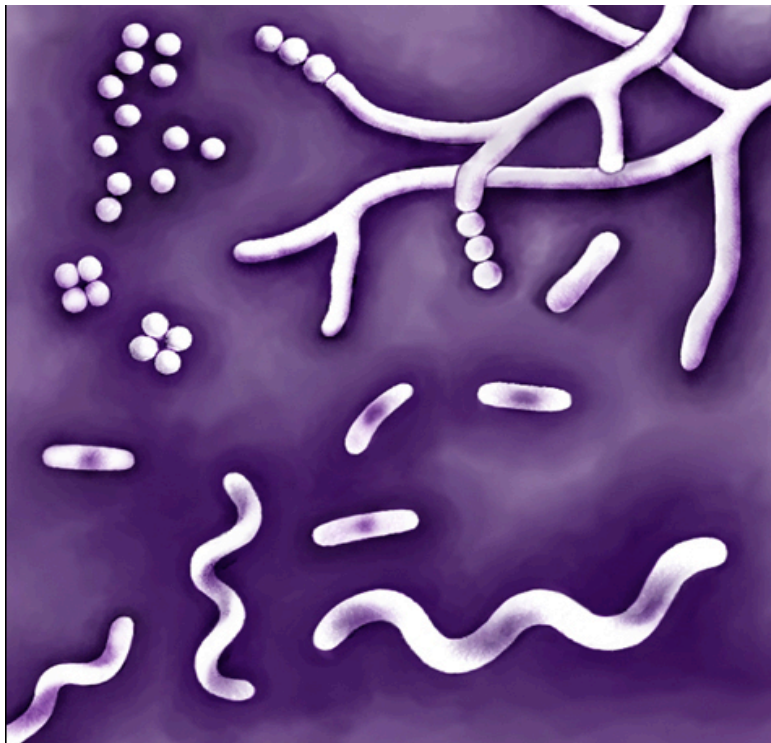


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Department of Biological Sciences

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All papers contained in this journal represent original work by the authors. The editorial staff provided minimal revision prior to publication.

Cover photo:

An illustration of various types of bacteria in an indirect stain. (H. Wilson)

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Isolation of *Neisseria canis* From Dog Saliva - Why it Might Not Be Such A Good Idea to Kiss Your Dog

MELANIE BLANK

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Received 26 June 2007/Accepted 15 July 2007

A Bacterial culture identified as *Neisseria canis* was isolated from the saliva of a domestic mixed breed canine. Identification was based primarily on the 16S rRNA gene nucleotide sequencing results showing 99% similarity with a gene bank sample of this species. Colony and cell morphology were determined and enzymatic tests were completed. The potential pathogenicity of *Neisseria canis* in humans if acquired by a dog “kiss” was investigated.

INTRODUCTION

Many Americans are so crazy about their dogs, that they allow their dogs to “kiss” them. Other dog lovers allow their dogs to eat food off their fork or spoon, and then continue using the utensil without thinking about what microorganisms are in the dog’s saliva, which is probably entering their oral cavity. Evidence provided by this investigation shows pet owners why kissing their dog might not be such a good idea, i.e., that certain bacteria transmitted to humans through dog saliva could cause disease. The *Neisseria canis* isolated from dog saliva during this investigation is recognized as an opportunistic pathogen.



Neisseria canis isolated from this canine

MATERIALS AND METHODS

Isolation of the subject culture of *Neisseria canis* was accomplished by collecting dog saliva with a sterile cotton swab and streaking a blood agar plate. A pure culture was obtained by transferring samples taken from well-isolated colonies onto two additional blood agar plates.

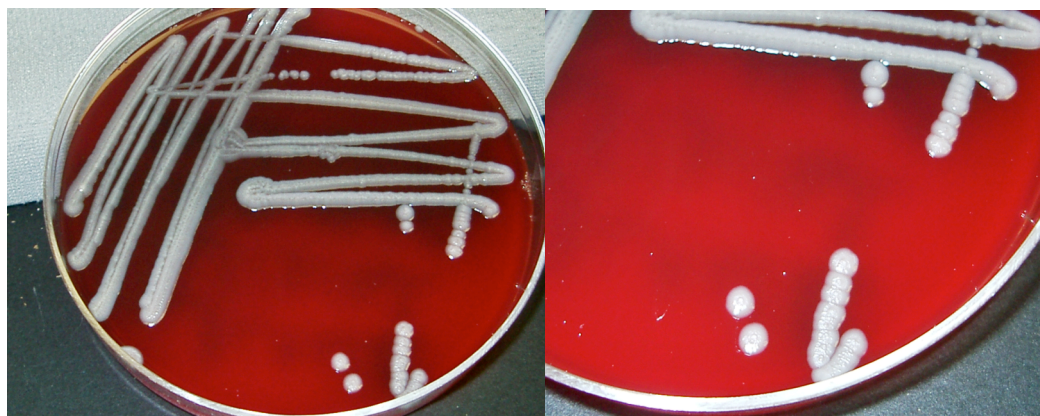
Cell wall composition was determined by completing a Gram stain. Nigrosin and Congo Red indirect stains were performed to confirm cell shape and size. Enzymatic tests for glucose and sucrose acid production (fermentation) were also performed, along with a catalase test.

DNA analysis was performed using the following methods: 1) Extraction of DNA by boiling cells in 10mM Tris buffer (pH 8.0); 2) Amplification of 16S ribosomal-DNA using the PCR and Taq Master Mix (Qiagen); The primers used were Bacteria 8-Forward and Universal 1492-Reverse; 3) Gel electrophoresis of PCR product DNA (total volume), using agarose and TBE buffer; 4) Purification of DNA samples using QIAquick Gel Purification Kits (Qiagen); 5) submission of purified DNA to the ^{uc}DNA Sequencing Facility, Storer Hall, University of California, Davis; The Sequencing primers used were bacteria 8-Forward and universal 1492-Reverse; 6) Electropherogram evaluation and editing using Mac OSX and 4Peaks; 7) Comparison of sequence data to information available in public databases through the NCBI BLAST algorithm.

RESULTS

A moderate growth pure culture was obtained on the second blood agar plate after incubation at 37 degrees, for 24 hours. Isolation of the pure culture resulted in the formation of yellowish pigmented, circular, opaque colonies, rough in texture, 2 to 4 mm in size. The organisms were non-hemolytic (demonstrated gamma-hemolysis) on blood agar.

Colonies of *Neisseria canis*

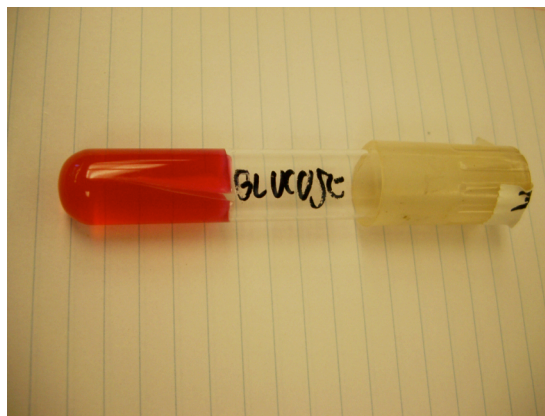
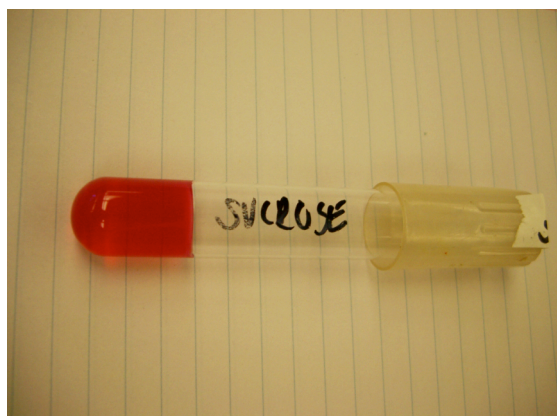


The Gram stain showed small Gram-negative diplococci. Cell shape (cocci) and size (0.8-1.5µm in diameter) was confirmed using Nigrosin and Congo Red indirect stains. The culture did not acidify glucose or sucrose agar deeps. The catalase test was positive. These

laboratory diagnostic tests were consistent with the identification of *N. canis*, according to the Bergey's Manual of Systematic Bacteriology, Second Edition, Volume 2, Part C.

SUMMARY TABLE – *Neisseria canis* as indicated in Bergeys Manual Vs project isolate

	Bergey's Manual	isolate
Gram stain	-	-
cell shape	cocci	cocci
sucrose (acid) production	-	-
glucose (acid) production	-	-



Finally the segment of the isolate's 16S ribosomal-RNA gene subject to an NCBI BLAST analysis was found to have significant similarity (99%) with a 1462 base Gene Bank representative identified as *Neisseria canis* - isolate VA25810gg_03 (AY426974.1).

DISCUSSION

Bacteria identified as *Neisseria canis* are part of the normal flora of domestic dogs' mouths, and in spite of the species name, are also found in the throats of cats. There have been reported cases of *Neisseria canis* being isolated from human clinical samples. In one case, a cat transmitted these bacteria to a patient through a bite wound. In another case study a 50-year old man in good health, presented with a purulent wound on the sole of his foot, after the patient had stepped on a dog bone a few days before. A swab of the culture was taken, *N. canis* was isolated and a course of antibiotics commenced (metronidazole and amoxycillin/clavulanic acid). Seven days later the patient made a full recovery.

These case reports demonstrate the potential of *Neisseria canis* as a human pathogen when transmitted through a bite or puncture wound. Isolations of *N. canis* in humans are rare (perhaps due to under reporting); however, these organisms should be considered as opportunistic pathogen after septic inoculation, such as occurs with a bite wound. Further

investigation is suggested to determine the potential pathogenicity of these bacteria when transmitted to healthy humans through direct oral contact with dog saliva.

In summary, dogs can pass microorganisms to humans through their saliva. While you are not likely to get seriously ill from kissing, touching, or owning dogs, it is important to remember that *Neisseria canis* is often present in dog saliva, and is an opportunistic pathogen. Therefore, in order to protect yourself from illness, it is advisable not to let your dog kiss you, and to thoroughly wash your hands with soap (surfactant) and water, after contact with dogs.

ACKNOWLEDGEMENTS

This investigation could not have been conducted without the assistance of professor Harriet Wilson, and funding provided by the Sierra College Foundation and the North Valley and Mountain Biotechnology Center, at American River College. Personnel in the ^{UC}DNA Sequencing Facility have also been extremely helpful in providing data used to confirm the identity of the isolate.

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Isolation and Identification of Micrococcus on an Air Plate From the Sierra College Microbiology Laboratory (Room S114)

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Microorganisms are everywhere, and the Sierra College Microbiology laboratory is no exception. The unknown culture was identified through isolation, morphological observation, Polymerase Chain Reaction, gel electrophoresis, and sequencing completed at the ^{UC}DNA Sequencing Facility. The nucleotide sequence returned from Davis was edited using 4peaks and electropherogram evaluation, and was then compared to data available through the National Center for Biotechnology Information (NCBI) using BLAST. The culture was identified as *Micrococcus luteus*, and physiological tests were performed to validate these results through application of the Bergey's Manual of Systemic Bacteriology Vol. 2.

INTRODUCTION

The goal of this investigation was to expose a nutrient agar plate to open air for one hour in the microbiology laboratory at Sierra College (Room S114) and assess what organisms would develop. Many colorful colonies were present after several days of incubation ranging in color from red, black, and, grey to white and yellow. From the bouquet of colonies present, a yellow sample was chosen, with coloration resembling a golden yellow pigment. Through careful observation and application of the scientific method, the unknown microorganisms were identified as *Micrococcus luteus* (2) .

MATERIALS AND METHODS

A nutrient agar plate was used initially to collect the microorganisms from the air. This plate was opened and exposed to air in the laboratory for approximately one hour. After the exposed agar was incubated for 72 hours at room temperature, many colonies had formed. A pure culture was obtained by taking a single colony from the original plate and streaking it on new nutrient agar. This process was repeated multiple times. When observation of a Gram stained sample indicated only one type of organism was growing on the plate, DNA was extracted from the sample using a method involving boiling in Tris buffer and beating with glass beads. The Polymerase Chain Reaction was used to amplify the 16S ribosomal-DNA by using Taq polymerase and the oligonucleotide primers Bacteria 8 forward and Universal 1492 reverse. After a 1484 bp region of the bacterial chromosome was amplified, gel electrophoresis was run to isolate the PCR product, and this was cut out and purified using a purification kit made by Qiagen. The purified PCR product DNA was then sent to the ^{UC}DNA Sequencing Facility at UC Davis. The gene sequences sent back were evaluated and edited with the 4peaks

program using Mac OSX. The final sequence was then compared with the National Center of Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST), resulting in a 99% identification match (2). To confirm these results the Bergey's Manual of Systematic Bacteriology was consulted and the indicated tests were performed to verify the organism's true identity (3).

