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Genetic Requirements for Conjugational Recombination in the Presence of λ Gam Protein in *Escherichia coli*

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Summary

The Gam protein of phage λ is a well-known inhibitor of the enzymatic activities of the RecBCD enzyme, the major enzyme involved in homologous recombination in bacteria *Escherichia coli*. In this work, we studied (*i*) the effect of the RecA loading-deficient *recB* (*recB*^{D1080A}) mutation on conjugational recombination in the presence of λ phage Gam protein and (*ii*) additional genetic requirements for the RecBCD-Gam-mediated conjugational recombination. For this purpose, we introduced Gam$^+$ and Gam$^-$ expressing plasmids into wild type cells and different mutants of *E. coli* (*recJ*, *recB*^{D1080A}, *recB*, *recN*, *recF*, *recR*, *recO*, *recD*), and determined the yields of recombinants after Hfr mediated conjugation. The obtained results suggest that RecA loading activity is not inhibited by Gam and that conjugational recombination in the presence of Gam is partially dependent on *recJ* and *recO* gene products.

Key words: *Escherichia coli*, phage λ, conjugational recombination, RecBCD enzyme, Gam protein

Introduction

RecBCD enzyme is a multifunctional protein complex that plays a central role in homologous recombination, DNA repair, degradation of foreign or damaged DNA, and cell viability in *Escherichia coli*. The enzyme is a heterotrimer composed of the products from *recB*, *recC*, and *recD* genes. *In vitro*, it possesses ATP-ase, DNA helicase, double-strand (ds) DNA exonuclease, single-strand (ss) DNA exonuclease and ssDNA endonuclease activities (*1*). It also recognises the recombination hotspot sequence, χ site (5′-GCTGGTGG-3′), which stimulates recombination and regulates the function of the RecBCD enzyme (*2*). RecBCD enzyme is responsible for the presynaptic processing steps of recombination. It binds to linear ds DNA with blunt or nearly blunt ends and produces 3′ ssDNA overhang. Initially, the enzyme starts the unwinding and cutting of DNA, preferentially on the 3′ strand. However, the polarity of degradation is reversed when RecBCD encounters a χ site in a 3′ to 5′ direction, *i.e.* it results in an attenuation of the 3′ to 5′ nuclease activity (*3*). This nuclease modification allows the production of 3′ single-stranded tail, which is the substrate for RecA protein. The χ site also induces the RecBCD en-

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zyme to coordinate the loading of RecA protein onto ssDNA (*4,5*). An alternative route for the loading of RecA protein onto ssDNA coated with SSB protein can be provided by the RecFOR proteins (*6*). In that case, the gene products of *recJ*, *recQ* and *recN* participate in the preparation of ssDNA (*1*).

Another modification of the RecBCD enzyme can result from its interaction with λ phage Gam protein. Gam plays an essential role in normal λ development and it is responsible for the inhibition of RecBCD nuclease activities during σ replication of phage λ. *In vitro*, Gam inhibits all the known enzymatic activities of the RecBCD enzyme (*7*). When produced *in vivo*, Gam has little effect on the host's ability to act as a recipient in conjugational recombination, although other RecBC phenotypes such as UV sensitivity, cell viability, DNA degradation and the ability to plate T42$^-$ were displayed (*7,8*). On the other hand, the presence of Gam protein in wild type (*wt*) bacteria enhances the cellular capacity for repair of γ or X-ray induced DNA lesions, *i.e.* double strand breaks. This phenomenon is known as *gam* dependent radioresistance (GDR) (*9,10*). It depends on *recB*, *lexA*, *recD*, *recJ*, *recQ* and *recN* gene products (*9,10*). Gam also increases the plating efficiency of unmodified phage λ (restriction alleviation) (*11*).

To explain the conflicting data from *in vitro* and *in vivo* studies, it was assumed that the RecBCD enzyme possesses an additional »unknown« activity that is resistant to or induced by Gam (*7,12*). Paškvan *et al.* (*10*) proposed that the RecBCD-Gam complex possesses RecA loading activity. This possibility has not been tested yet either *in vivo* or *in vitro*.

Using the specific *recB^{D1080A}* mutant (*13*), we wanted to test if the RecBCD-Gam complex has RecA loading activity *in vivo*. In addition, we studied the effects of several other mutations on RecBCD-Gam mediated conjugational recombination.

Materials and Methods

Bacterial strains, media and antibiotics

The strains of *E. coli* K-12 used in this study are listed in Table 1.

