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ADVANCED MOLECULAR AND MICROBIAL TECHNIQUES-
A COMPLETE LABORATORY NOTEBOOK

THESIS

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Carmen Lydia Brito-Rodriguez, B.A.

Denton, Texas

May, 1998

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The purpose of this project is to produce a complete and thorough notebook that may be used to supplement laboratory coursework. Its intent is to be used primarily by the students to aid them in understanding background information and the proper laboratory procedures involved in various types of experiments.

The laboratory notebook is a summation of all the experiments and procedures used in the six-credit hour Advanced Microbial and Molecular Biology (BIOL 5160) course offered during the summer semester at the University of North Texas. This class is a team taught effort by Professors O'Donovan and Kunz. The course is constructed as an intensive practice exercise to teach the student about gene mutations, biosynthetic pathways, preparation and analysis of plasmid DNA, and many other topics included in the notebook.

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TABLE OF CONTENTS

	Page
LIST OF ILLUSTRATIONS	vi
Chapter	
UNIT I: BASIC LABORATORY TECHNIQUES	
1. COLONY MORPHOLOGY AND STREAK ISOLATION	1
2. SMEAR PREPARATION	3
3. HANGING DROP EXPERIMENT	5
4. GRAM STAINING	8
5. ACID-FAST STAINING	11
6. ENDOSPORE STAINING	13
7. BIOCHEMICAL TESTS	15
8. GROWTH CURVE EXPERIMENT	23
UNIT II: BACTERIAL MUTAGENESIS	
9. 5-FLUOROURACIL EXPERIMENT	26
10. UV EXPERIMENT	28
11. EMS MUTAGENESIS	31
12. NTG MUTAGENESIS EXPERIMENT	36
13. MUTAGENESIS EXPERIMENT: CHECKING FOR METABOLIC BLOCKS IN A PATHWAY	41
14. NTG MUTAGENESIS: CRUDE METHOD	47

15. NTG MUTAGENESIS: CRUDE METHOD #2	49
UNIT III: ENZYME KINETICS	
16. BREAKING CELLS FOR ENZYME ASSAYS	51
17. PROTEIN ASSAY (BRADFORD)	53
18. ASPARTATE TRANS-CARBAMOYLASE ASSAY	55
19. DIHYDROOROTASE ASSAY	58
UNIT IV: PLASMIDS	
20. SOIL EXPERIMENT	61
21. RAPID PLASMID PREPARATION	64
22. AGAROSE GEL PREPARATION	67
23. INDIVIDUAL ISOLATION	69
24. STERILE DISK EXPERIMENT	70
25. ANTIBIOTIC SENSITIVITY EXPERIMENT	72
26. COMPETENT CELLS	75
27. COLONY LIFT EXPERIMENT	77
28. TRANSFORMATION	82
29. COLONY SCREENING METHOD FOR THE DETECTION OF A PARTICULAR CLONE	85
30. COLONY LIFT EXPERIMENT	90
31. "KNOCK-OUT" GENE REPLACEMENT TECHNIQUE	92
32. LIGATION AND ELECTROPORATION	94

33. TRANSFORMATION EXPERIMENT	97
34. PCR (POLYMERASE CHAIN REACTION)	100
35. BOOMERANG EXPERIMENT	104
UNIT V: GENE ANALYSIS AND CHARACTERIZATION	
36. PREPARATION OF MEDIA	106
37. PHENOTYPING	109
38. RAPID PLASMID PREPARATION	111
39. TRANSPOSON MUTAGENESIS	114
40. MOLECULAR ANALYSIS OF T _N 5-INDUCED MUTANTS	118
41. RAPID ISOLATION OF PLASMID DNA BY ALKALINE ANALYSIS- ANALYTICAL SCALE	120
42. SYNTHESIS OF NUCLEIC ACIDS DURING GROWTH	124
43. CALCULATIONS	129
44. INDUCTION OF ENZYME ACTIVITY	131
45. FRACTIONATION OF CATECHOL-2,3-OXYGENASE	137
46. PLASMID PURIFICATION	139
47. CONJUGATION	144
48. SDS GEL ELECTROPHORESIS OF PROTEINS	149
REFERENCES	152

LIST OF ILLUSTRATIONS

	Page
1. REPLICA PLATE – 50 COUNT	35
2. PYRIMIDINE PATHWAY	45
3. ARGININE PATHWAY	46
4. ANTIMICROBIAL ZONE OF INHIBITION EVALUATION	74
5. pUC 18 PLASMID	80
6. pBK 188 PLASMID	81
7. TOL PATHWAY	116
8. REPLICA PLATE – 24 COUNT	117

UNIT I

BASIC LABORATORY TECHNIQUES

There are many basic techniques used in a microbiology laboratory that are essential in good laboratory practices. Learning to identify a colony on an agar plate, learning to stain bacteria, and learning to identify the bacteria through various biochemical tests are all fundamentally important procedures one must learn in order to acquire more advanced laboratory techniques. Units I - IV are a demonstration of various methods and procedures learned in the first half of an intensive laboratory class taught by Dr. Gerard O'Donovan.

In this first unit, various experiments are introduced in order to learn about the type, motility, and identity of several different species of bacteria. In addition, the nature of bacteria will be learned through a myriad of biochemical testing procedures. With this, one will proceed in learning the biochemistry behind these tests and how these tests aid in identifying various bacteria. Several staining procedures will be discussed in detail in order to observe the different characteristics of bacteria.

CHAPTER 1

COLONY MORPHOLOGY AND STREAK PLATE ISOLATION

Purpose: The objective of this experiment is to observe the differences in colony morphology among various organisms and to isolate different types of bacteria into separate colonies on agar plates. This is useful for distinguishing between different species in a mixed culture and allows for the study of colony development. To some extent, colony morphology can aid in the identification of an organism. An agar plate provides the bacteria its basic nutritional needs. When a single bacterial cell is deposited on an agar surface, it begins to grow and divide. Eventually, this mass of cells becomes visible and is called a colony.

The general shape of the colony (i.e. edges and margins) can be determined by looking down at the top of the colony on an agar plate. The colony elevation may be determined by viewing the side of the plate at eye level. There are a great variety of bacterial forms and characteristics. The basic categories used to differentiate bacterial morphology include shape, margin, elevation, color, texture, and sometimes, odor. In addition, cells from a colony may be transferred to a sterile medium to begin a pure culture. Pure colonies can be obtained by doing a streak plate isolation. In this technique, bacteria are transferred onto an agar plate with an inoculating loop and then streaked out over the surface in such a way that it gives rise to separate colonies.

Materials:

Agar plates

Different strains of bacteria

Inoculating loop

Flame Source

Procedure:

1. View an agar plate from the top and look down onto the colony to determine the edges and margins of the colony.
2. View the side of the plate at eye level to determine the colony elevation.
3. Open the lid to determine if the bacteria have any characteristic smell (i.e. *Pseudomonas* has a characteristic odor).
4. Visually divide a plate into four quadrants.
5. Remove a loopful of bacteria and make about 30 streaks in the first quadrant on the plate.
6. Flame the loop to kill any remaining bacteria.
7. Streak quadrants 2,3,and 4, making sure to flame the loop each time. While streaking quadrants 3 and 4, be careful not to let the streaks run into each other because the idea is to get single colony isolations.

CHAPTER 2

SMEAR PREPARATION

Purpose: The purpose of this procedure is to learn how to prepare a good bacterial smear from a strain of bacteria. A bacterial smear is a dried preparation of bacterial cells on a glass slide. A good bacterial smear is one in which the bacteria are evenly distributed on the slide, the bacteria are not washed off during staining procedures, and the bacterial form is not distorted. Smears can be prepared from both solid media and liquid media. If solid media are used, then a small amount of bacterial growth is transferred to a drop of water on a glass slide and mixed, and the mixture is spread out evenly over a large area of the slide.

Materials:

Bacterial cultures (solid and liquid)

Glass slide

Inoculating loop

Microscope

Flame Source

Procedure:

From liquid medium:

1. Flame the inoculating loop and allow it to cool for a second or two.

2. Place a loopful of medium on the center of the glass slide.
3. Using the loop, spread the bacteria over a large area of the slide to ensure a thin preparation.
4. Allow the slide to air dry.
5. Heat-fix the slide by passing it through the flame 2-3 times.

From solid media:

1. Place a drop or two of water on a slide.
2. Flame the loop and allow it to cool.
3. Take a small amount of bacteria from one colony using the loop and mix it with the drop of water on the slide.
4. Spread the mixture over the slide making a thin smear.
5. Allow the slide to air dry.
6. Heat-fix the slide by passing it through the flame several times.

Note: If the smear preparations are not air-dried and heat-fixed, the smear will be washed away during the following stain procedures and no bacteria will be seen. When heat-fixing, be careful not to leave the slide in the flame too long or the smear will be charred.

CHAPTER 3

HANGING DROP EXPERIMENT

Purpose: The aim of this experiment is to learn the techniques used to make a hanging drop slide to study motility. Most bacterial microscopic preparations kill the organisms. The hanging drop slide technique allows the observation of living cells to determine cell size, arrangement, and shape.

In addition, the hanging drop technique is also used to demonstrate the motility of bacteria. True motility in bacteria has been recognized and involves several different mechanisms. Bacteria that have flagella exhibit flagellar motion. Helically shaped bacteria, like spirochetes, have axial filaments and move in a corkscrew-type motion. Other bacteria simply slide over moist surfaces in a gliding motion.

If a hanging drop slide is used to determine motility, the observer must be careful to distinguish between true motility and Brownian motion. Brownian motion is due to the random motion of the water molecules bombarding the bacteria and causing them to move. With true motility, cells will exhibit independent movement over greater distances.

Materials:

Depressed glass slide

Cover slip

Petroleum jelly

Inoculating loop

Flame source

Broth of bacterial culture containing both motile and non-motile bacteria

Microscope

Procedure:

1. Apply a thin layer of petroleum jelly to the four edges of a cover slip.
2. Place a drop of broth containing the bacteria to the center of the cover slip using a sterilized inoculating loop.
3. Place a depression microscope slide over the cover glass so that the drop is hanging undisturbed over the depression in the slide.
4. Invert the entire slide so that the cover slip is on top.
5. Place the slide under the microscope for examination.
6. Once the image is visible under low power, swing the high power objective into position and readjust the lighting. Since most bacteria are drawn to the edge of the drop by surface tension, focus near the edge of the drop.
7. To use the oil immersion lens, add immersion oil to the cover slip before viewing.
8. Keep lighting to a minimum because living bacteria are almost impossible to see under a bright light.

Note: The idea behind using petroleum jelly is that it causes the cover slip to adhere to the slide so that the preparation may be picked up and inverted without the loss of material. The petroleum jelly forms an air-tight seal that prevents drying of the drop and

allowing a long period for the observation of cell size, shape, and motility.

CHAPTER 4

GRAM STAINING

Purpose: The aim of the Gram staining procedure is to learn the techniques involved in using this type of stain and to separate bacteria into two categories. The Gram stain is the most widely used differential stain. It divides bacteria into two groups: Gram positive (Gram+) and Gram negative (Gram-). This technique was first used by Hans Christian Gram, a Danish scientist, and is, therefore, for whom the staining procedure was named after.

The primary stain is the basic dye, crystal violet, which stains the bacteria purple. The iodine solution acts as a mordant, hence, it increases the interaction between the bacterial cell and the dye so that the dye is more tightly bound and the cell is more strongly stained. The ethanol is used as a decolorizing agent. The phospholipids in the cell wall of the Gram- bacteria get dissolved, so they lose the crystal violet-iodine complex and become colorless while the Gram+ bacteria retain the complex. The counterstain, safranin, stains the colorless Gram- bacteria pink, but it does not alter the color of the Gram+ bacteria, which stay purple.

Note: Most bacteria can be stained using this procedure, but some bacteria require a special staining technique. For example, the genus *Mycobacterium*, which is acid fast due to the presence of mycolic acid in its cell walls, require an acid fast staining technique.

Materials:

Cultures of Gram⁺ and Gram⁻ bacteria

Crystal violet

Gram's Iodine

Safranin

Distilled Water

95% Ethanol

Microscope

Microscope slides

Inoculating loop

Flame Source

Immersion Oil

Forceps

Gloves

Procedure:

1. Prepare a smear using any desired bacterium.
2. Air-dry the smear.
3. Heat-fix the slide.
4. Flood the smear with crystal violet and allow it to stand for 1 minute.
5. Rinse with distilled water.

6. Flood the slide with Gram's Iodine and allow it to stand for 1 minute.
7. Do not rinse with distilled water.
8. Decolorize with 95% ethanol for about 5 to 10 seconds.
9. Rinse with distilled water.
10. Counterstain with safranin and allow it to stand for 1 minute.
11. Rinse with distilled water.
12. Blot dry and examine under the oil immersion lens.

CHAPTER 5

ACID-FAST STAINING

Purpose: The objective of this procedure is to learn the methods and principles of acid-fast staining, which is a special staining technique used to stain certain types of bacteria. For example, a few species in the genera *Mycobacterium* and *Nocardia*, in addition to the parasite *Cryptosporidium*, do not readily stain with simple stains.

Mycobacterium does not stain using a simple staining technique due to the presence of a high concentration of lipids in their cell wall. However, these organisms can be stained by heating them with carbolfuscin. Once these acid-fast organisms have taken up this dye, they do not readily decolorize and, thus, appear red. Microorganisms that are not acid-fast will stain blue due to the counterstaining with methylene blue.

Materials:

Cultures of *Mycobacterium*

Carbolfuscin

Acid alcohol

Methylene Blue

Distilled water

Glass beaker

Glass slides

Cover Slip

Paper towels

Flame Source

Beaker stand

Inoculating loop

Microscope

Immersion oil

Forceps

Gloves

Procedure:

1. Prepare a smear using a strain of *Mycobacterium* (e.g. *M. smegmatis*).
2. Air-dry and heat-fix the smear.
3. Place the slide on top of a beaker of boiling water.
4. Cover the smear with a small piece of paper towel.
5. Continually flood the smear with carbol fuchsin for 5 minutes. Do not allow the paper towel to dry out.
6. Rinse with distilled water.
7. Decolorize with acid alcohol for 30 seconds.
8. Rinse with distilled water.
9. Counterstain with methylene blue.
10. Rinse with distilled water.
11. Blot and observe under the oil immersion lens.

CHAPTER 6

ENDOSPORE STAINING

Purpose: The purpose of this procedure is to learn the principles and methods behind endospore staining. Two of the major genera that form endospores are *Bacillus* and *Clostridium*. Under favorable conditions of growth, most of these bacteria are in the vegetative state and do not form spores. However, in conditions that are not favorable for growth, the bacteria form spores to help them survive in the harsh environment. This structure develops within the bacterial cell and is capable of surviving for long periods in an unfavorable environment and then giving rise to a new cell.

Spores found inside the bacterial cell are called endospores. They are spherical to elliptical in shape and may be smaller or larger than the bacterial cell. Based on its position in the cell, it is called central, subterminal, or terminal.

Endospores do not stain readily. Heat is used to enable penetration of the dye, malachite green. Once stained, the endospores strongly resist decolorization. The rest of the cell is then decolorized and counterstained a light red by safranin.

Materials:

Cultures of *Bacillus subtilis* and *Bacillus stearothermophilus*

Malachite green

Safranin

Distilled water

Glass beaker

Paper towels

Beaker stand

Immersion oil

Microscope

Forceps

Flame Source

Gloves

Procedure:

1. Make a smear on a slide using the appropriate bacteria.
2. Air-dry and heat-fix the smear.
3. Place the slide on top of a beaker containing boiling water.
4. Cover the smear with a small piece of paper towel.
5. Continually flood the smear with malachite green for 5 minutes. Do not allow the paper towel to dry out.
6. Remove paper towel and rinse with water for 15-30 seconds.
7. Counterstain with safranin for 30 seconds.
8. Rinse the slide with distilled water.
9. Blot and view the slide under the oil immersion lens.

CHAPTER 7

BIOCHEMICAL TESTS

Purpose: There is a variety of tests that can be used to identify and study bacteria. In addition to various staining procedures, the following biochemical tests can be used to categorize different strains and species of bacteria.

- (1) **Glucose Fermentation Test-** This test is used to determine whether an organism can ferment glucose to produce acid or acid and gas. The indicator is phenol red at pH 6.8 (yellow) and pH 8.6 (red). Fermentation is anaerobic partial oxidation of glucose. Glucose is converted to pyruvate, which is then reduced to lactic acid or ethanol. The final electron acceptor for lactic acid is pyruvate, and the final electron acceptor for ethanol is acetaldehyde. Only 2 ATP per glucose molecule are formed. All enteric organisms will ferment glucose. Respiration is the complete oxidation of glucose.

In the case of *Pseudomonas*, which is aerobic, no oxygen is available when the end of electron transport is reached. Then, they use NO_3 in lieu of O_2 as the final electron acceptor. This is known as anaerobic respiration.

A small tube, called a Durham tube, is inverted into a tube containing the broth and traps any gas that may be produced as a result of fermentation.

- (2) **Triple Sugar Iron (TSI) Test:** This test is generally used for the

identification of enteric bacteria. It is also used to differentiate *Enterobacteriaceae* from other Gram negative intestinal bacilli. This is due to their ability to catabolize glucose, sucrose, or lactose. In addition, these bacteria also have the ability to liberate sulfides from ferrous ammonium sulfate or sodium thiosulfate.

This medium contains: 0.1% glucose

1% lactose

1% sucrose

TSI is inoculated by doing a fish tail inoculation on a slanted surface and then a stab into the medium using a loop.

The indicator is phenol red, and there are several different results that may need to be interpreted.

- a If the medium turns yellow, it is indicative of an acid. If the medium turns red, it means it is alkaline.
- b If the butt of the tube is yellow with a red slant, it is due to glucose fermentation. The slant remains red due to limited glucose in the medium and limited acid formation.
- c If the butt of the tube is yellow with a yellow slant, it is due to the fermentation of lactose and/or sucrose.
- d If the butt of the tube is red with a red slant, this indicates that none of the sugars was fermented. Also, neither gas, nor H_2S , was produced.
- e If splitting of the agar occurs, gas formation is noted.

f If blackening of the agar occurs, gas formation by H_2S is noted.

(3) SIM (Sulfide Indole Motility)-Hydrogen Sulfide and Indole Production and Motility

Test: This test is used to test for motility, as well as for H_2S production. In addition, this test also indicates the production of indole from tryptophan.

SIM medium is inoculated by stabbing with a needle. If the organism is motile, it can be seen growing away from the line of the stab. If the organism produces H_2S , a black precipitate will be seen.

(4) IMVC Tests:

a. Indole test- Bacteria that contain tryptophanase can hydrolyze tryptophan to its metabolic products: indole, pyruvic acid, and ammonia. The bacteria utilize pyruvic acid and ammonia to satisfy their nutritional needs. Indole is not used and accumulates Kovac's reagent, which reacts with the indole, producing a bright red color on the medium. Bacteria which produce a red color with Kovac's reagent are indole positive. Bacteria which do not produce a red color means tryptophan was not hydrolyzed, and the bacteria are indole negative.

b. Methyl Red test- All enteric bacteria oxidize glucose for energy, but the end products vary. Methyl red is a pH indicator that detects the presence of end products, such as lactic, acetic, succinic, and formic acids. Fermenters, such as *E.coli*, produce a mixed fermentation and, thus,

acidify the medium. Butanediol fermenters, such as *Enterobacter aerogenes*, form butanediol, acetoin, and a few organic acids. The pH does not fall as low during mixed acid fermentation. At pH 4, the methyl red turns the medium red, which indicates a positive test. At pH 6, the indicator turns yellow, and this indicates a negative test.

