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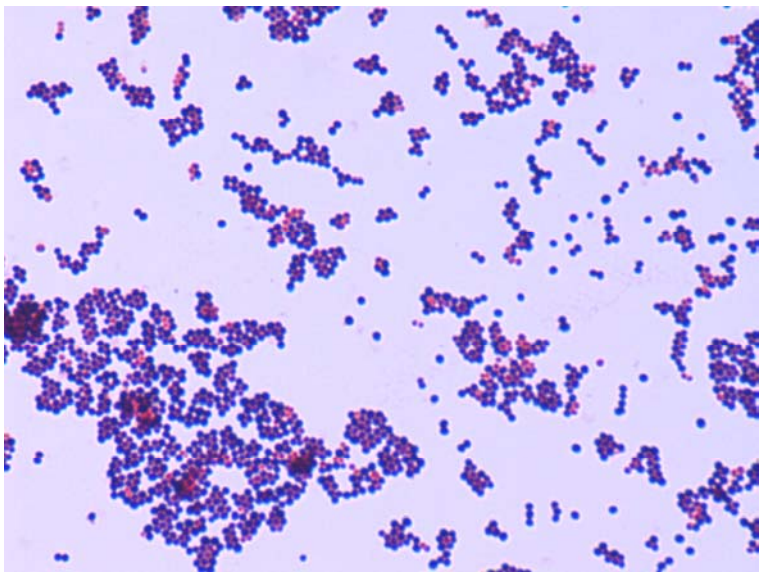
# *Sierra College Journal of Microbiology*



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# SIERRA COLLEGE JOURNAL OF MICROBIOLOGY

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**Editors:** SASHA WARREN and HARRIET WILSON

**Contributors:** Biology 4 & 8B students – Spring Semester, 2008

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**Cover photo:**
A Gram-stained smear of *Micrococcus varians*, isolated by Iryna Vlasik and Amber Andronico (pg. 24). This isolate was obtained from tap water that had been filtered with a PUR filter. Magnification: 1000x. (Photomicrograph: S. Warren)

# SIERRA COLLEGE JOURNAL OF MICROBIOLOGY

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# Isolation and Identification of an Unknown Organism in A Water Sample Obtained from an Outdoor Children's Playset in Auburn, California.

JENNIFER AGUDELO, LEISHA BAEZ FLORES, AND CHERECE MITCHELL

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Received 2 May 2008/Accepted 9 May 2008

**A water sample was obtained from an outdoor children's playset in Auburn, California. This sample was used to isolate and identify an unknowm organism we hypothesized to be *Escherichia coli*. Tests were done to establish the characteristics of the organism, identify it, and determine the potential health concerns related to the unknowm organism. The isolated organism was found to be a gram negative cocco-bacilli. Test results were compared to *Enterobacter aerogenes*, *Escherichia coli*, and *Klebsiella pneumoniae* which were it's closest relative, according to the National Center for Biotechnology Information website. These tests proved the unknown organism to be *Enterobacter aerogenes*, which is an opportunistic pathogen that can be associated with certain intestinal disorders. The information used for this project was obtained from the Microbiology Lab Syllabus, Bergey's Manual of Systematic Bacteriology, and the National Center for Biotechnology Information website.**

## INTRODUCTION

A small pool of water had settled in an outdoor children's playset, located in Auburn, California. The children playing with this toy will have physical contact with organisms in this water. Due to concerns about the health risks associated with children playing with outdoor toys, a water sample was collected for lab testing. The goal is to isolate and identify an unknown organism from the water sample. We hypothesized to find *E. coli* since it's commonly found in water supplies and looked for when testing water. If *E. coli* or any coliform is found on an outdoor playset, it raises an alarm that fecal matter from humans or animals is present.

## MATERIALS AND METHODS

Using a sterile loop we streaked a portion of a water sample, from an outdoor playset, on a TSA plate for colony growth and an EMB plate to test for *E. coli*. Both plates were incubated for about 24 hours at 37$^{o}$C. We chose a white colony versus an off white colony to re-streak on a TSA plate for isolation because it looked shiny and pretty (Wilson, 2007). That plate was then placed in the incubator for 24 hours at 37$^{o}$C. An indirect stain was performed to visualize cell morphology(Wilson, 2007). A gram-stain and KOH test was done to determine if the organism was gram negative or gram positive for cell wall morphology(Wilson, 2007).

A colony sample was used to isolate chromosomal DNA for analysis by vortex and heat. The goal of using PCR to amplify rDNA is to isolate the 16S ribosomal DNA using primers

Bacteria 8-forward and 1492-reverse. PCR was then run on a 1% agarose gel and the 1500 bp product was purified, using the QIA quick Gel Purification Kit. Purified rDNA was sent to the Division of Biological Sciences sequencing facility in UC Davis and sequenced using primer Bacteria-8-forward. This gave us one electropherogram back. The sequence from the isolated organism was compared to those in the database at National Center for Biotechnology Information website using the Basic Local Alignment Search Tool. Based on sequencing results we performed SIM, MRVP, urease, citrate tests (Wilson, 2007).

## RESULTS

There were two different types of colonies that grew on the TSA plate we first streaked. One was a shiny white color and the other an off white color. The EMB plate had dark red colonies with no color change of metallic green. We isolated the white colony in a new TSA plate and observed shiny, white, circular, entire, opaque and convex colonies that were about 1-3mm in diameter. The indirect stain revealed our organism to be cocco-bacilli (Wilson, 2007). The gram stain showed us pink organisms, and the KOH test gave us a "snotty" result (Wilson, 2007). After comparing our rDNA sequence to National Center for Biotechnology Information using the Basic Local Alignment Search Tool our rDNA closely matched *Enterobacter aerogenes* (accession # AF395913.1). The rDNA nucleotide sequence showed a 97% similarity, with a score of 1096, a ratio of 638/815 of matching nucleotides. There were two other organisms which had a 97% similarity, and they were *Escherichia coli* and *Klebsiella pneumoniae* (NCBI, 2008). We performed more tests in order to differentiate which species our rDNA matched. The SIM test resulted in a yellow tube agar with growth away from the stab line (Wilson, 2007). The MR test result was golden brown and the VP test result was cherry red (Wilson, 2007). The urease test stayed peach (Wilson, 2007). The citrate test turned blue (Wilson, 2007).

Figure 1- SIM tube result.

## DISCUSSION

The water sample collected for this project was first streaked on a TSA plate for colony isolation of an unknown organism. An EMB plate was also streaked due to our hypothesis of finding *Escherichia coli*. There was no metallic green color anywhere in the EMB plate to indicate that *Escherichia coli* was present. A white colony was chosen for isolation over an off-white colony and was re-streaked on a new TSA plate. An indirect stain was done to determine cell morphology which was cocco-bacilli. A gram stain and KOH test confirmed that the organism is gram negative and lysed when KOH was added due to that gram negative cell wall structure. Results from the NCBI BLAST revealed that *Enterobacter aerogenes*, *Escherichia coli*, and *Klebsiella pneumoniae* were closely matched to our rDNA sequence (NCBI, 2008). The SIM test indicated that our organism didn't make indole or $H_2S$, and was motile (Wilson, 2007). The MRVP revealed that the organism performed butanediol fermentation (Wilson, 2007). The citrate test showed that the organism can utilize citrate and the urease test confirmed that the organism doesn't have the enzyme urease (Wilson, 2007). These results concluded that our organism was more closely matched to *Enterobacter aerogenes* (Wilson, 2007).

| TEST | *Enterobacter aerogenes* | *Escherichia coli* | *Klebsiella pneumoniae* | Our Isolate |
|---|---|---|---|---|
| **Indole (SIM)** | - | + | - | - |
| **Motility (SIM)** | + | + | - | + |
| **Methyl Red** | - | + | + | - |
| **Voges Proskauer** | + | - | - | + |
| **Citrate** | + | - | + | + |
| **Urease** | - | - | + | - |

Table 1- Comparison of test results from unknown organism and closest matching species.

These results proved our hypothesis to find *Escherichia coli* wrong. *Enterobacter aerogenes* is an opportunistic pathogen, a fecal indicator, and is found in soil, human and animal feces, and also dairy products (Richard, 1984). The pathogenicity of the *Enterobacter* group is of a low order, but they can be associated with certain intestinal disorders and other syndromes (Bailey, 1966).

## ACKNOWLEDGEMENTS

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# Isolation of Common Bacteria Found on an Out-patient Exam Table and the Effectiveness of the Approved Disinfectant

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**A study was conducted to test the sterility of patient exam tables in outpatient clinics. Swab samples were taken from two doctor's exam tables before and after they were sterilized. The results show the presence of bacterial growth prior to sanitizing and no growth following sanitizing, thus the disinfectant agent PDI Sani- cloth germicidal disposable wipes removed all viable cells. From the cultures grown in lab, a bacterium was isolated and analyzed from our contaminated plate (prior to sanitizing). Through utilizing PCR, DNA sequencing and the Bergey's Manual, the isolated bacterium was identified as *Bacillus subtilis*, an organism that is not pathogenic to humans.**

## INTRODUCTION

In everyday life humans are surrounded and exposed to bacteria. There are some bacteria that act in favor to the host they survive on, in contrast to the good there is also a bad. Some bacteria that individuals are exposed to can lead to various life-threatening or life changing diseases. The severity of illness is dependent upon many factors. For example, the quantity or the strain of bacteria that an individual comes in contact with may have an adverse effect. Therefore, we set out to discover what types of organism are thriving on exam tables in an outpatient clinical setting.

Based on literature review regarding contamination of exam tables in patient rooms, one may predict that the results of the bacteria collected and analyzed will show a presence of many common bacteria (Environment Protection Agency, 1997). On average there are 4 to 10 patients seen between the cleaning of the exam tables. The only barrier between the patient and the exam table is a paper lining. In some situations the beds are cleaned by wiping with PDI Sani-cloth immediately following an exam when the patient has a known or suspected infectious disease such as MRSA or HIV. We would like to test the effectiveness of the PDI Sani-cloth germicidal disposable wipes.

The bacteria most likely to show up in the samples gathered and tested in this experiment will be normal flora such as; *Staphylococcus aureus, Staphylococcus epidermis, Corneybacterium diphtheria*, and *Micrococcus luteus* (Microbial Flora of Skin, 2008). In addition, there are common opportunistic pathogens that are often found in healthcare environments, such as: *Streptococcus pneumoniae, Klebsiella pneumoniae, Escherichia coli, Haemophilus influenza*, and *Acinetobacter baumannii* (Maley, 2000). Are patients really safe from exposure to the contaminated surfaces of these tables? The goals of this work are: to isolate bacterial organisms from patient exam tables and to discover the effectiveness of the disposable sanitizing wipes.

## MATERIALS AND METHODS

A patient exam table in an out patient clinic was sampled after six patients were seen and came in contact with the exam tables. The exam table was sanitized prior to the six patients. After the six patients were seen, the edge of the table (which has the most exposure to contact) was swabbed with a sterile Q-tip like applicator. The applicator was then submerged into a small sterile tube containing sterile water. The next steps were to plate out the water/organism mixture onto TSA plates and incubate them at 37°C for 48 hours. After the 48 hour incubation period there were approximately 10 different organisms present on our TSA plate. We chose to work with one organism in particular because of its colony morphology.

The following tests were used to identify the cellular morphology: Indirect stain, Gram stain, Endospore stain, Acidfast stain, Capsule stain, KOH test, and a Wet Mount.

The next step was to isolate the DNA. We isolated the chromosomal DNA by conducting a PCR reaction. Our goal in using the PCR reaction was to amplify and make a lot of copies of our organism 16s ribosomal RNA gene (rDNA). We used the primer Bacteria 8-F and 1492 reverse. Once we had many copies of the rDNA gene, a gel containing 1% agarose could be set up and the 1500Bp product was ready to be purified removing everything (primer, taq polymerase…) but the gene. The purification step was done by using the QIAquick kit which is made by Qiagen. After using this kit our end product was many copies of the purified 1500Bp rDNA gene and nothing else.

The purified gene was then sent to the Division of Biological Science Sequencing Facility at UC Davis. The gene was then sequenced using the primer Bacteria 8-F. We then received an electropherogram of our organisms DNA sequence and we compared it to other known sequences in the National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST).

Based on the results from the DNA sequencing we conducted the following tests: Indirect Stain, Gram Stain, Endospore Stain, Acidfast, Capsule, KOH, Wet mount, and Catalase.

## RESULTS

The DNA results show our organism is 99% identical to *Bacillus subtilis* accession # A4825035.1 with 99% accuracy. The ratio of identical matching nucleotides is 872/878 and the Bit Score is 1589. The colony morphology of the organism was: filamentous form, undulate margin, umbonate elevation, opaque optical character, whitish beige pigment, dry and wrinkled surface texture, and a size range from 3mm x 3mm – 6mm x 7mm. All of the colonies also displayed a white ridged design in the middle. Our isolate had long single celled rods that are motile and are 1.5 micrometers x 1 micrometer in size, and is catalase positive.

We preformed the following tests and obtained the following results: KOH: no snot, KOH negative. Gram stain: purple, Gram +. Acidfast stain: blue, no mycolic acid, and non-acid fast. Endospore stain: spores present, the organism forms endospores under starvation and other extreme environments/conditions. Capsule stain: capsules present; the organism does have capsules. After comparing our organism to the Bergey's Manual, all of the cellular, colony and physiological (catalase) characteristics matched (Garvie1872).

## DISCUSSION

From our literature reviewed, *Staphylococcus aureus, Staphylococcus epidermis, Corneybacterium diphtheria,* and *Micrococcus luteus* are the most common bacteria found on the epidermis (Microbial Flora of Skin, 2008; Maley 2000). We presumed by swabbing an out-patient exam table the culture would produce one or more of the bacteria listed above. Our plate grew out several different bacterial colonies, from these colonies one bacterium was then isolated and identified as *Bacillus subtilis* which is naturally found in the environment. The DNA results matched to *B. subtilis* and the biochemical test results matched exactly to *B. subtilis'* morphological and biochemical characteristics listed in Bergey's Manual (Garvie 1872). *Bacillus subtilis* has been found to be a hearty bacterium that can endure harsh environments and starvation (Todar, 2008). The only way to rid of this bacterium completely is through autoclaving. *B. subtilis* is also known to be a competent cell allowing it to undergo transformation. This may allow the bacterium to take up genes that will increase its survival rate as well as being used for our purposes regarding recombinant DNA technology (Todar, 2008).

Prior to our experiment we expected to find a potential pathogen; however, our results demonstrated a non- pathogenic bacterium that is prevalent in our environment. We believe we did not find one of the above listed bacteria due to the fact that we only isolated one out of several different colonies.

Prior to our education in microbiology we may have once shared the idea that all microbes cause disease. The average individual in our society associates the word microbe as something harmful. What most individuals do not know is that microbes are very important to our eco-system and they aid in our existence, for example, recombinant technology, bioremediation, and the medical field.

## ACKNOWLEDGEMENTS

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# Isolation and Identification of Potential Pathogen on Children's Playground Equipment

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Received 2 May 2008/Accepted 9 May 2008

**We tested local playground equipment looking for potential pathogenic organisms like *E. coli*. We did this by collecting samples, culturing, testing and isolating them to find a Gram-negative organism. Playgrounds seemed like a good place to find *E.coli* because children play in diapers, don't wash their hands and frequently have immune systems less developed than adults. Although we did not find *E. coli*, we did find the organism *Acinetinobacter haemolyticus,* which is potentially pathogenic to immune compromised individuals.**

## INTRODUCTION

Many forms of bacteria are dangerous to humans. Anytime we eat, drink or touch our hands to things that come into contact with bacteria, there is risk of ingesting them. We wanted to look to see if we could find potentially dangerous organisms in what most people assume to be safe environments like playgrounds. Was there any risk to children being exposed to bacteria like *Escherichia col:* 0157:H7 from simple playground equipment? Playgrounds seemed like a likely place to find *E.coli* because children play in diapers, don't wash their hands frequently and have immune systems less developed than adults. We collected samples from playground equipment at Rocklin Memorial Park on October 28, 2007 to see what potentially lived where children were playing.

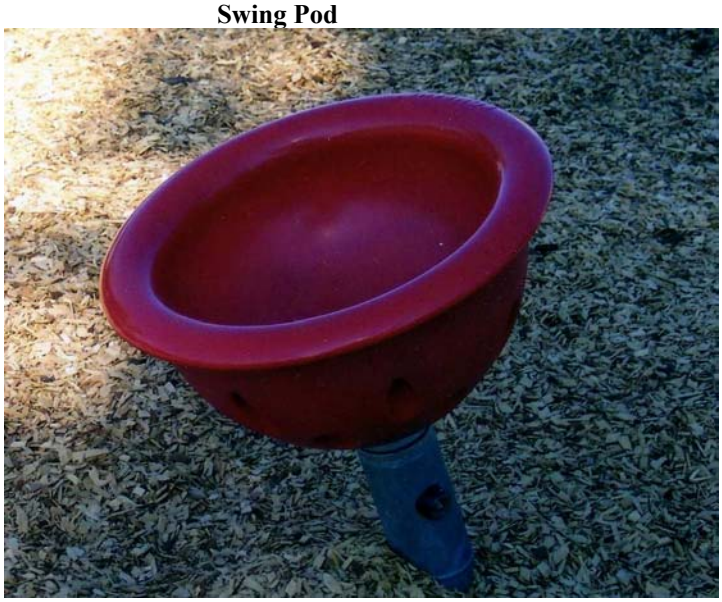
**Rocklin Memorial Park**

## MATERIALS AND METHODS

Samples were collected from four different pieces of playground equipment by swabbing with sterile cotton swabs and swirling swabs in small sterile distilled water vials. The contents of the small vials were poured onto 4 separate Nutrient agar plates and incubated for 48 hours at 37 degrees Celsius.

Of the 4 plates, using colony morphology, we identified and selected one colony from the Agar Plate containing cultures taken from Swing Pod playground equipment and transferred this with a sterile loop to MacConkey's agar (MAC). MAC being selective for Gram negative organisms like *E. coli* was used to further identify our unknown. We also continued to maintain a pure culture of this isolated organism on Nutrient Agar for future references.

**Swing Pod**



Once a pure culture was established, colony and cellular morphology was observed, the following cellular stains were performed: Gram Stain, Endospore, Acid-Fast, Capsule (Wilson & Warren, 2007).

Isolation of the organism's chromosome was achieved by boiling and vibrating with a vortex machine. Polymerease Chain Reaction PCR was used to amply the organism's DNA coding for 16S r-RNA. Oligonucleotide primers Universal 1492 reverse and Bacteria 8 forward were use to select for these base pair sequences. PCR reaction was run on a 1 % agarose gel and the 1500bp product was purified using the QIAquick gel Purification kit (Qiagen). Purified rDNA was sent to the D.B.S. Sequencing Facility at U.C. Davis and was sequenced with the primer Bacteria 8 forward.

