Presumptive identification of the corynebacterium genus

Abstract.

The experiment occurred over the course of a spring school term (January into April) with the objective of isolating and purifying an environmental sample. The environmental sample was obtained from the tile flooring on the third floor by room 0339 of George Lynn Cross Microbiology Building located on the University of Oklahoma campus. Through a series of 35 total physiological, biochemical, and morphological tests the isolate was able to be presumptively identified to the genus level. Among these tests, the acid-fast stain, catalase test, Gram-Stain, and endospore test, were most useful in the identification of the bacteria. The tests revealed that the environmental isolate (EI) is a non-sporing, non acid-fast, gram-positive, strict-aerobe, and catalase positive microorganism. These characteristics are common of the genus *Corynebacterium* . Thus, it is presumed that the environmental isolate is that of the *Corynebacterium* genus.

Introduction.

All bacteria share one common characteristic, ubiquity. This ubiquitous nature of bacteria allows them to grow in vast variety of places such as beneath the ground soil, on desk chairs, on door handles, in lakes, and in/on countless of other surfaces. Common locations such as public bathrooms, grocery stores, and other shared facilities are exposed to several variations of microbes. Like other organisms, different species of bacteria are able to grow in the same place/environment or atop the same surface making difficult to determine what type of bacteria are most prevalent (1). Based on

this knowledge, an experiment was performed over the course of a school semester.

An environmental sample was collected at the George Lynn Cross Microbiology building located on the University of Oklahoma campus from the tile flooring on the third floor by room 0339. Since the EI was obtained from flooring, it is presumed that the identity will most likely be of a bacterial species that typically resides in natural environments such as ground soil or water. This is because many students walk across grass fields or through puddles on their way to class and the bacteria found in these environments could be brought inside by students or professors. Past studies have shown that the most abundant groups of soil bacteria are *Arthrobacterium, Bacillus cereus, Bacillus mycoides, and Pseudomonas fluorescens* (2). One researcher by the name of Sergei Winogradsky had observed that soil bacteria don't typically exist within isolated colonies but rather adhered colonies found in the soil, which can be tracked inside by the shoe of students (3).

After obtaining the environmental sample it was environmentally streaked onto a TSA plate and then sub-cultured further in order to obtain a pure culture of the environmental isolate. The goal of the experiment was to identify the EI through various tests to at least the genus level. Aseptic techniques and quadrant streaking were practiced throughout the semester to maintain the pure culture of the EI. This would allow for more accurate results after performing tests on the EI. The identification process consisted of a large variety of tests outlined in the lab manual and canvas slides. These tests included biochemical tests such as " Ex. 5-2 Phenol Red," morphological tests such as " Ex. 3-5 Simple Stain," and physiological tests

like “ Ex. 2-6 Deep Agar Stab” or “ Ex. 2-10 Effect of pH on Microbial Growth” (4). Additional tests that may have been helpful in the presumtive identification of the environmental isolate are scanning electron micrographs which are useful in determining distinctive morphologies of the cells (2). Some advanced tools not available for use at the time such as tools for mass spectrometry or newer software analysis would have also aided in the identification process (5)

Methods.

The protocol “ Ex. 2-1 Ubiquity of Microorganisms” and all preceding tests outlined in the lab manual, *Microbiology Laboratory Theory and Application, 3rd Edition* by Leboffe and Pierce, were followed with a few modifications (4). The environmental sample was taken from the tile flooring on the third floor near room 0339 in George Lynn Cross Microbiology Building on the University of Oklahoma Campus. An environmental streaking pattern was used to inoculate the sample onto a Tryptic Soy Agar (TSA) plate and then incubated for five days at 37 degrees celsius. In order to isolate a pure sample from the large mixed culture that had grown on the TSA plate, three colonies were chosen based on visible morphology to be subcultured onto new TSA plates using aseptic techniques and incubated at 37 degrees celsius for 48 hours. Out of the three species that were subcultured, the EI that was chosen had the most easily identifiable morphology pattern and grew the best. From that point forward, the EI was sub-cultured by quadrant streaking method and incubated at 37 degrees celsius for 48 hours twice a week (if lab was not in session due to inclement weather, the EI was not subcultured until the next lab period). Below all physiological, biochemical, and morphological

tests performed on the EI are listed with any notable modifications, materials (mainly dyes), and controls that were used.

