Protein Expression in Response to Oil in a Marine Hydrocarbon Degrading Bacterium Isolated from Tampa Bay, Florida

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Abstract

Bacteria that are capable of oil degradation play a large role in bioremediation of hydrocarbon based pollutants in their environment. After the Deepwater Horizon oil spill, there has been a push to discover the species of hydrocarbon-degrading bacteria naturally present in an area and their precise role in bioremediation in a hydrocarbon-degrading bacterial consortium. As part of our preliminary data, we cultured, isolated, and identified through 16S rRNA gene sequencing, marine oil degrading bacteria from the coastlines of Central Florida. We selected a species of *Oceanobacillus* from this screen for further study, as other *Oceanobacillus* species are known to degrade hydrocarbons. However, the proteins utilized for hydrocarbon metabolism in *Oceanobacillus* and other facultative oil degraders are currently unknown. Our study focused on the differences in protein expression when a opportunistic oil-degrading bacterium, *Oceanobacillus sp.,* was exposed to an oil enriched or unaltered marine growth medium. These differences can provide insight into proteins that may be critical for oil degradation in *Oceanobacillus* sp. and other facultative oil degrading bacteria. Understanding the mechanism behind hydrocarbon degradation in this and other facultative hydrocarbon degrading species will provide insight into how a complex marine bacterial consortium can metabolize naturally occurring and pollutant hydrocarbons.

Introduction

Hydrocarbons are a common pollutant in marine areas that are frequently used as shipping centers, near coastal cities with heavy vehicular traffic, or adjacent to coal-processing facilities (Cerniglia, 1993). Hydrocarbons may also be introduced to the environment in the form

of crude oils released from tanker or drilling accidents. Regardless of their source, hydrocarbons have a devastating effect on the local marine environment. Plankton are often severely affected and larger fauna that survive the spill may accumulate hydrocarbon compounds in their tissues, affecting their lifespan and/or reproductive capabilities (Rice, 1978). Furthermore, larger hydrocarbons such as Benzo[a]pyrene are proven to be carcinogenic in humans (Cerniglia, 1993). Bioremediation of these pollutant hydrocarbons by naturally occurring oil-degrading bacteria is one possible strategy to minimize the effect of these chemicals.

A large variety of marine bacteria, at least 79 different genera, are able to metabolize some components of crude oil (Head, Jones, & Röling, 2006). Obligate oil-degrading bacteria such as *Alcanivorax sp.* are more uncommon than facultative species but they do make up a considerable amount of the bacterial biomass in areas with heavy hydrocarbon pollution (Beilen *et al.*, 2004) Much of the research into bacterial hydrocarbon metabolism has thus far focused on these obligate species that are easily lab culturable. Facultative hydrocarbon-degrading bacteria are able to use alternative carbon sources in the absence of an oil substrate and are often detectable in areas that are not experiencing a pollution event.

Aerobic bacteria that can metabolize hydrocarbons in crude oil commonly target either *n*-alkanes or aromatic hydrocarbons (Head, Jones, & Röling, 2006). The majority of oil-degrading bacteria, at least 65 genera, are able to utilize alkanes as their sole carbon source (Nie, *et al.*, 2015). Research into the metabolism of oil-degrading bacteria has also primarily focused on these alkane degraders. Genes encoding enzymes utilized in alkane degradation are known as alkane hydroxylase (*alk)* genes. (Sei, *et al.*, 2011). Despite the large amounts of research completed on alkane-degrading bacteria, further research is still required to uncover all

of the genes utilized in alkane degradation. For instance, *Alcanivorax borkumensis alkB1* and *alkB2* knock-out mutants were still able to utilize some alkanes as a carbon source, suggesting there are other genes involved in the production of alkane degrading enzymes (Sabirova *et al.,* 2006).

Instead of alkanes, microbes may metabolize the aromatic rings of other hydrocarbons in crude oil. All bacteria that metabolize aromatic hydrocarbons have some sort of dioxygenase protein to break the ring structure of the molecule. (Cerniglia, 1993). The most common aromatic degradation pathway encoding dioxygenase enzymes is a *nah*-like gene cluster. This gene organization is common is *Pseudomonas* and related species. *phn* genes recently discovered in a species of *Burkholderia* also encode for a low molecular weight aromatic hydrocarbon degradation pathway (Laurie & Lloyd-Jones, 2000). There may be other pathways encoding dioxygenase enzymes that are yet to be discovered.

