Journal of Experimental Microbiology and Immunology (JEMI) Vol. 7:43-48

Potential Synergistic Effects of Rifampicin Resistance and *rec* Mutations on Growth Rate in *Escherichia coli*

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Both the RecBCD pathway, known for its involvement in homologous recombination and repair of double stranded DNA breaks, and the RecFOR pathway may recognize the linear fragments of DNA that are introduced to the cell during transposition, affecting the transposition frequency. The exact nature of how Rec pathways are involved in transposition is a current topic of research. In this study, using a suicide plasmid system, we attempted to compare transposition frequencies of Tn*5* in various *Escherichia coli rec* mutants. Unfortunately, our results did not provide further insight into the involvement of RecBCD and RecFOR in transposition. However, they do suggest that rifampicin should not be used as a selectable marker for future studies involved in the Rec pathways.

Transposons are mobile segments of DNA that are integral to the horizontal gene pool. Movement of these genetic elements can result in various DNA rearrangements. While the insertion of a transposon can be advantageous to the recipient, for example by introducing genes conferring antibiotic resistance, it can also be potentially dangerous. Insertion within a gene generates mutations and insertion upstream of a gene can result in the activation of the gene (19). Consequently, transposition is maintained at a low frequency to ensure the survival of the host and, therefore, the survival of the transposon (21, 10).

In essence, these elusive transposons are simply a segment of DNA sequence flanked by two inverted repeats. Transposase, the enzyme responsible for excision and insertion of the transposon, is usually encoded within the inverted repeats (21). Tn*5* is a specific transposon that is excised by the conservative cut and paste transposition (10) mechanism. When it is excised from the donor DNA molecule, it exists as a linear, double stranded DNA (dsDNA) fragment until it is inserted into the recipient DNA molecule by the transposase (21).

The RecBCD pathway, which is known for its involvement in homologous recombination and repair of dsDNA breaks, may also recognize the linear fragments of DNA that are introduced to the cell by transposition. RecD is a subunit of the RecBCD complex that is responsible for activating the nuclease function of the RecBC complex. Without RecD, the RecBC complex has helicase activity, but no nuclease activity (12, 15).

The role of RecBCD and RecFOR enzyme pathways in the process of transposition has not been extensively studied. One would expect that the linear, dsDNA molecule involved in transposition may be seen by the RecBCD and RecFOR pathways as a dsDNA break (26). Therefore, one would anticipate the transposition frequency in *recD*$^-$ mutants to be higher than in wild type (WT) because RecD cannot activate the nuclease function of RecB to degrade the transposon. However, recent research into this topic has shown a decreased transposition frequency in *recD*$^-$ mutants (1).

Interestingly, it has been shown that *recD*$^-$ mutants are still able to catalyze homologous recombination and DNA repair at high rates. It appears that in these cells, the nuclease function is being overtaken by the RecJ exonuclease of the RecFOR pathway (26). The RecFOR pathway usually mediates recombination initiated at single stranded DNA breaks, but it can also act at dsDNA breaks. However, it has not been experimentally shown whether this process is involved during transposition. Therefore, we hypothesize that the decrease in transposition frequency observed by Acedillo et al. (1) in *recD*$^-$ mutants may be due, in part, to the exonuclease function of RecJ in the RecFOR pathway. This study attempted to examine the role of *recF* and *recJ* in the transposition pathway and the interaction with *recD*.

MATERIALS AND METHODS

Media, Reagents, and Antibiotics. Luria-Bertani (LB) medium comprised of 1% tryptone (w/v), 0.5% yeast extract (w/v), and 1% NaCl (w/v) at pH 7. Supplemental antibiotics and chemicals were added to the medium as appropriate. These included: ampicillin (100 μg/ml), rifampicin (25 μg/ml), kanamycin (50 μg/ml), tellurite (60 μg/ml), and 1% citrate (w/v).

