UNIVERSITY OF CALIFORNIA SAN DIEGO

Adaptive laboratory evolution of Gram-positive *Carnobacterium sp.* AT7 under high hydrostatic pressure.

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in

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by

Flora Tang

Committee in charge:

Professor Douglas H. Bartlett, Chair
Professor Justin Meyer, Co-Chair
Professor Sergey Kryazhimskiy

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The Thesis of Flora Tang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

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ABSTRACT OF THE THESIS

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Professor Douglas H. Bartlett, Chair

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The ocean makes up over 97% of the Earth's biosphere and much of it is located within deep-sea habitats. Life at these depths must possess adaptations for growth at high hydrostatic pressures. Microorganism that grow preferentially at elevated pressures are termed "piezophiles." Although prior studies have examined the piezophilic adaptations of Gram-negative microbes, little attention has been given to Gram-positive bacteria. In this project, I examined one of the few Gram-positive piezophiles, *Carnobacterium* sp. AT7. Two lineages of AT7 were subjected to progressively increasing hydrostatic pressures over the course of 130 generations. From these adaptive laboratory evolution (ALE) lineages, I

obtained mutant strains which possessed improved rates of growth at high pressure and increased upper pressure limits. During these experiments, it was discovered that incubation of AT7 under stressful, high hydrostatic pressure for approximately 200 hours resulted in the acquisition of greatly improved high pressure growth in a single incubation cycle. Interestingly, cells derived from these incubations did not display stable high pressure growth and are presumed to have gained this ability through a physiological rather than genetic mechanism. Because it is well known that high pressure growth requires the production of sufficient proportions of membrane unsaturated fatty acids, it was investigated whether the long-term incubation cultures display increased unsaturated fatty acid fractions. Surprisingly, the *Carnobacterium* high pressure growth mutants do not produce increased proportions of unsaturated fatty acids. It was likewise discovered that these ALE-derived mutants do not possess alterations in cell size or shape. This thesis provides data and strains with which to begin to shed light on the processes employed by Gram-positive bacteria to adapt to elevated high pressure conditions.

INTRODUCTION

The ocean makes up over 97% of the biosphere, making it a crucial environment for understanding life on earth. When scientists study the deep ocean, environmental factors such as nutrient availability, temperature, pH, and oxygen levels must be considered. Another critical parameter influencing marine microbial growth is hydrostatic pressure (Dworkin 2006, Yayanos 2001). At sea level, the atmospheric pressure is 1 atm, or 0.1 megapascals (MPa). Hydrostatic pressure increases by approximately 0.1 MPa per 10 meters below sea level. With a mean ocean depth of 3,800 meters, the average hydrostatic pressure of the ocean is approximately 38 MPa (Abe 2001), which is 380 times the amount of pressure faced under what we, as humans, consider "normal" pressure conditions. At such high pressures, marine organisms must find ways to survive and reproduce. Microbes that grow optimally under high hydrostatic pressures are termed "piezophiles."

The word "piezophile" is one of many terms used to describe the various bacterial responses towards growth under pressure. As the name indicates, piezosentitive microbes are sensitive to high pressures and grow best at 0.1 MPa. Piezotolerant organisms grow preferentially at atmospheric pressure, but are capable of growth at higher pressure. Finally, obligate piezophiles grow optimally under high pressure and are incapable of growth at atmospheric pressure (Abe 2001). Many piezophiles also thrive in low-temperature conditions, hydrothermal vent thermophiles being a notable exception, and are referred to as "psychropiezophiles" (Birrien 2011, Yayanos 1995).

Hydrostatic pressure can interrupt many important functions in the cell. These include protein synthesis, transcription, translation, nutrient uptake, and phospholipid membrane fluidity (Abe 2007). Piezophiles must adapt to high hydrostatic pressures to combat these detrimental

effects. At stressful elevated hydrostatic pressures which do not preclude growth, piezosensitive microbes develop elongated filaments (ZoBell 1964). Under these conditions, the FtsZ-rings, large multimers of tubulin-like proteins that localize to the site of cell division, are absent or reduced in numbers and cell division is inhibited (Ishii 2004). Other proteins are also affected throughout the cell when faced with high pressure conditions. Altered protein function may result from hydration changes and void volume compression, which in turn affects the overall structure and function of enzymes. Relatively little is known about the evolution of improved protein function in piezophiles. However, the accumulation of certain compatible solute osmolytes appear to provide a counter balance to hydrostatic pressure (Bartlett 1999, Ichiye 2018). The Gram-negative bacteria *Photobacterium profundum* sp. SS9, a model piezophile, accumulates oligomers of beta-hydroxobutyrate under high pressure conditions (Martin 2002). Deep-sea animals are well known to accumulate high levels of trimethylamine-N-oxide, which are thought to stabilize the hydration of proteins (Yancey 2014, Yancey 2018).

High pressure also affects DNA structure and function. For example, stressful pressures can lead to nucleoid condensation (Sato 2002, Wilton 2008). In the case of *Escherichia coli*, DNA synthesis is inhibited at around 50 MPa, perhaps because of the inhibition of the initiation of replication (Welch 1993, Wilton 2008, Yayanos 1969). In the case of the piezophile *P. profundum* SS9, disruption of the gene for the DiaA positive regulator of DNA synthesis results in pressure-sensitive growth and conversely, disruption of the gene for the SeqA negative regulator of DNA synthesis results in high pressure-enhanced growth (El-Hajj 2009). Translation has also been observed to be inhibited by pressure in *E. coli,* cutting off at about 58 MPa. This appears to result from effects on both ribosome stability and translocation along mRNA (Gross 1990, Schwarz 1972, Welch 1993). The helices of the 16S rRNA are elongated in most

piezophiles, including *P. profundum* SS9 and *Shewanella* species, which may correlate to improved ribosome assembly and function. (Lauro 2007). *Pyrococcus yayanosii*, an obligate piezohyperthermophile found at hydrothermal vents, increases the production of tRNA and ribosomal units with pressure, perhaps as an adaptation to reduced ribosome and tRNA function under high pressure conditions (Michoud 2016). Transcription, the process by which RNA polymerases synthesize messenger RNA, seems to be least affected by pressure; it is only inhibited in *E. coli* around 77 MPa (Welch 1993).

By far, the most well-studied trait of piezophiles is altered membrane fatty acid content. Phospholipid membranes perform a variety of critical cell functions including the exchange of molecules with the extracellular environment, and a location for certain protein-protein interactions, cell signaling, and cell division (Parsons 2011). However, under high pressures, the phospholipids are laterally compacted and shift from a liquid crystalline phase toward a gel phase, causing the entire membrane to become more rigid. This compromises many aspects of membrane function, including electron and proton transport, cell division, ect... (Bartlett 1992)

Fatty acids have a terminal carboxyl group connected to a hydrocarbon chain, and can be classified by the length of the carbon chain and number of double bonds present. The presence of double bonds in the hydrocarbon chain separates fatty acids into two major groupings: saturated and unsaturated fatty acids. Saturated fatty acids, or SFAs, lack double bonds and are generally organized as linear molecules. Because of their linear shape, SFAs stack tightly, thereby encouraging greater membrane rigidity. However, unsaturated fatty acids, UFAs, have one or more carbon-carbon double bonds. These double bonds create more rigid bonds in the carbon chain, which give the molecule a “bent” shape. Because of this, unsaturated fatty acids are unable to stack together as tightly as the linear saturated fatty acids, thereby enhancing

membrane fluidity. If there is more than one double bond, fatty acids are referred to as polyunsaturated fatty acids (PUFAs). All characterized piezophilic bacteria use the properties of unsaturated fatty acids to their advantage by increasing the ratio of UFAs (including PUFAs) to SFAs under high hydrostatic pressure. With an increased concentration of UFAs, packing of phospholipids is diminished and membrane fluidity is restored. *P. profundum* SS9 has been shown to require UFAs, specifically monounsaturated fatty acids 16:1 and 18:1, for growth under high hydrostatic pressure. Eicosapentaenoic acid, a 20:5 PUFA known as EPA, also increases in concentration at high pressure in SS9 and other piezophiles such as *Psychromonas* strain CNPT3 and *Shewanella violacea* (Allen 1999, DeLong 1985, Usui 2012). Curiously EPA is not critical to high pressure growth of *P. profundum* SS9 but is for *S.violacea*. EPA is commonly synthesized in cells through the *pfaA-D* genes (Allen 2002, Usui 2012). The production of unsaturated lipids for adaptation in high hydrostatic pressure environments is not limited only to piezophilic Gram-negative bacteria, but also extends to deep-sea archaea and deep-sea eukaryotic organisms, such as fish (Cario 2015, Cossins 1989).

