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REPORT

FROM EXPERIMENTS IN BACTERIAL GENETICS AND GENE TECHNIQUE

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Assignment:

To decide the sequence where the transposon *Tn5090* is inserted into the plasmid *RPI*.

Introduction:

An increasing problem today is the development of antibiotic resistance in bacteria. This will lead to difficulties in treating common illnesses that today are easily treated with antibiotics. This resistance in bacteria can arise in different ways, either from mutations in the bacterial genome or from acquirement of resistance genes. These received genes are often located on plasmids. The resistance that is arised in this way is of special interest since plasmids can be transferred from one bacterial strain to another.

One way in which the plasmids can move from one bacterium to another is by a process called *conjugation*. The transfer of the plasmid requires cell-cell contact, which in *E-coli* is made possible by the presence of pili. The resistance genes in plasmids are often located in *transposons*. Tranposones are able to move from one location on a DNA molecule to another location on the same, or on a different, DNA molecule. The *conjugation*- and *transposition frequency* can be determined to estimate the effectiveness of the transfer of these genetic elements.

In this laboration a “mating-out assay” is made to study the transposition of transposon *Tn5090*. *Tn5090*, which has a gene coding for trimetoprim resistance, is located in the chromosome of *JSR58b*. In the same bacterial strain the plasmid *RPI*, with a gene coding for kanamycin resistance, is present. If *Tn5090* moves to *RPI* it's possible to move the transposon into another bacterial strain because *RPI* is able to conjugate. This makes it possible to separate the transposons located in plasmids from those located in the chromosome. The bacterial strain that the plasmid with transposon is transferred to is *C600*. *C600* is natural resistant to nalidixan.

To be able to find out where *Tn5090* is located in *RPI* *RPI::Tn5090* must be extracted and the part of the plasmid to be sequenced must be cloned. This is accomplished by cleaving the interesting part out of *RPI::Tn5090* with restriction enzymes. In this part of *RPI::Tn5090* there is a gene coding for trimetoprim resistance which makes it possible to select the plasmids which carry this segment from those who doesn't. In addition this part also represents the junction between one of the inverted repeats of *Tn5090* and target site of *RPI*. This makes it easy to detect the target site of *Tn5090* in *RPI* wich is the main goal of the laboration. This part is ligated into the plasmid *pUC19*. *pUC19* has a gene coding for ampicillin resistance. This plasmid is transferred to the bacteria *DH5 α* by transformation in the purpose of multiplying the plasmid to get the amount required for sequencing.

Material and methods:

Day 1

- ✓ Casting of the agar plates, with different combination of antibiotics, needed during the laboration according to work order (see appendix 1). Each antibiotic concentration 50 µg/ml.
- ✓ Starting up conjugation due to separate instructions. In the beginning the transposon *Tn5090* will be in the bacteria *JSR58b*. The transposon will be translocated to the plasmid *RP1* that will conjugate to the bacteria *C600*. The purpose of this procedure is to be able to locate the conjugated plasmid. This is possible because of the different antibiotic resistance in *Tn5090* (Tp^R), *RP1* (Km^R) and *C600* (Nal^R). The bacteria *C600* that have the transposon *Tn5090* in the plasmid *RP1* are relevant for the laboration. These bacteria will be resistant to trimetoprim, kanamycin and nalidixan.

Day 2

- ✓ After incubation over night in 30°C the conjugation was ended. The culture was diluted and the plates were prepared according to the work order (see appendix 1). The plates were incubated over night in 30°C. The reason for incubation in 30°C is that *JSR58b* is sensitive to heat. Later in the laboration, when these bacteria is removed the incubation temperature can be raised to 37°C.

Day 3

- ✓ Determination of conjugation and transposition frequency.
- ✓ Cultivation of one cfu from plate C on a new plate of type C and incubation over night. Initiation of over night culture with bacteria from the same colony according to the work order (see appendix 1). The medium for the over night culture consisted of ISB with kanamycin, nalidixan and trimetoprim. ISB is used since all components in it are known. This medium is especially suitable when trimetoprim and sulfonamide are added for selection. Each antibiotic concentration 50µg/ml. A double sample was made to be sure of getting results.

