriction enzymes will cleave incoming DNA if it has not been modified by a cognate or another appropriate methyltransferase. Consequently, it has been widely believed that RM systems have been maintained by bacteria because they serve to defend the cells from infection by foreign DNAs. A contrasting idea for the maintenance of RM systems is based on the observation that several RM gene complexes in bacteria are not easily replaced by competitor genetic elements because their loss leads to cell death (*postsegregational killing* or *genetic addiction*) (46, 59). If an RM gene complex is lost, the cell's descendants will contain fewer and fewer molecules of the modification enzyme because of dilution. Eventually, the modification enzyme's capacity to protect the many recognition sites on newly replicated chromosomes from attack by the remaining pool of restriction enzyme becomes inadequate. Chromosomal DNA will then be cleaved at these exposed sites,

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which leads to cell death (unless the break is repaired, as we see below). This finding led to the proposal that these complexes may represent one of the simplest forms of life, similar to viruses, transposons, and homing endonucleases. This *selfish gene hypothesis* (35, 46) is now supported by many lines of evidence from experimentation and genome analysis (29, 30).

The individual specificity and the overall diversity of sequence recognition by the RM systems can be explained by their competition for recognition sequences, which was demonstrated for type II systems in the absence of any invading DNAs (35). The variety of mechanisms of gene regulation by RM systems may be interpreted in terms of their behavior as selfish mobile genetic elements, namely, that they have to establish themselves in a new host cell without killing it, maintain themselves, and engage in postsegregational host killing when they are threatened (30). The interference between two regulatory systems may results in mutual exclusion (super-infection exclusion) between two RM systems (47). Likewise, two type III systems, EcoP1 and EcoP15, are exclusive because of the similarity of the methyltransferases (56).

The decoding of several bacterial genomes has provided ample evidence of potential mobility of RM systems (REBASE: http://rebase.neb.com/rebase/rebase.html). Various types of evolutionary analyses suggest that many RM genes have undergone extensive horizontal transfer between distantly related groups of bacteria and archaea. Some of the bacterial and archaeal genomes have a large numbers of RM gene homologues. A feature that is common to some of those genomes is their capacity for natural transformation. This mechanism allows an RM gene complex to move between genomes of a population by means of homologous recombination. Chromosomal genes would be frequently replaced by incoming homologous stretches of DNAs. However, the RM gene complexes will resist their loss by host killing as has been demonstrated (21, 59).

Close examination of the genomic neighborhood of RM gene homologues and its comparison with a closely related genome provide hints as to how RM gene complexes can enter a genome. The RM gene complexes are often found on a variety of mobile genetic elements: plasmids, prophages, transposons, conjugative transposons, genomic islands and integrons (29). They may stabilize the maintenance of these mobile elements. Some RM gene homologues are found flanked by long (in the order of 100 bp) direct repeats. The comparison of two genomes has suggested that this duplication is generated when an RM gene complex inserts itself into the genome (49). This type of long direct repeats allows virus genome-like multiplication of an RM gene complex (59), the discovery of which favors the selfish gene point of view. Linkage of a restriction modification-homologue and a genome rearrangement inferred from genome comparison strongly suggests involvement of an RM gene complex in genome rearrangement (29). Indeed attempts to replace a chromosomal RM gene complex led to recovery of rearranged genomes (21, 59).

There are signs of coevolution between RM systems and their target genomes in the genomes and genome dynamics. For example, signs of strong selection against palindromic sequences that are the targets of attack by many type II RM systems are seen in the genomes (*restriction avoidance*) (13, 59). The genome of bacteriophage T7 carries many EcoP1 recognition sequence in only one orientation (42). Some bacteriophages as well as some bacteria carry a solitary methyltransferase that defends their genome against restriction (*molecular vaccination*) (67). Bacteriophage P1 particle carries DarAB protein that protects injected DNA from type I restriction (26, 33). Similar mechanisms, called Ard, are identified in several conjugative plasmids (5). Ral, encoded by the *ral* gene of bacteriophage lambda (39), and its analog *lar*, encoded by Rac prophage (28), alter the activity of a type I methyltransferase to efficiently methylate DNA. Ocr protein, encoded by gene *0.3* of bacteriophage T7, through DNA mimicry strategy, binds to EcoKI enzyme (type I) and blocks restriction and modification (74). In contrast to the T7 case, gene *0.3* of bacteriophage T3 and ϕYeO3-12 encodes *S*-adenosylmethionine hydrolase, which decreases type III restriction activity (34, 52). Also, it was reported that EcoP1 restriction was drastically decreased by mutation in *rpsL* gene that encodes ribosomal protein S12, probably due to reduced translation efficiency (56). These antirestriction processes are sometimes called restriction alleviation and often detected as changes in plaque formation efficiency of tester bacteriophage.

Some restriction alleviation processes are related with DNA repair and recombination. DNA damage by UV irradiation alleviates type I restriction. This prevents attack on newly synthesized unmodified DNA during repair (70, 71). Furthermore, UmuDC, induced by the SOS response, alleviates type I restriction (24). Another form of type I restriction alleviation occurs when RecBCD inhibitor is overproduced (61) for the reason we describe below.

The properties of the bacterial major homologous recombination machinery, RecBCD pathway of *Escherichia coli* appear well adapted to the behavior of RM systems as selfish elements. It destroys invading bacteriophage DNAs (nonself DNA) after restriction cleavage (10), but repairs bacterial chromosomes (self DNA) after restriction cleavage during postsegregational host killing (19). The RecBCD enzyme starts degrading DNA from a restriction break but switches to recombination repair when it encounters a specific sequence, called Chi, on the genome (19). The specific sequence varies among bacterial groups and likely serves as an identification marker of the genome of a group. This exonuclease-based system may represent another mechanism that allows the genome to distinguish between self and nonself, similar to the endonuclease-based RM systems.

