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Gamma-irradiated RecD overproducers become permanent *recB*$^{-}$/*C*$^{-}$ phenocopies for extrachromosomal DNA processing due to prolonged titration of RecBCD enzyme on damaged *Escherichia coli* chromosome

Damir Đermić [a,*], Edyta Đermić [b], Davor Zahradka [a], Mirjana Petranović [a], Nella Lerš [a]

[a] *Department of Molecular Biology, Ruđer Bošković Institute, Bijenička 54, P.O. Box 180, 10002 Zagreb, Croatia*
[b] *Department of Plant Pathology, Faculty of Agriculture, Svetošimunska 25, 10000 Zagreb, Croatia*

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Abstract

The RecBCD enzyme of *Escherichia coli* consists of three subunits RecB, RecC and RecD. RecBCD enzyme activities are regulated by its interaction with recombination hotspot Chi. Biochemical and genetic evidence suggest that interaction with Chi affects RecD subunit, and that RecD polypeptide overproduction antagonizes this interaction, suggesting that intact RecD replaces a Chi-modified one. We used bacteria with fragmented chromosomes due to double-strand breaks inflicted by UV and γ-irradiation to explore in which way increased concentrations of RecBCD's individual subunits affect DNA metabolism. We confirmed that RecD overproduction alters RecBCD-dependent DNA repair and degradation in *E. coli*. Also, we found that RecB and RecC overproduction did not affect these processes. To determine the basis for the effects of RecD polypeptide overproduction, we monitored activities of RecBCD enzyme on γ-damaged chromosomal DNA and, in parallel, on λ and T4 *2* phage DNA duplexes provided at intervals. We found that γ-irradiated wild-type bacteria became transient, and RecD overproducers permanent *recB*$^{-}$/*C*$^{-}$ phenocopies for processing phage DNA that is provided in parallel. Since this inability of irradiated bacteria to process extrachromosomal DNA substrates coincided in both cases with ongoing degradation of chromosomal DNA, which lasted much longer in RecD overproducers, we were led to conclude that the RecB^{-}/C^{-} phenotype is acquired as a consequence of RecBCD enzyme titration on damaged chromosomal DNA. This conclusion was corroborated by our observation that no inhibition of RecBCD activity occurs in γ-irradiated RecBCD overproducers. Together, these results strongly indicate that RecD overproduction prevents dissociation of RecBCD enzyme from DNA substrate and thus increases its processivity.

Keywords: Double-strand break repair; λ *red gam* phage recombination; T4 *2* phage plating; RecBCD enzyme processivity

1. Introduction

A vast majority of homologous recombination and double-strand break (DSB) repair in wild-type *Escherichia coli* is initiated by RecBCD enzyme (for reviews, see [1,2]). That is why *recB* and *recC* null mutants, deprived of all RecBCD enzyme activities, are extremely sensitive to DNA damaging agents (such as UV and gamma (γ)-irradiation) [3], inefficiently recombine DNA molecules [4], and are poorly viable [5]. RecBCD enzyme is a powerful heterotrimeric helicase/nuclease (ExoV), which processes DNA duplexes containing blunt or nearly blunt dsDNA end(s). Upon binding to a dsDNA end, the enzyme unwinds the DNA molecule driven by helicase subunits RecB and RecD [6,7]. The unwinding is accompanied with vigorous degradation of a 3′-ended strand at the entry site, and with weaker degradation of a 5′-ending strand [8].

RecBCD enzyme activities are regulated by its interaction with a specific, properly oriented, 8-nt DNA sequence named Chi. When RecBCD encounters a Chi sequence, it pauses tem-

Abbreviations: DSB, double-strand break; EOP, efficiency of plating; γ, gamma rays; kbp, kilo base pair; MOI, multiplicity of infection; UV, ultraviolet light.

* Corresponding author. Tel.: +385 98 169 5370; fax: +385 1 4561 177.
E-mail address: dermic@irb.hr (D. Đermić).

