

## Exercise 14

# USE OF SELECTIVE & DIFFERENTIAL MEDIA & REPLICA PLATING TECHNIQUES

### Introduction

**Heterotrophic** organisms require preformed organic compounds for their sources of carbon, while **autotrophic** forms can synthesize all or nearly all of their essential organic compounds from inorganic materials. Organisms that use dead, decaying organic materials are called **saprotrophs**, while those using living materials are likely to be **parasites**; obligate **intracellular parasites** are referred to as **hypotrophs**. Most of the microorganisms worked with in this microbiology class are chemoheterotrophs and can be cultured on various mixtures of nutritive materials. Such mixtures are called **media** (singular **medium**) and have long been an integral part of microbiology. Some important ingredients of media include, carbon and energy sources, nitrogen sources, vitamins, growth factors, and essential minerals. Culture media must be adjusted to a suitable pH, and have osmotic pressures compatible with bacterial cells (usually isotonic). Various types of media can be categorized as follows:

- Complex media** = Media containing the nutrients required for microbial growth, but in crude form, i.e., the various components and their exact quantities are not known (e.g., Nutrient agar, Brain Heart Infusion, Tryptic Soy agar, etc.).
- Defined media** = Media providing required nutrients in relatively pure chemical form and in specified concentrations (e.g., *Pseudomonas* and *Azotobacter* enrichment media).
- Enriched media** = Media containing cells, tissue or tissue fractions (e.g., Blood agar and Chocolate agar).
- Differential media** = Media supporting the growth of various types of microorganisms while providing an environment that makes it easy to distinguish various different forms (e.g., EMB, MAC, T-7, MSA, etc.).
- Selective media** = Media providing a means of isolating a particular species or type or microorganisms, while inhibiting the growth of others (e.g., EMB, MAC, T-7, MSA, etc.).

### Some Examples of Selective and Differential Media:

#### Mannitol Salt Agar (MSA)

Mannitol Salt Agar (MSA) is a selective medium for the isolation of pathogenic and non-pathogenic Staphylococci. Growth of most other organisms is inhibited by a high salt content (7.5% NaCl). MSA also acts as a differential medium for the differentiation of organisms that can ferment mannitol from those that cannot. *Staphylococcus aureus* colonies grow readily and are surrounded by yellow zones (can ferment mannitol), while non-pathogenic staphylococci such as *Staphylococcus epidermidis* produce smaller colonies surrounded by red or pink zones (cannot ferment mannitol). The color differences visible in MSA are due to the presence of **phenol red**, a pH indicator. Acid produced by mannitol fermenting bacteria will cause the color of the phenol red indicator to change from red (neutral pH) to yellow (acidic = low pH).

## MacConkey's Agar (MAC)

MacConkey's agar (MAC) is a medium that tends to select for the growth of Gram-negative organisms while inhibiting the growth of Gram-positive forms. At the same time, it is a differential medium that allows for the differentiation of organisms that can ferment lactose from those that cannot. MacConkey's Agar is recommended for use in the detection and isolation of all types of dysentery, typhoid, and paratyphoid bacteria (*Salmonella*). It promotes the development of these organisms and at the same time differentiates them from lactose-fermenting Gram-negative bacilli such as *Escherichia coli*. Isolated colonies of lactose fermenting bacteria (coliforms) are opaque pink in color, while *Salmonella* colonies are uncolored and translucent. Differentiation is most clear with well-isolated colonies. The pH indicator present is **neutral red**, and accumulates within and sometimes around colonies able to ferment lactose.

**Note** – MAC sorbitol agar (MacConkey's agar made with sorbitol instead of lactose) may be used to differentiate *E. coli* strain O157: H7 (Enterohemorrhagic *E. coli*) from other strains because they cannot ferment sorbitol.

## Eosin Methylene Blue Agar (EMB)

Eosin Methylene Blue (EMB) is a medium that tends to select for the growth of Gram-negative rods (bacilli). At the same time it provides a distinct differentiation between lactose fermenting colonies, and those that cannot ferment lactose. Lactose fermenting colonies appear dark (sometimes having a metallic green sheen) or have dark centers with light peripheries, while non-fermenting forms remain uncolored and nearly transparent. Fermentative colonies lower the pH, absorb the dyes present, and then refract light causing **iridescent color** (the metallic green sheen).

## Tergitol 7 Agar (T-7)

Tergitol 7 agar is a selective medium for *Escherichia coli* and other members of the coliform group. This medium contains the pH indicator **Bromothymol blue**, and permits the unrestricted growth of all coliform bacteria while inhibiting Gram-negative spore-formers and Gram-positive organisms. *E. coli* grown on Tergitol 7 produces yellow colonies surrounded by yellow zones, *Enterobacter* produce large mucoid colonies usually surrounded by yellow zones, and *Salmonella*, *Proteus*, and other non-lactose fermenters produce clear (transparent) colonies usually surrounded by blue zones. Bromothymol blue is green when the pH is neutral, turns yellow in acid and blue in alkaline environments.

