Bacterium strain identification from soil sample from Lake Forest Beach, IL

Petra Urgacova
Department of Biology
Lake Forest College
Lake Forest, Illinois 60045

Purpose

The purpose of this experiment is to determine the strain of bacterium isolated from soil samples from Lake Forest beach, IL. The bacterium is known to be antibiotically resistant as it will be taken from a McConkey agar plate with 3 µg/ml of Tetracycline concentration. We will use amplification of gene 13S ribosomal RNA which is common across prokaryotes to aid us in its identification. Sequenced data will be searched through gene databases. Techniques used include bacterium restreaking, genomic DNA isolation, Polymerase Chain Reaction (PCR), and gene sequence analysis using APE plasmid editor. We expect to find a close or identical match to a bacterium strain with known antibiotic resistance (Delventhal, 2022).

Procedures

1. Bacterial Restreak

A soil sample from Lake Forest beach IL was taken and bacteria from this sample was grown on a MacConkey agar with different concentrations of Tetracycline antibiotic (Urgacova, 2022). The bacterium that was restreaked came from a plate Tet3 that contained 3 µg/ml of Tetracycline and 1/10 dilution of the original soil sample (Figure 3). The single bacterium colony was restreaked on sterile agar media using a toothpick. The agar plate was then incubated for 72 hours at 28 °C. Afterwards the petri dish was sealed with parafilm and stored at 4°C (Delventhal, 2022).

2. Genomic DNA Isolation

DNA isolation was performed using “Genomic DNA Purification from Gram-negative Bacteria” kit (NEB #T3010). First step was to lyse the bacteria cells. 90 µl of cold PBS was put into a microcentrifuge. Then using a pipette tip, a singular isolated colony was picked from the petri dish and put into the microcentrifuge. The solution was then pipetted up and down to get the bacteria off the tip using a P200 pipette. Next, 10 µl of Lysozyme solution was added and vortexed before adding 100 µl of Tissue Lysis Buffer and vortexing again. The microcentrifuge tube was then incubated for 5 minutes at 37 °C, as the solution did not turn completely clear. 10 µl of Proteinase K was added and briefly vortexed. The microcentrifuge tube was then incubated at 56 °C for 30 minutes. During that time, circa every 5 minutes, the microcentrifuge tube was mixed by inversion. After 30 minutes, 3 µl of RNase A was added and briefly vortexed. Then the microcentrifuge was incubated for 5 minutes at 56 °C and mixed twice by flicking (Delventhal, 2022).

The next steps included filtering out protein and RNA, before finally obtaining a pure genomic DNA sample. 400 µl of gDNA (genomic DNA) Binding Buffer was added to the sample and mixed by vortexing to ensure the sample is thoroughly mixed. Next, 600 µl of the sample was carefully transferred to the gDNA Purification Column inserted into a collection tube. The sample was then centrifuged for 3 minutes at 1,000 x g to allow the gDNA to bind to the column and then 1 minute at maximum speed (12,000 x g). Afterwards, the flow through was discarded (Delventhal, 2022).

The column containing the gDNA sample was then transferred into a new collection tube, 500 µl of gDNA Wash Buffer was added, and the sample was centrifuged for 1 minute at maximum speed. After discarding the flowthrough, the column was reinserted into the collection tube and the wash procedure was repeated. Afterwards, the collection tube with flowthrough was discarded (Delventhal, 2022)

The column was then put into a DNase-free 1.5 ml microcentrifuge and 50 µl of preheated gDNA Elution Buffer was added, allowing it to incubate at room temperature for 1 minute. Afterward, the sample was centrifuged at the maximum speed for 1 minute. Lastly, the gDNA amount was quantified using a Biotek spectrometer (Delventhal, 2022).

3. Polymerase Chain Reaction (PCR)

To identify the bacteria strain, the sequence for 16S ribosomal RNA (rRNA) was selected for and amplified using PCR. This was done because the rRNA is a part of the 30S small ribosomal subunit which is common across most prokaryotes. However, they are still different enough that different strains of bacteria can be identified using this method (Janda and Abbott, 2007). The designed primers for 16S rRNA amplification were taken from a paper by Weisburg et al. (1991) (Figure 1).

Primer 27F: 5’-AGA GTT TGA TCC TGG CTC AG-3’
Primer 1492R: 5’-TAC GGG TAC CTT GTT ACG ACT T-3’

Figure 1. Forward primer (27F) and reverse primer (1492R) for highly conserved region of 16SrRNA ribosomal subunit (Lane, 1991).

