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Analyzing how transposon Tn3E.F. influences antibiotic resistance in *E. coli* strains S17.1 and C2110

TBT4146 Molecular Genetics

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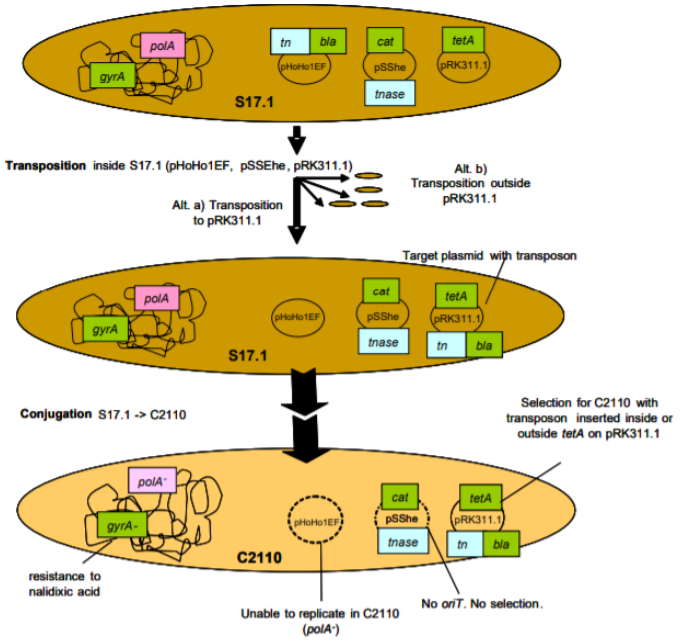

Figure 1: Overview of the experiment used to analyze how transposon Tn3E.F. influences the antibiotic resistance in E. coli strains S17.1 and C2110 (1).

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Abstract

In this experiment we learned how to use transposons as mutagens to inactivate a gene. During transposition inside *Escherichia coli* (*E.coli)* strain S17.1 the transposon Tn*3*E.F. inserts itself into different sites in the target plasmid pRK311.1. This transposon has the *bla* gene which contains ampicillin resistance. The pRK311.1 plasmid is the target and is resistant against tetracycline. During transposition, the transposon can be inserted into the *tet*A gene. If this happens the plasmid loses its tetracycline resistance and gains ampicillin resistance. When the insertion occurs outside the *tet*A gene, the plasmid is resistant to both tetracycline and ampicillin. The use of transposons as mutagens was demonstrated by analyzing the inactivation of the *tet*A gene.

After inoculation *E. coli* S17.1 and C2110 were plated out on LA+Amp+Tet+Nal. As expected, no growth was observed. Then both strains were brought together so that conjugation could take place. This mixture was diluted in a 10-fold series and plated out. As expected, growth was observed on all plates. The conjugation efficiency and transfer rate of transposon pRK311.1 were calculated.

Additionally, the results of the same experiment, conducted at UNIGEN, were analysed showing the different possible outcomes of this experiment.

A low conjugation efficiency of 7% and a low transfer rate of transposon 8% was obtained.

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1. Theory/Principles

Transposon

A transposon or transposable element (TE) is often called a jumping gene. It is a DNA sequence that moves within the genome. Numerous types of transposable elements exist and can be differentiated from one another based on the mechanism they use. Retrotransposons, class 1 TEs, require a specific enzyme called reverse transcriptase, that mediates the transcription of RNA into DNA. They use a copy-paste system. DNA transposons, class 2 TEs, do not require this enzyme in order to transpose (2). DNA transposons encode the enzyme transposase, required for the insertion and excision of the genomic sequence. This is referred to as a cut and paste method. These sequences move to different locations in the genome without copying themselves. Autonomous or functional transposons contain an internal genetic sequence consisting of genes that encode protein necessary for transposition. Non-autonomous elements do not encode these proteins and are therefore dependent on autonomous transposons for mobilization (3). The main reason for this is the absence of reverse transcriptase in class 1 transposons and transposase in class 2 transposons.

