UNIVERSITY OF CALIFORNIA SAN DIEGO

Cold and Stressed: Adaptive Laboratory Evolution of Antarctic Surface Seawater Microbial Communities and Isolates Under High Hydrostatic Pressure

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by

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The thesis of Sixuan (Christine) Li is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

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Master of Science in Biology

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The majority of the deep ocean is an extreme environment characterized by low temperature and high pressure, which drives bacteria to evolve unique adaptations in the deep sea. Analyses of cold-adapted microbes, psychrophiles, have suggested that their adaptations, which increase fluidity in membrane composition are similar to the adaptations of high-pressure-adapted microbes, piezophiles. To determine the adaptation mechanics of Antarctic seawater microbes, I performed adaptive laboratory evolution experiments using microbes from Antarctic surface seawater samples, by gradually increasing the incubation pressure of the microbes. ALE experiments were carried out with both the original microbial communities and bacterial strain isolates from the samples, by gradually increasing the incubation pressure of the microbes. This

paper presents the evaluations of changes in community structures through the ALEs and characterizations of the strains isolated at high pressure.

In all the ALEs, I observed decreased growth rates at high pressure for both microbial communities and isolates, as well as decreased diversities within microbial communities at high pressure, suggesting that high pressure hinders microbial growth. However, further growth analysis of *Psychrobacter* isolate ALE at 50 MPa after 6 rounds of transfers, surviving bacteria were capable of growing better at a higher pressure compared to the ancestral strains. Genome resequencing of the *Psychrobacter* strains suggested that the bacteria could have possibly adapted to growing at a higher pressure through mutations of proteins coding for transmembrane transport, membrane proteins and peptidoglycan production. In conclusion, this thesis project presents primary analysis that shows psychrophilic bacteria were capable of adapting to higher pressure environments, which might suggest that bacteria adapted to both low temperature and high pressure environment, such as the deep sea, could have first evolved to become psychrophilic which in turn enabled them to adapt to high pressure.

INTRODUCTION

The majority of the ocean is an environment of high pressure and cold temperature. The average depth of the ocean is 3800 m with the deepest being the Challenger Deep of the Marianna Trench, which is deeper than 10,000 m (Gardner et al., 2014). The depth of 10,000 m corresponds to a pressure of higher than 100 MPa, which is 1000 times higher than atmospheric pressure (Yayanos, 1995). The deep sea also has a low temperature with an average of 2 °C (Raminez-Llodra et al., 2010). While the high pressure and cold environment of the deep sea is considered uninhabitable by humans, a diversity of microbes, termed "psychropiezophiles", meaning cold and pressure loving (psychro - cold, piezo - pressure, phile - loving), are able to live under the extremes of both cold temperature and high pressure (Sogin et al., 2006, Yayanos, 1995).

Microbes have evolved unique adaptations to survive in the cold and high pressure environment. Piezophiles are microbes that have optimal growth at a pressure higher than atmospheric pressure (Yayanos, 1995). High hydrostatic pressure can inhibit many cell functions including translation, transcription, protein synthesis and cell membrane fluidity (Bartlett and Bidle, 1999). Under high hydrostatic pressure, the cell membrane loses its fluidity due to the lipids being compressed which affects nutrient and particle import and export (Bartlett and Bidle, 1999). Piezophiles have been found to increase their membrane fluidity by modifying the fatty acid composition in their membranes to include more poly unsaturated fatty acids (PUFAs), which introduce kinks into the membrane structures and expand their volumes compared to the linear structures of saturated fatty acids (Bartlett, 1992).

Similar adaptation to counteract the effect of rigidity from environmental stress on cell membranes are also found in psychrophiles, which are microbes that have an optimal growth at 15 ℃ or below. In comparison, psychro-tolerant microbes are microbes that are able to grow at 15 ℃ or below but has an optimal growth temperature above 15 ℃ (D'Amico et al., 2006). Psychrophiles have to overcome challenges at cold temperature including decrease in membrane fluidity, enzyme activity, potential protein denaturation and intracellular ice formation (D'Amico et al., 2006). Low temperature makes the cell membrane rigid and therefore loses its fluidity. Similar to piezophiles, psychrophiles also increase membrane fluidity by increasing the production of PUFAs and shorter fatty acid chains (D'Amico et al., 2006). Given the similarity in their adaptations of the cell membranes, I ask the question - could microbes have first adapted to the cold temperature before evolving to adapt to high pressure?

Microbes in Antarctica surface seawater are ideal for studying this hypothesis, not only because of their psychrophilic and non-piezophilic properties, but also because microbes from the surface Antarctic seawater could naturally be travelling with the water current through brine rejection and Antarctic Bottom Water formation, eventually reaching the deepest part of the ocean and adapting to the high pressure environment in the deep sea (Speer et al., 2000). The thermohaline circulation of the ocean, which is the mixing of heat and salt of different masses of water in the ocean, incorporates the surface seawater into the deeper part of the ocean (Rahmstorf, 2006). This process could also contribute to the migration of microbes, adapting microbes in surface seawater to the deep and cold environment of the deep ocean. A significant part of the thermohaline circulation is the formation of Antarctic Bottom Water (AABW), which is mostly made up of brine rejection, salty and dense seawater rejected by ice formation during the annual freeze-thaw cycle (Speer et al., 2000). Brine rejection happens when the top layer

freezes to form sea ice, the denser saltier brine is rejected for the sea ice formation and sinks down the water column (Speer et al., 2000). Geophysical studies have shown that surface Antarctic seawater is incorporated into AABW which spreads northward along the ocean floor (Johnson, 2008). Previous studies on the composition of seawater at the bottom of the ocean suggests that AABW makes up 70% or more at some of the deepest part of the ocean such as the South Sandwich Trench, Kermadec-Tonga Trench and Marianna Trench, indicating that the particles and organisms originated from Antarctica surface seawater could be travelling to the deepest part of the ocean (Johnson, 2008).

One way to study adaptations of Antarctic microbes to high pressure is through Adaptive Laboratory Evolution experiments (ALE) which are used in microbiology to select for specific phenotypic adaptations to certain environmental stressors under a controlled laboratory environment (Dragosits and Mattanovich, 2013). ALE experiments are used as a tool to study the changes in phenotypic adaptation and allow researchers to evaluate changes in the genome through the adaptation process (Dragosits and Mattanovich, 2013). Richard Lenski and his team performed the famous long-term ALE on *E. coli* which observed adaptations and mutations to different environmental stressors (Lenski and Burnham, 2008). While most ALE has been done using well defined lab-isolated bacteria such as *E. coli*, similar experimental design can be used on other microbes, including microbes isolated from environmental samples and potentially microbial communities (Lenski and Burnham, 2008). Previous high pressure characterization have been performed using *E. coli* and a piezophile *Photobacterium profundum* SS9 to identify the growth characteristics of these strains under high pressure (Zobell and Cobet, 1962, Welch et al., 1993, Allen et al., 1999). Characterization at high pressure using *E.coli* found that at 62 MPa, cell morphology changed from a rod shape to filamentous; fatty acid analysis showed more

PUFAs and genetic mutations were found in genes associated with fatty acid synthesis suggesting mesophiles alter their membrane structures to adapt to high pressure (Zobell and Cobet, 1962). Previous experiments have also characterized the high pressure growth of *P. profundum* strain SS9, which is a psychrotolerant, piezophilic microbe which found a change in the membrane fatty acids proportion to include more unsaturated fatty acids (Allen et al., 1999). However, high pressure ALE has not previously been done on psychrotolerant or psychrophilic microbes that are not already piezophilic. Comparing ALE results of non-piezophilic psychrophiles to ALE of non-piezophilic mesophiles, bacteria not adapted to cold temperature, such as *E.coli*, can provide a better understanding of how psychrophiles adapt to high pressure compared to mesophiles.

Using high pressure ALE, I attempt to further our understanding of psychrophiles and piezophiles. While there are similarities between the adaptations of psychrophiles and piezophiles, there has not been any high pressure ALE that was performed using non-piezophilic psychrophiles to determine if psychrophilic adaptations in membrane structure could be advantageous for the microbes to adapt to high pressure. I also attempt to consider the effects of bacteria interactions by not only using isolated strains but also bacterial communities for ALEs - while a mixed bacterial community better mimics the microbial community in nature, all previous ALE experiments have been done using a single microbial strain that does not account for microbial interactions that might contribute or restrict the adaptations of microbes. Therefore, the objective of my thesis is two-fold: 1) To evaluate if psychrophiles could have an advantage over mesophiles at adapting to high pressure given that their membrane composition that resembles piezophiles, and 2) To determine if ALE conducted with a mixed bacterial culture would yield different outcomes compared to ALE conducted using only single strains.

Using non-piezophilic psychrophilic bacteria from Antarctica surface seawater samples, I gradually increased the incubation pressure and to slowly adapt the bacteria to higher and higher pressure. In doing so, I was able to characterize the growth and adaptation mechanisms of these psychrophilic microbes at high pressure. Comparing to the previous studies of high-pressure ALE of mesophiles which suggested adaptations in membrane structures. 16S tag sequencing analysis of the changes in diversity of the bacterial communities suggested that most diversity was lost during the initial incubation at atmospheric pressure, therefore, only the few surviving genera were still present in the culture for high pressure characterization. Whole genome sequencing of high pressure isolates suggested genetic mutations that could be associated with the enhanced growth at high pressure.

MATERIALS AND METHODS

Samples Collection

Two samples were collected from Ross Sea, Antarctica in January 2018. The 10 m seawater samples were collected on Jan. 17th. 2018 from a sampling hole drilled at the coordinate 77 51.059 S, 166 48.559 E. The samples were collected using a Niskin bottle, 90 mL of sample was then filtered using a 0.2 µm membrane filter and resuspended in 2 mL of original seawater. The plankton net sample was collected on Jan. 26th. 2018 from the ice edge in Ross Sea using a plankton net tow. 1.5 mL of each sample was preserved in 20% glycerol and stored at -80 °C.

Media

Difco 2216, 75 % strength of BD Difco™ Dehydrated Culture Media: Marine Broth 2216, Cat no. DF0791-17-4. 20µM of MOPS buffer at pH 7.4 were added to the liquid media for ALE experiments.

AMS1 medium was prepared similarly to Carini et al. (2013). For some ALE experiments sodium pyruvate, MOPS buffer (pH 7.4) and $NaNO_3$ were added to the liquid medium to final concentrations of 20 mM, 20 mM and 100 mM, respectively. For other ALE experiments pyruvate was replaced with D-glucose, also at a final concentration of 20 mM.

All agar plates were made with the above recipes with an addition of 1.5% agar.

