EXPLORING MICROBIAL GROWTH OF A MODEL EXTREMOPHILE, *Archaeoglobus fulgidus* AT ELEVATED PRESSURES

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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ABSTRACT

Deep-sea vent and subsurface microorganisms are metabolically diverse and often display unique adaptive strategies that operate under elevated pressure conditions. However, because high hydrostatic pressure (HHP) laboratory cultivation has not been widely adopted, knowledge of how these microorganisms function in native high-pressure environments is limited. To explore how elevated pressures affect the metabolism and physiology of deep-sea and subsurface microorganisms, growth of a model extremophile, *Archaeoglobus fulgidus* (type strain VC16), was investigated up to 98 MPa in batch cultures for both chemoorganoheterotrophic and chemolithoautotrophic metabolisms. *A. fulgidus* is an anaerobic, hyperthermophilic sulfate reducing archaeon, first isolated from a shallow marine vent but has been commonly identified in high-pressure marine environments (to 2-4 km below sea level, 20-40 MPa), including deep-sea hydrothermal vents, deep geothermal wells, and deep oil reservoirs. In heterotrophic HHP cultivation experiments, exponential growth was observed up to 60 MPa. Cell densities were comparable from 0.1-40 MPa, while lower cell densities were observed at 50 MPa and 60 MPa and growth was inhibited at 70 MPa. *A. fulgidus* displayed both piezotolerance and moderate piezophily under certain heterotrophic HHP conditions. In autotrophic HHP conditions, *A. fulgidus* displayed piezotolerance with similar growth rates and maximum cell densities observed at up to 40 MPa and little to no growth was observed at 60 MPa. *A. fulgidus* biofilm production was observed in certain heterotrophic conditions from 0.1-50 MPa under HHP batch cultivation conditions due to both low calcium concentrations in the growth medium and the presence of a stainless steel needle that created a nucleation site. This suggests that biofilm production here was a response to growth medium chemistry and surface area, and was not related to the elevated pressure conditions. Here, *A. fulgidus* was shown to grow, and in some

cases also produce biofilm, over a range of elevated pressure conditions. To the extent of our knowledge, piezotolerance to HHP for both heterotrophic and autotrophic metabolisms have not been previously measured for a single species. *A. fulgidus'* metabolic plasticity and capacity for biofilm production reflects adaptive mechanisms that lend insight into how this species thrives in extreme and fluctuating environments.

1. Deep biosphere life at elevated pressures

Elevated pressure conditions are inherent to all deep biosphere environments. The deep biosphere collectively hosts the majority of bacterial and archaeal (microbial) life on Earth (Whitman, Coleman, & Wiebe, 1998; Jebbar, Franzetti, Girard, & Oger, 2015; Bar-on, Phillips, & Milo, 2018) and is confined to environments that experience 10 MPa of pressure or more, and thus begins at different depths as pressure increases ~10 MPa/km in the water column, ~15 MPa/km in subseafloor sediments, and ~25 MPa/km in oceanic and continental crust (Jannasch & Taylor, 1984; Oger & Jebbar, 2010; Picard, Testemale, Wagenknecht, Hazael, & Daniel, 2015). While most microbial life likely lives in high-pressure environments, very few communities or species have been studied under high-pressure conditions. It is currently estimated that there are ~1 trillion different microbial species on Earth (Locey & Lennon, 2016). Of these, only about 15,448 species have been isolated (List of Prokaryotic names with Standing in Nomenclature (LPSN) database in the last mini review, Parte, 2018) and ~200 studies have measured the effects of elevated pressure on natural and pure cultures from surface and deep environments (data in Picard & Daniel, 2013). Furthermore, there are only ~55 species known to preferentially grow at or have suboptimal growth at elevated pressure conditions (Picard & Daniel, 2013; Jebbar et al., 2015). Therefore, despite the extent of the deep biosphere, we know relatively little about the metabolic potential and physiological adaptations of the microbes that populate these high-pressure environments.

Our current understanding of metabolic diversity, activity, and potential mostly comes from studies based on microbial communities and pure cultures found in surface environments (Edwards, Becker, & Colwell, 2012) or those grown at surface pressure conditions (i.e. ~0.1

MPa). The extent of microbial growth and diversity under other extreme conditions (e.g. high and low temperatures, extreme in pH, extreme in salinity, etc.) has been studied rather extensively, and yet the pressure effects on microbial growth are poorly constrained compared to other environmental extremes (Jebbar et al., 2015). This lack of knowledge stems from the difficulty in both sampling from these environments and replicating those conditions in the laboratory for enrichment and isolation. Though the use of molecular and phylogenetic techniques on deep biosphere environmental samples have greatly advanced our knowledge of the microbial communities present and their potential activity in deep-sea and subsurface environments, there are still relevant questions about specific metabolic functionality or unique physiological characteristics from individual species left unanswered from using these methods alone, questions that can be explored through cultivation. Through batch cultivation, a range of metabolic capabilities and adaptive strategies can be explored in great lengths for a variety of environmental parameters.

Batch cultivation is a common microbiological technique used to grow microorganisms under specific environmental conditions (reviewed in Maier, 2009). In batch culture, a single environmental parameter can be isolated to measure the effect of that parameter on growth of pure cultures, co-cultures or natural environmental samples. Microbial growth follows a predictive pattern in batch cultures as microbes are inoculated into a closed system with a specific initial amount of nutrients and cell densities are measured over time as the nutrients are consumed, and when these nutrients become limited, cell growth declines. From this, microbial growth is characterized by its standard growth curve, which shows the four growth phases of batch culture (lag phase, exponential or logarithmic phase, stationary phase and death phase). These curves generally show lag phases, which reflect how long it takes the microbial species or

co-culture to sense and adjust to its new environmental conditions before exponential growth can occur. The slope of exponential growth phase indicates how fast an organism grows under each specific growth condition. The plateau of the curve represents the stationary phase, in which cell growth and cell death rates are equal. Finally, the slight decline at the end of each curve represents the death phase, where death rates start to exceed growth rates. This cultivation technique is effective for comparing microbial growth over a range of pressure conditions, while keeping temperature and growth medium chemistry constant, to find the maximum and optimum growth conditions for the specific set of parameters explored.

High-pressure growth experiments in the last ~70 years have made great progress to better understand how microorganisms found in both surface and subsurface environments cope with high-pressure conditions. These studies have revealed that pressure tolerance is specific to each microorganism and that some microbial growth rates and cell densities are negatively impacted by high-pressure conditions (piezosensitive), while others can grow over a specific range of pressures before growth is negatively impacted (piezotolerant), some have optimum growth at elevated pressure (piezophiles), and others require elevated pressure for growth (obligate piezophiles; e.g. Jannasch & Taylor, 1984; Kato, Nogi, & Arakawa, 2008; Fang, Zhang, & Bazylinski, 2010; Figure 1). Since a broad range of microorganisms respond differently to pressure, it makes it difficult to isolate specific high-pressure adaptive mechanisms from all other environmental parameters. Therefore, to better understand how elevated pressures alone affect microbial growth, controlled laboratory experimentation is needed. In the studies presented in the subsequent chapters, batch cultivation was used to measure the effects of increasing pressures on microbial growth of a model extremophile, *Archaeoglobus fulgidus*.

Archaeoglobus fulgidus is one of the most well studied hyperthermophilic archaea, it is metabolically versatile, and is commonly found in both surface and deep environments. *A. fulgidus* was one of the first archaea to have its genome sequenced (Klenk et al., 1997), and it is one of the few Archaea capable of sulfate reduction and acetogenesis (Stetter, Lauerer, Thomm, & Neuner, 1987; Burggraf, Jannasch, Nicolaus, & Stetter, 1990; Henstra, Dijkema, & Stams, 2007), which are metabolisms originally thought to be specific to Bacteria. *A. fulgidus* was initially isolated from a shallow marine hydrothermal system (Stetter et al., 1987; Stetter, 1988), but has since been isolated and genetically identified from both shallow and deep high temperature environments. It has been found in deep-sea hydrothermal vents (Stetter et al., 1993; Beeder, Nilsen, Rosenes, Torsvik, & Lien, 1994; Thauer & Kunow, 1995; Nilsen, Beeder, Thorstenson, & Torsvik, 1996; Reysenbach, Longnecker, & Kirshtein, 2000; Nercessian, Bienvenu, Moreira, Prieur, & Jeanthon, 2005), deep marine oil reservoir waters (Stetter et al. 1993; Beeder et al. 1994), a continental oil reservoir (L'Haridon, Reysenbach, Glénat, Prieur, & Jeanthon, 1995), and a deep geothermal well (Fardeau et al., 2009). This archaeon is a strict anaerobe and can grow as both a chemoorganoheterotroph and a chemolithoautotroph utilizing various substrates for growth (Stetter et al., 1987; Hartzell & Reed, 2006).

The study of microbial metabolism allows for understanding the combined set of reactions that both generate energy (catabolism) and consume energy (anabolism) for microbial growth. *A. fulgidus* cycles carbon and sulfur through both heterotrophic and autotrophic metabolisms (Stetter, 1988; Stetter, 1992; Hartzell & Reed, 2006). During heterotrophic catabolism, *A. fulgidus* can couple the oxidation of a variety of organic substrates (e.g. lactate, formate, formamide, pyruvate, fatty acids, *n*-alkenes, and *n*-alkanes) via sulfate or thiosulfate reduction where CO_2 and H_2S are generally the main products of growth (e.g. Stetter et al., 1987;

Stetter, 1988; Khelifi et al., 2010; Khelifi et al., 2014). Previously, trace amounts of methane have been detected, likely a result of incomplete oxidation in the methyl branch in the oxidative acetyl-CoA pathway (Stetter et al., 1987; Stetter, 1988; Möller-Zinkhan & Thauer, 1990; Klenk et al., 1997). For its heterotrophic anabolism, *A. fulgidus* can breakdown those same organic substrates for biosynthesis. For autotrophic catabolism, *A. fulgidus* can reduce thiosulfate with molecular hydrogen to produce H_2S and water, or it can reduce sulfate with CO with the formation of H_2S, CO_2, acetate, and formate (Stetter, 1988; Henstra et al., 2007). For its autotrophic anabolic reactions, it can fix CO_2 or CO into cellular structures for biomass production. As described, *A. fulgidus* can utilize many metabolic strategies, but growth for these metabolic strategies have only been demonstrated under surface conditions. One previous study had reported growth at 30 MPa and 42 MPa of *A. fulgidus* strain T25, isolated from a deep offshore oil reservoir, where crude oil was metabolized as a sole carbon source (Stetter et al., 1993). However, cell densities for a range of growth pressure or a maximum growth pressure range for different metabolisms have yet to be reported for *A. fulgidus*. This information would better constrain the depths at which this species can inhabit and show if it could still metabolize various substrates at depth as it does at the surface.

In the upcoming chapters, *A. fulgidus* growth over a range of elevated pressures was tested for two different metabolisms using various high hydrostatic pressure cultivation techniques. In the second chapter, the construction of two high temperature and high-pressure batch cultivation systems with experimental designs and protocols for growing *A. fulgidus* at elevated pressures with and without sample decompression is described in detail. The third chapter is dedicated to the results of *A. fulgidus* heterotrophic and autotrophic growth over a range of pressures. Additionally, *A. fulgidus* biofilm formation at ambient and elevated pressures

was explored. The fourth and final chapter focuses specifically on the effects of sample decompression on *A. fulgidus* heterotrophic growth using two high-pressure techniques. As a whole, these results show that elevated pressures and decompression affect microbial growth and that high-pressure growth patterns in one species can fluctuate depending on the specific environmental conditions as *A. fulgidus* displays piezophilic, piezotolerant, and piezosensitive behaviors. These results exemplify the importance of laboratory cultivation conditions better reflecting *in situ* environmental parameters for studying extremophiles found in the deep biosphere and are especially relevant to scientific endeavors in modern and ancient biogeochemical modeling, origin and evolution of life, habitability of life on ocean worlds, and biotechnology.

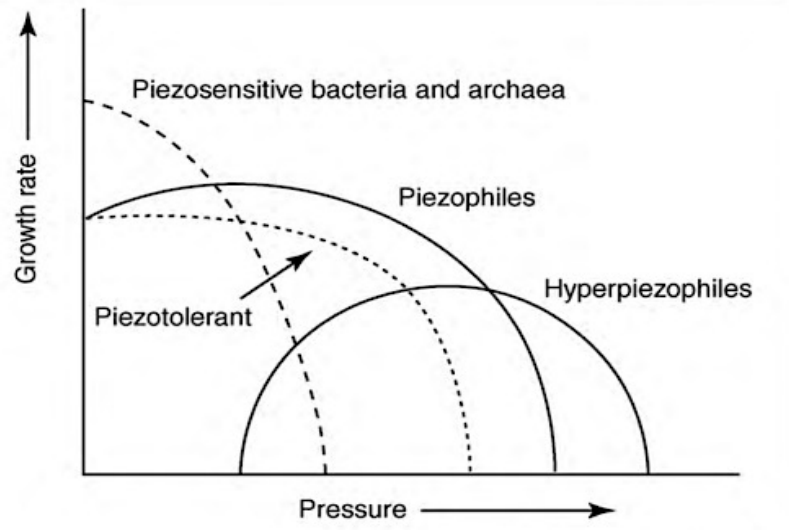

Figure 1.1. A schematic concept of the microbial growth characteristics as a function of pressure (Fang et al., 2010) modified from an initial concept in Jannasch & Taylor (1984).

1.1 References

Bar-On, Y. M., Phillips, R., & Milo, R. (2018). The biomass distribution on Earth. *Proceedings*

of the National Academy of Sciences, *115*(25), 6506–6511. 10.1073/pnas.1711842115

Beeder, J., Nilsen, R. K., Rosnes, J. T., Torsvik, T., & Lien, T. (1994). *Archaeoglobus fulgidus* isolated from hot North Sea oil field waters. *Applied and Environmental Microbiology*, *60*(4), 1227–1231.

Burggraf, S., Jannasch, H. W., Nicolaus, B., & Stetter, K. O. (1990). *Archaeoglobus profundus* sp. nov., represents a new species within the sulfate-reducing archaebacteria. *Systematic and Applied Microbiology*, *13*(1), 24–28.10.1016/S0723-2020(11)80176-1

Edwards, K. J., Becker, K., & Colwell, F. (2012). The deep, dark energy biosphere: intraterrestrial life on earth. *Annual Review of Earth and Planetary Sciences*, *40*(1), 551–568. 10.1146/annurev-earth-042711-105500

Fang, J., Zhang, L., & Bazylinski, D. A. (2010). Deep-sea piezosphere and piezophiles: geomicrobiology and biogeochemistry. *Trends in Microbiology*, *18*(9), 413–422. 10.1016/j.tim.2010.06.006

Fardeau, M.-L., Goulhen, F., Bruschi, M., Khelifi, N., Cayol, J.-L., Ignatiadis, I.,… Ollivier, B. (2009). *Archaeoglobus fulgidus* and *Thermotoga efii*, thermophilic isolates from deep geothermal water of the paris basin. *Geomicrobiology Journal*, *26*(2), 119–130. 10.1080/01490450802674970

Hartzell, P., & Reed, D. W. (2006). The genus *Archaeoglobus*. The *Prokaryotes*, *3*, 82–100. 10.1007/0-387-30747-8

Henstra, A. M., Dijkema, C., & Stams, A. J. M. (2007). *Archaeoglobus fulgidus* couples CO oxidation to sulfate reduction and acetogenesis with transient formate accumulation.

Environmental Microbiology, *9*(7), 1836–1841. 10.1111/j.1462-2920.2007.01306.x

Jannasch, H. W., & Taylor, C. D. (1984). Deep-sea microbiology. *Annual Review of Microbiology*, *38*, 487–514.

Jebbar, M., Franzetti, B., Girard, E., & Oger, P. (2015). Microbial diversity and adaptation to high hydrostatic pressure in deep-sea hydrothermal vents prokaryotes. *Extremophiles*, *19*(4), 721–740. 10.1007/s00792-015-0760-3

Kato, C., Nogi, Y., & Arakawa, S. (2008). Isolation, cultivation, and diversity of deep-sea piezophiles. In C. Michiels, D. H. Bartlett, & A. Aertsen (Eds.), *High-pressure microbiology* (pp. 203–218). Washington, DC: ASM Press.

Khelifi, N., Amin Ali, O., Roche, P., Grossi, V., Brochier-Armanet, C., Valette, O.,… Hirschler-Réa, A. (2014). Anaerobic oxidation of long-chain n-alkanes by the hyperthermophilic sulfate-reducing archaeon, *Archaeoglobus fulgidus*. *The ISME Journal*, *8*(11), 3057–60. 10.1038/ismej.2014.58

Khelifi, N., Grossi, V., Hamdi, M., Dolla, A., Tholozan, J. L., Ollivier, B., & Hirschler-Réa, A. (2010). Anaerobic oxidation of fatty acids and alkenes by the hyperthermophilic sulfate-reducing archaeon *Archaeoglobus fulgidus*. *Applied and Environmental Microbiology*, *76*(9), 3057–3060. 10.1128/AEM.02810-09

Klenk, H. P., Clayton, R. A., Tomb, J. F., White, O., Nelson, K. E., Ketchum, K. A.,… Venter, J. C. (1997). The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. *Nature*, *390*(6658), 364–70. 10.1038/37052

L'Haridon, S., Reysenbach, A.-L., Glenat, R., Prieur, D., & Jeanthon, C. (1995). Hot

subterranean bioshphere in a continental oil reservoir. *Nature*, *377*, 223-224.

Locey, K. J., & Lennon, J. T. (2016). Scaling laws predict global microbial diversity. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(21), 5970–5. 10.1073/pnas.1521291113

Maier, R. M. (2009). Bacterial Growth. In R. M. Maier, I. L. Pepper, C. P. Gerba (Eds.), *Environmental microbiology* (pp. 37–54). Burlington, MA: Academic Press. Inc.

Möller-Zinkhan, D., & Thauer, R. K. (1990). Anaerobic lactate oxidation to 3 CO2 by *Archaeoglobus fulgidus* via the carbon monoxide dehydrogenase pathway: demonstration of the acetyl-CoA carbon-carbon cleavage reaction in cell extracts. *Archives of Microbiology*, *153*. 10.1007/BF00249070

Nercessian, O., Bienvenu, N., Moreira, D., Prieur, D., & Jeanthon, C. (2005). Diversity of functional genes of methanogens, methanotrophs and sulfate reducers in deep-sea hydrothermal environments. *Environmental Microbiology*, *7*(1), 118–132. 10.1111/j.1462-2920.2004.00672.x

Nilsen, R. K., Beeder, J., Thorstenson, T., & Torsvik, T. (1996). Distribution of thermophilic marine sulfate reducers in north sea oil field waters and oil reservoirs. *Applied and Environmental Microbiology*, *62*(5), 1793–1798.

Oger, P. M., & Jebbar, M. (2010). The many ways of coping with pressure. *Research in Microbiology*, *161*(10), 799–809. 10.1016/j.resmic.2010.09.017

Parte, A. C. (2018). LPSN - List of prokaryotic names with standing in nomenclature (Bacterio.net), 20 years on. *International Journal of Systematic and Evolutionary*

Microbiology, *68*(6), 1825–1829. 10.1099/ijsem.0.002786

Picard, A., & Daniel, I. (2013). Pressure as an environmental parameter for microbial life - A review. *Biophysical Chemistry*, *183*, 30–41. 10.1016/j.bpc.2013.06.019

Picard, A., Testemale, D., Wagenknecht, L., Hazael, R., & Daniel, I. (2015). Iron reduction by the deep-sea bacterium *Shewanella profunda* LT13a under subsurface pressure and temperature conditions. *Frontiers in Microbiology*, *5*, 1–8. 10.3389/fmicb.2014.00796

Reysenbach, A. L., Longnecker, K., & Kirshtein, J. (2000). Novel bacterial and archaeal lineages from an *in situ* growth chamber deployed at a mid-atlantic ridge hydrothermal vent. *Applied and Environmental Microbiology*, *66*(9), 3798–3806. 10.1128/AEM.66.9.3798-3806.2000

Stetter, K. (1992). The genus *Archaeoglobus*. *The Prokaryotes*, *2*, 707-711.

Stetter, K. O. (1988). *Archaeoglobus fulgidus* gen. nov., sp. nov.: a new taxon of extremely thermophilic archaebacteria. *Systematic and Applied Microbiology*, *10*(2), 172–173. 10.1016/S0723-2020(88)80032-8

Stetter, K. O., Huber, R., Blöchl, E., Kurr, M., Eden, R. D., Fielder, M.,… Vance, I. (1993). Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. *Nature*, *365*(6448), 743–745. 10.1038/365743a0

Stetter, K. O., Lauerer, G., Thomm, M., & Neuner, A. (1987). Isolation of extremely thermophilic sulfate reducers: evidence for a novel branch of archaebacteria. *Science*, *236*(4803), 822–824. 10.1126/science.236.4803.822

Thauer, R. K., & Kunow, J. (1995). Sulfate-Reducing Archaea. In L. L. Barton (Ed.), *Sulfate-reducing bacteria* (pp. 33–45). New York: Plenum Press.

Whitman, W. B., Coleman, D. C., & Wiebe, W. J. (1998). Prokaryotes: The unseen majority. *Proceedings of the National Academy of Sciences*, *95*(12), 6578–6583. 10.1073/pnas.95.12.6578

2. High temperature and high hydrostatic pressure incubation systems for batch cultivation

2.1 Abstract

High temperatures (HT) and high hydrostatic pressures (HHP) are characteristic of deep-sea hydrothermal vents and the subseafloor of submarine vents. These environments host a vast and diverse microbial population yet only a small fraction of those populations have been successfully cultured. In part, this is due to the difficulty of sampling and maintaining *in situ* conditions and then replicating those high temperature and high-pressure conditions in the laboratory. In an effort to encourage more HT-HHP cultivation, we present two HT-HHP batch culture incubation systems for cultivating deep-sea vent and subsurface thermophilic and hyperthermophilic microorganisms. One HT-HHP system can be used for batch culture up to 110 MPa and 121°C that requires sample decompression. The second HT-HHP system can be use to culture microorganisms up to 100 MPa and 160°C with pressure-retaining vessels that negate whole sample decompression during subsampling. Here, we describe both how to build cost effective heating systems for these two types of high-pressure vessels and the protocols of HT-HHP microbial batch cultivation in both systems with a model extremophile, *Archaeoglobus fulgidus*.

2.2 Introduction

Deep-sea hydrothermal vents and subseafloor environments are highly productive environments in the deep biosphere and with the average black smoker deep-sea vent depth at

~2.1 km (i.e. ~21 MPa; Von Damm, 1990), they reside under high-pressure conditions (Fry, Parkes, Cragg, Weightman, & Webster, 2008; Jebbar, Franzetti, Girard, & Oger, 2015). The microbial communities in these environments are metabolically diverse and often display unique adaptive strategies that operate under elevated pressure conditions. Currently only ~0.1% of the microbial populations sampled from the subsurface are successfully cultured (D'Hondt et al., 2004; Parkes et al., 2014). In most cases, the isolates obtained from these environments are cultivated at *in situ* temperatures and 0.1 MPa and their growth under elevated pressure conditions are not usually characterized. Therefore, our view of deep-sea vent and subseafloor microbial diversity is biased for those microbes able to withstand drastic pressure changes from sampling decompression and our knowledge of microbial physiology and metabolism is base on growth under high temperatures at ~0.1 MPa. This is in large because high temperature (HT) and high hydrostatic pressure (HHP) microbial cultivation is not a widely adopted laboratory technique. HT-HHP microbial cultivation usually requires expensive equipment, specialized laboratory training, and it is more labor intensive compared to ambient pressure experiments. But HT-HHP cultivation is necessary to increase the number of isolates and to better understand high-pressure adaptation, unknown metabolic and physiological strategies that are relevant to biotechnology, geo-engineering, and the biogeochemical sciences. Thus, we suggest some practical HT-HHP batch culture experimental options to encourage more laboratories to adopt these long known techniques and expand the collective knowledge of how life thrives in deep-sea vents and in the subsurface.

We present two simple yet effective systems for routine HT-HHP batch cultivations. The first HT-HHP batch-culturing system was designed and built for four vessels, where sample decompression and repressurization is required for subsampling. Even though decompression-

repressurization cycles are unavoidable, tests can be done to measure the affects of decompression and the experimental design could minimize the amount of decompression-repressurization cycles (e.g. Yayanos, 1995; Yayanos, 2001). Alternatively, if enough vessels are available to sample from for every sampling period, each vessel could only go through one cycle of pressurization and decompression. Additionally, laboratories have come up with inventive ideas to fix the microbial samples at pressure and slowly decompress the culture after fixation (i.e. Marietou, Nguyen, Allen, & Bartlett, 2015). The second system was built for vessels that allow for subsampling without decompressing the entire microbial culture. These pressurized underwater sample handler (PUSH) vessels, built upon previous designs (Bianchi, Garcin, & Tholosan, 1999; Tamburini, Garcin, & Bianchi, 2003), were purchased through TOP Industrie© and were designed so that microbial cultures could be grown in a polyether ether ketone (PEEK) reservoir with a floating piston and pressure could be maintained to the whole sample during subsampling by connecting the vessel to a HHP pump. Furthermore, the PUSH vessel HT-HHP system was built for mobility to allow for anaerobic preparations in an anaerobic chamber without losing heat so that stable pressures could be achieved quickly.

High-pressure microbial batch cultivation is not a new idea and variations of vessels and culturing containers have been designed over the last ~70 years. The same initial HHP batch cultivation techniques described in Zobell and Oppenheimer (1950) are still in use today (e.g. Picard, Testemale, Hazemann, & Daniel, 2012; this study). These systems presented can be easily modified and applied to other vessel configurations. For example, vessels used for batch cultivation with sample decompression described in Yayanos (1982 and 2001) would be ideal and they can be fabricated at a lower cost when purchased in bulk. Microorganisms in fluid medium or sediment can be grown in these types of vessels at HT-HHP using a variety of

cultivation techniques in syringes (Baross & Deming, 1983; Marteinsson et al., 1999; Takai et al., 2008), glass tubes with rubber stoppers (Bowles, Samarkin, & Joye, 2011; Tasumi, Yanagawa, Miyazaki, & Takai, 2015), heat sealed plastic bags (Berger & Tam, 1970), and plastic bulbs (Bartlett & Welsh, 1995; Marietou et al., 2015). For HT-HHP systems that negate whole sample decompression, custom modifications can be made to vessels, like the PUSH vessels, to also cultivate sediment samples. Currently the PUSH vessels are designed for microbial batch cultivation in fluid medium.

Batch cultivation is a common and effective microbiological technique that is used to answer many questions regarding microbial growth, metabolism and physiology (e.g. Kell, 1987; Maier, 2009). In the HT-HHP systems presented here, growth can be measured in batch culture for a range of temperature and pressure conditions for limitless geochemical conditions. These affects can be measured to find out how microbial physiology and metabolism vary over these deep-sea vent and subsurface conditions. Furthermore, we have given the protocols and experimental design for cultivating a model extremophile, *Archaeoglobus fulgidus* with these HT-HHP growth systems.

2.3 HT-HHP system with sample decompression

2.3.1 Vessels

A heating instillation system was built for four High Pressure Equipment Co.© (HiP©), OC-1 O-Ring series 125 mL volume pressure vessels for batch cultivation experiments. The working temperature range of this system was 25-121°C and pressure range was 0.1-100 MPa.

Temperature and pressure limits were based on the maximum working capacities of the HiP© vessels and OMEGA™ heating jackets (see below).

2.3.2 Design objectives and schematics

The objective for the HT-HHP cultivation system with decompression design was to build an installation where each pressure vessel could be secured in place, heated, insulated and pressurized. Each vessel needed a temperature-controlled system, a high-pressure line to the pump, and an easily detachable and movable pressure gauge (Figure 2.1). Additionally, the bottom of each vessel was slotted to fit into a vice in the case that additional torque would be necessary to open the vessel lid. Therefore, custom machined parts were needed in the design to fit the vessel base shape to stabilize the vessel when opening the vessel lid. Finally, the length, width, and height of this system design considered table space availability and usability by researchers.

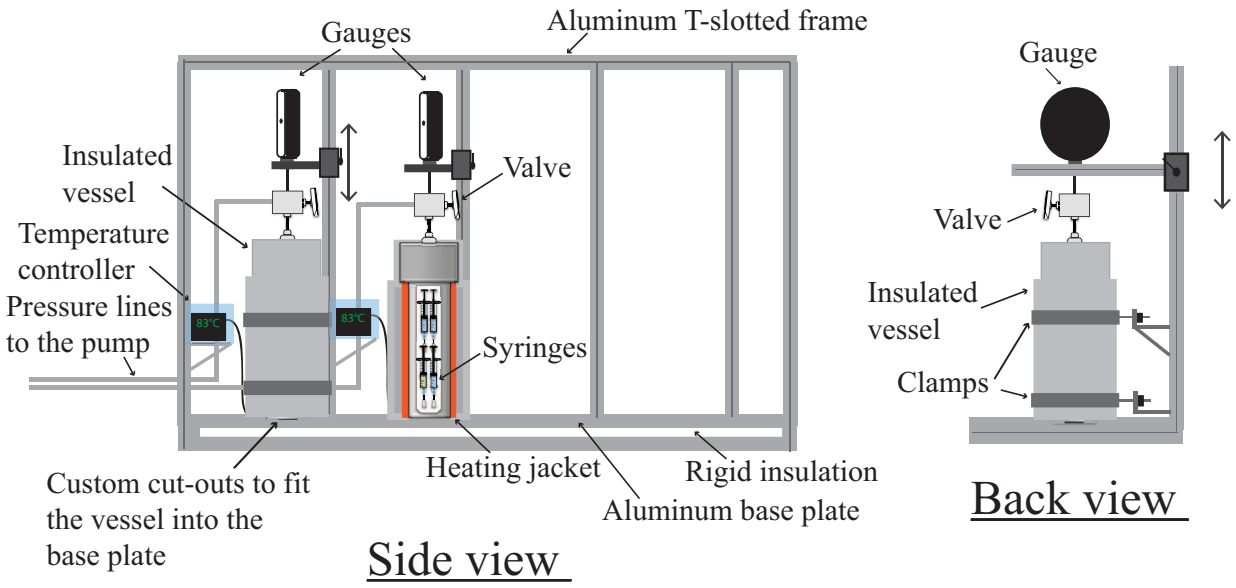

Figure 2.1. A design schematic of the HT-HHP system installation with sample decompression. The side view shows an insulated vessel and a cross section of an insulated vessel to show the heating element and syringe sample loading. The back view highlights the adjustable gauge and vessel security.

2.3.3 Materials

The installation frame was constructed from 80/20® Inc. T-slotted aluminum framing and accessories. T-slotted framing profiles, the 31-inch long by 9-inch wide aluminum base plate, nuts, bolts, fasteners, mounts, and brackets were purchased from 80/20® Inc. Clamping hangers, rigid galvanized standard duct, insulation (fiberglass and high temperature calcium silicate blocks), screws, fuses, power cords, and clear Nema boxes were purchased from McMaster Carr. Temperature controllers (CNi16D33), silicone rubber heating jackets, thermocouples (type J) were purchased from OMEGA™. High pressure gauges, gauge hex nut reducer, ¼ inch medium pressure tubing, valves and connections were purchased from Swagelok® and all rated to 137.89 MPa.

2.3.4 Temperature controller assembly and program

Thermocouples, heating jackets, and power cord wires were attached to the temperature controllers by terminal connections according to OMEGA™ instructions manual for the CNi16D33 temperature controllers. Thermocouples were first calibrated in a 0°C ice bath and then the controllers were set to 83°C and auto-tuned. After auto-tuning, fine calibration was done following the OMEGA™ manual, 83°C temperatures were reached with ± 0.5°C fluctuation.

2.3.5 Construction

First, four custom cutouts were machined out from the aluminum baseplate so that each vessel could be fitted into that baseplate. This allowed for vessel stability and resistance for additional torque needed to open the vessels when necessary. Rigid calcium silicate insulation was placed under each baseplate cutout to insulate the bottom of each vessel and additional insulation was placed underneath the entire baseplate. Unfortunately, small areas of the stainless steel vessels were in contact with the aluminum base plate resulting in heat loss. This issue is addressed in section 2.3.6.

The installation frame was assembled and bolted to the workspace (Figure 2.2). Each pressure vessel was placed into their respective cutout on the baseplate. Temperature was monitored by thermocouples (J type) placed in the middle of each vessel and was connected to a programed temperature controller (OMEGA™). Each controller was calibrated and programmed following the described procedures in section 2.3.4. Each vessel was wrapped in a 7-inch x10-inch silicone rubber-heating jacket (OMEGA™) and insulated (Figure 2.1). Heating jackets and insulation were wrapped around each vessel with hook and loop fasteners. Five-foot rigid

standard duct was cut to one-foot length sections to cover the vessel heating jacket and insulation. The vessels were secured to the aluminum frame by wrapping two threaded rod-clamping hangers around the rigid duct (Figure 2.1).

Each vessel was equipped with a pressure gauge that was secured on an extended arm off of the main installation frame over each pressure vessel. The extended arms were made to be vertically adjustable so that the gauge could be connected and disconnected from the vessel to remove the vessel lid. The high-pressure gauge was fitted to the pressure line by a hex nut connection. The hex nuts were machined to fit into the 80/20® Inc. mounts and were then fastened to the t-slotted extension arm off the main t-slotted frame. The four extension arms for each pressure gauge were connected to the main frame with single mount Unibearing™ assemblies. The pressure gauges were connected to a three-way valve, one way to the vessel, one way to the pressure gauge, and one way to the high-pressure line. Four high-pressure lines were constructed for each vessel from ¼-inch high-pressure stainless steel tubing (Swagelok®) that was measured, cut, bent, coned, and threaded so that each vessel could be connected to the high-pressure screw pump rated to 206 MPa made by HiP© (Figure 2.1 and 2.2).

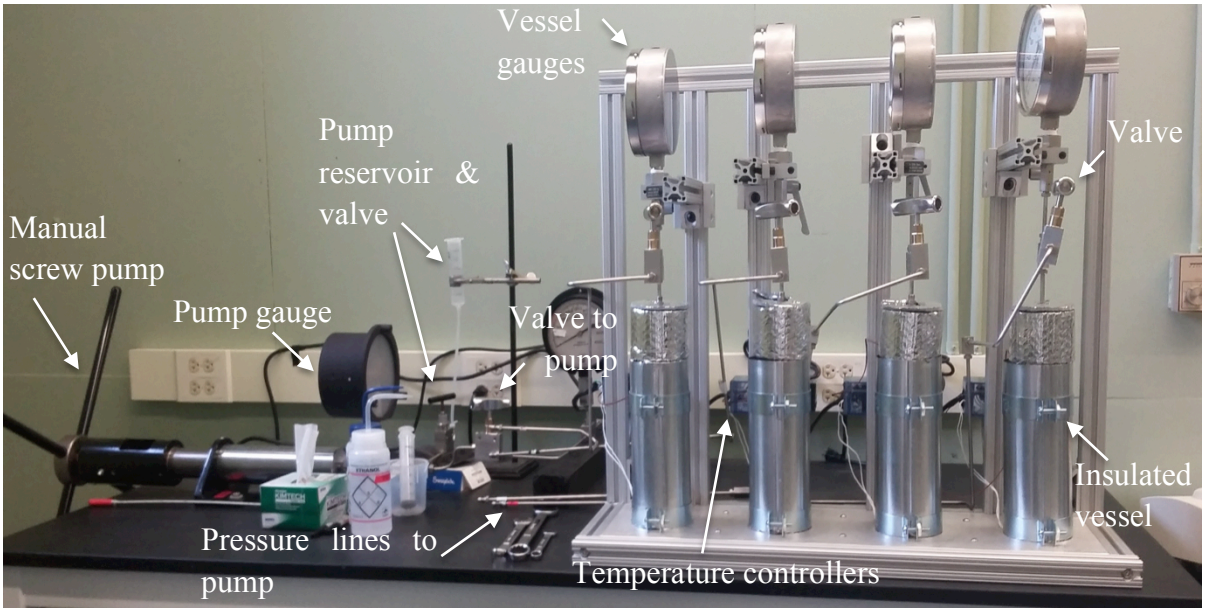

Figure 2.2. A picture of the HT-HHP system with sample decompression with temperature controlled insulated vessels.

