In Vivo Studies on the Interaction of RecBCD Enzyme and λ Gam Protein

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The interaction between the RecBCD enzyme of *Escherichia coli* and the λ Gam protein was investigated. Two types of experiments were done. In one type, Gam protein was produced by transient induction of the cells lysogenic for λ cI857gam⁺. The presence of Gam protein, which inhibits RecBCD nuclease, enabled these cells to support the growth of a gene 2 mutant of bacteriophage T4 (T4 2). The lysogens overproducing the RecB subunit of RecBCD enzyme could titrate Gam protein and thus prevent the growth of T4 2. In contrast, the lysogens overproducing either RecC or RecD retained their capacity for growth of T4 2. It is therefore concluded that the RecB subunit is capable of binding Gam protein. In the second type of experiments, Gam protein was provided by derepressing the *gamS* gene on the plasmid pSF117 (S. A. Friedman and J. B. Hays, Gene 43:255-263, 1986). The presence of this protein did not interfere with the growth of wild-type cells (which were F⁻). Gam protein had a certain effect on *recF* mutants, whose doubling time became significantly longer. This suggests that the *recF* gene product plays an important role in maintenance of viability of the Gam-expressing cells. Gam protein exerted the most striking effect on growth of Hfr bacteria. In its presence, Hfr bacteria grew extremely slowly, but their ability to transfer DNA to recipient cells was not affected. We showed that the effect on growth of Hfr resulted from the interaction between the RecBCD-Gam complex and the integrated F plasmid.

Like other viruses, bacteriophages make use of host functions for the majority of their needs. To ensure optimal development, they often specify the products that modify certain host functions. The *gam* gene product (Gam protein; see reference 45) of phage λ is an example. Its interaction with the RecBCD enzyme of *Escherichia coli* (43) is required for the transition from the early stage to the late stage of λ DNA replication. In the absence of this interaction, RecBCD enzyme precludes late replication, during which DNA concatemers (polymers of several DNA units) are formed. DNA concatemers are subjected to packaging-dependent cleavage and thus promote viral encapsidation (14). It is therefore clear that Gam-mediated modification of the host RecBCD system plays an important role in the physiology of phage λ .

Gam protein has also been shown to interact with the *sbcC* gene product of *E. coli* (19). This interaction allows growth of the palindrome-containing phage, which is otherwise unable to plate on the *sbcC*⁺ host (10). It is not known, however, whether the *sbcC* gene product has any effect on the physiology of wild-type λ . Nor is it known whether this product has any effect on the physiology of wild-type *E. coli*. Studies of the host metabolism in the presence of Gam protein are therefore focused on the processes mediated by the modified RecBCD enzyme.

RecBCD enzyme consists of three subunits coded for by the *recB*, *recC*, and *recD* genes (for a review, see reference 35). It possesses a variety of activities both in vivo and in vitro. In vitro, it exhibits ATPase, DNA unwinding, and DNase activities (39). RecBCD DNase cleaves single-stranded DNA endonucleolytically and degrades both single-

and double-stranded DNA exonucleolytically. During DNA unwinding, it also introduces specific cuts near the so-called Chi sites (31, 37), which are known to be recombinators in the RecBCD pathway for genetic recombination (20). All these catalytic activities of RecBCD enzyme are inhibited by Gam protein (18, 27, 33, 43). Thus, the biochemical data suggest that Gam protein converts *recBCD*⁺ cells to *recBCD*⁻ phenocopies.

In vivo, RecBCD enzyme is involved in numerous cellular functions. It participates in the principal pathway of homologous genetic recombination (12), DNA repair (12), degradation of foreign or damaged endogenous DNA (11), and maintenance of cell viability during normal growth (8). Gam protein is known to modulate these functions. Inhibition of the in vitro activities of RecBCD enzyme cannot, however, account for all of its in vivo effects. For example, Gam protein inhibits Chi's activity (27, 32, 36), but its presence does not affect recombinational frequencies following Hfr-mediated conjugation and P1-mediated transduction (16, 27). With respect to the effect of Gam protein on DNA repair, the data in the literature have led to conflicting conclusions. It seems, however, that Gam protein sensitizes the cells to UV light (15, 27; but see reference 4), whereas it enhances the cellular capacity for repair of X-ray- or gamma-ray-induced DNA lesions (23, 41, 42). On the other hand, there is general agreement that its presence within *E. coli* strongly inhibits degradation of foreign or damaged endogenous DNA (15, 16, 32, 42). With respect to the maintenance of the viability of Gam-expressing cells, the data in the literature are scarce. A decrease in the colony-forming ability of these cells has been observed in experiments with the *recF* strain (15).

