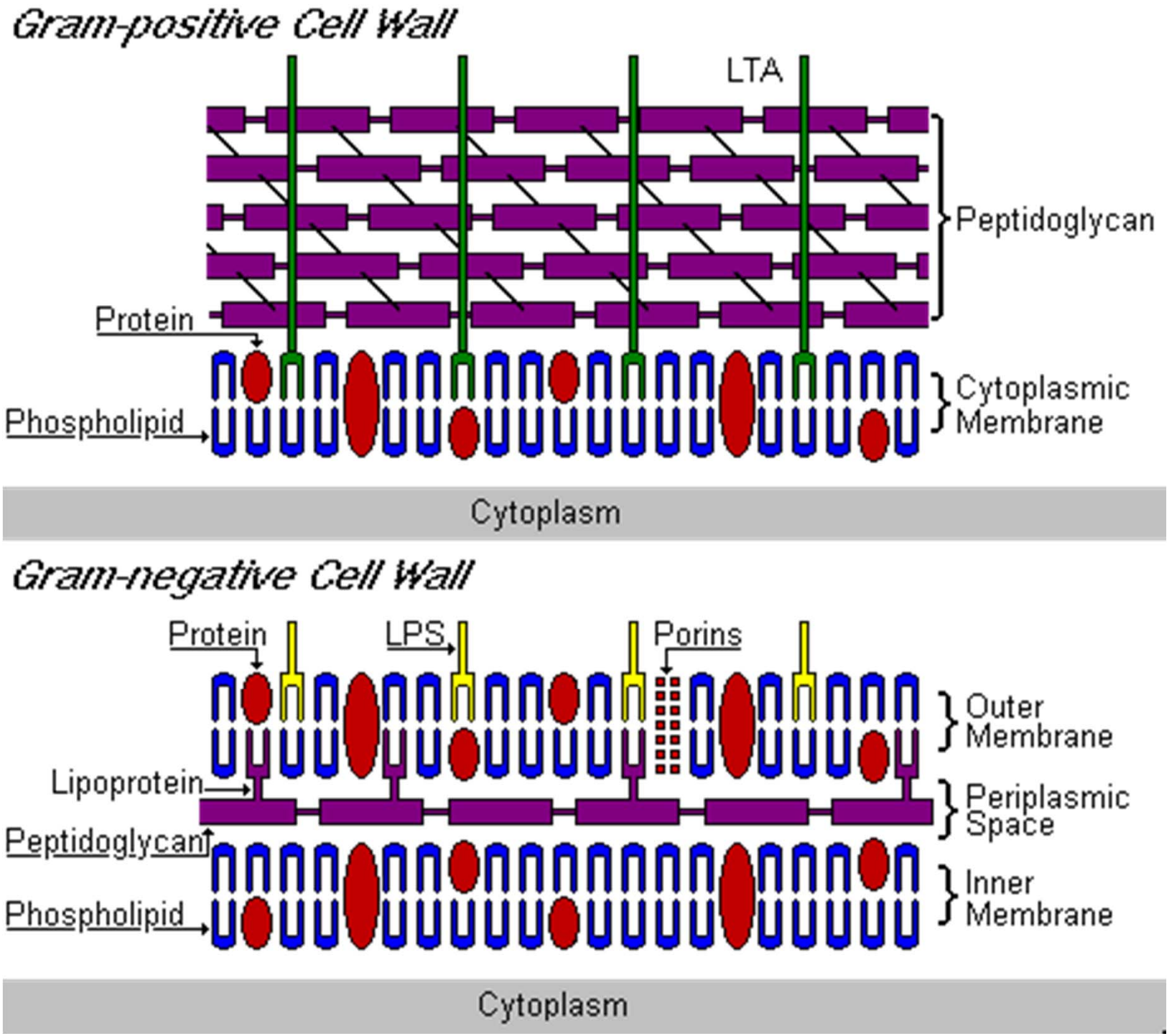
Previous Class

- Protein Sequencing, 3D structure determination
- Protein Microarray – Analytical, Functional and Reverse phase
- Introduction to microbes
- Gram staining
- Shapes of bacteria
- Intro to Fungi

Comparison of **gram +** and **gram -** bacteria

Cell wall structure	**Gram + bacteria (stain purple)**	**Gram – bacteria (stain pink)**
Complexity	Simple	Complex
Amount of **peptidoglycan** (protective sugar network)	Large amount	Small amount
Peptidoglycan placement	In outer layer of bacteria	Covered by outer membrane (protects from antibiotics)
Outer membrane	Absent	Present with lipopolysaccharides (toxic to host) attached


Gram-positive Cell Wall
LTA
Peptidoglycan
Protein
Phospholipid
Cytoplasmic Membrane
Cytoplasm
Gram-negative Cell Wall
Protein
LPS
Porins
Outer Membrane
Lipoprotein
Periplasmic Space
Peptidoglycan
Phospholipid
Inner Membrane
Cytoplasm