2.3.6 Equipment testing

The high-pressure lines were continually monitored for leaks before HHP experiments. Internal vessel temperatures were monitored by thermometer and the controllers were set to the temperature that reflects the internal vessel temperature of 83°C (±0.5°C). Since there were small areas of the vessel in direct contact with the aluminum base, heat was lost at the bottom of the vessels. As a result, there was a temperature gradient at the bottom two inches of the vessel. The temperature difference was ~4-6 degrees cooler in the bottom two inches of the vessel reservoir. Therefore, samples were always placed above this temperature gradient where a constant temperature was measured.

2.3.7 *A. fulgidus* HT-HHP growth in syringes

Various types of syringes, many custom made, have been used for HHP growth of deep-sea samples. These methods and other HHP cultivation containers have been reviewed in Yayanos (2001), though they are primarily for growing heterotrophs. Methods in Takai et al. (2008) and Tasumi et al. (2015) were developed for autotrophic HHP batch cultivation of anaerobes in gas tight glass syringes, this method could also be applied to anaerobic heterotrophs. Here, plastic syringes have been used to grow a model extremophile, *Archaeoglobus fulgidus*. Plastic syringes can be used for HHP heterotrophic growth of facultative anaerobes or anaerobic sulfate reducing microorganisms. The advantages of plastic syringes for HHP growth is that they are cost effective, you can use multiple syringes in each pressure vessel, and they can save time and energy. Since plastic syringes are not gas tight, small amounts of oxygen can penetrate the culture medium. However, in the case of *A. fulgidus* growth, the sulfide that was used to reduce the medium prior to inoculation and the sulfide within the inoculum maintained reduced conditions and *A. fulgidus* HHP growth was not affected by oxygen stress (see below). But in *A. fulgidus* experiments where lower inoculation volumes were used, glass gas tight syringes were needed for growth because of oxygen contamination that lead to cell death. Therefore, cell densities and cell morphology needs to be compared in plastic syringes and in a gastight growth containers for all anaerobic cultures to be sure there is no biases in HHP growth from oxidative stress.

Below, the protocols for setting up a HT-HHP growth curve experiment for *A. fulgidus* using plastic syringes are described. *A. fulgidus* growth reached stationary phase 18-20 hours after inoculation and it was important to sample every four to five hours during the first 24 hours of growth to obtain a robust standard growth curve. The four vessels with samples went through a maximum of four decompression and repressurization cycles to obtain the time points. This

protocol could be modified to suit the needs of the hypothesis or the convenience of the experimentalist.

2.3.7.1 Medium preparation

1. A. fulgidus is an anaerobic marine hyperthermophilic archaeon and is grown in lactate and sulfate rich medium for its heterotrophic metabolism. *A. fulgidus* couples the oxidation of organic compounds (i.e. lactate) with sulfate reduction. Therefore, the medium used for growth is from Hartzell and Reed (2006) as follows per liter: 0.34 g KCl, 15.142g $MgSO_2$•$7H_2O$, 2.75g MgCl • $6H_2O$, 0.25g NH_2Cl, 0.14g $CaCl_2$ • $2H_2O$, 0.0137g K_2HPO, 17.8g NaCl, 0.0039g Fe $(NH_4)_2$ (SO_4) • $6H_2O$, 2.1g sodium lactate, 1g yeast extract, 3.36g PIPES (piperazine-*N*,*N*′bis [2-ethanesulfonic acid), 0.1mL Resazurin (0.1% solution), 1ml of Wolfe's trace element solution following Hartzell & Reed (2006) at pH 6.7.

2. Medium is boiled under N_2 and transferred into Balch tubes or serum bottles. The Balch tubes or serum bottles are capped, crimped, and autoclaved for 15 minutes at 121°C for sterilization (Balch et al. 1979). After sterilization, anaerobic medium conditions are achieved by adding 0.1 mL sulfide from a 2.5 % (w/v) anaerobic solution for every 10 mL of medium.

3. Thirteen serum bottles filled with 10 mL of sterile medium are reduced before inoculation for each growth curve experiment in triplicate. Twelve serum bottles are used for *A. fulgidus* growth and one serum bottle of uninoculated medium is used for a negative control (Figure 2.3 A).

2.3.7.2 Strain and Precultures

1. *Archaeoglobus fulgidus* strain VC-16 (DSM 4304) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). Stocks of *A. fulgidus* cells are made from a fresh culture grown from the DSMZ culture. To prepare frozen stocks, an *A. fulgidus* culture are mixed with 20% dimethyl sulfoxide (DMSO) and stored at -80°C for up to two years. Before performing a growth experiment, *A. fulgidus* must be grown from a frozen stock by inoculating it into sterile anaerobic medium. Once that culture is grown, an aliquot of logarithmic phase cells are transferred to inoculate three precultures for HT-HHP growth.

2.3.7.3 Syringe preparation

1. Becton-Dickinson (BD) plastic syringes and .23 gauge BD needles were used for HT-HHP growth. Three, five, and ten milliliter syringes are used most often depending on the sample size needed. Best growth has been observed in 5 mL and 10 mL syringes. Before inoculation and transfer, each syringe and attached needle needs to be flushed with N_2 3-4 times. Nitrogen is left in the syringe until the transfer of inoculated medium. The needle is then embedded in the middle of a sterile butyl rubber stopper or silicone stopper. If silicone is used, be sure that the needle does not puncture all the way through since silicone does not self-seal like butyl rubber.

2. For our installation system, we can fit three 5 mL syringes plus one 3 mL syringe in each of the four vessels. When preparing a growth curve experiment in triplicate, 16 syringes are flushed and prepared. Twelve 5 mL syringes are used to grow *A. fulgidus* at HT-HHP and four 3 mL syringes are used or negative controls, one for each vessel (Figure 2.3 A).

2.3.7.4 Inoculation, transfer and HT-HHP incubation

1. Twelve serum bottles filled with reduced sterile medium are inoculated from three separate precultures. Four serum bottles are inoculated with pre-culture #1, four serum bottles are inoculated with pre-culture #2, and finally four serum bottles are inoculated with pre-culture #3. Medium is inoculated with 3% (v/v) logarithmic phase *A. fulgidus* cells to a final cell concentration of ~1.25 x 10^7 cells/mL. The inoculated medium is then shaken to homogenize the distribution of *A. fulgidus* cells before 5 mL from each serum bottle is transferred into N_2 flushed syringes. For negative controls, 12 mL from one serum bottle filled with uninoculated medium are transferred into four separate 3 mL syringes. Then, four syringes (triplicate experiments and one negative control) are placed in four heated vessels filled with ultrapure water (18.2 MΩ), and pressurized (Figure 2.3 B). Each vessel has 5 mL syringes with inoculated medium from precultures #1, #2, and #3 plus a 3 mL syringe with uninoculated medium as a negative control (Figure 2.3 A and B). Additionally, the 5 mL of inoculated medium remaining in the serum bottles are placed in an oven at 0.1 MPa as positive controls for HT-HHP growth.

2.3.7.5 Sampling with decompression

1. Here, each vessel is decompressed at an average rate of 19 MPa/minute. Syringes are taken out of the heated vessels and subsamples of *A. fulgidus* cells, usually aliquots of 0.5 mL, are fixed in 2.5% gludaraldehyde. Syringes can then be placed back into the heated vessels and pressurized. Samples go through a maximum of four decompression and repressurization cycles with at least six hours in between each cycle. *A. fulgidus* fixed cells are placed into a 4°C refrigerator until they are analyzed.

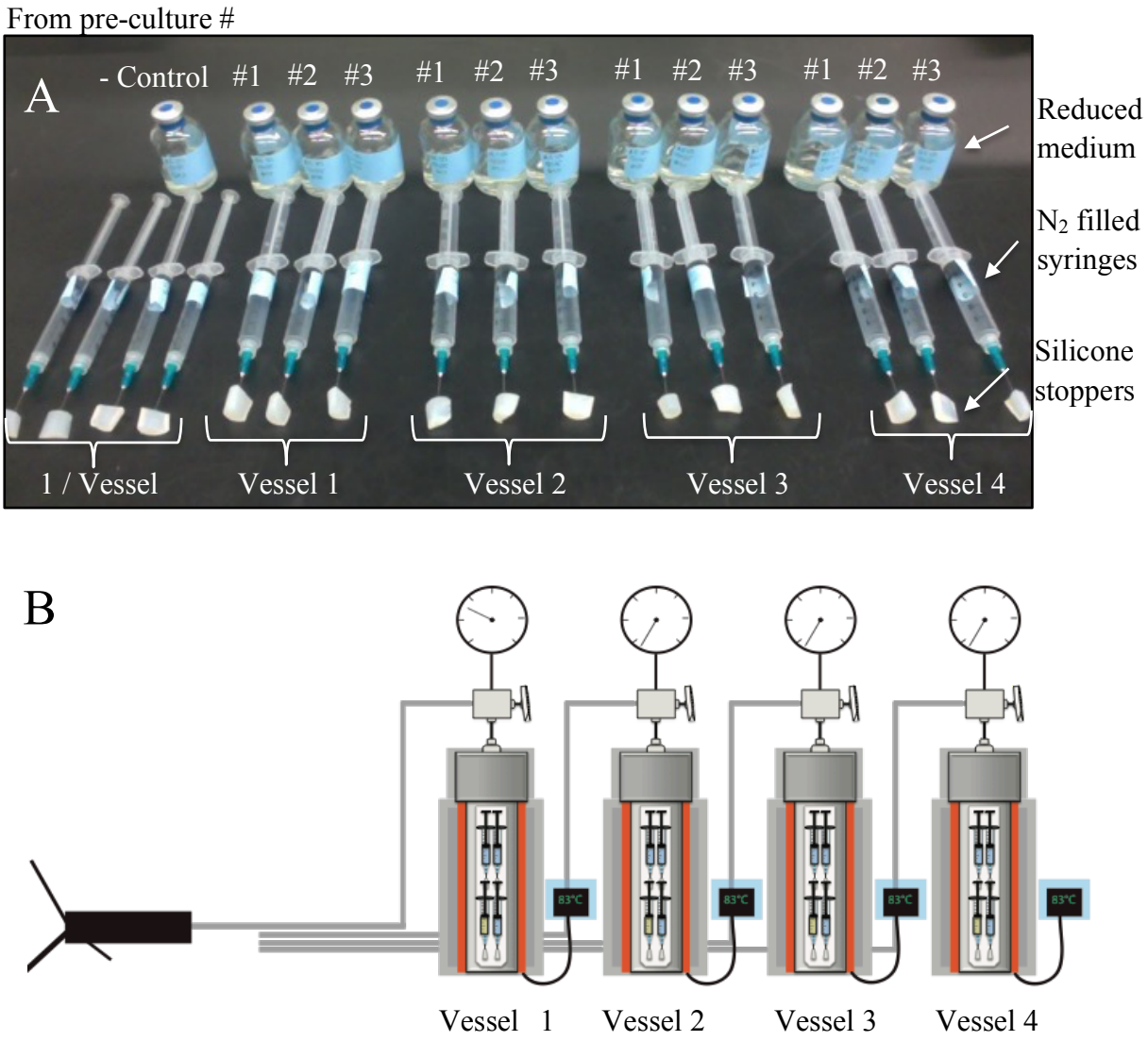

Figure 2.3. (A) A picture of serum bottles filled with reduced sterile medium for inoculation from three separate pre-cultures and flushed plastic syringes prepared for anaerobic transfer of inoculated medium. (B) A schematic of the four heated pressure vessels with syringe samples. This illustrates an example of how to prepare for a HT-HHP growth curve experiment done in triplicate with syringe distribution into each vessel.

2.4 Pressurized underwater sample handler (PUSH) vessel HT-HHP system

2.4.1 Vessels

A mobile HT-HHP system was built for eight pressure-retaining vessels with reservoir sizes of 50 mL for batch culture. These pressurized underwater sample handler (PUSH) vessels were made by TOP Industrie© and have a maximum pressure and temperature range of 0.1-100 MPa and 25-160°C respectively. Each vessel included a main PUSH vessel body, a polyether ether ketone (PEEK) reservoir with a floating piston and lid, two screw caps (one screw cap for the PEEK piston side and one screw cap to the PEEK reservoir side), two sets of valve connections and two valves (Figure 2.4 A, B, & C). For preserving the lifetime of the PEEK reservoir, the working pressure range was suggested for use up to 80 MPa (TOP Industrie©). It was highly recommended (from experience) to use an anti-seizing agent on the screw caps for routine batch culturing on the laboratory using these vessels. Seizing has occurred with regular usage and no lubricating agent.

2.4.2 Design objectives and schematics

The objectives for the second HT-HHP cultivation system design were to build portable heating systems that could be taken on and off of the PUSH vessels, create a portable power source for the heating systems for heating in an anaerobic chamber, and build an installation for eight vessels so that each vessel could be pressurized from one or two hydraulic pump(s). Overall, this heating system needed to be portable, removable, and suitable for continually heating the vessels in the anaerobic chamber. Again, the length, width, and height of this system design considered table space availability and usability by researchers.

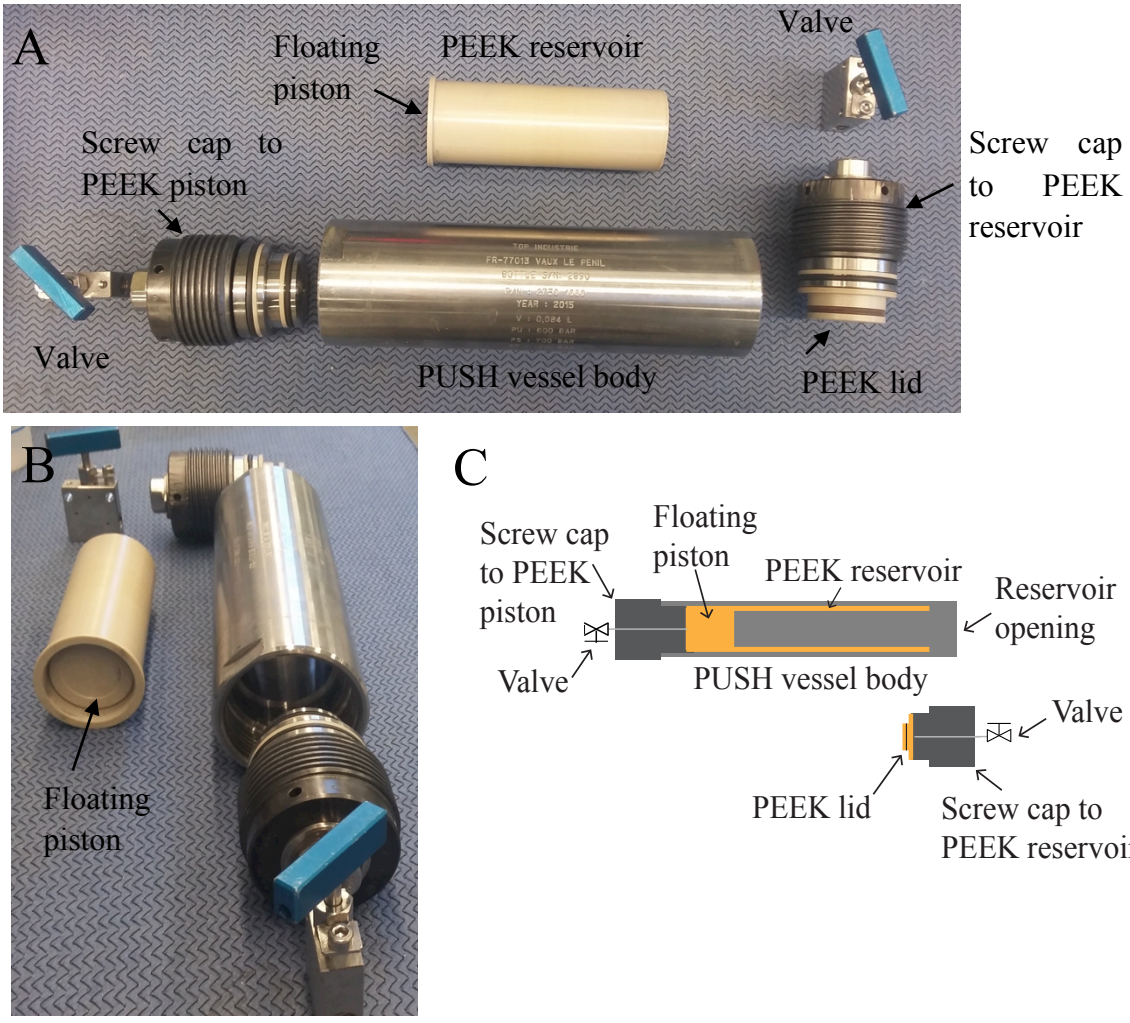

Figure 2.4. Picture of a disassembled Pressurized Underwater Sampler Handler (PUSH) vessel and individual parts (A), a side view of the PUSH showing the floating piston in the PEEK reservoir (B), and a schematic of the PUSH vessel (C).

2.4.3 Materials

The second installation frame was also constructed from 80/20® Inc. T-slotted aluminum framing (Figure 2.5). Clamping hangers, L-brackets, hinges, latches, clear Nema boxes (Figure 2.5), rigid galvanized standard duct, insulation (flexible foam sheets and rigid bubble insulation), 12-volt rechargeable large cell battery, battery charger, electrical wires, screws, fuses, and power cords were purchased from McMaster Carr. Eight additional temperature-controlled OMEGA®

systems previously described (section 2.3.4) were purchased and assembled for up to eight PUSH vessels. The only differences in the heating system were the heating jacket size (7x7 in. silicone rubber heaters) and the insulation (see 2.4.4). Flexible 1/16-inch tubing and 1/16 to 1/8 inch adapters were purchased from HiP© to make a high-pressure line that could connect the pump to each vessel. Also, 1/8-inch high-pressure tubing, one needle valve (HiP), and one micrometering valve (TOP Industrie©) were purchased to construct a decompression line.

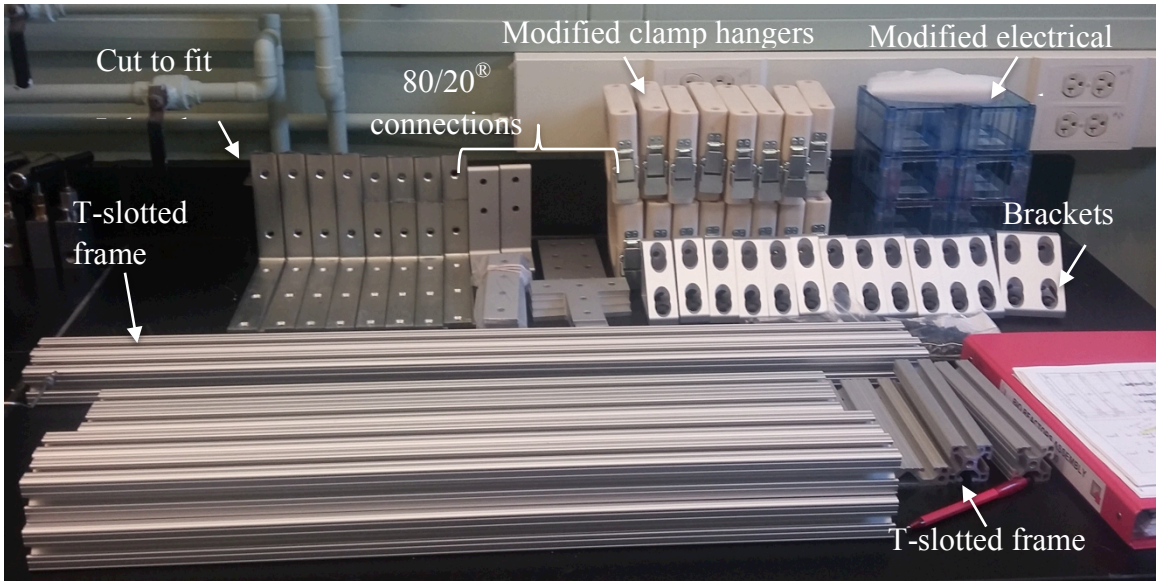

Figure 2.5. A picture of the HT-HHP installation system parts showing the T-slotted frame, connections, and brackets from 80/20® and modified materials from McMaster Carr.

2.4.4 Construction

Modifications were made to most of the materials to fit the installation design (Figure 2.5). To make supports and enclosures for each vessel, the following modifications were made: 8x8 inch L-brackets, used to support the vessels, were cut down to 4.5 inches on one side to fit the frame, hinges and latched were screwed into the clamping hangers, and eight 7-inch cylinders

were cut from a 10-foot stainless steel standard duct, each cylinder was cut in half and deburred. Each half of the stainless steel duct cylinder was screwed onto each side of the clamping hanger, and the clamping hanger was bolted to the L-bracket. Each vessel enclosure was made from two clamping hangers bolted to a set of L-brackets (Figure 2.6). The Nema electrical boxes were cut to fit the temperature controllers and holes were drilling through the boxes to enclose the heating and power element wires and a bottom hole was drilled in for stability onto the installation. Finally, the installation was made up of two vertically stacked sections of four vessels (Figure 2.6).

For the vessel heating system, the thermocouple was placed onto the middle of a PUSH vessel, then the heating jacket and insulation was wrapped around the vessel and thermocouple and was secured with two hook and loop fasteners. Each vessel had a double insulation wrap made from a flexible foam insulation sheet sealed to a less flexible bubble insulation sheet (Figure 2.6). These heating wraps were made to be easily taken on and off of vessels. For powering the heaters in the anaerobic chamber, a DC to AC power converter was purchased and connected to a 12-volt rechargeable lead-acid battery with a fuse attached to the positive cable (Figure 2.7).

Finally, the high-pressure line and decompression line was assembled. The high-pressure line was made with flexible 1/16-inch high-pressure tubing and was connected to a 1/16-inch to 1/8-inch adapter on both ends of the tubing. This was done because the connections to the PUSH vessels and the pump have 1/8-inch connections. The decompression line was made to minimize the difference of pressure in the vessel from within the decompression line and diminish shear stress applied to cell samples during decompression. This was made from using 1/8-inch high-pressure tubing and connecting it to a needle valve (HiP©) and to a micrometering valve (TOP

Industrie©). A plastic Luer-lok valve/connection was placed at the end of the decompression line to connect any slip tip or Luer-lok syringes for sampling cell cultures.

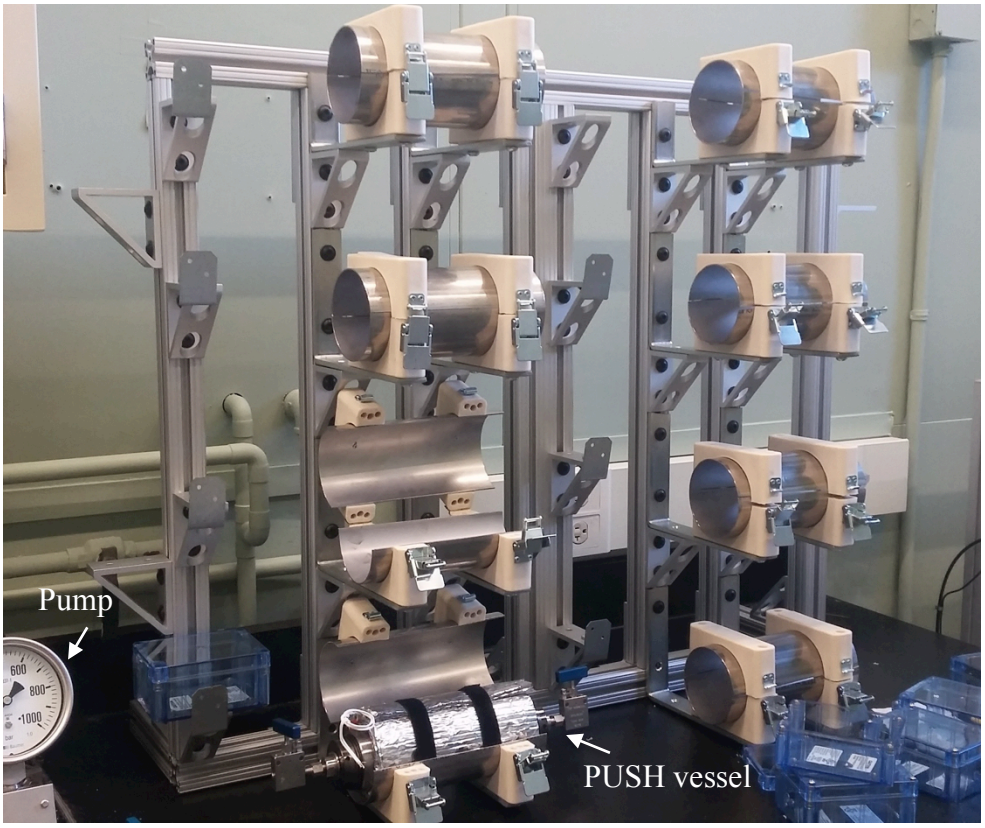

Figure 2.6. The main assembly of the HT-HHP installation frame with cut-to-fit stainless steel vessel holders, and for reference a hydraulic screw pump is shown in the bottom left and a PUSH vessel wrapped in a heating jacket and insulation is shown bottom center.

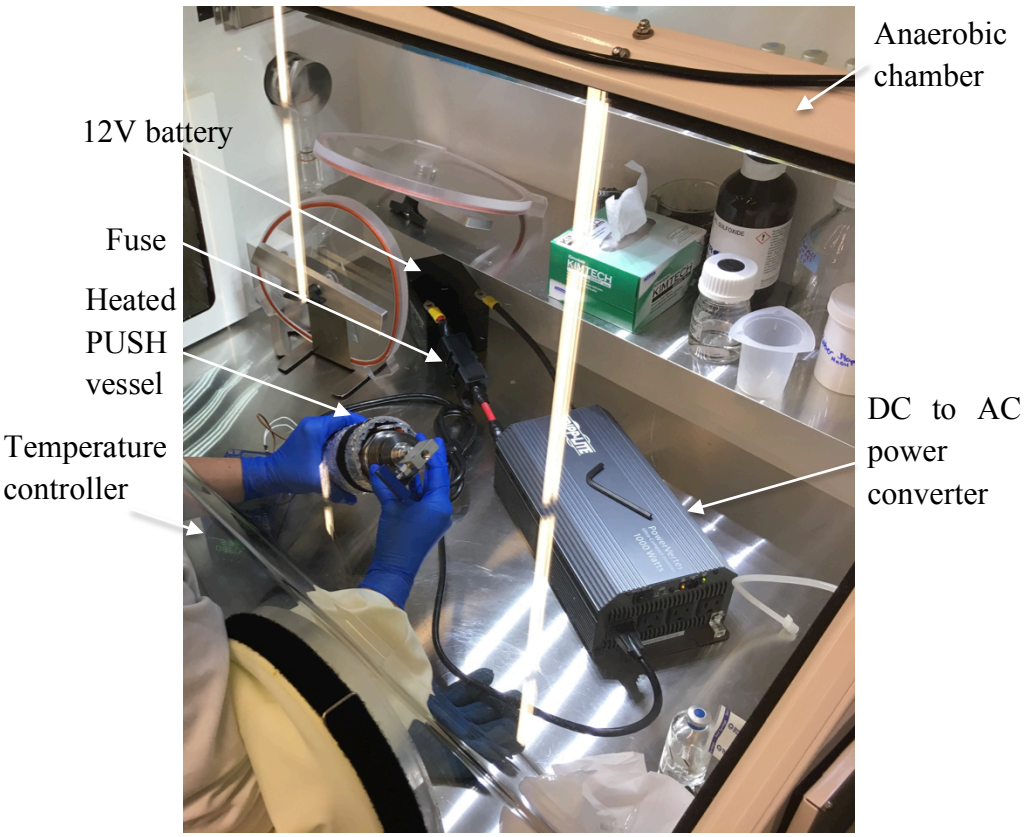

Figure 2.7. A heated and insulated PUSH vessel in the anaerobic chamber. The PUSH vessel and temperature controller system is plugged into the DC to AC power converter ran by a 12-volt battery.

2.4.5 Equipment testing

The internal PUSH vessel temperatures were monitored by directly measuring the ultrapure water (18.2 MΩ) fluid temperature inside the heated vessels. The controllers were set to the temperature that reflected an internal vessel temperature of 83°C (±0.5°C). The heat loss during the experimental procedure (see below) was at most 15°C and was reestablished within five minutes.

2.4.6 *A. fulgidus* growth in the PUSH vessels without sample decompression

Different pressure retaining vessels for microbial sample collection from the deep-sea and HT-HHP cultivation have been designed over the last fifty years (reviewed in Tamburini 2006), and a few of those designs were specifically made to cultivate hyperthermophilic piezophiles (Kyo, Tuji, Usui, & Itoh, 1991; Cangenella, Gonzalez, Yanagibayashi, Kato, & Horikoshi, 1997; Kato, 2006; Parkes et al., 2009). Here, we present a small scale HT-HHP system with PUSH vessels to grow ~50 mL *A. fulgidus* batch cultures without culture decompression during sampling. The protocols for running a growth experiment in triplicate with this HT-HHP system using PUSH vessels are given here.

2.4.6.1 PUSH vessel preparation

1. Four PUSH vessels are needed to run a growth experiment in triplicate. Three vessels are for inoculated medium and one vessel is for sterile medium as a negative control. To sterilize the vessels, each vessel must be partially assembled before being placed in the autoclave. To do this, the PEEK reservoir must be slid into the vessel and the screw cap to piston must be tightened to the vessel (Figure 2.8 A). Foil is placed over the opening to the PEEK reservoir and vessel, and then the foil is sealed with autoclave tape. Additionally, the screw cap to PEEK reservoir and attached PEEK lid, valve opening on the PUSH vessel, and valve connections all must be covered in foil and placed in the autoclave (Figure 2.8 A). The four vessels and connections are then autoclaved for 15 minutes at 121°C for sterilization.

2. After sterilization, each vessel is assembled under a biosafety cabinet (Figure 2.8 A). The screw cap to PEEK reservoir needs to be partially screwed onto the main body to maintain

sterility but not tightened or else the PEEK lid will seal onto the reservoir (Figure 2.8 B). The PEEK lid must be unsealed to later add the inoculated medium. Next, the valve on the PEEK reservoir side is closed until transport to the anaerobic chamber. After assembly, the vessels need to be pre-heated in the heating jackets and insulating wraps (Figure 2.8 C). Once the vessels reach 83°C, they can then be transported to the anaerobic chamber with their heating systems.

2.4.6.2 Media preparation and precultures

1. Media preparation and steps for preculture preparation here follows the same protocol as growth in syringes (see section 2.3.6). The only modification is the use of four serum bottles filled with 50 mL of sterile reduced medium for triplicate experiments and one negative control. The serum bottles filled with sterile medium are decrimped and placed in the anaerobic chamber with the precultures.

2.4.6.3 Powering the PUSH vessels inside the anaerobic chamber

1. The DC to AC power converter, cables, and fully charged 12-volt battery are transported into the anaerobic chamber and the battery is then connected to the power converter (Figure 2.7 and 2.9 A). All four vessel-heating systems can be plugged into the power converter so that the vessels are continually heated throughout the inoculation process and the vessels will not lose too much heat before pressurization (Figure 2.7 and 2.9 A).

2.4.6.4 Inoculation and transfer in the anaerobic chamber

1. Once in the anaerobic chamber, the heated vessels are plugged into the DC to AC power converter. Three of the four prepared sterile reduced medium serum bottles are inoculated with 1% (vol/vol) logarithmic phase *A. fulgidus* cells to a final concentration of ~5.0 x 10^6 cells/mL. Each serum bottle is inoculated with a separate preculture for triplicates experiments. Inoculated medium should be shaken, then the butly rubber stopper is removed, and finally each ~50 mL of inoculated medium is poured into a PUSH vessel (Figure 2.9 A and B). After the addition of inoculated medium, vessels are closed with the screw cap to PEEK reservoir and the respective valve is closed. After all of the medium is transferred, the heating systems are unplugged and the vessels are taken out of the anaerobic chamber.

2.4.6.5 Sample pressurization

1. All four vessels are taken to the HT-HHP installation frame to be secured into a designated vessel holder (Figure 2.10). There, each vessel is pressurized using a HHP pump (TOP Industrie©) connected to a flexible high-pressure line made from HiP© parts (Figure 2.10). To do this, the pump first needs to be filled with water (reservoir size, 50 mL) by closing the pressure valve and opening the reservoir valve. Once the pump is filled, the reservoir valve is then closed. The high-pressure line on the HHP pump is then connected to the valve on the piston side of the PUSH vessel. Then both, the pressure valve and valve to the piston on the PUSH is opened. Once the desired pressure is obtained, the valve to the piston is closed. This procedure is done to all four vessels. Pressure stability is checked regularly within the first 2-3 hours after inoculation.

2.4.6.6 Subsampling without decompressing the whole culture

1. The decompression line is cleaned with ethanol and ultrapure water (18.2 MΩ) water before subsampling. After cleaning, both valves on the decompression line are closed before starting the subsampling process. The decompression line is fastened to the valve connected to the PEEK reservoir side of the PUSH vessel (Figure 2.11). The decompression line is then secured in place with a stand and clamp holder (Figure 2.11). Next, the high-pressure line is connected to the valve to the piston side on the PUSH vessel. The pump is refilled and the pump reservoir valve is then closed. The pressure valve on the HHP pump is opened and the high-pressure line to the pressure of the vessel is then pressurized to the targeted pressure. Once the pressure is the same on the line as it is inside the vessel, the valve to the piston is then opened. The pressure should not change as the valve to the piston is opened.

2. To subsample, a sterile syringe is placed on the end on the decompression line (Figure 2.11). The valve on the PEEK reservoir is opened and the pressure is adjusted if necessary using the HHP pump. This is not a drastic pressure change (~5-10% pressure loss). Next, the first valve on the decompression line is opened (Figure 2.11). Again, the pressure is adjusted if needed. Then, the microvalve on the decompression line is slowly opened as the *A. fulgidus* culture fluid is sampled. While sampling, the pressure is maintained in the PUSH vessel using the HHP pump. The first 3-4 mL of fluid is discarded since it has come in contact with water and ethanol. After the decompression line has been flushed with culture, a 0.5 mL aliquot of *A. fulgidus* is taken and fixed into 2.5% gludaraldehyde and is stored until it can be analyzed.