The actions of transposable elements thus lead to mutations and genomic rearrangement. This includes insertions, deletions, duplication, inversions, and translocations. Transposition can increase or decrease the size of the genome. When a transposon is integrated into the open reading frame of a gene, it leads to gene inactivation. The gene can no longer encode proteins as the nucleotide sequence has been modified by the insertion of the transposon. The copy-paste mechanism can lead to duplication when the transposon is copied from and pasted into the same genome. Translocations occur when genetic sequences are moved from one chromosome to another. This generates novel chromosomes and is correlated with the development of diseases such as cancer (4). Transposition is often imprecise and thus affects neighboring sequences in both host and target DNA. This is a key process for evolution in the genomes of both eukaryotes and prokaryotes (5).

In this experiment transposon Tn3E.F., found in the *E. coli* strain S17.1, is used for mutagenesis. This non-autonomous transposon contains the *bla* gene, conferring ampicillin resistance (1). Tn3E.F. will be inserted from plasmid pHoHoE.F., unable to replicate in *E. coli* C2110, into the target plasmid pRK311.1 in S17.1. This plasmid contains the *tetA* gene, conferring tetracycline resistance. It is mobile by conjugation. S17.1 also contains plasmid

pSShe, which encodes the transposase enzyme. Plasmid pSShe contains the *cat* gene, conferring chloramphenicol resistance. This allows Tn3E.F. to transpose. After transposition, both E. coli strains are mixed together for conjugation of plasmid pRK311.1 to occur.

Conjugation

Conjugation is the process by which bacteria transfer genetic material through direct cell-to-cell contact. It is a form of horizontal gene transfer. The donor, containing the F-factor, transfers its genetic material to the recipient organism. The F-factor or fertility factors allows bacteria to produce a sex pilus (6). This tube-like structure brings the donor and recipient cell into direct contact and thus allows the transfer of genetic material. The F-factor can occur as an autonomous plasmid but it can also be integrated into the bacterial genome. Hfr bacteria, standing for the high frequency of recombination, carry the F-factor integrated into their genome. Therefore during conjugation, not only a copy of the F-factor will be transferred, but a segment of the bacterial genome can be transferred as well. The sex pilus is structurally fragile which explains why the genetic transfer is often incomplete. While the goal is to transfer a Hfr bacterium's entire genome, only a small section will be transmitted (7). In F^+ bacteria the F-factor lies in an autonomous plasmid. DNA from an F^+ bacterium is transferred to an F^- bacterium (8). Conjugation is often used to transfer antibiotic resistance genes.

Conjugation is important in this experiment as it prevents other transposition events to occur after Tn3E.F is transposed (1). By isolating pRK311.1 derivatives from other S17.1 DNA, analysis of transposition events is limited to C2110 cells. Another crucial role of conjugation in this experiment is that it allows to isolate bacteria with specific resistances on antibiotic plates.

Antibiotic selection

Antibiotic agents kill or interfere with the growth of organisms. Bacteria acquire antibiotic resistance both naturally and artificially (9). Scientists can artificially create a DNA strand containing an antibiotic resistance gene. This recombinant DNA can then be introduced into the target organism. As a consequence, the organism becomes resistant to the antibiotic. Spontaneous mutations in the DNA can also lead to resistance. Since bacteria reproduce asexually and thus make exact copies of their own genome, they can pass this resistance on to their offspring. Horizontal gene transfer ensures the transfer of genetic material between different bacteria. This occurs through processes of transduction, transformation, and conjugation.

To determine which bacteria have antibiotic resistance genes, culture plates with specific antibiotics are used. If the organism contains the antibiotic resistance gene, it will grow on this plate. Surviving bacteria will grow rapidly and, when reproduced asexually, make clones that also contain the gene. They form a colony, visible on the plate. If bacteria do not contain the gene, on the other hand, they will not be able to grow on the plate.

