Effects of recB, recC and recF mutations on Tn10 Transposition in Escherichia coli

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Both RecBC and RecF are involved in the regulation of Tn10 in the genome of transposed Escherichia coli cells. Depending on the conditions to which cells are exposed, either pathway could play the dominant role in the precise excision of the transposon. Previous studies have suggested that the RecF pathway plays a greater role in the precise excision of Tn10 from the E. coli chromosome. In the current study, we attempted to understand the role of RecF in transposon excision by comparing the transposition frequencies between wild type, a recBC mutant and a recBCF mutant. Our results indicate that the RecBC excision pathway has a greater effect on Tn10 transposition frequency. Varying MOIs and tetracycline concentrations also had an effect upon observed transposition frequencies. We propose that both RecBC and RecF excision pathways are activated in the recD mutant. It is possible that the RecBC-related DNA recombination machinery, which is required for Tn10 excision DNA, is partially functional in the recD mutant; at the same time, the RecBCD inhibition on the RecF pathway has been lifted, allowing this pathway to be activated as well.

The RecBCD enzyme in Escherichia coli plays an important role in the exclusion of foreign genetic material from the host cell’s genome (22). This heterotrimeric enzyme complex is comprised of one copy of RecB, RecC, and RecD (3). RecBCD, also known as exonuclease V (ExoV) is the most potent exonuclease in E coli; it also serves as a DNA helicase, DNA-dependent ATPase, and an ATP-dependent single-stranded DNA (ssDNA) exonuclease (14, 21,23). Such potent catalytic activity allows RecBCD to play crucial roles in the homologous recombination and repair of DNA, and the degradation of linear foreign DNA (3, 14). In a previous study, Acedillo et al. (1) used a recD mutant to investigate the role of RecBCD in the transposition of Tn10 in E. coli. They hypothesized that RecBCD might play a role in the regulation of transposon mobilization by degrading these transferable exogenous DNA fragments, hence lowering transposition frequency. Such regulation is critical to the bacteria’s survival because allowing non-replicative transposons such as Tn10 to freely mobilize between cells may generate lethal gaps in their genomes (16). In their study, Acedillo et al. (1) found that transposition frequency of Tn10 in recD mutants was unexpectedly lower than that of the wild type. They attributed these results to a Tn10 excision pathway involving RecF. Acedillo and his colleagues also postulated that this pathway is inhibited by functional RecBCD, thus less transposed Tn10 were removed from the genome of wild type E coli. In contrast, RecF Tn10 excision is no longer inhibited in the recD mutant, in which RecBCD is non-functional.

According to Nagel and Chan (25), both RecBCD and RecF participate in the precise excision of Tn10. In addition, they suggest that the RecB or RecF excision pathway may impart a greater effect upon transposition frequency, depending on environmental conditions the cells are exposed to (24). Acedillo et al.’s (1) results suggest that the RecF pathway plays a greater role in Tn10 excision among cells that have not been subjected to DNA damaging conditions. They have shown that in wild type E. coli, where the RecBCD excision pathway is predominant, excision frequency is lower compared to that which is observed in the recD mutant, in which the RecF pathway is predominant. In the current study, we attempted to validate Acedillo et al.’s (1) findings by comparing the transposition frequency of Tn10 using a lambda phage delivery system in three E. coli strains containing either wild type recBC and recF, mutant recBC, or mutant recBCF. Studies have demonstrated that either mutated recB or recC will mimic recD mutations because RecB and RecC are both essential components of RecBCD (3, 28). If Acedillo et al.’s (1) findings can be replicated, a higher transposition frequency in the wild type E. coli compared to the recBC mutant should be observed. We also hypothesized that the recBCF mutant will have the highest transposition frequency because both the RecBCD and RecF excision pathways are disrupted. Our results indicate that the recBC mutant has a higher transposition frequency than the wild type, which contradicts with Acedillo et al.’s data. We suggest that the low transposition frequency observed in the recD mutant is due to activity from both the RecBC and RecF excision pathways, as studies have shown that
that RecBC DNA recombination machinery, which is required for the precise excision of Tn10, is still functional in this mutant. Moreover, E. coli with recBCF showed the lowest transposition frequency out of the three strains. This unexpected result could be attributed to cell death caused by lethal amount of the TetA proteins expressed from Tn10 in the recBCF mutants (8).