Isolates Characterization

Thirteen bacterial colonies were isolated from the McMurdo aquarium and jetty seawater folloed by incubation on Difco 2216 plates at 4 °C. These colonies were then grown to mid-log phase in liquid Difco 2216 media. The cultures were cryopreserved in 16% glycerol and shipped

to lab in cryovials from Antarctica. The taxonomies of the isolates were determined using Sanger sequencing of 16S rRNA with primers 27F and 1492R which spans close to the whole ribosomal RNA sequence (Lane, 1991). These 13 isolates were used for characterizing the temperature and pressure sensitivities of microbes cultured from the environment.

The growth temperature sensitivity of each of the 13 strains was evaluated following incubations at 5 °C, 17 °C and 21 °C for 72 hours. Initially cryovial frozen stocks were inoculated into 10mL of liquid Difco 2216 media in glass culture tubes. After these cultures reached stationary phase, they were diluted 1:200 and growth followed via optical density (A 600 nm) measurements using a GENESYSTM 10S UV-Vis Spectrophotometer.

The growth pressure sensitivity of each of the 13 strains was evaluated following incubations at atmospheric pressure (0.1 MPa), 30 MPa and 50 MPa. Initially cryovial frozen stocks were inoculated into 10mL of liquid Difco 2216 media in glass culture tubes. After these cultures reached stationary phase, they were diluted 1:200 and inoculated into 5 mL polyethylene transfer pipette bulbs. The growth was followed via optical density (A 600 nm) measurements using a GENESYSTM 10S UV-Vis Spectrophotometer.

Culturing and Adaptive Laboratory Evolution Experiments (ALE)

50 μL of both frozen stocks were inoculated into liquid cultures that were allowed to grow to mid-log phase, at an OD 600 between 0.5 and 0.7, which were then used as the starting culture of the community ALE experiments. Additional 50 μL of frozen stocks of 10 m seawater and plankton tow samples were spread plated individually onto agar plate of all media. Single isolates of different morphologies were inoculated into liquid media and allowed to grow to mid-

log phase, which were called the ancestral strains and were used as the starting cultures of the single strain ALE experiments.

Adaptive laboratory evolution experiments (ALE) were performed using 15mL polyethylene transfer pipette bulbs that were heat sealed and pressurized in pressure vessels or incubated in brown plastic bottles at atmospheric pressure. Along with the 15mL polyethylene transfer pipette bulbs, numerous 5mL bulbs are incubated to track the growth of the inoculum periodically by cutting open one 5mL bulb and measuring the OD 600 value using a GENESYSTM 10S UV-Vis Spectrophotometer. Community ALE experiments were set up in triplicates labelled with vial numbers of stock cultures and each replicate was labeled A, B and C. Isolates ALEs were set up in duplicates labelled with the genus name and each replicate was labeled A and B. The initial culturing of all ALE experiments was incubated at a pressure that would challenge but not inhibit cell growth. When there was visible biomass in the bulbs, the inoculums are transferred to 50 mL centrifuge tubes for subculturing. The optimal density of absorbance at 600 nm (OD 600) was measured using a GENESYSTM 10S UV-Vis Spectrophotometer. 1.125 mL of the cultures were preserved with 25% glycerol and stored at -80 °C for further analysis. Using serial dilution, 100 µL of 10^{-5} dilution was plated onto agar plates to examine colony morphologies and account for diversity in community ALEs. 250 µL of the culture was inoculated into 50mL of fresh media to reach a 1:200 dilution for subculturing. The inocula were transferred into 15 mL polyethylene transfer pipette bulbs and pressurized to a pressure 5 or 10 MPa higher than the previous incubation. At selected pressure of the isolates ALEs, 1mL of 10^{-5} diluted culture was fixed with formaldehyde and filtered onto an Isopore™ 0.2 µm polycarbonate membrane filter (Ref No. GTTP02500) to observe the cell morphologies.

If no growth was observed at a certain pressure after 30 days of incubation, the pressure of the incubation was decreased to the previous incubation pressure without transferring the cultures to new media.

Whole Genome Sequencing and Genome Assembly

Whole genome sequencing was used for the DNA of all the "ancestral" strains that were isolated from the seawater frozen stocks. The inoculum single colonies are allowed to grow to mid-log phase. At mid-log phase, the DNA was extracted using Qiagen DNeasy® UltraClean® Microbial Kit (Cat No./ID: 12224-250) following the standard protocol. Sanger Sequencing of the 16S region using primers 27F and 1492R was used to determine the taxonomies of the isolates (Lane, 1991). The sequencing library was prepared using Illumina® Nextera DNA Flex Library Prep Kit (Cat No. 20018704) following the standard protocol. Four strains were pooled using the standard protocol and sequenced on an iSeq 100 system using paired end with 150 cycles each (2 x 150 bp).

The raw sequencing data was examined using FastQC (Andrews, 2010). With the raw sequencing data, I trimmed the reads to remove the adapters and any low-quality reads using Sickle with the pair-end and sanger sequencing option with default parameters (Joshi and Fass, 2011). The trimmed sequences were examined using FastQC again (Andrews, 2010). The estimated coverage of the genomes were calculated using the total number of trimmed reads multiplied by the read length, 151 bp, then divided by the genome size of the reference genome used for genome comparison. I then assembled the trimmed reads using SPAdes with kmers size 21, 33, 55, 77, and 89 (Bankevich et al., 2012). The SPAdes output was examined using QUAST (Gurevich et al., 2013). I then used SSPACE-standard to assemble the contigs into scaffolds

using a minimal contig size of 100, minimal coverage of 30, the minimum number of links required for a contig pair to be considered was set to 5 and the maximum overlap ratio allowed for contig combination was set to 0.7 (Boetzer et al., 2011). The assembled genomes were annotated using PATRIC (Wattam et al., 2017). PATRIC was also used to find the unique genes in each of the Antarctic isolates, each proteome of the isolates was compared to the proteome of the closest related species according to the BLAST results of 16S Sanger sequencing, which also had a whole genome sequence published on NCBI (Altschul et al., 1990, Wattam et al., 2017). The four genomes used are *Loktanella salsilacus* strain DSM 16199, *Shewanella frigidimarina* NCIMB 400, *Psychromonas profunda* strain 2825 and *Psychrobacter fozii* strain CECT 5889.

Whole Genome Resequencing and Growth Analysis

The 50 MPa culture of *Psychrobacter* 2216 ALE at 21°C were plated out on a Difco 2216 plate. One colony was inoculated into 10mL of fresh Difco 2216 media and allowed to grow to mid-log phase. DNA was extracted from 1.8 mL of the inoculum using Qiagen DNeasy® UltraClean® Microbial Kit (Cat No./ID: 12224-250) following the standard protocol. The genus was confirmed using BLAST of 16S Sanger sequencing with primers 27F and 1492R before the whole genome was sequenced on an iSeq 100 machine (Altschul et al., 1990). The raw sequencing data was trimmed using fastq_quality_filter in the fastx toolkit keeping reads that have 80% of the bases with a quality score of higher than 20 (Hannon, 2010). *Breseq* was then used to find the mutations in the evolved strains using the default setting with the assembled ancestral genome as the reference genome (Deatherage and Barrick, 2014). The quality trimmed Illumina reads of the ancestral strain was also mapped to the assembled genome using Breseq to account for any assembly errors that could appear as mutations in *breseq* output.

Genes with insertion mutations were further analyzed by translating the DNA to protein and modelling the protein of both the ancestral strain and the high pressure derived strain. The DNA was translated in CLC workbench and selecting the same reading frame as the ancestral strain. The protein sequences were then used to create protein structure models on SWISS modeling (Waterhouse et al., 2018).

Growth were compared between the ancestral *Psychrobacter* strain and two isolates from each lineage of 50 MPa culture of *Psychrobacter* 2216 ALE at 21 °C. Frozen stocks of the ancestral strain and the 50 MPa cultures were inoculated into fresh Difco 2216 media. 10 mL of the inoculums were incubated in test tubes at atmospheric pressure and 40 mL of the inoculums were inoculated into 5mL polyethylene transfer pipette bulbs and pressurized at 50 MPa in the same pressure vessel. OD600 readings were measured on a Spectronic 20 for the test tubes and on a GENESYSTM 10S UV-Vis Spectrophotometer for the high pressure incubations.

16S tag Sequencing and Analysis

16S tag sequencing was performed on the DNA of 10m seawater sample extracted in Antarctica, the frozen stocks of the 10m Seawater sample and plankton net sample as well as the starting ALE cultures after the initial incubation in Difco 2216 and AMS1 pyruvate. To minimize the effect of glycerol in the frozen stocks on DNA extraction, all 1.5 mL of the glycerol preserved frozen stocks was washed with 9 mL of artificial seawater and centrifuged at 1100 rpm to collect the cells. The washing step was repeated twice. After the supernatant is removed, the cell pellets were used to extract DNA using the DNeasy PowerSoil Kit (cat. 12888-100) following the standard protocol. Using the extracted DNA, the hypervariable region V4 in 16S rRNA was sequenced using the primers 515F: GTGYCAGCMGCCGCGGTAA and 806R:

GGACTACNVGGGTWTCTAAT, on an Illumina MiSeq machine (Walters et al., 2016). The raw sequencing data was imported into Qiime2, the forward and reverse reads were paired and trimmed using dada2 denoise-paired (Bolyen et al., 2019, Callahan et al., 2016). The sequences were classified using Greengenes classifiers 13.8 with 99% sequence identity (DeSantis et al., 2006). The taxonomy graph was created using taxa barplot in Qiime2 (Bolyen et al., 2019).

RESULTS

Hydrostatic pressure has been suggested to negatively impact the growth of non-piezophilic microbes as was seen in high pressure ALE of *E.coli* (Marietou et al., 2015). To evaluate the effects that high hydrostatic pressure has on psychro-tolerent, non-piezophilic bacteria isolated from Antarctic surface seawater, high pressure ALEs were carried out using both communities from 10m seawater, plankton tow samples and isolates of the genera *Loktanella, Psychrobacter, Psycrhomonas* and *Shewanella*. Cultures were transferred to a higher pressure of 5 or 10 MPa increase, depending on the growth of the bacteria, when they reached the mid-log phase of growth, as measured by using the OD 600 readings of the 5mL bulbs.

Isolates Characterization

Cultures of 13 samples (Sample #1- #13, Table 1) obtained from jetty seawater and McMurdo aquarium seawater and incubated at 4 ºC on Difco 2216 agar plates were characterized with regard to phylogeny and growth as a function of temperature and pressure. The results of 16S rRNA gene sequence analyses revealed that five of the strains belong the genus *Colwellia*, one belongs to the genus *Psychromonas*, five belong to the genus *Moritella* and one belongs to the genus *Pseudoalteromonas* (Table 1). One of the samples contained a mixed culture of *Pseudoalteromonas* and *Moritella*.

