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Investigating the Role of RecD in *Escherichia coli* and its Effects on Tn*10* Transposition

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RecD of the RecBCD enzyme complex is required for exonuclease activity. *RecD* mutants lacking in nuclease activity have been commonly used in the transformation of linear DNA to increase its stability in the cytoplasm, allowing for higher transformation frequency. Since transposition involves a linear DNA intermediate, this suggests that the RecD exonuclease activity may decrease the frequency of transposition. In this study, using a λ phage delivery system, we compared the transposition frequency of Tn*10* in wild type and mutant *recD* strains of *Escherichia coli.* Contrary to our expectations, our results showed a 15–to–20 fold increase of Tn*10* transposition in wild type *recD* as compared to the *recD* mutant *E. coli.* We propose that other host factors linked to the activity of the RecBCD complex, such as RecF, may be involved in the excision of the incorporated Tn*10* from the host genome in the absence of RecBCD activity.

Bacteria are often exposed to adverse environmental conditions that reduce survival rates. One way that bacteria can increase competitiveness in adverse conditions is through incorporation of foreign DNA material. Competitiveness is enhanced when this foreign DNA carries genes conferring favorable traits and is incorporated into the genome of the recipient bacteria (24). Hence, many bacterial genera are proficient in horizontal DNA transfer. However, incorporation of foreign DNA can also lead to detrimental effects. Therefore, bacteria have evolved mechanisms that prevent the persistence of foreign DNA in their cytoplasm.

One cytoplasmic enzyme involved in the degradation of foreign DNA is the multifunctional protein complex, RecBCD (19). RecBCD has several catalytic properties, including ATP-dependent DNA helicase activity, single stranded DNA (ssDNA) endonuclease activity, and ATP-dependent ssDNA and double-stranded DNA (dsDNA) exonuclease activities (1, 8, 19, 27). The numerous catalytic properties of RecBCD enables it to function in homologous recombination, recovery from DNA damage, maintenance of cell viability, and exclusion of foreign DNA (20, 26). RecBCD operates in foreign DNA exclusion through its degradation of linear ssDNA and dsDNA. The nuclease activity in the RecB active site requires the RecD subunit (15, 19). *RecD* mutants have been shown to be transformable with linearized plasmids, unlike their wild type (WT) counterparts (23). Thus, RecD mediated degradation of exogenous DNA is a potent inhibitor of linear DNA transformation. However, the role of RecD in the movement of transposable elements, another linear DNA species, is not well understood.

Transposons are mobile DNA elements that can insert at random or specific target sites within chromosomes (7, 12, 21). They are flanked by inverted repeats and encode for the transposase enzyme, which mediates their insertion and excision (12). Some carry additional genes that encode for antibiotic resistance and thus confer selective advantage to the recipient. However, transposons are also potentially damaging to host DNA. For example, some transposons, such as Tn*7* and Tn*10*, are mobilized via a non-replicative mechanism that generates gaps in the donor DNA (10, 12). When the donor DNA is the bacterial chromosome, transposon mobilization is potentially lethal (10). To minimize these potentially harmful effects while retaining the ability to gain favorable traits, transposon mobilization in bacterial systems is tightly regulated (16).

In the present study, we compared the transposition frequency of Tn*10* in *Escherichia coli* strains with WT *recD* or mutant *recD*. Transposons were introduced into the bacteria using a lambda phage delivery system. Results obtained were unexpected. Instead of a higher transposition frequency due to a lack of exonuclease activity in *recD* mutants, we observed an approximate 15–to–20 fold decrease as compared to the WT. These unexpected results could have been due to the influence of other recombination complexes and pathways that are affected by the presence or absence of a fully functional RecBCD complex. We suggest that the RecF pathway may be involved in the enhancement of Tn*10* excision in mutant *E. coli* strains lacking a functional RecD subunit, leading to the decrease in detectable Tn*10* transposition events based on tetracycline resistance screening.