There are two separate categories of fatty acid biosynthesis, the FASI pathway and the FASII pathway. The FASI pathway is primarily used by animals, while prokaryotes use the FASII pathway. Gram-positive and Gram-negative bacteria have differences in their FASII systems. For example, in Gram-negative bacteria, both the FabA and FabB enzymes are necessary for the for desaturation of saturated fatty acids (Cronan 2009). However, these genes are not present in Gram-positive bacteria. They must find substitutes to produce the unsaturated fatty acids necessary for regulating membrane fluidity. *S. pneumoniae*, a Gram-positive pathogen most well-known as a leading cause of pneumonia, utilizes a FabM isomer to introduce double bonds into the fatty acid chain at a later step in fatty acid biosynthesis pathway (Marrakchi 2002,

Cronan 2009). Other bacteria, such as *Bacillus subtilis,* produce fatty acid desaturases, which are also capable of synthesizing unsaturated fatty acids in an oxygen-dependent manner (Aguilar 1998, Cronan 2009). Meanwhile, *Enterococcus faecalis*, a Gram-positive lactic-acid bacteria, relies on FabZ and FabF homologs that are capable of introducing the double bonds necessary for unsaturated fatty acids into the carbon chains. The FabZ homolog, a dehydratase enzyme, was found to have similar functions to FabA and the FabF homolog was predicted to be a potential replacement for FabB function (Wang 2004, Cronan 2009).

Once the acyl-ACP molecules are made the acyl groups must be attached to glycerol-3-phosphate (G3P), a product of the Calvin Cycle. Most Gram-negative microbes accomplish this using the PlsB pathway, while Gram-positive bacteria use the PlsX/Y pathway. The PlsX/Y pathway converts acyl-ACP into acyl-phosphates, which can initiate the phospholipid formation by acylating G3P (Lu 2006, Yao 2017).

The first step in understanding microbial adaptation to stress is to find the genes and proteins involved. Genome-wide collections of deletion or insertion mutants of piezosensitive *E. coli* and piezophilic *P. profundum* SS9 have been used to identify genes whose loss of function affects growth at high pressure (Lauro 2008, Black 2013). *P. profundum* SS9 transcriptome and proteome studies have also been used to identify genes and proteins regulated by hydrostatic pressure (Le Bihan 2013, Campanaro 2012). Comparative genomics of piezophiles and related non-piezophiles is yet another approach (Pradel 2013, Zhang 2019, Vezzi 2005).

A complementary approach to finding genes and gene products involved in stress adaptation is the use of adaptive laboratory evolution (ALE). ALE involves the sequential culturing of microbial populations under a set of defined conditions for an extended period (Dragosits 2013). The most famous ALE experiment is Richard Lenski's long-term *E. coli*

experimental evolution project, which has extended to over 60,000 generations over a 30+ year time span (Lenski 2018). ALE has a variety of applications. Strains can be developed which possess improvements for biotechnology utilization. ALE populations and strains in concert with genome re-sequencing can be used to follow evolutionary processes of fundamental significance, and to identify mutations in genes which correlate with improved fitness. If the strains being used for ALE are genetically tractable then the possible role of gene alterations of interest can later be experimentally addressed, and those changes which are necessary and perhaps sufficient for adaptation discovered.

In a 2014 study, scientists performed adaptive laboratory evolution of piezosensitive *E. coli* under high hydrostatic pressure. They successfully obtained a single mutant, AN62, that showed improved growth at high hydrostatic pressures, including those beyond the pressure limit of the parental strain, and decreased growth ability at atmospheric pressure. AN62 also possessed an overall increase of unsaturated fatty acids, specifically cis-vaccenic acid (18:1). Since *E. coli* is one of the most common model organisms in microbiology, its genetic makeup has been extensively characterized, making it easy to examine genes of interest for mutations. In this case, PCR amplification and sequencing revealed a mutation in AN62 in the *acP* gene, which codes for the acyl carrier protein. This is a phosphopantetheine-containing protein that covalently binds all fatty acid intermediates and is involved in their chain extension during fatty acid biosynthesis. The changes in growth and fatty acid composition found in the 2014 study fits well with our current knowledge of piezophilic adaptations. However, this project did not include genome wide analyses of mutations in AN62 or any experimental verification of mutations required for improved high pressure growth (Marietou 2014).

Though high pressure studies have placed their focus on Gram-negative bacteria such as *Photobacterium* sp. SS9 and *E. coli*, little attention has been given to Gram-positive microbes. Gram-positive bacteria largely fall into two phyla, Firmicutes and Actinobacteria (Woese 1986). While Gram-negative microbes have two lipid membranes and a thin peptidoglycan protein layer in-between, Gram-positive bacteria have a single lipid membrane with an incredibly thick peptidoglycan layer (Madigan 2006). Astonishingly, there are not many known Gram-positive piezophilic species. Previously, the only known Gram-positive piezophiles were *Carnobacterium* sp. AT7 and AT12 (Lauro 2007) It wasn't until 2017 that more Gram-positive piezophiles were discovered and isolated from deep-subsurface sediments (Fang 2017). It is unknown why there is a higher proportion of Gram-negative piezophiles than Gram-positives. The difference in membrane bilayers and the thickness of the peptidoglycan layer are two factors of interest in Gram-positive adaptation to pressure.

Although no information yet exists on the mechanisms of Gram-positive bacteria adaptation to growth at high pressure, studies have addressed the sensitivity of Gram-positive bacteria to killing at ultra-high hydrostatic pressures, such as that used during pascalization of foods. These experiments demonstrate that Gram-positive strains are more resilient to ultra-high hydrostatic pressure than Gram-negative bacteria (Considine 2008, Smelt 1998). These results make it even more surprising that only a few Gram-positive organisms piezophiles have been cultured from deep-ocean samples (Fang 2017).

This thesis focuses on a deep-sea member of the *Carnobacterium* genus. Many *Carnobacterium* species have been observed to grow under many types of extreme environments. These include the guts of fish, in permafrosts, wetlands, food products, polar lakes, etc (Leisner 2007). One of the first Gram-positive piezophiles to be discovered,

Carnobacterium sp. AT7, was isolated in a water column sample from the Aleutian Trench 2500 meters below sea level (Lauro 2007). Though it grows and reproduces optimally at 15 MPa, AT7 has been documented to be capable of growth between 0.1 MPa and 60 MPa (Lauro 2007, Sebert 2010). It is also an ideal organism for studying mutations arising during ALE experiments due to its small genome size of 2.4 Mb, encoding 2388 proteins (www.genomesonline.org, Liolios 2006).

Since there are very few Gram-positive piezophiles, an objective of this study was to use ALE to force *Carnobacterium* sp. AT7 past its current pressure limit for growth and to utilize the resulting strain derivatives as a resource to better understand the process of high pressure adaptation in Gram-positive bacteria. Through adaptive laboratory evolution, hydrostatic pressure was slowly increased for two sets of AT7 lineages to select for mutants that showed increased high pressure growth fitness beyond the limit of the parental AT7 strain. Two mutants were characterized regarding growth, fatty acid composition, and morphology.