Strains were grown at 37 °C in high-salt Luria broth (LB) medium composed of 1 % bacto-tryptone, 0.5 % yeast extract, 10 % NaCl; plate media contained 1.5 % agar. M9 medium contained 0.5 g of NaCl, 1 g of NH_4Cl, 3 g of KH_2PO_4, 7.5 g of $Na_2HPO_4 \cdot 2H_2O$, 4 g of glucose, 120 mg of $MgSO_4$, 10 mg of $CaCl_2$, and water up to 1000 mL. For minimal selective plates, M9 medium was supplemented with appropriate amino acids, 1 μg/mL of thiamin and 16 g of agar (*16*). To obtain Gam$^+$ and Gam$^-$ producing cells, all strains were transformed with the multicopy plasmids pSF117 (*gamS*$^+$) and pSF119 (*gamS201*) (*8*). The antibiotic ampicillin (50μg/mL) was added to the overnight culture media of strains resistant to the ampicillin.

Crosses and measures of recombination

The procedures for conjugational crosses were those described previously (*17*). Matings were performed in LB broth for 30 min, mixed in a 1:10 donor to recipient ratio using recipient and donor cells grown to an OD_{650} of 0.4. Exconjugant mixture was interrupted by vigorous agitation, serially diluted and plated on appropriate minimal agar containing 100 μg/mL of streptomycin to counter select donor cells and 50 μg/mL of ampicillin for selection of plasmid-containing recombinants. Measurements of cell viability relate to the number of CFU in the recipient cultures at an OD_{650} of 0.4, as determined with nonselective LB agar supplemented with 50 μg/mL of ampicillin (*18*). The absolute recombination frequency is the ratio of the number of recombinants to the number of donors in conjugational mixture. All values are expressed as relative frequencies of recombination (the frequency of recombination in wild type bacteria is 1).

Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant genotype	Source or reference
a) Bacterial strains related to AB1157		
AB1157	F$^-$ *thr-1 leuB6* D(*gpt-proA*)*62 hisG4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 supE44 rpsL31 kdgK51 rfbD1 mgl-51* λ$^-$ *rac*$^-$	(*14*)
N4634	+ *recB270*::*Km*	R. G. Lloyd
RIK174	+ *recB^{D1080A}*	(*13*)
IRB103	+ *recO1504*::Tn*5*	(*10*)
JC12123	+ *recJ284*::Tn*10*	J. Clark
AM208	+ *recR256*::Tn*5*	(*15*)
WA576	+ *recF400*::Tn*5*	W. Wackernagel
SP254	+ *recN262*	R. G. Lloyd
RIK144	+ *recD1903*::Tn*10d(Tet)*	(*13*)
b) Other		
Hfr3000	Hfr *supQ80*? e-14 *relA1 spoT1 thi-1* λ$^-$	(*14*)
Plasmids		
pSF117	*Ap^R gamS*$^+$ in vector pBR322	(*8*)
pSF119	*Ap^R gamS201* in vector pBR322	(*8*)

The frequency of conjugational recombination for each strain was corrected to minimise the effect of recipient's viability.

Results and Discussion

The conjugational system of DNA transfer has been used extensively to study the genetics of recombination in *E. coli* (*19*). More than 20 genes have been linked with recombination in this organism, but only three of them, namely *recA*, *recB* and *recC*, reduce recombination severely. The fragment of Hfr DNA transferred to the recipient cell in conjugational crosses is initially single-stranded and is subsequently converted to ds DNA by lagging--strand synthesis (*20*). In *wt E. coli* conjugational recombination is almost completely dependent on the RecBCD enzyme. RecBCD is involved in the initiation of recombination, and its enzymatic activities, helicase, $5' \rightarrow 3'$ exonuclease and RecA loading are essential for this process (*13*,*21*).

One particular point mutation in the RecB nuclease centre, *i.e.* $recB^{D1080A}$, abolishes nuclease and RecA loading activities of RecBCD enzyme, but retains its helicase activity *in vitro* (*22*,*23*). *In vivo*, it causes a strong recombination-deficient phenotype, similar to that produced by a *recB* null mutant (*21*). It was assumed that this phenotype is due to a RecA loading defect since the missing $5' \rightarrow 3'$ exonuclease activity can be replaced by the RecJ protein which is also a $5' \rightarrow 3'$ exonuclease (*13*). It was shown that Gam inhibits the helicase as well as all nuclease activities of RecBCD enzyme *in vitro* (*7*). Contrary to this, genetic studies have shown that RecBCD-Gam complex is not biologically inert (*7*,*10*,*12*). The presence of Gam protein has a slight effect on conjugational recombination, while the recombinational repair after γ-irradiation mediated by the RecBCD-Gam complex is even more efficient than repair mediated by RecBCD enzyme (phenomenon of GDR) (*9*,*10*).