The sequence from the isolated organism was compared to those in the database at the National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST). Based on results from sequencing, we performed a Wet Mount Motility and O/F (Wilson &Warren, Microbiology Laboratory Syllabus, July, 2007)

## RESULTS

On MacConkey's Agar, our pure culture organism was circular in form, had raised elevations and the margin's were entire. It had an opaque look, white and glistening and was approximately 2 mm in size. A KOH test produced sticky pasty snot and a Gram Stain showed red cells. The Acid-Fast test produced blue coccibacilli cells and the Endospore stain revealed green endospores. White capsules were seen in the Capsule stain test.

**Colony Morphology from Pod-Swing MacConkey's Agar Plate:**

| | | | |
|---|---|---|---|
| **Form** | *Circular* | **Pigmentation** | *White* |
| **Margin** | *Entire* | **Size in mm** | *2 mm* |
| **Elevation** | *Raised* | **Surface Texture** | *Glistening* |
| **Optical Character** | *Opaque* | | |

**Stain & KOH Test Morphology from Pod-Swing:**

| Stain or Test | Result |
|---|---|
| *KOH* | *Gram -* |
| *Gram Stain* | *Gram -* |
| *Acid-Fast Stain* | *Non-acid fast cells* |
| *Endospore Stain* | *Many endospores* |
| *Capsule Stain* | *Glycocalyx layer in cell wall; many capsules* |
| *O/F Test* | *Non-fermenter* |
| *Wet Mount (Motility)* | *Non-motile* |

**DNA Data**

| | |
|---|---|
| Organism identified | *Acinotobacter haemolyticus* |
| Accession # | EU352764 |
| Bit Score | 97% |
| Match | 888/907 |
| Sequence of my organism is | 97% identical |

Our testing revealed our organism to be a Gram-negative, non-motile coccibacilli. It produces endospores, has a glycocalyyx capsule layer, and is nonfermentative. Our DNA data identified our sequence to be 97% identical to *Acineotobacter haemolyticus.*

## DISCUSSION

According to Dr. L. Marcus, in his newsletter from the Lancet Laboratories this *Acinetobacter* is a nonfermentative, Gram-negative, encapsulated coccobacilli that grows in various lengths (Marcus, 2005). This is supportive of our test results and DNA results.

We began our project looking for *E. coli*. Although we were unable to isolate *E. coli*, we were able to isolate a Gram-negative organism *Acinetobacter haemolyticus*. *A. haemolyticus* is

typically considered to be a non-pathogenic to healthy individuals; however, it is potentially pathogenic to immune compromised individuals. Since children's immune systems are less developed than adults, precautions as with any potential pathogen would be advisable.

## ACKNOWLEDGEMENTS

Sierra College Science Department
Dr. Sasha Warren

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# Isolation of *Kocuria rhizophila* from Shopping Cart Handles

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Received 2 May 2008/Accepted 9 May 2008

**Samples were taken from shopping cart handles to determine what types of organisms were present. After streaking the samples onto TSA plates, over twenty separate colonies were observed. One colony was isolated and determined to be *Kocuria rhizophila* by DNA sequencing of the 16s rDNA gene. The organism was subjected to several physiological tests in an effort to confirm its identity. This was done by comparison of our test results to the results for that organism as recorded in Bergey's Manual of Determinative Bacteriology. The identity of the organism was confirmed to be *Kocuria rhizophila* using these techniques. *Kocuria rhizophila* is a normal flora and thus, not pathogenic.**

---

## INTRODUCTION

Almost a decade has passed since the alarm was sounded about harmful bacteria being present on shopping cart handles. Back in 1999, a team of microbiologists at the University of Arizona reported the results of a major health study measuring "levels of contaminants on frequently touched public surfaces that can transmit infectious diseases (Business Editors and Health/Medical Writers, 1999)." Shopping carts were one of the worst offenders. In November 2006 several research studies concluded, "the typical shopping cart handles 1 million germs, shopping carts have more germs than a public rest room and food-borne bacteria cause 75 million illnesses annually (PR Newswire, 2006)." A lot of concern was raised by the alarm; however, a simple solution was offered to the American public – use sanitary wipes. "According to Seideman's lab tests, a simple, inexpensive cleansing wipe took a bacteria count in the thousands to almost zero – in a matter of seconds (Channel KOCO Oklahoma, 2004)." As a result of the above studies, we expect to find that there are several different kinds of microbes present on the handles of shopping carts. We expect these will include normal flora as well as pathogens and that we will be able isolate and identify one organism.

## MATERIALS AND METHODS

The following swab samples were taken of shopping cart handles: 2 samples of 1 handle from a shopping cart at Costco, Roseville, CA; 1 sample from 1 handle of a shopping cart from Longs Drugs in Auburn, CA; and 1 sample from 1 shopping cart handle from Save-Mart in Auburn, CA. The organisms collected from each of the 4 swab samples were immediately placed into individual tubes, each containing 1ml sterile water. On February 13$^{th}$ each of the 4

swab samples were streaked onto plates of Tryptic Soy Agar (TSA) media and incubated at 37°C for 48 hours. When removed from incubation, 20 different colonies were observed on the 4 TSA streak plates (Table 1). Of the 20 different colonies, 5 well-isolated colonies were streaked onto individual TSA plates and then incubated at 37°C for 48 hours. Pure cultures were observed on each of the 5 TSA plates. Two KOH tests were performed; 1 on the isolated colony present on TSA plate #1 and 1 on the isolated colony present on TSA plate #3 (Table 1). The decision was made to focus on the organism residing on TSA plate #1 due its pigment and KOH test results (Table 1- highlighted).

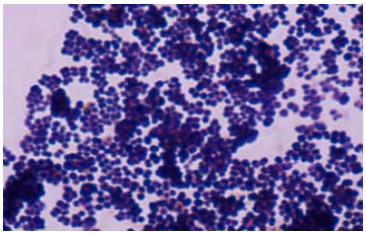
The boil method was utilized to extract chromosomal DNA. Dr. Warren set up the Polymerase Chain Reaction to amplify rDNA (goal was to isolate the 16s Ribosomal RNA gene (rDNA). Primers used were Bacteria-8-Forward and 1492 Reverse. PCR was run on a 1% agarose gel and the 1500 base pair product was purified using the QIAquick Gel Purification Kit (Qiagen). Purified rDNA was sent to the Division of Biological Sciences Sequencing Facility at UC Davis and sequenced using primer Bacteria-8-Forward.

The sequence from the isolated organism was compared to those in the database at National Center for Biotechnology Information using the Basic Local Alignment Search Tool. Based on the sequencing results, the following tests were performed: Gram Stain, Catalase, Oxidation/Fermentation test and the organism was streaked onto Mannitol Salt Agar media.

## RESULTS

Although 20 different colonies were originally isolated from the shopping cart handles, with 15 of them having unique colony morphology (Table 1), only one was chosen for isolation and identification. Colony morphology of the isolated organism showed circular, entire, pulvinate colonies, which were opaque, shiny and 1.5-2.0mm in diameter with yellow pigment. The KOH test results were ambiguous; the first test showed a small amount of viscocity and the second showed no viscocity when KOH was applied to the cells. The Gram Stain showed the bacterial cells to be Gram-positive cocci in irregular clusters and tetrads with no visible endospores (Fig. 1). Catalase tests showed the organism to be catalase positive. The Oxidation/Fermentation test gave a positive result shown by the medium turning yellow in the anaerobic environment of the tube sealed with vaspar (Fig. 2). The Mannitol Salt Agar test showed only weak growth when originally removed from 37°C incubation, but later showed substantial growth when the plate was allowed to sit at room temperature (approx. 25°C). The agar remained red at all temperatures. The bacteria grew readily on Tryptic Soy Agar (TSA) when incubated at 37°C for 48 hours. When removed from the incubator the colonies had an off-white, yellowish pigment. After the bacteria were removed from the incubation temperature of 37°C and allowed to sit at room temperature (approximately 25°C) for >24 hours, the organism produced a bright yellow pigment (Fig. 3).

DNA sequencing results were run through the Basic Local Alignment and Search Tool on the NCBI website and the organism was determined to be *Kocuria rhizophila* (Accession number Y16264). The sequence was compared with 6,672,150 sequences in the NCBI database and returned a match with 99% accuracy with 1470/1471 nucleotides matching.



1 -Gram Stain showing Gram-Positive cocci.

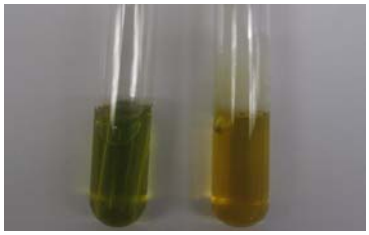


Figure 2- Oxidation/Fermentation test. Results show the organism can ferment glucose.

Figure 3- The plate on the left shows the pigment when the organism is first removed from the incubator. The plate on the right is the pigment after 48 hours at approx 25°C .

| | Costco 1 | Costco 2 | | Longs | | Save-Mart |
|---|---|---|---|---|---|---|
| No. Colonies | 1 Colony | 2 Colonies | | 2 Colonies | | 15 Colonies |
| Colony # | Colony 1* | Colony 1* | Colony 2 | Colony 1 | Colony 2 | Colony 1 |
| Form | Irregular | Irregular | Circular | Circular | Circular | Irregular |
| Margin | Undulate | Undulate | Entire | Entire | Entire | Entire |
| Elevation | Flat | Flat | Flat | Pulvinate | Umbonate | Umbonate |
| Surface Texture | Dull | Dull | Dull | Shiny | Shiny | Rough/Shiny |
| Optical Character | Translucent | Translucent | Opaque | Opaque | Opaque | Opaque |
| Pigmentation | White | White | Red* | Yellow* | White | Off-White |
| Size (mm) | 13x14 | 13x14 | 0.25 | 2 | 2 | 2 |
| Streaked Y/N | N | N | N | N | N | N |

| | Save-Mart | | | | | |
|---|---|---|---|---|---|---|
| No. Colonies | 15 Colonies | | | | | |
| Colony # | Colony 2* | Colony 3/4 | Colony 5 | Colony 6/7 | Colony 8 | Colony 9 |
| Form | Irregular | Circular | Irregular | Circular | Circular | Circular |
| Margin | Undulate | Entire | Undulate | Entire | Entire | Entire |
| Elevation | Flat | Pulvinate | Flat | Flat | Umbonate | Flat |
| Surface Texture | Dull | Shiny | Rough/Shiny | Shiny | Shiny | Dull |
| Optical Character | Opaque | Opaque | Translucent | Opaque | Opaque | Opaque |
| Pigmentation | White | Yellow* | Brown | Brown/Orange | Lt. Orange | Red* |
| Size (mm) | 13x14 | 2 | 7 | 2 | 2 | 0.25 |
| Streaked Y/N | N | Y(#1) | Y(#4) | N | Y(#3) | Y(#2) |

| | Save-Mart | | | | | |
|---|---|---|---|---|---|---|
| No. of Colonies | 15 Colonies | | | | | |
| Colony # | Colony 10 | Colony 11 | Colony 12 | Colony 13 | Colony 14 | Colony 15 |
| Form | Circular | Circular | Circular | Circular | Irregular | Circular |
| Margin | Entire | Entire | Entire | Entire | Entire | Entire |
| Elevation | Umbonate | Convex | Umbonate | Convex | Flat | Umbonate |
| Surface Texture | Dull/Rough | Glistening | Shiny | Shiny | Shiny | Dull |
| Optical Character | Translucent | Opaque | Translucent | Opaque | Opaque | Opaque |
| Pigmentation | Colorless | White | Dark Yellow | Yellow | White | Tan |
| Size (mm) | 4 | 3 | 1.5 | 5 | 2x3 | 2 |
| Streaked Y/N | Y(#5) | N | N | N | N | N |

**Table 1 –** Above is a chart of the twenty colonies that were present on the four TSA plates streaked with the shopping cart handle cultures.

## DISCUSSION

The genus *Kocuria* was originally part of the genus *Micrococcus* (Kovacs *et al*., 1999). We used the Bergey's Manual of Determinative Bacteriology to determine the physiological characteristics of the genus *Micrococcae.* These characteristics were used in considering which tests to conduct in an effort to confirm the organism's identity. Our tests results confirmed the organism is a Gram-positive cocci and it has the enzyme catalase which is in agreement with the findings in Bergey's (Baird-Parker, 1975). The organism's metabolism was found to be facultatively anaerobic as it can ferment glucose to acid in the absence of oxygen without producing gas as shown by the O/F test. This was confirmed when the organism was streaked onto MSA which produced a negative result for acid production. This finding was unexpected because < 5% of the organisms in the genus *Micrococcus* were fermentative (Baird-Parker, 1975). Although unexpected, this finding does not conflict with the identification of the organism as *Kocuria rhizophila*. It was also determined that the organism is able to grow in the presence of salt concentrations at or above 7.5%. The optimum temperature for growth for *Kocuria rhizophila* is 28°C (Kovacs *et al.*, 1999) which was confirmed by the observed changes in pigment and increased growth on MSA at the lower temperatures. Even though the KOH tests were inconclusive, it did not affect our ability to identify this organism. This identification agrees with our test results and the description of this bacterium in Bergey's Manual. It is conclusive that our organism is *Kocuria rhizophil*a.

As can be seen from the data in Table 1, there were many different types of microbes present on the shopping cart handles, although only one was isolated and identified in this study. These findings are in agreement with expected findings as initially stated. *Kocuria rhizophila* is a normal flora and thus, not pathogenic (Johnson *et al.,* 2002).

## ACKNOWLEDGEMENTS

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Vol. 2, No. 2

# Isolation and Identification of *Staphylococcus pasteuri* from a Presumed Clean Fingernail

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**In order to find out what grows under fingernails, an organism was isolated from underneath a female's fingernail and grown in a laboratory. The organism was identified through a series of morphological and physiological tests. The 16s rDNA was amplified and sequenced as well. The bacterium was identified as *Staphylococcus pasteuri*. This bacterium is relatively new so there is not an abundance of information to assist with the confirmation of its genetics. *Staphylococcus pasteuri* is considered to be among the normal flora of the human skin.**

## INTRODUCTION

You eat with them, you paint them, you touch things with them, and you clip and clean them and constantly use them throughout the day. "What are they," you ask? They are your hands of course. Throughout your daily activities, your hands come into contact with various objects and people. They accumulate various germs and bacteria; some are good and some are not. To keep the harmful ones at bay, you wash your hands and think that is enough, but you might want to think again. You might want to take a closer look at what lurks under your fingernails. Your nails store all types of flora that continually roam on your hands. Watching others eat with their hands, touch money, scratch their head and then bite their nails, makes one wonder what exactly is going into their mouth. Among the normal flora of the skin are *Pseudomonas areuginoasa, Staphylococcus aureus, and Streptococcus imitis.* In order to determine what types of flora are crawling under "clean" fingernails, we isolated bacteria from a group member's fingernail. After colonies developed, we chose a colony assuming it was *Staphylococcus aureus* because we had seen it before in lab. According to an article in the International Journal of Systemic Bacteriology, about 20% of people are persistent carriers, 60% are intermittent carriers, and approximately 20% never carry *S. aureus.* The article goes on to say that because of the increase of infections due to MRSA (methicillin-resistant *S.aureus*), treatment and preventing infections has become more of a problem. Individuals that are at a higher risk are those with HIV, AIDS, intravascular devices, on dialysis, and already colonized with MRSA (Kluytmans 1997). Our reasoning for isolating this organism was to see if it was in fact *S.aureus*. If our assumption was wrong, we wanted to know what the organism was and whether or not it was harmful to humans.

## MATERIALS AND METHODS

To begin this project we obtained forceps and a beaker with 95% ethanol in it. The forceps were sterilized using the ethanol before obtaining a sample from underneath the group member's fingernail (Figure 1). An autoclaved Q-tip that was soaked in sterile water was used to

swab the sample from the forceps. The sample was then streaked onto a TSA plate and incubated at 37 degrees Celsius for 48 hours. The plate was examined and two different colonies grew; one was white and the other was yellow. We chose the yellow colony based solely on its color. The chosen organism was streaked for verification of a pure organism. It was continually restreaked to keep the organism from dying. The colony morphology of the isolated organism growing on TSA medium was observed. A KOH test was performed to determine if the cells were Gram positive or Gram negative. To confirm the results of the KOH test a Gram stain was done. Cellular morphology was determined by performing the following tests: an indirect stain, an endospore stain, an acid fast stain, a capsule stain, and a wet mount (Wilson 2007). The next step was to isolate chromosomal DNA from the organism to use in the PCR reaction. Cells were put into a small tube with glass beads, boiled for ten minutes and vortexed for an additional ten minutes. The primers used were Bacteria 8 Forward and Bacteria 1492 Reverse to amplify the 16s rDNA. Next, the PCR was run on 1% agarose gel and 1500 base pairs were purified using the QIAquick Gel Purification Kit (Qiagen). Purified rDNA was sent to the Division of Biological Sciences Sequencing Facility at UC Davis and sequenced using primer Bacteria 8 Forward. The sequence from the isolated organism was compared to those in the database at the National Center for Biotechnological Information using a Basic Local Alignment Search Tool. Based on the sequencing results, we performed a series of tests. These tests were an oxidase, coagulase, urease, hemolysis, arabinose, maltose, sucrose, raffinose, arabinose, and lactose test, and streaked onto a MSA plate (Wilson 2007). In order to complete the carbohydrate tests aerobically, each carbohydrate deep was melted and set onto the wooden slant box to cool.

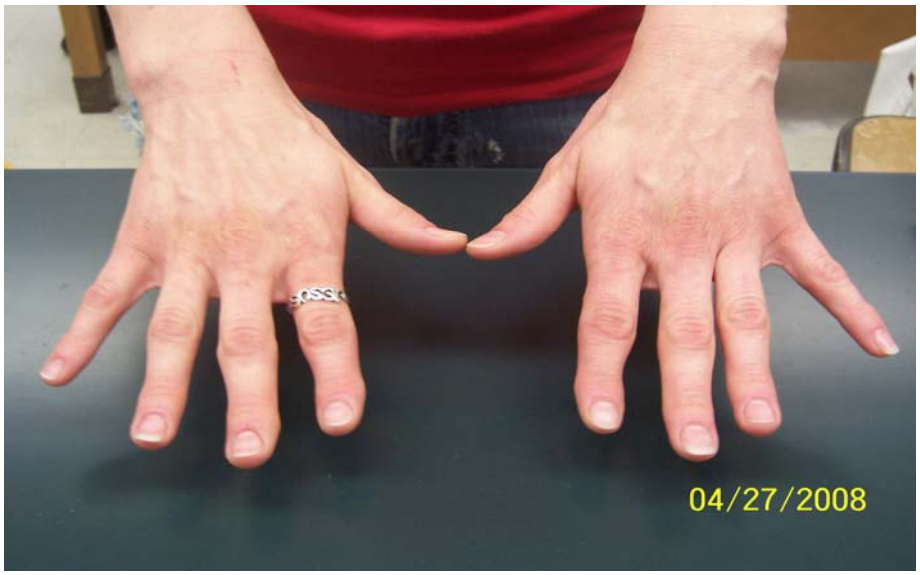

Figure 1. Subject's fingernails – the source of our isolate.

