Spring 4-19-2006

Housing Price Determinants in the Lynchburg, Virginia Area

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Confirming Identification of *Pseudomonas aeruginosa* through biochemical means from cultures collected from water from College Lake, Lynchburg, Virginia.

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In partial fulfillment of the requirements for Honors in Biology

Undergraduate

21 April 2006

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ABSTRACT

Identifying and confirming the presence of aquatic bacteria by molecular means presents many obstacles for a scientist. One bacterium that has been extensively studied because of its ability to adapt to and colonize a wide variety of ecological environments is *Pseudomonas aeruginosa.* The bacterium *P. aeruginosa* is a gram-negative bacterium that, in this case, was cultured from the water of College Lake on the campus of Lynchburg College in Lynchburg, Virginia. Samples of College Lake water were added to a biofilm reactor, and were run for three days. At that point slides were removed from the reactor, nutrient agar plates streaked and individual colonies isolated for strain and biochemical analysis. The purpose of this study was to confirm the identity of the bacterium as *P. aeruginosa* within the biofilms created, and begin preparation for determining the specific strain of *P. aeruginosa* present. First, chemical tests were carried out within a laboratory setting to confirm the bacterium's identity and then bacterium samples were isolated and frozen to create a stock supply of the bacterium. In future research, the DNA of the bacterium stock supply will be isolated using Trizol – phenol/chloroform extraction. The quantity and purity of the DNA will then be determined spectrophotometrically and finally a test will be carried out to determine the presence of *P. aeruginosa* using Polymerase Chain Reaction (PCR) and a microplate reader. At that point in the research the DNA could be run on a sequencer to determine which specific strain of *P. aeruginosa* is present.

INTRODUCTION

It has been estimated that approximately 30,000 different species of bacteria have been identified in the world; however, it is also estimated that approximately two-million species of bacteria exist in the sea, and a ton of soil contains about four- million different species. It is clear that the majority of bacteria present on planet Earth, both on land and in water remain unidentified ("Microbial diversity..." 2004). One problem that is being faced in the study of microbiology is the fact that the only known way of isolating bacteria is on nutrient agar plates; unfortunately, not all species of bacteria will grow on a nutrient agar plate. (Wilson, 1999). In the case of this research project, diversity of aquatic bacteria was the main focus. Water samples were collected from College Lake in Lynchburg, Virginia, biofilms were created and the bacteria from those biofilms were isolated and identified to determine what bacteria were present.

Many variables have contributed to the contamination and bacterial presence in College Lake. Since being created in 1934 by damming Blackwater Creek, sedimentation build up has increased from run-off caused by area construction projects. Due to this run-off and the presence of sewage in the lake due to overflow of pipes, the water quality has diminished and created a habitat favorable to a diverse population of bacteria (USACE, 2004). In the case of bacteria within an aquatic environment, some bacteria have been determined to be beneficial to the aquatic ecosystem. Some bacteria carry on such tasks as breaking down sewage in water or being an important contributor in the carbon cycle (Deacon, Sutherland, and Skillman 2004). A previous study was done by Lynchburg College graduate Matt McCarroll in 2004 in which he determined which bacteria were present in a water sample taken from the lake. From the water sample that was collected five different species of bacteria were determined to be present:

Bacillus, Lactobacillus, Corynebacterium, Pseudomonas, and Proteus (M McCarroll, 2004, unpublished data; unreferenced).

Pseudomonas aeruginosa is a gram-negative bacterium and an opportunistic pathogen which is capable of infecting humans and animals with bacteremia and is the bacterium of interest in the case of this research project. This bacterium is most prevalent in patients with cystic fibrosis and is a major cause of morbidity and mortality. It was determined that vegetables in hospitals are a main source for *P. aeruginosa*, which is unusual because the vegetables are washed in a one percent hypocholoride solution (Khan, and Cerniglia 1994). In a patient who is immune suppressed initially because of medical conditions or treatments, only 10 to 100 cells are needed to lead to gut colonization. The main habitat of *P. aeruginosa* has not been determined because it has been isolated from water, soil, plants, such as barley roots and stored onions, and a gasoline-contaminated aquifer (Morgan, et. al 1999). One thing for sure is that cultures of *P. aeruginosa* have been found in both clinical and environmental habitats; however, it is not known if these two different environments produce two entirely different types of *P. aeruginosa* or if they are the same strain (Morgan 1999).

