Classification and Antibiotic Properties of Chromobacterium

Honors Thesis

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Abstract:

Antibiotic resistance is an increasing problem throughout the world. The increased use of antimicrobial and antibacterial products on a day-to-day basis has allowed for more prevalent growth of resistant organisms. It has become necessary to find new antibiotics to deal with these more resistant bacteria. The source of most antibiotics is other microorganisms including bacteria and fungi that compete with these organisms in natural environments with many of our current antibiotics originating from soil microorganisms. In order to increase the chance of finding new microorganisms, soil samples should be taken from unique environments. Soil samples were previously isolated from fertile tropical rainforest soil of Volcanoes National Park on the big island of Hawaii. An antibiotic-producing organism of the Genus Chromobacterium was isolated. This research project will focus on the extensive characterization of this organism through fatty acid methyl ester (FAME) analysis, biochemical characterization, and a thorough sequencing of multiple housekeeping genes.

Introduction:

Since their discovery, antibiotics have been used to heal millions of people. However, there is a serious threat posed to the medical world today, and that threat is known as antibiotic resistance. While the extensive use of antibiotics has saved many lives, it has also allowed targeted bacteria to adapt and become resistant to the drugs that kill them. Antibiotics are very

rare, and it is difficult to create drugs that not only kill harmful bacteria, but pose no threat to the human body as well (Spellberg, et al., 2008). Furthermore, there is an extreme lack of funding for the discovery of new antibiotics, for pharmaceutical companies do not see the appeal in investing in treatments that are used for a week or two then discontinued (Spellberg, et al., 2008).

The Chromobacterium genus is from the family Chromobacteriace, in the class Betaproteobacteria, under the phylum Proteobacteria. This genus was discovered over a century ago by a man named Bergonzini (Mingxiong et al., 2016). For a long period of time, the well-characterized species Chromobacterium *violaceum* was thought to be the only species of the genus. However, since 2007, nine more species of this genus have been identified (Bajaj et al., 2016). Therefore, in comparison to other genera, this genus is relatively small. When viewed under a microscope, Chromobacterium are known to be gram-negative, rod shaped organisms. One can find this organism in a variety of aquatic and soil environments (Bajaj et al., 2016). Chromobacterium *violaceum* produces a characteristic violet pigment when streaked onto an agar plate. (Bajaj et al., 2016). A large amount of literature shows that this species produces a variety of antibiotics. More studies need to be performed on other species of Chromobacterium to confirm antibiotic activity, because C. *violaceum* is currently one of the only species studied and confirmed for this ability (Durán and Menck, 2001).

The organism used for this Honors Thesis was obtained from a lava tube on March 18, 2018 in Volcanoes National Park on the big island of Hawaii. Once collected, the organism was streaked onto a tryptic soy agar plate and incubated at room temperature. When streaked onto agar, this organism presents as irregular, cream colored colonies. The primary goal of the researchers who collected this organism was to test for any antibiotic producing properties. Therefore, they screened the organism against various test organisms to see if any growth

inhibition occurred. It was found that this organism was able to inhibit the growth of S. *aureus* and E. *aerogenes.* After this discovery, the researchers were prompted to use 16s ribosomal RNA sequencing to try to classify the organism. While this type of sequencing will not narrow down a specific species, it is beneficial in genus classification. The results of this initial sequencing test was as follows: A 96.58% match to the species Chromobacterium *alkanivorans*, a 96.17% match to Chromobacterium *aquaticum*, a 96.01% match to Chromobacterium *rhizoryzae,* a 95.9% match to Chromobacterium *haemolyticum*, and a 94.13% match to Chromobacterium *sphangi*. While these percentages might seem like high probability matches to these organisms, in this type of sequencing, any matches under 97% have the possibility to be novel species.

