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Identification and Analysis of Pathogenic Bacteria from Imported Frozen Tilapia

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Abstract

Tilapia is one of the most commonly eaten fish in the United States, yet little is known about the bacteria that it could carry. Because the vast majority of tilapia are raised and processed outside the United States, it is possible that there is less control of its bacterial content due to a higher chance of exposure to environments that could lead to the retention and/or introduction of pathogenic bacterial species. Therefore, it is important to determine if the tilapia sold commercially in the United States carry pathogenic bacteria that could pose a significant health risk to consumers. To address this issue, a study was initiated to find potential bacterial pathogens in locally-purchased tilapia. Of the bacteria that have been isolated so far, two were studied in greater depth, *Citrobacter freundii* and an isolate from the *Pseudomonas* genus. The goal was to identify the species of the *Pseudomonas* isolate and to start the process of determining if the isolates were resistant to antimicrobials and carried virulence factors. After finding conditions for running robust PCR reactions, DNA was isolated from the bacteria and used to amplify the 16S rRNA gene. This amplified gene from the *Pseudomonas* species was sequenced and the sequence was compared to the NCBI database. The results suggest that this isolate is either a strain of *Pseudomonas lundensis* or an entirely new species. In testing for antimicrobial resistance, both isolates were found to be resistant to Cefoxitin and Cefazolin. Additionally, *C. freundii* was resistant to Streptomycin and the *Pseudomonas species* was resistant to Ampicillin. Virulence factors, some of which are connected to antimicrobial-resistance genes, that have previously been found in *C. freundii* are a major focus of future research.

Keywords: *Citrobacter freundii*, tilapia, *Pseudomonas*, antimicrobial resistance

Introduction

In 2010, tilapia was the fourth most commonly eaten fish in the United States, after shrimp, tuna and salmon (NOAA, 2016). The sale of this imported fish is valued at over 35 million dollars a year (Stewart, 2015). However, all of that money *could* be getting consumers more than just fish from their local supermarket. It is hypothesized that bacterial species will be found in raw, locally bought tilapia and the bacteria will show resistance to antimicrobials.

This is a problem because of the approximately 9.5 million annual cases of domestically acquired foodborne illnesses in the United States, 142,000 were caused by bacterial infections (Painter, et al., 2013). Unfortunately, several bacterial species have been found residing in tilapia fillets in the past. In 2013, there were 33 illnesses and six hospitalizations due to food borne illness involving the consumption of tilapia (CDC, 2013). The source of these bacteria is often chalked up to contaminated water and poor sanitation in fish farms. Additionally, it could be due to improper cleaning and handling of the fillets prior to matriculation onto the supermarket shelves.

One study found that the main concern of tilapia consumption is the potential cross-contamination from contaminated organs during fish processing. Also, they found that the water quality effected the fish immune response: lower water quality increased bacterial infection in the fish (El-Shafai, et al., 2004).

There has been little research done on the possibility of pathogenic bacteria on locally raised tilapia and tilapia raised in the United States in general. However, one study, in Botswana, compared bacteria in catfish, frozen tilapia, whole tilapia, and gutted tilapia. The most commonly found bacteria in all four samples were *Citrobacter braakii* and *Staphylococcus* species. The most common bacteria found in frozen tilapia fillets, were *Citrobacter braakii, Citrobacter freundii,* and *Staphylococcus* species. These were hypothesized to be attributed to poor manufacturing conditions, as well as high levels of fecal coliforms in the samples (Mhango, et al., 2010).

Another study identified pathogenic bacteria from tilapia in Zimbabwe. It was found that there was 25.6×10^6 cfu/g total plate count of bacteria present on the skin of tilapia. Unfortunately, it was not stated whether or not all of the present bacteria were pathogenic. There was also a large amount of bacteria present in the intestines, gills, and mouth of the fish. The most common bacterial species found was a species of *Pseudomonas*. There were also a significant number of fecal coliforms present in the intestines, gills, and mouth of the fish. Although these are not generally the parts of the fish that are consumed, during handling and cleaning of the entire fish, there is likely some form of contamination. The presence of these coliforms suggests pollution of the habitat (Sichewo, et al., 2013). This is an important note because it is a possible cause of the bacterial species presence on these fish.

C. freundii is an Enterobacteriaceae bacteria that can be found in soil, water, sewage and food. It has also been found in different organs of diseased *and* healthy animals and is considered to be an opportunistic pathogen (Toranzo, et al., 1994). This particular species of *Citrobacter* has been known to cause several different health disorders. *C. freundii* is known to cause bacterial

meningitis in infants with a 25 to 50% mortality rate (Badger, 1999). Additionally, one outbreak of *C. freundii* in a nursery in India infected 17 infants, with one fatality. The infants showed symptoms of diarrhea and septicemia. The child with septicemia did not survive (Bai, et al., 2012).