The purpose of this project was to confirm McCarroll's identification of one of the five different species identified from the original water sample taken from College Lake (M McCarroll). Four plates were obtained from the previous study: three determined to be unknown and the other a *Pseudomonas spp.* The *Pseudomonas spp.* was thought to be *P. aeruginosa,* and therefore biochemical tests were performed to confirm the presence of that bacterium. Once that bacterium was positively identified as *Pseudomonas aeruginosa* in two of the four original nutrient agar plates, preparations began for future research in which genomic DNA was prepared

for a molecular confirmation using the bacterium's deoxyribonucleic acid (DNA) which would eventually be analyzed using a DNA sequencer.

MATERIALS AND METHODS

Growth on nutrient agar

First, fresh nutrient agar plates were made by mixing 11.5 grams of nutrient agar media with 500 milliliters of distilled water in a 1,000 milliliter Erlenmeyer flask. The mixture was then placed into the autoclave at 121°C at pressure of six to ten atmospheres for twenty minutes to insure sterilization. The mixture was then removed from the autoclave to cool. A sterile countertop was prepared, where new empty culture plates were placed. The lids of the plates were then opened half-way and the nutrient agar mixture was poured into the fresh plates to measure about one-forth of an inch deep. The lids were left halfway off and the plates were left undisturbed while hardening. Once hardened, the lids were put on and the plates were then placed into the refrigerator at 4°C to be used at a later time.

Four bacteria cultures were obtained from previous stocks in which the identifications of the bacteria obtained from water samples were determined by a series of chemical tests. The first plate obtained was labeled sample A-1, which a previous study could not determine what specific bacterium was present. A single colony was isolated with a sterile loop and streaked onto a fresh nutrient agar plate. The same procedure was performed with the second plate, B-1, which was identified in the previous study to be a *Pseudomonas spp* (McCarroll). Both plates were placed in an incubator at 37°C for forty-eight hours. At that time the plates were removed from the incubator, sealed with Parafilm and placed in the refrigerator for further use. The same procedure was then carried out with plates A-3 and E-3, both of which were determined to be unknown in the previous research; these two plates were not carried throughout the entire

project. The A-3 and E-3 nutrient agar plates were streaked to create a new stock to preserve for further research.

Storage of Bacterial Samples

In preparation for running Polymerase Chain Reaction (PCR), samples of the *P. aeruginosa* strain were prepared for freezing to create a stock of bacteria. The nutrient agar plates that had been cultured in the previous steps were obtained as well as four newly prepared nutrient broth tubes. To begin a storage buffer was prepared using a procedure for deep cold storage of bacteria (Hanahan, 1985). In an Erlenmeyer flask, 1000 milliliters of water were mixed with 7.4 grams of KCl, 7.5 grams of $CaCl_2 \cdot 2H_20$, 100 grams of redistilled glycerol, and 10 milliliters of a 1 molar stock solution of potassium acetate at pH of 7.5. Once all mixed together, the final pH of the buffer was adjusted to a pH of 6.2 (NaOH was added to make the solution more basic or HCl was added to make the solution more acidic). The storage buffer was then covered with Parafilm for use at a later time. For each of the nutrient agar plates that have been used to grow *P. aeruginosa* (A-1, A-3, B-1and E-3), two nutrient broth tubes were labeled. The sterile loop was obtained by flaming in a Bunsen burner and then one colony was obtained from each of the nutrient agar plates for each of the corresponding nutrient broth tubes. Once the colony was on the loop, it was then submerged in the nutrient broth of the appropriate tube and quickly swirled in the broth. The loop was removed from the broth and then re-sterilized in the flame from the Bunsen burner. This process was repeated for each tube of nutrient broth (two for each nutrient agar plate, for a total of eight tubes). After loosely capping the tubes, the tubes were placed in a test tube rack and put into a shaking incubator at 37°C for approximately 72 hours.