- c. Voges-Proskauer (VP) test- The VP test identifies bacteria that ferment glucose, leading to 2,3-butanediol accumulation in the medium. Addition of 40% KOH and a 5% solution of alphanaphthol in absolute ethanol will detect the presence of acetoin (acetylmethylcarbinol), which is a precursor in the synthesis of 2,3-butanediol. In the presence of reagents and acetoin, a cherry red color develops. A positive test is the development of a red color within 15 minutes following the addition of the reagent. A negative test is the absence of the red color.
- e. Citrate Utilization test: This test determines the ability of the bacteria to use citrate as a sole carbon source for their energy. This depends on the presence of the citrate permease protein that facilitates the flow of citrate into bacteria. Once inside the bacteria, citrate is converted to pyruvic acid and CO₂. This test is done on slants since O₂ is required for citrate utilization. When bacteria utilize citrate, they remove it from the medium and liberate CO₂. CO₂ combines with sodium (from sodium citrate) and water to form sodium carbonate, an alkaline

product. This raises the pH, and the indicator turns the medium into a blue color. This represents a positive test. Absence of a blue color is a negative test. Citrate negative cultures also show no growth in the medium.

- (5) Urease test: This test is used to distinguish members of *Proteus* from other non-lactose fermenting enteric bacteria. Urease is detected by growing bacteria in a medium containing urea and an indicator, such as phenol red. When urea is hydrolyzed, ammonia accumulates in the medium and makes it alkaline. This increase in pH causes the indicator to change from orange-red to deep pink or purplish-red. This is a positive test for urea hydrolysis. Failure of a deep pink color to develop is a negative test.

- (6) Lysine Iron Agar: Decarboxylation is the removal of a carboxyl group from an organic molecule. For example, decarboxylation of the amino acid lysine results in the production of an amine and CO_2 . Decarboxylation of lysine can be detected by culturing bacteria in the desired medium, amino acids, glucose, and pH indicator. The acids produced by the bacteria from the fermentation of glucose will initially lower the pH of the medium and cause the pH indicator to change from purple to yellow. The acidic pH activates the enzyme that causes the decarboxylation of lysine and subsequent neutralization of the medium. This results in a color change back from yellow to purple. The indicator, bromocresol blue, turns yellow as the test

becomes positive.

- (7) Starch Hydrolysis: The starch molecule consists of amylose, an unbranched glucose polymer, and amylopectin, a large, branched polymer. Both are rapidly hydrolyzed by certain bacteria via their α -amylases to yield dextrans, glucose, and maltose.

Gram's iodine can be used to indicate the presence of starch. Hydrolyzed starch does not produce a color change. If a clear area appears after adding Gram's iodine to a medium containing starch and bacterial growth, this indicates that α -amylase has been produced by the bacteria. If there is no clearing, then starch has not been hydrolyzed.

- (8) Catalase test: Many bacteria possess enzymes that afford protection against toxic O_2 products. Obligate aerobes and facultative anaerobes usually contain the enzymes superoxide dismutase and either catalase or peroxidase. The superoxide dismutase catalyzes the destruction of the superoxide radical, $O_2^{\cdot -}$. Most strict anaerobes lack both enzymes and, therefore, cannot tolerate O_2 . The H_2O_2 produced is detoxified by catalase.

Catalase production and activity can be detected by adding the substrate H_2O_2 to an appropriately incubated tryptic soy agar slant. If catalase is produced by the bacteria, free O_2 gas is liberated. Bubbles of O_2 represent a positive catalase test, and the absence of bubble formation indicates a negative catalase test.

- (9) Oxidase test: Oxidase enzymes play an important role in the operation of the electron transport chain system during aerobic respiration. Cytochrome oxidase uses O_2 as an electron acceptor during the oxidation of reduced cytochrome a_3 to form either water or hydrogen peroxide. The ability of bacteria to produce cytochrome oxidase can be determined by the addition of oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride) or an oxidase disc (p-aminodimethylaniline) to colonies. A positive reaction is pink (if dimethyl is used) or blue (if tetramethyl is used). The color change should be immediate to be considered a positive test. No color change indicates a negative test.
- (10) Mannitol Salt Agar (MSA): This is a selective medium used for the isolation of pathogenic staphylococci. The medium has a pH=7.4, and its indicator is phenol red. *Staphylococcus aureus* appears yellow on the media, while *Staphylococcus epidermidis* appears red.
- (11) Hectoen Enteric Agar (HE): This is a differential, selective medium used for the isolation and differentiation of *Salmonella* and *Shigella* from other Gram- enteric pathogens. The prepared medium plates appear green with a yellowish cast. *Enterobacter aerogenes* appears a salmon-orange color on HE medium, while *Escherichia coli* appears orange. Strains of *Salmonella* and *Shigella* appear greenish-blue.
- (12) Eosin Methylene Blue (EMB) Agar: This is a differential medium used for the

detection and isolation of Gram- enteric bacteria. *Salmonella* and *Shigella* appear translucent and amber colored or colorless. Coliforms that utilize lactose and/or sucrose are blue-black with a green metallic sheen. The coliform *Enterobacter* forms pink, mucoid colonies.

CHAPTER 8

GROWTH CURVE EXPERIMENT

Purpose: The objective of this experiment is to observe the growth of *Escherichia coli* bacteria in each of its different growth phases. The normal growth of bacteria has four phases:

- (1) Lag phase- during this phase, the bacterial growth is almost none.
- (2) Log phase- this phase shows exponential growth, when plotted on semi-log paper, indicating maximum growth of the organism during this phase.
- (3) Stationary phase- this phase shows an almost straight line because the number of bacteria that are growing are equal to the number of bacteria that are dying. Many bacteria can be identified based on the stationary phase. For example, *Pseudomonas aeruginosa* has a very long stationary phase, while *E.coli* has a shorter stationary phase.
- (4) Exponential death phase- during this phase, the death of bacteria is at its maximum.

Materials:

Pipettes

Micropipettes

Spectrophotometer

Cuvettes

Agar plates

Centrifuge tubes

Linear paper

Semi-log Paper

Procedure:

1. Inoculate *E.coli* in one tube of 5ml TSB broth.
2. Incubate overnight at 37°C while shaking.
3. Take 1 ml of the *E.coli* broth, and put it into 100 ml of TSB broth.
4. Take an initial absorbance reading of the inoculated TSB broth at 600 nanometers in the spectrophotometer.
5. Incubate the flask at 37°C while shaking.
6. Take absorbance readings every 30 minutes (by removing 500 µl into the cuvette) for 4-5 hours at A_{600} .
7. Remove 100µl every hour and place it in a 1.5ml centrifuge tube. Then, plate dilutions as follows: 10^{-2} , 10^{-4} , and 10^{-6} .
8. Incubate the plates overnight at 37°C.
9. Count the number of colonies on each plate.
10. Construct 2 tables:
 - (a) time vs. # of colonies every hour
 - (b) time vs. A_{600} every half-hour

11. To calculate the viable cell count (VCN), use the following formula:

$$\text{VCN} = \# \text{ colonies} \times \frac{1}{\text{dilution factor}}$$

$$\text{VCN units} = \text{cells/ml}$$

12. Construct another table:

(c) VCN vs. Time

13. Determine the generation time (or doubling time) for *E.coli*. This is the time it takes the bacteria to double its number.
14. Draw a graph on semi-log paper using the data in Table (a) with time on the y-axis and log # of colonies on the x-axis.
15. Pick an arbitrary number on the x-axis and note its corresponding time on the y-axis. Label it t_1 .
16. Take double the value of the arbitrary number selected in the above step and find its corresponding time on the y-axis. Label it t_2 .
17. Calculate the generation time using the following formula:
- $$\text{Generation time} = t_2 - t_1$$
18. To find out the number of cells at a particular time, draw a graph on semilog paper of absorbance (y-axis) vs. time.
19. Find 1.0 on the y-axis and find the corresponding time on the x-axis.
20. Draw a graph on semilog paper of VCN vs. time.
21. Take the time (x) and find the corresponding VCN on the y-axis. The y = # of cells/ml at time x.

UNIT II

BACTERIAL MUTAGENESIS

Mutagenesis experiments are used extensively in the microbiology and molecular laboratories for a variety of different reasons. Whether it is to identify mutant strains, to detect the presence or absence of enzymes, to observe the effects of different mutagenic agents, to aid in identifying particular genes, or to check for blocks in a certain biochemical pathway, mutagenesis experiments play a diverse role in advanced microbiology.

In this unit, several different types of mutagenic agents are introduced. For example, ultraviolet light, EMS, and NTG provide a variety of ways to make mutant bacteria, and each agent helps one to understand important aspects of bacteria. This includes learning how mutagenesis plays a role in biochemical pathways, learning about bacterial metabolism, and learning about its advantages in molecular biology as well.

CHAPTER 9

5-FLUOROURACIL EXPERIMENT

Purpose: The 5-fluorouracil experiment is used to detect the presence or absence of the enzyme uracil phosphoribosyltransferase in bacteria. In most bacteria, uracil can be utilized through a pyrimidine salvage pathway. 5-fluorouracil is not a mutagen, however, it is a toxic compound. When 5-fluorouracil (FU) is present, it is utilized in the salvage pathway similarly to uracil. Hence, when FU is taken up by the bacterial cell, it gets converted to FUMP by the enzyme, uracil phosphoribosyl transferase (UPRTase) encoded by *upp*. Then, the FUMP gets incorporated into the RNA of the bacterial cell and kills the cell. In this experiment, if the bacteria have a mutation in the *upp* gene, it does not get killed in the presence of FU.

Materials:

Culture of *Pseudomonas aeruginosa*

Pseudomonas minimal media (Psmm) plates (2)

Psmm broth in a flask

5-fluorouracil crystals

Forceps

Flame source

Hockey stick

Alcohol

Turntable

Inoculating Loop

Pipettes

Micropipettes

Procedure:

1. Inoculate *Pseudomonas aeruginosa* in a 5 ml Psmm broth.
2. Incubate overnight at 37°C while shaking.
3. Spread plate 100µl and 200µl of the culture on two Psmm plates.
4. Place a small crystal of 5-fluoro-uracil in the center of both of the spread plates.
5. Incubate right side up at 37°C.

CHAPTER 10

UV EXPERIMENT

Purpose: The objective of this experiment is to see the effects of the exposure of a given bacterial strain to a mutagenic agent, such as ultraviolet radiation. Streptomycin is an antibiotic that binds to the 30S ribosome, which is composed of, yet, other subunits. Normally, when a bacterial cell is exposed to streptomycin, the streptomycin will bind with the S_{12} subunit of the 30S ribosome in the cell and will cause a kink. Thus, the anticodon cannot recognize the codon. Therefore, there is no translation, and the cell dies. Hence, the bacteria cannot grow in the presence of streptomycin.

The ultra-violet radiation is an attempt to mutate and modify the S_{12} subunit so that the “kink” will be corrected. When the bacteria are exposed to a mutagenic agent, such as UV light, a mutation may result in the S_{12} subunit, thus, causing a kink in the S_{12} subunit. So, when streptomycin binds with the mutated S_{12} subunit, it corrects the kink. Then, these bacteria grow on a plate of TSA with streptomycin on a dark plate (a plate that is not exposed to light). When the bacteria on the TSA with streptomycin plate are exposed to light, the light corrects the kink in the mutated subunit. Then, when streptomycin binds with the S_{12} subunit again, it will kill the bacteria. Therefore, the organism is now able to grow on the TSA plate with streptomycin. However, it does not grow on TSA alone.

Materials:

Culture of *E.coli*

TSA plate (3)

TSA plate with 30 µl streptomycin per ml (3)

Nutrient broth

0.1M MgSO₄ buffer

Small Erlenmeyer flasks (2)

Aluminum foil

UV lamp

Sterile petri dish

50 ml conical tubes (3)

Alcohol

Turntable

Hockey stick

Vortex

Pipettes

Micropipettes

Flame source

Procedure:

1. (Per Group) Take 100ml of an overnight culture of *E.coli* and divide into two 50ml conical tubes.

2. Centrifuge at 4000 x g for 10 minutes at 4°C.
3. Pour off the supernatant.
4. Resuspend by vortexing both pellets (together) in 10ml of MgSO₄ buffer. (At this point, make two master control plates by spreading 100µl of cell suspension onto a TSA plate and a TSA plate containing streptomycin).
5. Pour cell suspension into sterile petri dish.
6. Take off the petri dish lid and expose to UV radiation for the following time points:
 - Group 1 - 60 seconds
 - Group 2 - 120 seconds
 - Group 3 - 180 seconds
 - Group 4 - 240 seconds
7. At the appropriate time point, remove 1ml of cell suspension from the petri dish and place in a sterile 50 ml conical tube containing 19ml of nutrient broth.
8. Spread plate 100µl on TSA and TSA strep.
9. Divide the 20ml cell suspension into two small Erlenmeyer flasks (10 ml in each flask).
 - Wrap one of the two flasks with aluminum foil to prevent the sample from being exposed to light.
10. Incubate the flasks at 37°C for one hour while shaking.
11. Plate 100µl from each flask (light and dark) onto a TSA plate and a TSA strep plate (four plates total).
12. Incubate all plates at 37°C overnight.

CHAPTER 11

EMS MUTAGENESIS EXPERIMENT

Purpose: The purpose of this experiment is to expose the bacteria to EMS and to identify mutant strains. EMS (Ethyl Methane Sulfonate) is an agent which causes mutants in DNA. EMS is a purine “specific” alkylating agent, which causes GC→AC transitions. In this experiment, bacteria are exposed to EMS. An attempt to identify and grow the strain of bacteria that has undergone a mutation will be made.

Materials:

5ml *Pseudomonas* minimal medium (Psmm) broth in a flask

Pseudomonas aeruginosa culture

50 ml Psmm in a flask

3ml of 4% EMS in a flask

18ml of Psmm in a flask (3)

Uracil

Arginine

20% dextrose

Psmm plates (5)

TSA plates (2)

Psmm plate + U (1)

Psmm plate + R (1)

Psmm plate +U + R (2)

Inoculating Loop

Flame Source

Turntable

Hockey stick

Alcohol

Pipettes

Micropipettors

Procedure:

1. Inoculate 5ml Psmm broth with *Pseudomonas aeruginosa*.
2. Incubate at 37°C overnight while shaking.
3. “Reinoculate” the next morning (12-24 hours) with 2 ml of the overnight culture into 50ml Psmm.
4. Incubate at 37°C for about 5 hours while shaking.
5. Take 3 ml of this 5 hour culture and put it into a flask containing 3ml of 4 % EMS solution (2% final concentration).
6. Shake at 37°C for 2 hours.
7. Put 2ml of each of the following into 3 conical flasks to make a 1:10 dilution:
 - (a) 18ml Psmm
 - 100µl uracil
 - 400µl 20% dextrose

(b) 18ml Psmm

100µl arginine

400µl 20% dextrose

(c) 18ml Psmm media

100µl uracil

100µl arginine

400µl 20% dextrose

8. Incubate at 37°C overnight while shaking.

Note: If there is no growth, add an additional 400µl of 20% dextrose to each flask and incubate at 37°C while shaking.

9. If there was growth, from each flask, make the following plates using Psmm plates for a total of 9 plates:

a. four quadrant streak plate

b. 10µl spread plate

c. 30µl spread plate

10. Incubate the plates overnight at 37°C.

11. Using individual colonies from these plates, do replica plating (grid plating) on 8 different plates as follows (Figure 11.1):

Set 1:

a. Psmm

b. Psmm + U

c. Psmm +U + R

d. TSA

Set 2:

a. Psmm

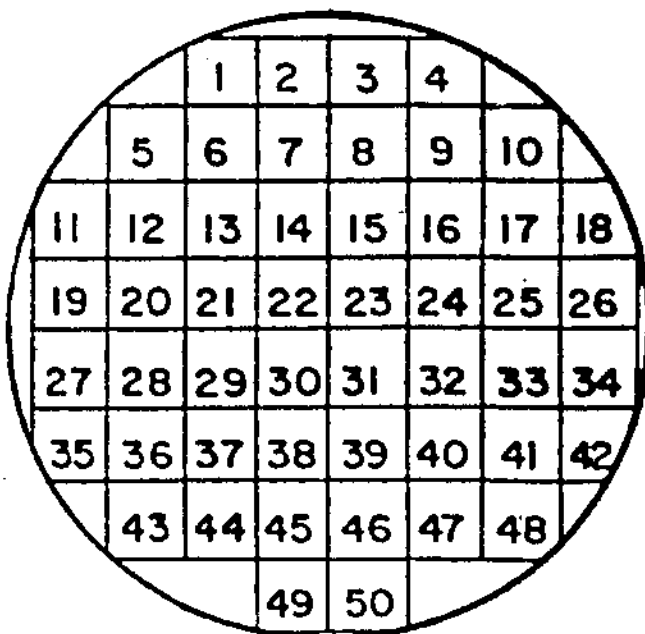
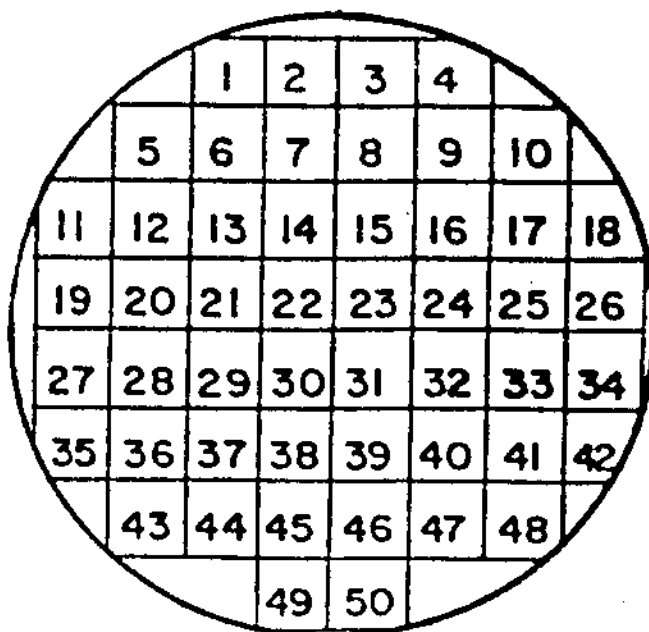
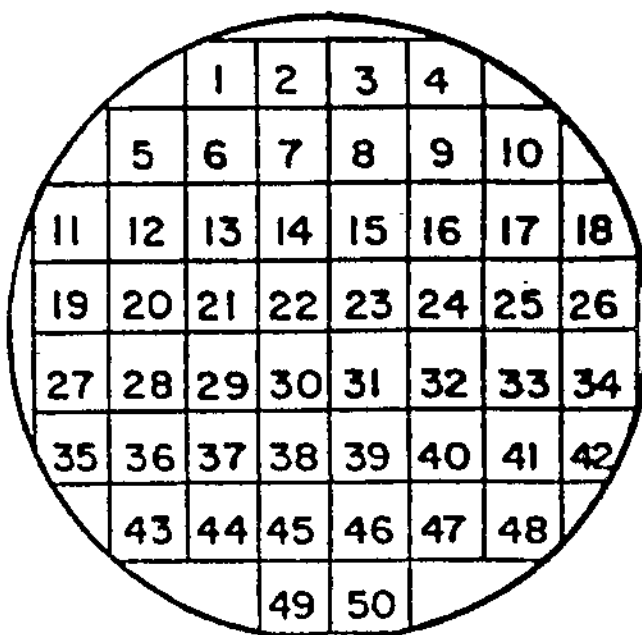
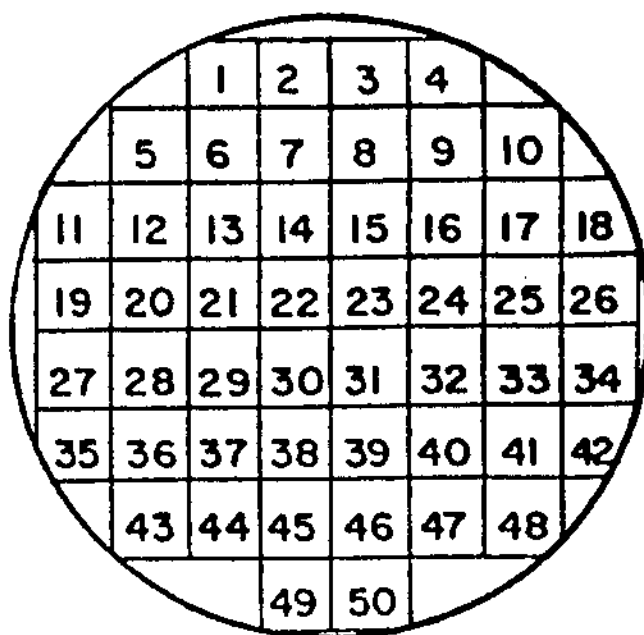
b. Psmm + R

c. Psmm +U + R

d. TSA

If the bacteria grew on all the plates of Set 1, then it would indicate a wild-type of bacteria. However, if it did not grow on one of the plates (for example, the uracil plate), it would indicate that a mutant came from the colony that was selected.