The polar molecules of oil are known as resins and asphaltenes. These molecules are relatively resistant to bioremediation and are often some of the last components to be degraded in the environment after a contamination event (Head, Jones, & Röling, 2006). The metabolic processes of resin and asphaltene degradation by any microbe is currently unknown and there has been no confident proof that there are any bacterial species able to utilize these compounds as a sole carbon source (Uribe-Alvarez, *et al.,* 2011).

Overall, different genes encode enzymes that allow bacteria to degrade hydrocarbons of different types and molecular weights and allows a microbial community to metabolize all of the components of crude oil (Cerniglia, 1993). Complete mineralization of oil pollutants through the metabolization of all components is the most desirable outcome to bioremediation. Incomplete

mineralization may result in the buildup of hydrocarbons that are still harmful to the marine environment (Meyer *et al.,* 1999).

A preliminary study, conducted during the summer of 2017, sought to identify the naturally-occurring oil-degrading bacteria from the coastal waters of Central Florida. Previous studies have identified the bacteria present in hydrocarbon-degrading microbial communities throughout the Florida panhandle and Gulf of Mexico after the 2010 Deepwater Horizon oil spill. However, no previous study had examined the microbial communities of Central Florida. Furthermore, the Atlantic coast is particularly poorly represented. After research identified a large variety of naturally occurring bacteria in Central Florida waters (Table 1), the metabolisms of which many had never been studied, the study was expanded to include the differential protein expression of a selected bacteria cultured from our initial research. All of the bacteria identified are facultative oil degraders, allowing a unique opportunity to study differences in protein expression with and without the presence of hydrocarbons as a carbon source. Ultimately, the *Oceanobacillus* strain isolated from Hillsborough Bay, Tampa, Florida adjacent to the Bay Palms Golf Complex was selected for further metabolic and proteomic studies (Figure 1)(Table 2). This organism was selected for its non-pathogenic nature, known oil degradation in the genus, and the large protein database of closely related organisms, specifically *Oceanobacillus iheyensis*.

The isolated *Oceanobacillus* strain, hereafter referred to as TB-8, is a 99% sequence accession match for the 16S rRNA genes of both *Oceanobacillus iheyensis* and *Oceanobacillus kimchii*. *Oceanobacillus iheyensis* is a deep sea halotolerant and alkaliphilic bacterium isolated from the Iheya Ridge (Lu, 2001). *Oceanobacillus kimchii* was isolated from the traditional korean food, fermented kimchi. *Oceanobacillus kimchii* is considered a close phylogenetic

relative to *iheyensis,* with a 16S rRNA sequence match of 98.9% (Whon et al., 2010). TB-8 is therefore likely a strain of one of these species or an, as of yet, unidentified close relative. Neither *Oceanobacillus iheyensis* or *kimchii* have been assessed for or shown evidence of hydrocarbon degradation capabilities.

Despite the lack of evidence for oil degradation of closely related species of *Oceanobacillus,* other members of the genus are involved in hydrocarbon degradation. Notably, *Oceanobacillus sp.* strain BR 10 was found to degrade 63% of crude oil after a period of 27 days. Furthermore, this species produced a biosurfacent found to enhance crude oil degradation (Jadhav et al., 2013). The proteins responsible for oil degradation in this strain, however, were not determined. Investigating whether or not TB-8 differentially expresses proteins in response to oil exposure will provide further insight into the metabolism of Oceanobacillates. To further explore the metabolism of TB-8, an API-20E test strip (bioMérieux), along with an oxidase and catalase test, will also be utilized.

Methods

Isolation and Identification

Oceanobacillus strain TB-8 was originally isolated as part of summer research student-faculty collaborative to identify the oil degrading bacteria naturally present in Central Florida's coastal waters. TB-8 was isolated from water samples collected from the Tampa Bay region (Figure 1, site 9). Samples were collected from surface waters, incubated in a solution of marine broth and 1% sterilized Texas crude oil, and serially diluted on to marine agar plates overlaid with 100μl of oil. Morphologically distinct colonies were transferred from the patch

plates and isolated with quadrant streaking to create pure cultures. DNA was extracted from each

culture and PCR was performed using universal prokaryotic primers to isolate the 16s rRNA

gene. After gel electrophoresis to confirm successful amplification, samples were sent to a

biotechnology company, GENEWIZ, for Sanger sequencing. Nucleotide sequences were

compared against the BLAST database of 16S rRNA bacterial gene sequences to determine a

genus and/or species match. We identified at least 17 distinct species of oil degrading bacteria