The growth temperature sensitivity of the strains was evaluated by following turbidity changes over 72 hours at 5 °C, 17 °C and 21 °C. The OD 600 readings suggested that all the isolates from jetty seawater were not able to grow at 21 °C. It is concluded that 4 of the isolates could be psychrophilic, having the best growth at 5 °C (Table 2). The remaining 9 strains are

psychrotolerant but not likely to be psychrophilic as all of them are capable to grow at a temperature lower than 15 °C but have better growth at temperature above 15 °C (Table 2). The growth pressure sensitivities of the strains were evaluated by following incubations at atmospheric pressure, 30 MPa and 50 MPa at 5 °C and the OD 600 was followed up to 35 days. The results indicated that except for *Pseudoalteromonas* strain SL006 which showed no growth at 30 MPa. All remaining strains showed some growth at 30 MPa but grew better at atmospheric pressure (Table 2). Note that the analyses of growth as a function of pressure were performed in sealed bulbs which resulted in reduced oxygen availability and largely fermentative growth. Under these conditions growth rates and yields. were greatly reduced. It is concluded that all strains are piezotolerant, except for *Colwellia* strain SL012 which is piezosensitive.

Whole Genome Assembly of Isolates Used in ALEs

Psychromonas strain SL002

The genus *Psychromonas*, belongs to the class of Gammaproteobacteria, include species that have psychrophilic, piezophilic and halophilic adaptations (Mountfort et al., 1998, Xu et al., 2003). The 16S rRNA sequence of *Psychromonas* strain SL002 has a percent identity of 98.67% compared to *Psychrobacter profunda*, which is a psychropiezophile that was isolated from deep Atlantic sediment (Xu et al., 2003). The whole genome sequencing yielded an estimated coverage of 44. Upon evaluation of the assembly graph, there are many short contigs which could suggest that there are many repeat regions in the genome. The strain might need long reads sequencing to build a better assembly of the ancestral strain.

Loktanella strain SL015

The genus *Loktanella*, belongs to the class of Alphaproteobacteria, has previously been isolated from Antarctica and shallow-water sediment (Van Trappen et al., 2004, Tanaka et al., 2014). The 16S rRNA sequence of *Loktanella* strain SL015 has a percent identity of 100% compared to *Loktanella salsilacus*, which is a psychrophile that was isolated from microbial mats in the McMurdo Dry Valleys, Antarctica. The whole genome sequencing yielded an estimated coverage of 13 which is not enough coverage to assemble the genome and therefore no genome was assembled, and no comparison is done at this time. Additional sequencing data was generated during the second iSeq run. A better assembly can be generated by combining the existing sequencing data with the new data.

Psychrobacter strain SL016

The genus *Psychrobacter*, belongs to the class of Gammaproteobacteria, that are psychrophilic or psychrotolerant (Bowman 2006). Species within the genus have also been found in the deep sea such as *Psychrobacter pacificensis* that was isolated from the Japan Trench (Maruyama et al., 2000). The 16S rRNA sequence of *Psychrobacter* strain SL016 has a percent identity of 100% compared to *Psychrobacter fozii* strain CECT 5889, which was also isolated from Antarctica (Bozai et al., 2003). The assembled genome size from SPAdes is 4,144,712 bp with a coverage of 17. Using *Psychrobacter fozii* strain CECT 5889 as reference, PATRIC annotated 2084 genes, excluding hypothetical proteins. Excluding hypothetical proteins, 121 genes are unique to *Psychrobacter* strain SL016. In all 121 unique genes, 7 are associated with phage sequences, which could suggest horizontal gene transfer from phages, and one gene that codes for a nickel-binding accessory protein.

Shewanella strain SL017

The genus *Shewanella*, belongs to the class of Gammaproteobacteria, is commonly found in aquatic and marine environment (Kato and Nogi, 2001). Known psychro-piezophiles in the genus include isolates from deep sea environments such as *Shewanella profunda*, *Shewanella benthica* and *Shewanella violacea* (Kato and Nogi, 2001). The 16S rRNA sequence of *Shewanella* strain SL017 has a percent identity of 99.71% compared to *Shewanella frigidimarina* NCIMB 400, which was isolated from the North Sea near the coast of Aberdeen, UK (Reid and Gordon, 1999). The assembled genome size from SPAdes is 4,944,551 bp with a coverage of 17, the coverage is not enough coverage to assemble the genome and therefore no genome was assembled, and no comparison is done at this time.

Adaptive Laboratory Evolution Experiments with Isolated Strains

To understand the high pressure evolutionary potential of Antarctic microbial species free from interspecies competition, ALE experiments were also conducted using the aforementioned strains *Loktanella* strain SL015*, Psychrobacter* strain SL016*, Psychromonas* strain SL002 and *Shewanella* strain SL017. ALEs were conducted at 5 °C in three media: a peptide-rich marine medium, Difco 2216, and defined marine media, AMS1, containing pyruvate or glucose as carbon and energy substrates. ALE experiments were also conducted at 21 °C for *Loktanella* strain SL015*, Psychrobacter* strain SL016 as the strains grew significantly better at the higher temperature.

Over 40 generations in the both replicates (43 generations in Lineage A and 45 generations in Lineage B) of incubation in Difco 2216 and at 21 °C at high pressure were

completed during the ALE experiment, reaching a pressure of 50 MPa. The growth of *Psychrobacter* strain SL016 was restricted at high pressure compared to atmospheric pressure (0.1 MPa) (Figure 1). It took 53 days for the strain to reach an OD 600 of 0.1 during the 40 MPa incubation which was more than twice as long when compared to both earlier incubations at lower pressure and subsequent incubations at higher pressures which had an average incubation time of 25.8 days (Figure 1). Thus, the growth of *Psychrobacter* strain SL016 was negatively impacted by high pressure and seemed to have been most restricted when the hydrostatic pressure of the ALE reached 40 MPa. After this bottleneck was overcome, improved growth was observed in both lineages, first at 45 MPa and then after transfer to 50 MPa (Figure 1). While the specific pressure varied for each strain, similar trends of a "bottleneck" pressure were also observed in all the other single strains ALE experiments indicating that there is a certain pressure which requires a longer incubation time for each strain to reach an OD 600 reading of 0.1. All the bacterial strains seemed to resume a similar growth rate compared to before the "bottleneck" pressure at subsequent incubations compared to incubations preceding the decreased growth rate. Incubation of this *Psychrobacter* strain SL016 had been shown to continue accumulating biomass at 55 MPa, but no living cell was able to be recovered after an unintended incubation period of over 3 months due to the COVID-19 pandemic (Appendix A).

***Psychrobacter* strain SL016 Genome Resequencing and Growth Analysis**

Both duplicate lineages of *Psychrobacter* strain SL016 were further characterized through genome re-sequencing and analysis. DNA was extracted from clonal isolates, *Psychrobacter* strain SL016 50A and *Psychrobacter* strain SL016 50B, from both lineages of the ancestral strain, *Psychrobacter* strain SL016, at the 50 MPa transfer and were sequenced using

the whole genome sequencing techniques with a coverage of higher than 50 on an Illumina iSeq 100 machine. *Breseq* analysis of these 50 MPa isolates indicated that both lineages evolved to have mutations compared to the ancestral strains. Breseq results using reads from *Psychrobacter* strain SL016 50A, *Psychrobacter* strain SL016 50B and the ancestral strain *Psychrobacter* strain SL016 showed that all three strains had a 10 bp insertion of "GTTCAACAAC" in a gene that codes for DNA polymerase III subunits gamma and tau using the assembled genome from ancestral strain as reference. In addition, lineage A also acquired a 5 bp insertion "GTTAT" in the *PvuC* gene, starting at the 607 bp of the 1076 bp gene. The protein translated from the mutated sequence suggested a truncated protein reducing the size from 358 amino acid to 220 amino acid. Protein modeling showed that the protein model of the ancestral strain had 10 helixes compared to only 6 helixes in *Psychrobacter* strain SL016 50A (Figure 8). The second mutation found in lineage A is a point mutation from T to C in the intergenic region between a putative membrane protein and a cold shock protein of CSP family. The third mutation is an insertion in the intergenic area of *msrB* that codes for Peptide-methionine (R)-S-oxide reductase and another gene that codes for alanine transaminase (Table 3) (Grimaud et al., 2001). In lineage B, other than the shared mutation, it also acquired a missense mutation in *murE*, which codes for UDP-N-acetylmuramoyl-dipeptide--2,6-diaminopimelate ligase (EC 6.3.2.13), from CCT to CTT resulting in a change of the 122nd amino acid of the gene from proline to leucine (Table 3) (Ogata et al., 2001).

Growth of the ancestral strain, *Psychrobacter* strain SL016, and the evolved strains of *Psychrobacter* 50 MPa lineages, *Psychrobacter* strain SL016 50A and *Psychrobacter* strain SL016 50B, were compared at atmospheric pressure and 50 MPa to analyze the changes in optimal incubation pressure. The results indicate that the ancestral strain grows better than the

two evolved strains at atmospheric pressure (Figure 2), while the two evolved strains are both able to grow better than the ancestral strain at 50 MPa, reaching a stationary OD600 reading more than twice as high as that of the ancestral strain (Figure 3).

High Pressure Characterization with Communities

To understand how the Ross Sea pelagic microbial community composition changes with exposure to gradually increasing pressure, high pressure characterization similar to Adaptive Laboratory Evolution (ALE) experiments with isolates were performed. The source material was either filtered and cryopreserved 10 m depth seawater or a cryopreserved concentrated plankton tow collection.

The untreated 10 m seawater sample contained 16S rRNA sequences of 51 families including 1 family of archaea and 50 families of bacteria with 1.75% of sequences with no assigned families (Figure 6a). After cryopreserved in 20% glycerol, the 10 m seawater frozen stock sample contained 16S rRNA sequences of 48 families without the family of archaea and two families of the class gammaproteobacterial that were present in the untreated sample (Figure 6b). The percentages of each order in the samples also changed between the untreated sample and the frozen stock sample. The percentage of *Flavobacteriales* increased from 33.1% to 46.8%, while the percentage of second most abundant family in the untreated seawater, *Rickettsiales*, dropped from 17.6% to 5.8%. Other orders of bacteria also had changes in their percentages within the samples. The frozen stock was diluted at 1:200 dilution and cultured at 5 °C in two media: a peptide-rich marine medium, Difco 2216, and defined marine media, AMS1, containing pyruvate as carbon and energy substrates. Illumina 16S tag sequencing suggested that only four genera of bacteria remained after the initial incubation in Difco 2216 at

atmospheric pressure, including 86.71% of *Shewanella,* 7.31% of *Flavobacterium,* 5.88% of *Psychrobacter* and 0.09% of *Pseudomonas,* with 0.01% of sequences not assigned to any bacterial genera through the Qiime2 pipeline (Figure 6d). There were 5 genera present after the initial incubation in AMS1 pyruvate, including 92.96% of *Shewanella,* 6.05% of *Pseudoalteromonas,* 0.63% of *Flavobacterium,* 0.24% of *Alteromonas,* 0.11% of *Pseudomonas,* with 0.01% of sequences not assigned to any bacterial genera through the Qiime2 pipeline (Figure 6c). When plated out on Difco 2216 plates, the 35 MPa Difco 2216 transfer of all replicates only had pink colonies and the 16S rRNA gene sequencing of 200 colonies suggested that the entire culturable community had shifted to the same species, most closely related to *Shewanella vesiculosa*. When plated out on AMS1 pyruvate plates, the 25 MPa transfer of AMS1 pyruvate incubation showed some diversity in colony morphologies. Sanger sequencing of 200 isolated colonies suggested that 66% of the colonies sequenced were *Pseudoalteromonas*, 30% were *Shewanella* and 4% were *Flavobacterium*. The pressure limit for 10m seawater sample Difco 2216 at which growth was observed was at 40 MPa over an incubation period of 32 days. Further culturing attempts at both 45 MPa for 31 days and subsequently at 42 MPa for 186 days were unsuccessful (Figure 4). Growth resumed during the 45 MPa incubation once the pressure was decreased back to 40 MPa.

