Biology Honors Thesis

TEMPERATURE STUDY AND GENOME ANALYSIS OF MARINE PSEUDOALTEROMONAS SP.

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TEMPERATURE STUDY AND GENOME ANALYSIS OF MARINE *PSEUDOALTEROMONAS* SP.

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I hereby declare I am the sole author of this thesis. It is the result of my own work and is not the outcome of work done in collaboration, nor has it been submitted elsewhere as coursework for this or another degree.

Signed:  Date: 4/26/2023

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Temperature study and genome analysis of marine *Pseudoalteromonas* sp.

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Abstract: Aluminum is a metal prized for its versatility. In the marine environment, aluminum is used in a variety of applications including ship building and oceanic equipment. However, constant seawater exposure corrodes aluminum and allows for biofouling, or the accumulation of organisms on the surface, which can damage the integrity of marine structures over time. To discourage this, man-made coatings are often applied, but serve as a source of environmental concern due to toxic chemical leach. Marine *Pseudoalteromonas* sp., isolated from an aluminum coupon submerged in the Pamlico River, is a bacterium thought to form uniquely protective biofilms which could prevent degradation and excrete anti-biofouling compounds. This property would make the bacteria a potential source for a natural aluminum-preserving coating *in situ*. To determine the optimal conditions for growth, the *Pseudoalteromonas* sp. was measured over twenty-four hours using an optical density plate reader at temperatures representative of a variety of marine environments; 22℃, 25℃, 30℃, and 35℃. DNA from the isolate was extracted and sequenced using fluorescence-based detection and PCR amplification to better understand the metabolism, anti-biofouling properties, and toxicity resistance unique to the isolate. The resulting DNA contigs were then analyzed into an annotated genome with Rapid Annotation using Subsystem Technology (RAST). When compared to isolates from the same source, the *Pseudoalteromonas* sp. displayed fewer iron-regulatory genes, more water-soluble nutrient metabolism genes, and a tendency to secrete antibacterial peptides (ABPs). Being able to

recognize the environments associated with *Pseudoalteromonas* sp., and understanding on a genomic level what sets it apart, we can begin to answer the questions posed for research in addition to facilitating methods for preserving and prolonging the existence of current and future marine structures in ways that are environmentally sound.

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Introduction

Aluminum is a uniquely versatile metal. It is malleable, corrosion resistant, and highly conductive with a low density, and its abundance makes it one of the most inexpensive metals available. Due to this, aluminum is used in a variety of contexts including the marine environment. Underwater, aluminum is used in ship hulls, oil rigs, and floorbed tubing, but constant exposure to seawater corrodes aluminum over time so, in the 1960s, magnesium and silicon were used to make a marine grade aluminum alloy that was resistant to degradation. However, according to Wahid's (2020) study, due to differences in the chemical and mechanical properties of the alloys, marine grade aluminum can pose a serious threat of injury to welders during fabrication. The vast differences in the chemical and mechanical properties exhibited by the alloys can cause radiation burns, thermal burns, and produce harmful fumes. Leaching of the alloys into marine environments is also of significant environmental concern. Compounding on, aluminum structures made prior to the invention of marine grade alloys and structures made without the intention of seawater exposure, like early oceanic equipment and plane wreckages, do not have resistance to corrosion from seawater and thus are slowly disappearing.

In addition to corrosion, most man-made structures submerged in water face a phenomenon known as biofouling. Biofouling describes the accumulation of microbes, plants, algae, or other fouling organisms on an artificial surface exposed to water. This accumulation of organic material is problematic as chronic accumulation can compromise both the integrity of marine structures and the performance of ocean-going vessels through increased drag. The fuel consumption of a vessel affected by biofouling has been estimated to increase by as much as 40% (Yebra, et al. 2004). Furthermore, the toxic metal coatings used to discourage biofouling on ships and various equipment serve as a source of harm to the ocean and its organisms. From the

1970s until 2003, when the International Maritime Organization banned it for collapsing local marine organism populations, tributyltin (TBT) coatings were used with great success to prevent biofouling (Munk, et al. 2009). In Munk's (2009) study, the modern chemical coatings, reformulated to contain minimally toxic biocides if any at all, were found to be less effective at preventing biofouling. Copper, recognized by the U.S. Environmental Protection Agency as "the first antimicrobial metal" (Montero, et al. 2019), has been implemented into the antibiofouling ship coatings with marked success in preventing buildup. However, copper pollution caused by leaching from these coatings contributes to ocean acidification and interrupts the life cycles of marine keystone species, such as kelp forests (Leal, et al. 2018).