METHODS AND MATERIALS

Strains and Genotypes. Bacteria and phage strains used in this study are described in Table 1. E. coli AB1122 carries wild type (WT) recB, recC, and recF genes, while JT1 and JC8111 carry mutations in the recB, recC, recF, and recB, recC genes, respectively. LNK561 phage carries the Tn10 translocatable insertion element encoding for a tetracycline resistance marker.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotypic Characteristics</th>
<th>Source or Reference</th>
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<tbody>
<tr>
<td>E. coli</td>
<td>recB::Tn10, argE7::Tn10, 32684::Km, 32684::Tc</td>
<td>CCGC (112)</td>
</tr>
<tr>
<td>AB1122</td>
<td>recB::Tn10, argE7::Tn10, 32684::Km, 32684::Tc</td>
<td>CCGC (112)</td>
</tr>
<tr>
<td>JC8111</td>
<td>recB::Tn10, argE7::Tn10, 32684::Km, 32684::Tc</td>
<td>CCGC (112)</td>
</tr>
<tr>
<td>JT1</td>
<td>recB::Tn10, argE7::Tn10, 32684::Km, 32684::Tc</td>
<td>CCGC (112)</td>
</tr>
<tr>
<td>LNK561</td>
<td>E. coli B21111317h: Tn10::hC37 LamA::Pha102d</td>
<td>(17)</td>
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Media and Reagents. ‘Regular’ LB medium was comprised of a solution of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl, adjusted to pH 7.0 with 1N NaOH. Phage LB medium consisted of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, and 1 mM MgCl2, adjusted to pH 7.0 with 1N NaOH. Phage titering plates were comprised of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, and 1 mM MgCl2, adjusted to pH 7.0 with 1N NaOH. Phage titers were obtained by plating 0.1 mL of the 4h culture, and incubated for 15 min at 37°C. After incubation, the phage-bacteria mixture was added to 5 mL of molten top agar maintained at 48°C, and poured onto bottom agar plates pre-warmed to 37°C. Plates were incubated at 37°C overnight and inspected for plaques the following day.

Transposition Assay. Escherichia coli strains AB1122, JT1, and JC8111 were grown overnight at 37°C in LB with supplements. Cell densities were determined through spectrophotometric measurements of optical density at 660 nm (Spectronic 20D+, Milton Ray), and normalized to 1 x 10^7 cells/mL by diluting with Phage LB with supplements. 500 µL aliquots of each strain culture were infected with LNK561 phage at multiplicities of infection (MOI) of 1, 3, 7, and 0 (mock infection), and incubated at 37°C for 1h to allow for transposition mobilization. To eliminate unbound phage, cells were washed three times by pelleting at 11,000xg for 1.5 min, followed by resuspension in 500 µL of LB. To determine transposition frequencies, cultures were plated at final plated dilutions (FPD) of 10^-5, 10^-6, 10^-7, and 10^-8 g/mL and 10^-9 g/mL. 50 µL of transposition events, and to determine corresponding viable cell counts, respectively. 50 µg/mL and 10 µg/mL of tetracycline were used for the first and second trials, respectively. Plates were incubated for 24 to 48 h at 37°C.

Lysogen Superinfection Assay. WT A phage preparation was obtained from heat induction of a lysogenic culture of E. coli W3350(λ). Lysates were clarified of cell debris via centrifugation at 11,000 xg for 2 min, and followed by chloroform treatment. Tetracycline resistant colonies from the transposition assay were streaked on ST plates, and three drops of each lysate were deposited at particular internals along each streak line. Plates were incubated at 37°C overnight, and observed for areas of clearing along each streak line.