The homologous recombination machinery carried by bacteriophages appears to be particularly well adapted to counteracting attacks by a variety of RM systems (31). Lambdoid bacteriophages, such lambda and Rac (prophage), may repair the restriction break by a DSB repair mechanism, in which a DSB is repaired by copying homologous DNA with or without associated crossing-over of the flanking sequences (66). RecE and RecT, encoded by Rac prophage, or Redα and Redβ, encoded by lambda phage, are responsible for the process (36. 66). The recombination may be nonconservative in the sense it generates only one progeny DNA out of two recombining DNAs (69). The chromosomal mutations leading to functional expression of *recET* include the following: chromosomal large deletions (*sbcA8* and *sbcA81* [6a]); chromosomal point mutations (*sbcA5* and *sbcA23)* (40); chromosomal transposon insertions (*sbcA111::*Tn*5*, *sbcA119::*Tn*5*, *sbcA117::*Tn*5*,

TABLE 1. Bacterial strains

Name	Another name	Genotype	Source, reference(s)
AB1157	BIK788	*supE44 thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 tsx-33 galK2 hisG4 rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1* λ^- F^-	3, 77
BIK733		As AB1157, but Δ*recA306*::Tn*10*	K. Yamamoto, 8
JC5519	BIK751	As AB1157, but *recB21 recC22*	T. Kato, 75
JC8679	BIK813	As AB1157, but *recB21 recC22 sbcA23*	A. J. Clark, 14
JC8691		As JC8679, but *recE159*	A. J. Clark, 14
KF1503	ME8582	HfrPO45 (*thyA-serA*)::*recT*::Tn*10 sbcA111*::Tn*5 thr-300 ilv-318*	A. Nishimura (NIG)
BNH884		As JC8679, but *recT*::Tn*10*	P1 (KF1503) to JC8679
JC9604		As JC8679, but *recA56*	A. J. Clark, 14
BNH931		As JC9604, but *recT*::Tn*10*	P1 (BNH884) to JC9604
DH5	BIK771	*recA1 endA1 hsdR17*	Laboratory collection, 3, 16
DH10B	BIK1291	F^- *araD139* Δ(*ara leu*)*7697 lacX74 galU galK mcrA* Δ*(mrr-hsdRMS-mcrBC) rpsL deoR* ϕ80*dlacZ*Δ*M15 endA1 nupG recA1*	Y. Kitamura, 16

*sbcA118::*Tn*5*, and *sbcA83::*IS*50*) (40); and chromosomal mutations of unknown structure, *sbcA6* (3a) and *sbcA20* (14). The plasmid rearrangements leading to functional expression of *recET* include the following: pRAC3 (6, 40), pRAC7 (6, 40), and pJC980 (7).

In the present work, we found that type III restriction is alleviated by homologous recombination function of a lambdoid bacteriophage. Interestingly, the alleviation was observed under the single infection condition in a simple plaque assay.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. All the bacterial strains used here are *E. coli* K-12 derivatives and are listed in Table 1. The bacteriophage lambda *vir* was used (laboratory collection) for an assay of restriction activity. Phages were prepared by a standard plate method. *E. coli* strains, JC8679 and DH10B, were employed as hosts for preparation of EcoKI-modified and EcoKI-unmodified lambda phage, respectively. Phages are EcoKI-modified unless otherwise stated.

Plasmids used in this work are listed in Table 2. Figure 1 shows a relevant region of Rac prophage and its parts placed on plasmids. Plasmids were constructed by standard molecular biology techniques (62). Restriction-negative constructs of EcoP1 and EcoP15 (pNH224 and pNH225) were made by disrupting a HindIII site in the restriction gene of either pNR201 or pNR301 with T4 DNA polymerase after partial digestion with HindIII. A unique AgeI site in the *lar* gene of pRAC3 was converted to BglII site using a BglII linker (5′-pC-C-G-G-G-T-A-A-G-A-T-C-T-T-A-C). Then, a BamHI fragment (~1 kb) containing kanamycin resistant gene of pUC4K was inserted into the BglII site of this plasmid (pNH270), and the resulting plasmid was named pNH271. To construct pNH263, AgeI- and HpaI-digested pRAC3 was self-ligated after blunting of the end.

Our sequence analysis of the junction between the *recT* gene and their vector (pBR322) in pJC980 revealed that the product of its *recT* gene (*recT950*) has lost the C-terminal four amino acid residues and obtained 15 residues from the vector sequence. Similarly, the 169th proline residue of the *recT* gene product was connected with an additional arginine residue (*recT951*) in pJC1501. The 55th methionine residue of the *recT* gene product became the initiating methionine of the beta-lactamase (*recT959*) in pJC1509.

Assay of restriction activity. Bacterial strains harboring (or not harboring) plasmids were grown to the stationary phase in L broth with appropriate antibiotic selection. Then, the culture was diluted to 1/100 to 1/50 and grown in tryptone broth (1.0% Bacto-tryptone and 0.5% NaCl), supplemented with 0.2% maltose, 10 mM $MgSO_4$, and 10 μg/ml vitamin B_1, to log phase. The culture (~2×10^8 cells/ml) was used as a host for measuring the plaque formation efficiency of unmodified lambda *vir* phage at a multiplicity of infection of less

TABLE 2. Plasmids

Name	Origin	Genes	Drug resistance	Comment	Source, reference(s)
pNR201	pACYC184	EcoP1I r^+ m^+	Cml		T. A. Bickle, 25
pNH224	pACYC184	EcoP1I r^- m^+	Cml	ΔHindIII site of R in pNR201	This work
pNR301	pACYC184	EcoP15I r^+ m^+	Cml		T. A. Bickle, 25
pNH225	pACYC184	EcoP15I r^- m^+	Cml	ΔHindIII site of R in pNR301	This work
pBR322	pBR322		Amp		Laboratory collection
pUC18	pBR322		Amp		Laboratory collection
pUC4K	pBR322		Amp, Kan		Laboratory collection, 73
pIK187	pBR322	*lar*$^+$	Amp	PstI fragment of pRAC3 ligated with pUC18	Gift from K. Kusano
pRAC3	pBR322	*recE*$^+$ *T*$^+$ *lar*$^+$	Amp		76
pJC980	pBR322	*recE*$^+$ *T*$^{+/-}$ *lar*	Amp		A. J. Clark, 7
pJC1501	pBR322	*recE*$^+$ *T*$^-$ *lar*	Amp		A. J. Clark, 7, 35
pJC1509	pBR322	*recE*$^+$ *T*$^-$ *lar*	Amp		A. J. Clark, 7, 35
pNH263	pBR322	*recE*$^+$ *T*$^+$ *lar*	Amp	ΔHpaI-SphI fragment of pRAC3	This work
pNH271	pBR322	*recE*$^+$ *T*$^+$ *lar*	Amp, Kan	ΔAgeI site in *lar* of pRAC3 and ligated with Kan^r fragment of pUC4K	This work
pIK172	pSC101ts	*Eco*RI r^+ m^+	Amp, Cml		46
pIK173	pSC101ts	*Eco*RI r^- m^+	Amp, Cml		35