## RESULTS

After a pure culture was obtained, colony morphology on TSA medium displayed shiny, opaque, yellow, circular colonies that were entire with a flat elevation, and about 2mm in diameter. Cellular morphology revealed a cluster of cocci with no motility. Instead, the cells

moved about by Brownian motion. Both, the KOH test and the Gram stain concluded the organism was Gram positive (Figure 3). The acid-fast stain concluded a negative result with blue cocci. The organism did give negative results for oxidase and coagulase tests, but showed positive for the urease test. The results for the carbohydrates showed positive results for maltose and sucrose and negative for arabinose, raffinose and lactose (Figure 2 and Table 1). When streaked onto a blood agar plate there was no breakdown of the medium around the isolated organism. On the MSA plate, the organism did not ferment mannitol, but the organism was still able to grow (Figure 4). The results from BLAST matched the sequence of *Staphylococcus pasteuri* strain ZA-b3 (Accession number AF532917) with 99% identity, a nucleotide ratio of 886/894, and a bit score of 1600. The lineage for this organism is Bacteria; Firmucuties; Bacillales; Staphylococcus. Pigmentation was yellow and the colonies measured to be less than 5mm. Both parts of the MRVP test were positive meaning the organism does mixed acid fermentation and produces acetoin. There was no fermentation of lactose, but there was fermentation of maltose (Table 1). There was no snot formed when the KOH test was done (Table 1). The table below summarizes all tests and results.

Table 1. Physiological comparison of fingernail isolate and *Staphylococcus pasteuri*

| **TESTS** | **ISOLATE** | ***S. pasteuri*** |
|---|---|---|
| MR Test | Positive | Positive |
| VP Test | Positive | Positive |
| Lactose | Negative | Negative |
| Maltose | Positive | Positive |
| Gram Stain | Gram positive | Gram positive |
| KOH | Negative | ND* |
| Acid-Fast Stain | Negative | ND* |
| Endospore Stain | Negative | ND* |
| Capsule Stain | Positive | ND* |
| Wet mount | Not motile | ND* |
| Oxidase Test | Negative | ND* |
| Coagulase Test | Negative | ND* |
| Urease Test | Positive | ND* |
| Hemolysis | Negative | ND* |
| Arabinose | Negative | ND* |
| Sucrose | Sucrose | ND* |
| Raffinose | Negative | ND* |
| MSA plate | Negative | ND* |

* NOT DETERMINED

Figure 2. Aerobic catabolism of carbohydrates.

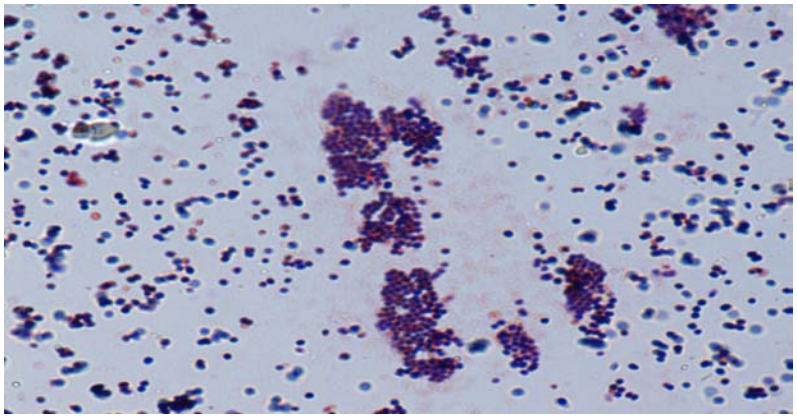
Figure 3. Gram Stain

Figure 4. Growth of isolate on MSA medium

## DISCUSSION

We set out to confirm the growth of *S. aureus* as normal flora, and found a new one. The BLAST results matched the DNA sequence with 99% accuracy down to the strain. *Staphylococcus pasteuri* did not match with any other organism in the BLAST database. To further confirm these findings, we went to the Bergey's Manual in search of physiological tests. Unfortunately, our organism was not in the manual which meant it was a relatively new strain. This forced us to seek out a scientific article(s) that had information about our newly found bacteria. In the meantime, we performed a series of tests that later turned out to be unnecessary, but thought would still be useful in helping to identify the new strain. The tests in the article were for pigmentation, acetoin production, lactose, maltose, and colony diameter (Chesneau, 1993). *Staphylococcus pasteuri* does not make endospores, but is capsulated. It only moves by Brownian motion. The organism does not use cytochrome C in its ETC, nor does it coagulate rabbit serum, as both of these tests were negative. It does however have the ability to break down urease; the medium turned hot pink (Figure 4). There was no breakdown of RBC's on the blood agar plate. The MSA plate turned hop pink, meaning *S, paseuri* does not ferment mannitol, but will still grow because of the salt in the medium. This indicates the organism is salt tolerant. *S. pasteuri* does ferment sucrose, but does not ferment arabinose or raffinose (Figure 2).

In the beginning of this project, we expected to find our chosen organism to be *Staphylococcus auereus*, but found that it was infact *Staphylococcus pasteuri.* This organism has been isolated from humans, food and animals (Chesneau 1993). *Staphylococcus pasteuri* was found to be one of 66 resistant staphylococci isolates from a study done from a swine meat production chain. Antibiotic resistant genes that are found in staphylococci are becoming a danger to humans because they aid in the spread of antibiotic resistant genes. The study concluded that this chain was a source of antibiotic-resistant staphylococci (Simeoni 2008).

## ACKNOWLEDGEMENTS

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# Isolation of *Micrococcus varians* From Tap Water Filtered With a PUR Filter.

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**A bacterium was isolated from tap water filtered from PUR filtration water system. Various biological tests such as Urease test, Citrate test, MR-VP, MSA, Esculin Hydrolysis, and more were used to determine that our isolated organism from filtered tap water was *Micrococcus varians*. This organism is said to be a contaminant found on human skin; however, has also been detected in beach sand, and water.**

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## INTRODUCTION

Our bodies are made up of primarily water, a vital necessity for all human survival. Did you ever stop to think about what might be in the water you drink? First we will take a look at some possibilities related to the tap water we use and drink everyday. According to California American Water Agency's 2006 Annual Report, microbial contaminants such as viruses and bacteria may be present in tap water (Turner, 2006). They say this may be caused by water runoff in streams and on land that pick up contaminants from animals, livestock, and sewage. According to the Centers for Disease Control, tap water in the United States passes all Federal and State regulations; however still has the possibility to pose a threat to immunocompromised people. Such diseases as *Giardia* and Guinea Worm Disease may still be present in drinking water (PUR filtration Systems). Although, drinking water is said to be safe, we would still like to find out for ourselves exactly what we are drinking. PUR filtration system is a device that attaches to your sink at home. It filters the tap water so you may drink it. In theory, you could assume that good marketing techniques have made PUR filtration system a household name. We are here to prove that there may still be microorganisms present in drinking water even after being filtered through PUR filtration systems.

## MATERIALS AND METHODS

We collected our sample of tap water in one of the apartments located in Antelope CA. The water sample was then filtered with a "PUR" water filter. We used double strength phenol red lactose broth and added equal amounts of filtered water to it. This method is used to identify *E.coli.* Then the sample was put into the incubator at 37 degrees Celsius. Four days later we streaked from the broth onto TSA medium and incubated at 37 degrees Celsius. the sample yielded us four different organisms. We picked one of the four microorganisms to identify. The organism we picked, was bright and pretty. We performed a KOH test, Gram stain, and Acid-fast stain. We attempted to do the DNA sequencing, however it did not work. Then we were left with the option of identifying our organism using the Bergey's Manual. In order to do that, we

performed the following tests: Urease test, Citrate test, Voges-Proskaur test, Methyl Red test, Esculin Hydrolysis test, Oxidation/Fermentation test, Catalase test, and Oxydase test. We streaked our organism on MSA medium to find out if it could tolerate the salt.

## RESULTS

The Double Strength Phenol Red Lactose Broth changed to a yellow color in the sample before and after filtration. Also, the tube had the air bubble on the top, some white sediment on the bottom and an oxygenated pellicle. We streaked from the broth to a TSA plate, which yielded 2 colony types. After we picked one organism to identify, we streaked it on TSA medium. The colonies we observed were a light yellow (Figure 1).

Figure 1. Water Isolate on TSA.

The morphology of the organism looks circular, margin entire, convex in shape. The organism is glistening and smooth. The cells are around 1-1.5 micrometers in diameter. The KOH test had no snot and the Gram stain appeared purple. (Figure 2)

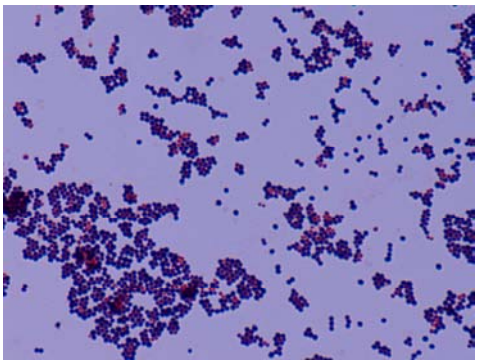

Figure 2. Gram Stain of Isolate

The Oxidase test did not show blue. After performing the Oxidation/Fermentation Test, both tubes were green. Then we performed a Catalase test. The results showed bubbles. We did a wet mount and the organism did not show motility.

## DISCUSSION

According to the test performed we determined we may have two different organisms: *Planococcus* and *Micrococcus*. In order to determine between the two, we grew our organism on Mannitol Salt Agar to find out if it could tolerate salt. The organism was salt tolerant. Esculin Hydrolysis yielded the result that the organism cannot hydrolyze Esculin. The next test performed was the Methyl Red-Voges Proskaur Tests; which told us the organism does not perform mixed acid fermentation nor butanediol fermentation. We also performed the Citrate test, which yielded negative results. The Urease test was positive which shows us that the organism has Urease. The KOH test that was performed showed no snot and the Gram Stain appearing purple tell us the Organism was Gram Positive. The Oxidase test did not show any blue or purple which tells us that the organism does not use Cytochrome C in its electron transport chain. With both tubes resulting green in the Oxidation/Fermentation test, concluded that the organism does not ferment or make a gas. The Catalase test showed no bubbles which concludes that the organism does not have catalase. All data obtained was compared to the Bergey's Manual and led us to the conclusion that our organism is probably *Micrococcus varians*. (Migula, 1900).

| Test Name | Water Isolate | *M. varians* |
|---|---|---|
| Urease Test | + | + |
| Citrate Test | - | ND* |
| Voges Proskaur | - | ND* |
| Methyl- Red Test | - | ND* |
| 7.5% Salt Agar | + | + |
| Esculin Hydrolysis | - | - |
| Oxidative/Fermentation | - | ND* |
| Catalase Test | + | ND* |
| Oxidase Test | - | - |
| Wet Mount | - | - |

Table 1 Comparison of Water Isolate and *M. varians*
*Not Determined

The Bergey's Manual states that the organism is found on mammalian skin, beach sand, and water. (Migula, 1900) Another source such as, the University of Texas-Houston Medical School revealed that *Micrococcus varians* is relatively harmless to humans, in fact it is a common contaminant found on human skin. (Centers for Disease Control) This organism is harmless to humans due to the saprophytic lifestyle they maintain. Although PUR filtration systems is mostly effective, small amounts of bacteria may still be present.

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# Isolation of Bacteria Taken from a Water Sample from Lake Wildwood, California

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**Water samples were taken from various areas of Lake Wildwood, in order to isolate an unknown organism and to determine if that organism poses a serious threat to humans and/ or the environment. By isolating this organism in the laboratory and subjecting it to sequencing it was determined that the unknown organism was Bacillus pumilus. In addition to sequencing, other tests included: KOH, Lysine Decarboxylation, and Esculin Hydrolysis. Carbohydrate deeps were prepared to test for fermentation of Arabinose, Glucose, and Mannitol. A Gram-stain was performed as well as a wet mount to test for motility. All results were found to be conclusive except for the Esculin Hydrolysis test. Bacillus pumilus exposure was found not to be a serious threat to humans and/or the environment.**

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## INTRODUCTION

In and around Lake Wildwood people both live and play. Where there is a large body of water people tend to engage in various water sports. Needless to say, this brings people in close contact with water and everything that may reside in that water. Knowing what is contained in that environment is of great importance, especially if what lives in that environment could be hazardous to one's health. By taking water samples from various locations in Lake Wildwood, I intend to find out if the waters contain unwanted pathogens. I do not expect to find any

pathogens, but it is important to be sure. Freshwater lakes contain a large quantity of microbiota. Generally microorganisms found in freshwater include the genera Micrococcus, Pseudomonas, Serratia, Flavobacterium, Chromobacterium, and Achromobacter. My water samples were taken at the littoral zone where the water contains various soil bacteria, fungi, and algae (Lim, 2002). When samples are cultivated at aerobic conditions Alpha, Beta, Gamma, and Delta Proteobacteria, Firmicutes, Actinobacteria, Bacteriodetes, and Planctomyces tend to be the predominant phyla (Tamaki, 2005). Lake Wildwood is surrounded by many inhabitants and various animals and has had a history of coliform contamination. Water samples taken from lakes of the Sierra Nevada have shown to grow colonies of Escherichia coli (Derlet, 2006). While I don't expect to find any coliforms in my sample I can't completely rule it out. Algae and Cyanobacteria tend to be the dominant microorganisms at the littoral zone (Tortura, 2007). However, with the media and laboratory conditions in use I don't expect to grow cultures of either. Some type of soil bacteria would seem to be the most likely to grow in the laboratory conditions I intend to use.

## MATERIALS AND METHODS

Water samples from Lake Wildwood were taken from various spots at the water surface and sealed in a ziplock bag for transport to the laboratory. 500 microliters of water sample were spread out evenly over a Tryptic Soy Agar plate. This plate was incubated for 24 hours at 37 degrees Celsius. Using this sample I proceeded to streak for isolation three TSA plates and incubate for 24 hours at 37 degrees Celsius.

KOH test was performed (Wilson, 2007, p.64). Colony morphology, and cellular morphology were observed. PCR was used to amplify rRNA (goal was to isolate the 16s ribosomal RNA gene (rDNA). Used primers Bacteria 8F and 1492 Rev. PCR was run on a 1% agarose gel and the 1500 bp product was purified using the QIAquick Gel Purification Kit (Qiagen). Purified rDNA was sent to the division of Biological Sciences Sequencing Facility at UC Davis and sequenced using primer Bacteria 8 Forward. The sequence from the isolated organism was compared to those in the data base at the National Center for Biotechnology Information using the Basic Local Alignment Search Tool.

Based on the sequencing results I performed the following tests: Arabinose, Glucose, Mannitol (Wilson, 2007, p.172-173), Lysine Decarboxylation (Wilson, 2007, p.180), and Esculin Hydrolysis (Wilson, 2007, p.174-175). Finally a Gram stain was performed (Wilson, 2007, p.62-63).

## RESULTS

Colony morphology was observed using Tryptic Soy Agar as media. Form was circular, pigmentation was off-white, margin was serrate. Approximate size of the individual colonies was 4 millimeters. Elevation was flat, surface texture was shiny and optical character was opaque.

KOH stain returned negative results, and Gram stain returned dark purple cells. Shape of cells were bacilli, with single cell arrangement. Approximate size of cells were 2 micrometers in

length and 1/2 a micrometer in width (Figure 1). Using a wet mount I observed motility of individual cells.

Based on the return of BLAST results, my sequence matched the sequence of Bacillus pumilus (Accession number DQ833752) with 98% identity. Ratio of identical nucleotides was 698 out of 709.with a bit score of 1280.

Esculin hydrolysis test returned a negative result. Carbohydrate deeps of Arabinose, Glucose and Mannitol returned positive results. Both control tube and lysine tube turned yellow following Lysine Decarboxylation test.

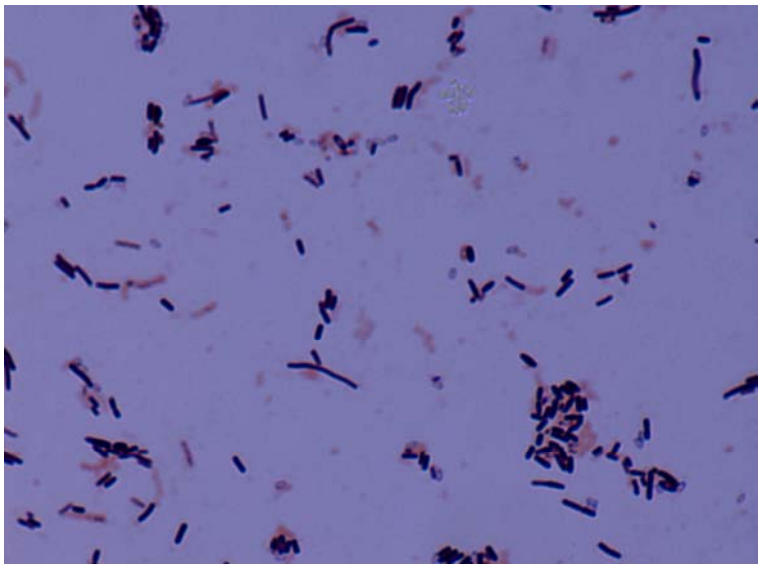

Figure 1.

## DISCUSSION

Based on my findings I believe that the organism I have isolated from my water samples is Bacillus pumilus. This organism is Gram-positive and grows aerobically. Like most Bacillus species it moves via peritrichous flagella (Todar, 2008). PCR results matched Bacillus pumilus with a 98% identity. There were no other organisms that matched that sequence with a higher identity.

Based on sequencing results I performed tests that coincided with the identity of Bacillus pumilus. Results proved that this organism could ferment Arabinose, Glucose, and Mannitol. Also consistent with Bacillus pumilus is it's inability to decarboxylate lysine. One of my tests resulted in conflicting data, Bacillus pumilus is supposed to be able to hydrolyze esculin; the esculin I used in my slant contained bile salts, so I am unable to determine whether Bacillus pumilus can hydrolyze esculin. (Claus and Berkeley, 1872).

The purpose of identifying an unknown organism in various water samples of Lake Wildwood was to find out whether the organism in question might pose a risk to humans. Bacillus pumilus has been used successfully in pesticides to prevent proliferation of harmful fungi in plant species, by preventing germination of fungal spores. Bacillus pumilus exposure is

not harmful to humans, however on occasion it has known to cause infections (Todar, 2008). Furthermore, Bacillus pumilus does not pose a threat to the environment (USEPA, 2003).

