

**Isolation, Characterization, and Identification of a Single Bacterial
Isolate from Wild Samples**

Anastasia Ignashkina

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PID: 6196542

Introduction

In science, it is important to know how to properly isolate and identify the bacterial sample because it allows us to better understand the structure and functions of a known organism, or to discover a new one. In this experiment, an unknown sample was taken from the human environment. After morphology examination and gram staining procedures, it was determined that the unknown is gram-positive cocci since the purple round shape cultures were observed under the microscope during the first examination. The culture was taken from the wild environment, subjected to a dilution gradient, and incubated on agar growth media for a week. Later, the plate was evaluated by cellular and colonial morphology, Gram staining results, colony transparency or opacity, colony size, and biochemical reactions in growth media. After that, new isolation strikes were performed to confirm one single species colony was morphologically uniformed to perform biochemical tests later. It is crucial to perform such tests since there is a large amount of different bacterial species that need to be narrowed. In this lab, biochemical tests included gelatin hydrolysis, Kligler's Triple Sugar Iron Agar, OF Medium, Litmus Milk, Starch Hydrolysis, Urease, Nitrate Reduction, Catalase, and Oxidase Tests, Methyl Red Test, and Voges-Proskauer Test. (Dr. Richardson, Dr. Makemson (2015)), Those are basic biochemical tests, required for the bacteria species determination. However, to achieve more accurate results more advanced biochemical tests must

be performed. In the given experiment, those tests included selective/differential media and API20 tests. Mannitol Salt Agar, Eosin Methylene Blue Agar, Blood Agar, and MacConkey Agar were used to test the ability of bacteria to reproduce and to show enzymatic activities in different environments (Dr. Richardson, Dr. Makemson (2015)). The final results were determined and, later on, confirmed by the API20 test and Bergey's Manual.

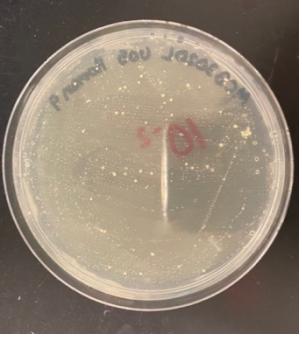
Results

The first step of the experiment was the isolation and inculcation of a sample.

During this stage, the main goal is to find a pure isolated colony for further isolation. It is crucial to do all procedures carefully and pay attention to every single detail because if the first mistakes occur at this step, the entire experiment can be ruined.

After the first growth appeared, they were analyzed by colony basic morphology: size, shape, color, margins, elevation, surface, and opacity (Dr. Richardson, Dr. Makemson (2015)). Additionally, the number of unique colonies was recorded. A colony is considered unique if it is growing alone, away from the other colonies. This number is important for the viable titer. This method allows for identifying the number of living cells in a sample. However, it is important to keep in mind that in the CFU, or colony-forming units, the number must be countable: there must be at least 20 colonies on the plate but less than 200.

Table 1: A population survey of the environmental sample

Environment	Picture	Description	Dilution Factor	Viable Titer
Human		<p>Population Survey <i>Unique colonies: >200</i></p> <p>Colony Morphology: <i>Size: small, medium Shape: circular, punctiform Color: white, creamy-white, yellow Margins: even Elevation: flat Surface: wrinkle Opacity: opaque Other: many colonies, some are spaced out, some are not</i></p>	10^{-2}	CFU: N/A Viable Titer: $\frac{CFU}{mL} \times DF$ <i>Calculations.</i> <i>Not available because there are too many CFU</i>
		<p>Population Survey <i>Unique colonies: 54</i></p> <p>Colony Morphology: <i>Size: small Shape: circular Color: white Margins: even Elevation: flat Surface: smooth Opacity: transparent Other: Most of the colonies are spaced out</i></p>	10^{-4}	CFU: 54 Viable Titer: $\frac{CFU}{mL} \times DF$ <i>Calculations</i> $\frac{54}{0.1} \times 10^{-4} = 5.4 \times 10^{-2}$
		<p>Population Survey <i>Unique colonies: -- 12</i></p> <p>Colony Morphology: <i>Size: small, medium, large Shape: circular, irregular Color: white, creamy-white Margins: even, wavy Elevation: flat Surface: smooth, dull Opacity: opaque Other: colonies differ from each other</i></p>	10^{-6}	CFU: 12 Viable Titer: $\frac{CFU}{mL} \times DF$ <i>Calculations</i> $\frac{12}{0.1} \times 10^{-6} = 1.2 \times 10^{-3}$



*As the dilution factor gets smaller,
the number of unique colonies decreases.*

Table 2: Gram Stains characterization of chosen colonies

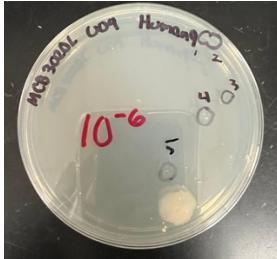
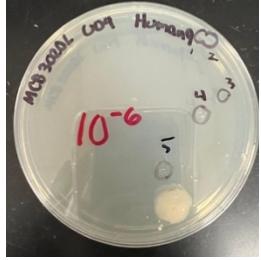
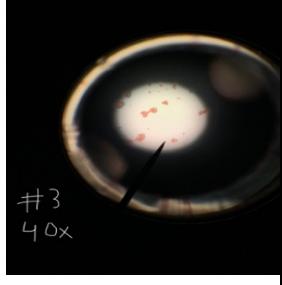
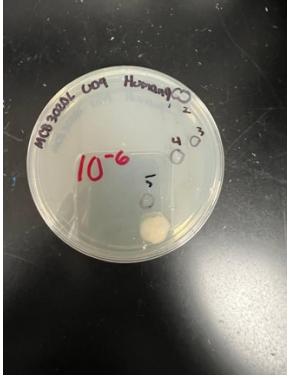
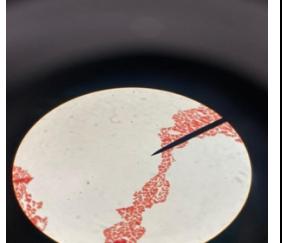
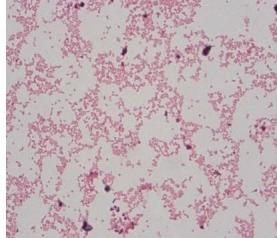
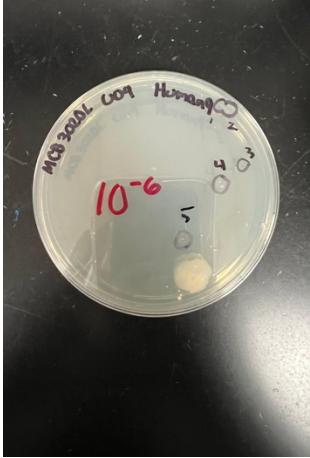
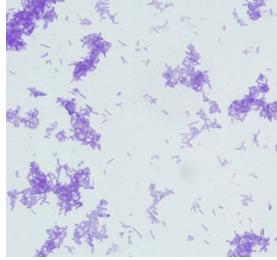
Isolated Colony	Dilution	Picture	Description	Picture	Description
Section: U05 Group: 9 Colony #1	10^{-6}		Colony Morphology: <i>Size: small</i> <i>Shape: circular</i> <i>Color: White</i> <i>Margins: even</i> <i>Elevation: flat</i> <i>Surface: smooth</i> <i>Opacity: opaque</i> <i>Other: Close to colony #2</i>		Cellular Morphology: <i>Size: medium</i> <i>Shape: rhizoid</i> <i>Arrangement: some are circular, some are more linear</i> <i>Color: purple</i> <i>Staining characteristics: gram-positive</i> <i>Other: not a good quality a picture</i>
Colony #2	10^{-6}		Colony Morphology: <i>Size: small</i> <i>Shape: circular</i> <i>Color: white</i> <i>Margins: even</i> <i>Elevation: flat</i> <i>Surface: smooth</i> <i>Opacity: opaque</i> <i>Other: close to colony #1</i>		Cellular Morphology: <i>Size: small</i> <i>Shape: punctiform</i> <i>Arrangement: close to each other</i> <i>Color: purple</i> <i>Staining characteristics: Gram-positive</i> <i>Other: Some pink dots are present</i>