In instances where this bacterium does lead to different health issues, it is important to be able to treat them. However, *C. freundii* has been found to be resistant to several antimicrobial agents. Due to antibiotics being used in fish farms, drug resistance occurs. It has previously been resistant to ampicillin, erythromycin, streptomycin, and sulfafurazole, as well as tetracycline, oxytetracycline, and chloramphenicol, which are all used to treat disease in fish (Toranzo, et al., 1994). It is very likely that our isolate of *Citrobacter freundii* will be resistant to the same antimicrobials.

This project aimed to identify what species of pathogenic bacteria are in frozen tilapia imported into the united states, analyze the possible antimicrobial resistance to antimicrobial resistance previously found, and characterize virulence factors that the bacteria may possess. It is hypothesized that bacterial species will be found in raw, locally bought tilapia and the bacteria will show resistance to antimicrobials. This will be tested through bacterial growth, PCR reaction, and sanger sequencing.

Materials & Methods

Growth conditions for bacterial isolates

The first step in the process of identifying, classifying, and analyzing the *Citrobacter freundii* and *Pseudomonas* isolates was to determine the best conditions for growth. To do this, several media were prepared and autoclaved on the liquid cycle, poured into plates for agar-based media, and flamed to pop surface bubbles. The media used were: Eosin Methylene Blue agar (EMB) (Oxoid), Tryptic Soy Agar (TSA) (Bectin, Dickinson), Blood Agar (Remel), Nutrient Agar slants (NA) (Bectin, Dickinson), and Brain Heart Infusion slants (BHI) (Oxoid). The plates and tubes were placed at several different temperatures (23°C, 30°C, and 37°C) for 24 hours. This was done to determine the conditions that led to the maximal amount of growth

The next step was to streak the media using aseptic technique. The bacteria were streaked onto EMB media that was selective and differential for gram negative bacteria, and/or media that contained many of the necessary elements for most bacterial species to grow.

Bacterial sample preparation

Samples were frozen back for storage in BHI broth and 40% glycerol. First, 500 uL of 40% glycerol was transferred to each of four cryogenic tubes, two for *Citrobacter freundii* and two for the *Pseudomonas* isolate. Next, 500 uL of each of the 18-24 hour broth cultures were aliquoted into the appropriately labeled tubes, then mixed. One tube of each bacterium was placed directly into a box in the -81°C freezer. The other two samples were placed in a dry ice/ethanol solution for flash freezing, then placed in the same box in the -81°C freezer.

Isolation of DNA

A Zymo Miniprep Kit (Zymo Research) was used to isolate the bacterial DNA from BHI overnight broth cultures. The first step was to combine 200 uL of sterile H_2O with 0.009 grams of *Citrobacter freundii* bacteria and 200 uL of sterile H_2O with 0.0137 grams of the *Pseudomonas* isolate to Bashing Bead Lysis Tubes. Next, 750 uL of Lysis Solution was added to each of the two tubes and both were placed on a bead beater at max speed for five minutes. The tubes were then centrifuged for one minute at 10,000 x g. The supernatant was transferred to Zymo Spin IV Spin Filters in collection tubes and centrifuged at 7,000 rpm for one minute. Next, 1200 uL of Binding Buffer was added to the filter. 800 uL was transferred to two columns (provided in the kit) which were then centrifuged at 10,000 x g for one minute. The next steps were to add 200 uL of Prewash Buffer and centrifuge once more at 10,000 x g for one minute, add 500 uL of Wash Buffer and centrifuge at 10,000 x g for one minute, and transfer to new columns. The final step was to add 100 uL of Elution Buffer and centrifuge for 30 seconds at 10,000 x g. The samples were then analyzed with the Nanodrop in order to measure the purity and concentration of the DNA. This was done by first cleaning the instrument with 2 uL of sterile water, and then blanking with another 2 uL of sterile water. The same amount of each sample was then placed on the instrument and read for concentration and purity.

PCR conditions and additions

PCR reactions were done in total volumes of 50 (Table 1) or 25 uL (Table 2). All ingredients except for the water and the DNA were combined into one tube and aliquoted into 250µl PCR tubes. Since pipetting error could result in insufficient liquid for every tube, the amount of each component of the final solution was increased by 1 tube for every 8 tubes used in the reaction. Therefore, if one was using 6 tubes, enough of each component was added for 7 tubes. If 10 tubes were used, enough of each component was added for 10 tubes. This was done as a general rule to account for error. Three different sources of Taq Polymerase were used: Invitrogen (100 units), Thermo Scientific (5 U/uL), and the Taq from a colleague (Dr. Finnerty). It was determined that the amount of Finnerty's Taq needed was between 2 uL and 5 uL.