Wild-type S17.1 strain contains resistance to ampicillin, tetracycline and chloramphenicol (1). C2110 is resistant to nalidixic acid. When transposon Tn3E.F. is transposed to prK311.1, which is then conjugated to a C2110 bacterium, this organism becomes resistant to ampicillin. If plasmid pRK311.1 is conjugated, the C2110 will show tetracycline resistance. Combining both, the C2110 bacterium can be resistant to ampicillin and tetracycline only if the transposon Tn3E.F is successfully moved to plasmid pRK311.1 outside of the *tetA* gene. If, however, the transposon is inserted in the *tetA* gene, ampicillin resistance is gained at the expense of tetracycline resistance. Thus plating out bacteria on plates with the antibiotics ampicillin (Amp), tetracycline (Tet), and nalidixic acid (Nal) shows whether transposition and conjugation were successful. Additionally, it shows whether the transposon was inserted inside or outside the *tetA* gene (if transposition occured). Plasmid or gene transfer in bacteria are natural processes. They require specific conditions. If the conditions are not optimal, the efficiency of these processes is low. Both conjugation efficiency and transfer rate of transposon Tn3E.F are evaluated in this report (Appendix).

2. Materials and methods

The materials and methods were used and carried out as described in the lab compendium (1). With the exception of step 4 on day 1. We rubbed the mixture on the plate. This could have had a negative influence on the conjugation as it is better to leave the cells as close to each other as possible to enhance transfer of genetic material through the pilus. We did get growth so this deviation from the compendium did not influence our results.

3. Results

The step in the experiment was conjugation. *E. coli* S17.1 and C2110 were inoculated by the course leaders in a shaking incubator. Each of the cultures were plated out on LA+Amp+Tet+Nal and incubated overnight. Both strains didn't grow which was expected, as neither has resistance to all of the antibiotics on the plates. The cultures were centrifuged, resuspended in LB, and afterwards plated on one LA plate (no antibiotics present). They *were* rubbed on the plate due to a misinterpretation of the instructions. We therefore anticipated some issues in the conjugation as the cells should be as close together as possible for this to happen optimally. Interestingly, after incubation overnight, the plate did display large growth indicating conjugation was successful despite the rubbing of the bacteria on the plate.

After conjugation of *E. coli* S17.1 and C2110, the mixture was diluted in a 10-fold series down to a 10^{-7} -dilution and plated out according to the table in the lab manual (page 29). The plates were incubated overnight. The results are given in Table 1. For the calculation of the average number of cells, only plates with 30 to 300 colonies were used. The plate with Amp and Nal is the only exception. Here a total of 26 colonies was counted and used for the calculations as no other reliable numbers were obtained.

Table 1: Results after plating out of the conjugation mixture on plates with different antibiotics in the agar plate. TNTC = Too Numerous To Count.

Plate type	Dilution	# cells /100 µL	Average # cells / 100 µL
Without antibiotics	10^{-6}	704	$820*10^6$
	10^{-7}	82	
Nal	10^{-6}	396	$400*10^6$
	10^{-7}	40	
Tet + Nal	10^{-2}	TNTC	$26*10^6$
	10^{-4}	TNTC	
	10^{-6}	26	
Amp + Nal	undiluted	TNTC	$2.26*10^6$
	10^{-2}	TNTC	
	10^{-3}	916	
	10^{-4}	226	
Amp + Tet + Nal	undiluted	TNTC	$0.420*10^6$
	10^{-2}	TNTC	
	10^{-3}	42	
	10^{-4}	348	

Ten colonies from the LA+Amp+Nal plate and ten colonies from the LA+Tet+Nal plate were transferred to an LA plate without any antibiotics and an LA+Amp+Tet+Nal plate. These plates were incubated at 37°C in a thermal cabinet overnight. The results are shown in Table 2. Thus all colonies picked from the Amp+Nal plate also appear to have tetracycline resistance. The colonies picked from the Tet+Nal plate did not grow on the Amp+Tet+Nal plate, indicating that they are not resistant to ampicillin.