Table 3: Other characterized colonies

Isolated Colony	Dilution	Picture	Description	Picture	Description
Colony #3	10^{-6}		<p>Colony Morphology:</p> <p><i>Size: medium</i> <i>Shape: punctiform</i> <i>Color: white</i> <i>Margins: wavy</i> <i>Elevation: flat</i> <i>Surface: smooth</i> <i>Opacity: opaque</i> <i>Other: 3 colonies close to each other</i></p>		<p>Cellular Morphology:</p> <p><i>Size: small</i> <i>Shape: punctiform</i> <i>Arrangement: spread out</i> <i>Color: pink</i> <i>Staining Characteristics: gram-negative</i> <i>Other: colonies are together, but spread out.</i> <i>The potential error could be excess amount of the dye. Thus, not the actual result but color of the dye was observed</i></p>
Colony #4	10^{-6}		<p>Colony Morphology:</p> <p><i>Size: medium</i> <i>Shape: irregular</i> <i>Color: creamy white</i> <i>Margins: wavy</i> <i>Elevation: flat</i> <i>Surface: smooth</i> <i>Opacity: opaque</i> <i>Other: 2 colonies close to each other</i></p>		<p>Cellular Morphology:</p> <p><i>Size: small</i> <i>Shape: circular</i> <i>Arrangement: some are close to each other, so are far</i> <i>Color: pink</i> <i>Staining Characteristics: gram-negative</i> <i>The possible error could be initially contaminated</i></p>

					slide, since the box with the rest of the slides were open all the time, slides could get contaminated, and, thus, dust was taken as a bacterial colony
Colony #5	10^{-6}		<p>Colony Morphology:</p> <p>Size: medium Shape: punctiform Color: creamy-white Margins: wavy Elevation: flat Surface: smooth Opacity: opaque Other: several colonies next to each other</p>	 	<p>Cellular Morphology:</p> <p>Size: small Shape: punctiform Arrangement: spread out Color: purple Staining Characteristics: gram-positive Other: not too many colonies The error could happen when the slide was passed over the flame for too long, and some of the colonies could burn</p>

Once the first results were recorded, and the pure colony was selected, new isolation streaks were performed on this bacterium. This is necessary for obtaining a “pure” culture as many different colonies can grow on the same plate (Dr. Richardson, Dr. Makemson (2015)).

After new streaks were performed and the growth appeared, the sample was taken for the gram stain analysis. Gram test allows for identifying the cell morphology of a bacterium. The bacteria can be either gram-positive or gram-negative. For the gram-positive result, the cell appears purple, while gram-negative bacteria show pink color. There are several aspects by which gram-positive and gram-negative bacteria differ, but the main difference is the thickness of a peptidoglycan barrier. Gram-positive bacteria have a thick peptidoglycan barrier, and for gram-negative it's thin (Tsang, J. (2022, February 20)).

Isolation and Gram Stain.

Sample	Picture	Description
Chosen nutrient agar plate with an isolated streak		<p>Colony Morphology:</p> <p><i>Size: small</i> <i>Shape: circular</i> <i>Color: creamy-white</i> <i>Margins: even</i> <i>Elevation: flat</i> <i>Surface: smooth</i> <i>Opacity: opaque</i> <i>Other: colonies are well-visible</i></p> <p><i>This colony matches with the previous one from the previous isolation streak because, compared with the first streak, no new growth appeared, which tells us that colonies do match. However, some new growing did appear, but, after visual examination, no differences between the previous and new colonies were detected, which serves as additional proof of the colony's matchings.</i></p>

		<p><i>This colony is pure because, after visible examination, the colonies looked the same, and the plate is not contaminated. Additionally, all procedures were performed accurately as the manual stated. Moreover, the tools were well sterilized every time, which reduces the risk of getting impurities. Thus, based on the evidence listed above, it can be concluded that the culture is pure so far, or, at least, no impurities are noticed at the moment. However, so many people were using the plate for their second streak, that it could get contaminated, and the growth will appear later.</i></p>
Gram stain	40x objective	<p><u>Cellular Morphology:</u> <i>Size: small Shape: circular Arrangement: colonies are grouped Color: purple Staining characteristics: gram-positive Other: coccus</i></p> <p><i>Some potential sources of error could affect the experiment: To begin with, since many people were using our sample, it could get contaminated after new streaks were made. The next source of error could be a lack of staining experience. First, the crystal violet was on slide not for 1 minute but 1 minute 3 seconds, and those 3 seconds could affect the result. For example, the result is gram-positive, which is purple. However, it could turn purple not because of the true result but because of the excess dye.</i></p>
	100x objective	<p><i>The 40x and 100x pictures match because the pictures show a purple sample, which indicates a gram-positive result. However, with 100x magnification, the results can be seen more clearly and tell more details. For example, under 40x magnification, the spherical form of bacteria is not that clear, but under 100x magnification observation, the conclusion was confirmed – the sample is coccus bacteria.</i></p> <p><i>This Gram stain result matches the previous colony (obtained from the isolation streak last week) because comparing both colonies, they do match. This can be proven by the fact that both colonies showed clear purple color after staining, which indicated a gram-positive result.</i></p>

Biochemical Tests

Biochemical tests are crucial in the bacterial identification process. Visible examination of a colony and gram stain can only provide the limited information regarding the unknown bacteria, because many bacteria have similar shapes and morphology characteristics. For example, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Neisseria gonorrhoeae* are all sphere-shaped bacteria. In that case, it is necessary to perform a series of biochemical tests for a more accurate result since the results of such tests create a unique “fingerprint” for the individual bacterium (Dr. Richardson, Dr. Makemson (2015)).

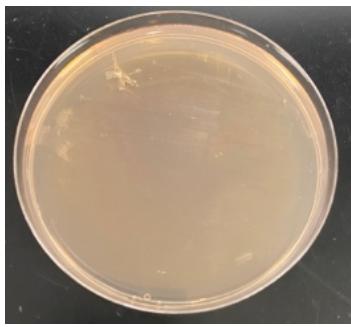
Test	Picture	Result	Description
OF Medium	Oil 	<p><u>Tube with Oil (anaerobic)</u>: The test is invalid. No changes were noticed, so it can be said that the reaction is negative, or, in other words, invalid.</p> <p><i>Reaction</i>: No growth or color change occurred, so, thus, there was no reaction.</p> <p><i>Color</i>: No color change. The starting color was green, and the oil was colorless.</p>	<p><i>Metabolic reaction</i>: Since no changes were observed, there is no reaction occurred. The green layer stayed at the bottom, just like when where it was during preparation, and the oil layer is floating at the top.</p> <p><i>Enzymatic activity</i>: Since no reaction occurred, there is no</p>

