1 Bacterial Transformation

Introduction

Microorganisms or microbes are divided into three groups: prokaryotes, eukaryotes, and viruses. Prokaryotes include bacteria, which are divided into two groups: gram-positive bacteria such as *Staphylococcus aureus* (*S. aureus*) and gram-negative bacteria such as *Escherichia coli* (*E. coli*). Eukaryotes include protozoa such as Giardia (which causes giardiasis, a severe diarrhea), fungi such as unicellular yeasts (which cause oral thrush), and algae such as blue-green algae. Viruses include those like the measles virus and the herpes virus.

Bacterial Nutrients

In nature, microorganisms search for their own nutrients or, in some instances, synthesize their nutrients. In the laboratory setting, however, we need to multiply or grow microbes. The term *grow* refers to an increase in the number of microorganisms, not their physical size. Microbes including bacteria grow quickly if we provide them with their optimum growth requirements. The right amount or concentration of nutrients, oxygen, water, pressure, and pH are some of the bacterial growth requirements.

Bacterial growth nutrients could be in the form of liquid (so-called broth) or solid (so-called agar). Microbial growth nutrients, either broth or agar, are also called media or medium. There are a variety of media available for microbial growth. Broth media are contained in sterile glass tubes. However, agar media can be stored in sterile glass tubes or Petri dishes (or simply agar plates).

Bacterial Cultures

A bacterial culture is the end result of bacterial multiplication in artificial media in the laboratory. Obtaining a pure culture is essential in guaranteeing accurate and reliable laboratory experiments. Because bacteria are numerous and small, they can easily be mixed together. Thus, cross contamination is common.

To ensure a pure culture, we must start with a single bacterium. There are several techniques available to achieve this. The multiplication of a single bacterium on agar plates appears as a colony. Each colony can be seen by the naked eye, while a single bacterium requires a microscope for observation. Bacterial colonies can appear as different colors or even different shapes or textures on different agar media plates.

Bacterial Transformation

Transformation is one method of introducing foreign genetic materials to cells. In nature, the process of transformation is accomplished without our intervention, but in the laboratory, we can make some gram-negative bacteria to accept the foreign genetic materials. The process of transformation in the laboratory can be summarized as three steps: synthesizing the plasmids containing the foreign genes, making the host cells ready for transformation, and introducing the plasmids to the cells.

Transgenic cells (or transgenic animals or plants) are those that have accepted foreign genes. Because bacterial hosts are not readily convertible to transgenic cells, they need to be treated chemically (with calcium chloride) and physically (with heat shock). The prepped cells that are ready for transformation are called competent cells. Among the bacteria, gram-negative bacteria like *E. coli* can be manipulated to accept the recombinant plasmid vectors. Genetically modified organisms (GMOs) are organisms with modified genetic materials. Transgenic *E. coli* are an example of GMOs.

Eukaryotic single cells (yeasts) and eukaryotic mammalian cells (such as green monkey kidney cells) are susceptible to transformation as well.

Plasmids

Plasmids are small, circular, double-stranded DNA that replicate independently from chromosomal DNA. They are located in the cytoplasm of bacteria. Plasmids contain a number of genes with different functions. Some plasmids contain genes that confer an antibiotic-resistant ability to their host. Thus, an antibiotic-sensitive host can acquire plasmid-mediated antibiotic resistance. Once introduced to the new host, the plasmids (recombinant or not) replicate independently of the host chromosome. Not all plasmids replicate at the same pace. Some laboratory-modified plasmids replicate at much higher rates and synthesize many copies of the recombinant genes.

Plasmids as Vectors

Plasmids can be modified in the laboratory to carry foreign genes. These modified plasmids are called recombinant plasmids. The recombinant plasmids are considered vectors because they can serve as vehicles to deliver foreign genes to the desired host. Whenever we have DNA from two different sources, it is referred to as recombinant DNA. Cloning, in broader terms, means to make an identical copy. Gene cloning means to obtain many copies of the same gene. Thus, in practice, isolating the gene from the donor, inserting it in the plasmid and making recombinant plasmid, and introducing it to the recipient host to make many copies of that gene is a cloning technique. Applying plasmid vectors is one method to introduce and replicate the donor gene in the host.

What is the advantage of cloning a gene? Having multiple copies of a gene makes it possible to visualize the DNA fragments, to determine their nucleotide sequences, and to harvest and study their products. For example, human insulin is synthesized in the laboratory by cloning the human insulin gene in a plasmid vector and introducing it to *E. coli*. The transgenic *E. coli* harboring the recombinant plasmid replicate exponentially, which allows for synthesizing a huge supply of human insulin, the final product of gene expression.

