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Impacts of high hydrostatic pressure on the activity of hydrocarbon-degrading bacteria

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by

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ABSTRACT OF THE THESIS

Impacts of high hydrostatic pressure on the activity of hydrocarbon-degrading bacteria

by

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Deepwater Horizon oil spill was the world's largest marine oil spills in history. This spill was unique in that a large proportion of the hydrocarbon was released into the deep ocean. Microbial communities present in the Gulf of Mexico rapidly responded to the oil spill. In order to understand the process of hydrocarbon degradation by deep-sea microbes, it is important to

determine when and under which conditions they are metabolically active. Here we report on the impacts of high pressure on the microbial growth and protein synthesis activity of hydrocarbon-degrading microbes isolated from the Gulf of Mexico, which included *Halomonas titanicae*, *Shewanella indicae* and *Alcanivorax xenomutans*. Bioorthogonal noncanonical amino acid tagging (BONCAT) is a method that uses a methionine analog to fluorescently tag cells undergoing active protein synthesis. We use BONCAT to follow the activity of microbes under atmospheric and high pressure. Actively growing cells that take up these methionine analogs are subsequently fluorescently tagged using click chemistry, and assessed by flow cytometry. The results showed that all three stains grown best in hexadecane with corexit. High hydrostatic pressure (10 and 25 MPa) inhibited the aerobic growth of *A. xenomutans*, while didn't show clear impacts on the growth of *H. titanicae* and *S. indica*. BONCAT results demonstrated that high hydrostatic pressures had negative impacts on the metabolic activity of *A. xenomutans* and *H. titanicae,* while did not show significant effects on the protein synthesis of *S. indica*. In general, the effects of high pressure on the microbial activity are species specific.

INTRODUCTION

Deepwater Horizon Oil Spill

On April 20, 2010, high-pressure oil and gas escaped from BP's Deepwater Horizon (DWH) exploratory well in Mississippi Canyon Block 252 (MC252), which was located 77 km offshore. After the explosions and fire, the Deepwater Horizon drilling rig burned and ultimately sank in 1500 m of water on April 22nd, leading to an uncontrolled release of oil and gas into the deep waters (Atlas and Hazen, 2011). By the time the well was capped in mid-July, over 210 million gallons of oil and some 250,000 metric tons of natural gases had been released into the Gulf (Joye, 2015), and more than 2 million gallon of chemical dispersants were added as a response treatment (Lubchenco *et al*., 2012). In addition to dispersants, other tools like controlled burns, skimming, siphoning from the well-head, and beach sand mixing were used broadly as well (Atlas and Hazen, 2011). As a result, a plume of dissolved and dispersed hydrocarbons was formed in the deep sea. This event was the largest and deepest marine oil spill in the history (The Federal Interagency Solution Group, 2010).

The impacts of hydrocarbon exposure to the Gulf's biological community were extensive, reaching from water column to the seafloor. Fernandez *et al*. (2016) provided direct evidence for incorporation of methane and petroleum into the suspended particulate organic materials at the 1000-1200m depth in the hydrocarbon plume, using stable isotopes and radiocarbon. There was also evidence showing that petrocarbon was incorporated to fish and invertebrate tissue, based on the observation of a west-to-east gradient in tissue ^{14}C content (Wilson *et al*., 2016). These studies demonstrated that large quantities of hydrocarbon released in the DWH event were incorporated into the food web. The accumulation of large amounts of marine particles and oil on the seafloor, largely through sedimenting marine oil snow, affected the benthic ecosystems

(Montagna *et al*., 2013). Damaged and decreased deepwater coral communities were found beneath the path of a previously documented plume emanating from the Macondo well, supporting that oil impacted deep-water ecosystems (White *et al*., 2012).

The well-head was located at 1544m below sea surface (mbs). The extreme depth of the blowout made it difficult to degrade the large volumes of oil released. The primary initial mitigation strategy was to inject the oil dispersant Corexit 9500 directly at the wellhead (Joye, 2015), which increased the hydrocarbon-water interferes and the rates of microbial degradation (Atlas *et al*, 2011). Stimulated biodegradation of oil with the addition of dispersant Corexit 9500 was demonstrated in previous studies (Swannell *et al.,* 1999, Lindstrom *et al.*, 1999; Richard *et al.,* 1999). However, the effects of using dispersants are controversial. Some studies proved that Corexit 9500 was toxic to microorganisms, including microzooplankton (Almeda *et al.*, 2014) and hydrocarbon-degrading bacteria (Hamdan *et al.*, 2011). In contrast, Lindstrom *et al.* (2002) showed that Corexit 9500 could result in either increases or decreases in the toxicity of residual oil. Have a better understanding of the effects of oil dispersants Corexit 9500 leads to more research on the microbial degradation of dispersed hydrocarbons at environmental conditions.

Microbial degradation after the DWH oil spill

Oil degrading bacteria have evolved over millions of years and are ubiquitous in the marine environment (Schedler *et al.,* 2014). Substantial bacterial blooms were observed in deep water in the months following the blowout (Valentine *et al*., 2010; Hazen *et al*., 2010; Redmond and Valentine 2012). Thousands of natural gas and oil seeps are distributed in the Gulf of Mexico basin (Abbriano *et al*., 2011), priming the microbial community for oil degradation and perhaps explaining the quick response to the DWH oil spill (Fernandez *et al*., 2016).

Crude oils are some of the most complex and diverse organic mixtures found on earth, containing thousands of different hydrocarbon compounds (Overton *et al*., 2016). These compounds differ in solubility and volatility and are degraded at different rates. Many saturated hydrocarbons are easily biodegraded, while other larger ones with more substituted groups are more persistent and toxic, like polycyclic aromatic hydrocarbons (Head *et al*., 2006). The ability to degrade hydrocarbons is found many different types of bacteria, and most of them are responsible for the degradation of particular types of hydrocarbons. For instance, *Oceanospirillales* (including the genus *Alcanivorax*) are primarily alkane degraders (Hara *et al*., 2003); *Cycloclasticus* specializes in the degradation of aromatic compounds (Head *et al*., 2006); *Colwellia* is directly linked to the oxidation of ethane, propane and benzene (Redmond and Valentine 2012). Along with the hydrocarbon compounds altered, the microbial community response to the DWH oil spill changed over time with different genera dominating the microbial community. Initially, the plume was dominated by members of *Oceanospirillales* and *Pseudomonas*, which are able to consume different types of alkanes; 16 other groups of *Gamaproteobacteria* were also enriched in plume samples (Hazen *et al*., 2010). In June, the plume was dominated by two different groups of *Gamaproteobacteria*, *Colwellia* and *Cycloclasticus*, which were able to degrade aromatic hydrocarbons (Valentine *et al*., 2010). After the oil well was completely capped, these groups were much less abundant and the dominant microbial community shifted toward methylotrophs (*Methylococcaceae*, *Methylophaga*, and *Methylophilaceae*), *Flavobacteria*, *Alteromonadaceae*, and *Rhodobacteraceae*, which may act as a dynamic biofilter that responds rapidly to large-scale methane inputs into the deep ocean (Kessler *et al*., 2011).

The DWH drilling rig well was located in the deep ocean, with a temperature of 4℃ and a hydrostatic pressure of 15megapascals (MPa) (Marietou *et al*., 2018). This deep-sea environment is characterized by low temperature and high hydrostatic pressure. Temperature has been demonstrated to be a significant influence on the final microbial community compositions in response to oil (Margesin *et al*., 1997; Bargiela *et al*., 2015). Prudhoe Bay crude oil dispersed by Corexit 9500 was proven to be biodegraded more rapidly at 20°C than at 5°C (Venosa and Holder, 2007). *Colwellia* from DWH oil spill was much more abundant in crude oil enrichments at 4°C than at room temperature (Redmond and Valentine., 2012). However, the effect of high pressure on the microbial hydrocarbon degradation is still unclear, despite several studies explored the microbial community response to hydrocarbons under high pressure (Grossi *et al*., 2010; Scoma *et al.,* 2016a&b). Schedler *et al*. (2014) showed that both bacterial growth and hydrocarbon-degrading activity were inhibited under high pressure. Marietou *et al.* (2018) described the microbial response to the DWH oil spill under in situ pressure and temperature conditions, and suggested that pressure acts synergistically with low temperature to slow microbial growth and thus oil degradation in deep-sea environments. All of the studies focused on the microbial growth and hydrocarbon degradation in response to oil. This study aims to improve our understanding of the impact of high hydrostatic pressure on the protein synthesis activity of hydrocarbon-degrading microbes.

Three hydrocarbon degrading bacteria

This study focused on three species isolated from DHW oil spill in the Gulf of Mexico: Bead 18 (related to *Alcanivorax xenomutans*), Bead 36 (related to *Shewanella indica*) and Bead 10BA (related to *Halomonas titanicae*) (Table 1).