Gam-expressing bacteria are at least superficially similar to cells carrying the *recD* mutation. In the absence of the

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Description	Reference or source
Bacterial strains		
AB1157	<i>thr-1 leuB6 lacY1 supE44 rfbD1 thi-1 ara-14 galK2 xyl-5 mtl-1 mgl-51 Δ(gpt-proA)62 hisG4 (Oc) kdgK51 argE3</i>	2
JC3916	As AB1157, also <i>recF143</i>	A. J. Clark
IRB53	AB1157 lysogenized with <i>λ</i> C1857 <i>red3</i>	This work
3000	Hfr (Hayes PO1) <i>relA1 spoT1 thi-1</i>	2
NM92	As 3000, also <i>recD1903::minitet</i>	This work
DM456	Wild type	26
Plasmids		
pDWS2	Ap ^r <i>recBCD</i> ⁺ in vector pBR322	31
pKI13	Ap ^r <i>recD</i> ⁺ in vector pUC18	7
pPB520	Cm ^r <i>recC</i> ⁺ in vector pACYC184	3
pPB700	Ap ^r <i>recB</i> ⁺ in vector pBR322	3
pSF117	Ap ^r <i>gamS</i> ⁺ in vector pBR322	15
pSF119	Ap ^r <i>gamS201</i> in vector pBR322	15

functional *recD* gene product, in vitro activities characteristic of the RecBCD enzyme are either reduced or abolished (30). Unlike *recB* and/or *recC* cells, *recD* mutants are nonetheless recombination proficient, resistant to UV light, and fully viable (1, 11, 21, 22, 35). As for Gam-expressing bacteria, *recD* mutants do not respond to Chi recombinators (11, 35); also, they are unable to degrade foreign or damaged endogenous DNA (6, 11). The question therefore arises whether Gam protein binds to the RecD subunit or whether it interacts with some other component of the RecBCD enzyme. In the present work, we attempted to answer this question. Furthermore, we studied the in vivo effects of the binding of Gam protein to the RecBCD enzyme, in particular the effect on bacterial growth.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phage. Bacterial strains and plasmids used in this study are described in Table 1. Strain IRB53, the *λ*C1857*red3* lysogenic counterpart of AB1157, was prepared in our laboratory. (*λ*C1857*red3* is characterized by thermoinducibility and by the failure to produce λ exonuclease and β protein.) NM92 was constructed by transducing strain 3000 to Tet^r with P1*vir* grown on PM90 (5). A gene 2 amber mutant of phage T4 (T4 2) was kindly supplied by R. Devoret (Gif-sur-Yvette, France). Phage ϕ 2, which specifically attacks a male (F⁺ or Hfr) host, was from our laboratory collection.

Culture media and growth conditions. M9 medium contained 0.5 g of NaCl, 1 g of NH₄Cl, 3 g of KH₂PO₄, 7.5 g of Na₂HPO₄ · 2H₂O, 4 g of glucose, 120 mg of MgSO₄, 10 mg of CaCl₂, and water to 1,000 ml. In all our experiments, this medium was supplemented with 1 μ g of thiamine per ml. Appropriate amino acids were added as needed. For M9 plates, M9 medium was supplemented with 16 g of agar (Serva) and 1 μ g thiamine per ml. When needed, appropriate amino acids were also added. TP broth contained 5 g of tryptone (Difco), 8 g of nutrient broth (Difco), and 5 g of NaCl per 1,000 ml of water. For TP plates, TP broth was supplemented with 3 g of Bacto Peptone (Difco) and 16 g of agar (Serva). Ampicillin (50 μ g/ml) or chloramphenicol (25

μ g/ml) was added to the overnight culture media of strains resistant to the respective antibiotic. Unless otherwise stated, the temperature of incubation was 37°C. This temperature allowed expression of the plasmid-borne *gam* gene (23).