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porarily and then continues moving along the DNA, although with reduced rate [9] and exhibiting weaker nuclease activity of switched polarity (from predominant 3′–5′ to 5′–3′) [10]. This change in extent and polarity of RecBCD nuclease activity enables a Chi-modified enzyme to produce a 3′-terminating single strand overhang, onto which it starts loading RecA protein, thereby creating a nucleoprotein filament [11]. As a consequence, most of the recombinational exchanges in wild-type *E. coli* are focused at Chi [12], which is therefore called a recombination hot spot. Genetic and biochemical evidence [9,13–16] have suggested that Chi imposes its influence on RecBCD enzyme via the RecD subunit, which becomes modified upon interaction with Chi. This modification is probably not ejection (since in vitro studies failed to detect RecD subunit detaching from Chi-modified enzyme [17,18]) but rather inactivation. This conclusion is based on remarkable similarities in behavior of RecBC enzyme, which is devoid of the RecD subunit, and Chi-modified RecBC(D). RecBC does not recognize Chi (resembling Chi-modified enzyme, which is unable to recognize another Chi site provided *in cis* or *in trans* [19]), but catalyzes homologous recombination with efficiency similar to that of Chi-modified RecBC(D) [20]. Recombinational exchanges in *recD* mutants are focused at dsDNA ends instead at a Chi site [21], apparently due to constitutive loading of RecA protein by RecBC onto the 3′-terminating overhang that it produces [22]. RecBC enzyme also lacks detectable nuclease activity and unwinds DNA with reduced speed [23] and processivity [24], compared to RecBCD. Furthermore, when in wild-type *E. coli* RecBCD enzyme is saturated with Chi sites (provided on plasmid or on fragmented chromosomal DNA), these cells become *recD*$^-$ phenocopies, i.e. the Chi-modified enzyme looses its nuclease activity (enabling an efficient T4 *2* mutant phage plating) and ability to interact with another Chi site while catalyzing recombination exchanges focused at dsDNA ends in λ crosses [15,16]. Some of these phenotypic changes can be antagonized by a RecD polypeptide overexpression. An excess of RecD polypeptide reverses the Chi effect, causing recombination exchanges in λ crosses not to be focused at dsDNA ends any more [15]. These results suggest that an intact RecD polypeptide in excess can replace a Chi-modified RecD subunit by mass action. The importance of RecD inactivation upon RecBCD interaction with Chi, and subsequent reversal of that reaction by RecD polypeptide overproduction is outlined by distinct phenotype of RecD overproducing bacteria. RecD overproducers have impaired DSB repair, increased DNA degradation [25], lower viability [26] and reduced recombination efficiency [15]. The RecBCD enzyme activities are apparently deregulated in RecD overproducers (resulting in "reckless" degradation of γ-irradiated chromosomes [25]), suggesting that overexpression of RecD antagonizes the Chi effect by mass action and thus changes the behavior of Chi-modified RecBCD, which affects cellular physiology. A crystal structure of RecBCD-DNA complex revealed a structural basis for exchange of RecD subunits in Chi-modified enzyme since tight contacts between RecB and RecC subunits were observed, while RecD was much more loosely connected to RecC subunit only [27]. However, the means by which an excess of RecD polypeptide imposes its effect on *E. coli* cell is still unclear.

The aim of this study was to elucidate the mechanism by which an excess of RecD polypeptide affects RecBCD enzyme behavior in *E. coli*. Furthermore, since increase in concentration of RecBCD and RecBC enzymes and also of RecD polypeptide in *E. coli* results in deterioration of DNA repair and homologous recombination [28,15,25], we wanted to examine the effect of increased concentrations of RecB or RecC polypeptide on some DNA metabolic processes, namely DNA repair and degradation.

2. Materials and methods

2.1. Bacterial strains, plasmids and phages

We used AB1157, a standard recombination- and repair-proficient strain (F$^-$ *thr-1 ara-14 leuB6 Δ[gpt-proA]62 lacY1 tsx-33 supE44 galK2* λ$^-$ Rac$^-$ *hisG4* [Oc] *rfbD1 mgl-5 rpsL31 kdgK51 xyl-5 mtl-1 argE3* [Oc] *thi-1 qsr*$^-$ [29]), and its *recB268*::Tn*10* derivative DE101 [30]. They were transformed as previously described [31] with the following plasmids: pKI13 is a pUC18 *recD*$^+$ derivative, which increases the cellular concentration of RecD polypeptide about 60-fold [25]; pDW11 (kindly provided by Dr. Wilfried Wackernagel) and pDWS2 are pBR322 derivatives that carry *recC*$^+$ and *recBCD*$^+$ alleles, respectively [32]. The expression of *recB*, *recC* and *recD* genes in these plasmids is controlled by their natural (noninducible) promoters, meaning that the amount of RecC or RecBCD proteins the plasmids produce depends entirely on their copy-number and is therefore expected to be elevated about 25-fold [32]. The gene expression from these plasmids was confirmed by complementation tests: pDW11 rendered *recC* mutants resistant to UV radiation, capable of degrading T4 *2* phage DNA and fully viable, while no effect was observed on *recB* mutants (not shown). Plasmid pDWS2 reduced the efficiency of T4 *2* phage plating on both *recC* and *recB* mutants to the level of wild-type, but only partially restored their resistance to UV irradiation, which is typical for bacteria with increased concentration of RecBCD enzyme [28]. Plasmid pPB700 contains *recB*$^+$ gene under control of *Ptac* inducible promoter [33]. Wild-type cells harboring pPB700 plasmid plated λ *red* mutant phage with reduced efficiency when induced by IPTG (not shown). This effect is due to titration of λ Gam protein by an overexpressed RecB polypeptide [30].