Microorganisms as Tools

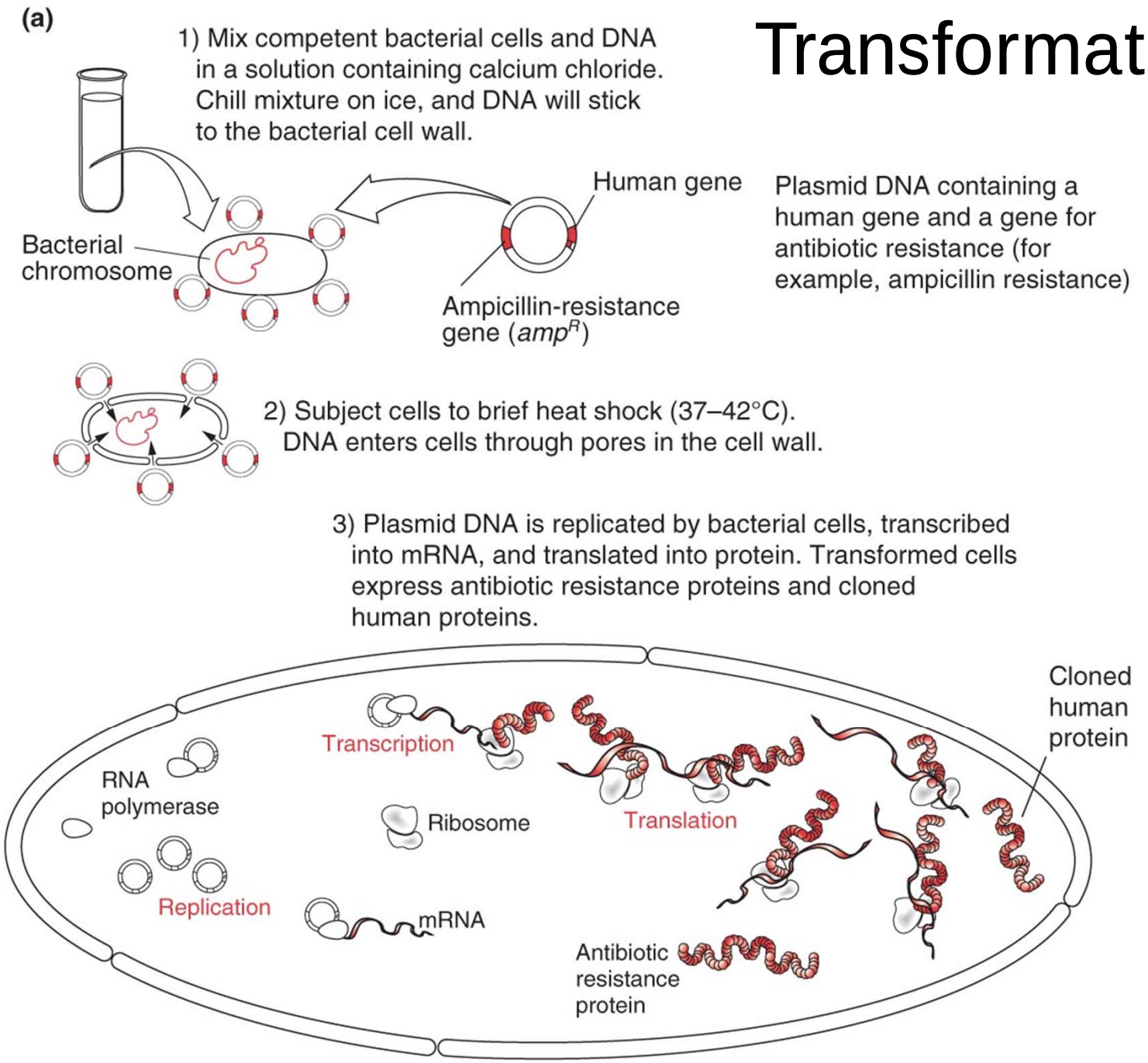
- Microbial Enzymes
  - Used in applications from food production to molecular biology research
  - Taq DNA polymerase
    - Isolated from a thermophile, *Thermus aquaticus*
  - Pfu polymerase
    - Thermostable enzyme, used for PCR, derived from *Pyrococcus furiosus*
  - Cellulase (produced by *E.coli*)
    - Degrade cellulose, a plant cell wall polysaccharide
    - Makes animal food more easily digestible
    - Stone-washed jeans
      - Treated with cellulases from fungi (*Trichoderma reesei* and *Aspergillus niger*). The cellulases mildly digest cellulose fibers in cotton and make softer fabric.
  - Subtilisin
    - Derived from *Bacillus subtilis*
    - Laundry detergents
    - Degrade protein stains from clothes