Plasmid pUC19: Marker Genes

There are a number of plasmids available as commercial vectors that can be purchased. The plasmids designated pUC18 or pUC19 have been used extensively. Let's consider pUC19. This plasmid has been modified to carry a gene for resistance to ampicillin (the so-called amp-r gene), which confers ampicillin resistance to the host. It also includes a beta-galactosidase gene that, upon transformation, can complement the defective host beta-galactosidase gene. The resulting gene

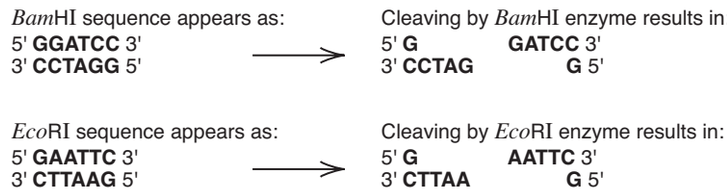
product, the enzyme beta-galactosidase, can break down x-gal, the substrate embedded in the agar medium. Consequently, the transformed cells with intact pUC19 will appear as blue colonies on the agar plates containing ampicillin and x-gal. However, insertion of exogenous DNA in the beta-galactosidase gene makes the beta-galactosidase gene defective. Thus, upon transformation, the bacteria containing the recombinant pUC19 will not be able to form blue colonies. In this scenario, they form white colonies on the medium containing ampicillin and x-gal.

What is the purpose of having the gene for amp resistance in this plasmid? It serves as a marker gene. If the transformation is successful, both the host harboring the recombinant plasmid and the unmodified plasmid (the control) should be able to grow on agar plates containing ampicillin antibiotic. But how can we distinguish the transformed cells containing intact pUC19 from those harboring the recombinant gene? As mentioned above, interruption of the beta-galactosidase gene leads to formation of white colonies. Thus, in pUC19, the beta-galactosidase gene serves as a second selecting marker, and the double selection ensures that all the necessary steps (gene isolation, insertion, transformation, and selection) are done correctly.

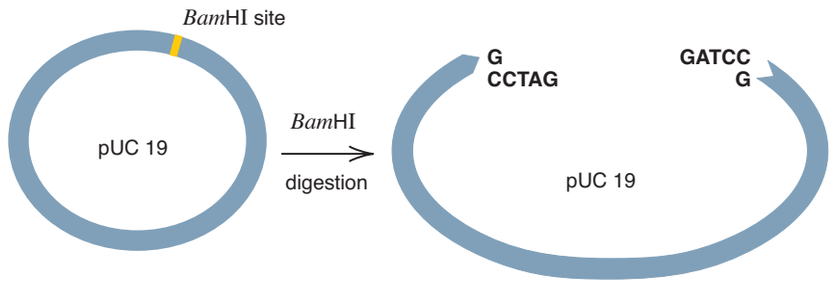
Plasmid pUC19 Multiple Cloning Sites (MCS) for Insertion of Exogenous DNA

There is a stretch of DNA within the beta-galactosidase gene called polylinker or multiple cloning sites (MCS). The presence of MCS makes it possible to insert exogenous DNA in the beta-galactosidase gene with a variety of different terminal sequences. Molecular biotechnology takes advantage of a series of enzymes called restriction endonucleases to cleave (break) DNA at specific nucleotides. The restriction enzymes can leave behind smooth ends (blunt ends) or sticky ends (overhang). Note that the standard writing of nucleic acid sequences starts at the 5' end and terminates at the 3' end.

Let's examine the MCS of pUC19, which include the following restriction enzyme sites: *Hin*dIII, *Pst*I, *Sal*I, *Bam*HI, *Sma*I, and *Eco*RI.



Cleaving *Bam*HI with the corresponding enzyme, *Bam*HI on pUC19 will result in opening the circular plasmid to a linear DNA with the following sticky ends, as seen above.



The following is an example of a stretch of donor DNA:

5' ATCCCCC**GGATCC**AAATTCGGCCTTAAC**GGATCC**GGGGT 3'
3' TAGGGGG**CCTAGG**TTTAAGCCGGAATTG**CCTAGG**CCCCA 5'

If you carefully examine the terminal sequences of this DNA, you can detect *Bam*HI sites at both ends. If you digest this stretch of DNA with *Bam*HI restriction enzyme, you will get three fragments. Focus on the main segment (the middle fragment), which is the DNA of interest and has the following sticky ends:

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5' ATCCCCCG          GATCCAAATTCGGCCTTAACG          GATCCGGGGT 3'
3' TAGGGGGCCTAG          GTTTAAGCCGGAATTGCCTAG          GCCCCA 5'
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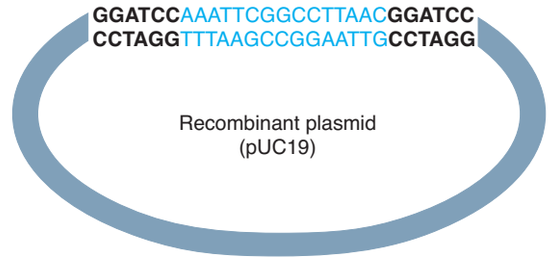
Insertion of the linear middle fragment in the digested pUC19 (opened with *Bam*HI restriction enzyme) requires a series of steps. First, the desired DNA needs to be isolated from the other fragments (see the section on DNA Fragment Detection). Then the purified DNA is ligated (attached) to pUC19 using enzyme ligase. The ligase takes advantage of the sticky ends and brings these two DNA together as following:



The final product is a closed, circular pUC19 with the exogenous DNA inserted in the *Bam*HI. Thus, a recombinant plasmid is synthesized with the inactivated beta-galactosidase gene.

Note that there is a possibility that the linear (opened) pUC19 plasmid can be ligated without picking up the foreign gene. The insertion of the exogenous DNA could happen in one of the two possible directions; one direction leads to a functional gene and the other to a non-functional gene.

What do you think the next step would be? It is to confirm that the resultant plasmid is the right plasmid. But we need enough of this plasmid DNA to do the confirmation studies. Thus, the competent *E. coli* needs to be transformed with the recombinant plasmids. Then some of the white colonies from the ampicillin plates need to be cultured to obtain a tangible amount of recombinant plasmids for further studies.

DNA Fragment Detection/Gel Electrophoresis

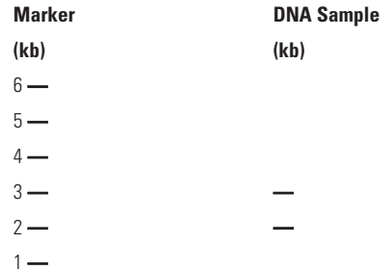
The backbone of DNA contains repeating units of phosphate and sugar. The two backbones of DNA (the two strands) are connected to each other by base pairs. The unit of DNA measurements is kilobase (kb). For example, if a DNA fragment consists of 5,000 base pairs, it is written as 5,000 bp or 5 kb.

The presence of phosphates in the DNA backbones confers a negative charge to DNA fragments. Scientists took advantage of this characteristic of DNA and invented a technique called agarose gel electrophoresis that allows electrical current to move the DNA fragments through gel from positive charge toward negative charge. In this technique, DNA fragments are separated from each other based on their size. The smaller the size, the farther they move along the gel. Different concentrations of gel are used depending on the size of the DNA sample. The concentration of the gel and the size of the fragments are inversely proportional. A lower concentration of gel is required for separating the larger DNA fragments. Roughly a 1% gel is used for DNA fragments up to 10 kb.

For simplicity, you can compare making the agarose gel to making a gelatin dessert from powder at home. While pouring the hot liquefied gel, insert a comb in the top of the gel to make space for loading samples upon solidification. DNA samples are mixed with a dye for two purposes: (1) to give color to the colorless DNA and thus make it easier to load DNA into the wells, and (2) to keep samples from floating out of the wells (that is, to prevent losing them). The dye also allows for visual tracking of the sample migration along the gel. The electrical current should be stopped when the dye gets closer to the bottom of the gel. To make the DNA fragments visible, stain the gel.

When the DNA samples are loaded, a standard DNA ladder marker (available commercially) is loaded simultaneously. The standard marker fragments serve as a visual aid to compare the size of unknown DNA samples. For example, if the unknown DNA fragment lines up with the 3 kb fragment in the maker ladder, the unknown DNA is roughly 3 kb.

In practice, at the conclusion of agarose gel electrophoresis, the gel needs to be stained. There are a variety of staining options such as ethidium bromide and commercially available fluorescent dyes. The gel is exposed to ultraviolet light, allowing us to visualize the stained DNA. If the inserted fragment is 2 kb and the vector plasmid is 3 kb, then after the restriction digestion, the resulting two fragments should appear as two bands compared with the DNA ladder marker as following:



Case 1

A Fictional Case

A large medical center has been dealing with a strange case of gonorrhea, a sexually transmitted disease (STD). *Neisseria gonorrhoeae* (*N. gonorrhoeae*) belong to gram-negative bacteria and cause gonorrhea, a painful disease. These bacteria have an attachment organ, called fimbriae, which allows *N. gonorrhoeae* to attach to the urethra. In this case, the patient who has been admitted to the infectious disease ward has been having excess bleeding, which is unusual for gonorrhea. Fortunately, the infectious disease department was able to successfully determine the etiology (cause) of this alarming bleeding. The scientists identified that unusually high numbers of *N. gonorrhoeae* isolated from this patient have a modified fimbriae that attaches to the urethra by penetrating the mucous membrane rather than merely attaching to it. Upon detachment, the tissue injury leads to bleeding.