Bacterial Strains and Plasmid. All *E. coli* strains used in the experiment and their genotypes are listed on table 1. The strains AB1157 (*rec*$^+$), BT125 (*recD*$^-$), and AK34 (*recD*$^-$ *recJ*$^-$) are isogenic. Strain R594 (*rec*$^+$) is isogenic to V355 (*recD*$^-$) and JC13031 (*recJ*$^-$) is isogenic to JC9239 (*recF*$^-$). The map of pUTel is shown in figure 1.

Donor Transformation and Amplification. Competent *E. coli* S17-1 λ_{pir} cells (25 μl) were thawed on ice and added to a sterile chilled electroporation cuvet containing plasmid DNA (pUTel; 10ng). The system was electroporated at 1.25 kV (MicroPulser Electroporator, Bio-Rad), followed by an one hour incubation in LB media (0.5 ml) at 37°C with shaking. The samples were spread plated on LB agar plates supplemented with ampicillin (100 μg/ml) at various concentrations to obtain isolated colonies (23).

TABLE 1. *Escherichia coli* strains and genotypes.

Strains		Genotype	Reference
Donor	S17-1 λpir	*Tp^r, Sm^r, recA, thi, pro, hsdR$^-$M$^+$ RP4:2-Tc:Mu:Km Tn7, λpir*	(25)
rec$^+$			
	R594	*lac-3350, galK2(Oc), galT22, λ$^-$, IN(rrnD-rrnE)1, rpsL179(strR)*	(5)
	AB1157	*F$^-$ λ$^-$ rac$^-$ thi-1 hisG4 Δ(gpt-proA)62 argE3 thr-1 leuB6 kdgK51 rfbD1 araC14 lacY1 galK2 xylA5 mtl-1 tsx-33 supE44(glnV44) rpsL31(strR)*	(2)
recD$^-$			
	V355	*lac-3350, galK2(Ox), galT22, λ$^-$, IN(rrnD-rrnE)1, rpsL179(strR), recD1014(Nuc-)*	(8)
	BT125	*recD1011*	(22)
recF$^-$			
	JC9239	*thr-1, araC14, leuB6(Am), Δ(gpt-proA)62, lacY1, tsx-33, qsr'-0, glnV44(AS), galK2(Oc), λ$^-$, Rac-0, hisG4(Oc), rfbD1, mgl-51, rpoS396(Am), rpsL31(strR), kdgK51, xylA5, mtl-1, recF143, argE3(Oc), thi-1*	(11)
	V66	*tsx-33, galK2(Oc), λ$^-$, Rac-0, hisG4(Oc), argA21, rpsL31(strR), kdgK51, xylA5, mtl-1, recF143, Met-(ts)?, bglR17*	(24)
recJ$^-$			
	JC13031	*thr-1, araC14, leuB6(Am), Δ(gpt-proA)62, lacY1, tsx-33, glnV44(AS), galK2(Oc), λ$^-$, Rac-0, hisG4(Oc), rfbD1, mgl-51, recJ153, rpsL31(strR), kdgK51, xylA5, mtl-1, argE3(Oc), thi-1*	(18)
	STL114	*recJ2003::mini-Tn10kan*	(17)
recD$^-$recJ$^-$	AK34	*recD1011 recJ2003::mini-Tn10kan*	(14)

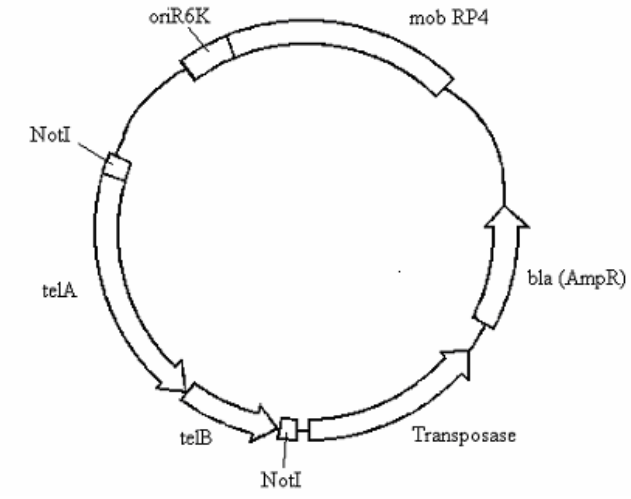

FIG. 1 Map of pUTel plasmid

Recipient Preparation. All nine *E. coli* test strains were plated on LB agar plates supplemented with rifampicin (25 μg/ml) only (R594, AB1157, V355, BT125, JC9239, V66, JC13031) or with both rifampicin (25 μg/ml) and kanamycin (50 μg/ml) (STL114, AK34) to create rifampicin resistant (rif^R) recipients.

