

Unknown Assignment Background: Tests for Biochemical Activities and Creating a Dichotomous Key

There are many different ways to identify an unknown bacterium. Bacterial identity is frequently analyzed now using genotypic approaches - often by carrying out 16S rRNA gene sequencing. However, phenotypic approaches that assess the cell shape, gram stain reaction and biochemical activities of a bacterium can also be used for identification. In this virtual assignment you will be using phenotypic approaches to identify 2 unknown bacteria that are present in a virtual mix. With the images provided you will be able to analyze cell shape and gram stain reaction of your two different bacteria. You will also be able to analyze the results of a number of differential tests that look at the metabolic requirements and enzyme production (the biochemical activity) of your two unknowns. The tests for biochemical activity we will be looking at are the **Triple Sugar Iron test**, **carbohydrate fermentation test**, the **IMViC series of tests**, the **catalase test**, the **coagulase test** and the **DNAse test**. How these tests work and some typical results for some common bacteria are described below. There is also a brief description of creating a dichotomous key for identifying microbes using phenotypic approaches (shape, gram stain reaction, results of biochemical activity tests). You will be creating such a key for identifying your unknowns as part of this exercise.

Tests for Biochemical Activities

1. Triple Sugar Iron (TSI) Test

TSI agar is a differential media that can be used to differentiate bacteria based on their ability to ferment glucose, sucrose and lactose as well as produce hydrogen sulfide and gas. It is most often used to help identify **gram-negative rods in the family Enterobacteriaceae** (often referred to as enteric bacteria) based on their abilities to ferment the sugars glucose, lactose and sucrose. Fermentation is carried out by chemoorganoheterotrophic microbes (microbes that use organic carbon as their carbon, electron and energy source) in order to generate ATP, the energy currency of the cell. Instead of using an electron transport chain and exogenous electron acceptors such as oxygen, an endogenous organic molecule such as pyruvate is used. This acceptor is typically an intermediate of the catabolic pathways used to oxidize the organic energy source. The ability of microbes to ferment different carbohydrates and the type of products formed after fermentation can be useful in the identification of microbes. For the TSI test, the medium contains a small amount of glucose (0.1%), abundant amounts of lactose and sucrose (1%), as well as ferrous sulfate (this is the iron part of the TSI) and the phenol red pH indicator. It is typically prepared as a slant, which is inoculated by first streaking the slant and then stabbing to the bottom of the tube (referred to as the butt). If the organism can ferment glucose as well as 1 or both of the sugars lactose and sucrose, it will produce abundant acidic byproducts that will cause the pH indicator to change from red to yellow throughout the medium (both the slant and the butt, this is referred to as an acid (A) slant and butt. If an organism can only ferment the small amount of glucose in the medium only a small amount of acidic byproducts will be produced and only the butt will turn yellow (A) while the slant will remain red (alkaline; K). If an organism is capable of reducing the sulfate in the TSI media, hydrogen sulfide gas will be produced which will react with the iron in the media to form a black precipitate of ferrous sulfide in the butt. If gas is

produced during the fermentation of the 3 sugars, it can be visualized as cracks (lifting of the agar) or gas bubbles in the agar.

Alcaligenes faecalis is a gram-negative rod that is an obligate aerobe (must use oxygen as its terminal electron acceptor) and does not have a fermentative metabolism. A K butt, K slant, no H₂S and no gas is expected. *Escherichia coli*, *Enterobacter aerogenes*, *Shigella flexneri* and *Proteus hauseri* are all facultatively anaerobic (prefer oxygen but will use fermentation or anaerobic respiration if oxygen is not present) gram-negative rods. *E.coli* and *E. aerogenes* can both ferment glucose and lactose and are expected to produce an A slant, A butt, no H₂S and gas. In contrast *S. flexneri* and *P.hauseri* only ferment glucose, and are expected to both produce a K slant and an A butt and no gas. *P. hauseri* however generates hydrogen sulfide gas, and therefore will cause blackening of the butt as well.