Previous research conducted by members of Dr. Erin Field's lab working on a related project (Price, et al. 2020), isolated and identified two main microbes found on aluminum coupons submerged in the Pamlico River. The two microbes; *Bacillus* and *Pseudoalteromonas* sp., were hypothesized to have different interactions with the surface they grew on. The *Bacillus* sp. was thought to use the aluminum coupon as a habitat and possibly cause corrosion, while the *Pseudoalteromonas* sp. was thought to use the coupon as a point of attachment to create a biofilm to then collect nutrients from its environment and potentially protect the metal from corrosion. These hypotheses were supported by previous literature and studies of these organisms.

In a 2019 study (Supardy, et al. 2019), *Pseudoalteromonas* sp. displayed natural antifouling properties that significantly discouraged the growth of several species of fouling organisms. Analysis of the bacterium discovered several potential bioactive compounds that could contribute to the antimicrobial nature of the *Pseudoalteromonas* biofilms. While the species of *Pseudoalteromonas* studied by Supardy is not the exact same as the one isolated from

the Pamlico River that is the focus of this research, it sets an interesting precedent of what *Pseudoalteromonas* bacteria can achieve.

Following previous studies into the microbial communities associated with various aluminum alloys, there are many facets of research available to explore. In Price's preliminary work, the two microbes isolated and studied were *Bacillus* and *Pseudoalteromonas* species, and how temperature may affect how they grow in increasing amounts of aluminum.

I focused my research solely on the effects of temperature on the growth of the marine *Pseudoalteromonas* species isolated in Price's study, as well as sequencing and analyzing its genome. Depending on location, season, and depth, ocean temperatures can vary widely, and microbes found in the cold Arctic Ocean are not typically found in warm tropical waters. By researching the temperatures that the *Pseudoalteromonas* sp. can grow in, a better understanding is gained of geographically where the bacteria has potential for use. Through genome analysis, we can gain insight into the use of aluminum by the isolate and the possible antibiofouling nature used to outcompete other bacterial species.

Being able to recognize the environments associated with *Pseudoalteromonas* sp., and understanding on a genomic level what sets it apart, we can begin to answer the questions posed for research in addition to facilitating methods for preserving and prolonging the existence of current and future marine structures in ways that are environmentally sound.

Methods

To address the questions posed for research, there were two phases of experimentation and data collection: the temperature study and the genome analysis.

Temperature Growth Experiments

The first phase, temperature study, began with growing cultures of the *Pseudoalteromonas* sp.. In an Erlenmyer flask, 50mL of marine broth (Difco) was inoculated with 0.50mL of *Pseudoalteromonas* stock. The flask was then kept on a shaker for twenty-four hours at twenty-five degrees Celsius. To keep consistency and prevent error from over-culturing, the culture was used within an hour of the twenty-four hour cycle finishing. The *Pseudoalteromonas* culture used in this project was sourced from the stock isolated from the Pamlico River in Price's (2020) study.

In a 24-well plate, three wells acted as the experimental wells. In the experimental wells, 0.25mL (10% of the total volume) of isolate was pipetted into 2.25mL of marine broth. To act as controls, three wells contained only the isolate, and three contained only marine broth, 2.50mL in each well. The outside ring of wells on the plate acted as a physical buffer against evaporation from the absorbance plate reader, and contained 2.50mL of only deionized water. This configuration of the wells can be seen in Figure 1. In the controls, the isolate and marine broth was added to the wells using a pipette to be as accurate as possible. In the evaporation ring, water was added using a serological pipette as accuracy was less important.

