Identification of an Unknown Microorganism

Unknown Sample # 571

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Introduction

Microbiology is a fascinating (and oftentimes overlooked) realm of biological science. Although microorganisms may be too small to directly see with the naked eye, their behavior significantly influences our global ecosystems, the practice of medicine, and impacts global economy/commercial industries. From infectious disease, to brewing beer, to environmental testing and everything in between we often take for granted the fact microbes impact our daily lives. Likewise, microbial activity can be observed, tested and analyzed in a variety of ways. The main purpose of this study was to practice various laboratory skills and apply microbiological knowledge acquired throughout the semester in an effort to successfully determine the identity of an unknown bacterium.

Materials & Methods

At the beginning of the semester unknown bacterium sample # 571 was randomly assigned by the laboratory professor. Using careful aseptic technique, the bacterium was subjected to a variety of microbiological tests. The results (mostly qualitative) were photographed, recorded in writing and summarized in Table 1. A list of possible bacterial species identities were also provided. Test results were verified for the possible species using *Bergey's manual of determinative bacteriology* and via Google search results.

A Gram stain was the first laboratory technique performed in this study followed by observation of the unknown under a compound light microscope. This differential stain was used to determine if the sample was Gram-positive or Gram-negative; the most common cell morphology and arrangement among the sample was also observed. An Oxidative-Fermentation test was performed to determine whether bacteria metabolizes carbohydrates via oxidation or fermentation. To determine the unknown's fermentative properties three differential mediums of

phenol red broth each containing a different carbohydrate (glucose, lactose, and sucrose) were inoculated with the unknown bacterium. Methyl Red and Voges Proskauer (MR-VP) combination broth solution was utilized to detect the ability of mixed acid fermentation in the unknown bacterium. A nitrate reduction test was performed to differentiate species of *Enterobacteriaceae* which perform one-step reduction of nitrate to nitrate from other microbes which either do not reduce nitrate or further reduce it to other compounds.

To determine whether the unknown bacterium is a facultative anaerobe member of Enterobacteriaceae a citrate utilization test was performed to detect if the bacterium can use citrate as a carbon source and undergo citrate fermentation. A urea hydrolysis test was performed to determine whether the unknown bacterium possesses the enzyme urease for the ability to hydrolyze urea to ammonia. SIM medium was used as a combination test to detect the production of sulfur, indole production, and motility. This medium is particularly useful in differentiating members of the *Enterobacteriaceae* family.

A triple sugar iron agar (TSIA) slant, which creates an aerobic and anaerobic environment, was inoculated with the bacterium to differentiate whether the unknown belong to the family *Enterobacteriaea* depending on the fermentation of a carbohydrate rich medium. Agar plates were inoculated with the unknown bacterium with one plate incubated inside a plastic jar to create an anaerobic environment while the other plate was incubated outside of the anaerobic jar. The growth on the two plates was then compared to classify the microbe as an obligate aerobe, obligate anaerobe, or facultative anaerobe. A catalase test was used to detect whether the unknown bacterium produces the catalase enzyme as a differential test for the catalase-positive family of *Micrococcaceae.* An oxidase test was used to determine the presence of the cytochrome c oxidase enzyme in the unknown bacterium. This differential test was useful in

determining if the bacterium possibly belonged to the family *Enterobacteriaceae* or the family *Pseudomonadaceae.* A starch agar plate was inoculated with the unknown bacterium to determine its ability to hydrolyze starch and detection of the presence of enzymes α-amylase and oligo-1,6-glucosidase. A DNA hydrolysis test was performed by inoculating a DNase plate with the unknown bacterium and observing the plate after incubation to determine if DNase is present based upon whether a clearing in the agar appeared around the microbe's growth. A lipase test was utilized by inoculating tributyrin agar and examining the plates for the presence or absence of a clearing around the growth to determine lipid hydrolysis activity.

Bile esculin agar (BEA) was used as both a selective and differential medium to determine whether the unknown bacterium is a possible member of Group D *Streptococcus* or *Enterococcus*. Mannitol salt agar was used to determine whether the unknown bacterium is a *Staphylococcus* species based on the ability to grow in a salty environment and the ability to ferment mannitol. MacConkey agar was used to determine the unknown's ability to ferment lactose and as a possible member of the *Enterobacteriaceae* family. The effect of temperature on the growth of the unknown bacterium sample was assessed by inoculating a broth tube and incubating the bacterium at the five different temperatures: 10° C, 25° C, 35° C, 45° C, and 60º C. The turbidity of the tubes compared to the temperatures was used to classify the organism's temperature range for optimal growth. Lastly, the effect of pH on the growth of the unknown bacterium was evaluated by inoculating five pH adjusted soy broth plates ranging from pH 2 to pH 5.