(Table 1). Bacterial strains were then preserved in a glycerol and marine broth solution and
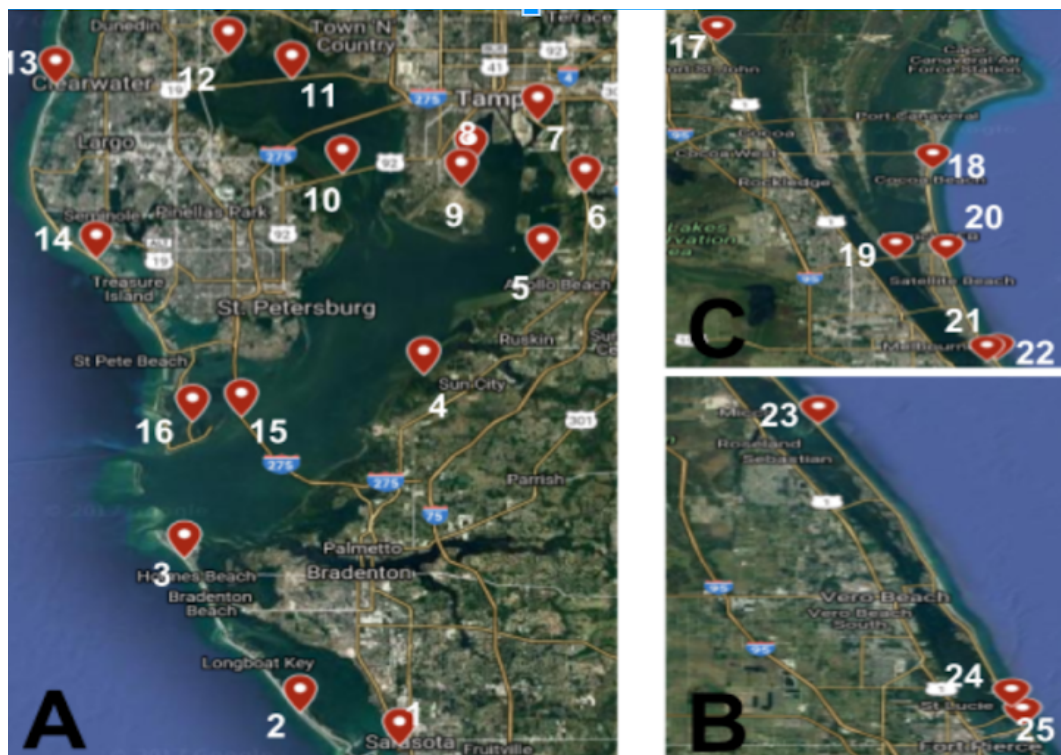
frozen at -80°C.

Figure 1. Central Florida seawater collection sites. A) Tampa Bay and the Gulf of Mexico, **B)** North-Central Atlantic Coast, **C)** South-Central Atlantic Coast)

Table 1. Identification of oil-degrading bacteria, by location. Green) Gulf of Mexico Sites **Blue)** Tampa Bay Sites **Yellow)** Atlantic Sites

Location	Bacteria Identified
1. Bayfront Park	Identification pending
2. Longboat Key	Identification pending
3. Holmes Beach	Vibrio sp., Family Campylobacteraceae, Arcobacter sp., Vibrio sp.
4. Cockroach Bay Aquatic Reserve	Bacillus firmus, Shewanella sp.
5. Apollo Beach Nature Preserve	Vibrio fluvialis
6. Williams Park Boat Ramp	Vibrio fluvialis
7. Mckay Bay Nature Park	Vibrio fluvialis, Bacillus gibsonii, Psuedoaltermonas sp.
8. Ballast Point Park	Vibrio fluvialis, Oceanobacillus sp.
9. Bay Palms Golf Complex	Oceanobacillus sp.
10. Gandy Bridge	Vibrio alginolyticus
11. Courtney Camble Causeway	Vibrio alginolyticus
12. Safety Harbor	Vibrio alginolyticus, Shewanella haliotis
13. Clearwater Beach	Identification pending
14. Madeira Beach	Bacillus firmus
15. Sunshine Skyway Bridge	Shewanella sp. Vibrio sp.
16. Fort Desoto	Identification pending
17. NASA Causeway, Indian River	Arcobacter sp., Vibrio sp.
18. Cocoa Beach	Micrococcus sp., Ferrimonas sp.
19. Satellite Beach	Psuedoalteromonas sp.
20. Merritt Island, Banana River	Bacillus kochii
21. Melbourne Beach (West Coast)	Marinobacterium stanieri
22. Melbourne Beach (East Coast)	Arcobacter sp., Marinobacterium sp.
23. Sebastian Inlet State Park	Vibrio alginolyticus
24. Jack Island State Preserve	Paracoccus sp., Vibrio alginolyticus
25. Fort Pierce State Inlet Park	Arcobacter sp., Vibrio alginolyticus