## ACKNOWLEDGEMENTS

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# Isolation and Identification of a Gram-negative Organism found in Creek Water at Empire Mine in Grass Valley, California

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**A water sample obtained from a mining area used for recreational purposes was investigated to determine the identity of one of the organism types present and the possible health risks posed by these organisms. Following isolation, biochemical tests were performed to identify the genus and species of the bacteria present in the culture. The organisms were determined to be Gram-negative bacilli with a respiratory type of metabolism. They were identified as a species in the Genus *Flavobacterium,* and although several closely related species were compared, no absolute species match was obtained. Health risks presented by bacteria in the Genus *Flavobacterium* appear restricted to disease and mortality in fresh water fish. These organisms are found worldwide and are common in cold and fresh water environments.**

## INTRODUCTION

The Empire Mine, which is now a California State Park, is one of the oldest and most prolific gold mines in California. Many individuals born and raised in the vicinity of the mine have toured the grounds on multiple occasions and used the extensive recreational trails for jogging and biking. Certain areas of the mine property have been fenced off over the years and warning signs have been posted identifying these as toxic. Education provided in local schools and through park tours, informs the public that mining operations can yield toxic forms of minerals such as arsenic. For many years, the park has been working to clean up the effected areas, since it is heavily used for recreational and educational purposes. The Grass Valley region, located in the Sierra Nevada foothills, has many sources of water originating from natural underground springs, mountain rivers and overflowing mine shafts that have filled with water. The Empire Mine area includes 367 miles of abandon mine tunnels and many shafts, most of which are filled with water. There are many creeks, containing very cold water, running through the park and exiting at lower elevations. The water sample used for this investigation was taken from one of these creeks. The objective was to identify organisms adapted to the environmental conditions presented by the mine, including toxic chemicals and very cold underground water.

## MATERIALS AND METHODS

A sample of water was collected from a stream exiting the Empire mine and transported to the microbiology laboratory in a clean zip-lock bag. A 100µl sample of this water was

transferred onto the surface of a nutrient agar plate with a sterile pipet, and the plate was kept at room temperature for 48 hours. After growth was visible on the agar plate, a chosen colony was re-streaked on both TSA and Nutrient agar plates in an attempt to isolate the organisms present and establish pure cultures. Samples from the pure cultures were then tested with 3% potassium hydroxide (KOH) to determine whether the organisms had Gram-positive or Gram-negative type cell walls. Additional samples were used to prepare Gram-stains and indirect stains.

A 2mm blob of cells from one pure culture was added to 500µl of Tris buffer, vortex mixed, and then placed in boiling water for 10 minutes to release chromosomal DNA and denature cellular enzymes. The 16S ribosomal-DNA from this sample was then amplified through the Polymerase Chain Reaction (PCR), using the primers 8-forward and 1530-reverse. A Bio-Rad thermal cycler was used for this process with a series of temperature fluctuations that were repeated 34 times. This series was initiated by exposing the DNA to 94° C for 4 minutes and then repeating a sequence of 55° C for 45 seconds, 72° C for 2 minutes, and 94° C for 30 seconds. To make certain the Taq polymerase was provided sufficient time for the building of the DNA, the sample was subject to 72° C for 20 minutes at the end of the last cycle.

Following amplification, the total volume of the PCR product was run in an electrophoresis gel and then stained with ethidium bromide. The DNA was cut from the gel, the strip of agar including the DNA was weighed, and then the DNA was purified using a QIAquick PCR purification kit (Qiagen). The purified DNA sample was then taken to the University of California at Davis (UCD) for sequencing. Ultimately, sequence data was obtained with three primers; bacteria 8-forward, internal 533-forward and 1530-reverse. Nucleotide sequences were returned electronically, where edited to remove errors and were combined to create a single sequence. The National Center for Biotechnology Information website was accessed and then the Basic Local Alignment Search Tool was used to compare this sequence to others in the gene banks.

Organisms that had been identified to the species level and were described within published articles where noted and the articles were accessed in order to identify additional tests that could be performed in order to characterize the particular species of the organisms obtained from the mine water. The enzymatic tests ultimately performed were the KOH test, Oxidation/Fermentation or O/F test, citrate utilization, TSI, SIM and utilization of a series of different carbohydrates as single carbon sources.

## RESULTS

The mine water isolate grown on TSA formed colonies that were irregular, entire, raised/convex, shiny, rough, opaque, 2-3mm in diameter and orange-yellow pigmented. When grown on nutrient agar the colonies were irregular, entire, raised, shiny, rough, translucent, swarming, effuse and yellow pigmented. The cells were variable in size, but most appeared to be 0.5µm in diameter and ranged from 0.5 to 3.0µm in length. Their arrangement was usually single cells. In the Gram-stain these cells appeared pink, so were Gram-negative rods. The KOH test resulted in observed viscosity, confirming a Gram-negative type cell wall.

The BLAST results obtained with the 1530-reverse primer alone showed 98% similarity between the unknown mine organisms and various other species of *Flavobacterium* found in

cold water environments throughout the world. The following organism types yielded 98% similarity with 259 bases out of 262 matching pairwise: *Flavobacterium hercynium*, assession number AM265623; *Flavobacterium xanthum*, Assession number AJ601392; *Flavobacterium hibernum*, assession number L39067.1; and *Flavobacterium limicola*, assession number AB075232. When three sequences were combined, the BLAST results were somewhat different. The entire sequence was 1444 bases in length, and still showed 98% similarity with gene bank sequences from *Flavobacterium limicola*, accession number AB075230, and *Flavobacterium hibernum*, accession number L39067. In addition, the sequence showed 98% similarity with a 1501 base gene bank sequences from *Flavobacterium johnsoniae*, and from *Flavobacterium columnare*, accession number AY747592.1. When comparisons were made using the longer sequence, *Flavobacterium limicola* (AB075232) showed only 97% similarity, while *Flavobacterium hercynium* and *F. xanthum* were not listed at all.

## DISCUSSION

Streak plates were made from the mine isolates using two different media types, nutrient agar and tryptic soy agar (TSA), in order to determine their preferred growing conditions. It was established that the organisms grew well on both media, but with different physical characteristics. When grown on nutrient agar, the culture showed a strong tendency to form effuse growth, with colony margins spreading across the agar in a translucent light yellow layer. This growth habit was interpreted as gliding motility. When grown on tryptic soy agar, the culture formed raised irregular, opaque, dark orange-yellow colonies with little tendency to spread. When the colonies from a pure culture were subjected to 3% KOH, they were determined to be Gram-negative because of the increased viscosity. The cell mass also changed color from yellow to bright orange. The wall type was confirmed as being Gram-negative (thin peptidoglycan) with a Gram-stain which resulted in pink rods.

After obtaining sequencing results from the U.C. Davis laboratory and using the NCBI BLAST, additional tests were performed in order to characterize the mine isolates. When compared to data obtained from various reference sources, the results of these tests were determined to be inconclusive relative to establishing the true identity of the mine organisms. This was partially due to insufficient testing of the mine isolates and partially due to the lack of similar data in the reference documents describing the characteristics of identified organisms. The growth patterns described for *Flavobacterium limicola* on both nutrient agar and TSA, as documented in the International Journal of Systematic and Evolutionary Microbiology, was very similar to the growth patterns of the *Flavobacterium* species found at the Empire Mine; however, these organisms did not show gliding motility, and did not form aerobic acid when grown on carbohydrates. Similar growth patterns were also described for *Flavobacterium johnsoniae* (formerly *Cytophaga johnsonae*) in the Bergey's manual of systematic bacteriology; however, according to the Bergey's manual, a mass of cells from *Flavobacterium johnsoniae* would quickly turn purple-brown when exposed to KOH, and cells from the mine isolate turned bright orange instead.

| TEST | Mine sample | F. johnsoniae | F. hercynium | F. xanthum | F. hibernum | F. limicola |
|---|---|---|---|---|---|---|
| Growth on: | | | | | | |
| Nutrient agar | + | + | + | + | + | + |
| Tyrosine agar | | | + | + | | + |
| Tryptic soy agar | + | + | + | + | + | + |
| Marine agar | | | | + | | – |
| Gliding motility | + | + | + | | + | – |
| Acid formation | + | + | | + | + | – |
| H2S production | – | – | | + | | – |
| Utilization of: | | | | | | |
| Xylose | + | + | | | | |
| Maltose | + | + | | | | |
| Glucose | + | + | | + | + | + |
| Sucrose | + | + | – | | | |
| Raffinose | + | + | – | | | |
| Rhamnose | + | | | | | |
| Arabinose | + | + | + | | | |
| Inositol | + | | | | | |
| Triple Sugar Iron | | | | | | |
| Glucose | – | | | | | |
| Sucrose | – | | | | | |
| Lactose | + | | – | | | |
| Iron sulfide | – | – | | | | |
| SIM | | | | | | |
| Iron sulfide | – | – | | | | – |
| Indole | – | – | | | | + |
| Motility | – | – | + | – | + | – |
| Citrate utilization | – | | | | | – |
| Oxidase | + | + | + | | | + |

Further testing will be necessary in order to identify the *Flavobacterium* species collected at the Empire mine. There are currently ten recognized species in the genus *Flavobacterium*, and three newly proposed species. It is possible that the isolate collected from the Empire mine represents a novel species, not yet characterized. Several species of *Flavobacterium* are known to cause disease in freshwater fish such as Salmon and Trout, so the culture isolated might pose a threat to fish species. Though there are no fish in this particular portion of the creek, the creek water does flow into areas where fish are present.

## ACKNOWLEDGEMENTS

Thank you to Harriet Wilson, microbiology instructor at Sierra College, and her dedication to helping students understand the world of microbiology and the activity and effect

of organisms on our lifecycle. Thank you to the personnel at UC Davis for their cooperation and assistance in the process of identification of our organisms.

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# Isolation and identification of a Gram-positive Organism discovered from an Air Plate

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**Isolation of an unknown type of organism was accomplished by streaking a nutrient agar plate with a sample of an isolated colony taken from an air plate. The tentative identification of this air plate sample was then determined by completing multiple tests. The tests performed for the analysis of 16S ribosomal-DNA included the Polymerase Chain Reaction, Gel electrophoresis, DNA purification and sequencing, electropherogram evaluation and editing and comparison of nucleotide sequences using the BLAST program on the NCBI website. Multiple enzymatic tests were also completed including MR-VP, catalase, citrate utilization, gelatin and starch hydrolysis, SIM, and the ability to form aerobic acid from glucose, arabinose and mannitol. Based on the results obtained, the culture's identification was tentatively determined to be either *Bacillus subtilis or Bacillus mojavensis*.**

## INTRODUCTION

The object of this investigation was to accomplish the identification of a specific type of microorganism found growing on an air sample plate exposed in an apartment where a little girl lives and suffers from asthma. Asthma is a chronic condition resulting from type 1 hypersensitivity reactions. It typically involves constriction of the airways due to inflammation and excess accumulation of mucous. This leads to breathing difficulty, coughing, wheezing and sometimes severe anxiety. Asthma can be caused by a variety of allergens including cigarette smoke, perfumes, pet dander, pollen grains and various types of microorganisms. Microorganisms are everywhere and are therefore inescapable. Testing the apartment air to verify that no harmful microorganisms currently existed there seemed to be a logical course of action.

## MATERIALS AND METHODS

An air sample was obtained by exposing the agar surface of a nutrient agar plate to the air in the subject apartment for one hour. The sample was then incubated at room temperature for two weeks. Upon examination of the different types of microorganisms growing on this plate, an isolated colony was selected and a sample of this was streaked onto a new nutrient agar plate so that a pure culture could be grown. Once a pure culture was obtained, a Gram stain and a Nigrosin Indirect stain were prepared and observed. A KOH test was also conducted to verify the type of cell wall present.

After these initial tests were performed, a DNA extraction was completed by boiling a small sample of cells in 500μL of 10mM Tris buffer (pH 8.5) for 10 minutes. About twenty

2mm glass beads were added and the sample was vortexed for 10 minutes to break the peptidoglycan walls. A PCR was run to amplify the 16S ribosomal DNA using the oligonucleotide primers Bacteria 8 forward and 1530 reverse. The reaction mixture used contained 25µL of Taq-DNA polymerase master Mix, 5.0µL of primer mix, 5.0µL of the template DNA, and 15µL of sterile distilled water (pH 8.0). A thermal cycler was used to amplify the DNA; the settings were 94ºC for 4 minutes followed by a repeating cycle of 55ºC for 45 seconds, 72ºC for 2 minutes, and 94ºC for 30 seconds. This cycle was repeated thirty four (34) times. After these cycles, there was another exposure to 55ºC for 45 seconds, followed by 72ºC for 20 minutes.

The PCR product DNA was then processed through gel electrophoresis accompanied by the tracking dyes xylene cyanol and bromphenol-blue. Once the gel was run, the DNA was stained with ethidium bromide for visibility. This material was then viewed using an ultra violet light that clearly showed where the DNA was located in the agarose gel. The gel was then cut with a razor blade and the strip containing the DNA was weighed. Purification of this DNA was performed using a QIAquick gel purification kit. Chemicals in the kit and exposure to hot water dissolved the agarose and then the DNA was captured in a filter. The filter was rinsed with ethanol, tris buffer was added and then the sample was spun to remove the DNA from the filter. It was captured in a clean sterile tube and was then taken to the College of Biological Sciences DNA Sequencing Facility of UC Davis. The sequencing primers used were Bacteria 8-forward, 533-forward and 1530-reverse.

When sequencing was completed, the personnel at UC Davis sent three electropherograms in the form of ab1 files. These were accessed with the software program 4Peaks run on an iBook computer using operating system X. The sequences were evaluated, edited, combined and then submitted for comparison to data in the gene banks through the National Center for Biotechnology Information (NCBI). The algorithm used was the Basic local Alignment Search Tool (BLAST). The BLAST results were then examined in order to obtain a tentative identification for the air isolate.

Using the tentative identification as a guide, the Bergey's Manual of Determinative Bacteriology was accessed, and a series of enzymatic tests was initiated. An article available through NCBI was also accessed to provide additional information. The tests determined as appropriate and available were: MR-VP, testing for fermentation through acid and acetoin production; 3% hydrogen peroxide, testing for the presence of catalase enzymes; Simmon's Citrate agar testing for citrate utilization and ammonia production; Starch agar, testing for the ability of organisms to hydrolyze starch; SIM, testing for hydrogen sulfide and indole formation and for motility; Nutrient Gelatin testing for the presence of proteolytic enzymes; and carbohydrate slants (Glucose, Arabinose and Mannitol), testing for the formation of aerobic acids. All of the media involved were inoculated with samples from the air culture and incubated for 48 hours at room temperature.

## RESULTS

When grown on nutrient agar, the air isolate displayed a distinctive colony morphology. Colonies were irregular in form, with entire or smooth edges. Their elevation was flat or slightly raised with a smooth texture and dull to slightly shiny appearance. The optical characteristic was

opaque and most colonies were pigmented a light tan or beige. Their size varied from approximately 1 to 3mm in diameter.

Analysis of the cell morphology indicated the cells to be Gram-positive bacilli approximately 1.0µm in diameter and 4.0µm in length. In the KOH test, no viscosity was formed. The arrangement of the cells ranged from a few single bacillus, to diplobacilli and a few streptobacilli. Some cells contained endospores within but many had already released their spores and were dead. A photograph obtained from a nigrosin indirect stain provides a display of the cell morphology as shown in Figure 1 below.

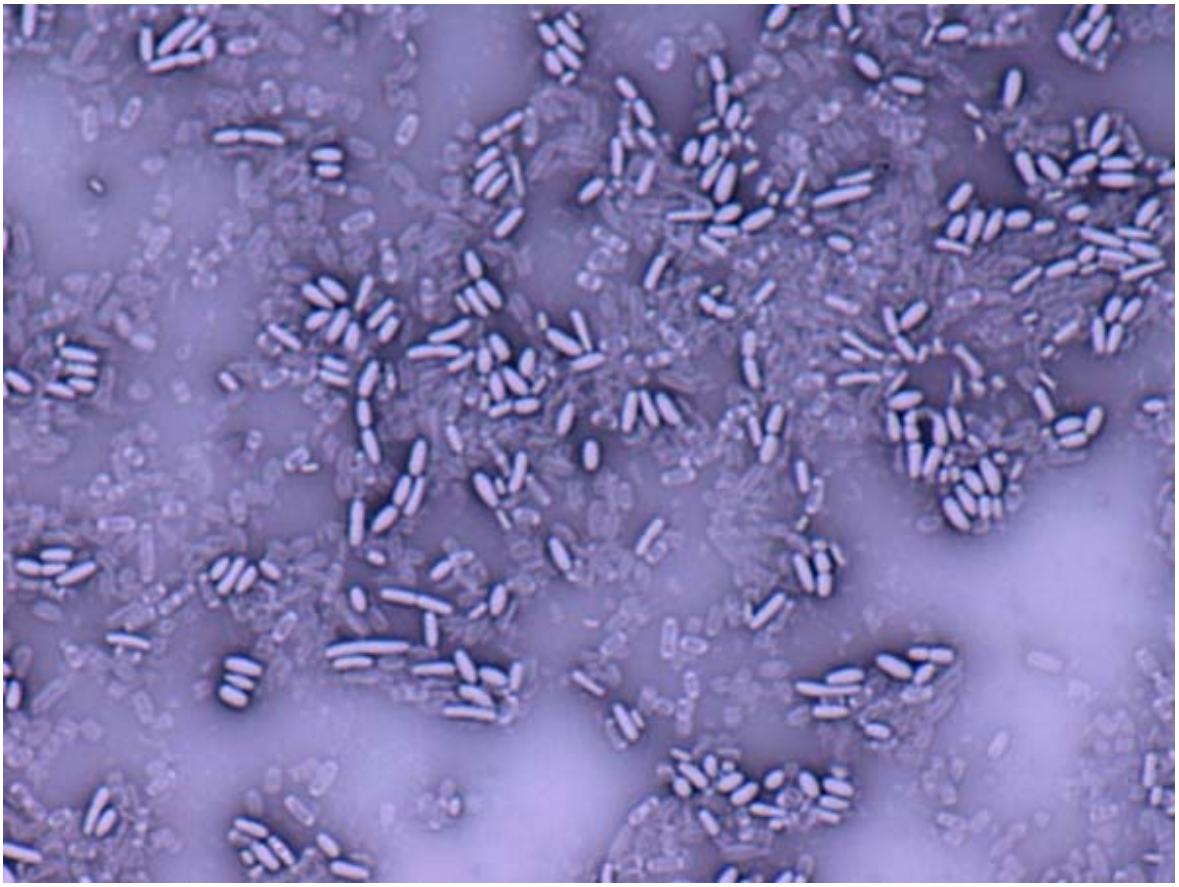

Figure 1 – Photomicrograph of a nigrosin indirect stain

Results obtained with the NCBI BLAST showed that the 16S ribosomal-DNA nucleotide sequence from the air isolate matched 100% with a gene bank sequence from *Bacillus mojavensis* strain BCRC 17531 (DQ993678). There were 1481/1481 bases matching and a bit score of 2736. This sequence also showed 99% similarity with a gene bank sequence from *Bacillus subtilis* strain MO1 (AY553094) with 1480/1481 bases matching and a bit score of 2732.