Table 1:

Solution	uL per tube
10x buffer (100mM Tris HCl pH 8.3, 500mM KCl)	5
MgCl2 (50 mM)	1.5
Fisher Scientific dNTPs (2mM)	5
forward primer (10pm/uL)	2.5
reverse primer (10pm/uL)	2.5
Invitrogen Taq polymerase (100 units)	4
DNA + sterile H_2O	29.5
Total	50

To ensure each tube had the desired volume, 27.5 uL of sterile H2O were added to all tubes. An additional 2µl of either H_2O (for the negative control), *E. coli* DNA (for the positive control), and either *C. freundii* or *Pseudomonas* species DNA (20 pm/uL) were added to the corresponding tubes (Table 2).

Table 2:

Ingredient	uL per tube
10x buffer	2.5
25mM MgCl2	1.5
forward primer	1
reverse primer	1
Thermo Scientific (100 mM) dNTP mix	1
Taq – (Finnerty)	2
DNA + sterile H_2O	16
Total	25

The samples were run through a PCR machine (Bio-Rad MyCycler) at 95°C for 4 minutes and then 30 cycles at 95°C for one minute, 50°C for 30 seconds and 72°C for either thirty seconds (for 1492R), one minute (for 1492R ALT) and then held at a 4°C until stored in the -20°F freezer. To detect the amplified product, a 1% agarose gel in TBE was made with 0.5 grams of agarose and 50 mL of TBE. The solution was heated until all agarose had dissolved, was allowed to cool, and was poured into a gel tray to solidify before any samples were loaded. Lane one was loaded with 6 uL of 100 bp ladder DNA marker (*Axygen Biosciences*) and combinations of 1 uL (10X) loading dye and 5 uL samples from PCR tubes in the other lanes. It was run at 106 volts for 45 minutes. The gel was then stained with Ethidium Bromide (10 ug/mL) for 15 minutes, de-stained with water for 5 minutes, and viewed and photographed under ultraviolet light using a UVP BioDoc IT Imaging System.

The PCR was re-run with the same additions and temperatures, but with dNTPs from Thermo Scientific and once more with the same additions and temperatures but Taq Polymerase from Dr. Finnerty. This Taq was derived from *E. coli* which contained a plasmid that has a gene that can encode Taq. This was done to try and determine which aspect of the procedure was at fault for the inconclusive results.

For 25µl PCR reactions, different amounts of the ingredients were used (see Table 2), but the conditions for how the reactions were set up and run were the same as described above.

The positive control bacteria (*E. coli)* were used for all five tubes and sterile water was used for the negative control tube. Sterile water was then used to bring all 6 tubes up to 25 uL. The reaction was run beginning with four minutes at 95°C and then 30 cycles of 95°C for one minute, 55°C for one minute, and 72°C for 30 seconds, followed by a 4°C holding temperature. And finally, a 1% TBE agarose gel was run.

One more PCR reaction was run to see if the primers 1492R and 1492R ALT both were able to successfully amplify the bacterial DNA (Table 4). The only difference was the annealing temperatures. The 1492R & 8F pair had an annealing temperature of 52°C and the other pair had an annealing temperature of 55°C. A gel was then run.

All primer's that matched the 16S rRNA gene and were used for PCR reactions were made at IDT and are listed in (Table 4).

Table 4:

Name	Concentration	Sequence 5' – 3'	Annealing Temperature °C (T_a)
533R	100 pm/uL	TTACCGCGGCTGCTGGC	55
8F Class	100 pm/uL	GAAGAGTTTGATCATGGCTCA	55
27F	100 pm/uL	AGAGTTTGATCCTGGCTGAG	55
1492R	100 pm/uL	CGGTTACCTTGTTACGACTT	52
1492R ALT	100 pm/uL	ACGGCTACCTTGTTACGACTT	55
515F	100 pm/uL	GTGCCAGCAGCCGCGGTAA	60
786F	100 pm/uL	GGATTAGATACCCTGGTAGTC	47
1054F	100 pm/uL	CATGGCTGTCGTCAGCTCGTG	60
1070R	100 pm/uL	GAGCTGACGACAGCCATGCAG	50

The number of each primer refers to the position of the 5' end of the primer in the 1541 bp *E. coli* 16S rRNA gene (Baker et al. 2003).

The original amplification using the primer pairs 27F & 1492R and 27F &1492R ALT and the BioLine Taq yielded no results for positive control or the *C. freundii* and *Pseudomonas* DNA. Both primer pairs were tested to see if the one nucleotide difference between R and R ALT affected how the DNA was amplified. Additionally, the same primer pairs and Taq but different dNTPs also yielded no results. The only bands present were those of the ladder. The next reaction was run with different Taq polymerase but still, no bands were seen besides the DNA ladder. This was likely due to the extension time being too short.