RESULTS

Micrococcus luteus formed golden yellow, circular, convex, smooth-shiny colonies with an entire margin. The largest colony was 3mm in diameter. The Gram stain results identified *M. luteus* as Gram positive. Growth on nutrient agar with 7.5% NaCl (Mannitol Salt Agar) was positive, and growth at 37° C on nutrient agar was positive. There was also growth on Simmons citrate agar. The ability of *M. luteus* to produce aerobic acid from glucose was negative, proving these microorganisms to be obligate aerobes. The Esculin hydrolysis test was negative, and the Oxidase test was positive. The Motility test showed *M. luteus* to be negative for motility (1).

DISCUSSION

Micrococcus luteus are usually regarded as non-pathogenic and are commonly described as contaminants in various settings. Though often found growing on air plates, these bacteria should be recognized as potentially pathogenic to immune compromised individuals. According to the Bergey's Manual, *M. luteus* and *M. varians* are the only species of *Micrococcus* to form bright yellow colonies on nutrient agar. Being highly resistant to reduced water potential, tolerant to drying, and to high salt concentrations, these bacteria are adaptive and frequently colonize along with others as the normal flora associated with mammalian skin. *M. luteus* are also found commonly in the soil, dust, water, and air (4).

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The Isolation of *Macrococcus equipericus* from a Canine Mouth

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The project covers the isolation of *Macrococcus equipericus* from a canine mouth in order to understand some of the natural flora that abides there. *Macrococcus equipericus* were originally part of the genus *Staphylococcus* but were given a unique genus along with other species due to the lack of teichoic acid in their cell walls, unique features of their 16S ribosomal-RNA, and differences in G+C concentration. These bacteria may be further distinguished as part of a unique species through various phenotypic characteristics observed through enzymatic and antimicrobial testing and cell and colony morphology.

INTRODUCTION

The following was conducted as an investigation of the identity of an organism commonly found in a canine mouth; specifically, a female, half-English pointer, half-mutt, family pet of Tim and Joanne Brown, known as “Lucy.” The project was begun due to the common misunderstanding that dogs have relatively clean mouths that may even contain antibacterial, cleansing, or “healing” qualities. After running tests on one culture obtained from a typical dog, it was concluded that bacteria, and all manner of microbial life, seem to maintain a healthy, if not happy, residence in the canine population

MATERIALS AND METHODS

A sample of *Macrococcus equipericus* was isolated by swabbing the inside of a canine mouth with a sterile cotton swab. This was then streaked onto a plate of blood agar, and after 24 hours incubation, a well-isolated colony of the, then, unknown *Macrococcus equipericus* was restreaked onto a 2nd blood agar plate to establish a pure culture.

The phenotypic characterization of the organism was observed in a Gram-stain, while cell morphology and arrangement were determined in a nigrosin indirect stain. Colony morphology and pigmentation were observed on nutrient, blood agar, and tryptic soy agar plates. The organisms were tested for catalase, oxidase, coagulase, and hemolytic activity, acid and acetoin production with MR-VP tests, fermentation of arabinose, raffinose, sorbitol, mannitol, sucrose, and lactose carbohydrates. They were also tested for urease and esculin hydrolysis activity. Antimicrobial sensitivity testing was performed with a Kirby-Bauer test on Mueller Hinton agar with Ampicillin, Bactricin, Penicillin, Polymyxin B, Streptomycin, Tetracycline, and Triple sulfa.

A PCR was run on the DNA, and the product sequenced for comparison with gene bank sequences to determine the specific genus and species. The DNA was extracted by boiling the cells in 10 mM Tris buffer (pH 8.0) and beating them with glass beads. The PCR and Taq

Master Mix (Qiagen) was used for the amplification of the 16S ribosomal-DNA, using bacteria primers Bacteria 8-Forward and Universal 1492-Reverse. A gel electrophoresis was then run of the PCR product DNA (total volume) in agarose and TBE buffer. The purification of the samples was completed using a QIAquick Gel Purification Kit (Qiagen). The product was then submitted to the ¹³C-DNA Sequencing Facility, Storer Hall, University of California, Davis. Electropherogram evaluation and editing was performed using Mac OSX and 4Peaks. The final comparison of the sequence DNA data was compared to the information available in public databases through the NCBI BLAST algorithm.

RESULTS

The cell morphological qualities of *Macrococcus equipercicus* are Gram-positive cocci arranged in staphylococci and tetrads, the cells are 1-2 microns in diameter. Colonies exhibit morphological features on TSA that are circular to irregular, serrate, flat, smooth, glistening, opaque, with light orange pigmentation and a wavy surface. Colonies are 1-6 mm in diameter.

The culture demonstrated positive catalase and oxidase activity. In a Methyl red test these organisms did not exhibit large quantities of acid production, enough to overcome the buffers present in the solution, neither did they exhibit acetoin production. They showed minimal hemolytic activity on blood agar and tested coagulase, urease, and esculin negative. For carbohydrate fermentation, the culture showed preference for mannitol, testing positive, but showed no activity in arabinose, raffinose, sorbitol, sucrose or lactose. The organisms did not grow on mannitol salt agar.

In the antimicrobial sensitivity or Kirby-Bauer test, this *Macrococcus equipercicus* strain demonstrated sensitivity to Ampicillin, Bactricin, Penicillin, Polymyxin B, Streptomycin, Tetracycline, and Triple sulfa.

Comparison of the 16S ribosomal-DNA to information in the public databases with the NCBI BLAST algorithm indicated the organisms in this strain showed 99% alignment with *Macrococcus equipercicus*.

DISCUSSION

The genus *Macrococcus* is a fairly new delineation from the species *Staphylococcus caseolyticus*. It was separated from the latter genus in 1998 and is said to be related to but distinctly separate from other known members of the genus *Staphylococcus* and should therefore be given a separate, unique genus. When compared to *Staphylococcus*, these organisms harbored much less in similarity of their 16S ribosomal-RNA and contained higher G+C content in their DNA. Organisms now in the genus *Macrococcus* also do not contain teichoic acids within their cell walls (Kloos Delimiting).

Organisms from the new genus *Macrococcus* may also be distinguished from their closely related *Staphylococcus* strains on the basis of more phenotypic characteristics such as their larger Gram-stain size and positive oxidase activity. The strain *Macrococcus equipercicus* may be specifically distinguished by acid production from mannitol, and its failure to produce acid from sucrose (Kloos).

Although *Macrococcus equiperdus* are also known to be distinguishable as a species based on their strong urease activity (Kloos), the strain obtained for this investigation displayed none at all, i.e., it tested urease-negative.

According to Kloos and others, the species *M. equiperdus* is traditionally found from the skin of horse species – mostly on Shetland ponies. It is a consideration that perhaps this particular specimen was found in a canine mouth as a result of “Lucy’s” premium dog chow, or perhaps from the stirring of dust and soil in the air of Grass Valley, CA, landing on Lucy’s skin, and being transmitted to her mouth during licking.

ACKNOWLEDGEMENTS

Special thanks to - The North Valley and Mountain Biotechnology Center, American River that supplied the grant for sequencing. UC Davis, College of Biological Sciences, DNA Sequencing Facility. Harriet S. Wilson, Professor of Microbiology for her unending work and excellent instruction. Jim Wilson, Sierra College Biology Department Webmaster. Tim and Joanne Brown who supplied the use of their dog Lucy, the habitat of the culture studied.

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Das Praputium – And a Potential Pathogen Associated With Urinary Tract Infections

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Received 22 June 2007/Accepted 15 July 2007

Staphylococcus lugdunensis was obtained from a male uncircumcised penis in an attempt to determine the correlation between the bacteria found on the penis that is covered by foreskin and the occurrences of bladder infections. A pure culture was obtained to isolate the bacteria, and then a number of enzymatic tests were run to determine the physiological characteristics of the culture. In addition, the DNA was extracted to run a PCR. The amplified DNA was used for a gel electrophoresis test. Lastly purified DNA was sent to the "DNA Sequencing Facility. When the results were received they were analyzed and edited using Mac OSX and 4Peaks, electropherograms were edited and combined and the final sequence was compared to data available in public databases using the BLAST at the NCBI website. The potential pathogen identified was determined to be part of the normal human flora, often found in the inguinal region.

INTRODUCTION

This independent project involved swabbing an uncircumcised penis below the rim of the head. The purpose of this project was to determine what type of bacteria resides in this region and if there is a correlation between this bacteria and bladder infections. Individuals who get more than five bladder infections during a six-month period are often recommended a maintenance dose of antibiotics over an extended period of time. Individuals with reoccurring bladder infections that are recommended antibiotics each time to kill the infectious agent may become inhabited by organisms that are resistant to the antibiotics. More importantly antibiotics that are taken over a long period of time may be damaging to ones normal flora and select for potential pathogens. A person's health is very important and long-term use of antibiotics is potentially damaging.

MATERIALS AND METHODS

A sterile cotton swab, a tube of sterile water and a blood agar plate were obtained for this project. The swab was placed into the sterile water, the foreskin was pulled back past the head of the penis and the area right below the head was swabbed. The cotton swab was placed back into the sterile water and the water was used to streak the bacteria onto a blood agar plate. The bacteria grew and in order to obtain a pure culture a second blood agar plate was used to restreak the culture. The plate was observed for colony morphology and a number of enzymatic tests were performed as well as a differential staining technique. These include a Gram stain, a KOH

test, catalase test, a coagulase test, and an MRVP test. Next, chromosomal DNA was extracted by putting cells in tris buffer, boiling them for ten minutes and then beating them with glass beads for ten minutes. Then a PCR (Polymerase Chain Reaction) procedure for amplifying the gene for 16S ribosomal-DNA using Taq Master Mix was completed. The bacteria primers used were Bacteria 8-forward and 1492-reverse. A Gel Electrophoresis of the amplified DNA was completed to isolate the PCR product, and this was cut out of the gel with a razor blade. After this, the PCR product was cleaned up using a QIAquick Gel Purification Kit. Finally it was sent to the ^{UC}DNA Sequencing Facility, Storer Hall, University of California, Davis. After the electropherogram was returned via email, it was analyzed and edited using Mac OSX and the 4Peaks program. The combined and edited sequence was then compared to data available in public databases using the BLAST at the NCBI website.

RESULTS

The results of the blood agar plate showed circular, entire, raised, shiny colonies, measuring 1-2 mm in diameter and displaying a sticky consistency. There was also a distinct odor associated with these colonies. The Gram stain showed the bacteria to be Gram-positive cocci. The KOH test confirmed the fact that the culture was Gram positive. The catalase test was positive and the coagulase test was negative. The MR portion of the MR-VP test was negative for acid formation and the VP portion was positive for acetoin. Electropherogram analysis and gene bank comparison indicated that the culture isolated was *Staphylococcus lugdunensis*. The sequence showed 99% similarity with a gene bank sequence 1492 bases in length, matching 1435/1443. Lineage for *Staphylococcus lugdunensis* is Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus.

DISCUSSION

Staphylococcus lugdunensis is a member of the genus *Staphylococcus*, consisting of Gram-positive bacteria with spherical cells appearing in clusters. The species was first described in 1988 and was recorded as a cause of serious human infections (4). *Staphylococcus* cells appear to be found over the entire surface of human skin and have the ability to establish primary infection in cutaneous or deep sites or to participate in mixed infections (2). The frequency of these microorganisms in urine cultures is unknown; however, research was conducted on five hundred *Staphylococcus* isolates categorized as coagulase-negative staphylococci (1). It was determined that thirty-one of the 500 isolates were *Staphylococcus lugdunensis*. Twenty-nine out of the thirty-one *S. lugdunensis* isolates were part of mixed pathogenic flora. Thirty of the thirty-one patients had medical records available for analysis and none of these patients had any underlying medical disorders. Twenty-one of the thirty patients evaluated were not treated with antibiotics that might be effective against *S. lugdunensis*. *Staphylococcus lugdunensis* may be an unrecognized yet infrequent cause of urinary tract infections (1).

Skin infections due to *Staphylococcus lugdunensis* have occurred at a number of different sites although the region below the waist is most commonly involved. *Staphylococcus lugdunensis* carriage may be in the perineum. Associations have been reported between *S. lugdunensis* endocarditis and inguinal skin breaks (3). Both women and men have been found to

be carriers of *S. lugdunensis* in both the left and the right inguinal folds and overweight patients were not more likely to be carriers. From the information gathered, it can be concluded that the isolate identified, *Staphylococcus lugdunensis*, can cause of bladder infections. Although the findings do not provide 100% proof that these organisms are responsible for the bladder infections in question, there is a distinct possibility that they do.

ACKNOWLEDGEMENTS

The assistance of the Sierra College foundation, the North Valley and Mountain Biotechnology Center at ARC and Harriet Wilson, microbiology professor at Sierra College is gratefully acknowledged.