Research on the background of possible species that this unknown species could be has been done in the order of the highest matches from ribosomal RNA sequencing. Chromobacterium *alkanivorans* is a motile bacterium that presents as circular, yellowish-cream colored colonies when streaked onto agar. A unique feature of the species is its ability to breakdown chlorinated pesticides, as discovered from a study done in 2016 (Bajaj et al., 2016). Chromobacterium *aquaticum* is an aerobic bacterium that is also motile due to its possession of a single, polar flagellum. It presents as smooth, tan colored colonies when streaked onto agar (Young et al., 2008). Biochemical testing has proven that this organism is hemolytic, meaning that it is able to lyse red blood cells when streaked onto sheep blood agar. Furthermore, this organism has tested positive for the production of enzymes lecithinase and lectin, and it tests negative for the enzyme catalase (Young et al., 2008). Chromobacterim *rhizoryzae* presents as tan, smooth colonies when streaked onto agar, and it also has poly-beta-hydroxybutyrate granules located intracellularly. This organism is hemolytic, and has tested positive for the enzymes lipase, lecithinase, and catalase (Mingxiong et al., 2016). Lastly, Chromobacterium

haemolyticum presents as round, grey colonies. This organism is hemolytic and has tested positive for the enzymes catalase and oxidase (Okada et al., 2013).

A three-part approach was used to characterize this organism. I used biochemical testing, multi-locus sequencing analysis, and fatty acid methyl ester analysis to further classify our unknown Chromobacterium. I also performed additional antimicrobial screenings on this organism. These screenings exhibited that our organism was able to inhibit growth against a few gram-positive organisms and gram-negative E. *coli*. Multi-locus sequencing showed that our organism was most closely related to Chromobacterium *haemolyticum* and Chromobacterium *rhizoryzae.* Furthermore, a Basic Local Alignment Search Tool (BLAST) analysis from the organism's 16s rRNA sequence indicated a 99.11% identity match to Chromobacterium *rhizoryzae*. Several inconsistencies with biochemical testing and gene assessment call for more research to be done in classifying this organism.

Materials and Methods:

Unknown Chromobacterium sample:

The organism was taken from a lava tube on March 18, 2018 in Volcanoes National Park in Hawaii. Once collected, the organism was quadrant streaked onto a tryptic soy agar plate and incubated at room temperature.

Primer design:

Pathosystems Resource Integration Center (PATRIC) was used as the database for which the sequences of housekeeping genes *recA*, *gapA*, *rpoA*, *topA*, and *ftsZ* were acquired. These sequences were taken from C. *aquaticum*, C. *haemolyticum*, C. *violaceum*, C. *vaccini*, C. *rhizoryzae*, and C. *amazonense*. Clustal Omega is a multiple sequence alignment program that

was used to align these housekeeping genes according to similarity. Primers for PCR were designed from this alignment data.

Polymerase Chain Reaction (PCR):

Once primers for our unknown Chromobacterium species arrived in the mail, they were diluted to 10 µM. A master mix of 2x Taq mix, diluted primer mix, water, and 1 part Chromobacterium colony were made for PCR. The sequences of the primers used are:

Gene	**Forward reaction sequence**	**Reverse reaction sequence**
topA	5'-GAA ATC CTC GGC AAA TAC CA-3'	5'- ACC GTG CCC ATG TCT ATC TC-3'
recA	5'-TGA GCG ACA ACC AGA TCA AC-3'	5'-ATC ACT TCC TCG CCC TTC TT-3'
rpoA	5'-AGT GAC TAT CGC TGG CGT TT-3'	5'-TCT CAG CTT TCA GGC AGT TG-3'
gapA	5'-AGT CGG TAT CAA CGG TTT CG-3'	5'-CAT GCC GGT CAG CTT CTT-3'
ftsZ	5'-CCG AAG TGG CCA AGG AAA TG-3'	5'-AAC TTG ATC TGC GCC TCT TC-3'

After PCR, gel electrophoresis was used to confirm the success of the PCR reaction. 100 mL of 10x TAE buffer was mixed with 1 gram of agarose to form the gel. 10µL of methyl red was used with the gel and 10µL of HyperLadder 1kb was loaded into the first well for a molecular marker. 2µL of 5x DNA loading buffer was mixed with 8 µL of primer for gel loading. Gel electrophoresis was run for 1 hour at 100 V.

Multi-locus sequencing analysis:

Samples were sent to Genewiz Global Headquarters in South Plainfield, NJ to be sequenced. The sequences from our unknown organism were aligned with housekeeping genes *recA* and *gapA* from C. *aquaticum*, C. *haemolyticum*, C. *violaceum*, C. *vaccini*, C. *rhizoryzae*, and C. *amazonense* using Clustal Omega.