MATERIALS AND METHODS

Media and Reagents. Luria-Bertani (LB) medium was comprised of 1% tryptone (w/v), 0.5% yeast extract (w/v), 1% NaCl (w/v), adjusted to pH 7 with 1 N NaOH. Phage LB medium comprised of 1% tryptone (w/v), 0.5% yeast extract (w/v), 0.5% NaCl (w/v) and 1 mM $MgCl_2$, adjusted to pH 7 with 1 N NaOH. Top agar consisted of 0.75% agar and was made with Phage LB. Bottom agar consisted of 1.5% agar, made of regular LB.

Strains and Genotypes. All bacteria and phage strains and genotypes used are listed in Table 1. *Escherichia coli* strain R594 encodes the WT *recD* gene while V355 carries the mutant *recD* and confers an exonuclease V–negative phenotype. Lambda λNK561 carries a Tn*10* that encodes for tetracycline resistance.

TABLE 1. Strains and genotypes

Strains	Genotype	Reference
E. coli		
R594	*lac-3350, galK2(Oc), galT22, λ-, IN(rrnD-rrnE)1, rpsL179(strR)*	(4)
V355	*lac-3350, galK2(Oc), galT22, λ-, IN(rrnD-rrnE)1, rpsL179(strR), recD1014(Nuc-)*	(6)
SG265	*ara, argE*am, *gyrA, thi,* Δ(*gpt-proAB-argF-lac*)XIII, *supP(P1cry*), *rpoB*	(17)
λ phage		
Wild-type		(9)
NK561	*b*221 *c*I171::Tn*10 c*1857 *O*am29 *P*am80	(17)

Phage amplification. *Escherichia coli* SG265 was grown overnight in LB broth. Two hundred microlitres of the overnight culture was mixed with 3 mL of molten top agar (held at 45°C) and poured onto Phage LB plates (bottom agar). To ensure solidification, plates were refrigerated at 4°C for 1 h. Thawed λNK561 lysate was streaked on the solidified top agar and then incubated overnight at 37°C. Five to six plugs were removed with Pasteur pipettes from the edges of the clearing of the bacterial lawn. Plugs were resuspended in 40 mL of LB broth and incubated overnight with shaking at 37°C. The following day, the phage-bacteria culture was spun at 4,000×g for 10 min and the supernatant collected constituted the phage lysate. Chloroform was added to the lysate at a final concentration of 5% (v/v). Secondary amplification was performed on the lysate using serial dilutions to achieve complete clearance of the bacterial lawn grown on Phage LB plates. The top agar of the cleared plates was scrapped and 5 mL Phage LB was added to allow for phage diffusion. The plates were incubated overnight at 4°C. The top agar lysate suspension was collected and centrifuged at 5000×g for 10 min. The lysate was filtered through a 0.2 μm-diameter pore-sized filter and stored at 4°C until further use.

Phage Titering. *Escherichia coli* SG265 was grown overnight in LB at 37°C. A one-in-sixty dilution of the overnight culture was used to inoculate 120 mL of 0.2% maltose-supplemented LB broth, which was grown at 37°C in a shaking water bath for 4h. Phage lysate was diluted at 10-fold increments with Phage LB. One hundred microlitres of each dilution was added to 500 μL of the 4 h bacteria culture. This phage-bacteria mixture was incubated at 37°C for 15 min. Following incubation, the phage-bacteria mixture was added to 3 mL of molten top agar and poured onto the bottom agar. These plates were incubated overnight at 37°C and plaques were enumerated the following day.

Transposition Assay. *Escherichia coli* strains V355 and R594 were grown overnight in LB at 37°C. One-in-sixty dilution of the overnight cultures were used to inoculate 120 mL of 0.2% maltose, 50 μg/mL streptomycin supplemented Phage LB and grown for 4 h at 37°C to a concentration of approximately 4.5 x 10^9 cells/mL. Cell density was determined by measuring the optical density (OD) by spectrophotometry at 660 nm. One milliliter aliquots of each bacterial strain were treated with phage at a multiplicity of infection (MOI) of 1, 7, and 0 (mock infection). Each mixture was incubated for 1 h at 37°C to ensure phage adsorption and subsequent transposon mobilization. The phage-bacteria mixtures were washed three times with LB, and then the cells were resuspended in l mL of LB. Cells were plated at final plated dilutions (FPD) of 10^{-1} to 10^{-5} onto LB agar plates supplemented with 50 μg/mL streptomycin and 50 μg/mL tetracycline (ST plates). Resuspended cells were

also plated on LB plates supplemented with 50 μg/mL streptomycin (S plates) at an FPD of 10^{-6}, 10^{-8}, and 10^{-10}. All were incubated at 37ºC for at least 24 h.