Morphological Tests

The procedure outlined in " Ex 2-2 Colony Morphology" in the lab manual except the environmental isolate obtained from the tile flooring of third floor of George Lynn Cross Microbiology building on the University of Oklahoma campus was used. The example controls viewed were *Proteus mirabilis, Serratia marcescens, Escherichia coli, Micrococcus luteus, Bacillus subtilis, Staphylococcus epidermidis, and Alcaligenes faecalis.*

The protocol " Ex 3-5 Simple Stain" outlined in the lab manual was followed except for a few modifications. The controls used were *Escherichia coli, Staphylococcus epidermidis, Bacillus subtilis* and crystal violet, safranin, and methylene blue were used.

The protocol " Ex 3-6 Negative Stain" outlined in the lab manual was followed except for a few modifications. The controls used were *E. coli, S. epidermidis, B. subtilis* and Nigrosin was used.

The protocol " Ex 3-7 Gram Stain" outlined in the lab manual was followed except for a few modifications. The controls used were *E. coli, S. epidermidis, B. subtilis.* Materials used were Crystal Violet, Safranin, Gram Stain, and ethanol.

The protocol " Ex 3-8 Acid-Fast Stain (Kinyoun)" outlined in the lab manual was followed except for a few modifications. The controls used were *Mycobacterium Smegmatis and S. epidermidis.* The Zeehl-Nielson method

was not performed, instead a quick demonstration was presented by the Teaching Assitant (TA) and prepared slides were viewed under microscopes. The Kinyoun stain was done and used.

The protocol " Ex 3-8 Capsule Stain" outlined in the lab manual was followed except for a few modifications. The controls used were *S. epidermidis* and *N. Capaulation.* Materials used were congo red dye and maneval stain.

The protocol " Ex 3-10 Endospore Stain" outlined in the lab manual was followed except for a few modifications. The controls used were 24 hour *E. coli,* 24 hour *Bacillus,* and 5-day *Bacillus.* Materials used were malachite green stain and safranin. The stain was steamed over water for 5 minutes.

Biochemical Tests

The protocol " Ex 5-6 Catalase Test" outlined in the lab manual was followed except for a few modifications. The controls used were *S. epidermidis* and *Streptococcus sanguinis.* Hydrogen peroxide was used and the slant test was not performed.

The protocol " Ex 5-7 Oxidase Test" outlined in the lab manual was followed except for a few modifications. The controls used were *E. coli* and *A. faecalis* . The test was performed on a BBL DrySlide.

The protocol " Ex 5-2 Oxidation Fermentation Test" outlined in the lab manual was followed except for a few modifications. The controls used were *E. coli* and *Pseudomonas aeruginosa.* The materials used were mineral oil, bromothymol blue, and O-F Glucose tubes.

The protocol “ Ex 5-3 Phenol Red” outlined in the lab manual was followed except for a few modifications. The controls used were *A. faecalis, E. coli, S. epidermidis,* and *Providencia stuartii.* Instead of five tubes of sucrose, lactose, and glucose a total of six tubes each were used. Phenol red indicator was used.

The protocol “ Ex 5-4 Methyl Red and Voges-Proskauer Test” outlined in the lab manual was followed except for a few modifications. The controls used were *E. coli* and *Enterobacter aerogenes.* Instead of three tubes of MR-VP Broth a total of four were used. 5 drops of methyl red reagent was used and 15 and 5 drops of VP reagents A (alpha-naphthol) and B (potassium hydroxide) were used respectively.

The protocol “ Ex 5-8 Nitrate Reduction Test ” outlined in the lab manual was followed except for a few modifications. The controls used were *E. coli, A. Faecalis,* and *P. aeruginosa.* Instead of four tubes of nitrate broth, a total of five tubes were used. Nitrate reagents A and B and zinc powder were also used.

The protocol “ Ex 5-9 Citrate Test” outlined in the lab manual was followed except for a few modifications. The controls used were *E. coli* and *E. aerogenes.* There were a total of 4 slants of Simmons Citrate used.

The protocol “ Ex 5-11 Decarboxylation Test ” outlined in the lab manual was followed except for a few modifications. The controls used were *E. coli, P. stuartii,* and *E. aerogenes.* Mineral oil, five tubes of decarboxylase base media and, five tubes lysine, five tubes of arginine, and five tubes of ornithine were used.

