Physiological Tests for Characterization of Bacteria

1. Culture Characteristics

2. Fluid Thioglycolate: Oxygen Requirements

**Purpose:** The use of thioglycolate broth permits growth of anaerobic bacteria. Growth patterns can help distinguish aerotolerance and oxygen requirements of bacteria.

**Media:** Contains glucose, cysteine, and sodium thioglycolate (oxygen reducing agent)

**Reagents/Indicators:** Resazurin

**Mechanism/reactions:** This is a nutritive medium with a reducing agent (sodium thioglycolate) which, due to a chemical reaction, removes oxygen from the broth. A chemical indicator is included in the broth- resazurin. The pinkish color indicates the presence of oxygen.

**Directions:** Deep stab needle inoculation.

**Interpretation:** Aerobic, Microaerophilic, Facultative anaerobe, Anaerobic

3. Novobiocin Sensitivity

**Purpose:** Selective identification of Staphylococci

**Media:** Mueller-Hinton Agar

**Reagents/Indicators:** Novobiocin 30 antibiotic disk

**Mechanism/reactions:** *Staphylococcus epidermidis* is sensitive for Novobiocin, *Staphylococcus saprophyticus* is resistant.

**Directions:** Swab small M-H Agar plate with broth culture. Use individual disk dispenser to place one Novobiocin disk on the center of the plate. Incubate 24-48h. After incubation, measure the zone of inhibition.

**Interpretation:**

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4. EMB Agar

**Purpose:** Selective and differential medium for enteric organisms

**Media:** Eosin, Methylene Blue, lactose, sucrose

**Reagents/Indicators:** Eosin Y and Methylene Blue

**Mechanism/reactions:** Selects for Gram Negative bacteria, and differentiates those enterics which ferment lactose (coliforms) from those which do not ferment lactose (non-coliforms). Indicators form a dark purple precipitate at low pH (due to fermentation products) and also inhibit gram
positive bacteria. E. coli will often produce a green metallic sheen due to strong fermentation and precipitation of acid and indicator complex.

**Directions:** Streak agar in a straight line and incubate for 24 – 48 hours.

**Interpretation:** (+) = Lactose fermentation, dark purple colonies with dark center. Weak fermenters will have pink mucoid growth.

- Green sheen = vigorous fermentation of lactose
- (-) = non-lactose fermenters, colorless growth.

5. **MacConkey Agar**

**Purpose:** Selective identification of Enterobacteriaceae

**Media:** Contains bile salts to inhibit most Gram (+) bacteria except *Enterococcus* and some species of *Staphylococcus*, peptone, and lactose.

**Reagents/Indicators:** Contains crystal violet with inhibits Gram (+) bacteria, neutral red dye which stains microbes fermenting lactose

**Mechanism/reactions:** By utilizing the lactose available in the medium, Lac+ bacteria such as *Escherichia coli*, *Enterobacter* and *Klebsiella* will produce acid, which lowers the pH of the agar below 6.8 and results in the appearance of red/pink colonies. Non-Lactose fermenting bacteria such as *Salmonella*, *Proteus species* and *Shigella* cannot utilize lactose, and will use peptone instead. This forms ammonia, which raises the pH of the agar, and leads to the formation of white/colorless colonies.

**Directions:** Streak agar in a straight line and incubate for 24 – 48 hours.

**Interpretation:**
- (+) = Lactose fermentation, re/pink colonies
- (Slow) = Some organisms ferment lactose slowly or weakly, and are sometimes put in their own category – these include *Serratia* and *Citrobacter*
- (-) = non-lactose fermenters, white/colorless growth

6. **Durham Tube Sugar Fermentations: Dextrose, Lactose, Sucrose**

**Purpose:** To distinguish carbohydrate fermenters from non-fermenters. To detect and distinguish utilization of specific carbohydrates by the products formed.

**Media used:** 0.5% to 1% carbohydrate broth - Dextrose, Lactose, or Sucrose, Peptone, with Phenol Red and an inverted Durham tube for detection of gas.

**Reagents and/or indicators:** Phenol Red

**Mechanism/reaction:** Carbohydrate fermentation results in acid and sometimes gas production causing a pH change and possibly gas being trapped in the Durham tube.

**Directions:** Inoculate tubes and incubate at 35°C for 24 – 48 hours.

**Interpretation:** Observe for color change and gas production.

- Positive (+)= color change from re to yellow, pH < 7.0
- Negative (-)= no color change, pH = or > 7.0
(Note: Color may change to a darker red than an uninoculated tube. This darker color indicates alkaline metabolic products due to the utilization of the peptone instead of the sugar.)