However, to obtain optimal results an 16S rRNA primer mix was used which was created by Frank et al. (2008) (Figure 2).

5’-AGA GTT TGA TYM TGG CTC AG-3’
5’-AGA ATT TGA TCT TGG TTC AG-3’
5’-AGA GTT TGA TCC TGG CTT AG-3’
5’-AGG GTT CGA TTC TGG CTC AG-3’

Figure 2. Mix of 27F primers used from 18S rRNA PCR amplification (Frank et al., 2008)

For the experimental condition, 7 µl of dH2O was put into the PCR reaction tube followed by 1 µl of 1492R primer (10 µM), 1 µl of 27F primer mix (10 µlM), 1 µl of isolated genomic DNA (217.6 ng/µl) and 10 µl Phusion Master Mix (MM). For the positive control, 7 µl of dH2O was put into the PCR reaction tube followed by 1 µl of 1492R primer (10 µlM), 1 µl of 27F primer mix (10 µM), 1 µl of known DNA template, and lastly 10 µl Phusion MM. For the negative/no template control, 8 µl of dH2O was put into the PCR reaction tube followed by 1 µl of 1492R primer (10 µlM), 1 µl of 27F primer mix (10 µM), and lastly 10 µl Phusion MM (Delventhal, 2022).

The PCR reaction was run in the thermocycler in the following conditions: 1 cycle of 98°C for 30 seconds, 30 cycles of 98°C for 10 seconds, 48°C for 30 seconds, and 72°C for 1 minute and 30 seconds. 1 cycle of 72°C for 10 minutes. After the PCR reaction was completed, the samples were stored at -20°C.

4. Gel Electrophoresis and Imagining

Gel electrophoresis was performed to evaluate whether the desired 16S rRNA sequence was amplified in the PCR. A 1% agarose gel solution with 0.5 µg/ml agarose was premade by the laboratory instructor. It was heated in the microwave and after it cooled down enough to touch it, was poured into the gel tray and a well comb was put in place. After the gel solidified, TBE buffer was poured into the gel box up to the fill line as for the gel to be covered (Delventhal, 2022).

To prepare the running samples, 5 µl of the sample was put into a new PCR tube followed by 1 µl of 6x loading dye. Samples were mixed by pipetting up and down. (Delventhal, 2022). 5 µl of each sample (i.e. experimental conditions, positive control, and negative control) was loaded into the gel alongside 5µl of 1kb DNA ladder. The gel was run at 125 – 150 V for approximately 30-60 minutes. The expected size of the amplified region of 16S rRNA was 1450 base pairs (bp). The gel was then imaged and analyzed (Delventhal, 2022).

5. PCR Amplicon Clean Up for DNA Sequencing

The goal of this experiment was to determine the tetracycline resistant bacterial strain obtained from the soil sample. To do this, a DNA sequence analysis was necessary. Therefore, an isolated amplified DNA sample was cleaned prior to sequencing. Approximately 1.5 µl exo I and 3 µl of rSAP was added to 15 µl of the PCR product in

a microcentrifuge tube. Afterwards the sample was placed in a thermocycler and incubated at 37 °C for 15 minutes and then at 80 °C for 15 minutes. The samples were then sent to University of Chicago Sequencing Facility (Delventhal, 2022).

6. DNA Sequencing Analysis Using ApE Plasmid Editor

The DNA sequence was analyzed using the ApE Plasmid Editor. Low quality sequences at the beginning and end were cut. The first 46 base pairs were deleted so the edited sequence started with "ACTTCTT". The end sequence at the 1012th base pair was cut. So, the end sequence was "TGCTGCGG". The sequence for analysis ended up being 967 base pairs in length (Figure 7). To identify the corresponding bacteria the sequence was run in the Ribosomal Database Projects (RDB) by Michigan State University. Additionally, the sequence was also run through the NCBI BLAST database. All matches were then evaluated to determine bacterial strain.

Results

The bacterium that was restreaked is shown in Figure 3, outlined in red with a center of yellow color. Figure 4 shows the restreaked plate and the red circle signifies the single colony of bacterium that was taken. The restreaked plate had a unique horizontal growth through the agar (Figure 4). However, the isolated colony was not a part of that region.

