INTRODUCTION

In the Program Introduction, you learned that the increase in diabetes in the United States has resulted in a great demand for its treatment, insulin. You also learned that the best way to meet this demand is to insert the human insulin gene into bacteria, enabling the bacteria to produce the insulin protein in quantities large enough to meet the demand. Chapter 1 gave you a chance to work with two physical tools and techniques of genetic engineering that are used to clone a gene: the micropipette and gel electrophoresis. In this chapter you will work with two other important genetic engineering tools—*plasmids* and *restriction enzymes*. These "tools" are actually biomolecules found in many bacteria, and their discovery was crucial to genetic engineering. With these tools, scientists can modify organisms to make human insulin and other medicines. You will now learn more about these tools and carry out the first steps in your quest to clone a gene.

CHAPTER 2A GOALS

By the end of this chapter, you will be able to do the following:

- Describe the characteristics of plasmids
- Explain how plasmids are used in cloning a gene
- Describe the function of restriction enzymes
- Explain how to use restriction enzymes to create a recombinant plasmid

WHAT DO YOU ALREADY KNOW?

Discuss the following questions with your partner and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these questions will help you think about what you already know about DNA, plasmids, and restriction enzymes.

1. What is the structure and function of DNA? Describe in words or a drawing the structure of a DNA molecule. Be as detailed as possible.
2. All living organisms contain DNA. In what ways is DNA from different organisms the same, and in what ways does it vary?
3. Using your understanding of genes and how they are expressed, explain why it is possible for a bacterial cell to make a human protein from the instructions encoded in a human gene.
4. As described in the Program Introduction, scientists use two biological tools to engineer organisms to make new proteins: plasmids and restriction enzymes. What do you remember about how these tools are used?

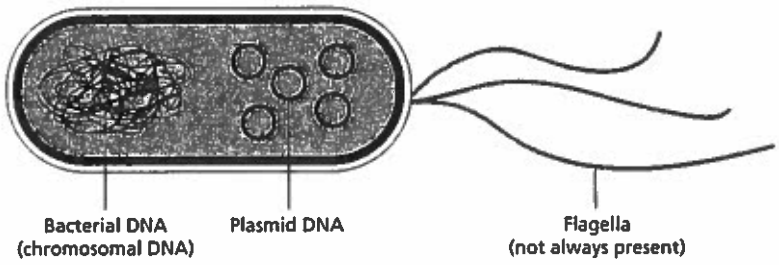
PLASMIDS AND RESTRICTION ENZYMES

The discovery of plasmids and restriction enzymes in bacteria is a classic example of how findings from basic research can revolutionize a field. Without the discovery of these biomolecules, major breakthroughs in understanding fundamental processes of life and in developing life-saving products might never have occurred.

PLASMIDS

Many different types of bacteria carry two forms of DNA: (1) a single chromosome made up of a large DNA molecule that contains all the information needed by the organism to survive and reproduce, and (2) plasmids, which are small circular DNA molecules, ranging in size from 1,000 to 200,000 *base pairs*—two nitrogenous bases joined to connect complementary strands of DNA—that are present in multiple copies separate from the chromosomal DNA (see Figure 2A.1). Some bacteria carry as many as 500 plasmids in each cell.

Figure 2A.1: DNA in bacterial cells



Several characteristics of plasmids make them ideal *vectors* (vehicles for carrying DNA sequences from one organism to another) for genetic engineering, for example:

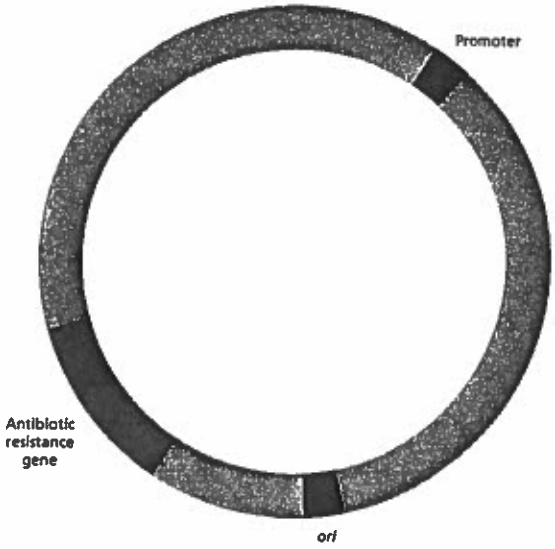
- The ability to replicate, that is, to make copies of itself independently of the bacterial chromosome. In order to do this, plasmids have a specific sequence where the host cell DNA synthesis enzymes bind and initiate *DNA replication* (a biological process that occurs in all living organisms to make copies their DNA). This sequence is called the *ori* (*"origin of replication"*) site.
- The ability to initiate *transcription* (the process by which information encoded in DNA is transferred to *messenger RNA* using the host cell RNA polymerase). This ability requires another specific sequence, called the *promoter* sequence. The promoter sequence binds RNA polymerase; this is

where transcription is initiated. All genes have promoter sequences located next to them in the DNA. In order for genes such as the insulin gene to be expressed in bacteria, they must be inserted in the plasmid next to the promoter sequence.

- A gene or genes that code for resistance to *antibiotics*, a class of compounds that kill or inhibit the growth of microorganisms. These genes code for proteins that inhibit the action of antibiotics secreted by microorganisms and can confer a selective advantage in nature to plasmid-containing bacteria in a microbial population in which bacteria compete for survival.

Figure 2A.2 illustrates some of the characteristics of plasmids that make them ideal vectors for genetic engineering.

Figure 2A.2: A plasmid vector



The basic components of a plasmid are the *ori* site for initiation of DNA replication, a promoter for the initiation of transcription, and a gene for *antibiotic resistance* (the state in which bacteria are no longer sensitive to an antibiotic and will continue to grow and divide in the presence of the antibiotic).

The plasmids you will work with in this and subsequent labs contain the genes for resistance to the antibiotics ampicillin and kanamycin. These genes produce proteins that inactivate the target antibiotic by chemically modifying its structure.

CONSIDER: Use what you know about natural selection and evolution to describe how plasmids might confer a selective advantage to their host bacteria.

A fourth feature of plasmids that is critical for genetic engineering is that they can be passed on from one bacterial strain to another in a process called *bacterial conjugation*, which enables bacteria to share and exchange genetic information. When a plasmid with a gene for antibiotic resistance is taken in by bacteria lacking that plasmid, the bacteria will then become resistant to that specific antibiotic. In nature, conjugation occurs with a very low efficiency; that is, only a small percentage of bacteria in a population can take in plasmid DNA at any point in time. The presence of an antibiotic resistance gene on the plasmid vector allows us to identify the small percentage of bacteria that took in the plasmid. Bacteria that did not take in the plasmid will be killed by the antibiotic. Those that have the plasmid with the gene of interest will survive and grow.

In developing techniques for cloning genes in bacteria, scientists had a powerful tool in plasmids—a vector that can be taken in by bacteria, that replicates in bacteria to produce many copies of itself, that has a promoter sequence for transcription of an inserted gene, and that carries a gene for antibiotic resistance. If you carry out the lab in Chapter 5A, you will take advantage of these features of plasmids when you transfer your recombinant plasmid into bacteria.

Once scientists recognized the power of plasmids as a potential vector, the next challenge was to determine how to incorporate a foreign gene of interest, such as the insulin gene, into the plasmid DNA.

RESTRICTION ENZYMES

In the early 1950s, scientists observed that certain strains of *E. coli*, a common bacterium found in the human gut, were resistant to infection by *bacteriophage* (viruses that infect bacteria by injecting their DNA into the cell and commandeering the host cell's molecular processes to make more bacteriophage). Investigation of this primitive "immune system" led to the discovery of *restriction enzymes*, proteins that restricted the growth of bacteriophage by recognizing and destroying the phage DNA without damaging the host (bacterial) DNA. Subsequent studies demonstrated that restriction enzymes from different strains of bacteria cut at specific DNA sequences, called *recognition sites*.