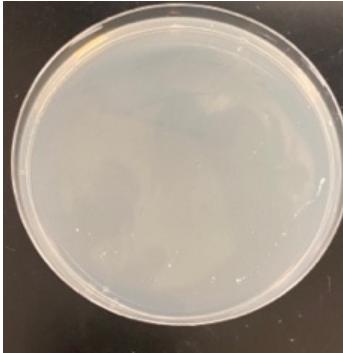
	No oil	<p></p> <p>After one week, the color of the tube remained green, and the color of the oil remained colorless. No new colors were detected.</p> <p><i>pH:</i> Since the result showed green color, which is an indicator of a negative test, it can be concluded that the pH value did not change and remained neutral. Neutral pH lies in the range of 7</p>	<p><i>enzymatic activity happened. Otherwise, there would be changes in the tube, which is a sign of reaction and, as follows, enzymatic activity. Because such activities produce reactions</i></p> <p><i>Other:</i> Both tubes remained green from the moment of their preparation. Thus, no enzymatic activities and, as follows, reactions in both tubes occurred. So, the test is negative, and a bacterium is not able to metabolize glucose either oxidatively, not fermentatively, or both.</p> <p><i>Reaction:</i> Since no changes were observed, there was no reaction in a tube without oil</p> <p><i>Color:</i> No color change. The color remained bright green from the moment of inoculation</p> <p><i>pH:</i> Since the result showed green color, which is an indicator of a negative test, the pH value did not change and remained neutral. Neutral pH lies in the range of 7</p>
		<p><u>Tube without Oil (aerobic):</u> No visible changes in color or structure were noticed. Thus, the test is neutral</p>	<p><i>Additionally, no byproducts, such as gas were detected (otherwise there would be a crack in agar)</i></p>

Kligler's Triple Iron Agar		<p><i>Reaction: -- positive/negative?</i></p> <p><i>Since the growth has only appeared on the surface of the slant, and there was no color change, the reaction did occur, and it is positive for alkaline end-products from the metabolism of nitrogenous nutrients</i></p> <p><i>Color: The entire test tube remained a bright red color. However, the surface of the slant turned pinkish</i></p> <p><i>pH: Due to the positive reaction to the alkaline end-products, the pH value is greater than 7.</i></p> <p><i>Other: The bacteria is an obligate aerobe because the entire tube is red and the growth is only on the surface ace of the slant, and there was no color change in the tube</i></p>	<p><i>Metabolic reaction:</i></p> <p>No growth on the slant was noticed, and the bacteria is an obligate aerobe. Next, bacteria are only able to utilize glucose and lactose. Based on that, metabolism of nitrogenous nutrients did occur</p> <p><i>Enzymatic activity:</i></p> <p>Based on the metabolic reaction, which includes the metabolism of nitrogenous nutrients in the presence of the nitrate/nitrite reductase enzyme has been proven</p> <p><i>Other:</i></p> <p>No gaps-cracks were not noticed there was no gas production by anaerobe.</p>
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Litmus Milk		<p><i>Reaction: -- positive/negative? The result showed a purple color with some light solid precipitate at the bottom. Thus, lactose was not fermented, and the reaction is negative. However, based on a purple color, the reaction produced casein (protein) hydrolysis.</i></p> <p><i>Color: Darker purple took approximately 2/3 of the tube, and at the bottom, some light purple-white precipitate appeared</i></p> <p><i>pH: Based on the purple color, it can be concluded that the pH condition is alkaline. The alkaline (basic) pH range typically lies in the range of greater than 7.</i></p> <p><i>Other: some solid precipitate was formed at the bottom. The solid precipitate can be a sign of a Curd-Acid formation as a byproduct</i></p>	<p><i>Metabolic reaction:</i> According to the results, lactose was not fermented, and the bacteria is not able to ferment the lactose to lactic acid. However, Casein was used, which is a sign of an Alkaline condition.</p> <p><i>Enzymatic activity:</i> Since no lactose fermentation occurred, the lactase enzyme is either absent or not functioning properly in the given bacteria</p> <p><i>Other:</i> The usage of Casein tells is a sign of the alkaline condition. There could also potentially be a reduction of litmus because the precipitate is brighter than the tube itself. However, it is more light purple, rather than white, so the suggestion is rejected. Additionally, neither frothing, nor coagulation was observed, so there are no gas or rennet as byproducts.</p>
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Nitrate Reduction		<p><i>Reaction: -- positive/negative? It is hard to say if the reaction is positive or negative because only a slight color change was noticed after the addition of zinc powder. However, the tint of the tube w/ NO₃ was a little bit darker, so there still could be a positive reaction, and the result is positive</i></p> <p><i>Color: The tube with NO₃ was slightly darker after the zinc powder was added, but it's hard to see. However, the color did not turn red.</i></p> <p><i>pH:</i></p> <p><i>Other: Zinc did react with nitrate, but it's almost not visible</i></p>	<p><i>Metabolic reaction:</i> Since the color change was still observed, the bacteria are still able to breakdown nitrates into nitrites</p> <p><i>Enzymatic activity:</i> To break down nitrates into nitrites, bacteria must have the enzyme nitrate reductase. Thus, in this reaction, the nitrate reductase was activated. However, if there is no color change, the nitrite reductase was activated as well</p> <p><i>Other:</i> Due to the poor change in color, it is hard to say which exact enzyme was activated – nitrate reductase or both: nitrite and nitrate reductase. So, the results are not accurate. To be confident in the result, the obvious color change from yellow to red must be observed.</p>
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Gelatin Hydrolysis		<p><i>Reaction: -- positive/negative?</i> <i>Since no liquefaction occurred, the conclusion is – bacteria are not able to breakdown gelatin, and the reaction is negative</i></p> <p><i>Color: Since no visible results appeared, the color remained constant, even after placing the tube in the ice bath</i></p> <p><i>pH: since no changes were seen, the pH value remained the same as the pH of the gelatin, which is approximately 4.8</i></p> <p><i>Other: Gelatin was not liquefactive</i></p>	<p><i>Metabolic reaction:</i> Due to the negative results – gelatin remained solid; the bacteria cannot show any metabolic activities regarding the gelatin breakdown</p> <p><i>Enzymatic activity:</i> Since the metabolic reaction did not occur, and the gelatin remained solid, the gelatinase (enzyme) was not taking place in the reaction.</p> <p><i>Other:</i> Solid gelatin after 10 mins in an ice bath. Thus, proteolysis (the process by which the bacterial enzyme gelatinase could break down gelatin in a test tube) did not occur. Because all procedures were followed as precise as possible, the conclusion is accurate</p>
Urease		<p><i>Reaction: -- positive/negative?</i> <i>Based on the fact that the agar remained clear throughout the reaction, our conclusion is - No reaction occurred, so the test is negative.</i></p> <p><i>Color: Neither growth nor color change appeared</i></p> <p><i>pH: Since the reaction is negative, and no metabolic/ enzyme</i></p>	<p><i>Metabolic reaction:</i> Because the reaction is negative, it was proven that no metabolic reaction happened, and the bacteria are not able to produce and utilize urease</p> <p><i>Enzymatic activity:</i> No activities were detected. It was explained by the negative reaction and</p>