<u>Metabolic Testing</u>

All metabolic tests were performed on isolated colonies of TB-8 cultured on plain marine media. Oxidase testing was carried out by swabbing a colony onto filter paper and depositing one drop of Kovacs oxidase reagent onto the bacteria. The test was then observed for changes in color. Catalase testing was performed by selecting another colony and depositing it onto a glass microscope slide. A drop of hydrogen peroxide was placed on the bacteria and the solution observed for evidence of oxygen gas production. Analytical Profile Index 20E testing was completed using a colony suspended in sterile saline. The bacterial suspension was pipetted into each well on the test strip. Sterile oil was overlaid on the ADH, LDC, ODC, H2S and URE compartments to create an anaerobic environment. The tray was then covered and placed in a 37°C incubator for 24 hours. One drop of ferric chloride and one drop of Kovacs reagent were added to the TDE and IND compartments respectively. Voges–Proskauer reagents one and two were added to the VP compartment. Wells then were observed for color changes indicative of positive results according to the manufacturer's (bioMérieux) protocol.

<u>Protein Extraction</u>

TB-8 was removed from glycerol storage and quadrant streaked onto plain marine agar (Difco^{TM} marine media with 2% agar concentration). Bacteria were allowed to grow at 30°C until isolated colonies sufficient for passage were observed. Isolated colonies were transferred to fresh marine agar plates and also allowed to grow at 30°C until plateau, approximately 48 hours. Plates were then refrigerated at 4°C and subcultured periodically to maintain strain viability. For protein extraction, colonies were heavy streaked onto marine agar overlayed with 100 microliters of 1M sodium pyruvate or filter sterilized Texas crude oil. A negative control was also streaked onto

plain marine agar. Plates were incubated for 36 hours at 30ºC. Bacteria were then swabbed from the entire surface of the plate, suspended in 1x phosphate buffered saline, and centrifuged at 10,000 rpm for 2 minutes to pellet cells. The supernatant was removed and the process repeated for a total of three PBS washes to remove residual pyruvate and oil. Cells were pelleted once more with a centrifugation at 5,000xg for ten minutes. The supernatant was removed and 2 µl of lysozyme, 1 µl of DNase, and 200 µl of B-PER reagent (Thermo Scientific) were added to the pellet. The mixture was homogenized through tituration and incubated for 15 minutes at room temperature. Finally, the mixture was centrifuged for 5 at 15000xg and the supernatant, along with the solubilized proteins, was transferred to a new microcentrifuge tube. The supernatant was frozen at -30ºC. A BCA assay was performed with Thermo Scientific's Pierce BCA assay kit with standard test tube protocol to estimate extracted protein concentration.

Polyacrylamide Gel Electrophoresis

A polyacrylamide gel was prepared with 10% acrylamide concentration. Protein samples were diluted with 4x Laemmli sample buffer and 35 µl loaded into wells. This resulted in a final protein concentration of 14.9 ug for oil, 20.7 for pyruvate, and 20.4 for the plain marine negative control. 4 µl of BioRad Protein Kaleidoscope ladder was also loaded. Unused wells were filled with 5 µl of 4x sample buffer. The gel was run for 20 minutes at 80 volts to move proteins through the stacking gel. The voltage was then increased to 100 volts for one hour for movement through the resolving gel. The gel was then removed from the spacer plates, stained in Coomassie for one hour, and allowed to destain overnight. The gel was then imaged with white light.