Table 2: Results of the incubation of the plates after transferring ten colonies from the LA+Amp+Nal plate and ten colonies from the LA+Tet+Nal plate to an LA plate without any antibiotics and an LA+Amp+Tet+Nal plate.

From	To	# Colonies
Amp + Nal	Amp + Tet + Nal	10
	without antibiotics	10
Tet + Nal	Amp +Tet + Nal	No growth
	without antibiotics	10

The values from Table 1 were used to calculate the conjugation efficiency and the transfer rate of transposon Tn3E.F. The calculations can be found in the appendix.

Conjugation efficiency = 7%
Transfer rate of transposon = 8%

At UNIGEN, the same experiment was carried out. Eleven samples with transposon insertion and one sample without transposon insertion were digested with restriction endonucleases *Hind*III and *Eco*RI (separately or in combination). The resulting fragments were then separated by agarose gel electrophoresis (1). This can be seen in Figure 2. Track 11 shows bands at 23.1, 9.4, 6.6, 2.3, and 2.0 kb. This is a marker. From the plasmid map of pRK311.1 in the lab manual (page 26), it can be calculated that, if only HindIII is used as an enzyme, a single band at 35.4 kb is expected. If only EcoRI is used, one band at 18.5 kb, one at 8.9 kb (insert) and one at 8 kb (vector) is expected. If both enzymes are used, bands are expected at 13.3 kb (vector), 5.2 kb (insert), 8.9 kb (insert), and 8 kb (vector). If any of these fragments do not appear, this indicates transposon Tn3E.F. was inserted. Additional proof of the presence of the insert is the appearance of an extra fragment if *Hind*III was used as the transposon has a restriction site for this enzyme. To know where the transposon was inserted, we look at the specific fragments from the vector and the insert. The results are shown in Table 3.

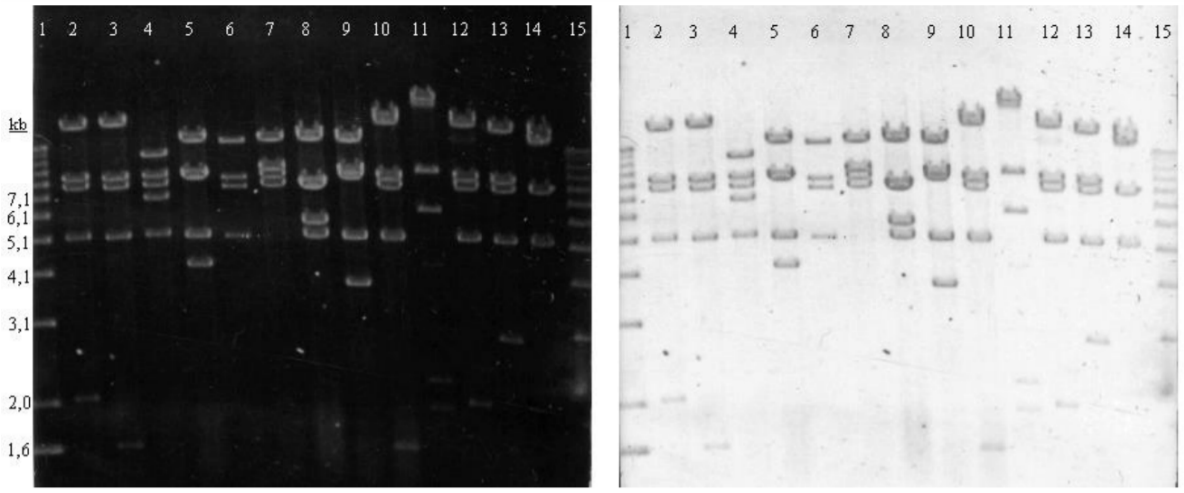

Figure 2: Plasmids with (11 samples) or without (1 sample) transposon insertion. The DNA was digested with HindIII + EcoRI. The two tracks on the outer edges are molecular weight markers and the values in kb are: 12.2, 11.2, 10.2, 9.2, 8.1, 7.1, 6.1, 5.1, 4.1, 3.1, 2.0 and 1.6. Track 11 is also a marker, with the molecular weights 23.1, 9.4, 6.6, 2.3 and 2.0. The negative of the gel is also included. Here you can clearly see where the bands are localized.