The *Alcanivorax* genus was firstly described by Yakimov and coworkers, who isolated and proposed *A. borkumensis* SK2 as the type strain (Yakimov *et al*., 2007). Later on, this ubiquitous genus was recognized to be dominant in oil-contaminated marine waters all over the world (Head *et al*., 2006). Three *Alcanivorax* species isolated from 10,400m water in Mariana Trench are demonstrated to be able to efficiently degrade hexadecane under conditions simulating the deep sea (Liu *et al*., 2019). *A. xenomutans* was first isolated from a sediment sample collected from a shrimp cultivation pond in Tamil Nadu (India), and the cells were Gram-negative, motile rods (Rahul *et al*., 2014). In 2018, Fu *et al.* published a complete genome of *A. xenomutans* P40, which was isolated from deep seawater. They demonstrated that the genes of *A. xenomutans* P40 involved in alkane degradation, heavy-metal resistance, stress response and so on, indicating its potential use in the bioremediation of oil polluted and heavy metal-contaminated environments (Fu *et al*., 2018).

Shewanella is the sole genus included in the marine bacteria family *Shewanellaceae*. Members of this genus have been described from diverse habitats, including deep cold-water marine environments, shallow Antarctic Ocean habitats, hydrothermal vents and freshwater lakes (Dikow, 2011). They have the capacity to use many compounds as terminal electron acceptors, which makes them distinguished in anaerobic respiration (Serres and Riley, 2006). In addition, their ability to reduce metals and metal oxides in the environment makes it possible that *Shewanellae* could serve as decontaminating agents in the river and potable groundwater supplies (Tiedje, J.M. 2002). Iron-reducing bacteria *Shewanella putrefaciens* is reported to be ubiquitously present in oil field fluids (Semple *et al*., 1987). *Shewanella indica* KJW27T was first isolated from the marine sediments of Karwar jetty, west coast of India. The cells were Gram-negative, facultatively anaerobic, rod-shaped, catalase- and oxidase-positive bacterium,

motile by means of a single polar flagellum, but this strain was not showed to have connection with hydrocarbon degradation (Verma *et al*., 2011).

Halomonas titanicae phylogenetically belongs to the Family *Halomonadaceae* within genus *Halomonas*. The majority of this genus are aerobes, but some *Halomonas* species are able to grow anaerobically with nitrate, nitrite or fumarate as an electron acceptor (Mata *et al*., 2002). They have been isolated from many different water and soil environments, predominantly from marine, hypersaline or alkaline habitats (Sánchez-Porro *et al*., 2013). The genus *Halomonas* contains several species that are reported to be able to degrade hydrocarbons, including *Halomonas organivorans* (Garcı´a *et al*., 2004), *Halomonas shengliensis* (Wang *et al*., 2007a), *Halomonas gudaonensis* (Wang *et al*., 2007b), and *Halomonas daqingensis* (Wu *et al*., 2009). *Halomonas titanicae* BH1 was first isolated from a sample of rusticle obtained from the RMS titanic in 1991. They found the cells are gram-negative, heterotrophic, aerobic, halophilic, non-endospore-forming, peritrichously flagellated and motile (Sánchez-Porro *et al*., 2010). In 2013, Sánchez-Porro *et al.* reported the genome of this species, which showed properties related to solute and ion transport, with genes putatively encoding solute-binding proteins, 18 sodium/solute symporters and transporters and osmolyte-related genes (Sánchez-Porro *et al*., 2013).

Methods that used to detect the active microbes

In order to understand the environmental processes, the distinction between active, inactive and dead microbial cells is essential. Until now, several different tools have been used in detecting microbial activity targeting a variety of physiological processes, like DNA-SIP, BrdU labeling, Metatranscriptomics and Raman microspectroscopy (Singer *et al*., 2017).

The first method that yields activity-labeled samples take advantage of incorporation of bromodeoxyiridine (BrdU) into newly synthesized DNA of a viable cell (Urbach *et al*., 1999). This method has been used in different studies of bacterial and archaeal (Borneman, 1999; Yin *et al*., 2000), as well as fungal (Urbach *et al*., 1999) DNA synthesis activity in response to various changing environmental conditions. However, the variability of ability and rate of BrdU incorporation among microorganisms may mispresent the active microbial population in a given environment (Singer *et al*., 2017). In 2000, stable isotope probing (SIP) labeling technology came into use. This technique was firstly demonstrated by Radajewski and co-workers, who added stable isotope ^{13}C-enriched carbon sources to soil and subsequently identifying active methylotrophs (Radajewski *et al.,*2000）. The disadvantage of this method includes long incubation time, cross-feeding problems and potential for enrichment bias. Nevertheless, DNA-SIP is still the best current method for sequence-based characterization of key populations synthesizing DNA(Singer *et al*., 2017). Metatranscriptome studies provide another perspective of microbial activity, enabling the analysis of expressed gene in environmental communities. High-throughput sequencing technology for mRNA analysis was first used in the studies of microorganisms in soil and marine environments (Leininger *et al*., 2008; Urich *et al*., 2008). Compared to other methods, metatranscriptomics is favorable because samples can be frozen immediately, thereby enabling delayed processing back at the lab. Another approach to monitor activity of microbes is to incorporate stable isotopes, such as ^{15}N, deuterium and ^{13}C into microbial biomass, then detected by secondary ion mass spectrometry (Orphan *et al*., 2009) or Raman microspectroscopy (Haider *et al*., 2010).

Another recently developed method that yield activity-labeled samples in situ is Bioorthogonal noncanonical amino acid tagging (BONCAT). This technique uses a noncanonical

amino acid analog to fluorescently tag cells undergoing active protein synthesis using click chemistry (Beatty *et al*., 2006). Rather than studying the bulk proteome, BONCAT is able to specifically target proteins that have been expressed in response to an experimental condition. This technique has been applied to a variety of research fields, from studies of proteomic and protein expression of mammalian cells (Best, 2009) to analysis of protein synthesis in native plant tissues (Glenn *et al*., 2017). There are a serious of biorthogonal amino acids that have been shown to successfully compete with native amino acids, but only a small subset of them is able to exploit the substrate promiscuity of the native translational machinery without the need for genetic modification of the host cell (Ngo and Tirrel, 2011). The commonly used biorthogonal amino acids are L_azidohomolanine (AHA) and homopropargylglycine (HPG), both of which are surrogates of L-methionine (Met). Hatzenpichler *et al.* demonstrated this method using azide-bearing AHA to be effective in labeling the proteomes of bacteria and archaea (Hatzenpichler *et al*., 2014). Later on, a separate method to measure protein synthesis rates in natural planktonic microbial assemblages using the alkyne-bearing HPG was developed (Samo *et al*., 2014). These previous studies all used fluorescence microscopy to detect and quantify the fluorescently labeled active cells. Since 2016, fluorescent active cell sorting (FACS) has been coupled to BONCAT, making detection and quantification of active cells faster and more objective, and easier for subsequent sequencing of interested populations (Hatzenpichler *et al*., 2016). The new developed technique of BONCAT-FACS was successfully applied to study the active fraction of a soil microbiome (Couradeau *et al*., 2019). In this thesis, I have used BONCAT coupled with flow cytometry (FCM) to follow the activity of protein synthesis of microbes under atmospheric and high pressure. This has made it possible to address questions regarding the cellular translational activities of oil-degrading bacteria in response to high pressure.

In general, this study aims to 1) characterize the hexadecane degradation ability of the 3 selected DWH strains, 2) examine the growth properties of each strain on hexadecane, Corexit or the mixture, at a range of pressures, 3) explore the effects of high hydrostatic pressure on the translational activity of these strains grown on hexadecane.

MATERIAL AND METHODS

Bacterial strains and culture conditions

The bacterial strains used in this work are *Alcanivorax xenomutans, Shewanella indica,* and *Halomonas titanicae*, obtained from Dr. Romy Chakraborty at Lawrence Berkeley National Laboratory. They were originally isolated from the DWH oil spill (Table 1). The three isolates were cultured aerobically at room temperature in ONR7a medium (a mineral salts medium made up according to Dyksterhouse *et al*., 1995) with other carbon sources. Three carbon source treatments were tested in this study: (i) 1.45% vol/vol hexadecane (50mM, 0.2-μm-filtered), (ii) 0.03% Corexit 9500, or (iii) a 1:50 Corexit-oil mixture (hereinafter referred to as hexadecane, Corexit, and dispersed-oil treatments, respectively). The concentration of hexadecane to employ was based on the culture tests at a variety of substrate concentrations (two of the three strains, *S. indica* and H. *titanicae* were found to grow best in 50mM hexadecane). The 1: 50 ratio of crude oil and dispersant mixture is at the lower end of the range of ratios recommended by the EPA for dispersing oil. The cultures were shaken in glass bottles with at least 50% headspace to promote sufficient aeration. Bacteria cultured in pressurizable polyethylene bulbs at 0.1MPa, 10MPa and 25MPa, were supplemented with 100mM HEPEs buffer and 100mM Nitrate in addition to hexadecane and Corexit (same concentration detailed above), in order to facilitate their growth under the low oxygen conditions that develop in the absence of an air headspace. All experiments were performed with triplicate cultures.