Lysogen Superinfection Assay. Three vertical streaks of WT λ phage lysate were made on ST plates. Colonies positive for tetracycline resistance (see above section) were streaked perpendicularly across the lysate streaks. Plates were incubated overnight at 37°C and observed for clearing at the intersections between the lysate and bacterial streaks.

RESULTS

Effect of MOI and RecD on transposition frequency. Figure 1 represents the transposition frequency of Tn*10* using a λ phage (NK561) delivery system with *E. coli* R594 (WT *recD*) and V355 (*recD1014*) strains as the recipients. Selection of bacterial cells with Tn*10* incorporation into chromosome was indicated by growth on ST plates. For the WT *recD* strain, infection at MOI of 7 conferred a transposition frequency that was 3-fold higher than that at MOI of 1. For the *recD1014* strain, we observed a 5-fold difference in frequency when infecting with an MOI of 7. This suggests that infection with a higher MOI correlates with a higher transposition frequency. Figure 1 also shows the difference in transposition frequency between the WT *recD* and *recD1014* strains. At MOI of 7, we observed approximately 16-fold greater frequency in WT *recD* than in *recD1014*. Similarly, at MOI of 1, we observed a 23-fold greater frequency in WT *recD* than in *recD1014*. This suggests that there is a decrease in the transposition frequency in the mutant *recD* strain compared to the WT *recD*. As expected, the mock infection (data not shown) produced no growth of tetracycline-resistant colonies.

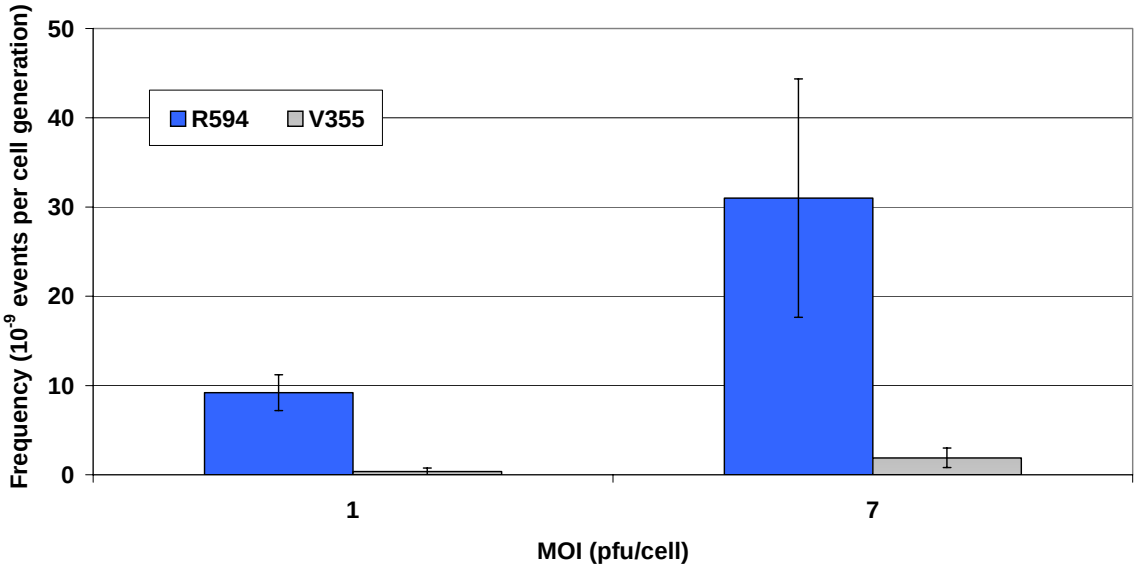

Figure 1. Frequency of Tn*10* transposition events in *E. coli* R594 (WT *recD*) and V355 (*recD1014*) strains. Error bars represent ± one standard deviation.