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Isolation of *Staphylococcus saprophyticus* From a Cat's Foot

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Received 29 June 2007/Accepted 15 July 2007

A bacteria culture isolated from a cat's foot was investigated and found to be *Staphylococcus saprophyticus*. *Staphylococcus saprophyticus* is a common human pathogen often coupled with acute urinary tract infections in females. While it is rarely found in association with healthy humans it is commonly isolated from animals. Due to the numerous and distinct characteristics of this organism, it can be readily identified through morphology, enzymatic testing and nucleic acid analysis. Biochemical tests including catalase, coagulase, MR-VP, urea hydrolysis, hemolysis reactions on sheep blood and aerobic acid formation from carbohydrates were used, along with 16S r-DNA nucleotide sequencing to identify this cat foot culture.

INTRODUCTION

Performing the Effectiveness of Hand Washing exercise in the microbiology laboratory stimulated interest in the types of microorganisms that might be growing on a cat's feet. Contemplating this activity sparked curiosity about the types of organisms growing on a specific, domestic cat's paws, since they are seldom washed (except by the cat) and have never had antiseptics applied to them. Since the cat in question has free reign over the house, the author became inquisitive and somewhat apprehensive about what microorganisms he might be tracking and where.

In order to determine what variety of microorganisms a one-year old cat was tracking throughout the house on his feet, the investigator chose to isolate one organism from a nutrient agar plate inoculated by stamping it with the cat's foot. After colonies had developed on the agar surface, one well-isolated colony was selected and a sample from this was streaked on another nutrient agar plate. This successfully established a pure culture that was used to run all tests and procedures relating to the identification.

MATERIALS AND METHODS

Identification of this microorganism required that several procedures and tests be completed. Initially, colonies were formed by stamping the front left paw of the cat being investigated onto the surface of a nutrient agar plate. Selecting a single, well-isolated colony from this plate and streaking a sample from it onto a second plate of nutrient agar resulted in a pure culture. This successful inoculation and isolation allowed for testing to begin. Identification initially involved tests to determine the shape and structural characteristics of the microorganisms and included a nigrosin indirect stain and a Gram stain. Enzymatic tests were then performed to determine the phenotype of the organism; these included a potassium

hydroxide (KOH) test, a catalase test, and methyl red and Voges-Proskauer (MR-VP) tests.

DNA analysis was the next step in the identification process, and involved a procedure requiring several stages. The first step was the extraction of DNA with cells boiled in 10mM Tris buffer and beaten with glass beads. The 16S-ribosomal DNA was then amplified using the polymerase chain reaction and Taq Master Mix. The bacterial primers used were Bacteria 8-Forward and Universal 1492-Reverse. Gel electrophoresis of the PCR product DNA then took place in agarose and TBE buffer. Purification of the DNA samples was achieved with a QIAquick Gel Purification Kit. The purified PCR product was then submitted to the ^{UC}DNA Sequencing Facility, Storer Hall, at the University of California, Davis where the DNA was sequenced using the primers bacteria 8-forward and universal 1492-reverse. Once the sequences were returned electronically, electropherogram evaluation and editing was completed using Mac OSX and 4Peaks software. The resulting nucleotide sequences were then compared to those available in the public databases utilizing the NCBI BLAST algorithm.

The data obtained from the NCBI BLAST provided a tentative identification and indicated the appropriate direction for additional research using the Bergey's Manual of Systematic Bacteriology. Based on the information gathered there, additional tests were preformed to confirm the identity of the isolate. These tests included: coagulase and hemolysis reaction on sheep blood agar, growth on mannitol salt agar (MSA), urea hydrolysis and tests for aerobic acid formation on arabinose, raffinose, sucrose, mannitol and lactose carbohydrate slants.

RESULTS

Results were determined based on the completion of the above tests and through observation and evaluation. Colony morphology was determined from colonies apparent on nutrient agar. Colonies were circular in form with entire margins, raised, shiny in texture, opaque with off-white pigment and approximately three to four millimeters in diameter. Cell morphology was determined by means of indirect staining and observations made of prepared slides under 1000X magnification. The organisms were revealed to be cocci in shape and arranged as single cells, in pairs (diplococci), and frequently in chains (streptococci). The average cell size was approximately 1.0-1.5 micrometers in diameter. The Gram stain results indicated the isolates were Gram positive and pH testing revealed the culture had a slightly acidic pH of 6.0 when grown in MR-VP broth.

Results of enzymatic testing:

Test being conducted:	Results
Aerobic Growth on nutrient agar plates	Positive
Anaerobic Growth in vaspar-sealed medium	Positive
Arabinose - aerobic acid formation	Negative
Catalase – determined with 3% H ₂ O ₂	Positive
Coagulase – determined in rabbit plasma	Negative
Hemolysis – determined on sheep blood agar	Negative
KOH – testing for resistance to lysis in 3% KOH	Positive
Lactose - aerobic acid formation	Positive
Mannitol - aerobic acid formation	Positive

Mannitol Salt Agar (MSA) - growth on 7.5% salt	Positive
Methyl Red (MR) – quantitative test for acid	Negative
Oxidase – testing for the presence of cytochrome C	Negative
Raffinose – aerobic acid formation	Negative
Sucrose – aerobic acid formation	Positive
Urease – hydrolysis of urea and ammonia formation	Positive
Voges-Proskauer (VP) – acetoin formation	Positive

Results obtained through DNA sequencing and comparison of sequence data through the NCBI BLAST indicated the organisms isolated were *Staphylococcus saprophyticus*. The query sequence obtained after electropherogram evaluation and editing showed 99% similarity with a gene bank sequence from this species. The lineage provided by NCBI was: Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus.

Based on the enzymatic test results supported by the data given for *Staphylococcus saprophyticus* in the Bergey's Manual of Systematic Bacteriology, Volume Two, and the DNA sequence similarity obtained by comparing the 16S r-DNA nucleotide sequence for the isolate to those available in the public databases through NCBI, the unknown culture was successfully identified as *Staphylococcus saprophyticus*.

DISCUSSION

Staphylococcus saprophyticus is recognized as a major human pathogen. It is the second most commonly encountered microorganism associated with acute urinary tract infections (10-20%) in young, sexually active females ages 17-27 and can often be isolated in their urine (3). Clinical isolates of *S. saprophyticus* have been shown to have developed resistance to the antibiotic novobiocin, a characteristic that is often relied upon in laboratory identifications (4). These organisms are rarely found in healthy humans, but are most commonly isolated from animals and their carcasses (4). Urinary tract infections due to *Staphylococcus saprophyticus* are most commonly treated with Quinolones and new PCR-based assays are being developed for the specific identification of this species (1).

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Isolation and Identification of an Unknown Organism Found in Water Obtained from Bittney Springs in Grass Valley, Ca.

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A water sample was obtained from an established spring commonly used as a source for drinking water. A sample of this water was used to inoculate media and provided the unknown organisms selected as the subjects of this investigation. Tests were run to identify, establish characteristics of, and determine the potential health concerns related to the unknown organisms. The culture isolated was identified from DNA analysis as *Aquitalea magnusonii* a newly discovered type of organism. These bacteria were determined to be Gram Negative bacilli with a fermentative type of metabolism. Test results from this culture were compared to those of *Chromobacterium violaceum*, it's closest relative, according to the NCBI web site. Health related information was not available for the genus due to the recency of its discovery, and the fact that the original isolation was not associated with a clinical sample. Information utilized during this investigation was obtained from the Bergey's Manual of Systematic Bacteriology, Volume One, NCBI and the Microbiology Laboratory Syllabus.

INTRODUCTION

Bittney Springs is a developed, natural spring found in Nevada County, California. Since many people choose to use this water source as their drinking water, in preference to using treated water, the spring provided an interesting topic for investigation. Is the water available at Bittney Springs safe for drinking, or is it not? For many years people have been observed stopping at the spring and filling a large number of five-gallon bottles for use as their drinking water. People who do this often let some bottles sit at room temperature for several weeks before using the water contained in them. The belief that water sitting still in these bottles, for long periods of time, might allow for the growth of microorganisms stimulated an interest in examining the spring water to determine what was present and potentially being ingested. Curiosity was gratified by collecting a sample of spring water in a sterile tube and taking it to the laboratory for testing purposes.

MATERIALS AND METHODS

A sample of water was collected from Bittney Springs in a sterile tube. A portion of this sample was then added to a tube of double-strength phenol red lactose broth in order to dilute the medium concentration by half. The broth sample was observed until growth was revealed, and then two nutrient agar plates were streaked to establish isolated colonies. One plate was placed in an incubator at 37°C for 24 hours and the other plate remained at room temperature for 24

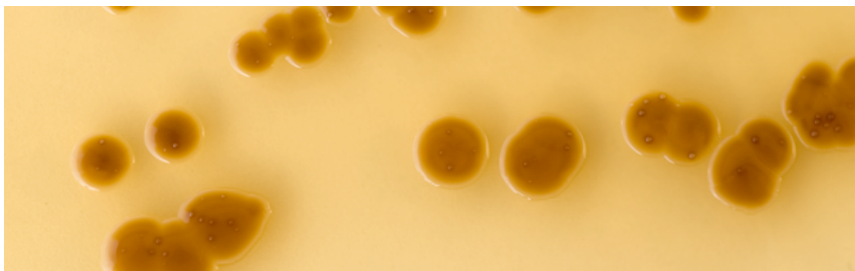
hours. A sample from an isolated colony growing on the plate maintained at room temperature was then re-streaked and kept at room temperature to establish a pure culture. Sample colonies from the pure culture were used to prepare a Gram stain and to perform a series of enzymatic tests. These involved the use of rhamnose, lactose, sucrose, raffinose, inositol, mannitol, and glucose carbohydrate deeps, Simmon's citrate agar, Triple Sugar Iron agar, Sulfur Indole Motility medium, Kovac's reagent, lysine decarboxylase medium and a lysine control tube, an oxidase test plate and two oxidation/fermentation (O/F) agar deeps.

A sample colony was used to obtain chromosomal DNA for analysis. DNA was released from the cells present by agitating them and then boiling them in Tris buffer for ten minutes. A region of the 16S ribosomal-RNA gene was then amplified with the polymerase chain reaction using primers bacteria 8-forward and universal 1492-reverse. The amplified DNA was then subjected to gel electrophoresis and purified prior to being sent to U.C. Davis for sequencing. The sequencing primers used were identical to those used in the PCR. The ^{UC}DNA Sequencing Facility established the sequences and forwarded results back to the Sierra College laboratory for evaluation. The two sequences were edited and combined to form one long sequence that was then used for identification on the National Center for Biotechnology Information website. The Basic Local Alignment Search Tool feature was used to compare the sample sequence to known sequences and to identify the unknown organisms. When results from the BLAST had provided a tentative identification for the isolate, the Bregey's Manual of Determinative Bacteriology was used to identify tests that would be useful for further analysis, and these were carried out as listed above.

RESULTS

The colonies that formed on the Nutrient agar were light brown, circular, undulate, raised, smooth, glistening, shiny and were 1-3mm in diameter. The Gram stain revealed pink Gram-negative rods, 1.5-2.5µm long and approximately 1.0µm wide. The O/F test revealed this culture to be fermentative. The SIM test results showed motility, the production of indole, and a lack of ability to produce hydrogen sulfide. The oxidase test returned a positive result confirming the presence of cytochrome c. The citrate utilization test showed that this culture has the ability to utilize citrate as a sole source of carbon. The BLAST results identified the unknown organisms as *Aquitalea magnusonii*, a type of Gram-negative bacteria originally isolated from a humic lake in northern Wisconsin, USA. The DNA nucleotide sequence from the isolate showed 99% similarity with a 1502 base gene bank sequence from *Aquitalea magnusonii*, strain TRO-001DR8, accession number DQ018117. The lineage for this sample was Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Aquitalea. The closest relative to the genus was determined to be *Chromobacterium violaceum*, with 95% sequence similarity.

Fig. 1 – Colonies of *Aquitalea magnusonii* on Tryptic Soy Agar



Test	<i>Aquitalea magnusonii</i>	<i>Chromobacterium Violaceum</i>
Mannitol fermentation	Negative	Negative
Raffinose fermentation	Negative	Negative
Citrate utilization	Positive	Positive
TSI - Lactose fermentation	Negative	Negative
Glucose & Sucrose acid	Positive	Positive
SIM - Motility	Positive	Positive
SIM - H ₂ S production	Negative	Negative
SIM – Indole formation	Positive	Negative
Inositol	Negative	Negative
Oxidase	Positive	Positive
Gram Stain	Negative	Negative
Cell shape	Bacilli (rods)	Bacilli (rods)

**Comparison of test results from unknown spring water isolate and closest relative.

DISCUSSION

The culture isolated in association with this project was initially grown in double-strength phenol red lactose broth, a medium commonly used for presumptive tests in the bacteriological examination of water. Some testing was required to identify the preferred growing conditions for the culture, and included streaking two nutrient agar plates and placing them in different environments to establish temperature preferences. It was established that this culture grew better at room temperature than in the 37° C incubator. After obtaining isolated colonies, a sample of the culture was transferred onto tryptic soy agar (TSA) for isolation and purification. A Gram stain was prepared, and then multiple tests were run to identify the phenotypic characteristics of the subject organisms. It was determined that these organisms had a fermentative type metabolism based on the results obtained from the oxidation/fermentation (O/F) test. Additional tests commonly used in the identification of fermentative, Gram-negative cultures were then run, but the results were inconclusive with respect to identifying the culture. Ultimately, results obtained from the nucleotide sequencing of PCR product were required for the identification. The BLAST results obtained with the edited and combined sequences from two primers returned with 99% similarity to a relatively newly identified organism type called *Aquitalea magnusonii*. This genus was not identified in the Bergey's Manual of Systemic Bacteriology due to its recent discovery (April, 2006).