Phage λ crosses were made with *red gam* mutant phages MMS555 (*Jam6 b1453 c*I*857* χ) and MMS754 (*b1453 c*I*857* χ*D Rts129*), kindly provided by Dr. Richard S. Myers. A gene *2* amber mutant of phage T4 was from our laboratory collection.

2.2. Media and growth conditions

Bacteria were grown in LB broth and on LB plates [34] at 37 or 34 °C. The strains containing plasmids were grown in

broth and on plates supplemented with 100 μg ml^{-1} ampicillin. The *recB*$^+$ gene expression was induced in bacteria carrying pPB700 by growing them overnight in medium containing 0.5 mM IPTG. For λ crosses cells were grown in tryptone broth with 0.3% maltose [35] at 34 °C.

2.3. Irradiations

Both UV and γ-irradiations were done as described [28]. For UV irradiation, fresh overnight cultures were serially diluted in 67 mM phosphate buffer (pH 7.0) and aliquots spread onto LB plates supplemented with ampicillin, when required. The plates were immediately irradiated with various doses of UV light at the dose rate of 3.3 J m^{-2} s^{-1}, and then incubated in the dark for 24 hours, at 37 °C. The source of UV light (254 nm) was a low pressure Hg germicidal lamp.

Fresh overnight cultures were exposed to various doses of γ-rays from a ^{60}Co source, with a dose rate of 10.3 Gy s^{-1}, at 0 °C. The irradiated cells were serially diluted in 67 mM phosphate buffer (pH 7.0) and samples plated on LB plates containing ampicillin, when necessary. Colonies of survivors were scored after 24 h of incubation at 37 °C.

2.4. Chromosomal DNA degradation

The cells were grown overnight in LB medium supplemented with 0.05 MBq [^{14}C]thymidine (specific activity 2. 18 GBq $mmol^{-1}$; Amersham Pharmacia Biotech, UK), 100 μg ml^{-1} deoxyadenosine and 100 μg ml^{-1} ampicillin, when required. Unincorporated [^{14}C]thymidine was washed out and the cultures were divided into two parts. One part served as a control, while the other was γ-irradiated with 400 Gy, at 0 °C. After irradiation, the cultures were incubated at 34 °C, duplicate samples were collected at intervals and assayed for acid-precipitable radioactivity as described [28]. Acid-precipitable radioactivity was measured by scintillation counting (1209 Rackbeta, Pharmacia Wallac) and corresponded to the amount of intact DNA.

2.5. λ Lytic crosses

The crosses were performed according to a described procedure [36], with some modifications. A fresh overnight culture was diluted in tryptone broth with 0.3% maltose to a density of 10^8 cells ml^{-1} (an OD_{600} of 0.25). The culture was divided into two parts. One part was γ-irradiated (with 400 or 600 Gy), at 0 °C, while the other was not irradiated and served as a reference. The irradiated bacteria were incubated at 34 °C with aeration. At intervals, 0.3 ml of this culture was mixed with 0.1 ml of phage mixture. The multiplicity of infection (MOI) was 10 for MMS555 (*Jam*), and 0.2 for MMS754 (*Rts*), to reduce variations in recombination frequencies due to fluctuation in phage populations [36]. The mixture was incubated for 10 min at 34 °C and then 1.6 ml of tryptone, prewarmed to 34 °C, was added. Unadsorbed phages were removed by centrifugation and infected cells resuspended in 1 ml of cross broth (tryptone broth with Nozu supplements [35]) and gently aerated for 90 min at 34 °C. To lyse the remaining cells, 0.2 ml of chloroform was added, the mixture was vortexed and then aerated for 10 min at room temperature. Supernatant, containing the phages, was separated from cell debris by centrifugation. The phage titers were determined on V371 (*recA56 recC1010 srl300*::Tn*10* Su°; provided by Dr. Richard S. Myers) host strain at 34 °C for total Am^+ phage, and at 42 °C for Am^+Ts^+ recombinants. Plaques were counted after 18–24 h of growth on tryptone plates containing Nozu supplements. The frequency of recombinants was calculated as the recombinant titer divided by the total (Am^+) titer. The frequency of λ recombination in irradiated part of each bacterial culture was expressed as a fraction of recombination in unirradiated part of the culture.

2.6. T4 2^- plating

Fresh overnight bacterial cultures were infected with T4 *2* phage at MOI 0.1. Each bacterial culture was divided into two parts, of which one served as a control and the other was irradiated with 400 Gy of γ-rays at 0 °C. Both parts of the culture were then incubated at 34 °C, with aeration. At intervals, 0.1 ml samples were mixed with the phages and incubated for 5 min at 34 °C. The resulting phage-bacteria complexes were serially diluted in 67 mM phosphate buffer (pH 7.0) and then mixed with an indicator strain (AB1157). LB soft agar was added, and the mixtures were plated on LB plates and incubated at 34 °C for 16 h. The efficiency of plating (EOP) of T4 *2* on irradiated bacteria was calculated relative to the EOP on unirradiated part of the culture.