Figure 11.1: Replica Plate – 50 Count



CHAPTER 12

NTG MUTAGENESIS EXPERIMENT

Purpose: The purpose of this experiment is to expose the bacteria to NTG and to identify mutant strains. NTG (N-methyl-N'-nitro-N-nitrosoguanidine) is an agent which causes mutations in DNA. It is a methylating agent and a very strong mutagen (NTG is a more powerful mutagenic agent than EMS, ethyl methane sulfonate), which causes GC→AT > AT→GC transitions. It also causes transversions. In this experiment, the bacteria are exposed to NTG and an attempt to identify and grow the strain of bacteria, which have undergone a mutation, will be made.

Materials:

Pseudomonas aeruginosa culture

Pseudomonas minimal media (Psmm) broth

50ml Psmm in a flask

10ml Psmm

6ml Psmm

50ml conical flask (1)

citrate buffer (pH=5.5)

NTG solution (1mg/ml)

18μl Psmm in flask (3)

Uracil

Arginine

20% dextrose

Psmm plates (11)

Psmm + U plate (1)

Psmm media + R plate (1)

Psmm media + U + R plate (2)

TSA plate (2)

Alcohol

Gloves

Flame Source

Inoculating Loop

Turntable

Hockey stick

Toothpicks

Pipettes

Micropipettes

Procedure:

1. Start one 5ml Psmm broth with *Pseudomonas aeruginosa*.
2. Incubate at 37°C overnight while shaking.
3. “Reinoculate” after 12-24 hours of the 2ml overnight culture into 50ml Psmm.

4. Incubate at 37°C for about 5 hours while shaking.
5. Pour 20ml of cell culture into a 50ml conical flask.
6. Centrifuge at 3000 x g for 5 minutes at 4°C.
7. Pour off the supernatant and resuspend the cell pellet in 9ml citrate buffer (pH 5.5).
8. Add 1 ml of NTG solution (1mg/ml) so that the final concentration is 100µg/ml.
9. Incubate at 37°C for 30 minutes (without shaking).
10. Centrifuge at 3000 x g for 5 minutes at 4°C to collect cells.
11. Pour off the supernatant into the waste container.
12. Resuspend in 10ml Psmm to wash cells.
13. Resuspend in 6ml Psmm media
14. Put 2ml of cell suspension into 3 flasks containing 18ml Psmm each so that the total volume in each flask is 20ml.
 - a. 18ml Psmm
100µl uracil
400µl 20% dextrose
 - b. 18ml Psmm
100µl arginine
400µl 20% dextrose
 - c. 18ml Psmm
100µl uracil
100µl arginine
400µl 20% dextrose

15. Incubate overnight at 37°C while shaking.
16. Using the three flasks from above, make the following 3 plates for a total of 9 Psmm plates:
 - a. four quadrant streak plate
 - b. spread plate with 10µl Psmm
 - c. spread plate with 30µl Psmm
17. Incubate the plates at 37°C.
18. Do two sets of replica plating. Using a pen, mark the paper with the grid and the Petri dish with the medium in the same place. Once the plate is removed from the paper, it insures that the plate corresponds to the numbers on the grid correctly. Using a sterile toothpick, take an individual colony from any plate and make a line on all four plates of Set 1 in the same place. Pick 50 different colonies. Repeat the same procedure on the second set of plates. Make replica plates as follows using the grid in Figure. 11.1:
 - Set 1:
 - a. Psmm
 - b. Psmm + U
 - c. Psmm + U + R
 - d. TSA
 - Set 2:
 - a. Psmm
 - b. Psmm + R
 - c. Psmm + U + R

d. TSA

19. Incubate the plates at 37°C overnight.

Note: If there is growth on all 4 plates of a particular set at the same place on the replica plate, that indicates a wild type bacteria with no mutation. The Psmm plate is important because any strain that has undergone a mutation would not grow on this plate.

CHAPTER 13

MUTAGENESIS EXPERIMENT: CHECKING FOR METABOLIC BLOCKS IN A PATHWAY

Purpose: The purpose of this experiment is to take different strains of bacteria that have undergone a mutation at different levels in the pyrimidine or arginine pathways and identify the level in which the mutation has occurred. In this experiment, various strains of *Pseudomonas aeruginosa* will be used to check for blocks in the pyrimidine pathway (Figure 13.1). To check for blocks in the arginine pathway (Figure 13.2), strains of *Escherichia coli* will be used.

The intermediates of the arginine pathway are able to enter the cells easily while it is not the same with the intermediates of the pyrimidine pathway. Carbamoyl aspartate, DHO, and orotic acid are intermediates of the pyrimidine biosynthetic pathway, while uracil, cytosine, cytidine, and uridine act through the salvage pathway. Nucleoside permease (nup) is non-specific and transports both uridine and cytidine. In *Pseudomonas*, cytosine is then deaminated to form uracil. Growth of organisms on these plates is seen in the substances occurring after mutation.

Materials:

Bacterial cultures of:

Pseudomonas putida PPN1137 *pyrB*⁻

Pseudomonas aeruginosa PAO483 *pyrF*⁻

Pseudomonas fluorescens pfcw1013 *pyrC*⁻

Escherichia coli TB2 *pyrB*⁻, *arg*⁻

Crystals of:

1. carbamoylaspartate
2. dihydroorotate
3. orotate
4. uracil
5. cytosine
6. cytidine
7. uridine
8. glutamate
9. ornithine
10. citrulline
11. argininosuccinate
12. arginine

Pseudomonas minimal media (Psmm) plates (3)

Psmm + uracil plate (1)

Thiamine

Casamino acids

Razor blade

Hockey stick

Alcohol

Turntable

Pipettes

Micropipettors

Toothpicks

Forceps

Gloves

Flame Source

Procedure:

1. The four different types of bacterial strains that are used are:

(a) *P. putida* PPN1137 *pyrB*⁻

(b) *P. aeruginosa* PAO483 *pyrF*⁻

(c) *P. fluorescens* pfcw1013 *pyrC*⁻

(d) *E. coli* TB2 *pyrB*⁻, *arg*⁻

2. Take 3 Psmm plates and one plate Psmm+uracil plate.

3. Spread 150µl thiamine on the the Psmm+uracil plate. Allow to dry.

4. On the other three plates, spread 300µl casaminoacid (CAA).

5. Then spread 0.1ml of the appropriate strain of bacteria on each plate:

Plate 1: Psmm + 300µl CAA + 0.1ml PPN1137

Plate 2: Psmm + 300µl CAA + 0.1ml PAO483

Plate 3: Psmm + 300 μ l CAA + 0.1ml pfcw1013

Plate 4: Psmm + uracil + 150 μ l thiamine + 0.1ml TB2

6. Divide the first three plates into seven different areas, and the fourth plate into five different areas. Cut a small well into the agar between them. There should not be any continuation between each area. This is to prevent diffusion from one part of the plate to another.

7. In plates 1, 2, and 3, we are tracing:

- (a) carbamoylaspartate
- (b) dihydroorotate
- (c) orotate
- (d) uracil
- (e) cytosine
- (f) cytidine
- (g) uridine

In plate 4, we are tracing:

- (a) glutamate
- (b) ornithine
- (c) citrulline
- (d) arginiosuccinate
- (e) arginine

8. Place a crystal on each of the above using a toothpick on the appropriate plate.

9. Incubate plates 1 and 3 at 30°C and plates 2 and 4 at 37°C.

Pyrimidine Biosynthetic Pathway

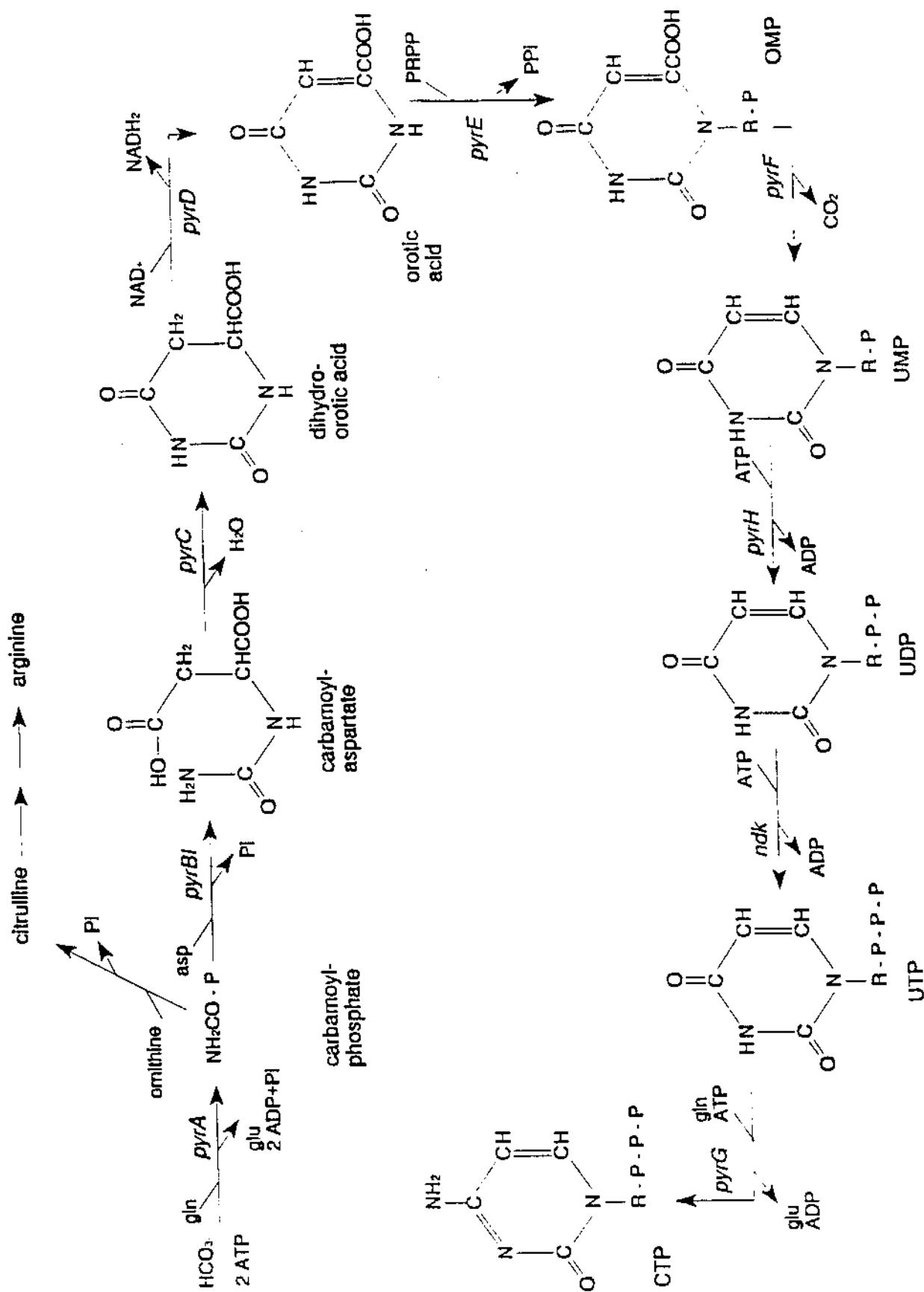
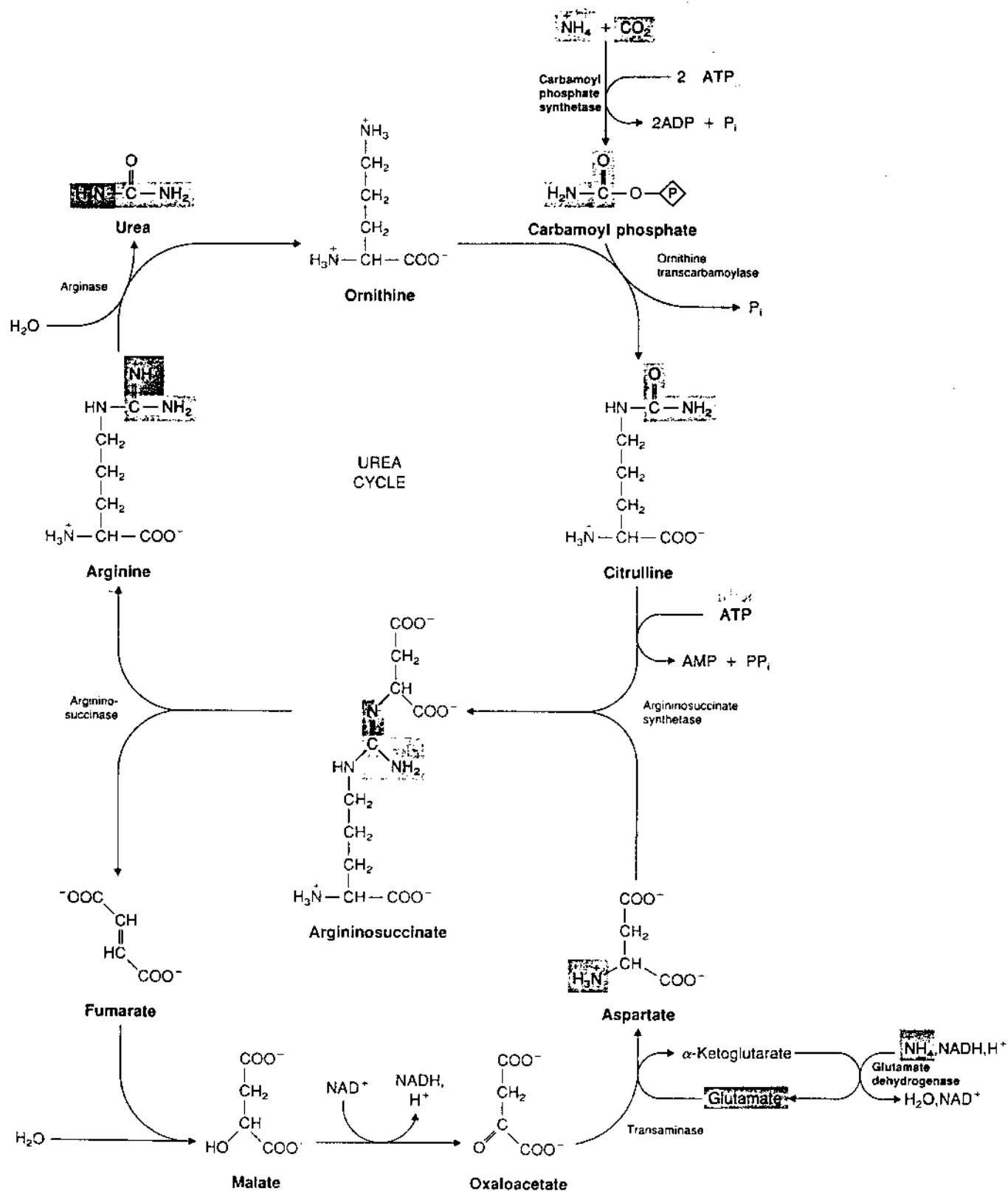


Figure 13.2: Arginine Pathway



CHAPTER 14

NTG MUTAGENESIS: CRUDE METHOD

Purpose: The purpose of this experiment is to expose the given strain of bacteria, *Pseudomonas putida* PPN1137, which is *pyrB*⁻ and *upp*⁻, to a mutagenic agent, NTG (N-methyl-N'-nitro-N-nitrosoguanidine). The resulting bacteria are tested to see if a mutation has occurred at *pyrB* and *upp* to make it *pyrB*⁺ and *upp*⁻. When the *pyrB*⁻ undergoes a mutation to become *pyrB*⁺, the *upp*, which is close to *pyrB*, is also thought to undergo a mutation and become *upp*⁺. The organisms that grew are then tested to see whether they are *upp*⁺ or *upp*⁻ by doing a fluorouracil experiment, as described earlier.

Materials:

Pseudomonas minimal media (Psmm) plates (2)

Casamino acid (concentration = 0.2%)

Cultures of PPN1137

NTG crystals

Toothpicks

Gloves

Turntable

Hockey stick

Alcohol

Flame source

Procedure:

1. Take two plates of Psmm and spread plate using a 0.2% concentration of casamino acids.
2. Incubate at 37°C for 15 minutes.
3. Spread plate 0.1ml PPN1137 onto each plate.
4. On one plate, place a crystal of the NTG in the center using a toothpick.
5. If after incubation the bacterial colonies are seen growing in the zone of killing (Plate1) around the NTG crystal, these bacteria are tested to see if they are upp⁺ or upp⁻ by doing the fluorouracil experiment.

CHAPTER 15

NTG MUTAGENESIS: CRUDE METHOD #2

Purpose: The purpose of this experiment is to expose the given strain of *pyrC*⁺ bacteria, *Pseudomonas fluorescens*, to NTG (N-methyl-N'-nitro-N-nitrosoguanidine) to test to see if the resulting strain is *pyrC*⁺.

Note: A test can also be done to see if the resulting strain is *arg*⁻ because the *arg* gene is located next to the *pyrC* gene. If a mutation occurs, *pyrC*⁻ becomes *pyrC*⁺ and *arg*⁺ becomes *arg*⁻.

Materials:

Pseudomonas minimal media (Psmm) plates (4)

0.1% Casamino acid (CAA)

pfcw1013 strain of *Pseudomonas*.

NTG crystals

Uracil

Toothpicks

Gloves

Turntable

Flame source

Alcohol

Hockey stick

Procedure:

1. Take 2 Psmm plates and spread plate 300µl of 0.1% casamino acid (CAA) on each.
2. Place the plates in the incubator at 37°C for 10-15 minutes so that the liquid is soaked into the medium.
3. Spread plate 0.1ml of pfcw1013 (a strain of *P. fluorescens* which is pyrC-).
4. Put a NTG crystal in the center of one plate.
5. Incubate the plate at 30°C.
6. If there is growth on Plate 1 (i.e. the plate with the NTG crystal), then the colonies in the zone of killing or at the edge of the zone should be used to do a replica plate. Do one Psmm replica plate using 300µl CAA + uracil and one Psmm plate using 300µl CAA without uracil.
7. Incubate the plates at 30°C.

UNIT III

ENZYME KINETICS

Enzyme kinetics is a fundamental aspect in learning about different microorganisms. Enzyme assays are used extensively in the laboratory to determine the regulation of enzyme synthesis. The steps leading up to performing an enzyme assay are fundamental procedures one must learn, and they are explained in detail in this unit. For example, breaking cells so that they can be used in assays is an important part in learning how assays work and it is essential for a good assay.

In this unit, the aspartate transcarbamoylase assay, the dihydroorotase assay, and a protein assay, also known as the Bradford assay, are introduced. These assays aid in learning more about the biochemical nature of different bacterial strains and also promote discussion about enzyme kinetics.

CHAPTER 16

BREAKING CELLS FOR ENZYME ASSAYS

Purpose: The purpose of breaking cells in this experiment is to prepare a sample of cell extract, which is necessary for enzyme assays.