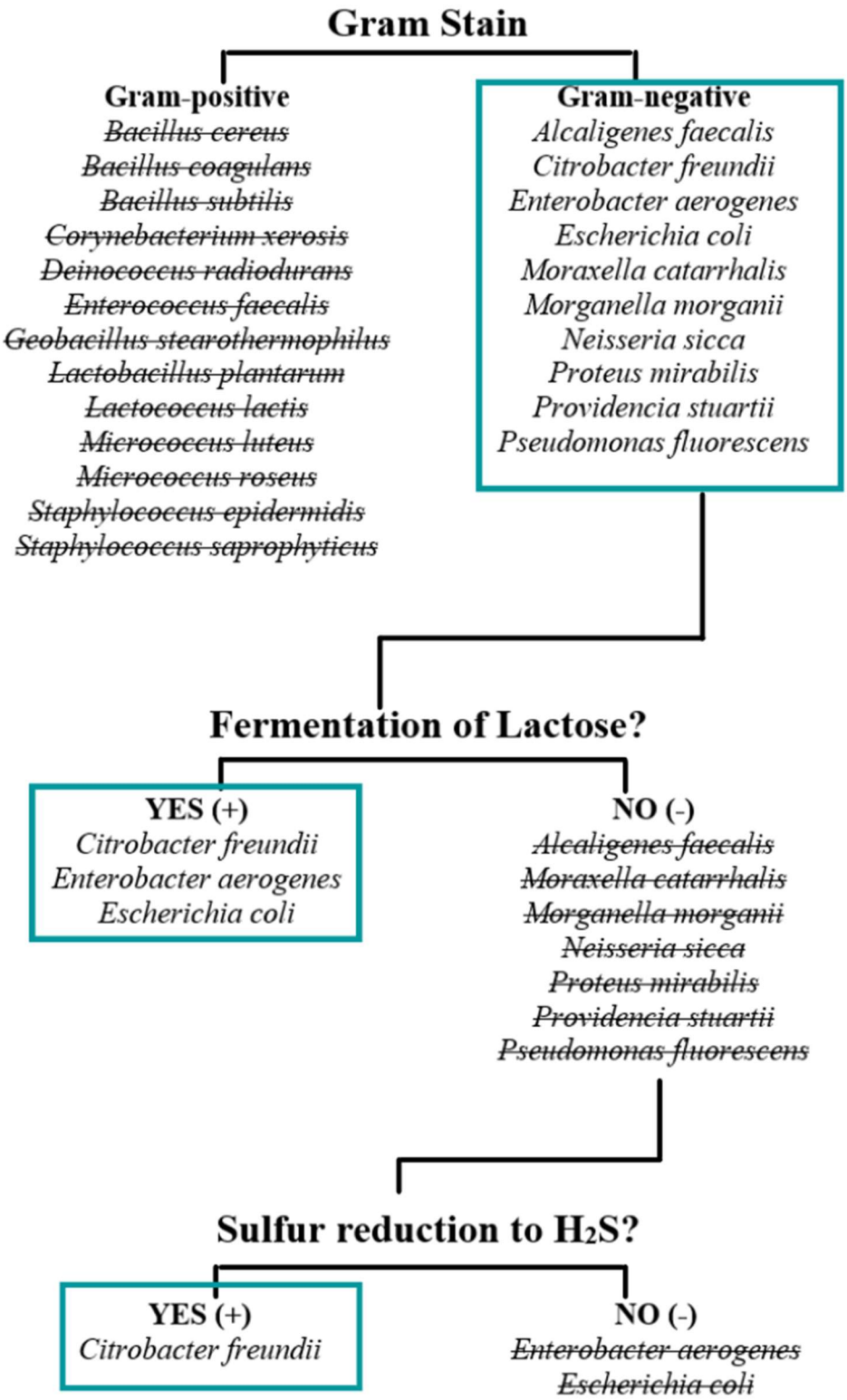
Results

Table 1. Summary of microbiological tests detailing the observations, and interpretations for unknown bacterium sample #571.