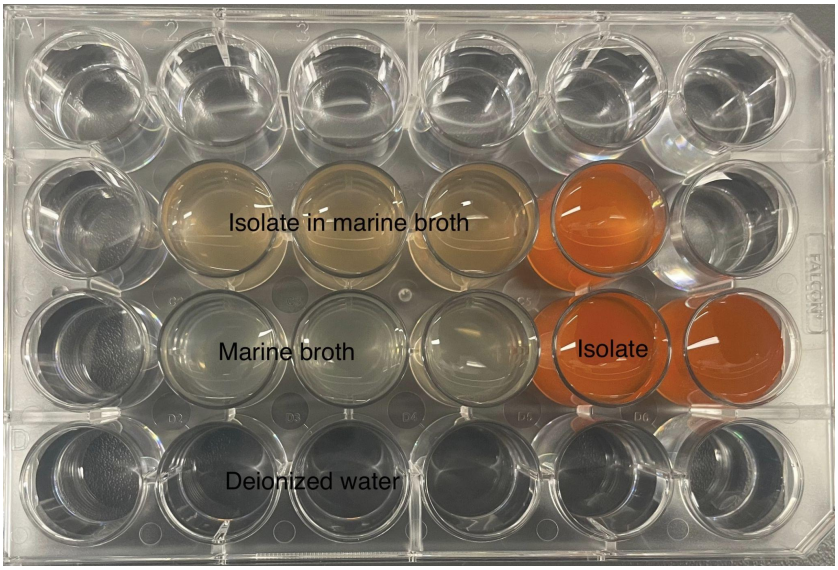

Figure 1: 24-well plate prior to reading. Contains evaporation ring (2.50mL deionized water), experimental wells (0.25mL isolate in 2.25mL marine broth), and control wells (2.50mL marine broth and 2.50mL isolate).

To quantify the cell density in each well, the optical density, at 600 nanometers, of the plates will be read every ten minutes for twenty-four hours by a plate reader. The plate reader will hold each 24-well plate at a temperature representative of a marine environment where the bacteria could potentially grow. The temperature 22℃, the lowest the plate reader heats, represented the cooler northern marine environments. 25℃ and 30℃ represented the average range of oceanic temperatures. 35℃ represented the warmer marine temperatures near the equator and during summer months. Each temperature was performed in triplicate.

Genome Analysis of *Pseudoalteromonas sp.*

For the second phase, genome analysis, DNA had to first be sequenced from the *Pseudoalteromonas*. DNA was previously sequenced by the Field lab using the Illumina MiniSeq and sequences were assembled into contigs. A contig is a set of overlapping DNA sequences

used to create a map that reconstructs the original DNA. The contig list was then uploaded into Rapid Annotation using Subsystem Technology (RAST), which produced an annotated genome that was further analyzed for genes. Special attention was paid to genes that potentially were responsible for the unique properties of the *Pseudoalteromonas* sp., such as antimicrobial genes and ones pertaining to the metabolism of the isolate.

Results

To compare the growth of isolate, the optical density, which positively correlates with cell growth, was averaged for each well type at the beginning and end of twenty-fours hours for all four temperatures. Error bars were created from the standard deviation calculated from each replicate and then averaged.