Results

Metabolic Testing

TB-8 reacted positively to the oxidase testing, producing an indigo color when exposed to

Kovacs reagent. It is therefore a producer of the enzyme, cytochrome oxidase. Colonies of TB-8

also produced gas in response to hydrogen peroxide, a positive indicator for the presence of

catalase enzymes. TB-8 reacted negatively to all of the substrates contained in the API-20E test.

Polyacrylamide Gel Electrophoresis

Protein samples from each test condition successfully produced bands when subjected to

gel electrophoresis and Coomassie staining. Many of the bands were identical in protein mass

and relative intensity when compared to each other and the control sample. Protein expression

bands between TB-8 exposed to crude oil and the control sample were identical. However, TB-8

exposed to pyruvate exhibited dramatic differential protein expression compared to the control

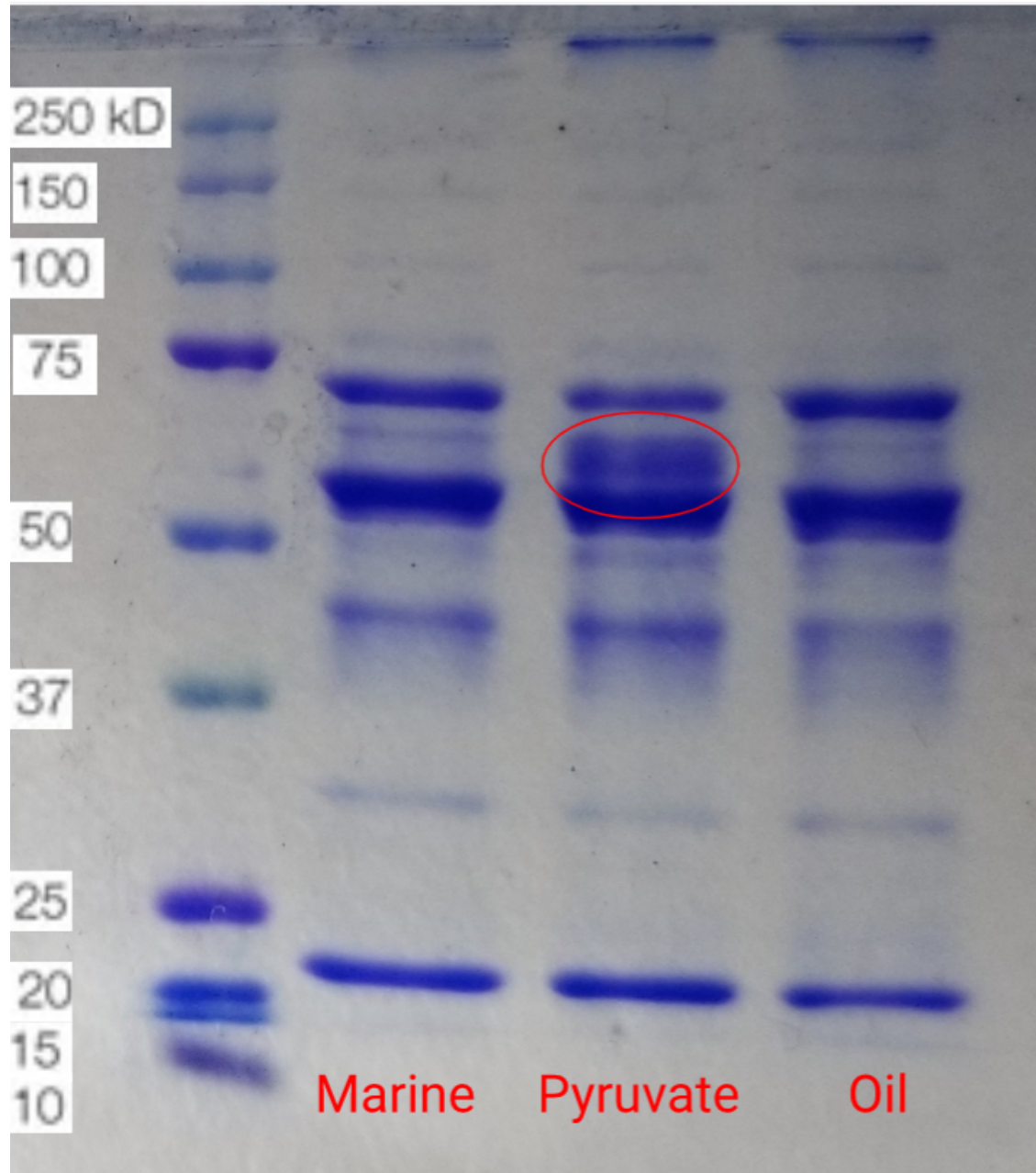(Figure 2). Differential expression was found in the mass range of 50 - 75 kDa.