The next reaction was run as a 25 uL reaction with the primer pair 8F class & 533R and the different Taq. Under ultraviolet light, bands were seen at 500bp for the negative control, positive control, and each of the DNA samples. The same amounts were run to ensure 1492R and 1492R ALT worked, there were very faint bands around 1500bp.

DNA purification for sequencing

In order to purify the amplified product from the 16S rRNA gene, another PCR reaction was run using 50 uL reactions (Table 5).

Table 5:

10x buffer	5 uL
MgCl2 (25mM)	3 uL
8F class primer	2 uL
1492RALT	2 uL
Thermo Scientific (5mM) dNTP mix	2 uL
Thermo Scientific (5U/uL) Taq	0.4 uL

A newly ordered Taq, from Thermo Scientific, was used because it was not contaminated with *E. coli.* For 10 tubes, 14.4 uL of the mixed solution was added per tube. Next, 3 uL of sterile H2O was added to the negative control, 3 uL of the positive control bacteria, and Pseudomonas DNA was added to the other eight tubes. They were run at 94°C for 4 minutes, 30 cycles of 95°C for 1 minutes, 55°C for 1 minute, and 72°C for either 30 seconds or one minute, followed by a 4°C holding temperature.

Eight tubes of amplified 16S rRNA from the *Pseudomonas* species were combined into four samples, 90 uL each. They were purified by following the protocol from the GenCatch PCR Purification Kit (Epoch Life Science), except that the purified DNA was resuspended in sterile water not Elution Buffer. The Nanodrop was then used to measure the purity and concentration of the samples for sequencing.

The sample was sent to Idaho State for Sanger sequencing (Bioinformatics, 2016). It was sent with six primers: 533R, 515F, 786F, 1054F, 1070R, and 1492RALT (Table 4).

The primers were diluted with sterile water to 3.2 pm/uL. These primers were chosen because they cover all but one of the variable regions of the 16S rRNA gene. The only variable region missing is V9, at the end of the gene (Baker, 2003). The results were analyzed with a program called 4Peaks (Nucleobytes, 2001).

Antimicrobial sensitivity testing

In an effort to determine any resistance to antimicrobials the Kirby Bauer test was performed in triplicate (Hudzicki, 2013**).** This was done by inoculating one Mueller-Hinton (MH) agar plate (15x100mm) with *C. freundii* and one MH plate with the *Pseudomonas* isolate. The following disks were then placed at an equal distance apart on the plate (Table 6).

Table 6:

Antibiotic	Abbreviation
Nalidixic Acid	NA30
Cefazolin	KZ30
Erythromycin	E15
Piperacillin	TZP110
Imipenem	IPM10
Gentamycin	GM10
Vancomycin	VA30
Cefoxitin	FOX30
Clindamycin	DA2
Streptomycin	S10
Ampicillin	AMP10
Ciprofloxacin	GIP
Kanamycin	K
Tetracycline	TE
Chloramphenicol	C

The abbreviations are for identification of the antimicrobial and the numbers refer to the concentration of each antimicrobial disk. The *C. freundii* plate was grown for 24 hours at 37°C and the *Pseudomonas* plate was grown for 24 hours at 23°C.

Virulence factor detection tests

The Hly gene was tested for. One blood agar plate was struck with Pseudomonas and another blood agar plate with *Citrobacter freundii*. The plates were then grown at room temperature for 48 hours and 37°C for 24 hours, respectively.

A urease test was performed by inoculating urea broth with both bacteria and growing in the shakers at room temperature and 37°C.

The motility of *Citrobacter freundii* was tested by inoculating Sulfur Reduction Indole Production (SIM) motility media with an inoculating needle.

The motility of the *Pseudomonas* isolate was tested by inoculating motility media with an inoculating needle.

An Enterotube was inoculated with *C. freundii* by touching one end of the needle to a previously grown colony, pulling the needle through all of the compartments, and poking holes in the aerobic compartments. It was grown at 37°C for 24 hours.

Results

Growth conditions

Citrobacter freundii did not grow at room temperature. It grew best at 37°C but also grew at 30°C on all media tested. *Pseudomonas* did not grow at 30°C, but did grow on all media tested at room temperature (Table 7).

Table 7:

Bacteria	Growth	No growth
Citrobacter freundii	EMB 30°C	23°C
	Blood agar 37°C	
	NA slant 37°C	
	BHI slant 37°C	
Pseudomonas sp.	EMB 23°C	30°C
	TSA 23°C	37°C
	Blood agar 23°C	
	NA slant 23°C	
	BHI slant 23°C	

Sample preparation

Upon isolating the *Citrobacter freundii* and *Pseudomonas* DNA the concentrations and purity were identified (Table 8).