3. Results

3.1. An excess of RecB or RecC polypeptide does not affect DNA repair in wild-type cells

The effect of an increased concentration of RecB, RecC and RecD polypeptides on DNA repair in *E. coli* cells was assessed by measuring their UV and γ-ray survivals. The plasmids pPB700, pDW11 and pKI13 were used to increase concentration of RecB, RecC and RecD polypeptides, respectively, in wild-type strain AB1157. As shown in Fig. 1, γ-ray survival of bacteria with overproduced RecB or RecC polypeptides was unaffected compared to cells with physiological levels of these polypeptides. On the other hand, a RecD protein overproduction sensitized wild-type cells to γ-irradiation (Fig. 1), confirming an earlier report [25]. The RecD overproducers were about twofold more radiosensitive than wild-type when comparing survival slopes. The difference in survival was about 10-fold at the highest dose tested. Moreover, a RecD overproduction did not have any effect on γ-survival of DE101, a *recB* deficient derivative of AB1157 (Fig. 1), suggesting that RecD polypeptide overproduction affects only RecBCD-dependent DNA metabolic processes.

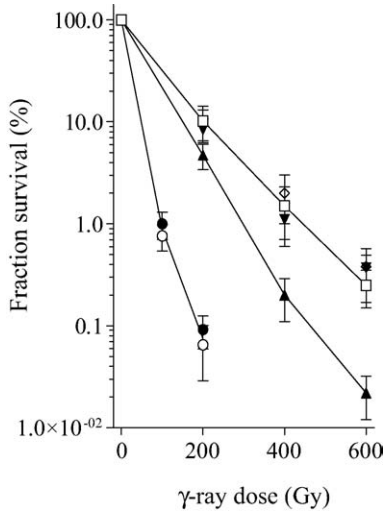

Fig. 1. Survival of γ-irradiated RecB, RecC and RecD overproducers. Fraction survival is given as a fraction of the unirradiated control. Each value is a mean of three independent experiments. Error bars represent standard deviations. Bacteria bearing the *recB*$^+$ plasmid pPB700 or its parental plasmid pBR322 were grown overnight in medium containing 0.5 mM IPTG. Symbols: (□) AB1157 (wild-type) with plasmid pBR322 (for clarity, essentially the same results, obtained with AB1157 harboring plasmid pUC18 or pBR322 + IPTG, are not presented); (◊) AB1157 carrying the *recB*$^+$ plasmid pPB700; (▼) AB1157 harboring the *recC*$^+$ plasmid pDW11; (▲) AB1157 bearing the *recD*$^+$ plasmid pKI13; (○) DE101 (*recB268*::Tn*10* derivative of AB1157) with plasmid pUC18; (●) DE101 containing the *recD*$^+$ plasmid pKI13.

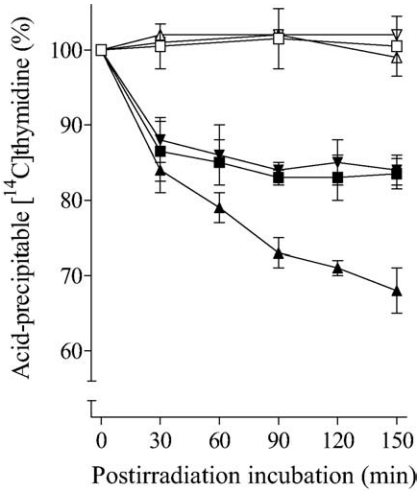

Fig. 2. Time course of [^{14}C]thymidine-labeled chromosomal DNA degradation in γ-irradiated AB1157 cells containing plasmids pBR322 (for clarity, essentially the same results, obtained with AB1157 carrying plasmid pUC18, are not presented) (□, ■); or the *recC*$^+$ plasmid pDW11 (▽, ▼); or the *recD*$^+$ plasmid pKI13 (Δ, ▲). Bacterial cultures were divided into two parts; one part served as a control (open symbols) while the other was irradiated with 400 Gy (closed symbols). Each value is a mean of at least three independent experiments. Error bars represent standard deviations.

The UV survival of bacteria carrying either RecB, RecC or RecD polypeptides in excess was not affected (data not shown).

These data show that RecB or RecC overproduction does not have any effect on DNA repair in otherwise wild-type bacteria, while RecD overproduction impairs repair in γ-irradiated wild-type bacteria but not in *recB* mutants nor in UV irradiated wild-type cells.