3. To close the PUSH vessel, the valve to the PEEK reservoir is closed and disconnect from the decompression line. The vessel should still be at the targeted pressure. If it is not, the pressure is adjusted and then the valve to the piston side is closed. Once both valves on the PUSH vessel are

closed, the pump reservoir valve is opened to release the pressure on the HHP pump. Then the high-pressure line is disconnected from the PUSH vessel.

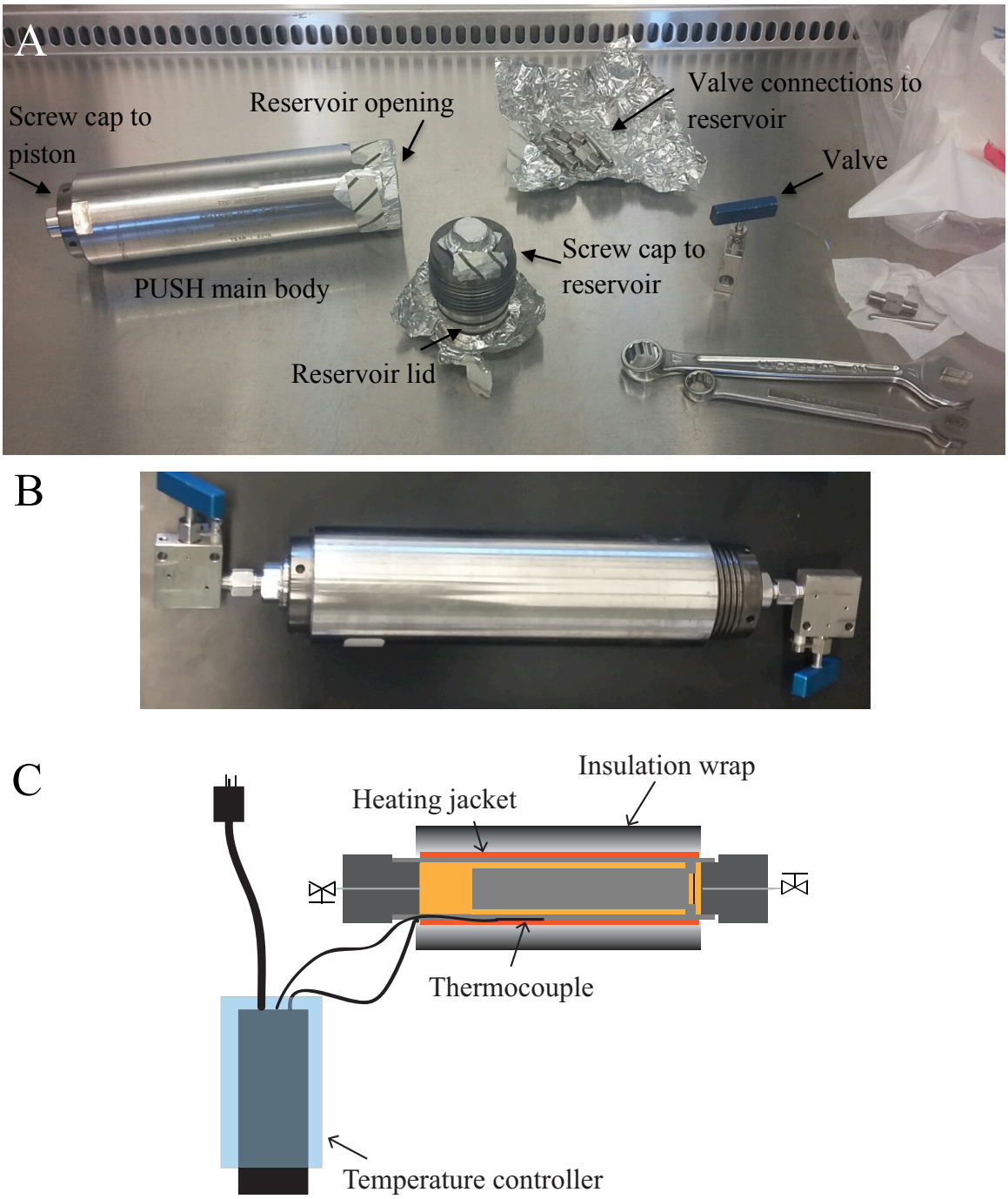

Figure 2.8. A picture of a sterile PUSH vessel and individual parts in a biosafety cabinet (A) for vessel assembly under sterile conditions. (B) An assembled PUSH vessel with the screw cap to the PEEK reservoir left partially unthreaded to later open for sample loading. (C) A schematic of a heated and insulated PUSH vessel with the temperature controlled system.

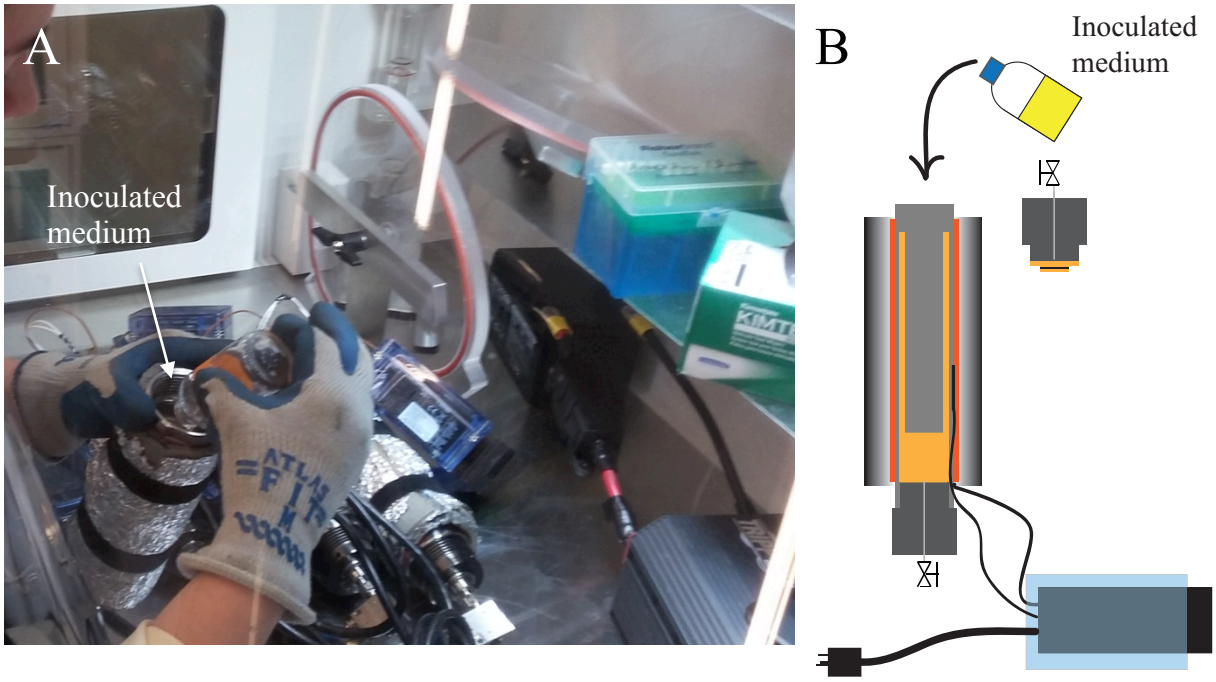

Figure 2.9. Picture of inoculated medium poured into a heated PUSH vessel in the anaerobic chamber (A). Three vessels were filled with inoculated medium and one vessel was filled with the sterile medium for triplicate experiments and a negative control (A). A schematic for pouring incoculated medium into a heated PUSH vessel (B).

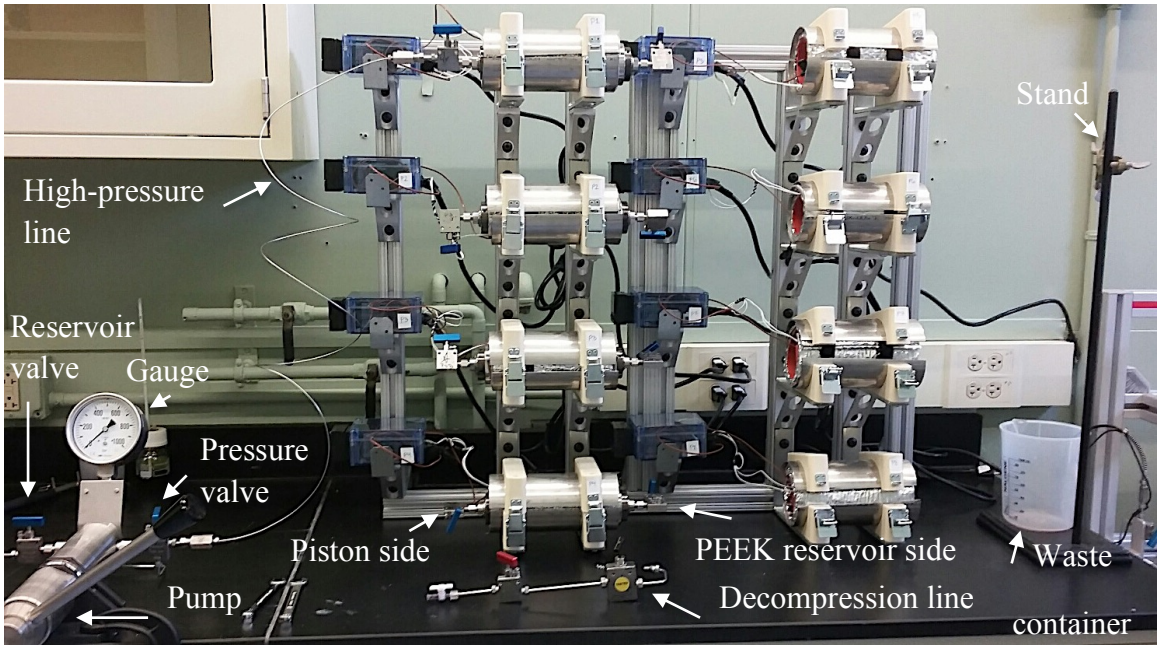

Figure 2.10. A picture of four heated and insulated PUSH vessels in the installation system secured in place with the manual screw pump (bottom left), high-pressure line, decompression line (bottom center), decompression line stand (right), and waste container (bottom right).

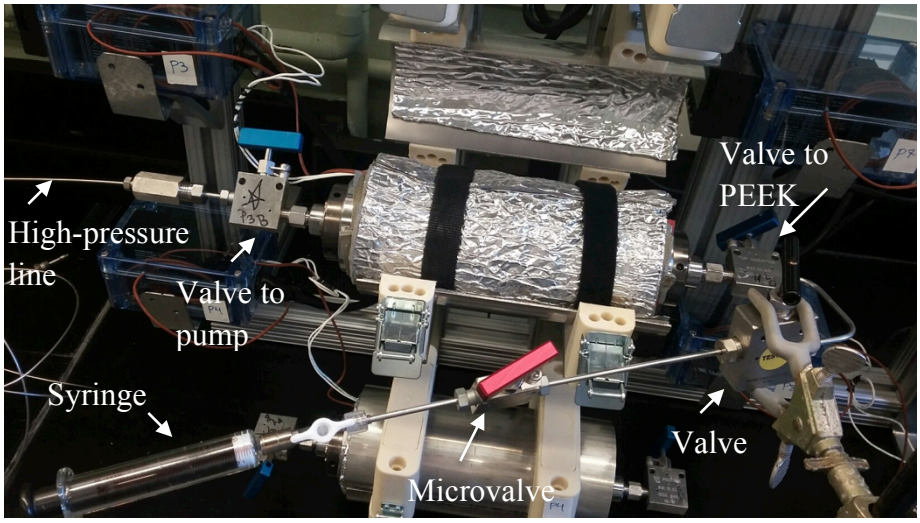

Figure 2.11. A picture of a heated and insulated PUSH vessel connected to a manual screw pump (left) and the decompression line (right) with a sampling syringe.

2.5 Concluding remarks

Here, we presented two high temperature (HT) and high hydrostatic pressure (HHP) batch cultivation systems and basic example protocols for growing a model extremophile, *Archaeoglobus fulgidus*. This was done in hopes to inspire other laboratories into adopting HT-HHP techniques when growing extremophiles from deep-sea vents and hot subseafloor environments. Previous HT-HHP microbial cultivation has highlighted the fact that relatively little is known about the physiology and metabolism of deep-sea and subseafloor microorganisms. Fluctuating temperature, pressure, and geochemical conditions all have an impact on microbial functionality. Therefore, a large effort is needed to grow deep-sea and subseafloor microorganisms under various HT-HHP and geochemical conditions to better replicate *in situ* conditions.

2.6 Acknowledgements

We would like to give our many thanks Dr. E. Bruce Watson for gifting the Habitability and Extreme Life Laboratory (HELL) four high-pressure vessels that were used for HT-HHP cultivation with sample decompression.

2.7 References

Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R., & Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiological Reviews*, *43*(2), 260–296. 10.1016/j.watres.2010.10.010

Baross, J., & Deming, J. W. (1983). Growth of `black smoker' bacteria at temperatures of at least 250 °C. *Nature*, *303*(5916), 423–426. 10.1038/303423a0

Bartlett D, H., & Welch T, J. (1995). OmpH gene expression is regulated by multiple environmental cues in addition to high pressure in the deep-sea bacterium Photobacterium species strain SS9. *Journal of Bacteriology*, *177*(4), 1008–1016. 10.1128/jb.177.4.1008-1016.1995

Berger, L. R., & Tam, L. Q. (1970). A method to grow obligately aerobic bacteria at increased hydrostatic pressure. *Limnology and Oceanography*, *15*(3), 483–485.

Bianchi, A., Garcin, J., & Tholosan, O. (1999). A high-pressure serial sampler to measure microbial activity in the deep sea. *Deep-Sea Research Part I: Oceanographic Research Papers*, *46*(12), 2129–2142. 10.1016/S0967-0637(99)00039-4

Bowles, M. W., Samarkin, V. a., & Joye, S. B. (2011). Improved measurement of microbial activity in deep-sea sediments at *in situ* pressure and methane concentration. *Limnology and Oceanography: Methods*, *9*, 499–506. 10.4319/lom.2011.9.499

Canganella, F., Gonzalez, J. M., Yanagibayashi, M., Kato, C., & Horikoshi, K. (1997). Pressure and temperature effects on growth and viability of the hyperthermophilic archaeon *Thermococcus peptonophilus*. *Archives of Microbiology*, *168*(1), 1–7. 10.1007/s002030050462

D'Hondt, S. (2004). Distributions of microbial activities in deep subseafloor sediments. *Science*, *306*(5705), 2216–2221. 10.1126/science.1101155

Fry, J. C., Parkes, R. J., Cragg, B. A., Weightman, A. J., & Webster, G. (2008). Prokaryotic biodiversity and activity in the deep subseafloor biosphere. *FEMS Microbiology Ecology*, *66*(2), 181–196. 10.1111/j.1574-6941.2008.00566.x

Hartzell, P., & Reed, D. W. (2006). The genus *Archaeoglobus*. The *Prokaryotes*, *3*, 82–100. 10.1007/0-387-30747-8

Jebbar, M., Franzetti, B., Girard, E., & Oger, P. (2015). Microbial diversity and adaptation to high hydrostatic pressure in deep-sea hydrothermal vents prokaryotes. *Extremophiles*, *19*(4), 721–740. 10.1007/s00792-015-0760-3

Kato, C. (2006). 31 Handling of piezophilic microorganisms. *Methods in Microbiology*, *35*(5), 733-741. 10.1016/S0580-9517(05)35031-8

Kell, D. B. (1987). Forces, fluxes and the control of microbial growth and metabolism. *Journal of General Microbiology*, *133*, 1651–1665.

Kyo, M., Tuji, Y., Usui, H., & Itoh, T. (1991). Collection, isolation, and vultivation system for deep-sea microbes study: concept and design. *The Institute of Electrical and Electronics Engineers*, 419-423.

Maier, R. M. (2009). Bacterial Growth. In R. M. Maier, I. L. Pepper, C. P. Gerba (Eds.), *Environmental microbiology* (pp. 37–54). Burlington, MA: Academic Press. Inc.

Marietou, A., Nguyen, A. T. T., Allen, E. E., & Bartlett, D. H. (2014). Adaptive laboratory evolution of *Escherichia coli* K-12 MG1655 for growth at high hydrostatic pressure. *Frontiers in Microbiology*, *5*, 1–8. 10.3389/fmicb.2014.00749

Marteinsson, V. T., Birrien, J., Reysenbach, A., Vernet, M., Marie, D., Gamba, A.,…Prieur, D. (1999). *Thermococcus barophilus* sp. nov., a new barophilic and hyperthermophilic archaeon isolated under high hydrostatic pressure from a deep-sea hydrothermal vent. *International Journal of Systematic Bacteriology*, (49), 351–359.

Parkes, R. J., Cragg, B., Roussel, E., Webster, G., Weightman, A., & Sass, H. (2014). A review of prokaryotic populations and processes in sub-seafloor sediments, including biosphere: Geosphere interactions. *Marine Geology*, *352*, 409–425. 10.1016/j.margeo.2014.02.009

Parkes, R. J., Sellek, G., Webster, G., Martin, D., Anders, E., Weightman, A. J., & Sass, H. (2009). Culturable prokaryotic diversity of deep, gas hydrate sediments: first use of a continuous high-pressure, anaerobic, enrichment and isolation system for subseafloor sediments (DeepIsoBUG). *Environmental Microbiology*, *11*(12), 3140–3153.

Picard, A., Testemale, D., Hazemann, J. L., & Daniel, I. (2012). The influence of high hydrostatic pressure on bacterial dissimilatory iron reduction. *Geochimica et Cosmochimica*

Acta, *88*, 120–129. 10.1016/j.gca.2012.04.030

Takai, K., Nakamura, K., Toki, T., Tsunogai, U., Miyazaki, M., Miyazaki, J., et al. (2008). Cell proliferation at 122°C and isotopically heavy CH_4 production by a hyperthermophilic methanogen under high-pressure cultivation. *Proceedings of the National Academy of Sciences*, *105*(31), 10949–10954. 10.1073/pnas.0712334105

Tamburini, C., Garcin, J., & Bianchi, A. (2003). Role of deep-sea bacteria in organic matter mineralization and adaptation to hydrostatic pressure conditions in the NW Mediterranean Sea. *Aquatic Microbial Ecology, 32*, 209–218.

Tamburini, C. (2006). Life under pressure: Deep-sea microbial ecology. In J. Seckbach (Ed.), *Life as we know it.* (pp. 125-143). Dordrecht, The Netherlands: Springer.

Tasumi, E., Yanagawa, K., Miyazaki, J., & Takai, K. (2015). In vitro high-pressure incubation and activity measurement of deep-sea methanogenic archaea, In T.J. McGenity et al (Eds.), *Hydrocarbon and lipid microbiology protocols* (pp. 51–64). Berlin, Germany: Springer.

Von Damm, K. L. (1990). Seafloor hydrothermal activity : black smoker. *Annual Review of Earth and Planetary Sciences*, (18), 173–204. 10.1146/annurev.earth.18.1.173

Yayanos, A. A. (1995). Microbiology to 10,500 meters in the deep sea. *Annual review of Microbiology*, *49*, 777–805. 10.1146/annurev.micro.49.1.777

Yayanos, A. A. (2001). 30 Deep-sea piezophilic bacteria. *Methods in Microbiology*, *30*, 615–637.

Yayanos, A. A., & Dietz, A. S. (1982). Thermal inactivation of a deep-sea barophilic bacterium, isolate CNPT-3. *Applied and Environmental Microbiology*, *43*(6), 1481–1489.

10.1016/0198-0254(82)90334-X

Zobell, C. E., & Oppenheimer, C. H. (1950). Some effects of hydrostatic pressure on the multiplication and morphology of marine bacteria. *Journal of Bacteriology*, *60*(0), 771–781.

3. The rate and extent of growth of a model extremophile, *Archaeoglobus fulgidus,* at elevated pressures

3.1 Abstract

High hydrostatic pressure batch cultivation of a model extremophile, *Archaeoglobus fulgidus*, was performed to explore how elevated pressures might affect microbial growth and physiology of microorganisms in the deep biosphere. Though commonly identified in high-pressure marine environments (up to 2-5 km below sea level, 20-50 MPa pressures), *A. fulgidus* growth at elevated pressure has not been previously characterized. Here, exponential growth of *A. fulgidus* was observed up to 60 MPa when supported by the heterotrophic metabolism of lactate oxidation coupled to sulfate reduction, and up to 40 MPa for autotrophic growth coupled to thiosulfate reduction. Maximum growth rates for this heterotrophic metabolism were observed at 20 MPa, suggesting that *A. fulgidus* exhibits moderate piezophily under these conditions. However, piezotolerance was observed for this autotrophic metabolism, as growth rates were similar from 0.3 MPa to 40 MPa. Additionally, *A. fulgidus* produced biofilm under heterotrophic cultivation from 0.1 MPa- 50 MPa. This physiological response was not induced by elevated pressures but rather was associated with low calcium concentrations and as well as the micro niche created by the presence of a stainless steel needle in contact with the growth medium. These results show that *A. fulgidus* can inhabit a wide range of high-pressure environments in the deep biosphere exploiting a variety of metabolic strategies and will produce biofilm that is advantageous in extreme and fluctuating environments.

3.2 Introduction

High-pressures (≥ 10 MPa, megapascals) are an inherent characteristic of all deep biosphere ecosystems (Jannasch & Taylor, 1984; Oger & Jebbar, 2010), however the effects of elevated pressure on the physiology of the diverse microbial populations that inhabit these systems are largely unknown. These deep-sea and subsurface microbial communities are the foundations of deep ecosystems as the primary producers and consumers, yet most microorganisms recovered from the deep biosphere are cultivated at ambient pressures (i.e. 0.1 MPa) and relatively few have been cultivated under elevated *in situ* pressures. Therefore our understanding of how subsurface microbes cycle mass and energy is based on their physiology under surface conditions instead of the high-pressure conditions of their natural habitats. High-pressure microbial enrichment cultivation, though first introduced by ZoBell and Oppenheimer in 1950, has been performed for decades but it has still not been widely adopted, largely due to costs and complexities of developing specialized high-pressure equipment (Jebbar, Franzetti, Girard, & Oger, 2015). Previous studies have shown that high-pressure does indeed impact microbial growth (e.g. Zobell & Oppenheimer, 1950; Yayanos, Dietz, & Boxtel, 1982; Jannasch, Wirsen, Molyneaux, & Langworthy, 1992; Bartlett, 2002; Takai et al., 2008) and that individual species respond differently to increasing pressures (e.g. Kato, Nogi, & Arakawa, 2008; Fang, Zhang, & Bazylinski, 2010; Jebbar et al., 2015). Thus, realistic models of the subsurface microbiome require characterization of subsurface microbial physiology under HHP that better reflect *in situ* conditions.

Here a model extremophile, *Archaeoglobus fulgidus*, found in both surface and deep environments, was used to explore the effects of high hydrostatic pressure (HHP) on microbial growth. *A. fulgidus,* one of the best described *Archaeoglobus* species (Achenbach-Richter, Stetter, & Woese, 1987; Klenk et al., 1997), is a marine hyperthermophilic archaeon that cycles carbon and sulfur via dissimilatory sulfate reduction (Stetter, Lauerer, Thomm, & Neuner, 1987). Even though *A. fulgidus* was first isolated from a shallow marine vent (Stetter et al., 1987), it has been isolated from a deep-sea hydrothermal vent, deep oil reservoirs, and deep geothermal wells (Stetter et al., 1993; Beeder, Nilsen, Rosnes, Torsvik, & Lien, 1994; L'Haridon, Reysenbach, Glenat, Prieur, & Jeanthon, 1995; Fardeau et al., 2009) and has been identified via molecular tools in various deep-sea vents and shallow terrestrial hot springs (e.g. Takai & Horikoshi, 1999; Schrenk, Kelley, Delaney, & Baross, 2003; Nercessian, Reysenbach, Prieur, & Jeanthon, 2003; Table 3.1). *A. fulgidus* was the first hyperthermophilic sulfate reducing archaeon isolated and characterized (Stetter, 1988; Thauer & Kunow, 1995; Rabus, Hansen, & Widdel, 2013), and other species of the genus *Archaeoglobus* remain the few known hyperthermophilic sulfate reducing archaea (Rabus et al., 2013). *A. fulgidus* and other hyperthermophilic sulfate reducing archaea (optimum growth temperatures between 75-90°C) often fill a specific ecological niche in hot sulfate-rich anoxic marine environments between the thermophilic sulfate reducing bacteria (optimum growth temperatures typically between 40°C to 70°C; Rabus et al., 2013) and the hyperthermophilic sulfur metabolizing archaea (optimum growth temperatures typically between ~70-100°C; Reysenbach, Longnecker, & Kirshtein, 2000; Huber, Huber, & Stetter, 2006; Hartzell & Reed, 2006) and theses microbes play an important role in the biogeochemical cycling of sulfur and carbon (Hartzell & Reed, 2006; Rabus et al., 2013). Therefore, it is critical

to investigate if *A. fulgidus* is able to cycle carbon and sulfate compounds in the deep biosphere environments it has been identified in as efficiently as it does in surface pressure conditions.

HHP growth has been reported for isolate *A. fulgidus* TF2 up to 35 MPa (Stetter et al., 1993) and other studies have suggested piezophilic behavior for *Archaeoglobus* species from Guyamas Basin hydrothermal sediments (Kallmeyer, Ferdelman, Jansen, & Jørgensen, 2003). The common occurrence of *A. fulgidus* in subsurface, high-pressure environments suggests that *A. fulgidus* is at least piezotolerant. However, the pressure range for growth as well as growth rates and cellular densities at elevated pressure of this model extremophile are unknown. Here the potential for *A. fulgidus* type strain VC-16 to grow in batch culture at elevated pressures was investigated at pressures up to 70 MPa. Growth rates and cell densities are reported for both a chemoorganoheterotrophic metabolism coupling lactate oxidation with sulfate reduction and a chemolithoautotrophic metabolism in which thiosulfate is reduced to H_2S with H_2.

Table 3.1. *Archaeoglobus* Isolates.

Species, Strain	Environment	Depth	Location	Reference
A. fulgidus				
VC-16†	Shallow vent	1-10 m	Vulcano, Italy	Stetter et al., 1987
Z	Shallow vent	1-10 m	Vulcano, Italy	Zellner et al., 1989
NS70-A	Deep-sea vent	~968 m	Iheya North Fields	Nakagawa et al., 2005
TF2	Oil reservoir	2-4 km	North Sea	Stetter et al., 1993
7324	Oil reservoir	2-4 km	North Sea	Beeder 1994
SL5	Oil reservoir	1.6 km	Paris Basin, France	L'Haridon et al., 1995
L3 & L4	Geothermal system	1.9 km	Paris Basin, France	Fardeau et al., 2009
A. profundus				
AV18	Deep-sea vent	2 km	Guaymas Basin, Mexico	Burggraf et al., 1990
NI85-A	Deep-sea vent	~968 m	Iheya North Fields	Nakagawa et al., 2005
A. veneficus	Deep-sea vent	3.5 km	Mid-Atlantic Ridge	Huber et al., 1997
A. infectus	Deep-sea vent	1.4 km	Izu-Bonin Arc	Mori et al., 2008
A. sulfaticallidus PM70-1	Black rust (borehole)	2.65 km	Juan de Fuca Ridge	Steinsbu et al., 2010
'A. lithotrophicus'	Oil reservoir	2-4 km	North Sea	Stetter et al., 1993

† Type strain

3.3 Methods

3.3.1 Heterotrophic and autotrophic batch cultivation

3.3.1.1 Microorganism and heterotrophic media preparation

Archaeoglobus fulgidus type strain VC-16 (DSM 4304) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). Heterotrophic batch culture medium composition was as follows per liter: 0.34g KCl, 15.142g

$MgSO_4$•$7H_2O$, 2.75g $MgCl$•$6H_2O$, 0.25g NH_2Cl, 0.056g $CaCl_2$•$2H_2O$, 0.0137g K_2HPO_4•$3H_2O$, 17.8g NaCl, 0.0039g $Fe(NH_4)_2(SO_4)$•$6H_2O$, 2.1g sodium lactate, 1g yeast extract, 3.36g PIPES (piperazine-*N*,*N*′bis [2-ethanesulfonic acid), 0.1 mL Resazurin (0.1% solution), and 1 mL of Wolfe's trace element solution following Hartzell & Reed (2006). The inorganic sea salt solution with Resazurin was autoclaved at 121°C for 20 minutes and cooled to room temperature prior to the addition of lactate, 10 mM PIPES solution and yeast extract by filter sterilization under a biosafety cabinet and the pH was adjusted to 6.7. The sterile medium was aseptically distributed into sterile Balch tubes (10 mL of medium), capped with butyl stoppers and crimped. The Balch tube headspaces were flushed with N_2 and Na_2S•$9H_2O$ was added to a final concentration of 0.1% prior to inoculation to obtain anaerobic conditions (Cario, Jebbar, Thiel, Kervarec, & Oger, 2016). For batch cultivation, sterile media was inoculated with 3% (v/v) logarithmic phase *A. fulgidus* cells from three separate precultures to a final cell concentration of ~1.25 x 10^7 cells/mL. All experiments conducted in Balch tubes were amended with a 0.3 MPa N_2 headspace following inoculation. All HHP batch culture experiments were performed in headspace-free syringes (see below). Growth experiments at ambient pressure were performed either under traditional batch cultivation techniques in Balch tubes (0.3 MPa; Balch, Fox, Magrum, Woese, & Wolfe, 1979), or in HHP batch culture methods in headspace-free syringes (0.1 MPa).

3.3.1.2 Heterotrophic HHP batch cultivation experiments

To perform HHP batch cultivation, the batch culture experiments, contained in stoppered syringes, were distributed across four static pressure vessels that required decompression during subsampling. To minimize decompression-repressurization cycles, each vessel contained

triplicate growth experiments and a single uninoculated negative control and the experiments in any vessel were only decompressed for subsampling at every third sampling interval. To obtain at least eleven samples for the standard growth curve, each vessel went through a maximum of four decompression-repressurization cycles and there was at least six hours in between subsampling times for each vessel. Since previous studies have shown that sample decompression can negatively impact microbial growth during high-pressure cultivation (Park & Clark, 2002), a suite of high-pressure experiments were performed to test the affects of subsampling decompression and repressurization on observed *A. fulgidus* cell densities (Chapter 3 Appendixes; Figure 1S).

For HHP batch cultivation, triplicate batch culture experiments and a single uninoculated control were conducted at each pressure. For each triplicate, 30 mL of sterile, anaerobic medium was inoculated with logarithmic phase *A. fulgidus* cells from a separate preculture. After inoculation, a total of 20 mL from each inoculated triplicate was transferred aseptically into four separate 5 mL N_2 flushed syringes (BD medical) with floating pistons. After transfer, excess N_2 gas was expelled from each syringe before the needle was embedded into a silicone stopper to maintain a closed system. A total of twelve 5 mL syringe cultures, four syringes from each triplicate for each vessel, and four 3 mL syringes filled with uninoculated medium for negative controls, were prepared for each HHP experiment. The syringes were placed inside the pre-heated stainless steel vessels, filled with deionized water, and pressurized using a hydraulic screw pump (HiP Equipment Company) to the desired hydrostatic pressure (e.g. 0.1-70 MPa in 10 MPa increments) at 83°C in a temperature controlled oven (Figure 3.1; Yayanos, 2001). HHP batch culture experiments were subsampled ~11 times over 36-52 hours to obtain standard growth curves. Subsamples for cellular enumeration were taken every 2-4 hours after inoculation

for 36 hours for 0.1- 40 MPa experiments and up to 52 hours for 50-70 MPa experiments. Each vessel was decompressed to subsample then re-pressurized at most four times. Cells were decompressed at an average rate of 19 MPa/minute and 0.5 mL subsamples were fixed with 2.5% gludaraldehyde.

Lastly, *A. fulgidus* cell recovery after a 52-hour exposure at 70 MPa for the heterotrophic metabolism was tested. *A. fulgidus* cells were incubated in triplicate at 70 MPa and 83°C for 52 hours and then ~1.1 x 10^7 cells/mL was transferred into sterile anaerobic medium in Balch tubes at 0.3 MPa. The HHP exposure at 70 MPa was done in triplicate using one heated vessel. Because the effects of decompression on *A. fulgidus* cells at 70 MPa were unknown, this exposure experiment was consistent with all of the previous HHP batch cultivation experiments described where *A. fulgidus* cells exposed to 70 MPa were also decompressed and repressurized four times at 18, 24, 36, and 52 hours after inoculation. After exposure and cell transfer to 0.3 MPa, growth was monitored visually until the culture became turbid after 64 hours and directs counts of triplicate experiments were taken.

3.3.1.3 Autotrophic medium preparation

A. fulgidus VC-16 was adapted from a heterotrophic metabolism to an autotrophic metabolism (see Appendixes). The autotrophic growth medium was similar to heterotrophic medium with the following modifications; sulfate, lactate, and yeast extract were omitted, magnesium chloride was increased to 6.38g/L $MgCl \cdot 6H_2O$ to maintain similar salinity, and calcium chloride was increased to 0.14g/L $CaCl_2 \cdot 2H_2O$ for all autotrophic batch culture experiments, PIPES was replaced with 2 g/L $NaHCO_3$, and a sterile anaerobic thiosulfate stock

solution was added after reducing the medium (see below) to a final concentration of 47 mM (Hocking, Stokke, Roalkvam, & Steen, 2014). The pH was adjusted from heterotrophic growth (6.7) to 7.3 to compensate for the expected decrease in pH from the addition of 80% H_2 and 20% CO_2 dissolved gas at 0.3 MPa and under HHP conditions into the growth medium. To obtain anoxia, the medium was boiled under N_2 atmosphere and 20 mL were transferred into 30 mL serum bottles under flowing N_2. Each serum bottle was then stoppered with butyl rubber, capped, crimped, and sterilized by autoclaving at 121°C for 20 minutes. Following sterilization, the headspace was vacuumed and flushed with 80% H_2: 20 % CO_2 gas mixture at least 5 times. To completely reduce the medium, a final concentration of 0.1% $Na_2S.9H_2O$ was added prior to inoculation (Cario et al., 2016). After the medium was reduced, 0.2 mL of 2.5% (w/w) sodium thiosulfate solution (sterile and anaerobic) was added to each serum bottle. All cultures were inoculated with 1.5% (v/v) logarithmic phase *A. fulgidus* cells from three separate precultures to a final cell concentration of ~3 x 10^6 cells/mL. All HHP batch cultures, from 0.3 MPa to 60 MPa, were performed in glass syringes (see below) and all ambient cultures were performed in serum bottles with a 0.3 MPa (80% H_2: 20 % CO_2 gas mixture) headspace.

3.3.1.4 Autotrophic HHP batch cultivation experiments

Autotrophic HHP batch cultivation of *A. fulgidus* was carried out in glass gastight syringes (Hamilton, 5 mL syringes) following methods of Takai et al. (2008) and Tasumi et al. (2015), so that additional gas (H_2 and CO_2) could be added to the syringe headspace to support autotrophy. Five static high-pressure vessels were used for these HHP batch culture experiments, avoiding multiple decompression-repressurization cycles. Each vessel held two 5 mL glass

syringes and samples were pressurized once and decompressed once upon sampling. Each HHP batch culture experiment was performed in triplicate with a negative control.