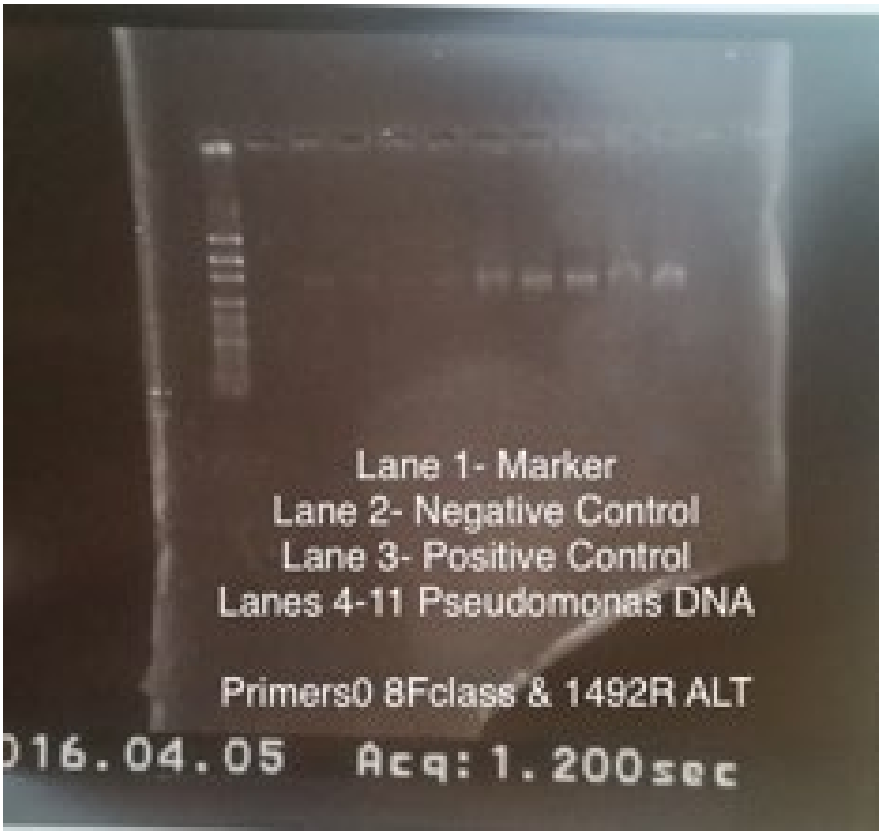
Table 8:

	Concentration (ng/uL)	260/280	260/230
Citrobacter freundii	46.2	1.61	0.64
Pseudomonas sp.	100.2	1.52	0.64

DNA purification from the PCR reaction

The first PCR reaction of the *Pseudomonas* isolate was used to ensure the required amount of DNA for sequencing and to ensure a purified amplified product of the 16S rRNA gene. This reaction was a 50 uL reaction with brand new Taq polymerase. The only band seen was the positive control band. The next reaction increased the time at 72°C to one minute and yielded positive results in all 8 Pseudomonas DNA lanes, as well as the positive control lane. This likely worked due to the extended extension time (Figure 1).

Figure 1:



Upon amplification of the samples, the concentrations and purity were assessed (Table 9).

Table 9:

Sample number	Concentrations (ng/uL)	260/280	260/230
1	4.2	2.61	0.5
2	7.6	2.27	0.68
3	19.2	1.65	0.84
4	19.6	2.06	1.65

When the Sanger sequencing was completed, each individual primer sequence was compared to the Blast database **(**Blast, 2016). The primer 533R had a 100% identity match with *Pseudomonas fragi*, strain P121. The primer 786F had a 99% identity match (550/551) with *Pseudomonas lundensis*. Primer 1054F had a 99% match with *Pseudomonas fragi,* 1070R had a 99% match with *Pseudomonas lundensis*, and 1492R ALT had a 100% match with *P. lundensis.* Essentially, what this means is that the numbering systems matched for a few different potential bacteria. These results led to the conclusion that the *Pseudomonas* isolate was most likely either *P. fragi* or *P. lundensis.* The primer 515F was not a usable sequence. Because the other primers yielded usable results, the primer itself was likely the problem. All five of the primer sequences that were usable overlapped and were combined into one sequence using 4Peaks (Figure 2).