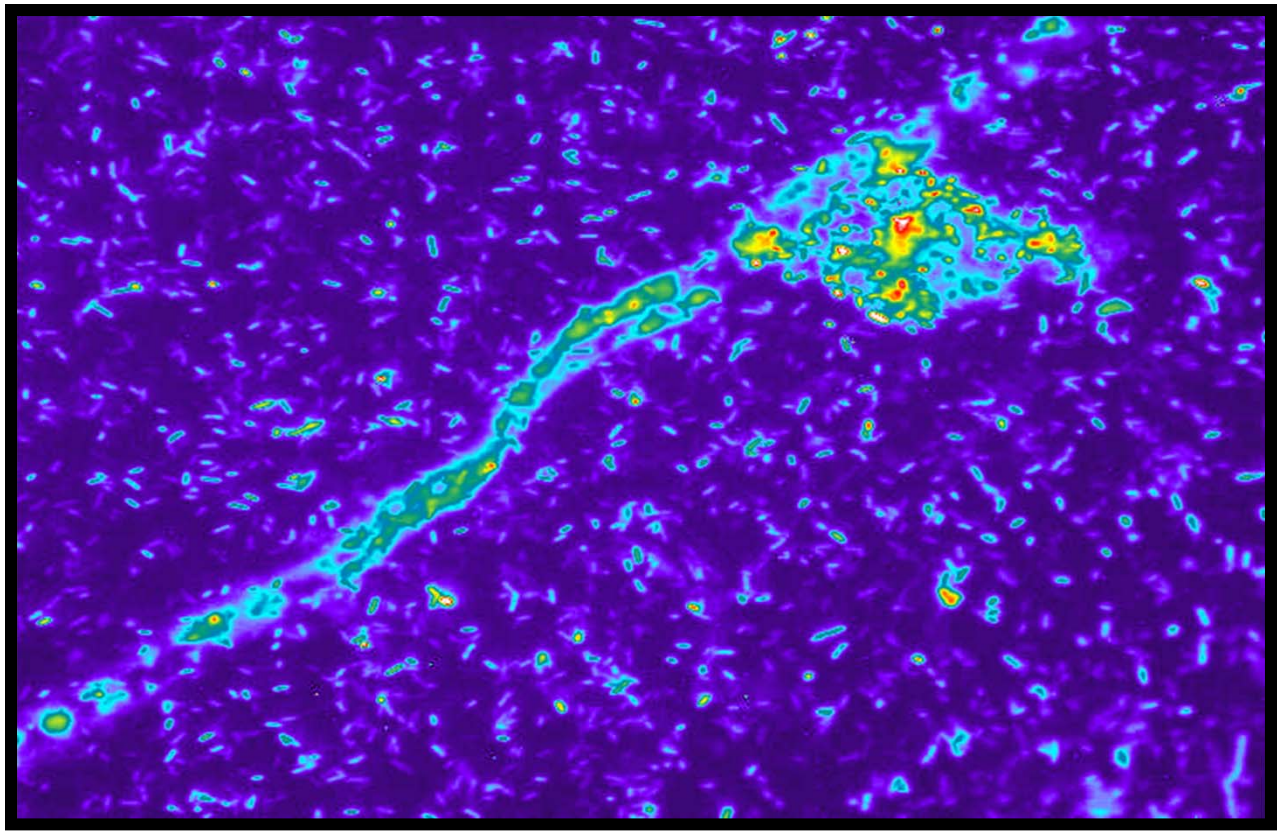
Microorganisms as Tools

- **Transformation** – the ability of bacteria to take in DNA from their surrounding environment
  - Essential step in the recombinant DNA cloning process
  - **Competent cells** are cells that have been treated so they are ready to take up DNA easily
    - Treat cells with ice-cold solution of calcium chloride
    - low temperature create gaps in lipid structure of cell membrane and Ca^{2+} ions neutralize the negative charges of phosphates in the cell membrane and the DNA

- **Transformation**
  - Target DNA is introduced into a plasmid containing one or more antibiotic resistance genes (Ligation)
  - Plasmid vector is mixed in a tube with competent cells and placed on ice
  - **Competent cells** are heated briefly (heat shock) to allow DNA to enter cell
  - Growth in liquid media
  - Plate on agar plates containing antibiotics