3.2. Chromosomal DNA degradation is not affected in bacteria that overproduce RecB or RecC polypeptides

The chromosomes of bacteria irradiated with γ-rays become fragmented due to DSBs inflicted by this agent. DSBs are the entry sites for RecBCD, the strongest exonuclease in *E. coli*, which leads to chromosomal DNA degradation, but also to its repair. To evaluate the effect of an excess of RecBCD's subunits on DNA degradation, we exposed RecB, RecC and RecD overproducing bacteria to γ-rays and followed the breakdown of their radioactive isotope-labeled chromosomes. DNA degradation in γ-irradiated bacteria with the excess of RecC (Fig. 2) or RecB polypeptide (not shown), was equal to that in the wild-type bacteria. After 150 min of postirradiation incubation, they degraded about 15% of their DNA (Fig. 2). On the other hand, bacteria with increased concentration of RecD polypeptide degraded much more of their chromosomal DNA. About 35% of their genome was made acid-soluble after 150 min (Fig. 2). The kinetics of chromosomal DNA degradation was also different in RecD overproducers. Whereas in wild-type cells DNA degradation stopped 30 min postirradiation, RecD overproducers degraded their DNA continuously during 150 min after being irradiated (Fig. 2).

3.3. Recombination in λ lytic crosses is permanently inhibited in γ-irradiated RecD overproducers

Since the excess of RecD polypeptide increases both the extent and duration of DNA degradation (Fig. 2; [25]), it is evident that RecD overproducers process their damaged DNA in a different way than bacteria with physiological level of that polypeptide. In bacteria overproducing RecD polypeptide nuclease activity of RecBCD enzyme is deregulated.

According to the current models of regulation of the RecBCD enzyme activities, the excess of RecD polypeptide reverts (antagonizes) Chi-effect by replacing a Chi-modified RecD subunit by mass action ([15,16,36]; see 1. Introduction). There are two ways by which RecD polypeptide in excess could replace Chi-modified RecD subunit in γ-irradiated *E. coli*, resulting in increased chromosomal DNA degradation. An excess of RecD polypeptide might either: i) refurnish a Chi-modified RecBC(D) enzyme released from its chromosomal DNA substrate (a newly-assembled RecBCD enzyme would then be capable of binding and degrading damaged DNA again), or ii) replace inactivated/lost RecD subunit while a Chi-modified enzyme is still bound to DNA it is repairing. The reconstituted RecBCD complex would then continue degrading chromosomal DNA without being released to cytoplasm. Both hypotheses have testable predictions. They can be differentiated by introducing another DNA substrate in γ-irradiated RecD overproducers and assessing whether RecBCD would be able to process it.

Recombination of λ *red gam* mutant phages in wild-type *E. coli* depends entirely on RecBCD enzyme (reviewed in

Ref. [2]). Therefore, by measuring the efficiency of recombination in λ *red gam* mutant phage crosses in γ-irradiated bacteria one can determine whether RecBCD enzyme resides in cytoplasm and is therefore capable of efficiently processing λ DNA, or it is trapped on chromosomal DNA and, consequently, unable to recombine λ (a $recB^{-}/C^{-}$ phenocopy). Therefore, we determined a time course of recombination activity in λ lytic crosses in γ-irradiated *E. coli*. First, we confirmed a RecBCD dependence of λ *red gam* phage recombination. A *recB* mutant recombined λ phages with low efficiency of about 3% of that of the wild-type. Irradiation of *recB* bacteria with γ-rays did not affect that recombination. It was still about 30-fold lower than λ recombination in unirradiated wild-type, and did not change during incubation (Fig. 3).

When wild-type strain AB1157 received 600 Gy of γ-rays, its λ *red gam* recombination activity declined rapidly, reaching its minimum after 10 min, when it was about fivefold lower than in unirradiated control (the yield of $J^{+}R^{+}$ recombinants fell from 32% to 6%), and came close to the recombination level in *recB* control (a partial $recB^{-}/C^{-}$ phenocopy) (Fig. 3). However, this inhibition of λ recombination was only transient; it recovered quickly, equaling unirradiated control 30 min postirradiation. From then on, γ-irradiated wild-type bacteria exhibited high λ recombination activity (Fig. 3). We may thus infer that