Figure 2:

5'-
TATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGT
AGAGAGGTGCTTGCACCTCTTGAGAGCGGCGGACGGGTGAGTAATACCTAGGAATC
TGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTA
CGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGAT
TAGCTAGTTGGTGAGGTAATGGCTCACCAAGGC**T**ACGATCCGTAACTGGTCTGAGA
GGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA
GTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAA
GAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTAACCTAATA
CGTTAGTG**C**TTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAG
CCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGC
GTAGGTGGTTTGTTAAGTTGAATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATC
CAAAACTGGCAAGCTAGAGTATGGTAGAGGGTAGTGGAATTTCCTGTGTAGCGGTG
AAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACTACCTGGACTGATA
CTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGTCAACTAGCCGTTGGGAACCTTGAGTTCTTAGTGGCGCAGC
TAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA
AGAACCTTACCAGGCCTTGACATCCAATGAACTTTCCAGAGATGGATTGGTGCCTTC
GGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGG
GTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGG
CACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTC
ATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTT
GCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGT
CTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACG
GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGC
ACCAGAAGTAGCTAGTCTAACC**C**TCGGGAGGACGGTTACCACGGTGTGATTCATGA
CTGGGGTGAAGTCGTACG -3'

The three bolded nucleotides were not identical to any of the comparable bacterial sequences. It is important to note that this is not the entire gene. The primer closest to the 5' end of the gene began at the 8th nucleotide. However, the first few nucleotides from sequencing were inconclusive so the results of the sequencing reactions start at about the 15th nucleotide of the sequence. Additionally, the V9 variable region is missing so the sequence stops at about 1492 bp.

Antimicrobial sensitivity

Upon testing for antimicrobial resistance, *Citrobacter freundii* was resistant to Cefazolin, Cefoxitin, Streptomycin, and Ampicillin. *Pseudomonas species* was resistant to Cefazolin, Cefoxitin, and Ampicillin (Table 10).

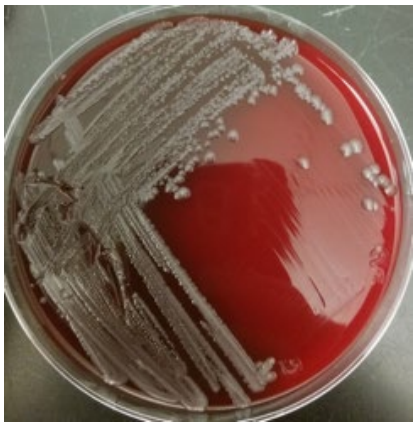
Table 10: S= sensitive, I= intermediate, R= resistant.

	C. freundii Trial 1 (mm)	*C. freundii* Trial 2 (mm)	*C. freundii* Trial 3 (mm)	*C. freundii* Trial 4 (mm)	*Pseud* Trial 1 (mm)	*Pseud* Trial 2 (mm)	*Pseud* Trial 3 (mm)	*Pseud* Trial 4 (mm)
NA	19.5 S	19 S	18 S		18 I	15 I	16 I	
KZ	12 R	10 R	10 R		0 R	0 R	0 R	
E	7 R				0 R			
TZP	22 S	25 S	25 S		26 S	28 S	30 S	
IPM	17 I	20 I	18 I		28 S	35 S	34 S	
GM	17 S	18 S	18 S		20 S	22 S	22 S	
VA	0 R				0 R			
FOX	0 R	0 R	0 R		0 R	0 R	0 R	
DA	0 R				0 R			
S	10 R	9 R	10 R		16 S	18 S	18 S	
AMP	10 R	17 I	18 I		0 R	0 R	0 R	
GIP	32 S	35 S	35 S		35 S	32 S	35 S	
TE		19 S	20 S	18 I		19 S	20 S	21 S
K		18 S	18 S	17 I		25 S	25 S	25 S
C		20 S	20 S	20 S		15 I	16 I	14 I

Virulence factors

The Hly gene, an extra-cellular toxin that destroys erythrocytes via inflammation, was tested for (Coggan, et al., 2008). It was found that for *Citrobacter freundii* no hemolysis was seen on the blood agar plate (Figure 3).

Figure 3:



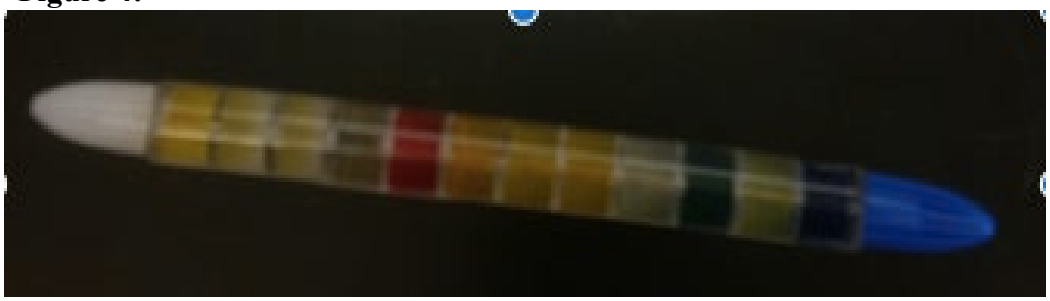
When inoculated with *C. freundii* bacteria, neither of the two tubes of urea broth changed colors after incubation. Additionally, neither of the two tubes inoculated with *Pseudomonas* bacteria changed color upon growth.

