Introduction: Cloning (DNA Ligation) & Transformation

Overview

During the last decade, there has been a technological revolution in the field of molecular genetics; new research techniques have allowed scientists to explore and "engineer" changes in the genomes of a variety of organisms.

With the help of **restriction endonucleases** (special bacterial enzymes that cut DNA at specific restriction sites), foreign DNA can now be inserted into bacterial plasmids (small, circular pieces of extrachromosomal DNA) and can be replicated. Bacterial cells that contain foreign DNA can express the genetic information and make the gene products. Thus, by cloning these cells, we can learn about the structure and operation of genes. Commercially, we can produce large amounts of rare proteins and other specific gene products. We can also use such plasmids to **transform** the genetic constitution of other organisms.

Plasmids used for DNA cloning or bacterial transformation experiments are usually those that carry a gene for antibiotic resistance. The presence of the antibiotic-resistance gene makes it possible to **select** bacteria containing the plasmid of interest: the bacteria that have the plasmid will grow on a medium that contains the antibiotic, whereas bacteria lacking the plasmid will not be resistant to the antibiotic and will die.

In this lab, you will investigate some of the basic principles of genetic engineering. Plasmids containing specific fragments of foreign DNA will be made and used to transform *E. coli* cells, conferring both antibiotic (ampicillin) resistance and the lac+ phenotype (the ability to metabolize lactose) to recipient cells.

E. *coli* is an ideal organism for genetic manipulation and has been used extensively in recombinant DNA research. It is a common inhabitant of the human colon and can easily be grown in standard nutrient mediums.

The single circular chromosome of *E. coli* contains 5 million DNA base pairs ($1/600^{th}$ the total amount of DNA in a human cell). In addition, the cell contains small, circular, *extrachromosomal* (outside of the chromosome) DNA molecules called **plasmids**. These fragments of DNA, 1,000 to 200,000 base pairs in length, also carry genetic information. Some plasmids replicate only when the bacterial chromosome replicates and usually exist only as single copies within the bacterial cell. Others replicate autonomously and often occur in as many as 10 to 200 copies within a single bacterial cell. Certain plasmids, called R plasmids, carry genes for resistence to antibiotics such as ampicillin, kanamycin, or tetracycline.

In nature, genes can be transferred between bacteria in three ways: conjugation, transduction, or transformation. **Conjugation** is a mating process during which genetic material is transferred from one bacterium to another "sexually" different type. **Transduction** requires the presence of a virus to act as **vector** (carrier) to transfer small pieces of DNA from bacterium to another. **Bacterial transformation** involves transfer of genetic information into a cell by direct absorption of the DNA from a donor cell.

Through the process of bacterial transformation a bacterium can acquire a new trait by incorporating and expressing foreign DNA. In the lab, the DNA used most commonly for transformation experiments is bacterial plasmid DNA.

Transformation can occur naturally but the incidence is extremely low and is limited to a relatively few bacterial strains. In the growth cycle of these strains, there is a stage called **competence** when the bacteria are most receptive to uptake of foreign DNA. Competence to absorb DNA develops toward the end of the logarithmic growth phase, just before cells enter the stationary phase in culture. The mechanism by which competence is acquired is not completely understood, but experimentally, the competent state can be induced by treating bacterial cells with divalent cations (Ca^{2+}, Mg^{2+}) or by heat shock.

Part I: DNA ligation – constructing recombinant plasmids

Plasmids can transfer genes such as those for antibiotic resistance which are already a part of the plasmid, or plasmids can act as carriers for introducing foreign DNA from other bacteria, plasmids, or even eukaryotes into bacterial cells. Restriction endonucleases are used to cut and insert pieces of foreign DNA into the plasmid vectors.