Results obtained with the NCBI BLAST revealed that *Chromobacterium violaceum* was the closest identified relative to *Aquitalea magnusonii*. Information about *Chromobacterium violaceum* was then used to compare the characteristics of these two organism types. The enzymatic test results revealed that most of the characteristics were the same with the one significant difference being the production of indole by *Aquitalea*. The cultural characteristics of these two genera are also significantly different in that *Aquitalea magnusonii* lacks the purple pigment present in *Chromobacterium violaceum* colonies, and is brown instead. Because of the lack of information available on this genus, it is impossible to discuss its impact on human health or the environment.

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Sophie's Nasal Unknown Friend – *Rothia Nasimurium*

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An unknown variety of Gram-positive bacteria collected from a pot-bellied pig was identified as *Rothia nasimurium*. These bacteria are normally found in the natural flora of the human mouth and nasal cavity (3). Sophie, a pot-bellied pig, roots in the ground and completely packs her nostrils with dirt on a daily basis. The possibility that this pig would have some really nasty bacteria growing up her nose was the basis for this investigation. Surprisingly, there was relatively little bacterial growth on the initial agar plates used for isolating the subject culture, and the bacteria identified were completely normal for the nasal cavity. Comparative 16S r-RNA gene sequencing demonstrated that the unknown isolate was in the family *Micrococcaceae* and had phylogenetic correlation (96%) with *Rothia mucilaginosa*. The DNA sequence analysis also demonstrated 99% similarity with a *Rothia*-like species that in the year 2000 was tentatively identified as *Rothia nasimurium* (3).

INTRODUCTION

Pigs have quite the reputation for being unclean animals; therefore, a domesticated pot-bellied pig (Sophie) seemed like a good candidate for investigation, i.e., would provide material suitable for identifying unknown microorganisms. Ultimately, identifying organisms collected from the dirtiest part of the pig, the nostril, became the objective of this project. For the most part, the pot-bellied pig is quite a clean animal; however, the natural urge to “root around” results in quantities of dirt collecting on and up the nose. The dirt where the pig lives is clay, very hard and must be moist or wet in order for rooting to commence. Since moist or wet environments often provide good conditions for bacterial growth, especially during the summer months when temperatures are warm, many types of bacteria were expected to reside within the pig's nostril. In order to determine the prominent type of bacteria present in the pot-bellied pig's mucous, a swab sample was obtained and used to inoculate media.

MATERIALS AND METHODS

A sample of mucous from the pig's nasal cavity was collected with a sterile cotton swab, and then streaked onto a blood agar plate. A wire loop was used to dilute the sample and then the plate was incubated at 37 degrees Celsius for around 24 hours. Relatively little growth appeared on the plate, but there was an abundance of one type of colony. An isolated colony of the dominant type was chosen and a sample from this was streaked onto another blood agar plate in order to establish a pure culture. Once a pure culture was established, a series of morphological and enzymatic tests were conducted.

The first series of tests performed included a Gram stain, a nigrosin indirect stain, a catalase test, a wet mount (to determine motility), an MR-VP test and a KOH test. Subsequent to performing these tests, chromosomal DNA was extracted from the unknown culture by taking some cells from a well-isolated colony and putting them into 500µl of 10mM Tris buffer with some glass beads. This mixture was vortex mixed to separate the cells, was boiled for ten minutes and was then vortex mixed again for ten minutes to break open the Gram-positive cell walls. Amplification of 16S ribosomal-DNA was completed by adding Taq Master Mix (Qiagen), the primers Bacteria 8-Forward and Universal 1492-Reverse, water and a sample of the culture mix to a tube and subjecting this to the PCR. The amplification process occurred in a Bio-Rad, My Cycler, thermal cycler. The total volume of PCR product DNA was then put into an agarose gel with TBE buffer in order to complete Gel Electrophoresis. The amplified DNA was then cut from the gel and purification of the PCR product was completed with a QIAquick Gel Purification Kit (Qiagen). The purified DNA sample was then submitted to the ^{UC}DNA Sequencing Facility, Storer Hall, University of California, Davis. Once the results of the nucleotide sequencing were received, electropherogram evaluation and editing was completed using Mac OSX and 4Peaks. The resulting sequence was then sent to the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) algorithm for comparison with nucleotide sequence data available in public databases (4).

Based on the results of the DNA analysis and biochemical tests, and after referring to the Bergey's Manual of Systematic Bacteriology, (1), (2) the following additional enzymatic tests were performed: TSI, oxidase test, urea, esculin and starch hydrolysis, and acid and gas production from glucose, sucrose, mannitol, raffinose, soribitol, arabinose.

RESULTS

Colony morphology, as observed on the blood agar plate, included circular colonies up to 2mm in diameter with entire to lobate margins and a slightly raised elevation. The colonies appeared to be opaque, smooth, shiny, and dirty, milky white to tan in color. Cells were Gram-positive and appeared as clusters of cocci ranging in size from 0.5 – 1.5µm in diameter.

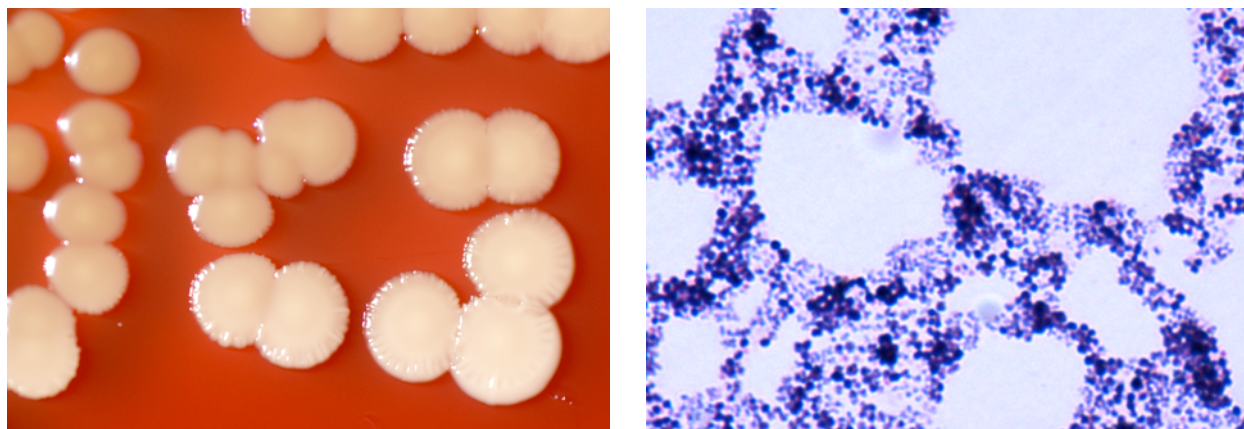


Fig. 1 – Pig nostril, *Rothia nasimurium* colonies on blood agar and cells in a Gram stain

The culture isolated on the blood agar plate tested negative for citrate utilization, starch hydrolysis, esculin hydrolysis, acetoin formation (VP) and acid production from the fermentation

of raffinose, sorbitol, and arabinose. The culture was also negative for the presence of cytochrome c as determined with the oxidase test. The culture tested positive for urea hydrolysis (urease activity), catalase activity, and the production of acid from the fermentation of glucose (MR), sucrose and mannitol. The fermentation of lactose was determined to be positive in TSI, but hydrogen sulfide production was negative. Results from the KOH test confirmed that the culture was Gram-positive. Observation of a wet mount confirmed that the cells were not motile.

Tests performed	Expected Results	Actual Results
Gram stain	Gram-positive	Gram-positive
Wet Mount	Non-motile	Non-motile
Citrate utilization	(-)	(-)
Starch Hydrolysis	(-)	(-)
TSI Fermentation of Lactose	(+)	(+)
TSI Hydrogen Sulfide Production	(-)	(-)
Urease – urea hydrolysis	(-)	(+)
Esculin Hydrolysis	(+)	(-)
Glucose Fermentation	(+)	(+)
Raffinose Fermentation	(-)	(-)
Sorbitol Fermentation	(-)	(-)
Arabinose Fermentation	(-)	(-)
Sucrose Fermentation	(+)	(+)
Mannitol Fermentation	(+)	(+)
Oxidase – Cytochrome c	(-)	(-)
Catalase – Action on H ₂ O ₂	(+)	(+)
Voges Proskauer (VP)	(-)	(-)
KOH test for wall type	Gram-positive	Gram-positive
Methyl Red (MR)	(+)	(+)

Results obtained with the NCBI BLAST indicated that the nucleotide sequence obtained from the pig isolate showed 99% similarity with a *Rothia*-like species CCUG 35957, 16S r-RNA gene (accession number AJ131121). The nucleotide sequence from this *Rothia*-like species was found to cover 100% of the query sequence. The proposed name for these *Rothia*-like bacteria was found to be *Rothia nasimurium*, according to an article published in May of 2000.

DISCUSSION

Comparison of 16S r-RNA gene sequences using the NCBI BLAST demonstrated that the unknown culture was a member of the family *Micrococcaceae* and that it had 96% similarity with *Rothia mucilaginosa*, as well as 99% similarity with *Rothia nasimurium*. The Bergey's Manual of Determinative Bacteriology available in the Sierra College microbiology laboratory is an edition from the year 1986 and does not have *Rothia nasimurium* listed, because this *Rothia*-like species was not identified until May of the year 2000. In the article where *Rothia nasimurium* was characterized, *Stomatococcus mucilaginosa* was reclassified as *Rothia mucilaginousa* (3). Therefore, in order to find additional information about the pig nose bacteria, the section of the Bergey's manual addressing *Stomatococcus mucilaginosa* was used. The Bergey's Manual of Determinative Bacteriology Volume Two (1986), contained information about both *Stomatococcus mucilaginosa* and *Rothia mucilaginosa*, so both sections were utilized during the investigation.

Of the tests available in the laboratory, and indicated in the Bergey's Manual, most provided results consistent with those expected for *Stomatococcus/Rothia mucilaginosa*. Two tests provided unexpected results, and these were the esculin hydrolysis test and the urease test. *Rothia* species described in the Bergey's manual and in the May, 2000 article showed positive results for esculin hydrolysis and negative results for urease activity. The *Rothia nasimurium* isolated from the pig nostril showed negative results for esculin hydrolysis and positive results for urease (2). The gene sequencing data placed *Rothia mucilaginosa* as the closest species to *Rothia nasimurium*, but the unweighted pair group average linkage of correlation coefficients between whole-cell protein patterns of *Rothia nasimurium* and some relatives, (3) indicated that this species is some distance away from *Rothia mucilaginosa*. This could be the reason not all of the testing correlated as expected.

Bacteria identified as *Stomatococcus/Rothia mucilaginosa* were originally described as part of the normal flora found in the human mouth, throat and nasopharynx (2). An unknown type of bacteria isolated from the nose of a healthy mouse was investigated, and based on the results of a taxonomic study, characterized as a new species, *Rothia nasimurium* (3). Although these bacteria were initially isolated from a mouse nose, they have now been isolated and identified from the nose of a pot-bellied pig. These findings suggest that *Rothia nasimurium* are not confined to only one type of animal.

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Pot-bellied pig test subject, ie. Sophie

Isolation and Identification of *Bacillus simplex* from the Environment

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Bacteria identified as *Bacillus simplex* were obtained from a bathroom air plate, exposed in an attempt to identify the type of organisms living in that environment. A pure culture was established and then several enzymatic tests were completed in order to determine the physiological characteristics of these bacteria. Chromosomal DNA was extracted from the culture and a portion of the 16S r-RNA gene was amplified with the PCR. The results from the PCR were made visible using gel electrophoresis, were cut from the gel and purified. Then the purified DNA sample was sent to the ¹³C DNA Sequencing Facility at the University of California, Davis. When the sequencing results were received, an electropherogram evaluation and editing were completed using Mac OSX and 4Peaks. The sequence data was then compared to information made available through the NCBI BLAST algorithm and public databases.

INTRODUCTION

This independent project involved a nutrient agar plate that was opened and left exposed to air in an unclean bathroom for one hour. The purpose of this project was to determine the type of bacteria living in the bathroom and if or not they are pathogenic. Many individuals are fearful about germs that may reside in the bathroom. An individual with such a phobia might obsessively clean the area after every use for fear of contracting some type of illness. Obsessing over pathogens that may or may not be present in the bathroom can be damaging to ones mental health. Determining the types of bacteria actually present could alleviate such concerns.