After 72 hours the tubes were removed from the incubator and placed on a sterile countertop. Growth was determined by cloudiness of the nutrient broth. Only the tubes that became cloudy were used in the next step of the procedure. The storage buffer that was prepared previously was obtained as well as enough 2.0-mL cryo-centrifuge vials with screw caps for each nutrient broth tube (eight total). Each of the 2.0mL centrifuge vials was labeled appropriately and then each tube received 200 microliters (2 milliliters) of storage buffer. Next, using individual sterile pipettes 1800 microliters (18 milliliters) of each bacterium were pipetted into each corresponding centrifuge tube. Once all the tubes were tightly capped, they were all placed into a rack and placed into a small box. Paper towels were used to insulate the tubes inside the box to ensure that the bacteria mixed with sample buffer slowly froze. The box with tubes inside was then placed into a -20°C freezer overnight. The next day, the box was moved to a -70°C freezer and remained there for a week. After that week's time period the tubes were put into liquid nitrogen to finish the freezing process. One of each of the samples (A-1 and B-1) were picked to unfreeze prior to undergoing liquid nitrogen treatment to insure that the bacteria was not damaged during freezing. The two tubes that were to be unfrozen were placed into a water bath at 50°C to thaw quickly. Four fresh nutrient broth tubes were obtained and labeled (A-1.1, A-1.2, B-1.1 and B-1.2), two for each centrifuge tube. One milliliter of storage buffer/bacterium solution was pipetted into each of the nutrient broth tubes. Once all tubes had been pipetted with the once frozen solution, the tubes were placed into a test tube rack and placed into a shaking incubator at 37°C for 48 hours. The nutrient broth tubes were then observed for cloudiness and the tubes that exhibited cloudiness were streaked onto individually labeled fresh nutrient agar plates with a sterile loop and incubated at 37°C for 24 hours. After the 24 hours the plates were

removed from the incubator and compared to the original nutrient agar plates to insure the same bacteria was still present.

The remainder of the biochemical testing was done with the four nutrient agar plates obtained from streaking the bacteria from the frozen samples.

Gram Stain

This differential staining technique was done to determine if the bacteria present was gram positive or negative and also the shape of the bacteria. A heat fixed smear of each bacterium was prepared on separate slides. The slides were then placed on a staining rack over a waste container. Crystal violet was applied to each smear for 30 seconds, and then rinsed for 5 seconds with deionized water. Then Gram's iodine was applied to each smear for 1 minute, then rinsed with DI water for 5 seconds. Each smear was then decolorized with 95% ethanol for about 20 seconds and then rinsed with DI water for 5 seconds. Finally, safranin was applied to each smear for about 1 minute and rinsed with DI water once more. Each slide was then blotted dry with bibulous paper and observed under the microscope to determine what size the bacteria were and if the bacteria present was gram positive or negative.

Differential Media

These media distinguish between different groups of bacteria. All of the media used was prepared according to directions obtained from the containers of stock powder obtained from DIFCO Labs in Detriot, Michigan.

Eosin methylene blue (EMB) plates were prepared from stock powder. One plate was streaked (from nutrient agar plates cultured from frozen stock bacteria), and labeled (A-1.1, A-1.2, B-1.1, and B-1.2) for each bacterium and placed into the 37°C incubator for 24 hours. After

that time period the plates were observed for lactose fermentation which would have been evident by dark bacterial growth with a green sheen appearance.