Day 4

- ✓ Preparation of plasmid-DNA according to special instructions. In the first step an eppendorf tube was filled and centrifuged. The supernatant was discarded and the tube was filled again. After centrifugation the supernatant was removed. This was repeated a third time. The following material was used:

- ♦ GTE is added to, with help from Mg^{2+} , weaken the cell walls.
- ♦ NaOH/SDS: NaOH raises the pH and SDS denaturates the genomic and plasmid DNA and proteins.
- ♦ KAc precipitates SDS. KAc can be used in many purposes. It precipitates genomic DNA, proteins, cell wall components and some RNA.
- ♦ CPI purifies the supernatant and removes proteins and some RNA.
- ♦ EtOH precipitates DNA.
- ♦ RNase brakes down RNA.

This was made to extract the plasmid from C600.

- ✓ Cleavage of plasmid with EcoRI to confirm that *RPI* with *Tn5090* was present. The cleavage was made due to work order (see appendix 1). Buffer volume mustn't be more than 1/10 of the total volume. The same goes for the enzyme. The enzymes that were used in this laboration have the concentration 2 units/ μ l. One unit of restriction endonuclease will completely digest 1 μ g of substance DNA in a 50 μ l reaction in 60 minutes. If the solution contains the plasmid with the transposon EcoRI will cleave two times in *Tn5090* and one time in *RPI*. This results in two large fragments and one small fragment, with the size of about 3.5 kbp.

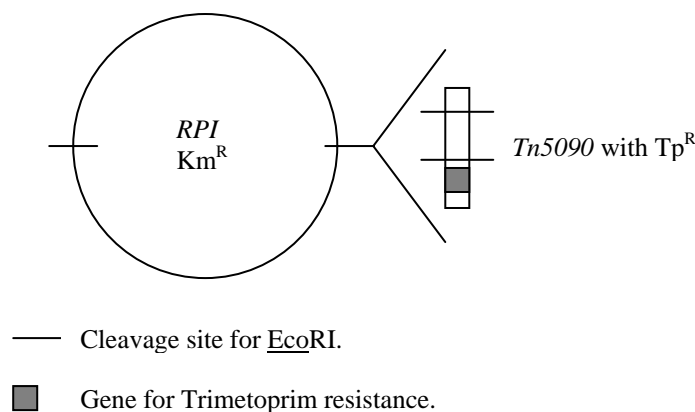


Figure 1 Simplification of *RPI* with *Tn5090*

- ✓ Agarose gel electrophoresis. To establish the result from the cleavage above, an agarose gel electrophoresis was made according to special instructions. λ was used as reference.

The part of the plasmid, that wasn't cleaved, was saved for day 5. The sample was stored in the cold room.

- ✓ Initiating an over night culture with *DH5 α* in 2xTI.¹

¹ The over night culture with *DH5 α* in 2xTI was initiated by the laboratory assistants.

Day 5

- ✓ Preparative cleavage of DNA and *pUC19* with PstI and EcoRI due to work order (see appendix 1). The concentration of each enzyme is determined in the same way as in day 4. *pUC19* is a plasmid that will carry *Tn5090* into the bacteria *DH5α*. The cleavage will result in a part of *Tn5090* being cut out from *RPI*, although a small fragment of *RPI* will be cut out together with *Tn5090* because the cleavage is not made exactly in the end of the transposon.

The part of the transposon that is interesting carries a Tp^R gene. This makes it possible to select the *DH5α* with *pUC19* carrying *Tn5090*. Since *pUC19* is cleaved with the same enzymes the sticky ends of the DNA and *pUC19* will recognize each other and ligation is possible.

- ✓ Incubation in 37°C for 2 hours.