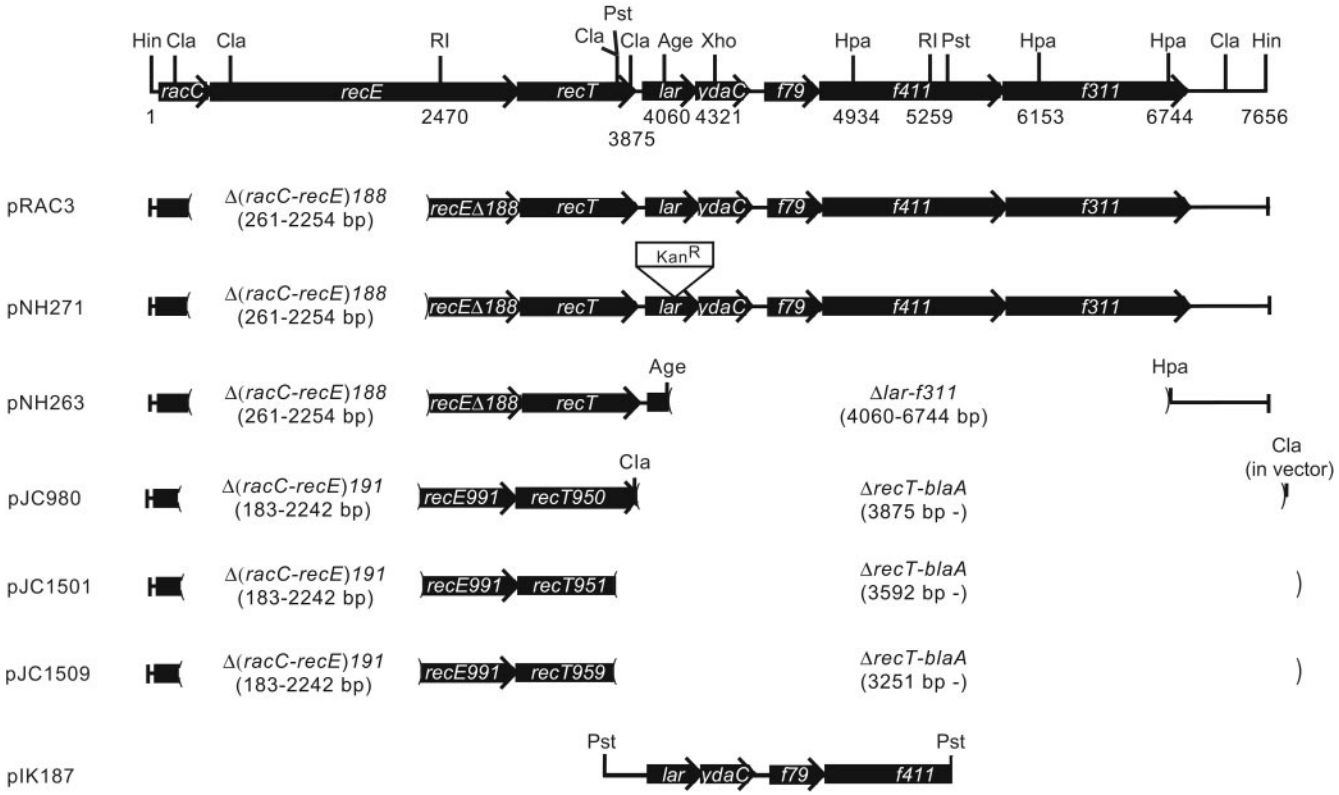

FIG. 1. Gene organization of *recET-lar* region of Rac prophage. pRAC3 carries a deletion that connects the N terminus of *racC* with the C terminus of the *recE* gene. This fused gene encodes a functional RecE (6, 7, 76). The kanamycin resistance gene is inserted into the *lar* gene in pNH271. The C terminus of *lar* is deleted in pNH263. The C-terminal region of pJC980 derivatives is deleted by ClaI digestion, and connected with the ClaI site of the vector. Hin: HindIII; Cla: ClaI; RI: EcoRI; Pst: PstI; Age: AgeI; Xho: XhoI; Hpa: HpaI.

than 0.1 with serial dilutions. After ten minutes incubation at room temperature, the infected culture was mixed with top agar and poured onto tryptone plates (1.0% Bacto-tryptone, 0.5% NaCl and 1% agar) at 37°C for overnight. The plating efficiency of lambda *vir* was calculated as the ratio of the titer on the strain being measured to the titer on a restriction-negative strain as indicated in the figure legends. The results of duplicate measurements in two independent experiments are presented.

RESULTS

Type II restriction in a plaque assay is not influenced by the presence of the *recE* or *recT* gene. Restriction enzyme expressed in a bacteria cell protects the host against bacteriophages with unmodified genome. Homologous recombination is initiated by DNA cleavage and causes its repair. In order to investigate the influence of recombination on restriction, restriction activities were analyzed in two recombination-proficient *E. coli* strains in which the major RecBCD pathway (AB1157) or DSB repair type of recombination (JC8679) is activated. JC8679 is *recBC* but *sbcA* and is therefore functional for RecET. AB1157 is $recA^+$ $recBCD^+$ and functional for host RecBCD. With the EcoRI RM system classified as type II, there was no difference between these two strains in the restriction efficiency to unmodified lambda phage in the plaque formation assay (Fig. 2). This was also the case with the PaeR7I RM system, another type II system (data not shown). Therefore, we concluded that type II restriction in the plaque assay is not influenced by the presence of *recE* and the *recT* genes, which were shown to contribute to repair of restriction breaks on plasmid in a transformation assay (37). Presumably, under the single-infection conditions, a broken phage genome cannot find a homologue with which to recombine.