Effect of phage infection on cell viability. We sought to determine whether phage infection affected the viability of WT *recD* and *recD1014* cells. Table 2 shows the bacterial concentration (cfu/mL) of both strains at various MOIs grown on S plates. The average bacterial concentration ranged from 5.9 to 7.4 x 10^9 cfu/mL for both strains at varying MOIs. This suggests that neither phage infection nor the differences in the genotype between the two strains affected cell viability significantly and cannot account for the observed differences in transposition.

Lysogen Superinfection Assay. To rule out the possibility that the observed transposition frequency was affected by lysogeny, we performed an assay to determine if the tetracycline-resistant cells contained the λNK561 lysogen. Lysogen-containing cells are resistant to superinfection. Cell lysis was observed after infection with WT λ phage (data not shown), indicating the absence of a lysogen.

Table 2. *E. coli* bacterial concentration after infection with λNK561 at different MOIs

MOI (pfu/cell)	Strain ($\times 10^9$ cfu/ml ± 1 SD*; n=4)	
	R594	**V355**
1	7.4 ± 1.8	7.2 ± 1.9
7	6.0 ± 1.7	5.9 ± 1.5
0 (control)	5.7 ± 1.7	5.9 ± 1.6

DISCUSSION

We hypothesized that λNK561::Tn*10* infection of a *recD1014 E. coli* strain, with attenuated exonuclease activity, would result in the prolonged existence of the Tn*10* element within the cytosol, thereby increasing the likelihood of a transposition event. However, the results shown in Figure 1 were contrary to our hypothesis. The transposition frequency in the WT *recD* strain was about 15-20-fold greater than in the *recD1014* strain.

There has not been any literature that suggests a direct link between the functions associated with the presence of RecD in the RecBCD complex and the transposition of Tn*10*. The functions of the RecBCD complex are presumably involved in homologous recombination and DNA repair. Studies have shown that Tn*10* transposition is linked to DNA repair mechanisms through recombination when double stranded breaks occur due to transposon mobilization (2, 16). However, *recD* mutants are hyperrecombinogenic (3), which may imply more efficient DNA repair of double stranded breaks. Inefficient repair of double stranded breaks results in lower cell viability, which may lead to an underestimation of the true transposition frequency. This implies that the WT would have a lower observed transposition frequency. Therefore, our experimental results cannot be explained by this proposed mechanism.

Research using RecBCD mutants have found that residual recombination is mediated by RecF and its associated proteins such as RecO and RecR (11, 19). However, the exact details of how RecF or the RecB recombination pathways are involved have yet to be found (5). Studies suggest that the RecF system increases the recombination efficiency to near the WT level in cells without RecBCD nuclease and exonuclease I (SbcB) activity (29). The RecBCD complex is involved in the degradation of a DNA intermediate of the RecF pathway, inferring that the absence of RecBCD nuclease activity promotes the RecF pathway. Studies have shown that enhanced RecF pathway leads to the induction of precise excision of Tn*10* from the host chromosome (22). Based on this model, we hypothesize that our *recD1014* mutant has an enhanced RecF pathway, which promotes Tn*10* excision, thus resulting in a lower number of cells retaining the transposon with tetracycline resistance. The extent to which the RecF pathway is enhanced due to nuclease attenuation remains unknown, and will require further study. The actual mechanisms behind this model may be more complex than what we have described above. Studies have correlated the RecF enhanced excision of Tn*10* with a *sbcB* (exonuclease I) mutation (14, 19). However, we do not know the significance of exonuclease I in our experimental system since no studies have been done to directly correlate Tn*10* to *sbcB*.