2. Carbohydrate fermentation tests

Another way to determine which sugars a bacterium is able to ferment is to simply perform individual carbohydrate fermentation tests. For Virtual Lab #2, glucose, lactose and sucrose fermentation tests were performed. In these tests the medium contains just the one type of sugar as well as the pH indicator phenol red. Microbes able to ferment the sugar in the medium will produce organic acids. These acid byproducts of fermentation will be released into the medium and will lower its pH, resulting in the phenol red undergoing a colour change from red to yellow. CO₂ gas is produced during some carbohydrate fermentation pathways. Gases produced during fermentation can be detected by using a small, inverted tube, called a Durham tube within the liquid culture medium. Gas produced during carbohydrate fermentation will collect in the tube, displacing liquid in the tube and producing an easily visible bubble. Note that the individual carbohydrate fermentation tests can be used to support the results of the TSI test, and vice versa. You may find that it is easier to see the results of the carbohydrate fermentation tests in the images provided.

3. IMViC Tests

The IMViC series of tests (Indole, Methyl Red, Voges-Proskauer and Citrate) are, like the TSI test, most commonly used to differentiate enteric bacteria. Each test in this series is described below.

a. Indole Test

The indole test is used to determine whether a bacterial species can convert the amino acid tryptophan into indole. Some bacteria have the ability to hydrolyze tryptophan using an enzyme called tryptophanase. Indole, pyruvic acid and ammonium are byproducts of this reaction. Production of indole can be detected using Kovac's reagent. This reagent contains hydrochloric acid, p-dimethylaminobenzaldehyde (DMABA) and n-amyl alcohol. If indole is present it reacts with DMABA to form a red compound (rosindole dye). This test is carried out using SIM media, a semi-solid agar differential medium that also can test for sulfur reduction (black precipitate indicates sulfur reduction) and motility (turbidity throughout the semi-solid agar in the tube indicates motility). In the Indole Test, when Kovac's reagent is added to the surface of a SIM deep inoculated with bacteria, a bright red ring on the surface of the agar is a positive indole

reaction (a negative reaction will give a brownish ring). This indicates that the bacteria express tryptophanase and can hydrolyze tryptophan to form indole.

b. Methyl Red (MR) Test

All enteric bacteria catabolize glucose for their energy needs, however the metabolic pathways they utilize to do so are different. In the methyl red test, the degree of acidity of the end products of fermentation are assessed. Following growth in a supportive media, the pH indicator methyl red is added to the culture. This pH indicator is red in acidic conditions under pH 4.4 and yellow at a pH above 6.2. Mixed acid fermenters produce a mixture of fermentation acids and acidify the media resulting in the methyl red indicator turning dark red – this is a positive methyl red test. In contrast butanediol fermenters form butanediol, acetoin and much fewer organic acids resulting in less acidification of the medium and a yellowish-orange colour– this is a negative methyl red test.

c. Voges-Proskauer (VP) Test

Like the methyl red test, the Voges-Proskauer (VP) test differentiates enteric bacteria based on their pattern of glucose metabolism. It identifies bacterial species that are butanediol fermenters, capable of breaking down glucose to 2,3-butanediol. Following growth in a supportive media, Barritt's reagent (40% KOH and a 5% solution of alpha-naphthol in absolute ethanol) is added to the bacterial culture to detect the presence of acetoin. Acetoin is an intermediate in the pathway leading from glucose to 2,3-butanediol. If acetoin is present, a dark red colour will develop when Barritt's reagent is added – this is a positive VP test. Absence of a dark red colour is a negative VP test (some reddish-brown colour is expected due to the colour of the Barritt's reagent).

d. Citrate Test

Simmon's citrate agar is a chemically defined medium used to test for the ability of enteric bacteria to use citrate as their sole carbon source. This agar contains sodium citrate as the carbon source, ammonium as a nitrogen source and the pH indicator bromothymol blue. Following inoculation of a Simmon's citrate agar slant, use of citrate by the bacterium will increase the pH of the medium to above 7.5 which causes the bromothymol blue indicator to change from dark green to bright royal blue – this colour change indicates a positive citrate test. If the media remains dark green, the bacterium is citrate negative.