Type III restriction is alleviated by the presence of Rac prophage or its part. However, when we investigated EcoP1 and EcoP15, both classified as a type III RM system, in the same plaque formation assay, we found that type III restriction activity in JC8679 strain was dramatically reduced compared with two isogenic strains, AB1157 and JC5519 (Fig. 3). In the *E. coli* rec^+ strain AB1157, the strongest restriction was ob-

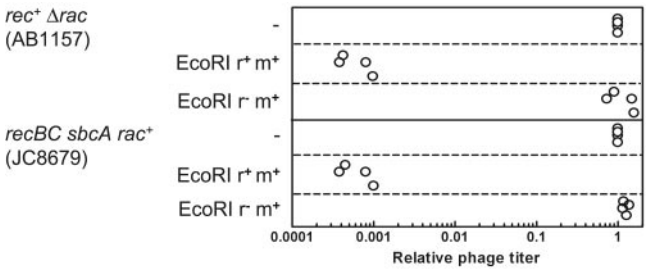

FIG. 2. Type II restriction activities in the presence and absence of Rac prophage. The bacterial strains carry no plasmid, an EcoRI plasmid (pIK172) or its restriction-negative version (pIK173). The titer of EcoRI-unmodified lambda on each strain relative to that on the strain without any plasmid is shown.

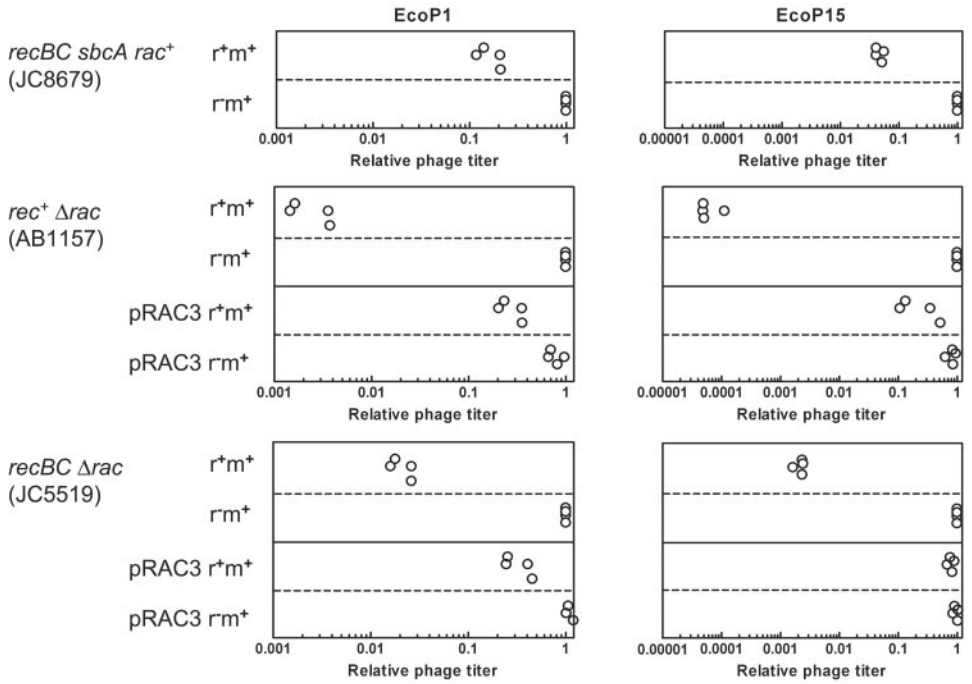

FIG. 3. Type III restriction activities in bacterial strains with different recombination genotypes. The relative titer of EcoP1 and EcoP15-unmodified lambda on the strain is shown. The left graphs are for *E. coli* carrying the EcoP1 plasmid (pNR201) or its restriction-negative version (pNH224). The right graphs are for *E. coli* carrying the EcoP15 plasmid (pNR301) or its restriction-negative version (pNH225). pRAC3, which carries the *recET-lar* part of Rac (Fig. 1), is present in some of the strains. The values were normalized by the titer on AB1157 carrying pNH224. The recombination or alleviation function coded by the plasmid is indicated in Fig. 1.

served—the plaque-forming efficiency in the presence of either EcoP1 or EcoP15 restriction was 3.0×10^{-3} and 6.0×10^{-5}, respectively. When strain JC5519 (*recBC*, defective in the major recombination pathway) was used, the restriction was slightly weaker (1.5×10^{-2} for EcoP1 and 2.3×10^{-4} for EcoP15). On the other hand, EcoP1 and EcoP15 restriction was dramatically alleviated, around 50- and 700-fold, respectively, when strain JC8679 was used.

AB1157 and JC5519 lack Rac prophage, while JC8679 carries Rac prophage, some genes of which are activated by an *sbcA* mutation (Fig. 1). When a plasmid carrying a part of Rac prophage (pRAC3) was introduced into AB1157 and JC5519, alleviation to the level of JC8679 or more was observed (Fig. 3). The region carried by this plasmid includes *lar*, which is responsible for type I restriction alleviation (28), and functional *recE* and *recT*, which are responsible for homologous recombination (RecE pathway) (76).

Though it is established that strain JC8679 is proficient in the double-strand break repair of type II restriction breaks (68), there might be no DNA stretch that could be recombined with the broken phage chromosome in the plaque formation assay involving infection of a cell by a single phage particle. We will come back to this issue in the Discussion. It is noteworthy that host recombination enzyme (RecBCD enzyme) seems to enhance type III restriction in contrast to the bacteriophage-encoded RecET recombinational pathway, which reduces restriction.

When an RM system is transferred into a new bacterial host, restriction activity is often found decreased by a mutation in the restriction gene. The weaker restriction activity will make possible symbiosis of the RM system and its host bacterial line. We examined whether type III restriction alleviation we observed is due to such mutations in the cells. The EcoP1 and EcoP15 plasmids (pNR201 and pNR301, respectively) were recovered from JC8679 and AB1157 carrying pRAC3 and investigated for restriction activity in a new host cell. These plasmids showed the same level of restriction activities (data not shown). Therefore, type III restriction alleviation by Rac prophage is not due to mutational inactivation of the restriction gene in the majority of the cells.

Type III restriction alleviation does not need or depend on *lar* on Rac unlike EcoKI (type I) restriction alleviation. Lar, encoded by Rac prophage, is known to mediate alleviation of EcoKI (type I) restriction (64, 72). We examined whether type III restriction alleviation depends on *lar* or not.

We first confirmed EcoKI restriction alleviation in our constructs (Fig. 4A). The plasmid pIK187, in which *lar* gene was connected to *lac* promoter (Fig. 1), showed strong EcoKI alleviation as in the previous work (28). This *lar* plasmid, however, did not show type III restriction alleviation at all (Fig. 4B). Therefore, *lar* is not sufficient for type III alleviation. Absence of the *lar* gene from a plasmid capable of type III restriction alleviation did not affect restriction alleviation (Fig. 5A). There was no influence when *lar* was knocked out by insertion (Fig. 1 and Fig. 5A). Therefore, *lar* is not necessary for type III restriction alleviation.