Prior to *A. fulgidus* HHP autotrophic growth experiments, the H_2 loss from glass syringes at elevated temperature (83°C) and pressures (up to 60 MPa) conditions was measured. In test syringes containing 6.5 mL ultrapure water (18.2 MΩ) and 2 mL H_2, at most 0.2 mL H_2 loss was measured volumetrically over 150 hours. Therefore, ~0.5 mL of 100% H_2 was added to all autotrophic HHP batch cultures in glass syringes, so that these experimental conditions were more comparable to traditional batch cultures carried out in Balch tubes with a 0.3 MPa 80% H_2 and 20% CO_2 headspace.

The detailed experimental protocol for autotrophic HHP batch cultivation is outlined in Figure 3.2. For sterilization, clean and dried 5 mL glass syringes (Hamilton, Reno, NV), chromatography needles, butyl stoppers, Teflon tape, and butyl rubber tape for sealing the needle to syringe connection, were placed under UV light for 1 hour in a biosafety cabinet (Figure 3.2, Step 1). During syringe sterilization, anaerobic media was prepared in 30 mL serum bottles (Figure 3.2, Step 2). After sterilization, syringes were assembled to maintain gastight conditions during incubation, and then syringes were flushed with N_2 using an over-pressurized bottle (0.3 MPa) in the biosafety cabinet to maintain anaerobic and sterile conditions (Figure 3.2, Step 3). Each serum bottle was then inoculated and the headspace was over-pressurized with 80% H_2: 20% CO_2 gas mixture to 0.3 MPa and left to equilibrate for up to 5 minutes (Figure 3.2, Step 4). Next, 6.5 mL of inoculated medium (containing dissolved H_2 and CO_2) was transferred into each syringe from a serum bottle (Figure 3.2, Step 5). Finally, 0.5 mL 100% H_2 gas was added to each syringe and the needles were then embedded into butyl rubber stoppers to maintain a closed system (Figure 3.2, Step 6). All of the syringes were then placed into pre-heated vessels at 83°C

and pressurized following the same method described in section 3.3.1.2 (Figure 3.2, Step 7). The inoculated medium remaining in the serum bottles was simultaneously placed in 83°C ovens to monitor growth under ambient conditions. Lastly, *A. fulgidus* growth at 0.3 MPa in serum bottles with a ~15 mL headspace was compared to growth at 0.3 MPa in 5 mL glass syringes with ~0.5 mL gas phase incubated in the static pressure vessels. To achieve such low pressures in these vessels, an additional pressure gauge was placed onto the pressure line. This gauge was rated up to 0.7 MPa so that 0.3 MPa could be reached more precisely.

A. fulgidus autotrophic HHP batch culture experiments were performed from 0.3-60 MPa in ~20 MPa increments with minimal sample decompression to obtain up to five samples for cellular enumeration over ~145 hours for standard growth curves. Each vessel was pressurized once and decompressed once for subsampling. Cells were decompressed at an average rate of 19 MPa/minute and 0.5 mL subsamples were fixed with 2.5% gludaraldehyde.

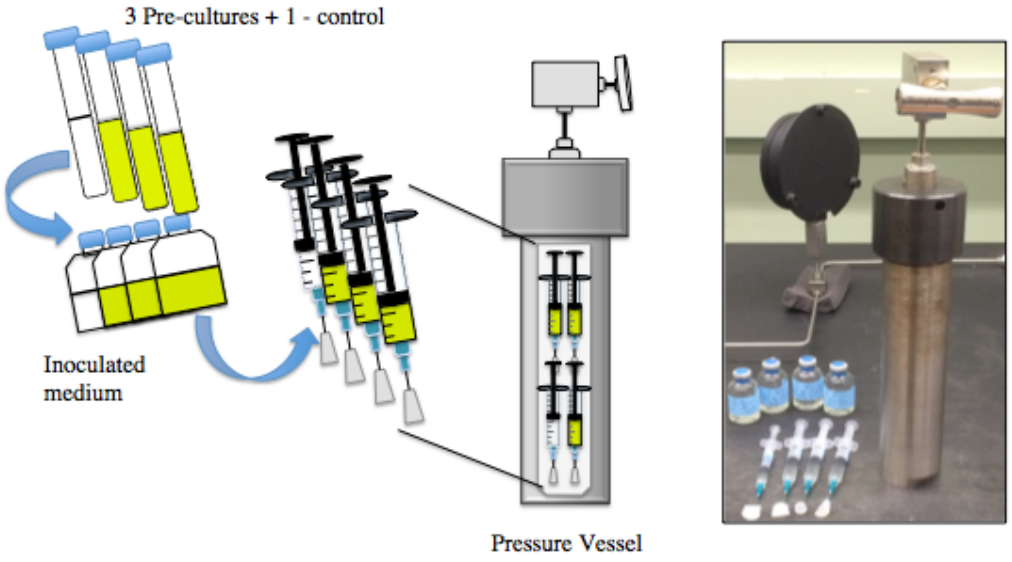

Figure 3.1. *A. fulgidus* HHP heterotrophic growth procedure for triplicate experiments at 83°C.

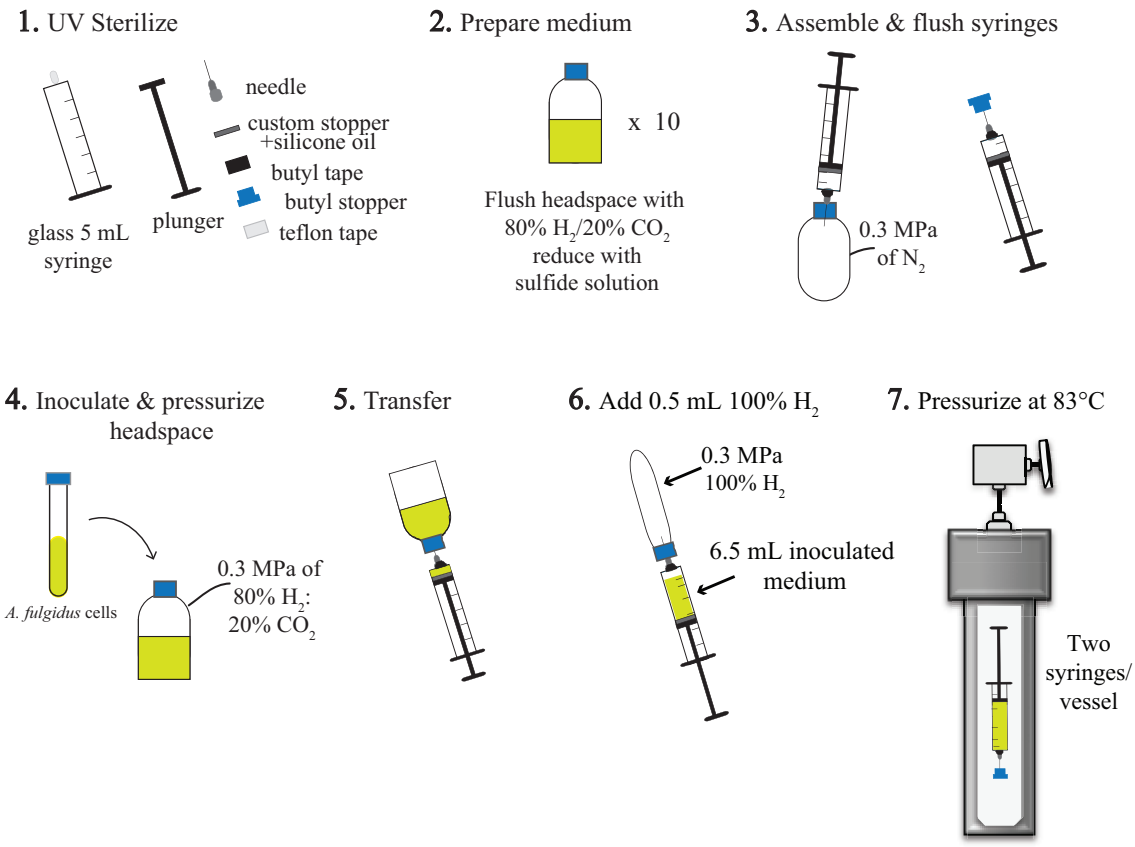

Figure 3.2. *A. fulgidus* HHP autotrophic growth procedure for a single growth curve experiment following Takai et al., (2008).

3.3.2 Microscopy

For heterotrophic growth, cell enumeration was estimated by direct counting of fixed cells in 2.5% (v/v) gludaraldehyde filtered onto 0.2µm black polycarbonate membranes (EMD Millipore) and stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Sigma Aldrich; Kepner & Pratt, 1994), under 200x magnification on an Olympus XM10 fluorescence microscope. For autotrophic growth, cell density was estimated by direct counts from fixed cells in 2.5% (v/v) gludaraldehyde on a Thoma-chamber (depth: 0.02mm; Brand, Wertheim, Germany) using a light microscope (model XM: Olympus) under 80x magnification (e.g. Huber, Woese, Langworthy, Fricke, & Stetter, 1989; Hei & Clark, 1994; Blöchl et al., 1997; Cario et al., 2016). Maximum cell densities were observed from stationary phase for all pressure conditions and error bars indicate standard deviation from at least triplicate experiments. Growth rates were calculated from the logarithmic growth phase slopes from triplicate batch culture experiments using the LINEST function in Excel. Error bars indicate the standard error from linear regressions of triplicate experiments.

3.3.3 *A. fulgidus* cell aggregation

During the growth experiments described above, cell aggregation and extracellular polymeric substances (EPS) formation was visibly evident in *A. fulgidus* batch culture experiments in syringes from 0.1 to 50 MPa. However, there was no visible EPS production for similar growth in Balch tubes, which contain 16 mL over pressurized N_2 headspace (0.3 MPa). Further, cell aggregation was not observed during autotrophic growth. Biofilm formation in *A. fulgidus* has been documented previously and was attributed to various stress conditions

including elevated trace metal concentrations and high pH (≥ 7.5; LaPaglia & Hartzell, 1997), but the potential for elevated pressure to induce biofilm formation in this species has not been documented. Because aggregation was observed in some but not all of the HHP experiments, it was not clear if the observed biofilm was due to elevated pressures or other factors. To determine the cause of the biofilm formation observed here, we also tested additional chemical and physical growth conditions including *(i)* variations in growth media composition; *(ii)* chemical changes resulting from the formation of metabolic products; and *(iii)* physical differences in growth conditions, specifically the presence of a large-volume (>50%) headspace in the ambient pressure Balch tubes. Several comparative growth experiments were conducted to constrain the specific growth conditions that might account for the biofilm formation observed here, and to determine if elevated pressures contributed to this phenomenon.

3.3.3.1 Comparative growth experiments to investigate biofilm formation

Sulfide, pH and pressure: Production of sulfide and corresponding increases in pH due to *A. fulgidus* sulfate reduction might have induced biofilm formation and the chemical impacts on cell growth could have manifested differently for cell cultivation in Balch tubes and syringes at ambient and elevated pressures. Therefore, *A. fulgidus* cell growth, biofilm formation, sulfide concentrations, and pH were measured in batch culture experiments conducted in headspace-free syringes at 0.1 MPa and 20 MPa and compared to 0.3 MPa batch cultivations in Balch tubes with 16 mL of headspace. Aliquots for each triplicate experiment were retrieved 18, 30, and 42 hours after inoculation, corresponding to mid- to late-exponential phase and the early stationary phase of growth.

Leaching of trace metals: Previous studies have shown that elevated trace metal concentrations can induce biofilm formation in *A. fulgidus* (LaPaglia & Hartzell, 1997), and the HHP batch cultivation experiments all used a stainless steel needle inserted into a butyl stopper to maintain a closed system. We conducted leaching experiments to determine if leaching of these needles was increasing trace metal concentrations in the growth medium during these growth experiments. To minimize analytical interferences, the complex growth medium was replaced with ultrapure water (18.2 MΩ), or ultrapure water (18.2 MΩ) amended with 25 mM sodium sulfide, corresponding to the maximum sulfide concentration possible from complete reduction of sulfate in the standard growth medium. To put an upper limit on the potential for trace metal leaching, 10 sterile needles were placed into 20 mL of ultrapure water (18.2 MΩ) or 20 mL of 25 mM sodium sulfide solution in Balch tubes flushed with N_2 to maintain anoxic conditions. Tubes were incubated for 24 hours at 83°C and trace element concentrations were measured in the fluids using inductively coupled plasma mass spectrometry (ICP-MS; see below).

Varying $CaCl_2$ concentrations: Previous experiments (data not shown) suggested that *A. fulgidus* was sensitive to $CaCl_2$ concentrations, so the potential for biofilm production during growth in three different growth media with varying concentrations of $CaCl_2$ was tested. The standard growth medium used throughout the HHP batch cultivation experiments described here contained 0.38 mM $CaCl_2 \cdot 2H_2O$. We also tested a growth medium containing 0.95 mM $CaCl_2 \cdot 2H_2O$, and an additional medium that contained no additional $CaCl_2 \cdot 2H_2O$, but ~3 µM $CaCl_2$ derived from the yeast extract used (BD Bacto™ Difco™ ultra filtered). Growth of *A. fulgidus* in all three types of media was monitored during cultivation at ambient pressures in Balch tubes and syringes in triplicate.

Headspace and nucleation surfaces: Results of the comparative growth experiments outlined above (described in section 3.4.2) suggested that several parameters affected biofilm formation including $CaCl_2$ concentrations, the presence of a gas phase, and the presence (but not leaching) of a stainless steel needle. To explore these effects in more detail, several variations in cultivation protocols were designed. To test the presence of a gas phase, *A. fulgidus* was grown in both 10 mL plastic syringes, and in 26 mL Balch tubes, with and without an N_2 headspace at 0.1 MPa. Those with a gas phase had a 1:2 liquid to gas ratio by volume. Additionally, the presence of the stainless steel needle acting as a nucleation site for biofilm formation was also tested by including a needle in the growth experiments in Balch tubes. These were comparative to growth experiments in syringes, in which a needle used to maintain a closed system was also in contact with the growth medium. A last test was done to further explore if either the metal or the plastic hub on the needles stimulated biofilm production. This was done by separating the needle metal from the plastic hub and placing them into two separate Balch tubes with 10 ml of medium under sterile and anaerobic conditions. Each culturing variation was conducted at all three $CaCl_2$ concentrations, and all growth experiments were conducted in triplicate.

3.3.3.2 Cell imaging and chemical analyses

Scanning electron microscopy (SEM) was used to image planktonic *A. fulgidus* cells and cell aggregation with extracellular polymeric substances (EPS). *A. fulgidus* cells from late logarithmic growth phase were filtered onto 0.2μm black polycarbonate membranes (EMD Millipore) and washed with 5 mL of 0.1M phosphate buffer solution (PBS). Cells were fixed by incubating the filter in 2.5% gludaraldehyde in 0.1M PBS overnight at 4°C, and rinsing with

0.1M PBS. Filters were dehydrated using an anaerobic ethanol series (10%, 25%, 50%, 75%, 100%) and dried in a critical CO_2 freeze drier (Tousimis Autosamdri-815) for 2 hours. Samples were sputter coated with platinum and imaged on a FEI Versa 3D Dual Beam Scanning Electron Microscope with a 2 kV acceleration voltage.

Congo red dye, which binds to acidic polysaccharides, was used to verify the production of extracellular polymeric substances (EPS) that makes up a biofilm matrix (Hartzell, Millstein, & LaPaglia, 1999; Stoodley, Sauer, Davies, & Costerton, 2002). The relative levels of acidic polysaccharide production from *A. fulgidus* planktonic cells and biofilm forming cells were determined following Hartzell et al. (1999). Aliquots of 5 mL of *A. fulgidus* cultures were centrifuged at 16,000g for 15 minutes at 4°C. Cells were washed with a 0.39M sea salt base and centrifuged where the remaining supernatant was discarded. Wet cell slurries were weighed to approximately 0.002 ± 0.0008 g (~2 x 10^9 cells) and incubated with 10 µg/mL Congo red in 75% (v/v) ethanol. Samples were vortexed for 10 seconds and incubated for 2 hours at 4°C. After incubation with Congo red, samples volumes were adjusted to 1 mL with ultrapure water (18.2 MΩ), vortexed for 10 seconds and then centrifuged at 14,000g for 20 minutes at 25°C. This removed cells and EPS with bound Congo red from the supernatant. Supernatant transmittances were measured on a SpectraMax Plus 384 Absorbance Plate Reader at 500 nm. Standard calibration curves were obtained from 2 µg/mL, 4 µg/mL, 6 µg/mL, 8µg/mL, 10 µg/mL Congo red standard solutions in 1 mL with ultrapure water (18.2 MΩ), and 0 µg/mL Congo red (i.e. ultrapure water 18.2 MΩ). The amount of Congo red, in µg/mL, left in the supernatant (i.e. not bounded in polysaccharides) was calculated by subtracting the measured sample value from the negative control that contained 10 µg/mL Congo red in 1 mL of ultrapure water (18.2 MΩ). The subtracted measured values were presented as percent transmittance, where 0% representing the

negative control (10 μg/mL Congo red in 1 mL with ultrapure water, 18.2 MΩ) and 100% signifying that all of the Congo red was removed from the supernatant and bound by *A. fulgidus* produced polysaccharides. Congo red measurements were conducted on triplicate experiments, and standard deviations were calculated from the mean of measured values.

Sulfide concentrations were determined using the methylene blue assay (NPDES Method 376.2, 1978). Total aqueous sulfide concentrations and pH were measured in *A. fulgidus* culture samples taken after 18, 30, and 42 hours after inoculation in cultures grown at 0.3 MPa in Balch tubes with planktonic cells and in cultures grown at 0.1 MPa and 20 MPa in syringes with EPS. Sulfide standards were prepared by making dilutions from a 20 mM anaerobic $Na_2S•9H_2O$ stock solution. Standard curves were obtained from 3.25 mM, 1.95 mM, 1.30 mM, and 0.65 mM sulfide standard concentrations. Measurements were performed on a SpectraMax Plus 384 Absorbance Plate Reader at 670nm (NPDES Method 376.2, 1978). *A. fulgidus* sample pH was estimated with pH paper concurrently with sulfide measurement sampling. All measurements were done in triplicate and error was calculated as the deviation from the average.

Trace metal concentrations from fluids in the leaching experiments were measured using inductively coupled plasma mass spectrometry (ICP-MS). Fluids from each tube were analyzed on a Bruker 820-MS ICP-MS. Standards and sample dilutions were prepared in volumetric flasks rinsed with distilled 5% HNO_3 and ultrapure water (18.2 MΩ). Fluid samples from the tubes containing needles in ultrapure water (18.2 MΩ) and the sulfide solution were diluted 200 times and in an additional analysis, the needles in ultrapure water (18.2 MΩ) were diluted 10 times. The mass scan setup for determination for trace metals was ^{27}Al, ^{35}Cl, ^{39}K, ^{43}Ca, ^{49}Ti, ^{52}V, ^{52}Cr, ^{53}Cr, ^{55}Mn, ^{57}Fe, ^{58}Ni, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{67}Zn, ^{88}Sr, ^{90}Zr, and ^{98}Mo. The trace elements of interest were Cr, Mn, Ni, Cu, Zn and Al, since these were previously associated with biofilm in *A.*

fulgidus or were of interest for comparison with the trace element solution (LaPaglia & Hartzell, 1997; Hartzell & Reed, 2006). Samples were standardized to a ^{45}Sc internal standard and the multi-element 1V-stock-1643 standard from Inorganic Ventures was used for calibration. The limits of detection for Cr, Mn, Ni, Cu, Zn and Al were 1.0 ppb, 0.3 ppb, 1.8 ppb, 3.1 ppb, 12 ppb, and 1.5 ppb respectively for three fluid measurements.

3.4 Results

3.4.1 *A. fulgidus* growth at elevated pressure

A. fulgidus grew heterotrophically from 0.1-60 MPa under HHP batch cultivation at 83°C (Figure 3.3 A). Standard growth curves show similar growth up to 30 MPa, slower growth was observed from 40-60 MPa, and no notable increases in cell densities were measured at 70 MPa (Figure 3.3 A). Growth rates and maximum cell densities were similar from 0.1-30 MPa and decreased with pressure above 40 MPa (Figure 3.4 A & 3.5 A). The maximum growth rate for comparable cultivation techniques (e.g. syringes without headspace) was observed at 20 MPa (0.15 ± 0.005 hr^{-1}), which was faster than growth at 0.1 MPa (0.13 ± 0.008 hr^{-1}), suggesting moderate piezophily. While, the fastest growth rate was measured at 0.3 MPa (0.17 ± 0.015 hr^{-1}; Figure 3.4 A), this traditional batch culture experiment was conducted in Balch tubes in the presence of a gas headspace and biofilm was not detected. Therefore, these traditional batch cultures do not have a similar HHP counterpart and growth cannot be directly compared to growth in the syringes. Growth rates and maximum cell densities of *A. fulgidus* began to decline at 40 MPa (Figure 3.4 A and 3.5 A), and growth was barely detectable at 60 MPa (0.014 ± 0.004 hr^{-1}; Figure 3.4 A & 3.5 B). No growth was measured at 70 MPa and the maximum cell density

(9.82 ± 1.78 x 10^6 cells/mL) was only slightly higher than initial values (9.04 ± 1.3 x 10^6 cells/mL). Cell elongation and deformation was observed at 50 MPa - 70 MPa (Appendixes, Figure 2S). Even though growth did not occur at 70 MPa, cells exposed to 70 MPa for 52 hours that were subsequently transferred to 0.3 MPa recovered to high cells densities (5.6 ± 0.25 x 10^8 cells/mL) after 64 hours of growth at 0.3 MPa (Figure 3.5 A). The HHP batch cultivation experimental design described in section 3.3.1.2 required minimal cycles of decompression and repressurization and these did not appear to impact growth from 10-50 MPa (Appendixes, Figure 3S).

A. fulgidus grew autotrophically from 0.3 MPa-40 MPa at 83°C under HHP batch cultivation (Figure 3.3 B). Similar growth trends were observed from 0.3 MPa to 40 MPa, though growth at 40 MPa had a longer lag phase. Only slight growth was observed at 60 MPa (Figure 3.3 B). *A. fulgidus* was grown autotrophically at 0.3 MPa in serum bottles with a ~38% by volume headspace and also grown using HHP methods in syringes with minimal headspace of ~0.5 mL of H_2 at 0.3 MPa and no headspace from 10-60 MPa. Comparing growth only in HHP batch cultures, similar growth rates were observed from 0.3 MPa – 40 MPa (0.0195 ± 0.002 hr^{-1} – 0.0241 ± 0.002 hr^{-1}), suggesting that *A. fulgidus* is a piezotolerant autotroph (Figure 3.4 B). Slightly higher growth rates were observed in traditional batch culture at 0.3 MPa (0.0329 ± 0.001 hr^{-1}; Figure 3.4 B open circle). Likely due to the presence of an H_2/CO_2 phase in traditional batch culture in serum bottles. Again, cultivation in serum bottles at 0.3 MPa is not directly comparable to growth in syringes under HHP culture conditions. For HHP batch cultivation, similar maximum cell densities were measured at 0.3 MPa- 40 MPa (9.22 ± 1.33 x 10^7 cells/mL - 1.38 ± 0.215 x 10^8 cells/mL; Figure 3.5 B). Minor growth was measured at 60 MPa with an average maximum cell density that reached 8.67 ± 1.29 x 10^6 cells/mL from an average

inoculum cell density of 3.16 ± 1.65 x 10^6 cells/mL (Figure 3.5 B). As expected, autotrophic growth was slower with lower cell densities compared to heterotrophic growth (Prieur & Marteinsson, 1998). Nevertheless, *A. fulgidus* shown here was able to grow on this metabolic strategy up to 40 MPa.

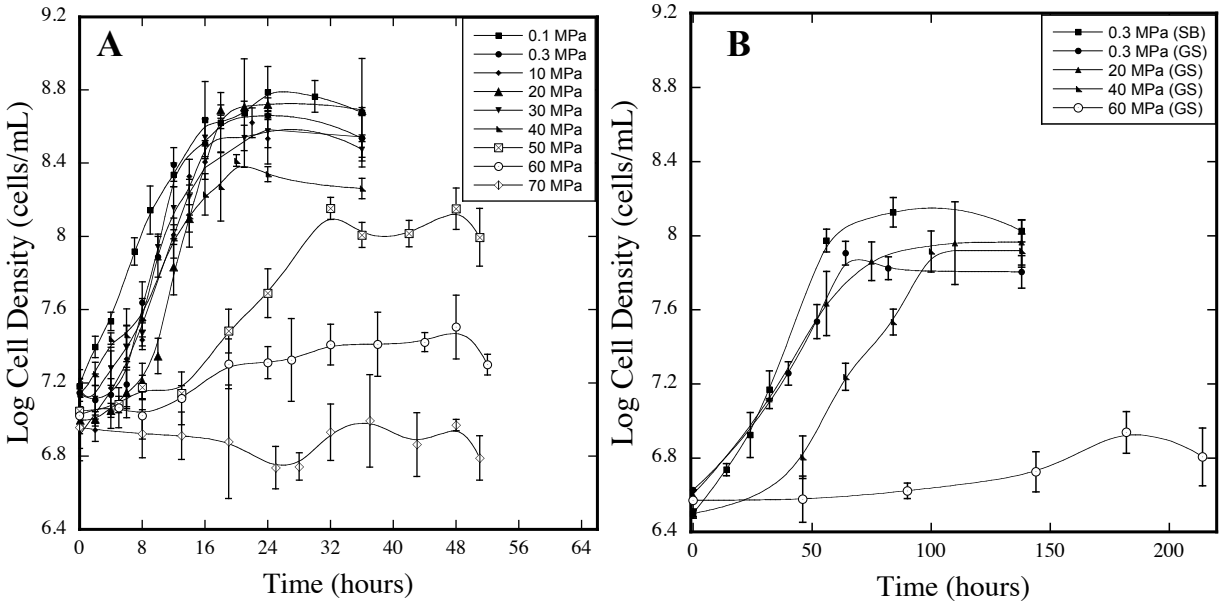

Figure 3.3. *A. fulgidus* heterotrophic HHP growth curves from 0.1-70 MPa (A) in syringes and Balch tubes at 0.3 MPa and *A. fulgidus* autotrophic HHP growth curves from 0.3-60 MPa (B) in glass syringes (GS) and in serum bottles (SB). Error bars are standard deviations from the average of at least triplicate experiments.

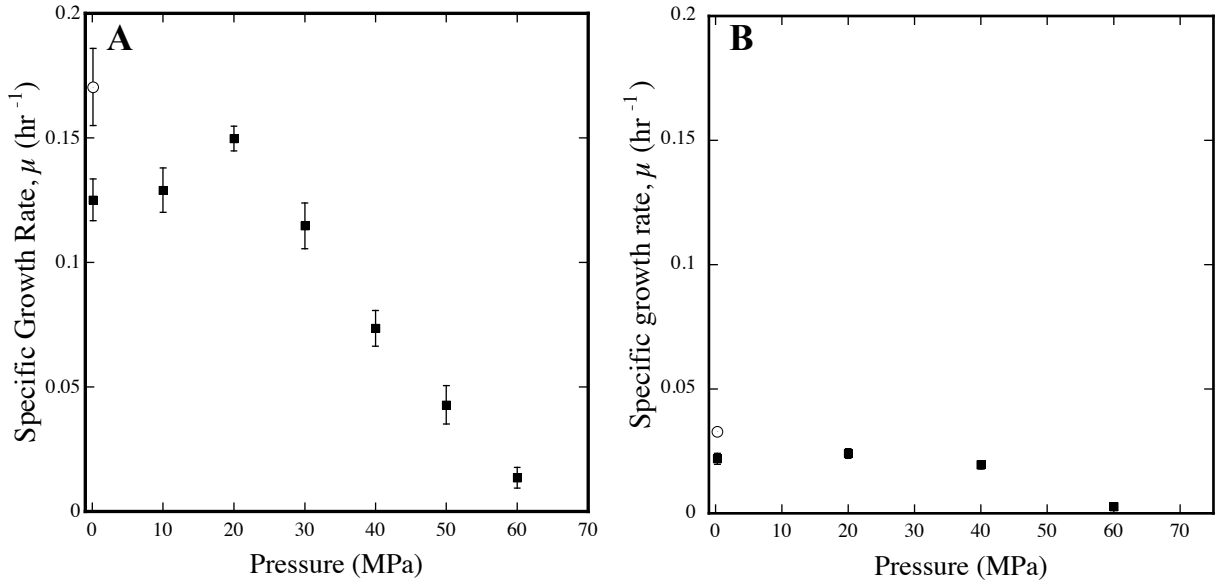

Figure 3.4. The effects of elevated pressure on *A. fulgidus* heterotrophic (A) and autotrophic (B) growth rates, estimated from exponential growth phases (Figure 3.3 A & B). Growth rates were estimated from 3-5 data points and error bars represent the standard deviation from at least triplicate experiments.

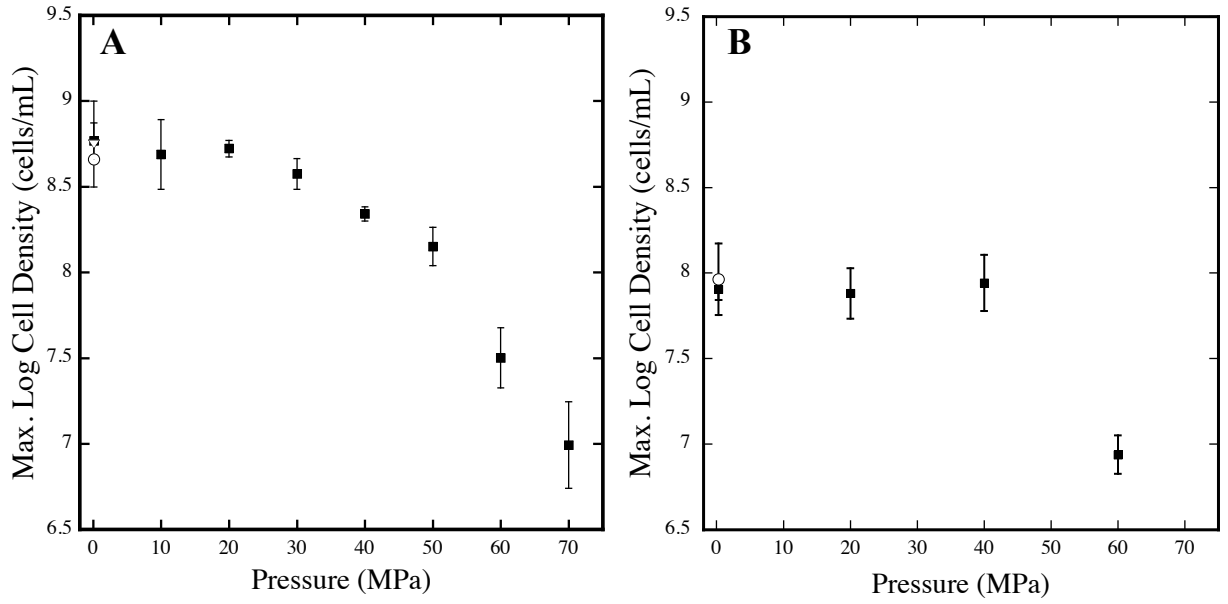

Figure 3.5. *A. fulgidus* heterotrophic (A) and autotrophic (B) maximum cell densities in logarithmic scale grown in syringes (closed squares), balch tube or serum bottle (open circle), and at 0.3 MPa after exposure to 70 MPa (open triangle in A). No growth was observed at 70 MPa in A. The error bars indicate the discrepancy between triplicate experiments.

3.4.2 Biofilm formation

Cell aggregation and extracellular polymeric substances (EPS) were observed from *A. fulgidus* growth in the heterotrophic HHP batch culture from 0.1 MPa-50 MPa. HHP does not seem to cause this response since cell aggregation and EPS were also observed under both ambient growth pressures and HHP in syringes. Biofilm formation began during the early stationary phase (16-24 hours of growth) and became more dense with time. When syringes were removed from of the pressure vessels, *A. fulgidus* aggregated cell mass was observed at the syringe and needle interface. This physiological response was not observed in traditional batch culture experiments performed in Balch tubes with a 0.3 MPa gas headspace.

Three growth conditions were chosen to explore the nature of biofilm formation that was observed during heterotrophic batch culture. To explore if elevated pressure affected biofilm formation *A. fulgidus* was gown in headspace-free syringes at both ambient pressure (0.1 MPa), and 20 MPa, the pressure that yielded that highest growth rates for this method. Additionally, cultivation in Balch tubes (60% headspace by volume over pressurized with N_2) at 0.3 MPa was also carried out for comparison. The goal behind these experiments was to first characterize the nature of *A. fulgidus* cell aggregation and EPS formation. Second, these growth experiments were chosen to help to identify the specific environmental conditions that were causing this physiological response. It is generally thought that *A. fulgidus* biofilm is a response to environmental stress (LaPaglia & Hartzell, 1997), and in addition to pressure, we hypothesized that the accumulation of aqueous sulfide, particularly during cultivation in headspace-free syringes, could have been a contributing factor. The formation of biofilm in both experiments conducted in headspace-free syringes (0.1 MPa and 20 MPa) was confirmed visually and these results replicated the original biofilm observations that biofilm was not a response to HHP

conditions. Further, no cellular aggregation or biofilm formation was observed in Balch tubes. These results were confirmed by SEM imaging. The SEM images of *A. fulgidus* planktonic cells (0.3 MPa in Balch tubes) showed evenly distributed individual cells (Figure 3.6 A), whereas growth in syringes yielded cells that were completely embedded within the EPS matrix (Figure 3.6 B and C). Furthermore, the Congo red binding to acidic polysaccharides within cells and EPS showed that *A. fulgidus* planktonic cells grown in Balch tubes bound less Congo red than *A. fulgidus* cells in EPS (Figure 3.7 B). Correspondingly, cell aliquots from Balch tube cultivation had a much lower transmittance (26-40%, Figure 3.7 A) than either sample that formed biofilm in syringes. The much higher transmittance values (maximum 84.3-89%, Figure 3.7 A) for both 0.1 MPa and 20 MPa growth in syringes reflects the binding of Congo red dye by EPS. While transmittance values were similar for 0.1 MPa and 20 MPa growth at 30 and 42 hours, transmittance for the 0.1 MPa culture was higher than the 20 MPa culture, at 18 hours, suggesting that biofilm formation started earlier in the ambient pressure culture (Figure 3.7 A).

These results suggest that it is not elevated pressure that induces biofilm formation, but instead the cultivation technique could have been the differentiating factor. The obvious difference, the presence or absent of a gas phase, could certainly affect how volatiles, specifically H_2S, might accumulate in the growth medium during *A. fulgidus* sulfate reduction creating high pH conditions. Elevated sulfide concentrations are known to be toxic or inhibitory for growth of many sulfate reducers (Reis, Almeida, Lemos, & Carrondo, 1992), and high pH ($\geq$7.5) has previously been shown to induce *A. fulgidus* biofilm (LaPaglia & Hartzell, 1997), however it is not clear if these stresses could induce biofilm here. This hypothesis was explored by measuring aqueous sulfide concentrations and pH during *A. fulgidus* growth in the three experiments described in section 3.3.3.1. For all three growth experiments investigated, both

sulfide concentrations and pH increased with time (Figure 3.8). Values of pH increased similarly in all three experiments reaching ~8.5 after 42 hours. Maximum sulfide concentrations increased more quickly in the 0.1 MPa syringe cultivation experiment. This suggests a higher rate of metabolism in this culture compared to the others, and is consistent with the higher EPS production values noted for this experiment (Figure 3.7 A). Despite this correspondence, similar maximum sulfide concentrations and pH values measured for all three cultivation conditions cannot account for the observed difference in biofilm formation.