		<p><i>activities were detected, the environment is acidic, which lies in the range of below 7 on the pH scale</i></p> <p><i>Other: The agar plate has a specific smell. However, the suggestion is – since no reaction occurred, the smell can be explained by the natural composition of a given agar</i></p>	<p>the absence (or poor functioning) of the enzyme urease, which is responsible for the positive reaction in the given experiment</p> <p><i>Other: Agar plate stayed completely clear, so neither carbon dioxide, water, nor ammonia was produced. Also, in this reaction, as can be noticed, the agar plate was used instead of the test tube.</i></p>
Starch Hydrolysis		<p><i>Reaction: -- positive/negative? Due to the lack of color change, the reaction is negative, and the bacteria are not able to produce the alpha-amylase enzyme</i></p> <p><i>Color: No color change from orange. The zone around bacteria remained clear after the iodine was added.</i></p> <p><i>pH: based on the negative results, it was concluded that the pH remained neutral (around 7). However, the iodine could give a slight effect, and change the pH value, but it was not proven.</i></p> <p><i>Other: Since the bacteria are not able to produce the alpha-amylase enzyme, it is</i></p>	<p><i>Metabolic reaction:</i> The negative results showed no metabolic activities in the relation to starch.</p> <p><i>Enzymatic activity:</i> No activities were noticed, so the bacteria do not have the alpha-amylase enzyme. Or, if it does, the enzyme does not function properly since it's not able to breakdown the starch</p> <p><i>Other:</i> There was no starch present. This can be explained by the fact that the zone around the bacterial colony was clear after the addition of iodine. However, the colonies were almost</p>

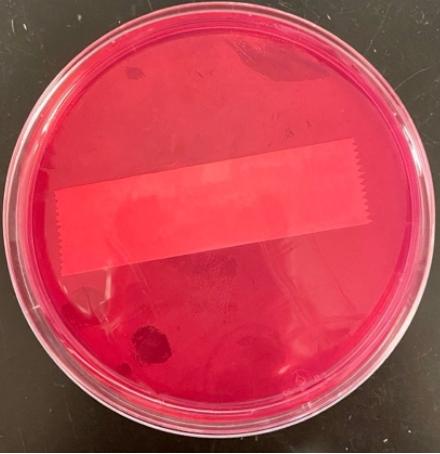
		<i>not able to breakdown the starch</i>	not seen, so there could be a source of error during the bacteria incubation, which could lead to the wrong results
Methyl Red (48 hours)		<p><i>Reaction: -- positive/negative? The reaction did not show any changes, so the conclusion is – it is negative.</i></p> <p><i>Color: No color change to red. The tube remained yellow</i></p> <p><i>pH: No acid production and the reaction is negative, so the pH is greater than 6</i></p> <p><i>Other: The conclusion should be right because the results were taken when the tube was observed for longer than 24 hours. Otherwise, the results could be false</i></p>	<p><i>Metabolic reaction: No reaction was observed, and the acidic-end products were not produced. So, the bacteria are not able to go through fermentation to breakdown dextrose/glucose into pyruvate</i></p> <p><i>Enzymatic activity: No enzymatic activity was detected. Thus, the enzyme is either absent or not functioning.</i></p> <p><i>Other: No color change appeared. The mixed acid fermentation pathway was not taken, and the acid was not produced.</i></p>
Voges-Proskauer (48 hours)		<p><i>Reaction: -- positive/negative? No changes occurred, so the reaction is negative, and the 2,3 butanediol fermentation pathway was not used to ferment glucose</i></p> <p><i>Color: No color change and the tube remained yellow</i></p>	<p><i>Metabolic reaction: No glucose fermentation through the 2, 3 butanediol pathway. Thus, there is no metabolic reaction required for this test.</i></p> <p><i>Enzymatic activity: Due to the negative results, no enzymatic</i></p>

		<p>pH: Potassium hydroxide is basic, so the pH should stay basic due to the negative results.</p> <p>Other: The presence of yellow color indicates that the acetone is not present, which is weakly acidic. This is another proof of the basic pH.</p>	<p>activities were detected.</p> <p>Other: No visible changes. Thus, this bacterium does not produce 2,3 butanediol end product. Otherwise, there would be a brownish-red/pink color. However, for the negative result, the test tube must be brownish green/yellow. The test tube showed only pure yellow color, without any brown tint. Thus, there could be a potential source of error. Probably, during incubation.</p>
Catalase		<p>Reaction: -- positive/negative? During the reaction, it was expected to see bubbles instantly. However, nothing was observed. Thus, the reaction is negative.</p> <p>Color: no reaction was observed, including the color change, which remained the same as it was before the reagent was placed.</p> <p>pH: Because no reaction occurred, and no visible changes (bubbling were noticed), the pH value stayed the same as H₂O₂ pH, which is typically around 6.2</p> <p>Other: No changes after more than 1 minute. However, it</p>	<p>Metabolic reaction: The main sign of metabolic reaction is the presence of bubbles, which was not observed. Thus, there is no metabolic reaction</p> <p>Enzymatic activity: The main enzyme that needs to be produced and utilized is catalase. However, in this trial, it was not due to the absence of bubbles. So, thus, no enzymatic activities occurred. Even though the results are instant, we still would have time to "catch" the reaction.</p>

		<p><i>does not necessarily mean that “non-aerobic” or “anaerobic only” as there are different Cytochrome molecular variants in respiratory pathways</i></p>	<p><i>Other:</i> As it can be seen, the slide is clear. The photo was taken right after the reagent was placed (less than 30 seconds time difference)</p>
Oxidase		<p><i>Reaction: -- positive/negative? Since no color change from colorless to blue was noticed, the reaction is negative.</i></p> <p><i>Color: The color did not turn blue, or any other color, so the conclusion is – no color change happened</i></p> <p><i>pH: The negative result is the sign of the pH value around 5</i></p> <p><i>Other: No dark blue color appeared. However, even though, the results are negative, does not necessarily mean “non-aerobic” or “anaerobic only” because cells have various Cytochrome molecular variants in respiratory pathways</i></p>	<p><i>Metabolic reaction: Since the reaction is negative, bacteria are not able to produce any metabolic activities</i></p> <p><i>Enzymatic activity: No activity was noticed. Thus, the bacteria are not able to produce and utilize the enzyme called Cytochrome - C</i></p> <p><i>Other: There is no color at all on the slide, so nothing turned blue, and the overall reaction is negative.</i></p>

Selective/ Differential Media

Occasionally, even with a unique bacterial “fingerprint”, it is hard to determine a specific bacterial type. In that case, more advanced biochemical tests must be performed. Those tests often include the preparation of a specific environment, in which some bacteria can grow, while others cannot. The media can be selective, differential, or both at the same time (Dr. Richardson, Dr. Makemson (2015)). Differential media tell apart bacteria by causing a color change in a plate, while selective media “selects for/ against” specific bacteria based on the chemical properties of the medium. As was mentioned earlier, the media can be combined as selective and differential at the same time. An example of such a case is MacConkey Agar. This type of agar is selective in a way that only gram-negative bacteria can grow on this media, and differential for lactose utilization.