Type I restriction is affected by the episome on which it resides (61). While we inserted type III restriction-modification gene complex to pACYC184 replication unit in the above

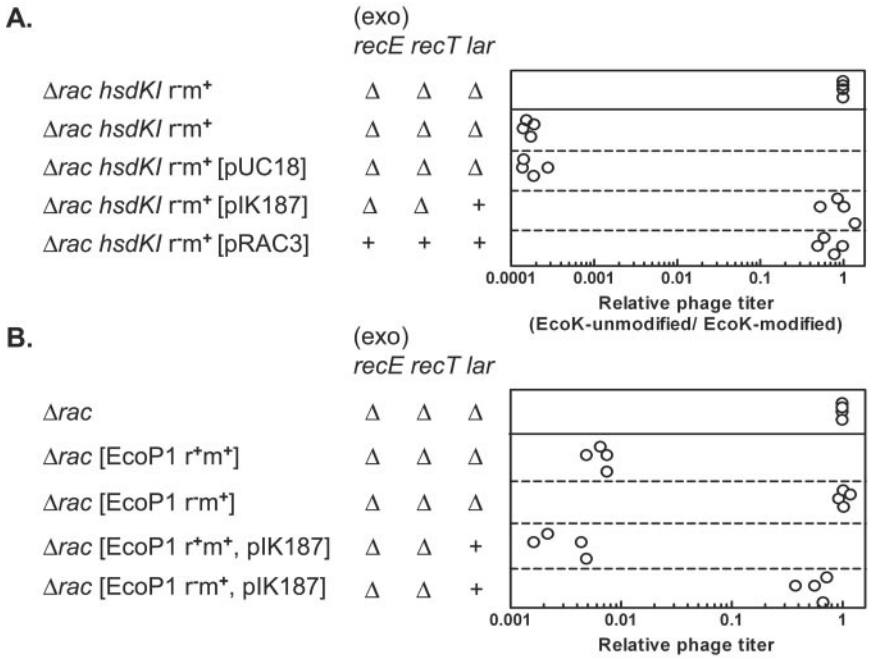

FIG. 4. Effects of *lar* on type I and type III restriction. A. Type I (EcoKI) restriction alleviation by Lar, encoded by Rac prophage. These values were normalized to the titer of DH5 in which EcoKI restriction is defective. All of the remaining strains are derived from AB1157. B. Type III restriction is not affected by Lar. Restriction activities were measured in strains harboring the EcoP1 plasmid (pNR201) or its restriction-negative version (pNH224) in the presence or absence of *lar*-expressing plasmid (pIK187). All of the strains are derived from AB1157. The ratio of the number of lambda plaques to that of the strain without any plasmid is shown.

studies, we observed comparable type III restriction alleviation with pBR322 derivatives (data not shown).

A combination of *recE* and either *recT* or *recA* is necessary for type III restriction alleviation. The *recE* and the *recT* gene products of Rac prophage (7, 17) together promote DSB repair type of homologous recombination that can repair type II restriction breaks (37). The *recE* gene product, called Exonuclease VIII, degrades double-strand DNA and leaves 3′ overhang. The *recT* gene encodes a protein that anneals complementary single-stranded DNAs (17, 18).

Possible contribution of these recombination functions to type III restriction alleviation was examined. Examination of a truncated *recT* (pJC1501 and pJC1509, Fig. 1) demonstrated that the *recT* gene product is not required in a *recA*$^+$ background (Fig. 5A) but is required in an isogenic *recA*-negative background (Fig. 5B). Thus, RecT function seems exchangeable with RecA function in the phenomenon of type III restriction alleviation. We also observed that a truncated *recT* gene, of which 4 amino acids in C-terminal region are deleted, in pJC980, did not cause type III restriction alleviation in the *recA* background (Fig. 5B), although this plasmid was once treated as *recET*$^+$ (7). The pJC980 restored the UV resistance to *recBC* mutant cells. Nevertheless its DSB repair capacity was not comparable with that of pRAC3 (N. Handa, K. Kusano, and I. Kobayashi, unpublished observation).

The gene requirement for type III restriction alleviation was also examined with bacterial mutants (Fig. 6). A *recE* mutation decreases restriction alleviation. A combination of a *recA* mutation and a *recT* mutation decreases restriction alleviation more than a *recA* mutation alone or a *recT* mutation alone does. Therefore, we concluded that RecE and either RecT or RecA are required for type III restriction alleviation.

Type I restriction is partially alleviated by *recE* and/or the *recT* gene. It is known that type I restriction is alleviated in *sbcA* cells (28). The phenomenon depends on the product of *lar* gene placed downstream of the *recT* gene on Rac prophage as mentioned (Fig. 1). Finally, we investigated whether this Rac recombination pathway causes type I restriction alleviation or not. As shown in Fig. 7, partial alleviation of the restriction was observed by the presence of *recE* and the *recT* genes even in the absence of the *lar* gene product. We concluded that *recE* and *recT* are able to contribute to type I restriction alleviation. Even in the *recA*$^+$ background, little difference between pJC980 and pNH271 was observed. While the *lar* gene is completely deleted in pJC980, an N-terminal portion of the *lar* gene product may be expressed in the pNH271 construct. It is possible that such a truncated Lar has a small effect on type I restriction, for example.

DISCUSSION

Type III restriction is alleviated by bacteriophage-mediated homologous recombination function, but is enhanced by bacterial RecBCD-mediated homologous recombination function. We demonstrated here that Rac prophage or *recET* dramatically decreased EcoP1 and EcoP15 restriction. In a previous work, type III restriction was slightly reduced by the *dam* mutation, although the effect was more significant for EcoKI, a type I restriction enzyme (11). In this work, we demonstrated that type III restriction of EcoP1 and EcoP15 is alleviated by