Transformation

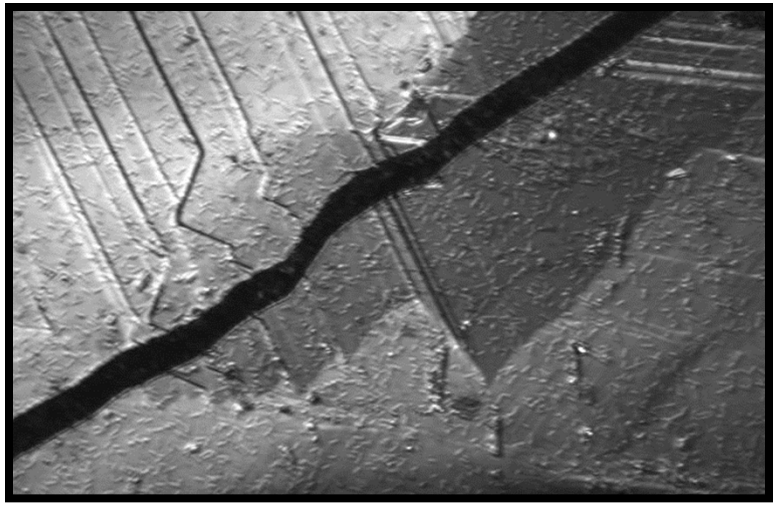




E. coli bacterium transformed with a gene from a jellyfish

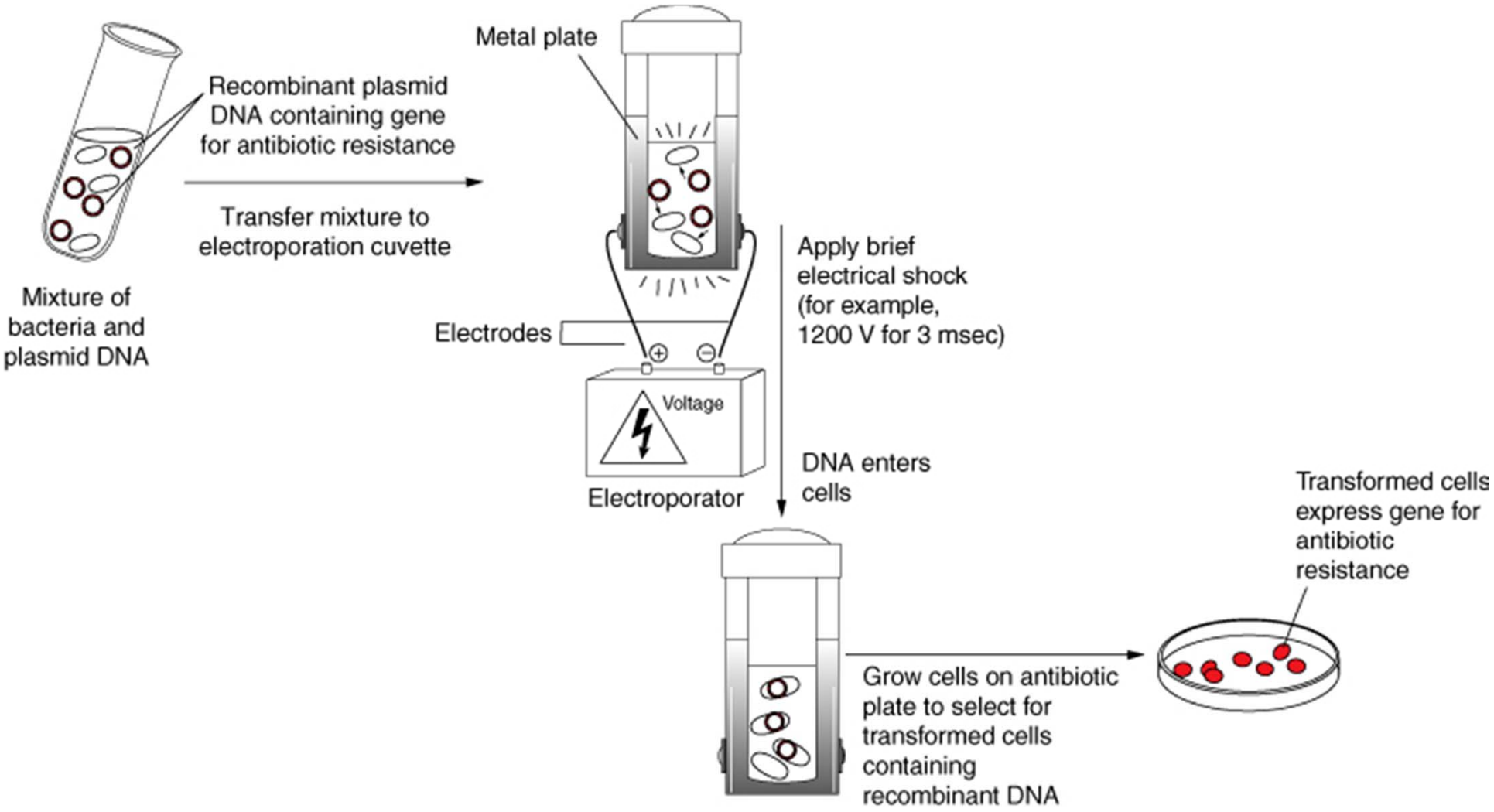
The jellyfish gene encodes a “green fluorescent protein” (GFP) which allows you to easily see a bacterial cell that has been transformed and expresses the protein

In this case, the gene is referred to as a reporter gene because it is reporting on the location of the bacterium.



Electroporation

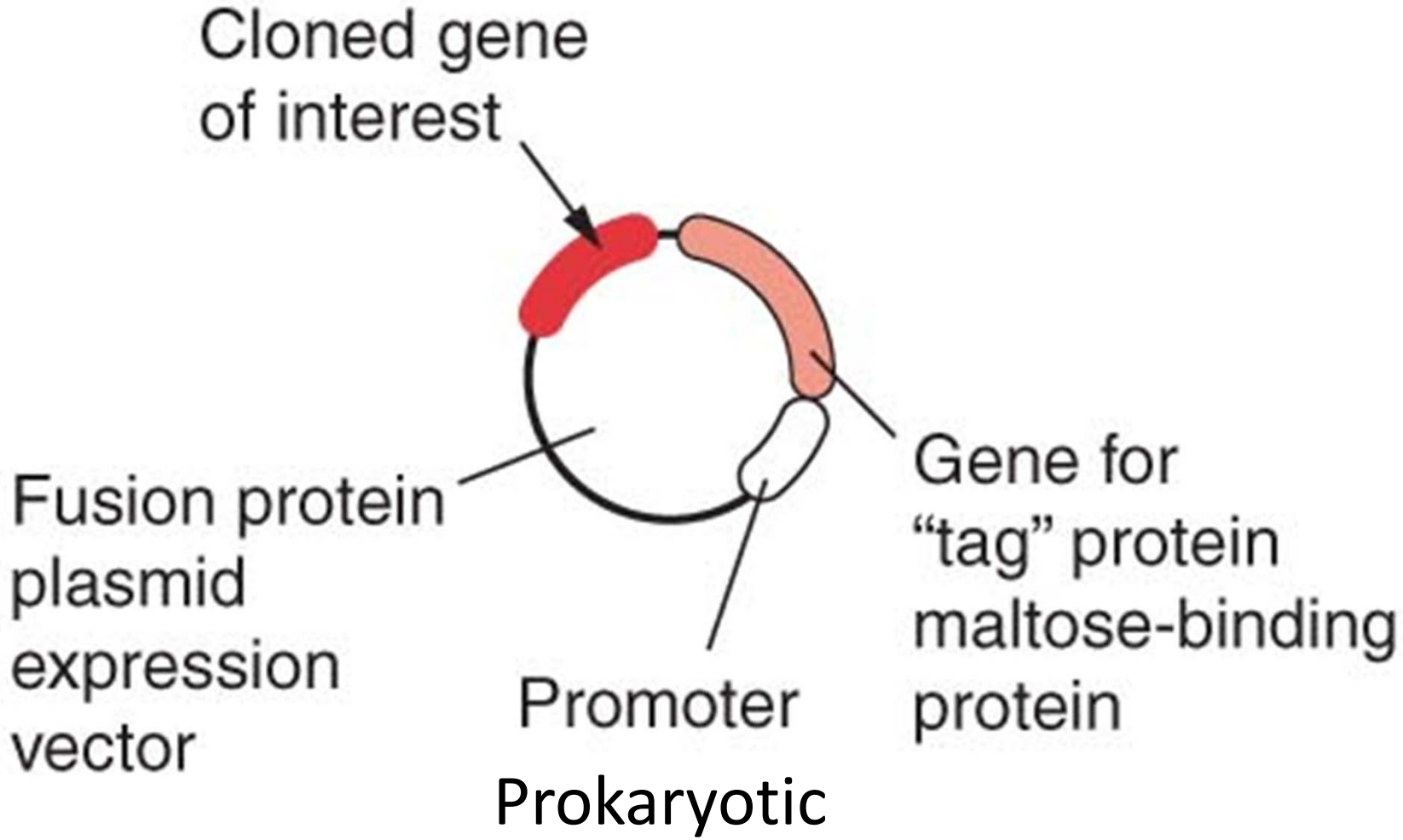
- An instrument called an electroporator produces a brief electrical shock that introduces DNA into the cells without killing them
- Advantages
  - Rapid
  - Requires fewer cells
  - Can be used to introduce DNA into other cell types
  - More efficient process