A. fulgidus is known to produce biofilm in the presence of elevated metal concentrations (LaPaglia & Hartzell, 1997), and it has also been shown that sulfate reducers can corrode stainless steel (e.g. Hamilton, 1985; Angell & White 1995, Enning and Garrelfs, 2014), potentially leaching trace metal constituents into the surrounding fluid. Here, we tested the potential for trace metal leaching. Maximum potential leached trace metal concentrations were evaluated via leaching experiments in either ultrapure water (18.2 MΩ) or a 25 mM sulfide solution at 83°C for 24 hours. Resulting concentrations of Mn, Ni, Cu, and Zn were all well below the concentrations of these metals in the growth medium, Al concentrations were comparable to those concentrations in the growth medium from the trace element solution, and the Cr concentration measurements were null (Appendixes; Figure 4S), suggesting that metal leaching is negligible and unlikely to be stimulating biofilm formation. It should be noted that we were unable to account for localized surface interactions on the stainless steel needle itself or at the needle hub, where biofilm was often observed in previous experiments.

Finally, biofilm formation was tested in three heterotrophic media with 0.95 mM, 0.38 mM, and ~3 μM $CaCl_2 \cdot 2H_2O$ concentrations in traditional cultures and in HHP culture conditions, with and without a gas headspace, and with the presence of a needle as a nucleation

site. Here, *A. fulgidus* biofilm was not observed in medium containing 0.95 mM $CaCl_2 \cdot 2H_2O$ concentrations under any of the cultivation conditions tested (Figure 3.9). However, biofilm was observed in all of the conditions tested in medium with ~3 μM $CaCl_2 \cdot 2H_2O$ concentrations (Figure 3.9). Yet for the medium used for the HHP batch cultivation experiments with the intermediate calcium concentrations (0.38 mM $CaCl_2 \cdot 2H_2O$), *A. fulgidus* biofilm was stimulated in the presence of a needle in contact with the growth medium (Figure 3.9 & Figure 3.10). Additionally where *A. fulgidus* biofilm was observed, it was observed in both glass and plastic syringes, suggesting that this is not a response to glass or plastic materials. Furthermore, biofilm formed whether the needle shaft or the needle hub alone was present in the growth medium and the presence or absence of a gas phase was not causation. Here, *A. fulgidus* biofilm was not a response to HHP but rather is associated with low calcium concentrations, and at intermediate concentrations (0.38 mM $CaCl_2 \cdot 2H_2O$) there had to be an additional surface or micro niche for biofilm attachment.

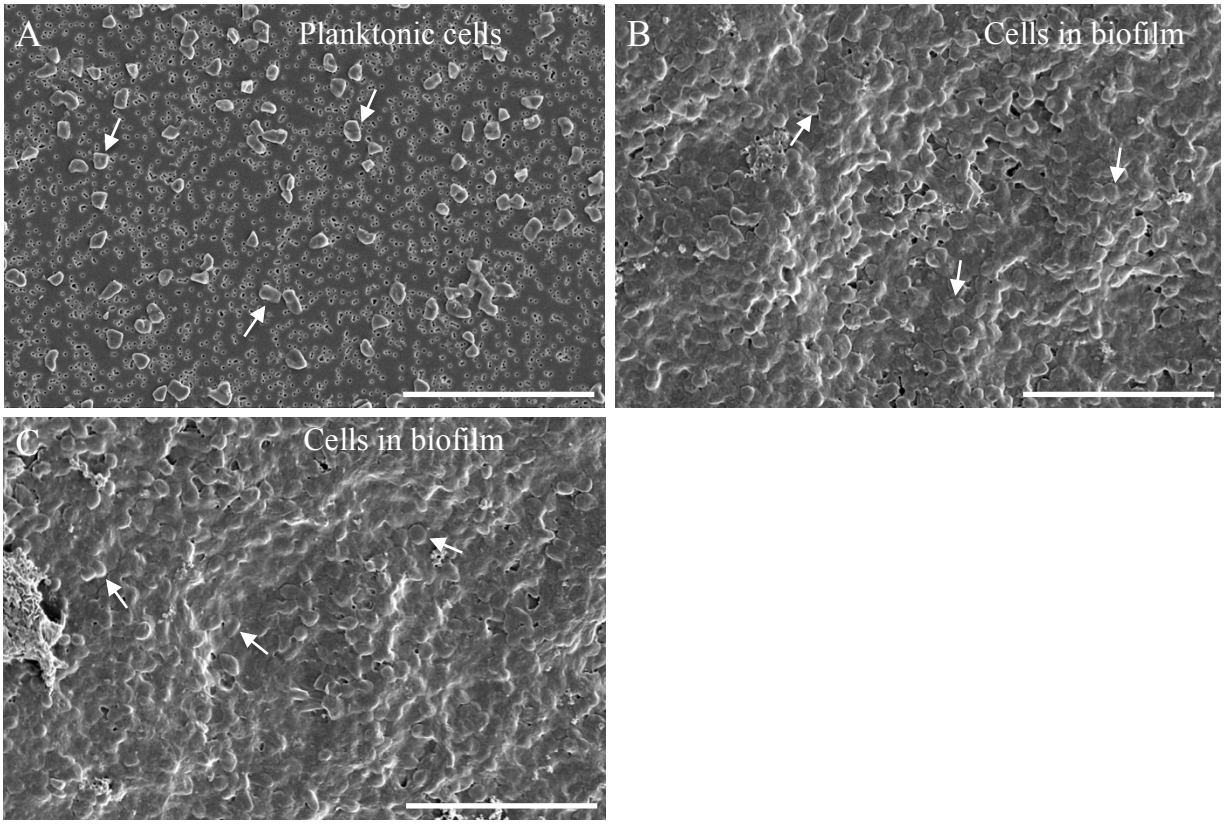

Figure 3.6. Scanning electron micrographs of *A. fulgidus* planktonic cells grown in Balch tubes at 0.3 MPa (A) and biofilm forming cells grown in syringes at 0.1 MPa (B) and 20 MPa (C). White arrows point to single *A. fulgidus* cells. Scale bars are 10 µm.

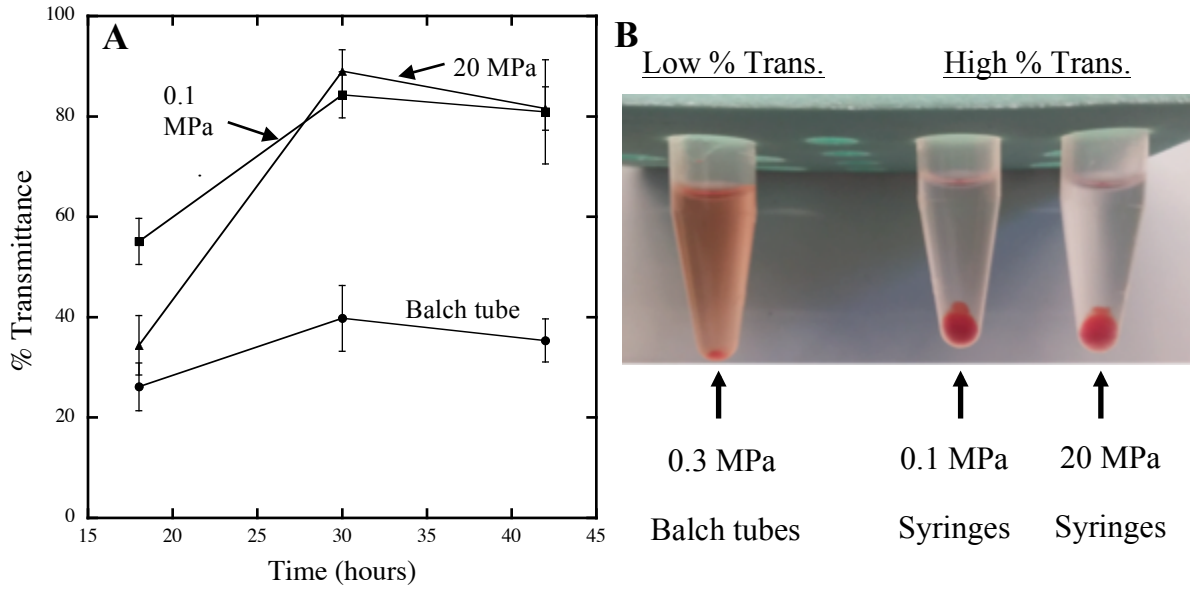

Figure 3.7. Percent transmittance (A) of the remaining Congo Red (CR) dye left in the supernatant from binding to *A. fulgidus* grown heterotrophically in syringes at 0.1 MPa (squares) and 20 MPa (triangles) and *A. fulgidus* grown in balch tubes with a 0.3 MPa gas headspace (circles) sampled after 18, 30, and 42 hours after inoculation.

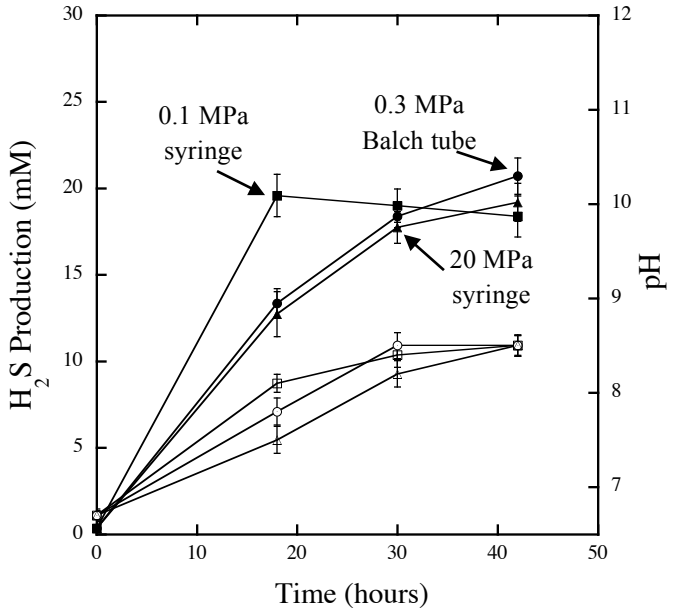

Figure 3.8. Sulfide concentrations (solid) and pH (open) measurements of *A. fulgidus* cultures grown in Balch tubes with a 0.3 MPa N_2 headspace (circles), at 0.1 MPa (squares) and 20 MPa (triangles) in syringes after 18, 30, and 42 hours after inoculation.

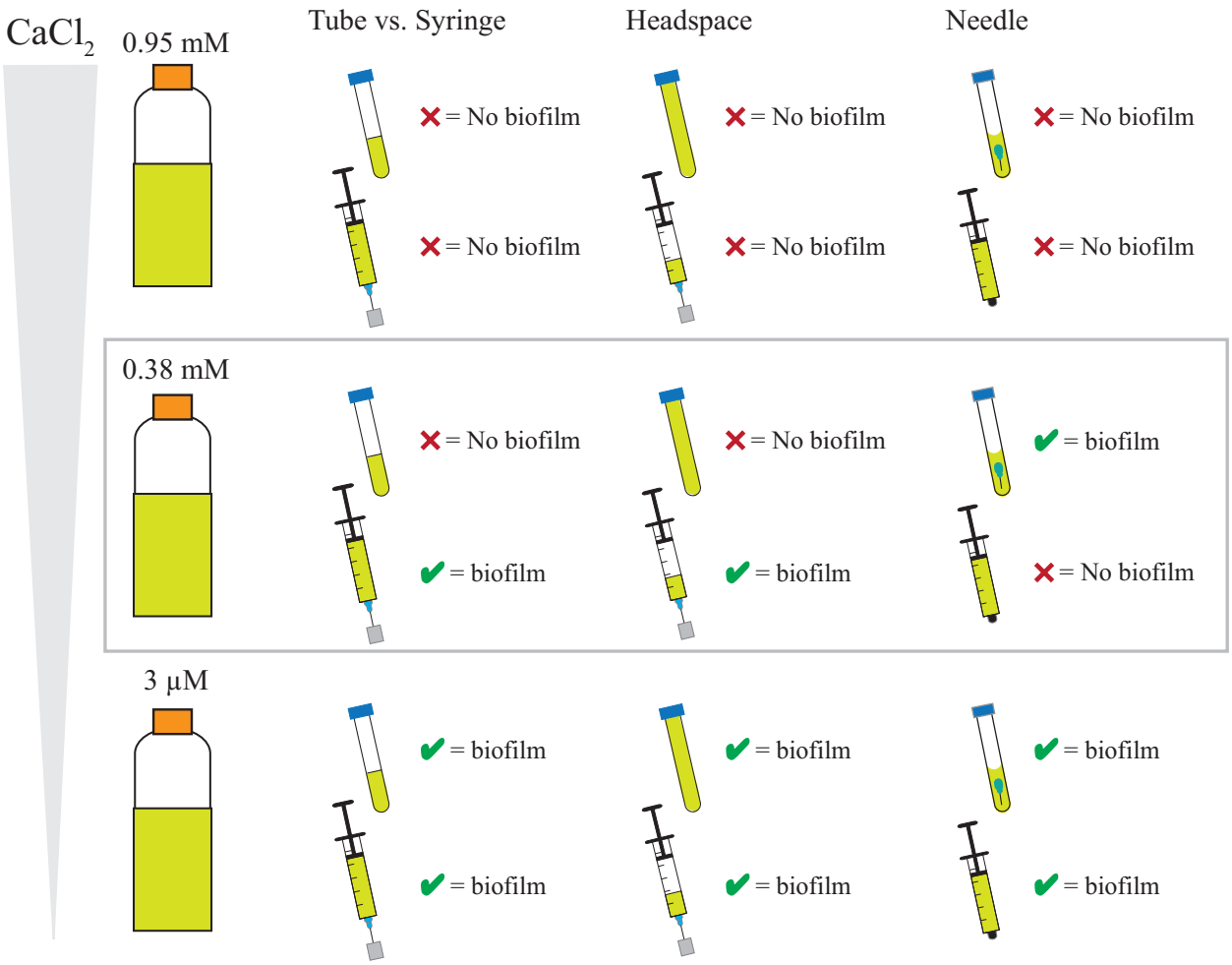

Figure 3.9. Cultivation variations in Balch tubes and syringes, both with and without a headspace, or in the presence of a stainless steel needle in media containing 0.95 mM, 0.38 mM, and ~3 µM calcium chloride dihydrate concentrations to test for *A. fulgidus* biofilm formation at 0.1 MPa.

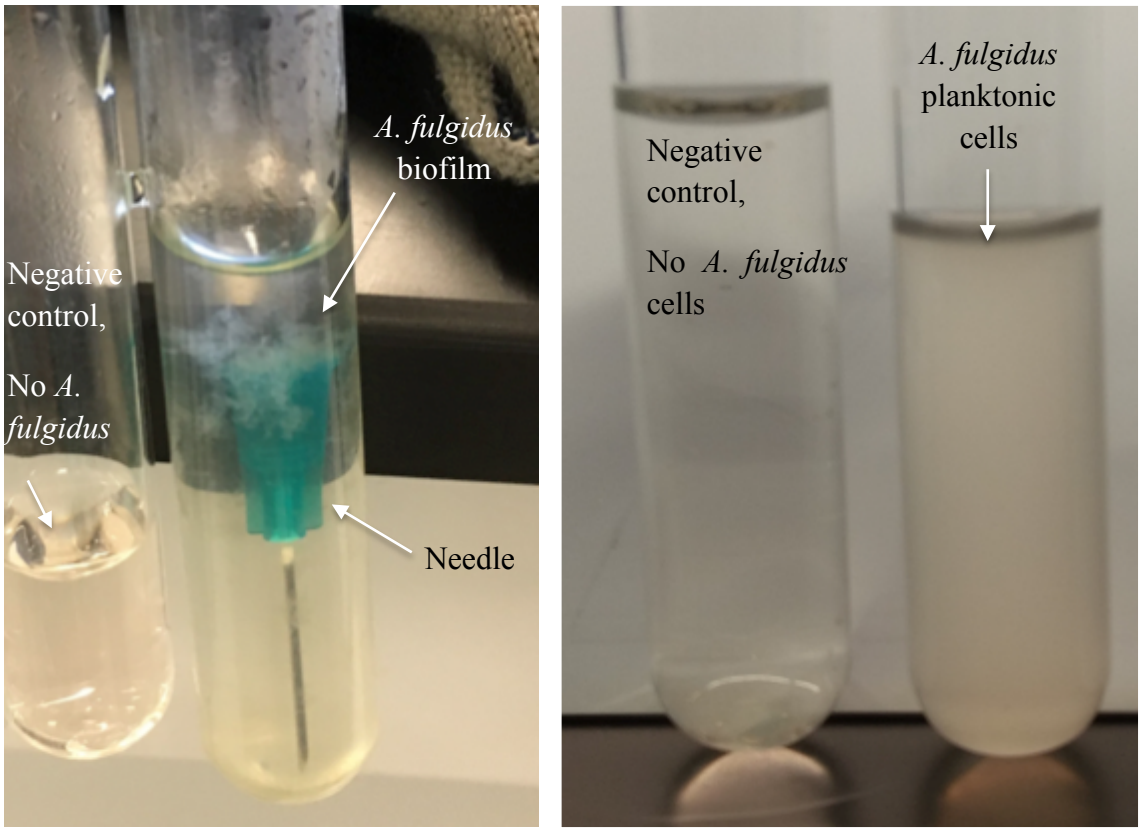

Figure 3.10. *A. fulgidus* biofilm forming culture in the presence of a needle in a Balch tube with a negative control (A). *A. fulgidus* planktonic culture grown in a Balch tube with a negative control (B).

3.5 Discussion

3.5.1 *A. fulgidus* HHP growth for heterotrophic and autotrophic conditions

A. fulgidus (type strain VC16) is capable of growing at HHP conditions both heterotrophically and autotrophically. Here, *A. fulgidus* displays both piezophilic and piezotolerant behavior depending on metabolic strategy. Growing heterotrophically *A. fulgidus* is moderately piezophilic, with maximum specific growth rates measured at 20 MPa for the particular conditions explored here. On the other hand, *A. fulgidus* growth rates and stationary phase cell densities were similar for autotrophy from 0.3 MPa-40 MPa. Interestingly, growth

rates under traditional batch culture generated different results from HHP culture conditions at 0.1 MPa or 0.3 MPa for both metabolisms, highlighting that different cultivation methods can impact microbial growth patterns. Taken together, these results suggest that *A. fulgidus* is capable of both heterotrophic and autotrophic growth at elevated pressures of various surface and deep biosphere environments. To our knowledge, piezotolerance to elevated pressures has not been directly measured for both heterotrophic and autotrophic metabolic strategies for a single species (from data in Jebbar et al., 2015). *A. fulgidus* serves as a model extremophile for further enzymatic or bioenergetic investigations for HHP growth for multiple metabolisms over the full growth pressure range. The results of such studies might elucidate why the maximum growth pressures for heterotrophic growth is greater (60 MPa) than autotrophic growth (between 40 MPa and 60 MPa) and identify proteins more sensitive or tolerant to increasing growth pressures that govern this response.

Microbial response to HHP is highly dependent on growth temperatures and growth medium chemistry. *A. fulgidus'* slight piezophilic behavior and piezotolerance in this study depended on the specific metabolic strategy investigated at 83°C. Other studies have shown both piezotolerant and piezophilic behaviors in a single species and the variations in patterns have more commonly corresponded to temperature changes. For example, Nogi et al. (1998) found that *Moritella japonica* grew optimally as a psychrophilic piezophile at 15°C and 50 MPa but displayed piezotolerance when grown at 10°C with an optimum growth pressure at 0.1 MPa. Similarly, the hyperthermophilic archaeon, *Thermococcus eurythermalis,* exhibited piezophily at 95°C and piezotolerance at 85°C (Zhao, Zeng, & Xiao, 2015). All of the HHP experiments described here were carried out at 83°C, which is this strain's optimum temperature at 0.1 MPa (Stetter, 1988). Thus, it remains unknown if piezotolerance or piezophily of *A. fulgidus* VC-16

would also change with temperature. Nevertheless, these results highlight that HHP growth characteristics may also change depending on metabolism, and not just with changing temperatures.

Characterizing microbial growth in terms of both temperature and pressure can better constrain the environments they can inhabit. *A. fulgidus* VC-16 has a reported temperature range of 60-95°C (Hartzell & Reed, 2006) and here we expand the heterotrophic growth pressure range to 0.1-60 MPa (corresponding to 0 to 6 km below sea level) and autotrophic growth pressures range to 0.1-40 MPa (corresponding to 0 to 4 km below sea level). These pressures encompass the average ocean floor depth, 3.7 km (i.e. ~37 MPa, National Oceanic and Atmospheric Administration, 2018), depths of many oil field reservoirs ranging form ~1-3 km depth (Pinder, 2001), and the average depth of black smoker hydrothermal vents at ~2.1 km (i.e. ~21 MPa). *A. fulgidus* has been identified in multiple deep oil reservoirs at ~2-4 km depths corresponding to ~20 MPa-40 MPa (Stetter et al., 1993; Beeder et al., 1994; L'Hardion et al., 1995), in deep-sea vents at Juan de Fuca ridge (Schrenk et al, 2003), and the East Pacific Rise (Reysenbach et al., 2000) from depths between ~2 to 3.5 km at ~20-35 MPa pressures. In this study, *A. fulgidus* HHP growth was unaffected by sample decompression and repressurization up to 40 MPa. Perhaps the reason this species has been isolated from multiple deep environments is in its ability to survive decompression upon sampling from high-pressure environments. The only other HHP growth performed on this species was *A. fulgidus* strain TF2 (Stetter et al., 1993). This strain was isolated from an oil reservoir at ~3 km depth and was reported to grow at 42 MPa on crude oil as a sole growth substrate (Stetter et al., 1993), reflecting its adaptiveness to the deep, hot oil reservoir it was sampled from. Also, an archaeal strain (L3), most closely related to *A. fulgidus*, was isolated from a 2 km depth (~25-30 MPa) geothermal well and successfully cultivated at

~0.2 MPa under autotrophic conditions (H_2 and CO_2 as a sole carbon source in the presence of thiosulfate; Fardeau et al., 2009). This strain had phenotypic differences from the *A. fulgidus* type strain VC-16 and required yeast extract for growth (Fardeau et al., 2009), but the finding from this study suggest that this species is capable of autotrophy (without yeast extract supplement) at elevated pressure conditions of the deep biosphere. Furthermore, *A. fulgidus* HHP growth under these two drastically different metabolic conditions, in a high nutrient lactate and sulfate rich medium supplemented with yeast extract for this heterotrophic metabolism and in autotrophic medium without yeast extract or vitamin supplementation, highlight that *A. fulgidus* can likely metabolize a variety of substrates at elevated pressures as it does under surface pressures.

A. fulgidus type strain VC-16 was isolated from a shallow marine vent and was initially thought to be the "shallow" dwelling *Archaeoglobus* species compared to *Archaeoglobus profundus,* isolated from hot sediments from Guaymas Basin at ~2 km depth (~20 MPa; Burggraf et al., 1990). Yet over the past ~30 years, other *A. fulgidus* strains have been isolated from various deep environments (Table 3.1) and this has expanded our view of the distribution of this species. *A. fulgidus* has rarely, if ever, been discovered or identified in deeper environments. HHP results shown here suggest that *A. fulgidus* is capable of actively growing in deeper high-temperature environments depending if the temperature and chemical conditions are favorable for growth. Additionally, other *Archaeoglobus* species that have been isolated from high-pressure environments (Table 3.1) are likely to be better adapted to higher growth pressures, along with other *A. fulgidus* 'deep' dwelling species, but this will remain unknown until HHP growth experiments have been tested for more *A. fulgidus* stains and other *Archaeoglobus* species. Furthermore, it would be advantageous to use molecular techniques to compared

different *A. fulgidus* strains under HHP growth conditions to see any differences translated in the transcriptome or proteome to better understand how this species regulates growth at 0.1 MPa versus HHP. More broadly, further HHP batch cultivation performed on other extremophiles is necessary to contribute to our understanding of how microorganisms regulate pressure changes and how that governs their distribution on Earth.

3.5.2 *A. fulgidus* heterotrophic biofilm

A. fulgidus biofilm production was observed in cultures grown from 0.1–50 MPa in HHP culture conditions. Elevated pressures do not induce *A. fulgidus* biofilm formation, instead this appears to be a response to two concurrent stimuli. Biofilm here is associated with low calcium concentrations at 0.38 mM $CaCl_2 \cdot H_2O$ with an interface for biofilm attachment. In HHP culture conditions, the interface was created between the cultivation syringe and needle. Our hypothesis is that the connection of the syringe to the needle is creating an interface or micro niche that stimulates aggregation, adhesion and biofilm formation at ambient pressures and HHP conditions. Since calcium is so integral in microbial physiological functionality (Domínguez, Guragain, & Patrauchan, 2015), it is possible that the low $CaCl_2$ concentration was low enough to induce stress in *A. fulgidus,* and that the interface initiated biofilm formation for attachment r settlement. Yet, the exact mechanism that promotes a sessile mode of life from a planktonic mode in these experiments is unclear. Further investigation of cellular surface charge or overall cellular hydrophobicity would help to better constrain this behavior and help predict what types of materials and surfaces *A. fulgidus* could colonize onto (Costerton et al., 1995; Toole, Kaplan, & Kolter, 2000).

A. fulgidus biofilm has previously been characterized as a stress response by LaPaglia and Hartzell in 1997. They reported that various stressful conditions induced *A. fulgidus* biofilm formation including high pH (≥7.5), UV exposure, high temperatures, low temperatures, and high metal concentrations (LaPaglia & Hartzell, 1997). The pH levels in our experiments reached 8.5, but were not considered to induce biofilm formation because these elevated pH values were measured in both *A. fulgidus* planktonic and sessile cells. Furthermore, LaPaglia and Hartzell (1997) had found that high chromium (≥ 25 ppm), copper (≥ 3 ppm), and nickel (≥ 3 ppm) concentrations induced *A. fulgidus* biofilm formation. Here, we tested the possibility of metal leaching from the needle into the growth medium and estimated null to low concentrations for chromium (2 ± 1.94 ppb), copper (17 ± 31.2 ppb), and nickel (9 ± 5.91 ppb). It is unlikely that there could be a localized toxic level of metals reaching up to similar concentrations that found in LaPaglia and Hartzell (1997). But, because the actual micro niche created in these experiments could not be directly measured, this remains elusive.

The ability of *A. fulgidus* to produce protective biofilm up to 50 MPa suggests that it may inhabit the deep biosphere in multicellular structures or biofilms. Direct observations from an active sulfide chimney (Finn) sampled off the Mothra Vent Field on the Juan de Fuca Ridge identified archaeal clone sequences that most closely related to *A. fulgidus* (Schrenk et al., 2003). These microbial communities at Juan de Fuca Ridge were found thriving within microbial biofilm and cell clusters at 2.27 km (~22 MPa, Schrenk et al., 2003). Exploring how environmental variations influence physiology of extremophiles, like *A. fulgidus,* are important to understanding how species-scale processes operate and vary so that better predictions of their behavior can be made for natural settings. Since deep-sea and subsurface environments are prone

to drastic environmental fluctuations, *A. fulgidus* biofilm formation might lend insight to deep biosphere microbial adaptive strategies.

3.6 Concluding remarks

A. fulgidus type strain VC-16, originally isolated from a shallow marine vent, has shown that it is a model extremophile for learning more about adaptive strategies in both surface and subsurface environments. It is able to grow as a chemoorganoheterotroph and chemolithoautotroph under both ambient and HHP conditions and forms a biofilm under specific heterotrophic conditions up to 50 MPa. Since the ability to grow both heterotrophically and autotrophically is relatively rare for a single species, *A. fulgidus* HHP growth represents an important opportunity to investigate how elevated pressures impact microbial metabolism. These HHP growth results suggest that *A. fulgidus* can likely metabolize a variety of substrates at elevated pressures as it does under ambient pressure conditions. *A. fulgidus'* metabolic flexibility and capability to produce biofilm sheds light into why this species is ubiquitous in extreme and fluctuating environments at the surface and in the deep biosphere. *A. fulgidus* would be an ideal subject for a vast array of analyses (molecular and biogeochemical) over a range of pressure conditions. For instance, research on *A. fulgidus'* membrane structure and functionality under different pressure, decompression, biofilm forming, and metabolic conditions may lend some insight into this species' ability to regulate changing pressure conditions. The results of such studies would have implications in not only in HHP microbiology but also in biotechnology, oil industry, early life chemical and fossil identification, and origins of life research.

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3.8 References

Achenbach-Richter, L., Stetter, K. O., & Woese, C. R. (1987). A possible biochemical missing link among archaebacteria. *Nature*, *327*(6120), 348–349. 10.1038/327348a0

Angell, P., & White, D. C. (1995). Is metabolic activity by biofilms with sulfate-reducing bacterial consortia essential for long-term propagation of pitting corrosion of stainless steel? *Journal of Industrial Microbiology*, *15*(4), 329–332. 10.1007/BF01569987

Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R., & Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiolological Reviews*, *43*(2), 260–296. 10.1016/j.watres.2010.10.010

Bartlett, D. H. (2002). Pressure effects on in vivo microbial processes. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, *1595*(1), 367–381. 10.1016/S0167-4838(01)00357-0

Beeder, J., Nilsen, R. K., Rosnes, J. T., Torsvik, T., & Lien, T. (1994). *Archaeoglobus fulgidus*

isolated from hot North Sea oil field waters. *Applied and Environmental Microbiology*, *60*(4), 1227–1231.

Blöchl, E., Rachel, R., Burggraf, S., Hafenbradl, D., Jannasch, H. W., & Stetter, K. O. (1997). *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113 degrees C. *Extremophiles : Life Under Extreme Conditions*, *1*(1), 14–21. 10.1007/s007920050010

Burggraf, S., Jannasch, H. W., Nicolaus, B., & Stetter, K. O. (1990). *Archaeoglobus profundus* sp. nov., represents a new species within the sulfate-reducing archaebacteria. *Systematic and Applied Microbiology*, *13*(1), 24–28. 10.1016/S0723-2020(11)80176-1

Cario, A., Jebbar, M., Thiel, A., Kervarec, N., & Oger, P. M. (2016). Molecular chaperone accumulation as a function of stress evidences adaptation to high hydrostatic pressure in the piezophilic archaeon *Thermococcus barophilus*. *Scientific Reports*, *6*, 1-8. 10.1038/srep29483

Costerton, J. W., Lewandowski, Z., Calkwell, D. E., Kober, D. R., & M.Lappin-Scott, H. (1995). Microbial biofilms. *Annual Review of Microscopy*, *49*, 711–745.

Domínguez, D. C., Guragain, M., & Patrauchan, M. (2015). Calcium binding proteins and calcium signaling in prokaryotes. *Cell Calcium*, *57*(3), 151–165. 10.1016/j.ceca.2014.12.006

Enning, D., & Garrelfs, J. (2014). Corrosion of iron by sulfate-reducing bacteria: New views of an old problem. *Applied and Environmental Microbiology*, *80*(4), 1226–1236. 10.1128/AEM.02848-13

Fang, J., Zhang, L., & Bazylinski, D. A. (2010). Deep-sea piezosphere and piezophiles: geomicrobiology and biogeochemistry. *Trends in Microbiology*, *18*(9), 413–422. 10.1016/j.tim.2010.06.006

Fardeau, M.-L., Goulhen, F., Bruschi, M., Khelifi, N., Cayol, J.-L., Ignatiadis, I.,… Ollivier, B. (2009). *Archaeoglobus fulgidus* and *Thermotoga efii*, thermophilic isolates from deep geothermal water of the paris basin. *Geomicrobiology Journal*, *26*(2), 119–130. 10.1080/01490450802674970

Hamilton, W. A. (1985). Sulfate-reducing bacteria and anaerobic corrosion. *Annual Review in Microbiology*, *39*, 195–217. 10.1146/annurev.mi.39.100185.001211

Hartzell, P. L., Millstein, J., & Lapaglia, C. (1999). Biofilm formation in hyperthermophilic archaea. *Methods in Enzymology, 310,* 335-349. 10.1016/S0076-6879(99)10027-2

Hartzell, P., & Reed, D. W. (2006). The genus *Archaeoglobus*. The *Prokaryotes*, *3*, 82–100. 10.1007/0-387-30747-8

Hei, D. J., & Clark, D. S. (1994). Pressure stabilization of proteins from extreme thermophiles. *Applied and Environmental Microbiology*, *60*(3), 932–939. 10.1038/nature06899

Hocking, W. P., Stokke, R., Roalkvam, I., & Steen, I. H. (2014). Identification of key components in the energy metabolism of the hyperthermophilic sulfate-reducing archaeon *Archaeoglobus fulgidus* by transcriptome analyses. *Frontiers in Microbiology*, *5*(MAR), 95. 10.3389/fmicb.2014.00095

Huber, H., Huber, R., & Stetter, K. O. (2006). Thermoproteales. The *Prokaryotes*, *3*, 10–22. 10.1007/0-387-30743-5_2

Huber, H., Jannasch, H., Rachel, R., Fuchs, T., & Stetter, K. O. (1997). *Archaeoglobus veneficus* sp nov, a novel facultative chemolithoautotrophic hyperthermophilic sulfite reducer, isolated from abyssal black smokers. *Systematic and Applied Microbiology*, *20*(3), 374–380. 10.1016/S0723-2020(97)80005-7

Huber, R., Woese, C. R., Langworthy, T. A., Fricke, H., & Stetter, K. O. (1989). *Thermosipho africanus* gen. nov., represents a new genus of thermophilic eubacteria within the "Thermotogales." *Systematic and Applied Microbiology*, *12*(1), 32–37. 10.1016/S0723-2020(89)80037-2

Jannasch, H. W., & Taylor, C. D. (1984). Deep-sea microbiology. *Annual Review of Microbiology*, *38*, 487–514.

Jannasch, H. W., Wirsen, C. O., Molyneaux, S. J., & Langworthy, T. A. (1992). Comparative physiological studies on hyperthermophilic archaea isolated from Deep-sea hot vents with emphasis on Pyrococcus strain GB-D. *Applied and Environmental Microbiology*, *58*(11), 3472–3481.

Jebbar, M., Franzetti, B., Girard, E., & Oger, P. (2015). Microbial diversity and adaptation to high hydrostatic pressure in deep-sea hydrothermal vents prokaryotes. *Extremophiles*, *19*(4), 721–740. 10.1007/s00792-015-0760-3

Kallmeyer, J., Ferdelman, T. G., Jansen, K. H., & Jørgensen, B. B. (2003). A high-pressure thermal gradient block for investigating microbial activity in multiple deep-sea samples. *Journal of Microbiological Methods*, *55*(1), 165–172. 10.1016/S0167-7012(03)00138-6

Kato, C., Nogi, Y., & Arakawa, S. (2008). Isolation, cultivation, and diversity of deep-sea

piezophiles. In C. Michiels, D. H. Bartlett, & A. Aertsen (Eds.), *High-pressure microbiology* (pp. 203–218). Washington, DC: ASM Press.