The results of the enzymatic tests run were as follows: pH paper indicated that the pH of the methyl red medium was 7 after incubation. The VP medium took on a pink color when

Barrit's reagents were added and the culture sample was shaken, so the VP test was positive. Bubbles were formed when the culture was exposed to 3% hydrogen peroxide, so the culture was catalase-positive. The Simmon's Citrate medium turned blue, so citrate was utilized. Both starch and gelatin were hydrolyzed but hydrogen sulfide and indole were not formed. The cells appeared to be motile within the SIM medium. Aerobic acid was formed from glucose, turning the slant yellow, but not from arabinose or mannitol, where the slants stayed red. A summary of these results is indicated in Table 1 below and compared to data from *Bacillus subtilis* and *Bacillus mojavensis*.

| Test Being Run | *Bacillus subtilis* | *Bacillus mojavensis* | Air plate sample |
|---|---|---|---|
| Methyl-Red | pH<6 d, >7 = (-) | pH<6 = d, >7 = (-) | pH = 7 |
| Voges Proskauer | (+) | (+) | (+) |
| Catalase Test | (+) | (+) | (+) |
| Citrate utilization | (+) | (+) | (+) |
| Starch hydrolysis | (+) | (+) | (+) |
| Gelatin hydrolysis | (+) | (+) | (+) |
| Indole formation | (-) | (-) | (-) |
| $H_2S$ formation | | | (-) |
| Acid on glucose | (+) | (+) | (+) |
| Acid on arabinose | (+) | (+) | (-) |
| Acid on mannitol | (+) | (+) | (-) |

Table 1 – Summary of Enzymatic Test Results

## DISCUSSION

According to the results obtained from the DNA analysis, the microorganisms isolated from the air plate were identified as *Bacillus mojavensis* or *Bacillus subtilis.* Both of these organism types are aerobic, endospore-forming, Gram-positive bacteria that are non-pathogenic. *Bacillus mojavensis* and *Bacillus subtilis* are *Bacillus* strains that show almost identical results with respect to the identification methods used in this investigation. Their 16S ribosomal DNA nucleotide sequences varied by only one base pair, and their enzymatic test results were identical. The taxonomy of both is also mirrored until the species category, being: Prokaryote; Bacteria; Firmicutes; Bacilli; Bacillales; *Bacillus*; Species: *mojavensis* or *subtilis.* According to information provided by Wang (2007) these two species cannot be differentiated by either 16S ribosomal-DNA sequence comparison or enzymatic testing, and a better method for differentiating them involves analysis of the gene encoding DNA-gyrase (*Gyr*-B).

The enzymatic tests used in this investigation were based on the test recommendations from the Bergey's Manual of Systematic Bacteriology for Gram-positive microbes in the genus *Bacillus*. The results obtained matched those expected for *Bacillus subtilis* except for the negative results obtained for aerobic acid formation on both Arabinose and Mannitol. The negative results obtained here were difficult to explain but might be attributed to an error in the preparation of the media with respect to pH adjustment. Both were considered to be failed tests.

A tube of SIM medium was inoculated to test for indole formation, but was also used to assess motility and $H_2S$ production. No $H_2S$ production or motility data was available in the Bergey's manual to confirm or contradict these results.

When researching these two types of microorganisms, considerable information was found. *Bacillus subtilis* was originally named *Vibrio subtilis* until 1835 when Christian Gottfried Ehrenberg renamed it *Bacillus subtilis*. These organisms can be found in soil and are also used in food preparation and for medicinal purposes. One very interesting fact was the discovery of the medicinal use of *Bacillus subtilis* in 1941 by the Nazi German army medical corps and their troops in Africa. Many German soldiers were dying of uncontrollable dysentery, and sulfur was not a treatment option at that time as it was not yet available in an ingestible form. Microbiologists were sent to resolve the mystery of the unknown agent causing the fatal disease. While working with the local residents, medical personnel learned of an old folk remedy involving the eating of fresh, warm camel or horse dung. This practice would cure the dysentery. Upon testing the dung, the medical corps found *Bacillus subtilis* to be present in large numbers, and capable of killing the organisms causing dysentery. After being grown in pure cultures, *Bacillus subtilis* bacteria were dried and put into ingestible capsules. After ingesting these capsules, the soldiers made a quick and full recovery and the mortality rate dropped from hundreds weekly to zero. According to rense.com, these bacteria are still being used today for medicinal purposes; "*Bacillus subtilis* remains one of the most potent and beneficial of all health-promoting and immune-stimulating bacteria."

Bacteria identified as *Bacillus mojavensis* were first described in 1994 by Roberts et. al. and are very similar to *Bacillus subtilis*. These bacteria are used more widely for disease control in corn than in humans. Recent discoveries indicate that these bacteria can play a major role as biosurfactant-producing microorganisms and may play an important role in the accelerated bioremediation of hydrocarbon-contaminated sites. *Bacillus mojavensis* can also be used in enhanced oil recovery and may be considered for other potential applications in environmental protection.

Both of the microorganism types described here have been shown to be human friendly and one is a potential bioremedial alternative. With either result, the identification of the microorganisms from the air plate proved to be highly educational. Proving beyond a doubt the specific type of bacteria growing in the air culture would be possible only by running a whole-cell fatty acid composition test and a divergence in the DNA sequence analysis with the *Gyr*-B gene, neither of which can be accomplished at Sierra College. Since these tests could not be performed, a tentative identification for this isolate proved to be enough to satisfy the original intent of the investigation; to identify one of the types of bacteria growing on the original air plate and determining if these organisms would or would not harm the family or the little girl with asthma living in the apartment in Auburn, California. The results obtained and historical information accessed proved these bacteria are harmless and possibly beneficial.

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# Identification and Analysis of Bacteria found in an Argentine Tegu's Mouth

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## ABSTRACT

**A sterile cotton swab was used to sample material taken from an Argentine Tegu's mouth, and was streaked on a nutrient agar plate. After colonies had formed on the agar surface, a sample was taken from one of the most common types present, and a pure culture was established on a new nutrient agar plate. Following isolation, DNA was extracted from the pure culture, a PCR was used to amplify the 16S ribosomal-RNA gene, gel electrophoresis was used to observe the PCR product, this was purified and the nucleotide sequence was determined. The NCBI BLAST was used to compare this nucleotide sequence to those in the gene banks, and then enzymatic testing was completed to determine what the prevalent type of mouth bacteria was. The mouth isolate was ultimately identified as *Janibacter melonis*.**

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## INTRODUCTION

The Argentine Tegu, also known as a Black and White Tegu, is a large, South American lizard. These lizards are normally found in rain forests or in open grasslands along the margins of where these two different types of habitats meet. They are natives of Argentina, but are also found in parts of Brazil and Urugauy. Argentine Tegus are readily maintained as pets; they reach full size (about four feet long) in three years or less, and adapt readily to human surroundings. They are very intelligent. The aim of this study was to determine what type of bacteria was most prevalent in the mouth of a healthy, adult, male Argentine Tegu named Rufus.

## MATERIALS AND METHODS

A fresh, sterile, cotton swab was used to take a sample from the inside of an Argentine Tegu's mouth. One quadrant of a nutrient agar plate was streaked with this swab and then the sample was streaked over the remaining plate surface with a sterile wire loop. The plate was placed into a lab drawer and maintained at room temperature until colonies appeared. One of the most common colony types was then sampled, and used to prepare a new streak plate. Once a pure culture had been isolated, the cell and colony morphology was recorded. The isolate was also grown on both a Tryptic Soy Agar (TSA) and a Brain Heart Agar (BHA) plate. A Gram

stain was prepared and observed and a KOH test was conducted to determine the cell wall type of the mouth isolate.

Once the cell wall type had been determined, DNA analysis was begun. DNA was extracted by having a sample of cells boiled in 10mM Tris buffer (pH 8.0) and beaten with glass beads. The Polymerase Chain Reaction (PCR) with Taq Master Mix (Qiagen) was used to amplify the 16S ribosomal-DNA from these cells. The primers used were Bacteria 8-Forward and 1530-Reverse. Gel electrophoresis of the PCR product DNA (total volume) was conducted using a agarose gel in TBE buffer. The DNA sample was then cut from the gel and purified with QIAquick Gel Purification Kit (Qiagen). The purified sample was then submitted to the $^{UC}$DNA Sequencing Facility, Storer Hall, University of California, Davis. The sequencing primers used were 8-Forward, 533-Forward and 1530-Reverse. After obtaining electronic versions of the sequence information (ab1 files), Mac OSX and 4peaks were used to complete the electropherogram evaluation and editing. The sequence was then compared to information available in public databases through the NCBI BLAST algorithm.

After obtaining a tentative identification through NCBI, a series of enzymatic tests were conducted to confirm the identification. Aerobic acid production from carbohydrates was determined with carbohydrate slants. The carbohydrate media used included arabinose, sorbitol, glucose, mannitol, sucrose, rhamnose, lactose, raffinose, and inositol. The organism's ability to take in and utilize citrate as a sole carbon source was determined with a citrate utilization test. To determine if or not the bacteria were likely to be pathogenic, an esculin hydrolysis test was performed. To test for proteolytic enzymes a gelatin hydrolysis test was completed. Methyl red and Voges–Proskauer tests were conducted to determine the type of metabolism the culture was using, and motility was determined with a wet mount.

## RESULTS

When the unknown culture was grown on Nutrient, Tryptic Soy, and Brain Heart Agar media it grew readily, forming many colonies. The appearance of these colonies did not vary significantly. Their morphology was determined to be circular, entire, convex, cream-colored, opaque, smooth to glistening, and 1-2mm in diameter. The cells appeared to be cocci, 0.5-1.0μm in diameter arranged in lose clusters. The Gram stain results indicated they were Gram-positive. The results obtained with 3% potassium hydroxide (KOH test) indicated these organisms had thick peptidoglycan walls.

The 16S ribosomal DNA from the Tegu mouth isolate showed 99% sequence similarity with a DNA sample from *Janibacter melonis*, a type of bacteria isolated from an "abnormally spoiled" oriental melon in Korea. The accession number for this entry was AY522568. There were 1450 out of 1454 bases matching and the bit score was 2663. The taxonomic lineage given for the melon isolate was Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Intrasporangiaceae; *Janibacter*. All the carbohydrate slants used to test for aerobic acid production stayed pink, indicating that acid formation did not occur. The inoculated slant from the Citrate test stayed green indicating that citrate utilization did not occur within these bacteria. The slant from the esculin hydrolysis test turned a very dark brown, almost black color indicating that the microorganisms did hydrolyze esculin. The nutrient gelatin in the gelatin hydrolysis test solidified when placed in an ice bath after 4 days of incubation, indicating

that gelatin hydrolysis did not occur. The MR-VP medium remained yellow when methyl red was added, and did not form a wine-red color when treated with Barrit's reagents. This indicated that not enough acid was formed to test positive in the Methyl Red test and that the culture could not produce acetoin. The lack of obvious motion in the wet mount indicated that this culture was non-motile.

## DISCUSSION

After DNA analysis and enzymatic testing were completed it was found that the most prevalent bacteria type present in the Argentine Tegu's mouth was *Janibacter melonis*. The 16S ribosomal-DNA nucleotide sequence was 99% similar with only 4 bases out of 1454 not matching. The results obtained from the enzymatic testing indicated the Tegu mouth isolate was a non-fermentative strictly aerobic culture that did not form aerobic acids from any of the carbohydrates tested. The culture was positive for esculin hydrolysis, but negative for both gelatin and citrate utilization. Although these characteristics were mostly consistent with those described for *Janibacter melonis*, the melon culture was reported to be positive for citrate utilization, so this might indicate the Tegu mouth isolate was a different strain.

Research indicated that *Janibacter melonis* was originally isolated from a spoiled oriental melon (*Cucumis melo*) from a cultivation field in Korea. (Yoon et al., 2004) The genus *Janibacter* was originally proposed by Martin et al. (1997) At this point, the genus is made up of three species, *Janibacter limosus* (Martin et al., 1997), *Janibacter terrae* (Yoon et al., 2000) and *Janibacter melonis* (Yoon et al., 2004). The tegu that this culture was taken from doesn't eat any melon. His diet consists of raw turkey, raw eggs, and rats. Tegus in general can eat melon, but Rufus doesn't like eating fruit. Since some Tegus do eat melon, it might be possible for bacteria living in a lizard's mouth to become associated with an overly ripe melon, but Tegus are not native to Korea, so this seems unlikely.

Although the Tegu mouth isolate seems likely to be *Janibacter melonis*, a couple of questions have been raised by this investigation. One question is why the culture isolated from the Tegu mouth was not able to utilize citrate while *Janibacter melonis* can. The bigger question is why a type of bacteria found originally in an oriental melon would be found in the mouth of a lizard that only eats eggs and meat.

## ACKNOWLEDGEMENTS

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# Isolation and Identification of *Kocuria rhizophila* From Air

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**In order to collect a sample of microorganism from air, a nutrient agar plate was opened and exposed to air for one hour on a kitchen counter. One of the several microbes collected was then isolated using streak plate inoculation and identified using a combination of the following methods: observations of morphological features, Polymerase Chain Reaction, gel electrophoresis, nucleotide sequencing, electropherogram analysis, BLAST comparison and enzymatic testing. The results of these tests and analyses indicated that the organisms isolated from kitchen air were *Kocuria rhizophila*.**

## INTRODUCTION

The average person is exposed to many different types of microorganisms every day. In addition to normal flora associated with humans and animals, we come in contact with an unknown number of potentially pathogenic microbes. The purpose of this project was to determine the identity of one of the types of microorganisms residing in the air, particularly in the air in a typical kitchen, where most people store, prepare, and sometimes consume, food. In order to accomplish this, an "air plate" was obtained by exposing the agar surface of a nutrient agar plate to air for one hour on a kitchen counter. A single bacterial culture was then isolated from this air plate and the steps necessary for making an accurate identification were initiated.

## MATERIALS AND METHODS

A nutrient agar plate was placed agar-side-down on a kitchen counter in Colfax, California. The plate was opened, and the agar surface was exposed to air for one hour. This "air plate" was then incubated in a laboratory drawer at room temperature for several days. At the end of this time period, when a variety of different looking microorganisms were observed to be growing on the agar surface, one specific type was chosen for identification. A new pure culture was then isolated using streak plate inoculation and a new nutrient agar plate. (Wilson, 2007, pg 44-45)

After obtaining a pure culture, a KOH test was conducted to determine the cell wall type present, and then chromosomal DNA was extracted. A glob of cells was placed into a sterile microfuge tube with 500µL of tris buffer and boiled for 10 minutes. About 20 sterile glass beads were added and the mixture was vortexed for ten minutes to break open the cells. A PCR was run using the primers Bacteria 8 forward and Enteric 1530 reverse and a My-Cycler thermal cycler (Bio-Rad). These primers were designed to amplify 16S ribosomal-DNA. (Wilson, 2007, pg 191-195) Following amplification, the PCR product DNA was run in an electrophoresis chamber in an agarose gel and then stained with ethidium bromide. The DNA was then cut from

the gel and purified using a QIAquick gel purification kit (Qiagen). The purified DNA was then taken to the UC Davis $^{UC}$DNA Sequencing Facility. Gene sequences were returned in the form of electronic files, which were then evaluated and edited using an iBook computer running OSX and then software program 4 PEAKS. The edited gene sequences were compared with existing gene sequences using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) website, providing a tentative identification of the unknown culture.

Finally, several enzymatic tests were performed to verify the identification of the air isolate, including MRVP, Catalase, Oxidase, Citrate utilization, urea hydrolysis, Esculin hydrolysis, Starch hydrolysis, Gelatin hydrolysis, SIM, TSI, and several carbohydrates prepared as agar slants and/or as single carbon sources. (Wilson, 2007, pg 171-189)

## RESULTS

Colony morphology was determined using direct observation and the colonies were found to be circular with entire margins, convex elevation, smooth, shiny surface texture, opaque optical character and yellow pigmentation. Cell morphology was determined from a Gram stain of the culture. The cells were observed to be Gram-positive cocci, occurring as pairs, tetrads, or as single cells. The culture grew in MR-VP medium did not produce acid or acetoin. Bubbles were formed when the culture was exposed to 3% hydrogen peroxide but no color change occurred when it was exposed to oxidase test reagent. The culture was able to take in and catabolize citrate and gelatin, but not urea, esculin, or starch. When grown on Sulfur, Indole, Motility medium (SIM) it did not form hydrogen sulfide ($H_2S$) or indole and was not motile. When grown on Triple-Sugar Iron agar (TSI) the culture formed some acid but did not form hydrogen sulfide ($H_2S$). Aerobic acid was not formed when the culture was grown on

| **Test Performed:** | **Result:** |
|---|---|
| MR | Negative (-) |
| VP | Negative (-) |
| Catalase | Positive (+) |
| Oxidase | Negative (-) |
| Citrate Utilization | Positive (+) |
| Urea hydrolysis | Negative (-) |
| Esculin hydrolysis | Negative (-) |
| Starch hydrolysis | Negative (-) |
| Gelatin hydrolysis | Positive (+) |
| SIM – Indole formation | Negative (-) |
| SIM – $H_2S$ | Negative (-) |
| SIM – Motility | Negative (-) |
| TSI – acid formation | Positive (+) |
| TSI – $H_2S$ | Negative (-) |
| **Carbohydrate Slants** | |
| Lactose – acid formation | Negative (-) |
| Maltose – acid formation | Negative (-) |
| Mannitol – acid formation | Negative (-) |
| **Sole Carbon Source** | |
| Inositol | Negative (-) |
| Arabinose | Negative (-) |
| Sorbitol | Negative (-) |
| Mannitol | |
| **Table 1: Enzymatic Test Results** | |

lactose, maltose or mannitol agar slants, and no growth was observed when it was streaked on agar slants containing inositol, arabinose, sorbitol or mannitol as single carbon sources. The results of various enzymatic tests performed are summarized in Table 1.

## DISCUSSION

Results of enzymatic test data as stated above were compared to data reported in other sources. (Kovács, 1999 and Tang, 2003) This comparison, combined with the results of the PCR, electropherogram analysis, and enzymatic testing performed, indicated that the unknown organisms obtained from the air plate are likely to be *Kocuria rhizophila*. However, some results reported for enzymatic testing, specifically for production of acid from catabolism of glucose and fructose did not agree with testing performed as a part of this project. (Kovács, 1999) This discrepancy in reported results of enzymatic testing may indicate that additional analyses may be warranted.

Species in the genus *Kocuria* are generally considered to be non-pathogenic and are commonly found in the environment and on human skin as normal flora. However, various species in the genus *Kocuria* have been known to be human pathogens. *Kocuria kristinae* was found to be the cause of infection in a case of catheter related bacteremia. (Ma, 2005) This is significant given the widespread distribution of *Kocuria* species and the ability of bacteria to be opportunistic pathogens.

## ACKNOWLEDGEMENTS

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# Careful Where You Cook; You Never Know What's Lurking in Your Kitchen Air.