Figure 2. Polyacrylamide Gel Electrophoresis of Proteins Extracted from TB-8 Under Different Growth Conditions. Marine) plain marine agar serving as negative control, **Pyruvate)** marine agar supplemented with 100 µl of 1M pyruvate , **Oil)** marine agar supplemented with 100 µl sterile crude oil. Ladder weights (kDa) are provided and differential expression circled in red.

			Species			
Reaction Tested	1	2	3	4	5	6
Urease Hydrolysis	-	-	+	+	-	-
β - Galactosidase	-	-	+	+	-	-
Arabinose Utilization	-	-	-	-	-	-
Mannitol Utilization	-	-	+	+	-	+

Table 2. Metabolic tests of TB-8 compared to other *Oceanobacillus* species. Species 1) TB-8; 2) *O. oncorhynchi oncorhynchi* 3) *O. neutriphilus* 4) *O. oncorhynchi incaldanensis* 5) *O. sojae* 6) *O. locisalsi.* Data from strains other than TB-8 obtained from Liu & Yang, 2014.

Discussion

Metabolic results from oxidase, catalyse, and API-20E tests were not unexpected for a species of *Oceanobacillus.* The closely related *Oceanobacillus iheyensis* is similarly catalase positive, however it is oxidase variable (Lu, 2001). It is not impossible TB-8 is also oxidase variable and further testing would need to be conducted under different growth conditions to confirm exclusively positive oxidase results. The API-20E test is designed to distinguish gram negative enterobacteria. As TB-8 is gram positive and a member of bacillaceae, it was not expected to react positively to many of the test reactions. Some *Oceanobacillus* species do react positively to the metabolic tests and allowed a comparison of the metabolism of TB-8 and other *Oceanobacillus* (Table 2). It was, however, interesting that TB-8 did not share some of the metabolic properties of *Oceanobacillus iheyensis* and *kimchii,* given the close relation implied by 16s rRNA gene sequencing. For instance, both species are able to hydrolyze gelatin, while TB-8 cannot (Lu, 2001)(Whon et al., 2010). These findings may indicate that TB-8 may be a

subspecies of one of these organisms or a seperate, unidentified, species. Further metabolic and genetic studies would need to be conducted on TB-8 to determine species identification and phylogenetic relations.

Through polyacrylamide gel electrophoresis, TB-8, was not found to dramatically change protein expression in response to the presence of crude oil. To support these results, more experiments utilizing gels of varying acrylamide concentrations should be conducted. Furthermore, more sensitive staining methods than Coomassie, such as a silver stain, could be used to rule out small up or down regulations in protein. A 2-D gel, separating proteins by both isoelectric point and mass could also be utilized for better protein separation.

Although TB-8 did not exhibit noticably changes in protein expression when exposed to crude oil, changes in expression were observed when the bacterium was exposed to sodium pyruvate. The differentially expressed proteins are between 50 and 75 kDa in mass (Figure 2). These proteins may be osmoregulatory in nature and produced in response to the increased sodium concentration of sodium pyruvate. When subjected to osmotic stress, halotolerant species increase the production of proteins involved in solute transport, such as glycine and proline transporters (Rubiano-Labrador, 2015). The mass of proteins involved in glycine betaine transport in *Oceanobacillus iheyensis*, however, are consistently less than 50 kDa (Takami, 2002). It is more probable that the differentially expressed proteins in TB-8 are involved in pyruvate metabolism. The pyruvate carboxylase enzyme, responsible for metabolism of pyruvate to oxaloacetate, of *Oceanobacillus iheyensis* is 63 kDa in mass (Takami, 2002). The actual identity of these differentially expressed proteins can only be speculated, however, without confirmation through mass spectrometry.

Overall, TB-8 is an *Oceanobacillus* species isolated from Tampa Bay capable of hydrocarbon degradation. It is closely related, by 16S rRNA gene sequence, to *Oceanobacillus iheyensis* and *kimchii* but reacts differently to some metabolic tests. It does express dramatic differential protein expression when exposed to pyruvate but does not detectably alter protein expression when exposed to crude oil for 36 hours.