MATERIALS AND METHODS

Growth conditions

Carnobacterium sp. AT7 was isolated in a water sample from the Aleutian Trench at 2500 meters below sea level (DeLong 1986). Three different media – TSY, TSBYS, Difco 2216 (Nicholson 2015, Nicholson 2013, Lauro 2007) – and three different temperatures – 17°C, 23°C, 30°C – were tested to determine the best growth conditions. This was done at atmospheric pressure. After these tests, all cultures were grown in TSBYS (trypticase soy broth yeast extract salt) at 23°C (Nicholson 2013). High pressure cultures were grown in stainless steel, pin-closed pressure vessels (Yayanos 2001).

Adaptive laboratory evolution

Adaptive laboratory evolution was set up in duplicate lineages (A/B), along with a negative control (Fig. 1). These cultures were labeled with initials (FT), transfer number, and lineage (A/B). For example, the first culture was labeled FT1A and FT1B. The initial incubation was at 20 MPa and in 15 mL polyethylene transfer pipette bulbs. When biomass was observed, the bulbs were removed and the optical density (A600 nm) was tested using cuvettes and a GENESYSTM 10S UV-Vis Spectrophotometer. The optical density and incubation time of each transfer was used to estimate cell concentration and doubling time, which was then used to calculate the number of generations during each period. At each sub-culturing step, 15% glycerol frozen stocks were made for future analysis and stored at -80C. In addition, 16S rRNA gene PCR analysis and sequencing was done to double check to make sure there was no contamination. The remaining culture was diluted 1:100 and re-incubated at high pressure. When the optical density of the culture was above 0.400, the pressure utilized for the next cycle of high pressure

incubations was increased by an increment of 5 MPa (Fig. 1). At the fifteenth transfer (FT15), the cultures were grown in 50 mL polyester Kapak SealPAK bags with a 1:50 dilution.

Growth Analysis of Select Evolved Strains

To determine the growth characteristics of strains within the ALE lineages, colony isolates were sub-cultured and growth curve analyses were performed at both atmospheric pressure and elevated pressure. For these experiments, nine time points were taken in triplicate using 5 mL polyethylene pipette bulbs for each optical density (A600 nm) measurement. The growth curves were performed at 60 MPa. The average at each time point and standard error was calculated.

An aliquot of the parental and FT14B1 strain from the 200+ hour incubation at 60 MPa was used to prepare frozen stock and streak plates. Colony isolates grown on these plates were sub-cultured and the growth curve analysis at 0.1MPa and 60MPa was repeated for another incubation cycle.

Colony and Cell Morphology

To view any differences in colony morphology, cultures were streaked on TSBYS plates and incubated at room temperature for 48-72 hours. Colony size, color and texture were visually inspected. Images were captured with a Samsung S9.

To view any changes in cell morphology, cultures were grown and fixed under *in situ* pressure conditions (Marietou 2014, Fig. 2). The positive control, *E. coli* K-12 MG1655, was grown in 20 mL of LB media at 30 MPa and 37C for three days, and was fixed with 37% formalin (2.20 mL of formalin in 17.80 mL LB), so that the final concentration would be 2% formalin. The fixed culture was diluted 1:200 and 1:500 and filtered onto 0.2 um filters, which were stored at 4C. For microscopy, filters were stained with 4’6-diamidino-2-phenylindole

(DAPI) stain, and viewed under an Olympus BX51 fluorescence microscope. Images were captured with an iPhone X.

Fatty Acid Composition Analysis

Fatty acid composition was analyzed for the parental strain, and selected strains FT14A1 and FT14B1. For a single sample, two 15 mL bulbs were filled with inoculated media and grown until estimated mid-exponential phase at either atmospheric or elevated pressure/60 MPa, for a total volume of 30 mL of culture. The two bulbs were removed from the pressure vessels and transferred to a sterile 50 mL falcon tube, which was spun down and decanted to isolate a cell pellet. The pellets were freeze dried for 5-6 hours. The fatty acids were extracted through a methyl esterification derivation protocol and the samples were run on an Agilent 5977B GC-MSD (Allemann 2018). The GC/MSD ran on spit mode, not spit-less, as mentioned in the FAME GC-MS protocol; all temperatures and other settings remained the same (Allemann 2018). The area under each peak was calculated on the connected software and copied onto an excel file for further analysis. This procedure was performed in triplicate for each sample. The positive control for these measurements was *Photobacterium* sp. SS9R, since its fatty acid characteristics has been previously determined (Allen 1999). It was incubated under atmospheric pressure and 30 MPa at 17ºC for 72 hours. The media used for SS9R was Difco Marine Broth 2216 with glucose (22 mM) and HEPES buffer (100 mM). For each fatty acid peak, the area was calculated based on the area under the curve over the total area of all peaks. Since this was performed in triplicate, the average of peaks was calculated, as well as standard error.

Genetic Analysis / Bresequencing

To determine the potential evolutionary changes in the variant genome that allowed for better growth at high hydrostatic pressure, the genome of FT14A1 and B1 were compared to the

parental strain, along with the whole reference genome via bresequencing. Cultures from frozen stock were streaked onto a TSBYS agar plate and select colony isolates were grown in fresh media. The DNA was extracted from six of these cultures – FT1A1, FT1B1, FT14A1, FT14B1, FT16A1, FT16B1 -- using the DNeasy UltraClean Microbial kit and the genomes were sequenced through Illumina HiSeq 4000 with 50-100X coverage. The sequences will be tested against the reference genome, currently 69 contigs, in breseq version 0.33.1 (Deatherage 2014).

RESULTS

Overview of cultivation through adaptive laboratory evolution

Cultures of lineages A and B began at 20 MPa and, by the seventeenth transfer, reached 70 MPa. Both underwent adaptive laboratory evolution for a period of 381 days. At each point of transfer, the optical density was measured to estimate the number of generations (black solid line, Fig. 3). In this time span, the cultures underwent approximately 130 generations (red dotted line, Fig. 3). Both lineages showed rapid growth from 20 MPa to 55 MPa. Starting from 60 MPa, the lineage growth rates exhibited more dramatic reductions. The cultures were continued at 60 MPa for another five sets of transfers before the decision was made to further increase the pressure to 65 MPa. At the seventeenth transfer, cultures were moved from 15 mL bulbs to 50 mL bags and the dilution was decreased from 1:200 to 1:50 to increase the chance for further adaptive mutations in the populations to be transferred from one round to the next. At the most recent transfer, FT20, at 70 MPa, cultures continued to show growth, but not enough to warrant an increase in pressure (Fig. 3).

Growth characteristics of the fourteenth transfer, FT14, for lineage A and B

After the 14th transfer and around 95 generations, colonies were isolated from both lineages and individual strains, designated FT14A1 and FT14B1, were selected for further characterization (Fig. 4). Both evolved strains exhibited similar growth to the parental strain at 23ºC and 0.1 MPa in bulb cultures, reaching stationary phase in about 50 hours (Fig. 4A-B). In contrast, at 60 MPa, FT14A1 and FT14B1 reached stationary phase in about 100 hours, during which time the parental strain displayed little growth. Thus, it appeared that the evolved strains had developed a higher upper pressure limit for growth. However, after prolonged incubation, starting at about 200 hours, the parental strain's growth rapidly increased, and by 300 – 400

hours the cultures had achieved densities nearly as great as that of the evolved strains. The improved high pressure growth of the parental strain was not stable. However, the FT14B1 strain sub-culture retained its enhanced high pressure growth phenotype. Curiously, the high pressure sub-culture of FT14B1 exhibited reduced growth ability at 0.1 MPa (Fig. 5).

Cell and colony morphology

To assess whether the FT14 evolved strains acquired morphological changes in conjunction with their improved high pressure growth capabilities their colonies and cells were further examined. Streak plates were obtained after each transfer to look for potential changes in colony morphology. However, there were no observed changes in colony size or shape for either lineage at all transfer stages, including strains FT14A1/B1 (Fig. 6).