Modified egg yolk agar test:

A recipe taken from Hardy Diagnostics ("Egg Yolk Agar, Modified") was used for the modified egg yolk agar test. 10 g of TSA, 1.25 g of yeast, 25 mL of egg, and 5 g of agar were autoclaved and poured to produce the modified egg yolk agar. B. *cereus* was used as a positive control for lecithinase, S. *aureus* was used as a positive control for lipase, and our unknown organism was streaked onto the agar as well. All of the plates were incubated at 30 degrees Celsius for two days.

Crude organic extract for antibiotic screening:

Our unknown Chromobacterium sample was heavily streaked onto three TSA plates. After incubation at 30 degrees Celsius for four days, the plates were divided into small squares and put into vials. Dry ice with ethanol was used to freeze the vials for thirty minutes. 20 mL of ethyl acetate and 10 mL of water were added. The vials were then shaken for an hour each and put back in the freezer overnight. The next day, liquid from the vials was put into smaller scintillation vials. The organic phase was separated from the aqueous phase, and the organic phase was put under the hood to evaporate. Twenty-four hours later, the organic phase was resuspended with 500 µL of ethyl acetate. 10 µL of the organic extract was put on one half of a petri dish while 10 µL of ethyl acetate was used as a control. Test organisms P. *aeruginosa*, P. *mirabilis,* E.*coli*, S. *aureus*, B. *cereus*, B. *subtilis*, E. *aerogenes*, and S. *marcescens* were suspended in liquid TSA and poured over the extract and control. The plates were incubated at 30 degrees Celsius for a day.

Results:

A crude organic extract of our organism exhibited weak inhibition of growth on gram-positive organisms such as S. *aureus* and B. *cereus* and the gram-negative organism E. *coli.* The most growth inhibition was seen on B. *subtilis*. This organism did not impede growth on P. *mirabilis*, E. *aerogenes,* or P. *aeruginosa.* The negative result against E. *aerogenes* conflicts with earlier testing that showed growth inhibition.

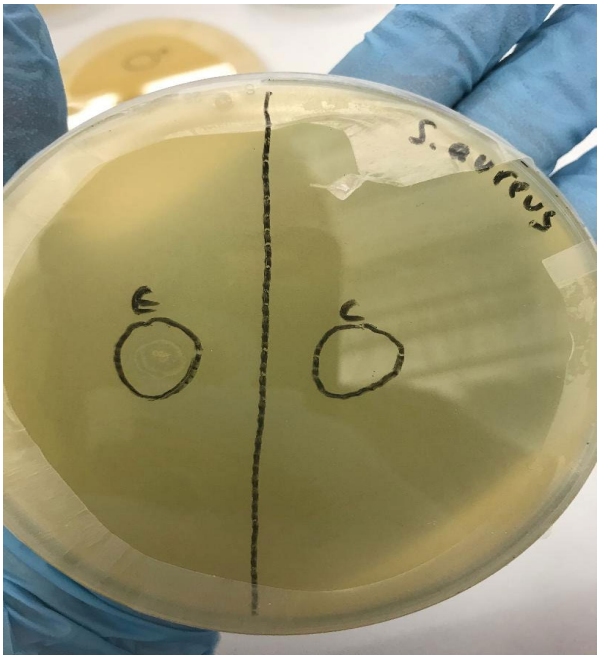

Figure 1: Organic extract tested against S. aureus. *Weak inhibition of growth can be seen against the test organism S. aureus compared to the ethyl acetate control*