RESULTS

Effect of MOI on transposition frequency. Figure 1 shows the transposition frequencies of Tn10 in E. coli AB1122 (WT recBC), JT1 (recBC) mutant, and JC8111 (recBCF) mutant following LNK561 infection. Transposition events were indicated by growth on ST plates. For the WT and recBC mutant, the transposition frequencies at an MOI of 1 were 2 and 5 times higher than that at MOIs of 3 and 7 respectively. For the recBCF mutant strain, a 3-fold difference was observed between infections at MOIs of 1 and 7. This suggests...
Effect of tetracycline concentration on transposition frequency. At a tetracycline concentration of 10 µg/mL, the transposition frequency for our WT strain was, on average, approximately 3-fold higher across the three MOIs relative to treatment at 50 µg/mL (Fig. 1A, 1B). Similarly, a 7-fold increase in frequency at the lower tetracycline concentration was observed for the recBC mutant. For the recBCF mutant, transposition frequency was 2 times higher at the lower concentration at an MOI of 1, although there was no significant difference at the other MOIs. Overall, the dominant trend suggests that there is a negative correlation between tetracycline concentration and transposition frequency.

Effect of RecBC and RecF on transposition frequency. Figure 1 also shows the differences in transposition frequencies of the WT, recBC mutant, and recBCF mutant strains. For the cells subjected to 10 µg/mL of tetracycline, the transposition frequency of the recBC mutant, at MOI of 1, was 5 and 8 times greater than the WT and recBCF mutant respectively. At MOI of 3, there was a 3 and 11-fold increase in frequency for the recBC mutant relative to WT and the recBCF mutant. A similar trend is observed at MOI of 7, as well as for the cells cultured at a tetracycline concentration 50 µg/mL. This suggests that the recBC mutant has the highest transposition frequency, followed by the WT and recBCF mutant.

Effect of MOI on cell viability. Table 2 lists the cell concentrations of E. coli AB1122 (WT), JT1 (recBC), and JC8111 (recBCF) infected by λNK561 at different MOIs, as determined by plating on media with streptomycin. Results were averaged between two trials, with the same cell samples plated each time. We felt this approach was reasonable, as the only parameter that was changed between the two trials was the concentration of tetracycline on the ST plates, which had no direct influence on the survival rates of cells grown on S plates. Across all strains, cell viability declined as MOI was increased. Compared to an infection using an MOI of 1, a 77% decrease in colony forming units per mL of WT occurred when an MOI of 3 was used, and a 94% lower cell concentration was found at an MOI of 7. Similarly, there was a 12% decrease in cell viability of the recBC mutant at MOI=3 and a 40% level of cell loss at an MOI of 7, relative to an MOI of 1. Displaying the same trend, recBCF mutant showed 31% and 89% lower cell viability at MOIs of 3 and 7, respectively.

Superinfection Assay. To ensure that all instances of acquired tetracycline resistance observed were due to Tn10 transposition and not λNK561 lysogeny, a superinfection assay was carried out. Lysogenized cells are immune to lysis by subsequent lambda phage infections. No lysis was observed for all tested colonies; however, uninfected control colonies were
Table 2: Bacterial Concentrations of E. coli following infection with λNK561 at varying MOIs.

<table>
<thead>
<tr>
<th>MOI (pfu/cell)</th>
<th>Strain (× 10⁶ cfu/mL)</th>
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<tbody>
<tr>
<td></td>
<td>AB1122</td>
</tr>
<tr>
<td>1</td>
<td>32.4</td>
</tr>
<tr>
<td>3</td>
<td>7.4</td>
</tr>
<tr>
<td>7</td>
<td>2.1</td>
</tr>
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</table>

not lysed by the phage either, suggesting that insufficient phage concentrations were used to perform the assay. It should be noted that the $cI$ gene, required for lysogenic growth, was inactivated in $\lambda$NK561 (17). Furthermore, CI possesses an additional property as a suppressor of lytic growth in lysogenized cells (28). Thus, in the unlikely event of lysogenization, lysis would not have been prevented regardless, due to a deficiency in $cI$ (12).