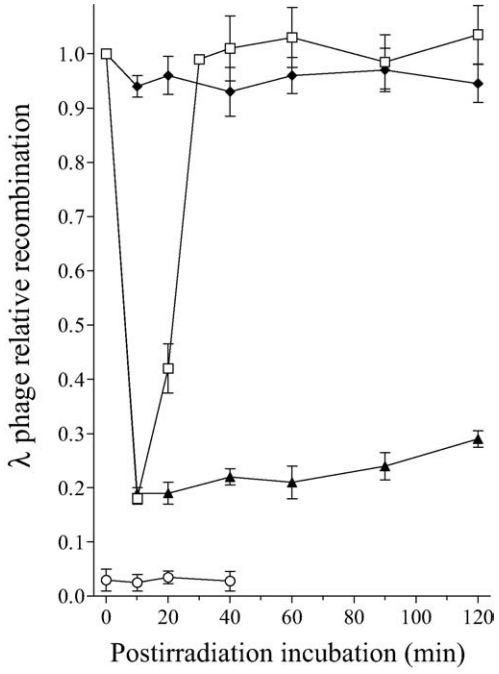

Fig. 3. Time course of recombination in λ lytic crosses in bacteria γ-irradiated with 600 Gy. Bacterial cultures were divided into two parts; one part was irradiated while the other served as a control. Irradiated bacteria were infected with a mixture of two λ phages at intervals and $J^{+}R^{+}$ recombinants selected. The efficiency of λ recombination in irradiated bacteria was expressed in relation to recombination in unirradiated control, which was 32% ± 5 for wild-type cells. Each value is a mean of at least three independent experiments. Error bars represent standard deviations. Symbols: (□) AB1157 bearing plasmid pUC18 (for clarity, essentially the same results, obtained with AB1157 harboring plasmid pBR322, are not presented); (▲) AB1157 with the $recD^{+}$ plasmid pKI13; (♦) AB1157 harboring the $RecBCD^{+}$ plasmid pDWS2; (○) DE101.

γ-irradiation renders wild-type *E. coli* a transient $recB^{-}/C^{-}$ phenocopy concerning λ recombination.

Recombination of λ *red gam* phages in RecD overproducing bacteria (yielding about 27% $J^{+}R^{+}$ recombinants) was also affected by γ-irradiation. When γ-irradiated with 600 Gy, RecD overproducers lost the ability to recombine λ phages. As shown in Fig. 3, the extent and the timing of the effect were similar to that in wild-type bacteria. However, in RecD overproducers, unlike wild-type, λ recombination was not restored for at least 120 min postirradiation (Fig. 3). Therefore, we conclude that γ-irradiated RecD overproducers become permanent $recB^{-}/C^{-}$ phenocopies for λ recombination. λ lytic crosses in which recombination was inefficient also yielded less phage progeny (not shown). We also found the inhibition of λ recombination to be dose-dependent. Irradiation of wild-type bacteria and RecD overproducers with 400 Gy of γ-rays, caused about threefold decrease in their λ recombination activity, while the kinetics of the effects were the same as in bacteria that received 600 Gy (not shown).

The decrease of λ recombination activity in γ-irradiated wild-type and RecD overproducing cells can be attributed to the lack of available RecBCD enzymes since γ-irradiated RecBCD overproducers (which exhibit about 25-fold increased ExoV activity [32]) did not become $recB^{-}/C^{-}$ phenocopies for λ recombination (Fig. 3). Irradiated RecBCD overproducers generated $J^{+}R^{+}$ recombinants with about the same efficiency of about 30% as their unirradiated control.

Also, the inhibition of λ recombination in γ-irradiated wild-type and RecD overproducing bacteria coincided with degradation of their damaged chromosomal DNA (compare Figs. 2 and 3), suggesting that their inability to recombine λ phages is caused by titration of RecBCD enzyme molecules on chromosomal DNA that they are repairing. Since the titration of RecBCD enzymes on γ-irradiated chromosomal DNA lasted much longer in RecD overproducers than in wild-type bacteria, this supports the hypothesis according to which an excess of RecD polypeptide replaces inactivated/lost RecD subunit of a Chi-modified RecBC(D) while the enzyme is still bound to its DNA substrate, thereby preventing it from being released to the cytoplasm.

3.4. An excess of RecD polypeptide in γ-irradiated E. coli does not restore RecBCD's nuclease activity on infecting T4 2 phage DNA

To confirm that the observed prolonged inhibition of λ phage recombination in γ-irradiated RecD overproducers is due to long-lasting titration of RecBCD enzyme on damaged chromosomal DNA, we infected irradiated cells with T4 *2* phage. The linear DNA duplex genome of T4 phage mutant *2* is sensitive to digestion by RecBCD enzyme because it lacks pilot protein 2. This protein is bound to the ends of phage T4 genome, blocking entry by RecBCD enzyme [37]. The nuclease activity of RecBCD enzyme in the cell can thus be determined by measuring the T4 *2* phage EOP. T4 *2* plates with low efficiency on wild-type cells, while on mutants deficient in all

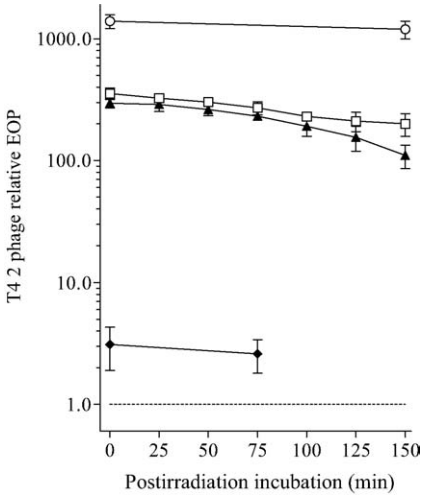

Fig. 4. Time course of T4 *2* phage EOP in bacteria γ-irradiated with 400 Gy. Irradiated bacteria were infected with T4 *2* phage at intervals and its plating efficiency determined. It was expressed in relation to EOP on unirradiated controls, which is represented by dashed line. Each value is a mean of at least three independent experiments. Error bars represent standard deviations. Symbols: (□) AB1157 carrying plasmid pUC18 (for clarity, essentially the same results, obtained with AB1157 harboring plasmid pBR322, are not presented); (▲) AB1157 containing the *recD*$^+$ plasmid pKI13; (♦) AB1157 containing the *RecBCD*$^+$ plasmid pDWS2; (○) unirradiated DE101.