Crosses. The procedures for conjugational crosses were those described by Miller (25). Donor and recipient strains were mixed in a ratio of 1:20 and incubated for 60 min at 37°C. Before the strains were plated on agar selective for transconjugants, matings were interrupted by vigorous agitation with a vibrating mixer. To counterselect donors, we used streptomycin at 100 μ g/ml in the plate.

Determination of the stability of the Hfr character. In one type of experiment, strain 3000/pSF117 was tested for stability of maintenance of its Hfr character. The following procedure was used (cf. reference 24). A single colony of 3000/pSF117 was inoculated into M9 medium containing ampicillin and then incubated at 37°C until the stationary phase of growth was reached. Thereafter, the bacterial culture was diluted 1,000- to 2,000-fold into fresh M9 medium with ampicillin (the zero-generation time point of our experiments). After 12 h of growth (10 to 13 generations), the cell suspension was subcultured in the same way. This procedure was continued for 80 generations of growth. At each dilution, aliquots were plated for colony count determination. About 100 colonies from each plate were then tested for their Hfr character. For this purpose, single colonies were suspended in 0.5 ml of M9 medium and then gridded onto a lawn of AB1157 F⁻ bacteria on the plates selective for Thr⁺ Leu⁺ Str^r recombinants. Only those regions of these plates onto which cells with the Hfr character had been gridded showed confluent growth.

Test for male phage sensitivity. The test for male phage sensitivity was used to distinguish Hfr and F⁺ cells from F⁻ bacteria. The response of bacterial cells to the male phage ϕ 2 was examined by the cross-streaking method (25).

Rate of bacterial multiplication. The rate of bacterial multiplication was determined on the basis of viable cell measurements during exponential growth of the bacterial culture. With some modifications, we used the procedure described by Capaldo-Kimball and Barbour (8). The overnight culture of each strain was diluted between 1:50 and 1:100 into TP broth and allowed to grow in a shaking 37°C water bath for 3 h. A 1-ml sample of this suspension was then mixed with 75 ml of fresh TP broth in a 250-ml flask, and incubation continued at 37°C for additional 3 h. During this period, i.e., during the actual growth experiment, samples were taken every 30 min and, after appropriate dilutions in 0.067 M phosphate buffer, plated on TP agar. The plates were incubated at 37°C, and the colonies were counted the next day.

RESULTS AND DISCUSSION

Genetic analysis of the interaction of Gam protein with the individual subunits of RecBCD enzyme. We used a simple genetic approach to determine whether Gam protein interacts with any of the individual subunits of RecBCD enzyme (RecB, RecC, RecD). The appropriate plasmids (see Materials and Methods, Table 1) were introduced into strain IRB53. Due to the presence of the thermoinducible prophage *λ*C1857*red3* in IRB53, all the transformants thus constructed can be transiently induced by a brief heat treatment (e.g., 5 to 6 min at 42°C). Such a treatment does not affect their colony-forming ability (40), but it enables the cells to make some λ products, including Gam protein. This protein inhib-

TABLE 2. Effects of plasmids overproducing various components of RecBCD enzyme on the titer of phage T4 2 plated on pulse-heated and unheated λ C1857 gam^+ lysogen IRB53

Plasmid	Efficiency of plating on ^a :	
	Unheated IRB53	Heated IRB53
None	0.01	1.0
pDWS2 (<i>recBCD</i> ⁺)	0.02	0.07
pKI13 (<i>recD</i> ⁺)	0.01	1.2
pPB520 (<i>recC</i> ⁺) ^b	0.02	0.89
pPB700 (<i>recB</i> ⁺) ^b	0.007	0.007

^a IRB53 and its derivatives containing various plasmids were grown at 30°C to the stationary phase, diluted 10-fold in TP medium at 30°C (unheated IRB53) or 42°C (heated IRB53), and incubated for 6 min. T4 2 was then plated on the lawns of these bacteria. Efficiency of plating on heated IRB53 is taken as 1.0.

^b See reference 3. To achieve high intracellular levels of RecC (in the presence of pPB520) or RecB (in the presence of pPB700), the cells were grown to the late log phase and then treated with 1 mM IPTG. After 2 h of additional growth, the cells reached the stationary phase and were used in the experiment.

its RecBCD nuclease, which normally prevents the growth of phage T4 2 (29). T4 2 is therefore expected to plate at high efficiency on the pulse-heated λgam^+ lysogens. This increased plating efficiency may be less pronounced or even eliminated if the cells overproduce the component of RecBCD enzyme that exerts the titration effect on Gam protein.