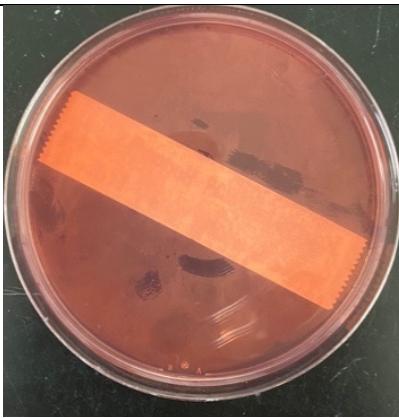
Test	Picture	Result	Description
Mannitol Salt Agar (MSA)		<p><i>Reaction:</i> Based on the fact that neither color change, nor colony growth was detected, the conclusion is – the reaction is negative</p> <p><i>Color:</i> No color change occurred from Pink/Red to yellow</p> <p><i>pH:</i> Negative result shows</p>	<p><i>Metabolic reaction:</i> Mannitol Salt Agar is used for the mannitol fermentation test. Thus, given bacteria is not able to undergo this process</p> <p><i>Enzymatic activity:</i> The MSA agar is selective for halophile cultures. Since the result is negative, the bacteria are not able to grow and reproduce in a salty environment</p>

		<p><i>neutral pH for the given media</i></p> <p><i>Other: This type of agar is both selective (7.5% NaCl) and differential (mannitol and red phenol)</i></p>	<p><i>Other: As a selective part, the MSA agar tests whether the bacteria is halophile or not, and by differential, it tests whether the bacterium can ferment the mannitol.</i></p> <p><i>Since, in this experiment, no changes occurred, the unknown is not a halophile and is not able to ferment the mannitol</i></p>
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Eosin-Y Methylene Blue Agar (EMB)		<p>Reaction: positive/negative? <i>Since the moment of inoculation and incubation, no visible changes appeared, and the reaction is negative</i></p> <p>Color: No changes in color occurred, the color remained red</p> <p>pH: If the result is negative, and no reactions occurred, the pH value stays neutral (around 7)</p> <p>Other: At the very beginning of the project, it was detected that the unknown is gram-positive. Since mostly gram-negative bacteria grow on this type of agar, and no cultures were observed, this can be another proof of the initial gram-positive bacteria identification</p>	<p>Metabolic reaction: Neither color change, nor colony growth were observed, thus, no metabolic reactions occurred.</p> <p>Enzymatic activity: Since neither cream/colorless/light purple/pink colonies appeared, the bacteria are not non-lactose ferment. Additionally, relying on the fact that no dark purple colonies appeared, the bacteria is not the lactose ferment as well</p> <p>Other: Since no green-like growth was seen, there is no rapid fermentation + strong acid production occurred</p> <p>Based on the selective properties, the result showed the presence of gram-positive bacteria, which can be proven by the absence of any possible reactions.</p>
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Blood Agar		<p>Reaction: <i>Compare to the initial agar plate at the moment of incubation, the white colonies appeared, which is an indicator of a positive reaction</i></p> <p>Color: <i>The color of agar stayed red, but the colonies appeared white</i></p> <p>pH: <i>Due to the positive result that looks like gamma-hemolysis positive, and the agar was delivered from the sheep blood, the pH should be around 7.2 to 7.6</i></p> <p>Other: <i>Some colonies are isolated, but some of them are together. However, they are all identical in terms of the actual examination. Thus, the suggestion is – the colony is pure. However, additional testing needs to be performed</i></p>	<p>Metabolic reaction: <i>The presence of white colonies indicates gamma-hemolysis</i></p> <p>Enzymatic activity: <i>The gamma-hemolysis is the type of result that tells that there is no hemolysis occurred</i></p> <p>Other: <i>This type of agar is not selective, but differential. It is differential for the red blood cells hemolysis identification</i></p> <p><i>As it was mentioned earlier, the blood agar is only differential. The results showed that the unknown is a gamma-hemolysis bacteria. Most of the gamma-hemolysis bacteria include facultative or aerobic cocci</i></p>
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MacConkey Agar



Reaction: Based on the absence of color change and new colony growth, it can be concluded, that the reaction is negative

Color: The color remained light pink since the moment of inoculation and incubation

pH: No changes in the pH value occurred, it stayed neutral

Other: The most important part of this test is that only gram-negative bacteria can grow on MacConkey agar. Thus, since the unknown is gram-positive bacteria, negative results were expected.

Metabolic reaction: Because this agar is only for gram-negative bacteria, and the sample is gram-positive,

Enzymatic activity: No activities

Other: This agar is only differential. It is used to test the utilization of lactose

API Test

API testing is another common method in bacteria determination. This system has been developed for the quick identification of bacteria that is clinically related. However, the biggest disadvantage of this method is that in the API test, only known bacteria can be identified.

In general, this test includes 20 wells with several biochemical tests, and all of them have to be inoculated with a pure culture cell suspension in sterile saline. For the API test, several types of kits are available, which must match a specific bacterium. For example, in this experiment, it was determined that the unknown sample is gram-positive cocci bacteria, which is negative for the catalase test, thus, the API that was used is API20Strep, and so on (Dr. Richardson, Dr. Makemson (2015)). However, if the result would show the different bacterium – for example, if the catalase reaction was +, then the API that would be used is API20Staph.

API test results before inoculation and incubation:

All walls were filled with suspension and, later on, incubated for a week. Once the process of incubation was over, the first results have been recorded. However, some of the walls required the addition of different chemical reagents. The procedures for the API20Strep included:

- 1) The suspension was distributed in the first half of the strip (tests VP through ADH) using the following methods:

100 microliters were distributed into each tube for the tests VP to LAP

For the ADH test, only the tube was filled

- 2) For the second half of the strip (tests RIB through GLYG) the following procedures have been met:

An API GP Medium ampule has been opened and the rest of the suspension has been transferred into it. The obtained suspension was distributed into the tubes.

- 3) All underlined tests cupule (ADH to GLYG) have been additionally filled with mineral oil.

- 4) Finally, the lid was placed, and the strip was incubated.



API test results interpretation:

The strip was incubated in aerobic conditions at 36°C. To obtain the first results, it is necessary to wait approximately 4 – 4.5 hours. However, the waiting time for the second results is 24 hours (Dr. Richardson, Dr. Makemson (2015)). To read the results properly, the following procedures must be completed:

- 1) 1 drop of VP 1 and 1 drop of VP 2 must be added to the VP test
- 2) 1 drop of ZYM A and ZYM B (*) must be added to the PYRA, alpha-GAL, beta-GUR, beta-GAL, PAL, and LAP test



As the strip was filled with necessary reagents, and the first visible results appeared, the following conclusions have been made:

VP: color change from orange to pink, positive

HIP: color change from red to dark blue, positive

ESC: no color change, negative

PYRA: no color change, negative

Alpha – GAL: no color change, negative

Beta – GUR: no color change, negative

Beta – GAL: no color change, negative

PAL: no color change, negative

LAP: color changed to darker orange/brown, positive

ADH: no color change, negative

RIB: no color change, negative

ARA: no color change, negative

MAN: no color change, negative

SOR: no color change, negative

LAC: no color change, negative

TRE: no color change, negative

INU: no color change, negative

RAF: no color change, negative

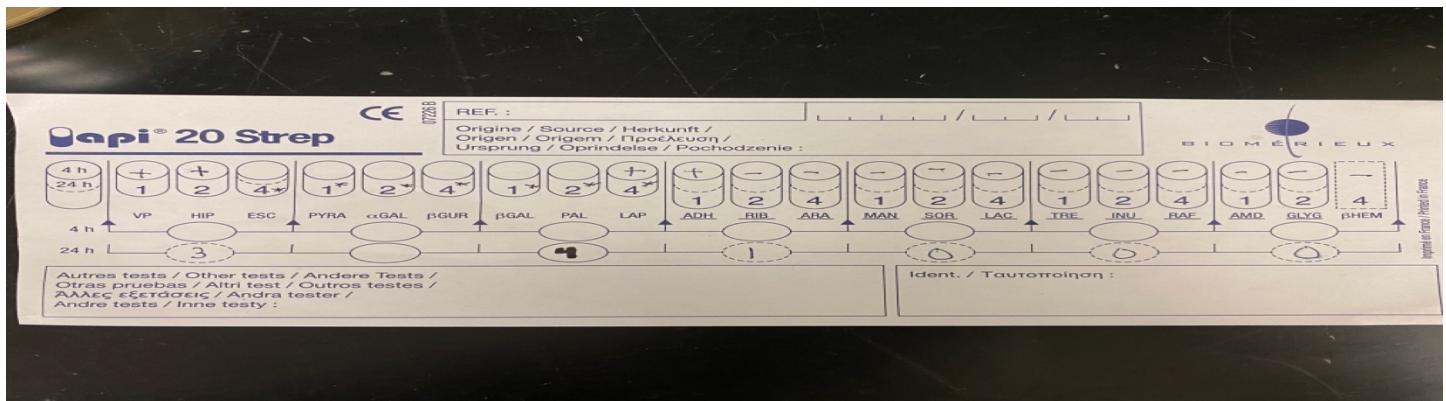
AMD: no color change, negative

GLYG: no color change, negative

Web Results

The API Website is a very powerful programming interface, which helps to identify the bacteria based on the API strip results. After completing the API test results interpretation, the results must be recorded on the reading sheet blank to put them in the system later (Aryal, S. (2019, April 12)).

Initial



However, the website resulted in an identification that was “not acceptable”, so the API20 Strep results had to be changed slightly to achieve better results and be able to work with them. The only change that has been made was - changing Negative Beta-GAL to positive Beta GAL

Thus, more reliable results have been obtained. As it can be seen, based on the APIWeb, the probability that the unknown is *Aerococcus urinae* is 84.7%. However, there is also a probability of the presence of another bacteria, which is *Leuconostoc* spp, and the value is 10.3%, which is not significant. Thus, the final APIWeb conclusion was – the unknown is *Aerococcus urinae*

Final Web Results

The screenshot shows a computer screen displaying the APIweb software interface. The title bar reads "apiweb" and the URL is "eb.biomerieux.com/strip/6". The main menu includes "Printout", "Export", "New test", and "Modify". The "REFERENCE" field is empty, and the "DATE" is set to "6/30/22". The "COMMENT" field is also empty.

ACCEPTABLE IDENTIFICATION

Strip	API 20 STREP V8.0				
Profile	3 0 5 1 0 0 0				
Note	POSSIBILITY OF Str.acidominimus OR Str.pluranimalium				

Significant taxa

Significant taxa	% ID	T	Tests against		
<i>Aerococcus urinae</i>	84.7	0.59	VP	3%	

Next taxon

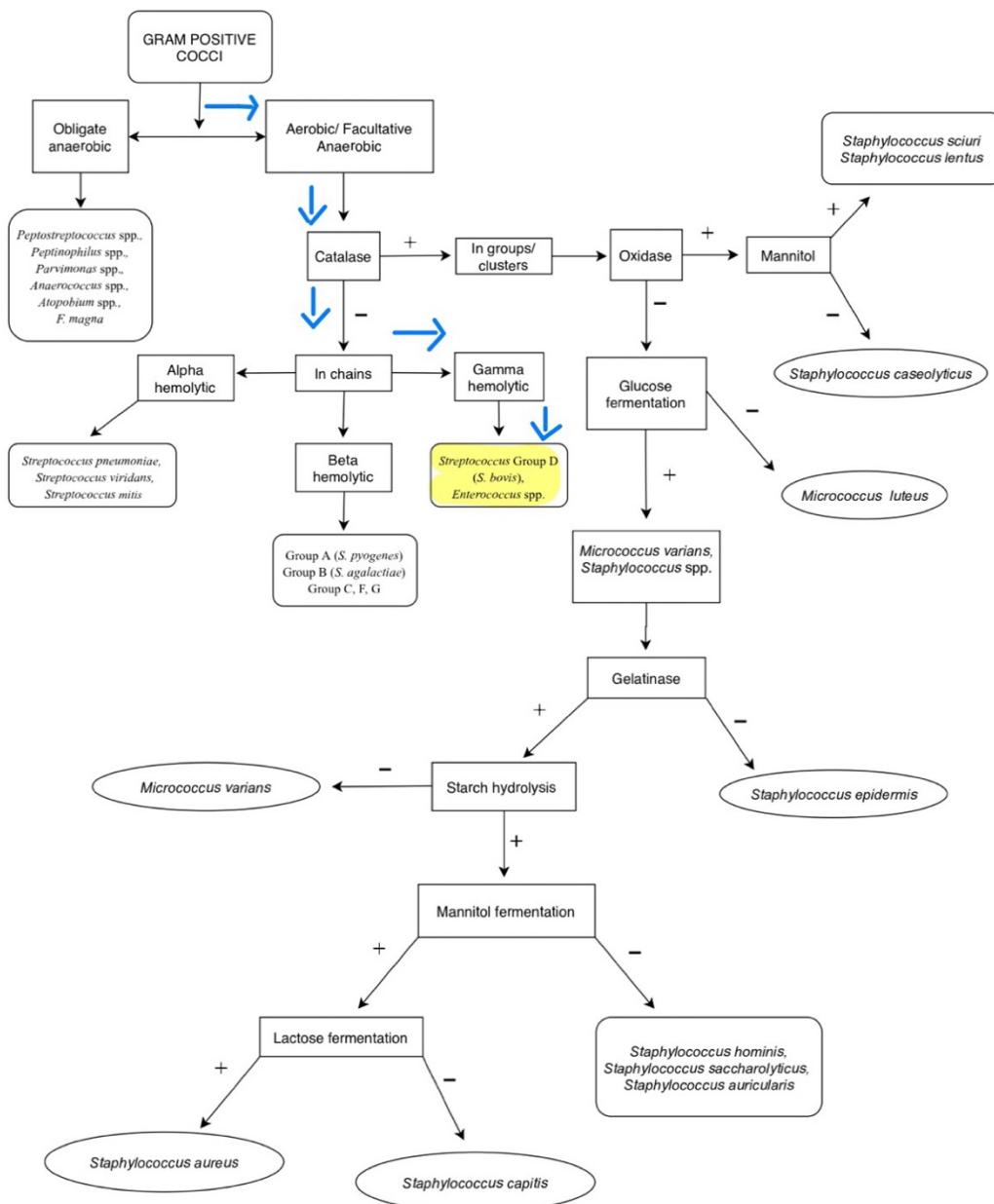
Next taxon	% ID	T	Tests against		
<i>Leuconostoc</i> spp	10.3	0.43	HIP	1%	ADH 10%

Bergey's Manual

Bergey's Determinative and Systematic Bacteriology Manuals are another tool that microbiologists use for placing an unknown bacterium into major taxa. This source provides all necessary information about bacteria classification based on their biochemical properties. In this experiment, based on Bergey's Manual, it was determined that the unknown is *Streptococcus* Group D (*S. bovis*), *Enterococcus* spp. All pathways of determination are shown below.

First of all, the gram stain test provided fundamental information about bacterial morphology- the bacteria are gram-positive cocci. Thus, a corresponding manual has been used. Later on, after the first biochemical tests result, specifically OF Medium test, it was found that the bacteria are aerobic/ facultative anaerobic. As following, the next step was a consideration of the catalase test, which gave a negative result. For the last step, the blood agar test was used, which showed that the sample is gamma hemolytic. Thus, based on all those conclusions, the final result was determined and recorded.