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**Three different bacterial cultures were isolated from kitchens in the Roseville, California area. Using 16S rRNA gene sequencing, NCBI-BLAST nucleotide comparison, enzymatic testing and information contained within the Bergey's Manual of Determinative Bacteriology, the three organism types were tentatively identified as *Pseudomomas putida*, *Micrococcus luteus* and *Arthrobacter agilis*. Although these three types of bacteria are not generally considered to be pathogenic when associated with food, they are common in water, air and on human skin, so could easily get into food left sitting out in a kitchen.**

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## INTRODUCTION

Becoming aware of exactly what is living in the place where meals are prepared was the purpose behind this investigation. The kitchen is typically where food is kept, where meals are created and sometimes even where foods are presented to people who will consume them shortly thereafter. This is not the kind of place that should contain any sort of organism that has the potential of being pathogenic; however, one can never be sure what exactly has taken up residence in a seemingly clean kitchen. Finding a type of organism that could be potentially pathogenic or harmful if ingested or breathed in could affect not only the owner of the kitchen it was found in, but also human health in general. This investigation was conducted in order to determine exactly what kinds of organisms were living in some typical kitchens and to be certain there was nothing of the pathogenic sort to be found there. After collecting organisms from various locations in three Roseville kitchens, it was expected that the organisms found would not be potentially pathogenic or hazardous to human health in any way.

## MATERIALS AND METHODS

Three separate nutrient agar plates were opened and exposed to the air in three different kitchens in Roseville, California. Each plate was exposed to the air for approximately one hour and then closed and returned to the microbiology laboratory. After four days of incubation at room temperature, several different-looking colonies were easily observed growing on the agar surface within each plate. Using aseptic technique, a single isolated colony was taken from each plate, and each was streaked onto a new nutrient agar plate. After a few more days of incubation, diagnostic tests were begun on the pure cultures grown to determine what types of organism were present. First the colony morphology and color visible to the naked eye was

documented. An indirect stain was then prepared and observed to identify cell morphology (Wilson, 2008), then a Gram stain was prepared to determine the composition of the cell walls (Wilson, 2006). Following the initial staining, a KOH test was conducted. Acid fast and endospore stains were also completed at this time (Wilson, 2008).

DNA extraction was completed using a boil and beat method for the cultures found to be Gram-positive, and a ten-minute boiling method for the culture found to be Gram-negative. The Polymerase Chain Reaction (PCR) was used to amplify the 16S ribosomal-DNA from each isolate using the primers Bacterial 8-forward and 1530-Reverse. Gel electrophoresis was used to separate the approximately 1500-base pair PCR product from other DNA segments and to verify that the PCR was successful. Each DNA sample was then cut out of the gel and purified using a QiAQuick gel Purification Kit (Qiagen). The purified DNA was then taken to the College of Biological Sciences DNA Sequencing facility at UC Davis. The sequencing primers used were Bacteria 8-forward, 533-forward and 1530-reverse. Once the sequence information was received it was analyzed and edited using 4 Peaks software and Mac OSX. The sequences were then compared with those contained in the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) algorithm.

After obtaining a tentative identification for each isolate, the Bergey's Manual of Determinative Bacteriology was consulted to determine the types of enzymatic tests appropriate for characterizing them. Different tests were performed depending on the specific isolate type, but for the Gram-positive cultures included: MR-VP, motility determination (wet mount), oxidase test, citrate utilization (Simmon's citrate), aerobic acid formation from glucose and lactose, urea hydrolysis and esculin hydrolysis (Wilson, 2008). These cultures were maintained at room temperature but one plate of each was also incubated at $37^{o}$ C. Tests run on the Gram-negative culture included the Oxidation/Fermentation or O/F test, gelatin and starch hydrolysis tests and a series of tubes designed to determine if or not the culture could utilize specific carbohydrates as sole sources of carbon. This culture was also grown on Mueller Hinton agar in order to detect pigment production.

## RESULTS

One of the air isolates grown on nutrient agar formed circular, entire, convex, smooth colonies that were yellow in color and ranged in size from 1-3mm in diameter. The indirect stain showed these organisms formed cocci-shaped cells arranged in clusters and tetrads. Most of the cells observed were 1-1.5µm in diameter (Fig. 1). In the Gram stain these bacteria appeared as purple-colored cocci-shaped cells arranged in clusters and tetrads. The KOH test resulted in no change in viscosity. The acid-fast stain showed blue-colored cocci and the endospore stain showed pink-colored cocci; there were no endospores. There was no movement observed in the wet mount prepared for the motility test.

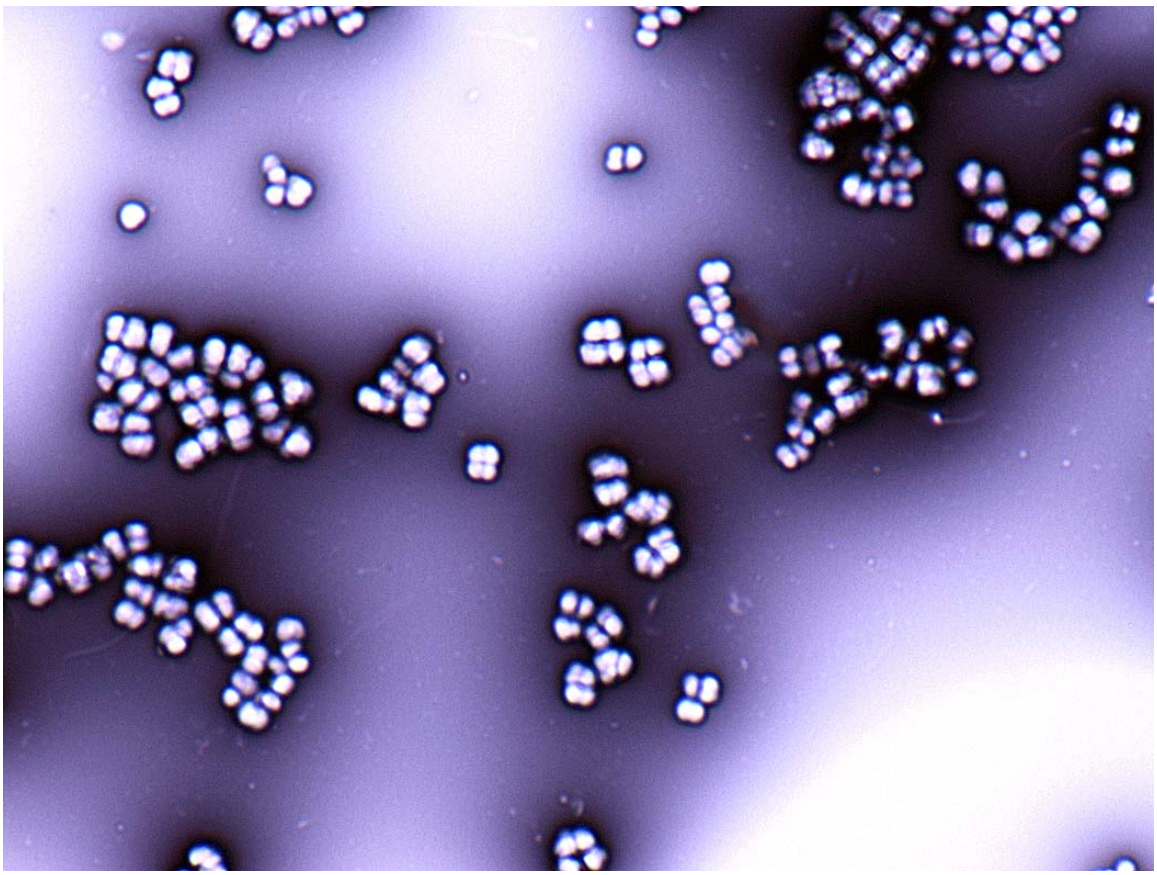

**Figure 1.**

The second isolate formed pink, circular, slightly convex, smooth textured, opaque colonies with regular margins. These ranged in size from 1-4mm in diameter. The Gram stain revealed these to be Gram-positive cocci ranging in size from 0.5 to 1.0μm in diameter. There was no organized arrangement. The KOH test did not reveal sticky goo, therefore confirming that the culture was Gram-positive.

The third isolate formed pale off-white, circular, entire, low-convex, shiny-looking colonies that were translucent on both nutrient agar and Mueller-Hinton agar and reached a maximum size of 5-6mm in diameter. These colonies did not form a colorful pigment on nutrient agar, but when grown on Mueller-Hinton agar produced a light-yellowish, water-soluble pigment. When the culture on the Mueller-Hinton agar was placed under ultra violet light, it was fluorescent green. The Gram-stain showed these cells to be pink, Gram-negative bacilli. In the indirect stain they appeared to be 0.5-1.0μm in diameter and 2-4μm in length. In the KOH test, these organisms formed a thick gooey mass, suggesting they had thin cell walls.

The NCBI BLAST results showed the 16S ribosomal-DNA nucleotide sequence from the first air culture matched most closely with a gene bank sequence from *Micrococcus luteus* isolate SO-139 (AM237388). The query had 99% sequence similarity with 1454/1455 bases matching pairwise. The bit score was 2682. The lineage was Bacteria, Actinobacteria, Actinobacteridae, Actinomycetales, Micrococcineae, Micrococcaceae, *Micrococcus*. The nucleotide sequence from the second culture matched best with a gene bank sequence taken from *Arthrobacter agilis*

(X80748) a culture formerly called *Micrococcus agilis*. This sequence showed a 99% similarity with 1455/1460 bases matching pairwise. The bit score was 2667. The lineage for this culture was Bacteria, Actinobacteria, Actinobacteridae, Actinomycetales, Micrococcineae, Micrococcaceae, *Arthrobacter*. The nucleotide sequence from the third air culture showed 99% sequence similarity with a gene bank sequence from *Pseudomonas putida* strain GB-1 (CP000926). In this case 1459/1466 bases were found to be matching pairwise and the bit score was 2665. The lineage was Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas*.

Similar results were obtained for both of the Gram-positive cultures. The carbohydrate slants that were inoculated and incubated did not change color and stayed dark pink. The MR-VP tests showed no color change for either culture. When the organisms from the first isolate were place on the filter paper surface for the oxidase test they turned purple in color, but those from the second isolate did not. Neither culture produced a color change on urea agar and neither showed any motility when viewed in a wet mount. The culture forming yellow-colored colonies was capable of growing at 37$^{o}$ C, but the culture forming pink-colored colonies was not. When the Gram-negative culture was grown in the O/F medium, both tubes remained green and there were no bubbles in the Durham tube. Of the ten carbon sources tested, only five showed growth and these were xylose, inositol, mannitol, arabinose and glucose. There was no growth visible on the slants containing sorbitol, raffinose, maltose, sucrose or rhamnose as the only carbon sources. The gelatin hydrolysis medium, after a week of being kept in a lab drawer, remained solid when placed in an ice bath.

## DISCUSSION

The bacteria chosen as the first isolate from the nutrient agar exposed to air in a kitchen were Gram-positive cocci that were not acid-fast and did not form endospores. These cells were not motile and were not capable of fermentation. They were unable to form aerobic acid from glucose or lactose, but did form cytochrome C. These organisms were able to grow at room temperature and at 37◦C. The information collected was in agreement with that recorded in the Bergey's Manual for *Micrococcus luteus*. Given that the BLAST comparison showed a 99% match with *Micrococcus luteus*, this is the most likely identity of the first isolate.

The culture chosen as the second isolate were also Gram-positive, not acid-fast and unable to form endospores. According to *Principles of Microbiology*, 2$^{nd}$ edition, these organisms exhibit a life cycle in which there is a change in form from rod-shaped to coccoid cells, but only cocci were observed. These cells were also not motile, were aerobic and were unable to form aerobic acids from glucose or lactose. This culture could not form cytochrome C, and although they grew well at room temperature, did not grow at 37◦C. Since the results of the BLAST comparison showed a 99% match with *Arthrobacter agilis*, this is the most likely identity of the second kitchen air isolate.

The third kitchen air isolate was Gram-negative, and according to the O/F test was strictly respiratory. This culture was capable of using xylose, inositol, mannitol, arabinose and glucose as their only source of carbon, but could not use sorbitol, raffinose, maltose, sucrose or rhamnose. The organisms grew very well at room tempature and on the Mueller-Hinton agar

produced a yellow-green fluorescent pigment visible under ultra violet light. With the BLAST results showing a 99% match with *Pseudomonas putida*, it was determined that this was the most likely identity of the third isolate.

*Micrococcus luteus* are generally considered to be non-pathogenic bacteria appearing as part of the normal flora on the skin of humans and other animals. They can sometimes cause opportunistic infections in immune-compromised individuals, including skin infections and sub-acute bacterial endocarditis. *Arthrobacter agilis* are non-pathogenic to man, plants and animals. They are commonly isolated from dust, water, skin surfaces and salt containing foods. Bacteria identified as *Pseudomonas putida* are commonly found in water and soil. They are sometimes used in bioremediation and to prevent a plant disease called "damping off" that is caused by fungi in the genus *Fusarium*. None of the organisms isolated from air found in kitchens was found to present any hazard to the people residing there.

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# Isolation and Identification of a Gram-positive Bacillus, Corynebacterium auriscanis from an Air Plate

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**A relatively new species, *Corynebacterium auriscanis*, was isolated from a nutrient agar plate exposed to indoor, bedroom air in Penryn, California. Analysis of 16S ribosomal-DNA indicated the air isolate was highly similar (99-100%) to strains of *Corynebacterium auriscanis* originally isolated from dogs. Enzymatic tests were chosen based on those listed for the genus *Corynebacterium* in the Bergey's manual (Vol.2, 1988), and those described in the referenced article (Collins, 1999). Observation of cellular features and the results obtained during enzymatic testing supported the identification, but raised some interesting questions, e.g., do these organisms form part of the normal microbiota of dogs, or could they reside elsewhere? Are they highly virulent or are they only opportunistic pathogens? While the original isolate had a canine origin, being specifically from an infected ear; Zena, a dog residing in the home tested, was never allowed in the bedroom. Evidently, either the dog snuck in, or the organisms were introduced by way of clothing or shoes previously in contact with her. There was some evidence suggesting the air isolate might represent a new strain, since two of the enzymatic tests conducted yielded results unlike those expected. Additional testing would be required to confirm this possibility.**

## INTRODUCTION

A colony observed growing on a nutrient agar plate exposed to air in a teenager's somewhat messy bedroom was chosen as the subject for this investigation. Although the agar surface within the plate had been exposed to air for only one hour, there were many colonies to choose from. Potential sources for microbial inhabitants appeared to include human skin, shoes, dirty dishes, books, papers and various items of previously worn clothing; however, pollen grains and mold spores from the outside environment could not be excluded. Overall, it was anticipated there could be some very interesting organisms present, especially considering the state of the room.

## MATERIALS AND METHODS

The colony selected for this investigation was originally cultured on nutrient agar, but was later isolated on tryptic soy agar (TSA) and then maintained on Blood agar. All transfers were accomplished with a sterile wire loop and each new plate inoculated was streaked to obtain

isolated colonies. Following isolation, colony morphology was determined through direct observation; a Nigrosin indirect stain and a Gram stain were performed (Wilson, 2007 pg. 54-55, 62-63), followed by a wet mount preparation to determine motility. Observations were made with an American Optical compound microscope using 1000X magnification.

Chromosomal DNA was extracted from the culture by boiling a 2mm sample in 10mM Tris buffer (pH 8.0) for ten minutes and then beating the sample for ten minutes with 2mm glass beads. Amplification of 16S ribosomal-DNA was accomplished using the Polymerase Chain Reaction (PCR) involving Taq Master Mix (Quiagen) and the primers Bacteria 8-Forward and 1530-Reverse. Purification of the PCR product with a QIAquick Gel Purification Kit (Quiagen) followed gel electrophoresis of the total volume in agarose and TBE buffer. Personnel in the $^{UC}$DNA Sequencing Facility, Storer Hall, University of California, Davis sequenced the purified DNA sample using the primers Bacteria 8-forward, 533-forward and 1530-Reverse. Electropherograms were evaluated and edited using a Macintosh iBook computer running OSX and 4Peaks. Comparison of the DNA sequence data with that stored in public databases was accomplished using the NCBI BLAST algorithm.

Following tentative identification based on the results obtained with the NCBI BLAST, a number of enzymatic tests were performed, including the catalase and oxidase tests, gelatin, starch, esculin and urea hydrolysis, MR-VP, and the production of aerobic acid from specific carbohydrates (glucose, lactose, sucrose, mannitol, raffinose, arabinose and maltose). A CAMP test was performed on 5% sheep blood agar using a stock culture of *Staphylococcus aureus* (ATCC-25923) as the source for α and β-hemolysin. The results from most tests were read after 4 days of incubation at 37$^{o}$ C (Wilson, 2007). The results for the esculin hydrolysis test were read after 14 days of incubation at room temperature.

## RESULTS

The original colony observed growing on the nutrient agar plate was circular, lobate, raised, smooth-shiny, opaque, cream-colored, and 1.5mm in diameter after two weeks of incubation at room temperature. Colonies on TSA were similar to those grown on nutrient agar, but were convex, yellow-colored and grew to 1.5-2mm in diameter. When grown on blood agar the colonies were circular, entire, pulvinate, smooth-shiny, opaque, cream-colored and grew to 1.5mm in diameter after 48 hours at 37$^{o}$ C. In the indirect stain preparation the cells appeared as small, irregular bacilli, 1.5μm in length and 0.75μm in diameter occurring as single cells, but also in pairs and clusters. In the Gram stain, the cells appeared as dark purple or Gram-positive, and when mixed with 3% KOH, the cell suspension did not increase in viscosity (the KOH test was negative). When observed in a wet mount the cells were non-motile.

The edited and combined 16S ribosomal-DNA nucleotide sequence from this culture was 1453 bases in length, and when compared to data available in the gene bank showed 100% similarity with a 1424 base sequence from the 16S r-RNA gene of *Corynebacterium auriscanis* strain CCUG 39783 (AJ243820.1) with 1407/1407 bases matching. The bit score was 2538. The sequence also showed 99% similarity (1395/1396) with a genebank sequence from the originally described strain of *Corynebacterium auriscanis* (Y13777). The bit score was 2515. The species showing the next closest match (98%) was *Corynebacterium resistens*, a multi-drug

resistant strain found in association with human infections. In this case, only 1388/1414 bases matched and the bit score was 2408.

Results obtained from the enzymatic tests conducted indicated that the culture being investigated was catalase-positive (bubbles formed), but oxidase-negative (no color change appeared on the test paper). The MR and VP tests were both negative (no red coloration appeared when test reagents were added), as was the CAMP test (no enhanced hemolysis was observed). The culture tested positive for urea hydrolysis, showing a bright pink medium after incubation. The bile esculin medium did not turn black, no clear zones appeared around colonies growing on starch agar and exposed to Gram's iodine, and the nutrient gelatin remained solid, indicating that hydrolysis of these substances did not occur. There was no evidence of aerobic acid formation from any of the carbohydrates tested (all media remained red in color).