**DISCUSSION**

We hypothesized that if Acedillo et al.’s (1) results were replicated in the current study, a lower transposition frequency should be observed in the recBC mutant when compared to the wild type, as the recBC mutant should produce a similar phenotype as a recD mutant (3, 28). From our results (Fig. 1A, 1B), the recBC mutant, in which the RecF Tn10 excision pathway predominates, exhibited transposition frequencies 3 to 11 fold higher than those of the wild type, which utilized the RecBC Tn10 excision pathway instead. These results imply that RecBC normally plays a greater role than RecF in inducing precise excision of Tn10 because the wild type showed a much lower frequency, compared to the recBC mutant, where RecF predominated. Acedillo et al.’s (1) findings suggested the opposite.

The discrepancies between the data of the two studies led us to further investigate the phenotypes of recBC and recD mutations. Although essential for the exonuclease activity of RecBCD, RecD is not required by E. coli to carry out DNA recombination and repairing, mechanisms (2, 24). RecBC by itself is a potent helicase capable of interacting with RecA, the key protein in homologous recombination (4). Null mutations in the recB and recC genes, however, will eradicate all enzymatic activities of RecBCD, thus decreasing recombination proficiency by 100 to 1,000 fold (4, 24). Although the exact mechanism is not well understood, the recombination proficiency of a cell is related to its ability to carry out precise excisions of transposons. Machinery involved in recombination, replication, and DNA repair are involved in the spontaneous deletion events akin to transposon excisions (25). It is therefore possible for the recD mutant to exhibit Tn10 excision via the RecBC pathway, since RecBC still possess recombination functions. At the same time, the RecF pathway is also activated because it is no longer fully inhibited by functional RecBCD (21). We suggest that the combined Tn10 excision capacity conferred by the suboptimal activation of these two pathways is greater than the excision proficiency expressed by the RecBC or RecF pathway alone. This model explains why the recD mutant had the lowest transposition frequency compared to wild type and the recBC mutant.

Results from the current study also indicate that the recBCF mutant had the lowest transposition frequency. These results suggest that RecBC and RecF may also play a role in the initial transposition of Tn10 into the E. coli genome, which normally involves transposase encoded by the IS10 elements of Tn10 (27). However, according to our hypothesis, the opposite outcome was expected, as the combined mutations of recBC and recF should have additive effects in diminishing Tn10 excision (25). One possible explanation for this unexpected result is that under conditions of reduced excision, multiple transposed Tn10 may have persisted in the genome of the recBCF mutant. Upon exposure to tetracycline, expression of the tetA gene encoding the tetracycline efflux pump is induced, hence conferring antibiotic resistance to the host cell (15, 26, 28). However, as the number of copies of Tn10 present in the cell’s genome increases, more TetA will be expressed and inserted into the cell membrane, thus
resulting in altered and compromised membrane potential and integrity, ultimately leading to cell death (8). Following this logic, the recBCF triple mutant likely experienced the highest rate of Tn10 transposition owing to the lack of functional Tn10 excision mechanisms; however, many of these cells may have been killed due to a lethal amount of TetA incorporated into their cellular membranes. This cell death significantly decreased the number of colonies observed and the transposition frequency.

Contrary to Acedillo et al.’s (1) results, we also found that infecting E. coli with a higher MOI decreased both the transposition frequency (Fig. 1A, 1B) and cell viability (Table 2). For all three strains, the transposition frequencies at an MOI of 1 were 2 to 5 times higher when compared to those observed at MOIs of 3 and 7. Cell viability declined between 40% to 94% as cells were infected with higher MOIs; this trend was observed across all three strains. As phages are suspended freely amongst the E. coli cells, the number of infections per cell, at a given MOI, should theoretically follow a Poisson distribution (6, 18, 19). Based on such a distribution, the chances of a cell becoming infected by multiple phages during the course of incubation is 24%. This figure rises to 80% with an MOI of 3, and the odds of multiple infections essentially reaches 100% when an MOI of 7 is applied. Therefore, it is counterintuitive for transposition frequency to decrease with increased MOI, as increasing phage numbers should correspond to higher transposition.