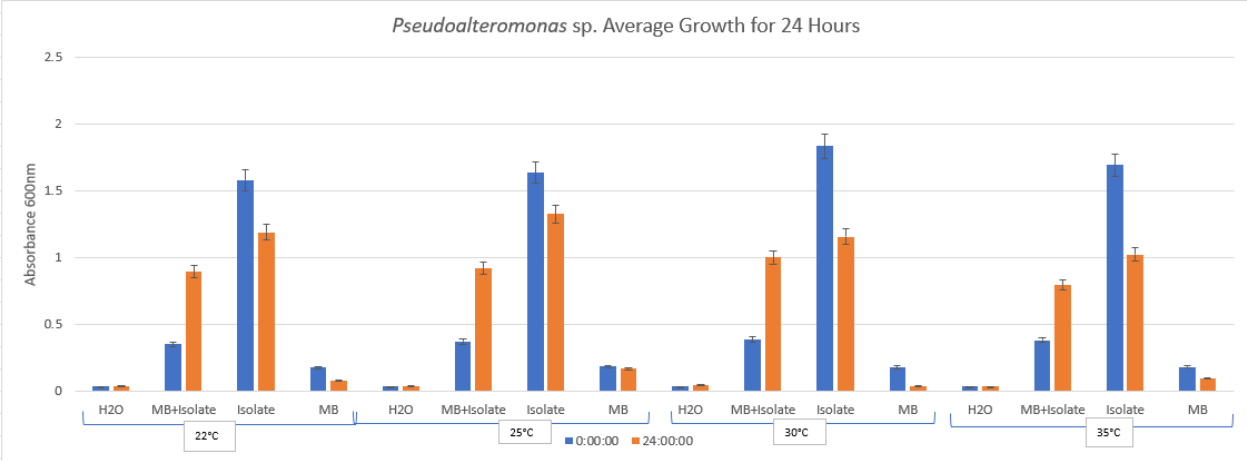

Figure 2: Average optical density for each well type at each temperature.

The highest optical density is seen on Figure 2 in the isolate-only wells. This is to be expected as these wells contained undiluted culture. However, because there was no growing medium in the isolate-only wells, the cells had no access to nutrients and were unable to continue growing, creating the sharp decline in density from start to finish.

OD 600									
22C Avg	H2O	MB+Isolate	Isolate	MB	**30C Avg**	H2O	MB+Isolate	Isolate	MB
0:00:00	0.03713	0.35511	1.58478	0.17789	0:00:00	0.03707	0.389332	1.83811	0.18122
24:00:00	0.03827	0.89556	1.19133	0.08411	24:00:00	0.04789	1.0043333	1.15722	0.04071
Avg Overall	0.00113	0.39004	-0.39344	-0.09378	Avg Overall	0.00022	0.6150013	-0.68089	-0.12644
25C Avg	H2O	MB+Isolate	Isolate	MB	**35C Avg**	H2O	MB+Isolate	Isolate	MB
0:00:00	0.03718	0.374889	1.63967	0.18778	0:00:00	0.03691	0.383889	1.69589	0.18211
24:00:00	0.03811	0.9213333	1.33	0.17211	24:00:00	0.03669	0.7966667	1.02522	0.09789
Avg Overall	0.00093	0.5464447	-0.30966	-0.01567	Avg Overall	-0.00082	0.412778	-0.67067	-0.08422

Table 1: Numerical average optical density and overall difference in optical density for each cell type at all four temperatures.

In Table 1, the necessity of the evaporation ring is demonstrated best by looking at the average overalls for the deionized water wells. At 22℃, 25℃, and 30℃, there is a small positive increase in the optical density of the water wells, but at the warmest temperature, 35℃, there is a small negative decrease in optical density, likely due to evaporation.