Quantification of bacterial growth

When hexadecane was added alone into the medium, it was noted that only the water accommodated fraction and some mechanically dispersed oil droplets will reach the bacteria and

thus impact microbial communities, while the remainder floats on the medium surface. In addition, even the blank medium turned cloudy with the addition of Corexit. Because of this effect, it is not possible to accurately measure the optical densities of cell cultures growing with hexadecane and Corexit. Instead, biomass accumulation over time was measured as total cellular protein. Protein quantification utilized a modified version of growth quantification based on Overholt *et al* (2016). Cultures were grown in 100ml of ONR7a medium, and were supplemented with hexadecane, dispersed oil, or Corexit alone, as detailed above. Negative controls included an uninoculated control (nonbacterial control) for each substrate and an inoculated control with no added carbon source but containing bacterial inoculum in the same volume as in the carbon treatments (noncarbon control). All treatments were performed in triplicate.

Treatment cultures (3 replicates each) were sacrificed at each time point. 5ml of culture medium was added to a 15-ml Falcon tube, which was centrifuged at 3,200g for 20 min. A preliminary protein assay test on both 5ml and 10ml of samples showed that 5ml presented the same trend of growth curve as 10ml, and thus the smaller volume was used in this study to lessen disturbance to the remaining cultures. After centrifugation, the supernatant was removed without disturbing the cell pellet, and the samples were stored at -20 °C until further analysis. Total cellular protein was extracted using 1ml of 2% SDS lysis buffer (50mMTris-HCl buffer with 2% [wt/vol] SDS) followed by room temperature incubation for 20 min. Samples were sonicated (Fisher Scientific Sonic Dismembrator model 550, amplitude of 4) for 15 s total (half a second on, half a second off). The samples were then centrifuged at 3,200g for another 20 min. Total cellular protein was quantified by following the Pierce bicinchoninic acid protein assay protocol according to the instructions(Life Technologies, Grand Island, NY).

Quantification of the Hydrocarbon biodegradation

During the growth of the cultures, the remaining hexadecane was extracted with dichloromethane and determined using gas chromatography mass spectrometry (GC-MS) according to the method described by Marietou *et al*.(2018) with the following modifications. 0.5 ml samples were extracted with 5 ml dichloromethane by vigorous mixing at room temperature for 30 min. The mixture was allowed to sit for 2 h and then 0.5 ml of the solvent-oil phase (bottom layer) was transferred to a new glass vial where another 2 ml of dichloromethane were added. Then the new mixture was shaken for 30 min and allowed to sit for 2 h. The solvent-oil phase was transferred to a clean vial where anhydrous sodium sulfate was added and allowed to stand for 30 min at room temperature to remove any residual water. Finally, the sample was collected and stored at -20 ºC till further processing. As a control the same concentration of hexadecane oil (1.45% v/v) in ONR7a medium with no bacteria added was used. Experiments were done in the dark to prevent hydrocarbon photooxidation (Widdel F. 2010). An Agilent 5977B Gas Chromatograph-Mass Selective Detector (GC-MSD) instrument was used for GC-MS analysis. The GC was equipped with an Agilent DB-5MS UI column (30 m length, 0.25mm internal diameter). All analyses were carried out with the split ratio of 10:1. Helium was used as the carrier gas with a flow rate of 1.2 ml/min. The injection temperature was 250 ºC. The oven temperature program was as follows: an initial temperature of 60 ºC was increased to a final temperature of 270 ºC at a rate of 15 ºC /min, and hold for 2 minutes at 270 ºC, with a final 2min hold at 280 ºC. The mass spectrometer transfer line temperature was held at 250 ºC and the ion source temperature at 230 ºC (Table 2).

Quantification of active cells fraction using BONCAT

BONCAT was performed following the established protocol of Hatzenpichler *et al*.(2016) with following modifications. The cultures were initially set up in 10ml sealed serum glass vials for three strains under atmosphere pressure and high pressures, respectively, to promote growth. When they reached the mid-log phase, as determined by prior protein assay-based growth curve analysis, HPG was added. Specifically, 1 µl HPG (50mM) was added into the 10 ml enriched cultures, and inverted gently to mix (final concentration of HPG is 5µM). The vials were then labeled, and re-incubated at the desired pressures. Samples (10 ml) were withdrawn as a function of time, fixed with 8.8 µl paraformaldehyde to a final concentration of 3% (15 minutes at room temperature, in the dark). Then the cells were pelleted via centrifugation (16, 900 g or max, for 5mins at RT), supernatants removed, and the cell pellets resuspend in 100 µl PBS. This cell suspension was pelleted as above to withdrawn any remaining paraformaldehyde, resuspended in 100µl 1:1 PBS: EtOH, and stored at -20 ^{o}C until further treatment. For click chemistry, the samples were thawed and pelleted as above, followed by removal of the supernatant and resuspension in 100 µL PBS. The "click cocktail" was always freshly mixed according to the instructions of the click kit (Click-iT HPG Alexa Flour Protein Synthesis Assay Kits, Life Technologies). One hundred microliters of this cocktail solution was added into each sample, and incubate in the dark at room temperature for 30 minutes. After click chemistry, the cells were washed with 100 µl rinse buffer and 3% BSA respectively, via centrifugation (16, 900 g or max, for 5mins at RT). Finally, the cells with resuspended in 500 µl PBS, and DNA stain was added. In particular, Hoechst 33342 was used in this study, diluted to final concentration of 10 µg/ml. The stained cells with subsequently examined with flow cytometry.

Flow Cytometry

Samples were run on a ZE5 Cell Analyzer (BioRad) equipped with the small-particle detection module. DNA stain Hoechst 33342 (excitation = 361 nm, emission = 497 nm) was excited off the 355nm laser (50mW) and fluorescence was collected through a 447/60 nm band-pass filter, while the Alexa Flour 488 azide dye (excitation = 490 nm/ emission = 525 nm) was excited off the 488 nm laser (100mW) and fluorescence collected through a 523/30 nm band-pass filter. Sample delivery was by a calibrated peristaltic pump allowing for precise measurement of absolute counts. The PBS buffer used for the final sample resuspension was used as a negative control to exclude background particles. The first gate was drawn on the Hoechst positive (Hoechst 33342+) particles, under the assumption that this would capture the cells (Fig. 3). Hoechst 33342+ events accounted for >95 % of the events depending on the samples. The BONCAT positive (BONCAT +) and BONCAT negative (BONCAT -) where further gated as a subfraction of the Hoechst 33342+ cells based on the Alexa Fluor 488 azide dye fluorescence. The samples that were not incubated with HPG were used as negative controls to define the level of nonspecific BONCAT stain fluorescence, the BONCAT- gate was drawn under that line and BONCAT + gate was such that <1% of negative control cells were in it. The fraction of BONCAT + cells was determined for a time course for both 0.1MPa, 10 MPa and 25 MPa. Flow cytometry data were analyzed using the program FlowJo (FlowJo LLC, BD).

RESULTS

Bacteria Aerobic Growth on oil and dispersant

Bacterial aerobic growth assay for *Alcanivorax xenomutans*, *Halomonas titanicae*, and *Shewanella indica*, hereinafter referred to as *Alcanivorax*, *Halomonas* and *Shewanella*, respectively, were conducted using the five treatments detailed above, which includes hexadecane, Corexit, dispersed oil, nonbacterial control and noncarbon control. In all assays, negative controls showed no growth, and thus are not shown in the growth curves. Overall, *Alcanivorax* demonstrated higher growth rates and a higher maximum biomass accumulation than other two strains.

Initially, *Alcanivorax* showed higher growth yields when grown on dispersed oil. The maximum growth yields under this treatment presented after 2days, and the total protein was 220.94 +39.34 μg/ml. Subsequently, the hexadecane treatment reached its maximum growth yields, which was 225.33 + 47.33 μg/ml after 7 days. Compared to growth curve of dispersed oil treatment, a lag was observed in the hexadecane treatment (Fig. 4A). Significant growth of *Alcanivorax* was observed with Corexit as the sole carbon source (maximum 116.06+15.97 μg/ml total protein). Overholt *et al* did similar assays on *Alcanivorax sp.* P2S70, and found that strain showed similar maximum growth yields when grown on dispersed oil and on crude oil, and they reached maximum growth yields after approximately 2 days (Overholt *et al*., 2016). Their growth on Corexit alone showed lower than that of this study. But it needs to be noted that they are using MC252 crude oil, while we only use hexadecane as the carbon source. In addition, the strain used in the study is not exactly same.

Halomonas demonstrated maximum growth rates and biomass accumulation when it was grown on dispersed oil (3 day-point time, 133.13 + 13.98 μg/ml) (Fig. 4B). It produced 41% less

protein than dispersed oil and had a lower growth rate when hexadecane served as the sole carbon source. *Halomonas* was also capable of growth on Corexit alone (maximum reached after 5 days, 98.97+14.44 μg/ml). And it showed higher growth yields on Corexit alone than hexadecane alone. Although *Halomonas* only produced 60.18% growth yields of *Alcanivorax*, both of them revealed best growth on the hexadecane and Corexit mixture.