Table 3: Results of the UNIGEN experiment. Table was constructed using the information visible in Figure 2. From left to right, it was determined whether the track was a sample or a marker, which enzyme was used to digest the DNA, whether or not an insertion could have happened, and if it was in the insert or vector.

	Sample/Marker	Enzyme(s) used	Insertion	Insert/Vector
1	Marker	/	/	/
2	Sample	*Hind*III & *Eco*RI	Yes	Vector
3	Sample	*Hind*III & *Eco*RI	Yes	Vector
4	Sample	*Hind*III & *Eco*RI	Yes	Vector
5	Sample	*Hind*III & *Eco*RI	Yes	Vector
6	Sample	*Hind*III & *Eco*RI	No	/
7	Sample	*Eco*RI	Yes	Insert
8	Sample	*Hind*III & *Eco*RI	Yes	Insert
9	Sample	*Hind*III & *Eco*RI	Yes	Vector
10	Sample	*Hind*III & *Eco*RI	Yes	Vector
11	Marker	/	/	/
12	Sample	*Hind*III & *Eco*RI	Yes	Vector
13	Sample	*Hind*III & *Eco*RI	Yes	Vector
14	Sample	*Hind*III & *Eco*RI	Yes	Insert
15	Marker	/	/	/

4. Discussion

E. coli S17.1 and C2110 were inoculated and afterwards plated out on LA+Amp+Tet+Nal. Strain S17.1 was not expected to grow as it is not resistant to nalidixic acid. C2110 is resistant to nalidixic acid, but not to ampicillin, tetracycline and chloramphenicol. Therefore a culture of C2110 as such is not expected to grow on plates with LA+Amp+Tet+Nal.

S17.1 can donate plasmids by conjugation and C2110 can accept these. Therefore, mixing both strains can, through conjugation, create genetic variation through horizontal gene transfer. On a plate without antibiotics, S17.1 and C2110 as such can grow, as well as transconjugants. This explains the high (average) number of cells (820*106) on these plates.

On a plate with nalidixic acid C2110 will grow. This strain has the resistance gene. Transconjugants are also expected to grow on these plates. The transposition does not influence the *gyrA*- gene that confers resistance to nalidixic acid. This explains the second highest amount of cells (400*106) counted on the LA+Nal plates.

On a plate with tetracycline and nalidixic acid, only transconjugants will grow. There are two important prerequisites for these transconjugants. First, the conjugation of pRK311.1 must occur. This plasmid contains the *tet*A gene, conferring tetracycline resistance. Another requirement is that the transposition of Tn3E.F. transposon, conferring ampicillin resistance, was not inserted into the tetA gene. If this is the case, ampicillin resistance is at the expense of tetracycline resistance. Thus, on these plates cells in which the transposition didn't occur but the conjugation did, grow. And, cells in which transposition did occur and the bla gene was not inserted into the tetA gene, also grow on these plates.

To grow on a plate with ampicillin and nalidixic acid, cells require the genes *bla* and *gyrA*-. Thus, this requires conjugation of pRK311.1 to C2110 cells. Transposition of Tn3E.F. is necessary for the *bla* gene. In this case it doesn't matter whether this transposon is inserted inside or outside the *tetA* gene. After all, these plates do not contain tetracycline, which means that resistance to this antibiotic is not a requirement. The additional requirement to grow on these plates explains why fewer colonies were counted on these plates ($2{,}26*10^6$) than on the LA+Tet+Nal plates ($26*10^6$).