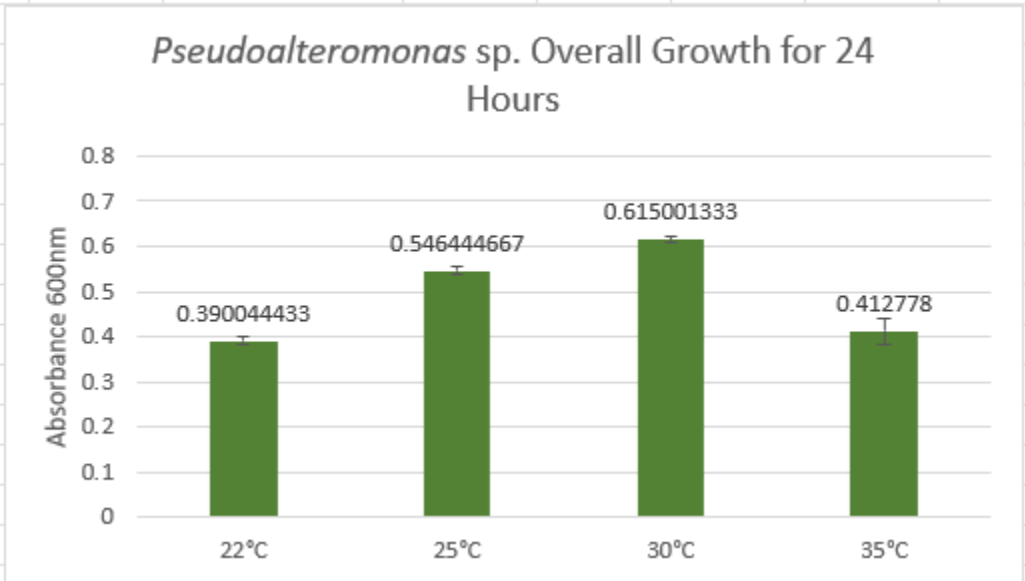

Table 2: Average difference in optical density from beginning to end for experimental wells at all four temperatures.

On Table 2, the largest average increase in optical density of the experimental wells was seen at 30℃ with an increase of 0.615, followed by 25℃ with 0.546, and the smallest at 35℃ and 22℃ with increases of 0.413 and 0.390 respectively. At 35℃, the largest error bars are seen, likely from the increased evaporation risk associated with higher temperatures.

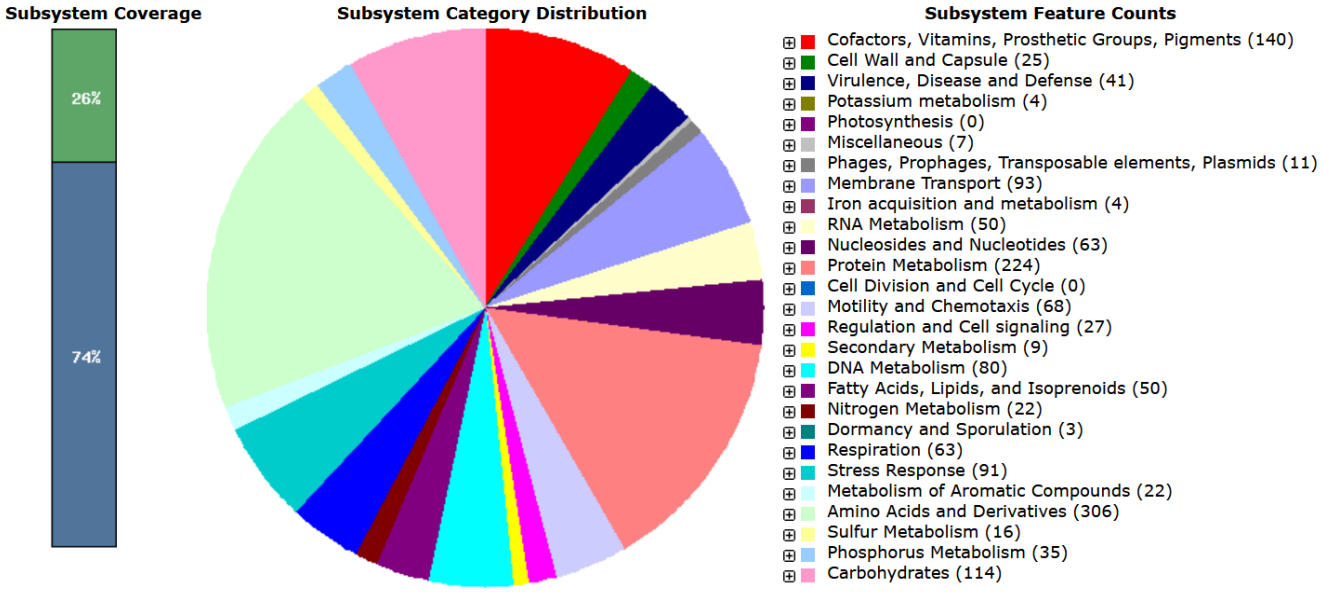

Figure 3: Distribution breakdown of genes in the *Pseudoalteromonas* sp. genome, provided by RAST.