Since RecA loading activity is essential for recombination, we wanted to test if this activity is preserved in the RecBCD-Gam complex *in vivo*. This could explain the high recombination proficiency of *wt* cells in the presence of Gam. To do this, we compared recombination proficiencies of *wt* and $recB^{D1080A}$ mutant in the presence of Gam protein. We supposed that bacteria with $RecB^{D1080A}CD$-Gam complex ($recB^{D1080A}$ mutant) would show reduced frequency of recombination compared to bacteria containing RecBCD-Gam complex (*wt*) if RecA loading activity of RecBCD-Gam complex is preserved. We transformed *wt* and $recB^{D1080A}$ cells with plasmids that express or do not express Gam, and determined the yields of recombinants after Hfr-mediated conjugation. The results are shown in Table 2.

Data are first presented as recombination proficiencies relative to *wt* strain in the absence of Gam, *i.e.* RecBCD pathway. This is instructive for detecting the effects of Gam protein on recombination in each mutant background. Alternatively, all recombination data concerning the RecBCD-Gam pathway are also presented as recombination proficiencies relative to the *wt* cells in the

Table 2. Recombination in Hfr crosses

Strain	Relevant genotype	Function[e]	Recombination frequency[c]	Recombination frequency[d]
AB1157[a]	wild type	Gam^+	0.68 (±0.38)	1
		Gam^-	1[b]	-
N4634[a]	*recB*	Gam^+	0.033 (±0.017)	0.048
		Gam^-	0.072 (±0.036)	-
RIK174[a]	$recB^{D1080A}$	Gam^+	0.028 (±0.006)	0.041
		Gam^-	0.33 (±0.19)	-
RIK144[a]	*recD*	Gam^+	0.71 (±0.42)	1.04
		Gam^-	0.68 (±0.08)	-
JC12123[a]	*recJ*	Gam^+	0.246 (±0.13)	0.36
		Gam^-	2.2 (±0.47)	-
WA576[a]	*recF*	Gam^+	0.94 (±0.36)	1.38
		Gam^-	2 (±0.44)	-
IRB103[a]	*recO*	Gam^+	0.17 (±0.08)	0.25
		Gam^-	1.2 (±0.48)	-
AM208[a]	*recR*	Gam^+	0.5 (±0.32)	0.73
		Gam^-	2.1 (±0.47)	-
SP254[a]	*recN*	Gam^+	0.27 (±0.18)	0.40
		Gam^-	0.41 (±0.21)	-

[a] Mating was done with Hfr3000 donor, the selected marker was Pro^+

[b] Wild-type frequency of 1.0 = 35 exconjugants per 1000 donors

[c] Values are means of at least three independent experiments, corrected for the viability of recipients; standard deviations are given in parentheses

[d] Recombination frequency relative to *wt* in the presence of Gam

[e] Gam^+ cells contain pSF117 plasmid, Gam^- cells contain pSF119 plasmid

presence of Gam. This is useful for detecting the specific quantitative effect of each mutation on RecBCD-Gam mediated recombination.

The presence of Gam reduces the frequency of conjugational recombination in the *recB*^{D1080A} mutant to the level of a *recB* null mutant (Table 2). Since the recombination proficiency of *wt* cells expressing the Gam protein is high (only slightly weaker than in *wt* in the absence of Gam), we suggest that the RecBCD-Gam complex has RecA loading activity *in vivo*. This activity is probably responsible for such a high recombination level. However, according to our data, the *recB*^{D1080A} mutation reduces recombination by RecBCD pathway just 3-fold, which is an unexpectedly weak effect in comparison with the strong effect of the same allele in the study of Amundsen *et al.* (*21*). A possible explanation for this discrepancy could be different experimental conditions used. In the report by Amundsen *et al*. (*21*), the RecB^{D1080A}CD enzyme was overexpressed since the *recB*^{D1080A,} *recC*, and *recD* genes were provided on a multicopy plasmid. Contrary to this, we used the *recB*^{D1080A} mutation located on the chromosome, which is a more natural condition. Since the *recFOR* gene products have no effect on the RecBCD pathway of recombination (*1*), it is possible that RecA loading in *recB*^{D1080A} mutant in the absence of Gam requires additional factors. If the RecBCD-Gam complex has RecA loading activity, it would be expected that it also retains a weak helicase activity since RecA loading is coordinated with DNA unwinding. An alternative interpretation for a relatively high recombination proficiency of *recB*^{D1080A} strain in the absence of Gam and its low recombination proficiency in the presence of Gam might be that Gam inhibits the helicase activity of the RecB^{D1080A}CD enzyme. Future experiments are needed to solve this problem.