Gram Positive Coccis



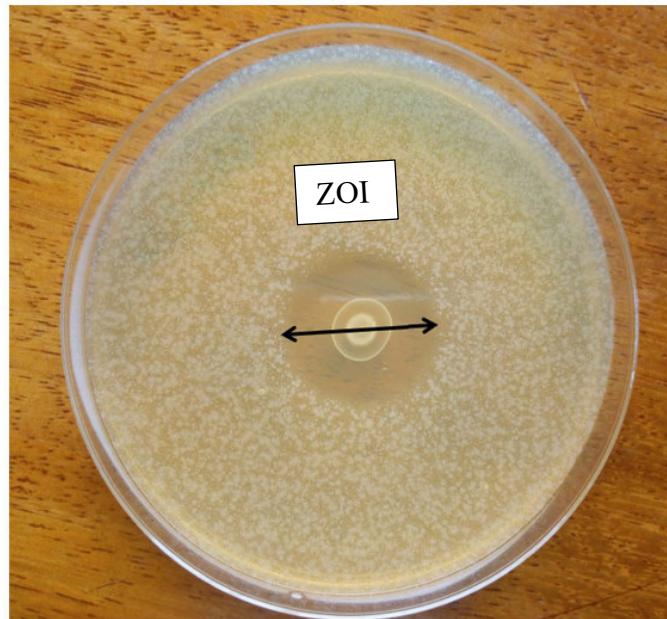
Blue arrows are showing the pathways, and the final result is highlighted in yellow.

Kirby Bauer

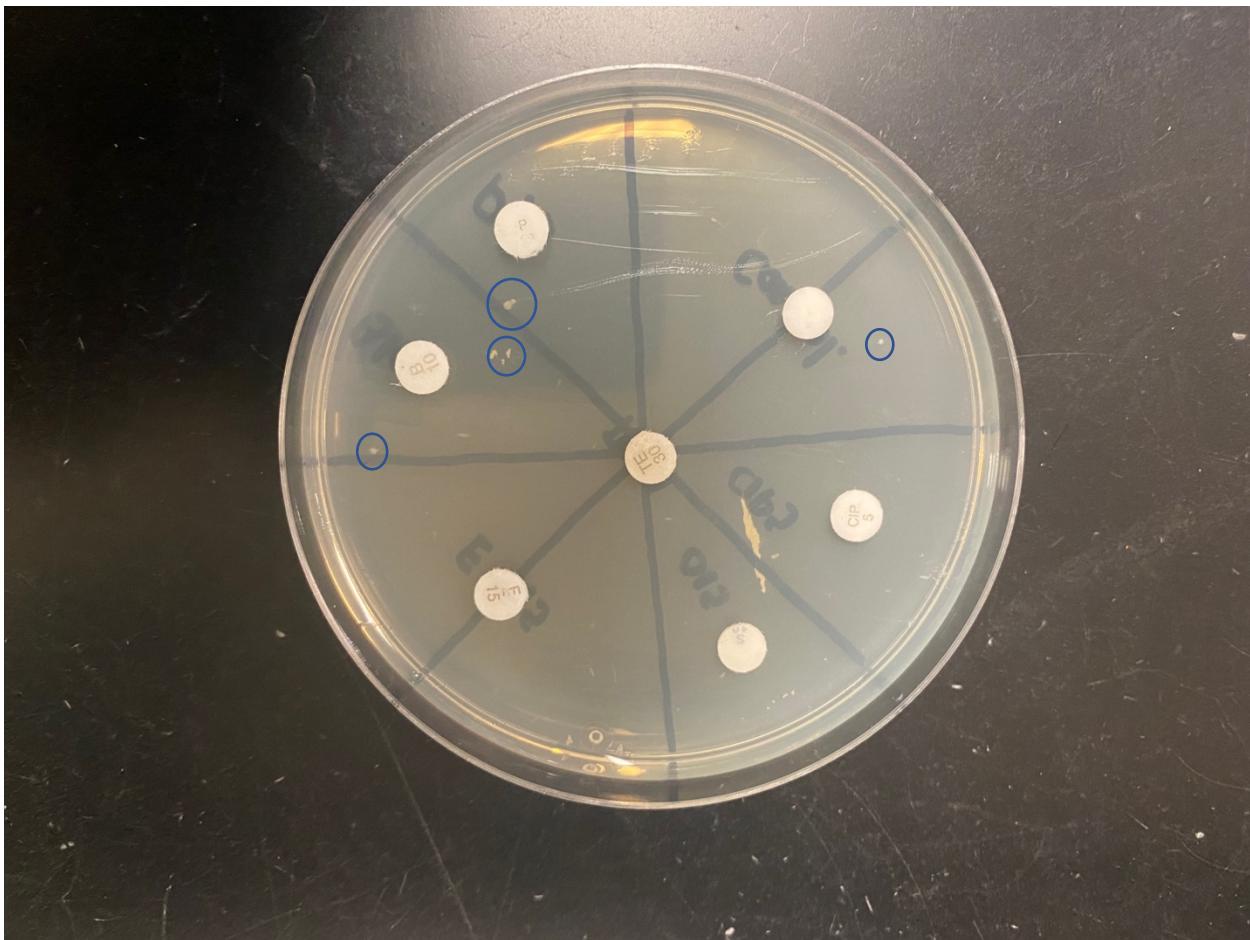
Kirby antibiotic assay is a very powerful and important tool in medicine. It allows us to identify how effectively different antibiotics work against a certain bacterium.

During the Kirby Bauer assay, the small disk that contains antibiotics is placed on a lawn of bacterial culture, and then the plate is incubated. This assay is based on the minimum inhibitory concentration (MIC), or, in other words, the lowest concentration of antibiotics, which will be able to prevent the bacterial growth. The effect of antibiotics can be measured by the zone of inhibition (ZOI) around the disk. ZOI is an area where bacteria are not able to grow due to the presence of antibiotics (Dr. Richardson, Dr. Makemson (2015)). The zone of inhibition is measured by the diameter of this blank area.

An example of the zone of inhibition



In this experiment, no zones of inhibition have been detected. This can be explained by two different possibilities. First, all antibiotics are effective against this bacterium, or, second, the inoculation and incubation processes were not performed properly. However, looking at the picture carefully, there are still several tiny colonies that appeared. On the other hand, this bacterium is considered antibiotic susceptible and, thus, the absence of growth is explainable.



Comparison of obtained results and expected results

Test	Obtained Result	Expected Result
		<i>Insert the name of bacterial species obtained from the API system here</i> <i>Aerococcus urinae</i>
OF medium	Negative, because no changes were noticed in either test tube with oil or test tube without oil	Negative. This bacterium does not metabolize glucose oxidatively, fermentatively, or both
Kligler's Triple Iron Agar	Positive for alkaline end-product because some growth did appear on the surface of the slant, so the	Negative. These bacteria do not metabolize glucose, lactose, or sucrose

	reaction did happen	
Litmus Milk	Negative because no lactose fermentation happened.	Negative. This bacteria does not ferment lactose and Casein used
Urease	Negative, because neither change in color nor any growth were noticed. Thus, there was no reaction	Negative. This bacteria cannot proceed and utilize urease
Starch Hydrolysis	Due to the lack of color change and any new growth, the reaction is negative	Negative. No ability to produce alpha-amylase enzyme
Nitrate Reduction	It is hard to say if the reaction is positive or negative because only a slight color change was noticed after the addition of zinc powder. However, since slight changes did happen, the reaction should be positive	Negative. It is hard to conclude due to unclear results
Gelatin Hydrolysis	Negative because gelatin remained solid after the ice bath	Negative. This bacterium is not able to break down gelatin via the enzyme gelatinase
Methyl Red	No changes occurred, so the reaction is negative	Negative. This bacterium does not go through fermentation to ferment glucose and produce one or more stable organic acid as a byproduct
Voges-Proskauer	The color of the test tube stayed the same. Thus, the reaction did not occur, and the result is negative	Negative. This bacteria is not using the 2,3 butanediol fermentation pathway to ferment glucose
Oxidase	No color change from colorless to blue was noticed, so the result is negative	Negative. This bacteria does not produce and utilize the enzyme Cytochrome - C
Catalase	No bubbles were observed. Based on that, the reaction is negative	Negative. This bacterium does not produce and utilize catalase.
Blood Agar	White colonies appeared on the plate, which indicates a positive result	Positive. This bacteria goes through gamma-hemolysis
MacConkey Agar	No changes were noticed. The reaction is negative	Negative. This bacteria is not gram-negative
EMB (Lactose)	No visible changes occurred, so the reaction is negative	Negative. This bacteria is not utilized lactose, and it is gram-positive