The NK561 phage used contains amber mutations in the lambda O and P genes, which prevent lytic growth, as well as a mutation in the cI gene, which prevents the phage from lysogenizing (17). No other phage gene deletions or changes were described. It is likely, then, that the remaining unaltered phage genes were expressed at normal levels during NK561 infection of E. coli. Despite the phage being unable to replicate, with more copies of the λNK561 genome in cells infected at higher MOIs, more resources are devoted to synthesizing viral proteins, potentially inhibiting cell growth and leading to host killing (19). These factors could explain the progressive decline in the number of viable cells observed on both S and ST plates as the starting MOI was increased. Also, cells infected at higher MOIs were more likely to die when they were exposed to tetracycline because of compromised membrane integrity due to high expression of TetA (as described above). This further cell death may have caused the transposition frequencies to decrease as MOI was increased.

The concentration of tetracycline also had an effect on transposition frequency. Cells grown under lower a lower tetracycline concentration (10 g/ml) showed frequencies 3 times higher on average compared to cells grown under a higher tetracycline concentration (50 g/ml) (Fig. 1A, 1B). It is possible that the TetA efflux pumps in the cells become saturated under higher tetracycline concentration (>30 g/ml), causing tetracycline, which enters the cell via passive diffusion, to accumulate within the cell and cause cell death (5, 8, 11, 28).

It is important to note that some of the plated colony counts used to determine transposition frequency are out of the reliable range of 30 and 300 cfu/plate. However, the accuracy of these counts was probably acceptable for the current study, as most of the trends were observed across the two sets of plating.

The results from the current study suggest that RecBC normally plays a bigger role in Tn10 transition than RecF, even though RecF could potentially play a bigger role under other growth conditions (25). We propose that both excision pathways are activated in the recD mutant, thus conveying higher Tn10 excision proficiency compared to wild type and the recBCD mutant where only the RecBC and RecF pathway are activated respectively. We also noted a correlation between the observed transposition frequency and experimental conditions such as MOI and antibiotic frequencies.

**FUTURE EXPERIMENTS**

To validate the current model regarding the activation of both RecBC and RecF excision pathways in the recD mutant, we could conduct an experiment comparing the transposition frequency of a recD mutant, a recDF mutant, and a recBCD mutant. If the recDF and recBCD mutants exhibit higher transposition frequencies (hence lower excision proficiency) than the recD mutant, it will be safe to infer that both RecBC and RecF are involved in the precise excision of Tn10 in recD. Alternatively, if recBCD and recD exhibit the same levels of transposition frequency, then it is likely that the recombination proficiency conferred by RecBC in the recD mutant has no effect on Tn10 excision. The discrepancies between data from Acedillo et al’s study and the current experiment then have to be explained by a new model.

Furthermore, in order to eliminate any negative effects imposed by unnecessary lambda viral gene expression on cell viability, we propose performing this experiment using a suicide plasmid delivery system, instead of the current λNK561 phage transduction setup. Ideally, a non-replicating plasmid encoding a mini-Tn10, from which the transposase gene has been deleted, would be used, with the functional transposase encoded at a separate site on the plasmid. This would guarantee that only those factors involved in Tn10 transposition are present, minimizing disturbances that
could otherwise impair normal cell health and behaviour.

Additionally, having the transposase located apart from the transposon itself and remaining on the vector at all times would ensure that no secondary transposition events could occur once the initial Tn10 insertion took place (10). It was suggested that the lambda genome, which is linear prior to infection, gets degraded when it enters the host cell, and hence a plasmid would confer the additional advantage of providing more time for a transposition event to occur (1). However, it has been demonstrated that the lambda genome immediately circularizes at its cos sites upon infection, and thus degradation should not be of major concern (23).

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