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

Bacterial fusion proteins

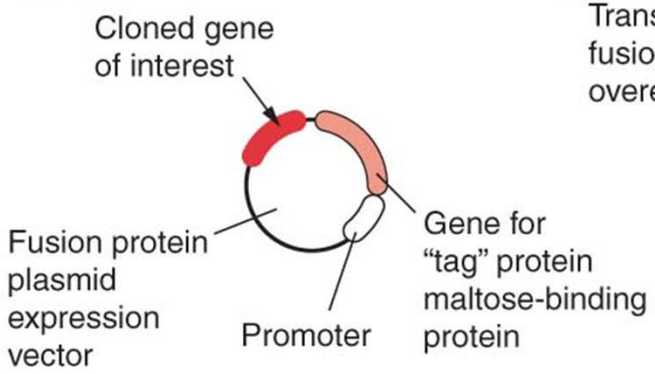
- Bacteria can be used to mass-produce proteins
  - **Bacterial fusion proteins**
    - Gene for protein of interest is inserted into a plasmid containing a gene for a well-known protein that serves as a "tag"
    - The tag protein allows for the isolation and purification of the recombinant protein as a fusion protein
    - Plasmid vectors used are often called **expression vectors**
      - Incorporate prokaryotic promoter sequences
      - Commonly used expression vectors carry – maltose binding protein, glutathione S-transferase, luciferase, green fluorescent protein, and β-galactosidase.

Expression Vectors



Prokaryotic

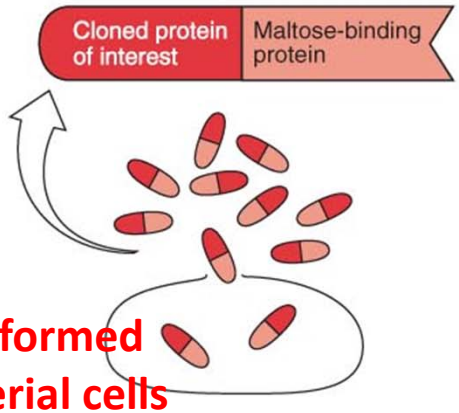
Step 1



Express fusion protein in expression vectors

Step 2

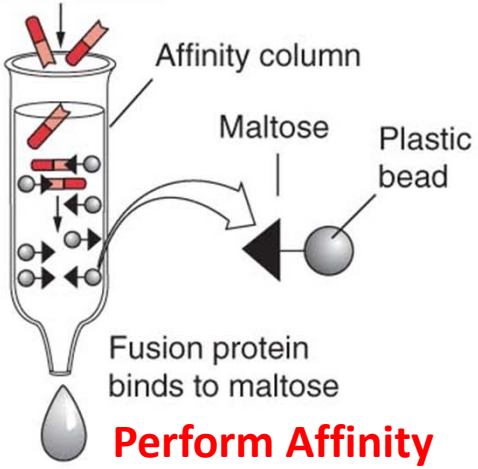
Transform bacteria and express fusion protein. Lyse cells to release overexpressed protein.



Lyse transformed bacterial cells

Step 3

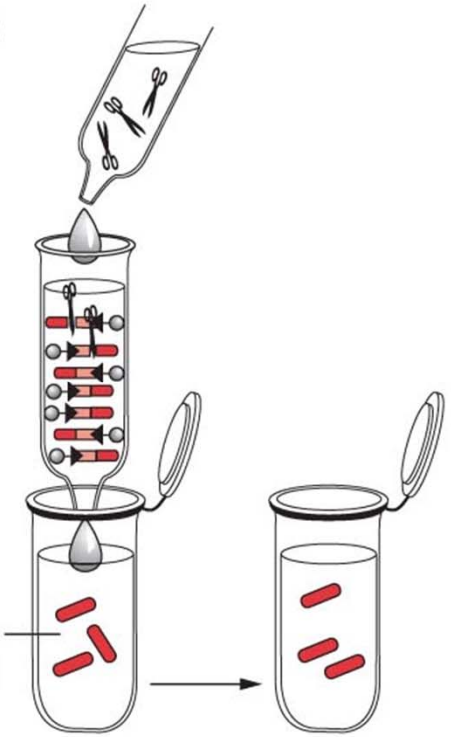
Add cell lysate to maltose plastic beads



Perform Affinity chromatography

Step 4

Cleave fusion protein with site-specific protease (for example, Thrombin or Factor Xa).

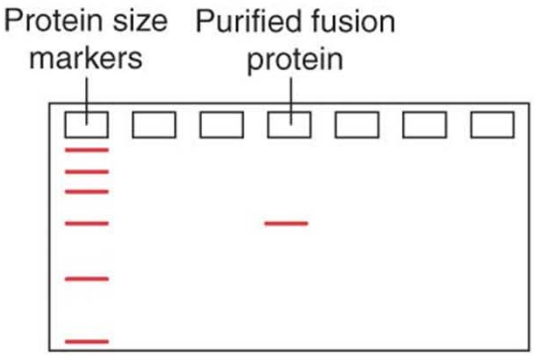


Protease cleavage to separate tag from protein if interest

Collect eluate–contains cloned protein of interest without tag proteins.