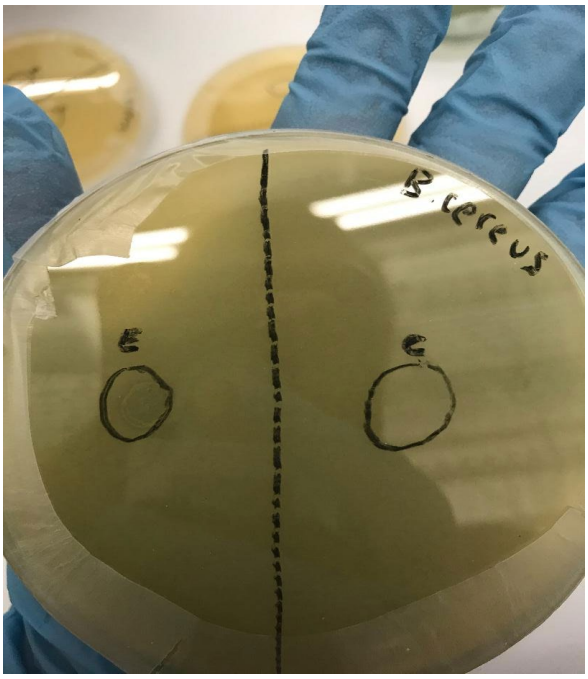

Figure 2: Organic extract tested against B. cereus. *Weak inhibition of growth can be seen against the test organism B. cereus compared to the ethyl acetate control.*

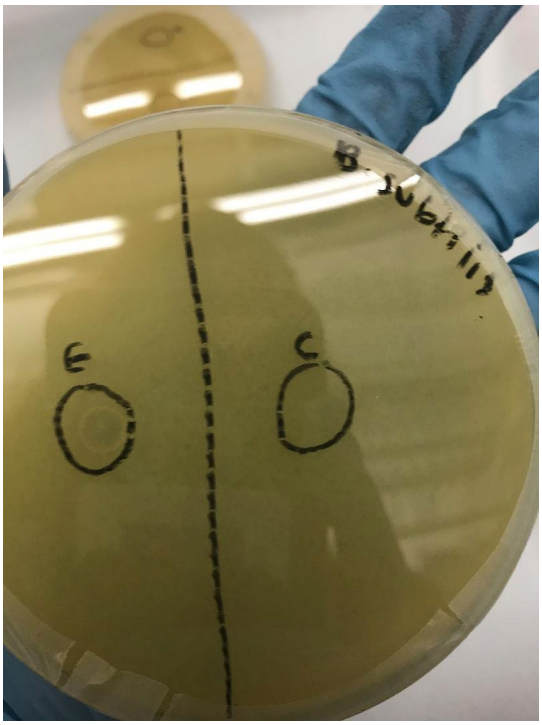

Figure 3: Organic extract tested against B. subtilis. *Inhibition of growth can be seen against the test organism B. subtilis compared to the ethyl acetate control.*

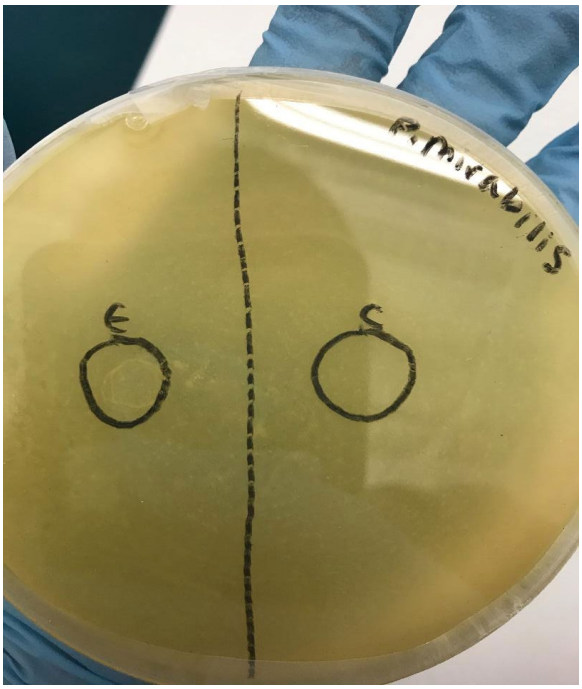

Figure 4: Organic extract tested against P. mirabilis. *No inhibition of growth can be seen against the test organism P. mirabilis compared with the ethyl acetate control*