Shewanella demonstrated a similar growth trend to the *Halomonas*. Its best growth rate and highest biomass were also in the dispersed oil treatment (71.10+6.2 μg/ml) (Fig. 4C). Compared to the growth yields on dispersed oil, 35% less protein was produced in hexadecane alone. *Shewanella* had the ability to grow on Corexit alone as well (maximum reached after 6 days, 59.53+8.65 μg/ml). It also showed higher growth yields on Corexit alone than hexadecane alone. *Shewanella* showed the least biomass accumulation among the three strains. This was consistent with its utilization of other hydrocarbons. It was noted that *Shewanella* always had the difficulty to proliferate in ONR7a medium coupled with other hydrocarbon sources, but grew well on ONR7a supplemented with glucose or peptone, which indicated it has no inherent limitation to growth in a ONR7a medium.

On the whole, all three strains grew best when supplemented with a 1:50 Corexit and hexadecane mixture.

Quantification of hexadecane degradation

Triplicates cultures of *Alcanivorax, Shewanella,* and *Halomonas* were incubated aerobically on 50 mM hexadecane at room temperature. Total hexadecane was extracted from the oil-amended treatments. Bacterial treatments were set up in triplicate.

The uninoculated control exhibited a slightly decrease after 5 days incubation (Fig. 6). The three strains showed different capabilities in oil degradation. *Alcanivorax* degraded or transformed 71% of the hexadecane relative to the level degraded in the uninoculated control treatment after two days of incubation (Fig. 5). When incubations were extended to 5 days, *Alcanivorax* transformed or removed 78% of the hexadecane. No significant change of oil degradation was observed in the *Alcanivorax* cultures between 2 and 5 days of incubation. *Shewanella* and *Halomonas* cultures only degraded or transformed 31% and 36% of hexadecane, respectively, after 2 days. However, *Halomonas* presented a 2-fold increase in oil degradation (72%) after 5 days incubation (Fig. 5). *Shewanella* cultures degraded or transformed another 23% hexadecane after 5 days. The obvious oil degradation occuring during its incubation indicated that *Shewanella* does have the capability of oil degradation, regardless of its poor growth in hexadecane or dispersed oil (Fig. 4C). The 3 time points overlapping hexadecane chromatography peaks detected by GC-MS are showed for the uninoculated control and the 3 strains (Fig. 6). These results clearly demonstrate that all strains have the ability to degrade the model alkane hydrocarbon hexadecane, and that this ability was most rapid, as expected, in the case of *Alcanivorax*.

Growth in Hexadecane/Corexit as a function of pressure

Because all three strains grown best in ONR7a medium with dispersed oil, bacterial growth as a function of pressure was conducted using the same carbon source treatment. But in addition to hexadecane and Corexit, the medium was supplemented with HEPES buffer and nitrate (which served as alternate electron acceptor). Incubations were set up under three different pressure conditions replicating the pressure at (i) the sea surface (0.1MPa), (ii) 1000

meters below surface (10MPa), (iii) 2500 meters blow surface (25 MPa). Overall, aerobic growth rate and biomass accumulation at atmosphere pressure was always better for all strains than the hypoxic growth rates associated with incubation conditions in pressurizable bulbs, regardless of the pressures used. However, this was least evident for the *Shewanella*, which exhibited the slowest growth rates and biomass levels.

The bulbs cultures of *Alcanivorax* were found to grow 2-fold better at atmosphere pressure (8 days-time point, 177.5 +20.35 μg/ml) than at high pressures (e.g., 8 days-time point, 92.33 + 7.67 ug/ml at 10MPa). Its growth behavior at 10MPa and 25MPa were both similar (Fig. 7A). Like *Alcanivorax*, *Halomonas* produced maximum protein when it was grown with certain amount of oxygen at atmosphere pressure, which was 1.5 fold more than that of the growth in sealed bulbs at the same pressure (Fig. 7C). However, high pressure didn't clearly impact its growth. The slow-growing *Shewanella* also appeared to be relatively pressure-resistant for growth in bulbs from 0.1-25 MPa (Fig. 7E).

Quantification of active cells using BONCAT

Cells pre-incubated to mid-log phase were used to be labeled with the amino acid tag HPG. Samples were harvested as a function of time along the HPG incubation period. No HPG labeled cells were set up as negative controls as well. After click chemistry, fluorescent cells counts were quantified with flow cytometry.

For *Alcanivorax and Halomonas*, it was always the active cells percentage under aerobic conditions higher than other conditions, only for the 24h time point of *Alcanivorax* was the exception (Fig. 7B&D). It needs to be noted that the cells grown in bulbs are not being grown anaerobically, although they do reduce the dissolved oxygen level in the medium as they grow. It

is speculated that initially *Alcanivorax* was not that sensitive to the hypoxia conditions in bulbs. Results with *Alcanivorax* showed that during the incubation, the fraction of active cells were higher at atmosphere pressure than that of 10MPa, and 25MPa was the lowest. (Fig. 8B). Like *Alcanivorax*, high pressure negatively impacted the activity of *Halomonas*. The difference between these two strains is that the limitation of high pressure existed significantly throughout the incubation of *Halomonas* (Fig. 8D). When it comes to *Shewanlla*, its fraction of active cells under high pressures (both 10 and 25 MPa), was similar to that of at atmosphere pressure, especially when looking at the variance (standard deviation) of data (Fig. 8F). In contrast to its less growth under hypoxia conditions, the fraction of active cells in bulbs was higher than that of aerobic conditions. Given that this strain exhibits the poor growth on hexadecane and Corexit under all growth conditions, this suggests that the fraction of BONCAT positive cells is a poor reflection of its growth ability.

DISCUSSION

Effects of oil dispersants on biodegradation

Chemical dispersants are considered to be one of the main tools in the response to oil spills. Dispersants Corexit 9500 was widely used in the BP Deepwater Horizon spill, which was used to increase the hydrocarbon-water interferes areas and the rates of biodegradation (Atlas *et al*, 2011). However, the effects of using dispersants are controversial.

In contrast to most prior studies which focused on the mixed microbial populations, we investigated how specific bacterial strains responsd to oil and dispersed oil to elucidate their impacts on bacterial growth and proteins synthesis. In this study three isolated strains from the DWH oil spill were tested.

We found that all of strains grow best on hexadecane in the presence of dispersants, and among the strains *Alcanivorax,* not surprisingly, showed the best growth, as it is famous for degrading alkanes (Hara *et al*., 2003). This stimulated biodegradation of oil with the addition of dispersant Corexit 9500 is consistent with other studies (Swannell *et al.,* 1999, Lindstrom *et al.*, 1999). Richard *et al.* (1999) also demonstrated that the addition of dispersants can increase the rate of oil biodegradation by promoting the growth of indigenous hydrocarbon-degrading bacteria. *Overholt et al*. (2016) did similar tests on another *Alcanivorax sp.* and they confirmed that the potential for oil degradation or transformation was significantly higher with dispersed oil treatment. However, they also demonstrated a *Acinetobacter sp.* strain exhibited a better growth in crude oil alone, rather than with dispersed oil, and thus it is not appropriate to conclude that hydrocarbon-degrading bacteria uniformly respond positively to dispersed oil (Overholt *et al*., 2016). Kleindienst *et al.* (2015) provided evidence that dispersants applied to either surface water or deep water from the Gulf of Mexico did not stimulate oil biodegradation.

We further found that all three stains have the capacity to grow on Corexit as the sole carbon source, although *Shewanella* appeared to grow much slower than *Alcanivorax* and *Halomonas* (Fig 4). These results are confirmed by previous studies that showed that microbial consortia were able to degrade both hydrocarbon fraction and the dioctyl sodium sulfosuccinate (DOSS) fraction of Corexit (Campo *et al.*, 2013). Lindstrom *et al*. (2002) pointed out that the dispersant may help degrade only some particular components of crude oil, like dodecane and 2-methyl-naphthalene, and the toxicity of residual oil depends on the selective microbial degradation of hydrocarbons. This specific microbial degradation was confirmed in another study, which showed that after 3 days of incubation at 14°C, the microbial communities from two different deep waters became dominated by well-known oil degrading bacteria, and the overall microbial community diversity drastically decreased (Liu *et al*., 2017). Techtmann *et al.* (2017) indicated that several operational taxonomic units (OTUs) were inhibited by the addition of Corexit 9500, while a number of OTUs were stimulated, many of which were identified as know hydrocarbon-degrading bacteria. It is speculated that Corexit itself is not toxic to the microbes, instead, the dispersed oil after specific microbial degradation leads to increased or decreased toxicity of residual oil.

Degradation of hexadecane by three strains

A useful method to follow microbial degradation of hydrocarbons is to employ GC-MS (Schedler *et al*., 2014; Overholt *et al*., 2016). I tested the three study strains and an uninoculated control for hexadecane degradation with hexadecane as the sole carbon source.