CONSIDER: How do bacteria that carry a restriction enzyme avoid cutting up their own DNA?

Table 2A.1 provides examples of restriction enzymes isolated from different strains of bacteria and the DNA sequences they cut. In the examples shown, the enzymes cut asymmetrically on the strands of DNA, leaving single-stranded overhanging sequences at the site of the cut. For example, a cut (or digestion) with EcoRI will leave an AATT overhang (or "sticky end") on one strand and a TTAA sticky end on the other strand.

Table 2A.1: Restriction enzymes used in this laboratory

Source	Restriction enzyme	Recognition site
Escherichia coli	EcoRI	5′ G↓AATTC 3′ 3′ CTTAA↑G 5′
Bacillus amyloliquefaciens	BamHI	5′ G↓GATCC 3′ 3′ CCTAG↑G 5′
Haemophilus influenzae	HindIII	5′ A↓AGCTT 3′ 3′ TTCGA↑A 5′

The symbols ↓ and ↑ indicate where the DNA is cut.

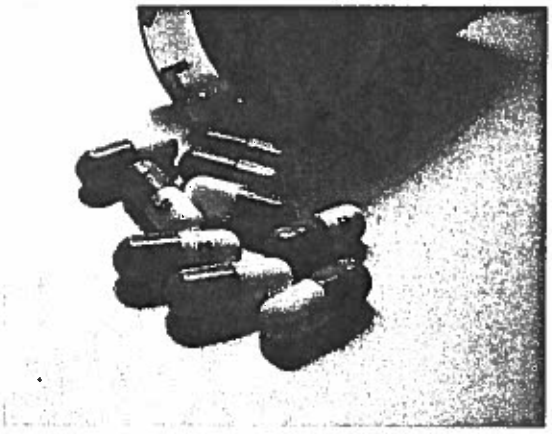
CONSIDER:

- **What is the sequence of the sticky end that results when DNA is cut with BamHI? With HindIII?**
- **Scientists can modify plasmids to have a single restriction enzyme site. Imagine that you have a plasmid with a single EcoRI site. Draw the structure of the plasmid after it has been cut with the enzyme, and show the nucleotide sequences left at the site of the cut. If you wanted to insert a gene from a plant at this site, what enzyme would you use to cut the plant DNA with? Explain your response.**

DID YOU KNOW?

The Rise of Antibiotic-Resistant Bacteria

Antibiotics and similar drugs have been used for the last 70 years to treat patients who have infectious diseases. When prescribed and taken correctly, antibiotics are enormously valuable in patient care. However, these drugs have been used so widely and for so long that the infectious organisms the antibiotics are designed to kill have adapted to them, making the drugs less effective. Antibiotic resistance occurs when some bacteria in a population are able to survive when exposed to one or more antibiotics. These species that have become resistant cause infections that cannot be treated with the usual antibiotic drugs at the usual dosages and concentrations. Some have developed resistance to multiple antibiotics and are dubbed multidrug-resistant bacteria or "superbugs."



Antibiotic resistance is a serious and growing phenomenon and has emerged as one of the major public health concerns of the 21st century. Drug-resistant organisms may have acquired resistance to first-line antibiotics, requiring the use of second-line agents. Typically, the first-line agent is selected on the basis of several advantages, including safety, availability, and cost; the second-line agent is usually broader in spectrum, may be less beneficial in relation to the associated risks, and may be more expensive or less widely available.

CLONE THAT GENE

You now know about two biological tools for cloning a gene:

1. A plasmid that has several important features:
    - A restriction enzyme site or sites that opens the plasmid circle and enables insertion of the gene of interest into the plasmid DNA
    - A sequence for the initiation of DNA replication, called the *ori* site, that allows the plasmid to replicate in the bacteria using the host DNA synthesis enzymes
    - A promoter sequence for initiating transcription of the inserted gene
    - A gene encoding a protein for antibiotic resistance, which allows for identification of bacteria that have taken in the plasmid

2. Restriction enzymes for the digestion of both the plasmid and the human DNA containing the gene of interest (such as insulin) to be cloned