Materials:

Four different bacterial cultures

ATCase buffer

Sonicator

Microfuge tubes

Ice

Pipettes

Micropipettes

Procedure:

1. Each group is given a grown culture:

Group 1: *upp*⁻ PRS 2000 (-UMP)

Group 2: *upp*⁺ PRS 2000 (+UMP)

Group 3: *upp*⁺ PRS 2000 (-UMP)

Group 4: *upp*⁻ PRS 2000 (+UMP)

2. Spin the cells down at 3000 x g for 5 minutes. Do this twice.

Note: After the first 5 minutes, pour off the supernatant. Fill the tube again with the given culture and spin for an additional five minutes.

3. Resuspend in 1ml of ATCase buffer.
4. Sonicate for 3 minutes.
5. Spin at 3000 x g for 10 minutes.
6. Remove the supernatant and transport it to a new microfuge tube.
7. Keep the tube on ice until it is ready to be used in an enzyme assay (i.e. ATCase assay or DHOase assay).

CHAPTER 17

PROTEIN ASSAY (BRADFORD)

Purpose: This protein assay is done to find out the amount of proteins present in a given cell extract (in $\mu\text{g}/\mu\text{l}$).

Materials:

Sample of cell extract to be used for the assay

Coomassie reagent

Test tubes

Procedure:

1. Take four tubes with the cell extract (the test sample used in the ATCase and DHOase assay) and prepare the tubes as follows.

Tube 1: 100 μl H₂O

Tube 2: 5 μl cell extract + 95 μl H₂O

Tube 3: 50 μl cell extract + 50 μl H₂O

Tube 4: 100 μl cell extract

2. Add 5ml of Coomassie reagent to each of the tubes.
3. Mix each tube by inverting several times.
4. Read at an absorbance of 595 nm.

5. Calculate the μg proteins/ μl from the standard curve.
6. Construct a standard curve. To plot the standard curve, use the BSA values.
7. Take the absorbance reading, which falls between 0 and 1.
8. Find the corresponding value from the graph. This will give the amount of proteins in the sample in $\mu\text{g}/\mu\text{l}$.

CHAPTER 18

ASPARTATE TRANSCARBAMOYLASE ASSAY

Purpose: This assay is used to detect the presence of ATCase. Aspartate transcarbamoylase (ATCase) is an enzyme in the pyrimidine biosynthetic pathway (Figure 13.1). It catalyzes the formation of N-carbamoylaspartate and Pi from carbamoyl phosphate and aspartate.

UTP is thought to control the expression of the ATCase in the pathway. If UTP is present in an increased amount, the amount of ATCase present will be decreased.

Materials:

Premix

3 tubes labeled “+”, “-“, and “test”

Cell extract

Carbamoyl phosphate (0.1M)

Stop reagent

Water bath set at 65°C

Vortex

Distilled water (dH₂O)

Ice

Tubes

Pipettes

Micropipettes

Procedure:

1. Add 400 μ l premix into each of 3 tubes labeled “+”, “-“, and “test”.
 2. Add 50 μ l of the appropriate cell extract to each tube.
 - + tube: 50 μ l cell extract (control)
 - tube: 50 μ l dH₂O
 - test tube: 50 μ l test sample
 3. Vortex each tube to mix.
 4. Incubate the tubes at 37°C for three minutes.
 5. Add 50 μ l of 0.1M carbamoyl phosphate. Vortex to mix.
 6. Incubate at 30°C for 20 minutes.
 7. Add 1.5ml of stop reagent. Vortex to mix.
 8. Incubate at 65°C for one hour in the light. Do not cover the water bath. This step allows the color to develop.
 9. Read the tubes at an absorbance of 466nm.
- Note: An increase in yellow color would indicate the presence of increasing amounts of ATCase.
10. Do the following calculations:

To calculate n moles of carbamoyl/aspartase formed:

$$Y = 184.85x + 7.3409$$

where: x = reading of sample at 466nm

$$y = n \text{ moles CAA mode}$$

$$\text{Specific activity of ATCase} = \frac{n \text{ moles CAA mode/min}}{\text{mg protein}}$$

11. Use the following calculations for the ATCase Assay:
12. Calculate the concentration of protein from the standard curve in $\mu\text{g}/\mu\text{l}$.
 - b. The amount of test sample used = $50\mu\text{l}$
 - c. $\frac{\text{protein concentration} \times \text{amount of test sample used}}{1000} = \text{amount of protein (mg)}$
13. Let the color develop for about 20 minutes.
14. Calculate the specific activity of ATCase.

CHAPTER 19

DIHYDROOROTASE ASSAY

Purpose: Dihydroorotase (DHOase) is the third enzyme in the pyrimidine de novo synthesis pathway (Figure 13.1). It catalyzes the condensation reaction that transforms carbamoyl aspartate (CA) into dihydroorotate (DHO). The reaction is readily reversible ($CA \leftrightarrow DHO$) and is the assay most commonly used to detect the presence of DHOase, which uses the end product, DHO, as a substrate. The amount of CA produced from DHO is detected by adding a stop solution that reacts with the CA to form a yellow color and is the measured colorometrically by taking the absorbance at 466 nm.

Materials:

Cell extract

Tris buffer (pH 8.6)

EDTA pH 8.0

20mM dihydroorotase solution

Phosphate buffer

Stop solution

Marble

Water bath

Tubes marked “+”, “-“, and “test”

Distilled water (dH₂O)

Vortex

Ice

Pipettors

Micropipettors

Procedure:

1. Take 20μl of appropriate cell extract in 3 tubes marked "+", "-", and "test."
 - + tube: 20μl cell extract (control)
 - tube: 20μl dH₂O
 - test tube: 20μl test sample
2. Add 200μl Tris buffer, pH 8.6, 1 Molar solution to each of the three tubes.
3. Add 200μl 10mM EDTA pH 8.0 to each of the three tubes.
4. Add 1380μl dH₂O to each of the three tubes.
5. Vortex the tubes and incubate them at 30°C for 10 minutes.
6. Place the tubes on ice and add 200μl of DHO solution.
7. Vortex the tubes and incubate at 30°C for 20 minutes.
8. Place tubes on ice and add 1.5ml of color mix to each tube.
9. Carefully vortex each sample and place a marble on top of each tube.
10. Incubate in the light at 60°C for 1.5 hours. Do not cover the water bath because this reaction requires light.
11. Read the absorbance of the samples at OD₄₆₆.

Note: The presence of yellow color would indicate the presence of DHOase. Increasing amount of DHOase would cause an increase in the color intensity.

12. Do the following calculations:

To calculate n moles carbamoyl/aspartase formed:

$$Y = 184.85x + 7.3409$$

Where X = reading of sample at OD466

$$Y = n \text{ moles CAA mode}$$

$$\text{Specific Activity of DHOase} = \frac{n \text{ moles CAA mode/min}}{\text{mg protein}}$$

13. Use the following calculations for the DHOase assay:

14. Calculate the amount of protein using the standard curve in $\mu\text{g}/\mu\text{l}$.

b. The amount of test sample used = 20 μl

c. $\frac{\text{Concentration of protein} \times \text{amount of test sample}}{1000} = \text{amount of protein (mg)}$

15. Calculate the specific activity of DHOase.

UNIT IV

PLASMIDS

The role of plasmids in microbiology and molecular biology are extensive and vary greatly. This unit includes many different aspects in studying plasmid DNA and includes a variety of methods used to study plasmids. In addition, all of the steps leading up to and following the study of plasmids is discussed in this unit. Techniques such as ligation and digestion are introduced in this unit, as well as methods such as colony lifting and replica plating. Other procedures, including making competent cells and transformation, also play a key role in learning about plasmids. From learning how to prepare an agarose gel to learning how to do PCR, all of these procedures play an essential role in learning advanced laboratory techniques.

CHAPTER 20

SOIL EXPERIMENT

Purpose: The purpose of this experiment is to identify the organisms present in the soil and to determine whether they can degrade linseed oil, gasoline, and toluene. This procedure is also used to determine whether or not these organisms can grow on kanamycin.

Materials:

Soil Sample

100ml flask

TSB broth

Test tubes (5)

TSA plates (5)

Gram's Iodine

Crystal violet

Safranin

95% ethanol

Saline

Distilled water (dH₂O)

Inoculating loop

Microscope

Balance

Pipettes

Gloves

Forceps

Flame source

Procedure:

1. Obtain a sample of soil.
2. Save 1g of soil and put the rest in a 100ml flask.
3. Fill to the 50ml line.
4. Place the 1g of soil into a bottle containing 99ml of dH₂O. From this, make the following plate dilutions on TSA: 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , and 10^{-8} .
5. Incubate all of the plates at 37°C (Set A).
6. Do a colony morphology and Gram stain of at least 5 different colonies from the plates of Set A.
7. Grow up a 5ml culture (TSB) overnight while shaking with any of the 5 organisms.
8. With the flask containing the 1gm of soil, in each group place one of the following:
Group 1: gasoline
Group 2: linseed oil
Group 3: toluene
Group 4: Glucose + Kanamycin (50mg/ml)

9. Take one of the TSA plates (i.e. 10^{-5} dilution), and pour saline into it.
10. Pour it into the flask containing gasoline (linseed oil, kanamycin etc.)
11. Incubate at 37°C for about a week.
12. Do serial dilutions and spread plates as follows: 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , and 10^{-8} .
13. Incubate all the plates at 37°C overnight (Set C).
14. Using the plates of Set C, do a colony morphology and Gram stain of any 5 colonies.
15. Using the colonies from the plates of Set C (the same ones used for the gram stain),
grow up a 5ml culture (TSB) overnight while shaking (Set D).
16. Using the TSB culture (i.e. Set B), do a rapid plasmid preparation.
17. Also do a rapid plasmid preparation of Set D and run them on a gel.

CHAPTER 21

RAPID PLASMID PREPARATION

Purpose: This is a modified alkaline lysis procedure, which is fast and reliable. This is used to prepare plasmids for electrophoresis.

Materials:

Soil solution

TENS solution

Sodium acetate

TE buffer

RNase

70% ethanol

100% ethanol

Distilled water (dH₂O)

Microfuge tubes

Vortex

Dry ice

Hot water bath

Pipettes

Micropipettes

Procedure:

1. Spin 1.0ml of overnight culture for 10 seconds in a microcentrifuge to pellet cells.
(This increases the size of the cell pellet.)
2. Gently decant the supernatant (TSB), leaving 50-100µl together with the cell pellet.
3. Vortex at high speed to resuspend cells completely. In order to do this, 50-100µl of supernatant should be left, but not too much in the case of dilution of TENS solution in Step 4.
4. Add 300µl of TENS solution to break open the cell walls. Vortex the mix 2-5 seconds until the mixture becomes sticky. If more than ten minutes are needed before moving to the next step, it is better to set samples in ice to prevent them from the degradation of chromosomal DNA, which may be coprecipitated with plasmid DNA in Steps 7 and 8.

TENS

TE buffer	3.75ml
0.1 N NaOH	1.0 ml 0.5M NaOH
0.5% SDS	<u>0.25ml 20% SDS</u>
Total	5.0ml TENS

5. Add 150µl of 3.0M sodium acetate (pH 5.2) to precipitate the DNA. Vortex 2-5 seconds to mix completely.
6. Spin for 2 minutes in the microcentrifuge to pellet cell debris and chromosomal DNA.

7. Transfer supernatant to a fresh tube. (At this time, phenol/ether extraction is possible.)
8. Mix well with 0.9ml of 100% ethanol, which has been pre-cooled to -20°C . (Put on dry ice for 10-15 minutes.)
9. Spin for 5 minutes to pellet plasmid DNA and RNA. (A white pellet is observed.)
10. Discard supernatant and rinse the pellet twice with 1ml of 70% ethanol to get rid of the salts. (Invert and pour off as much supernatant as possible without disturbing the pellet.) Dry the pellet under the vacuum for 5 minutes.
11. Resuspend the pellet in 30 μl of TE buffer or dH_2O for further use.
12. Freeze for five minutes and put in H_2O bath for 2-3 minutes at 42°C . This is called the Freeze-Thaw Method and is used to get DNA into solution.
13. Vortex to resuspend the pellet.
14. RNase Step:
 - (a) Add 2 μl of RNase (10mg/ml)
 - (b) Incubate at 65°C for 15 minutes (or 5 minutes at 60°C)
 - (c) Vortex
15. Load the sample into the gel.

CHAPTER 22

AGAROSE GEL PREPARATION

Purpose: The purpose of the agarose gel preparation is to determine the presence of plasmids in a sample.

Materials:

Agarose

TAE buffer

5X loading dye

Ethidium bromide

UV radiation

Electrophoresis tray

Microwave

Balance

Water

Gloves

Pipettes

Micropipettes

Procedure:

1. Take 1g agarose gel and add 100ml TAE buffer (Tris, acetic acid, EDTA).

2. Weigh it and then heat to dissolve the gel (in a microwave).
3. Weigh it again and make up the previous weight with water.
4. Add 2 μ l of ethidium bromide. (This is a mutagenic agent. It should be handled with gloves.)
5. The agarose gel is poured into the electrophoretic tray filling it to about 2/3rd full.
6. Wait for the gel to solidify.
7. Pour TAE buffer in a tray and place the electrophoresis tray in it.
8. Take 16 μ l of the sample obtained after the rapid plasmid preparation and add 4 μ l of 5X loading dye to it so that the total volume is 20 μ l (The loading dye is made up of bromophenol blue, xylene cyanol and glycerol.)
9. Load this into the wells in the gel on the electrophoretic tray.
10. Run it at 60 volts for 1.5 hours.
11. Examine the tray under UV radiation.
12. Repeat the above procedure after doing a rapid plasmid prep using the TSB inoculum of Set D from the previous experiment.
13. Take 17 μ l of sample and add 3 μ l dye. Mix and spin for 0.5 minute.
14. Load sample into the gel.

CHAPTER 23

INDIVIDUAL ISOLATION

Purpose: The purpose of this experiment is to isolate bacteria from any source and to identify it using various stains and biochemical tests.

Materials:

Bacteria of choice

TSA plate

Gram stain materials

Catalase test materials

Oxidase Test materials

Starch plate

MSA plate

Microscope

Procedure:

1. Take a TSA plate and expose it to the atmosphere for 5 minutes.
2. Incubate the plate at 37°C overnight while shaking.
3. Do a colony morphology and Gram's stain of the organism.
4. Do a catalase and oxidase test.
5. Do a starch plate and MSA plate.

CHAPTER 24

STERILE DISK EXPERIMENT

Purpose: The purpose of this experiment is to inoculate discs that have been sterilized in an autoclave and to send these discs to other members in the class through the mail. Once the discs have been received in the mail, an attempt will be made to see if the bacteria can survive this method of transport.

Materials:

Any bacterial strain

TSB broth

10% glycerol

Filter paper

Microcentrifuge tube

Paper hole punch

Aluminum foil

Inoculating loop

Flame source

Envelope

Postage stamp

Lab partner's address

Toothpick

Procedure:

1. Cut out discs of filter paper using the paper hole punch and wrap them in aluminum foil.
2. Autoclave the disks.
3. Take a microcentrifuge tube and add 100µl of 10% glycerol.
4. Choose any bacterial colony with a sterile toothpick and mix it with the 10% glycerol.
5. Take 50µl of this inoculum and put it on the autoclaved disc. Wrap it in aluminum foil.
6. Send the disc to a lab partner through the mail.
7. After receiving the disc in the mail, put it in a flask of TSB broth.
8. Incubate at 37°C overnight while shaking.
9. Using this culture, streak a plate and incubate it at 37°C overnight.
10. Do the appropriate tests to determine the type of bacteria grown.

CHAPTER 25

ANTIBIOTIC SENSITIVITY EXPERIMENT

Purpose: The antibiotic sensitivity experiment is used to detect whether a certain strain of bacteria is susceptible or resistant to various antibiotics. This can be done using many different methods and any bacteria. One method is called the Mueller-Hinton method.

While doing this method, there are certain guidelines that must be followed:

- the amount of agar in each plate should be the same.
- use the 0.5 McFarland standard.
- use standardized amounts of antibiotic on each disc.
- incubate at standard conditions.

The McFarland standard consists of:

1% H_2SO_4 = 19.9ml

1% Barium chloride = 0.1ml (100l)

20ml Total

Materials:

Bacterial suspension

Mueller-Hinton plate

Cotton swab

Various antibiotic discs

Ruler

Forceps

Procedure:

1. Prepare a bacterial suspension (should be comparable to the McFarland control).
2. Take the Mueller-Hinton plate, and swab the plate with the bacterial suspension (in three different directions).
3. Put the antibiotic discs on the plate, spacing them out evenly. The antibiotic discs used are:
 - a. Oxacillin (o)
 - b. Aztreopam (ATM)
 - c. Cefoxitin (FOX)
 - d. Erythromycin (E)
 - e. Gentamycin (GM)
 - f. Tetracycline (TE)
 - g. Chloramphenicol (C)
 - h. Ampicillin (AM)
4. Incubate the plate for 18 hours at 37°C.
5. Measure the zone of clearing around each antibiotic disc.
6. Compare the readings obtained to the given standard values using Figure 25.1.
7. Determine if the bacteria are sensitive or resistant to a particular antibiotic.

Figure 25.1: Antimicrobial Zone of Inhibition Evaluation

Antimicrobial Agent	Disk Potency	R Resistant mm	I Intermediate mm	S Sensitive mm
Amikacin	10 mcg	<12	12-13	>13
Ampicillin				
Gram-negative organisms and enterococci	10 mcg	<12	12-13	>13
Staphylococci and penicillin G susceptibles	10 mcg	<21	21-28	>28
Bacitracin	10 units	< 9	9-12	>12
Carbenicillin				
For <i>Proteus spp.</i> and <i>E. coli</i>	50 mcg	<18	18-22	>22
For <i>Pseudomonas aeruginosa</i>	50 mcg	<13	13-14	>14
Cephalothin				
For cephaloglycin only	30 mcg	<15		>14
For other cephalosporins	30 mcg	<15	15-17	>17
Chloramphenicol	30 mcg	<13	13-17	>17
Clindamycin	2 mcg	<15	15-16	>16
Colistin	10 mcg	< 9	9-10	>10
Erythromycin	15 mcg	<14	14-17	>17
Gentamicin				
For <i>Ps. aeruginosa</i>	10 mcg	<13		>12
Kanamycin	30 mcg	<14	14-17	>17
Lincomycin (Clindamycin)	2 mcg	<17	17-20	>20
Methicillin				
(Penicillinase-resistant penicillin class)	5 mcg	<10	10-13	>13
Nafcillin	1 mcg	<11	11-12	>12
Nalidixic Acid	30 mcg	<14	14-18	>18
Neomycin	30 mcg	<13	13-16	>16
Nitrofurantoin	300 mcg	<15	15-16	>16
Novobiocin	30 mcg	<18	18-21	>21
Oleandomycin	15 mcg	<21	12-16	>16
Oxolinic Acid	2 mcg	<11		>10
Penicillin G				
For staphylococci	10 units	<21	21-28	>28
For other organisms	10 units	<12	12-21+	>21
Polymyxin B	300 units	< 9	9-11	>11
Rifampin (for <i>Neisseria meningitidis</i> only)	5 mcg	<25		>24
Streptomycin	10 mcg	<12	12-14	>14
Tetracycline	30 mcg	<15	15-18	>18
Tobramycin	10 mcg	<12	12-13	>13
Triple Sulfa	250 mcg	<13	13-16	>16
Vancomycin	30 mcg	<10	10-11	>11

CHAPTER 26

COMPETENT CELLS

Purpose: The purpose of this experiment is to make cells competent for use in other procedures. Competent cells are cells that are capable of taking up DNA. Certain cells are naturally competent, while some others can be made competent. In this experiment, the bacteria are treated with calcium chloride salt to make them competent.