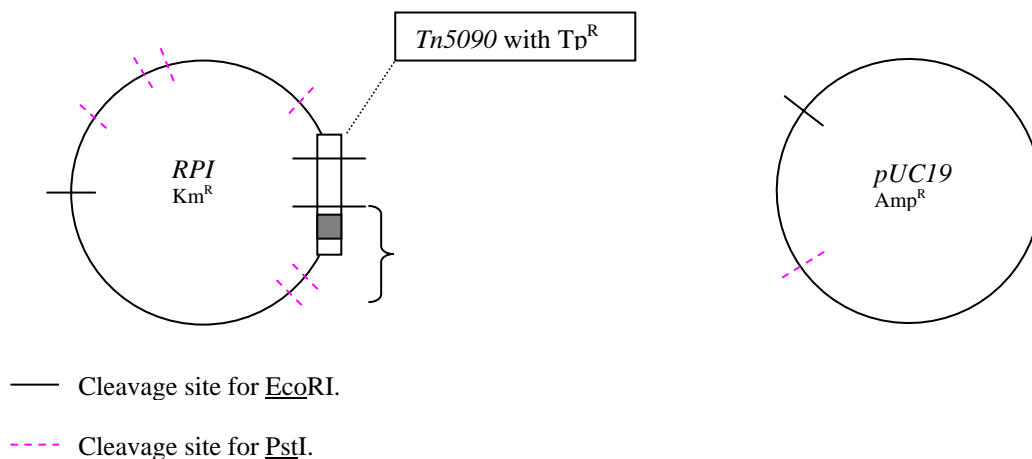


Figure 2 Simplification of *RPI* and *pUC19*

- ✓ After incubation the two samples (DNA and *pUC19* preparation) were mixed and phenol/chloroform extraction was performed according to special instructions.
 - ◆ Phenol was added to remove buffers, enzymes.
 - ◆ Chloroform is added to remove the phenol. Phenol is more soluble in chloroform than in water, and can easily be extracted from the water phase.
- ✓ Ligation. The pellet was resuspended in 16μl H₂O. 2μl ligase and 2μl ligase buffer was added. See special instructions. The concentration of T4 DNA ligase is 40 units/μl when delivered. 4 units/μl will be added since the ends were sticky (if the ends would have been blunt 40 units/μl would have been added).
- ✓ Incubation in 16°C over night since this is temperature for ligation.

- ✓ Preparation of competent cells according to special instructions.² Increased competence indicates that the cells are more able to receive DNA via transformation. CaCl_2 treatment is necessary to give the cells the opportunity to take up the plasmids. Ca^{2+} is believed to create a complex with DNA, which attach to the cell surface easier.

Day 6

- ✓ Transformation of 10 μl DNA due to special instructions. The heat shock initiates the uptake of the plasmids. This is made because the plasmid *pUC19* with a part of the transposon *Tn5090* is to be transferred into the bacteria *DH5 α* . The reason for using this bacteria instead of any strain of *E. coli* is that *DH5 α* doesn't recombine with foreign DNA. This makes it suitable for this transformation where the purpose is to multiply the plasmid without recombining it into the bacterial genome. The purpose is to multiply the plasmid.
- ✓ The transformants were spread on selective plates with ampicillin and trimetoprim. 1/10 on one plate and the rest on a second plate. This antibiotic combination was used since the part of *Tn5090* that is interesting carries a gene for Tp^{R} and *pUC19* carries an Amp^{R} gene.
- ✓ The plates were incubated in 37°C over night.

Day 7

- ✓ One cfu from the selective plate was transferred to an Eppendorf tube with 200 μl H_2O . 25 μl of the solution was transferred to a selective plate with ampicillin, 50 $\mu\text{g}/\text{ml}$.³ The plate was incubated in 37°C over night.⁴ The rest of the content of the tube was boiled for 5 minutes, to lyse the cells, and centrifuged for 30 seconds in order to clear the DNA from the rest of the cell components.
- ✓ Preparation of sample for PCR according to work order (see appendix 1) and special instructions. The concentration of each primer should be 1 μM and the concentration of dNTP should be 200 μM . 1 μl taq polymerase is used to 50 μl total volume. Half of the total volume was used in the PCR reaction.⁵
- ✓ PCR:
 - ◆ The temperature is raised to 94°C to denaturate the DNA.
 - ◆ The temperature is lowered to 66°C. This is a good temperature for the primers to bind. The primers used in this laboration are *uni* and *pSPH*.

² Preparation of competent *DH5 α* was made by other students.

³ It would have been better to use a plate containing both ampicillin and trimetoprim, but such a plate wasn't available.

⁴ This was made since there was too long time before next laboration day. If an over night culture would have been made this day, the bacteria wouldn't survive. Over night culture was initiated on an additional day (day 8) instead.

⁵ The reason for using only half the volume is that 10 μl DNA is too much, and preparing a volume with total volume of only 25 μl reduces the accuracy.

- ◆ In the third step of the cycle the temperature is raised to 72°. At this temperature the *Taq polymerase* is most effective.

This cycle is performed 25 times. The PCR was made to determine if the transformation was successful.

- ✓ The result from PCR was analyzed by using electrophoresis gel. λ was used as a reference.

Day 8

- ✓ Initiation of over night culture from the plate with ampicillin in ISB with ampicillin and trimetoprim. Each antibiotic concentration 50 μ g/ml. ISB is used since all components in it are known. This medium is especially suitable when trimetoprim and sulfonamide are added for selection.

