5

Microbial Biotechnology

Chapter 5 Introduction

- **Microbes (microorganisms)** are tiny organisms that are too small to be seen individually by the naked eye and must be viewed with the help of a microscope
  - Bacteria, fungi, algae, and protozoa
- Bacteria were the first life forms on earth and have existed for over 3.5 billion years
- Bacteria are estimated to comprise over 50% of the earth's living matter

Chapter 5 Introduction

- We are surrounded by bacteria.
  - They live in our mouths, on our skin and in our digestive tract.
  - There are approximately 10 times as many bacterial cells in the human body, than our own cells!

- Bacteria are adapted to living in some of the harshest environments on the planet:
  - Polar ice caps, deserts, boiling hot springs and under extraordinarily high pressure in deep-sea vents miles under the ocean's surface

Chapter 5 Introduction

- Humans have long used microbes in 'traditional biotechnology' and microbes are central to recombinant DNA technology

- Two exciting areas of microbial biotechnology are 'biofuels' and 'synthetic biology’

- Less than 1% of all bacterial species have been identified, cultured and studied, so we can only imagine the contribution of microbes to biotechnology in the future

The Structure of Microbes

- Eukaryotic microbes include yeast, algae and protozoans

- Prokaryotes are single celled microorganisms which include the domains Bacteria and Archaea
  - Archaea share properties of both eukaryotes and prokaryotes
  - They live in extreme environments and have unique metabolic properties
  - Those living in salty environments are called '**halophiles**' and those living in hot environments are called '**thermophiles**'

The Structure of Microbes

- Structural Features of Bacteria
  - Small (1–5 µm)
  - No nucleus; DNA is contained in a single, circular chromosome
  - May contain plasmids
  - Cell wall that surrounds plasma membrane contains *peptidoglycan*; provides rigidity for protection
  - Some bacteria contain an outer layer of carbohydrates in a structure called a *capsule*

The Structure of Microbes

- Bacteria are classified by the Gram stain
  - Gram + bacteria stain purple
    - Have simple cell walls rich in peptidoglycan
  - Gram – bacteria stain pink
    - Have complex cell wall structures with less peptidoglycan

The Structure of Microbes

- Single, circular chromosome is relatively small
  - 2–4 million base pairs

- Some bacteria contain plasmids as well
  - Plasmids often contain genes for antibiotic resistance and genes encoding proteins that form connecting tubes called *pili* which allow exchange of genetic information between cells.
  - Plasmids are an essential tool for cloning

The Structure of Microbes

- Bacteria grow and divide rapidly
  - Divide every 20 minutes or so
  - Millions of cells can be grown on small dishes of agar or in liquid culture media
- Easy-to-make mutant strains to be used for molecular and genetic studies

The Structure of Microbes

- Yeast – single-celled eukaryotic microbes; fungi
  - Sources of antibiotics and drugs that lower cholesterol
  - Mechanisms of gene expression resemble those in human cells

The Structure of Microbes

- Yeast
  - Can grow in the presence of oxygen **(aerobic)** or in the absence of oxygen **(anaerobic)**

  - *Pichia pastoris*
    - Grows to a higher density in liquid culture than other yeast strains
    - Has a number of strong promoters that can be used for production of proteins
    - Can be used in batch processes to produce large number of cells

Microorganisms as Tools

- Microbial Enzymes
    - Used in applications from food production to molecular biology research
    - Taq DNA polymerase
        - Heat stabile, isolated from a thermophile
    - Cellulase
        - Makes animal food more easily digestible
        - Stone-washed jeans
    - Subtilisin
        - Laundry detergents

- 'Bioprospecting', the discovery and development of new products from biological resources, promises to unearth other valuable microorganisms

Microorganisms as Tools

- **Transformation** – the ability of bacteria to take in DNA from their surrounding environment
  - Bacteria will naturally take up DNA from the environment.
  - In biotechnology, cells are treated so that they become **competent** and are able to take up DNA more easily.
  - Transformation of **competent cells** is used to introduced recombinant plasmids into cells so the bacterial can replicate these plasmids.
  - This process is called transformation because one can "transform" the properties of bacterial cells by introducing foreign genes.

Microorganisms as Tools

- There are two methods for transformation commonly used in biotechnology:

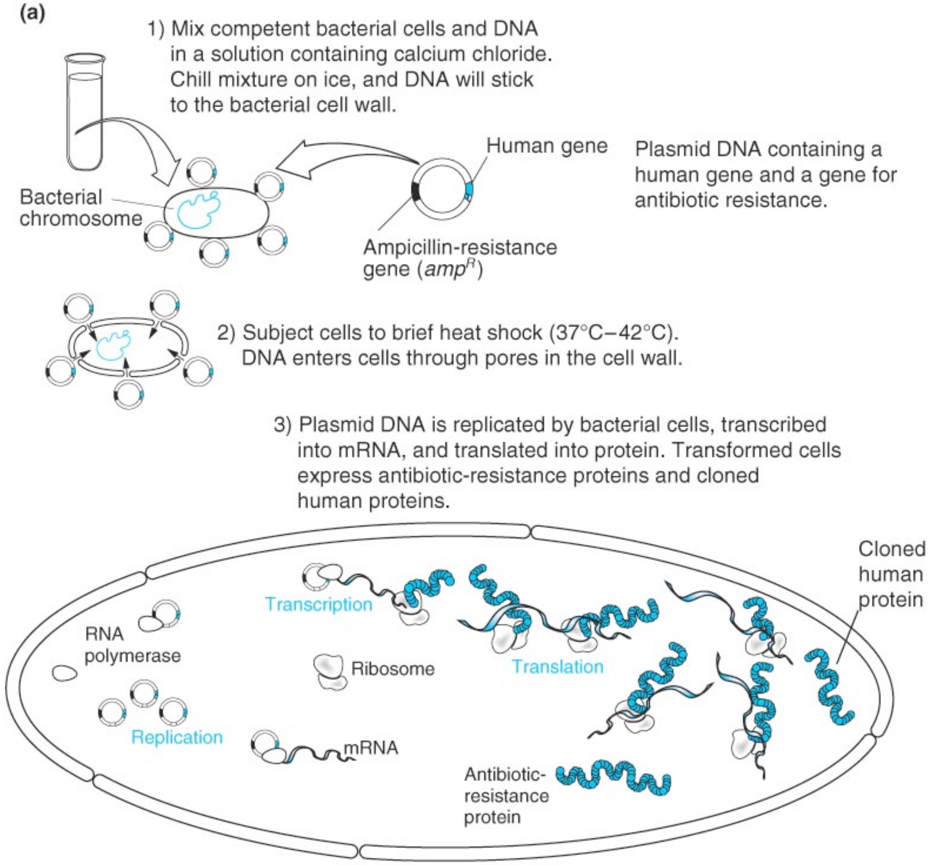
1. The 'calcium chloride' method involves treatment of cells with ice-cold solution of calcium chloride followed by a brief 'heat shock’