Materials:

E.coli DH5 α culture

TSB broth

50ml conical flasks

0.1M CaCl₂

Test tubes

Ice

Pipettes

Procedure:

1. Grow a 5ml culture of *E.coli* DH5 α cells in TSB broth.
2. Do a 1% inoculum.
3. Grow cells to A₆₀₀ = 0.2 to 0.3 at 37°C while shaking.
4. Transfer the culture to 50ml conicals

5. Keep on ice for 10 minutes.
6. Centrifuge for 5 minutes at 3000 rpm.
7. Pour off supernatant.
8. Resuspend cells in 20ml of 0.1M CaCl_2 . (Keep CaCl_2 ice cold.)
9. Keep on ice for 30 minutes to 1 hour.
10. Centrifuge for 10 minutes at 2000 rpm.
11. Pour off supernatant.
12. Resuspend in 1ml of 0.1 CaCl_2 and swirl gently.
13. Keep at 4°C until ready to use.

CHAPTER 27

COLONY LIFT EXPERIMENT

Purpose: The purpose of this experiment is to ligate different plasmids together. The colonies formed will be tested to see if a particular type of ligation has occurred. The process involves the digestion of plasmid DNA followed by a bacterial ligation.

pUC 18 (Figure 27.1) has a *Lac Z* site which has multiple cloning sites. This means that several enzymes can split the plasmid open in this area, but it can happen only once, thus, resulting in the plasmid being split open. The origin of replication for each organism is specific for that organism. It cannot act as an origin of replication for another bacteria. The ampicillin resistant (Amp^r) gene makes the bacteria resistant to ampicillin.

Restriction enzymes are enzymes that are capable of dividing a double-stranded DNA. When *SmaI* recognizes a particular sequence in the DNA, it cuts it resulting in a palendromic sequence. This means that the sequence in the two resulting genes will be the same on either side. *SmaI* cuts blunt ends, and *EcoRI* cuts DNA at sticky ends.

Methylation can occur in 2 ways: dcm methylase puts the methyl group on cytosine, while dam methylase will recognize a certain sequence and put a methyl group on adenosine.

In this experiment, pUC18 will be split open at a cloning site in the *Lac Z* gene. Similarly, *FspI* splits it into four different sized fragments. If the procedure is successful during the ligation process, *FspI* fragments will be attached to the split open pUC 18

plasmid and ligate into pBK188 (Figure 27.2). This blunt end ligation is more difficult to achieve than a sticky end ligation.

Materials:

Various bacterial digestions

Pipettes

Micropipettes

Ice

Procedure:

1. Prepare the following mixture for digestion of pBK 188 (300ng/ μ l):

1g DNA (3.3 μ l)

5 μ l 10X buffer

1 μ l *Fsp*I enzyme

40.7 μ l ddH₂O

50 μ l total volume

2. Incubate at 37°C for 2 hours.

3. Prepare the following mixture for digestion of pUC 18 (231 ng/ μ l):

1g DNA (5 μ l)

2 μ l 10X buffer (4)

1 μ l *Sma*I enzyme

12 μ l ddH₂O

20µl total volume

4. Incubate at room temperature for 2 hours.

5. Prepare the ligation as follows:

insert

vector

ATP

DTT

T4 DNA ligase

Note For blunt end ligation, keep at room temperature overnight.

For sticky end ligation, keep at room temperature for 4 hours.

Figure 27.1: pUC 18 plasmid

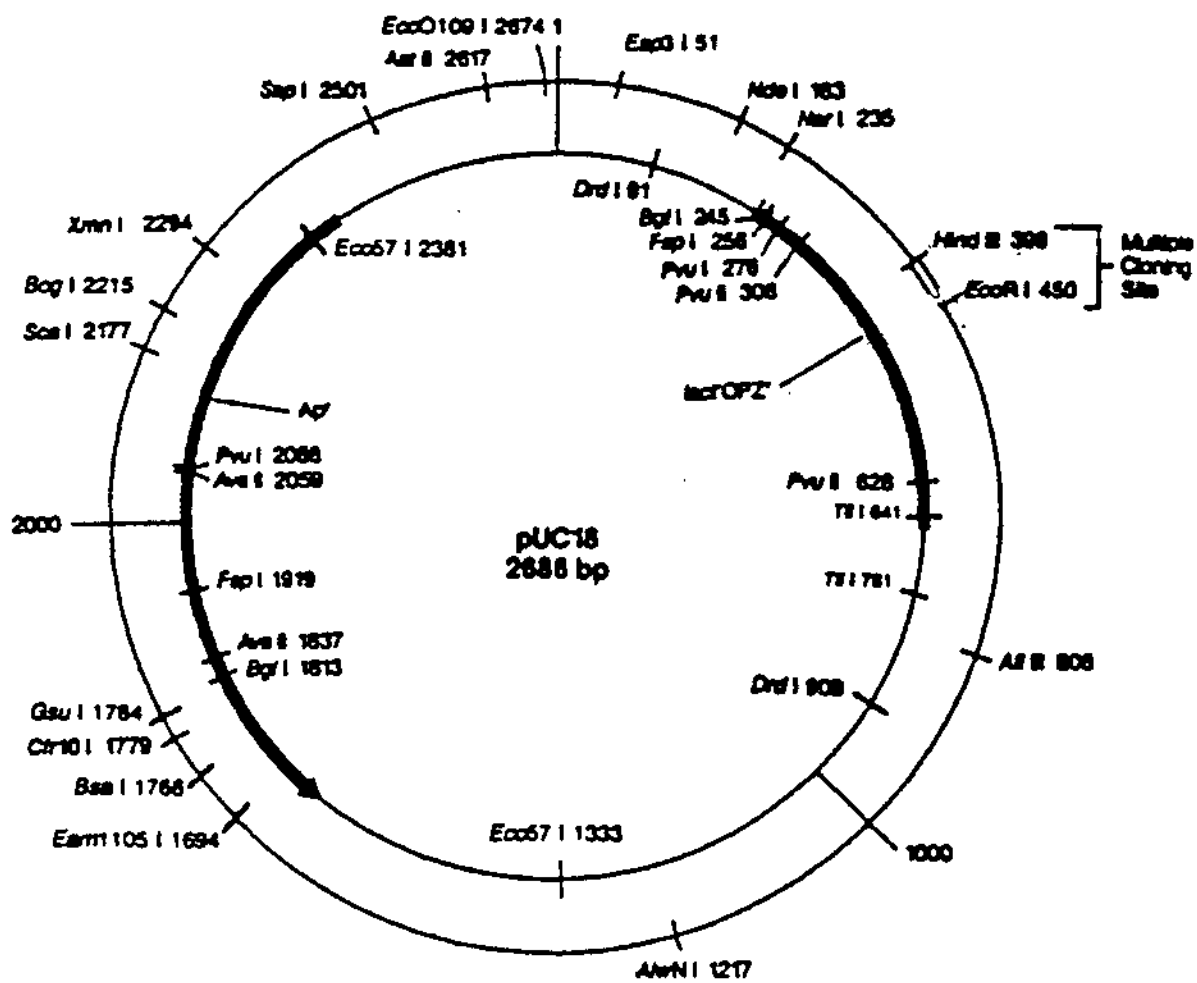
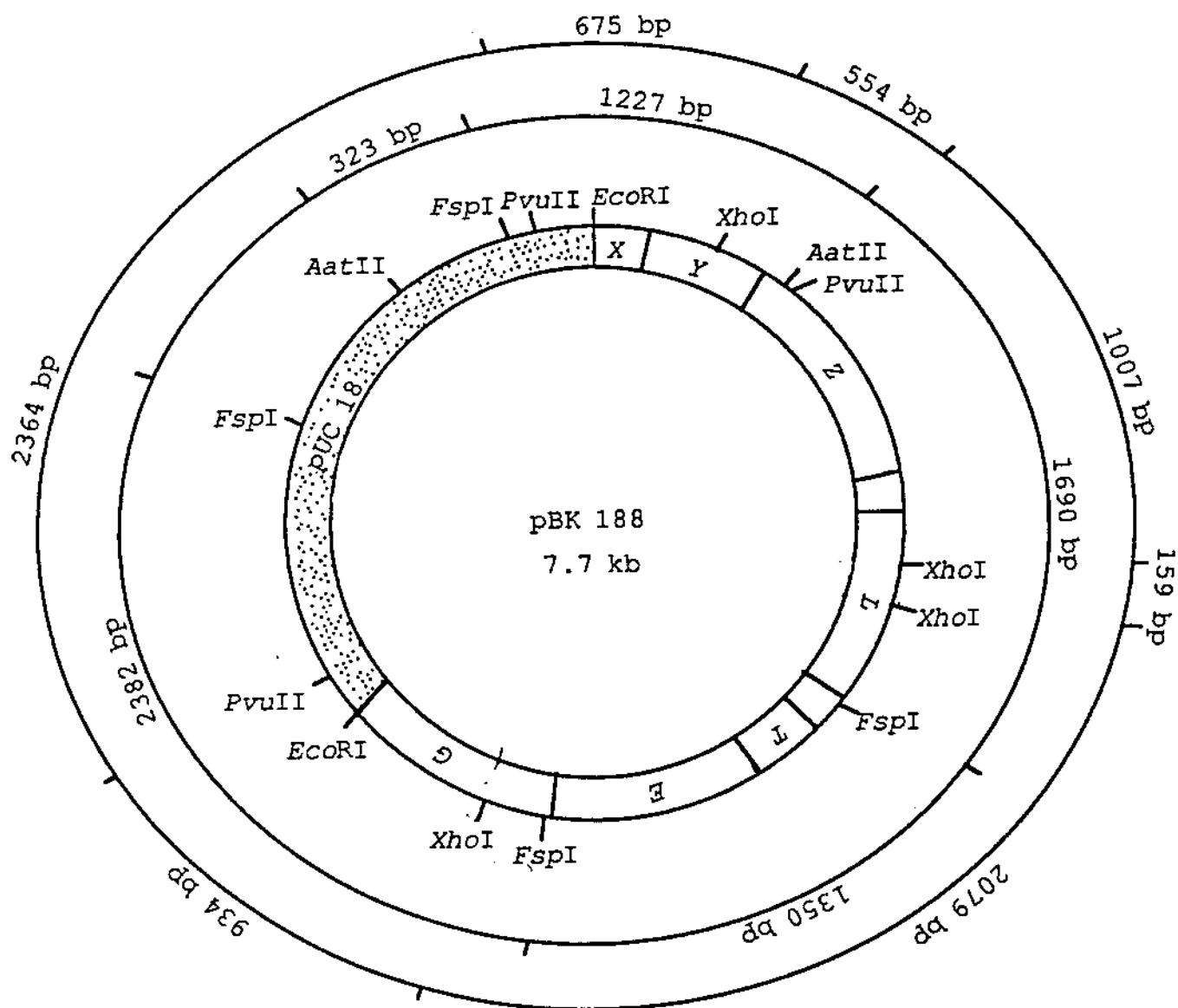


Figure 27.2: pBK 188 plasmid



Fsp I and *Aat* II digest
sizes (inner oval)

<i>Fsp</i> I	1350 bp	<i>Fsp</i> I
<i>Fsp</i> I	1690 bp	<i>Aat</i> II
<i>Fsp</i> I	1227 bp	<i>Aat</i> II
<i>Fsp</i> I	323 bp	<i>Aat</i> II
<i>Fsp</i> I	700 bp	<i>Aat</i> II
<i>Fsp</i> I	2382 bp	<i>Fsp</i> I

Xho I and *Pvu* II digest
sizes (outer oval)

<i>Xho</i> I	2079 bp	<i>Xho</i> I
<i>Xho</i> I	159 bp	<i>Xho</i> I
<i>Xho</i> I	1007 bp	<i>Pvu</i> II
<i>Xho</i> I	554 bp	<i>Pvu</i> II
<i>Xho</i> I	675 bp	<i>Pvu</i> II
<i>Pvu</i> II	2364 bp	<i>Pvu</i> II
<i>Xho</i> I	934 bp	<i>Pvu</i> II

CHAPTER 28

TRANSFORMATION

Purpose: The purpose of this procedure is to transform cells that have previously been digested and ligated into competent cells. The *Lac Z* gene in pUC18 (Figure 27.1) produces β -galactosidase, which breaks down lactose. IPTG acts as an inducer for the cell to make β -galactosidase. The presence of colonies on the plates indicates that a vector re-ligated without an insertion (i.e. the split pUC18 closed by itself without the insertion of the *FspI* fragment). Therefore, the *Lac Z* gene continues to produce β -galactosidase, which will split the chromagen, x-gal, and cause the colonies to turn blue in color.

During the ligation, one of the *FspI* pieces may be ligated to the split open pUC 18. Hence, β -galactosidase will not be formed as a result of x-gal not being split, and the colonies will remain white. The aim of this experiment will be to successfully produce these white colonies.

Materials:

Ligation mixture

DH5 α competent cells

TSB broth

LB Amp plates (4)

10mM IPTG

2% x-gal

Microcentrifuge tubes

Ice

Water bath

Turntable

Hockey stick

Alcohol

Flame Source

Pipettes

Micropipettes

Procedure:

1. Take a 1.5ml microcentrifuge tube and add 30 μ l of ligation mixture.
2. Place the tube on ice and add 200 μ l of competent DH5 α cells. (Do not vortex. This will kill the cells.)
3. Keep the tube in an ice slurry for 20 minutes. Put the tube in a floatie and place it in a water bath at 42°C for 2 minutes. (This is called heat shock and enables the plasmid to go into the cell.)
4. Add 1ml TSB broth to the tube and shake at 37°C for 1-2 hours.
5. Take 3 LB amp (100 μ g/ml) plates.
6. Spread plate the transformants on LB amp + IPTG + x-gal plates as follows:

Plate 1: 10 μ l

Plate 2: 50 μ l

Plate 3: 100 μ l

7. Spin down the rest of the fluid. Resuspend the cells and make a spread plate for Plate 4 using a LB amp + IPTG + x-gal plate.
8. Incubate all four plates at 37°C upside-down.

CHAPTER 29

COLONY SCREENING METHOD FOR THE DETECTION OF A PARTICULAR CLONE

Purpose: The purpose of this experiment is to demonstrate the colony lift technique. In addition, this procedure will be used to demonstrate a method for rapidly screening many bacterial colonies to see if they have the proper (desired) recombinant plasmid. In this experiment, we will be screening for those bacteria that contain a plasmid that has the *xyIE* gene cloned into it. First, this experiment begins by digesting the plasmid pBK188 (Figure 27.2) with the restriction enzyme *FspI*. Then, this digested DNA is ligated into pUC18 cut with *SmaI*. DH5 α is transferred with the recombinant plasmid mixture, and these cells are plated onto LB amp plates containing IPTG and x-gal. Blue colonies contain a plasmid with no insert. White colonies contain recombinant plasmids, but because there are at least four different types of recombinant plasmids possible, it would take a lot of rapid preps to find the desired one, which contains the *xyIE* insert. Hence, to rapidly screen all of the colonies, a colony lift procedure is done, and then the plasmid with the correct insert is probed.

Note: When handling nylon membranes, always wear gloves.

Materials:

Nitrocellulose membranes

1.5ml centrifuge tubes

Probe

Film

Tweezers

Gloves

Needle

India ink

LB amp + IPTG + x-gal plate

Denaturation solution

3M paper

Pipettor

Basin

2 μ l DNA (*xyIE*)

Distilled water (dH₂O)

Ice

Nucleotide mix

5 μ l primer

1 μ l Klenow enzyme

Prehybridization mix

1 x SSC

0.1% SDS

0.5 x SSC

Buffer A

Liquid blocking agent

1.2g BSA

50µl anti-fluorescein-AP conjugate

Procedure:

1. Using tweezers, place the nylon membrane onto the plate with colonies, starting at one edge and gently laying it down towards the other. Mark the orientation of the membrane to the plate by pricking the plate and membrane with a needle dipped in India ink. Leave the membrane on the plate for one minute.
 2. Carefully peel the membrane away from the plate using tweezers. Then, again carefully, lay the membrane down on a clean LB amp, IPTG, x-gal plate and mark your orientation (keeping it consistent with the previous plate). This is your replica plate.
 3. Now, place the membrane face (bacterial cell/DNA side) up on a piece of 3M sided in denaturation solution. Soak for 5 minutes. (This breaks open the cells and releases the DNA).
- Note: Do not allow the solution to get on top of the membrane. Only allow it to be soaked up through the membrane.
4. While the first membrane is soaking, take a second membrane and place it on the original plate. Mark your orientation and allow the membrane to sit on the plate for 5 minutes.
 5. Remove this filter and soak it face up in the denaturation solution for five minutes.
 6. After the denaturation step, neutralize the membranes by soaking them face up on 3M soaked in neutralization solution for 8 minutes.
 7. Remove the membranes from the neutralization solution and allow them to dry face up

on 3M paper. If using nylon membrane, the membranes may be dried in the oven at 50°C for 30 minutes. It is important that the membranes be dry before continuing any further.

Note: If using nitrocellulose membranes, do not put into the oven or they will disappear (combust spontaneously!).

8. Labeling the Probe:

- a) Place 2µl DNA (*xyI ET*) in a 1.5ml centrifuge tube.
- b) Add 18µl ddH₂O.
- c) Spin down.
- d) Boil for five minutes, and quickly plunge into ice for five minutes.
- e) Spin down.
- f) Add the following to a microfuge tube and keep it on ice:
 - sterile H₂O (14µl)
 - Nucleotide mix (10µl)-one of the nucleotides will be labeled with fluorescein
 - Primer (5µl)
 - Klenow enzyme (1µl)-does not have exonuclease activity
- g) Mix by flicking the tube, and spin for 1 minute.
- h) Incubate at 37°C for 1 hour.
- i) Boil it again and quickly plunge into ice.
- j) Place it into the box with the prehybridized membranes.

k) Let it shake at 60°C overnight.

9. Prehybridization of membranes procedure:

a) Prehybridize the dried membranes at 60°C for 1 hour, shaking gently.

b) Prepare the hybridization mix as follows:

-5 x SSC (Sodium Chloride + Sodium Citrate)

-0.1% SDS

-5% dextran sulphate (slows down movement of DNA)

-20 fold dilution of liquid block

-100µg/µl denatured salmon sperm DNA

10. Washing membrane procedure:

a) Prepare wash solution:

-1 x SSC, 0.1% SDS at 60°C for 15 minutes

-0.5 x SSC, 0.1% SDS at 60°C for 15 minutes

b) Pour off wash solution.

c) Incubate blots at room temperature for one hour with 500ml of a 1:10 dilution of liquid blocking agent in buffer A (100mM Tris HCl; 300mM NaCl at pH 9.5)

d) Pour off liquid block, and add 250ml anti-fluorescein-AP conjugate (Buffer A; 1.2g BSA; 50l anti-fluorescein-AP conjugate).

e) Incubate at room temperature for 1 hour with gentle agitation.

f) Expose to film.

CHAPTER 30

COLONY LIFT EXPERIMENT

Purpose: The purpose of this experiment is to use the colony lift procedure to demonstrate the desired recombinant plasmid. Colony screening is also used to rapidly screen many bacterial colonies.

Materials:

Plate with colonies

Tweezers

Nylon membrane

Hypodermic needle

India ink

LB amp + IPTG + x-gal plate

Denaturation solution

Procedure:

1. Using tweezers and wearing gloves, place the nylon membrane onto the plate with the colonies. Starting on one edge of the plate, gently lay the membrane down until it reaches the opposite edge of the plate.
2. Mark the orientation of the membrane to the plate by pricking the plate and the membrane with a needle dipped in India ink.

3. Leave the membrane on the plate for one minute.
4. Carefully peel the membrane away from the plate using tweezers.
5. Carefully lay the membrane down on clean LB Amp, IPTG, x-gal plate and mark the orientation (keeping it consistent with the previous plate). This is the replica plate.
6. Place the membrane face up (the side with the bacteria on it) on a piece of 3M paper soaked in denaturation solution. Soak for 5 minutes. This breaks open the cells and allows both the plasmid and chromosomal DNA to be released. Note: Do not allow the denaturation solution to get on top of the membrane. Only allow the solution to be soaked up through the membrane.
7. While the membrane is soaking, take a second membrane and place it onto the original plate.
8. Mark the orientation so that it corresponds to the previous markings. Leave the membrane on the plate for 5 minutes.
9. Remove the filter paper and soak it in the denaturation solution for 5 minutes.