The analysis of cell morphology was complicated by the fact that the high pressure-induced filamentation stress response can be quickly lost after decompression (Ishii 2004). As a result, the cells had to be fixed for microscopy while they were incubated at high pressure as described in the materials and methods. Cells were viewed by DAPI staining and fluorescence microscopy. *E. coli* was used as a positive control, as its filamentation response has been well studied (Ishii 2004, ZoBell 1964). At atmospheric pressure, *E. coli* did not show any filamentation; however, it was apparent at 30 MPa (Fig. 7). Microscopy of the *Carnobacterium* parental and FT14A1 and FT14B1 strains showed similar coccoid morphologies at low and high pressures, with no filamentation. At atmospheric pressure, the cells clustered together in large numbers. Under elevated hydrostatic pressure, the cells tended to be isolated, with little to no clustering (Fig. 7).

Fatty acid composition

The other phenotype that was assessed among the parental and FT14 evolved strains was their fatty acid profiles, since the production of increased unsaturated fatty acids with increasing pressure is a universal response among all tested piezophiles and the previously *E. coli* ALE at high pressure resulted in the generation of a strain with increased UFA levels (Marietou 2014). For these experiments *Photobacterium* sp. SS9 was used as a positive control. It displayed the expected phenotype; under high pressure, there was an increase in the saturated fatty acid 12:0, unsaturated fatty acids 16:1 and 18:1, and the polyunsaturated fatty acid 20:5. There was a decrease in saturated fatty acids 14:0, 16:0, and 18:0, and the unsaturated fatty acid 14:1. Overall, there was a 10.94% increase of unsaturated fatty acids when SS9 was incubated under high hydrostatic pressure (Table 3, Fig. 9).

The parental and FT14 strains showed no significant difference in fatty acid composition at atmospheric pressure. Under 60 MPa incubation, the mutant strain exhibited a decrease of unsaturated fatty acid 18:1. There was also an increase of saturated fatty acids 16:0 and 18:0. No PUFAs were detected in any of the samples (Fig. 8, Table 1, Table 2). Lineage B showed an overall 13.87% decrease in unsaturated fatty acids and lineage A showed a 16.50% decrease in unsaturated fatty acids (Table 1-2).

DISCUSSION

Previous studies have provided much insight into the adaptation of Gram-negative bacteria to the high-pressure conditions of the deep ocean. However, there have been no studies that have analyzed the mechanisms by which Gram-positive microbes grow at depth. Through adaptive laboratory evolution, two mutant strains from *Carnobacterium* sp. AT7 showing increased fitness under high hydrostatic pressure were successfully obtained. This was reflected in the mutant strains' ability to grow to a high optical density under high pressure conditions (60 MPa) that precluded significant growth of the parental strain, at least for about 200 hours. They are designated FT14A1 and FT14B1. The ALE experiments have continued beyond the transfer point in which these evolved strains were obtained. However, no strains from those generation periods have yet been examined.

Evolutionary adaptation versus physiological acclimation

Strains FT14A1 and FT14B1 exhibit stable, reproducible high pressure-enhanced growth phenotypes. It is reasonable to conclude that they have acquired mutations in their genomes that underlie these altered growth phenotypes. Though the pressure range of AT7 seems to have expanded beyond 60 MPa, it has not yet shifted away from atmospheric pressure growth.

But another high-pressure growth phenomenon may also be present in *Carnobacterium* sp. AT7. During single prolonged incubations, extending beyond 200 hours, it was discovered that it can gain the ability to grow at 60 MPa, which is at its previously described limit (Fig. 4, Lauro 2007). This does not appear to be the result of mutation, since subcultures of these populations exhibit the same extended growth delay at 60 MPa (Fig. 5). One possibility is that AT7 can eventually mount an adaptive response to an otherwise non-permissive high pressure. In further studies, after 300 hours, the 60 MPa cultures could be decompressed and immediately

diluted and re-incubated at high pressure, rather than allowing for the cells to re-acclimate to atmospheric pressure; this could potentially allow any physiological change to carry over into the next incubation cycle. It would also be interesting to further characterize this process, by examining changes in gene expression, protein synthesis, and osmolytes.

Morphology of cells

AT7 and the evolved strains all showed major differences in traits previously observed in Gram-negative piezophiles. For example, none of these cells appear to have a filamentation response at pressures below or above their pressure optima. Typically, cells show filamentation at stressful high hydrostatic pressure, at least at pressures which do not prevent biomass accumulation (ZoBell 1964, Ishii 2004). This indeed was observed in the case of *E. coli* (Fig. 6). Despite incubation and *in situ* fixation under atmospheric and high hydrostatic pressures, fluorescence microscopy of *Carnobacterium* sp. AT7 showed no change in cell morphology, and the cells retained their spherical shape. The only difference with pressure is that the cell clustering evident at low pressure did not occur at high pressure (Fig. 7). Colony morphology also remained unchanged (Fig. 6).

AT7 is not the first Gram-positive microbe to show a lack of change in the general morphology of the cell with pressure. Previously, *Leuconostoic mesenteroides*, another Gram-positive bacteria, showed no change in the overall shape of the cell under high pressure and only began to show blistering at the cell surface after 500 MPa treatment. The peptidoglycan layers also remained largely intact, even under the extreme pressure treatments (Kaletunç 2004). It should be noted that the pressure used in this case was far above that which would allow biomass accumulation to be possible.

Decrease of unsaturated fatty acids and increase of saturated fatty acids

Even more surprising than the lack of morphological changes with pressure among the *Carnobacterium* strains was the finding that the evolved strains do not increase their UFA/SFA ratios with increasing pressure. In fact, they decreased their UFA/SFA ratio, the complete opposite effect that has been seen in Gram-negative bacteria response to high pressure. Gram-negative piezophilic bacteria have previously been understood to increase the ratio of unsaturated to saturated fatty acids in response to high hydrostatic pressure, as a form of homeoviscous adaptation (Allen 1999, DeLong 1985, Usui 2012). Indeed, the analysis of *P. profundum* SS9 analysis, showed the fatty acid profile expected, increasing 16:1, 18:1, and 20:5, which are known, important unsaturated fatty acids for increasing membrane fluidity (Table 3, Fig. 9). It is also noteworthy that the evolved strains produced no PUFAs under any growth conditions (Fig. 8, Table 1-2). PUFAS are a characteristic of many, but not all Gram-negative piezophiles (DeLong 1986).

There are several possible explanations to be considered. One possible explanation is that some of the produced fatty acids are being applied to functions other than the cell membrane, such as storage (Cronan 2009). The extraction of fatty acids was not limited to the membrane, and so the analysis performed describe the overall fatty acid profile of the cell. Second, the differences in the outer layers of Gram-positive and Gram-negative bacteria could result in different needs for unsaturated fatty acids. Since Gram-negative bacteria have a thin peptidoglycan cell wall and two separate phospholipid membranes, the fluidity of the membrane might take on a greater role. In contrast, Gram-positive bacteria have one phospholipid membrane and a thick peptidoglycan layer. Typically, the peptidoglycan layer functions as a barrier from toxins in the extracellular environment, protection from osmotic pressure changes, and acts as a structural support for the cell (Madigan 2006). There have been very few studies

looking at the effects of high hydrostatic pressure to the peptidoglycan layer, since Gram-negative microbes have been the focus of piezophilic studies and the peptidoglycan layer has not yet shown any significant role in piezophilic adaptation (Kaletunç 2004). *Carnobacterium* sp. AT7, and potentially other Gram-positive bacteria, might rely more on the composition and thickness of the peptidoglycan layer for structural support and not be as reliant on membrane fluidity as an important factor in deep ocean adaptation. Finally, it may also be possible that *Carnobacterium* is employing something other than fatty acids to modulate its membrane fluidity. One example of such a type of molecule are hopanoid lipids (Kannenberg 1999). Evolved strain sequence analysis could provide some clues regarding high pressure membrane adaptation in this species.