Step 5

Analyze by SDS-PAGE to check purity.



Purification

Preferred hosts for expression vectors

- *E. coli* & *B. subtilis* are commonly used for creating fusion proteins
- *B. subtilis* is used for human proteins as it secretes proteins into growth media where they can be easily harvested.
- *B. subtilis* process proteins such that the 3D folding and function is maintained.

Microbial Proteins as Reporters

- **Bioluminescence** – method of producing light used by marine organisms to attract mates in dark ocean
  - Created by bacteria such a *Vibrio fisheri* that use marine organism as a host
  - Create light through action of *lux* genes encoding proteins subunits that form an enzyme **luciferase**

Microbial Proteins as Reporters

– *Lux* genes have been cloned and used to study gene expression
  - Clone *lux* genes into plasmid
  - *lux* plasmid can be used to produce a fusion protein
  - If inserted into animal or plant cells, will produce luciferase and will fluoresce, providing a visual indicator of gene expression – **reporter** gene

- Examples: the lux gene which produces luciferase
  - Used to develop a fluorescent bioassay to test for TB (the lux gene is in a virus that only infects *M. tuberculosis*). If the bacteria is present, the virus infects the cells and the bacterial cells glow!

Using Microbes for a Variety of Everyday Applications

- **Food Products**
  - **Rennin** enzyme used to make curds (solid) and whey in production of cheese
  - Recombinant rennin is known as **chymosin** (first recombinant food product approved by FDA)
  - Lactic acid bacteria (*L. lactis*, *L. acidophilus*) are used for coagulation for Cheese production
  - These microbes degrade casein and use lactase enzyme to break milk sugars which are used by bacteria for fermentation

Using Microbes for a Variety of Everyday Applications

- **Food Products produced by microbial fermentation**
  - Breads, yogurts, cheeses, sauerkraut
  - Beer, wines, champagnes, liquors
  - Cells obtain energy from carbohydrates

Glucose  Energy (ATP)

Electrons are transferred from sugar to carrier molecules (NAD^+)