Gas production (+) = bubble trapped in the inverted Durham tube
No gas production (-) = no bubble trapped in the inverted Durham tube

Record results as:
AG = acid with gas production
A = acid, no gas
(-) = negative for acid and gas

7. **Indole Test: Tryptophan Hydrolysis**

**Purpose:** To detect production of tryptophanase

**Media:** Tryptone Agar plates

**Reagents/indicators:** Indole dry slides

**Mechanism/reactions:** Tryptophanase causes the hydrolysis of Tryptophan → indole + pyruvic acid

**Directions:** Using a sterile wooden stick (do not use an inoculating wire) pick a small amount of bacteria from a TA plate and touch an area on one section of the dry slide. Look for the color change to pink/red within about 30 sec. DRY SLIDES CAN BE USED FOR MANY TESTS (4 / SQUARE, 4 SQUARES PER SLIDE. USE UP EACH SLIDE BEFORE OPENING A NEW SLIDE PACKET).

**Interpretation:** (+) = Color change to red within 30 sec.
(-) = No color change to red

8. **Methyl Red Test: Mixed Acid Fermentation**

**Purpose:** To determine mixed acid fermentation (lactic, acetic, formic, etc). Part of the IMViC test (IMViC is the acronym for Indole, Methyl Red, Voges Proskauer, Citrate)

**Media:** MRVP broth--buffered peptone glucose broth used for both MR and VP tests.

**Reagents/indicators:** Methyl Red- red in pH under 4.4, yellow in pH over 6.2, and orange in between

**Mechanism/reactions:** If the organism uses the mixed acid fermentation pathway and produces large amounts of organic acids from glucose, the acids will overcome buffers in the medium and the culture will be acidic.

**Directions:** Broth is inoculated and incubated for 48 hours – 5 days. After incubation, add 5 drops of Methyl Red indicator.

**Interpretation:** (+) = bright red color immediately upon the addition of methyl red (pH < 4.4)
(-) = yellow color (pH > 6.2)
Weak (+) = orange color

9. **Voges Proskauer Test: Butanediol Fermentation**

**Purpose:** To detect the production of acetoin (acetyl methyl carbinol) or 2,3 butanediol (acetoin is the precursor) from glucose broth. Part of the IMViC test.
Media: MRVP broth-- buffered glucose peptone broth used for both MR and VP test.

Reagents/indicators: Barratt’s reagents A- Napthol and B- Potassium Hydroxide (KOH)

Mechanism/reactions: Glucose + diacetyl + KOH + O₂ + arginine → pink color
(Acetoin is oxidized to diacetyl in the presence of KOH)

Directions: Inoculate broth and incubate 48 hours. After incubation add 20 drops of Barratt’s Reagent A (napthol) and 20 drops Barratt’s Reagent B (KOH). Shake well at frequent intervals and allow reaction to develop up to 1 – 2 hours if necessary.

Interpretation: (+) = red layer at top in 10 minutes. (earliest detection), progressing downward. (-) = no red color, disregard any copper or brownish-purple color.

10. Catalase Test

Purpose: Production of catalase.

Media: TSA

Reagents/indicators: 3% Hydrogen Peroxide (H₂O₂)

Mechanism/reactions: Catalase converts hydrogen peroxide, a by-product of oxidative respiration, to oxygen and water. Anaerobes and aerotolerant bacteria lack this enzyme.

Directions: Apply several drops of 3% hydrogen peroxide to growth from a TSA plate.

Interpretation: Vigorous bubbling due to the release of oxygen via catalase.

11. Oxidase Test

Purpose: The oxidase test identifies organisms that produce the enzyme cytochrome oxidase

Media: Use growth from a TSA plate or slant

Reagents/indicators: Oxidase dry slides

Mechanism/reactions: In organisms that use oxygen as the terminal electron acceptor in the electron transport chain, cytochrome oxidase transfers electrons to the oxygen. In the test, the reagent in the dry slide acts as the electron acceptor and changes from yellow to purple when it is oxidized.

Directions: Using a sterile wooden stick (do not use an inoculating wire) pick a small amount of bacteria from a TSA plate or slant and touch an area on one section of the dry slide. Look for the color change to purple within about 30 sec. DRY SLIDES CAN BE USED FOR MANY TESTS (4 / SQUARE, 4 SQUARES PER SLIDE. USE UP EACH SLIDE BEFORE OPENING A NEW SLIDE PACKET).