Analysis of the AT7 drafted genome sequence prior to breseq

The genomes of FT14A1 and FT14B1 have been sequenced and will be later compared to the draft genome sequence of *Carnobacterium* sp. AT7, present as 69 contigs. Prior to obtaining the fatty acid profiling data, it seemed logical to presume that the evolved strains would have some alteration in fatty acid biosynthesis. As mentioned previously, Gram-positive bacteria lack the *fabA* and *fabB* genes and must utilize substitutes for increasing production of unsaturated fatty acids (Cronan 2009). Analysis of the AT7 genome suggests that *Carnobacterium* does not employ desaturases, such as those found in *B. subtilis*, present (Aguilar 1998). *FabM* (Marrakchi 2002) likewise does not appear to be present. However, both the *fabZ* and *fabF* genes have been identified. FabZ and FabF are important for saturated fatty acid biosynthesis. In *E. faecalis,* homologs of these genes are involved in unsaturated fatty acid synthesis (Wang 2004). Both *Carnobacterium* sp. AT7 and *Enterococcus faecalis* are within the order *Lactobacillales,* making *fabZ* and *fabF* genes of interest.

Additional genes to examine in the evolved strains include those involved in the construction of the peptidoglycan layer, since it might play a large role in the protection of Gram-positive cells under high hydrostatic pressure. Teichoic acids provide structural support to the peptidoglycan layer (Brown 2013), and increases in their production might lead to increased resistance to high hydrostatic pressures. Genes that have currently been found to be involved with teichoic acid synthesis and integration include *CAT7_01155, CAT7_06458*, and *CAT7_08565*.

Future Studies

The comparative genomics of FT14A1, FT14B1 with one another and their parental *Carnobacterium* sp. AT7 strain is the top priority. After generating a list of gene mutations, the focus will be on the nature of those genes mutated in both evolved strains.

Since I could successfully perform ALE on a piezophilic Gram-positive microbe, a future study could attempt to repeat this experiment with a piezosensitive Gram-positive bacteria. By studying an already-piezophilic bacteria, I potentially miss prior evolutionary mutations that popped up to confer the mild piezophilic adaptation in AT7. Further continuation of the adaptive laboratory evolution of *Carnobacterium* sp. AT7 could also potentially lead towards the rise of obligate piezophily, which could be further characterized and compared to this study's mutant strains of interest.

Eventually, mutations of interest could be further examined to ascertain if they are necessary or perhaps even sufficient for the high-pressure growth phenotype. Their parental gene sequence could be crossed into FT14A1 or FT14B1, or mutations present in these strains could be crossed into the parental strain or into related piezosensitive *Carnobacterium* species.

The apparent physiological adaptation of AT7 to high pressure mounted after ~ 200 hours of 60 MPa incubation also warrants further study. The stability of this response following decompression for short or long periods could be examined, and changes in the transcriptome / proteome of these cells as a function of pressure and incubation times assessed. It's also possible that post-translational changes, e.g. protein phosphorylation, account for this response.

It is difficult to predict what other experiments might help define the evolved strain phenotypes. Certain microscopic techniques, including scanning and transmission electron microscopy and cryotomography might also provide insight into membrane or cell wall changes. It might also be useful to examine nucleoid structure, as well as DNA replication, and the production of mRNA and proteins. Osmolytes might also be of interest -- given their influences on macromolecule stability and turgor pressure (Abe 2007, Bartlett 1999).

TABLES AND FIGURES

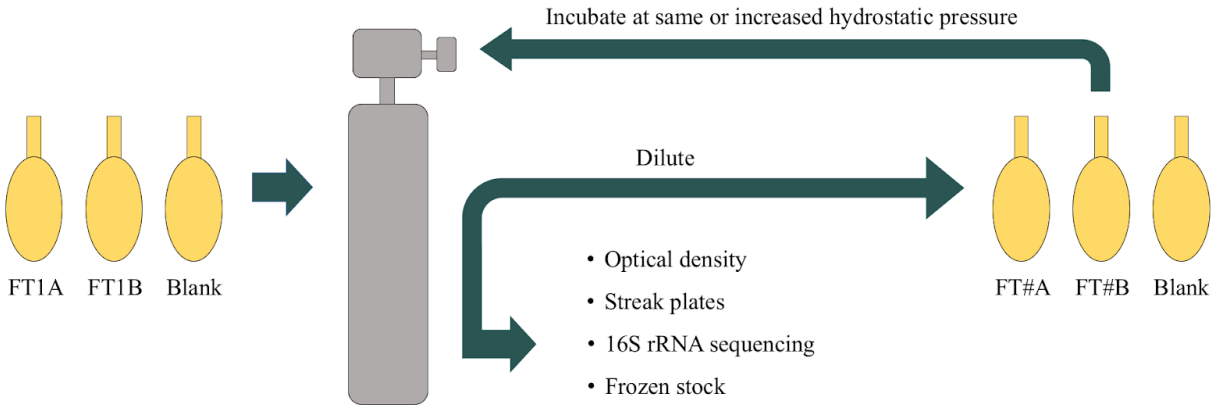


Figure 1. Diagram of adaptive laboratory evolution experiment under high hydrostatic pressure.

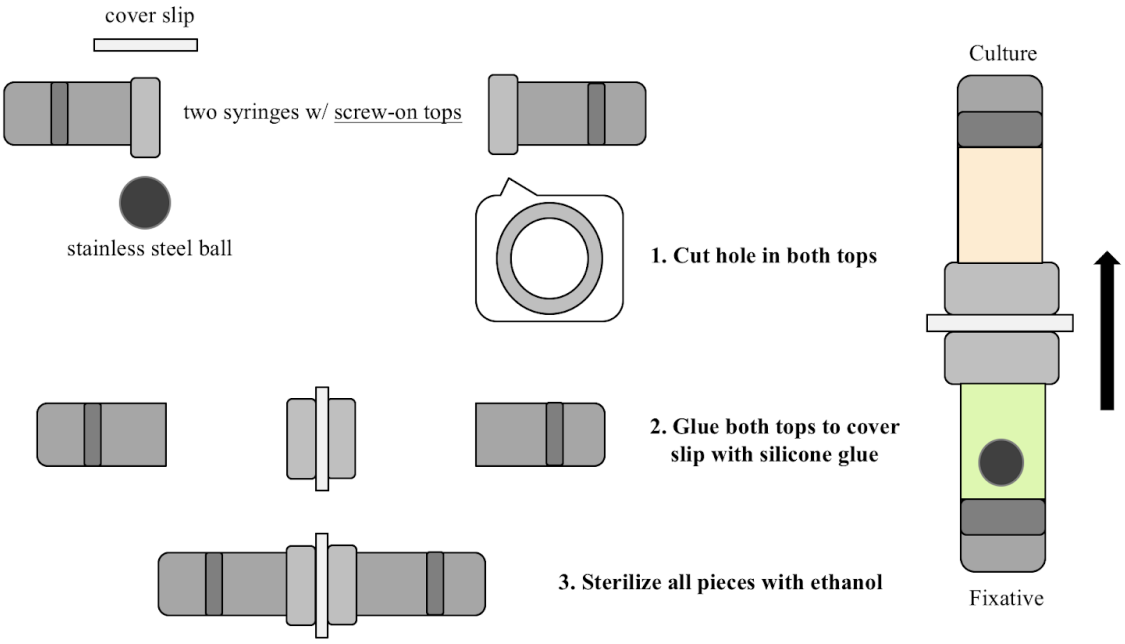


Figure 2. Diagram of *in situ* fixation under high hydrostatic pressure. The black arrow indicates the direction the incubation device should be placed under high pressure to prevent the stainless-steel ball from breaking the coverslip prematurely.