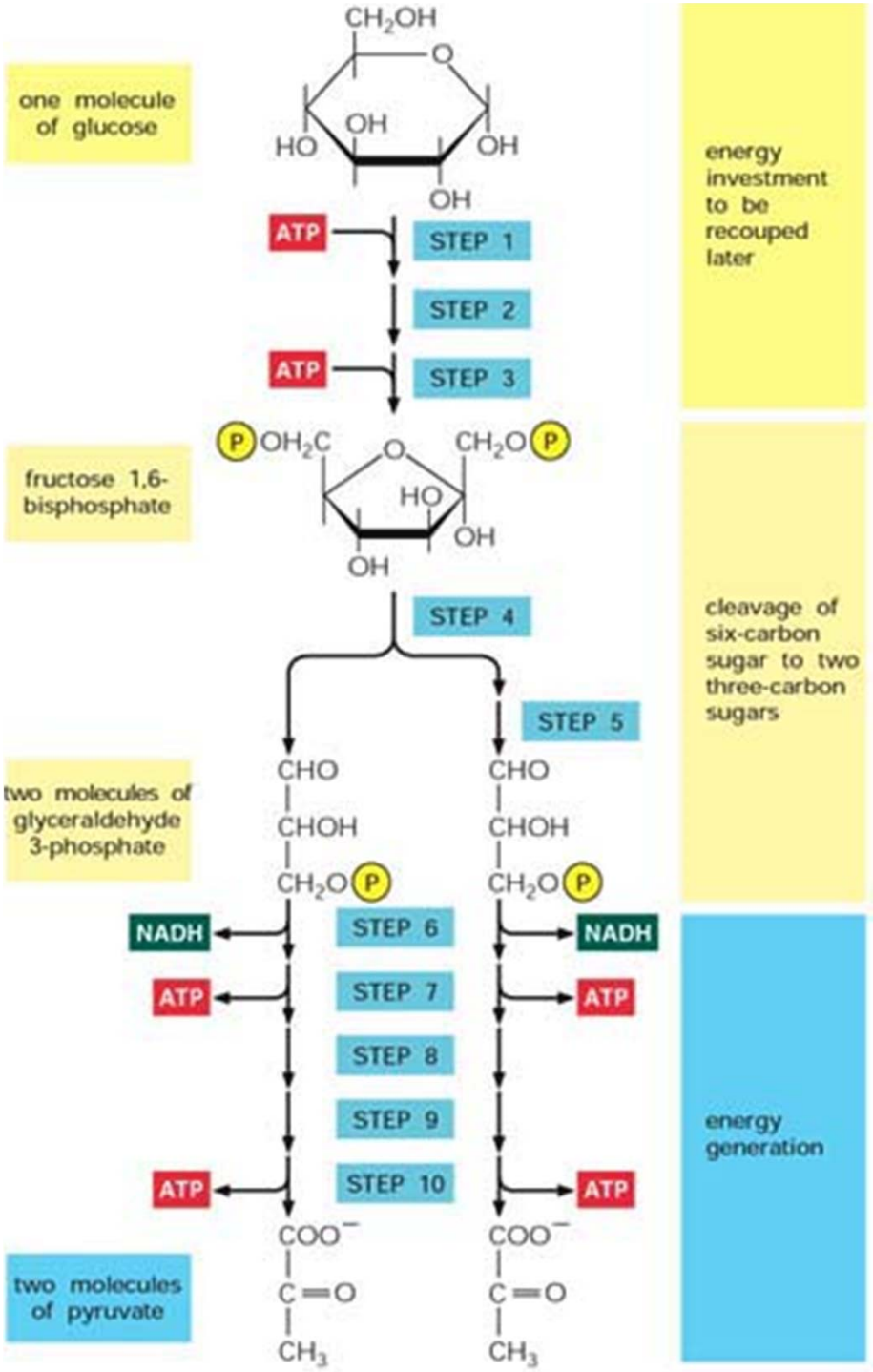
Glycolysis

Glucose

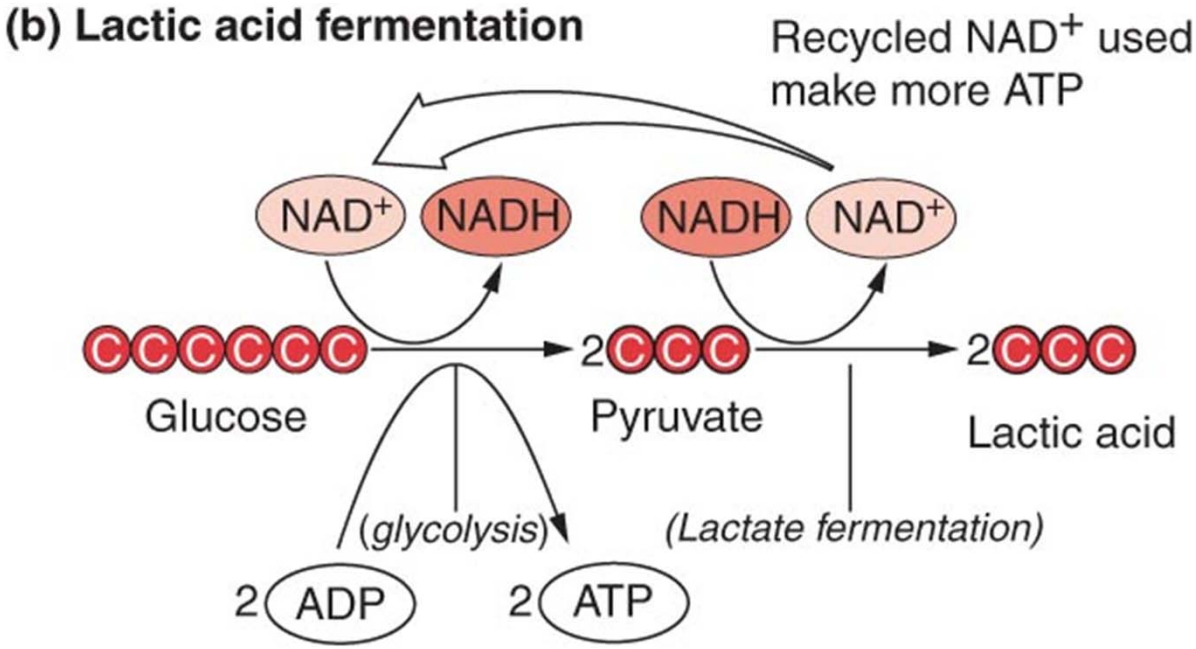
⇩

Pyruvate & ATPs

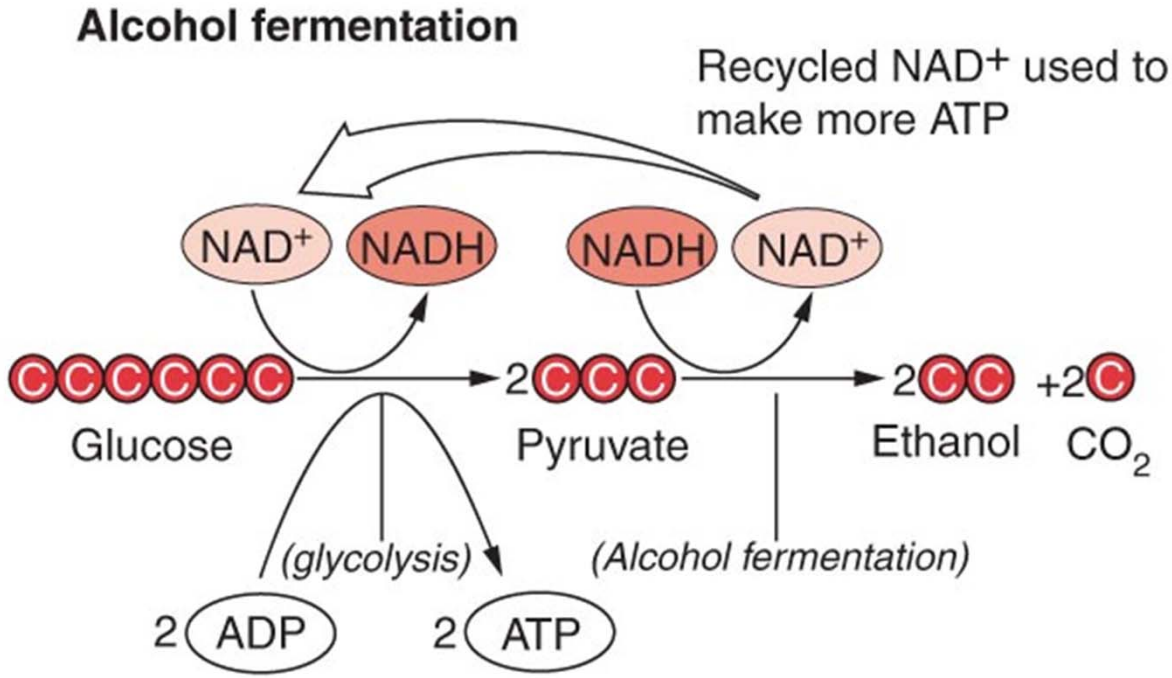
- Electrons are transferred from glucose to NAD+ producing NADH
- NADH transports e^- to subsequent reactions to produce ATP
- some bacteria utilize oxygen for this e^- transport reactions – **aerobic bacteria**