CHAPTER 31

"KNOCK-OUT" GENE REPLACEMENT TECHNIQUE

Procedure: The purpose of this experiment is to learn the "knock-out" gene replacement technique, which is a highly effective way of making mutants. This experiment uses a cassette to cause a mutation.

Materials:

pA 10 plasmid

LB plate with gentamycin (250 μ g/ μ l)

Gm cassette (1.8 kb)

LB plate with carbenicillin (300 μ g/ μ l)

Turntable

Hockey stick

Alcohol

Pipettes

Micropipettes

Flame Source

Procedure:

1. The pA 10 plasmid, which is an *E.coli* plasmid pUC18 plasmid vector carrying the *Pseudomonas aeruginosa* gene, is inserted with a Gm cassette.

2. Spread plate using a LB plate with gentamycin.
3. Incubate the plate at 30°C.
4. Using the newly grown colonies, do the following to spread plate onto two separate plates:
 - (a) LB plate with carbanicillin
 - (b) LB plate with gentamycin
5. To see if the cassette has been inserted in the right place (i.e. at *pyrC*), use PCR.

CHAPTER 32

LIGATION AND ELECTROPORATION

Purpose: The purpose of this experiment is to put together a known vector with an insert and expose it to an appropriate amount of electrical current in an effort to make a blunt end ligation. The electric current is used to make the cells competent. Salts, such as calcium chloride, cannot be used to make the cells competent because they are used in the electroporation. The time constant is the time taken for the current to pass from one side of the cuvette to the other. The time constant varies in different bacteria (i.e. in *E.coli*, it is 5-7 seconds, while in *Pseudomonas*, it is 9-10 seconds).

If during this experiment a blunt end ligation has taken place, then the organisms will become resistant to Gentamycin (as the Gm cassette was present in the insert). To see if ligation has occurred, plates will be spread with different dilutions. Therefore, the organisms that will grow are gentamycin resistant.

Materials:

pA 10 cut with *FspI*

Gm cassette

Ligation buffer (1mM MOPS, 15% glycerol)

T4 ligase

Double distilled water (ddH₂O)

Electrocompetent PAOAK 903

Pseudomonas aeruginosa (rec-)

TSB broth

LB Gent 250 plates (3)

Pipettes

Micropipettes

Procedure for Ligation:

1. Make the following ligation mixture:

1µl vector, 40ng (pA 10 cut with *EspI*)

2µl insert, 200ng (Gm cassette)

2µl 10X ligation buffer

3µl T4 ligase

12µl ddH₂O

20µl total volume

2. Incubate at room temperature for 4 hours.

Procedure for Electroporation:

1. Take the 20µl ligation mixture from above and add 100µl electrocompetent PAOAK 903.
2. Spin down the cells at 3000 x g for 10 minutes.
3. Resuspend the cells in 45ml buffer.

4. Spin down cells at 3000 x g for 10 minutes.
5. Repeat steps 2 and 3 four more times.
6. After the above 5 washes, resuspend in 250µl of buffer.
7. The cells are now ready for electroporation. Run electric current through the cells twice.
8. Add 500µl of TSB broth and shake at 37°C for 1.5 hours.
9. Plate out the following on three LB Gent 250 plates:
 - (a) 10µl of competent cells
 - (b) 100µl of competent cells
 - (c) Spin down the remainder of the cells and plate them.
10. Incubate at 37°C overnight.

CHAPTER 33

TRANSFORMATION EXPERIMENT

Purpose: This experiment is used to demonstrate how transformation can occur in nature.

Transformation is the uptake by a cell of a naked DNA molecule or fragment from the medium and the incorporation of this molecule onto the recipient chromosome.

Natural transformation is a random process by which organisms acquire DNA without any manipulations. These organisms include: *Streptococcus*, *Neisseria*, *Haemophilus*, and *Acinetobacter*. Other organisms can undergo artificial transformation in which their membranes are rendered more permeable by CaCl_2 .

The organisms to be used are as follows: *Acinetobacter calcoaceticus* ADP1, a wild type strain that can grow on p-hydroxybenzoate (POB) agar plate, and *Acinetobacter calcoaceticus* ADP6, which lacks the enzyme protocatechuate oxygenase and, therefore, cannot grow on POB agar plate.

Materials:

ADP1

ADP6

Lysis buffer

Pseudomonas minimal media (Psmm) buffer

H_2O bath

Crude lysate

POB plates (3)

pBK18

Plates containing catechol

Turntable

Alcohol

Hockey stick

Flame

Pipettes

Micropipettes

Microcentrifuge tubes

Procedure:

1. Take 1ml of ADP1 and 1ml of ADP6 in two microcentrifuge tubes and spin down for lab use. Decant liquid.
2. Resuspend cell pellet of ADP1 in 0.5ml lysis buffer and the cell pellet of ADP6 is Psmm broth. (Lysis buffer is sodium dodecyl sulfate in saline citrate buffer.)
3. Spin down. Decant liquid.
4. Resuspend the pellet as above for both ADP1 and ADP6.
5. Heat the ADP1 in a 65°C water bath for 1 hour.
6. Aseptically make dilutions of crude lysate in the lysis buffer (ADP1) as follows: 10^0 , 10^{-1} , 10^{-2} , and 10^{-3} .

7. Take 3 POB plates and do the following:
 - a. On plate 1, spread plate 100µl of ADP6.
 - b. On plate 3, spread plate 100µl of ADP6.
 - c. Divide plates 2 and 3 into four quadrants.
 - d. Label the quadrants 10^{-0} , 10^{-1} , 10^{-2} , and 10^{-3} .
 - e. Place a drop (10µl) of the 4 different dilutions of ADP1 in the appropriate quadrants on each plate.
8. Do a similar experiment of a plate containing catechol and using the DNA pBK 18 instead of ADP. Make appropriate dilutions of pBK 18 (10^{-0} , 10^{-1} , 10^{-2} , and 10^{-3}) and follow the above procedure.
9. The plates using POB (1st set) should be incubated at 37°C overnight, while the plates containing catechol (green in color-2nd set) should be incubated at 30°C.

CHAPTER 34

PCR (POLYMERASE CHAIN REACTION)

Purpose: This is a procedure used to amplify DNA. The amplification of the DNA is based on the number of cycles. In this experiment, DNA is amplified using different strains of bacteria. Water is used as a control to rule out any contamination.

During the process, a forward primer binds to the top strand, and a reverse primer binds to the bottom strand. The polymerase always needs a 3' end to extend from the primer.

Materials:

pMJS29

Pseudomonas chromosomal DNA

ppN1137 chromosomal DNA

Burkholderia cepacia chromosomal DNA

ppBO primer

ppBSB primer

PCR master mix

PCR tubes

Taq polymerase

PCR machine

Distilled water (dH₂O)

Micropipettes

Pipettes

Loading dye

Electrophoresis materials

UV light

Procedure:

1. Using PCR tubes, make the following five mixtures:

Tube A: pMJS29 (4.3ng/ml)-plasmid DNA

10ng (2.3μl) DNA

1μl ppBO primer

1μl ppBSB primer

Tube B: *Ps.* chromosomal DNA (650ng/μl)

1000ng (1.5μl) DNA

1μl ppBO primer

1μl ppBSB primer

Tube C: *Ps.* ppN1137 chromosomal DNA (600ng/μl)

1000ng (1.66=1.7μl) DNA

1μl ppBO primer

1μl ppBSB primer

Tube D: *Burkholderia cepacia*. chromosomal DNA (30ng/μl)

23µl DNA

1µl ppBO primer

1µl ppBSB primer

Tube E: Control (No DNA)

23µl H₂O

1µl ppBO primer

1µl ppBSB primer

2. Add enough water to each tube to make the volume- 25µl.
3. Add 25µl of the PCR mater mix to each tube.
4. Mix by flicking. Spin for 0.5 minute. The mixture is now ready for the PCR machine.
5. PCR involves 7 major steps:

Step 1: Denatures DNA- Set the machine at 95°C for 5 minutes.

Step 2: Denatures DNA- The machine is set at 95°C for 30 seconds.

Step 3: $T_m = 49^\circ\text{C}$ (The temperature at which 2 pieces of DNA come together. This is called annealing.) To calculate the T_m , give 4 points for every G and C, and give 2 points for every A and T.

eg: ppBO- TACCGCGACGACTTCAG

$$T_m = 54$$

ppBSB- ATGAATGGTTGGTGCTC

$$T_m = 50$$

The lower T_m value among both is 50°C. Program the PCR machine at 49°C.

Step 4: Polymerization: The machine is set at 72°C for 2 minutes. Taq polymerase is isolated from a thermophile, *Thermus aquaticus*, and is able to withstand higher temperatures.

Step 5: Go back to Step 2 and repeat the cycle 29 more times for a total of 30 cycles.

Step 6: The machine is set at 72°C for 5 minutes.

Step 7: The machine is kept at 4°C. The amplification of DNA in PCR is based on the number of cycles. (eg. If you start with 10ng and do 30 cycles you get a 2-30 amplification.)

6. To 25µl of each sample (A-E), mix 5µl of dye.
7. Flick mix and spin for 0.5 minutes.
8. Load in the wells of the electrophoresis tray and run a gel.
9. Read the gel under UV radiation.

CHAPTER 35

BOOMERANG EXPERIMENT

Purpose: In this experimental procedure, three strains of the same *Salmonella* bacteria (i.e. each has undergone a different mutation at different stages in the pathway) are used to see if they can grow utilizing the enzymes from each other in spite of having undergone mutation.

Materials:

Salmonella, pyrC⁻

Salmonella, pyrD⁻

Salmonella, pyrF⁻

E.coli minimal media plate with casamino acids

Cotton swab

Procedure:

1. Take a plate of *E.coli* minimal media with casamino acids. Use three types of

Salmonella:

-*Salmonella pyrC⁻*

-*Salmonella pyrD⁻*

-*Salmonella pyrF⁻*

2. Streak each organism onto the plate using a cotton swab. Make the streaks close to

each other but do not allow any contact between organisms.

3. Incubate at 37°C upside down.

UNIT IV

PLASMIDS

The role of plasmids in microbiology and molecular biology are extensive and vary greatly. This unit includes many different aspects in studying plasmid DNA and includes a variety of methods used to study plasmids. In addition, all of the steps leading up to and following the study of plasmids is discussed in this unit. Techniques such as ligation and digestion are introduced in this unit, as well as methods such as colony lifting and replica plating. Other procedures, including making competent cells and transformation, also play a key role in learning about plasmids. From learning how to prepare an agarose gel to learning how to do PCR, all of these procedures play an essential role in learning advanced laboratory techniques.

CHAPTER 36

PREPARATION OF MEDIA

Purpose: The purpose of making media is to grow and maintain bacterial strains. Strains need to be cultivated, and with media, one can select for different phenotypes of bacterial strains. There are two different types of media: complex media and minimal media. LA (Lennox Agar) and LB (Lennox Broth) media are called complex media because the exact composition and concentration of each component in the medium is not known. Minimal medium is chemically defined medium. The exact components are known, and only the minimum amount of substances is involved to allow for growth.

Materials:

tryptone

yeast extract

sodium chloride salt

agar

deionized water

KH_2PO_4

FeSO_4

MgSO_4

$(\text{NH}_4)_2\text{SO}_4$

Glucose

R-salts

PN (phosphate and ammonia)

balance

bottles (2)

flasks

Petri dishes

balance

graduated cylinder

scoopulas

metal spatulas

cotton

gauze

Procedure:

1. Place 50ml of PN into the first bottle (which is double the concentration) and place 50ml of water agar (which is water and agar at double strength) into a second bottle.
2. Make a cotton and gauze stopper to fit the top of each bottle.
3. Autoclave both bottles for 45 minutes.
4. Add other substances to the first bottle that may be needed for each particular experiment. This may include R-salts, carbon sources, growth factors (e.g. amino acids), antibiotics, etc.

5. Mix both bottles and add water to make the final volume 100ml.
6. Quickly pour into plates (25ml/plate).

Note: To calculate the amount of a substance that needs to be added, use the following:

$$\frac{\text{Required concentration} \times \text{Volume}}{\text{Original Concentration of Stock}} = \text{Volume to be Added}$$

CHAPTER 37

PHENOTYPING

Purpose: The purpose of this experiment is to phenotype mutant strains of bacteria by using different types of media. The first part of the experiment requires a master plate to be made of six different organisms. Next, the master plate is used to inoculate eight different types of media. A rapid plasmid preparation is then used for specific bacterial strains per group. Finally, a gel electrophoresis is run, and a growth curve is made from the results of the gel.

Materials:

Six different strains of bacteria:

E.coli JM101

E.coli JM101 (pBK 189)

E.coli DP2229

E.coli DP2229 (pBK 489)

P.putida PpC1 (TOL)

P.putida Paw 630 (RP1::TOL)

Eight different types of media plates:

m-tol/MM

m-toi/trp^{su}

LA/Ap⁵⁰

LA/Ap⁵⁰ + Km⁵⁰

Glu¹⁰/MM

Glu¹⁰/ pro²⁵, leu²⁵, thi^{2.5}, Ap²⁵

LA

LA/Ap⁵⁰ + Nm^{1.2}

Replica plate grid

Toothpicks

Flame source

Procedure:

1. Using toothpicks, make a master plate of the six different types of bacteria on a LA agar plate. Make six marks on the master plate per bacteria for a total of 36 marks.
2. Prepare the eight different media plates according to its specifications.
3. Using a toothpick, transfer bacteria from one area of the master plate grid to the corresponding grid (Figure 11.1) on the media plates.
4. Incubate the plates at 30°C for *Pseudomonas* strains and 37°C for *E.coli* strains overnight.
5. Pick from the master plate and inoculate 5ml of L-broth for a rapid plasmid prep.

CHAPTER 38

RAPID PLASMID PREPARATION

Purpose: The purpose of this procedure is to quickly detect plasmids from crude cell-lysates of bacteria. This particular preparation is a mild, alkaline lysis procedure used to detect small plasmids.

Materials:

Cells grown overnight

Solution A:

50.0 mM glucose

10.0 mM EDTA

25.0 Tris-HCl, pH 8.0

6.0 mg/ml lysozyme

Solution B:

0.2 N NaOH

1.0% SDS

5M potassium acetate, pH 4.8:

60.0ml 5M potassium acetate

11.5ml glacial acetic acid

28.5ml distilled water

100% ethanol

70% ethanol

TE buffer

Microfuge tubes

Pasteur pipettes

Micropipettes

Vortex

Water Bath

Ice

Agarose gel electrophoresis solutions and equipment

Procedure:

1. Transfer 1.5ml of cells (grown overnight) into a microfuge tube. Centrifuge for 1 minute in a microfuge centrifuge.
2. Remove the medium with a Pasteur pipette leaving the bacterial pellet as dry as possible.
3. Resuspend the pellet by vortexing in 100 μ l of an ice-cold solution A.
4. Store for 5 minutes at room temperature.
5. Add 200 μ l of an ice-cold solution B. Close the cap of the tube and mix the contents by inverting the tube rapidly two or three times. Store the tube on ice for 5 minutes.
6. Add 150 μ l of an ice-cold solution of potassium acetate. Close the cap of the tube and mix the contents by inverting the tube rapidly two or three times. Do not vortex! Store

the tube on ice for 5 minutes.

7. Centrifuge for 5 minutes at 10,000 x g.
8. Transfer the supernatant to a fresh tube.
9. Centrifuge for 5 minutes at room temperature.
10. Remove the supernatant. Add 1ml of ice-cold 70% ethanol. Vortex briefly and then recentrifuge for 5 minutes.
11. Remove all of the supernatant and resuspend the pellet in 30 μ l of TE buffer.
12. Place the tube in a 37°C water bath for 10 minutes.
13. Run a gel using the following conditions:
 - 0.56g agarose/80ml TBE buffer
 - 20 μ l sample + 5 μ l tracking dye
 - 120V for 2.5 hours

CHAPTER 39

TRANSPOSON MUTAGENESIS

Purpose: The purpose of this experiment is to isolate mutant strains of bacteria using transposons. Transposons are used to mutagenize DNA as a method of locating genes and physical mapping. Furthermore, Tn5, which codes for kanamycin resistance, is used to provide a convenient physical marker for mapping genes. In this experiment, Tn5 will be used to generate insertions of cloned TOL DNA (Figure 39.1), which is present in plasmid pBK489.

The *E.coli* strain DP2229 carries Tn5 in its chromosome. If the antibiotic concentration (i.e. neomycin) is increased, selection for transpositions from the chromosome onto the plasmid can occur by means of its amplification. Hence, by increasing the selective pressure, transposition onto the plasmid by virtue of its ability to amplify (e.g. 50-100 copies/cell) is possible.

In this experiment, DP2229 (pBK489) will be patched onto LA/Ap⁵⁰ + Nm^{1,2} plates. These plates will incubate for five to seven days. Then, Nm^r colonies will be picked and purified on the same medium. These colonies will then be subjected to further analysis to see if mutants were created. This will be achieved by observing if Tn5 has been inserted into the amplifiable pBK489 plasmid.

Materials:

DP2229 (pBK489)

neomycin plate (LA/Ap⁵⁰ + Nm) (3)

5ml tubes of LB broth (3)

*EcoR*I

Grids for patching

Toothpicks

Pipettes

Micropipettes

Agarose gel electrophoresis solutions and equipment

Flame source

Procedure:

1. Take DP2229 (pBK489) from a LA/Ap + Km plate and score it on a neomycin plate.
Score from the same clone on twelve different patches to obtain twelve different mutants (Figure 39.2).
2. Incubate the plate upside-down at 37°C for 24-48 hours.
3. Score the mutants on another neomycin plate for the first purification.
4. Repeat steps 2-3 two more times for a total of three purifications.
5. Select a patch and inoculate three separate tubes of 5ml LB broth.
6. Incubate at 37°C overnight.