2. Electroporation uses a brief electrical shock to introduce DNA into cells

Microorganisms as Tools

- **Calcium Chloride Transformation**
  - Target DNA is introduced into a plasmid containing one or more antibiotic resistance genes
  - Plasmid vector is mixed in a tube with competent cells and placed on ice
  - Cells are heated briefly (heat shock) to allow DNA to enter cell
  - Grow in liquid media
  - Plate on agar plates containing antibiotics
  - Only cells which have taken up the plasmid will be able to divide and produce colonies

(a)

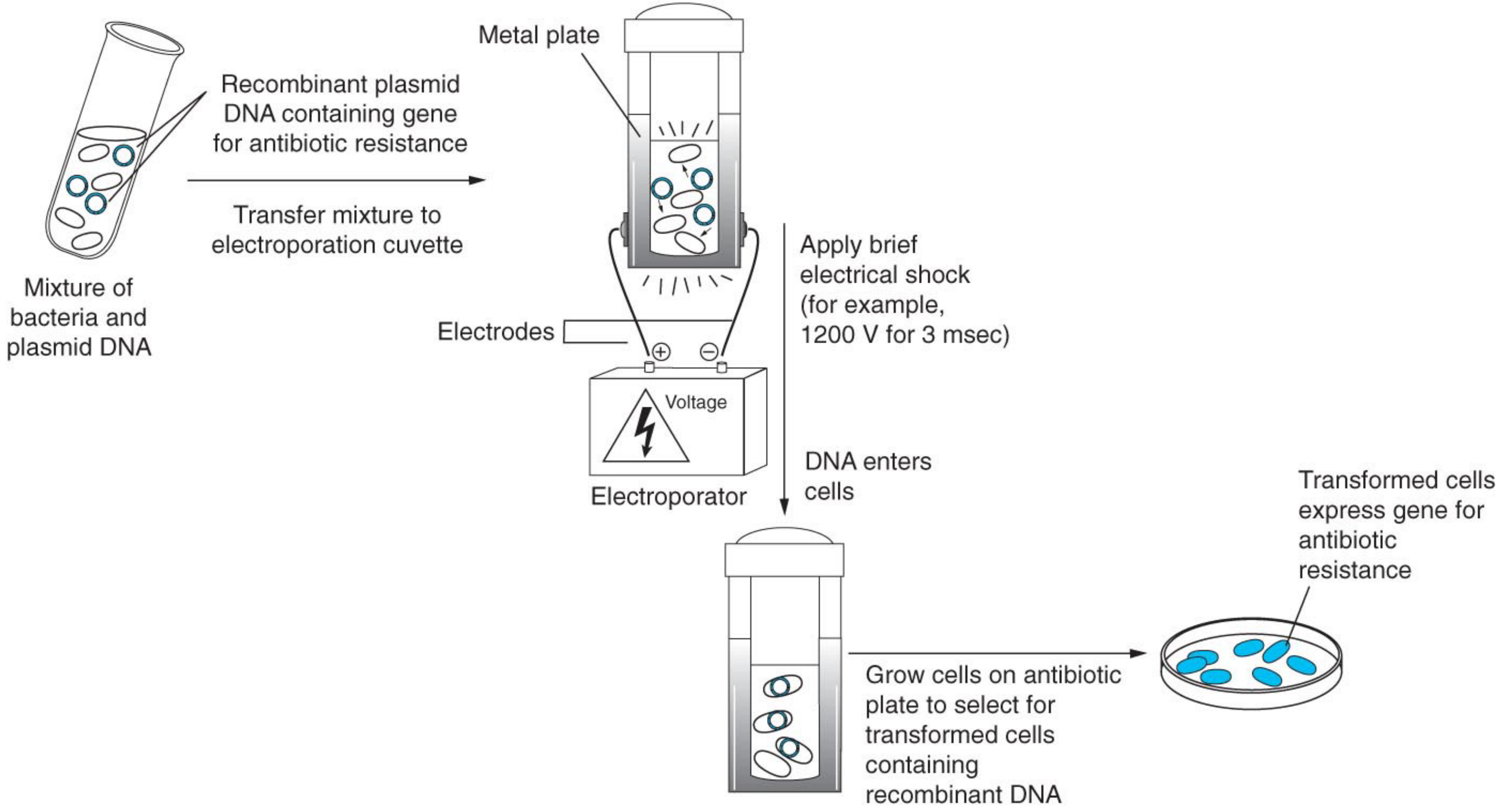

1) Mix competent bacterial cells and DNA in a solution containing calcium chloride. Chill mixture on ice, and DNA will stick to the bacterial cell wall.
Bacterial chromosome
Human gene
Plasmid DNA containing a human gene and a gene for antibiotic resistance.
Ampicillin-resistance gene (*amp*^{R})
2) Subject cells to brief heat shock (37°C–42°C). DNA enters cells through pores in the cell wall.
3) Plasmid DNA is replicated by bacterial cells, transcribed into mRNA, and translated into protein. Transformed cells express antibiotic-resistance proteins and cloned human proteins.
Transcription
RNA polymerase
Ribosome
Translation
Cloned human protein
Replication
mRNA
Antibiotic-resistance protein

Microorganisms as Tools

- **Calcium Chloride Transformation**
  - The exact mechanism for transformation is not understood
  - It is thought that when the cells are cold, DNA will stick to them and the cold creates gaps in the membrane that allow DNA to enter the cells when they are heat shocked