MATERIALS AND METHODS

A nutrient agar plate was obtained for this project and was left open in a bathroom that had not been cleaned for five days. The agar surface was exposed to air for approximately one hour. The bacteria landing on the nutrient agar were allowed to grow and reproduce for several days. Then the bacteria were streaked again onto a nutrient agar plate to obtain a pure culture. The pure culture was then used to observe colony and cell morphology and for several enzymatic tests, such as the catalase, MR-VP, oxidase, citrate, mannitol, gelatin, and starch test. Differential staining techniques such as the Gram stain and KOH test were also performed. Then DNA was extracted from the cells by boiling them for ten minutes and then beating them with glass beads in Tris buffer. Then a Polymerase Chain Reaction (PCR), using Taq Master Mix, was completed to amplify a section of the 16S ribosomal-DNA. The primers used were Bacteria 8-forward and 1492- Reverse. Gel electrophoresis and a QIAquick gel purification kit were used to purify the DNA samples and then these were sent to the ¹³C DNA Sequencing Facility. The

Sequencing Facility returned the resulting electropherograms via email, and these were then evaluated and edited using Mac OSX and 4Peaks. The final version of the nucleotide sequence was then compared to information provided in the public databases using the NCBI BLAST (4).

RESULTS

The pure culture established on nutrient agar showed irregular, filamentous, shiny, opaque colonies, measuring 0.1-2.0 millimeters in diameter. The Gram stain showed that these bacteria were rod-shaped and pink, suggesting they were Gram-negative; however, the KOH test determined them to be Gram-positive. The catalase test was positive, the MR test showed negative for acid formation and the VP test showed the culture was negative for acetoin. The oxidase test indicated these bacteria were positive for the presence of cytochrome-c. The gelatin test indicated they were also positive for the hydrolysis of gelatin. These bacteria were negative for starch hydrolysis and for aerobic acid formation from mannitol. They were positive for citrate utilization, but the positive result did not appear until a week after the test medium had been inoculated. Results obtained with the NCBI BLAST indicated the unknown isolate showed 99% similarity with *Bacillus simplex*, strain M4 (EF443164.1), with 1442/1445 bases matching pairwise. The length of the gene bank sequence was 1529. The lineage for *Bacillus simplex* was Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.

DISCUSSION

Bacteria identified as *Bacillus simplex* are Gram-positive organisms highly similar to *Bacillus sphaericus* (3). They were first described as a distinct taxon in 1988 by Priest. There was minimal information about *Bacillus simplex* found, however, due to the high similarities described in articles between *Bacillus simplex* and *Bacillus sphaericus*, a comparison of the results from the enzymatic tests for these two species was completed. Based on information provided in the Bergey's Manual of Systematic Bacteriology, *Bacillus sphaericus*, like *Bacillus simplex*, tested negative for the acid formation in the MR test and acetoin formation in VP test. *Bacillus sphaericus* does not form aerobic acid from mannitol and is catalase positive. The utilization of citrate resulting in ammonia formation is variable for *Bacillus sphaericus* (2).

Further research on *Bacillus sphaericus* indicated that some strains of *Bacillus sphaericus* contain proteins that are toxic to the larvae of mosquitoes (1). Neither protein is toxic alone, but when present in combination they are toxic to the larvae. These proteins target epithelial cells of the mosquito midgut. More specifically *Bacillus sphaericus* is used as a biological insecticide against mosquitoes in the genera *Culex* and *Anopheles*. Since *Bacillus sphaericus* is used as a biological insecticide and is highly similar to *Bacillus simplex* it might be concluded that *Bacillus simplex* is pathogenic to insects also, but additional research would be required to confirm this possibility.

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Isolation and Examination of *Leuconostoc mesenteroides* from Brie Cheese

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This project focused on the bacterial cultures found in cheese; specifically, a sample taken from a wedge of brie cheese. This was streaked onto a plate of Bromocresol purple lactose agar, and then a well-isolated bacterial colony was selected and isolated into a pure culture to provide samples for further testing in order to identify a specific type of bacteria from the cheese. The bacteria isolated were identified as *Leuconostoc mesenteroides* by means of morphology, enzymatic testing and DNA analysis. Certain subspecies of *Leuconostoc mesenteroides* such as *Leuconostoc mesenteroides ssp. cremoris* are commonly added to commercially produced buttermilk cultures and mixed starter cultures of soft ripened cheeses to enhance the cheeses' flavors by adding "buttery notes" without creating acidic flavors that are considered too strong. The exact subspecies present in this cheese sample could not be determined with the data obtained.

INTRODUCTION

This project was undertaken to gain a better understanding of the microbiology of cultured dairy foods. Microorganisms including bacteria and sometimes fungi (as secondary flora) are added during the production of dairy foods such as buttermilk, cheese, and yogurt, because the fermentation products of these microorganisms contribute to many of the cultured foods' characteristics such as textures, flavors, and aromas. In addition, public awareness of the presence of live microbial cultures in dairy foods is increasing as researchers learn more about the possible health benefits of consumption of cultured dairy products.

MATERIALS AND METHODS

First, a sample of Brie cheese was streaked onto a Bromocresol purple (BCP) lactose agar plate. A sample taken from a well-isolated colony was then transferred to another BCP lactose plate to obtain a pure culture. This culture was grown at room temperature in the microbiology laboratory. Cells from this culture were Gram stained and indirectly stained with nigrosin.

Second, DNA analysis was conducted using the following methods. Cells were boiled in 10mM Tris buffer (pH 8.0) and beaten with glass beads for DNA extraction. PCR and Taq Master Mix (Qiagen) were used to amplify 16S ribosomal-DNA. The primers used were Bacteria 8-Forward and Universal 1492-Reverse. The total volume of PCR product DNA was subjected to gel electrophoresis in agarose and TBE buffer. QIAquick Gel Purification Kits (Qiagen) were utilized for DNA purification. The purified DNA sample was submitted to the ¹³C DNA Sequencing Facility, Storer Hall, University of California, Davis. Sequencing primers were 8-Forward and 1492-Reverse. Mac OSX and 4Peaks were utilized for electropherogram evaluation and editing. The sequence data was compared to information available in public databases via the NCBI BLAST algorithm.

Third, various enzymatic tests were performed on cell samples including inoculation of the following carbohydrate deeps: glucose, inositol, lactose, mannitol, raffinose, rhamnose, and sorbitol. An MR-VP test, esculin hydrolysis test, catalase activity test, oxidase test, and a citrate utilization test were also performed.

RESULTS

The bacteria grew readily on BCP lactose agar and turned this violet medium to yellow indicating acid formation; however, the bacteria failed to grow on tryptic soy agar (TSA). On BCP lactose agar, the bacteria formed circular colonies measuring 1.5 to 2 mm in diameter. The margins were entire, and the elevations were between raised and convex. The surface texture of the colonies was shiny, and their optical character was opaque. The colonies' color appeared to be yellowish beige. The bacteria cells were ovoid cocci and measured between 0.5 and 0.75 microns in width and 1 to 1.5 microns in length. The cells were arranged as single cells. In addition, the cells stained Gram positive.

The NCBI BLAST algorithm indicated the cheese isolate bacteria showed 99% similarity with a gene bank sequence from *Leuconostoc mesenteroides* strain PC13 (EF579730).

The results of the enzymatic tests were as follows: acid production from carbohydrates – glucose positive, lactose-positive, inositol-negative, mannitol-negative, raffinose-negative, rhamnose-negative, and sorbitol-negative; citrate utilization-negative; esculin hydrolysis-positive; catalase-negative; Voges-Proskauer-negative; and, methyl red-negative.

DISCUSSION

Dairy foods are cultured by various bacteria collectively known as Lactic Acid Bacteria. These bacteria (most but not all) ferment the lactose in milk to form lactic acid and other end products that characterize the cultured foods' textures (the acids produced aid in coagulation), aromas, and flavors. During production, the milk is first pasteurized, and, commercially prepared bacterial starter cultures are added. Starter cultures can be mixed cultures containing a combination of different bacteria. Generally, these bacteria are Gram-positive, non-motile, non-spore forming, and catalase-negative. In addition, manufacturers adjust the composition of mixed starter cultures in an effort to increase resistance to bacteriocins, antibiotics, and bacteriophages.

Leuconostoc mesenteroides ssp cremoris is commonly used in mesophilic cheese making cultures, as it grows best between 20 and 30 degrees Celsius. Mesophilic cultures are used for soft ripened cheeses that do not require high temperatures to dry the curd. *Leuconostoc mesenteroides* are Gram-positive, facultatively anaerobic, and heterofermentative bacteria that can produce diacetyl and acetaldehyde which contribute to flavor notes described as “fresh” in buttermilk and “buttery” in cheese without producing an overly strong acid flavor.

Considering this information, it was expected that the isolated *Leuconostoc mesenteroides* bacteria would belong to the subspecies *cremoris*; however, there was a discrepancy regarding citrate utilization. According to the Bergey’s Manual, the subspecies *cremoris* utilizes citrate, which is associated with the production of diacetyl. The isolated bacteria tested negative for citrate utilization. This strongly suggests that this *Leuconostoc mesenteroides* strain is not of the *cremoris* subspecies. Unfortunately, it will not be possible to determine which subspecies these bacteria belong to with the data available. Additional tests might be used to clarify the subspecies in the future.

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Isolation of Culture Obtained from Beer Brewed with a Home Brewing Kit Using Aseptic Technique

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This experiment was designed to test the effectiveness of aseptic technique used while brewing beer with a home brewing kit. It was conducted in a home kitchen with home appliances. It was hypothesized that if the process was completed aseptically, there would be no microbial growth within the final beer product except the Brewer's Yeast that was known to be *Saccharomyces cerevisiae*. Samples were taken from the product at two different points in the process, and potatoe dextrose agar plates were streaked. Pure cultures were grown on both plates and samples of these were transferred to slides for observation. Chromosomal DNA was extracted from one of the cultures, a portion of the 16S r-RNA gene was amplified, run in a gel, purified and sent to the ^{UC}DNA Sequencing Facility at the University of California, Davis for sequencing. Results were compared to others using the National Center for Biotechnology Information (NCBI) web site and Basic Local Alignment Search Tool (BLAST) option. Results showed a 96% match with both *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*, meaning the aseptic technique used was adequate and successful.

INTRODUCTION

The importance of aseptic technique in the brewing process when not taken seriously, results in unpleasant, and undrinkable beer. The objective of this experiment was to test the aseptic technique used when brewing in a home kitchen. It was known that Brewer's Yeast (*Saccharomyces cerevisiae*) is the accepted and most commonly used yeast in the brewing of beer. Having home-brewed beer before, the author had experienced both good and bad tasting beer results. Using a home kitchen with home appliances and equipment it was thought that a cause of the bad tasting beer could have been the excess growth of bacteria in the beer batches as a result of an unclean or non-sterile environment. To test this idea, steps were recorded and aseptic technique was used throughout the preparation. Establishing pure cultures using samples from the beer and analyzing the microorganisms growing there would provide a means for determining if or not the aseptic technique used was sufficient.

MATERIALS AND METHODS

A stainless steel brewpot with lid was sanitized by boiling 5 gallons of water for 15 minutes at 100 degrees Celsius. All other equipment used (brew spoon, plastic bowl, glass carboys for 5 gal. and 7 gal., rubber stopper, hydrometer and thermometer) was sanitized with

Five Star Acid Sanitizer. Using a steeping bag, flavor grains were steeped in water at 68.3 degrees Celsius for 30 minutes and then removed. Malt syrup, dry malt, and extract was added to water and it was brought to a boil for another 10 minutes. Boiling hops were slowly added to boiling water and were boiled for another 30 minutes. During the last 10 minutes of the boil, the finishing hops were added. Irish moss and dextrines were also added at this point. When completed, this mixture became wort. The wort was cooled in a kitchen sink full of ice to a temperature in the range of 25 to 27 degrees Celsius. The Brewer's Yeast was rehydrated using a sanitized plastic bowl and sanitized water cooled to a temperature between 25 and 30 degrees Celsius for 15 minutes. During hydration, the bowl was covered with foil. The first sample was taken from this small bowl and put in a small sanitized jar. When the wort was cooled to the appropriate temperature, it was transferred to the sanitized five gallon carboy. The yeast was then funneled into the carboy and it was shaken twice during the first two hours. The second sample was taken from the carboy after this time period, and put into the other sanitized jar. The rubber stopper was then inserted along with the airlock and the brew was stored in a cool place for 1 week. Beer was then syphoned using a racking cane, funneled into the sanitized secondary carboy, and stored in a cool place for 1 more week. Priming sugar was then added and mixed thoroughly and the beer was syphoned for a second time into sanitized bottles and capped. These bottles were stored in a cool place for 2 weeks. Ethanol and carbon dioxide were formed during the fermentation process, and then the beer was ready to drink.