Day 9

- ✓ An Eppendorf tube was filled with over night culture and centrifugated for one minute. The supernatant was discarded and the tube was filled with over night culture a second time and centrifuged for one minute. The supernatant was discarded. This was repeated a third time.
- ✓ Preparation of plasmid DNA, with QIAprep method, from the over night culture according to special instructions.⁶ The purposes of the buffers used were the following:
 - ◆ The pellet was resuspended in buffer P1.
 - ◆ Buffer P2 was added to lyse the cells.
 - ◆ Buffer N3 was added to stop the lysing of the cells and to protect the DNA from denaturing.
 - ◆ Buffer PE was used to wash the DNA in the spin column.
 - ◆ Buffer EB was added to elute the DNA.

The spin column is positive and the DNA is negative. This attaches the DNA the column. The PE buffer removes salts in the sample which will disturb the electrostatic attraction between the column and the DNA making the elution of DNA possible.

- ✓ Sequencing according to special instructions. The conditions used were the following:
 - ◆ Heating to 95°C to denaturate the DNA
 - ◆ Lowering the temperature to 55°C so that the primer will bind.
 - ◆ Heating to 72°C. At this temperature the polymerase is most effective.
- ✓ Gel electrophoresis. The gel used was polyacrylamide gel, 6%.

⁶ The reason for using this preparation, and not alkaline SDS mini-plasmid preparation used day 4, is that the fragment to be prepared is too large for the SDS method.

In the sequencing radioactive labeled ddNTP are added to the transcription process. ddATP, ddCTP, ddGTP respective ddTTP was added to four different tubes with reaction mix. Together with each ddNTP all dNTPs are present. The reaction mix contained reaction buffer, DNA, primer, H₂O and polymerase. When the ddNTPs are inserted in the produced chain instead of dNTP the transcription is terminated. The pieces will have different sizes because the ddNTPs are inserted randomly into the chain. By analyzing on a gel the sequence can be determined since the short pieces will move faster than the long ones on the gel. Since there is only one type of ddNTP in each tube, all fragments on the gel from this sample will end with this specific ddNTP. The fragments are detectable by x-ray film because of the ddNTP's radioactivity. During sequencing it is important to have a carefully adjusted concentration of the ddNTPs. If the concentration is too high the fragments will all be short and this will result in the sequence being impossible to determine.

Results

Day 3

$$\text{Conjugation frequency} = \frac{B}{A - B} = \frac{(20 \times 10^6 + 180 \times 10^5) \times 0.5}{2200 \times 10^7 - (20 \times 10^6 + 180 \times 10^5) \times 0.5} = 8.6 \times 10^{-4}$$

$$\text{Transposition frequency} = \frac{C}{B - C} = \frac{501 \times 10^1}{((20 \times 10^6 + 180 \times 10^5) \times 0.5) - 501 \times 10^1} = 2.6 \times 10^{-4}$$

For explanation of what the plates consist of, see appendix 1 (day 1)
The values in the equations are cfu/ml.

Day 4

- ✓ Our control cleavage was successful; the photo from the agarose gel electrophoresis showed that we had two large fragments, about 30-35 kbp, and one small one, about 3.5 kbp. See appendix 2, figure 1.

Day 7

- ✓ After the transformation we didn't get any bacteria on our selective plates. We used the bacteria from another group to be able to proceed with PCR.
- ✓ The result from the agarose gel was good. The photo showed that we had a fragment of approximately 900bp. See appendix 2, figure 2.

Day 9

The gel electrophoresis didn't show anything, so we received a photo from the laboratory assistants. After studying the photo we could determine where in *RPI Tn5090* is inserted. The insertion position in *RPI* is 35021. The sequence in this area is:

CGCAATTTAAA|ACAGCAAAGT
RPI *Tn5090*

Discussion

When transposition- and conjugation frequencies were determined on day 3 the following observations were made: On the C plate diluted to 10^{-1} we didn't get any countable colonies. Plate A diluted to 10^{-5} was uncountable (the value was too high) and the plate diluted to 10^{-6} also was hard to count. We should have diluted it to 10^{-7} instead, to get a more accurate value. This makes our values less certain.

We didn't get any bacteria on our selective plates day 7 ☹. We don't really know the reason for this. Apparently, some step between day 5 and day 7 didn't work out the way it should have. After the phenol/chloroform extraction we had an "invisible" pellet that was washed with ethanol. Maybe we didn't remove the ethanol fast enough, so that the pellet was resuspended and the DNA was harmed. Another possible reason for the unsuccessful result is the end of the transformation. It was hard to resuspend the pellet in NaCl, and maybe we didn't do it well enough.

Our sequencing reaction was not successful. Since the control PCR was successful something must have failed, probably is the preparation of our DNA the source of the failure.