The protocol “ Ex 5-13 Starch Hydrolysis (Amylase Test) ” outlined in the lab manual was followed except for a few modifications. The controls used were *E. coli and B. cereus.* Instead of one plate of starch agar, two plates were used as well as gram’s iodine.

The protocol “ Ex 5-14 DNA Hydrolysis (DNase Test) ” outlined in the lab manual was followed except for a few modifications. The controls used were *E. aerogenes* and *S. epidermidis.* Two plates of DNase test agar were used instead of one.

The protocol “ Ex 5-16 Casein Hydrolysis (Casease Test) ” outlined in the lab manual was followed except for a few modifications. The controls used were *E. coli* and *S. marcescens.* Two plates of milk agar were used instead of one.

The protocol “ Ex 5-17 Gelatin Hydrolysis (Gelatinase Test)” outlined in the lab manual was followed except for a few modifications. The controls used were *E. coli* and *S. marcescens.* Four nutrient gelatin stabs were used instead of three.

The protocol “ Ex 5-18 Urea Hydrolysis (Urease Test) ” outlined in the lab manual was followed except for a few modifications. The controls used were *P. mirabilis* and *E. coli.* Slants were not used, only tubes of broth were used.

The protocol “ Ex 5-19 PYR Test (PYRase Test)” outlined in the lab manual was followed except for a few modifications. The controls used were *E. aerogenes* and *S. epidermidis.* A total of 4 PYR disks were used and one drop of PYR Reagent was placed on each disk.

The protocol “ Ex TSI Agar Test ” outlined in the lab manual was followed except for a few modifications. The controls used were *Morganella morganii, E. coli, Citrobacter freundii,* and *A. faecalis.* The Kligler Iron Agar test was not performed.

The protocol “ Ex Bacitracin, Novobiocin, Optochin Susceptibility Test” outlined in the lab manual was followed except for a few modifications. The controls used were *Streptococcus sanguinis* and *Streptococcus pneumoniae* for optochin. The controls were *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* for novobiocin. And lastly the controls were *S. epidermidis* and *M. Luteus* for bacitracin. Forceps were used to place the disks onto the agar plates. Optochin, novobiocin, and bacitracin disks were used.

The protocol “ Ex Coagulase Test” outlined in the lab manual was followed except for a few modifications. The controls used were *S. epidermidis* and *Staphylococcus aureus.* The slide tests were not performed.

The supplemental exercise Indole Test provided by the TA was followed except for a few modifications. The controls used were *E. coli* and *E. aerogenes.* 8 drops of Kovac’s reagent was used.

The supplemental exercise Beta-Lactamase given by the TA was followed except for a few modifications. The controls used were *E. coli* without a plasmid and *E. coli* with a plasmid. A nitrocefin slide was used.

The protocol “ Ex 7-3 Antimicrobial Susceptibility Test: DIsk Diffusion (Kirby-Bauer) Method” outlined in the lab manual was followed except for a few

modifications. The controls used were *E. coli* and *S. epidermidis.* Penicillin, Ciprofloxacin, Streptomycin, and Trimethoprim disks were used.

The protocol " Ex 5-25 Blood Agar" outlined in the lab manual was followed except for a few modifications. The controls used were *S. epidermidis, S. sanguinis, and S. aureus.* A total of 5 blood agar plates was used instead of one.

Physiological Tests

The protocol " Ex 5-28 Motility Test" outlined in the lab manual was followed except for a few modifications. The controls used were *S. epidermidis* and *Enterobacter aerogenes.*

The protocol " Ex 2-6 Deep Agar Stab" outlined in the lab manual was followed except for a few modifications. The controls used were *Clostridium sporogenes, A. Faecalis,* and *E. coli.*

The protocol " Ex 2-7 Fluid Thioglycollate Broth" outlined in the lab manual was followed except for a few modifications. The controls used were *Clostridium sporogenes, A. Faecalis,* and *E. coli.* A control tube that received no treatment was not used/made.

The protocol " Ex 2-8 Anaerobic Jar" outlined in the lab manual was followed except for a few modifications. The controls used were *Clostridium sporogenes, A. Faecalis,* and *E. coli.* An anaerobic bag was used in place of an anaerobic jar.