Conversely, in the WT *recD* strain we propose that the RecF pathway functions at a low basal level as compared to the enhanced level in *recD1014* mutant strain. Therefore, transposon excision from the host is decreased, leading to the observed retention of tetracycline resistance on the bacterial genome. The frequencies shown in Figure 1

suggest that net transposition is occurring at a higher rate in WT *recD* than in *recD1014*. However, the transposition frequency may have been underestimated in *recD1014* due to the increased excision of Tn*10* from the bacterial chromosome through the function of the RecF pathway. Consequently, the actual difference in transposition frequencies may have been masked by our proposed mechanism as outlined in Figure 2. In addition to considering the rate of transposon insertion into the bacterial chromosome, the rate of excision, and consequently, the loss of tetracycline resistance undermines our ability to screen for transposition events.

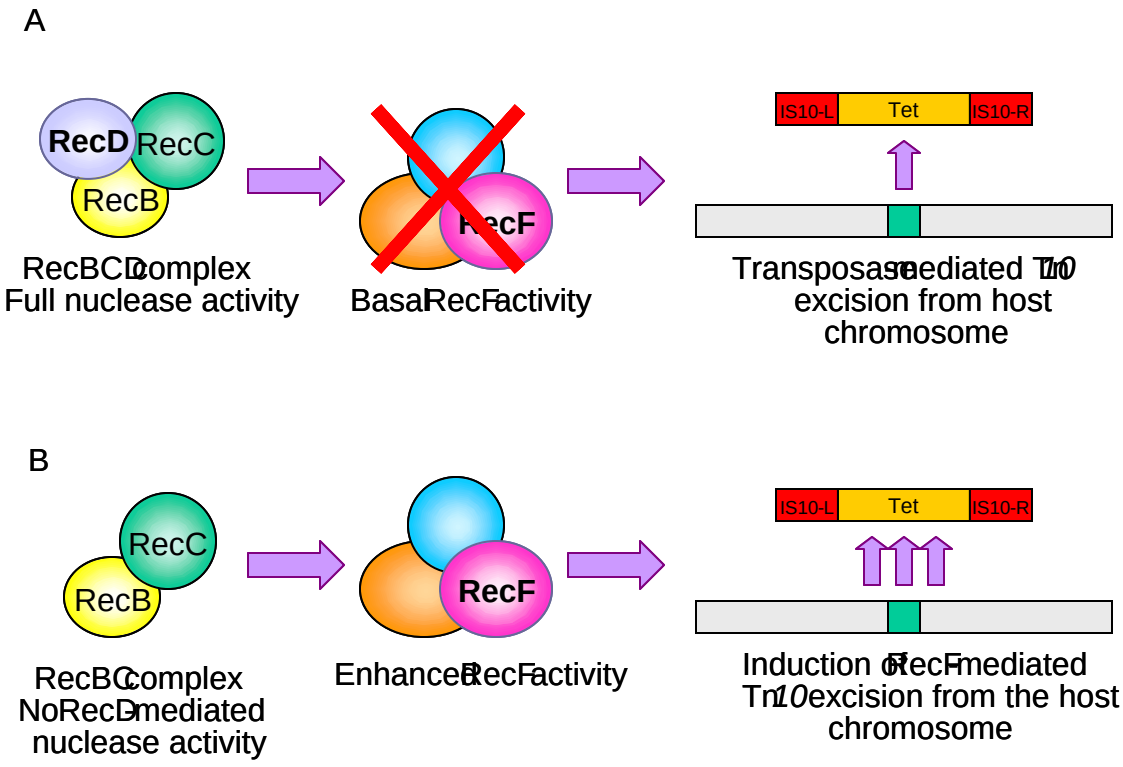

FIG.2 Model of Tn*10* excision through the RecF pathway as a consequence of reduced RecBCD nuclease activity. **A** Tn*10* excision is not induced by the RecF pathway due to the presence of complete RecBCD complex. **B** Tn*10* excision induced by the RecF pathway due to the lack of RecD-mediated nuclease activity.