Triple Sugar Iron Agar (TSIA) slants were prepared by first mixing 19.5 grams of TSIA media with 300 milliliters of water. The media was placed on a heat/stir plate and heated until almost boiling. Test tubes were filled with 10 milliliters of media and capped. The tubes were then placed into the autoclave for sterilization under the same conditions as the nutrient agar media. After removed from the autoclave, tubes were placed in a slanted position in which the butt of the tube remained covered and the media slanted up the side of the tube. After hardening occurred, an inoculating needle was obtained, and a single colony of each bacterium was scraped from each of the nutrient agar plates. The needle was then used to stab the butt of the agar and as removing the needle from the agar an "S" was made on the surface of the agar. The tubes (four total) were loosely capped and then placed in a test tube rack in a 37°C incubator for 24 hours. After that time period the tubes were observed for color change which would be caused by the fermentation of lactose or sucrose, this would be observed by a yellow color on the butt, on the slant of both. A black color in the butt would be indicated that H_2S was being produced.

Phenol red broth tubes with Durham tubes were obtained from the lab (four total). With a sterile inoculating tube a single of colony from each bacterium was obtained from the nutrient agar plates and submerged into individual phenol red broth tubes. The tubes were placed into a shaking incubator at 37°C for 24 hours. After the time period the tubes were observed for a color change based on the fermentation of glucose. A color change to yellow would indicate glucose fermented to produce acid, and a bubble within the Durham tube would indicate that gas was produced.

Pseudomonas F agar plates were made using the same procedure as the nutrient agar plates, but this time 3.5 grams of Pseudomonas F agar media was mixed with 100 milliliters of distilled water. Sterilization was achieved by using the autoclave and when cooled, the plates were poured, left undisturbed for fifteen minutes to allow hardening of the agar and then stored in the refrigerator until needed. Pseudomonas F Agar plates were obtained from the 4°C refrigerator and streaked with each bacteria (from nutrient agar plates from the frozen stock), two plates for each bacterium (eight total plates). One plate from each bacteria was placed in a 42°C incubator overnight and was then observed for growth tolerance at an increased temperature. The second plate for each bacterium was placed into a 37°C incubator and after 24 hours was observed under short wave UV light for fluorescence.

RESULTS

Growth on nutrient agar

The initial nutrient agar plates that were streaked from the prior research project grew the same. The A-1, B-1, A-3 and E-3 when observed all produced a white growth on the nutrient agar plates that were used for further studies.

Storage of Bacterial Samples

The four samples (A-1.1, A-1.2, B-1.1 and B-1.2) that were re-cultured after freezing, first in nutrient agar broth and then on nutrient agar plates exhibited growth after incubation. When compared to the *P. aeruginosa* that was cultured on nutrient agar plate previously (prior to freezing), the bacteria looked the same and the assumption was made that *P. aeruginosa* was present and undamaged from the freezing protocol (Figure 1).

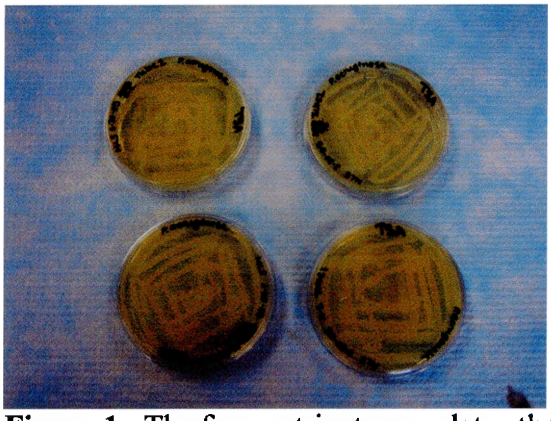

Figure 1. The four nutrient agar plates that were cultured (37°C for 24 hours) from the frozen stock bacteria. Starting at the right bottom and moving clockwise corner plates are: A-1.1, B-1.1, B-1.2, and A-1.2). A white growth was observed on all plates.

Gram Staining

When observed under the microscope all four samples, A-1.1, A-1.2, B-1.1, B-1.2, were seen as gram-negative rod shaped bacteria which would be characteristic of *P. aeruginosa*.

Eosin methlyene blue (EMB) agar

Four plates were streaked one for each of the frozen bacteria stock nutrient agar plates. After the incubation period the plates were observed for lactose fermentation. The plates all had growth that took the reddish brown color of the EMB agar and no sheen was observed. This means that the bacteria present was not a coliform and the bacteria present followed the characteristic pattern of *P. aeruginosa*.