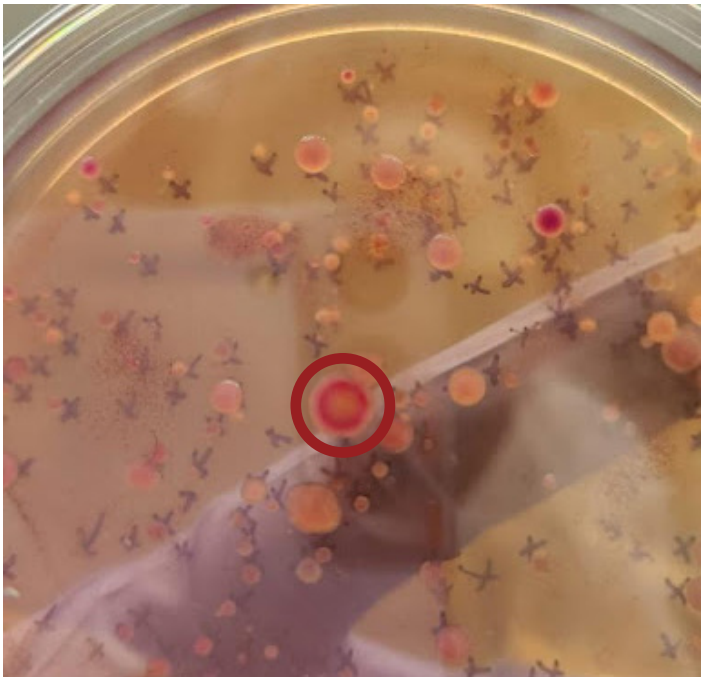

Figure 3. Tet3 McConkey agar plate containing 3 µg/ml of Tetracycline and 1/10 dilution of the original soil sample, the bacteria restreaked is in the red circle

The genomic DNA was isolated and purified using the method described above. The sample's concentration was 217.6 ng/µl. The DNA quantification machine also measured the purity of the sample. This purity, reported as the ratio 260/280, measured protein contamination which in this case was 1.952. This is higher than 1.8 which means protein contamination is in the good range (Delventhal, 2022).

Afterwards the PCR and gel electrophoresis was run. The gel was imaged and analyzed (Figure 5). The expected PCR sample length was 1,450 bp, so it was expected to have a thick band around 1,500 bp, which is shown in Figure 5 (Delventhal, 2022). This suggests that our sample has our expected product. The same results were found for the positive control, which had a known DNA template and was predicted to have PCR product around 1,500 bp. The Negative/no template control showed no amplification, as expected, as the sample had no DNA in it. This result suggests that we didn't have contamination in our sample.

The genomic DNA was isolated and purified using the method described above. The sample's concentration was 217.6 ng/µl. The DNA quantification machine also measured the purity of the sample. This purity, reported as the ratio 260/280, measured protein contamination which in this case was 1.952. This is higher than 1.8 which means protein contamination is in the good range (Delventhal, 2022).

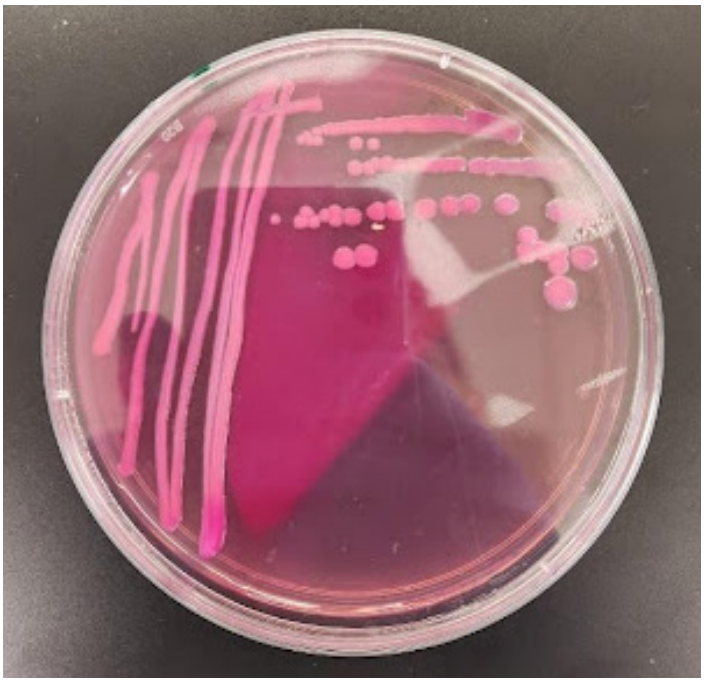

Figure 4. Restreaked bacteria on McConkey agar, the bacteria whose genomic DNA was isolated is in the red circle