In our system, we wanted to determine the effects of different MOIs on transposition frequency. Figure 1 shows that the transposition frequency appeared to be higher when infecting at MOI of 7 in both WT *recD* and *recD1014* strains compared to infecting at MOI of 1. Using the Poisson distribution, found on the Multiplicity of Infection Website (http://www.life.uiuc.edu/micro/316/topics/phage/moi.html), we calculated that at an infection of MOI 7, there is a greater than 99% chance that all bacterial cells would be infected with phage, whereas at an infection of MOI 1, there is an approximately 67% chance that all bacterial cells would be infected with phage. The increased infection rate suggests that transposition frequency should also increase by approximately 1.5-fold. In contrary, the observed transposition rate between MOI 7 and 1 increased by 3 fold in our experiment. However, the error analysis in Figure 1 shows uncertainty in transposition frequencies of the two MOIs falls in the range of a 1.5-fold difference. There was a concern that phage infection or the genotypic differences in our two strains would affect cell viability and consequently introduce bias into our results (28). Table 2 indicates that there is no significant difference in cell viability due to phage infection of the WT and *recD1014* strains. Therefore, the data is not biased by differences in cell viability due to phage infection.

Normal Tn*10* transposition frequency is approximately 10^{-7} transposition events per cell generation (16). However, our results showed a range of frequencies between 10^{-8} and 10^{-10}. The low transposition frequencies may have been due to methylation of the promoter region of the Tn*10* transposase gene during phage propagation by DNA adenine methylase in the host cells (12). Since DNA is temporarily unmethylated following replication, Tn*10* transposase expression on bacterial genomes is optimal during this period and accounts for the 10^{-7} transposition frequency previously observed (12). In our phage delivery system, λNK561 acts as a suicide vector, which remains unreplicated. λNK561 thus carries a transposase gene that is persistently methylated and thus reducing the transposition frequency.

Cells infected with lysogens are resistant to subsequent infection and lysis (13). Therefore, the re-infection of cells with a WT λ phage, would result in lysis of cells that did not contain a lysogen. The observed cell lysis in our phage superinfection assay indicated that the tetracycline-resistant cells resulted from the incorporation of a transposon, not of a lysogen, into the host chromosome. The assay results agree with the documented genotype the λNK561 phage, which contains a *b*221 mutation that negates the phage's ability to undergo lysogeny in *E. coli* (17). The data obtained from plated colony counts following the transposition assay are equally outside the reliable range of enumeration, which is between 30 and 300 cfu/plate. Despite this fact, the counts were still likely to be within the correct magnitude. This range of precision is acceptable for the purpose of this study.

It is apparent from the results of our study that *recD1014* mutation decreases the observed transposition frequency of Tn*10*. However, it remains to be determined whether the RecD dependent exonuclease activity directly affects transposition.

FUTURE WORK

In our experiment, one variable that was not controlled for was secondary transposition, or the intragenomic mobilization of the transposon following translocation from the phage genome into the bacterial genome. In addition, the observed transposition frequency from the phage genome into the host genome would be decreased due to loss of Tn*10* and its encoded tetracycline resistance. To eliminate this variable, we propose to repeat the experiment using a mini-Tn*10* system, where the transposase is encoded outside of the inverted repeats. The loss of the transposase gene following transposition into the host genome ensures the stability of the transposon within the recipient.

In addition, we propose to repeat the experiment using a circular suicide plasmid mini-Tn*10* delivery system rather than a phage delivery system. During phage infection, phage genome is injected as a linear DNA molecule, which may be susceptible to degradation by RecBCD exonuclease activity. Thus, transformation with a circular plasmid would eliminate the possibility of losing the transposon due to the degradation of the linear phage genome prior to the mobilization of the transposon.

Furthermore, to test whether RecF-mediated excision of Tn*10* increases in *recD*$^-$ mutants, we propose to carry out mutational analysis of RecF using an *E. coli* strain with inducible mutations (such as temperature sensitive mutations) in *recD*, *recF* and a mini-Tn*10* inserted in *lacZ*. If RecF-mediated excision of Tn*10* is increased in *recD* mutants, there would be an increased reversion from *lacZ*$^-$ to *lacZ*$^+$ upon precise excision of the mini-Tn*10*.

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