Conjugation. Overnight cultures of the nine recipient strains were grown in liquid LB medium. The donor was grown overnight in liquid LB containing ampicillin (100 μg/ml). Turbidity readings were taken at 600nm and each culture was diluted to an OD_{600} of 0.4. As a control, each recipient culture was sampled by plating on LB rifampin (LB rif) plates at final plated dilutions (FPD) of 10^{-5}, 10^{-6}, and 10^{-7} to estimate bacterial concentration. The donor culture was plated on LB ampicillin (100 μg/ml) + tellurite (60 μg/ml) at the same FPDs as the recipients. Ten microlitres of the donor culture and 100 μl of each recipient culture were mixed in 5 ml 1% sterile NaCl and filtered onto a membrane filter (25 mm diameter, 0.45 mm pore size membrane, Millipore Corporation) to bring cells in close contact. Each filter was placed cell side up on an LB plate and allowed to incubate overnight at 37ºC. To prepare test samples for the transposition assay, each filter was resuspended in 5 ml 1% NaCl.

TABLE 2. Growth of rif^R recipients in overnight culture in LB containing rifampicin.

Strain	Relevant genotype	Culture concentration (x 10^8 cfu/ml)
R594	*rec*$^+$	0
V66	*recF*$^-$	0.4
JC9239	*recF*$^-$	1.1
JC13031	*recJ*$^-$	0.4
V355	*recD*$^-$	1.3
AB1157	*rec*$^+$	0
STL114	*recJ*$^-$	0
BT125	*recD*$^-$	0
AK34	*recD*$^-$ *recJ*$^-$	0

* Cultures were grown for 26 hr in LB rif at 37°C. Culture concentrations were estimated by OD_{600} readings.

TABLE 3. Growth of rif^R recipients grown in LB only.

Strain	Relevant Genotype	Incubated (hr)	Culture concentration (x 10^7cfu/ml)
R594	*rec*$^+$	24	30 ± 5.6
V66	*recF*$^-$	120	0
JC9239	*recF*$^-$	24	8 ± 0.3
JC13031	*recJ*$^-$	24	1 ± 0.1
V355	*recD*$^-$	24	50 ± 43.1
AB1157	*rec*$^+$	120	0
STL114	*recJ*$^-$	120	0
BT125	*recD*$^-$	120	0
AK34	*recD*$^-$ *recJ*$^-$	120	0

*Cultures were grown in LB at 37°C. Culture concentrations were estimated by dilution plate counts. Concentration estimates are expressed as average $\pm$ one standard deviation.

Transposition Frequency Assay: Each test sample was plated at a FPD of 10^{-1}, 10^{-2}, 2 x 10^{-2}, 10^{-3}, 2 x 10^{-3}, 10^{-4}, and 10^{-5} onto LB rifampicin + citrate plates and LB rifampicin + tellurite + citrate plates. All were incubated at 37ºC for at least 24h. After performing plate counts, the transposition frequency was approximated by the number of rif^R and tellurite resistant (tel^R) colonies divided by the number of total rif^R colonies.

RESULTS

Overnight cultures of rif^R mutants. Our first attempt to grow the rif^R mutants in overnight culture yielded no growth for some strains (Table 2). Due to time constraints, the mutants were grown a second time in LB alone. All strains grew to an OD_{600} of ~0.5. The resulting cultures were diluted to OD_{600} 0.4 and plated on LB rif plates (or LB rif + kanamycin plates for strains STL114 and AK34) to determine cell concentration of the cultures (Table 3).