Kepner, R. L., & Pratt, J. R. (1994). Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. *Microbiological Reviews*, *58*(4), 603–615. 0146-0749/94/

Klenk, H. P., Clayton, R. A., Tomb, J. F., White, O., Nelson, K. E., Ketchum, K. A.,… Venter, J. C. (1997). The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. *Nature*, *390*(6658), 364–70. 10.1038/37052

L'Haridon, S., Reysenbach, A.-L., Glenat, R., Prieur, D., & Jeanthon, C. (1995). Hot subterranean bioshphere in a continental oil reservoir. *Nature, 377,* 223-224.

LaPaglia, C., & Hartzell, P. L. (1997). Stress-induced production of biofilm in thehyperthermophile *Archaeoglobus fulgidus*. *Applied and Environmental Microbiology*, *63*(8), 3158–63.

Mori, K., Maruyama, A., Urabe, T., Suzuki, K. I., & Hanada, S. (2008). *Archaeoglobus infectus* sp. nov., a novel thermophilic, chemolithoheterotrophic archaeon isolated from a deep-sea rock collected at Suiyo Seamount, Izu-Bonin Arc, western Pacific Ocean. *International Journal of Systematic and Evolutionary Microbiology*, *58*(4), 810–816. 10.1099/ijs.0.65422-0

Nakagawa, S., Takai, K., Inagaki, F., Chiba, H., Ishibashi, J. I., Kataoka, S.,… Sato, Y. (2005). Variability in microbial community and venting chemistry in a sediment-hosted backarc hydrothermal system: Impacts of subseafloor phase-separation. *FEMS Microbiology*

Ecology, *54*(1), 141–155. 10.1016/j.femsec.2005.03.007

Nercessian, O., Reysenbach, A. L., Prieur, D., & Jeanthon, C. (2003). Archaeal diversity associated with in situ samplers deployed on hydrothermal vents on the East Pacific Rise. *Environmental Microbiology*, *5*(6), 492–502. 10.1046/j.1462-2920.2003.00437.x

National Oceanic and Atmospheric Administration (2018). How Deep is the Ocean? Retrieved February 15, 2019 from https://oceanservice.noaa.gov/facts/oceandepth.html

Nogi, Y., Kato, C., & Horikoshi, K. (1998). *Moritella japonica* sp. nov., a novel barophilic bacterium isolated from a Japan Trench sediment. *The Journal of General and Applied Microbiology*. 10.2323/jgam.44.289

Oger, P. M., & Jebbar, M. (2010). The many ways of coping with pressure. *Research in Microbiology*, *161*(10), 799–809. 10.1016/j.resmic.2010.09.017

Park, C. B., & Clark, D. S. (2002). Rupture of the cell envelope by decompression of the deep-sea methanogen *Methanococcus jannaschii*. *Applied and Environmental Microbiology*, *68*(3), 1458–1463. 10.1128/AEM.68.3.1458

Pinder, D. (2001). Offshore oil and gas: Global resource knowledge and technological change. *Ocean and Coastal Management*, *44*(9–10), 579–600. 10.1016/S0964-5691(01)00070-9

Prieur, D., & Marteinsson, V. T. (1998). Prokaryotes living under elevated hydrostatic pressure. *Advances in Biochemical Engineering/Biotechnology*, *61*, 23–35. 10.1007/bfb0102288

Rabus, R., Hansen, T. A., & Widdel, F. (2013). Dissimilatory sulfate- and sulfur-reducing prokaryotes. In E. Rosenberg et al. (Eds), *The prokaryotes: prokaryotic physiology and biochemistry* (pp. 309–404). Berlin, Germany: Springer.

Reis, M. a, Almeida, J. S., Lemos, P. C., & Carrondo, M. J. (1992). Effect of hydrogen sulfide on growth of sulfate reducing bacteria. *Biotechnology and Bioengineering*, *40*(5), 593-600. 10.1002/bit.260400506

Reysenbach, A. L., Longnecker, K., & Kirshtein, J. (2000). Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a mid-atlantic ridge hydrothermal vent. *Applied and Environmental Microbiology*, *66*(9), 3798–3806. 10.1128/AEM.66.9.3798-3806.2000

Schrenk, M. O., Kelley, D. S., Delaney, J. R., & Baross, J. A. (2003). Incidence and diversity of microorganisms within the walls of an active deep-sea sulfide chimney. *Applied and Environmental Microbiology*, *69*(6), 3580–3592. 10.1128/AEM.69.6.3580-3592.2003

Steinsbu, B. O., Thorseth, I. H., Nakagawa, S., Inagaki, F., Lever, M. A., Engelen, B.,… Pedersen, R. B. (2010). *Archaeoglobus sulfaticallidus* sp. nov., a thermophilic and facultatively lithoautotrophic sulfate-reducer isolated from black rust exposed to hot ridge flank crustal fluids. *International Journal of Systematic and Evolutionary Microbiology*, *60*(12), 2745–2752. 10.1099/ijs.0.016105-0

Stetter, K. O. (1988). *Archaeoglobus fulgidus* gen. nov., sp. nov.: a new taxon of extremely thermophilic archaebacteria. *Systematic and Applied Microbiology*, *10*(2), 172–173. 10.1016/S0723-2020(88)80032-8

Stetter, K. O., Huber, R., Blöchl, E., Kurr, M., Eden, R. D., Fielder, M.,… Vance, I. (1993). Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. *Nature*, *365*(6448), 743–745. 10.1038/365743a0

Stetter, K. O., Lauerer, G., Thomm, M., & Neuner, A. (1987). Isolation of extremely

thermophilic sulfate reducers: evidence for a novel branch of archaebacteria. *Science*, *236*(4803), 822–824. 10.1126/science.236.4803.822

Stoodley, P., Sauer, K., Davies, D. G., & Costerton, J. W. (2002). Biofilms as complex differentiated communities. *Annual Review of Microbiology*, *56*(1), 187–209. 10.1146/annurev.micro.56.012302.160705

Takai, K., & Horikoshi, K. (1999). Genetic diversity of archaea in deep-sea hydrothermal vent environments. *Genetics*, *152*(4), 1285–1297. 10.1016/s0723-2020(87)80053-x

Takai, K., Nakamura, K., Toki, T., Tsunogai, U., Miyazaki, M., Miyazaki, J.,… Horikoshi, K. (2008). Cell proliferation at 122°C and isotopically heavy CH_4 production by a hyperthermophilic methanogen under high-pressure cultivation. *Proceedings of the National Academy of Sciences*, *105*(31), 10949–10954. 10.1073/pnas.0712334105

Tasumi, E., Yanagawa, K., Miyazaki, J., & Takai, K. (2015). In vitro high-pressure incubation and activity measurement of deep-sea methanogenic archaea, In T.J. McGenity et al (Eds), *Hydrocarbon and lipid microbiology protocols* (pp. 51–64). Berlin, Germany: Springer.

Thauer, R. K., & Kunow, J. (1995). Sulfate-Reducing Archaea. In L. L. Barton (Ed.), *Sulfate-reducing bacteria* (pp. 33–45). New York: Plenum Press.

Toole, G. O., Kaplan, H. B., & Kolter, R. (2000). Biofilm formation as microbial development. *Annual Review in Microbiology*, *54*, 49-79.

Yayanos, A. A. (2001). 30 Deep-sea piezophilic bacteria. *Methods in Microbiology*, *30*, 615–637.

Yayanos, A. A., Dietz, A. S., & Boxtel, V. (1982). Dependance of reproduction rate on pressure

as a hallmark of deep-sea bacteria. *Applied and Environmental Microbiology, 44*(6), 1356–1361.

Zellner, G., Stackebrandt, E., Kneifel, H., Messner, P., Sleytr, U. B., de Macario, E. C.,…Winter, J. (1989). Isolation and characterization of a thermophilic, sulfate reducing archaebacterium. *Archaeoglobus fulgidus* Strain Z. *Systematic and Applied Microbiology*, *11*(2), 151-160. 10.1016/S0723-2020(89)80055-4

Zhao, W., Zeng, X., & Xiao, X. (2015). *Thermococcus eurythermalis* sp. nov., a conditional piezophilic, hyperthermophilic archaeon with a wide temperature range for growth, isolated from an oil-immersed chimney in the Guaymas Basin. *International Journal of Systematic and Evolutionary Microbiology*, *65*(1), 30–35. 10.1099/ijs.0.067942-0

Zobell, C. E., & Oppenheimer, C. H. (1950). Some effects of hydrostatic pressure on the multiplication and morphology of marine bacteria. *Journal of Bacteriology*, *60*(0), 771–781.

4. Measuring the effects of decompression on microbial growth at high hydrostatic pressures

4.1 Abstract

The extent to which the full microbial diversity of the subsurface biosphere can be captured via cultivation techniques is likely hindered by the inevitable loss of cellular viability from decompression during sampling, enrichment and isolation experiments from high-pressure environments. Here, we explored the effects of decompression on microbial growth using two high hydrostatic pressure (HHP) cultivation techniques. The more conventional method uses static high-pressure vessels and necessitates whole sample decompression and repressurization cycles, while the newly developed pressurized underwater sample handler (PUSH) vessels maintain HHP during cultivation, sampling and transfer. A model extremophile, *Archaeoglobus fulgidus* (type strain VC-16), was cultivated in batch cultures from 0.1 MPa- 98 MPa using both HHP methods. For both methods of HHP batch cultivation, *A. fulgidus* showed exponential growth through 50 MPa, and up to 60 MPa using the PUSH vessel method without sample decompression. In both cases, *A. fulgidus* growth was impeded as pressure increased, however, the sensitivity to increasing pressure was more pronounced when cells were decompressed and repressurized multiple times. In conventional static pressure vessels, these negative effects, evidenced by slower growth rates and longer generation times, became more pronounced at pressures in excess of 40 MPa. Additionally, maximum cell densities, which were similar for both HHP methods up to 40 MPa, were lower for experiments with multiple decompression-repressurization cycles for pressures from 50-98 MPa. These results highlight that decompression can have a significant negative impact on observed cell growth and becomes

more pronounced away from optimal growth pressures, suggesting that decompression tolerance may depend on habitat depth. Additionally, *A. fulgidus* cells were grown and transferred under HHP. *A. fulgidus* was successfully grown at 50 MPa from cells initially grown at 20 MPa and subsequently transferred under 20 MPa. These results highlight that the PUSH vessels can be used to sample from deep-sea environments and transfer fluid samples for enrichment and isolation experimentation without sample decompression, thus increasing access to the portion of the deep biosphere that is sensitive to decompression and might otherwise escape cultivation.

4.2 Introduction

Most of the bacterial and archaeal biomass on Earth is in deep-sea and subsurface environments at elevated pressures (Whitman, Coleman, & Wiebe, 1998; Kallmeyer, Pockalny, Adhikari, Smith, & D'Hondt, 2012; Parkes et al., 2014; Bar-on, Phillips, & Milo, 2018). These microorganisms have been shown to be well adapted to the elevated pressures of their natural habitats (e.g. Somero, 1992; Allen & Bartlett, 2002; Simonato et al., 2006; Jebbar, Franzetti, Girard, & Oger, 2015; Peoples & Bartlett, 2017). Despite this, pressure seems to be one of the least explored parameters for microbial growth, as relatively few microorganisms from these deep environments have been isolated and/or grown under *in situ* pressures. To date, about 55 species have been characterized as piezotolerant (i.e. can grow over a range of pressures but optimum growth is at ambient pressures) or piezophilic (i.e. optimal growth at high-pressures; Picard & Daniel, 2013; Jebbar et al., 2015), and few obligate piezophiles (i.e. high-pressures are required for growth) have been identified (Bartlett, 2002; Zeng et al., 2009). As a consequence,

our knowledge of active microbial species, their physiological and metabolic potential, and community diversity in these deep environments is limited.

The paucity of knowledge with respect to high-pressure life stems from the difficulty in both sampling from high-pressure environments and replicating those pressure conditions in the laboratory in order to isolate and characterize piezophiles. For obligate piezophiles, high-pressure sampling and cultivation is the only route to isolate novel species (Yayanos & Dietz, 1983; Jannasch & Wirsen, 1984). However, facultative piezophiles and piezotolerant microorganisms can often tolerate lower pressure conditions during sampling and transfer (Jannasch & Wirsen, 1984). In these cases, the total change in pressure, duration of decompression, and number of subsequent decompression-repressurization cycles can impact the successful isolation of piezophiles (Yayanos, 2001; Park & Clark, 2002; Peoples & Bartlett 2017). The pressure condition of cultivation experiments may also impose a selection bias on enrichments and isolation experiments that favor species more tolerant to decompression or lower growth pressures (Jannasch, Wirsen, & Taylor, 1976; Yayanos, 1995; Grossart & Gust, 2009). Furthermore, Park and Clark (2002) showed that rates of decompression could impact microbial survival during decompression. Accelerated rates of decompression (26 MPa/second) caused the piezophile, *Methanococcus jannaschii* cells to rupture while slower rates of decompression (5.2 MPa/minute) over the same pressures increased viable cell yields (Park & Clark, 2002). Additionally, many piezophiles that have been recovered from the intestinal systems of deep-sea macro fauna (Yayanos, Dietz, & Boxtel, 1979; Deming, Tabor, & Colwell, 1981; Jannasch & Wirsen, 1984), these perhaps show a greater tolerance to sample decompression, however the loss of species from decompression has yet to be quantified and it is

unclear if tolerance to decompression of deep-sea species is correlated to habitat depth (Yayanos, 1978).

Deep biosphere species experience decompression both upon sample recovery from great depths and often during subsequent high-pressure cultivation because decompression-repressurization cycles are often necessitated by traditional high-pressure cultivation techniques. For enrichments and isolation, common methods involve growth in syringes, plastic bulbs, or heat sealed plastic bags, pressurized in hydrostatic vessels (reviewed in Yayanos, 2001). However, subsampling to monitor cell growth with time requires decompression of the entire system followed by repressurization for continuation of the growth experiment. Inoculating such devices for high-pressure enrichment experiments also requires sample decompression. Therefore, significant effort has gone into developing pressure-retaining vessels to sample from the deep ocean and cultivate samples for enrichment and isolation experiments without decompression (Tabor & Colwell, 1976; Jannasch & Wirsen, 1977; Yayanos, 1977; Cahet, Daumas, & Sibuet, 1990; Bianchi, Garcin, & Tholosan, 1999; Tamburini, Garcin, & Bianchi, 2003; Kato, Nogi, & Arakawa, 2008). Such vessels allow for subsampling while maintaining HHP and in some cases this is done through variable volume, floating piston devices that eliminate the decompression-repressurization cycle (Bianchi et al., 1999; Tamburini et al., 2003). Building on previous designs (Bianchi et al., 1999; Tamburini et al., 2003), we recently collaborated with TOP Industrie© (Vaux Le Peńil, France) to develop a high-pressure (up to 100 MPa), high temperature (up to 121°C), floating piston device that can hold up to 50 mL of fluid. These pressurized underwater sample handler (PUSH) vessels were designed to sample from deep-sea environments and enable subsequent HHP enrichment and isolation without decompression.

Here, this technology was used to explore how decompression affects microbial growth and growth patterns at HHP. These PUSH vessels were used to grow our model extremophile, *Archaeoglobus fulgidus*- an archaeon known to grow from 0.1-60 MPa (Oliver at al, in prep; section 3.4.1)- in batch culture from 0.1-98 MPa at 83°C (optimum temperature at 0.1 MPa) without decompression and compare this to HHP growth of *A. fulgidus* using traditional HHP methods that include multiple decompression-repressurization cycles. Additionally, *A. fulgidus* cells were transferred under pressure from one PUSH to another. These experiments not only tested the effects of decompression on *A. fulgidus* growth over the full working pressure range of the PUSH vessels, but they also tested vessel utility to transfer cell cultures under pressure for future deep-sea HHP sampling and subsequent HHP enrichment and isolation experiments.

4.3 Methods

4.3.1 High temperature, high hydrostatic pressure (HT-HHP) equipment

4.3.1.1 Pressurized underwater sample handler (PUSH) vessel HT-HHP batch culture system

The pressurized underwater sample handler (PUSH) vessels, similar to those vessels described in Bianchi et al. (1999) and purchased through TOP Industrie©, were used for high temperature (HT) and high hydrostatic pressure (HHP) microbial batch cultivation without whole sample decompression and repressurization cycles during subsampling periods (vessels described in Chapter 2.3.1). The PUSH vessels have a maximum pressure and temperature range of 0.1-100 MPa and 25-160°C respectively. To subsample from these vessels, a decompression line was constructed to mitigate the drastic pressure change from inside the PUSH vessel to the sampling syringe and decrease potential cell shearing or death from rapid decompression during

the subsampling process (Park & Clark, 2002; Foustoukos & Pérez-Rodríguez, 2015). The line was built with two valves, one being a micrometeringvalve to slowly subsample while operating the HHP screw pump to maintain vessel pressures.

For HT conditions, the temperature was controlled by a heating jacket and thermocouple system constructed for each PUSH vessel. The heating jackets, thermocouples, and temperature controllers were purchased from OMEGA™. Each vessel has an insulation wrap over the heating jacket and thermocouple. This heating system can be moved into an anaerobic chamber and plugged into a DC to AC converter powered by a 12-volt battery to maintain HT conditions during anaerobic preparations so that stable pressures could be reached upon pressurization (see section 2.4 for details).

4.3.1.2 Glass syringes in static pressure vessels for HT-HHP batch cultures

For cultivation of HT-HHP in static pressure vessels microbial growth experiments were conducted in glass syringes contained in heated static pressure vessels and were exposed to decompression and repressurization at each subsampling time point. Here, Hamilton© 10 mL, 1000 series luer lock gastight glass syringes were used for microbial cultivation. To secure sample enclosure without leakage at HT-HHP in these syringes, custom-made butyl rubber stoppers were made to create an extra seal between the fluid medium and the syringe piston. Syringe needles were embedded in butyl rubber stoppers before being transferred into the pressure vessels. For HHP growth, four High Pressure Equipment Co.© (HiP©), OC-1 O-Ring pressure vessels were used. Each 125 mL vessel can hold one 10 mL glass syringe for batch experiments. These vessels were equipped with individual gauges and heating systems, similar to

the heating systems described above for the PUSH vessels, but these systems were permanently installed. The temperature range of this system is 25-121°C and can accommodate pressures from 0.1-100 MPa. These limits are based on the maximum working pressure capacities of the HiP© vessels and the temperature range of the BUNA O-Rings.

4.3.2 Microorganism and growth medium

Archaeoglobus fulgidus strain VC-16 (DSM 4304) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). *A. fulgidus* VC-16 is an anaerobic, hyperthermophilic sulfate reducing archaeon. Though this type strain was isolated from a shallow marine hydrothermal system (Stetter, 1988), this species has also been commonly found in subsurface high-temperature terrestrial and marine environments with pressures ranging from ~10 to 40 MPa (e.g. Stetter, Lauerer, Thomm, & Neuner, 1987; Beeder, Nilsen, Rosnes, Torsvik, & Lien, 1994; L'Haridon, Reysenbach, Glenat, Prieur, & Jeanthon, 1995; Schrenk, Kelley, Delaney, & Baross, 2003; Nercessian, Reysenbach, Prieur, & Jeanthon, 2003; Fardeau et al., 2009). Here, *A. fulgidus* was grown chemolithoheterotrophically in a lactate-sulfate-rich medium. The composition of the culture medium followed Hartzell and Reed (2006) and sterile anaerobic conditions were maintained following Balch, Fox, Magrum, Woese, & Wolfe, (1979). The medium was reduced prior to inoculation by adding $Na_2S.9H_2O$ to a final concentration of 0.1% prior to inoculation (Cario, Jebbar, Thiel, Kervarec, & Oger, 2016).

4.3.3 HT-HHP batch culture experiments

4.3.3.1 Pre-cultures, growth medium, and inoculation

For triplicate HT-HHP batch culture experiments with and without sample decompression, pre-cultures and the culture medium were prepared for four PUSH vessels and four glass syringes. To prepare pre-cultures for each pressure experiment, *A. fulgidus* was grown from a frozen stock stored at -80°C and transferred once into 10 mL sterile anaerobic growth medium before inoculating three pre-cultures. Next, reduced sterile medium was prepared in four 55 mL serum bottles. Three of the four prepared medium-filled bottles were inoculated with 1% (v/v) logarithmic phase *A. fulgidus* cells to a final concentration of ~6.5 x 10^6 cells/mL and one medium-filled bottle remained uninoculated as a negative control. After inoculation, 8-10 mL from each medium-filled serum bottles was transferred into glass syringes and the remaining 45-47 mL were transferred into each PUSH vessel. *A. fulgidus* inoculation and transfer were all done in an anaerobic chamber. All growth experiments were carried out at 83°C.

4.3.3.2 PUSH vessel preparation, pressurization, and subsampling

For each batch culture HT-HHP experiment, four PUSH vessels were sterilized, assembled, and pre-heated before inoculation and transfer (Figure 4.1). To sterilize the PUSH vessels, each vessel was partially assembled with the PEEK reservoir inserted into the PUSH vessel body and the PEEK piston screw cap was attached (Figure 4.1 A). The PEEK reservoir screw cap and lid was left unattached and separately wrapped in foil. The valve connections and open PEEK reservoirs were also covered in foil. The foil-wrapped vessels, screw caps, and connections were sterilized by autoclaving at 121°C for 15 minutes. After sterilization, PUSH vessels were pre-assembled under a biosafety cabinet to maintain sterile conditions. The PEEK

reservoir screw cap was left partially unthreaded so that it could be removed later to fill the reservoir with inoculated growth medium. Finally, valves were attached to each side of the PUSH vessel. After pre-assembly, each PUSH was individually wrapped in an insulated temperature-controlled system with thermocouple and heating jacket to 83°C (Figure 4.1 B). Once pre-heated, all four vessels were transferred with their respective temperature-control systems into an anaerobic chamber (Bactron Shellab). In the anaerobic chamber, the four PUSH temperature-control systems were plugged into the DC to AC power converter. This allowed for continual vessel heating throughout the anaerobic inoculation process.

In the anaerobic chamber, the PEEK reservoirs of each of the four PUSH vessels were filled with ~45-47 mL of pre-inoculated growth medium (triplicate) or sterile growth medium (Figure 4.1 C). The PEEK reservoir screw cap and lid was then closed completely and the valves were closed while in the anaerobic chamber. The vessels were then transported out of the anaerobic chamber for pressurization. The vessels lost a maximum of 15°C during transfer in and out of the anaerobic chamber but regained stable 83°C temperatures within 3-5 minutes after connection to an AC wall outlet. Each vessel was then pressurized to the targeted pressure.

All four PUSH vessels were individually pressurized with one HHP screw pump and attached pressure gauge. To pressurize the PUSH vessels, the HHP screw pump was connected to the PEEK piston valve (Figure 4.1 D) and pressurized to the targeted pressure. Once pressurized, initial 0.5 mL subsamples were taken and fixed in 2.5% gludaraldehyde from each PUSH vessel using the decompression line to the PEEK reservoir valve (see below). After pressurization was achieved, the PUSH valve was closed and the HHP screw pump was detached. Initial sampling and pressurization of the all of the PUSH vessels and syringes were done within one hour after inoculation in the anaerobic chamber. To assure pressure stability,

potential pressure loss from vessel leakage was closely monitored for the first three hours after inoculation.

Up to nine subsamples were obtained from each PUSH vessel for every HT-HHP batch culture growth experiment. For subsampling, the HHP screw pump was connected to the PEEK piston valve and pressurized to the pressure of the vessel. Once pressurized, the PEEK piston valve was opened. Next, the decompression line with a sterile syringe attached was connected to the PEEK reservoir valve. The valves on the decompression line were closed. The PEEK reservoir valve was first opened and any pressure loss was regained using the HHP screw pump. The first valve on the decompression line was opened and again the target pressure was re-established. Finally, the microvalve was opened with an average decompression rate of 15-25 MPa/minute (Figure 4.1 E). The first 3 mL of medium sampled were discarded as waste from flushing the decompression line before taking a 0.5 mL aliquot sample for enumeration. A maximum of 10% pressure loss occurred during subsampling in all experiments but the pressure was rapidly re-established. The decompression line was cleaned with 70% ethanol and ultrapure water (18.2 MΩ) before and after subsampling each PUSH vessel.

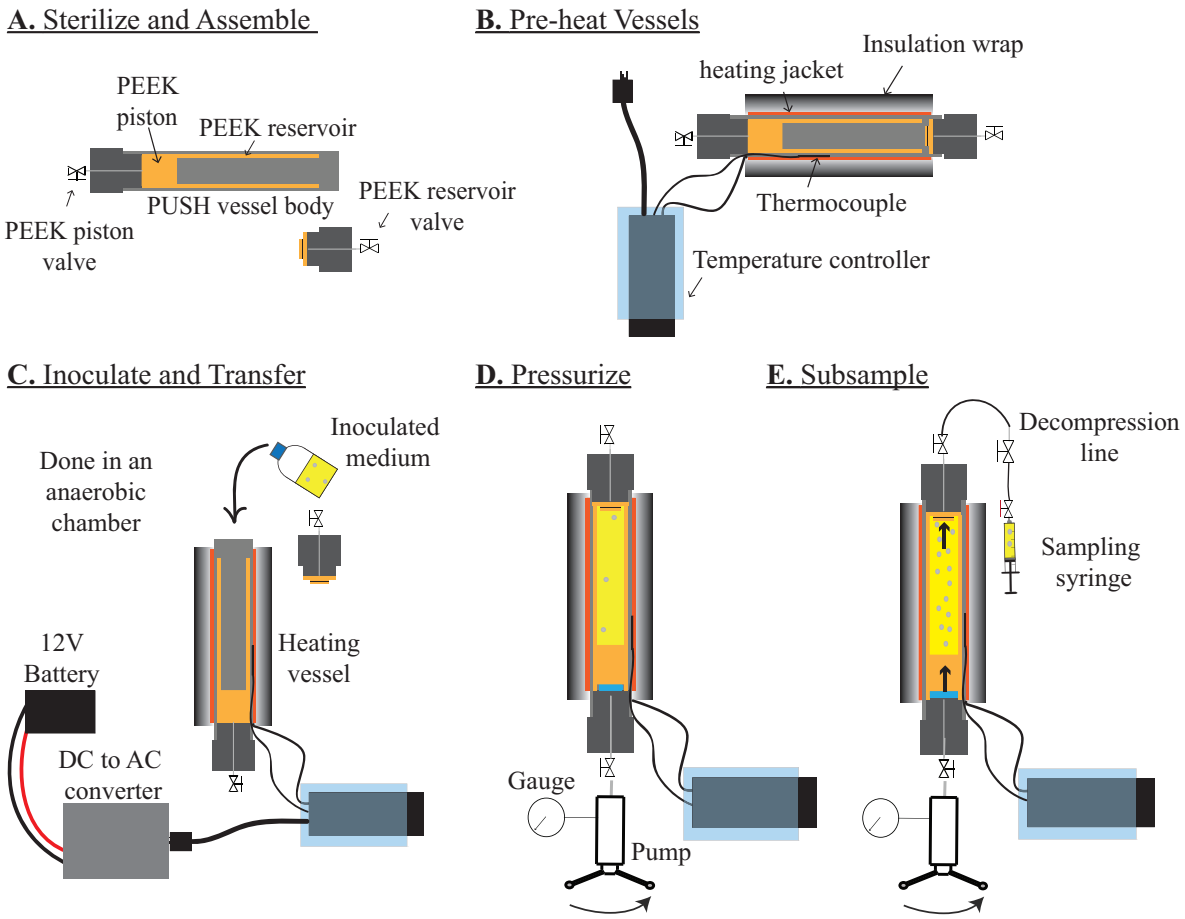

Figure 4.1. A schematic showing the procedures performed for preparing and running an *A. fulgidus* HT-HHP batch cultivation experiment in the PUSH vessels and temperature-controlled systems.

4.3.3.3 Glass syringe preparation, pressurization, and subsampling

Four 10 mL glass syringes were used for HT-HHP batch cultivation with sample decompression. To prepare the syringes for the experiment, the glass syringes, the syringe pistons, custom-made butyl stoppers, and needles were sterilized under a UV lamp for one hour in a biosafety cabinet (Figure 4.2 A). After sterilization, the syringes were assembled under the biosafety cabinet. The syringes were flushed with N_2 and the syringe needles were then

embedded into butyl stoppers (Figure 4.2 B). The assembled syringes were then transported to the anaerobic chamber. Once in the anaerobic chamber and after inoculation, 8-10 mL of inoculated medium was transferred into each of the three 10 mL glass syringes and 8-10 mL of sterile medium was transferred in the fourth 10 mL glass syringe as a negative control (Figure 4.2 C). The syringes were removed from the anaerobic chamber and an initial 0.5 mL subsample was fixed in 2.5% gludaraldehyde for enumeration. Finally, each syringe was placed in one of the four available preheated HiP© vessels, filled with water, and pressurized by connecting each vessel to a HHP screw pump to obtain the target growth pressure (Figure 4.2 D). For syringe subsampling, each vessel was decompressed at an average rate of 19 MPa/minute and a 0.5mL aliquot sample was taken and fixed in 2.5% gludaraldehyde from each syringe. After subsampling, the syringes were returned to the vessels and again pressurized to the targeted growth pressure. *A. fulgidus* cells grown in the glass syringes were decompressed and repressurized a maximum of nine times throughout each batch culture experiment.

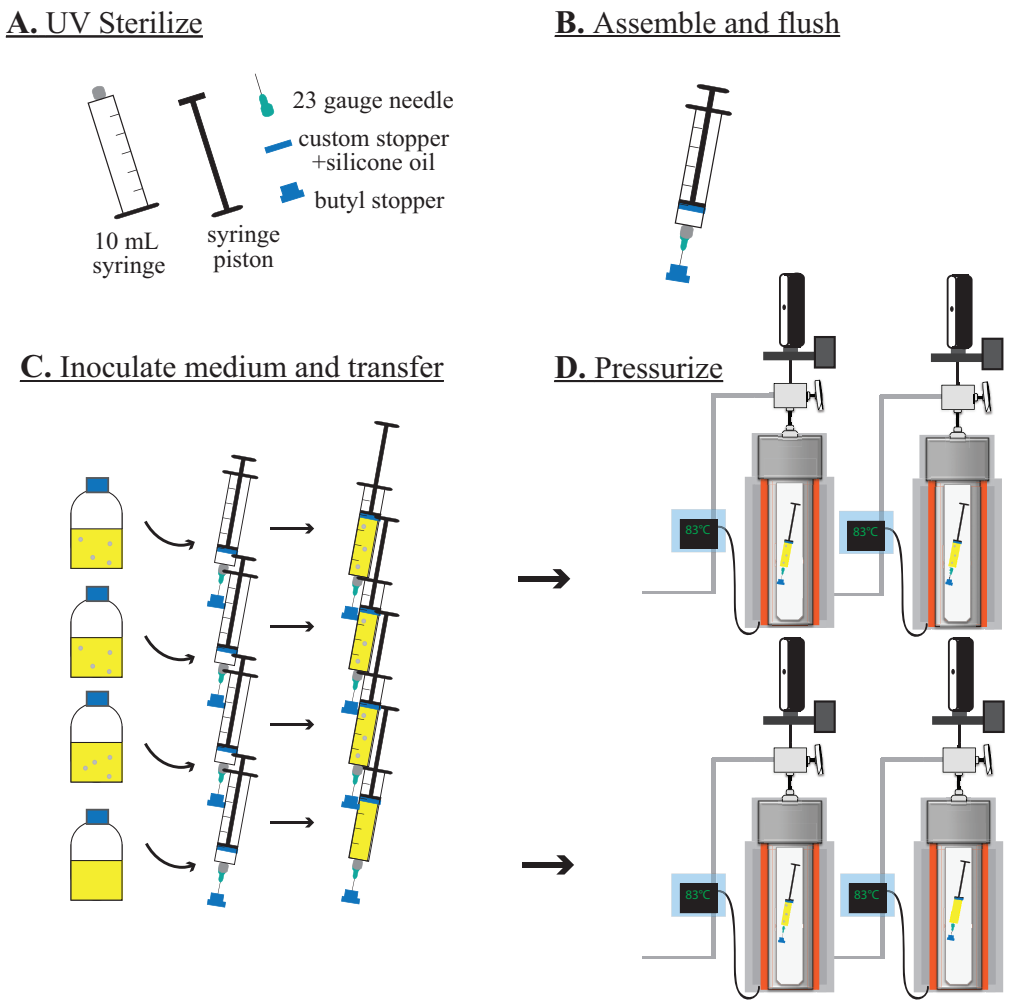

Figure 4.2. Glass syringe sterilization (A) and assembly (B) for batch culture growth experiments with sample decompression. Following inoculation, *A. fulgidus* cultures were transferred into 5 mL syringes (C) and placed in heated static pressure vessels filled with water and pressurized (D).

4.3.3.4 PUSH-to-PUSH culture transfer

The transfer of fluid medium from one PUSH vessel to a second PUSH vessel under pressure was tested with active *A. fulgidus* cultures. Here, *A. fulgidus* was grown at 20 MPa in a PUSH vessel as described above (section 4.3.3.2) and logarithmic phase cells were then transferred to a second PUSH vessel filled with sterile anaerobic medium already pressurized to 20 MPa. After HHP inoculation, the second PUSH vessel was pressurized to 50 MPa and growth

was measured over time described previously (section 4.3.3.2; Figure 4.3). Growth at 50 MPa from a HHP (20 MPa) culture was compared to growth at 50 MPa from a 0.1 MPa inoculation to measure any differences in growth patterns. This experiment was conducted to test the viability of *A. fulgidus* cells after being transferred from one vessel to another under pressure and experiencing a pressure increase to 50 MPa.

Additions and modifications to the HT-HHP batch culture experiments were made to prepare the PUSH-to-PUSH culture transfer. First, a 5-inch long 1/8-inch ID high-pressure tube was cut and fitted with valve connections. This tubing was then connected to the two PUSH vessels for fluid transfer. Additionally, the fluid volume that passed through the high-pressure tubing was calibrated (~0.6 mL of fluid transferred per HHP screw pump rotation). Medium and pre-culture preparations were performed as previously described (section 4.3.3.1). The PUSH vessels, connections, and the 5-inch tubing connector were sterilized as previously described (section 4.3.3.2). Additionally, magnetic stir bars were placed inside PEEK reservoirs before sterilization for mixing *A. fulgidus* at 50 MPa and stir bars were also placed in sterile anaerobic medium in serum bottles to be sure that the stir bar did not influence growth. The PUSH vessels were assembled and heated similar to methods described above (section 4.3.3.2) with the exception of connecting a micrometeringvalve to the PEEK reservoir screw cap end of the vessel that contained the 20 MPa *A. fulgidus* culture to diminish the pressure difference that might cause cell shearing during the transfer (Figure 4.3). Finally, a second HHP screw pump and gauge was used to pressurize the second PUSH vessel during inoculation under pressure (Figure 4.3).