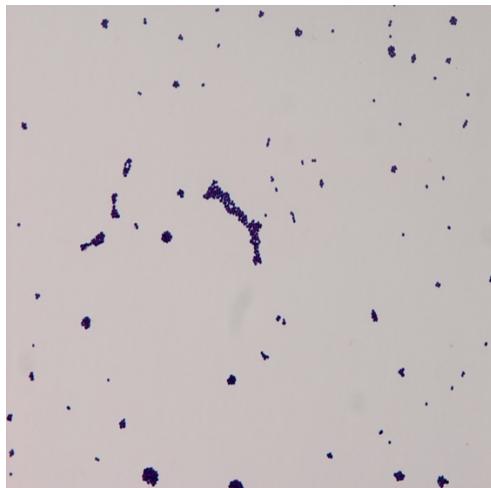
MSA (Mannitol)	Neither color change nor the appearance of new growth was noticed. Thus, the result is negative	Positive. This bacteria is supposed to be either halophile or it's supposed to produce acid from mannitol
<i>List the 20 (or 50) names from your specific API test kit in the following rows.</i>		
...VP	Color changed from orange to pink, positive	3
HIP	Color changed from red to dark blue, positive	99
ESC	No color change, negative	24
PYRA	No color change, negative	12
Alpha-GAL	No color change, negative	0
Beta – GUR	No color change, negative	52
Beta- GAL	No color change, negative	41
PAL	No color change, negative	50
LAP	Color changed to dark orange-brown, positive	92
<u>ADH</u>	No color change, negative	28
<u>RIB</u>	No color change, negative	28
<u>ARA</u>	No color change, negative	0
<u>MAN</u>	No color change, negative	32
<u>SOR</u>	No color change, negative	13
<u>LAC</u>	No color change, negative	56
<u>TRE</u>	No color change, negative	64
<u>INU</u>	No color change, negative	1
RAF	No color change, negative	1
<u>AMD</u>	No color change, negative	40
<u>GLYG</u>	No color change, negative	0

Discussion

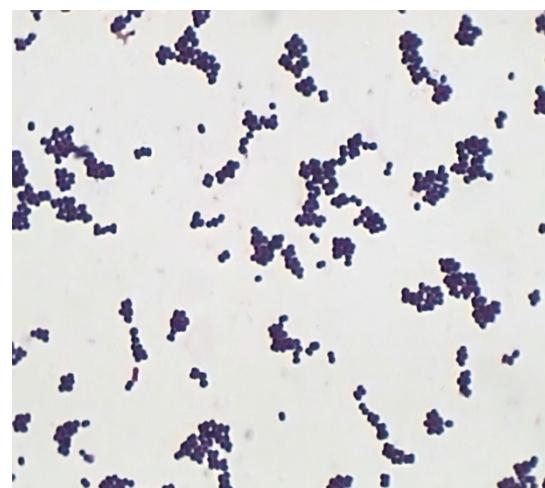
A clear exact conclusion cannot be made since the results vary, and there are different possibilities for the same bacteria.

Based on visual examination, gram stain results, biochemical tests, and Bergey's Manual, the unknown single bacterial isolate from the wild sample is

Streptococcus Group D (*S. bovis*), *Enterococcus* spp. However, the API test showed a different result, where the unknown is *Aerococcus urinae*. According to the API 20 Web page, this identification was acceptable and showed 84.7% ID for the given bacteria. The second possibility is *Leuconostoc* spp, which has 10.3 % ID. Both structures have similar morphology since both of them belong to the class coccus (Zhang, Q., Kwoh, C., Attorri, S., & Clarridge, J. E. (2000)).



Streptococcus Group D (*S. bovis*)



Aerococcus urinae

However, since the greatest number of tests were performed to achieve the *Streptococcus* Group D (*S. bovis*) result, it was considered the final decision. Moreover, when the actual results have been compared to the expected results, the only disagreement was the Mannitol agar test and the API tests. Thus, the results became more justified. Additionally, as it can be seen, API expected results had more mismatches with obtained results than biochemical tests. As follows, the unknown should be *Streptococcus* Group D (*S. bovis*), not *Aerococcus urinae*. However, since the conclusion is not 100% clear, there were some possible sources of error in the experiment. Those errors include lack of experience, inattentive reading of results, and, finally, contamination of the sample. Since it was the first experience in any of the microbiological procedures: inoculation, gram-stain, biochemical tests, reading the results, and so on, the probability of making mistakes is high. Additionally, many students were using the sample as their main culture, and it was impossible to monitor the proper procedure (for example, closed lid while the sample is being in use by someone else), and the plate could get contaminated. Finally, the amount of API test result mismatches is high. The potential source of error could be improper inoculation of the strip in the very beginning and, that is why the results are improper. All those sources of errors should be monitored and improved in the future. It can be proceeded by following the procedures more accurately and, if necessary, re-doing them. Unfortunately, in

this experiment, it was impossible to repeat any of the tests due to the lack of time. The only tests that were initially suspected incorrect are the oxidase test, catalase test, and API.

Streptococcus Group D (*S. bovis*) is a gram-positive bacterium that causes urinary tract infection, meningitis, sepsis, endocarditis, colorectal, and others. The most common transmission of this bacteria is through contaminated dust or by inhaling/swallowing the bacteria.

Streptococcus Group D (*S. bovis*) is resistant to many known antibiotics, including the ones that have been used in the experiment: Bacitracin, Ciprofloxacin, Erythromycin, Penicillin, Streptomycin, and Tetracycline (Zhang, Q., Kwoh, C., Attorri, S., & Clarridge, J. E. (2000)). This fact supports the final decision of identification of an unknown, as the second hypothesis – the unknown is

Aerococcus urinae is not reliable because this bacterium is sensitive to the most antibiotics. However, despite the characteristics of *Streptococcus* Group D (*S. Bovis*), it is still treatable. Most often, this bacterium is treated with penicillin, or, in case of unsuccessful therapy, the alternative medication is used. This medication is a third-generation cephalosporin, which can treat such infections as effectively as penicillin. If treatment is not successful, or the infection is not noticed in time, *Streptococcus* Group D (*S. bovis*) can produce a strong colorectal cancer (CRC), which leads to a lethal outcome.

Many children can easily catch *Streptococcus* Group D (*S. bovis*) as well. However, if for the adults the main signal of such infection is the presence of symptoms, kids can experience no symptoms at all, but still, be tested positive! Around 15 – 20% of children get *Streptococcus* Group D (*S. Bovis*). Nowadays, a lot of scientists are trying to answer the following question – how can these bacteria be detected and treated in children?

Reference

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