Procedure/Test	Observation/Result	Interpretation
Gram Stain	**Stain:** pink **Cell morphology:** bacillus (rod-shaped) **Cell arrangement:** single-celled	Gram-negative
Oxidative-Fermentation	**Sealed tube:** yellow throughout **Unsealed tube:** yellow throughout	Oxidation and Fermentation OR Fermentation Only
Phenol Red Broth	**PR-glucose:** yellow broth w/ bubble **PR-lactose:** yellow broth w/ bubble **PR-sucrose:** yellow broth w/ bubble	Capable of fermenting glucose, lactose, and sucrose with acid and gas end products
Methyl Red	Red	Mixed acid fermentation (+)
Voges Proskauer	No color change	No 2,3-butanediol fermentation; acetoin not produced (-)
Nitrate Reduction	No gas in Durham Tube; no color change after addition of reagents; no color change after addition of zinc	Nitrate reduction to nongaseous nitrogenous compounds; nitrate reductase is present (+)
Citrate Utilization	Blue	Citrate is utilized
Urea Hydrolysis	Orange/Yellow	No urea hydrolysis; urea is absent
H_2S Reduction	Black in medium	Sulfur reduction; H_2S production (+)
Indole Production	Reagent color is unchanged	Organism does not produce tryptophanase and tryptophan is not hydrolyzed (-)
Motility	Growth radiating outward from stab line	Motility

Triple Sugar Iron Agar	**Slant Color:** yellow **Butt Color:** black **Gas Produced?:** yes	H_2S production, glucose and lactose/sucrose fermentation
Anaerobic Jar	**Growth Aerobic Plate:** Abundant **Growth Anaerobic Plate:** Abundant (Organism grew abundantly on both plates but more lucious/thick on aerobic plate)	Possible facultative anaerobe
Catalase Test	Bubbles	Catalase is present
Oxidase Test	No color change to blue/purple occurred within 20 seconds	Cytochrome c oxidase is not present (-)
Starch Hydrolysis (Amylase Test)	No clearing around growth	Neither alpha-amylase or oligio-1,6-glucosidase are present (-)
DNA Hydrolysis (Dnase Test)	Clearing in agar (some loss of color around growth)	Dnase is present (+)
Lipid Hydrolysis	Clearing in agar around growth; loss of color in plate	Lipase is present (+)
Bile Esculin Agar	No darkening of medium after 48 hours	Presumptive determination: not a member of Group Streptococcus or Enterococcus
Mannitol Salt Agar	**Growth on MSA:** NONE **Color of Growth on MSA:** Red	Organism inhibited by NaCl; Not Staphylococcus
MacConkey Agar	**Amount of Growth on MAC:** good **Color of Growth on MAC:** pink/purple	Organism produces acid from lactose fermentation; probable coliform
Effect of Temperature on Growth	**Broth:** growth @ 25° C - 45° C **Plate:** growth @ 25° C - 45° C	mesophile
Effect of pH on Growth	Growth at pH range 6-10 (Best growth occurred at pH 8)	Appears to be mainly a neutrophile

Dichotomous Key



Conclusion

Following the above-referenced microbiological tests, unknown bacterium # 571 was determined to most closely align with the characteristics of the species *Citrobacter freundii.* The first procedure being a Gram stain successfully eliminated more than half of the possible bacterium identities. A few of the differential tests most (most notably MacConkey Agar, Oxidase Test, and SIM medium) were key in placing the unknown organism as a member of the *Enterobacteriaceae* family of bacteria which all but eliminated three possible organisms.

The test that determined the final species identity was a positive SIM test reading for sulfur reduction to hydrogen sulfide. Interestingly, there were a few discrepancies where the results of the unknown sample did not align with the expected result for *Citrobacterf freundii.* These discrepancies included the unknown sample yielding a negative result for urea hydrolysis, and positive results for both DNA hydrolysis and lipid hydrolysis – *C. freundii* is said to exhibit the opposite of these confounding results. Nonetheless, the the unknown sample most closely aligned with the temperature and pH ranges of *C. freundii* which boosted confidence in determining the final identity.

Discussion

Citrobacter freundii is a member of family *Enterobacteriacea* which exhibit a negative gram-stain, rod-shaped (bacillus) morphology, and are nonmotile or motile via flagella (Madigan *et al*., 2018). This facultative anaerobe is naturally present in soil, water, and the feces of both animals and humans as a typical inhabitant of the intestinal flora (Holt *et al*., 2000). Kus and Burrows (2007) note *C. freundii* can be pathogenic to both children and those who are immunocompromised as this species is associated with health issues including wound infections, urinary tract issues, sepsis, and meningitis. Unfortunately, some *Enterobacteria* species such as

C. freundii have become an increasingly problematic public health and environmental issue in connection with contaminated drinking water in certain parts of the world (De Boeck *et al*., 2012) even causing mass mortality of certain species of fish due to hatcheries infected with the pathogenic bacteria (De Boeck *et al., 2009)*.

References

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