The PUSH-to-PUSH culture transfer experiment involved several inoculation steps (Figure 4.4). Before growth in the PUSH vessels, *A. fulgidus* pre-cultures were grown at 0.1 MPa

to logarithmic phase (Figure 4.4 A). These cells were then inoculated into ~45-47 mL of sterile anaerobic medium and transferred into a heated PUSH vessel in an anaerobic chamber to a final concentrations of ~6.5 x 10^6 cells/mL. Simultaneously, ~45-47 mL of sterile, uninoculated anaerobic medium was transferred into an additional heated PUSH vessel to test for contamination from this method. Once filled, the heated PUSH vessels were closed and pressurized to 20 MPa. Additionally, the same *A. fulgidus* pre-culture cells were used to inoculate sterile anaerobic medium at 0.1 MPa to a final of ~6.5 x 10^6 cells/mL. *A. fulgidus* cells were then grown to logarithmic phase at 20 MPa and 0.1 MPa before transferring into a second PUSH vessel and serum bottles, both filled with sterile anaerobic medium (Figure 4.4 B1 and B2).

Next, *A. fulgidus* cells were transferred from the PUSH vessel at 20 MPa to a second PUSH vessel under pressure (20 MPa) for growth at 50 MPa, and to a serum bottle for growth at 0.1 MPa. To transfer *A. fulgidus* cells from the PUSH vessel at 20 MPa to a second PUSH vessel, the sterile 5-inch high-pressure tubing was connected to both of the closed PUSH vessel PEEK reservoir valves and each PUSH vessel was connected to a HHP screw pump (Figure 4.3). The PEEK reservoir valve on the PUSH vessel filled with sterile anaerobic medium was opened to the 5-inch tubing then the vessel and tubing was pressurized to 20 MPa. The PEEK reservoir valve on the PUSH vessel with *A. fulgidus* culture was then slowly opened and the HHP screw pump on that vessel was rotated ~1¾-2¼ turns to increase the pressure on both vessels. Finally, the HHP screw pump on the second PUSH vessel was rotated to decrease pressure back to 20 MPa and transfer *A. fulgidus* cells into that vessel. Once *A. fulgidus* cells were transferred, both vessels were closed and detached from their respective HHP screw pumps. The inoculated PUSH vessel with the magnetic stir bar was then taken to a stir plate to mix the cells in the vessel. Once

mixed, the inoculated PUSH vessel was again connected to the HHP screw pump and pressurized from 20 MPa to 50 MPa (Figure 4.3 and 4.4 C1). After inoculation and pressurization, *A. fulgidus* cells grown at 20 MPa were used to inoculate a serum bottle filled with sterile anaerobic medium and grown at 0.1 MPa to ensure growth at ambient pressure conditions (Figure 4.4 C2). Growth at 50 MPa was measured over time with initial and subsequent subsampling done as previously described (section 4.3.3.2).

The final inoculation step transferred *A. fulgidus* cells grown at 0.1 MPa to a PUSH vessel pressurized to 50 MPa following the previously described methods and to a serum bottle at 0.1 MPa (section 4.3.3.2; Figure 4.4 C3 and C4). Growth was also measured over time from the PUSH vessel at 50 MPa (Figure 4.4 C3). Additionally, a heated and pressurized PUSH vessel filled with uninoculated anaerobic medium was monitored over the incubation time to test for contamination from this method. The PUSH-to-PUSH culture transfers were done in triplicate experiments from three separate pre-cultures.

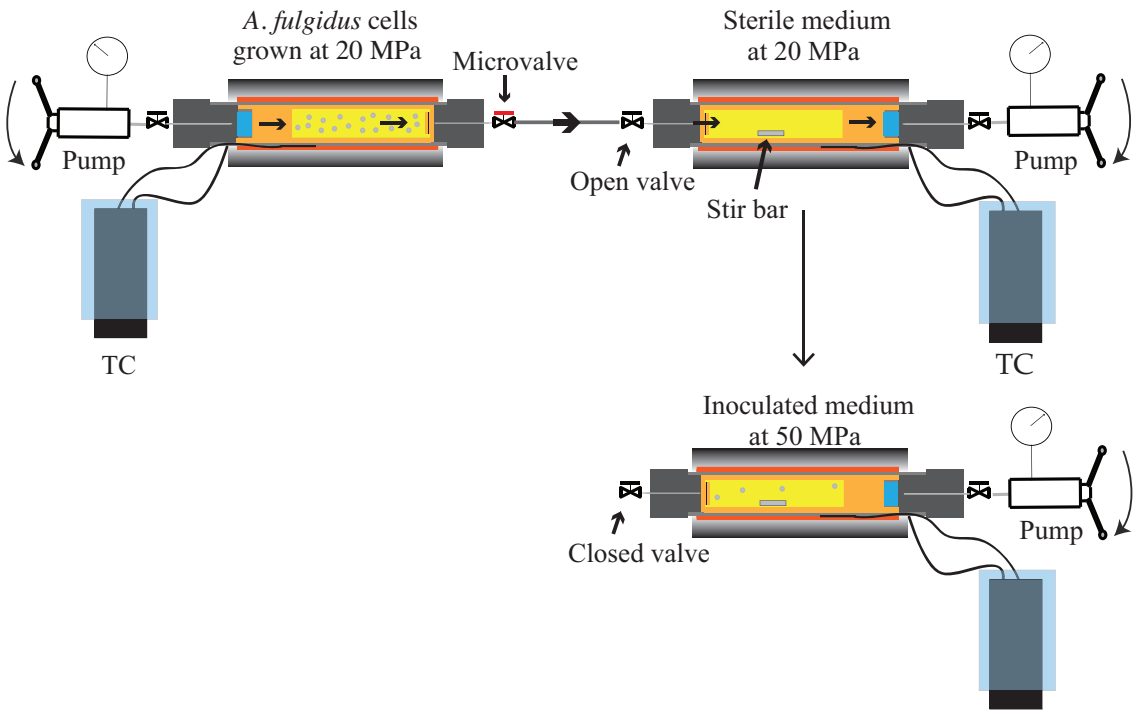

Figure 4.3. A schematic of experimental procedures for transferring *A. fulgidus* cells grown in a PUSH vessel at 20 MPa to a second PUSH vessel filled with sterile medium under pressure. Once *A. fulgidus* cells were transferred, the second PUSH vessel was closed and pressurized from 20 MPa to 50 MPa.

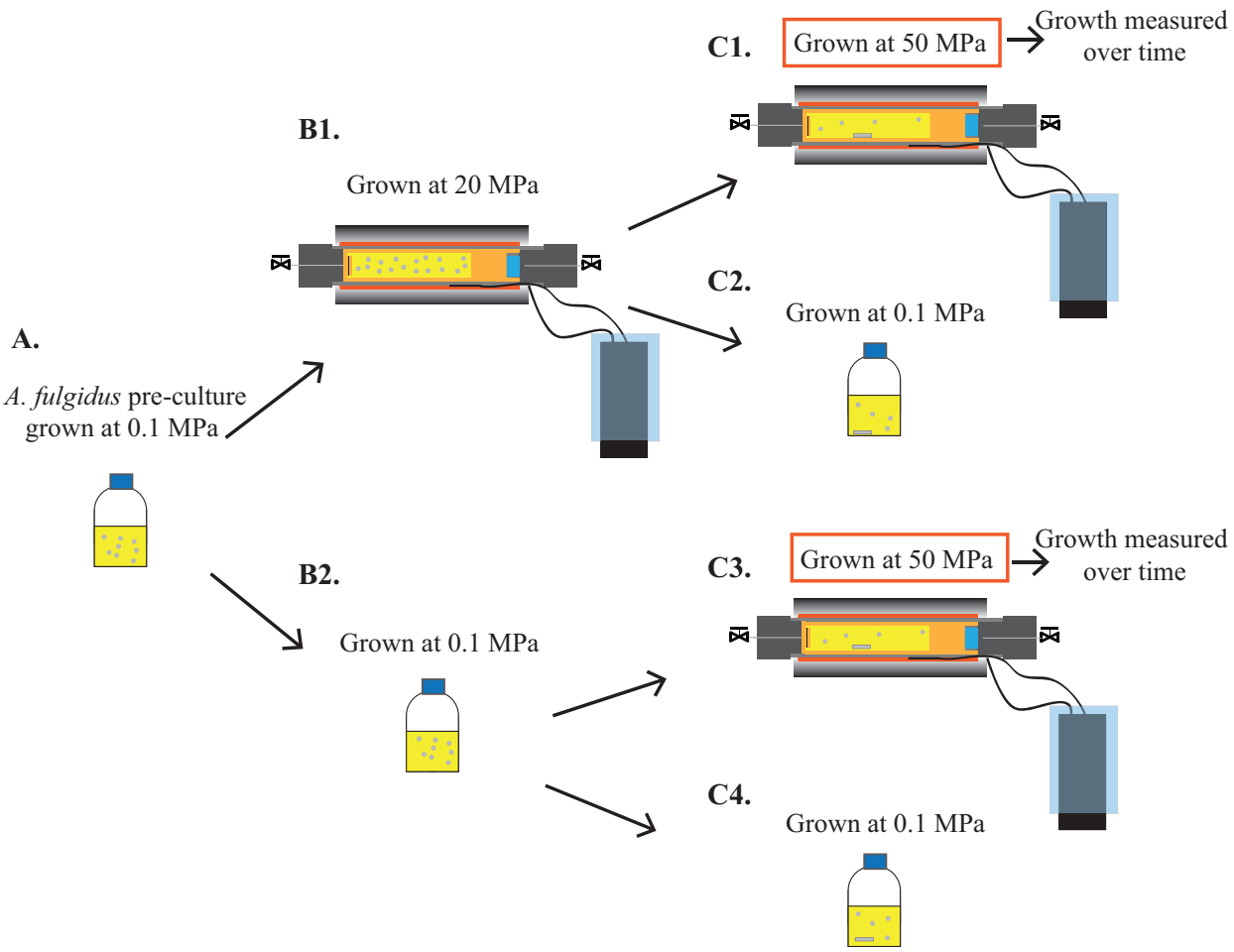

Figure 4.4. A schematic showing the experimental design for comparing *A. fulgidus* growth at 50 MPa (C1 and C3) from cells previously grown at either 20 MPa in a PUSH vessel (B1 to C1) or at 0.1 MPa (B2 to C3). To be sure *A. fulgidus* growth was observed at optimum conditions (0.1 MPa and 83°C), *A. fulgidus* was also transferred to sterile anaerobic medium at 0.1 MPa from cells previously grown at 20 MPa in a PUSH vessel (B1 to C2) and at 0.1 MPa (B2 to C4).

4.3.4 *A. fulgidus* HHP cell recovery experiment

A final batch cultivation experiment was conducted to evaluate if *A. fulgidus* cells could recover and grow at 0.1 MPa following exposure to extreme HHP. *A. fulgidus* cells were incubated at 80 MPa in the PUSH vessels following the previously described methods (section 4.3.3.2). Triplicate experiments of *A. fulgidus* cells were exposed to 80 MPa for 115 hours, then

1 mL of fluid medium with ~3.2 x 10^6 cells was subsampled from the PUSH vessel and inoculated into sterile anaerobic medium at 0.1 MPa and 83°C. Cell growth was monitored visually for 120 hours.

4.3.5 Light microscopy and cellular enumeration

Cell enumeration was estimated by direct counts from fixed cells (2.5% (v/v) gludaraldehyde) using a light microscope (model XM: Olympus) with a Thoma-chamber (depth: 0.02mm; Brand, Wertheim, Germany) under 80x magnification (e.g. Huber, Woese, Langworthy, Fricke, & Stetter, 1989; Hei & Clark, 1994; Cario et al., 2016). Specific growth rates (μ^{-hr}) were calculated from linear regressions of exponential growth from triplicate experiments using the LINEST function in Excel. Generation times were calculated as the $\log 2/\mu^{-hr}$. Error bars indicate the standard error from linear regressions of triplicate experiments. Maximum cell densities were either taken from stationary phase or, in the case of 70 MPa and 90 MPa experiments, maximum cell densities were taken after 20 hours to better represent cell densities for those pressures. Error bars indicate standard deviation from triplicate experiments.

4.4 Results

4.4.1 *A. fulgidus* HT-HHP batch cultures

HT-HHP batch cultivation of *Archaeoglobus fulgidus* VC-16 (type strain) was performed from 0.1 MPa-98 MPa in ~10 MPa increments at 83°C in PUSH vessels and in static pressure vessels. Over the growth period (48-115 hours), *A. fulgidus* cells grown in PUSH vessels did not

experience whole sample decompression upon subsampling, whereas cells grown in static pressure vessels were subjected to whole sample decompression and repressurization at each subsampling time. Growth was observed from 0.1 MPa-60 MPa in both the PUSH vessels and in static pressure vessels and measurable cell densities were recorded over the entire pressure range tested, up to 98 MPa (Figure 4.5 A and B). Definitive exponential growth of *A. fulgidus* was observed in the PUSH vessels up to 60 MPa and up to 50 MPa in static pressure vessels subjected to subsampling decompression. In static pressure vessels, minor increases in cell density ($1.34 \pm 0.11 \times 10^7$ cells/mL) relative to initial values ($6.03 \times 10^6 \pm 1.02$ cells/mL) were observed at 60 MPa, but were not consistent with a true exponential growth phase (Figure 4.5 B). In the PUSH experiments at 70 MPa, cell densities increased slightly to $1.37 \pm 0.36 \times 10^7$ cells/mL at 20 hours, before falling again to values close to the initial cell densities. Additionally, at 90 MPa in the PUSH vessels, cell densities also increased slightly ($1.02 \pm 0.09 \times 10^7$ cells/mL) at 10 hours. There was no initial increase in cell densities in static pressure vessels and overall cell densities decreased over time for cultures grown at 80-98 MPa and in the 80 MPa and 98 MPa PUSH vessel experiments (Figure 4.5 A and B).

Based on the cell density data shown in Figure 4.5 A and B, *A. fulgidus* growth rates and generation times were calculated for pressures between 0.1 MPa and 60 MPa (Figure 4.6 A and B). Additionally, the maximum observed cell densities are shown as a function of pressure between 0.1 MPa and 98 MPa (Figure 4.6 C). Overall, *A. fulgidus* growth characteristics showed similar trends with pressure, although growth was generally more robust in PUSH vessels compared to static pressure vessels, especially at the higher end of the pressure range (Figure 4.6, filled squares). The fastest growth rate in the PUSH vessels was measured at 0.1 MPa (0.141 ± 0.007 μ^{-hr}), though growth rates were only slightly slower from 10 MPa to 30 MPa ($0.131 \pm$

0.005 – 0.112 ± 0.012 μ^{-hr}). However, the average growth rate at 10 MPa (0.112 ± 0.016 μ^{-hr}) was slower and more variable than both 0.1 MPa (0.141 ± 0.007 μ^{-hr}) and 20 MPa (0.131 ± 0.004 μ^{-hr}; Figure 4.6 A). Also, slower growth rates were measured from 40-60MPa (0.078 ± 0.008 μ^{-hr} - 0.026 ± 0.002 μ^{-hr}; Figure 4.6 A). Generation times followed the same pattern with the shortest measured at 0.1 MPa (2.11 ± 0.17 hrs) and similar generation times recorded up to 40 MPa (2.29 ± 0.34 –3.71 ± 0.35 hrs). Significantly longer generation times were measured from 50 - 60 MPa (6.81 ± 0.58 – 11.51 ± 0.23 hrs; Figure 4.6 B). Similarly, maximum cell densities decreased only slightly from 0.1 to 40 MPa (6.45 x ± 1.34 x 10^8 cells/mL - 3.66 ± 0.62 x 10^8 cells/mL; Figure 4.6 C). A more notable decrease in maximum cell densities were observed between 50 and 70 MPa (9.58 ± 0.58 x 10^7 cells/mL - 1.14 ± 0.37 x 10^7 cells/mL), while cell densities fell below the initial inoculation cell densities from 80 to 98 MPa (6.75 ± 0.21 x 10^6 cells/mL – 6.37 ± 0.09 x 10^6 cells/mL), indicating net cell death at these pressures. The loss of cellular viability above 80 MPa was confirmed by exposing *A. fulgidus* cells to 80 MPa in the PUSH vessels for 115 hours. Cells were subsequently retrieved from the PUSH vessel and used as inoculum for a growth experiment at optimum conditions (0.1 MPa and 83°C). Finally, *A. fulgidus* cell recovery was tested after exposure to 80 MPa in the PUSH vessels. After *A. fulgidus* were exposed to 80 MPa for 115 hours, cells were subsampled from the PUSH vessel and inoculated into sterile anaerobic medium at 0.1 MPa and 83°C. These batch cultures were visually monitored for one week but growth was not seen at 0.1 MPa after being exposed to 80 MPa. Overall, growth rates and generation times measured here show that *A. fulgidus'* optimum growth is at 0.1 MPa but is tolerant to pressure up to 30 MPa and *A. fulgidus* was able to reach similarly high maximum cell densities up to 40 MPa. Additionally, these data illustrate that pressures above 40 MPa had an

overall negative affect on *A. fulgidus* growth when grown in the PUSH vessels without whole sample decompression upon subsampling.

The growth rates, generation times and maximum cell densities that correspond to *A. fulgidus* growth in static pressure vessels are shown as open circles in Figure 4.6. The fastest growth rate was measured at 0.1 MPa (0.145 ± 0.003 μ^{-hr}) and slower rates were measured with increasing pressures (Figure 4.6 A). There was a noticeably larger difference in growth rates from 30 MPa (0.095 ± 0.005 μ^{-hr}) to 40 MPa (0.045 ± 0.016 μ^{-hr}) in static pressure vessels compared to PUSH experiments and the growth rate at 40 MPa had greater deviation. Following growth rate trends, the shortest generation time was measured at 0.1 MPa (1.99 ± 0.40 hrs) and similar generation times were measured up to 30 MPa (2.48 ± 0.53 hrs – 3.18 ± 0.39 hrs). Progressively longer generation times were measured from 40-60 MPa (6.56 ± 1.51 hrs – 25.9 ± 0.34 hrs; Figure 4.6 B). Maximum cell densities were comparable from 0.1-40 MPa (7.46 ± 0.44 x 10^8 cells/mL - 3.20 ± 0.67 x 10^8 cells/mL; Figure 4.6 C). Additionally, measured maximum cell densities were significantly lower at 50 MPa – 70 MPa (4.97 ± 0.94 x 10^7 cells/mL – 9.90 ± 1.1 x 10^6) and cell densities fell below the initial inoculation cell densities at pressures above 70 MPa (Figure 4.6 C). Optimum growth of *A. fulgidus* in static pressure vessels with several cycles of decompression was at 0.1 MPa with decreasing growth rates and increasing generation times as pressure increased. Maximum cell densities from 10 MPa-40 MPa were less effected to multiple sample decompressions but generally, elevated growth pressures with multiple sample decompression and recompression cycles had a negative effect on *A. fulgidus* growth especially at and above 40 MPa.

Differences in growth patterns from *A. fulgidus* grown in the PUSH vessels compared to glass syringes in static pressure vessels increased with increasing growth pressures (Figure 4.6

A, B, and C). *A. fulgidus* growth curves from the two high-pressure methods were similar from 0.1-40 MPa (Figure 4.5 A and B), however the negative impact of sample decompression on growth was most notable at 50 MPa and 60 MPa pressure conditions (Figure 4.7 A and B). From 20 MPa- 60 MPa, growth rates measured from PUSH vessel cultures were faster than cells exposed to several cycles of decompression with greater differences measured from 40 MPa – 60 MPa (Figure 4.6 A). Generation times for the two high-pressure methods were similar from 0.1 MPa-30 MPa and the impact of sample decompression was not noticeable until 40 MPa. However, the difference in generation times between constant pressure equipment (PUSH vessels) and those subjected to several cycles of decompression (static pressure vessels) was much more pronounced at 50 MPa and 60 MPa (Figure 4.6 B). At the maximum growth pressure (60 MPa), the difference was ~15 hours, with *A. fulgidus* doubling in 11.51 ± 0.23 hours in the PUSH vessels, but needing 25.9 ± 0.34 hours when subjected to decompression (Figure 4.6 B). The negative impact of several decompression cycles was also apparent in values of maximum cell densities above 50 MPa. For both high-pressure growth methods maximum cell densities decreased with increasing pressure from 50 MPa to 98 MPa. However, higher cell densities were measured in *A. fulgidus* cultures grown in PUSH vessels without decompression compared to cells that experienced decompression (Figure 4.6 C). The most noteworthy disparities in maximum cell densities were for 50 MPa, 60 MPa, 80 MPa, and 90 MPa growth pressures (Figure 4.6 C). Although cell densities fell below the initial inoculation cell densities for 80 MPa -98 MPa in all experiments, more cells were recovered in PUSH vessel incubations than in static pressure vessels (Figure 4.6 C). Generally, sample decompression and repressurization at pressures above 30 MPa resulted in increasingly slower growth rates and longer generation times and these affects on maximum cell densities were seen at pressures above 40 MPa.

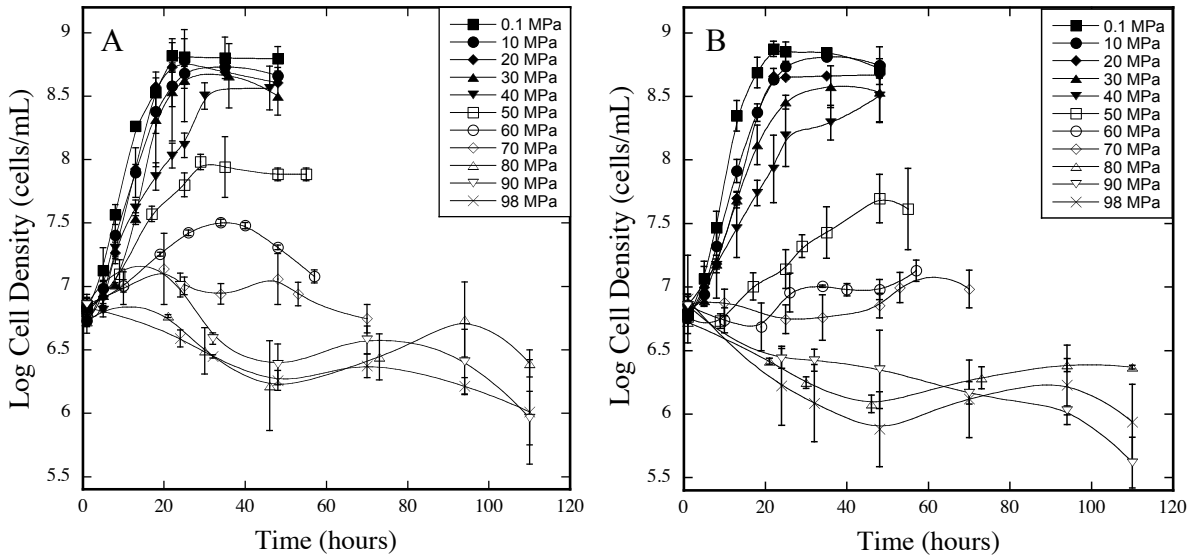

Figure 4.5. ***A. fulgidus*** **growth curves in the PUSH vessels without sample decompression (A) and in static pressure vessels with multiple sample decompression-repressurization cycles (B) from 0.1-98 MPa. Error bars are the standard deviations from the average of triplicate experiments.**

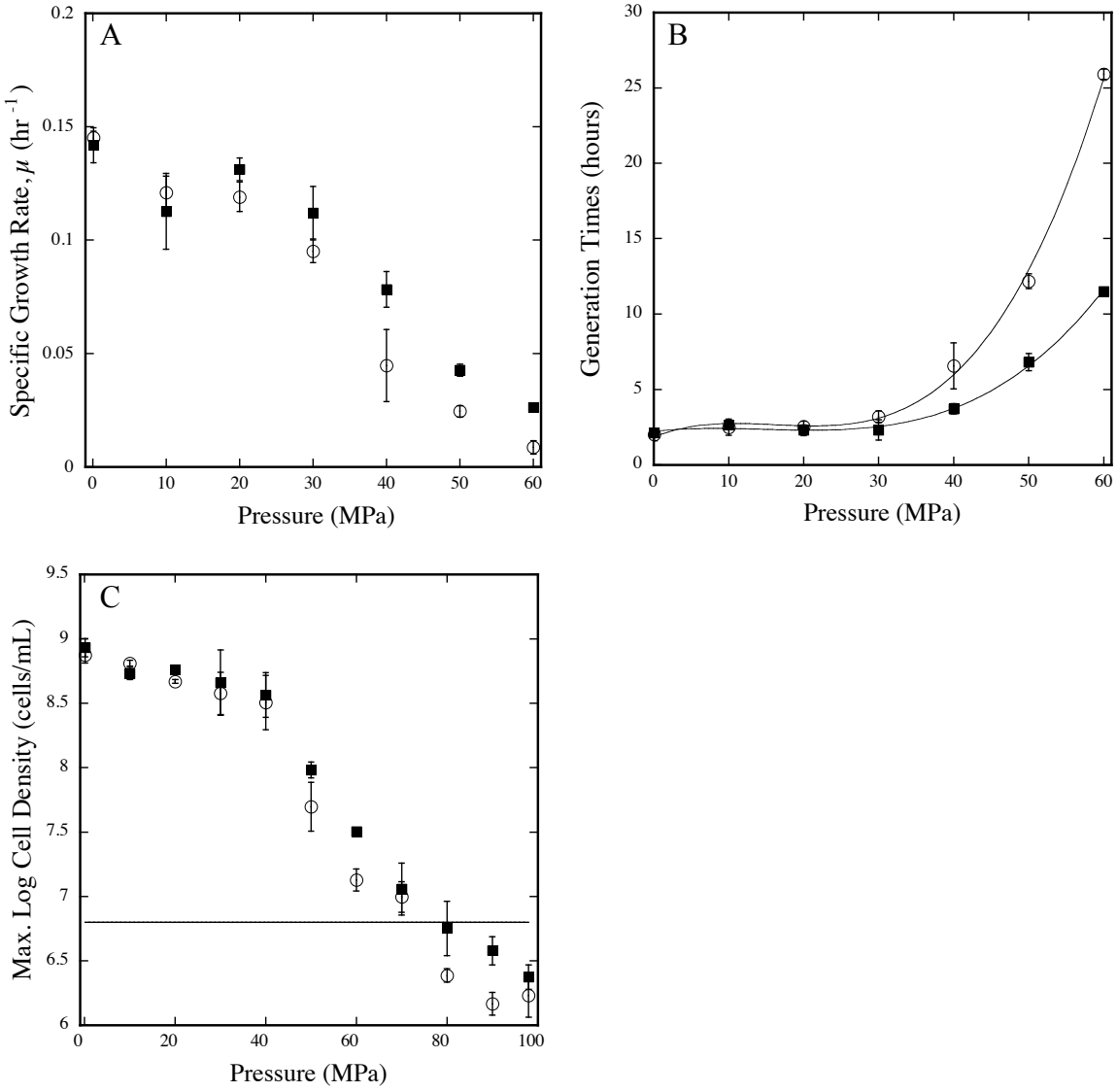

Figure 4.6. *A. fulgidus* growth rates (A), generation times (B), and maximum cell densities (C) grown in the PUSH vessels without whole sample decompression (filled squares) and in static pressure vessels with multiple sample decompression-repressurization cycles (open circles). The horizontal line at log cell density ~6.8 across graph C is the average initial cell density. Error bars indicate standard deviations from the average of triplicate experiments.

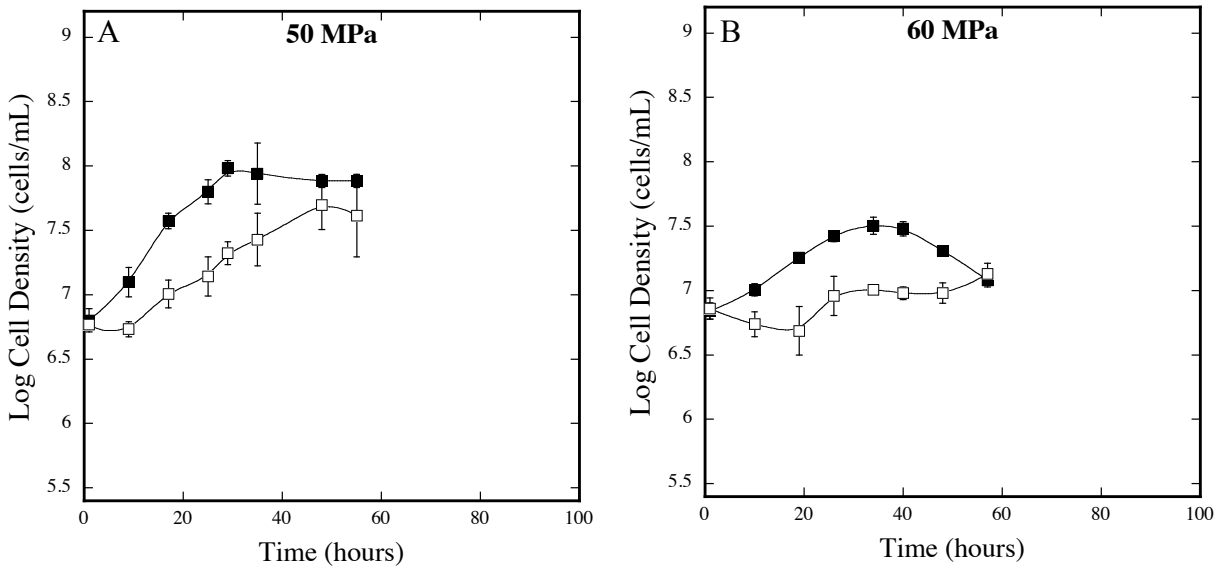

Figure 4.7. Comparison of *A. fulgidus* growth in the PUSH vessels (filled squares) versus growth in the static pressure vessels (open squares) at 50 MPa (A), 60 MPa (B). Error bars indicate standard deviations from the average of triplicate experiments.

4.4.2 PUSH-to-PUSH culture transfer under pressure

The viability of *A. fulgidus* cells transferred under pressure from one PUSH vessel to a second PUSH vessel was tested for initial growth at 20 MPa and subsequent inoculation and growth at 50 MPa. Several 0.1 MPa control experiments were conducted to insure growth at optimum conditions (Figure 4.4). Growth was observed from *A. fulgidus* cells grown at 50 MPa after being grown and transferred at 20 MPa (Figure 4.8, open diamonds). However *A. fulgidus* cells grown at 50 MPa with prior growth at 0.1 MPa grew to higher cell densities (Figure 4.8, filled squares). Nonetheless, *A. fulgidus* cells were successfully transferred under pressure from one PUSH vessel into another and growth was measured at a pressure known to be extreme for this strain.

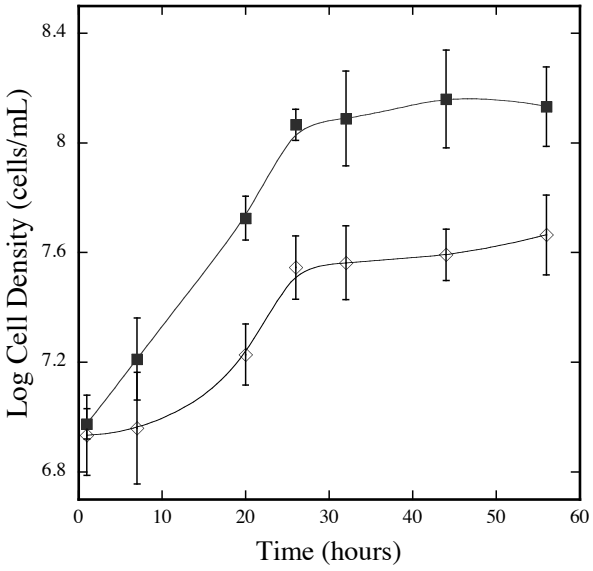

Figure 4.8. ***A. fulgidus*** **growth curves at 50 MPa from inoculum grown at 0.1 MPa (black squares) and at 20 MPa (open diamonds). Error bars are standard deviations from the average of triplicate experiments.**

4.5 Discussion

4.5.1 Effects of decompression

The effects of sample decompression on microbial growth at elevated pressures were explored using a model extremophile, *Archaeoglobus fulgidus*. *A. fulgidus* was cultivated at 83°C (optimum temperature) from 0.1 MPa to 98 MPa in the pressurized underwater sample handler (PUSH) vessels without whole sample decompression and in glass syringes in static pressure vessels where samples experienced multiple decompression-repressurization cycles during subsampling. Growth patterns and cellular densities were compared between the two HHP cultivation methods. Here, *A. fulgidus* exponential growth was measured from 0.1 MPa-60 MPa in both the PUSH vessels and in static pressure vessels, though growth at 60 MPa in

syringes was very low (Figure 4.5 B). Cell densities were measured up to 98 MPa for both cultivation methods. In general during exponential growth, *A. fulgidus* was mostly tolerant to sample decompression up to 30-40 MPa (Figure 4.5 A and B), although with increasing pressure, decompression had a more pronounced impact on growth rates and recovered cell densities (Figure 4.5 A and B).

The negative impacts of decompression on *A. fulgidus* HHP growth were evident in growth rates, generation times, and cell densities. Significantly slower growth rates and longer generation times (Figure 4.6 A and B; open circles) were observed in *A. fulgidus* subjected to decompression from 40 MPa -60 MPa. There was up to a 30% difference in growth rates and 30-70% difference in generation times from the two culturing methods from 40 MPa-60 MPa. The degree to which decompression negatively impacted growth seemed to correlate with the magnitude of pressure difference from ambient pressures (0.1 MPa). *A. fulgidus* growth at 50 MPa and 60 MPa was shown to be the most negatively impacted by sample decompression (Figure 4.7 A and B). These two pressures were previously reported to be extreme conditions for *A. fulgidus* growth (Oliver et al., In prep; section 3.4), yet these decompression results illustrate *A. fulgidus* sensitivity to pressure fluctuations at pressures conditions so different from its optimal growth at 0.1 MPa. Additionally, the affects of four decompression-repressurization cycles on *A. fulgidus* growth was previously tested, and reported that sample decompression did not affect observed cell densities from 10-50 MPa (Oliver et al., In prep, Appendixes, Figure 3S). The results shown here illustrate that *A. fulgidus* maximum cell densities were not drastically impacted by up to nine cycles of decompression-repressurization up to 40 MPa (Figure 4.6 C), further constraining the specific pressure range (0.1 MPa to 40 MPa) from which to recover the highest chances of viability. Further illustrating this, there was a substantial decrease in

maximum cell densities for both culturing methods from 40 MPa -50 MPa used here, but more cells were measured from PUSH vessel when samples were not decompressed. Although cell death was observed above 70 MPa, overall higher cell densities were recovered from incubations in PUSH vessels. In generally, these results show that *A. fulgidus* has a tolerance to pressure with sample decompression up to 30 MPa, but at higher pressures decompression increasingly impacted growth rates, generation times, and recovered maximum cell densities.

Though *A. fulgidus* type strain VC-16 was isolated from a shallow marine vent (Stetter et al., 1987), these results and our previously reported *A. fulgidus* HHP growth (Oliver et al, in prep, section 3.4) show that this strain is tolerant to HHP pressure and decompression up to ~30-40 MPa, which is consistent with the depths and pressures (1-4 km, ~10-40 MPa) of deep-sea and deep subsurface environments where it has been identified (Stetter et al., 1993; Beeder et al., 1994; L'Haridon et al., 1995; Nakagawa et al., 2005; Fardeau et al., 2009). *A. fulgidus* decompression tolerance up to 30 MPa-40 MPa supports why this species has often been identified from these depths. On the other hand, *A. fulgidus* has rarely been identified in deeper environments. The successful, albeit slower, growth of *A. fulgidus* up to 60 MPa suggests that this strain could inhabit deeper high-temperature environments. However, in such cases deep-sea sampling with decompression would likely inhibit successful isolation of *A. fulgidus* from environments at pressures between 50-60 MPa, although other factors (i.e. temperature, enrichment medium, pH etc.) also have a significant effect on successful isolation.