Microorganisms as Tools

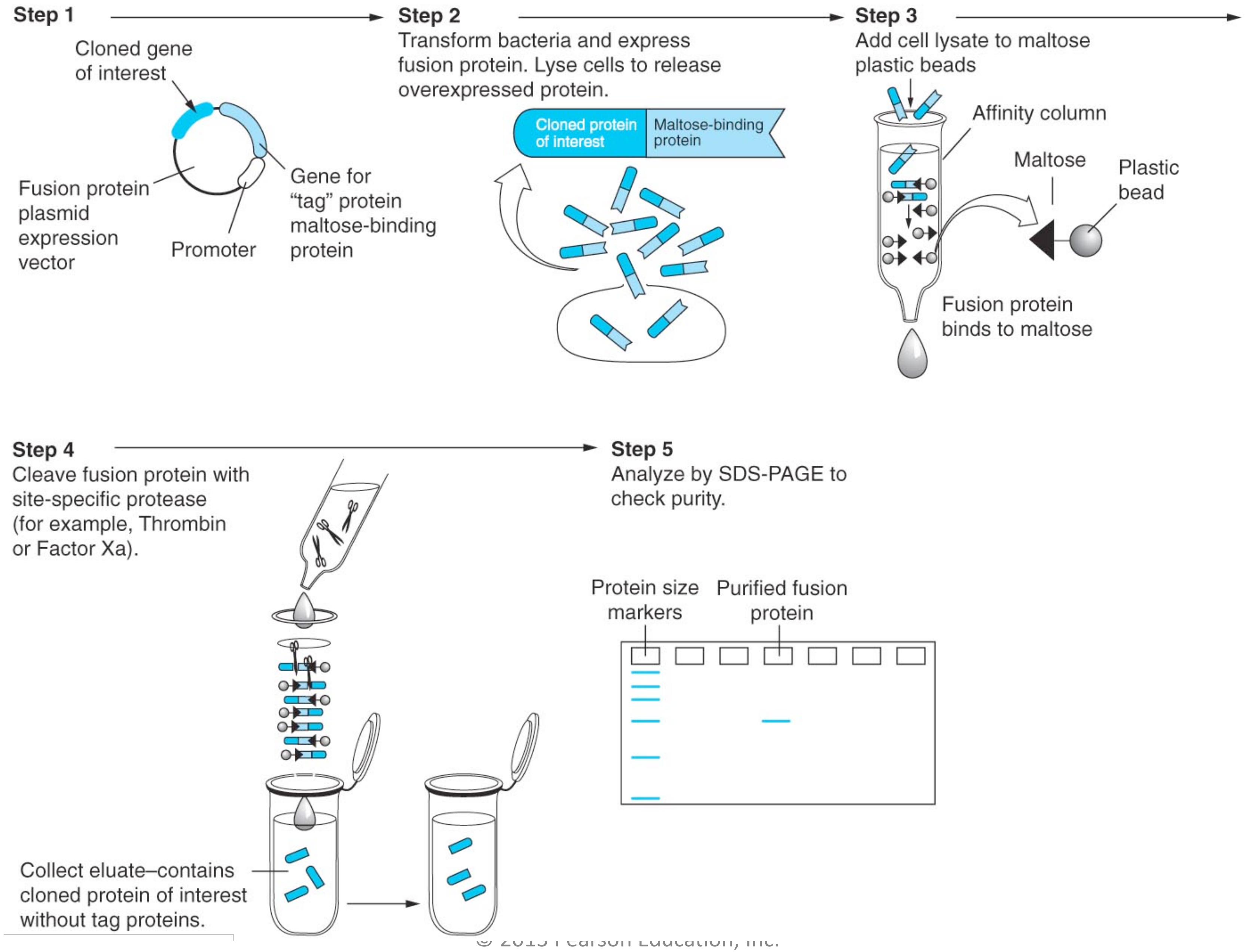
- **Electroporation**
  - An instrument called an electroporator produces a brief electrical shock that introduces DNA into the cells without killing them
  - Advantages
    - Rapid
    - Requires fewer cells
    - Can be used to introduce DNA into other cell types
    - More efficient process



Metal plate
Recombinant plasmid DNA containing gene for antibiotic resistance
Transfer mixture to electroporation cuvette
Mixture of bacteria and plasmid DNA
Electrodes
Voltage
Electroporator
Apply brief electrical shock (for example, 1200 V for 3 msec)
DNA enters cells
Transformed cells express gene for antibiotic resistance
Grow cells on antibiotic plate to select for transformed cells containing recombinant DNA

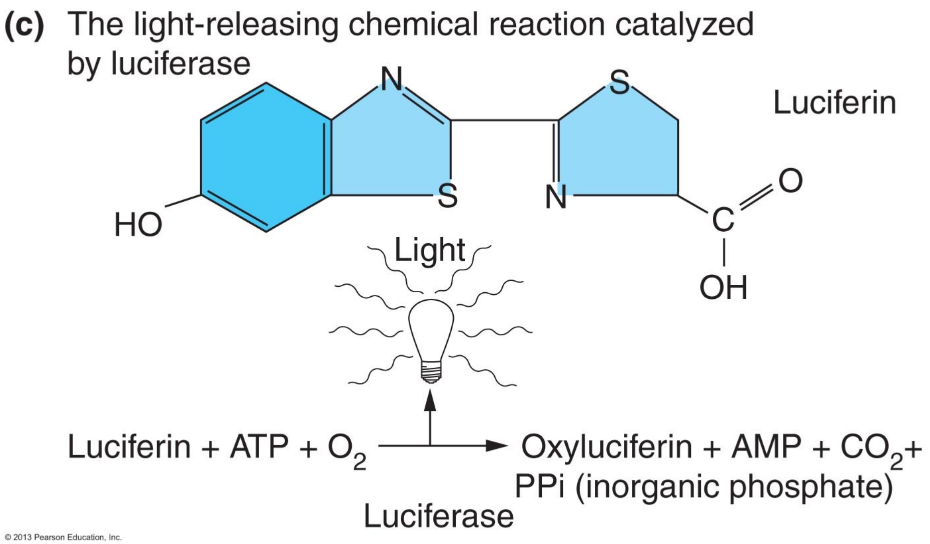
Microorganisms as Tools

- Bacteria can be used to mass-produce proteins
  - A useful way to express proteins that can be easily purified for use is to create **fusion proteins**
    - The gene for protein of interest is cloned into a type of protein known as an expression vector which has a gene for the "tag" protein downstream of a promoter
    - The gene for the protein of interest and the "tag" protein are expressed as a fusion protein which then can be purified by a technique called **affinity chromatography**