(*recB/C*) or just nuclease activities (*recD*) of RecBCD, it plates efficiently.

It is known that T4 *2* phage EOP increases in wild-type *E. coli* which chromosome is fragmented by γ-irradiation [38] or treatment with bleomycin [16]. We ran a time course of T4 *2* phage EOP on bacteria γ-irradiated with 400 Gy (this is a minimal dose that increases a T4 *2* titer maximally on wild-type *E. coli* [38]), to determine whether RecD overexpression reverts their ExoV$^-$ phenotype. As shown in Fig. 4, a T4 *2* phage titer increased more than 300-fold immediately after irradiation of wild-type bacteria and came close to the titer on a *recB* strain. An increased EOP on wild-type bacteria lasted for at least 150 min. On the other hand, the survival of T4 *2* phage on γ-irradiated bacteria overproducing RecBCD enzyme (by the plasmid pDWS2) increased only marginally, about three-fold (Fig. 4).

An excess of RecD polypeptide did not restore the ability of γ-irradiated wild-type bacteria to degrade T4 *2* phage genome. In γ-irradiated RecD overproducers (carrying plasmid pKI13), a T4 *2* phage EOP rose about 300-fold and remained high for at least 150 min (Fig. 4). This result mimics inability of γ-irradiated RecD overproducers to carry out λ phage recombination, arguing further for a *recB*$^-$/*C*$^-$ phenocopy resulting from an altered behavior of RecBCD enzyme in that genetic background.

4. Discussion

In this study, we examined the effects of imbalanced (increased) concentrations of individual subunits of RecBCD enzyme on *E. coli* DNA metabolic processes. We have shown that an excess of RecB or RecC polypeptide does not affect DNA repair and degradation in otherwise wild-type bacteria, in contrast to RecD overproduction. RecD overproducers exhibited impaired DNA repair and more processive DNA degradation (Figs. 1 and 2; [25]). Even mild RecD overproduction (about twofold), sensitized bacteria to γ-rays to the same extent as a 60-fold RecD overproduction (unpublished result). Phenotypic changes in RecD overproducers are due to altered activities of RecBCD enzyme because RecD overproduction does not affect DNA repair in *recB* mutant (Fig. 1).

It is known that RecBCD interaction with many Chi sites (generated by fragmenting chromosomal DNA) results in silencing of the enzyme's nuclease activity [16,38]. Two mechanisms have been proposed to explain this acquired ExoV$^-$ phenotype; (i) binding and processing of broken chromosomal DNA leads to titration of RecBCD molecules on substrate DNA [38], resulting in a transient *recB*$^-$/*C*$^-$ phenocopy, or (ii) the interaction of RecBCD enzyme with Chi sites on chromosomal DNA irreversibly inactivates the RecD subunit, making these cells permanent *recD*$^-$ phenocopies [16]. Both proposed mechanisms are suggested from our results, al least for wild-type cells. By comparing the kinetics of chromosomal DNA degradation in γ-irradiated wild-type *E. coli* with its ability to recombine λ phage DNA provided in parallel, we show that γ-irradiated bacteria become transient, partial *recB*$^-$/*C*$^-$ phenocopies concerning λ recombination, coincident with ongoing chromosomal DNA degradation, indicating that RecBCD enzymes are titrated on the chromosomal DNA that they are repairing and thus are unable to recombine λ (compare Figs. 2 and 3). This conclusion was corroborated by our observation that an excess of RecBCD enzyme alleviates that inhibition (Fig. 3), and also that the extent of the λ recombination inhibition is proportional to a γ-ray dose (i.e. to the number of DSBs) the cells suffered. In irradiated wild-type *E. coli*, chromosomal DNA degradation stopped at about the same time point at which λ recombination was restored (30 min postirradiation), suggesting that RecBCD molecules are being released from chromosomal DNA and thus disposed to recombine λ DNA (compare Figs. 2 and 3). During the period of restored λ recombination, irradiated bacteria were still unable to inhibit T4 *2* phage growth (an ExoV$^-$ phenotype), suggesting that they become *recD*$^-$ phenocopies (compare Figs. 3 and 4). Thus, we infer that in wild-type *E. coli* whose chromosome is fragmented by γ-rays RecBCD enzyme becomes sequestered on broken DNA (ExoV$^-$, *rec*$^-$), but soon dissociates from it. The enzyme released from its DNA substrate is devoid of RecD functions (ExoV$^-$, *rec*$^+$), mimicking RecBC enzyme.