The two samples in jars were taken to the microbiology laboratory at Sierra College and samples from these were streaked onto two potatoe dextrose agar plates. The cells streaked from the wort sample were then indirectly stained and Gram stained. DNA was then extracted from these cells by boiling them in 10mM Tris buffer with a pH of 8.0, and beating them with glass beads. Using the PCR and Taq Master Mix (Qiagen) a portion of 16S ribosomal DNA was amplified. The primers used were 0817 Forward and 1536 Reverse (Operon). The Gel Electrophoresis of the PCR product DNA was in agarose and TBE buffer. Purification of the DNA was completed with a QIAquick Gel Purification Kit (Qiagen). Purified DNA samples were sent to the ^{UC}DNA Sequencing Facility, Storer Hall, University of California, Davis. Sequencing primers were 0817 Forward and 1536 Reverse. Evaluation and editing of the Electropherogram results was completed using Mac OSX with the 4Peaks program. Comparison of DNA sequence data was analyzed using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) web site.

RESULTS

Isolation of a pure culture on potatoe dextrose agar showed rapid growth of beige-colored colonies. They were irregular to circular, entire to undulate colonies, and were flat, smooth, opaque and somewhat dull in appearance. When observed in the indirect stain, the cells were single cocci or coccobacilli. They ranged in size from 5 to 10 microns, and some showed small buds forming. The Gram stain showed some cells as Gram-negative, but the majority of the vegetative cells were Gram-positive. It was apparent that the colony and cell morphology of the pure culture sample matched with the characteristics of *Saccharomyces cerevisiae*, since this is a well known and thoroughly studied fungus.

After obtaining the DNA sequence data and using the BLAST feature on the NCBI web site, results showed the beer culture to have 99% sequence similarity with both *Saccharomyces*

cerevisiae and *Saccharomyces paradoxus*, without editing. When taste-tested the beer had a “pleasant” flavor as described by those who tested it.

DISCUSSION

These results showed that the aseptic technique used was adequate and the only microbe that grew within the beer was the yeast needed for fermentation to create the carbon dioxide and alcohol. The boiling technique as well as the Five Star Acid Sanitizer worked well in preventing invading microbes from getting into the beer throughout the brewing process. *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* are both yeast species used for fermentation in the making of both wine and beer. *Saccharomyces cerevisiae* is the most well known yeast used in brewing, but *Saccharomyces paradoxus* is its closest relative. Further research could be conducted on beer contamination affecting flavor with repetition of this experiment using a precise aseptic technique as well as a precise non-aseptic technique. Comparison of beer quality (tastes) resulting from the two techniques by various testers would also be necessary.

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Isolation of Organisms From a Reused Plastic Water Bottle

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With the production of plastic water bottles taxing dwindling oil reserves and the inability of most users to recycle them, these convenience items have become a major concern for environmentalists. Physicians are also becoming concerned about the increased use of these bottles, particularly when they are reused. News programs have reported a variety of pathogens theoretically growing inside these reused plastic bottles. The isolation of a bacterial culture from a water bottle refilled and reused for three days, resulted in the identification of *Bacillus subtilis spizizenii*, a type of bacteria found to be useful in oil extraction processes. Lipoprotein biosurfactants produced by these bacteria have been found to have significant potential for greatly reducing the cost and increasing the efficiency of methods used in mobilizing oil trapped deep in the pores of limestone oil reservoirs. Methods used in the isolation and identification of these bacteria are described, and include observation of cell and colony morphology, enzymatic testing and DNA analysis.

INTRODUCTION

Many people today are becoming more concerned about their health, and convenience is a high priority. As a result, the consumption of bottled water has skyrocketed in recent years, reaching nearly \$11 billion in sales in 2006 (2). There is growing concern that America's growing affinity for bottled water is doing damage to the environment. It is estimated that the making of water bottles for American consumption uses 1.5 million barrels of oil annually, enough to power 100,000 cars (2). Some sources report that as many as 90% of these bottles are not properly recycled and end up in landfills. A new concern over the use of plastic water bottles is the tendency for people to refill and reuse them. Many people will refill and reuse water bottles for several days or more, often letting them sit in their cars or other warm environments for hours at a time. This is a prime environment for microbial growth and recent news reports have claimed that many potentially pathogenic microorganisms have been isolated from such bottles and people are being told not to refill their water bottles any more. The object of this investigation was to isolate organisms from a reused plastic water bottle to determine what kinds of microorganisms, if any, inhabit the water many people are drinking and whether or not consumption of these organisms is really unsafe.

MATERIALS AND METHODS

A bottle of Arrowhead drinking water was purchased from a convenience store on a Friday and was repeatedly drunk from and refilled over the course of a weekend. The following Monday, 750 μ l of water from the bottle was transferred onto each of two agar plates, one nutrient and one Tergitol-7, using a digital pipette. Both plates were incubated at 37° C for 48 hours. When growth had become evident on the nutrient agar plate, a sterile wire loop was used to transfer a sample of the culture present onto a new nutrient agar plate. This plate was then incubated at 37° C for 24 hours. Once isolated colonies were observed, a Gram-stain and a Nigrosin-indirect stain were performed on samples taken from these colonies.

Results from the Gram-test were used to determine the PCR procedure used. DNA was extracted from a 2mm diameter sample of cells by boiling them in 10mM Tris buffer, and beating them with glass beads. A segment of 16s ribosomal-DNA was then amplified using the PCR, Taq Master Mix (Qiagen), and Bacteria 8-Forward and Universal 1492-Reverse primers. Gel electrophoresis of the PCR product DNA (50 μ l total volume) was run in an agarose gel and TBE buffer. The DNA sample was purified using a QIAquick Gel Purification Kit (Qiagen). The purified DNA sample was submitted to the ^{UC}DNA Sequencing Facility, Storer Hall, University of California, Davis. The sequencing primers used were Bacteria 8-Forward and Universal 1492-Reverse. The electropherogram was evaluated and edited using Mac OSX and 4Peaks. Sequences were compared to information available in public databases using the NCBI BLAST algorithm.

Information from the algorithm and Bergey's Manual of Systemic Bacteriology (1986) was used to determine the appropriate enzymatic tests. A catalase test was performed on a sample from an isolated colony on the nutrient agar (NA) plate using Hydrogen Peroxide. An MR-VP tube was inoculated with another sample and was incubated at 37° C for 48 hours. From this tube, 1ml of solution was transferred to a clean, screw top tube for a Voges-Proskauer test, to which 18 drops each of Barrit's reagent A and B were added. The remaining solution in the MR-VP tube was subjected to a Methyl Red test by adding 5 drops of methyl red indicator to the tube. Carbohydrate slants including, Glucose, Arabinose, and Mannitol were inoculated and incubated at 37° C and checked at 24 and 48 hours for signs of aerobic acid production. A plate of Mannitol Salt Agar (MSA) was streaked to observe the culture's ability to grow in a hypertonic environment and was incubated at 37° C for 24 hours. A slant of Simmons Citrate Agar was inoculated to observe the culture's ability to utilize citrate and was incubated for 48 hours at 37° C. A Gelatin Hydrolysis test was performed by inoculating a tube of nutrient gelatin, incubating this at 37° C and checking for liquifaction at 24 and 48 hours by putting the tube in an ice bath. A starch hydrolysis test was performed by streaking a starch agar plate, incubating this at 37° C for 24 hours, and then subjecting the culture present to a few drops of Gram's iodine to check for starch hydrolysis.

RESULTS

An off-white lawn culture was observed on the original nutrient agar plate after 48 hours while the Tergitol-7 plate showed no growth. After a 24-hour incubation, the second NA plate showed a number of well-isolated colonies ranging in size from 1.5 to 6.0mm in diameter. Colonies were opaque, dull, off-white, irregular in shape, ranging from flat to raised, with a

margin varying from lobate to filamentous. From the Nigrosin indirect stain many single-celled bacilli were observed under 1000X magnification using a compound microscope with an ocular micrometer and were found to range in size from 0.75 to 1.0µm in diameter and 1.5-3µm in length. From the Gram-stain slide prepared, the culture was found to be Gram-positive with many centrally located ellipsoidal endospores in non-swollen sporangia. There were also many poorly stained exospores visible.

Results obtained from the 16S r-DNA sequence comparison using the NCBI BLAST algorithm indicated the water bottle culture showed 100% similarity with a 1467 base gene bank sequence from *Bacillus subtilis* strain BZ15 (1446/1446) and 100% similarity with a 1475 base gene bank sequence from *Bacillus subtilis spizizenii* (1444/1444), along with two other *Bacillus subtilis* strains (AU30 and MO2).

Test conducted	Bergey's Manual <i>Bacillus subtilis</i>	Culture isolated from water bottle
Catalase	+	+
VP	+	+
MR	-	-
Glucose	+	+
Arabinose	+	-
Mannitol	+	+
Citrate	+	+
7.5% NaCl	+	+
Gelatin	+	+
Starch	+	+

Table 1 - Comparison of enzymatic test results listed in the Bergey's Manual of Systematic Bacteriology vol 2, for *Bacillus subtilis*, with those obtained for the water bottle isolate.

After conducting the enzymatic tests suggested in the Bergey's Manual of Systematic Bacteriology (1986) for *Bacillus subtilis*, results were observed and compared with those expected. Bubbles were observed after adding hydrogen peroxide to the sample, indicating the culture was catalase-positive. The Vogues-Proskauer tube turned red-wine color after adding Barritt's reagents and subjecting the tube to vigorous shaking, resting, then re-shaking. This indicated a positive result for acetoin production. The Methyl Red solution turned a deep yellow color with an orangish top indicating a negative result for acid production exceeding the buffer's capacity to neutralize. After 24 hours of incubation, the glucose and mannitol slants were bright yellow color with observable growth indicating positive tests for aerobic acid formation. At 24 hours the arabinose slant was still bright red indicating a negative test for acid formation. This slant was checked again at 48 hours with only a slight change in color confirming the negative result observed initially. The MSA plate was observed after a 24 hour incubation and showed many isolated colonies surrounded by yellow media indicating the culture's ability to grow in a high salt environment and confirming its ability to form aerobic acid on mannitol. The Simmons Citrate agar showed no change at 24 hours, remaining green. It was checked again at 48 hours

and showed a change in color to a deep blue, indicating a positive result for citrate utilization. The tube of nutrient gelatin was subjected to an ice bath for several minutes after 24 hours of incubation and readily re-solidified. It was then incubated for an additional 24 hours and placed in an ice-bath again remaining liquid, indicating a positive test result for gelatin hydrolysis. The streaked starch agar plate had many isolated colonies after 24 hours of incubation and showed clear zones in the agar surrounding the colonies when subjected to several drops of Gram's iodine indicating a positive result.

DISCUSSION

The colony and cell morphology recorded from the water bottle isolate were consistent with data recorded for *Bacillus subtilis* in the Bergey's Manual. Likewise, the results obtained with the enzymatic testing strongly correlated with those expected for *Bacillus subtilis*. The only variation was the negative result obtained for aerobic acid formation on arabinose, and perhaps if this had been incubated for a longer period of time, it would have been positive. Results obtained with the NCBI BLAST confirmed the identity of the water bottle culture as *Bacillus subtilis*, with 100% sequence similarity for 1446 bases.

Because the only organisms isolated from the used water bottle were identified as *Bacillus subtilis*, and nothing grew on the Tergitol-7 agar (a medium selective for Gram-negative bacteria), the water in the bottle was probably not dangerous to drink. *Bacillus subtilis* is not a species associated with any diseases or infections in humans or other animals. These bacteria are commonly found in soil (1), and may have been better able to withstand the chlorination of the tap water used to refill the bottle than some other, possibly pathogenic, bacteria would have been because of their ability to form endospores. An interesting feature of *Bacillus subtilis spizizenii* is its use in producing lipopeptide biosurfactants. When introduced to an oil reservoir along with nutrients such as glucose and sodium nitrate, *Bacillus subtilis spizizenii* will produce large amounts of a biosurfactant that will mobilize entrapped oil allowing for a much more thorough utilization of oil in reservoirs (5). It is quite a coincidence that the organisms isolated from a plastic water bottle (a product made with the oils from these reserves that are being depleted rapidly), would produce a compound capable of more complete oil utilization. Without the use of microbially enhanced oil recovery (MEOR), only about one-third to one-half of the oil originally present in an oil reservoir is recovered. Using *Bacillus subtilis spizizenii* to recover the oil from these reserves could greatly increase this percentage, while requiring considerably less energy and money than processes currently in use (5).