Afterwards the PCR and gel electrophoresis was run. The gel was imaged and analyzed (Figure 5). The expected PCR sample length was 1,450 bp, so it was expected to have a thick band around 1,500 bp, which is shown in Figure 5 (Delventhal, 2022). This suggests that our sample has our expected product. The same results were found for the positive control, which had a known DNA template and was predicted to have PCR product around 1,500 bp. The Negative/no template control showed no amplification, as expected, as the sample had no DNA in it. This result suggests that we didn't have contamination in our sample.

The cleaned genomic DNA was sent for sequencing. Figure 6 shows an example of how the sequence looked in the ApE software used for analysis. The first 46 bp were deleted as they were not assigned nucleotide bases. At the 1012th base, the end was cut because the peaks became very small and overlapping. The final DNA sequence was 957 bp which is shown in Figure 7.

When the sequence was run through the Ribosomal Database Project by Michigan State University it categorized this gene sequence into the family *Enterobacteriaceae*. However, it was classified further as unknown. So, the sequence was blasted through the NCBI database. As shown in Figure 8, the results gave out one 100% identical sequence. This sequence corresponded to the *Citrobacter tructae* strain SNU WT2 chromosome.

Discussion

This set of experiments achieved its goal of determining the strain of one of the tetracycline-resistant bacteria that was isolated from a soil sample obtained from Lake Forest beach, IL. The sequenced DNA from 16S rRNA gene matched 100% with Citrobacter tructae strain SNU WT2 chromosome. The DNA of this strain was sequenced in a paper by Jung et al. (2021) about a novel Citrobacter species. The researchers first sequenced this gene from a kidney of diseased Rainbow Trout that was bred on a trout farm. Jung et al. (2021) conducted a gene analysis and discovered that this strain has many antibiotic resistance genes and virulence factors, both on its chromosome and plasmid. If a trout was infected with this bacterium, its kidney would become infected and this infection would consequently kill it. According to the authors, the strain was also not susceptible to any of the antibiotics they tested in the study. Jung et al. (2021) also mention that because of the ineffectiveness of the antibiotic treatment, this particular strain

may cause a problem to rainbow trout fisheries.

Our question, however, is how did a Citrobacter tructae get to a soil sample from Lake Forest beach. Antibiotic resistant bacteria are more commonly found around livestock because of increased antibiotic use with livestock (Nogrado et al., 2021). It is possible that the bacteria entered into Lake Michigan through water waste, or that it may have originated in Lake Michigan itself. Additionally, there are Rainbow Trout living in Lake Michigan so it is possible that they may be carrying that antibiotic resistant bacteria (Rainbow Trout, n.d.). Either way this illustrates the danger of antibiotic overuse and how quickly novel mutated bacteria can spread into the environment. A strain that may have originated in a fishery that uses a lot of antibiotics can now be found in the lakes surrounding soil. Looking at Figure 3, the bacterium was one of the largest on the 3 µg/ml of Tetracycline McConkey agar plate, which also suggests higher antibiotic resistance.

During the procedure there were a few limitations. For example, looking at the restreaked plate in Figure 4, we can see both horizontal and vertical growth which is unusual. This may have been due to two different bacteria being accidentally restreaked on one plate. Additionally, during gDNA extraction, the column was not centrifuged at maximum speed which may have lowered overall purity of the sample. Even though our sample was within an acceptable range, in the future this can be a way to improve the results. Finally, another source of error could have included improper pipetting as the amounts were very small.

For future studies, there are a variety of ways forward. As the paper by Jung et al. (2021) suggested, Citrobacter tructae may become a problem for fisheries as they do not have an effective antibiotic to treat diseased Rainbow Trouts. Therefore, one future study would be trying a wider spectrum of antibiotics in order to figure out an effective treatment for this kidney disease. Another future study regards the ecological impact this strain may have. Because this bacteria was found on Lake Forest beach, this means it is not isolated to fisheries only but is in the wild as well. Therefore this raises the question, are Rainbow Trout in Lake Michigan infected with *Citrobacter tructae*? Sampling Rainbow Trout from Lake Michigan and testing whether they have been infected would be a way to see if this bacterium may have a larger scale impact on fish populations.