In our experiments, there was a slight hexadecane concentration decrease in the uninoculated control over time (Fig. 6A). Since hexadecane is not light sensitive, the possibility

of photooxidation should be low. I speculate that it is because of small amount of alkane volatilization during the incubation in the capped but unsealed test tube. All three strains demonstrated significant degradation of hexadecane within 5 days compared to the uninoculated control (Fig. 5). These results are consistent with the growth of *Alcanivorax* and *Halomonas,* which showed clear evidence of growth in hexadecane alone (Fig. 4). Curiously, although *Shewanella* also showed clear degradation of hexadecane, its growth was poor in hexadecane. This suggests that *Shewanella* has the ability to assimilate hexadecane, but perhaps not all the enzymatic machinery for complete oxidation. *Shewanella* was present in the samples taken from the Prestige oil spill (Martin-Gil *et al*., 2004). *Shewanella spp.* was one of the predominant phylotypes in the oil-treated microcosmos cultivated from Arctic Sea-ice (Gerdes *et al*., 2005). Neethu *et al.* (2019) also observed the presence of the genus *Shewanella* in oil contaminated seawater and sediment samples collected from Chennai coast of India, and they mentioned that *Shewanella* might be one of the globally distributed hydrocarbon degrading organisms (Neethu *et al*., 2019). However, there have been no studies on hydrocarbon growth or degradation within the genus *Shewanella*. The mechanism of this bacteria how to assimilate hydrocarbon is still unknown.

High pressure effects of microbial growth

In this study, *Alcanivorax, Halomonas,* and *Shewanella* were grown under aerobic and hypoxia conditions on hexadecane with Corexit under atmosphere and high pressures. Not surprisingly, I found that all three strains grown best with certain amount of oxygen than under hypoxia conditions in sealed bulbs. Among the three strains the relatively fast growing *Alcanivorax* exhibited the most pressure-sensitive (piezosensitive) growth, with clear reduction

in growth rate and yield at 10 and 25 MPa compared to 0.1 MPa cultures grown under otherwise identical conditions (Fig. 7A). In contrast, while the *Shewanella* and *Halomonas* strains exhibited slower growth, they also displayed more piezotolerant growth, with no discernable difference noted across the span of 0.1, 10 and 25 MPa pressures (Fig. 7C&E).

The results with these strains must be considered in the context of prior pressure studies on microbial oil degradation. Schwarz *et al.* (1975) discovered that a microbial community collected from the sediment-water interface of a core sample taken off the coast of Florida at the depth of 4940 m, was able to utilize n-hexadecane as a sole carbon source for growth at 50 MPa (4 °C), but showed decrease in rates of growth and hexadecane utilization of at 50 MPa compared to 0.1 MPa. The alkane-degrading bacterium *Rhodococcus qingshengii* was isolated from a seawater sample located beneath an ice cap during an expedition to Norway, and its growth on n-hexadecane was slightly reduced at 15 MPa (room temperature) (Schedler *et al*., 2014). A significant reduction in the final growth yields was seen after 4 days incubation of the hydrocarbonoclastic, piezosensitive *Alcanivorax sp.* at 10 MPa (20 °C) with n-dodecane as the sole carbon source (Scoma *et al*., 2016a). However, Grossi *et al.* (2010) found that high pressure 35 MPa (room temperature) did not inhibit the growth rate and hexadecane consumption of piezotolerant alkane-degrading bacterium, *Marinobacter hydrocarbonoclasticus* strain #5, isolated from deep Mediterranean seawater (3475m). It should be noted that our experiments were conducted at room temperature (25 °C), the approximate growth optimum of the strains. Marietou *et al*. (2018) cultured 1070-meter-deep water samples collected from DWH oil spill with Macondo oil (MC-252) and observed lower cell numbers at high pressures after 30 days of incubation at 15 and 30 MPa (4 °C). They demonstrated that the synergistic effect of high

pressure and low temperature appears to have an even more dramatic effect on the rates of microbial activity.

High pressure effects on protein synthesis activity

In order to understand and predict environmental processes, it is critical to distinguish active, inactive and dead bacteria. In this study we applied BONCAT-FACS to detect fraction of active cells incubated with hexadecane and Corexit under different pressures.

Alacnivorax and *Halomonas* showed lower fraction of active cells as pressure increased, while *Shewanella* didn't show significant difference among three pressure conditions, and much higher activity was seen on cell protein synthesis than the microbial growth in parallel compared to other two strains (Fig. 7). To our knowledge, this is the first report of studying the effects of high hydrostatic pressure on protein synthesis of hydrocarbon-degrading bacteria. High pressure can affect many important cell functions, including DNA structure and function, protein synthesis, transcription, nutrient uptake and membrane fluidity (Abe, 2007; Bartlett, 2002). Bartlett *et al.* (2007) mentioned that elevated pressure may lead to the impairment of cell division of mesophilic microbes. Lauro *et al*.(2008) described that the largest fraction of loci associated with pressure sensitivity were involved in chromosomal structure and function in the research of a deep-sea bacterium *Photobacterium profundum* strain SS9. In a review of the effects of high hydrostatic pressure (based on *E coli*), Abe mentioned that it is widely recognized that cell growth in organisms is inhibited by high pressure, and protein synthesis is notably susceptible to high pressure (Yayanos and Pollard, 1969). Scoma *et al.* (2016a) also suggested that protein synthesis was highly impacted already under mild high pressure (10MPa). Uncharged ribosomes (ribosomes without mRNA and tRNA) are dissociated accompanied by a

large negative volume change under high pressure, which thus limits cell viability and growth (Gross *et al*., 1993). Although it is unknown what is the most crucial factor limiting the growth of the strains under high pressure, it could well be the impaired DNA replication.

Michoud and co-workers found that *Pyrococcus yayanosii*, an obligate piezohyperthermophile found at hydrothermal vents, upregulated genes in translation to increases the production of tRNA and ribosomal units with pressure (Michoud *et al*., 2016). Scoma *et al.* (2016a) proposed that high pressure deeply affected the protein translation of the piezosensitive bacterium *Alcanivorax borkumensis* SK2. They found that expression of almost all ribosome subunits was upregulated under 10 MPa together with translation, elongation and tRNA modifying factors such as the pseudouridine synthase. It is puzzling that the *Shewanella* strain exhibits the most pressure-resistant protein synthesis of the three strains, given that it is the only one from shallow sea waters (46m) and thus would seem to be a priori less likely to possess piezotolerant growth or protein synthesis. Perhaps its slow growth under the conditions used exerts some of a controlling influence on cell growth and protein synthesis than pressure, thereby masking pressure effects on these processes. Peoples et al. (2018) previously reported experiments with piezophilic bacterial strains in culture that BONCAT analyses of the fraction of active cells correlated well with the degree of high pressures-adapted growth. But the strains examined were all capable of relatively rapid growth rates.

Finally, another factor that could affect the analyses of translation activity among the strains is the sensitivity of the technique itself. Samo and co-workers compared BONCAT results with ^{35}S-methionine microautoradiography, and discovered that a much larger fraction of a natural marine bacterial assemblage was observed to be actively synthesizing protein than that

observed via autoradiography (Samo *et al*., 2014). Similar results have been reported for soil samples (Couradeau *et al*., 2019).

On the whole, the effects of high pressure on the microbial activity are species-specific. More studies using genomics and transcriptomics can help to determine the exact mechanism of pressure effects on hydrocarbon-degrading bacteria. BONCAT coupled with flow cytometry has proven itself to be a valuable method for understanding the metabolic activity of microbial species under particular environmental conditions.

Future studies

After all of the growth and activity analyses, the next focus will be on the detection and analysis of functional genes, like alkane-1-monooxygenase (*alkB*), which is a key enzyme involved in bacterial alkane degradation. This would help to establish the genetic capability for hydrocarbon degradation among the strains. I have tried two sets of primers (AlkBF/AlkBR, and Rh alkB1-F2/ Rh alkB1-R1, Table 2) to detect the *alkB* gene for *Alcanivorax, Halomonas* and *Shewanella*. PCR (Table 3) and sequencing results didn't show the successful amplification of the *alkB* gene. This could because the variations in *alkB* nucleotide sequences, and thus leads to the high diversity of this gene. Jurelevicius *et al.*(2013) suggested that a combination of *alkB* primers was an efficient way to enhance the detection of the *alkB* gene, and for better characterizing the distribution of alkane-degrading bacteria in environmental samples.