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Isolation of *Staphylococcus Cohnii* from Hand-washing Exercise

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Abstract

A bacterial unknown was taken from a nutrient agar plate inoculated during a laboratory exercise assessing the effectiveness of hand washing. The unknown was chosen from a thumbprint pressed into nutrient agar after washing the subject's hands three times, using anti-bacterial soap and disinfectant. The culture was isolated on nutrient agar for the purposes of identification using PCR and DNA sequencing. A Gram stain was prepared and a variety of enzymatic tests were conducted including growth on MSA (a selective and differential medium), catalase test, Methyl Red & Vogues-Proskuer test, coagulase test and mannitol fermentation. Results obtained with the NCBI BLAST indicated the culture isolated to be *Staphylococcus cohnii*. Contamination of the original culture with a *Bacillus* species complicated the identification.

INTRODUCTION

This study involved the identification of an unknown bacterial culture taken from a plate inoculated on May 12, 2007, for the purposes of demonstrating the effects of using various hand-washing techniques/materials as a part of aseptic technique. A Petri dish containing nutrient agar was divided into four sections and a human thumb was used to inoculate each section following a specified procedure. The first quadrant of the Petri dish received a thumbprint from the subject's unwashed hand. The second quadrant received a thumbprint after washing the subject's hands with tap water. The third quadrant received a thumbprint after the subject had washed with antibacterial soap, and the fourth quadrant received a thumbprint after washing with de-ionized water and laboratory disinfectant.

The microbial sample taken was from the fourth quadrant after the bacteria on the Petri dish had been allowed to grow and colonize the agar for a period of two weeks. The colony chosen, when viewed with the naked eye, appeared to be white in color, opaque, circular with entire edges, convex in elevation and shiny in appearance. This sample was chosen for the investigation because it was surprising to find bacteria surviving after being subjected to three hand-washing episodes, one involving antibacterial soap and another involving disinfectant. In fact, more of this particular type of bacterial colony appeared, the more times the subject's hands were washed. For this reason, a study was initiated to determine the identity of this unknown, seemingly resistant, type of bacteria. It seemed important to determine if these bacteria could be pathogenic, or potentially disease-causing agents that should cause concern for the subject from which they were taken, or if they were just a part of the natural flora of the epidermis.

MATERIALS AND METHODS

Initially, a wire loop was flamed and a sample of the unknown bacterial culture was taken from a well-isolated colony growing on the fourth quadrant of the hand washing exercise plate. Using aseptic technique and an appropriate dilution method, a Petri plate containing nutrient agar was streaked for the purpose of establishing a pure culture. This plate was maintained in the lab drawer, and was checked often for bacterial growth. At the end of 72 hours, as a pure culture appeared to have been established, a Gram stain was performed on the unknown and results were recorded. A sample of the culture was used to conduct a catalase test, and to inoculate a tube of MR-VP broth medium.

At the end of seven days, a sample of the culture was taken for the purpose of amplifying 16S ribosomal-DNA by performing a PCR. A small sample of cells was added to 500 μ l of 10mM Tris buffer (pH 8.0) in a microfuge tube, 10-15 2mm glass beads were added, and the tube was set in a boiling water bath for ten minutes. Following the boiling process, the tube was strapped to the platform head of a vortex mixer and the cells were beaten for 10 minutes. Amplification of 16S ribosomal-DNA was accomplished by using Taq Master Mix (Qiagen), a sample of the beaten cell material, water and Bacteria 8-Forward and Universal 1492-Reverse oligonucleotide primers. These materials were added to a 2ml PCR tube and placed into a Bio-Rad My Cycler, thermal cycler. The total volume of the product DNA was added to agarose and TBE buffer to undergo gel electrophoresis following the PCR. Then the DNA sample was cut from the gel and purified using a QIAquick Gel Purification Kit (Qiagen). The purified DNA sample was submitted to the ^{UC}DNA Sequencing Facility, Storer Hall, University of California, Davis. Sequencing was completed on an ABI 3730 Capillary Electrophoresis Genetic Analyzer, using bacteria 8-Forward and 1492-Reverse as sequencing primers. Sequence results were received electronically, and the sequences presented on electropherograms were edited and combined using 4Peaks and Mac OSX. The resulting sequence data was then compared to DNA sequences available through the NCBI website using the BLAST algorithm.

After observing results obtained from the NCBI BLAST, the Bergey's Manual of Determinative Bacteriology, vol. 2, was consulted and a set of enzymatic tests were selected for verifying the culture's identity. At this point, the original pure culture plate was found to be badly contaminated, and unsuitable for testing. A sample from this plate was restreaked onto tryptic soy agar (TSA) and mannitol salt agar (MSA) plates in an attempt to eliminate the contaminant and reestablish pure cultures. A new Gram stain was prepared and compared to the original and a coagulase test was run, but time restraints prohibited the performance of additional enzymatic tests.

RESULTS

The colony morphology of the unknown culture on nutrient agar was white in color, opaque, circular with entire edges, convex in elevation and shiny in appearance. Individual colonies were 3-4mm in diameter. Gram staining of the original and subsequent samples showed the unknown cells to be Gram-positive, cocci in shape and arranged in grape-like clusters (staphylo).

The unknown bacterial sample yielded positive results in the catalase test, was positive for acid formation in the Methyl Red test, and positive for acetoin formation in the Voges-Proskauer test. The culture tested positive for the fermentation of mannitol when grown on mannitol salt agar (MSA) and negative for coagulase formation in the coagulase test.

Results obtained with the PCR and nucleotide sequencing using the NCBI BLAST showed the DNA from the unknown culture to have 94% sequence similarity with 16S r-RNA genes from *Staphylococcus cohnii*. The low percentage indicated the culture used for sequence analysis was not pure (the electropherograms from both the Bacteria 8-forward and 1492-reverse primers were messy and contained many regions with overlapping peaks). When the culture was inspected, it was found to be contaminated with what appeared to be some type of *Bacillus*. This appeared to have been introduced along the edge of the plate farthest from the original inoculation (possibly by dust mites), but had spread as effuse growth across the entire plate. The contaminating culture was more tan than white, was semi-translucent, shiny, and highly irregular, with lobate extensions along all of its margins. Where it grew between the original *Staphylococcus* colonies, it was more translucent, and difficult to detect.

When grown on mannitol salt agar (MSA), the colonies showed the same morphology in general as those on nutrient agar, but appeared as do pathogenic *Staphylococcus aureus*; i.e., yellow colonies surrounded by yellow zones indicating acid production. The unknown colonies changed the color of the phenol red pH indicator in the medium from red to yellow throughout the plate. This provided evidence that the unknown bacterial sample was potentially-pathogenic. The contaminant did not grow on the mannitol salt agar plate. The new culture grown on tryptic soy agar appeared pure after 24 hours at 37° C, but after a few days displayed the same contamination as had been observed before.

DISCUSSION

The bacteria isolated from the hand-washing exercise plate were identified as *Staphylococcus cohnii*, facultatively anaerobic organisms known to be one type of bacteria contributing to the normal, protective flora existing on the human skin. The taxonomic lineage for this culture is as follows: Bacteria; Firmicutes; Bacilli, Bacillales, Staphylococcaceae, *Staphylococcus*. The normal bacterial flora present on human skin help fight off competing microbes which may be more pathogenic and damaging to the superficial or deep tissues of the body, if allowed a method of entry such as a puncture or cut. *Staphylococcus cohnii* help fight off these potential pathogens by taking up the nutrients and space available on the skin surface as an individual defense for the bacteria and a natural defense for their human hosts. These organisms make it less likely that pathogenic bacteria will find space or nutrients in order to grow on the host's skin.

The human epidermis has its own natural defenses, including squamous epithelial cells that act as a seal or barrier keeping microbes out and preventing the desiccation of underlying host cells. However, as is apparent from the growth on the hand washing technique plate, many naturally occurring microbes are also present. Colonies formed by these organisms actually became more abundant with each hand washing, while microbes found in the first quadrant (likely to be competitive pathogens picked up from working in the laboratory setting), became less prevalent. This suggests that *Staphylococcus cohnii* inhabit the region under some of the

dead, highly keratinized surface cells of the epidermis, and that colonies from these normal flora increase in number after some of the dead surface cells have been removed by hand-washing.

Though generally considered non-pathogenic, *Staphylococcus cohnii* can act as rare opportunistic pathogens in immuno-suppressed individuals such as victims of HIV infection and AIDS. In these individuals, the penetration of *Staphylococcus cohnii* through lesions into the bloodstream or deep tissues can cause disease symptoms such as septicemia and sub-acute endocarditis. *Staphylococcus cohnii* are also known to be responsible for causing urinary tract infections and wound infections.

The contamination found on the original pure culture plate caused some difficulty during this investigation, and prevented the completion of enzymatic tests necessary for confirming the identification. Two different subspecies of *Staphylococcus cohnii* are listed in the Bergey's Manual, and additional enzymatic test results might have been used to distinguish between them. The contaminants present with the culture grown on nutrient agar grew on tryptic soy agar, but did not grow on mannitol salt agar. This indicated the contaminants were not halophiles, and that the selective feature of mannitol salt agar provided an effective means for reestablishing a pure culture. More consistent monitoring of the pure culture plate during the investigation would have significantly improved the potential for obtaining accurate results.

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The Isolation and Identification of *Streptococcus mitis* from the Oral Cavity of a Resident of Roseville, California.

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The human mouth is a cavity that contains several different types of microorganisms that are probably unknown to many people. In an attempt to isolate and identify one of the types of organisms found in the mouth, *Streptococcus mitis* was discovered. This type of bacteria is a mesophilic, alpha-hemolytic species of *Streptococcus* that inhabits the oral cavity and makes up to around 40% of the *Streptococcus* in saliva. They account for a large part of the normal microbial flora found in the mouth and upper respiratory tract. Steps used in the isolation and identification of this species are described, and include observation of cell and colony morphology, enzymatic testing and DNA analysis.

INTRODUCTION

For this independent project, a bacterial examination of the mouth, or oral cavity was initiated. Humans have several different kinds of microorganisms residing within their oral cavity, so for this project, a sterile cotton swab was used to sample an oral cavity and streak a blood agar plate. A sterile wire loop was then used to dilute the inoculum so a specific type of organism could be isolated and identified. Additional testing was planned to determine some of the biochemical qualities that bacteria living in the mouth possess.

MATERIALS AND METHODS

To begin this independent project, a sample was taken from an oral cavity with a sterile cotton swab. The saliva obtained on this swab was streaked onto a blood agar plate, and then diluted with a sterile wire loop and aseptic technique. After 24 hours of incubation at 37° C, the plate was observed for indications of growth. Alpha hemolytic colonies were discovered and were then restreaked onto a separate blood agar plate to obtain a pure culture. After a pure culture was established, a Gram stain was completed to discover if the organisms present were Gram-negative or Gram-positive, information essential for chromosomal DNA extraction in preparation for performing the Polymerase Chain Reaction or PCR.

In order to prepare for the PCR, DNA was extracted from the organisms by boiling them for 10 minutes in 10mM Tris buffer (pH 8.0) and beating them with glass beads. A reaction mixture for the amplification of 16S ribosomal-DNA was prepared using Taq Master Mix (Qiagen), the primers Bacteria 8-Forward and Universal 1492-Reverse, chromosomal DNA and water. The mixture was placed into a Bio-Rad My-Cycler thermal cycler, which alternately

raises and lowers the temperature to facilitate the Polymerase Chain Reaction. The temperature was first raised to 94°C, breaking hydrogen bonds and causing the two strands of the template DNA molecules to separate, or be denatured. The temperature was then lowered to 55°C allowing the primers to anneal, or hybridize. Lastly, the temp was raised to 72°C, allowing the Taq Polymerase to build new complimentary DNA strands by means of extension (5). This process was repeated over and over multiple times (37) resulting in millions of DNA fragments. Gel electrophoresis of the PCR product DNA (total volume) was then performed in agarose and TBE buffer. The DNA was cut from the gel and purified with a QIAquick Gel Purification Kit (Qiagen). After this was completed, the purified DNA sample was submitted to the ^{UC}DNA Sequencing Facility, Storer Hall, University of California, Davis; the sequencing primers used were 8-Forward and 1492-Reverse.

When the DNA sequencing results were returned, an electropherogram was evaluated and edited using Mac OSX and 4Peaks. The edited sequence was then compared to information available in public databases, using the NCBI (National Center for Biotechnology Information) BLAST algorithm. After the BLAST algorithm indicated a species match, research was conducted in the Bergeys Manual of Systematic Bacteriology, to find out a series of enzymatic tests that could be performed in order to verify the identification. The enzymatic tests performed on the oral cavity isolate included mannitol, sorbitol, raffinose and lactose carbohydrate deeps, esculin, starch, and urea hydrolysis, (VP) or acetoin formation, catalase test, and hemolysis reactions on blood agar.

RESULTS

When a pure culture was obtained on the blood agar, the colonies were circular, entire, convex, semi-translucent, milky white and fairly small, about 0.5mm in diameter. Also, each of these colonies was surrounded by a circle of green coloration, indicating α -hemolysis. The Gram stain performed and observed indicated the culture was Gram-positive. The stain showed purple, semi-ellipsoidal cells in pairs and short chains, with each cell measuring approximately 0.6 micrometers in diameter. When sequence data was put into the BLAST algorithm, the results indicated the oral cavity culture showed 99% similarity with a 1469 base gene bank sequence from *Streptococcus mitis* strain GCSS 1303 (AY485601). The lineage was as follows: Bacteria; Firmicutes; Lactobacillales; Streptococcaceae; Streptococcus.