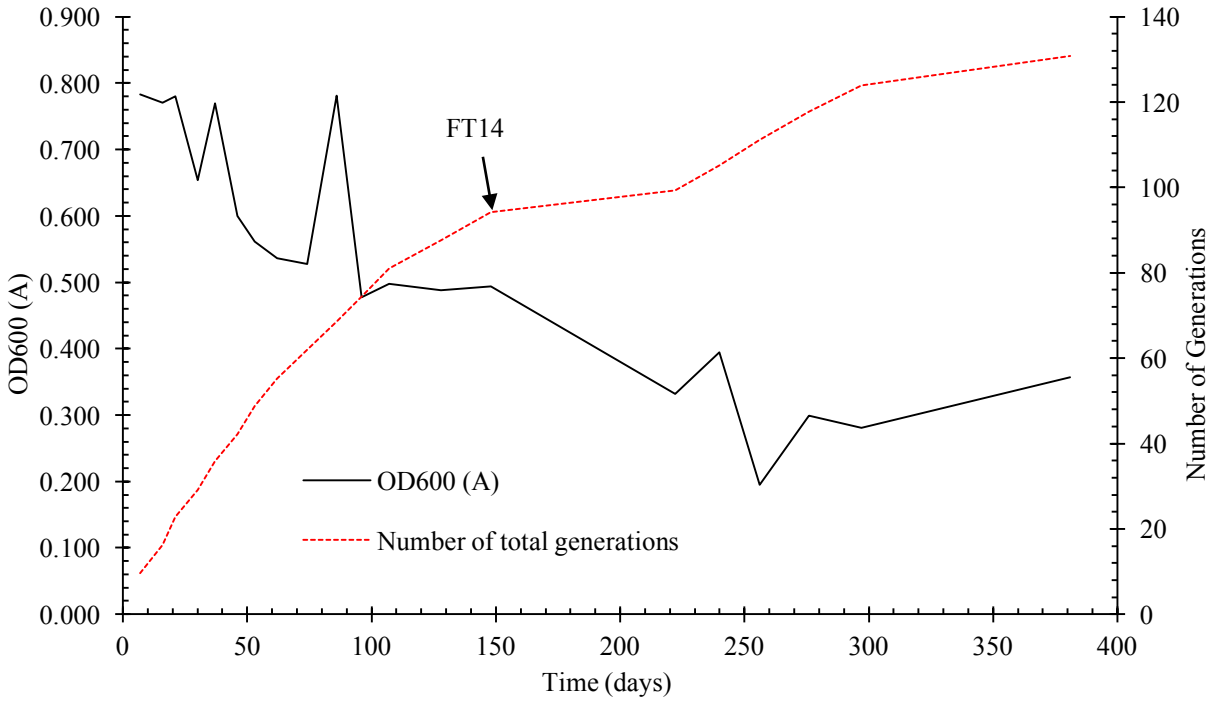


Figure 3. Overview of cultivation through adaptive laboratory evolution. The solid line represents the optical density at each transfer, while the dotted line represents the estimated number of generations – both in respect to time in days.

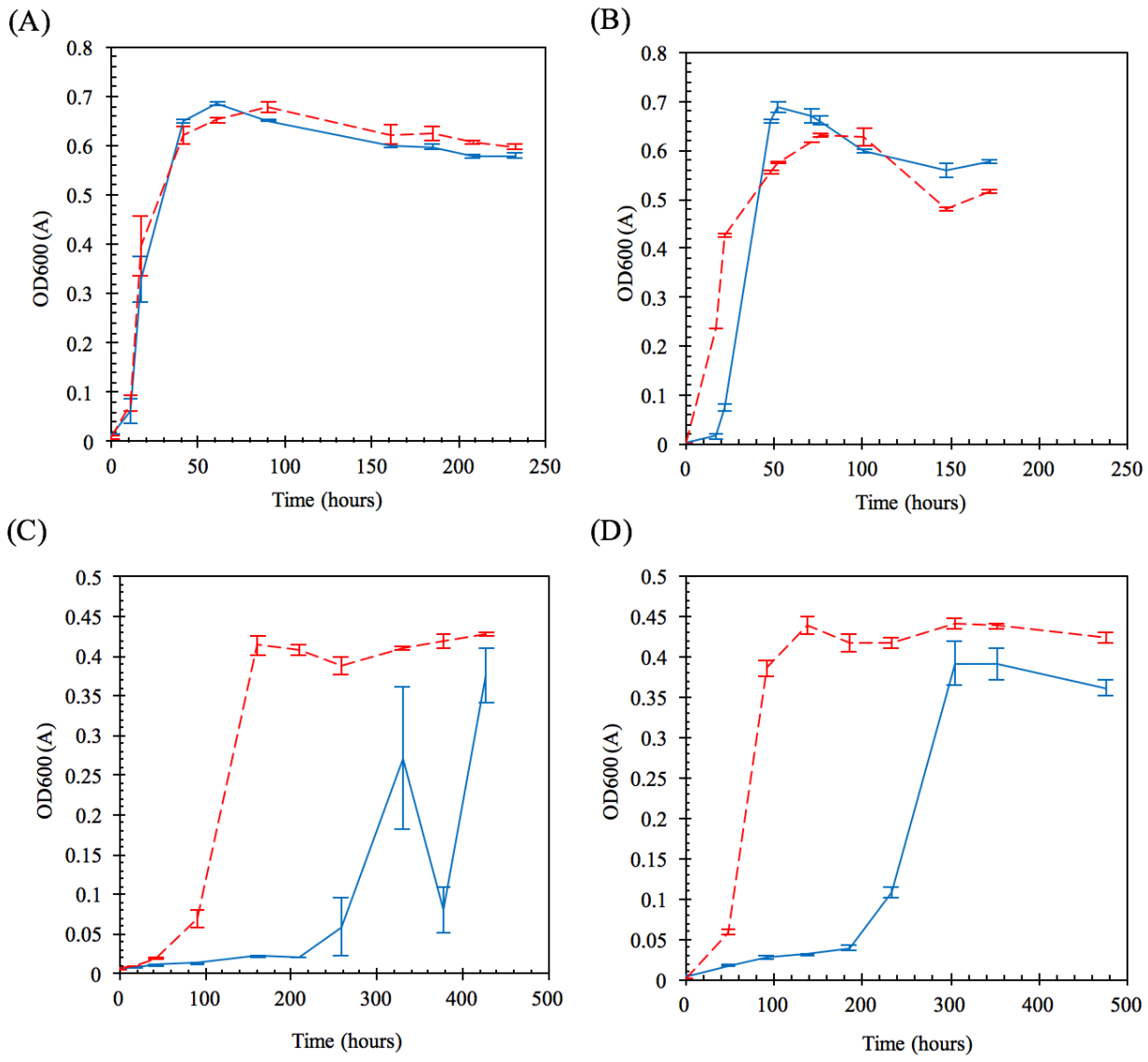


Figure 4. Growth curves of parental vs. mutant strains at atmospheric and elevated hydrostatic pressure. These four graphs show (A) parental vs. FT14A1 at 0.1 MPa, (B) parental vs. FT14B1 at 0.1 MPa, (C) parental vs. FT14A1 at 60 MPa, and (D) parental vs. FT14B1 at 60 MPa. The dotted lines represent the mutant strains, while the solid lines represent the parental strain. All cultures were incubated at room temperature, or 23°C.