## Hektoen Enteric Agar (HEK)

Hektoen Enteric agar is a selective and differential medium used for the isolation and differentiation of *Salmonella* and *Shigella* from other Gram-negative enterics. This medium contains the pH indicators Bromthymol blue and acid fuchsin. The presence of bile salts and indicators inhibits the growth of most Gram-positive organisms, while thiosulfate and ferric ammonium citrate cause H<sub>2</sub>S producing colonies to form black precipitate. Coliforms such as *E. coli*, that can rapidly ferment the carbohydrates present (lactose, saccharose and salicin), will form salmon pink to orange, opaque colonies surrounded by bile precipitate. *Shigella* species form semi-transparent green colonies and *Salmonella* (as well as some *Proteus*) species form green colonies with black centers.

## Xylose Lysine Desoxycholate Agar (XLD)

Xylose Lysine Desoxycholate agar is a selective plating medium designed for the direct isolation of *Shigella* and *Providencia* species from stool samples. The sodium desoxycholate present inhibits the growth of Gram-positive organisms and swarming of *Proteus* species. The pH indicator is phenol red. *Shigella* and *Providencia* colonies that ferment xylose slowly if at all will form semi-transparent red colonies. *Salmonella* colonies are also red, usually with black centers. Organisms such as *Proteus*, *Enterobacter* and *E. coli* form yellow opaque colonies with or without black centers.

### Some Examples of Enriched Media:

#### Tryptose Blood Agar Base with Sheep Blood (Blood Agar)

Blood agar is an enriched medium that supports the growth of fastidious organisms (such as Streptococci) and allows for differentiation on the basis of **hemolysis reactions**. Organisms that can cause the complete hemolysis of RBCs will form colonies surrounded by clear zones. This is referred to as **beta-hemolysis ( $\beta$ -hemolysis)**. Organisms that can convert hemoglobin to methemoglobin will form colonies surrounded by greenish zones. In this case the RBCs are not actually lysed, but the alteration in coloration is referred to as **alpha-hemolysis ( $\alpha$ -hemolysis)**. When colonies growing on blood agar cause no apparent change in the medium, they may be said to display **gamma-hemolysis ( $\gamma$ -hemolysis)** or no hemolysis reaction. This classification of hemolytic patterns is used primarily with Streptococci, but is sometimes applied to other genera.

*Staphylococcus aureus* produces toxins called **hemolysins** that cause a distinct pattern of hemolysis on blood agar. The  **$\alpha$ -hemolysin** of *S. aureus* causes complete hemolysis within 24 hours, while a second toxin known as  **$\beta$ -hemolysin** causes only partial hemolysis. If the culture is kept at a cold temperature (in a refrigerator) for another 24 hours, the area of partial hemolysis will become complete. This feature is sometimes referred to as **hot-cold lysis** and is sometimes useful in the diagnosis of staphylococcal infections.

The type of animal blood used in the preparation of blood agar may cause variation in the hemolysis reactions expected as can the presence of more than one organism. For example: group D Streptococci (enterococci) are  $\beta$ -hemolytic on horse and rabbit blood agar but  $\alpha$ -hemolytic on sheep blood. Certain species of *Streptococcus* and *Listeria* are only weakly hemolytic alone, but when grown in the presence of *Staphylococcus aureus* or its  $\beta$ -hemolysin will show enhanced hemolytic ability. This phenomenon is known as the **CAMP reaction** (named for the investigators who described it) and is sometimes used as a diagnostic test (the **CAMP test**).

#### Chocolate Agar Enriched (Chocolate Agar)

Chocolate agar enriched is a medium used in the isolation and identification of fastidious organisms that grow poorly or not at all on media made with whole blood. Disease causing agents such as *Neisseria gonorrhoeae*, *N. meningitidis* and *Haemophilus influenzae* are examples of such organisms. This medium contains hemoglobin as its primary nutrient source and includes a number of supplements that enhance the survival and growth of fastidious microbes. Modifications made to the formulation of chocolate agar have led to the development of additional media increasing the recovery of fastidious microbes.

## Replica Plating Technique

Replica plating is a transfer technique that may be used to produce multiple copies (replicas) of a particular colonial arrangement on a number of agar plates. With this technique, cells from bacterial colonies can be transferred from one plate (a master copy or template) to several new plates, and the relative arrangement of the colonies remains fixed. Replica plating may be used to test the ability of a variety of bacteria to grow on a number of different media. Since many different colonies can be transferred at once, it **saves time and materials**.