Figure 39.1: TOL Pathway

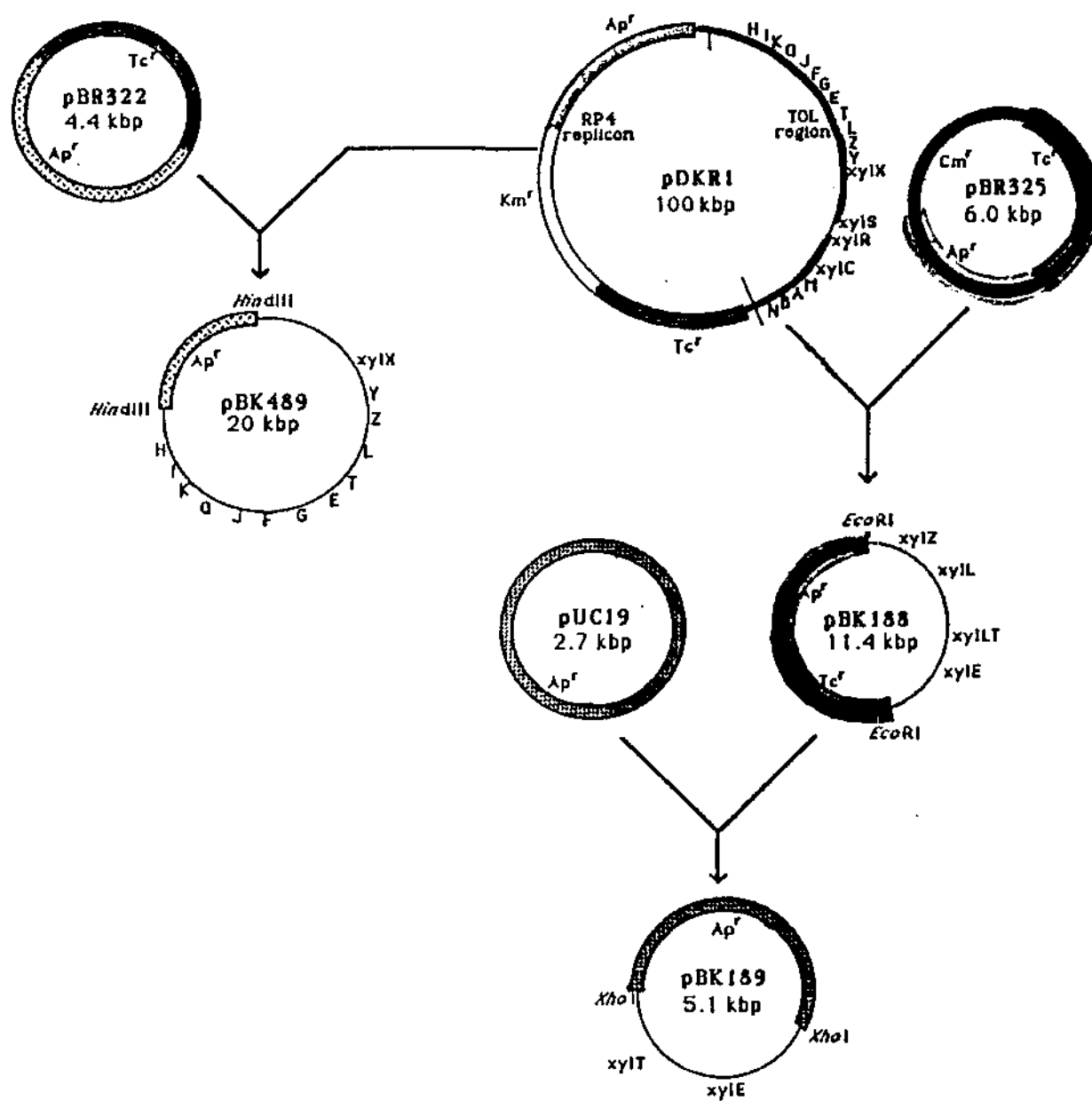
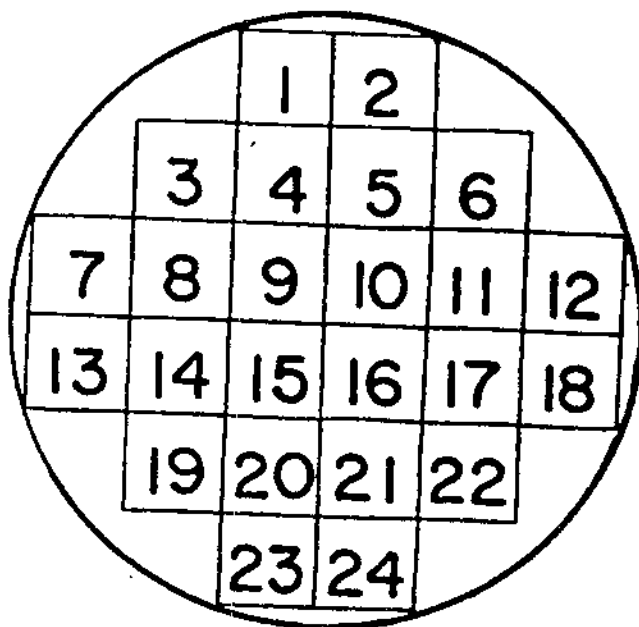
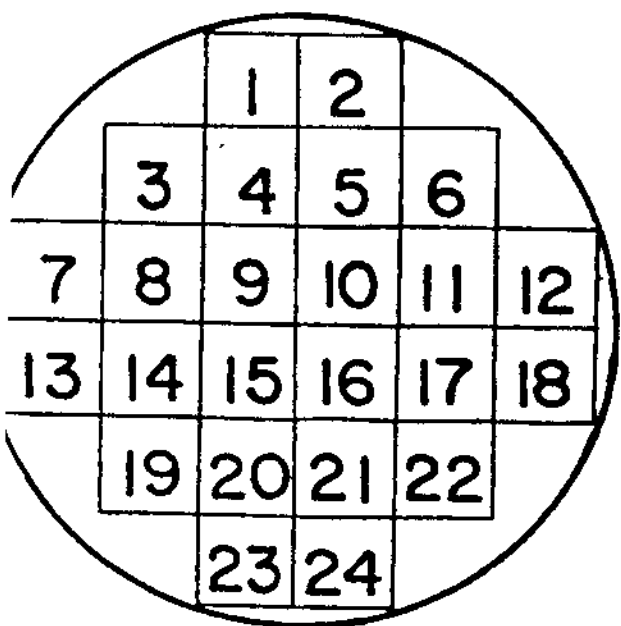
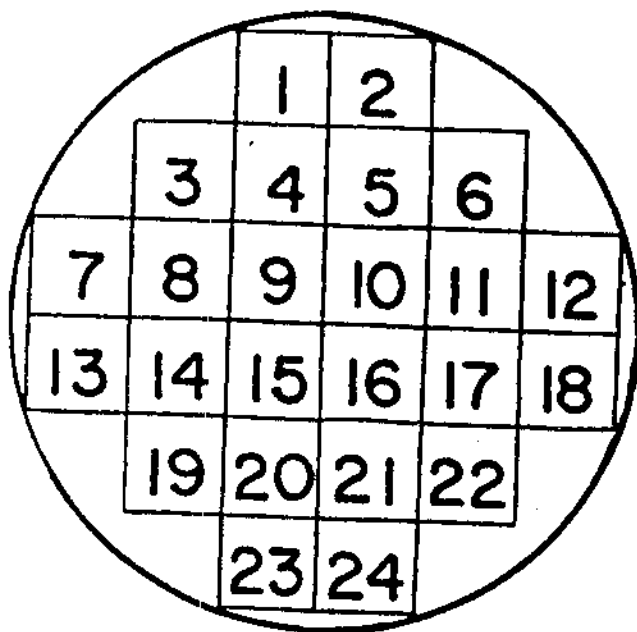
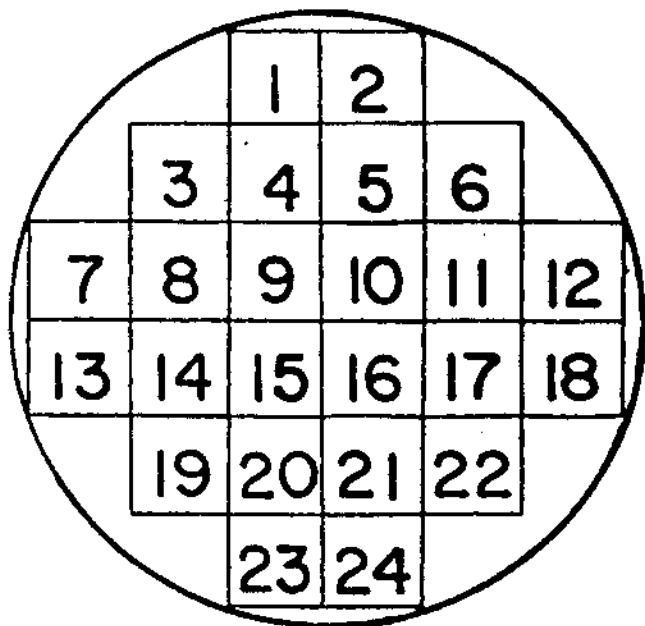


Figure 39.2: Replica Plate – 24 Count



CHAPTER 40

MOLECULAR ANALYSIS OF Tn5-INDUCED MUTANTS

Purpose: In this experiment, plasmid analysis will be carried out to see if mutants of cloned TOL genes that carry Tn5 inserts are isolated. First, plasmids from mutant strains of *E.coli* will be detected using a rapid crude-lysate procedure. From these crude lysates, a small amount of overnight culture will be used to quantitatively assay for the expression of catechol-2,3-oxygenase (C230). In addition, the crude lysates from the various mutants will be subjected to restriction endonucleases digestion analysis. Finally, the mutants will be analyzed by agarose gel electrophoresis to indicate any changes in size that have occurred to the native plasmids.

Materials:

Crude lysate

Microfuge tubes

0.5ml phosphate buffer

13 X 100 mm test tube

catechol (10mM)

1 μ l *Eco*R1

2 μ l restriction buffer (Tris, MgCl₂, NaCl)

5 μ l loading (tracking) dye

Pipettes

Micropipettes

Flame Source

Agarose gel electrophoresis solutions and equipment

Procedure for Crude Lysate Assay:

1. Remove 0.5ml of culture and spin in a microfuge.
2. Resuspend cells in 0.5ml phosphate buffer.
3. Place 0.2ml of cells into a clean 13 x 100mm test tube.
4. Add 20-50 μ l of catechol (10mM) and look for the formation of yellow color.

Procedure for Restriction Endonuclease Digestion:

1. To a microfuge tube, add the following components:
 - 17 μ l DNA (be sure sample is at bottom of tube)
 - 1 μ l *Eco*R1
 - 2 μ l restriction buffer
2. Allow the reaction to proceed at 37°C for 2 hours.
3. Stop the reaction by adding 5 μ l loading buffer.
4. Load the entire sample onto a horizontal agarose gel for overnight electrophoresis at 30V.
5. Stain and examine the overnight gels for restriction digestion patterns.

CHAPTER 41

RAPID ISOLATION OF PLASMID DNA BY ALKALINE LYSIS –

ANALYTICAL SCALE

Purpose: The purpose of this procedure is to prepare the crude cell-lysates of bacteria for the detection of plasmids. This cell-lysate is then analyzed by gel electrophoresis to see if any inserts are detected.

Materials:

Solution A:

50.0 mM glucose

10.0 mM EDTA

25.0 mM Tris-HCl

6.0 mg/ml lysozyme

Solution B:

0.2 N NaOH

1.0% SDS

5M Potassium Acetate, pH approximately 4.8

60.0ml 5M potassium acetate (not pH adjusted)

11.5ml glacial acetic acid

28.5ml distilled water

Phenol:Chloroform:

1 volume of saturated phenol

1 volume of chloroform

100% ethanol

70% ethanol

TE buffer (pH 8.0)

25 µg/ml heat-treated RNase A

Microfuge tubes

Paper towel

Pipettes

Micropipettes

Flame Source

Ice

Vortex

Agarose gel electrophoresis solutions and equipment

Procedure:

1. Inoculate 5ml of medium containing the appropriate antibiotic (or other selective medium) with a single bacterial colony. Incubate at 37°C (for *E.coli* cultures) overnight with vigorous shaking.
2. Remove 1.5ml of the culture to a microcentrifuge tube. Centrifuge for 1 minute at

10,000 rpm in a microcentrifuge centrifuge. Store the remainder of the overnight culture at 4°C.

3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.
4. Resuspend the pellet by vortexing in 100µl of ice-cold solution A.
5. Store for 5 minutes at room temperature. The top of the tube does not need to be closed during this period.
6. Add 200µl of a freshly prepared solution B. Close the top of the tube and mix the contents by inverting the tube rapidly two or three times. Do not vortex. Store the tube on ice for 5 minutes.
7. Add 150µl of an ice-cold solution of potassium acetate. Close the top of the tube and mix the contents by inverting the tube rapidly two or three times. Do not vortex. Store the tube on ice for 5 minutes.
8. Centrifuge for 5 minutes at 10,000 x g in a microcentrifuge at 4°C.
9. Transfer the supernatant to a fresh tube.
10. Add an equal volume (450µl) of phenol:chloroform. Mix by vortexing.
11. After centrifuging at 10,000 x g for 2 minutes in a microfuge centrifuge, transfer the supernatant to a fresh tube.
12. Add two volumes (750µl) of 100% ethanol at room temperature. Mix by vortexing. Stand at room temperature for two minutes.
13. Centrifuge at 10,000 x g for 5 minutes in a microfuge centrifuge for 5 minutes at room temperature.

14. Remove the supernatant. Stand the tube in the inverted position on a paper towel to allow all the fluid to drain away.
15. Add 1ml of 70% ethanol. Vortex briefly and recentrifuge for 5 minutes at 10,000 x g.
16. Again remove all the supernatant. Dry the pellet briefly in a vacuum desiccator.
17. Add 30 μ l of TE with 25 μ g/ml heat-treated RNase A. Vortex briefly.
18. Incubate at 37°C for 15 minutes.
19. Analyze the DNA by agarose minigel gel electrophoresis.

CHAPTER 42

SYNTHESIS OF NUCLEIC ACIDS DURING GROWTH

Purpose: The purpose of this experiment is to estimate the content of DNA and RNA in a cell using radioactive ^{32}P . Bacteria require phosphorus for growth and use it primarily for the synthesis of phosphate compounds such as ATP and nucleotides. Furthermore, viable cell counts and total cell counts can be used to determine other components of a cell, including chromosomes and ribosomes. Using the Petroff-Hausser grid, the number of living cells per milliliter can be determined. Then, a growth curve can be made using Klett units per time. The growth curve is done because macromolecular synthesis is a function of the growth rate. Finally, different equations are used to assemble a chart and to compare data.

In this experiment, two cultures of *E.coli* JM101, one containing a plasmid pBK189 and the other containing no plasmid, will be used. One culture will contain ^{32}P -ortho-phosphoric acid while the other will serve as a control to determine VCN (viable cell number).

Materials:

E.coli JM101

E.coli JM101 (pBK 189)

50ml LB (2)

50ml flasks (2)

$^{32}\text{P-PO}_4$ (6.6×10^6 CPM/ml)

KH_2PO_4 (100mM)

0.5 N NaOH

Scintillator

20% TCA (tri-chloro-acetic acid)

0.7% NaCl

5% TCA

2:1 chloroform/methanol

petri dishes

Petroff-Hauser grid

Filter paper

Semi-log paper

Water bath (60°C)

Ice

Test tubes (2)

Inoculating Loop

Pipettes

Micropipettes

Flame Source

Hockey stick

Alcohol

Turntable

Procedure:

1. Inoculate 2 50ml flasks of LB broth (labeled flask A and flask B) with 1ml of an overnight culture containing JM101 and JM101 (pBK189), respectively.
 2. Add 1ml of KH_2PO_4 to each flask.
 3. Use the culture in flask B to perform VCN and to monitor Klett readings. Take Klett readings and do VCN at the same time each 0.5ml aliquot is removed. Skip to step 12 for flask B and follow the remaining procedure.
 4. Add 0.4ml of $^{32}\text{PO}_4$ to flask A and follow steps 5-11 for flask A only.
 5. At time zero, remove 50 μl and spot on filter to determine ^{32}P of entire culture.
 6. At time 0, 2, and 3 hours, remove 2 1ml samples and place in separate tubes. Label them tube A and tube B.
 7. For tube A, add 2ml 0.5 NaOH and incubate in a 60°C water bath for 20 minutes. This degrades the RNA. Cool on ice and add 4ml 20% TCA.
 8. For tube B, add 2ml 0.7% NaCl and 3ml 20% TCA. This precipitates macromolecules.
 9. Collect samples by filtration by doing the following for both tubes:
 - a) wet the filter with 5% TCA
 - b) pour contents of tube onto filter
 - c) wash tube with 10ml TCA. Do this 2 times.

This process allows the macromolecules to adhere to the filter.
 10. Wash the filter several times with 10ml 2:1 chloroform/methanol. Do this 2 times.
- Then, wash the filter with 5% TCA. Phospholipids are broken down and washed

through the filter, and only DNA or DNA/RNA remain.

11. Measure counts per minute (cpm) in a scintillation counter.

Note:

Tube A filtrate = PO_4 incorporated into DNA

Tube B filtrate - Tube A filtrate = PO_4 incorporated into RNA

12. For flask A, determine the VCN. At the designated times (e.g. 0, 2, 3 hours), remove 0.5 ml of culture and dilute appropriately to obtain 10-100 colonies per plate. For example, few cells would be expected in the culture at time 0, so the culture would not have to be diluted much. Do the following dilutions at time = 0: 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} .
13. At time 2 hours, some cell duplication is expected. Do the following dilutions: 10^{-5} , 10^{-6} , 10^{-7} , and 10^{-8} .
14. Do dilutions for the 3 hour sample: 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} .
15. Calculate the cell concentration using the Petroff-Hauser counter:
 - a) Count the number of cells in 16 squares in 3 separate fields at 43X (i.e. 3 large squares [there are 25 total]).
 - b) Average cell number/field (e.g. 90 cells/field).
 - c) Calculate cell number/ml.

$$\text{e.g. } \frac{90 \text{ cells/field}}{\text{volume}} = \text{cell number/ml}$$

$$\begin{aligned} \text{and volume} &= 50\mu\text{m (side)} \times 50\mu\text{m (side)} \times 20\mu\text{m (depth)} \times 16 \text{ squares} \\ &= 8 \times 10^5 \mu\text{m}^3 \end{aligned}$$

$$= 8 \times 10^{-7} \text{ cm}^3$$

$$= 8 \times 10^{-7} \text{ ml}$$

$$\frac{90 \text{ cells/field}}{8 \times 10^{-7} \text{ ml}} = 1.12 \times 10^8 \text{ cells/ml}$$

then $1.12 \times 10^8 \text{ cells/ml} \times \text{dilution factor} = \text{cells/ml in original culture}$

16. On semi-log paper, draw a graph using Klett units vs. Time (hours).

17. Calculate the growth rate constant:

$$\mu = 1/t_d - \text{doubling time}$$

18. Calculate the following using the equations on the next page and assemble the values in a chart for analysis:

μ

KU

TCN

VCN

$\mu\text{mol DNA/ml}$

$\mu\text{mol RNA/ml}$

g DNA/ml

g RNA/ml

chromosome/ml

ribosome/ml

chromosome/cell

ribosome/cell

CHAPTER 43

CALCULATIONS

1. Total ^{32}P will be determined by taking a sample of the culture and applying it directly to a filter and counting it. This will give total cpm/ml.
2. Since the final phosphate concentration will be 2mM, then the cpm/ $\mu\text{mol PO}_4$ can be determined.
3. From the alkali treated tube of each culture, estimate the amount of ^{32}P incorporated into DNA.

ex.
$$\frac{\text{cpm DNA/ml}}{\text{cpm}/\mu\text{mol PO}_4} = \mu\text{mol DNA/ml}$$

4. From the other tube, we will estimate ^{32}P incorporate into both DNA + RNA.

ex.
$$\frac{\text{cpm DNA + RNA/ml}}{\text{cpm}/\mu\text{mol PO}_4} = \mu\text{mol DNA + RNA/ml}$$

5. $\mu\text{mol RNA (or DNA)/ml} \times 10^{-6} \text{ mol/ml} \times 300 \text{ g/mol (average weight of nucleotide)}$
 $= \text{grams RNA (or DNA)/ml}$

6. Parameters available:

Mass of *E.coli* chromosome is about 4×10^{-9} micrograms.

For each *E.coli* ribosome, there is about 3×10^{-12} micrograms RNA (rRNA + tRNA + mRNA).

7. So, determine the amount of DNA and RNA made:

$$\frac{\text{grams DNA/ml}}{\text{mass of } E. coli \text{ chromosome}} = \# \text{ chromosomes/ml}$$

$$\frac{\text{grams RNA/ml}}{\text{mass of } E. coli \text{ ribosome}} = \# \text{ ribosomes/ml}$$

8. Now, calculate how many cells there were in the culture obtained in the duplicate LB culture of each strain set up without ^{32}P .

$$\# \text{chromosomes/ml (or ribosomes)} / \text{viable cells/ml} = \# \text{ chromosomes (or ribosomes)} \\ \text{per unit cell.}$$

CHAPTER 44

INDUCTION OF ENZYME ACTIVITY

Purpose: The purpose of this experiment is to assay the enzymes of various bacteria to determine the regulation of enzyme synthesis. Two of the enzymes of the TOL pathway, catechol-2,3-oxygenase (C23O) and 1,2-dihydroxy-3,5-cyclohexadiene diol carboxylic acid dehydrogenase (DHCDH), will be assayed. In the C23O assay, the product itself, 20 HMS, is measured directly. In the DHCDH assay, the reduction of the desired co-factor, NAD⁺, is measured. To accomplish this, the cells must be grown under appropriate conditions to induce these enzymes. Then, the cells must be harvested and prepared before the break to measure for enzyme activity.