Acknowledgements

Literature Cited

Beilen, J. B. V. *et al*. Characterization of two alkane hydroxylase genes from the marine

    hydrocarbonoclastic bacterium Alcanivorax borkumensis. *Environmental Microbiology* **6**,

    264–273 (2004).

Cerniglia, C. E. Biodegradation of polycyclic aromatic hydrocarbons. *Current Opinion in*

    Biotechnology **4**, 331–338 (1993).

Head, I. M., Jones, D. M. & Röling, W. F. M. Marine microorganisms make

    a meal of oil. *Nature Reviews Microbiology* **4**, 173 (2006).

Jadhav, V. V., Yadav, A., Shouche, Y. S., Aphale, S., Moghe, A., Pillai, S., Arora, A., Bhadekar,

    R. K. Studies on biosurfactant from Oceanobacillus sp. BRI 10 isolated from Antarctic

    sea water. *Desalination* **318**, 64-71. (2013)

Laurie, A. D. & Lloyd-Jones, G. Quantification of phnAc and nahAc in Contaminated New

    Zealand Soils by Competitive PCR. *Applied and Environmental Microbiology* **66**,

    1814–1817 (2000).

Liu, W., & Yang, S. S. Oceanobacillus aidingensis sp. nov., a moderately halophilic bacterium.

    Antonie Van Leeuwenhoek, **105**, 801-808 (2014).

Lu, J. Oceanobacillus iheyensis gen. nov., sp. nov., a deep-sea extremely halotolerant and

    alkaliphilic species isolated from a depth of 1050 m on the Iheya Ridge. *FEMS*

    Microbiology Letters, **205**, 291-297 (2001).

Meyer, S., Moser, R., Neef, A., Stahl, U. & Kampfer, P. Differential detection of key enzymes of

    polyaromatic-hydrocarbon-degrading bacteria using PCR and gene probes. *Microbiology*

    145, 1731–1741 (1999).

Nie, Y. *et al*. Diverse alkane hydroxylase genes in microorganisms and environments. *Scientific Reports* **4**, (2015)

Penton, C. R. *et al*. Functional genes to assess nitrogen cycling and aromatic hydrocarbon degradation: primers and processing matter. *Frontiers in Microbiology* **4**, 279 (2013).

Rice, S. D. *et al*. EFFECTS OF PETROLEUM HYDROCARBONS ON ALASKAN AQUATIC ORGANISMS: A COMPREHENSIVE REVIEW OF ALL OIL-EFFECTS RESEARCH ON ALASKAN FISH AND INVERTEBRATES CONDUCTED BY THEAUKE BAY LABORATORY, 1970-8. 113

Rubiano-Labrador, C., Bland, C., Miotello, G., Armengaud, J., & Baena, S. Salt Stress Induced Changes in the Exoproteome of the Halotolerant Bacterium Tistlia consotensis Deciphered by Proteogenomics. *Plos One*, **10**, (2015).

Sabirova, J. S., Ferrer, M., Regenhardt, D., Timmis, K. N. & Golyshin, P. N. Proteomic Insights into Metabolic Adaptations in Alcanivorax borkumensis Induced by Alkane Utilization. *Journal of Bacteriology* **188**, 3763–3773 (2006)

Sei, K,. Sugimoto, Y., Mori, K., Maki, H. & Kohno, T. Monitoring of alkane-degrading bacteria in a sea-water microcosm during crude oil degradation by polymerase chain reaction based on alkane-catabolic genes. *Environmental Microbiology* **5**, 517–522 (2003)

Takami, H. Genome sequence of Oceanobacillus iheyensis isolated from the Iheya Ridge and its unexpected adaptive capabilities to extreme environments. *Nucleic Acids Research 30* (2002)

Uribe-Alvarez, C. *et al*. First evidence of mineralization of petroleum asphaltenes by a strain of

Neosartorya fischeri. *Microbial Biotechnology* **4**, 663–672 (2011)

Whon, T. W., Jung, M., Roh, S. W., Nam, Y., Park, E., Shin, K., & Bae, J. Oceanobacillus

kimchii sp. nov. Isolated from a traditional Korean fermented food. *The Journal of*

Microbiology **48**, 862-866 (2010)