## DISCUSSION

The culture isolated from the air plate appeared to be very similar to the *Corynebacterium auriscanis* strain described by Collins, 1999, but was not identical. Cell morphology and nucleotide sequences data showed little or no discrepancy (only one base out of the 1397 present in the nucleotide sequence did not match). Some variation was observed when enzymatic test results were compared. The air isolate was not capable of fermentation and did not produce aerobic acid from any of the carbohydrates it was grown on. The culture did not produce cytochrome C, but did contain catalase enzymes. Esculin, gelatin and starch were not hydrolyzed, but urea was, so urease enzymes were formed. According to Collins, 1999 the original strain of *Corynebacterium auriscanis* was negative for urea hydrolysis, variable for esculin hydrolysis and formed aerobic acid from glucose. Therefore, the urea hydrolysis results, along with aerobic acid formation from glucose were conflicting. Subsequent re-testing was not performed due to time constraints and in an attempt to be frugal with media; however, it would have been interesting to repeat those tests.

The original strains of *Corynebacterium auriscanis* (Collins, 1999) were obtained from clinical samples taken from dogs with ear infections. The one the air isolate was compared to specifically came from a "mixed culture with *Staphylococcus intermedius* and *Streptococcus canis* from a dog suffering from bilateral otitis" (Collins, 1999). Although some species of *Corynebacterium, e.g., Corynebacterium diphtheria,* are recognized as important pathogens, others live symbiotically without causing harm to their hosts. Since the dog assumed to be the source of the air isolate was not suffering from any type of infection, this might indicate that this species is not highly pathogenic.

## ACKNOWLEDGEMENTS

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Vol. 2, No. 2

# Isolation and Identification of two Different Types of Microorganisms Found in Arrowhead Bottled Water

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**Two different types of microorganisms were isolated from a 500μL sample of Arrowhead bottled water placed on a Nutrient Agar plate, and were then grown separately as pure cultures. Gram stains were prepared, morphological features were recorded, and then DNA extraction, PCR, gel electrophoresis, and nucleotide sequencing were performed to determine the identity of these microorganisms. Enzymatic testing was conducted to confirm the results obtained with DNA sequencing. Most of the cells contained in the water produced pink colonies determined to be punctiform, entire, low-convex, smooth/shiny/glistening, opaque, pink and 0.5-1mm in diameter. These bacteria were determined to be Gram-negative, bacilli or rod-shaped, arranged as single cells that ranged in size from 1.0-1.5μm in diameter and 3.0-5.0μm in length. They were tentatively identified as *Methylobacterium radiotolerans.* The second isolate from the water formed an irregular, undulate, rough, opaque, orange colony 3mm in diameter. The cells from this colony were determined to be Gram-positive, irregular short rods arranged as streptobacilli, diplobacilli and clusters. The cells were acid-fast and were tentatively identified as *Mycobacterium fortuitum.***

## INTRODUCTION

In the past several years, sales of bottled water have increased dramatically. This is due to the fact that most people prefer bottled water to tap water because of its perceived purity. For instance, Coca-Cola admitted in 2004 that they purify their brand name Dasani water by using a filtering process on ordinary tap water. While bottled water companies use a variety of purification techniques in their production, bacteria may still be present in their water. A four-year study conducted by the National Resources Defense Council (NRDC) found that one-third of 1,000 bottles of bottled water contained contamination. Interestingly, the FDA does not require bottled water that is packaged and sold within the same state to be regulated which accounts for about 65% of all bottled water in the United States (Natural Resources Defense Council, 1999). The primary purpose for this investigation then was to determine if or not there are living organisms present in bottled water sold to the general public. Another purpose was to focus specifically on Arrowhead water. Arrowhead bottled water has a distinctive taste that prompted the question of whether or not there are unique bacteria living in it. With this in mind, the examination of Arrowhead bottled water was initiated.

## MATERIALS AND METHODS

Using a 1000µL micropipette and a sterile tip, 500µL of water from a newly opened bottle of Arrowhead bottled water was transferred to the surface of a Nutrient Agar plate. The water was then spread evenly over the surface using a glass spreader, sterilized by being flamed before use. The Nutrient Agar plate was then inverted so that the agar side was up and placed in a lab drawer at room temperature.

After growth was evident on this plate, samples taken from two different looking colonies were aseptically transferred to new, separate plates in order to grow pure cultures with isolated colonies. A sample from the smaller, more numerous colonies was transferred to nutrient agar, while a sample from the single, larger colony was transferred to Tryptic Soy Agar (TSA). After 2-3 days of growth, a Gram-stain was performed on each culture (Wilson, 2008, pg. 44-45), to determine the cell wall composition and cell morphology of both isolates. An acid-fast stain and a Methyl Red – Vogues-Proskauer test were also performed on the large-colony isolate.

DNA analysis included the extraction of DNA from both cultures by boiling them in 500µL of 10mM Tris buffer, and in the case of orange colonies, beating the cells with glass beads. The amplification of 16S ribosomal-DNA was accomplished using the PCR and Taq Master Mix (Qiagen). The bacteria primers used were Bacteria 8-Forward and 1530-Reverse. The thermal cycler used to amplify the DNA went through the following series of heating and cooling: 94°C for 4 minutes, followed by 34 cycles of 55°C for 45 seconds, 72°C for 2 minutes, and 94°C for 30 seconds. After the last period of 55°C for 45 seconds, the samples were subjected to 72°C for 20 minutes. Following amplification, the total volume of PCR product DNA from each isolate was run in a separate agarose gel with TBE buffer for gel electrophoresis and then stained with ethidium bromide. Then the DNA samples were cut from the gels with a razor blade, weighed, and then a QIAquick gel purification kit was used to purify each sample. The purified DNA was then transported to and processed by the UC Davis DNA Sequencing Facility. After they sent back electronic files (ab1), the program 4 PEAKS and iBook computers running OSX were used to evaluate and edit the electropherograms. Finally, the BLAST algorithm of the NCBI public database was used and tentative identification was determined by comparing sequence data to the information available there.

## RESULTS

The nutrient agar plate initially exposed to 500µL of Arrowhead bottled water contained approximately 350, small, pink-colored colonies after the initial period of incubation at room temperature. When this culture had been isolated, the colony morphology was determined to be punctiform to circular, entire, convex, smooth/shiny/glistening, opaque, pink and 0.5-1mm in diameter. After the Gram-stain was prepared and observed, it was evident that these bacteria were Gram-negative. They displayed rod-shaped cells with a single arrangement and ranged in size from 1.0 to 1.5µm in diameter and 3.0-5.0µm in length.

Growth of the larger, orange-colored colony appeared on the original nutrient agar plate about two weeks after inoculating the plate with 500µL of Arrowhead water. When a sample of these cells was aseptically transferred to a plate of Tryptic Soy Agar, growth was apparent in three to four days. The colonies that formed were circular, entire, raised, glistening, opaque

orange, and measured from 3.0 – 5.0mm in diameter. The Gram stain for this culture resulted in short, purple-colored (Gram-positive) cells arranged as streptobacilli, diplobacilli, single cells and clusters. The acid-fast stain produced red-colored cells. When subjected to the Methyl Red Test, this culture formed a positive, red colored liquid while in the Vogues-Proskauer test they formed a negative, yellow-brown-colored liquid.

After using the NCBI BLAST, it was discovered that the Gram-negative bacteria from the Arrowhead water were most similar to *Methylobacterium radiotolerans*. The 16S ribosomal-DNA nucleotide sequence from these bacteria was 98% similar to a 1405-base gene bank sequence from *Methylobacterium radiotolerans* strain F47 (accession number AM910539), with 1405/1434 bases matching pairwise. The Gram-positive, acid-fast bacteria were found to be most similar to two strains of *Mycobacterium*. The 16S ribosomal-DNA nucleotide sequence from these bacteria showed 98% similarity with that from *Mycobacterium gilvum* strain PYR-GCK (part of a complete genome) and a 1472 base sequence from *Mycobacterium fortuitum* strain SCCTB40 (AY509249) with 1433/1455 bases matching pairwise in both cases.

## DISCUSSION

Many types of organisms are found in water so it is not surprising that *Mycobacterium gilvum/fortuitum* and *Methylobacterium radiotolerans* were present in the source water used by the Arrowhead bottling company. Various *Mycobacterium* and *Methylobacterium* species are commonly found in water and soil (Bergey's Manual*)*, so it makes sense that these organisms could make their way into bottled water. The *Methylobacterium* species in particular are ubiquitous in nature, common in air, and found in a wide variety of environmental, industrial and even clinical samples as part of transient flora or chance contaminants (Bergey's manual). What is somewhat more surprising is that these organisms survived the purification process used to prepare the bottled water for public consumption.

Organisms in the genus *Mycobacterium* have high levels of mycolic acid in their cell walls, and are relatively resistant to most of the broad-spectrum antibiotics (Bergey's manual). Most species are also capable of adapting to growth on very simple substrates, so might readily find the nutrients they need in spring water. As Gram-positive cells, they are more resistant to heat, pressure and radiation than are most Gram-negative bacteria, so might readily survive purification procedures. Organisms in the genus *Methylobacterium* are highly resistant to dehydration, freezing, chlorination, ultra violet light, ionizing radiation, and elevated temperatures (*Bergey's Manual)*. Some strains exhibit resistance to gamma radiation 10-40 times higher than that exhibited by other Gram-negative bacteria. This ability to survive in hostile environments might well explain why they also survived the purification process used on the Arrowhead water.

Although some *Mycobacterium* species cause disease in humans, *Mycobacterium gilvum* and *M. fortuitum* are not recognized as important human pathogens. According to an unpublished article listed with NCBI, at least one strain of *Mycobacterium fortuitum* was found to cause infection in Siamese fightingfish, *Betta splendens*. Although this investigation cannot prove with certainty that the *Mycobacterium* species isolated is either *gilvum* or *fortuitum*, it is relative unlikely that these organisms pose a health hazard to people drinking Arrowhead bottled

water. As for *Methylobacterium radiotolerans*, these are not associated with any disease in humans or other animals (Bergey's manual). They therefore do not pose a health hazard to humans drinking the water.

## ACKNOWLEDGEMENTS

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# Isolation Of Bacteria From the Sierra College Salad Bar.

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**Due to concern over the sanitary conditions of food displayed in the open air, an investigation of the cleanliness of food obtained in a college cafeteria was initiated. Bacteria were cultured from items purchased from a cafeteria salad bar. Three different types of bacteria were isolated and using extensive testing methods, some of their morphological and physiological characteristics were determined. The organism types were identified as *Pseudomonas gessardii*, *Janthinobacterium lividum* and *Flavobacterium frigidimaris*. These findings inspired important questions regarding the sanitation methods employed in the school cafeteria, and left a small portion of the hungry public wondering what else was sharing their meals. Microorganisms are everywhere and many are normal and safe for consumption by humans. Though the three species of bacteria discovered in this investigation were non-pathogenic, the possibility remains that pathogenic bacteria could contaminate our food if proper sanitation steps are not taken.**

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## INTRODUCTION

Everyday students, faculty and guests peruse the items available in the Sierra College cafeteria in search of a meal, assuming they are the only living organisms enjoying a satisfying cup of soup, burrito or tossed salad since the food has been cleaned and is devoid of "germs." Or is it? Watching people saunter down the salad bar line, picking at vegetables, adding some and putting some back, occasionally wiping a nose or coughing into a hand prompted an investigation into the types of microorganisms living in the salad materials which could be contamination by non-sanitary conditions. By displaying food items in the open air, as is typical with salad bars, and allowing customers to pick through the items using casual or non-existent means of cleanliness or aseptic techniques, concessionaires clearly increase the risk of bacterial contamination of the foods being provided. Determining whether or not potentially pathogenic bacteria could be detected on such food items was the object of this study.

## MATERIALS AND METHODS

A small sample of lettuce, tomatoes, cucumbers, kidney beans and carrots was purchased at the Sierra College cafeteria, and transported directly to the microbiology laboratory in a plastic box. To begin extraction of bacteria from these vegetables, they were placed into a large zip lock bag with 5ml of sterile de-ionized water. The bag was shaken to distribute the water, and then a 100µl sample of the water was extracted with a sterile pipette and transferred to a Petri dish containing nutrient agar. The sample was then spread over the agar surface with a sterile

glass spreader and the plate was placed into a lab drawer and left over time to allow the microorganisms to grow and colonies to form. Three distinctly different looking colonies were chosen as subjects for the investigation.

Samples from each of the three chosen colonies were taken from the original Petri dish and inoculated onto three new, fresh, Nutrient Agar plates to allow each to grow into a pure culture. The three new plates were left at room temperature to allow the microorganisms to grow, and observations were made during successive laboratory sessions. This process was repeated until three pure cultures had been obtained, and then a KOH test was performed on each culture to determine if the microorganisms present were Gram-positive or Gram-negative (Wilson, 2006). The KOH test was followed by a Gram-stain (Wilson, 2006) and an Indirect stain to determine the accurate cell size, shape and arrangement (Wilson, 2006). The cultural characteristics of colonies were determined using direct observation, and cellular features were determined by observing the stained preparations magnified 1000 times.

To complete the DNA analysis for the three chosen types of bacteria, a series of steps were required. Chromosomal DNA extraction was completed by placing 2mm culture samples into tubes containing 500µl Tris buffer, mixing the tube contents and then boiling these for 10 minutes. Amplification of 16S ribosomal-DNA using the PCR and Taq Master Mix (Qiagen) was then completed for each cell type. The primers used were Bacteria 8-forward and 1530-Reverse. Gel electrophoresis of the three PCR product DNA samples was then completed in agarose gels submerged in TBE buffer. DNA stained with ethidium bromide was cut from these gels and using QIA quick Gel Purification Kits (Qiagen), the purification of these DNA samples was completed. Purified DNA samples were submitted to the DNA Sequencing Facility, Sorer Hall, University of California, Davis. Following electronic return of the sequence data, electropherogram evaluation and editing was performed using Mac OSX and 4Peaks. Comparison of sequence data to the information available in public databases through the NCBI BLAST algorithm was then completed.

Information obtained through the NCBI BLAST was used to direct a search of the Bergey's Manual of Systematic Bacteriology (Volume 1 and Volume Two Part C, second edition), and enzymatic tests useful in characterizing each organism type were determined. Appropriate examples of the following enzymatic tests were then completed to determine additional characteristics of each bacteria type: Starch hyrolysis, Gelatin hydrolysis, Oxidation/Fermentation test (O/F test), Esculin Hydrolysis, Catalase activity test, Oxidase test for Cytochrome C, Citrate utilization, Triple Sugar Iron (TSI), Sulfur, Indole, and Motility (SIM), Urea Hydrolysis, growth on Tryptic Soy Agar (TSA) and Mueller-Hinton agar (MHA), the ability to ferment various carbohydrates within agar deeps, and the ability to utilize a variety of organic compounds as single carbon sources.

## RESULTS

When grown on nutrient agar, one of the isolates formed circular, filamentous, flat, dull, translucent, milky white colored colonies, 3-10mm in diameter. Similar looking colonies were formed on Mueller Hinton agar, but appeared more yellowish in color and emitted a pale yellow-green fluorescence when exposed to ultraviolet light. These organisms appeared as rod-shaped cells or bacilli 0.5-1µm wide by 1-2µm long. When Gram-stained, the cells appeared pink, and

some had a thin, pink area surrounding them. A second isolate produced dark purple, irregular shaped, opaque, shiny, wet looking colonies with undulate margins and raised to umbonate elevations. Some of these colonies had white edges or lighter colored margins bordering their purple centers. The colonies with white margins appeared more circular than their irregular counterparts. Colony sizes ranged from 0.5 – 10 millimeters in diameter. The Gram stain yielded pinkish-purple, single, diploid and streptobacilli with short chains, i.e., no more than three rods in a row. The indirect stain showed rods of 0.5-1μm in width and 1-3μm in length. The third isolate formed colonies that appeared bright yellow, translucent, irregular, undulate, low raised, shiny, and semi-wrinkled. These colonies were initially 2 to 4 mm in diameter but then grew together, indicating motility. When grown on Tryptic Soy Agar (TSA), these colonies were circular, low convex, bright orange-yellow, translucent, and shiny with diameters of 1 to 4 mm. These organisms were observed to be Gram-negative, very thin bacilli (rods) or coccobacilli arranged as single cells, pallisiding groups or in v-shaped or snapping pairs. (Figure 1). The indirect stain revealed rods 1μm in width and 1 to 5μm in length (Figure 2) appearing in streptobaccili, single, and diplobacilli arrangements.

