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ISOLATION AND CHARACTERIZATION OF *SALINIVIBRIO* SP. STRAIN LP-1 FROM AN IMPOUNDMENT USED FOR MARCELLUS SHALE WASTE WATERS

A Thesis

Submitted to the Bayer School of Natural and Environmental Sciences

Duquesne University

In partial fulfillment of the requirements for

the degree of Master of Science

By

Oliver B. Dugas

May 2014

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Oliver B. Dugas

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ISOLATION AND CHARACTERIZATION OF *SALINIVIBRIO* SP. STRAIN LP-1

FROM AN IMPOUNDMENT USED FOR MARCELLUS SHALE WASTE WATERS

By

Oliver B. Dugas

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF *SALINIVIBRIO* SP. STRAIN LP-1 FROM AN IMPOUNDMENT UTILIZED FOR THE CONTAINMENT OF MARCELLUS SHALE WASTE WATERS

By

Oliver B. Dugas

May 2014

Thesis supervised by Dr. John Stolz

A euryhalic, gram-negative, facultatively anaerobic bacterium was isolated from an enrichment culture found in an impoundment used for storing Marcellus Shale waste water. Designated strain LP-1, its cells were non-spore-forming, motile, curved rods with a single polar flagellum. Phylogenetic analysis using 16S rRNA gene sequences revealed it is a γ-proteobacerium closely related (98%) to *Salinivibrio costicola*. Carbohydrates serve as energy sources both aerobically and anaerobically. It grew anaerobically on nitrate with hydrogen, acetate, pyruvate, or lactate but not with formate, and did not use arsenate, sulfate, or thiosulfate as electron acceptors. Strain LP-1 grew optimally at 37°C (range 4-65°C), 10.0% NaCl (range 0-20.0%), and a pH of 7.5 (range 4.5-10.5). It is capable of forming pellicles in liquid culture and Strontium and Barium-containing precipitates. These results suggest that the recycling impoundments contain unique microbiota that have adapted to living in a broad range of conditions.

DEDICATION

To my sister Christina, my mother, my father, and Mimi. Thank you for the continued support over the years and enduring the insanity.

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Firstly, I would like to thank Dr. Stolz for the support, mentorship, guidance, and independence. You gave me the opportunity and developed me into a researcher. I would like to thank my committee members, Dr. Castric and Dr. Porter, for their guidance and advice that shaped this finished product. I would like to thank Dean Reeder for all his help and reassuring words throughout the deadline changes. I also would like to thank Dr. Kabala for all his assistance in revising some of my writing prior to my defense. Additionally, I would like to thank the Heinz Endowment for partially funding my research.

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Chapter 1 Introduction

1.1 Introduction

With the increased amount of energy required to sustain the exponentially growing global population and the rising number of modern, industrialized societies, shale gas presents itself as a viable option to meet the climbing energy demand. Large scale measures are being taken to facilitate the extraction of commercial quantities from natural gas plays all around the world. Due to the magnitude of this pursuit, it is imperative to understand all aspects that are involved within the drilling process.

One element that affects industrial activities are the diverse microbial communities that inhabit various stages of the extraction process. These organisms can contaminate many facets of extraction activities and, although they are small in size, they possess the ability to cause a profound impact both environmentally and economically. Microbes can create productivity difficulties by causing well-biofouling, bioplugging, formation souring, and the microbially induced corrosion of industrial equipment and well-infrastructure. Despite industry efforts to eradicate these organisms with biocide treatments, they continue to form adapted communities. Additionally, the potential transport of these organisms into the surrounding environment could possibly have unseen effects on the biomass and biodiversity within local ecosystems.

Whether it is to improve industry practices or preserve the environment's wellbeing, these potential issues warrant further research to identify and distinguish the microorganisms that inhabit the unique set of conditions that are present in the natural gas extraction process.

The fluids involved in shale drilling generate an extreme habitat for organisms to survive in. They are generally characterized by their high salinity concentrations and large quantities of total dissolved solids that can include, but are not limited to, metals such as barium, strontium,

and iron. In an effort to begin identifying the inhabitants of these particular environments, research has been conducted to analyze microbial communities as a whole. Generally, these communities are composed of halophilic species, some of which are capable of forming biofilms and precipitates.

In this study, the aim was to isolate and identify a strain that populates a waste water sample associated with shale gas extraction. This strain was then to be characterized physiologically. Biofilm, particularly pellicle, formation and the produced precipitates are of particular interest because they give insight into how the organism is interacting with the conditions that surround it.

Several produced water samples were acquired by Dr. John Stolz and analyzed by J Lucas Eastham, who provided the water chemistry and enrichment culture data (Eastham, 2012). The sample that was selected for this study was an enrichment culture collected from an impoundment water sample located in Washington County, Pennsylvania. The impoundment was aerated and the sample was collected by the land owner. This culture was designated as Lone Pine (LP).