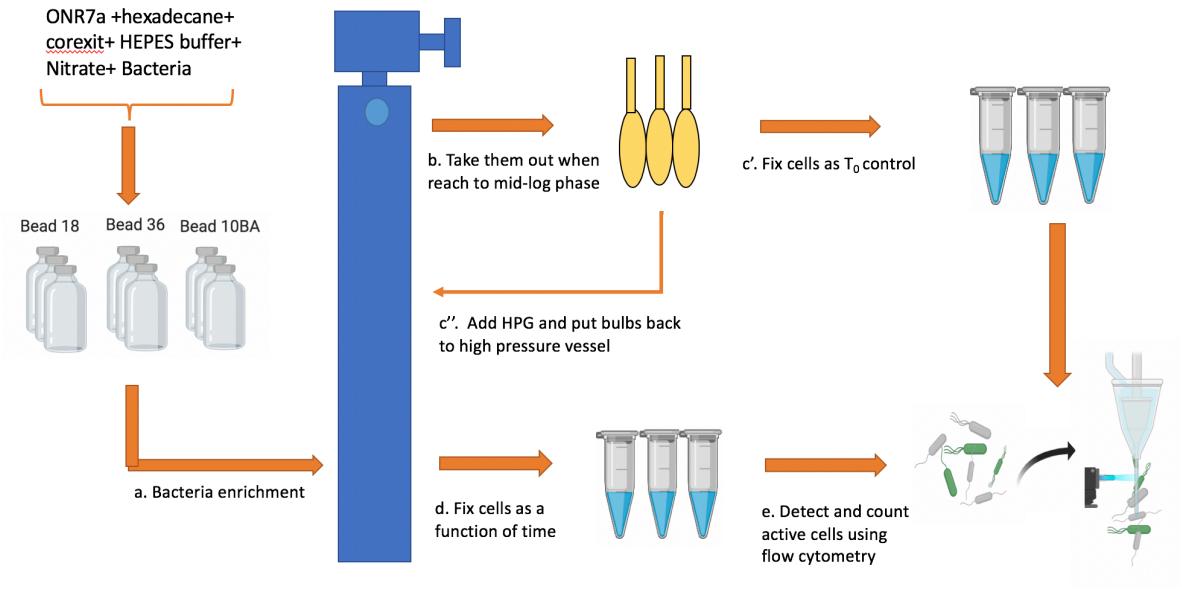


Figure 1. Diagram of bacteria enrichment under high hydrostatic pressure, sample fixation as a function of time, and subsequent BONCAT work for detecting and quantifying active cells.

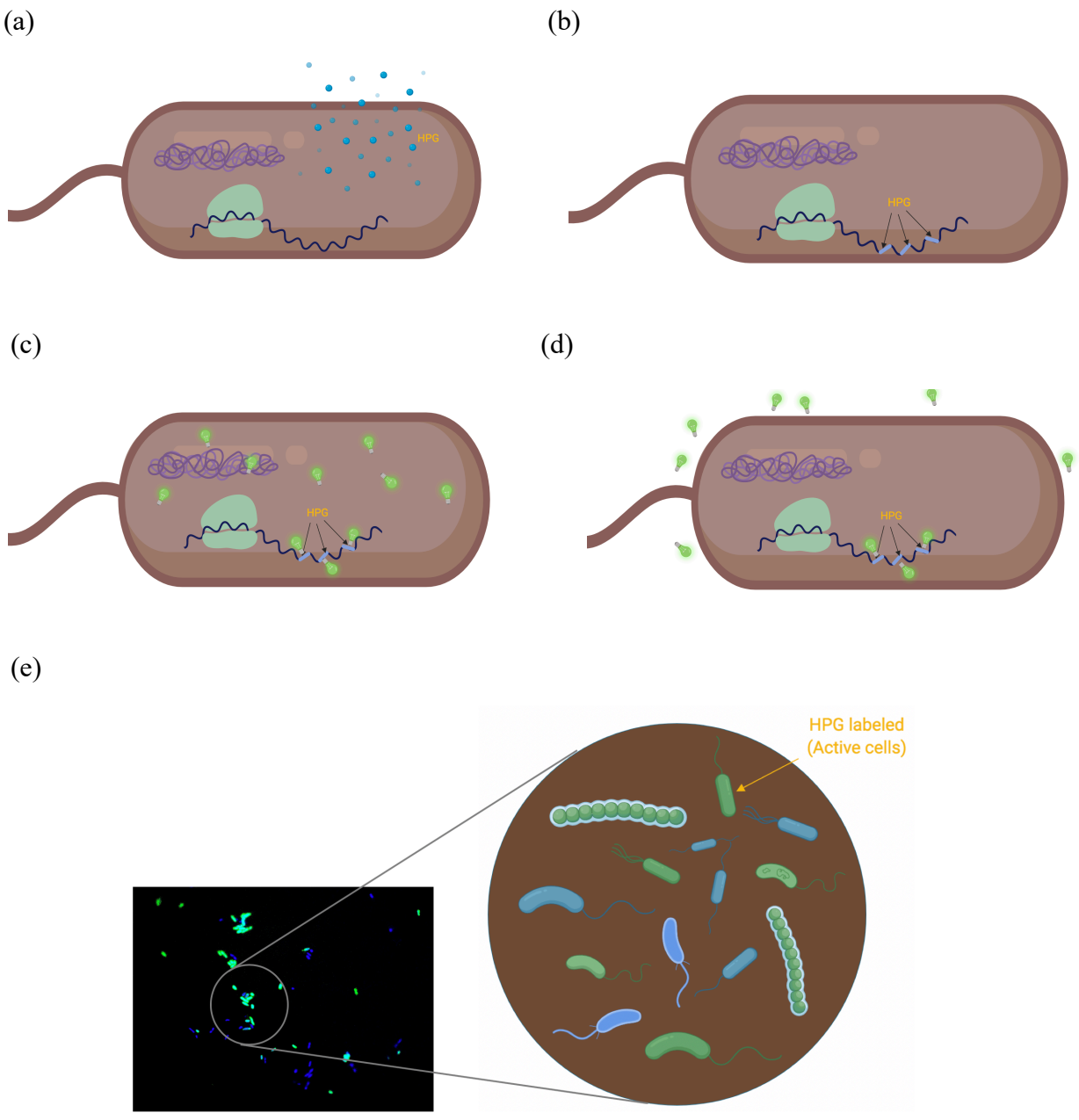


Figure 2. Concept for the visualization of translationally active cells.

(a) The bioorthogonal amino acid HPG is added to the culture medium, which is then incubated under in situ conditions. (b) After HPG has entered the cells, the exact process which is currently unknown, it competes with methionine for incorporation into newly made peptides. (c) HPG-containing proteins are then fluorescently labeled via a Cu(I)-catalyzed azide–alkyne click reaction (d) Extra 'Click Cocktail' are washed away (e) The cells that have undergone protein synthesis during time of incubation are fluorescent, which can be detected through fluorescent microscopy or FACS.

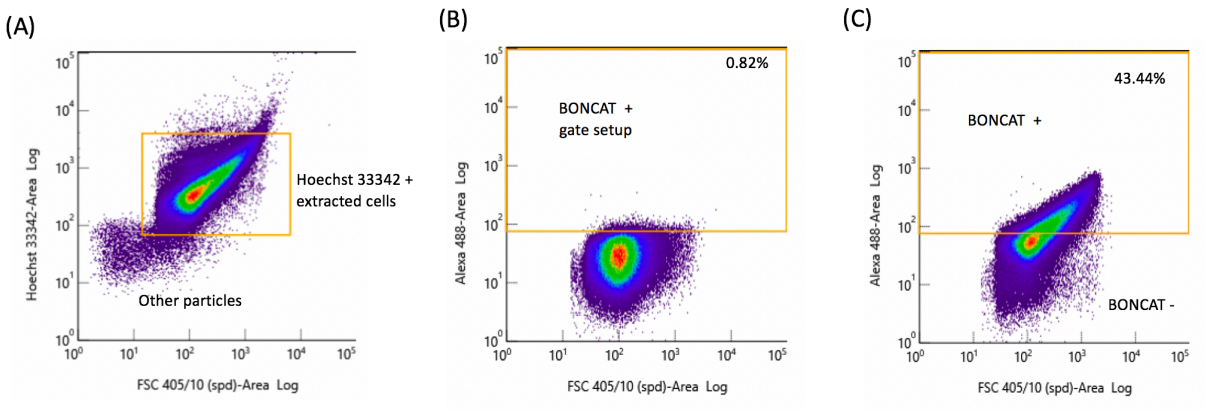
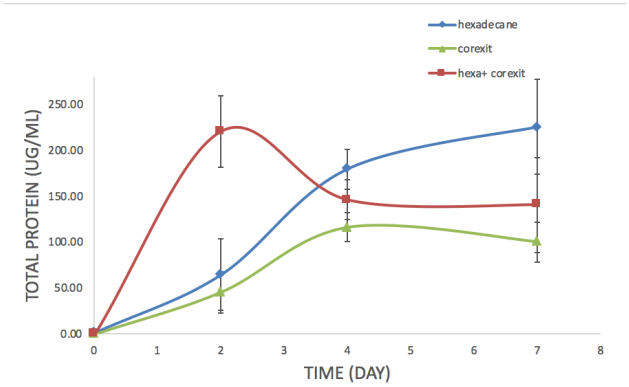


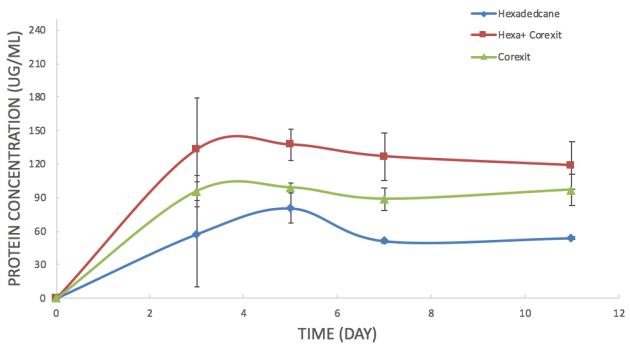
Figure 3. Analysis of images generated from BONCAT-FCM.