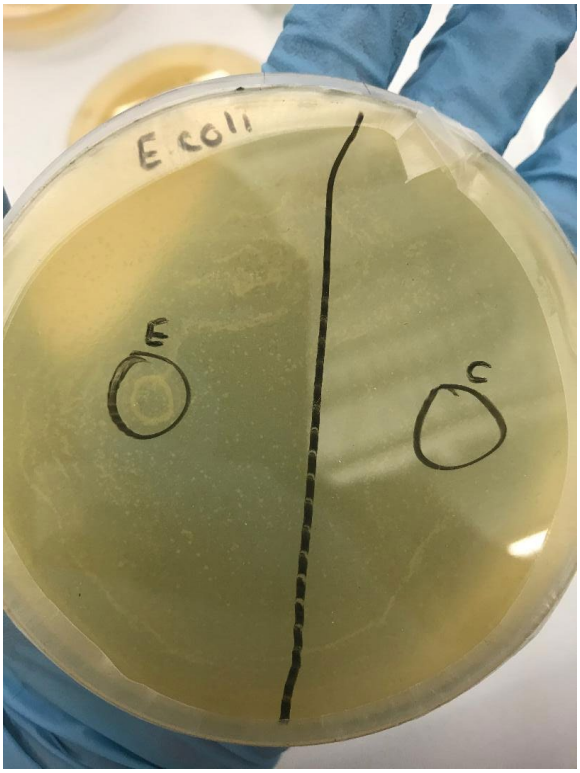

Figure 5: Organic extract tested against E. coli*. Inhibition of growth can be seen against the test organism E. coli compared to the ethyl acetate control.*

Multi-locus sequencing analysis using the housekeeping genes *ftsZ* and *recA* showed that our organism is most closely aligned with C. *haemolyticum*. A phylogenetic tree was generated by the multiple sequence alignment program Clustal Omega and showed our unknown organism as being distantly related to other Chromobacterium species. A percent identity matrix was also generated by this program and showed that our organism had a 97.38% percent identity match to C. *haemolyticum* and a 97.18% identity match to C. *rhizoryzae.* The 16s ribosomal RNA sequence for our organism was entered into BLAST and a 99.11% percent identification to C. *rhizoryzae* was found. C. *haemolyticum* was close behind with a 98.88% match.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident
Chromobacterium rhizoryzae strain LAM1188 16S ribosomal RNA, partial sequence	1609	1609	100%	0.0	99.11%
Chromobacterium haemolyticum strain MDA0585 16S ribosomal RNA, partial sequence	1598	1598	100%	0.0	98.88%
Chromobacterium alkanivorans strain IITR-71 16S ribosomal RNA, partial sequence	1592	1592	100%	0.0	98.77%
Chromobacterium aquaticum strain CC-SEYA-1 16S ribosomal RNA, partial sequence	1565	1565	100%	0.0	98.21%
Chromobacterium piscinae strain LMG 3947 16S ribosomal RNA, partial sequence	1530	1530	99%	0.0	97.54%