Table 2 shows the results of an experiment in which the effect of a brief heat treatment on the ability of IRB53 and its various plasmid-containing derivatives to support the growth of T4 2 was examined. Concerning these results, the following points need to be emphasized.

(i) Heating of the original λgam^+ lysogen IRB53 strikingly enhanced the cellular capacity for the growth of T4 2. (A mutation in the *gam* gene abolished this effect; data not shown.)

(ii) Only a slight increase in the efficiency of plating of T4 2 was observed after heating of the gam^+ lysogen harboring the *recBCD*⁺ plasmid pDWS2. Within strain AFT379, plasmid pDWS2 amplifies the level of RecBCD proteins about 25-fold (31). Radiobiological studies suggested that the same is true for AB1157 (and IRB53) carrying pDWS2 (cf. strain KBK90, reference 5). Thus, a 25-fold increase in the amount of RecBCD proteins nearly abolishes the activity of the lysogen-derived Gam protein. To assess the titration effect of the individually overproduced RecB, RecC, and RecD subunits, at least the same level of overproduction should be achieved.

(iii) Certain data hinted at the possibility that the RecD subunit of RecBCD enzyme interacts with Gam protein (23; see Introduction). Contrary to expectation, however, T4 2 produced a large number of plaques on the heated λgam^+ lysogen carrying the *recD*⁺ plasmid pKI13. Thus, the RecD subunit, whose amount is amplified 60-fold in the presence of pKI13 (7), shows no titration effect on Gam protein. One may speculate that it is unable to exert such an effect because its high intracellular levels can lead to the formation of insoluble aggregates (3). Under our experimental conditions, however, this is probably not the case. In AB1157 bacteria harboring pKI13, the RecD protein is functional: its elevated levels sensitize the cells to gamma rays and increase the rate of radiation-induced DNA degradation (7).

(iv) Overproduction of the RecC subunit of RecBCD enzyme was achieved by the overexpression *recC*⁺ plasmid

FIG. 1. Effect of the *gam*⁺ plasmid pSF117 on the rate of multiplication of wild-type cells. Symbols: ○, AB1157/pSF119; □, AB1157/pSF117.

pPB520 (3). Following induction with isopropyl-β-D-thiogalactopyranoside (IPTG), RecC in strain CSH/pPB520 may represent ≈10% of the total cellular protein. Such huge amounts of RecC were also obtained with other *E. coli* strains carrying pPB520 (3). Our data show that, upon heating, IPTG-induced λgam^+ lysogen harboring this plasmid could plate T4 2 at high efficiency. We may thus infer that, just as for RecD, the RecC subunit is unable to titrate away Gam protein from the cellular RecBCD enzyme.

(v) Overproduction of the RecB subunit was achieved by the overexpression *recB*⁺ plasmid pPB700 (3). This plasmid, which is closely related to pPB520, directs the synthesis of elevated amounts of RecB. In the IPTG-induced host strain, the overproduced RecB subunit may also occur at levels amounting to approximately 10% of total cellular proteins (3). Our data show that, in marked contrast to the results obtained with RecD and RecC, the RecB subunit exerts the titration effect on the lysogen-derived Gam protein. Upon heating, phage T4 2 plated at the same low efficiency on the IPTG-induced IRB53/pPB700 as it did on the unheated lysogen.

The results presented above strongly suggest that Gam protein binds to the RecB subunit of RecBCD enzyme. As already mentioned, Gam also interacts with the *sbcC* gene product (SbcC protein). It is of interest that RecB and SbcC resemble each other: they both are large polypeptides and have similar binding folds (28). In addition, both the *recB* and *sbcC* genes lie within the genetic regions of *E. coli* which are expressed rather poorly (17).