The expected results for the IMViC series of tests for a number of gram negative, facultatively anaerobic, lactose-fermenting rods is shown in the table below:

	Indole	Methyl Red	Voges-Proskauer	Citrate
<i>E. aerogenes</i>	-	-	+	+
<i>E. coli</i>	+	+	-	-
<i>K. oxytoca</i>	+	-	+	+

4. Catalase Test

Catalase is an enzyme that catalyzes the breakdown of hydrogen peroxide (H_2O_2) to oxygen and water. Hydrogen peroxide, as well as the hydroxyl radical and the superoxide radical, is a reactive oxygen species produced when proteins in a cell transfer electrons to oxygen, reducing it. These reactive oxygen species can be very damaging to cells, and therefore organisms exposed to oxygen must produce enzymes, such as superoxide dismutase, catalase and peroxidase, to break them down. The presence or absence of the catalase enzyme can be used to differentiate between groups of bacteria. Catalase production and activity can be detected by adding hydrogen peroxide to a tryptic soy agar slant culture. If catalase enzyme is present and active, H_2O_2 will be broken down to H_2O and O_2 , and extensive oxygen gas bubbles will be seen – this indicates a positive catalase test. The absence of gas bubbles indicates a negative catalase test.

Typically, organisms who are obligate aerobes or facultative anaerobes will express catalase enzymes, while organisms with strictly fermentative metabolisms (such as the gram positive bacterium *Enterococcus faecalis*) will not express catalase.

5. Coagulase test

Coagulases are enzymes produced by some pathogenic gram positive staphylococci species such as *S. aureus* that cause the formation of a fibrin clot around the microbe when it is present in the blood stream. Coagulase does this by triggering the natural cascade of clotting factors already present in the blood. By forming a clot around themselves the bacteria can evade host immune defenses. In the coagulase test, the ability of bacteria to cause a clot to form in rabbit plasma is tested. Plasma is the non-cellular portion of blood that still contains proteins called clotting factors that trigger clotting. Citrate is added to the rabbit plasma to act as an anti-coagulant and prevent spontaneous clotting that would yield false positive results. The clot that forms in a positive coagulase test appears cloudy and stays near the end of the tube when the tube is tipped to the side (as the tubes are in the images in your data set). The coagulase test and the DNase test which follows are differential tests used primarily to distinguish between pathogenic (coagulase positive and DNase positive, such as *S. aureus*) and non-pathogenic (coagulase negative and DNase negative, such as *S. epidermidis*) staphylococci species.

6. DNase test

In addition to coagulase production, most pathogenic strains of staphylococci (such as *S. aureus*) produce a nuclease enzyme called DNase, while non-pathogenic strains (such as *S. epidermidis*) do not. DNase degrades host DNA and increases the pathogenicity of staphylococci, streptococci and some *Serratia* spp. that produce it. To determine whether this enzyme is produced by a microbe, DNase test agar containing intact DNA can be used. If the bacteria inoculated onto the plate produces DNase it will hydrolyze the DNA in the plate into small fragments (oligonucleotides) or single nucleotides around the area of growth. Following addition of 1M HCl an obvious clear zone will be visible around bacteria that produce DNase while the rest of the DNase agar will turn opaque. This is due to the fact that the intact DNA is not soluble in the acid and will form cloudy looking precipitates, while the hydrolyzed DNA is soluble in the acid and will not form any precipitate. Note: the results of the DNase test can be used to support the coagulase test results.

Using a dichotomous key to determine bacterial identity

A dichotomous key can be used in biology to identify and classify an unknown organism. It presents the user with a series of characteristics that describe the possible organisms the unknown organism might be, starting with more general characteristics which may be shared by multiple organisms and moving towards more specific characteristics until only one choice of organism remains and the identity can be determined. Typically, there are 2 choices at each level in a dichotomous key, although this can vary. Prepare your own dichotomous key (using gram reaction, shape and the tests described above) that allows you to determine the identity of each of the potential bacteria in your unknown mix. A (very simple) example is shown below that could be used to identify a human from other living things! The simplest way to start designing your dichotomous key is to start with dividing your potential unknown bacteria into gram negative and gram positive, and then work from there. Don't forget to include cell shape.