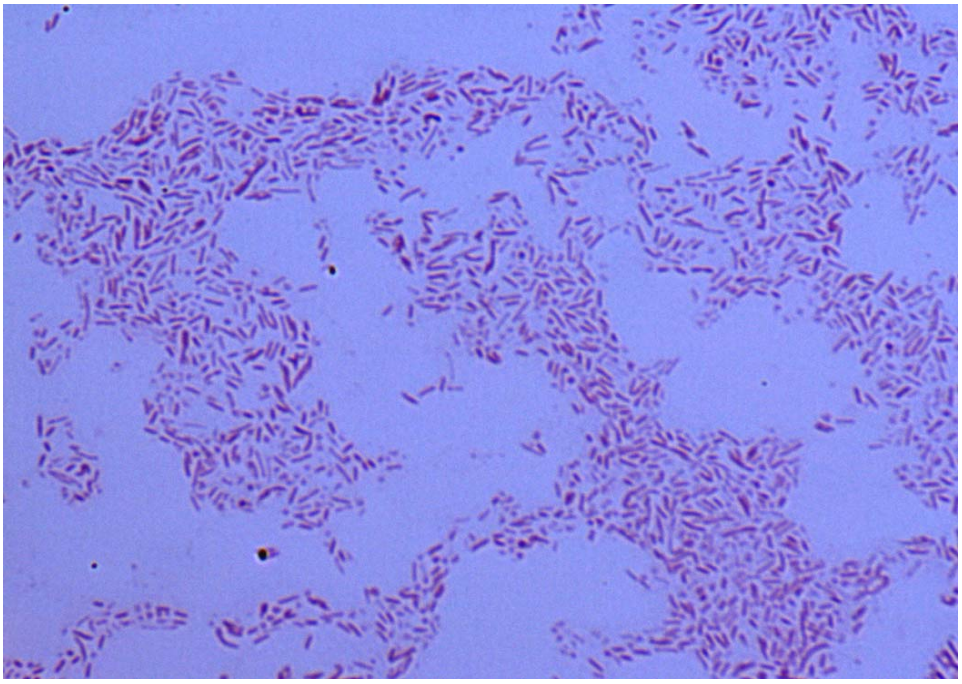

Figure 1 – Gram stain

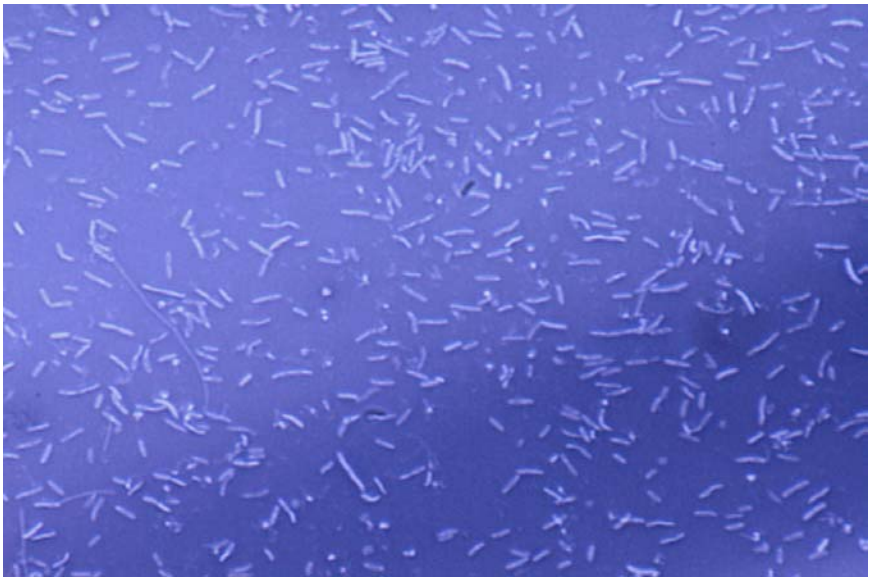

Figure 2 – Nigrosin indirect stain

The16S ribosomal-RNA gene sequence data obtained from the white colonies showed 99% similarity with a 1516 base gene bank sequence from *Pseudomonas gessardii* (AF074384) with 1463/1467 bases matching pairwise and a bit score of 2689. The sequence also showed 99% similarity with a 1489 base sequence from *Pseudomonas fluorescens* with 1464/1468 bases matching pairwise (bit score = 2688) and a 1516 base sequence from *Pseudomonas libanensis* (AF057645) with 1462/1467 bases matching pairwise and a bit score of 2682. The16S ribosomal-RNA gene sequence data obtained from the purple-colored colonies showed 99% similarity with a 1486 base gene bank sequence from *Janthinobacterium lividum* (AF174648) with 1459/1460 bases matching pairwise and a bit score of 2693. Two other strains of *Janthinobacterium lividum* also showed close sequence similarity. The16S ribosomal-RNA gene sequence data obtained from the yellow-colored colonies showed 99% similarity with a 1501 base gene bank sequence from *Flavobacterium johnsoniae* (unpublished) with 1435/1447 bases matching pairwise and a bit score of 2603. The sequence also showed 99% similarity with a 1424 base sequence from *Flavobacterium frigidimaris* (AB183888) with 1413/1416 bases matching pairwise and a bit score of 2597.

Enzymatic tests conducted with samples from the white colored colonies yielded the following: The oxidation/fermentation (O/F) medium remained green in the tube sealed with vaspar and in the unsealed tube. No gas bubbles were evident inside the Durham tube. The nutrient gelatin used to indicate gelatin hydrolysis remained solid and the starch agar medium used to indicate starch hydrolysis turned dark purple when Gram's iodine solution was added. For the single carbon source slants inoculated with the organisms, growth was evident on arabinose, xylose, maltose, glucose, mannitol, and inositol. No color change occurred when the culture was exposed to the oxidase test reagent. Bubbles appeared when the culture was exposed to 3% hydrogen peroxide. After incubation the SIM medium remained yellow, and the stab line appeared slightly cloudy. The Kovac's reagent added to the SIM medium surface remained yellow.

Enzymatic tests conducted with samples from the purple colored colonies yielded the following: Cells exposed to 3% potassium hydroxide (KOH) yielded snot-like, slimy goo indicative of Gram-negative bacteria. The purple pigment also changed to a deep blue color. The oxidation/fermentation (O/F) medium remained green in the tube sealed with vaspar and in the unsealed tube a layer of deep purple formed at the agar surface. No gas bubbles were evident inside the Durham tube. Cells taken from the pale margins of colonies exposed to the Oxidase test reagent turned dark purple. Bubbles were observed during the Catalase test, indicating that the bacteria were breaking down hydrogen peroxide and releasing water and oxygen. The green medium of a Citrate test turned blue, while the pale peach-colored medium of a Urea hydrolysis test stayed peach-colored. The SIM medium remained yellow, but became cloudy along the upper portion of the stab line; no color change occurred when Kovac's reagent was added to the medium surface. The bile esculin medium changed color from gray-brown to black, nutrient gelatin was liquefied and clear zones appeared behind colonies growing on starch agar when Gram's iodine was added. Agar slants containing maltose, glucose, arabinose and inositol as single carbon sources all showed growth of colonies. Enzymatic tests conducted with samples taken from the yellow-colored colonies yielded the following: The Oxidation/Fermentation (O/F) test medium remained green in both tubes and there was no evidence of gas being released. Yellow-colored medium at the top of the unsealed tube showed acid was formed where there was exposure to oxygen. Cells subjected to the Catalase test bubbled when hydrogen peroxide was added. The Oxidase test, which was conducted twice, confirmed that a dark purple color was formed, indicating the presence of cytochrome C. Inoculation and incubation of Esculin test medium resulted in the formation of a black precipitate from the bacteria hydrolyzing esculin. When grown on starch agar, these bacteria catabolized the starch leaving clear zones against a dark purple-colored medium when Gram's iodine was added. The nutrient gelatin was incubated at room temperature, and became almost completely liquefied after two weeks. The SIM medium remained yellow in color, as did the Kovac's reagent added to the medium surface; however, the SIM test for motility was positive because of the turbid medium and non-distinct stabline. Testing for aerobic acid formation on carbohydrate slants revealed a color change from red to yellow with Arabinose, Maltose, Mannitol, and Sucrose but not with glucose and raffinose. Utilization tests involving sugars as the only carbon source demonstrated that inositol, rhamnose, and sorbitol would not support growth and colony formation.

## DISCUSSION

The bacteria forming white colonies on nutrient agar were determined to be Gram-negative bacilli in the genus *Pseudomonas*. The 16S ribosomal-DNA nucleotide sequence data strongly suggested they were *Pseudomonas gessardii* (a newly identified species) or *Pseudomonas fluorescens*. These organisms used a respiratory type of metabolism, were catalase-positive, were unable to hydrolyze gelatin or starch, and were able to grow on media containing arabinose, xylose, maltose, glucose, mannitol and inositol as their only carbon source. According to the results obtained with SIM medium these organisms were motile, but did not produce hydrogen sulfide ($H_2S$) or indole. The results obtain with the oxidase test reagent

suggested they were unable to form cytochrome C, but this data may have been in error because most *Pseudomonas* species are oxidase-positive.

The culture forming purple-colored colonies on nutrient agar were also determined to be Gram-negative bacilli. The 16S ribosomal-DNA nucleotide sequence data indicated they were *Janthinobacterium lividum*. These bacteria used a respiratory type of metabolism, were catalase and oxidase-positive, were able to utilize sodium citrate as a single carbon source, but could not hydrolyze urea to form ammonia. They were able to hydrolyze starch, gelatin and bile esculin, but did not form indole or hydrogen sulfide ($H_2S$). They were motile and capable of utilizing maltose, glucose, arabinose and inositol as single carbon sources. According to information provided in the Bergey's manual of Determinative Bacteriology (Second edition, Volume 2, Part C) *Janthinobacterium lividum* produces a purple pigment called violacein, and has the same characteristics as those described for the purple-pigmented salad organisms except that they are negative for starch hydrolysis and produce aerobic acid from glucose. Atypical strains are described as being weakly positive for starch hydrolysis, variable for acid formation on glucose, but negative for gelatin and esculin hydrolysis. The salad culture, therefore, appears to be more similar to the typical strains of *Janthinobacterium lividum*, but not identical.

Like the other two cultures, the salad isolates forming yellow colonies were Gram-negative bacilli not capable of fermenting glucose, but able to use a respiratory (aerobic) type of metabolism. They produced aerobic acid in association with their glucose catabolism in O/F medium, but not when grown on a glucose slant containing phenol red. They did form aerobic acid from arabinose, maltose, mannitol and sucrose, but not from raffinose. When grown on agar slants containing single carbon sources, they showed growth on glucose, maltose, arabinose, sucrose, raffinose, and mannitol, but not inositol, rhamnose or sorbitol. These organisms were catalase-positive, oxidase-positive, and were capable of hydrolysis when grown on bile esculin, starch and gelatin. They did not form hydrogen sulfide ($H_2S$) or indole, but were motile when grown in SIM medium. According to a study by Nogi, et al (2005), *Flavobacterium frigidimaris* are psychrotolerant organisms isolated from Antarctic seawater that have the same characteristics as those of the yellow-pigmented salad bar isolates except for a negative Oxidase test, and will usually produce aerobic acid when grown on glucose and raffinose. The salad bar isolates did form aerobic acid from glucose in the O/F medium, so this characteristic is not inconsistent. The negative oxidase test described for *Flavobacterium frigidimaris* might be due to a difference in testing methods used, or perhaps the salad bar isolates represent mutant forms of *Flavobacterium frigidimaris* adapted to the harsh environment of the Sierra College cafeteria salad bar.

In our day-to-day lives, many of us tend to take sanitation and cleanliness of our food for granted, assuming that proper steps have been taken by the cafeteria or restaurant staff to ensure our safety and health. Unfortunately, there are common circumstances that lead to an increased risk of microbial contamination of our food which can result in illness as a consequence of ingestion of this tainted food. For example, salad bars where patrons can pick through the food and there are no rules against replacing an unwanted item from the customers' plate back into the salad bar, introduces the potential for infectious contact from handling the food. This investigation of vegetable items obtained from an open salad bar revealed the presence of three different species of organisms commonly associated with soil and water. Though these were not pathogens, there remains the possibility that our food could be contaminated from non-sanitary practices. We urge continued investigation of these conditions with the eventual goals of increasing the awareness of this hazard and changing the conditions and practices to ensure better sanitation.

## ACKNOWLEDGMENTS

We would like to thank Harriet Wilson and Sasha Warren for their extensive help and guidance throughout this project. We also appreciate the assistance of Jim Wilson and all of the assistants in the laboratory for their help in running information to UC Davis, taking photographs and setting up within the classroom and lab. The Microbiology sponsors are needed for the completion of certain procedures within the lab, and include the Sierra College Foundation, the North Valley and Mountain Biotechnology Center at American River College. We also appreciate the work completed by the personnel in the [UC]DNA Sequencing Facility at University of California, Davis.

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# Isolation and Identification of Selected Gram-positive Bacteria from Household Air in Lincoln, California

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**Two Gram-positive bacteria were isolated from indoor air in Lincoln, California. After the extraction of chromosomal DNA from these isolates, amplification of 16S ribosomal DNA was completed using the Polymerase Chain Reaction (PCR). Gel electrophoresis, DNA purification, DNA sequencing, and electropherogram analysis was accomplished, and then the data was compared to that available in the public databases through the NCBI BLAST. The two species showing greatest similarity to the two isolates were *Micrococcus luteus* and *Arthrobacter agilis* (formerly identified as *Micrococcus agilis*). Results obtained through enzymatic testing confirmed the tentative identification.**

## INTRODUCTION

This student project, conducted by a Biological Sciences major at Sierra College, initially began as a Microbiology lab assignment designed to demonstrate the variety of microbes present in air. A nutrient agar plate was opened and the medium surface exposed to air inside a Lincoln home for one hour. The plate was then returned to the laboratory. After a week of incubation at room temperature, two of the most colorful colonies present were selected as the basis for a semester project involving isolation and identification of unknown types of microorganisms.

## MATERIALS AND METHODS

A Nutrient Agar plate was opened, placed agar side down, and the medium surface exposed to household air for one hour. Subsequently, the lid was replaced and the plate was placed in a lab drawer, agar side up, for an incubation period of approximately one week at room temperature. The two colonies with the most interesting morphology were selected and samples from these were aseptically transferred (Wilson, 2007) and streaked onto separate Nutrient Agar plates for the purpose of establishing pure cultures. This procedure was repeated once more to obtain pure cultures, and the new plates were streaked in a manner that would yield isolated colonies (Wilson, 2007). These cultures were temporarily identified as culture A and culture B

After establishing pure cultures with well-isolated colonies, the process of DNA extraction was initiated. A Gram-stain (Wilson, 2007) was prepared, in order to determine whether to boil and beat or just boil the cells. This was followed by a KOH test (Wilson, 2007) as an alternate method for determining the cell wall type of each culture. DNA extraction involved taking a blob of cell material approximately 2mm in diameter from each culture and

adding these to separate 1.5ml tubes containing 500μL of 10mM Tris buffer (pH 8.5). These samples were boiled for 10 minutes and then beaten with 2mm glass beads to break up the thick peptidoglycan walls of the Gram-positive bacteria. The steps required to complete the Polymerase Chain Reaction (PCR) procedure were followed as described in "Application of the Polymerase Chain Reaction in Bacterial Identification" (Wilson, 2007), using Taq polymerase as the catalyzing enzyme. The oligonucleotide primers used were Bacteria 8-Forward and 1530-Reverse.

Gel electrophoresis was completed next, (Wilson, 2007) involving the total volume of each PCR product. These DNA samples were mixed with loading/tracking dye, carefully inserted into the wells of agarose gels, and exposed to electric fields. Following electrophoresis, the gels were stained with ethidium bromide, the DNA was cut out and then purified with QIAquick gel purification kits (Qiagen). The purified DNA samples were then taken to the DNA Sequencing Facility at UC Davis. After obtaining the sequence data electronically, the electopherograms were edited and combined, using 4Peaks software, as outlined in "Automated Nucleotide Sequencing and Electropherogram Evaluation" (Wilson, 2007). The resulting data was compared with other bacteria sequencing data by using NCBI's BLAST (Basic Local Alignment Search Tool) function.

After a tentative identification for each culture had been obtained, Volume 2 of the Bergey's Manual of Determinative Bacteriology was consulted to determine the types of enzymatic tests useful in characterizing each culture. A series of enzymatic tests were then completed on each culture and included: catalase test, oxidase test, MR-VP tests, esculin hydrolysis, starch hydrolysis, growth on media containing 7.5% NaCl (mannitol salt agar), and growth at 37$^{o}$ C. The specific steps necessary for completing these tests can be found on pages 171-186 of the Microbiology Laboratory Syllabus (Wilson, 2007). The results obtained from these tests were then compared to the data found in the Bergey's Manual, Volume 2, pages 1004-1007.

## RESULTS

The Gram-stains prepared showed that both organism types were Gram-positive. This was confirmed by a KOH test for each culture (no viscosity was observed). The cell morphology of the first culture (A) was determined to be spherical (cocci), 1-2μm in diameter, arranged generally in pairs (diplococci) and groups of four (tetrads). The colonies formed by this culture were circular, undulate to lobate, convex but very fluted/ridged/wrinkled on top, opaque, bright yellow, and 1-7mm in diameter. They were collected and maintained on nutrient agar. When grown on TSA, this culture formed colonies that were lighter in color (a pale yellow). This culture had beautifully arranged loops and folds on its colony surfaces. Some were more plainly ridged, but the larger ones were quite intricate and amazing to look at through the stereoscope. The species showing greatest similarity, based on BLAST results, was *Micrococcus luteus* isolate CV31. The length of the nucleotide sequence available in the gene banks was 1506 base pairs, and the accession number was AJ717367.

The second culture (B) appeared to produce both spherical cells and some elongated spheres or short rods. These were 0.5μm in diameter and 0.5-1.5μm in length, organized as diplococci and tetrads. The colonies were circular, entire, convex, shiny, opaque, dark red, and

0.5-1.5mm in diameter. They were collected on nutrient agar but were later maintained on TSA. This culture grew rather slowly, with initially punctiform colonies. These were a deep cherry red when grown on TSA, but were somewhat lighter in color when grown on nutrient agar. The species showing greatest similarity, based on BLAST results, was *Arthrobacter agilis*, also listed as *Micrococcus agilis*. The length of the nucleotide sequence available in the gene banks was 1482 base pairs, and the accession number was X80748.

The results obtained with the enzymatic testing of each culture are shown below in comparison with data found in the Bergey's Manual of Determinative Bacteriology Volume 2. The first culture (culture A) produced results consistent with those expected for *Micrococcus luteus*, while the second culture (culture B) produced results consistent with *Arthrobacter agilis*. The descriptions for colony color were more consistent when the cultures were grown on TSA than on nutrient agar.

| | *M. luteus* | *Culture A* | *M. agilis* | *Culture B* |
|---|---|---|---|---|
| MR (acid production): | (–) | (–) | (–) | (–) |
| VP (acetoin formation): | (–) | (–) | (–) | (–) |
| Catalase: | (+) | (+) | (+) | (+) |
| Oxidase – Cytochrome C: | (+) | (+) | (–) | (–) |
| Esculin hydrolysis: | (–) | (–) | (+) | (+) |
| Starch hydrolysis: | (–) | (–) | (+) | (+) |
| Growth at 37* C: | (+) | (+) | (–) | (–) |
| Growth on Mannitol Salt Agar: | (+) | (+) | (–) | (–) |
| TSA compared to NA: | yellow | pale yellow | pink | deep red |

## DISCUSSION

The tentative identifications made through the BLAST analysis were verified through the results obtained with the enzymatic testing, as shown in the comparison above. This suggests that the cultures isolated from the air plate were correctly identified as *Micrococcus luteus* and *Arthrobacter agilis*. A seeming disagreement regarding the name of culture A led to researching information regarding the classification of the genera *Micrococcus* and *Arthrobacter*. The 16S ribosomal DNA from 15 species within those two genera were phylogenetically analyzed, resulting in the conclusion that "The degrees of relatedness between *M. agilis* and certain members of the genus *Arthrobacter* were significantly higher than the degrees of relatedness between *M. agilis* and the other two *Micrococcus* species and even between many *Arthrobacter* species. This result suggested that *M. agilis* is a member of the genus *Arthrobacter* sensu stricto, but that its development is blocked so that only one stage of the life cycle typical of *Arthrobacter* species is observed (Koch, 1995, pg. 837)." A case can be made for this change in taxonomic rank due to the fact that, compared to the original time in which they were classified, much more complex information about these genera is now available through DNA analysis and the study of phylogeny.

According to studies done by M. Weiser and others, the species *M. luteus* exhibits great diversity in cell wall composition and biochemical properties. The study states, "To our

knowledge, no single bacterial species has been described that is characterized by such large differences in its quinone system and its peptidoglycan type (Weiser, 2002, p. 634-635)." The report states that, after much discussion, it was deemed to be premature to dissociate *M. luteus* into three subspecies. However, they stated that *M. luteus* should be dissected into *M. luteus* biovar I, II, and III, and stated criteria for making that differentiation. If more time had been available, the further study of the isolated specimen would have been an intriguing continuation of the identification process.

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