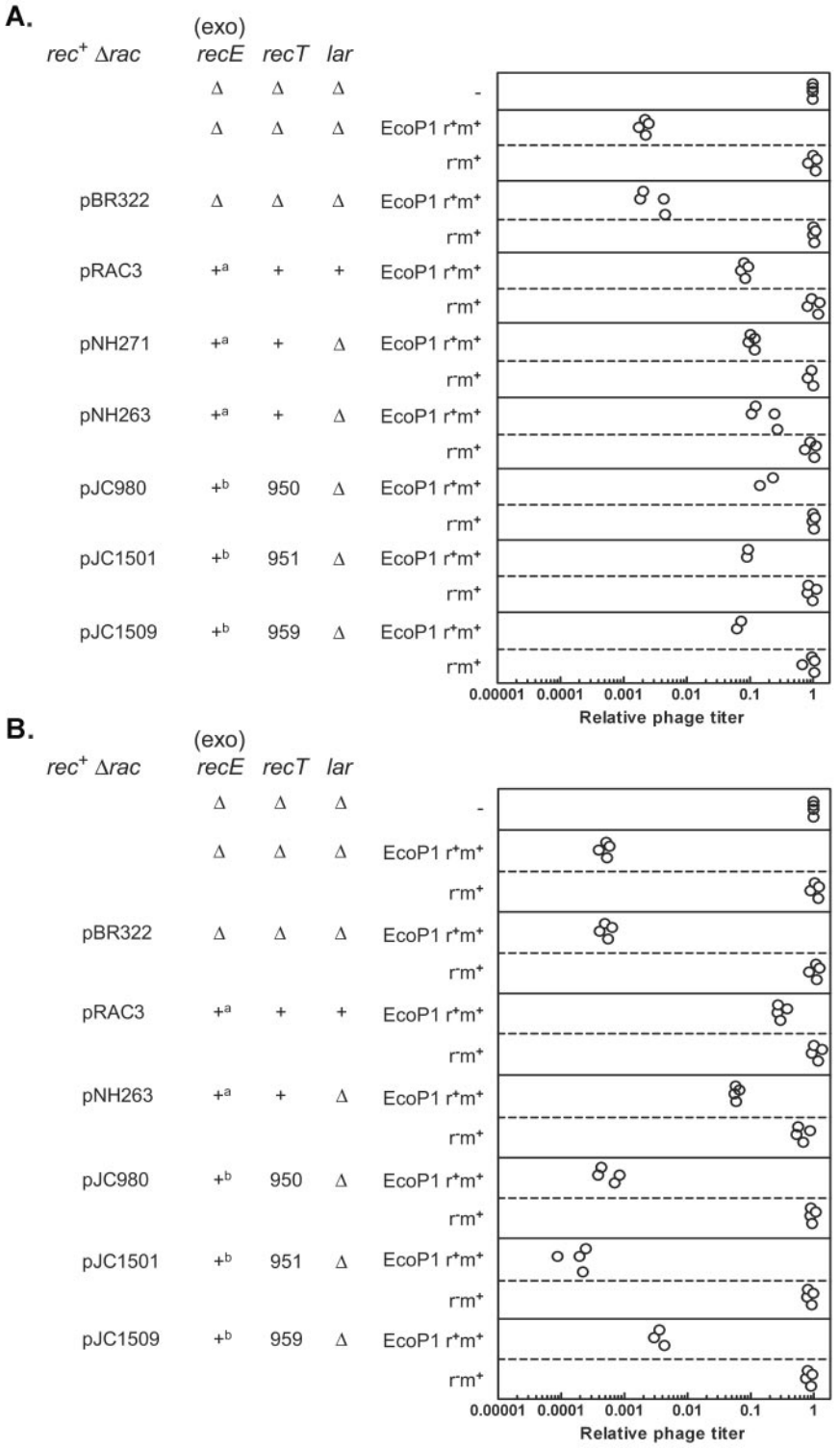

FIG. 5. Gene requirements for type III restriction alleviation. EcoP1 restriction activities were measured in strains that harboring EcoP1 plasmid (pNR201) or its restriction-negative derivative (pNH224) and a part of Rac prophage. The ratio of the number of lambda plaques on the strain to that of the strain without any plasmid is shown. A. *rec*$^+$ background (AB1157). B. *recA* mutant background (BIK733). In the *recE* (exonuclease) columns a and b, Δ(*racC-recE*)188 and Δ(*racC-recE*)191 showed the Exo$^+$ phenotype (Fig. 1) (6, 40, 76). In the *recT* column, 950, 951, and 959 are the allele names in Clark et al. (7) as shown in Fig. 1 (also see Materials and Methods).