The protocol “ Ex 2-9 Effect of Temperature on Microbial Growth” outlined in the lab manual was followed except for a few modifications. The controls used were *S. marcescens, P. fluorescens, E. coli,* and *Geobacillus stearothermophilus.* The growth of the bacterial species were measured over four different temperatures, 4°C, 37°C, 55°C, and room temperature instead of the two listed in the manual. This caused there to be a total need of 28 sterile tubes instead of 20 as listed. S. marcescens was quadrant streaked instead of environmentally streaked (zigzag pattern).

The protocol “ Ex 2-11 Effect of pH on Microbial Growth” outlined in the lab manual was followed except for a few modifications. The controls used were *S. epidermidis, A. Faecalis,* and *E. coli.* Instead of five tubes, a total of seven tubes were used to measure the growth at pH levels of 2, 4, 6, 8, and 10.

The protocol “ Ex 2-9 Effect of Osmotic Pressure on Microbial Growth” outlined in the lab manual was followed except for a few modifications. The controls used were *S. epidermidis* and *E. coli.* Instead of four tubes, a total of six tubes were used to measure the growth in saline medium at dilutions of 0%, 2%, 5%, 8%, and 11%.

Results.

Table 1. Morphological Test Data

Exercises	Observations
Ex 2-2 Colony	Smooth, creamy-white

Morphology	color, raised, circular, round edges, entire
Ex 3-5 Simple Stain	Single or paired rods
Ex 3-6 Negative Stain	Single or paired rods
Ex 3-7 Gram Stain	Purple rods, positive
Ex 3-8 Acid- Fast Stain (Kinyoun)	Blue, negative
Ex 3-9 Capsule Stain	Halos around rods, positive

Ex 3-10 Endospore Stain	Negative, pink

The table presents all morphological tests performed on the EI and the results of those tests.

As seen in Table 1, the morphology of the colonies for the EI are of a creamy-white color. They are raised, circular, with a smooth surface, round edges and entire. All the morphological tests indicated that the EI is a gram-positive organism and is rod-shaped. It is important to note the organism had a halo around the rods as indicated by the capsule stain. The EI is also a non acid-fast organism.

Table 2. Physiological Test Data

Exercises	Observations/ Interpretations
Ex 5-28 Motility Test	N/A
Ex 2-6 Deep Agar Stab	Growth at top of stab
Ex 2-7 Fluid Thioglycollat	Growth at

e Broth	top of broth
Ex 2-8 Anaerobic Jar	No growth
Ex 2-9 Effect of Temperature on Microbial Growth	15 º -40 º C optimal growth
Ex 2-10 Effect of pH on Microbial Growth	Ph 6-10 optimal growth
Ex 2-11 Effect of Osmotic Pressure on Microbial Growth	0%- 2% NaCl optimal growth

Table 2 shows all results for physiological tests done. Note that N/A results means that the test results were unable to be obtained or performed.

Table 2, it is important to note that the EI only grew on the top of the fluid thioglycollate broth and the deep agar stab and could not grow throughout.

It also did not grow in anaerobic conditions. The optimal range of growth for the EI was 15 degrees celsius to 40 degrees celsius, pH level of 6-10 and, within a 0%-2% NaCl solution.

Table 3. Biochemical Test Data

Exercises	Observations/ Interpretations
Ex 5-6 Catalase Test	Positive
Ex 5-7 Oxidase Test	Negative
Ex 5-2 Oxidation Fermentation Test	-Aerobic: green throughout tube, negative Anaerobic: green bottom, yellow top, acidic

	environmen t, positive, slow fermenter
Ex 5-3 Phenol Red	-Lactose: tube was a reddish- pink, K -Sucrose: negative -Glucose: negative
Ex 5-4 Methyl Red and Voges- Proskauer Test	-MR: positive, mixed acid fermentatio n -VP: negative, 2, 3- butanediol fermentatio n, acetonin

	not produced
Ex 5-8 Nitrate Reduction Test	No growth or gas
Ex 5-9 Citrate Test	negative
Ex 5-11 Decarboxylation Test	-Ornithine: negative -Arginine: negative -Lysine: negative
Ex 5-13 Starch Hydrolysis (Amylase test)	Negative, no zone of inhibition
Ex 5-16 Casein Hydrolysis	Negative, no zone

(Casease test)	
Ex 5-17 Gelatin Hydrolysis (Gelatinase test)	Negative, not liquid
Ex 5-14 DNA Hydrolysis (DNase test)	Negative, no zone
Ex 5-18 Urea Hydrolysis (Urease test)	Positive
Ex 5-21 TSI Agar Test	Positive
Ex 5-19 PYR test (PYRase test)	Negative
Ex 5-27 Coagulase Test	Negative