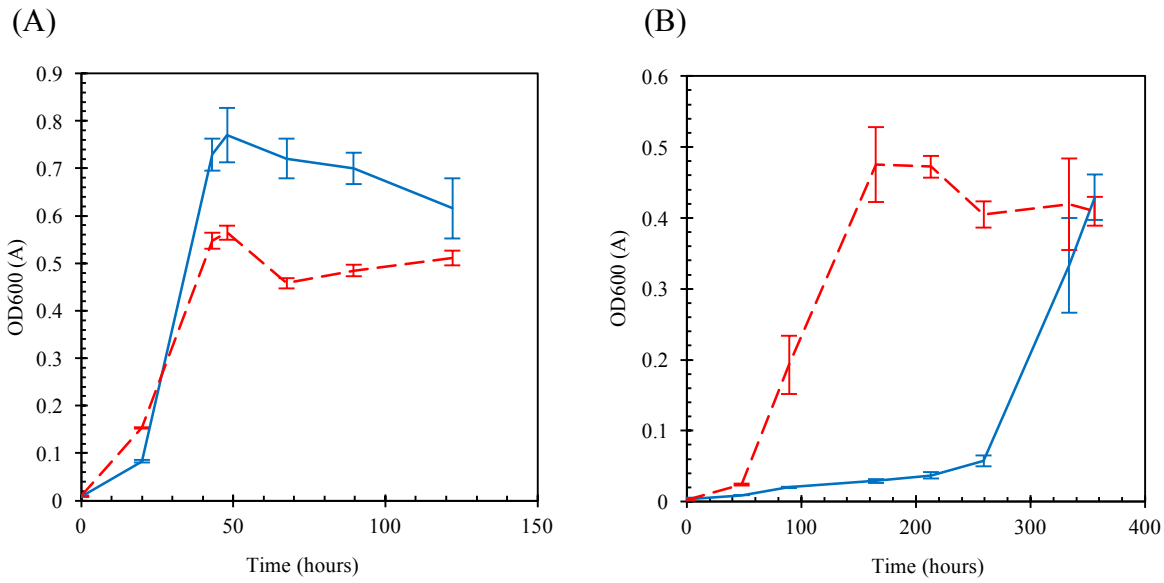


Figure 5. Growth curve of parental vs. FT14B1 at atmospheric and elevated hydrostatic pressure after prior incubation at 60MPa for 200+ hours. These two graphs show (A) parental vs. FT14B1 at 0.1 MPa and (B) parental vs. FT14B1 at 60 MPa. The red dotted lines represent the mutant strain, while the blue solid lines represent the parental strain. All cultures were incubated at room temperature, or 23°C.

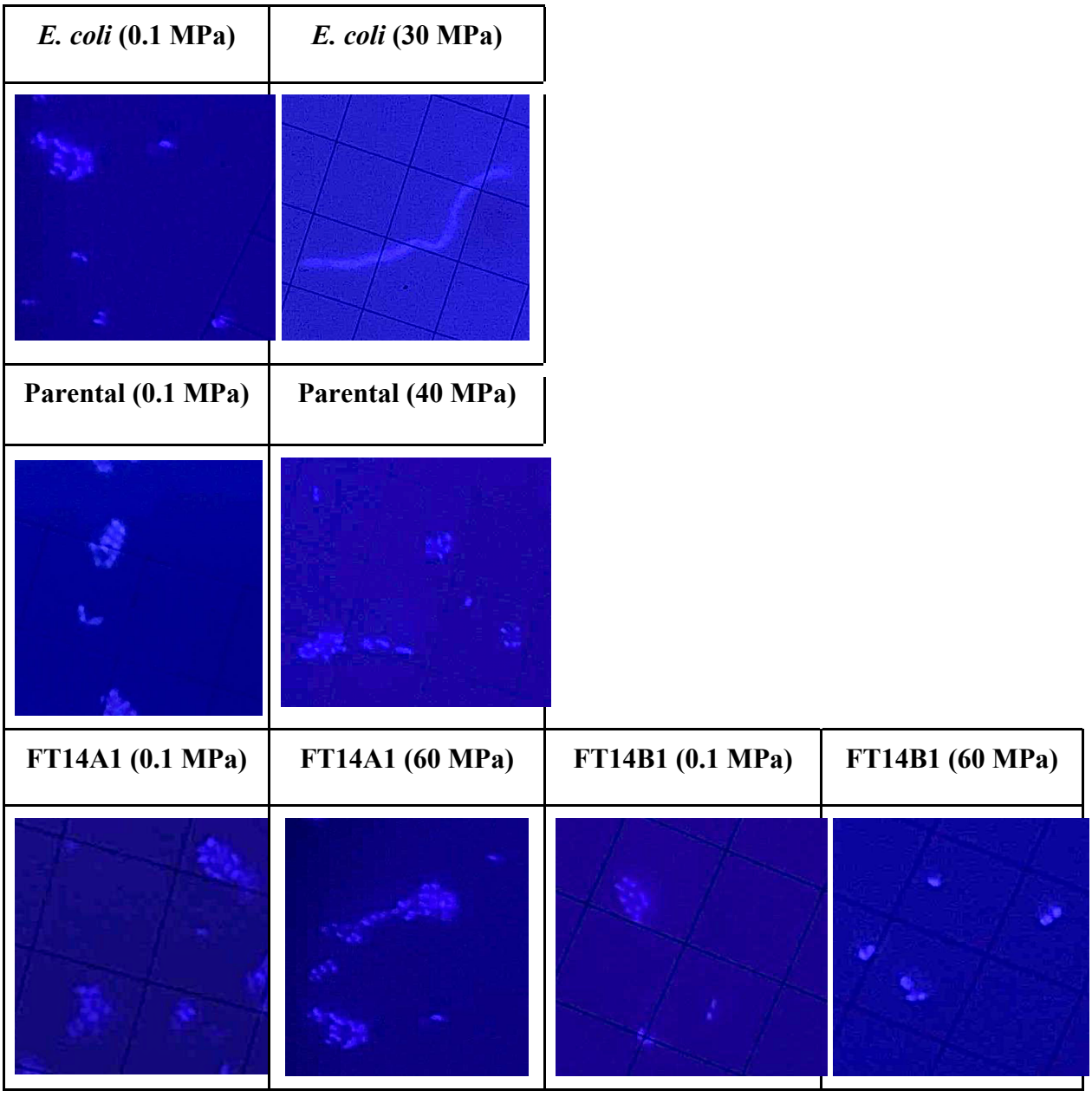
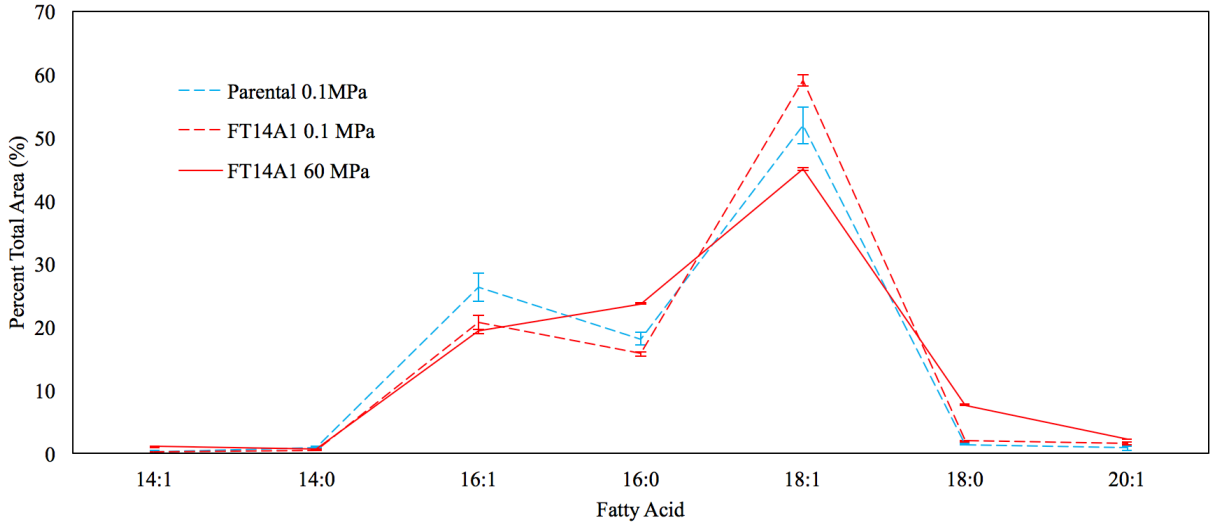


Figure 6. DAPI fluorescence microscopy of indicated strains at atmospheric pressure and under high hydrostatic pressure.