How do scientists use these two tools to create a recombinant plasmid, which contains the insulin gene (or any other gene of interest) inserted into a bacterial plasmid? One important step is choosing a restriction enzyme or enzymes that cut the plasmid and the human DNA. The restriction enzyme(s) must do all of the following:

- Cut the plasmid at a site or sites that allow for the insertion of the new gene.
- Cut the plasmid at an appropriate site to ensure that no important genes or sequences are disrupted, including the *ori* site, the promoter, and at least one of the genes encoding antibiotic resistance.
- Cut the plasmid near the promoter so that the inserted gene can be expressed.
- Cut the human DNA as close as possible to both ends of the insulin gene so that it can be inserted into the appropriate site in the plasmid DNA, without cutting within the insulin gene.

STOP AND THINK: Why is it important that the same enzyme or enzymes be used to cut both the plasmid and the insulin gene from the human DNA?

In this activity, you will make a paper model of a recombinant plasmid that contains an insulin gene. You have three tasks:

1. Cut the plasmid and the human DNA with the appropriate restriction enzyme
2. Insert the insulin gene into the plasmid DNA
3. Determine which antibiotic you would use to identify bacteria that have taken in the plasmid

HANDOUTS

- **Plasmid Diagram (RM 2)**
- **Human DNA Sequence (RM 3)**

PROCEDURE

1. On the **Plasmid Diagram (RM 2)**:
    - Use scissors to cut out the plasmid sequence and tape the ends together to make a paper model of the plasmid.
    - Locate the positions of the *ori* site, the promoter site, and the genes for antibiotic resistance.
    - Locate the positions of each restriction enzyme recognition site.
2. Choose the restriction enzyme that should be used to cut the plasmid. Verify that the restriction enzyme meets all the following criteria:
    - The *ori* site on the plasmid is intact
    - The promoter site is intact
    - At least one of the antibiotic resistance genes is intact
    - The enzyme cuts the plasmid only once
    - The cut is close to the promoter sequence
3. Review **Table 2A.1** on page C-7 and use scissors to cut the plasmid at the recognition site exactly as the restriction enzyme would cut it. Write the sequences of the nucleotides that are left on each end of the plasmid.
4. On the **Human DNA Sequence (RM 3)**, scan the human DNA sequence and determine where the three restriction enzymes, BamHI, EcoRI, and HindIII, would cut the DNA.
5. Determine whether the restriction enzyme you chose in step 2 is a good choice for cutting out the insulin gene from the human DNA by verifying that it meets all the following criteria:

- It does not cut within the insulin gene
- It cuts very close to the beginning and end of the gene
- It will allow the insulin gene to be inserted into the cut plasmid

6. Review **Table 2A.1** and use scissors to cut the plasmid at the recognition site exactly as the restriction enzyme would cut it. Write the sequences of the nucleotides that are left on each end of the insulin gene after it is cut from the human DNA.

7. Use tape to insert the insulin gene into the cut plasmid. Verify that the sticky ends will connect in the correct orientation. (In the lab, a third biological tool, *DNA ligase*, is used to permanently connect the sticky ends together.) This is a paper model of a recombinant plasmid that contains an insulin gene. Once the plasmid replicates (copies) itself, the insulin is also copied, or cloned!