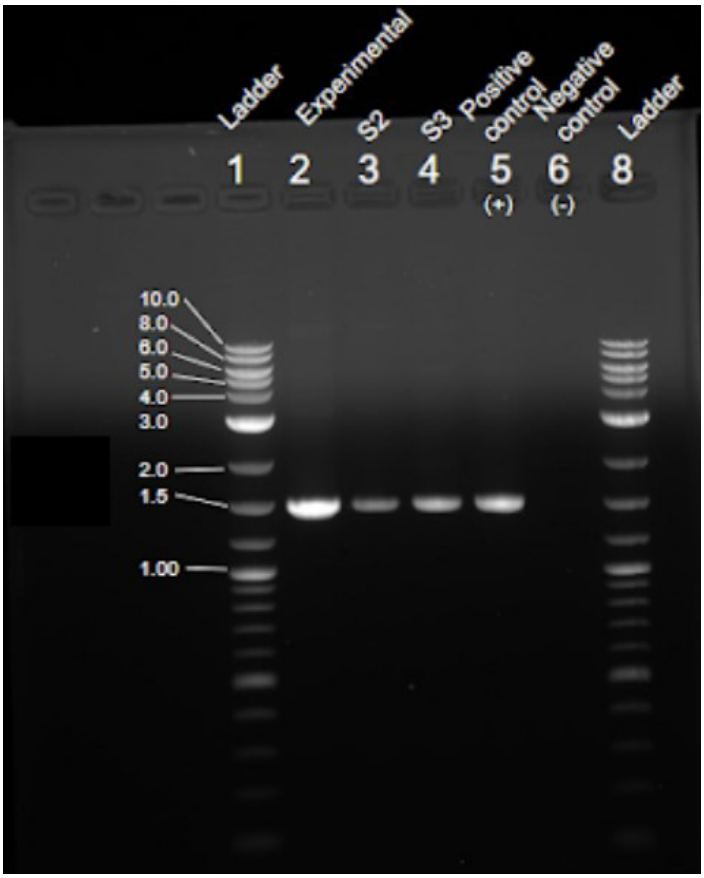

Figure 5. Imaged gel electrophoresis. Well 1 is DNA ladder, well 2 is the PCR product, well 3 and 4 are other classmates' samples, well 5 is positive control, well 6 is negative/no template control, S2 and S3 are other students' experimental PCR products

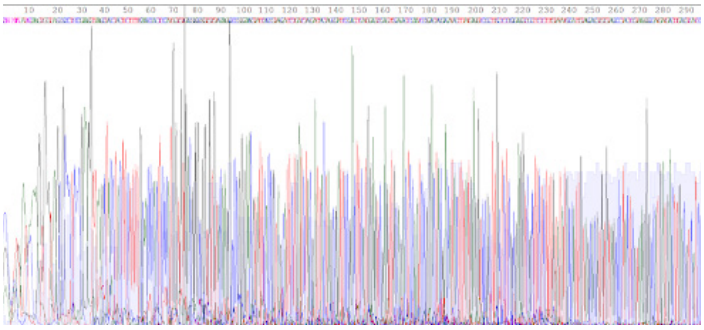

Figure 6. Sequenced DNA of PCR product (amplification of 16S rRNA gene) in APE plasmid editor

ACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTA-
CAAGGCCCGGGAACGTATTCACCGTAGCATTCTGATCTACGAT-
TACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATC-
CGGACTACGACATACTTTATGAGGTCCGCTTGCTCTCGCGAGGTC-
GCTTCTCTTTGTATATGCCATTGTAGCACGTGTGTAGCCCTACTC-
GTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTC-
CAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGAAC-
CGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACT-
TAACCCAACATTTCACAACACGAGCTGACGACAGCCATG-
CAGCACCTGTCTCACGGTTCCCGAAGGCACCAATTCATCTCT-
GAAAAGTTCCGTGGATGTCAAGAGTAGGTAAGGTTCTTCGC-
GTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGG-
CCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTC-
CCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACTCCT-
CAAGGGAACAACCTCCAAGTCGACATCGTTTACGGCGTGGAC-
TACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCT-
GAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTAT-
TCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTAC-
CCCCCTCTACAAGACTCTAGCCTGCCAGTTTCGGATGCAGTTC-
CCAGGTTGAGCCCGGGGATTTCACATCCGACTTGACAGACCG-
CCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCAC-
CCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGT-
GCTTCTTCTGCGAGTAACGTCAATTGCTGCGG