On a plate with ampicillin, tetracycline, and nalidixic acid, cells will only grow if they have the *bla*, *tet*A, and *gyr*A- gene. This is only possible if pRK311.1 is conjugated to C2110 cells, and transposition occured to transfer the *bla* gene to pRK311.1 outside the *tet*A gene.The multiple conditions explain why the number of cells counted on these plates ($0.420*10^6$) is lower than previous plates.

The transfer of the ten colonies from both Tet+Nal and Amp+Nal plates to plates with and without antibiotics didn't yield any surprising results. Growth on the plates without antibiotics was expected. As all the colonies grew on the plates without antibiotics, we can conclude nothing went wrong during the plating. These plates served as a control in the experiment.

Growth of the bacteria transferred from the Amp+Nal plate to the plate with Amp+Tet+Nal indicates that the bacteria successfully acquired the plasmid after transposition and conjugation *outside* of the tetA gene. If the transposon was inserted *inside* the *tetA* gene, the bacteria would have lost their resistance against tetracycline and therefore would have not survived on these Amp+Tet+Nal plates.

The selected bacteria, transferred from the Tet+Nal plate didn't grow on the Amp+Tet+Nal plates. This indicates the selected bacteria did not have ampicillin resistance. We can therefore conclude that in those cells, transposition of Tn3E.F. to pRK311.1 did not occur. These results are not in accordance with our expectations as we did expect growth. Unaware of the low efficiency of transposition, we expected to see about five colonies on the plate (50% efficiency). Perhaps, a bigger sample than 10 colonies would have shown growth.

Conclusion

The results from the experiment were used to calculate the conjugation efficiency and transfer rate of transposon Tn3E.F. A conjugation efficiency of 7% and a transfer rate of transposon 8% was obtained. These values are significantly lower than expected. They do however agree with real data. The low values were further confirmed by selecting ten random colonies and transferring these to plates with addinal antibiotics. Out of ten bacterial colonies from the Tet+Nal, none grew on the Amp+Tet+Nal plates. Thus no transposition occured in this sample of ten colonies.

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Appendix

Transfer frequency

To calculate the conjugation efficiency in this experiment, the amount of cells that are able to grow on plates with antibiotics are compared to the total amount of cells containing the recipient plasmid. This is because all resistance is due to conjugation in this experiment. C2110 cells grow on nalidixic acid without any antibiotics. If conjugation occurs with S17.1, C2110 transconjugants can grow on plates with antibiotics. To calculate the transfer rate of the transposon Tn3E.F., the number of cells growing on nalidixic acid and ampicillin are compared to the total amount of transconjugants.

$$conjugation\ efficiency\ = \frac{[(Nal{+}Tet) + (Nal{+}bla)] - (Nal{+}bla{+}Tet)}{Nal}$$

$$transfer\ rate\ of\ transposon\ = \frac{(Nal{+}bla)}{(Nal{+}Tet) + [(Nal{+}bla) - (Nal{+}bla{+}Tet)]}$$

Nal+Tet = all cells with pRK <u>except</u> for cells where *bla* is inside Tet
Nal+*bla* = all cells that have *bla* either outside **or** inside Tet
Nal+*bla*+Tet = all cells that have *bla* outside Tet

Calculation of the conjugation efficiency and transfer rate of transposon pRK311.1:

$$conjugation\ efficiency\ = \frac{[(Nal + Tet) + (Nal + bla)] - (Nal + bla + Tet)}{Nal}$$

$$conjugation\ efficiency\ = \frac{[(26\,000\,000) + (2\,260\,000)] - (420\,000)}{400\,000\,000} = 0.0696 \approx 0.07 = 7\%$$

$$transfer\ rate\ of\ transposon\ = \frac{(Nal{+}bla)}{(Nal{+}Tet) + [(Nal{+}bla) - (Nal{+}bla{+}Tet)]}$$

$$transfer\ rate\ of\ transposon\ = \frac{(2\,260\,000)}{(26\,000\,000) + [(2\,260\,000) - (420\,000)]} \approx 0.081 \approx 8\%$$