- Microbes living in oxygen deficient environments use fermentation for this purpose – **anaerobic microbes**
- **Fermentation** – process of deriving energy from sugars in the absence of oxygen
- Anaerobes recycle NADH to NAD^+ in fermentation
  - **Lactic acid fermentation**
  - **Alcohol fermentation**



Electrons from NADH are used to convert pyruvate to lactic acid which regenerates NAD^+



Electrons from NADH are used to convert pyruvate to ethanol which regenerates NAD^+. CO_2 is released as waste product

- Fermenting bacteria
  - Acetic acid in vinegar, citric acid in fruit juice, acetone and methanol
  - Alcohol fermentation – used to produce alcohol beverages
  - Lactic acid fermentation –
    - used to flavor yogurts, cheese, sour cream,
    - biopreservatives

Therapeutic Proteins

- Bacteria are used to produce medically important proteins
- For example, insulin (stimulate uptake of glucose into muscle cells, where it produces ATP)
- What is Type I diabetes (insulin-dependent diabetes mellitus)
  - Inadequate production of insulin by beta cells in the pancreas
- Earlier insulin was isolated from pancreas of pigs and cows
- 1978 : Recombinant insulin in bacteria

Human active insulin – made of chains **A** and **B**

Introduce into expression vector

Transform bacteria and express b-gal bound chains

Affinity chromatography

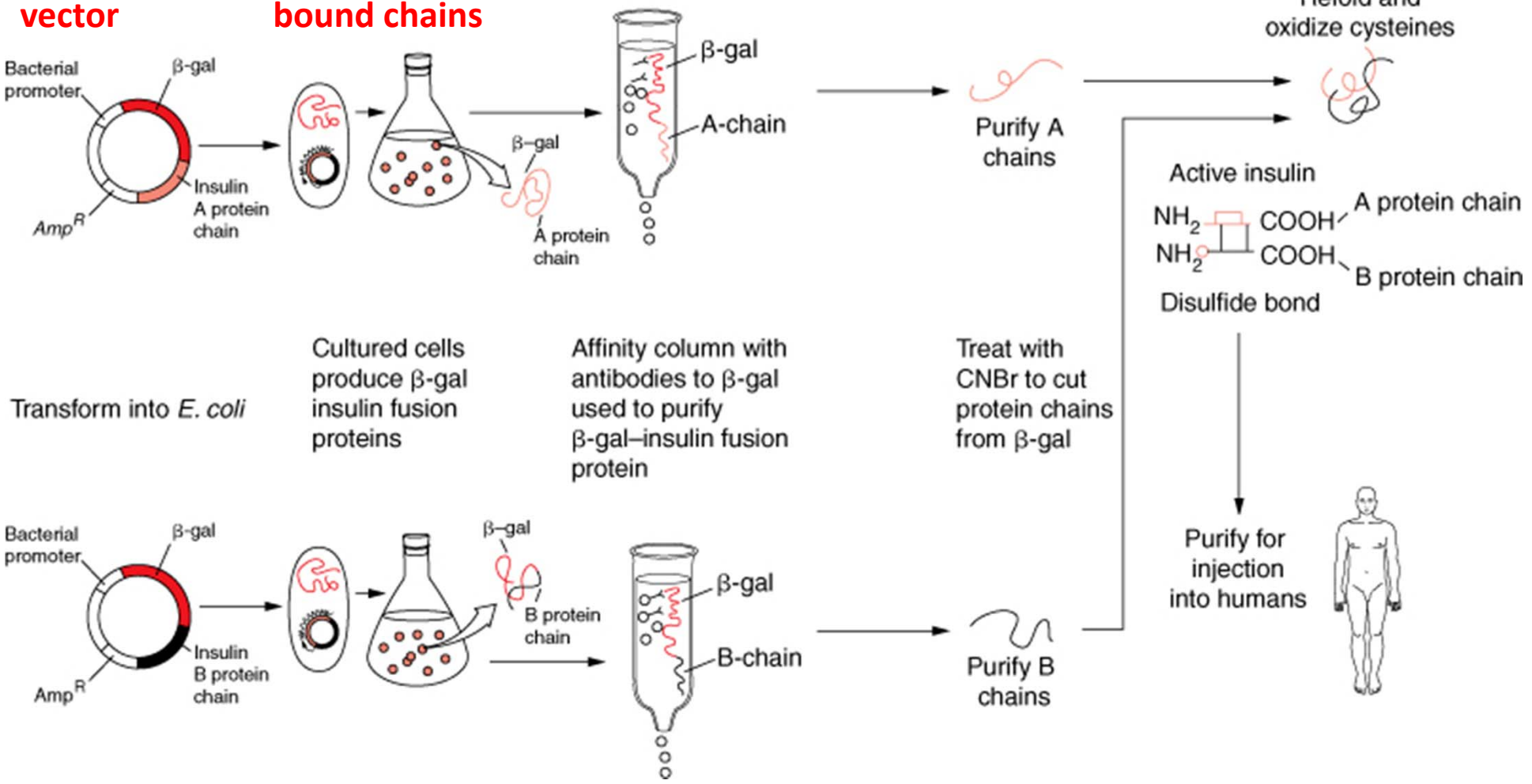


Table 5.1 THERAPEUTIC PROTEINS FROM RECOMBINANT BACTERIA

Protein	Function	Medical Application(s)
DNase	DNA-digesting enzyme	Treatment of cystic fibrosis patients
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 heart attack patients and stroke victims
Vaccines (e.g., Hepatitis B vaccine)	Stimulate 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