Interpretation: (+) = Color change to purple within 30 sec.
(-) = No color change, or a change after more than 30 sec.

12. Simmons’s Citrate

Purpose: To determine an organism’s ability to use citrate as the sole source of carbon. Part of the IMVIC test series (Indole, Methyl Red, Voges-Proskauer, Citrate)
Media: Simmon’s Citrate Agar- contains sodium citrate as sole carbon source, mineral salts, and pH indicator Bromthymol blue

Mechanism/reactions: Utilization of citrate leaves a sodium residue, increasing pH of the medium

Directions: Streak slant, cap loosely (this is an aerobic process), incubate 24 – 48 hrs

Interpretation: (+) = medium changes color from green to Prussian blue
(-) = no change, medium remains green
***Caution, Simmon’s Citrate can sometimes give a false positive result. If the media is blue, check for growth on the slant to confirm a positive.

13. Starch Hydrolysis

Purpose: To detect production of amylase

Media: Starch Agar plates (1% starch)

Reagents/indicators: Gram’s Iodine

Mechanism/reactions: When iodine comes in contact with starch it turns blue-black.

Directions: Streak starch agar in a straight line. After incubation add Gram’s iodine, drop wise, sparingly, just to cover growth and surrounding area on medium.

Interpretation: (+) = Colorless zone around colonies where starch has been hydrolyzed by amylases.
(-) = No zone, medium is blue-black immediately adjacent to growth

14. Urease Test: Urea Hydrolysis

Purpose: To detect production of urease

Media: Urea broth

Reagents/indicators: Phenol Red

Mechanism/reactions: Urease hydrolyzes urea to ammonia and carbon dioxide. Ammonia increases the pH of the culture causing the phenol red to go from yellow to bright pink.

Directions: Inoculate urea broth and incubate for 24 – 48 hours.

Interpretation: (+) = Red or bright pink color (pH > 8.4)
(-) = Yellow color (pH < 6)

15. Casein Hydrolysis (Skim milk)

Purpose: To detect the production of casease.

Media: Skim Milk Agar

Mechanism/reactions: Casease proteolyses casein into peptides and amino acids.

Directions: Streak agar in a straight line and incubate for 24 – 48 hours.
Interpretation:  (+) = Clear zone around growth indicating casein hydrolysis via casease
(-) = No clear zone.

16. Gelatin Liquefaction:
Purpose: To determine the production of gelatinase.

Media: Nutrient Gelatin Deep (12- 15% gelatin)

Mechanism/reactions: Gelatinase causes the breakdown and liquefaction of gelatin→polypeptides→amino acids.

Directions: Deep stab inoculation, ⅔ of the way down the center of the tube. After incubation, refrigerate for 1 hour before reading.

Interpretation:
(+): liquefaction (after refrigeration)
(-): gels when refrigerated, no liquefaction

This test does not detect weak positives and concentration of gelatin used may inhibit growth of some organisms.

17. Triple Sugar Iron Agar

Purpose: The differentiation of Enterobacteriaceae by their ability to ferment glucose, lactose, and sucrose, and produce gas and/or hydrogen sulfide.

Media: TSI contains ferrous sulfate, phenol red, glucose, lactose, sucrose.

Mechanism/reactions:
Fermentation:
Phenol red turns yellow in an acid environment.
If glucose is fermented, the butt of the medium turns yellow and the slant remains red.
If either lactose or sucrose is fermented the butt and slant both turn yellow.
Gas: Gas from fermentation may appear as breaks or cracks in the medium.
Hydrogen Sulfide: A few bacteria are capable of reducing the SO₄²⁻ to H₂S (hydrogen sulfide). The iron combines with the H₂S to form FeS (ferrous sulfide) a black compound. This will turn the butt black. Thus, a black butt indicates H₂S production.

Directions: Dual inoculation with a needle: streak surface of agar slant, then stab, incubate for 24 – 48 hours.

Interpretation: Slant/Butt, Gas, H₂S
ALK/A: (+) Dextrose = Alkaline slant (red) over acid butt (yellow)
A/A: (+) Lactose or Sucrose= Acid slant over acid butt
ALK/ALK: No change, alkaline slant over alkaline butt
G: Gas production
H₂S (+): = Hydrogen Sulfide production = black (produced in acid environment)