In addition to the strong effect of the *recB*^{D1080A} allele, the effects of *recJ* and *recO* mutations on RecBCD-Gam pathway are weak (2.5-4 fold). Possibly, the effect of *recJ* is stronger (9 fold) if one compares it with the recombination frequency in the absence of Gam. Such stronger effect of *recJ* is in agreement with the previous data (*7*,*10*). Since the RecBCD-Gam complex has no nuclease activities, a possible explanation for the effect of the *recJ* mutation is that RecJ, itself a $5' \rightarrow 3'$ exonuclease, replaces the missing activity of the RecBCD-Gam complex.

The role of alternative RecA loading by the RecFOR system is marginal for the RecBCD-Gam pathway. This view is supported by the data concerning *recF* and *recR* mutations, which definitely have a weak effect on RecBCD-Gam mediated recombination (Table 2). A possible exception could be the result with *recO* mutation because potentially it has a larger effect (7 fold) if compared to the recombination proficiency of a *recO* mutant in the absence of Gam. Possible requirement for RecO is unexpected since it usually displays the same phenotype as *recF* and *recR* mutants. Other data also support the view that RecFOR is not essential for the RecBCD-Gam pathway. As mentioned above, the RecA loading deficiency of the *recB*^{D1080A} mutant in the presence of Gam decreases recombination to the *recB* null mutant level (Table 2). This implies that almost the entire RecA loading activity during the RecBCD-Gam pathway is provided by the RecBCD-Gam complex itself. According to the data by Murphy (*7*), the RecF protein is not involved in RecBCD-Gam mediated conjugational recombination. Paškvan *et al.* (*10*) also demonstrated that GDR is not dependent on RecFOR proteins.

The *recN* mutation has a weak effect and the *recD* mutation has no effect on RecBCD-Gam mediated conjugational recombination. This behaviour of *recN* is different from the one in the RecBCD-Gam mediated recombinational repair where *recN* is required (*10*).

We also compared recombination frequencies in the presence and absence of Gam protein in all used genetic backgrounds. In addition to the *recD* background where the recombination proficiencies in the presence and absence of Gam are almost the same, in other mutants Gam reduces recombination proficiencies. In most cases Gam inhibits recombination slightly (1.5-4 fold; Table 2); exceptions are mutants with strong and moderate effects on the RecBCD-Gam pathway, *i.e. recB*^{D1080A}, *recJ* and *recO*. The inhibitory effect of Gam in these backgrounds is 7-12 fold.

Conclusions

From our results we made the following conclusions: (*i*) we suggest that RecA loading activity of the RecBCD enzyme is not inhibited by Gam *in vivo*, and this could explain the high recombination proficiency of *wt* cells in the presence of Gam; (*ii*) conjugational recombination in the presence of Gam is partially dependent on *recJ* and *recO* gene products.

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Genetička analiza produkata bakterije *Escherichia coli* koji sudjeluju u konjugacijskoj rekombinaciji u prisutnosti proteina Gam faga λ

Sažetak

Gam protein bakteriofaga λ je inhibitor enzimskih aktivnosti enzima RecBCD koji sudjeluje u homolognoj genetičkoj rekombinaciji u bakteriji *Escherichia coli.* U ovom su radu proučavani (*i*) učinak *recB* mutacije deficijentne u nanošenju proteina RecA ($recB^{D1080A}$) na konjugacijsku rekombinaciju u prisutnosti proteina Gam faga λ i (*ii*) učinak mutacija drugih rekombinacijskih gena na konjugacijsku rekombinaciju u bakterijama s kompleksom RecBCD-Gam. Zbog toga smo unijeli plazmide koji eksprimiraju Gam^+ i Gam^- u divlji tip i u različite mutante bakterije *Escherichia coli (recJ*, $recB^{D1080A}$, *recB, recN, recF, recR, recO, recD)*, te odredili prinos rekombinanata nakon Hfr-konjugacije. Dobiveni su rezultati pokazali da aktivnost nanošenja proteina RecA vjerojatno nije inhibirana proteinom Gam. U prisutnosti proteina Gam konjugacijska rekombinacija djelomično ovisi o produktima gena *recJ* i *recO*.