Restriction endonucleases are essential tools in recombinant DNA methodology. Several hundred have been isolated from a variety of prokaryotic organisms. In the nomenclature of restriction endonucleases, the letters refer to the organism from which the endonuclease was isolated. The first letter of the name stands for the genus name of the organism. The next two letters represent the initial letters of the second word of the species name. The fourth letter (if there is one) represents the strain of the organism. Roman numerals indicate whether the particular endonuclease was the first isolated, the second, and so on.

*Eco*RI	E = genus *Escherichia*
	co = coli
	R = strain RY13
	I = first endonuclease isolated

Each restriction endonuclease "recognizes" a specific DNA sequence (usually a 4- to 6-base-pair sequence of nucleotides) in double-stranded DNA and digests phosphodiester bonds at specific sites in the sequence. The result is an open circle (if circular DNA is cut at only one site) or DNA fragments (if the restriction endonuclease recognizes two or more sites on the DNA molecule). Each of the fragments has a phosphate at the 5' end and a hydroxyl at the 3' end. The length of each DNA fragment corresponds to the distance between restriction sites. Some restriction endonucleases cleave cleanly through the DNA helix at the same position on both strands to produce fragments with blunt ends. Other endonucleases cleave specific nucleotides on each strand to produce fragments with overhangs or "sticky ends." By using the same restriction endonuclease to "cut" DNA from two different organisms, complementary overhangs or "sticky ends" will be produced and can realign in a "template-complement" manner, thus recombining the DNA from the two sources.

In bacteria, restriction enzymes provide protection by breaking and destroying the DNA of invaders, such as that of bacteriophage viruses. However, since the recognition sites for restriction endonucleases also occur within the bacterial DNA itself, bacteria have a mechanism for preventing their own restriction enzymes from digesting their own DNA. For each restriction endonuclease produced by a bacterium, there is a corresponding enzyme that methylates the bacterial DNA at the specific recognition sites: addition of a methyl group to the nucleic acid base prevents a close association from forming between the restriction endonuclease and the recognition site. In this way, bacteria can break down the DNA of invaders while protecting their own genetic material from destruction.

READ LAB INSTRUCTIONS FOR DAY 1: DNA LIGATION

READ LAB INSTRUCTIONS FOR DAY 2: INNOCULATION AND PLATE PREPARATION

Part 3: Transformation

In this exercise, we will work with the plasmid pUC19. Two different restriction enzymes have been used to cleave the plasmid into fragments (EcoRI and PstI…keep in mind that PstI cleaves within the β-galactosidase gene…see below). Plasmid pUC19 includes the ampicillin resistance gene Amp^r. The bacterial cells in this study are not resistant to ampicillan, but can acquire resistance (be transformed) be insertion of the pUC19 plasmid carrying the Amp^r gene. Only transformed cells can grow on LB agar plates containing ampicillin. This allows us to select for the desired transformants.

In addition to the Amp^r gene, plasmid pUC19 also carries another fragment from a DNA bacteriophage. This fragment is responsible for directing the synthesis of the α-peptide of β-galactosidase, an enzyme necessary for the breakdown of galactose. (The gene, *lac z*, producing β-galactosidase is one of the three genes of the *lac* operon). When functional β-galactosidase is produced, bacteria can metabolize lactose and are *lac*+. Bacteria of mutant strain bacterial cells are lac^-: they are incapable of producing the α-peptide of β-galactosidase (due to a deletion in a portion of the *lac z* gene) and are thus unable to use lactose. However, the introduction of plasmid pUC19 into these cells can transform the bacteria to *lac*+. Transformed cells will turn blue (the blue phenotype is the manifestation of the altered genetic makeup of the recipient cells) when cultured on LB agar containing ampicillan and X-gal (which forms a blue-colored product when used by bacterial cells as a histochemical substrate for β-D-galactosidase) plus IPTG (an inducer of β-galactosidase activity). The expression of the blue phenotype verifies that the *lac z* gene of the plasmid is being expressed.

READ LAB INSTRUCTIONS FOR DAY 3: TRANSFORMATION