Step 1
Cloned gene of interest
Fusion protein plasmid expression vector
Promoter
Gene for "tag" protein maltose-binding protein
Step 2
Transform bacteria and express fusion protein. Lyse cells to release overexpressed protein.
Cloned protein of interest
Maltose-binding protein
Step 3
Add cell lysate to maltose plastic beads
Affinity column
Maltose
Plastic bead
Fusion protein binds to maltose
Step 4
Cleave fusion protein with site-specific protease (for example, Thrombin or Factor Xa).
Collect eluate–contains cloned protein of interest without tag proteins.
Step 5
Analyze by SDS-PAGE to check purity.
Protein size markers
Purified fusion protein

Microorganisms as Tools

- Microbial Proteins as Reporters
  - **Bioluminescence** – method of producing light used by marine organisms
    - Created by bacteria such a *Vibrio fisheri* that use marine organisms as a host
    - Create light through action of *lux* genes

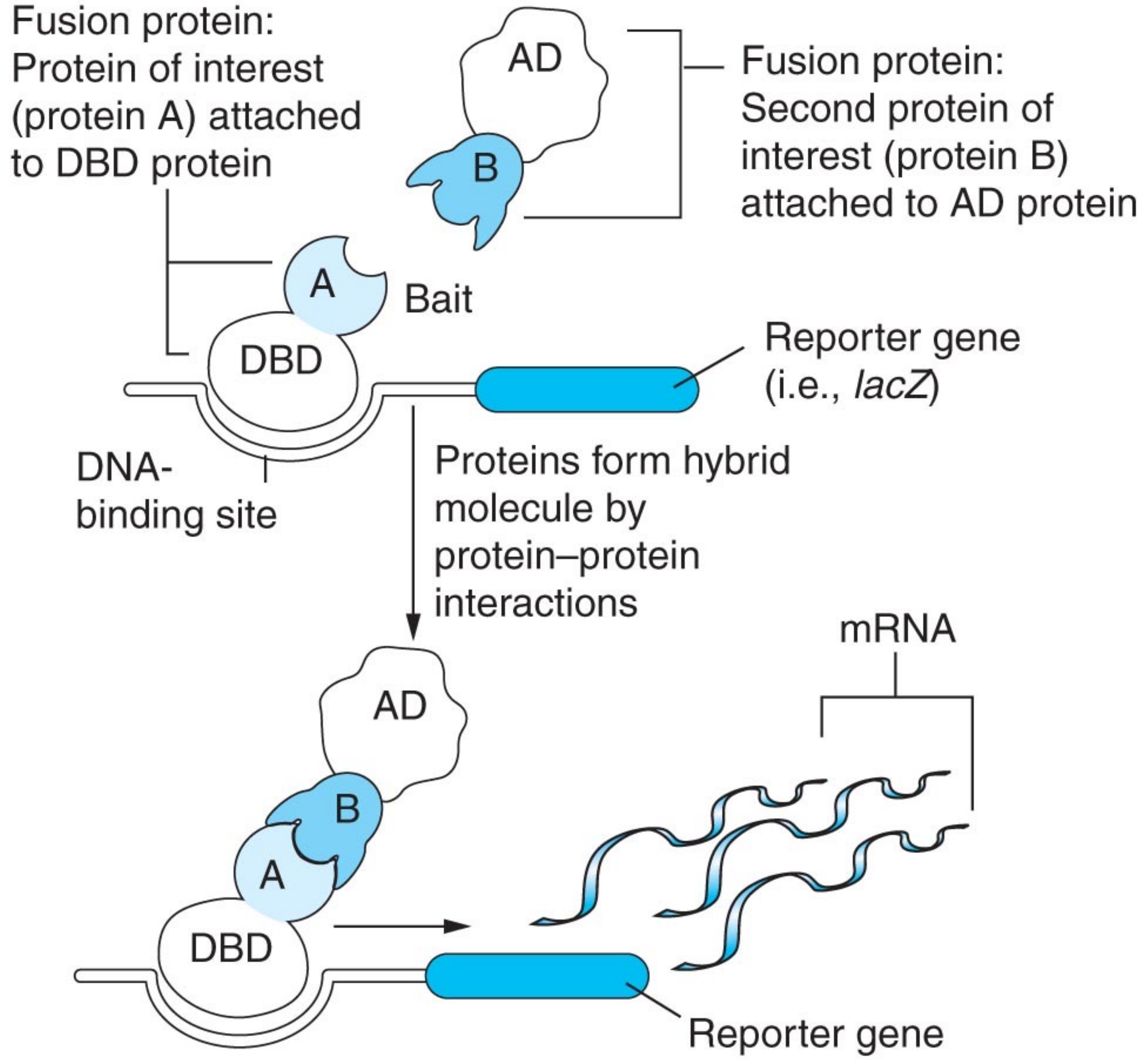


Microorganisms as Tools

- Microbial Proteins as Reporters
  - *Lux* genes have been cloned and used to study gene expression
    - Clone *lux* genes into plasmid
    - If inserted into animal or plant cells, will produce luciferase and will fluoresce, providing a visual indicator of gene expression
    - Lux genes have been used to develop a fluorescent bioassay to test for tuberculosis

Microorganisms as Tools

- **The Yeast Two-Hybrid System**
  - Say a research wants to know if proteins 'A' and 'B' interact with each other
  - Two fusion proteins are created
    - One is a fusion of a DNA binding domain (DBD) of a protein such a transcription factor with protein 'A'
    - The second is a fusion of an activator domain (AD) from a transcription factor and protein 'B'
  - If protein 'A' and 'B' interact, the DNA binding domain and the activator domain will be brought together, making a functional transcription factor, which interacts with the promoter for a reporter gene, such as lacZ, that be expressed



Hybrid protein can now stimulate transcription of reporter gene. Activity of protein from reporter gene mRNA can be measured.