Through the techniques of serial dilution and streak plating, a strain was isolated and the 16s gene was partially sequenced to obtain the relative identity of the microbe. The isolate was named strain LP-1 and it was characterized phenotypically. Following the characterization, other experiments were performed to determine if the isolate was capable of producing pellicles and precipitates. This information can distinguish one organism that inhabits the distinctive set of conditions that are housed within a surface impoundment that contained Marcellus Shale waste water. Data and further research should be conducted give insight into the ways in which the organism can interact with both the industrial and environmental aspects of shale gas extraction.

1.2 An overview of the Unconventional Gas Extraction Process

In the last decade the United States of America has experienced a significant increase in the production of natural gas due to the recent developments in extraction technologies, such as horizontal drilling accompanied by the use of hydraulic fracturing (Parliamentary Office of Science and Technology, 2011). Horizontal drilling is the process of drilling a gas well from a surface to a subsurface location vertically just above a natural gas reservoir. The well bore then deviates from the vertical plane around a curve to enter and traverse the gas reservoir horizontally at a slight inclination (Department of Mineral Resources). This process is utilized because most gas reserves are more extensive in their horizontal dimensions rather than their vertical dimensions (Department of Mineral Resources). This method is being used widely in shale gas extraction, which has increased from contributing only 2% of the US production of natural gas in 2000 to 14% in 2009 (Parliamentary Office of Science and Technology, 2011). It is predicted to continue rising to more than 30% of the total US natural gas production by 2020 (Parliamentary Office of Science and Technology, 2011).

Multilateral drilling is the newest technological advancement from horizontal drilling. This is the process of drilling several lateral wells in one well pad (Yanping et al., 2009). This allows for an increase of gas drainage area, improvement of well production, and greatly reduces reservoir development cost (Yanping et al., 2009). Prior to December 2006, there were more than 8,000 multilateral wells all over the world (Yanping et al., 2009). That number is continuing to grow. These multilateral wells can stem in multiple directions from underneath a drilling pad. These locations house wellheads for a number of horizontally drilled wells (EIA, 2012). The benefit of a drilling pad is that it increases efficiency for operators so they can drill multiple wells in a shorter amount of time rather than just one well per site (EIA, 2012).

Natural gas is extracted directly from shale, a sedimentary rock that is characterized by its low permeability. The gas is not easily released from the shale and so the rock must be fractured to free it, especially to yield commercial volumes (Parliamentary Office of Science and Technology, 2011). Hydraulic fracturing is used to fulfill this purpose by improving gas flow and yield. This is accomplished by pumping a solution of water, a propping agent, and chemicals into the well at very high pressures. When the pressure exceeds the rock's strength, the fluids open up and create a latticework of fractures in the shale adjacent to bore hole (Parliamentary Office of Science and Technology, 2011). Typically, these fractures will extend 100 meters or more away from the well (Parliamentary Office of Science and Technology, 2011). The propping agent, which is typically sand, is pumped into the fractures to keep them from closing when the pumping pressure is released (EPA, 2012).

Once the fracturing process is completed, the internal pressure of the geologic formation forces the injected fracturing fluids to rise to the surface through the well casing (Arthur et al, 2009). This process can last from a few days to a couple of weeks. The fluid recovered from the fracturing process is referred to as "flowback" water initially and as "produced" water subsequently; due to the elongated time within the formation, produced water begins to possess more characteristics within the shale formation (Rose et al., 2013). These fluids are generally characterized by their high salinity and total suspended solid (TSS) levels.

The waste water can be disposed of in a number of ways that include discharging into surface waters or underground injection (EPA, 2012). Additionally, the waters can be stored in tanks or pits prior to disposal or recycling.

1.3 The Geology of the Marcellus Shale

The Marcellus Shale is composed of organic-rich black shale that was formed from an oxygen-deficient marine environment during the Middle Devonian period, approximately 380 million years ago (Engelder and Lash, 2008). It has been a source for many conventional oil and gas reservoirs in the Appalachian basin, but recently is being used as an unconventional reservoir (PA DCNR). The formation covers an area of 240,000 km^2 (95,000 mi^2) encompassing a large portion of Pennsylvania; eastern West Virginia; and parts of New York, Ohio, and Maryland (Kargbo et al. 2010).

Black shale formations are typically created due to crustal loadings from fluctuating sea levels and tectonic movements (Engelder and Lash, 2008). These movements create deep to shallow evaporate conditions, where seawater becomes trapped between layers of sediment, not allowing for the complete migration of seawater through pore spaces (Engelder and Lash, 2008). It is believed that rather than forming through a true evaporation process, the Marcellus Formation was deposited in a deep inland sea basin under anoxic conditions when the reflux of seawater was limited (Blauch et al., 2009).