For this exercise we will use plastic blocks covered on one side by velvet. The tiny hair-like projections of the velvet readily pick up bacterial cells when the velvet is pressed firmly against bacterial colonies. Cells adhering to the velvet "hairs" are transferred to new agar plates by pressing these firmly against the plastic block (velvet side up). Since only a few cells tend to come off each time the velvet touches a new agar surface, many plates can be inoculated using a single master copy.

### Procedure: (Please work in groups of 2-3 people)

1. Obtain a nutrient agar plate (master or template) that has been inoculated with five different types of bacteria, and sterile plates (one each) of the various media provided. Label each plate with your group names, the date, and the type of medium present.
2. Using aseptic technique, assemble a replica-plating block from the materials provided.
3. Draw a copy of the bacterial colony arrangement present on your master plate in the space provided on the worksheet, and label the individual colonies as specifically as possible.  
**Note** – Your instructor will provide the names corresponding with the bacterial colonies present **after data and results have been obtained**.
4. Remove the lid from your master plate, and press the plate surface firmly (DO NOT MASH THE AGAR) against the replica-plating block (velvet side up). Be careful that you make good contact with each of the colonies present. Then remove the block carefully, and replace the plate lid. Do not allow the velvet surface of the block to touch any foreign surfaces between plates. **Hold it** in one hand (do not put it down on the bench surface).
5. Remove the lid of each sterile agar plate (one at a time) and press the plate onto the velvet surface as you did in step #4 above. Replace each lid as quickly as possible to minimize exposure of the agar surface to air.
6. Place all replica plates (including the master) in a stack, tape them together, and place them in the container provided. The best results will be obtained if these plates are incubated at 37° C for 24 hours. Your instructor will insure the proper incubation time until the next laboratory period.
7. Observe the replica plates and record the appearance of the bacteria colonies growing on each one. Use the information provided about pH indicators and dyes present to determine which bacteria ferment the carbohydrates present and form acidic end products. Colonies unable to ferment will often catabolize the peptone present within these media, resulting in the formation of alkaline end products. These can also influence the color of pH indicators.
8. Compare the last plate in your replica series (nutrient agar) to the replica master plate. What does the appearance of colonies on this plate tell you about the success of your transfer?

**Questions:**

1. What types of bacteria can grow on MacConkey's agar, on EMB, on T-7? Why?
2. What morphological differences can you detect between the colonies growing on MacConkey's agar and EMB? What do these morphological differences indicate?
3. Which media would you use to isolate the following: *Staphylococcus*, *Streptococcus* and *Neisseria*. Why did you choose the media you did?
4. What aspect of blood agar makes it a differential medium?

**NOTES, OBSERVATIONS & ADDITIONAL INFORMATION**

Name \_\_\_\_\_

Lab Section \_\_\_\_\_

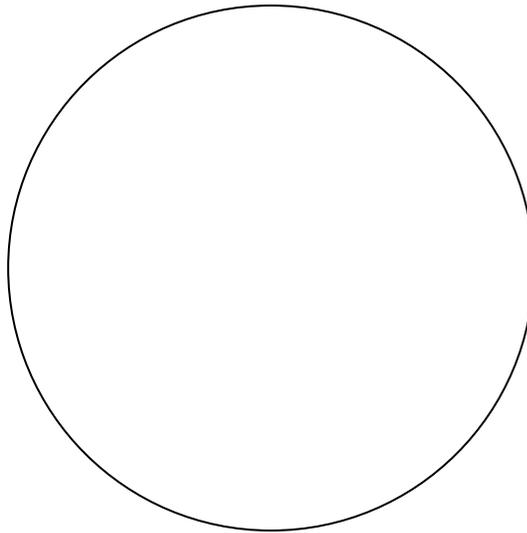
**WORKSHEET**  
**Exercise 14**  
**Replica Plating Techniques**

**Goals:** \_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

**Materials & Methods:**

Replica Plating Technique

**Master plate:** Draw a copy of the bacterial colony arrangement present on your master plate in the space provided below, and label the individual colonies as specifically as possible.



Media used (list IN ORDER of use): \_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

Incubation temperature: \_\_\_\_\_ Duration of incubation: \_\_\_\_\_

Additional Notes: \_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

**Data & Results:**

Complete the following table. Include whether or not the organisms grew on the medium as well as observations of colony and medium color.

Organisms	Type of Medium				

**Conclusions:**

Was the technique successful? \_\_\_\_\_ How do you know? \_\_\_\_\_

Based upon your data, what can you conclude about the following organisms?

*Staphylococcus aureus*: \_\_\_\_\_

*Staphylococcus epidermidis*: \_\_\_\_\_

*Bacillus*: \_\_\_\_\_

*Escherichia coli*: \_\_\_\_\_

*Salmonella*: \_\_\_\_\_