DNA extracted from the frozen stock of the plankton tow sample showed that 50.62% of the sequences belongs to the phylum *Cyanobacteria*, within which 33.07% belongs to the order *Stramenopiles* and 17.19% belongs to the order *Haptophyceae* (Figure 7a). Additionally, 17.29% of all sequences belong to the order *Flavobacteriales* and 12.12% of all sequences belong to the order *Rhodobacteraceae* (Figure 7a). Illumina 16S tag sequencing suggests that the genera of bacteria present after the initial incubation in Difco 2216 at atmospheric pressure are 65.29%

*Pseudoalteromonas,*33.97% *Shewanella,*0.64% *Marinomonas,*0.049% *Flavobacterium* and 0.049% *Psychrobacter* (Figure 7c). The genera of bacteria present after the initial incubation in AMS1 pyruvate are 89.42% *Shewanella*, 9.60% *Pseudoalteromonas*, 0.67% *Flavobacterium*, 0.17% *Marinomonas*, 0.098% *Colwellia*, 0.017% *Oleispira* and 0.006% *Pseudomonas* (Figure 7b). Only pink colonies were observed on spread plates of the Difco 2216 35 MPa transfer (Figure 7d). Biomass was observed at Difco 2216 40 MPa incubation, but no living biomass was recovered after a prolonged period of unintended incubation due to the COVID-19 pandemic.

DISCUSSION

Isolates Characterization

The thirteen isolates from both jetty seawater and McMurdo aquarium seawater on Difco 2216 plates in Antarctica were grown at various temperature and pressure conditions to characterize the growth at different temperature and pressure. The results from both characterizations suggested that these isolates are psychrotolerant and pressure sensitive which was expected of microbes that were collected from the cold and non-high-pressure environments in Antarctic seawater close to the sea surface.

Interestingly for the three isolates used in ALEs that were isolated from 10 m seawater sample and plankton net sample, all three genera made up for less than 0.1% of the frozen stock, suggesting that culturing the bacteria on agar plate is selective towards bacteria that are able to utilize the nutrients available.

Isolates Adaptive Laboratory Evolution Experiments

Previous Adaptive Laboratory Evolutions (ALE) experiments using *E. coli* have shown that high hydrostatic pressure can slow down or even inhibit cell growth by disrupting cellular functions such as nutrients transports and denaturing intracellular molecules (Marietou et. al. 2015). Our ALE experiments characterized the adaptations of Antarctica surface seawater microbes to high pressure and showed similar trends observed in previous studies (Figure 1). Growth rates decreased at higher pressure for all the ALE experiments, suggesting that cells were stressed at high pressure. However, compared to the ancestral strains at the start of the ALE experiments, the high-pressure adapted strains were capable of growing at a pressure higher than the pressure limit of the ancestral strains (Figure 3).The “bottleneck” pressure which entailed a

longer incubation time that was observed in all single strains ALEs seem to suggest that the bacteria had to overcome either phenotypic or genetic restraints in order to survive at the pressure and allow them to continue growing at high pressure (Figure 1).

Previous studies of high pressure characterization using *E. coli* and *P. profundum* strain SS9 have shown that strains capable of growing at higher pressure had changes in their cell membrane fatty acid composition (Zobell and Cobet 1962, Allen et al., 1999). In my experiments, the ancestral strains isolated from Antarctica samples were already adapted to cold environment which suggests that they have likely already acquired a more fluid-like cell membrane to counteract the rigidity induced by low temperature. Given this change in cell membrane, psychrophilic strains isolated from Antarctica samples that are capable of growing at low temperature may have had an advantage compared to mesophiles when undergoing changes in their membrane fatty acid composition to adapt to high pressure. The high-pressure strains from our ALEs could also have acquired mutations in their genomes that allowed them to grow better at higher pressure, which was found in ALE with *E. coli*, *P. profunda* strain SS9 and *Carnobacterium sp.* AT7 (Marietou et. al. 2015).

It is also possible that there were no changes in either the membrane compositions or the genome sequences of the bacteria. Previous experiments have suggested that increased amount of osmolytes such as trimethylamine-N-oxide (TMAO), which are small organic molecules capable of changing the fluidity of the cell, can reverse the adverse effects of high pressure on cellular molecules such as fragments of DNA and RNA in culturing flasks (Arns 2019). Similarly, the strains in our experiment could also be increasing the amount of osmolytes allowing survival at high pressure. This would mean that the strains may simply be acclimated to

higher pressure from accumulating osmolytes over prolonged exposure to high pressure, but do not have changes in cell membranes or genome differences compared to the ancestral strain. However, the original study on the effects of osmolytes were not measured *in vivo*, but rather only on DNA and RNA in an environment mimicking intracellular conditions (Arns et. al. 2019). It is uncertain if osmolytes would contribute to the adaptation of whole bacterial cells in a similar way. Measurements of osmolyte levels in ancestral and ALE-derived strains as a function of pressure might provide clues to a role for osmolytes *in vivo* during physiological adaptation or evolutionary selection at high pressure.

50 MPa *Psychrobacter* Genome Resequencing

Breseq is a bioinformatics analysis pipeline that is capable of identifying mutations in genome using next generation sequencing reads (Deatherage and Barrick, 2014). Using the assembled *Psychrobacter* genome from the ancestral sequences, the Illumina reads of the ancestral, and the two evolved 50 MPa lineages were aligned to identify mutations using *Breseq*. All three strains showed the same 10 bp insertion at the same position in a DNA polymerase III gene suggests that this is not a true mutation, but rather an assembly error of the ancestral strain.

Previous high pressure ALE study on *E.coli* has found mutations in genes that code for proteins associated fatty acid synthesis and cis-vaccenic acid production (Marietou et al., 2015). However, none of the mutations I found in *Psychrobacter* is in a gene that codes for similar function. This could suggest that compared to mesophile such as *E.coli*, psychrophilic bacteria such as *Psychrobacter* has acquired fatty acid synthesis ability that would allow them to grow at high pressure.

Three mutations were found in Lineage A, one or more of these mutations could be responsible for the increase in growth at high pressure. The first mutation found in lineage A is a 5 bp insertion in *pvuC* gene which codes for Ferrichrome ABC transporter. The *E. coli* homolog of this gene was found to encode for very hydrophobic polypeptide that is the transmembrane subunit of a Periplasmic Binding Protein (PBP)-dependent ATP-Binding Cassette (ABC) transporter that is responsible for the uptake of amino acids with branched-chain (Staudenmaier et al., 1989). The 5 bp insertion starts at the 607 bp of the 1076 bp gene which causes a frame shift and resulted in a shorter translated protein. Protein structure modeling using SWISS showed that the mutation resulted in a less completed protein structure which could mean that the mutated protein is likely non-functional (Figure 8). In this case, it could suggest that in order to adapt to high pressure, the cells acquired a mutation that resulted in a reduced or lose of function in *pvuC* gene. However, high pressure can result in more tightly packed protein structures, which means that the mutated protein with a less compact structure could be more efficient at molecule transport. In which case, the mutation found in this transmembrane transporter protein could be efficient in countering the decrease in nutrient transport resulted from high hydrostatic pressure (Bartlett and Bidle, 1999). Additionally, the hydrophobic protein coded could be enhancing the hydrophobicity of the protein to prevent unfolding at high pressure. Previous biophysical analysis has demonstrated that high hydrostatic pressure decreases the strength of hydrophobic interactions which results in unfolding of hydrophobic proteins (Herberhold et al., 2004). Another study of two different species of *Pyrococcus* suggested that the proportion of hydrophobic proteins was found to be higher in high-pressure adapted species found in the deep sea compared to the other species found in shallow seawater that is not adapted to high pressure (Afonnikov et al. 2011).

The second mutation found in lineage A is a point mutation in the intergenic region between a gene that codes for cold shock protein and a putative membrane protein. The cold shock protein sequence is 94% identical to a CspA-like protein in *Arthrobacter* sp. B5. CspA protein was identified as the major cold shock protein in *E.coli* (Jiang et al., 1997). Studies of CspA as well as other homologous cold shock proteins have suggested that they can bind to RNA and induce the productions of other genes in response to cold shock (Bae et al., 2000). The mutation could be upregulating the cold shock protein (csp) which allows the bacteria to adapt to high pressure environment. Previous study of a psychrophilic bacteria, *Listeria monocytogenes*, had found an increase in csp in cells that are able to survive high hydrostatic pressure (Wemekamp-Kamphuis et al., 2002). In similar ways, the mutation found in the high-pressure strain of *Psychrobacter* could also be upregulating csp in the cells to adapt to high hydrostatic pressure.

The third mutation found in lineage A is a 19 bp insertion in the intergenic region between *msrB* that codes for peptide methionine (R)-S-oxide reductase (EC 1.8.4.12) and a gene that codes for Alanine transaminase (EC 2.6.1.2). *MsrB* is known to be able to repair oxidative stress damages in peptides by reducing the oxidative form of methionine to the functional state (Singh and Moskovitz 2003). High pressure is known to increase cytoplasmic oxidative stress in bacteria that results in cell inactivation and cell death as was previously demonstrated in *E.coli* that were exposed to high pressure (Aertsen et al., 2004). The mutation could be upregulating the *msrB* gene which could counteract the effect of oxidative stress caused by high pressure. Alanine transaminase (EC 2.6.1.2) is a protein that is part of the synthesis of alanine, which are building blocks of the cell wall (Typas et al., 2012). The mutation in the intergenic region upstream of the

gene could be resulting in structural differences in the cell wall which in term allow the strain to grow better at high pressure.