The SIM media inoculated with *Citrobacter freundii* turned completely black after just 12 hours of incubation.

The motility media inoculated with *Pseudomonas species* bacteria showed a line of air bubbles and a slight color change where the inoculation needle entered the media.

The Enterotube showed the following results (Figure 4).

Figure 4:



The glucose/gas compartment had changed from red to yellow, indicating a positive result. There was also formation of gas. The Lysine and Ornithine compartments were both negative. The H_2S/Indole was positive, which was already know from the SIM media. The Adonitol was negative. The Lactose, Arabinose and Sorbitol were all positive, as they had all turned to a yellow color. The VP, Dulcitol, and Urea compartments were all negative. The last compartment, Citrate, was positive, as it had turned from dark green to dark blue. These results produced the code: 31561. When compared to the database, this code corresponded to *Citrobacter freundii*. After the addition of Kovac's reagent, it did in fact turn red, meaning the indole test was positive.

Discussion

Identification of growth conditions

Upon growth of Citrobacter freundii on several media and at several different temperatures, it was found that the ideal temperature is 37°C. Additionally, it was found that in just 24 hours, the bacterium grew on all media types tested. Upon growth of Pseudomonas species it was found that the ideal temperature for growth is room temperature (about 23°C) and that it grows best at 36 to 48 hours. It is not surprising that these two bacteria grew on the EMB because that media is selective and differentiable for gram negative bacteria. Also, it isn't surprising that there was ample growth on the nutrient agar, blood heart infusion agar, blood agar, and tryptic soy agar. This is because those media are infused with elements that allow many bacterial species to adequately grow (Cooper, et al., 1968). It is significant that the *Pseudomonas* isolate preferred colder temperatures because it is a psychrophilic bacterium, meaning it is cold-loving.

Bacterial sample preparation

With regards to the DNA isolation of the two bacterial species, the DNA concentrations after isolation were sufficient for subsequent PCR reactions. Regarding the purity of the samples, the 260/280 value relates to the proteins present in the sample. Around 280 nm is when the proteins are picked up by the UV spectrum. If a sample is significantly below 1.8, then there are too many proteins in the sample. These samples were lower than 1.8 but not significantly, at 1.61 and 1.52. The 260/230 ratio refers to the organic compounds present in the sample. Any number between 2 and 2.2 is considered pure. These samples were much lower, at 0.64, however, for our purposes, they were sufficient.

PCR conditions

There were several possible reasons that the original attempts at amplification were not successful. One reason is that some of the necessary solutions were left out of the freezer for an extended period of time. This could have affected the Taq Polymerase, the dNTPs, or any of the primers because all three need to be kept either on ice or in the freezer at all times. The culprit was identified as the stock Taq from Invitrogen, which was thrown out. Dr. Finnerty's Taq was used in it's place. It was then found that 0.2 uL was not sufficient and 2 uL of that Taq was needed for the amplification of these bacterium.

The next Taq that was used was a new batch from Dr. Finnerty. Upon running the PCR samples on a gel, it was found that there was a band present in the negative control lane. It was later discovered that the Taq was contaminated with *E. coli* DNA and possibly RNA from the breaking open of the cells in the process used to produce and isolate large quantities of the Taq Polymerase, hence the band in the negative control lane. In the future, this Taq can be used to test if primers and other ingredients work to amplify DNA and be used on nonbacterial DNA, but cannot be used to test for genes in other species of bacteria is those genes are normally carried by *E. coli*.

DNA purification

The first attempt at amplification of the Pseudomonas species DNA yielded no results, which suggested a problem with the PCR temperatures. Once the extending temperature was increased to a one-minute interval, positive results were obtained and used for preparation of amplified product for sequencing of the 16S rRNA gene. Prior to sending the samples to be sequenced, the concentrations were found. The concentrations for samples one and two were too low to be sent in for sequencing. Sample three had a low level of purification at 280 nm, suggesting proteins were present in the sample. The best sample to be sent out was number four, therefore, it was used to sequence the 16S rRNA gene of the unknown *Pseudomonas* species.

When the entire sequence was analyzed via a Blast search of the NCBI database, there were 12 differences with *P. fragi,* and only three differences with *P. lundensis* strain ATCC 49968. There were some additional identity differences in the *P. lundensis* sequence, but they were all "N"s. This suggests a problem with the sequencing of the *P. lundensis* sequence deposited in the database. The other three differences seen are bolded in the full sequence. These

differences could be due to the isolate sequence being a different strain than the ATCC. They could also be due to the isolate being an entirely new species. Another set of genes or the entire genome of the isolate will have to be sequenced to figure out if it is *P. lundensis* or a new species. Physiological assays will also have to be done to help in the process of understand this isolate.