Supplemental Exercise	N/A
Indole Test	
Ex 5-24 Bacitracin, Novobiocin, Optochin Susceptibility Test	N/A
Supplemental Exercise Beta-Lactamase Test	N/A
Ex 7-3 Antimicrobial Susceptibility Test: Disk Diffusion (Kirby-Bauer) Method	N/A
Ex 5-25 Blood Agar	N/A

Table 3 above shows all results from biochemical tests done. Note that test results indicated N/A were not obtained or performed.

Table 3 shows that the EI is unable to ferment or break down lactose, sucrose, and glucose sugars compared to others. It can preform mixed-acid fermentation and is unable to produce most proteins such as casease, amylase, gelatinase, DNase, PYRase, Beta-Lactamase, and oxidase. But it can produce catalase, urease, ammonia, and CO2. Decarboxylation tests were negative but proof of fermentation occurred as the tubes came back yellow after incubation.

Conclusion.

After performing all of these tests on the EI the most important tests used for presumptive identification were “ Ex 5-6 Catalase Test,” “ Ex 3-10 Endospore Stain,” “ Ex 3-9 Acid-Fast Stain,” and “ Ex 3-7 Gram Stain” (4). The gram-stain is deemed one of the important tests in the identification process because it was able to provide more information on the structure of the cell of the EI. There are two possible categories that the EI could have belonged to: Gram-negative and Gram-positive. The presence or absence of a peptidoglycan layer within the cell wall is the main difference between the two. If a peptidoglycan layer is present then the the layer is able to retain the crystal violet dye, which causes the organism to appear purple when examined under a microscope. If the layer is absent then the crystal violet dye is washed out by ethanol as the cell was too thin to retain it. This causes the organism to appear pink underneath a microscope the safranin. Since gram-positive organisms have that thick layer of peptidoglycan it is easy to determine that the EI was gram-positive (8). Some of the tests had weighed heavier in the presumptive identification process than other tests because the results are more straightforward in nature. The catalase test also proved

to be an important tests in determination because the EI had reacted with the reagent immediately to produce bubbles. This production leads to conclusion of a catalase positive organism. Other tests that lead to easily observed results were the Acid-Fast Stain and Endospore stain. Under a microscope, one was able to tell that the EI could not produce spores because of the absence of a green-colored center, which indicates spore formation. Tests like " Ex. 5-3 Phenol Red" did not aid in the process as it was much more difficult to interpret the results. For example, it could have been difficult to determine the reddish-pink from red or red from pink, leading to misinterpretation of results.

As noted, the EI is a non-spore forming organism, gram-positive, and non acid-fast. Additionally, the EI was catalase positive (9). Based on these results along with the other biochemical, physiological, and morphological tests the environmental isolate is most likely a part of the *Corynebacterium* genus. Common characteristics of members from this genus is non-spore forming, non-motile, gram-positive, non-acid fast, and rod-shaped bacteria (10). Most *Corynebacterium* are also catalase positive like the EI (11). Another characteristic of *Corynebacterium* is that they are aerobic which is the same as the results seen in Table 2. Physiological Test Data (10). The standard growth temperature for almost all of the test was 37 degrees celsius which is an ideal environment for *Corynebacterium* which are known to grow well in laboratory settings (11).

There is a total of 88 identified species of the genus *Corynebacterium* and 35 of those species have been in natural environments such as on animals, in soil, and in outdoor water samples. This correlates to the EI because it was

isolated from a surface that is often a contact with the shoes of students. Shoes are often in contact with outdoor soil and groundwater (11). Therefore, it is possible that dirt and groundwater containing a species of the genus *Corynebacterium* could have been tracked inside and found its way onto the tile flooring. The characteristics of the EI based on the tests are the similar to that of the genus. One of the results that was unable to obtained was that of the motility tests, which could have further provided proof or disproof of this presumptive identification. In the future, doing this tests would be useful in the identification process. It is important to note that many errors could have occurred throughout the experiment leading to incorrect interpretation of results. Additionally, contamination had appeared on the EI plates on some days which could have impacted the results of the tests performed. In order to get a more accurate presumptive, more advanced tools and analysis tests could have been performed.

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