Figure 7. Edited sequence of amplified 16S rRNA gene of unknown tetracycline resistant bacterium isolated from soil sample

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Citrobacter tructae strain SNU WT2 chromosome, complete genome	Citrobacter tructae	1786	14061	100%	0.0	100.00%	4840504	CP038469.1
Citrobacter sp. UIWRF0159 16S ribosomal RNA gene, partial sequence	Citrobacter sp. UIWRF0159	1777	1777	100%	0.0	99.79%	1364	KR189482.1
Citrobacter tructae strain SNU WT2 16S ribosomal RNA gene, partial sequence	Citrobacter tructae	1753	1753	100%	0.0	99.38%	1474	MN083886.1
Citrobacter freundii strain SL151, complete genome	Citrobacter freundii	1748	15442	100%	0.0	99.28%	5096586	CP016952.1
Citrobacter sp. UIWRF0906 16S ribosomal RNA gene, partial sequence	Citrobacter sp. UIWRF0906	1742	1742	100%	0.0	99.17%	1320	KR190248.1
Citrobacter freundii strain HVUzoo ww1 16S ribosomal RNA gene, partial sequence	Citrobacter freundii	1742	1742	100%	0.0	99.17%	1435	ON202906.1
Citrobacter braakii strain GX-GL-S6-T-2-2021 16S ribosomal RNA gene, partial sequence	Citrobacter braakii	1742	1742	100%	0.0	99.17%	1441	ON202730.1
Citrobacter sp. S_T_MRS_12 16S ribosomal RNA gene, partial sequence	Citrobacter sp. S_T_MRS_12	1742	1742	100%	0.0	99.17%	1430	JX860618.1
Citrobacter sp. RHBSTW-01013 chromosome, complete genome	Citrobacter sp. RHBSTW-0...	1742	13784	100%	0.0	99.17%	5217381	CP056185.1
Citrobacter sp. 40 16S ribosomal RNA gene, partial sequence	Citrobacter sp. 40	1742	1742	100%	0.0	99.17%	1506	HQ399664.1
Bacterium EMB231-3 partial 16S rRNA gene, strain EMB231-3	bacterium EMB231-3	1740	1740	99%	0.0	99.38%	1362	HG792118.1
Citrobacter freundii strain Upstream_1 chromosome, complete genome	Citrobacter freundii	1738	13910	99%	0.0	99.37%	4839493	CP038856.1
Citrobacter werkmanii isolate MCYG-HGIT-02535 genome assembly, chromosome: 1	Citrobacter werkmanii	1738	13882	99%	0.0	99.37%	4865099	LR699014.1

Figure 8. NCBI BLAST of the PCR product edited sequence, this sequence had an 100% perfect identity with *Citrobacter tructae*

References

Delventhal, B. (2022). BIOL221X 01 22F: Molecules, Genes, and Cells lab manual. Lake Forest College, Lake Forest, IL.

Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A., and Olsen, G. J. (2008). Critical Evaluation of Two Primers Commonly Used for Amplification of Bacterial 16S rRNA Genes. *Applied and Environmental Microbiology,* 74(8), 2461–2470.

Janda, J. M., and Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. *Journal of Clinical Microbiology*, 45(9), 2761–2764.
Jung, W. J., Kim, H. J., Giri, S. S., Kim, S. G., Kim, S. W., Kang, J. W., Kwon, J., Lee, S. B., Oh, W. T., Jun, J. W., and Park, S. C. (2021). Citrobacter tructae sp. nov. Isolated from Kidney of Diseased Rainbow Trout (Oncorhynchus mykiss). *Microorganisms*, 9(2), 275.
Nogrado, K., Unno, T., Hur, H.G., and Lee, J.H. (2021). Tetracycline-resistant bacteria and ribosomal protection protein genes in soils from selected agricultural fields and livestock farms. *Applied Biological Chemistry,* 64(1).
Rainbow Trout. (n.d.). Www.michigan.gov. Retrieved November 23, 2022.
Urgacova, P. (2022). BIOL 221: Antibiotic Resistance in Bacteria Colony Isolated from Soil Sample from Lake Forest Beach, IL.
Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, 173(2), 697–703.