Remarkably, lineage B was able to achieve better growth at high pressure through one single basepair missense mutation in *murE* which codes for the enzyme UDP-N-acetylmuramoyl-dipeptide--2,6-diaminopimelate ligase (EC 6.3.2.13). The homolog of this enzyme was characterized in *E.coli*, *Staphylococcus aureus* and *Verrucomicrobium spinosum* (Mengin-Lecreulx et. al., 1999, Patin et. al, 2010, McGroty et al., 2013). In these other bacterial species, the enzyme catalyzes the addition of the third residue during peptidoglycan synthesis (McGroty et al., 2013). This residue is involved in peptidoglycan cross-linking which helps maintain osmotic pressure of the cell (Mengin-Lecreulx et. al., 1999, Patin et. al, 2010). Peptidoglycan cross-linking has been associated with the change in pore sizes in Gram-negative bacterial cell wall (Furchtgott et. al, 2011). At high pressure, a larger pore size could counter the rigidness in cell membrane imposed by high pressure by creating a more fluid-like membrane and allow for more efficient nutrient transport (Bartlett and Bidle 1999). Future analysis of the fatty acid composition of the bacterial cell membrane by comparing the types of fatty acid in the ancestral strain and the evolved 50 MPa strains will be able to determine how this mutation could change the cell membrane structure (Allemann and Allen, 2018).

The missense mutation present in lineage B changes the amino acid from proline to leucine. Different from the ring structure of proline, leucine contains hydrophobic side chains. This change in amino acid could results in a significant structural change in the protein, as it was demonstrated in a study that showed a mutation from leucine to proline resulted in a structural change from a single helix to coil in the protein structure (Kundu et al., 2013). In addition to structural changes, the hydrophobic side chains of leucine could interact with other amino acids

in the enzymes in ways that are different from the ring structure in proline, which in terms influence the enzyme activity. This has been shown previously in a study that induced point mutations that substitute proline with other amino acids in a transport protein in *E.coli* (Pi et al., 1998). While no functional changes were observed in mutations occurring at some of the positions, one of the leucine substitutions reduced the transport activity to 26% (Pi et al., 1998).

Further analysis of protein structure through homology modeling and functional analysis of enzyme activities will give us a better understanding of how this mutation in lineage B affect the enzyme UDP-N-acetylmuramoyl-dipeptide--2,6-diaminopimelate ligase, and facilitate cell growths at high pressure.

50 MPa *Psychrobacter* strain SL016 Growth Analysis

Growth analysis of the two evolved lineages and ancestral strain were done at both 50 MPa and atmospheric pressure to evaluate the difference in growth between the evolved lineages and the ancestral strain, which could suggest changes in pressure tolerance. The 50 MPa growth analysis showed that the two evolved strains are able to grow to an OD that is more than five times as high as the ancestral strain compared to the ancestral strain (Figure 2). The increase in OD suggests that the evolved strains were growing better at 50 MPa compared to the ancestral strain which indicates that they have either expanded their range of pressure tolerance or they have evolved to grow at a different pressure range compared to the ancestral strain (Figure 2). To determine if the pressure range has shifted for the evolved strains, growth analysis between the three strains were also done at atmospheric pressure (Figure 2). The growth at 50 MPa showed that the ancestral strain was capable of reaching a higher OD at stationary phase compared to the two evolved strains. The decrease of growth in the evolved strains at atmospheric pressure

suggest that the evolved strains have shifted the pressure range for optimal growth compared to the ancestral strain by adapting to grow better at higher pressure.

High Pressure Characterization of Community Samples

10m seawater sample

Community diversities of the cryopreserved sample and the starting communities of ALEs were evaluated using Illumina16S tag sequencing. The cryopreserve process in 20% glycerol retained most of the diversity from the untreated samples, however, the percentage of each taxonomic group changed significantly for some groups (Figure 6). The percentage of *Flavobacteriales* increased by 13.7% while the percentage of *Rickettsiales* decreased by 11.8% (Figure 6). No archaea sequence was present in the frozen stock sample (Figure 6b). Future analysis of the alpha and beta diversity would be able to quantify the differences between the two samples. The majority of the diversity were lost during the initial incubation from cryopreserved frozen stock to the culturing media (Figure 6c, d). The two media, Difco 2216 and AMS1 pyruvate seem to have enriched for different bacteria genera. *Psychrobacter* was only present in Difco 2216 enrichment while *Pseudoalteromonas* and *Alteromonas* were only present in AMS1 pyruvate enrichment (Figure 6). Diversities of high pressure transfers were evaluated through colony morphology and 16S Sanger sequencing of 20 colonies. The community composition changed significantly at high pressure in Difco 2216, with only pink colonies likely belonging to the genus *Shewanella* remaining at 35 MPa in all three replicates of the 10 m seawater sample (Figure 6). Interestingly, *Shewanella* makes up only 0.116% of the community from frozen stock. At 25 MPa, the spread plate of the three replicates in AMS1 pyruvate still showed some diversities and the 16S Sanger sequencing results of 200 colonies were consisted of 66% of

Pseudoalteromonas, 30% of *Shewanella* and 4% of *Flavobacterium* (Figure 6). Comparing between the Difco 2216 and AMS1 pyruvate high pressure incubation, it suggests that the bacteria that wins at high pressure depends on the survival of the initial incubation at atmospheric pressure (Figure 6). *Pseudoalteromonas* was abundant at 25 MPa in AMS1 pyruvate sample, but not in Difco 2216 because no *Pseudoalteromonas* was present at the start of the Difco 2216 high pressure characterization (Figure 6). The selection at high pressure is also dependent on the abundance of the bacteria at the start of the high pressure incubation. *Shewanella* outgrew the other bacteria in Difco 2216 likely because of its abundance in the starting culture (Figure 6). However, diversity evaluation through colony morphology is not definite of the taxonomy of the bacteria, especially for bacteria that might not be able to grow on plates, or some of the genera of bacteria that share similar morphologies. Future analysis of high-pressure community diversities using 16S tag sequencing will be able to identify the bacteria as well as their proportion in the population.

Compared to the incubations in Difco 2216, some diversities still remained at AMS1 media at the final transfer, 25 MPa as colonies of different colors were observed on the plates. This seems to suggest that the rich undefined media, Difco 2216, was more selective towards *Shewanella* during high pressure ALEs. The differences in community diversity at high pressure could be due to the ingredients of AMS1 media, which do not contain complex sugars such as peptone and yeast extract in Difco 2216, which would favor bacteria that is able to occupy the most nutrients before giving the other bacteria a chance to grow.

Plankton tow sample

Cyanobacteria made up about half of the frozen stock community (Figure 7a). The abundance of *Cyanobacteria* is probably because it is the largest group of photosynthetic prokaryotes, which can be found in the same habitat with eukaryotic planktons (Stanier and Cohen-Bazire, 1977).

Similar to the 10 m seawater sample, the diversity in plankton tow sample decreased significantly after the initial incubation (Figure 7b, c). However, the diversity varied between Difco 2216 and AMS1 pyruvate. *Shewanella* was the most abundant genus in AMS1 pyruvate making up 89.42% of the all sequences, while *Pseudoalteromonas* was the most abundant in Difco 2216 making up 65.29% of the total sequences (Figure 7b, c). In Difco 2216, after 2 rounds of transfer, at 35 MPa, the plankton tow sample also only had *Shewanella* remaining in the culture (Figure 7d).

FUTURE DIRECTIONS

Adaptive Laboratory Evolution Experiments with Communities

Future students will continue community ALEs in AMS1 pyruvate and AMS1 glucose media until the communities reach its pressure limit or 60 -70 MPa, which is equivalent to the pressure at the hadal zone in the ocean (Gardner et al., 2014). Upon completion of the ALE incubations, 16S tag sequencing should be performed on the community according to Materials and Methods. When plating out the inoculum, if the diversity is low or if there is only one "winning" strain, whole genome sequencing of the strain should be performed and compared to the genomes assembled from ancestral strains that were isolated from the community samples and sequenced using Illumina iSeq.

Adaptive Laboratory Evolution Experiments with Isolates

Additionally, all three isolates ALEs should be continued using frozen stocks of the last transfer until the strains' pressure limits or 60-70 MPa. Whole genome sequencing and analysis should be performed on all the high pressure isolates using the same method described in Materials and Methods section. Identifying genetic mutations at high pressure could be done by using *Breseq* and separately analyzing the ancestral strain and the high-pressure strain. Alternatively, for isolates that do not have a closely related published genome, the genome of the ancestral strain could be constructed using De Novo Assembly tools in the same way that the genome was assembled of the *Psychrobacter* species. For genome with lots of repeats such as the *Pseudomonas* genome, long reads sequencing such as PacBio sequencing could be beneficial for constructing the genome.

To determine if there are physiological or genetic changes that account for improved growth of ALE-derived strains at high pressure, growth of the ancestral and ALE-derived strains must be compared to determine if the high-pressure isolates grow better compared to the ancestral strains, in the same set up as was done with the *Psychrobacter* strain by incubating both the ancestral strain and high pressure strains at high pressure to determine if the high-pressure isolates grow better compared to the ancestral strain. Comparison of the ratio of saturated fatty acid and unsaturated fatty acid (SFA/UFA) of the cell membranes as well as analysis of fatty acid structures will show if any changes occurred in membrane structure in the high-pressure stains which could advance the growth at high pressure (Allen et al., 1999). This analysis will compare the membrane composition of the evolved strains to the ancestral strains and determine if the isolates from Antarctica seawater also evolved to have more unsaturated fatty acid in their membranes as suggested by previous studies (Allen et al., 1999, Marietou et al., 2015).

Whole genome sequencing (WGS) could be performed on the rest of the evolved strains, using the same analysis pipeline as was done with the *Psychrobacter* strain to allow the identification of specific mutations contributing to increased growth at high pressure (Lind et. al. 2015). Furthermore, the use of molecular experiments, such as knock-down and silencing experiments, could confirm if the mutations identified in WGS are responsible for the increased growth at high pressure (Marietou et. al. 2015).

If genetics editing experiments such as knock-in using CRISPR-Cas9 tool or plasmids can be performed on isolates from ALEs to determine the functionality of each mutation. Mutations could be introduced into the ancestral strains one at a time or in combinations to determine if one or more of the mutations are responsible for high pressure adaptations. Alternatively, knock-out experiments could be done on high-pressure adapted strains by

knocking out the genes with mutations either one at a time or in combination and analyze the high-pressure growth of the mutant strains to determine if the gene and mutation is essential for high pressure growth.

Comparing *Shewanella* strain from Community ALEs and Isolate ALEs

Shewanella was the only genus present at 40 MPa in the Difco 2216 ALE of both 10m seawater sample and plankton net sample. Future studies could compare the pressure limits of the *Shewanella* strain in community ALEs and isolate ALEs. A difference in pressure limits could suggest that interspecies interactions can affect pressure adaptations in this *Shewanella* strain. However, the differences in pressure limits could also be due to different mutations acquired in the genome, which could be further analyzed using whole genome sequencing.