Figure 6: BLAST results from 16s rRNA sequence for Chromobacterium. *C. rhizoryzae came up as the highest percent identification at 99.11%. C. haemolyticum was a 98.88% match and C. alkanivorans showed a 98.77% match.*

```
#  Percent Identity  Matrix - created by Clustal2.1
#
#

     1: DNE            100.00   93.66   93.76   96.07   97.38   97.18   95.87
     2: C_violaceum     93.66  100.00   96.48   94.26   93.76   94.06   96.17
     3: C_amazonense    93.76   96.48  100.00   93.25   93.55   94.06   95.47
     4: C_aquaticum     96.07   94.26   93.25  100.00   95.77   95.67   95.27
     5: C_haemolyticum  97.38   93.76   93.55   95.77  100.00   98.39   96.07
     6: C_rhizoryzae    97.18   94.06   94.06   95.67   98.39  100.00   95.57
     7: C_vaccinii      95.87   96.17   95.47   95.27   96.07   95.57  100.00
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Figure 7: Percent Identity Matrix from Clustal Omega for unknown Chromobacterium species. *This matrix showed C. haemolyticum as having a 97.38% identity match to our unknown organism and C. rhizoryzae at a 97.18% match.*

Figure 8: Phylogenetic tree generated by Clustal Omega for unknown Chromobacterium species. *The tree shows the unknown organism as being more distantly related to other Chromobacterium species based on preliminary analysis.*

In the spring of 2019, an API strip was performed on our organism. API strips allow for a multitude of biochemical tests to be performed in a single setting. The results from this test showed that this organism is known to have a negative result for the enzymes citrate, indole, urease, and sucrose. A positive result was found for the enzyme oxidase. Results from this test also showed that this organism does not fully match any of the recommended species from the previous ribosomal RNA sequencing analysis. When the results were entered into a database, a species identification was not able to be obtained. Furthermore, the database revealed that our organism only had a 66.1% probability of being Chromobacterium *violaceum.* As stated before, C. *violaceum* produces a violet pigment when streaked, and our organism presents as cream-colored colonies.

Figure 9: API strip. *Our unknown organism tests negative for citrate, indole, urease, and sucrose. It tests positive for oxidase.*

A modified egg yolk agar test was performed on our unknown Chromobacterium species. B. *cereus* was used as a positive control for lecinthinase production. S. *aureus* was used as a positive control for lipase production. It was found that our organism did not produce lecinthinase but did produce lipase. It also showed proteolytic activity.

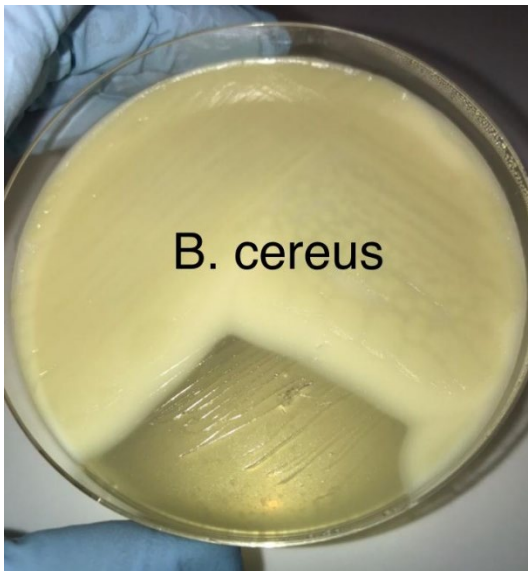

Figure 10: B. cereus was used as a positive control for lecithinase production on modified egg yolk agar. *The white opaque zone on the edge of the colony is indicative of lecithinase production.*

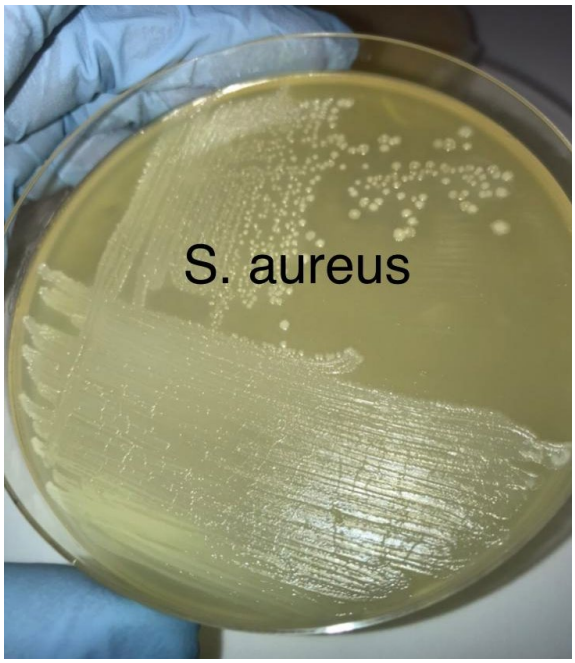

Figure 11: S. aureus was used as a positive control for lipase production on modified egg yolk agar. *The iridescent sheen (like oil in water) on the colony is indicative of lipase production.*

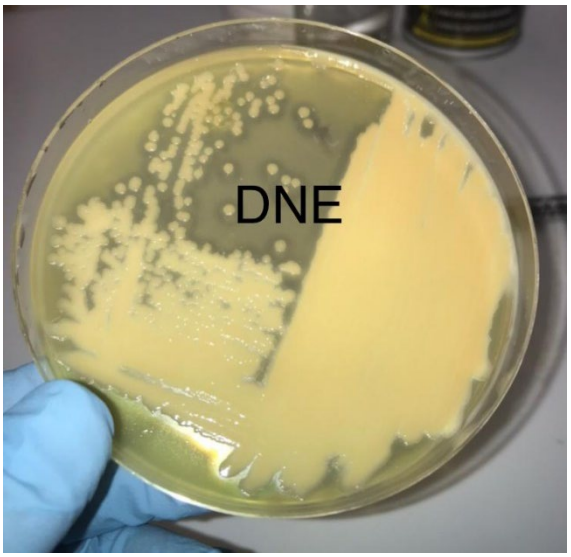

Figure 12: Modified egg yolk agar test for our unknown organism. *The iridescent sheen (like oil in water) on the colony is indicative of lipase production. The zones of inhibition around the colonies shows proteolytic activity. There is no white opaque zone that would indicate lecithinase production.*

Discussion:

Using an organic extraction technique, we tested the antibiotic properties of our unknown organism against a mix of gram-positive and gram-negative organisms. Earlier tests showed our organism inhibiting growth of S. *aureus* and E. *aerogenes*. We observed clear zones around B. *subtilis*, E. *coli*, S. *aureus*, and B. *cereus* compared to the ethyl acetate control. Growth inhibition was especially pronounced in B. *subtilis*, but only a small amount could be seen for the other organisms. Our unknown organism hindered more growth in gram-positive organisms compared to gram-negative organisms. While E. *aerogenes* was previously