Growth of Gam-expressing cells. Subsequent experiments were undertaken to determine the *in vivo* effect of Gam protein on bacterial growth. Clearly, Gam protein must be present within the cell throughout the growth experiment. To achieve this, we transformed the cells with the *gamS*⁺ plasmid pSF117 (15). The *gamS*⁺ gene codes for an 11.6-kDa polypeptide which might be a smaller version of the phage-derived Gam protein with a supposed mass of 16.4 kDa (15). It should be stressed, however, that *in vitro* activities of the *gamS* gene product do not differ from those of the phage-encoded Gam protein. Two versions of this protein, if they exist, are therefore expected to produce the same effect *in vivo*.

Figure 1 shows the results of an experiment in which the rate of multiplication of wild-type (AB1157) bacteria carrying the *gam*⁺ plasmid pSF117 was examined. Control (*Gam*⁻) cells contained the pSF119 plasmid, a *gamS201* derivative of pSF117. The increase in the number of viable

TABLE 3. Effect of the *gam*⁺ plasmid on conjugational transfer^a

Cross Hfr × F ⁻	Donor markers among recombinants (%)		
	<i>thr</i> ⁺ <i>leu</i> ⁺	<i>pro</i> ⁺	<i>his</i> ⁺
3000 × AB1157	42.0	21.1	0.2
3000/pSF119 × AB1157	50.0	21.0	0.6
3000/pSF117 × AB1157	98.9 ± 12.0	47.5 ± 9.3	0.9 ± 0.3

^a Strain 3000 transfers its genes in the order *thr*⁺ *leu*⁺, *pro*⁺, *his*⁺. The results are expressed as the number of recombinants per 100 viable donors. For the cross 3000/pSF117 × AB1157, all values are averages (±SEM) from six separate experiments.

cells per unit of time was the same in both bacterial cultures. Hence, the presence of Gam protein did not affect the growth rate of wild-type cells. The identical results were also obtained with the original strain AB1157 (data not shown). Under our experimental conditions, the growth rate of all plasmid-free strains was the same as that of the corresponding derivatives containing the control plasmid pSF119. In subsequent figures, the results with plasmid-free strains are therefore omitted.

In our experiments on the rate of multiplication of Gam-expressing wild-type cells, we also used strain DM456. Unlike AB1157, the growth of DM456 is not affected by the elevated temperature (42°C). Therefore, the effect of the presence of pSF117 on the rate of its multiplication can be studied at this temperature. The *gamS* gene, which is placed under the λP_R promoter and regulated by the λcI_{ts857} -coded repressor (15), is then fully expressed. Even in this case, the rate of bacterial multiplication remained unaffected (data not shown).

It is possible that the normal growth of wild-type cells bearing pSF117 depends on the presence of the functional *recF* gene product. The absence of this gene product was reported to decrease the viability of Gam⁺ cells (15). In our experiments with the *recF* strain JC3916, similar results were obtained (data not shown). On the basis of the results of four independent experiments, we calculated that the doubling time of the *recF* strain carrying the *gam*⁺ plasmid was 22% ± 0.6% longer than that of the Gam⁻ derivative. Apparently, the *recF* gene product plays an important role in maintenance of viability of the Gam-expressing wild-type strain. Clark (13) has recently postulated two RecF pathways of recombination, one resistant to the *sbcB* gene product (SbcB^r) and the other sensitive (SbcB^s). We speculate that the SbcB^r RecF pathway, which is operative in the wild-type strain, supports the normal growth of Gam-expressing cells.

We next investigated the effect of pSF117 on the behavior of an HfrH strain. While F⁻ cells carrying the *gam*⁺ plasmid behaved normally after mating (data not shown; cf. references 16 and 27), the cross between 3000 HfrH/pSF117 and AB1157 F⁻ gave surprising results (Table 3). The frequencies with which the donor markers appeared among recombinants were abnormally high. For example, the percentage yield of the Thr⁺ Leu⁺ recombinants was well above the theoretical value of 50%. The simplest explanation of this phenomenon is that there are some dead cells in the culture of 3000 HfrH/pSF117 and that they can participate in conjugational DNA transfer. We therefore determined the effect of the *gam*⁺ plasmid pSF117 on the rate of multiplication of 3000 HfrH cells. The results are presented in Fig. 2. It is seen that, indeed, the doubling time of strain 3000 HfrH/pSF117 is much longer (≈100%) than that of strain 3000 HfrH/pSF119.

FIG. 2. Growth curves in TP medium of HfrH cells. Symbols: ○, NM92; △, 3000 HfrH/pSF119; ▲, 3000 HfrH/pSF117.