(A) The gate drawing was done in two steps, first the cells were separated from the background particles based on their DNA dye staining Hoechst 33342 fluorescence (excitation = 361 nm, emission = 497 nm) as pictured by the orange gate in the center. The rest could be cell aggregates and other particles. (B) The Hoechst 33342+ cells were further analyzed for their BONCAT fluorescence with the Alexa Fluor 488 dye (excitation = 490 nm, emission = 525 nm). The middle panel shows an example of a control sample of *Alcanivorax* that was incubated without HPG and clicked, the BONCAT gate (on the top) was set such that less than 1 % of events would fall in that gate (false positive). (C) An example of how the BONCAT+ and BONCAT – gates where set in a HPG incubated sample. Note that the gate is the same as in the control sample.

(A) *Alcanivorax xenomutans* Bead 18



(B) *Halomonas titanicae* Bead 10BA



(C) *Shewanella inidica* Bead 36

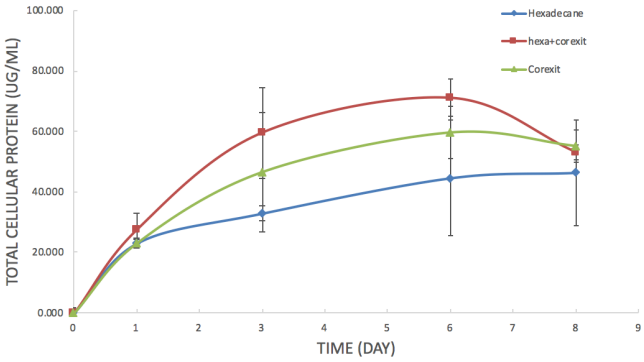


Figure 4. Growth curve under different conditions, determined using a Pierce bicinchoninic acid protein assay. (A) *Alcanivorax xenomutans* (B) *Halomonas titanicae* (C) *Shewanella indica*

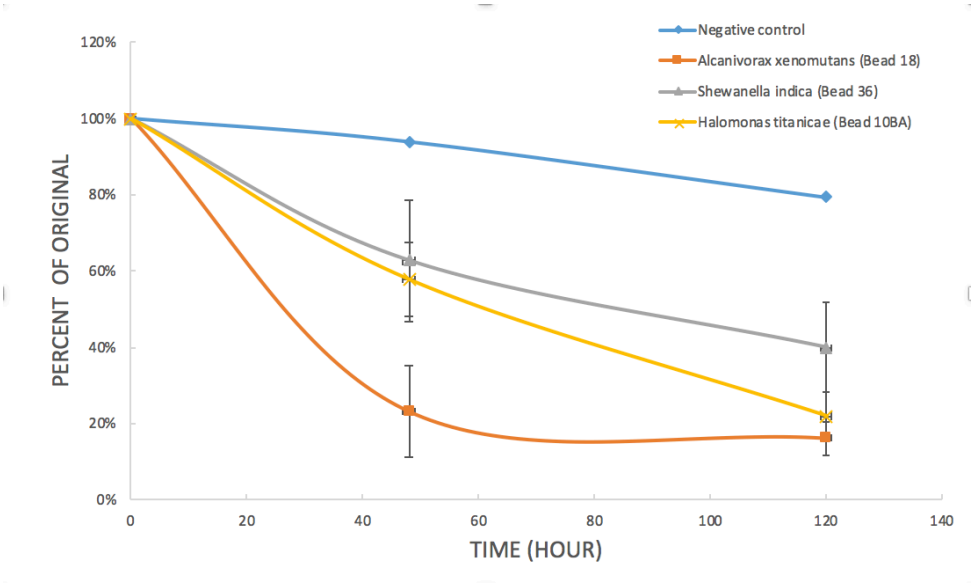
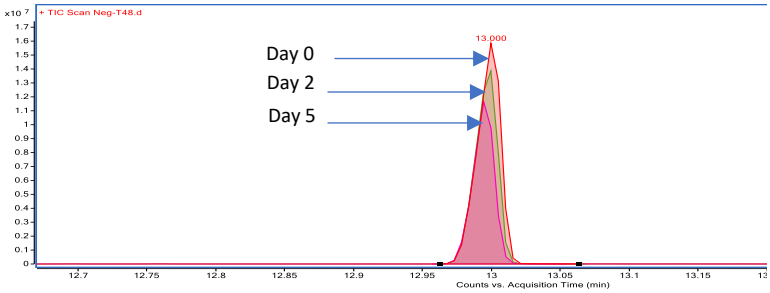
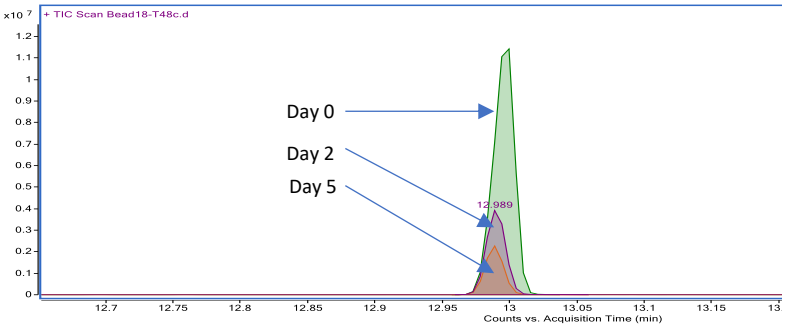


Figure 5. Hexadecane degradation percentage of three strains and uninoculated control during the time of incubation.

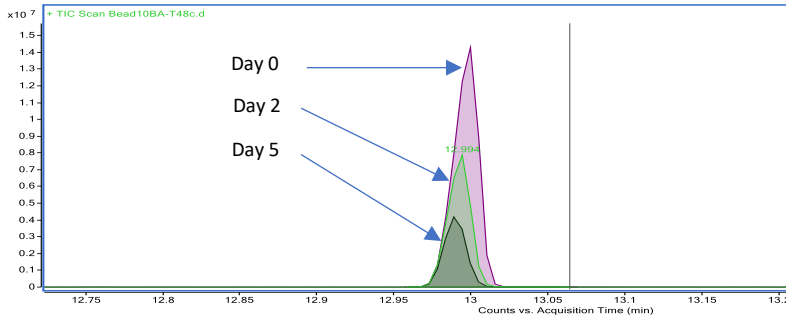
(A) Negative control



(B) *Alcnivorax xenomutans* Bead 18



(C) *Halomonas titanicae* Bead 10BA



(D) *Shewaenella indica* Bead 36

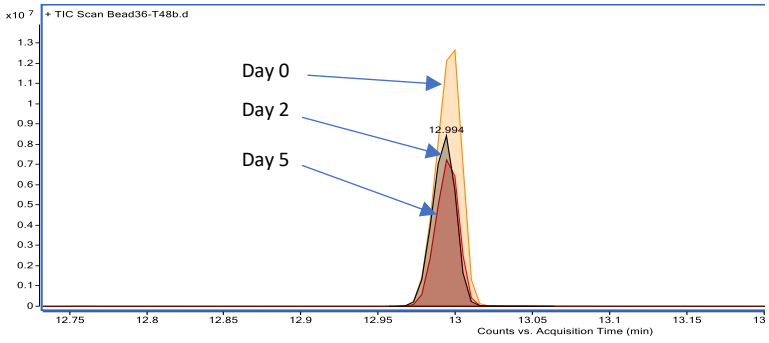
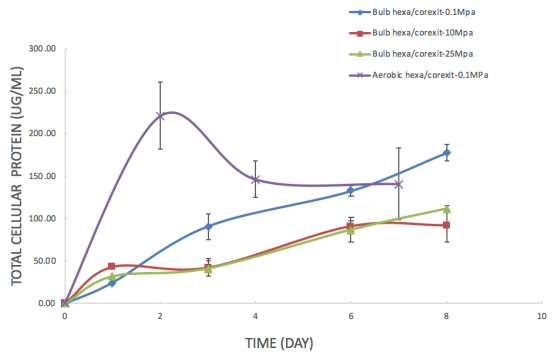
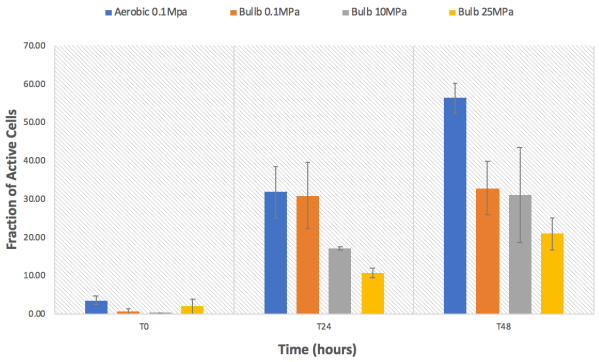


Figure 6. Decrease of hexadecane chromatography peak of three strains and uninoculated control during the time of incubation.