Black shale, like the Marcellus formation, is typically composed of mineralogical components such as quartz, feldspar, pyrite, mica, clay minerals, sulfides, organic matter, and minor amounts of carbonate, phosphate as well as other accessory minerals (Roen, 1984). The dark color of the shale comes from its rich organic content. The organic content of Devonian Shale ranges from 0.5% to nearly 20% (Roen, 1984). In the Appalachian basin, the organic content is believed to be established from marine as well as terrestrial sources (Roen, 1984). Phytoplankton were the primary source of organic matter in the Marcellus Shale (Riding and Awramik, 2000). Primary productivity from bacteria rapidly degraded the excess organic matter

left by the bloom, creating an oxygen depleted, benthic environment (Riding and Awramik, 2000). This precipitous microbial activity and high brine concentrations created dead-zones that preserved organic materials (Blauch, 2009). Additionally, density differences stratified the water column further, facilitating anoxic conditions in the benthic zone (Blauch, 2009).

The surplus of organic content accompanied by low quantities of oxygen provide a unique habitat for a multitude of life forms that survive using a variety of metabolic pathways throughout the stratified water column (Riding and Swarmik, 2000). Anaerobic heterotrophic bacteria dominate the degradation of the organic carbon below the redox boundary (Riding and Swarmik, 2000). These organisms can be metabolically facultative or obligate anaerobes that utilize degraded organic compounds. Electron acceptors employed in the anaerobic respiration process are sulfate, nitrate, bicarbonate, and carbon dioxide (Riding and Swarmik, 2000).

Production data suggest that recoverable reserves from Marcellus Shale could be as large as 489 trillion cubic feet (Tcf) (Kargbo et al. 2010). The potential for activity and output from this formation is very great and so it is important to understand the chemical and biological aspects within the shale play to determine how it interacts with both industry and the environment.

1.4 The Chemical Composition of Shale Waste Waters

Hydraulic fracturing is a stepwise process that utilizes millions of gallons of water-based fracturing fluids that are pumped into the target formation in a controlled manner (Arthur et al. 2008). The main component of these mixes is water, but they also contains a variety of functioning additives.

Acids, such as hydrochloric acid or muriatic acid, are used to dissolve sand and minerals to initiate cracks in the formation (Arthur et al. 2008, Murphy and Ramundo, 2010). Biocides

like glutaraldehyde are used to control bacterial growth and prevent the establishment of microbial communities that can damage industrial equipment and infrastructure (Arthur et al. 2008, Murphy and Ramundo, 2010). A gelling agent, such as guar gum or hydroxyethel cellulose, is included to increase the viscosity of the fluid (Arthur et al. 2008, Murphy and Ramundo, 2010). Sodium chloride is utilized as a breaker to delay the breakdown of the gelling agents (Arthur et al. 2008, Murphy and Ramundo, 2010). A corrosion inhibitor, such as N,N-dimethyl formamide, is used to prevent the corrosion of steel equipment (Arthur et al. 2008, Murphy and Ramundo, 2010). A fiction reducer like petroleum distillate is added to reduce the friction between the fluid and the pipes (Arthur et al. 2008, Murphy and Ramundo, 2010). An iron control agent, like 2-hydroxy-1,2,3-propanetricarboxylic acid, is utilized to prevent metal oxide precipitation (Arthur et al. 2008, Murphy and Ramundo, 2010). An additive called an oxygen scavenger, such as ammonium bisulfate, is used to remove oxygen to prevent further corrosion of equipment (Arthur et al. 2008, Murphy and Ramundo, 2010). A proppant agent, such as silica or quartz sand, is utilized to prevent the produced fractures from closing, allowing the gas within the shale to be released (Arthur et al. 2008, Murphy and Ramundo, 2010). An additive called a scale inhibitor, such as ethylene glycol, is used to reduce salt deposition on the pipes (Arthur et al. 2008, Murphy and Ramundo, 2010). Finally, a pH adjuster, such as potassium or sodium carbonate, is used to maintain pH for the functioning of the crosslinker (Arthur et al. 2008, Murphy and Ramundo, 2010). Some of these additives, such as the gelling agents and polyacrylamide-based friction reducers for example, are a readily available food source for the microbial communities that inhabit these flowback and produced waters (Fichter et al., 2012)

Data provided by the Palmerton Environmental Group (for the Pennsylvania Department of Environmental Protections), Blauch et al., 2009, and Eastham, 2012 suggests that flowback water has particularly high levels of total dissolved solids (TDS) in the form of chloride salts. Major ions include chloride, calcium, sodium, and significant quantities of barium and strontium. Additionally, flowback water contained quantities of metals and metalloids such as arsenic, lead, chromium, selenium, silver, lithium, and zinc (Palmerton Environmental Group) (Table 1.1).