found to be an organism that our Chromobacterium could inhibit, this was not the case in our tests. This could be due to issues with the crude organic extract itself. These findings are important because they point towards the possibility of a novel species. As stated before, C. *violaceum* is currently the only Chromobacterium species studied enough to confirm antibiotic activity. We have found no indication from biochemical tests or multi-locus sequencing that our unknown species aligns with C. *violaceum*, nor does our organism present as a violet colony. Therefore, it is possible that our organism could be a new, antibiotic producing species.

Further classification of this organism was performed by using sequences of multiple housekeeping genes from our unknown organism and comparing them to known sequences of other Chromobacterium species. Chromobacterium *violaceum*, *haemolyticum*, *rhizoryzae, amazonense,* and *aquaticum* have their genomes fully sequenced in an accessible online library. C. *alkanivorans* does not have an entirely sequenced genome. For multi-locus sequencing analysis, I chose five genes that are considered standard housekeeping genes that I wanted to isolate from our organism. Housekeeping genes are widely found in bacteria species and are essential for basic metabolic function. For example, genes such as *recA*, *rpoA*, *gapA*, and *topA*

have been used in multiple studies for multi-locus sequencing of the Vibrio genus (Sawabe et al., 2007). After choosing *recA*, *rpoA, topA*, *gapA*, and *ftsZ* as the genes I wanted to isolate, I took these genes from previously sequenced Chromobacterium genomes, aligned the genes according to similarity, and designed primers for a polymerase chain reaction (PCR) based off the results. After utilizing PCR to amplify the genes I wanted to isolate, I was able to compare them to the genomes of multiple Chromobacterium species. Difficulties with *gapA*, *topA*, and *rpoA* during the primer design process and PCR left me with only *recA* and *ftsZ* sequences to compare to other species. This is important to note, because the comparison of only two housekeeping genes as opposed to five can just pose as preliminary testing.

Comparison of our organism's *recA* and *ftsZ* genes to C. *violaceum*, C. *haemolyticum*, C. *aquaticum*, C. *rhizoryzae*, C. *amazonense*, and C. *vaccini* showed a 97.38% identity match to C. *haemolyticum* and a 97.18% match to C. *rhizoryzae*. It is interesting to note that on the percent identity matrix, species such as C. *rhizoryzae* and C. *haemolyticum* have a 98.39% identity match to one another, but they are confirmed to be different species. Average nucleotide identity is used to measure genetic similarity, and a range of 95%-96% is generally used as a cutoff value for potential new species. Data has shown that ANI values can differ between genomes, and this value range is used for fully sequenced genomes (Kim et al., 2014). Our percent identity matrix was based off the alignment of just two housekeeping genes, not an entire genome. Furthermore, the phylogenetic tree data generated by Clustal Omega showed our organism as being more distantly related to other Chromobacterium species rather than similar. A BLAST analysis of the 16s rRNA gene sequence of our unknown organism showed a 99.11% similarity to C. *rhizoryzae*. While this percentage is quite high, too many inconsistencies with biochemical tests and colony presentation are present to confirm that our species is C. *rhizoryzae*. As seen below in

Figure 13, C. *haemolyticum* looks similar to our organism biochemically, but its oxidase test is negative when it is positive for our organism. C. *aquaticum* has a negative catalase test and positive lecithinase test, but our organism has a positive catalase test and negative lecinthinase test. Overall, *Figure 13* shows that our organism does not completely match any known Chromobacterium species, so the possibility for a novel species is present.

Organism	Colony	ADH	LDC	ODC	CIT	H2S	URE	IND	VP	GEL	GLU	MAN
C. *alkanivorans*	Circular yellow-cream	P	N	N	P	N	P	N	N	P	P	N