The rates of enzyme reactions are a function of substrate concentration and the enzyme provided. It is always a good idea to perform assays at saturating substrate levels but at limiting enzyme levels. That is, rates should be roughly linear with respect to enzyme concentration. Therefore, in both of the assays, two different enzyme quantities will be added to show that the rate of reactions can be doubled with enzyme concentrations. Once the protocol for assaying the enzymes is complete, the absorbance of the extracts will be taken to calculate the specific activity (S.A.). The slopes of each graph are used to determine the S.A., and various calculations will be made to analyze the data.

Materials:

Different bacterial strains:

PpC1 (TOL)

PaW630 (RP1::TOL)

E.coli JM101 (pBK189) - *xylTE* (2)

E.coli DP2229 (pBK489)

Different media plates according to the respective bacteria:

m-toluolate⁵

Glut¹⁰ + m-xylene

Glycerol

LB + IPTG²⁵ μ g/ml (2)

Glutamate

LA plates (5)

Centrifuge tubes

2-liter Erlenmeyer flask

Phosphate buffer

Tared 50ml centrifuge tube (5)

0.05M KH₂PO₄

0.01 M Catechol

Tris buffer-100mM (pH 8.0)

NAD + 10mM

Crude diol prep (~5mM)

Distilled water (dH₂O)

Spectrophotometer

Scale

Cotton

Gauze

Ice

DNase

Inoculating loop

Pipettes

Micropipettes

Flame source

Procedure:

1. Prepare 500ml MM of the following media in a 2-liter Erlenmeyer flask fitted with a cotton plug according to the assigned bacteria and sterilize for 25 minutes:

PpC1 (TOL) | | m-toluate⁵

PaW630 (RP1:: TOL) | | Glut¹⁰ + m-xylene

E.coli JM101 (pBK189) - *xyITE* | | Glycerol

E.coli JM101 (pBK189) - *xyITE* | | LB + IPTG²⁵ μ g/ml

E.coli DP2229 (pBK489) | | LB + IPTG²⁵ μ g/ml

2. The day before harvesting the cells, inoculate flasks with 6ml of each respective

organism grown as a starter culture (to be provided) on glutamate only. Allow the organisms to incubate at 30°C overnight.

3. Just before harvesting, streak the bacterial culture to LA to check its purity.
4. Pour the contents of the culture into a centrifuge tube and spin cells in a Sorvall GSA (or GS3) rotor. Be sure to balance tubes against each other. Spin at 8,000 x g for 12 minutes.
5. Resuspend pellet in 30ml phosphate buffer in a tared 50ml centrifuge tube. Centrifuge the cells at 4,000 x g for 15 min. and weigh the wet pellet. Record the weight.
6. Resuspend the wet pellet in 2X volume of phosphate buffer and keep it on ice.
7. The suspension will then be passed through a French Press at 20,000 x psi and keep it on ice.
8. Add 1ml of commercial DNase to your sample. Allow the sample to stand at room temperature for 8 minutes.
9. Balance tubes and centrifuge in a SS34 (or SA600) rotor for 25 min. at 14,500 rpm.
10. Save the supernatant as crude extract.
11. Set up two cuvettes for the enzyme assays according to the following: (One cuvette will serve as a control while the other, the experimental, will be used to record the actual enzymatic reaction.)

For catechol-2,3-oxygenase (C23O) assay:

<u>Reagent</u>	<u>Expt.</u>	<u>Control</u>	<u>mol/rxn.mix</u>
0.05 M KH_2PO_4 (pH 7.5)	0.97	0.97	145
0.01 M Catechol	0.033	-	0.33
H_2O	-	0.033	
Crude Extract	5 μl	5 μl	

- (i) Scan the catechol spectrum.
- (ii) Add enzyme to both cuvettes (continue the first and then start monitoring immediately after addition to the experimental cuvette).
- (iii) Watch the appearance of an absorption band at 375nm (yellow)
- (iv) Scan the absorption spectrum of the reaction product (2-hydroxymuconic semialdehyde [20HMS]).
- (v) Activities calculated from ϵ for 20HMS of $33,000 \text{ M}^{-1} \text{ cm}^{-1}$.

Diol Dehydrogenase (DHCDH)

<u>Reagent</u>	<u>Expt.</u>	<u>Control</u>	<u>$\mu\text{mol/rxn.mix}$</u>
Tris buffer-100 mM (pH 8.0)	0.5	0.5	50
NAD^+ -10 mM	0.2	0.2	2
Crude diol prep (~5 mM)	0.1	-	~0.4
H_2O	0.2	0.3	
Crude Extract	10 μl	10 μl	

- (i) Scan the spectrum of the diol before adding NAD^+ to both cuvettes.
- (ii) Add enzyme (first to the control and then to the experimental).
- (iii) Follow the formation of NADH at 340nm.
- (iv) Scan the spectrum of NADH at 340nm.
- (v) Activities calculated from ϵ value of NADH of $6,220 \text{ M}^{-1} \text{ cm}^{-1}$.

CHAPTER 45

FRACTIONATION OF CATECHOL-2, 3-OXYGENASE

Purpose: Various methods exist for the fractionation and purification of proteins that are produced by bacterial cells. The method used depends on the protein of interest. In this experiment, an attempt to fractionate the proteins containing catechol-2,3-oxygenase activity will be made from crude cell lysates. The extent of fractionation will then be ascertained by gel electrophoresis.

Materials:

Cell extracts

DEAE-Sephacel

CM-Sephadex

Microfuge tubes

Water bath (55°C)

Pipettors

Micropipettors

Procedure:

1. Do a heat treatment by taking 1ml of each extract and heat in a water bath at 55°C for 10 minutes.
2. Spin the sample in a microfuge tube at full speed for 2 minutes.

3. Remove the supernatant and place it on ice. Save 200 μ l for C23O assays and protein determinations.
4. Do the C23O assay and dilute it 50-fold.
5. Place 0.3ml of supernatant on a column packed with DEAE-sephacel (anion-exchanger).
6. Place the loaded columns into adapters fitted for the SS-34 rotor and centrifuge the samples at low speed (2,000 x g) for 12 minutes.
7. Collect the eluate (~0.8ml), from each column.
8. Assay and determine the protein content of each fractionated sample.

CHAPTER 46

PLASMID PURIFICATION

Purpose: The purpose of this experiment is to isolate plasmids from two different bacterial strains using a high concentration of salts and to perform many centrifugations to get rid of everything but the plasmid DNA. A variety of techniques have been described for the isolation of plasmids from bacteria. Most of these take into account the differences in density between the plasmid and chromosomal DNA. Usually, a procedure involving the use of a strong base to denature the chromosomal DNA is incorporated along with steps to remove it through a combination of salt treatment and centrifugation. In this experiment, two different plasmid isolation procedures will be carried out: the isolation of moderately large plasmids from *Pseudomonas* (~100kb) versus the isolation of small plasmids from *E.coli* (~2-20 kb).

Materials:

1 liter *E.coli* cells

1 liter *P.putida* cells

25% sucrose solution (containing 0.05M Tris-HCl)

lysozyme solution (5mg/ml in 0.25M Tris-HCl, pH 8.0)

0.25M EDTA

Triton Detergent solution (2% Triton X-100 in 0.05M Tris-HCl, pH 8.0, 0.0625 EDTA).

CsCl

Ethidium Bromide (5mg/ml in H₂O)

5M NaCl

50% PEG-6000

TES buffer (TES = Tris + EDTA + Salt)

TE buffer (10mM Tris + 1mM EDTA)

Butanol (saturated with equal amounts of TE)

Sterile Falcon™ tubes

Centrifuge tubes

Crimping machine

250ml graduated cylinder

20G hypodermic needles

Sterile test tubes

Pasteur pipettes

Dialysis tubing

Rubber bands

1 liter beaker

Spectrophotometer

Ice Bath

Pipettes

Micropipettes

Goggles

Gloves

Agarose gel electrophoresis solutions and equipment

Procedure:

1. For the plasmid extraction and purification from *E. coli* cells:
 - (a) Harvest one liter of cells at 7,500 x g for 12 minutes.
 - (b) Resuspend cells in 12ml of 25% sucrose solution.
 - (c) Add 1ml lysozyme solution followed by 2ml 0.25 EDTA.
 - (d) Wait 5 minutes and add 8ml Triton Detergent solution.
 - (e) Hold at 0°C for 5-15 minutes.
 - (f) Clarify by centrifugation for 15 minutes at 40,000 x g in a SA600 rotor.
 - (g) Make CsCl gradients by adding the following:
 - 8ml of cleared lysate
 - 8g CsCl
 - 1ml ethidium bromide
 - (h) Transfer to centrifuge tubes. Centrifuge to equilibrium at 40,000 rpm for 40-60 hours in a DuPont Sorval T-1270 rotor.
2. For the *Pseudomonas* plasmid isolation procedure:
 - (a) Harvest cells in a GSA rotor at 7,000 rpm for 20 minutes.
 - (b) Resuspend pellet in 25ml Tris-sucrose per liter culture.
 - (c) Add 5ml of 5mg/ml lysozyme dissolved in 0.25M Tris (pH 8.0).
 - (d) Incubate on ice in the refrigerator for 20 minutes.

- (e) Add 5ml 0.25M EDTA (pH 8.0) at room temperature.
 - (f) Add a mixture of 12.5ml 5M NaCl and 10ml Sarkosyl-DOC at 10°C. Swirl gently while adding this solution. Let it sit for 1 minute.
 - (g) Incubate for 2 hours in an ice bath.
 - (h) Spin for 30 minutes at 27,000 rpm (T647.5 rotor).
 - (i) Pour off liquid supernatant into a 250ml graduated cylinder. Be careful not to dislodge the loosely-adhering gelatinous pellet.
 - (j) Add 0.25 volumes 50% PEG-6000, which will precipitate the DNA.
 - (k) Mix and store in the refrigerator overnight in the cylinder.
 - (l) Spin in the GSA rotor for 10 minutes at 10,500 rpm.
 - (m) Resuspend in 12ml TES buffer and swirl gently.
 - (n) Make CsCl gradients by adding the following:
 - 8ml of cleared lysate
 - 8g CsCl
 - 1ml ethidium bromide (use gloves)
 - (o) Transfer to centrifuge tubes. Centrifuge to equilibrium at 40,000 rpm for 40-60 hours in a DuPont Sorval T-1270 rotor.
3. To collect plasmid bands from CsCl gradients, insert a 20G hypodermic needle in the top of the centrifuge tube. Insert another 20G hypodermic needle directly below the second band in the centrifuge tube. (The second band is the plasmid, which is covalently closed circular DNA). Carefully extract the plasmid DNA only.
4. Transfer the plasmid DNA to a sterile test tube. The remainder of the procedure should

use sterilized equipment.

5. Wash the plasmid DNA by adding approximately an equal amount of butanol saturated with TE. (Ethidium bromide diffuses into the butanol changing the butanol red.) Using a Pasteur pipette, draw off the upper layer containing butanol and ethidium bromide. Continue washing the plasmid DNA in this manner until the upper layer remains clear, indicating that ethidium bromide is no longer present in the plasmid DNA.
6. Place a single layer of dialysis tubing across the top of each of the microfuge tubes. Secure the dialysis tubing with a rubber band. Place a plastic Pasteur pipette against the outside surface of the microfuge tube and secure it to the microfuge tube with the rubber band. Label the Pasteur pipette with the student's name.

Note: The dialysis tubing is permeable to CsCl and impermeable to large macro-molecules (i.e. DNA). Thus, CsCl will move into the TE buffer, and DNA will remain in the microfuge tube.
7. Place the microfuge tubes into a beaker containing a minimum of 1 liter of TE buffer for 24 hours. During the 24 hours, the TE buffer should be changed twice.
8. Aseptically transfer the contents of each microfuge tube to a sterile Falcon™ tube. Freeze Falcon™ tubes to keep the DNA from denaturing.
9. Determine the DNA concentration by reading the O.D. at 260nm.
10. Run a small amount of sample on a gel to determine the extent of contamination by chromosomal DNA.

CHAPTER 47

CONJUGATION

Purpose: The purpose of this experiment is to mate two bacterial strains to see if a plasmid would transfer from one to the other. A conjugation frequency can be calculated. Of the two bacterial strains, one is the donor and the other is the recipient. Dilutions of the bacterial cultures will be made according to the protocol. In this experiment, the conjugation frequency of the RP1::TOL plasmid will be measured under different selective conditions. In addition, the possibility of any genetic transfer in the pBK489 plasmid will be detected. In genetic mating procedures, the objective is to develop a selection procedure that allows plasmid transfer to be observed through quantitating the transfer of phenotypic traits.

Conjugation is a process whereby two cells of different mating types exchange genetic information. This is thought to occur by a physical bridge between the two for which pili are thought to play a role. Some plasmids are conjugative. This means that in addition to carrying traits such as antibiotic resistance or biodegradation, some can even transfer a plasmid between mating pairs. Plasmids may even mobilize a second plasmid if present. Conjugative transfer of plasmids can be measured by plating mating mixtures to selective media following genetic mating.

Materials:**Donor cells:**

PaW630 trp⁻ (RP4::TOL)

Ec DP2229 (pBK489)

PpC14 leu⁻, ileu⁻, val⁻, his⁻ (TOL)

Recipient cells:

Pp 503CA (plasmidless *P. putida*)

Ec JM101

Various media plates:

LA (26)

LA/Km⁵⁰ (3)

LA/Ap⁵⁰ (3)

Glu¹⁰/Km⁵⁰ (12)

Glu¹⁰/Ap⁵⁰ (2)

Glu¹⁰/Ap³⁰ (2)

m-tol⁵ (16)

m-tol⁵/trp⁵⁰ (3)

m-tol⁵/aa (3)

0.85% saline

Hockey stick

Alcohol

Turntable

Flame source

Procedure:

1. Mate the cells by contracting them on agar plates:

<u>Group #</u>	<u>Cultures</u>	<u>Media</u>
1	DP2229 X JM101	1 LA plate/spread
	DP2229	“
	PaW630	“
2	PaW630 X JM101	1 LA plate/spread
	JM101	“
3	PpC14 X 503CA	1 LA plate/spread
	PpC14	“
4	PaW630 X 503CA	1 LA plate/spread
	503CA	“

2. Incubate the plates for 24 hours at 30°C.
3. Add 2.0ml of sterile 0.85% saline to the surface of the Mating Mixture plate. Scrape the cells off and place them into a sterile test tube. Repeat so that the final volume of the cell suspension is 5ml.
4. Repeat separately for the donor and recipient cells alone.
5. Centrifuge the suspension in a desk-top centrifuge.
6. Pour off the supernatant aseptically and resuspend the pellet in 5ml 0.85% saline. The suspensions are ready for dilution and plating.
7. Plate cells to the plates outlined below and incubate all plates at 30°C. The following table summarizes the plates to which mating mixtures (and controls) will be plated:

<u>Group#</u>	<u>Tubes to be Plated</u>	<u>Medium</u>	<u>Dilution Plated</u>
1	Mating Mixture	LA	10^{-7} , 10^{-8}
	DP2229 x JM101	Glu ¹⁰ /Km ⁵⁰	10^{-1} , 10^{-2}
		Glu ¹⁰ /Ap ³⁰	10^{-1} , 10^{-2}
	Donor (DP2229)	LA	10^{-7} , 10^{-8}
		LA/Km ⁵⁰	10^{-6} , 10^{-7} , 10^{-8}
		LA/Ap ⁵⁰	10^{-6} , 10^{-7} , 10^{-8}
		Glu/Km ⁵⁰	10^{-1} , 10^{-7} , 10^{-8}
		Glu/Ap ⁵⁰	10^{-1} , 10^{-2}
	Donor (PaW630)	LA	10^{-7} , 10^{-8}
		Glu ¹⁰ /Km ⁵⁰	10^{-1} , 10^{-2}
		m-tol ⁵	10^{-1} , 10^{-2}
2	Mating Mixture	Glu ¹⁰ /Km ⁵⁰	10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}
	PaW630 x JM101	LA	10^{-7} , 10^{-8}
	Recipient (JM101)	LA	10^{-7} , 10^{-8}
		Glu ¹⁰ /Km ⁵⁰	10^{-1} , 10^{-2}
		Glu ¹⁰	10^{-7} , 10^{-8}
3	Mating Mixture	LA	10^{-7} , 10^{-8}
	PpC14 x 503CA	m-tol ⁵	10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}
	Donor (PpC14)	LA	10^{-7} , 10^{-8}

		m-tol ⁵	10 ⁻¹ , 10 ⁻² , 10 ⁻³
		m-tol ⁵ /aa	10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷
4	Mating Mixture	m-tol ⁵	10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵
	PaW630 x 530CA	LA	10 ⁻⁷ , 10 ⁻⁸
	Recipient (503CA)	LA	10 ⁻⁷ , 10 ⁻⁸
		m-tol ⁵	10 ⁻¹ , 10 ⁻²

8. Count the colonies on the LA plates and calculate the conjugation frequency.

$$\text{Conjugation Frequency} = \frac{\# \text{ transconjugants/ml}}{\# \text{ donor cells/ml}}$$

CHAPTER 48

SDS GEL ELECTROPHORESIS OF PROTEINS

Purpose: The purpose of this experiment is to become familiar with the SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) method of separating proteins by molecular weight. The separation of proteins is almost always carried out in polyacrylamide gels under conditions whereby the native protein is disassociated into its individual subunits or polypeptide chains. This is most usually accomplished with the strongly anionic agent, SDS. Polypeptides will migrate in an electrophoretic field just as will DNA with the migration distance being related to the size of the peptide and independent of its amino acid sequence. Thus, it is possible to estimate the molecular weight of a polypeptide chain by including size standards on the gel.

Materials:

Mini-gel (7 x 8 cm)

12% acrylamide

0.25M Tris (pH 8.8)

0.1% SDS

Solubilizing buffer containing:

150mM Tris buffer (pH 7.8)

150mM dithiothreitol

21% glycerol

0.003% bromphenol blue

6% sodium dodecylsulfate

Double distilled water (ddH₂O)

1cm 4.5% stacking gel

Comassie blue reagent

0.1% Comassie brilliant blue R-250

50% methanol

7% glacial acetic acid

20% methanol

Protein molecular weight standards (daltons):

Myosin (H-chain)	200,000
β-galactosidase	116,250
Phosphorylase b	97,400
Bovine Serum Albumin	66,200
Ovalbumin	45,000
Carbonic anhydrase	31,000
Soybean trypsin inhibitor	21,500
Lysozyme	14,400

Procedure:

1. A mini-gel is poured (12% acrylamide in Tris 0.25M [pH 8.8] containing 0.1% SDS).
2. After the gel has solidified, a stacking gel is poured onto the resolving gel containing 10

wells for the addition of samples. (This is called discontinuous SDS-PAGE).

3. Protein samples (2-5 μ l) are denatured by heating at 95°C for 3 minutes in 3 μ l solubilizing buffer. ddH₂O will be added to the denatured sample (~12 μ l, 20 μ g protein), and the sample is applied to a 1cm, 4.5% stacking gel and samples electrophoresed at 4°C.
4. Protein molecular weight standards (denatured) are also added (3ml commercial standards plus 3ml solubilizing buffer plus 6ml ddH₂O).
5. Electrophoresis is carried out at 4°C at 200 volts for 60 minutes.
6. After electrophoresis, the gel will be removed from the casting apparatus, and the protein band will be developed by staining with Coomassie Blue reagent.
7. Stain gel for 30 minutes in 0.1% Coomassie brilliant blue R-250, 50% methanol and 7% glacial acetic acids.
8. Destain overnight in 20% methanol, 7% glacial acetic acid, and 3% glycerol.

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