Isolation of SAR 11 Clade Microbes

SAR11 is a clade of alpha proteobacteria and is considered the most abundant bacteria in the ocean environment based on studies of the 16S rRNA sequences (Morris et al., 2002). To characterize SAR 11 Clade in the samples, preliminary work was done by plating the cryopreserved seawater samples onto agar plates of AMS1 pyruvate amended with 2% chitin. 10 of the colonies with different colony morphologies were sequenced using 16S Sanger sequencing, with two of which having top blast results being “Uncultured SAR11 cluster” and an additional two being “Uncultured alpha proteobacterium clone”. To obtain enough biomass to further analyze this group of bacteria, isolates could be transferred to 96-well plates with the same solid agar media.

CONCLUSION

My thesis presented preliminary findings on the adaptation mechanisms of psychrophilic, non-piezophilic bacteria to high hydrostatic pressure. Community diversity decreased most significantly after the initial incubation. The community diversity continued to decrease at increasing high pressure. Future experiments sequencing the genome of the "winning" strain and potentially comparing community changes in different media could answer the underlying question of why some bacteria survive at higher pressure but not the others. Isolates ALEs demonstrated that *Psychrobacter* and potentially the other three strains are capable of adapting to a higher pressure and even shifting the pressure range in the case of *Psychrobacter*. Genetic mutations were present in both *Psychrobacter* lineages and were identified to be in different genes than the mutations found in the ALEs of *E.coli, P. profunda* strain SS9 and *Carnobacterium sp.* AT7. The mutations in *Psychrobacter* could be suggesting differences in high pressure adaptation compared to non-psychrophilic bacteria. Future analysis of the membrane fatty acid composition as well as genetics experiments could provide more insights into the phenotypic and genetic changes that contribute to better growth at high pressure.

FIGURES

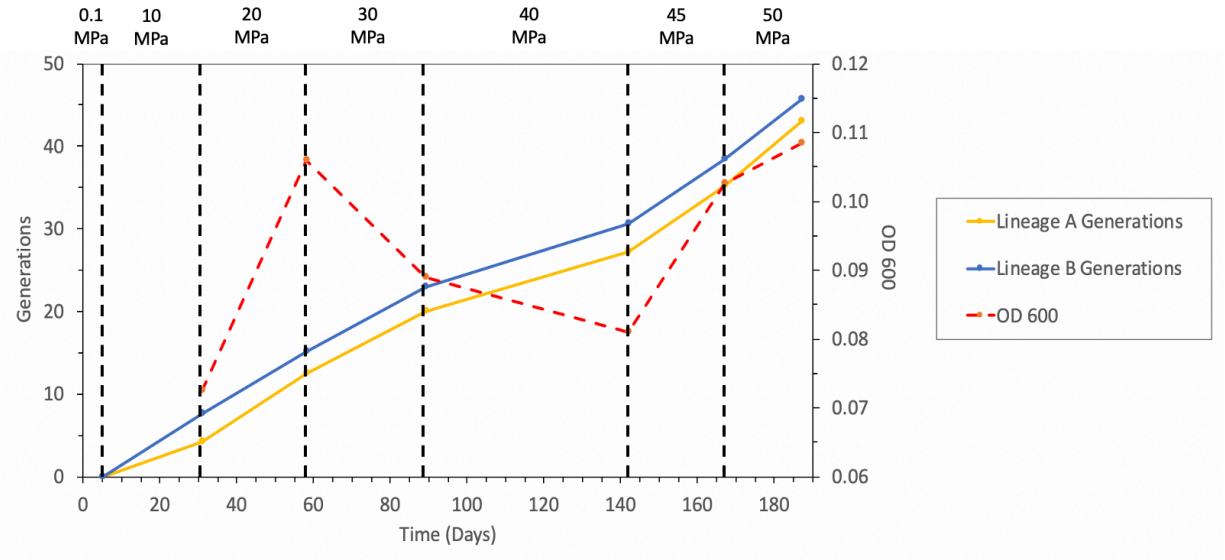


Figure 1. Growth of *Psychrobacter* strain SL016 through adaptive laboratory evolution at high hydrostatic pressure at 21 °C. Optical density readings at 600 nm (OD 600) and the number of generations in ALE were obtained when the cultures were transferred to a higher incubation pressure. Each vertical black dashed line represents a change in pressure following each transfer. The yellow dots represent the number of generations in Lineage A, the blue dots represent the number of generations in Lineage B and the red dots represent the average OD 600 readings at each transfer over the duration of the ALE experiment. The OD 600 readings were measured using a spectrophotometer and the numbers of generations were calculated using the OD 600 readings.

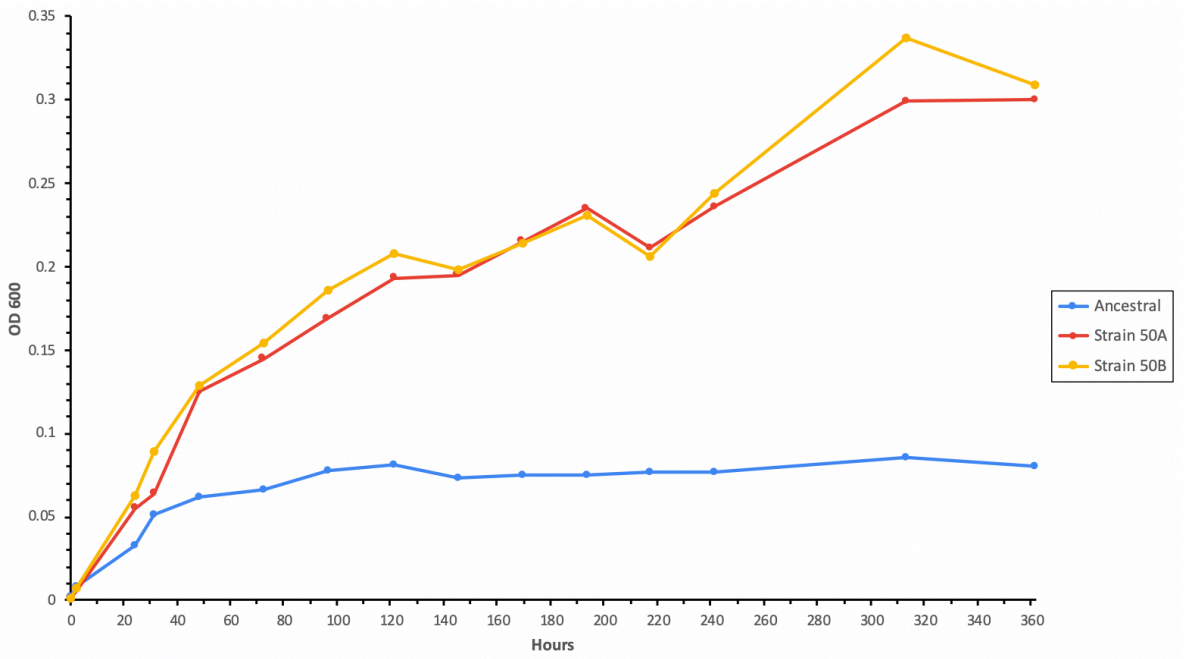


Figure 2. Comparison of the growth at 50 MPa of *Psychrobacter* strain SL016 and high pressure adapted isolates *Psychrobacter* strain SL016 50A and *Psychrobacter* strain SL016 50B. OD 600 readings were measured at different time points for the three strains grown in Difco 2216, at 50 MPa and 21 °C.

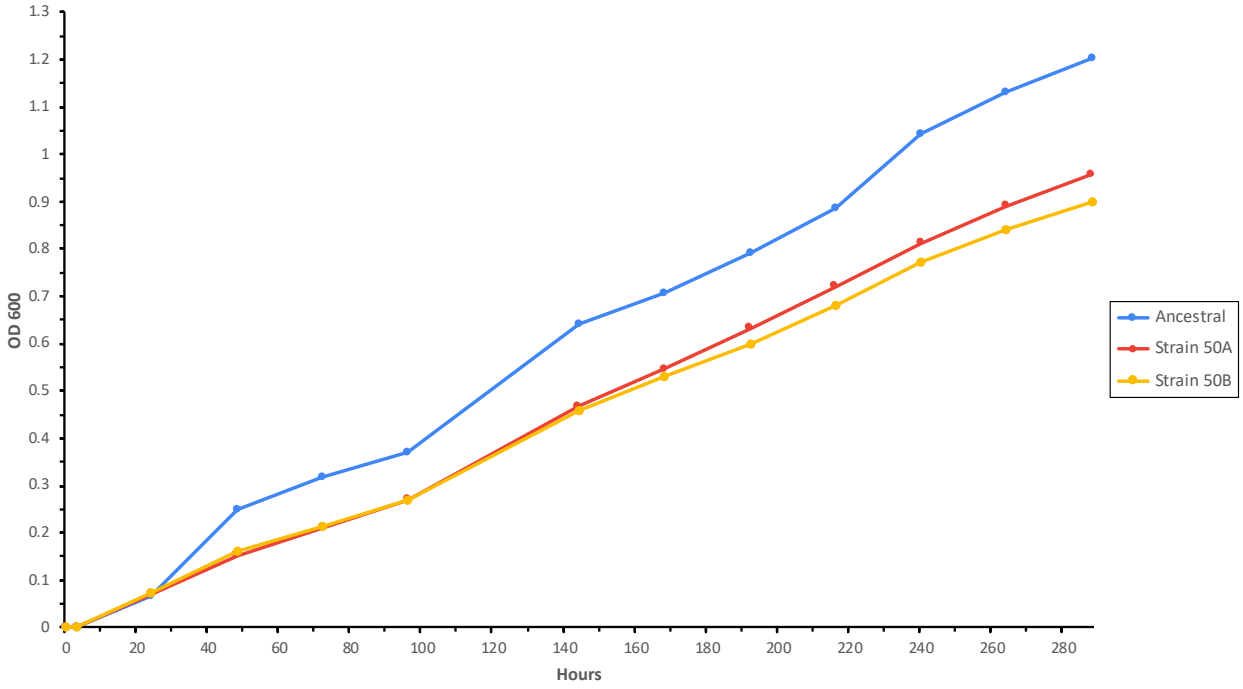


Figure 3. Comparison of the growth at 0.1 MPa of *Psychrobacter* strain SL016 and high pressure adapted isolates *Psychrobacter* strain SL016 50A and *Psychrobacter* strain SL016 50B. OD 600 readings were measured at different time points for the three strains grown in Difco 2216, at 0.1 MPa and 21 °C.