Once the contig list from DNA sequencing was uploaded into RAST, an annotated genome was created, seen in Figure 2, that broke down the identifiable genes into several categories. The largest distribution of genes was amino acids and derivatives with 306 genes, followed by protein metabolism with 224 genes.

Taking a closer look, over seventy water-soluble nutrient metabolism genes are found; phosphorus metabolism (35), nitrogen metabolism (22), and sulfur metabolism (16). Only four iron

acquisition and metabolism genes were identified, along with thirteen total genes for tolerance to metals like copper, cobalt, zinc, and cadmium. One bacteriocin-producing gene also was detected.

RAST identified the genetic closest neighbor of the *Pseudoalteromonas* sp. to be *Pseudoalteromonas haloplanktis* TAC 125. *P. haloplanktis* is one of the most commonly studied bacteria adapted to cold-water environments, originally isolated from seawater off the coast of the Antarctic.

Discussion

As observed, an increase in cell growth confers with an increase in optical density. Given that the largest increase in optical density was seen at 30℃, I can conclude that the optimal growing temperature tested of the *Pseudoalteromonas* isolate is at 30℃.. Taking into account the growth seen at the other three temperatures tested, and how sharply bacterial growth tends to decrease once past the optimal temperature, the specific actual optimal growing temperature of the *Pseudoalteromonas* sp. is between 30℃ and 35℃. The decrease in growing ability seen at the highest and lowest temperatures tested indicate that the *Pseudoalteromonas* sp. would not grow well too close or too far from the equator, contrasting greatly with its Antarctic-based closest neighbor and suggesting that there are several species links missing between the *Pseudoalteromonas* isolate and *P. haloplanktis*.

Bacteriocins are ribosomally-synthesized antibacterial peptides (ABPs). They act upon a cell to affect various processes, such as protein synthesis, gene expression, and at the cellular envelope, to ultimately kill their target, which are often colonies of different bacterial species that are competing for the same resources. Bacteriocins are similar to antibiotics, but differ in that antibiotics are secondary metabolites while bacteriocins are produced on the ribosomal surface. The presence of a bacteriocin-producing gene in the isolate's genome supports and accounts for the idea that the *Pseudoalteromonas* sp. is antibiofouling in nature.

Looking at the metabolism of the *Pseudoalteromonas* sp., the lack of iron metabolizing genes is significant in its implications. Iron is a major impurity in aluminum, and is often added to aluminum alloys for strength. Having so few iron metabolizing genes, coupled with the abundance of water-soluble nutrient genes, lends credibility to the hypothesis that the *Pseudoalteromonas* isolate does not use aluminum as a source of nutrients but rather as a point

of attachment to then collect nutrients from the surrounding marine environment. As aluminum itself is a toxic to cellular life, the multiple-metallic resistance to toxic metals displayed in the isolate's genome give an idea to how the *Pseudoalteromonas* sp. tolerates the the presence of aluminum so closely.

An important caveat to this is that the entire genome of the *Pseudoalteromonas* sp. has not been categorized, and so a lack of iron and aluminum metabolizing genes does not necessarily mean that there are none present at all, just that there are none that have been identified.

The investigation into the optimal growing temperature and genome analysis of the *Pseudoalteromonas* sp. provides a basis for future experimentation regarding how temperature affects the ability of the isolate to grow in increasing salinity and aluminum concentration, as well as testing the potency of the bacteriocin. Additionally, given that growth does not completely cease at 22℃ and 35℃, it is possible that the isolate continues to grow in more extreme temperatures and is worth investigating. In Price's 2020 study, she noted that the *Pseudoalteromonas* sp. grew differently on different aluminum alloys. Taking that into account, testing the growth of the isolate and its ability to form biofilms on various marine grade aluminum alloys could be an interesting future direction.

By researching the environments of the microbes and microbial communities that form on submerged aluminum, a better understanding of how these microbes contribute to the integrity of these structures and how preservation of historical, modern, and ecological significance may be achieved through cooperation with microbes.

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