The microbial diversity seen from molecular studies of deep-sea and subsurface environments is vast, yet only a small percentage of what we sample is successfully isolated (D'Hondt, 2004). A contributing factor for this is likely due to the loss of cell viability during sampling and subsequent transfers under ambient pressures or from repeated sample

decompression-repressurization cycles during enrichment and isolation. Many parameters affect the successful isolation of novel microorganisms and are accounted for during enrichment and isolation. For example, if obligate anaerobes are being selected for, then enrichment and isolation is done under anaerobic conditions so that samples are not exposed to conditions that cause cell death. However, this similar reasoning has not been applied to pressure. Our data suggests that exposure to pressures outside the range of growth can reduce cell viability and thus inhibit subsequent cell growth. Here, *A. fulgidus* did not recover at 0.1 MPa from incubations at 80 MPa in the PUSH vessels after 115 hours. This could have been due to the long exposure at 80 MPa leading to loss of viability or the inoculation cell density was too low for successful recovery. Previous data have shown that at after incubation at 70 MPa, *A. fulgidus* recovered to full cell densities at 0.1 MPa. These findings further exemplify that the greater the pressure difference is away from optimum growth, either increasing or decreasing pressures, the great chance of lost cell viability. Therefore, it is important to maintain sample pressures similar to *in situ* conditions during the sampling process and for subsequent enrichments and isolations from HHP environments to increase the likelihood of recovering novel piezophiles and obligate piezophiles.

Thus far, most of the obligate piezophiles identified have been psychrophilic bacteria sampled from depths greater that 6 km (i.e. ~60 MPa pressures; e.g. Yayanos, 1981; Deming et al., 1988; Kato et al., 1998; Nogi, Hosoya, Kato, & Horikoshi, 2004) and only one obligate piezophilic hyperthermophilic archaeon, *Pyrococcus yayanosii*, has been identified from a hydrothermal vent at 4.1 km depth (Birrien et al., 2011). Since many marine microorganisms are tolerant to some degree to HHP (Zhang, Li, Xiao, & Bartlett, 2015), and many can tolerate decompression-recompression cycles, like *A. fulgidus*, from 1-4 km depths (Jannasch & Wirsen, 1984), it is difficult to select for piezophiles from these depths. Even HHP sampling and

cultivation without decompression with equipment like the PUSH vessels could selective for piezotolerant and facultative piezophiles enrichment and isolation. Therefore, the pressure conditions of enrichment and isolation experiments are important considerations. Nevertheless, using HHP equipment like the PUSH vessels does increase the likelihood of collecting and maintaining more piezophiles and obligate piezophiles.

4.5.2 PUSH vessels for future deep-sea sampling, enrichments, and isolations

In order to accommodate the need for maintaining *in situ* pressures throughout sampling, enrichment, and isolation of deep subsurface microbes, fluid sample transfers at HHP are necessary. Therefore, *A. fulgidus* was used to develop and test a systematic technique using two PUSH vessels in series for transferring cell cultures under HHP. This suite of experiments monitored *A. fulgidus* growth at 50 MPa both from cells previously grown at 20 MPa and transferred under pressure and from cells previously grown at 0.1 MPa. *A. fulgidus* growth was measured in both HHP batch cultures at 50 MPa, though growth at 50 MPa was greatly diminished after growth and transfer at 20 MPa (Figure 4.7). *A. fulgidus* cells were successfully transferred under pressure using this method, but the loss of *A. fulgidus* cellular viability from this transfer, suspected from the observed diminished growth at 50 MPa using this method, is unknown. Both experiments performed at 50 MPa started with similar initial cell densities, but it is unclear if there was similar cellular viability from the cells inoculated under pressure to those grown at 0.1 MPa. Further experimentation using this method and monitoring live and dead cells would better elucidate if cellular viability was affected by the transfer of cells under pressure and subsequent increase in pressure. Alternatively, *A. fulgidus* was shown to have slightly slower

growth rates at 20 MPa and with the subsequent transfer to 50 MPa, an extreme growth pressure for this strain, could have resulted in the observed diminished growth after transfer to 50 MPa under pressure. However, if the former is the case and *A. fulgidus*, a piezotolerant archaeon, lost cellular viability, then this technique could be advantageously used for selectively isolating piezophiles or obligate piezophiles from samples collected from deep-sea and subsurface environments that most likely contain piezotolerant microorganisms. This technique of growth and transfer at *in situ* pressures in PUSH vessels with the subsequent increase in pressure could potentially filter out piezotolerant microbes as they loose cell viability with increased pressure, leaving the piezophiles and obligate piezophiles, which can withstand the increased pressure conditions, for further enrichment and potential isolation.

4.6 Concluding remarks

Sample decompression increasingly impacted *A. fulgidus* HHP growth as the difference in pressure away from optimal growth conditions increased. For *A. fulgidus*, growth was negatively impacted from sample decompression with increasing pressures. But for an obligate piezophile, sample decompression is likely to negatively impact cell viability with decreasing pressures. Therefore it is imperative to maintain the pressure conditions of the collected samples from deep subsurface environments. These HHP results show that the PUSH vessels are capable of sampling up to ~9-10 km water depth and could maintain sample *in situ* pressures upon retrieval to the surface. Circumventing whole sample decompression upon deep-sea and subsurface increases the chances of collecting and maintaining piezophilic and obligately piezophilic microorganisms. Furthermore, the methods described here for HHP batch cultivation

in the PUSH vessels and transfer of *A. fulgidus* cells under pressure connecting two PUSH vessels together could be advantageous for sampling, enrichment and isolation of novel obligate piezophiles.

4.7 References

Allen, E. E., and Bartlett, D. H. (2002). Piezophiles: Microbial adaptation to the deep-sea environment. *Extremophiles*, *3*, 231–255.

Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R., and Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiolological Review*, *43*(2), 260–296.

Bar-On, Y. M., Phillips, R., and Milo, R. (2018). The biomass distribution on Earth. *Proceedings of the National Academy of Sciences*, *115*(25), 6506–6511.

Bartlett, D. H. (2002). Pressure effects on in vivo microbial processes. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, *1595*(1), 367–381.

Beeder, J., Nilsen, R. K., Rosnes, J. T., Torsvik, T., and Lien, T. (1994). *Archaeoglobus fulgidus* isolated from hot North Sea oil field waters. *Applied and Environmental Microbiology*, *60*(4), 1227–1231.

Bianchi, A., Garcin, J., and Tholosan, O. (1999). A high-pressure serial sampler to measure microbial activity in the deep sea. *Deep-Sea Research Part I: Oceanographic Research Papers*, *46*(12), 2129–2142.

Birrien, J. L., Zeng, X., Jebbar, M., Cambon-Bonavita, M. A., Quérellou, J., Oger, P.,… Prieur,

D. (2011). *Pyrococcus yayanosii* sp. nov., an obligate piezophilic hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. *International Journal of Systematic and Evolutionary Microbiology*, *61*(12), 2827–2831.

Blöchl, E., Rachel, R., Burggraf, S., Hafenbradl, D., Jannasch, H. W., and Stetter, K. O. (1997). *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°C. *Extremophiles : Life Under Extreme Conditions*, *1*(1), 14–21.

Cahet, G., Daumas, R., & Sibuet, M. (1990). In situ experimentation at the water/sediment interface in the deep sea: 2. Biotransformation of dissolved organic substrates by microbial communities at 2000m depth in the Bay of Biscay. *Progress in Oceanography*, *24*(1–4), 169–178. 10.1016/0079-6611(90)90028-Z

Cario, A., Jebbar, M., Thiel, A., Kervarec, N., and Oger, P. M. (2016). Molecular chaperone accumulation as a function of stress evidences adaptation to high hydrostatic pressure in the piezophilic archaeon *Thermococcus barophilus*. *Scientific Reports*, *6*(April), 29483.

Deming, J. W., Tabor, P. S., and Colwell, R. R. (1981). Barophilic growth of bacteria from intestinal tracts of deep-sea invertebrates. *Microbial Ecology*, *7*(1), 85–94.

Deming, J. W., Somers, L. K., Straube, W. L., Swartz, D. G., & Macdonell, M. T. (1988). Isolation of an obligately barophilic bacterium and description of a new genus, Colwellia gen. nov. *Systematic and Applied Microbiology*, *10*(2), 152–160. 10.1016/S0723-2020(88)80030-4

D'Hondt, S. (2004). Distributions of microbial activities in deep subseafloor sediments. *Science*,

306(5705), 2216–2221. 10.1126/science.1101155

Fardeau, M.-L., Goulhen, F., Bruschi, M., Khelifi, N., Cayol, J.-L., Ignatiadis, I.,… Ollivier, B. (2009). *Archaeoglobus fulgidus* and *Thermotoga efii*, thermophilic isolates from deep geothermal water of the paris basin. *Geomicrobiology Journal*, *26*(2), 119–130. 10.1080/01490450802674970

Foustoukos, D. I., and Pérez-Rodríguez, I. (2015). A continuous culture system for assessing microbial activities in the piezosphere. *Applied and Environmental Microbiology*, *81*(19), 6850–6856.

Grossart, H. P., & Gust, G. (2009). Hydrostatic pressure affects physiology and community structure of marine bacteria during settling to 4000 m: An experimental approach. *Marine Ecology Progress Series*, *390*, 97–104. 10.3354/meps08201

Hartzell, P., & Reed, D. W. (2006). The genus *Archaeoglobus*. The *Prokaryotes*, *3*, 82–100. 10.1007/0-387-30747-8

Hei, D. J., and Clark, D. S. (1994). Pressure stabilization of proteins from extreme thermophiles. *Applied and Environmental Microbiology*, *60*(3), 932–939.

Huber, R., Woese, C. R., Langworthy, T. A., Fricke, H., & Stetter, K. O. (1989). *Thermosipho africanus* gen. nov., represents a new genus of thermophilic eubacteria within the "Thermotogales." *Systematic and Applied Microbiology*, *12*(1), 32–37. 10.1016/S0723-2020(89)80037-2

Jannasch, H. J., Wirsen, C., & Taylor, C. D. (1976). Undecompressed microbial populations from the deep sea. *Applied and Environmental Microbiology*, *32*(3), 360–367.

Jannasch, H. W., and Wirsen, C. O. (1977). Retrieval of concentrated and undecompressed microbial populations from the deep sea. *Applied and Environmental Microbiology*, *33*(3), 642–646.

Jannasch, H. W., and Wirsen, C. O. (1984). Variability of pressure adaptation in deep sea bacteria. *Archives of Microbiology*, *139*(4), 281–288.

Jebbar, M., Franzetti, B., Girard, E., and Oger, P. (2015). Microbial diversity and adaptation to high hydrostatic pressure in deep-sea hydrothermal vents prokaryotes. *Extremophiles*, *19*(4), 721–740.

Kallmeyer, J., Pockalny, R., Adhikari, R. R., Smith, D. C., and D'Hondt, S. (2012). Global distribution of microbial abundance and biomass in subseafloor sediment. *Proceedings of the National Academy of Sciences*, *109*(40), 16213–16216.

Kato, C., Li, L., Nakamura, Y., Nogi, Y., Tamaoka, J., and Horikoshi, K. (1998). Properties of hyper-barophilic bacteria isolated from the mariana trench at a depth of 11,000m. *the Review of High Pressure Science and Technology*, *7*(4), 1274–1276.

Kato, C., Nogi, Y., & Arakawa, S. (2008). Isolation, cultivation, and diversity of deep-sea piezophiles. In C. Michiels, D. H. Bartlett, & A. Aertsen (Eds.), *High-pressure microbiology* (pp. 203–218). Washington, DC: ASM Press.

L'Haridon, S., Reysenbach, A.-L., Glenat, R., Prieur, D., & Jeanthon, C. (1995). Hot subterranean bioshphere in a continental oil reservoir. *Nature, 377,* 223-224.

Nakagawa, S., Takai, K., Inagaki, F., Chiba, H., Ishibashi, J. I., Kataoka, S.,… Sato, Y. (2005). Variability in microbial community and venting chemistry in a sediment-hosted backarc

hydrothermal system: Impacts of subseafloor phase-separation. *FEMS Microbiology Ecology*, *54*(1), 141–155. 10.1016/j.femsec.2005.03.007

Nercessian, O., Reysenbach, A. L., Prieur, D., and Jeanthon, C. (2003). Archaeal diversity associated with in situ samplers deployed on hydrothermal vents on the East Pacific Rise. *Environmental Microbiology*, *5*(6), 492–502.

Nogi, Y., Hosoya, S., Kato, C., & Horikoshi, K. (2004). *Colwellia piezophila* sp. nov., a novel piezophilic species from deep-sea sediments of the Japan Trench. *International Journal of Systematic and Evolutionary Microbiology*, *54*(5), 1627–1631. 10.1099/ijs.0.03049-0

Park, C. B., and Clark, D. S. (2002). Rupture of the cell envelope by decompression of the deep-sea methanogen *Methanococcus jannaschii*. *Applied and Environmental Microbiology*, *68*(3), 1458–1463.

Parkes, R. J., Cragg, B., Roussel, E., Webster, G., Weightman, A., and Sass, H. (2014). A review of prokaryotic populations and processes in sub-seafloor sediments, including biosphere: Geosphere interactions. *Marine Geology*, *352*, 409–425.

Peoples, L. M., & Bartlett, D. H. (2017). Ecogenomics of deep-ocean microbial bathytypes. In *Microbial ecology of extreme environments* (pp. 7–50). San Diego, California: Springer.

Picard, A., and Daniel, I. (2013). Pressure as an environmental parameter for microbial life - A review. *Biophysical Chemistry*, *183*, 30–41.

Schrenk, M. O., Kelley, D. S., Delaney, J. R., and Baross, J. A. (2003). Incidence and diversity of microorganisms within the walls of an active deep-sea sulfide chimney. *Applied and Environmental Microbiology*, *69*(6), 3580–3592.

Simonato, F., Campanaro, S., Lauro, F. M., Vezzi, A., D'Angelo, M., Vitulo, N.,…Bartlett, D. H. (2006). Piezophilic adaptation: a genomic point of view. *Journal of Biotechnology*, *126*(1), 11–25. 10.1016/j.jbiotec.2006.03.038

Somero, G. N. (1992). Adaptations to high hydrostatic pressure. *Annual Review in Physiology*, *54*(1), 557–577.

Stetter, K. O. (1988). *Archaeoglobus fulgidus* gen. nov., sp. nov.: a new taxon of extremely thermophilic archaebacteria. *Systematic and Applied Microbiology*, *10*(2), 172–173. 8

Stetter, K. O., Lauerer, G., Thomm, M., and Neuner A. (1987). Isolation of extremely thermophilic sulfate reducers: evidence for a novel branch of archaebacteria. *Science*, *236*(4803), 822–824.

Stetter, K. O., Huber, R., Blöchl, E., Kurr, M., Eden, R. D., Fielder, M.,… Vance, I. (1993). Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. *Nature*, *365*(6448), 743–745. 10.1038/365743a0

Tabor, P. S., and R. R. Colwell. (1976). Initial investigations with a deep ocean in situ sampler. *Proceedings of the MTS/IEEE OCEANS '76*, Washington, D.C., 13D-1–13D-4.

Tamburini, C., Garcin, J., & Bianchi, A. (2003). Role of deep-sea bacteria in organic matter mineralization and adaptation to hydrostatic pressure conditions in the NW Mediterranean Sea. *Aquatic Microbial Ecology, 32*, 209–218.

Whitman, W. B., Coleman, D. C., and Wiebe, W. J. (1998). Prokaryotes: The unseen majority. *Proceedings of the National Academy of Sciences*, *95*(12), 6578–6583.

Yayanos, A. A. (1977). Simply actuated closure for a pressure vessel: Design for use to trap

deep-sea animals. *Review of Scientific Instruments*, *48*(7), 786–789. 10.1063/1.1135150

Yayanos, A. A. (1978). Recovery and maintenance of live amphipods at a pressure of 580 bars from an ocean depth of 5700 meters. *Science*, *200*(4345), 1056–1059.

Yayanos, A. A., Dietz, A. S., & Van Boxtel, R. (1979). Isolation of a deep-sea barophilic bacterium and some of its growth characteristics. *Science*, *205*(4408), 808–810. 10.1126/science.205.4408.808

Yayanos, A., Dietz, A. S., & Boxtel, R. V. (1981). Obligately barophilic bacterium from the Mariana Trench. *Proceedings of the National Academy of Sciences of the United States of America*, *78*(8), 5212–5215.

Yayanos, A. A., and Dietz, A. S. (1983). Death of a hadal deep-sea bacterium after decompression. *Science*, *220*(4596), 497–498.

Yayanos, A. A. (1995). Microbiology to 10,500 meters in the deep sea. *Annual Review Of Microbiology*, *49*, 777–805. 10.1146/annurev.micro.49.1.777

Yayanos, A. A. (2001). 30 Deep-sea piezophilic bacteria. *Methods in Microbiology*, *30*, 615–637.

Zeng, X., Birrien, J.-L., Fouquet, Y., Cherkashov, G., Jebbar, M., Querellou, J.,… Prieur, D. (2009). Pyrococcus CH1, an obligate piezophilic hyperthermophile: extending the upper pressure-temperature limits for life. *The ISME Journal*, *3*(7), 873–876.

Zhang, Y., Li, X., Xiao, X., and Bartlett, D. H. (2015). Current developments in marine microbiology: High-pressure biotechnology and the genetic engineering of piezophiles. *Current Opinion in Biotechnology*, *33*, 157–164.

APPENDICES

1S. Affects of decompression on *A. fulgidus* HHP growth

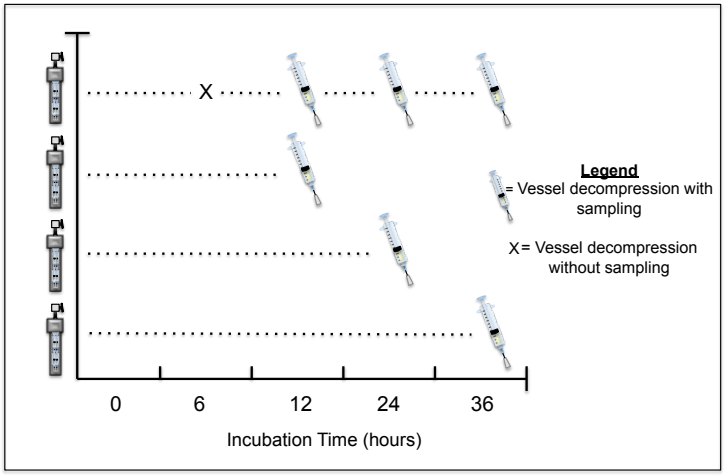

Figure 1S. Experimental setup for the suite of experiments performed to test if sample decompression affected growth cell densities.

To measure if sample decompression affected observed *A. fulgidus* growth, cell densities from cells pressurized and decompressed once were compared to cell densities from cells decompressed and repressurized multiple times. The experimental design made use of four static pressure vessels. These four static pressure vessels were filled with samples, inoculated from the same triplicate precultures, and pressurized at the same time. One vessel was decompressed without sampling after six hours, and repressurized. This vessel was then decompressed with sampling at 12 hours, 24 hours and 36 hours after inoculation for 10 MPa - 60 MPa growth pressures. The second vessel was decompressed once after 12 hours of growth and cell densities were compared to cells grown in the first vessel after 12 hours of growth. Next, the third was

decompressed after 24 hours and the fourth vessel was decompressed after 36 hours respectively and again cell densities were compared to those in the first vessel that experiences multiple cycles of decompression and repressurization.

2S. *A. fulgidus* metabolic adaptation procedure

A. fulgidus was successfully adapted to grow from a heterotrophic metabolism to an autotrophic metabolism through a series of growth experiments. First, *A. fulgidus* VC-16 was received from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and grown in a lactate and sulfate rich medium supplemented with yeast extract. The first adaption step was to decrease the amount of yeast extract from 2 g/L to 1 g/L in the growth medium and supplement *A. fulgidus* growth with 1% (v/v) vitamin solution (DSMZ medium 141). *A. fulgidus* was successfully grown three consecutive times and following growth of the third time, was transferred into medium with 0.5 g/L yeast extract and 2% (v/v) vitamin solution. *A. fulgidus* was successfully grown in medium with less and less yeast extract with at most 2% (v/v) vitamin solution until high cell density growth was observed without the addition of yeast extract. Next, *A. fulgidus* was adapted to grow without the vitamin solution in lactate and sulfate rich medium only.

A. fulgidus cells adapted to grow in the lactate and sulfate rich medium without a supplement were used to further adapt this strain to autotrophic growth with H_2 as an electron donor and thiosulfate as an electron acceptor, while fixing carbon from CO_2 into its biomass. Using consecutive transfers of cells, *A. fulgidus* was first inoculated into medium described in section 3.3.1.3 following the same procedures described in that section with the only amendment

being the addition of 2% (v/v) vitamin solution (DSMZ medium 141). *A. fulgidus* was successfully grown autotrophically and after three consecutive transfers in the same medium with vitamin supplementation, it was transferred into medium without vitamins. Finally, *A. fulgidus* was successfully grown autotrophically without yeast extract or vitamin supplementation. Standard cellular stocks were made with these autotrophic adapted *A. fulgidus* strain following methods described in section 2.3.7.2. *A. fulgidus* autotrophic HHP growth experiment pre-cultures were prepared from these standard cellular stocks.

3S. Cell elongation and deformation at HHP

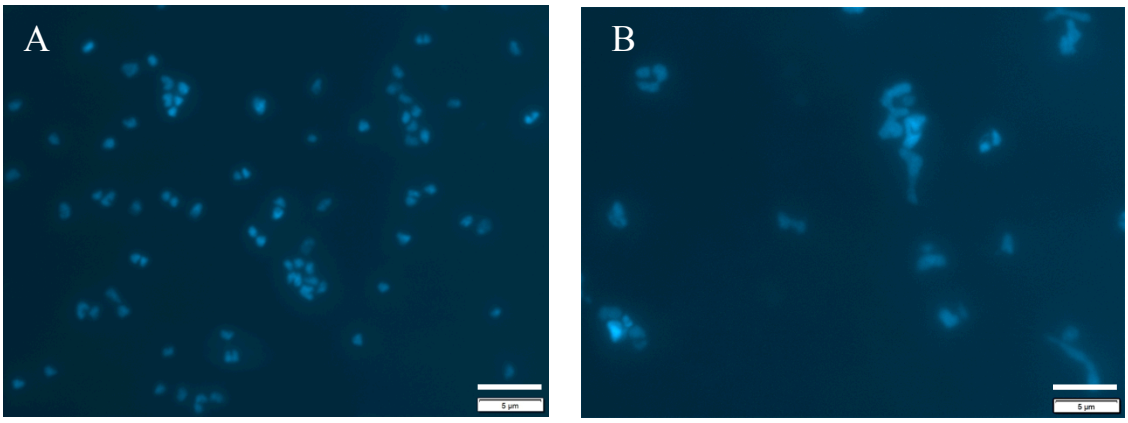

Figure 2S. DAPI stained heterotrophic *A. fulgidus* cells after 24 hours of growth at 0.1 MPa (A) in Balch tubes and at 50 MPa (B) in syringes. Dilution factors for were 20x (A) and 4x (B). Bar 5 µm.

A. fulgidus cells are normally coccus but at growth pressures above 40 MPa, cell morphology was noticeably different. Cells became irregular and elongated at 50 MPa and 60 MPa. These pressures could be impairing specific membrane proteins responsible for cell division but further investigation is needed to support this hypothesis.

4S. Affects of decompression on *A. fulgidus* HHP growth

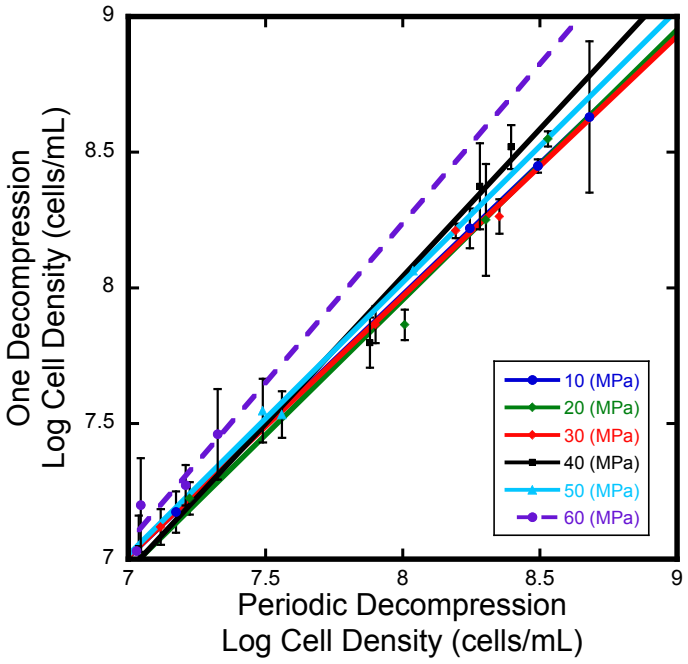

Figure 3S. One to one cell density ratios of *A. fulgidus* cells depressurized once versus cells decompressed multiple times for pressures from 10MPa to 60MPa. Error bars are deviations from the average of triplicate experiments. Low growth yields at 60 MPa contribute to a low R value and therefore difficult to discern any affects from subsampling decompression.

Since traditional high-pressure batch cultivation of microbial species usually requires short periods of decompression and cooling for thermophiles when subsampling (Park and Clark, 2002), a suite of experiments were conducted in order to test if these methods affected the observed growth yields. Repeated subsampling decompressions did not impact exponential growth from 10-50 MPa, with 1:1 cell density ratios ≈ 1 (Figure 2). Slightly higher growth densities at 10 MPa - 30 MPa for cells decompressed multiple times versus one decompression might suggest that recompression may have a positive impact on growth (Yayanos, 1995). Decompression may have had a negative impact on cells decompressed multiple times at 60 MPa since maximum cell densities at 60 MPa for cells decompressed once were $3.02 \pm 0.10 \times 10^7$

cells/mL, whereas maximum cell densities of cells decompressed multiple times were 2.15 ± 0.59 x 10^7 cells/mL. However, decompression affects at 60 MPa are hard to constrain here due to low cell densities. Nevertheless, these results have shown *A. fulgidus'* capability to grow up to 50 MPa with strong cellular viability unaffected by periodic decompressions and pressure tolerance at 60 MPa with subsampling decompressions. Additional observations after decompression during and past stationary phase included visible gas bubble formation in *A. fulgidus* cultures grown in syringes with HS-M and LS-M. Larger gas bubbles were observed in cultures grown in HS-M, but additional quantitative analyses need to be performed to measure gas exsolution after decompression.

5S. Trace metal concentrations for biofilm formation

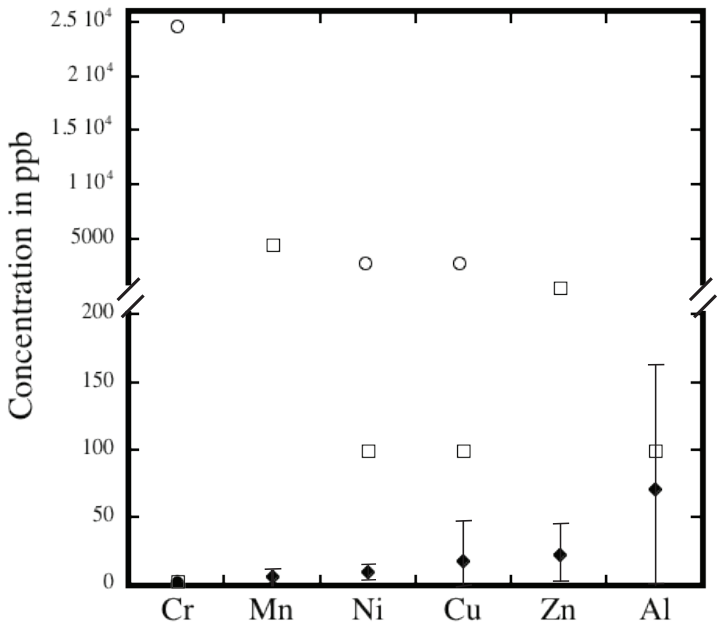

Figure 4S. The average Cr, Mn, Ni, Cu, Zn, and Al concentrations measured from fluids containing stainless steel needles in ultrapure water (18.2 MΩ) and in a sulfide solution (filled diamonds) with a 2σ standard deviation, open squares are the concentrations of these trace metals added to the growth medium (Hartzell and Reed, 1999), and the concentrations of Cr, Ni, and Cu (open circles) that previously induced *A. fulgidus* biofilm (LaPaglia and Hartzell, 1997).

Previously, *A. fulgidus* biofilm had been induced by various environmental stresses (LaPaglia and Hartzell, 1997). They found that high concentrations of Cr (≥ 25,000 ppb), Ni (≥ 3000 ppb), and Cu (≥ 3000 ppb) had induced biofilm. In this study, a stainless steel needle was attached to the syringes used for HHP cultivation and it was hypothesized that, in the presence of high sulfide concentrations, trace metals were leaching into the growth medium and inducing biofilm production. To test this hypothesis, 10 stainless steel needles were incubated in ultrapure water (18.2 MΩ) and a sulfide solution for 24 hours at 83°C. Figure 4S above shows low measured concentrations of the trace metals of interest that are far below the concentrations that were previously found to induce *A. fulgidus* biofilm (LaPaglia and Hartzell, 1997), and these

measured estimates are far below the concentrations already added into the growth medium from the trace element solution (Hartzell and Reed, 1999). Due to the method described in 3.3.3.2 section, these are estimates of the maximum concentrations that would possibly be coming from the needle in the HHP experiments. It is possible that there were unaccountable biases in these measurements and additional experiments would be needed if more precise concentrations were desired. Since the goal was to gather whether or not high concentrations, comparable to those found in LaPaglia and Hartzell, 1997, were coming from the stainless steal needle these estimations serve as a good approximation.

6S. *A. fulgidus* growth in DSMZ medium

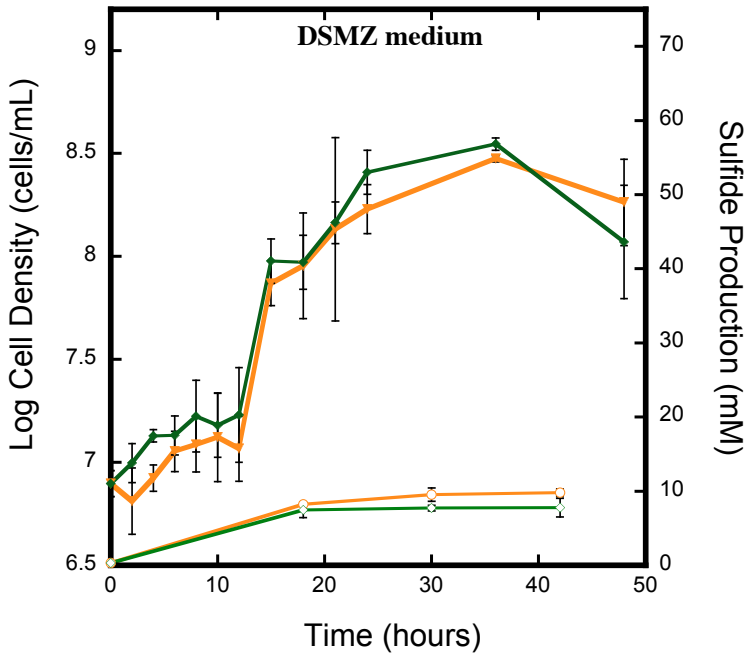

Figure 5S. *A. fulgidus* growth curves (closed squares and diamonds) and sulfide production (open squares and diamonds) from growth at 0.1 MPa in a Balch tube with a 0.3 MPa N_2 headspace (orange) and at 20 MPa in syringes (green) in a second lactate and sulfate rich medium following the DSMZ recipe (see below).

A. fulgidus growth was tested in a second lactate and sulfate rich medium to test for biofilm production in syringes at 20 MPa. The major differences in the growth medium following the DSMZ recipe was the lower amounts of lactate and sulfate (see recipe below). We initially tested this second medium to see if *A. fulgidus* biofilm was induced by pressure. Biofilm was never observed in this medium, likely because it contained 0.95 mM $CaCl_2 \cdot 2H_2O$ (see figure 3.9).

Table 1S. STL medium (section 3.3.1.1) and DSMZ medium recipes.

STL (per liter)		DSMZ (per liter)	
KCl	0.34 g	KCl	0.34 g
$MgSO_2$•$7H_2O$	15.142 g	$MgSO_2 \cdot 7H_2O$	3.45 g
$MgCl \cdot 6H_2O$	2.75 g	$MgCl \cdot 6H_2O$	4.00 g
NH_2Cl	0.25 g	NH_2Cl	0.25 g
$CaCl_2 \cdot 2H_2O$	0.05 g	$CaCl_2 \cdot 2H_2O$	0.14 g
K_2HPO	0.137 g	K_2HPO	0.14 g
NaCl	17.8 g	NaCl	18.01 g
$Fe(NH_4)_2 (SO_4) \cdot 6H_2O$	0.0392 g	$Fe(NH_4)_2 (SO_4) \cdot 6H_2O$	0.002 g
sodium lactate	2.1 g	sodium lactate	1.5 g
yeast extract	1 g	yeast extract	1 g
PIPES	3.36 g	PIPES	3.36 g
Resazurin (0.1% solution)	0.1 mL	Resazurin (0.1% solution)	0.1 mL g
$Na_2EDTA \cdot 2H_2O$	77.9 µM	Nitrilotriacetic acid	78.47 µM
$MnSO_4 \cdot H_2O$	29.583 µM	$MnCl \cdot 4H_2O$	25.265 µM
$CoCl_2 \cdot 6H_2O$	7.5652µM	$CoSO_4 \cdot 7H_2O$	6.3884 µM
$ZnSO_4 \cdot 7H_2O$	3.4777 µM	$ZnCl_2$	6.3103 µM
$NiCl \cdot 6H_2O$	0.462 µM	$NiCl \cdot 6H_2O$	1.262 µM
$CuSO_4 \cdot 5H_2O$	0.4 µM	$CuCl_2 \cdot 2H_2O$	0.3 µM
H_3BO_3	1.6173 µM	H_3BO_3	1.6173 µM
$KAl(SO_4)_2 \cdot 12H_2O$	0.21 µM	$KAl(SO_4)_2 \cdot 12H_2O$	0.42 µM
$Na_2MoO_4 \cdot 2H_2O$	0.413 µM	$Na_2MoO_4 \cdot 2H_2O$	0.413 µM
$NaSeO_3$	0.33 µM	$NaSeO_3$	0.133 µM
$NaWO_4 \cdot 2H_2O$	0.11 µM	$NaWO_4 \cdot 2H_2O$	0.44 µM
V_2O_5	0.439 µM	$FeSO_4 \cdot 7H_2O$	0.359 µM