Using Microbes for a Variety of Everyday Applications

- **Food Products**
  - Microbes are used with traditional and modern biotechnology to make many foods, including bread, yogurt, cheese and alcoholic beverages
  - The first recombinant DNA food ingredient approved by the Food and Drug Administration (FDA) is a recombinant form of an enzyme that is used to make cheeses
  - Curds to make cheeses are made traditionally from rennin, an enzyme which is extracted from the stomach of calves
  - **Chymosin**, is a rennin that was cloned and expressed in bacteria and is less expensive and easier to produce

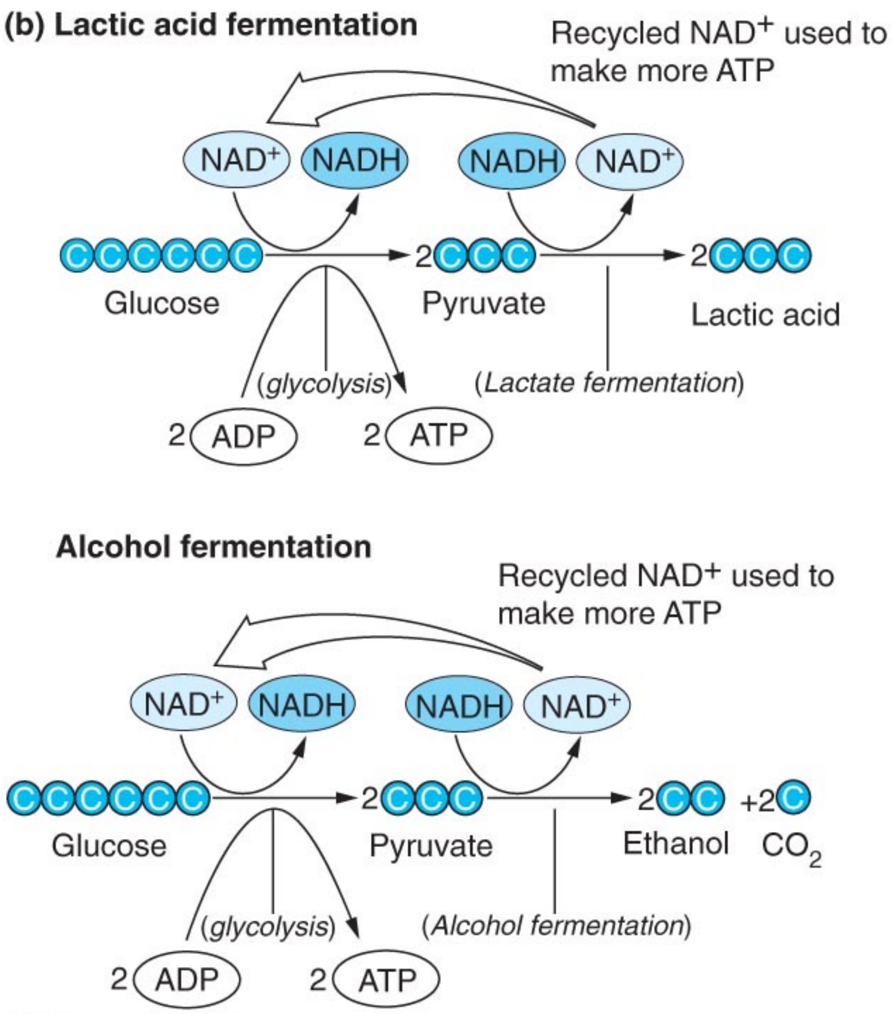
Using Microbes for a Variety of Everyday Applications

- **Fermentation** – process of deriving energy from sugars in the absence of oxygen

    1. **Lactic acid fermentation:** used to make yogurt, sour cream, sauerkraut, vinegar and certain cheese and breads.

    2. **Alcohol fermentation:** used to make beer, wine, champagne

Using Microbes for a Variety of Everyday Applications



Using Microbes for a Variety of Everyday Applications

- **Therapeutic Proteins**
  - Bacteria are used to produce medically important proteins
  - Insulin, the first recombinant molecule expressed in bacteria for use in humans is an excellent example:

Using Microbes for a Variety of Everyday Applications

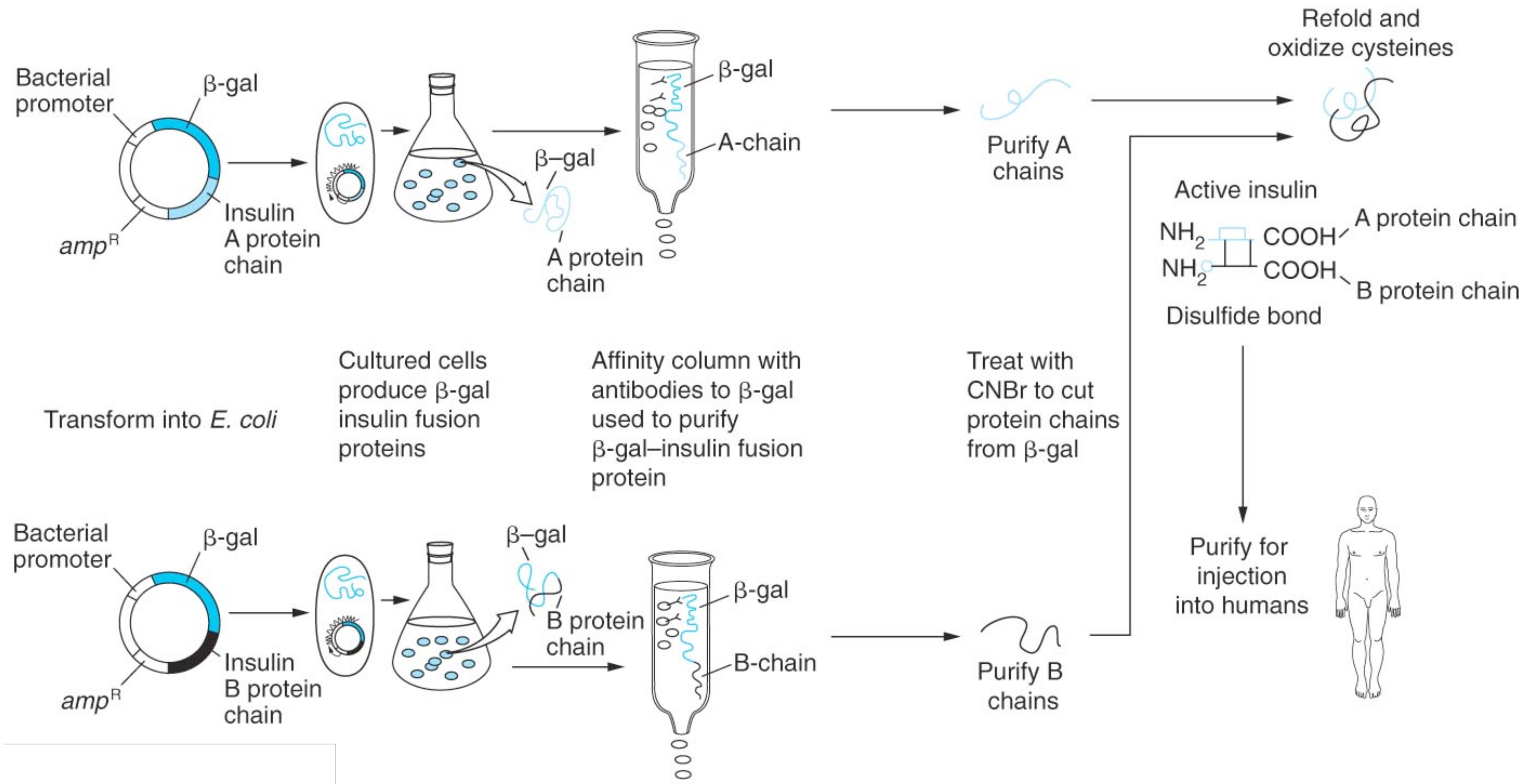
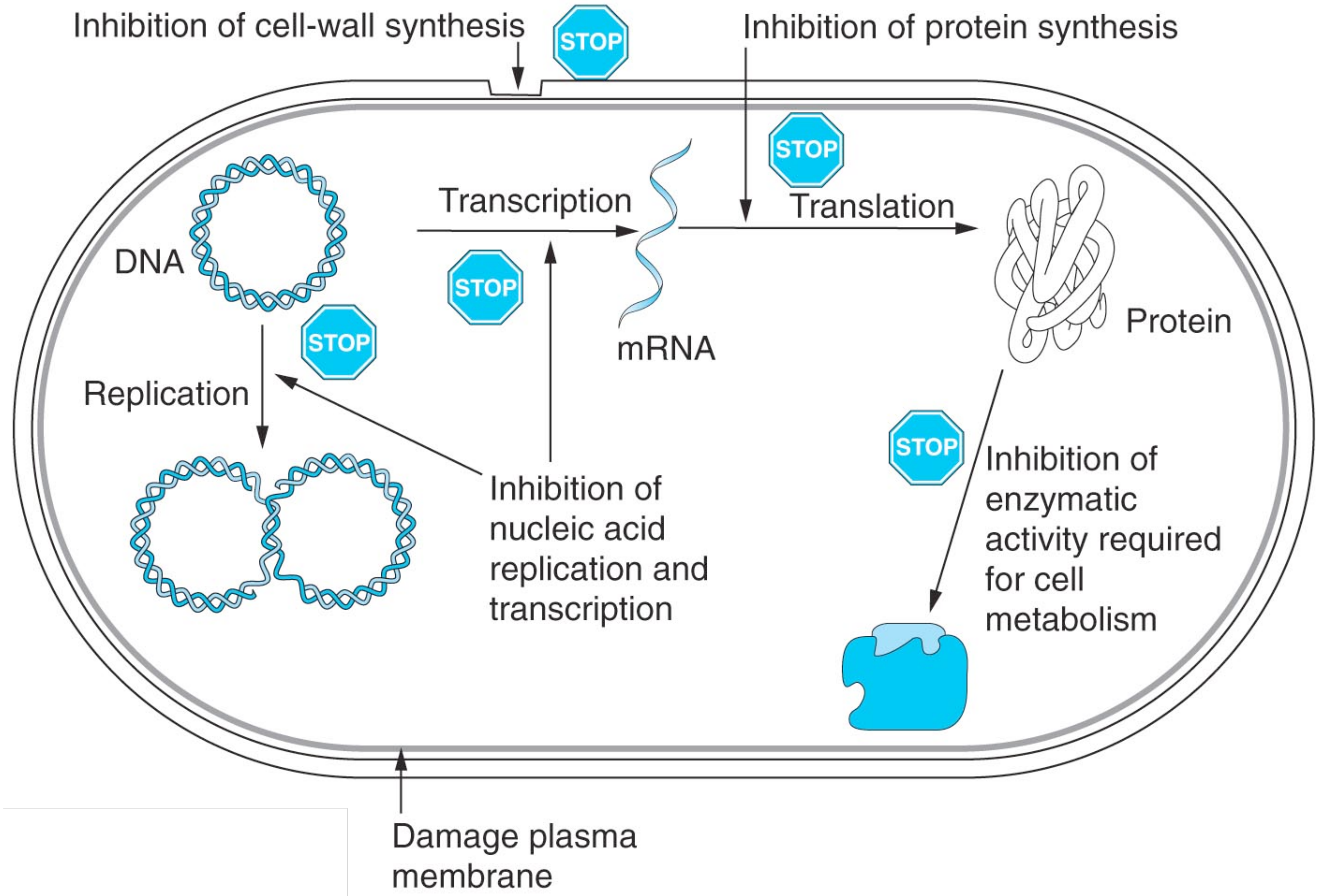


TABLE 5.1 THERAPEUTIC PROTEINS FROM RECOMBINANT BACTERIA

Protein	Function	Medical Application(s)
DNase	DNA-digesting enzyme	Treatment of patients with cystic fibrosis.
Erythropoietin	Stimulates production of red blood cells	Used to treat patients with anemia (low number of red blood cells).
Factor VIII	Blood clotting factor	Used to treat certain types of hemophilia (bleeding diseases due to deficiencies in blood clotting factors).
Granulocyte colony-stimulating factor	Stimulates growth of white blood cells	Used to increase production of certain types of white blood cells; stimulate blood cell production following bone marrow transplants.
Growth hormone (human, bovine, porcine	Hormone stimulates bone and muscle tissue growth	In humans, used to treat individuals with dwarfism. Improves weight gain in pigs and cows; stimulates milk production in cows.
Insulin	Hormone required for glucose uptake by body cells	Used to control blood sugar levels in patients with diabetes.
Interferons and interleukins	Growth factors that stimulate blood cell growth and production	Used to treat blood cell cancers such as leukemia; improve platelet counts; some used to treat different cancers.
Superoxide dismutase	An antioxidant that binds and destroys harmful free radicals	Minimizes tissue damage during and after a heart attack.
Tissue plasminogen activator (tPA)	Dissolves blood clots	Used to treat patients after heart attack and stroke.
Vaccines (e.g., hepatitis B vaccine)	Stimulate the immune system to prevent bacterial and viral infections	Used to immunize humans and animals against a variety of pathogens; also used in some cancer tumor treatments.