The enzymatic testing yielded several results; the organisms have the ability to ferment lactose, but do not have the ability to ferment mannitol, sorbitol, or raffinose. They have the ability to hydrolyze esculin and produce a grayish-black color in bile esculin medium. They were also positive for starch hydrolysis, allowing the agar behind or directly around the colonies to remain light-colored when Gram's iodine was added to the culture growing on the starch plate. These organism cannot hydrolyze urea, so do not form urease enzymes. The Vogues Proskauer test was also negative, indicating that the organisms do not have the ability to produce the neutral end product, acetoin. When grown on blood agar plate, the organisms were positive for alpha-hemolysis, producing a green coloration (met-hemoglobin) around the colonies. These organisms did not produce any bubbles when mixed with 3% hydrogen peroxide, indicating that they do not form catalase enzymes (5).

Enzymatic Test Run	Oral Cavity Isolate Results	<i>Streptococcus mitis</i> Results
Mannitol	(-)	(-)
Sorbitol fermentation	(-)	(-)
Raffinose fermentation	(-)	(-)
Lactose fermentation	(+)	(+)
Esculin hydrolysis	(+)	(+)
Starch hydrolysis	(+)	(+)
Urea hydrolysis	(-)	(-)
Acetoin formation	(-)	(-)
Hemolysis reaction (alpha)	(+)	(+)
Catalase test	(-)	(-)

Table 1 – Comparison of results obtained from the oral cavity isolate with those expected from *Streptococcus mitis* as indicated in the Bergey's Manual of Determinative Bacteriology.

DISCUSSION

The mouth is a perfect habitat for mesophilic microorganisms, and this is a very broad category of microorganisms; however, a successful attempt was made to identify at least one type of oral cavity microorganism and maybe shed some light on the type of biochemical characteristics associated with bacteria living in the mouth. Streptococci have traditionally been classified on the basis of colony morphology, hemolysis reactions on blood agar, and biochemical reactions (4). The hemolytic quality of the oral cavity isolates and their colony morphology were observed on sheep blood agar and correlated well with information provided in the Bergey's Manual of Determinative Bacteriology for *Streptococcus mitis*. Although the *mitis* species is considered difficult to identify by biochemical methods alone, because of the lack of reliable traits, organisms in this group have been found to be Gram-positive, nonmotile, nonsporeforming, facultatively anaerobic, catalase-negative cocci that appear in pairs or chains(4). They can form both smooth and rough colony variants and sometimes lack hemolysis ability. They typically form acid from glucose, lactose and sucrose, but not from mannitol, sorbitol or arabinose. These features also correlate well with the oral cavity isolates.

It was discovered that the oral cavity isolate's 16S r-DNA nucleotide sequence showed 99% similarity with seven strains of *Streptococcus mitis*, the highest scoring sequence matching 1441/1447 bases. All of the mismatched bases fell between 615 and 783, on the query, which is within an area of overlap for sequences generated by 8-forward and 1492-reverse primers, but well outside the 500 base limit suggested for accuracy with automated sequencing equipment. If a third sequence were generated using the primer 533-forward, it is highly likely that the similarity would be significantly higher, and potentially 100%.

The bacteria isolated from the oral cavity were found to be *Streptococcus mitis*, α -hemolytic organisms from the genus *Streptococcus* (1). These microorganisms predominate on human oral mucosa and may form, on the average, 34% of the streptococci in dental plaque, and 40% of the streptococci in saliva. This is because members of the *mitis* species adhere far better than other streptococci to the teeth and to buccal mucosa (2). These streptococci comprise a large part of the normal flora of the oral cavity and upper respiratory tract of humans, but can

also be involved, usually as opportunistic pathogens, in human diseases, such as tooth decay and bacterial endocarditis (3). Although *Streptococcus mitis* can be a potential and sometimes opportunistic pathogen, it is pretty harmless for the most part, serving as a member of the normal flora providing protection against more virulent organisms.

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Isolation of *Bacillus megaterium* from an Aquatic Turtle Tank

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An unknown type of bacterial culture isolated from an aquatic turtle tank was determined to be *Bacillus megaterium*. The use of DNA analysis involving 16S ribosomal DNA nucleotide sequencing, enzymatic tests including carbohydrate slants, citrate utilization, oxidase and catalase tests and different staining techniques assisted in this identification. The possibility of reptiles as pets and pathogen carriers was explored and the main bacteria responsible for causing human illnesses in association with turtles (*Salmonella*) were investigated. Strategies for proper human sanitation after reptile interactions are presented.

INTRODUCTION

This project was carried out to determine if reptiles undoubtedly assist in the transmission of bacterial pathogens to humans. Reptiles have been shown to be carriers of *Salmonella* species reported to have made many people ill, especially small children (1). Within the last five years turtles have become popular house pets, facilitating frequent reptile to human interactions within the home. Many people wonder if turtles are not the only organisms people are welcoming into their houses; proposing that pathogenic bacteria, specifically *Salmonella*, may also be present. The Association of Reptile and Amphibian Veterinarians has stated that not every reptile is a carrier of *Salmonella* or other pathogenic bacteria (1). The reptile/pathogen connection provides an interesting topic for investigation.

If turtles carry a variety of microorganisms, but do not always carry *Salmonella*, then an unknown, randomly selected culture taken from a turtle is unlikely to be *Salmonella*. With this hypothesis in mind, the object of this project was to isolate and identify an unknown, type of bacteria, randomly chosen from growth cultured from a turtle tank. If this culture was found to be non-pathogenic and not *Salmonella*, it would disprove the belief that reptiles alone are to blame for human bacterial infections.

MATERIALS AND METHODS

Multiple procedures were completed during this project in order to correctly identify the particular bacteria isolated. Most of these were found in the Microbiology Laboratory Manual (4). Initially, a Petri plate containing nutrient agar medium was laid agar side down on the screen of a turtle aquarium for one hour. Growth became visible within twenty-four hours and a streak plate was made from one of the colonies in order to establish a pure culture. Once a pure culture was obtained, multiple stains were executed including a Gram stain, an indirect stain, a

capsule stain, and an endospore stain. The indirect stain was carried out using nigrosin and the reagent used in the endospore stain was Malachite Green (4).

DNA Analysis was also completed and included the following preparations. Chromosomal DNA was extracted from a sample of cells by boiling them in 10mM Tris buffer (pH 8.0) and beating them with glass beads. Amplification of 16S ribosomal DNA was accomplished with the PCR using Taq Master Mix (Qiagen) and the bacteria primers Bacteria 8-Forward and Universal 1492-Reverse. Gel electrophoresis of the PCR product DNA was completed using agarose and TBE buffer. The sample was then purified using a QIAquick Gel Purification Kit (Qiagen). The purified DNA sample was then sent to the ^{UC}DNA Sequencing Facility, Storer Hall, University of California, Davis. Electropherogram evaluation and editing was completed using Mac OSX and 4Peaks software. The complete nucleotide sequence was then compared to data available in public databases through the National Center for Biotechnology Information (NCBI) using the Basic Location Alignment Search Tool (BLAST) algorithm (4).

Information gained through application of the NCBI BLAST was used to focus the investigation on a specific section of the Bergey's Manual of Systematic Bacteriology. Addition testing to determine phenotypic characteristics was then completed as indicated. The enzymatic tests used to verify the identity the unknown culture included glucose, arabinose and mannitol carbohydrate slants, an oxidase test, a citrate utilization test, and a catalase test (2).

RESULTS

The results obtained during this project indicated that the bacteria isolated from the turtle tank were *Bacillus megaterium*. Morphological features of colonies growing on nutrient agar were compared with those described for this species in the Bergey's Manual. These cultural characteristics are presented below in Table 1: Colony Morphology.

Table 1: Colony morphology of turtle tank isolate and *Bacillus megaterium*.

Colony Morphology	Turtle tank isolate	<i>Bacillus megaterium</i>
Form	circular to irregular	heaped – non-spreading
Margin	entire to undulate	not described
Elevation	raised	raised, sometimes wrinkled
Surface texture	shiny, waxy	glossy or moderately dull
Optical character	opaque	not described
Pigment	pale creamy yellow	yellowish, darker with age
Size (mm)	1-5mm, variable	not described
Medium	Nutrient Agar	Nutrient Agar

Cells observed in the Gram stain were found to be purple in color, indicating they were Gram-positive. The indirect stain results were used to determine cell shape and size, as indicated in Table 2 – Cell Morphology and Stain Characteristics, below. The capsule stain showed white capsules surrounding some of the cells. The endospore stain depicted ellipsoidal, centrally-located endospores with non-swollen sporangia.

Table 2: Cell Morphology and Stain Characteristics

Cell Morphology	Turtle tank isolate	<i>Bacillus megaterium</i>
Gram stain	Gram-positive	Gram-positive
Size (µm)	1.2- 1.5µm dia, 5µm length	1.2- 1.5µm dia, 2-5µm length
Shape	rods or bacilli	rods or bacilli
Arrangement	chains	not described
Endospores	central ellipsoidal	central, ellipsoidal or round
Capsule	yes	growth can be mucoid

The three carbohydrate slants (glucose, arabinose and mannitol) yielded positive results as indicated by a yellow color in the medium due to aerobic acid formation. The citrate utilization test yielded positive results with a dark blue color meaning that *B. megaterium* utilizes citrate as a sole carbon source. The catalase test was positive as indicated by copious amounts of bubbles produced when 3% H₂O₂ was added to a sample of the culture. Lastly, the oxidase test showed a negative result due to the unchanging white treated filter paper, meaning there was no presence of Cytochrome C enzymes. These results are indicated in Table 3: Enzymatic Testing, below.

Table 3: Enzymatic Testing

Tests	Turtle tank isolate	<i>Bacillus megaterium</i>
Glucose	Positive	Positive
Arabinose	Positive	Variable
Mannitol	Positive	Variable
Citrate	Positive	Positive
Oxidase	Negative	Variable
Catalase	Positive	Positive

The results obtained from the DNA analysis and BLAST indicated that the 16S r-DNA nucleotide sequence from the turtle tank isolate showed 100% similarity with five different gene bank sequences from *Bacillus megaterium* with 1448/1448 bases matching pairwise. The *Bacillus megaterium* strains identified were strain KL-197 (1522 bases), and strain GSP10 (1504 bases). The other three strains were not specifically identified.

DISCUSSION

The identity of the unknown, turtle tank isolate bacteria was determined to be *Bacillus megaterium*. *Bacillus* taxonomy in general has been significantly assisted by the analysis of 16S ribosomal RNA molecules by oligonucleotide sequencing (3); however *Bacillus megaterium* is one of the 34 species identified in the Bergey's Manual of Determinative Bacteriology, first edition, vol. 2 (1986). The taxonomic lineage for this species is: Bacteria; Firmicutes, Bacilli; Bacillales; Bacillaceae; *Bacillus*; *megaterium* (3). *Bacillus megaterium* require no growth factors such as amino acids or vitamins and can grow within twenty-four hours (3) on nutrient

agar. They are considered to be mesophiles, having an optimum growth temperature of 30-45 degrees centigrade (3). These particular bacteria can synthesize capsules made of polypeptide and polysaccharides, and because they form endospores, are very resistant to heat, radiation and other environmental factors (3).

According to the Bergey's Manual, the spores of *Bacillus megaterium* occur in soil, but they also occur in some aquatic environments. Reptile aquatic environments are typically maintained within the optimal temperature range for *Bacillus megaterium*, and since the endospores made by these organisms can survive many unfavorable conditions, *Bacillus megaterium* as residents of aquatic turtles are nearly impossible to get rid of. Based on the information found in Todar's Online Textbook of Bacteriology, cultures of *Bacillus megaterium* have rarely been isolated from human infections and these bacteria can be considered as pathogens of animals (3). Does this mean that turtle owners are seriously at risk for developing a bacterial infection caused by *Bacillus*? No, not necessarily. Usually the predominant bacterial infection humans receive from turtles is due to *Salmonella*, not *Bacillus megaterium* (1).

The public must remember that disease transmission not only involves having the vector, vehicle or reservoir available, but is also influenced by how specific individuals respond to that bacterial source. Even though *Bacillus megaterium* are potentially present on pet turtles, precautionary steps can be taken to decrease the chances of becoming infected from these unique household pets. The Association of Reptile and Amphibian Veterinarians website suggests adopting habits such as washing ones hands after every encounter, keeping children under five years of age away from the reptiles and not washing any reptile belongings in the kitchen where food is prepared (1). If these steps are religiously followed humans should not contract any bacterial diseases from reptiles despite the fact that reptiles can be carriers of potentially pathogenic bacteria.

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