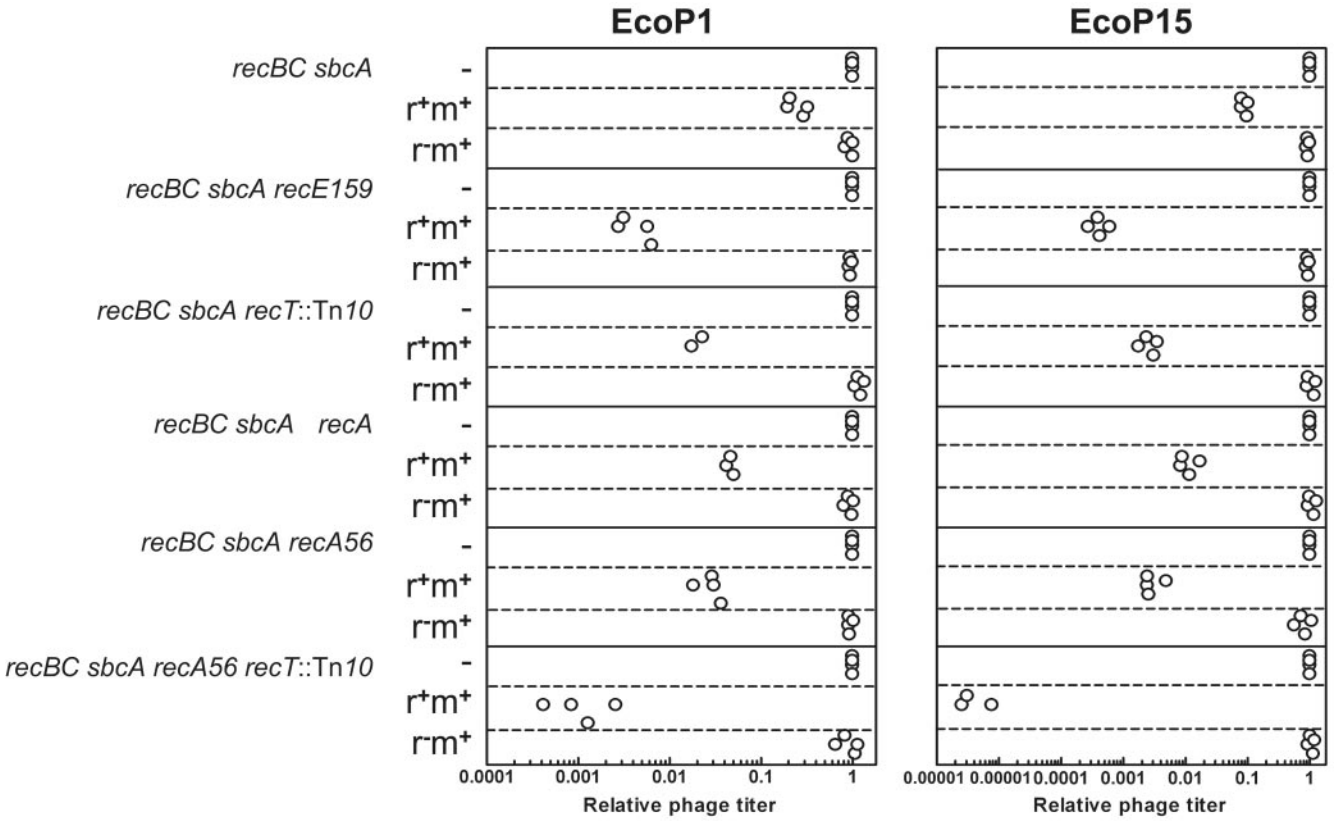

FIG. 6. Gene requirements for type III restriction alleviation. JC8679 and its recombination-negative derivatives were examined. Shown on the left is plaque formation in *E. coli* that carries the EcoP1 plasmid (pNR201) or its restriction-negative derivative (pNH224), while shown on the right is plaque formation in *E. coli* that carries the EcoP15 plasmid (pNR301) or its restriction-negative derivative (pNH225). Plaque formation efficiencies were normalized to that in the strain without a plasmid.

the RecE pathway of homologous recombination encoded by Rac prophage. While type I restriction of EcoKI also seems to be slightly alleviated by the RecE pathway (Fig. 7), the type II restriction enzyme EcoRI was not (Fig. 2). Type III restriction alleviation depends on the *recE* and *recT* genes in a *recA*-negative background, whereas the *recT* gene is not absolutely required in a *recA*$^+$ background. This suggests that the *recT* gene product can be substituted by the *recA* gene product in the alleviation phenomenon. In plasmid assay systems (36, 68), however, pJC1501 or pJC1509 did not restore the DSB repair even in a *recA*$^+$ background showing the requirements for both RecE and RecT for that assay (37).

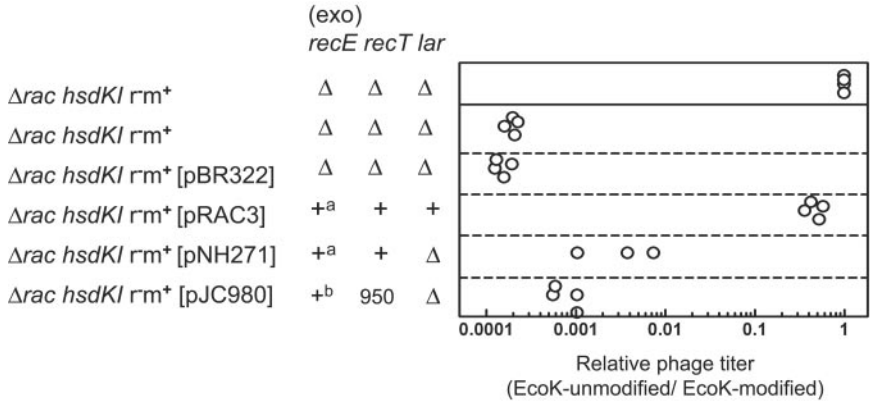

FIG. 7. Type I restriction alleviation by recE and recT recombination function. Restriction by EcoKI, a type I system, was measured in strains that express *recE*, *recT*, or *lar* from a plasmid. These values were normalized to the titer of DH5, in which EcoK restriction is defective. The remaining strains are derived from AB1157. a and b, shown in the *recE* (exonuclease) column, indicate Δ(*racC-recE*)188 and Δ(*racC-recE*)191, respectively. In the *recT* column, 950 is an allele name.

A partial type I restriction alleviation was shown by pJC980, though this plasmid has an extensive deletion after the *recT* gene (Fig. 1). Also, pJC1509, which has a large deletion of the *recT* gene, showed moderate type III restriction alleviation in the absence of RecA function. A similar plasmid, pJC1501, suggested RecT function is necessary under this condition (Fig. 5). The *recT* gene in pJC980 is missing the C-terminal four amino acids and connected with an additional 15 amino acid residues from the vector sequence (7). Whether the above observations have resulted from such small differences in the plasmid construction is unclear.

On the other hand, the presence of the RecBCD pathway enhances type III restriction (Fig. 3). Presumably, the RecBCD enzyme, a double-stranded DNA exonuclease, degrades the phage DNA from the double-stranded DNA break made by type III restriction endonuclease as found for type I restriction (10). Therefore, we conclude that a bacterial recombination pathway and an RM system fight together against invading selfish gene units, such as phage genomes, and maintain the integrity of the bacterial genome. We earlier demonstrated that RecBCD pathway repairs bacterial chromosomes attacked by a type II restriction system (19).

We suppose that this type of interaction among bacteriophages, RM systems, and bacteria may take place frequently under natural conditions because RM genes and prophages are quite abundant in many of the bacterial genomes sequenced. For example, pathogenic *E. coli* strain O157:H7 carries 24 prophages, and each of two closely related *Helicobacter pylori* strains turned out to carry more than 25 RM systems (22, 38, 48; http://rebase.neb.com/rebase/rebase.html).

One simple explanation for RecET-mediated restriction alleviation is recombination repair of restriction breaks by RecET-mediated homologous recombination. However, we should consider other possibilities. For example, RecET could directly inhibit or modulate the restriction/modification proteins. Or it could protect the cut ends against cellular exonucleases.

The *red*α and *red*β gene products of bacteriophage lambda are similar to the *recE* and the *recT* gene products, respectively, in the reaction mechanism. Homologous recombination through the DSB repair mechanism they promote can repair a type II restriction break when an intact homologous DNA is present within the same cell (66). We do know yet whether the Red recombination pathway also causes type III restriction alleviation. In all the restriction assays in the present work, we used strain lambda *vir*, which should express red^+ and gam^+, whose product is an inhibitor of RecBCD enzyme (61). When we used lambda *red gam* mutants instead, restriction by EcoP1 or EcoP15 was stronger than with lambda *vir*. The restriction was slightly alleviated by *recBC* mutations (data not shown).