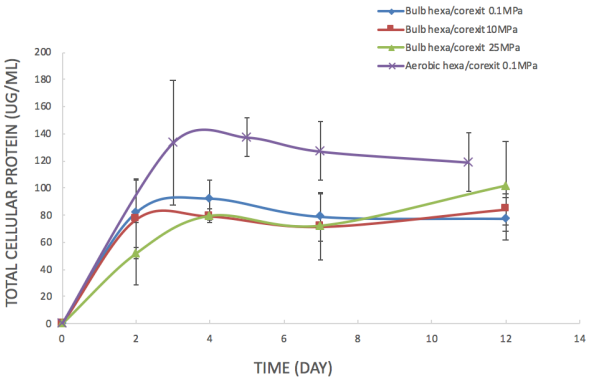
(A) *Alcanivorax xenomutans* growth curve



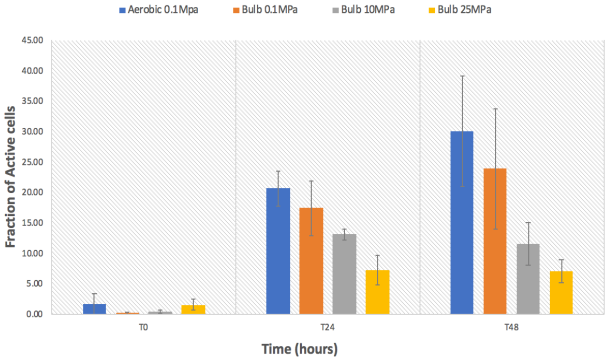
(B) *Alcanivorax xenomutans* BONCAT+



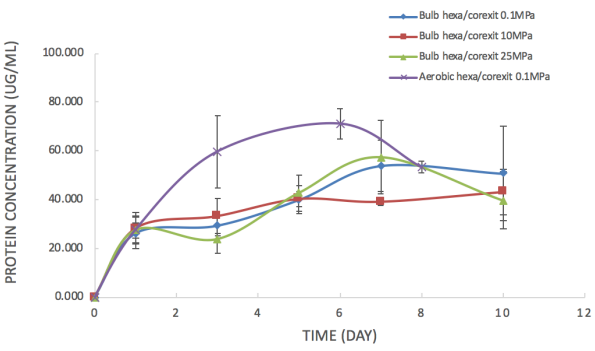
(C) *Halomonas titanicae* growth curve



(D) *Halomonas titanicae* BONCAT+



(E) *Shewanella inidica* growth curve



(F) *Shewanella inidica* BONCAT+

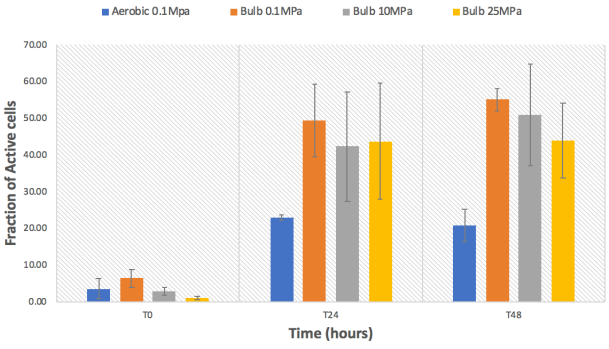


Figure 7. Left panel (A, C, E) : Growth curve in hexadecane and Corexit under different pressures 0.1,10 and 25 MPa. Right Panel (B, D, F): Temporal dynamics of BONCAT+ (express as a percent of the extractable cells) labeling (A, B) *Alcanivorax xenomutans* (C, D) *Halomonas titanicae* (E, F) *Shewanella indica*.

Table 1. Studies strains used in this study and their isolation depth within the Gulf of Mexico.

Strain names	**Closest Cultured Relative**	**Isolation Depth**
Bead 18	*Alcanivorax xenomutans*	1509m
Bead 36	*Shewanella indica*	46m
Bead 10BA	*Halomonas titanicae*	1509m

Table 2. Details of the GC-MS method used in this study.

GC	Column Name: Agilent DB-5MS UI, 30 meter, 0.25 mm ID, 0.25 um df. Cat # 122-5532UI
	Oven temperature gradient: held at 60 ^{0}C for 4 minutes, then increased to 270 ^{0}C at 15 C/min, and hold for 2 minutes at 270 ^{0}C. Post run: 280 C for 2 minutes
	Injection Temp.:250℃
	Injection Mode: Split
	Split Ratio: 10 split ratio
	Injection Volume: 1.0 ul
	Column Flow: 1.2 ml/min helium gas
	Total Run Time: 20 minutes
MS	Transfer Line Temperature: 250 C. Ion Source Temperature: 230℃
	Electron Impact Ion Source. m/z: 35-500

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APPENDIX

Appendix Table 1. List of *alkB*-targeting primers used in this study.

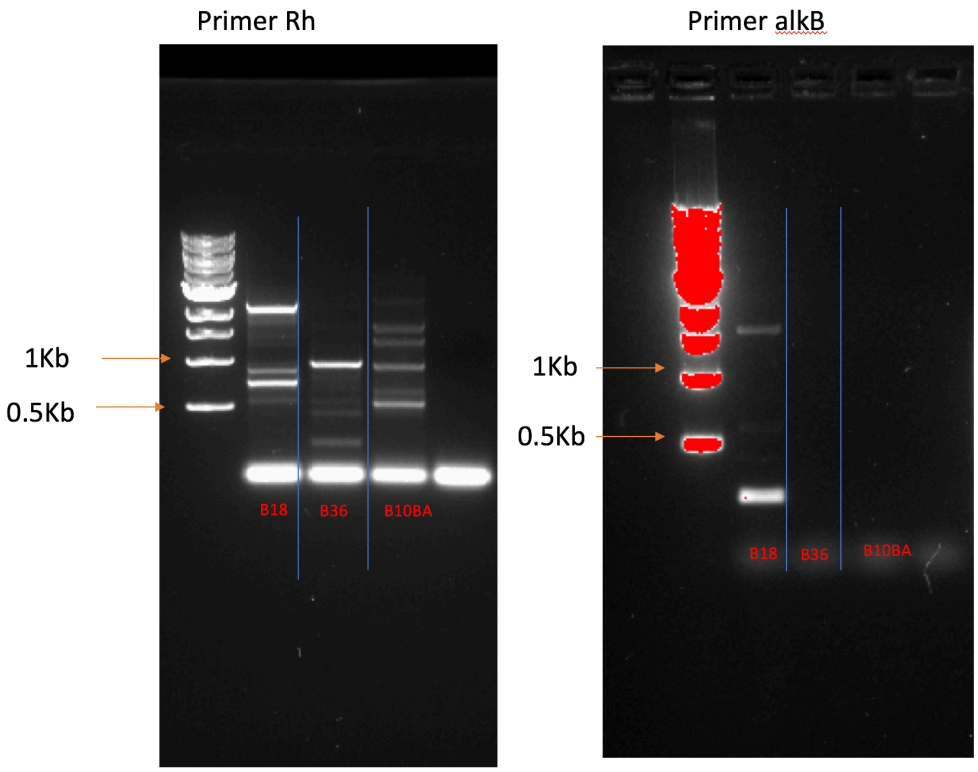
Primer code(a)	alkB-targeting primers	Primer sequences	Approxinate position of alkB fragments
a	Rh alkB1-F2	5' ATC TGG GCG CGT TGG GAT TTG AGC G 3'	331 to 950 nt (b)
	Rh alkB1-R1	5' CGC ATG GTG ATC GCT GTG CCG CTG C 3'	
b	AlkBF	5' CCT GCT CCC GAT CCT CGA 3'	170 to 911 nt
	AlkBR	5' TCG TAC CGC CCG CTG TCC AG 3'	

(a) Primer code used throughout the results section and figures
(b) nt = nucleotide

Appendix Table 2. PCR parameters needed to amplify the *alkB* genes based on fragment size and melting temperatures (Tm) of the primers.

RhalkB			
Segment	# of cycles	Temperature (°C)	Duration
Initial denaturation	1	95	4min
Denaturation	30	95	45sec
Primer annealing		65	1min
Extending		72	1min
Final extending	1	72	5min

AlkB			
Segment	# of cycles	Temperature (°C)	Duration
Initial denaturation	1	94	5min
Denaturation	30	94	45sec
Primer annealing		65	45sec
Extending		72	45sec
Final extending	1	72	7min



Appendix Figure 1. Gel results of *alkB* gene PCR using primer RhalkB and AlkB. No *alkB* gene band was detected for the three strains.

This thesis, in full, is co-authored with Mullen, Kelli. and Douglas, D.H. The thesis author is the second author of the paper.