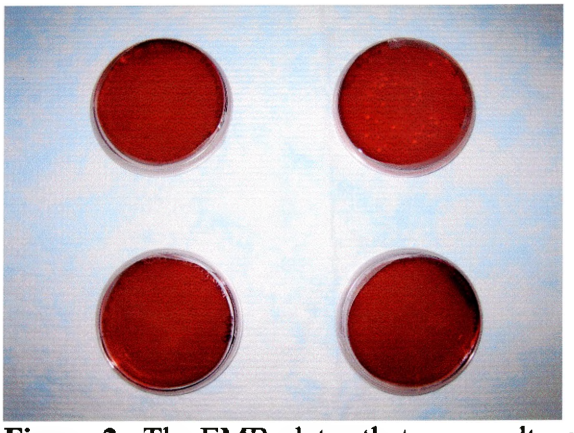

Figure 2. The EMB plates that were cultured (37°C for 24 hours) from the frozen stock bacteria that were grown on nutrient agar plates. Starting at the right bottom and moving clockwise corner plates are: A-1.1, B-1.1, B-1.2, and A-1.2. The red dots seen on A-1.2 are water droplets not growth. The growth that appeared on the plates took the color of the agar and no sheen was seen. Therefore, lactose was not being fermented and the bacteria present is not a coliform.

Triple Sugar Iron Agar (TSIA)

Four tubes were cultured one for each of the frozen bacteria stock nutrient agar plates. The butts of the experimental tubes did not change color, they remained red which means that no sugars were being fermented (glucose, lactose, sucrose). The slant had a faint hint of yellow when taken out of the incubator which may mean a small amount of glucose being fermented, but the yellow color later disappeared. The agar did not exhibit a black color which means that no H_2S was being produced and no gas was produced because the agar was not fragmenting.

Figure 3. The experimental TSIA tubes were compared to a control tube to determine if any fermentation had occurred (grown at 37°C for 24 hours). From far left to right the tube are: B-1.2, B-1.1, Control, A-1.1, A-1.2. The yellow color can not been seen in this picture because it disappeared once removed from the incubator.

Pseudomonas F Agar

A total of eight Pseudomonas F Agar plates were streaked with the bacteria from the frozen stocks. After incubation at 42°C of four of the plates, growth was observed on all four plates. After incubation at 37°C of the other four plates, all plates exhibited growth, but none of the plates exhibited fluorescence under short-wave UV light. Both of these results are characteristic of *P. aeruginosa* and distinguished this bacterium from other *Pseudomonas spp.*

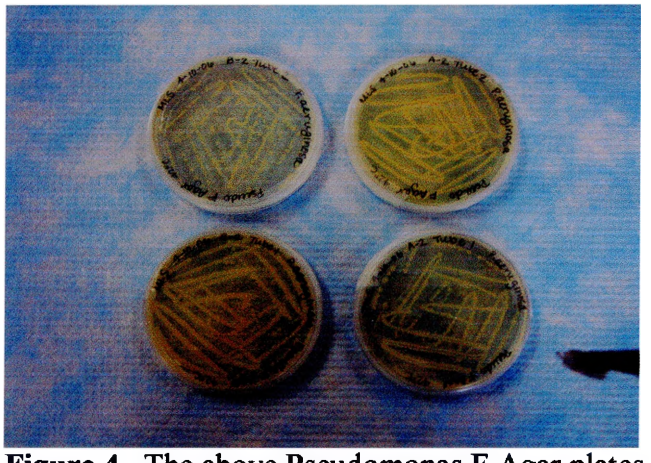

Figure 4. The above Pseudomonas F Agar plates were grown at 42°C, and all plates exhibited growth. Plates are labeled beginning in the right-hand bottom corner and going counter clockwise: A-1.1, B-1.1, B-1.2, and A-1.2.