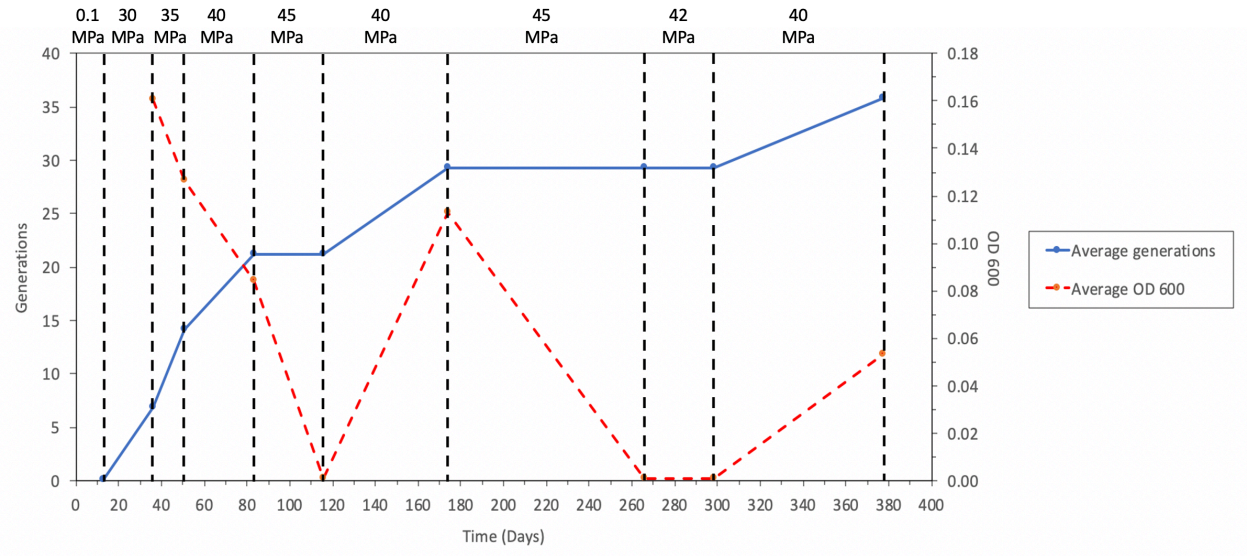


Figure 4. Growth of 10 m seawater community through adaptive laboratory evolution at high hydrostatic pressure at 5 °C. Optical density readings at 600 nm (OD 600) and the number of generations in ALE were obtained when the cultures were transferred to a higher incubation pressure. Each vertical black dashed line represents a change in pressure following each transfer. The blue dots represent the number of generations and the red dots represent the OD 600 readings at each transfer over the duration of the ALE experiment. The OD 600 readings were measured using a spectrophotometer and the numbers of generations were calculated using the OD 600 readings.

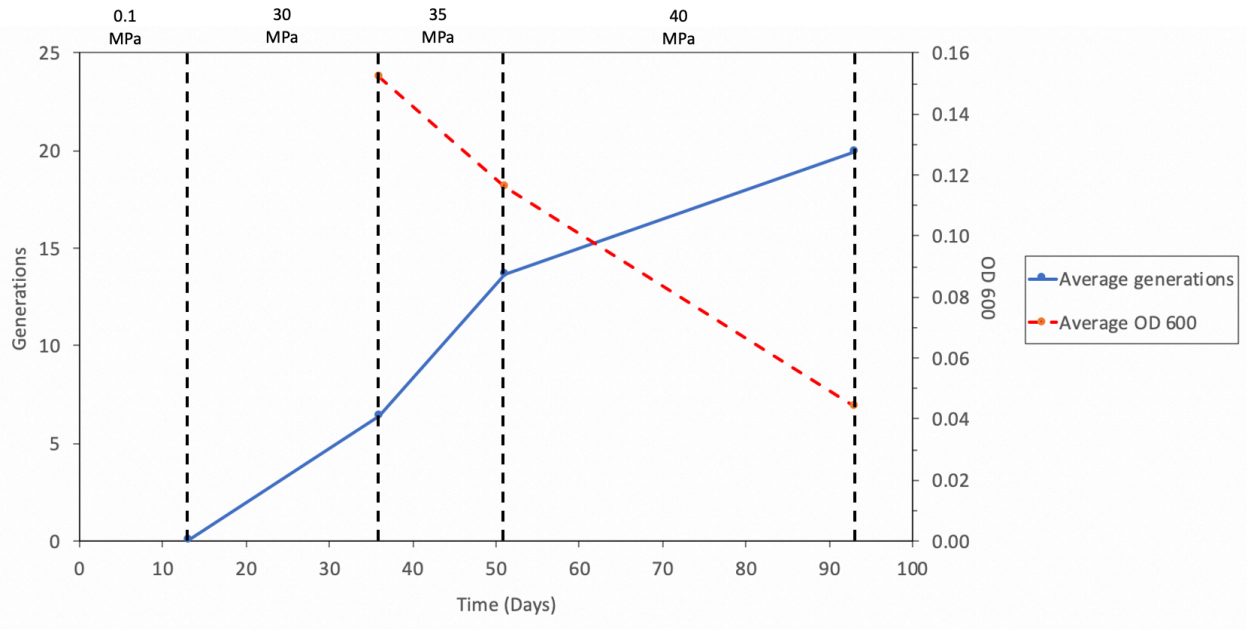
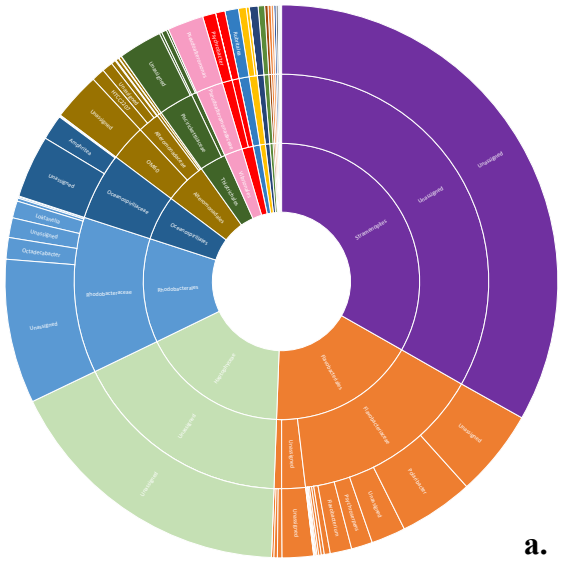


Figure 5. Growth of plankton tow community through adaptive laboratory evolution at high hydrostatic pressure at 5 °C. Optical density readings at 600 nm (OD 600) and the number of generations in ALE were obtained when the cultures were transferred to a higher incubation pressure. Each vertical black dashed line represents a change in pressure following each transfer. The blue dots represent the number of generations and the red dots represent the OD 600 readings at each transfer over the duration of the ALE experiment. The OD 600 readings were measured using a spectrophotometer and the numbers of generations were calculated using the OD 600 readings.

Figure 6. Changes in community diversity of 10 m seawater sample through ALE experiments **a.** Diversity of untreated10 m seawater sample evaluated using Illumina 16S tag sequencing. **b.** Diversity of 10 m seawater frozen stock evaluated using Illumina 16S tag sequencing. **c.** Diversity of 10 m seawater sample after initial incubation in AMS1 pyruvate evaluated using Illumina 16S tag sequencing. **d.** Diversity of 10 m seawater sample after initial incubation in Difco 2216 evaluated using Illumina 16S tag sequencing. **e.** Diversity of 10 m seawater sample after 25 MPa incubation in AMS1 pyruvate evaluated from 16S Sanger sequencing of 200 isolated colonies and colony morphologies. **f.** Diversity of 10 m seawater sample after 35 MPa incubation in Difco 2216 of 200 isolated colonies and colony morphologies.

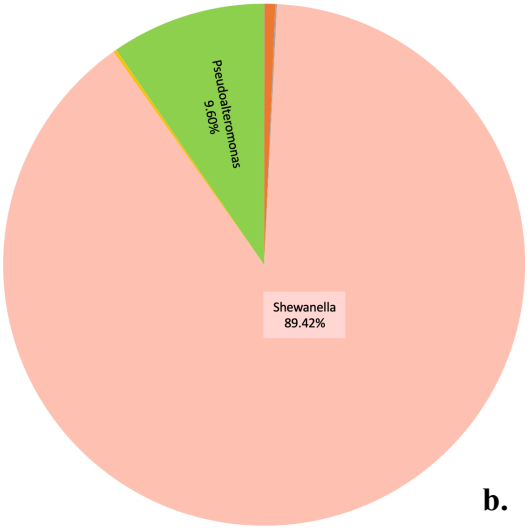

a.
b.
Acidimicrobiales
Bacteroidales
Cytophagales
Flavobacteriales
Sphingobacteriales
Saprospirales
Cryptophyta
Haptophyceae
Stramenopiles
Fibrobacterales
Phycisphaerales
Rhodobacterales
Rhodospirillales
Rickettsiales
Sphingomonadales
Methylophilales
Myxococcales
Sva0853
Alteromonadales
Chromatiales
Pseudoalteromonas
6.05%
Shewanella
92.96%
c.
Psychrobacter
5.875%
Flavobacterium
7.311%
Shewanella
86.71%
d.
Flavobacterium
4%
Shewanella
30.0%
Pseudoalteromonas
66.0%
e.
Shewanella
100.0%
f.
Unassigned
Flavobacterium
Alteromonas
Shewanella
Pseudomonas
Pseudoalteromonas
Psychrobacter

Figure 7. Changes in community diversity of plankton tow sample through ALE experiments
a. Diversity of plankton tow sample frozen stock evaluated using Illumina 16S tag sequencing. **b.** Diversity of plankton tow sample after initial incubation in AMS1 pyruvate evaluated using Illumina 16S tag sequencing. **c.** Diversity of plankton tow sample after initial incubation in Difco 2216 evaluated using Illumina 16S tag sequencing. **d.** Diversity of plankton tow sample after 35 MPa incubation in Difco 2216 of 200 isolated colonies and colony morphologies.

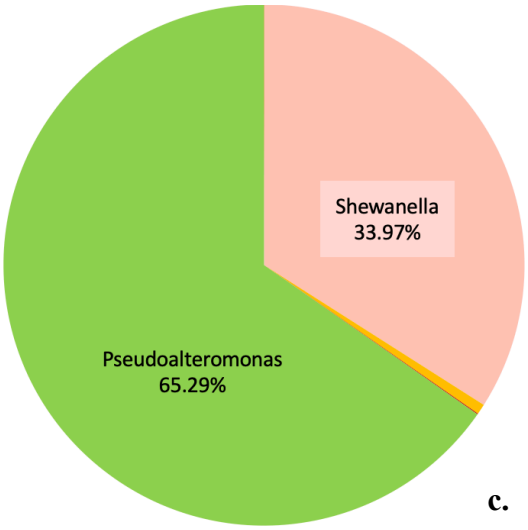


a.