Using Microbes for a Variety of Everyday Applications

- **Antibiotics**
  - Produced by microbes that inhibit the growth of other microbes
  - 1928 discovery of penicillin by Alexander Fleming
  - Majority are produced by bacteria, and inhibit the growth of other bacteria


Inhibition of cell-wall synthesis
STOP
Inhibition of protein synthesis
STOP
Transcription
Translation
DNA
STOP
mRNA
Protein
STOP
Replication
Inhibition of nucleic acid replication and transcription
STOP
Inhibition of enzymatic activity required for cell metabolism
Damage plasma membrane

Using Microbes for a Variety of Everyday Applications

- **Antibiotics**
  - Improper use of antibiotics has lead to dramatic increase in antibiotic-resistant bacteria
  - Since we can see that known antibiotics attack a bacterial cell in a limited number of ways, resistance to one antibiotic often leads to resistance to many other drugs.
  - New antimicrobial drugs that act in unique ways need to be developed
  - Microbiologists are bioprospecting in diverse habitats to identify sources of new anti microbial substances

Vaccines

- First vaccine developed in 1796 by Edward Jenner
  - Used live cowpox virus to vaccinate against smallpox

Vaccines

- Immune System and Antibodies
  - **Antigens** are foreign substances that stimulate an immune response
    - Whole bacteria, fungi, and viruses
    - Proteins, lipids, or carbohydrates
  - Immune system responds to antigens by producing antibodies
    - Called **antibody-mediated immunity**
    - **B cells**, with the help of **T cells**, recognize and bind to the antigen
    - B cells then develop to form **plasma cells** that produce antibodies

Vaccines

- Immune System and Antibodies
  - Antibodies are very specific
    - Bind to the antigen
    - Macrophage can then recognize the antigens coated with antibodies and "eat" them
  - Sometimes our natural production of antibodies is not enough to protect us from pathogens

Vaccines

- **Vaccines** – parts of a pathogen or whole organisms that can be given to humans or animals by mouth or by injection to stimulate the immune system against infection by those pathogens

Vaccines

- Three Major Strategies to Make Vaccines
  1. **Subunit vaccines** are made by injecting portions of viral or bacterial structures
  2. **Attenuated vaccines** use live bacteria or viruses that have been weakened through aging or by altering their growth conditions to prevent replication
  3. **Inactivated (killed) vaccines** are made by killing the pathogen and using the dead or inactivated microorganism for the vaccine

Types of Vaccines

- Vaccines can be categorized into one of several groups:

1. **Live attenuated bacteria**
    - (bacillus Calmette– Guèrin, BCG, used to immunize against tuberculosis)
2. **Dead or inactivated bacteria**
    - (e.g. cholera and pertussis vaccines)
3. **Live attenuated viruses**
    - (e.g. measles, mumps and yellow fever viral vaccines)
4. **Inactivated viruses**
    - (e.g. hepatitis A and polio (Salk) viral vaccines)
5. **Toxoids**
    - (e.g. diphtheria and tetanus vaccines)
6. **Pathogen-derived antigens**
    - (e.g. hepatitis B, meningococcal, pneumococcal and *Haemophilus influenzae* vaccines)
7. **Nucleic acid vaccines**
    - (e.g. COVID-19)

Types of Vaccines

Vaccine Type	What is it?	Challenges	Examples
Live Attenuated	Weakened version of living microbe that can't cause disease	Mutation; Storage	Measles, mumps, rubella, polio (Sabin vaccine), yellow fever
Inactivated or "killed"	Microbes killed with chemicals, heat or radiation	Weaker immune response; Need boosters	Cholera, flu, hepatitis A, Japanese encephalitis, plague, polio (Salk vaccine), rabies
Subunit	Include antigens (or epitopes) that best stimulate immune system	Identifying specific antigen takes time	Hepatitis B, pertussis, pneumonia caused by S. Pneumoniae
Toxoid	Formalin inactivated toxins used as vaccine	Used when main cause of illness is a bacterial toxin	Diphtheria, Tetanus
Conjugate	Specialized subunit vaccine where antigens are linked to polysaccharides	Most effective for immature immune system of infants	H. Influenzae type b, pneumonia caused by S. Pneumoniae
DNA	DNA of important Antigens introduced to cell	Experimental	influenza and herpes as well as HIV
Recombinant vector	attenuated virus or bacterium (vector) used to introduce microbial DNA to cells	Experimental	HIV, rabies, and measles

Vaccines

- Currently, a majority of subunit vaccines are made using recombinant DNA approaches in which the vaccine is produced in microbes
  - Hepatitis B
    - Genes for proteins on the outer surface of the virus are cloned into expression plasmids and transformed into yeast
    - Fusion proteins produced by the yeast are purified
  - Gardasil, which protects against four strains of human papillomavirus (HPV)