Also included in Fig. 2 are the results obtained with NM92, a *recD* derivative of 3000 HfrH. Although there are many similarities between Gam-expressing wild-type cells and *recD* bacteria, NM92 grew quite normally. Even more interestingly, its growth rate was not changed in the presence of the *gam*⁺ plasmid (data not shown).

One may speculate that the F plasmid integrated into the host chromosome somehow interferes with the normal growth of Gam-expressing cells. If this plasmid in its cytoplasmic state does not produce such an effect, the growing cultures of strain 3000/pSF117 will rapidly accumulate F⁺ cells. We therefore conducted an experiment in which strain 3000/pSF117 was tested for stability of maintenance of its Hfr character (see Materials and Methods). The results of such an experiment are presented in Fig. 3. After a lag, the percentage of Hfr bacteria in the culture began to fall rapidly. After 30 to 35 generations, about 60% of the cells lost their Hfr character. Unexpectedly, however, the fraction of Hfr bacteria increased during further growth.

We first analyzed the cells that had lost their Hfr character. These cells were sensitive to the male-specific phage f2, i.e., they carried the F plasmid in their cytoplasm. These cells could also support the growth of phage T4 2, i.e., they were Gam⁺. Electropherograms demonstrated the presence of two plasmids in their cytoplasm. In addition to pSF117, they contained the plasmid whose size (≈100 kb) corresponded to F.

We next analyzed those Hfr cells that appeared at later

FIG. 3. Effect of the *gam*⁺ plasmid pSF117 on stability of the HfrH character during growth of strain 3000. Symbols: ○, 3000/pSF119; △, 3000/pSF117. For details, see text.

FIG. 4. Growth curves in TP medium of strain SR4 (■) and SR10 (□).

stages of the growth experiment. These Hfr cells contained only one plasmid, which was smaller than pSF117. Furthermore, they were unable to support the growth of phage T4 2. Evidently, they became Gam⁻ as a result of a deletion of the plasmid-borne *gam* gene.

We examined the growth characteristics of both classes of the mutant cells isolated from the culture of strain 3000/pSF117. SR4, a Gam-expressing F⁺ isolate, and SR10, a Gam⁻ Hfr isolate, were typical examples of these two classes. As shown in Fig. 4, their rate of multiplication was normal or nearly normal (cf. the growth rate of 3000/pSF119 in Fig. 2). However, SR10 grew slightly faster than SR4. This slight difference in the growth rate may explain the eventual predominance of Gam⁻ Hfr cells in the bacterial culture.

The inhibitory effect of pSF117 on the growth of an HfrH strain must be a consequence of the interaction between the integrated F plasmid and Gam protein. The integrated F plasmid is clearly not in a passive state. Its transfer system is functional; moreover, nicking at *oriT*, i.e., at the site of initiation of conjugative DNA transfer, may occur (44). In certain cases, nicking at *oriT* somehow allows RecBCD enzyme to enter the duplex circular DNA molecule (9). In vitro and in vivo studies (5, 38) suggest that RecBCD enzyme enters duplex DNA only after a double-strand break has been formed. If such a break at *oriT* is generated, it may be repaired by the RecA-RecBCD recombination system (34). Perhaps RecBCD-Gam complex is unable to substitute the RecBCD enzyme in this type of repair. In Gam-expressing HfrH cells, the double-strand break would therefore become a lethal lesion. Another possibility is that even in the absence of mating pair formation, the RecBCD-Gam complex activates the *oriT*-dependent conjugative DNA synthesis. This synthesis may interfere with normal replication, thus decreasing cell viability. It is of interest that conjugative DNA synthesis is conceptually similar to the late (rolling-circle) mode of λ DNA replication (see Introduction). In conjunction with RecBCD enzyme, Gam protein might actively participate in this type of λ DNA replication as well as in the conjugative DNA synthesis.

Finally, it should be stressed that only the RecBCD-Gam complex exerts an inhibitory effect on the growth of an Hfr strain. As mentioned above, wild-type HfrH, HfrH *recD*, and HfrH *recD*/pSF117 grew quite normally. Thus, RecBCD-Gam complex possesses certain catalytic properties which differ from those of RecBCD enzyme, RecBC enzyme, and RecBC-Gam complex.

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