Phenol Red Broth

Four tubes were cultured, one for each of the frozen bacteria stock nutrient agar plates. When observed after incubation tubes B-1.1, A-1.1 and A-1.2 were a bright yellow color which meant that acid was produced by glucose fermentation. Tube B-1.2 changed to a light orangish-yellow color which meant that acid production began as a result of fermentation, but did not proceed at the intensity of the other three tubes. Gas production did not occur in any of the tubes, which was evident because none of the Durham tubes exhibited bubbles. These results are characteristic of *P. aeruginosa*.

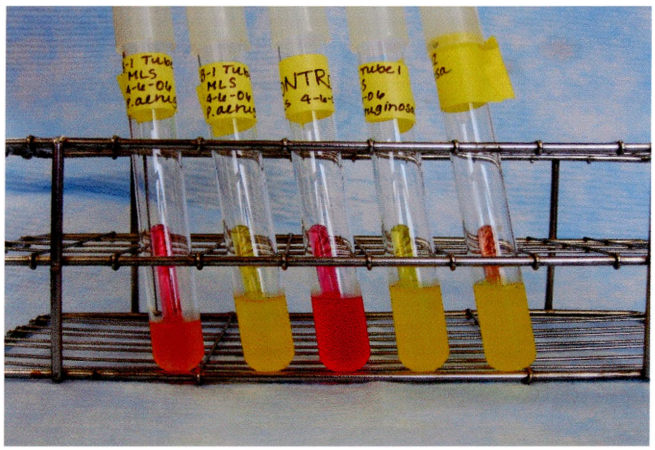

Figure 5. The above phenol red broth tubes were incubated at 37°C in a shaking incubator overnight. From far left to right the tube are: B-1.2, B-1.1, Control, A-1.1, A-1.2. The control tube was used to compare the results after incubation. Acid production is very evident in 3 of the 4 experimental tubes.

DISCUSSION

The diversity that exists in the world of bacteria can sometimes prove to be a cause for difficulty when attempting to identify an unknown bacterial specimen. In this experiment, preliminary tests were done to give a starting point for this research project. If those preliminary tests had not been done, tests would have begun with gram staining, a differential test that divides all bacteria into two different categories based on the cell wall that is present. Because the bacterium of interest in this project is a familiar one, identifying the bacteria through biochemical means was not the difficult issue. Issues arose when attempting to find primers that could be used to run Polymerase Chain Reaction (PCR) to amplify the DNA of the bacterium. Because of time and money constraints, biochemical confirmation of the bacterium present was the focus of the results in this research.