Actinomycetales Cytophagales Flavobacteriales Saprospirales Haptophyceae Stramenopiles Chroococcales Lactobacillales Clostridiales BD1-5
Victivallales Caulobacterales Rhodobacterales Methylophilales Alteromonadales Legionellales Oceanospirillales Pseudomonadales Thiohalorhabdales Thiotrichales

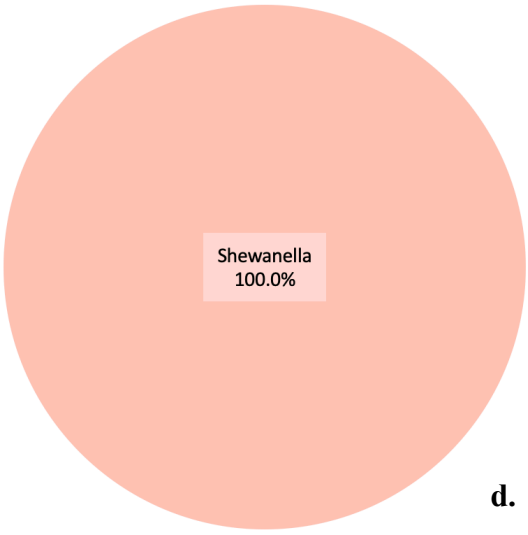


b.



c.

Unassigned Flavobacterium Colwellia Shewanella Marinomonas Oleispira Pseudomonas Pseudoalteromonas Psychrobacter



d.

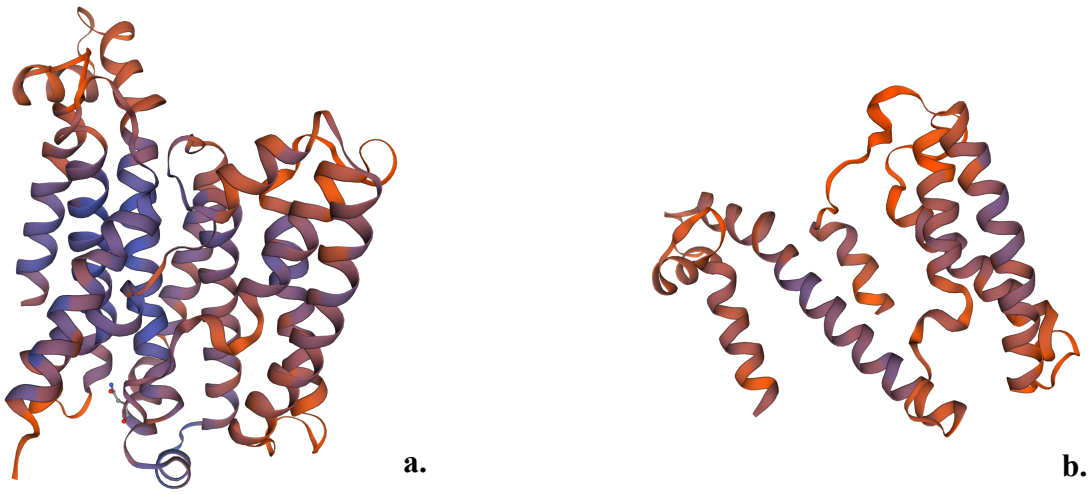


Figure 8. Modeled proteins of the *PvuC* gene in *Psychrobacter* strain SL016 and *Psychrobacter* strain SL016 50A. a. Protein structure modeled using SWISS modeling tool of the *PvuC* gene in *Psychrobacter* strain SL016. **b.** Protein structure modeled using SWISS modeling tool of the *PvuC* gene in *Psychrobacter* strain SL016 50A.

TABLES

Table 1. List of isolates used for characterization and ALEs

Sample #	Sample Origin	Strain Names
1	Jetty seawater	*Colwellia* strain SL001
2	Jetty seawater	*Psychromonas* strain SL002
3	Jetty seawater	*Colwellia* strain SL003
4	McMurdo aquarium seawater	*Moritella* strain SL004
5	McMurdo aquarium seawater	*Moritella* strain SL005
6	McMurdo aquarium seawater	*Pseudoalteromonas* strain SL006
7	McMurdo aquarium seawater	*Colwellia* strain SL007
8	McMurdo aquarium seawater	*Moritella* strain SL008
9	McMurdo aquarium seawater	*Pseudoalteromonas* strain SL009 + *Moritella* strain SL010
10	McMurdo aquarium seawater	*Moritella* strain SL011
11	McMurdo aquarium seawater	*Colwellia* strain SL012
12	McMurdo aquarium seawater	*Moritella* strain SL013
13	McMurdo aquarium seawater	*Colwellia* strain SL014
14	Plankton tow sample	*Loktanella* strain SL015
15	Plankton tow sample	*Psychrobacter* strain SL016
16	10m Seawater sample	*Shewanella* strain SL017

Table 2. Preferred growing temperature and pressure of the 13 samples used for characterization

Sample #	Sample Origin	Strain Names	Preferred Temperature	Preferred Pressure
1	Jetty seawater	*Colwellia* strain SL001	5°C	0.1MPa
2	Jetty seawater	*Psychromonas* strain SL002	5°C	0.1MPa
3	Jetty seawater	*Colwellia* strain SL003	17°C	0.1MPa
4	McMurdo aquarium seawater	*Moritella* strain SL004	5°C	0.1MPa
5	McMurdo aquarium seawater	*Moritella* strain SL005	5°C	0.1MPa
6	McMurdo aquarium seawater	*Pseudoalteromonas* strain SL006	17°C	0.1MPa
7	McMurdo aquarium seawater	*Colwellia* strain SL007	17°C	0.1MPa
8	McMurdo aquarium seawater	*Moritella* strain SL008	23°C	0.1MPa
9	McMurdo aquarium seawater	*Pseudoalteromonas* strain SL009 + *Moritella* strain SL010	17°C	0.1MPa
10	McMurdo aquarium seawater	*Moritella* strain SL011	23°C	0.1MPa
11	McMurdo aquarium seawater	*Colwellia* strain SL012	23°C	0.1MPa
12	McMurdo aquarium seawater	*Moritella* strain SL013	17°C	0.1MPa
13	McMurdo aquarium seawater	*Colwellia* strain SL014	17°C	0.1MPa

Table 3. *Breseq* predicted mutations in *Psychrobacter* strain SL016 50A and *Psychrobacter* strain SL016 50B

Predicted mutations in *Psychrobacter* strain SL016 50A					
mutation type	**scaffold name**	**position**	**mutation**	**annotation**	**gene description**
Insertion	scaffold10_size94658	87,913	$(TATTG)_{2\rightarrow3}$	coding (608/1077 nt)	Ferrichrome ABC transporter (permease) PvuC
Point Mutation	scaffold14_size71080	52,531	T→C	intergenic (+220/-157)	putative membrane protein/Cold shock protein of CSP family
Insertion	scaffold7_size112801	15,104	$(ATTATAAATCTCTCAATGC)_{1\rightarrow2}$	intergenic (-258/-213)	Peptide-methionine (R)-S-oxide reductase MsrB (EC 1.8.4.12) /Alanine transaminase (EC 2.6.1.2)

Predicted mutation in *Psychrobacter* strain SL016 50B					
mutation type	**scaffold name**	**position**	**mutation**	**annotation**	**gene description**
Point Mutation	scaffold16_size58137	25,879	C→T	P122L (CCT→CTT)	UDP-N-acetylmuramoyl-dipeptide--2,6-diaminopimelate ligase (EC 6.3.2.13)

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APPENDIX

Appendix A. **List of all high-pressure experiments.** Last column represents whether growth was observed after an unintended long period of incubation due to the COVID-19 pandemic. No living biomass was recovered from the final transfers.

All High Pressure Experiments						
Sample Name/Strain Name	**Media**	**Temperature (℃)**	**# Transfer**	**# Replicates**	**Last Incubation Pressure (MPa)**	**Growth Observerd (Y/N)**
Community transfers at atmospheric pressure						
10m SeaWater	Difco 2216	5	5	3	40	Completed
Plankton Net	Difco 2216	5	5	3	40	Completed
10m SeaWater	AMS1 Pyruvate	5	3	3	40	Completed
Plankton Net	AMS1 Pyruvate	5	4	3	40	Completed
Community ALE						
10m SeaWater	Difco 2216	5	5	3	40	Y
Plankton Net	Difco 2216	5	4	3	40	Y
10m SeaWater	AMS1 Pyruvate	5	4	3	30	Y
Plankton Net	AMS1 Pyruvate	5	3	3	25	Y
Single Strain ALE						
Psychrobacter	Difco 2216	23	6	2	55	Y
Psychrobacter	Difco 2216	5	5	2	35	Y
Loktanella	Difco 2216	23	4	2	35	Y
Loktanella	Difco 2216	5	4	2	35	Y
Psychromonas	Difco 2216	5	2	2	30	N
Psychromonas	AMS1 Pyruvate	5	2	2	30	Y
Psychromonas	AMS1 Glucose	5	2	2	30	Y
Shewanella	Difco 2216	5	3	2	35	Y
Shewanella	AMS1 Pyruvate	5	2	2	30	Y
Shewanella	AMS1 Glucose	5	2	2	30	Y
Psychrobacter	AMS1 Pyruvate	5	2	2	30	Y
Psychrobacter	AMS1 Glucose	5	2	2	30	N

Appendix B. List of Isolates and Corresponding Frozen Stock Position

Frozen Stock Position	Sample Origin	Strain Names
Bartlett Box 1 - 69	Jetty seawater	*Colwellia* strain SL001
Bartlett Box 1 - 70	Jetty seawater	*Psychromonas* strain SL002
Bartlett Box 1 - 71	Jetty seawater	*Colwellia* strain SL003
Bartlett Box 1 - 72	McMurdo aquarium seawater	*Moritella* strain SL004
Bartlett Box 1 - 73	McMurdo aquarium seawater	*Moritella* strain SL005
Bartlett Box 1 - 74	McMurdo aquarium seawater	*Pseudoalteromonas* strain SL006
Bartlett Box 1 - 75	McMurdo aquarium seawater	*Colwellia* strain SL007
Bartlett Box 1 - 76	McMurdo aquarium seawater	*Moritella* strain SL008
Bartlett Box 1 - 77	McMurdo aquarium seawater	*Pseudoalteromonas* strain SL009 + *Moritella* strain SL010
Bartlett Box 1 - 78	McMurdo aquarium seawater	*Moritella* strain SL011
Bartlett Box 1 - 79	McMurdo aquarium seawater	*Colwellia* strain SL012
Bartlett Box 1 - 80	McMurdo aquarium seawater	*Moritella* strain SL013
Bartlett Box 1 - 81	McMurdo aquarium seawater	*Colwellia* strain SL014
Christine Antarctica Box 1 - 22	Plankton tow sample	*Loktanella* strain SL015
Christine Antarctica Box 2	Plankton tow sample	*Psychrobacter* strain SL016
Christine Antarctica Box 2	High Pressure 2216 ALE isolate	*Psychrobacter* strain SL016 50A
Christine Antarctica Box 2	High Pressure 2216 ALE isolate	*Psychrobacter* strain SL016 50B
Christine Antarctica Box 1 - 33	10m Seawater sample	*Shewanella* strain SL017