Parental Strain	FT14A1	FT14B1
		

Figure 7. Colony morphology of parental and mutant strains. Parental and mutant streaked onto TSBYS plate and incubated at room temperature, atmospheric pressure.

(A)



(B)

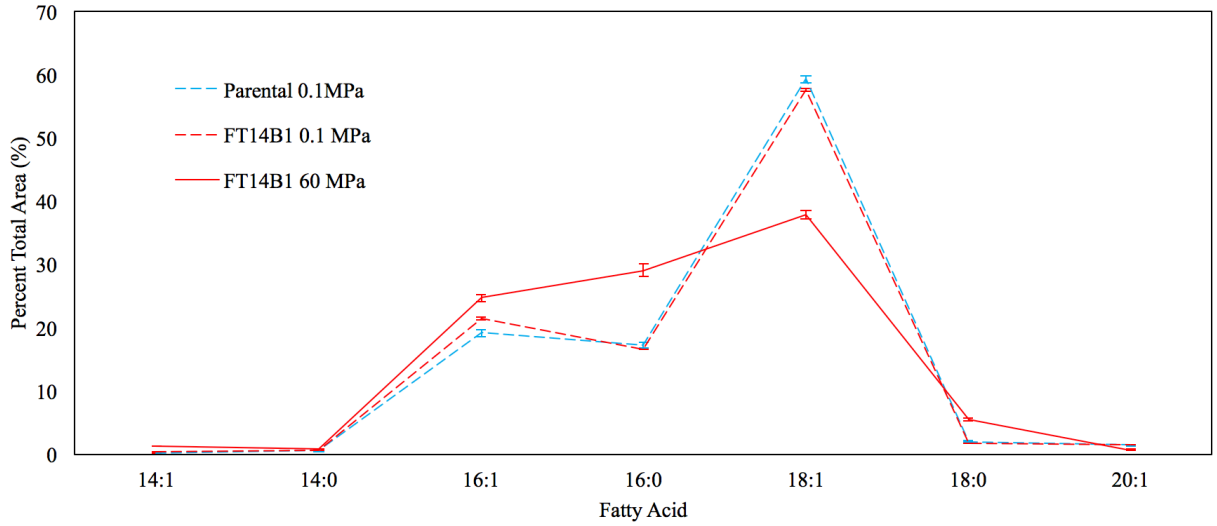


Figure 8. The membrane fatty acid composition of parental and evolved strains. The dotted lines represent strains grown under atmospheric pressure, while solid lines represent strains grown at 60 MPa. The blue line represents the parental strain and red lines represent the mutant strains. Both (A) FT14A1 and (B) FT14B1 were examined.

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APPENDIX

Table 1. Fatty acid composition of lineage A parental and mutant strain. Data provided as percent area composition of averages, N=3.

Fatty Acid	Parental (0.1 MPa)	Standard Error	FT14A1 (0.1 MPa)	Standard Error	FT14A1 (60 MPa)	Standard Error
14:1	0.37	0.04	0.24	0.06	1.10	0.06
14:0	0.94	0.11	0.54	0.06	0.78	0.02
16:1	26.28	2.21	20.74	1.07	19.41	0.34
16:0	18.12	1.01	15.77	0.36	23.71	0.12
18:1	51.99	2.84	59.03	0.93	44.97	0.20
18:0	1.43	0.11	1.99	0.17	7.70	0.09
20:1	0.86	0.40	1.68	0.19	2.33	0.00
Total % SFA	20.50	--	18.31	--	32.18	--
Total % UFA	79.50	--	81.69	--	67.82	

Table 2. Fatty acid composition of lineage B parental and mutant strain. Data provided as percent area composition of averages, N=3.

Fatty Acid	Parental (0.1 MPa)	Standard Error	FT14B1 (0.1 MPa)	Standard Error	FT14B1 (60 MPa)	Standard Error
14:1	0.20	0.04	0.31	0.03	1.27	0.05
14:0	0.55	0.05	0.69	0.00	0.90	0.04
16:1	19.21	0.56	21.54	0.24	24.70	0.48
16:0	17.21	0.48	16.60	0.07	j29.05	0.98
18:1	59.37	0.55	57.65	0.27	37.87	0.70
18:0	2.01	0.16	1.69	0.01	5.52	0.13
20:1	1.43	0.11	1.52	0.08	0.68	0.12
Total % SFA	19.78	--	18.98	--	35.48	--
Total % UFA	80.22	--	81.02	--	64.52	--

Table 3. Fatty acid composition of positive control *P. profundum* strain SS9. Data provided as percent area composition of averages, N=3.

Fatty Acid	SS9 (0.1 MPa)	Standard Error	SS9 (30 MPa)	Standard Error
12:0	2.43	0.18	2.90	0.07
14:1	1.58	0.12	0.39	0.02
14:0	9.08	0.04	3.43	0.06
16:1	38.02	0.31	39.64	0.38
16:0	44.48	0.49	38.37	1.04
18:1	2.45	0.20	8.35	0.23
18:0	0.72	0.08	0.60	0.01
20:5	1.23	0.18	5.84	0.33
Total % SFA	56.72	--	45.29	--
Total % UFA	43.28	--	54.22	--

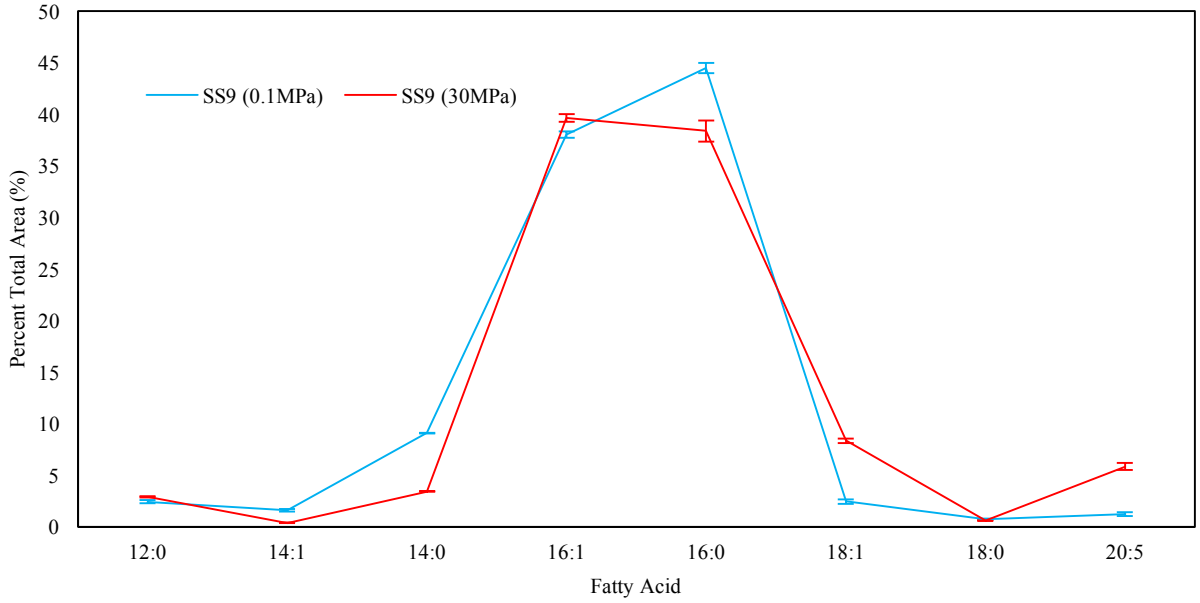

Figure 9. The membrane fatty acid composition of *P. profundum* SS9. The blue line represents SS9 grown at 0.1 MPa and red line represents SS9 grown at 30 MPa.