Type III restriction and DNA replication. In the present study, restriction activity was measured as efficiency of plaque formation of an unmodified phage. In this assay, less than one phage particle will infect a host bacterial cell so that only a single copy of the bacteriophage genome will be available in each cell. A restriction break on the single genome cannot be repaired by homologous recombination with an intact homologous DNA. Therefore, lack of alleviating effect of the bacteriophage recombination function on type II restriction was easily expected, while its alleviating effect on type III restriction was rather unexpected.

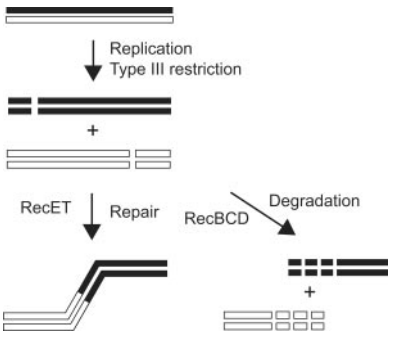

FIG. 8. Model for type III restriction alleviation. Infected lambda phage genome will be replicated prior to the type III restriction break. Restriction cleavage initiates homologous recombination with the sister chromosome, or the broken chromosome will be degraded by endogenous exonuclease (RecBCD enzyme).

Figure 8 illustrates a simple model that addresses this issue. The action of type III restriction enzyme is hypothesized to take place after DNA replication or somehow coupled to DNA replication. This leads to two (or more) genome copies each with a break (or breaks) at its unique loci. The homologous recombination between them reconstitutes an intact genome copy. On the other hand, the RecBCD enzyme, exonuclease V, degrades double-stranded DNA from the breaks.

Type II restriction enzymes cleave DNA at, or near, its recognition sequence. However, type I and type III restriction requires at least two recognition sites to cleave DNA. Type I and type III restriction enzymes possess all 7 helicase motifs in R subunit (9, 15, 41, 60). They bind to their recognition sequence and pull the DNA with motor activity powered by ATP (43, 63). A type III restriction enzyme cleavages DNA near its recognition site when it encounters with another enzyme molecule (43), while a type I restriction enzyme cleaves DNA at the site of such an encounter. Therefore DNA replication of bacteriophage could take place before a type III (or type I) restriction enzyme complex meets another enzyme on the single infecting DNA to cleave DNA. Lambda genome carries 49 EcoP1 sites and 72 EcoP15 sites. It would not be surprising if the resulting two daughter copies of the phage genome carry breaks at different loci. Homologous recombination would be able to reconstitute one intact copy from them. This is just one of many possible mechanisms for the relation between DNA replication and type III (type I) restriction, which should be tested in future experiments.

The reason why efficiency of alleviation was so different between type III and type I restriction is not understood. The requirement of two inverse target sites and effective DSB repair type of homologous recombination by RecET system could be a key to solve the question. The difference could be based on the expression level of type I and type II enzymes, target frequency, translocation mechanism, cleavage reaction, influence of proteolysis and so on. Possibly, the difference of linear diffusion of the enzyme complexes might affect their restriction and/or alleviation efficiency (27). Further investigation would be necessary to explain this question.

Type III restriction cannot cleave DNA during replication once it is fully methylated (42). However, methylation of infecting lambda DNA may be delayed relative to replication as in *E. coli* chromosome replication (4a). After at least one round of phage DNA replication, a homologous stretch of DNA is available, and DSB caused by restriction endonuclease may result in recombination-dependent replication (1, 32). In fact, recombination-dependent replication may help propagation of lambda phage (23, 65). This recombination-dependent replication as well as origin-dependent replication would lead to further restriction and recombination repair.

Among the 49 EcoP1 sites on lambda genome, 32 lie rightward and 17 lie leftward. Among 72 EcoP15 sites, 31 lie rightward and 41 lie leftward. We have not noticed any bias in their distribution with respect to the origin of DNA replication.

Double-strand break repair as adaptation to restriction by bacteriophages. The mechanism of homologous recombination by lambdoid bacteriophages appear well suited to fights against restriction (31, 69). As proposed in the DSB repair models, a restriction break is repaired through copying of a homologous DNA. If the template DNA lacks the restriction site, the recombination may result in a DNA region devoid of the particular restriction site and resistant to the restriction. This repair is often accompanied by crossing-over of the flanking sequences. Moreover, outcrossing involving crossing-over and gene conversion may take place between an incoming phage and a chromosomal prophage as well as between coinfecting phages and would generate various combinations in term of restriction sites, which can be regarded as deleterious mutations. Some of them would be more resistant to attack by the present RM systems than the others and would increase in number. Because a bacteriophage population encounters bacterial populations possessing various combinations of RM systems of diverse specificities, the repair process from restriction breakage and gene conversion with crossing-over must have an advantage over proliferation.

This hypothesis assumed the presence of a homologous copy of the infecting phage genome, either as a coinfecting phage or a prophage. The efficiency of homologous recombination frequency is reduced by even slight sequence divergence (12). How often a bacteriophage genome encounters with a sufficiently homologous DNA remains an open question. The present results, however, demonstrate that, even with single infection of a bacterial cell, bacteriophage-mediated homologous recombination can fight against type III and type I restriction. This further supports the concept that the DSB repair type of homologous recombination is well adapted to bacteriophage survival in the challenge of restriction (29, 54, 66).

Difference between plasmid double-strand break-repair and type III restriction alleviation. Although RecT does not share its amino acid sequence with RecA, RecT promotes renaturation of homologous single-stranded DNA in vitro, as RecA protein does (17, 44, 50, 51). Previously, we reported that RecE-mediated DSB repair depends on the *recE* and the *recT* genes even in *recA*$^+$ background (37). Though RecT was not exchangeable with RecA in such a plasmid assay, RecT could be substituted by RecA in type III restriction alleviation as presented here. It was also demonstrated that *recA* gene is necessary for the repair of spontaneous chromosomal double-strand breaks in *recBC sbcA* cells as well as the *recT* gene is (20). These observations may suggest that the combination of RecE and RecA or of RecE and RecT functions in different ways in the process. In the absence of replication, for example, the production of viable phage particles requires Red and RecA function (54). In other words, the different genetic requirements might reflect different mechanisms of repair for type II and type III restriction.

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