Perhaps the most interesting finding of this study is that an excess of RecD polypeptide changes the above-described pattern of RecBCD activities in γ-irradiated wild-type *E. coli*. Chromosomal DNA degradation in irradiated RecD overproducers is deregulated and thus occurred continuously for 150 min postirradiation, which again coincides with the inhibition of recombination in λ lytic crosses (compare Figs. 2 and 3). In agreement with this finding is the observed inability of an excess of RecD polypeptide to render γ-irradiated bacteria proficient for T4 *2* phage genome degradation (Fig. 4), suggesting that lack of ExoV activity in irradiated RecD overproducers is

not a consequence of inactivated RecD subunit (a *recD*$^-$ phenocopy), but rather, of sequestered RecBCD enzyme (a *recB*$^-$/*C*$^-$ phenocopy).

Together, these results strongly indicate that RecD overproduction prevents dissociation of RecBCD enzyme from its DNA substrate, causing its prolonged sequestration on the DNA it processes, which renders the enzyme unable to act on another DNA molecule. Since RecBCD enzyme's processivity is defined as "its ability to remain associated with its substrate" [39], we conclude that the long-lasting titration of the enzyme on DNA substrate represents its increased processivity.

Earlier, Köppen and al. [16] also noticed a certain reduction in λ phage recombination in bacteria whose chromosomes were fragmented by bleomycin treatment. However, this reduction was less pronounced (about 2.5-fold) compared to our results (about fivefold), probably due to the low dose of bleomycin used. They also observed only a marginal decrease of T4 *2* phage titer in RecD overproducers treated with bleomycin, consistent with our results. Although some of their observations are similar to ours, the authors reached different conclusions, i.e. that interaction of RecBCD enzyme with fragmented chromosome makes *E. coli* a *recD*$^-$ phenocopy only, and that RecD overproduction suppresses that effect. The same conclusions were reached by Myers et al. [15], after they failed to detect any reduction of λ recombination in cells harboring cosmid with Chi sequence. Distribution of recombinational exchanges in these λ crosses mimics that of *recD* mutants (bacteria become *recD*$^-$ phenocopies); unless RecD polypeptide overexpression antagonizes that phenotype, suggesting that intact RecD polypeptide replaces Chi-modified one [15]. These effects (an unaffected λ phage recombination and successful reversion of acquired RecD$^-$ phenotype by RecD overproduction) are clearly different from those observed in our study and the one of Wackernagel's group [16]. However, if the nature of DNA substrates used to expose RecBCD enzyme to Chi sites is considered, these results do not seem contradictory any more. While in the later two studies the fragmented bacterial chromosome was a substrate, in the former study a ~3 kbp cosmid (our estimate from data in Ref. [40]) was used. Such a small DNA molecule is certainly unable to trap RecBCD for a considerable time, unlike large chromosomal DNA fragments. It is thus not surprising that in experiments with cosmid DNA no RecB$^-$/C$^-$ phenotype was noted (the assay was apparently not timely enough), and that an excess of RecD polypeptide countered the effect of Chi on RecBCD.

Our finding that γ-irradiated RecD overproducers become partial *recB*$^-$/*C*$^-$ phenocopies due to long-lasting titration of RecBCD enzyme on damaged chromosomal DNA is analogous to an earlier observation concerning logarithmically growing *recA* mutants, a fraction of which are also *recB*$^-$/*C*$^-$ phenocopies due to "reckless" degradation of their chromosomal DNA [41].

How does RecD subunit regulate processivity of RecBCD enzyme? It is known that in vitro RecD polypeptide assembles with RecBC enzyme, thus creating RecBCD enzyme, which is about 10-fold more processive than RecBC (our estimate from the data in Refs. [24,42]). Here, we show that an excess of RecD polypeptide in vivo further increases processivity of RecBCD by refurnishing Chi-modified enzyme with intact RecD subunit while the enzyme is still bound to DNA duplex, thus preventing its dissociation from DNA substrate. There are two ways by which RecD subunit might increase the processivity of RecBCD enzyme: (i) RecD prevents creation of a nucleoprotein filament (by enabling nuclease activity and inhibiting RecA loading activity of the enzyme), whose pairing with homologous DNA duplex apparently causes release of the enzyme from its DNA substrate [43], and (ii) as RecD subunit is one of two helicase motors of RecBCD enzyme (along with RecB subunit), it is required that both of them are dissociated simultaneously to release the enzyme from DNA, which makes such an event much less likely (as proposed in Ref. [6]). The combination of these two RecD subunit functions equips RecBCD enzyme with almost unlimited processivity if no interaction with Chi site occurs (as in *recA* mutants [40,41]). But, upon interaction with Chi and subsequent modification of a RecD subunit, both of its regulating functions are seemingly turned off, resulting in the limited processivity of a Chi-modified RecBCD enzyme.

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