C. *aquaticum*	Smooth tan	P	N	N	P	N	N	N	N	P	P	N
C. *rhizoryzae*	Smooth tan	P	N	P	P	N	N	N	N	P	P	P
C. *haemolyticum*	Grey	P	N	N	P	N	N	N	N	P	P	P
Unknown	Cream	P	N	N	P	N	N	N	N	P	P	N

Organism	SOR	RHA	SAC	MEL	ARA	Catalase	Oxidase	Hemolysis	Lecithinase	Lipase
C. *alkanivorans*	P	N	N	N	N	P	N	N	N/a	N/a
C. *aquaticum*	N	N	N	N	N	N	P	P	P	P
C. *rhizoryzae*	N	N	N	N	P	P	N	P	P	P
C. *haemolyticum*	N	N	N	N	N	P	N	P	N/a	N/a
Unknown	N	N	N	N	N	P	P	P	N	P

Figure 13: Biochemical similarity between unknown organism and different Chromobacterium species. *Our organism does not align perfectly with any other Chromobacterium species.*

Biochemical testing is an old fashioned, but nevertheless efficient way to classify organisms. This type of testing was utilized in the time period before genomic sequencing was discovered in order to distinguish between different organisms. For this project, I used a modified egg yolk agar test to see if our unknown organism produces the enzyme lecithinase, lipase, or shows any proteolytic activity. B. *cereus* was used as the positive control for lecinthinase production. A positive result for this enzyme presents as having a white, opaque zone along the edge of the colony. S. *aureus* was used as the positive control for lipase production. Since lipase hydrolyzes fatty acids, producers of this enzyme will present with an

iridescent sheen over their colonies ("Egg Yolk Agar, Modified"). Our organism tested positive for lipase production, because a shiny gloss could be seen over the colonies. Although there were no white opaque zones for lecithinase production, clear zones around some of the colonies exhibited proteolytic activity. This biochemical test serves to further distinguish this organism from known species of Chromobacterium.

Along with multi-locus sequencing analysis and biochemical tests, fatty acid methyl ester analysis is being used in this project to further classify this organism. Samples of our organism have been sent out to be analyzed, so we do not have the results from this test yet. Fatty acid methyl ester analysis is a useful characterization technique, because fatty acid profiles taken from bacterial membranes are exclusive between different species of organisms (Ichihara et al., 2015). The company will be able to remove individual cells from our culture media and lyse these cells to liberate fatty acid from the cellular media. Once the fatty acids are freed, they will be methylated to form free fatty acid methyl esters. Then, these FAME's will be transferred from an aqueous phase to an organic phase, and the organic extract will be washed with aqueous solution. Chromatography will be performed on this organic extract, and the results of this test will be sent back to us (Ichihara et al., 2015). We will be able to compare our organism's fatty acid profile to different Chromobacterium species to discover any alignments.

Conclusion:

The purpose of this study was to classify an unknown Chromobacterium species using biochemical tests, multi-locus sequencing analysis, and FAME analysis. Antimicrobial screening was another major part of this project. Overall, we found that this organism does not align with a particular Chromobacterium species. Results from this study show a probability that our unknown organism could be a novel species due to inconsistencies in biochemical data and

antibiotic properties. A major limitation to this study is that C. *alkanivorans* is not fully sequenced, and we were not able to obtain the organism for comparison. Early 16s rRNA sequencing showed a 96% match to C. *alkanivorans*, so it would be beneficial to compare this organism's housekeeping genes to our unknown organism. Future research should focus on acquiring C. *alkanivorans* and performing multi-locus sequencing analysis. Furthermore, only *recA* and *ftsZ* genes were used in this project's MLSA, so further tests using over five housekeeping genes could provide more accurate results. More antibiotic tests could also be performed in order to fully understand the antimicrobial potential of this organism.

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