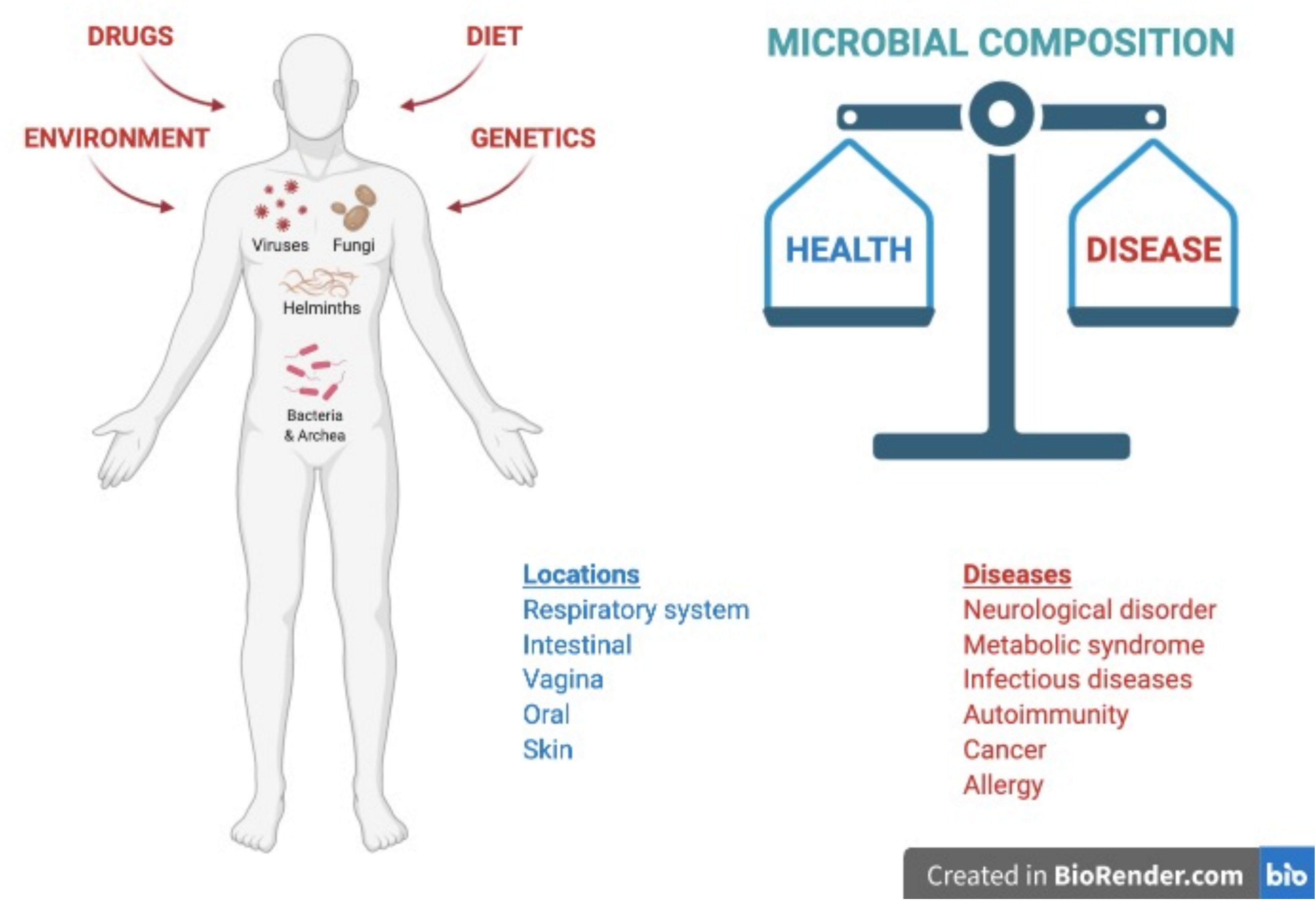
Microbial Genomes

- Why sequence microbial genomes?
  - Identify genes involved in bacterial cell metabolism, cell division, and genes that cause human and animal illnesses
  - Find new strains
    - For bioremediation or other tasks
    - Disease causing organisms

Microbial Genomes

- 2008 NIH announced plans for the Human Microbiome Project
  - Five year project to sequence 600 genomes of microorganisms that live on and inside humans

Microbiota & Microbiome

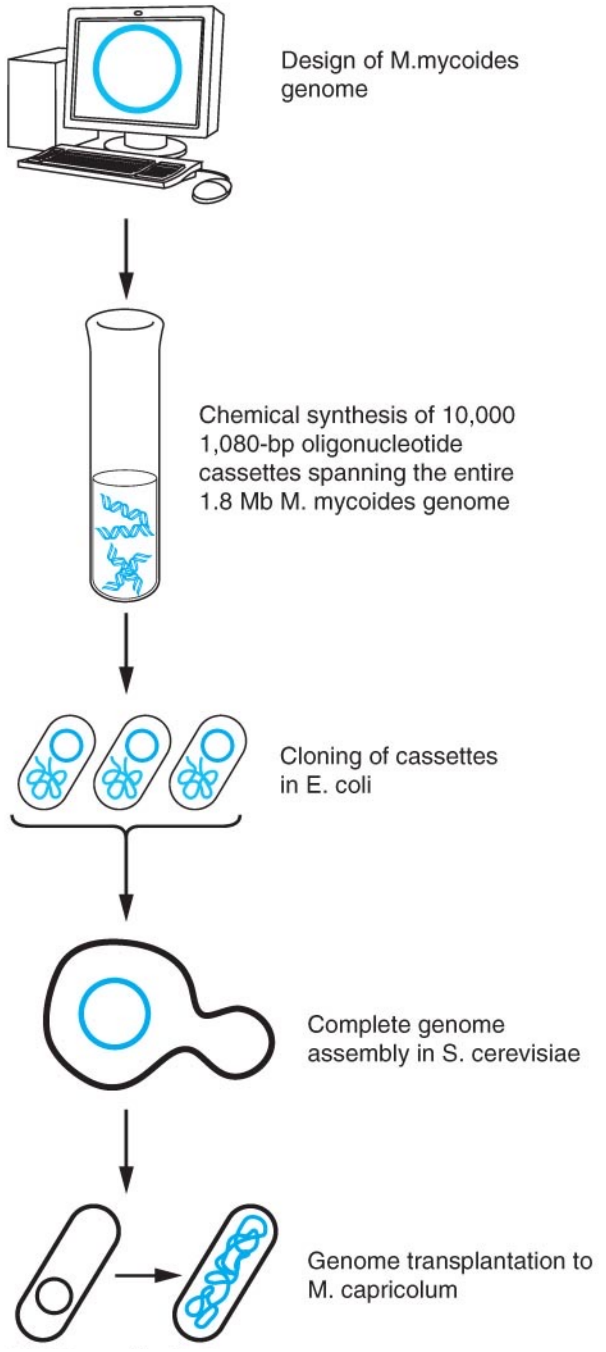


Microbial Genomes

- Goals of the Human Microbiome Project
  - determine if individuals share a core human microbiome
  - understand how we acquire and maintain microbial communities
  - understand how changes in the microbiome can be correlated with changes in health, and conditions that affect the microbiome
  - develop new methods for analysis of the microbiome
  - address ethical, legal and social implications raised by human microbiome research
    - Does this sound familiar?

Microbial Genomes

- Creating Synthetic Genomes: A Functional Synthetic Genome Is Produced for a Bacterial Strain



Microbial Genomes

- Creating Synthetic Genomes: A Functional Synthetic Genome Is Produced for a Bacterial Strain
  - The creation of M. mycoides JCVI-syn 1.0 because, while it did not create life from an inanimate object, it is a "proof" of concept the synthetic genomes can be produced.
  - It is speculated that new bacterial and other cells can be designed and programmed to be controlled as we want them to be for many uses.

Microbes for Making Biofuels

- Biorefineries could convert cellulose from stalks and other biomass into sugars that could be used to make ethanol sustainably.
- Microbes are being genetically engineered to more effectively break down cellulose to sugars, or converting sugars to ethanol.
- Bioprospecting efforts seek to identify microbes which produced other enzymes useful for making biofuels.