Microbiological demonstration of restriction enzymes and modifying enzymes in E.coli

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This experiment reproduces the classical proof performed by Arber, Nathans and Smith who postulated and demonstrated the existence of restriction enzymes and methyl transferases in the late 60s.

Two strains of bacteria, E.coli α and αR are used in the experiment, the latter encoding a restriction enzyme and a corresponding methyl transferase, which exerts a protective function on the restriction enzyme. One infects both strains with non-methylated DNA of Lambda bacteriophage of the α strain. In order to denote the origin of the phage, it will be termed $\Lambda\alpha$.

The efficiency of bacteriophage infection is analyzed by the ability to form bacterial plaques on agarose plates ("efficiency of plating"). The efficiency of plating is 2000-fold lower in E.coli αR as compared to E.coli α. Therefore, we can conclude that E.coli αR contains a protective system that prevents the strain from $\Lambda\alpha$ infection. However, some bacteriophages can circumvent the internal bacterial protection system. This is due to the methylation of αR DNA that was released from E.coli αR. The methylation of recognition sites of restriction enzymes by methyl transferases results in the tolerance of phage DNA by E.coli αR.

The initial role of methylation was to protect bacterial DNA from endogeneous degradation by endonucleases. During new infection and lysis cycles of E. coli α by bacteriophage $\lambda\alpha$R, newly synthetized DNA is not methylated and can therefore be recognized as foreign DNA by E.coli αR.

Experimental procedure

Microbiological demonstration of restriction enzymes in E.coli by bacteriophage Lambda infection (part I infectious cycle)

1.1 Preparation of bacteria (3 min)
Resuspend bacterial pellets (α and αR) in 500 µl LB media. Incubate the resuspended bacteria at 37°C for 2-20 hours in a waterbath or incubator at 37±2°C.

1.2 Preparation of LB Agar plates (15 min)

Add 3.5 g LB agar (powder) to 100 ml of destilled water in a sterile beaker or bottle.

Boil the mixture in a microwave oven until the solution is clear; avoid bubbles

Cool down solution for 3-5 min.

Label petri dishes:

A. α+2x$10^2\lambda\alpha$

B. α+2x$10^4\lambda\alpha$

C. αR+2x$10^2\lambda\alpha$

D. αR+2x$10^4\lambda\alpha$

Fill 25 ml of agar on each of four petri dishes. Leave the dishes open until the agar becomes solid.

Set waterbath to 45°C.

1.3 Bacteriophage infection of bacteria

Label 2 reaction tubes on the lid: phage lysate $\lambda\alpha$ (2x10^2 pfu/100µl) α and αR.

Label another 2 tubes on the lid: phage lysate $\lambda\alpha$ (2x10^4 pfu/100µl) α and αR.

Pipett 100 µl of the corresponding suspension into tube. Mix by inverting the tube, do not shake vigorously!

For adherence of phages onto host cells, mix samples for 30 min at 37°C.

1.4 Preparation of Top-agar (7 min)

Compared to normal agar, Top-Agar contains only 50% agar and therefore maintains liquid for a long period of time even at lower temperatures.

Pour agar into petri dishes that contain polymerized agar (step 1.2). Top agar is used for the fixation of infected bacteria/released bacteriophages. Using this technique, bacterial plaques can be easily identified on the plate.

Mix 0.5 g Agar in 20 ml destilled water (glass beaker)

Boil in microwave until the solution is clear.

Transfer 3 ml of Top agar into polystyrene tubes and incubate at 45°C.

Top agar should not become solid! You can boil the solution several times.

1.5 Plating of infected bacteria (10 min)

After incubation, mix samples with 3 ml prewarmed Top agar by pipetting up and down (avoid bubbles). Plate immediately onto LB agar plates and cover the whole dish with solution.

Air dry for 5 min at room temperature, keep lid open.

There is no risk of contamination.

1.6 Plaque formation (6h, preferably over night)

After the agar is solid, reverse the plate (lid down) and incubate at 30-37°C. Add a glass beaker containing 200 ml of water in order to prevent the plates from drying out over night.

1.7 Analysis

Count the plaques on the plates (if possible)

On the plate labeled α+2x$10^2\lambda\alpha$, roughly 200 single plaques should be visible. According to the number of plaques, you can calculate the concentration of infectious particles.

On the plate labeled α+2x$10^4\lambda\alpha$ “confluent lysis" occured. You will not be able to count the number of plaques anymore as they are no longer separated

On the plate αR+2x$10^2\lambda\alpha$ no plaques should be visible, as statistically only one out of 2000 bacteriophages will be infectious in the protected strain.

On the plate αR+2x$10^4\lambda\alpha$, 5-10 plaques should be visible. These phages managed to circumvent the bacterial restriction system. The bacterial genomic DNA contains methylated DNA sequences.

1.8 Storage/binning of plates

You can store the plates in the refrigerator for several weeks. Seal the plates with tape.

Before wasting them, incubate plates with 10 ml of ethanol for 10-15 min. Afterwards, plates can be put into normal waste containers. Petri dishes can be recycled for another experiment.

1.9 Troubleshooting

A. Lower number of plaques than expected:

1. Titer of infectious plaques is decreasing with storage time. Don’t expose the lysates to higher temperatures. Do not freeze the lysates.

2. The viability and infection capacity of bacteria decreases with time. Avoid multiple freeze/thaw cycles.

B. No separate plaques visible

Agar plates not dry enough, phage distribution in condensed water droplets. Wait until condensed water has evaporated from the plates prior to use. Close the lids after plates are dry.