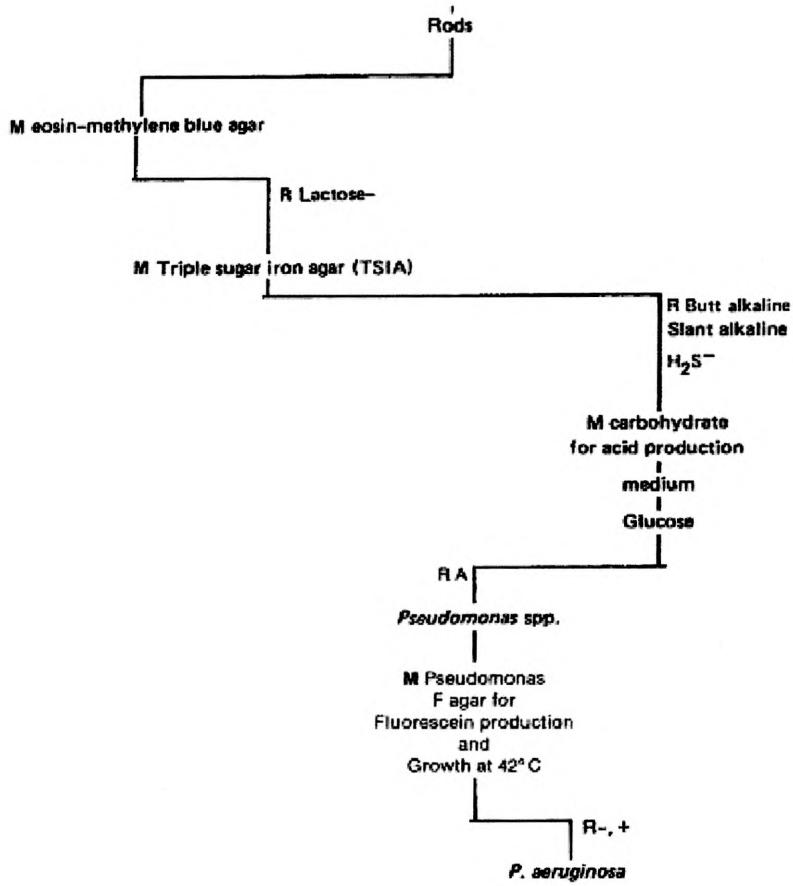

Figure 6. Pathway which was followed to determine which chemical tests were used to determine bacteria present. (Wistreich, 2003). The diagram above helped to determine which tests were performed to determine what bacteria were present. The bacteria was determined to be a gram-negative rod, so an EMB plate was then streaked to determine if lactose fermentation occurred at incubation at 37°C. Because no lactose fermentation occurred a TSIA test was done next. Based on those results a glucose test in phenol red broth was next and finally a test on the Pseudomonas F Agar plates was last.

In the previous study, plate A-1 was determined to not exhibit growth on triple sugar iron agar (TSIA), but according to the pathways provided in a lab manual, *P. aeruginosa* should have grown on that media (Wistreich, 2003). In this research project the bacteria was again grown on TSIA and the results obtained were different. The results given in previous research did not address the fermentation of sugars, but only that there was no growth (McCarroll 2004). When determining if *P. aeruginosa* was present, the concern is the color change of the agar due to

fermentation of sugar. The results collected with this research indicated that no fermentation occurred which supports the conclusion that *P. aeruginosa* is present (Leboffe, 2005).

The results obtained from tube B-1.2 for phenol red broth could have been interpreted by another researcher to be a negative result for glucose fermentation to produce acid. After looking in the Bergey's Manual of Determinative Bacteriology, it was determined that *P. aeruginosa* could either have a positive reaction for acid production and be observed yellow or not have a positive reaction and be observed as a pinkish-orange (Bergey, 1994). It was also read in other literature that a pinkish-orange color could result because the culture was incubated over 18 hours and a reversion occurred meaning that the organism exhausted all of the carbohydrate and the pH shifted causing the yellow that was not observed to turn pinkish-orange. Prior to 18 hours, the culture may have been yellow, but because the culture was left for 24 hours, accurate results may not have been collected (Leboffe, 2005). To obtain a definitive result, the test should have been performed again, but because of time constraints this was not able to be done.

The results from the other biochemical tests: EMB, and Pseudomonas F Agar allow the conclusion to be made that *P. aeruginosa* is in fact the bacterium that is present. The last test performed confirmed in fact that *P. aeruginosa* was present because the results singled out *P. aeruginosa* from other *Pseudomonas spp.* Growth at 42°C allows, *P. aeruginosa* to be differentiated from other bacteria because of its tolerance to grow at an extreme temperature. The fact that the bacteria did not produce fluorescence when exposed to short wave UV light also differentiated it from other *Pseudomonas spp.* (Wistreich, 2003).

The bacterial samples which were used to carry out the various biochemical tests were obtained from cultures grown from frozen bacterial stocks. The purpose of these stocks was to

prepare the bacterium to eventually be run through Polymerase Chain Reaction (PCR) to amplify the DNA and then run the bacterium on a sequencer to confirm that *P. aeruginosa* was present and then determine exactly which strain was present. If there had been more time each of the frozen samples should have been unfrozen to insure that no damage occurred, not just the two that were unfrozen in this project.

The next step for this research project, and unfortunately because of time constraints will have to be continued in the future, would be to isolate the DNA using Trizol – phenol/chloroform extraction. The quantity and purity of the DNA would then be determined spectrophotometrically and finally a test would be carried out to determine the presence of *P. aeruginosa* using PCR and a microplate reader. Eventually, following those procedures the DNA of the bacterium would be run on a sequencer to determine which strain of *P. aeruginosa* was present.

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