The gene for the wild-type (prototype) fimbriae has already been identified and has been designated *FIM* (not a real-life term). The scientists were able to identify the mutant gene for the fimbriae and designated it *mFIM* gene (a fictional mutant). Next, the researchers would like to clone this mutant gene (*mFIM*). The following questions will help you achieve this goal.

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QUESTIONS

Q1. What term would you use to describe making multiple copies of the same gene?

Q2. You are given plasmid pUC19 and a small amount of the *mFIM* gene DNA, the two main ingredients for cloning. How do you insert the *mFIM* gene in the plasmid?

Hint: You need to know what restriction enzyme sites are available on the plasmid and on the *mFIM* gene. Because the exact changes in the nucleotide sequences of the *mFIM* are not known to you at this early stage, use the nucleotide sequence of the *FIM* gene as guidance.

Q3. You are given the sequence of the nucleotides of the *FIM* gene. You closely examine the sequence of base pairs of the *FIM* gene, and you notice that the *FIM* gene has one of the following restriction sites on both ends: *Hin*dIII, *Pst*I, *Sal*I, *Bam*HI, *Sma*I, or *Eco*RI. Can you detect which restriction site is present on both ends of the *FIM* gene shown below?

Hint: See pUC19 MCS. It is either *Bam*HI or *Eco*RI.

5' ATCGAATTCGCCGATCCAAATTCGG/.../CCTTAACGCATGAATTCGGGT 3'
3' TAGCTTAAGCGGCTAGGTTTAAGCC\...\GGAATTGCGTACTTAAGCCCA 5'

Q4. Can you write down the sequence of the resulting fragments after digestion with that particular restriction enzyme? How many fragments will you expect?

Q5. Will you cut the pUC19 and the *mFIM* gene with the same restriction enzyme? Why or why not?

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Q6. If you cut the pUC19 with *Eco*RI, what would be the sequence of the sticky ends of the resulting linear DNA?

Q7. You are told that the *mFIM* gene has *Bam*HI sites at both ends as well. Can you use the *Bam*HI enzyme to achieve your goal of inserting *mFIM* gene in pUC19? Would you change your mind if you knew that the *mFIM* gene has a *Bam*HI site somewhere in the middle of gene in addition to both ends?

Q8. What is the advantage of having an enzyme that leaves behind sticky ends versus those with blunt ends?

Q9. After cutting (digesting) the pUC19 and the *mFIM* gene with the desired restriction enzyme, what enzyme do you need to connect (or ligate) the linear *mFIM* gene with the opened circular pUC19?

Q10. How many ways can the sticky ends of the plasmid and the digested *FIM* gene join together after the ligase enzyme is added?

Hint: One possibility is that the cut pUC19 is resealed to its intact original circle without picking up any foreign DNA fragment.

Q11. What would be the general name for pUC19 after inserting the *mFIM* gene in its MCS?

Hint: Whenever we have DNA from two different sources, it is considered a recombinant DNA.

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Q12. After ligating the *mFIM* gene in pUC19, do you think you have enough DNA materials to do additional exploratory experiments?

Q13. You decide that you do not have enough recombinant DNA, and you would like to expand the repertoire of your DNA. What is the general name for the next step?

Q14. You decide to use *E. coli* as a host to replicate your recombinant plasmid. What would be the first step in transformation?

Q15. How many ways can you make competent bacteria?

Q16. You transform the competent *E. coli*. How do you know you are dealing with the right transformed host?

Hint: The medium should contain what antibiotic?

Q17. You detect some colonies on the ampicillin agar plate. Does this confirm that the growing bacteria are harboring the recombinant plasmid? What would be another possibility?

Q18. How do you determine that the bacteria growing on the ampicillin plate contain the recombinant plasmid?

Hint: You would need agar plates containing ampicillin plus what other chemical?

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Q19. How can the color of colonies help you to decide which host is harboring intact pUC19 and which contains recombinant pUC19?

Q20. You identify one white colony on the plate. Does this indicate a pure culture?

Q21. What would be the next step in cloning the recombinant plasmid containing the *mFIM* gene?

Q22. You have a large culture of transformed *E. coli.* What would be the next step?

Q23. How you do you know you have cloned the right DNA fragment?

Q24. If the *mFIM* gene is 2 kb and pUC19 is roughly 3 kb, what DNA ladder marker would be helpful to confirm that you are dealing with the right size DNA insert fragment?

Hint: You need to compare your DNA fragments with what size standard DNA fragments?

Q25. What can you do next with this cloned mutant gene?

Hint: Compare it with a wild-type gene with respect to size, nucleotide sequence, amino acid sequence, functional differences, and treatment considerations.

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Q26. Transformation is one way for cells to obtain foreign genetic materials. What are the other two methods?

Hint: Think about direct bridging between bacteria for transfer of plasmids. The second one involves bacterial viruses known as _____. There is a specific name for each of these two methods.