Transposition frequency assay. Each conjugation mixture was plated onto LB rif + tel and LB rif to determine the frequency of transposition. Only one strain grew on the LB rif plate, and no strains grew on LB rif + tel plates (Table 4).

TABLE 4. Concentrations of conjugated *E. coli rec* mutants.

Strain	Relevant Genotype	Growth on rif plates (x 10^7 cfu/ml)	Growth on rif + tel plates (cfu/ml)
R594	*rec*$^+$	0	0
V66	*recF*$^-$	0	0
JC9239	*recF*$^-$	2.3 ± 0.1	0
JC13031	*recJ*$^-$	0	0
V355	*recD*$^-$	0	0
AB1157	*rec*$^+$	0	0
STL114	*recJ*$^-$	0	0
BT125	*recD*$^-$	0	0
AK34	*recD*$^-$ *recJ*$^-$	0	0

* All grown for 120 hours at 30°C. Concentration estimates are expressed as average ± one standard deviation.

DISCUSSION

Rifampicin is an antibiotic that targets the RNA polymerase (RNAP) of bacteria and inhibits RNA synthesis (7). To differentiate the recipient cells from the donor cells post conjugation, our mutants were plated onto LB rif plates in order to obtain rif^R mutants. Although the induction of rif mutations in recipients to enable the growth inhibition of the donor cells post conjugation is standard procedure in conjugation protocols (16), there may have been strong effects on the growth rate when we attempted to induce rif resistance in our *rec* mutants.

Rifampicin specifically binds the β subunit of the RNAP and inhibits transcription (6). It has been shown that DNA repair-deficient strains of *E. coli* are especially susceptible to rifampicin killing (7). This may explain why the induction and growth of rif^R *rec* mutants was very slow. Furthermore, when the rif^R mutants were grown overnight in LB containing rif, only some of the rif^R *rec* mutants grew, even after 26 hours (Table 2). The RecBCD pathway has been shown to promote the rescue of stalled or broken DNA replication forks. Normally the RNAP will stall at lesions or broken replication forks in the DNA (27). Rifampicin resistant mutants have also been shown to have a reduced half-life for RNAP-promoter binding (28). Rifampicin mutations are commonly in the *rpo* region of the gene encoding for the β subunit of the RNAP, effectively disrupting interactions essential for stable DNA binding of the RNAP (3). Therefore, *rpo** RNAP mutants are slower at making RNA transcripts for protein synthesis. The combination of poor RNAP binding along with the disrupted RecBCD pathway not properly resolving DNA lesions blocking the RNAP, may have caused slow RNA transcription rates and, consequently, slow protein synthesis and growth.

A possible answer for why the wild type *E. coli* strains did not grow may stem from the fact that rifampicin resistant mutations lead to a plethora of phenotypes. Rif^R mutants have been found to be temperature sensitive, uracil sensitive, able to support various bacteriophages, maintain F' episomes, and interact with mutant alleles (13). Since rifampicin resistance can have so many profound effects, the rif mutation in concert with the *rec* mutation would have been more detrimental to growth than either mutation alone. These mutations together could have exerted a greater pressure on the cells to revert to rif sensitivity.

Due to the slow growth observed in our first attempt at overnight culture, we grew our rif^R recipient strains a second time overnight in LB, without rif selection. We expected that when grown with no selection, the rif^R mutants would be less stressed, resulting in increased growth rates. As expected, all inoculated cultures grew to an OD_{600} of ~0.5 overnight (results not shown). When spread plated onto LB rif, no colonies grew, suggesting that the rif^R mutants had reverted to rif^S. Unknown at the time that these cultures of *rec* mutants were rif^S, they were conjugated with our donor *E. coli* in order to assess transposition frequency in *rec* mutants. Immediately after conjugation, the donor-recipient mixture was placed overnight on LB agar with no selection to allow for growth. The cells were later

plated onto LB rif and LB rif + tel to determine the frequency of transposition. Once again, the plated cells did not give rise to colonies (Table 4) providing further evidence to the hypothesis that the rif^R mutants had reverted to rif^S after growth with no selection. A rif^S revertant in the culture would have had a faster generation time, enabling it to outcompete the compromised rif^R mutant, and, therefore to quickly overtake the culture.