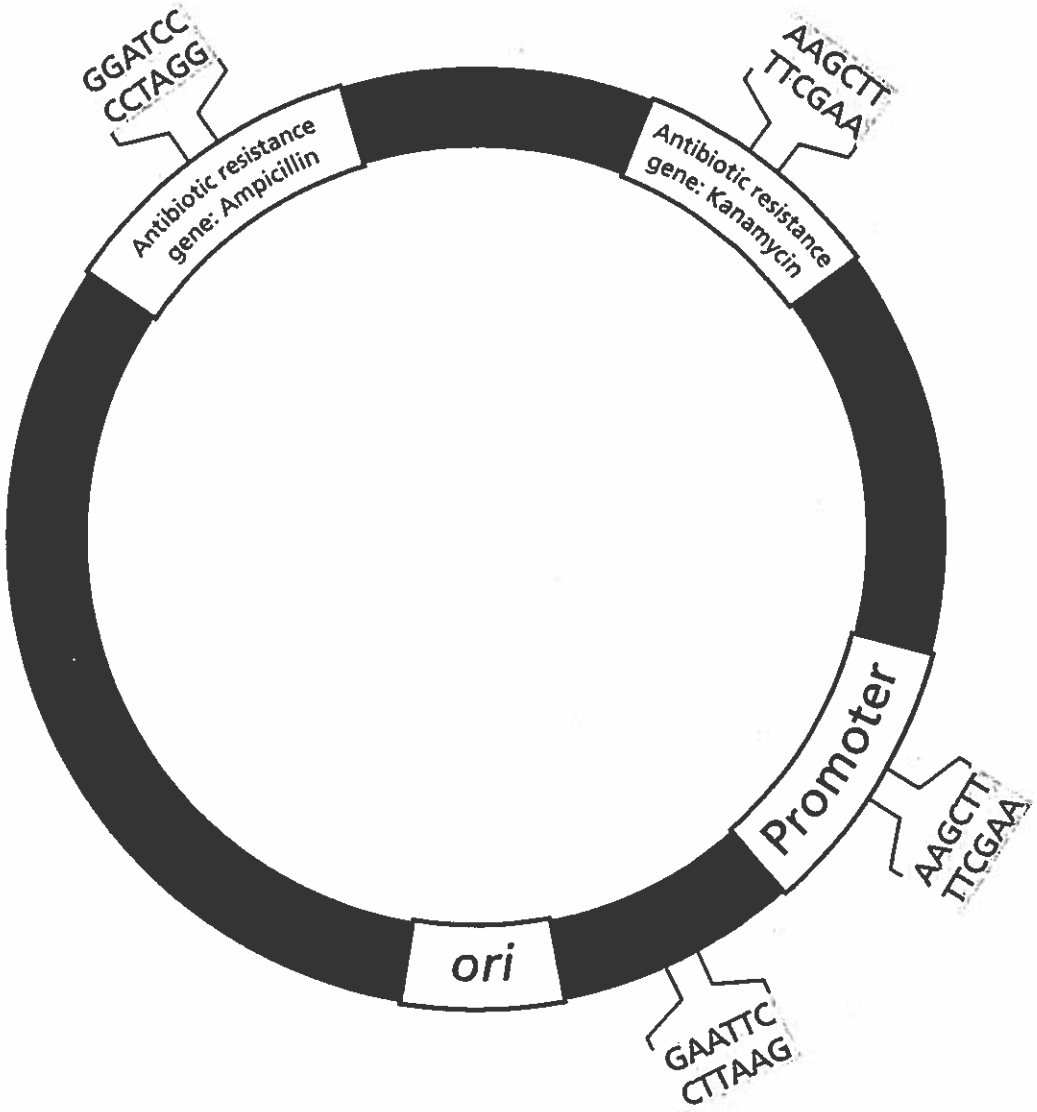
ACTIVITY QUESTIONS

1. Which restriction enzyme did you choose? Why did you choose that one?

2. Where would you insert the insulin gene and why?

3. Which antibiotic would you use to determine if the recombinant DNA was taken in?

CLONE THAT GENE: PLASMID DIAGRAM



Restriction Enzyme Recognition Sites

BamHI	GGATCC CCTAGG
EcoRI	GAATTC CTTAAG
HindIII	AAGCTT TTCGAA

CLONE THAT GENE: HUMAN DNA SEQUENCE

Human Insulin Gene

TAAGCTTCCATAGAATTCGCACGTTACAAGGATCCCGTGAAGGTAAAAGAATTCCTTA
ATTCGAAGGTATCTTAAGCGTGCAATGTTCCTAGGGCACTTCCATTTTCTTAAGGAAT

Restriction Enzyme Recognition Sites

BamHI	GGATCC CCTAGG
EcoRI	GAATTC CTTAAG
HindIII	AAGCTT TTCGAA