Antimicrobial sensitivity

The *Pseudomonas species* was found to be resistant to Cefoxitin, Ampicillin, and Cefazolin. Citrobacter freundii was found to be resistant to Cefoxitin, Ampicillin, Cefazolin, and Streptomycin. It makes sense that it is not resistant to both Nalidixic acid and ciprofloxacin because they are both in the same family, quinolones. It is most significant that the bacteria are resistant to Ampicillin, Streptomycin, Cefoxitin, and Cefazolin because they are broad spectrum antibiotics and should work against gram negative bacteria (Horvart, 2006). This suggests that the bacteria have developed resistance to these commonly used antimicrobials, possibly due to one or more antibiotic resistant genes.

Virulence factors

No hemolysis on the blood agar suggests that the Hly gene is not present in these bacterial species. This crosses one gene off the list of potential antibiotic resistant genes. No color change in the urea broth suggests that neither bacteria produces urease. This is important because it eliminates the ability of the bacteria to colonize in a host organism. The SIM media turning all black means that the bacteria are motile and therefore have flagella. This suggests that it is easier for the bacteria to metastasize throughout a host organism. If the bacteria were not motile, it would not have spread throughout the media. It also means that the bacteria reduce sulfur to hydrogen sulfide, which then combines with iron to form ferric sulfide (the source of the black precipitate) (Hardy Diagnostics, 1996).

The results of the Enterotube were interpreted. A positive result in the glucose/gas compartment suggests the bacteria fermented glucose. The positive result in the H_2S compartment suggests the reduction of sulfur. The positive result of Indole production via Kovac's reagent suggests the breakdown of Tryptophan. The positive results for Lactose, Arabinose, and Sorbitol suggest the bacteria ferments all three. The positive result in the Citrate compartment suggests a utilization of Citrate as an energy source (Kaiser, 1999).

Conclusions

Due to the potential pathogenic properties of *C. freundii*, it is crucial to identify the sources of the possible virulence factors in order to effectively be able to treat any issues that may arise after consumption of these bacterium. Past research of this bacterial species has noted the presence of many virulence genes that could cause it to be harmful to a number of animal species.

The chromosomal *eae* gene is a causative agent of murine colonic hyperplasia in laboratory mice. This is a disease that causes epithelial cell proliferation due to the production of attaching and effacing (AE) lesions. These lesions are present on the outer membrane and have been known to cause diarrhea in humans and rabbits. Presence of the gene was found in *Citrobacter freundii* biotype 4280 (Schauer & Falkow, 1993). This suggests that there is potential for this gene to be present in our strain of *C. freundii*.

Another virulence factor, the Vi Antigen, is a heat-labile somatic antigen. It has been found in *Citrobacter freundii* and is said to be able to shield bacteria from the host's immune system (Houng, et al, 1992). Because this antigen has been associated with pathogenicity, the *C. freundii* isolate needs to be tested to determine if it carries the gene that makes this virulence factor.

Integrons are genetic units that may carry antibiotic resistance and virulence genes in various bacterial species. The class I integron has been found in *Citrobacter* species including *Citrobacter freundii* (Pepperell, et al., 2002). Because our *C. freundii* sample has exhibited significant resistance to a number of antimicrobials, it would not be surprising if it contained class I integrons. Integrons are important in the transfer of resistance genes and virulence factors to other bacteria via horizontal gene transfer (Pepperell, et al., 2002).

This research project has led to the finding of antimicrobial resistance to multiple antimicrobials. This is a serious problem because of the possible pathogenicity of *C. freundii*. If this particular strain is capable of having negative effects on animals, there needs to be a way to properly treat and rid the body of any infection. However, with so much antimicrobial resistance, the list of potential drugs to treat such infections is shortened.

Regarding the *Pseudomonas species* sample, after careful analysis of the sequences received and without any other identifying information, it can be concluded that the most likely candidate for the *Pseudomonas* isolates is *Pseudomonas lundensis*. However, further analysis is necessary in order to assess if the species can be narrowed down to a specific strain or to see if the species is something else entirely. To do this, another gene/genes or the entire genome should be sequenced.

Pseudomonas lundensis is known to lead to biofilm formation on meat, fish, milk, and cheese, causing spoilage (Liu, 2015). It is indirectly related to possible health risks, meaning that ingesting spoiled foods can be dangerous to human's and animal's health, particularly children, the elderly, and the immune compromised.

It is important to continue the research into these two bacterial species found in store bought frozen fish because there are potential health risks involved. Future research could look into identifying the presence of a number of virulent genes. Biofilm formation is another aspect of the bacterium that should be studied further.

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