Mutations resulting in antibiotic resistance usually provide resistance at the cost of decreased fitness and, therefore, decreased rates of bacterial replication. Growth with no selection could result in either reversion to antibiotic sensitivity or a compensatory mutation that increases the fitness of the bacteria without the loss of resistance (4, 20). It has been shown, however, that rif^R mutants of *E. coli* grown for ~200 generations in the absence of rifampicin did not revert to rif sensitivity. After growing these strains for several generations, they show an increased level of fitness, but no reversion to rif sensitivity, suggesting that a second site mutation had occurred allowing for increased fitness without the loss of rif resistance (20). Compensatory mutations are more common than reversions since they can happen at many different sites (4). Our results, however, do not show any evidence of compensatory mutations. Most likely, the increased growth that we observed was due to reversion of our rif^R mutants to rif^S.

However, a turbidity of 0.5 OD_{600} for an *E. coli* overnight culture in LB was lower than expected. In addition, limited growth under rif selection was observed in both the *rec* mutants and rec^+ strain. These two results suggest that something else may be limiting the growth. Since growth conditions were optimized for temperature and pH, the slow growth rate may more likely be due to an intrinsic factor rather than extrinsic. Further investigation is required to elucidate the cause of this growth limitation.

Although our results did not provide insight into the roles of RecBCD and RecFOR in transposition, we have shown support for the role of the Rec pathway in DNA repair based on our difficulty culturing rifampicin resistant mutants and would therefore suggest that rifampicin resistance should not be used as a marker when studying mutants in the Rec pathways.

FUTURE EXPERIMENTS

Due to problems associated with rifampicin resistance, we propose that in the future, this experiment can be repeated using erythromycin resistance as a selectable marker instead of rifampicin resistance. Erythromycin binds to the 50S ribosomal subunit and disrupts peptide bond formation (9). Since erythromycin inhibits protein synthesis, it should not result in synergestic effects with the Rec enzymes.

We also suggest using a different method of conjugation. The system that we used involves filtering a mixture of donor and recipient cells and allowing the conjugation to proceed on the filter placed overnight on LB media without selection. The purpose of the filter was to facilitate conjugation by bringing the donors and recipients in close contact. However, this system also has its faults. For example, not all of the mixture could be pushed through the filter resulting in the loss of some of cells. Also, removing the filter from the filter case was problematic, resulting in further loss of cells. Therefore, another possible reason for getting no growth on LB + rif + tel plates after the conjugation may have been that the conjugation did not work and the recipient strains did not receive the transposon encoding tellurite resistance. This filtration step may be improved by using smaller filter holders attached to a vacuum flask, which would provide better seal. Also, a different conjugation system could be used in which donor and recipient cells were combined in a 1:10 ratio and spotted onto solid agar on which conjugation would occur. This would avoid loss of cells due to the filter.

However, the use of any antibiotic marker will ultimately slow down the growth of the *rec* mutants. In the future, the time it takes to grow these mutants should be accounted for in the experimental agenda. It may be desirable to go back to the λ phage system (1) or use the system used in this study with replica plating to screen for and eliminate the donor cells rather than the induction of a mutation to confer antibiotic resistance.

ACKNOWLEDGEMENTS

We would like to sincerely thank Carly Huitema and Dr. Lindsay Eltis for providing the pUTel plasmid and E. coli S17.1 λ_{pir} and also for experimental advice and help in the lab. We also thank Mary Berlyn from the *E. coli* Genetics Stock Center at Yale for sending *E. coli* strains R594, V355, JC9239, V66, and JC13031; and Dr. Andrei Kuzminov from the University of Illinois for sending *E. coli* strains AB1157, BT125, STL114 and AK34. We would finally like to thank Dr. William Ramey, Jennifer Sibley, and Karen Smith for their insight and helpful suggestions. This study could not have been done without the help of the staff of the UBC Media Room. This work was supported by the Department of Microbiology and Immunology at the University of British Columbia.

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