Table 1.1 Produced Water Analyses for Marcellus Shale Play

(Eastham, 2012)

	Unit	*PADEP - Water Analysis Palmerton Group 2010*		*Blauch et al. 2009 Southwest PA Well*
		Frac Fluid (Before)	Flowback (After)	Late Stage Flowback Water
Specific Conductivity	umhos/ cm	362 -34,600	8,740 - 570,000	NA
pH		5.2-7.8	5.5 -7.8	6.0
Alkalinity	mg/L	126	37 -577	880
Sulfate	mg/L	ND - 2,920	3.1 - 348	24
TDS	mg/L	221-27,800	5,090 -264,000	NA
Bromide	mg/L	ND - 107	35.5 -1,600	NA
Chloride	mg/L	30.7 - 3,560	2,460 - 181,000	60,925
Barium	ug/L	0.075 - 9.81	0.243 - 5150	87,000
Calcium	mg/L	9.9 -329	11.6 -16,4000	8,360
Iron	ug/L	0.137 - 14.3	0.192 - 242	70 (mg/L)
Magnesium	mg/L	1.36 - 235	1.84 -1,380	524
Potassium	mg/L	ND - 0.0574	0.177 - 2,820	1,288
Strontium	ug/L	0.206 -439	9.58 - 5,410	1,441
Sodium	mg/L	25.7 - 6,190	0.58 - 64,900	30,503
Metals:				
Arsenic	ug/L	ND - 0.111	0.112 -14	
Lead	ug/L	ND - 0.111	0.003 - 0.596	
Lithium	ug/L	ND - 14.9	0.0338 - 201	
Silver	ug/L	ND - 0.125	0.0007 - 0.123	
Selenium	ug/L	ND - 0.0353	ND -0.0956	
Chromium	ug/L	ND - 0.050	0.0075 - 0.359	
Zinc	ug/L	0.0348 - 0.0457	0.0257 - 2.93	

It is proposed that the high levels of TDS in the produced waters is a result of the dissolution of shale content from the added acid or because of the mixing with the brine solution that is prevalent in the Marcellus Shale formation (Blauch et al. 2009). This is supported by a study conducted by Rose et al., 2013 that stated that the chemistry of late-stage flowback water, or produced water, was similar to in-situ brine typically found in conventional oil and gas wells (Rose et al., 2013).

The hydraulic fracturing operations and the storage of the shale waste waters are being met with rising public health concern and regulatory challenges. This is due to the potential environmental impacts, especially in regards to surface and ground water quality, increasing demand for fresh water, and the lack of safe disposal mechanisms (Blauch et al. 2009). Locally, elevated levels of chloride, an indicator of elevated TDS levels, have been observed in the Monongahela River (Rose et al. 2013). This can be supported by the Warner et al., 2013 study that reviewed the impacts of Shale Gas waste water disposal on the water quality in Western Pennsylvania. This was accomplished by analyzing the discharge eluents from the Josephine Brine Treatment Facility and the stream water and sediments of both upstream and downstream regions from the discharge site on a stream named Blacklick Creek, located in the Ohio River Basin (Warner et al., 2013).

They found that the discharge of wastewater eluent to surface water had a discernible impact on the water quality (Warner et al., 2013). The chloride concentrations 1.7 km downstream of the treatment facility were 2−10 times higher than any chloride concentrations recorded in any background western PA streams that were examined (Warner et al., 2013). The calculated average yearly enrichment factor (EF) of chloride in the stream was 4.6 times background concentrations (Warner et al., 2013). This data supports recent studies that suggest

that treatment facilities have an impact on concentrations of chloride throughout western PA. The results also demonstrate that even the 500−3000 dilution of the wastewater eluent is not sufficient to reduce bromide content to background levels (Warner et al., 2013). This means that the discharge of wastewater could potentially increase the concentrations of Br downstream drinking-water treatment facilities (Warner et al., 2013). Their data displayed that the geochemical signature of Marcellus wastewater is apparent, even after treatment, in the eluents from the treatment facility and in the downstream water and sediments (Warner et al., 2013). The majority of elemental chemistry and isotopic ratios in the treated waste water eluents during 2010 and 2011 were similar to the compositions of flowback and produced waters from the Marcellus shale gas operations (Warner et al., 2013). It was found that despite treatment, the isotopic ratios in the eluent can still be used as tracers for delineating the sources of oil and gas waste waters (Warner et al., 2013).

This is a direct reflection of the unique regional problem that is being experienced in the state of Pennsylvania. The limited capacity for underground injection wells and the fact that there are currently no water treatment facilities that are equipped to treat these highly saline flowback and produced waters, accompanied by the increased drilling activity and the shrinking regulated discharge limits, restrict the ability to dispose of these fluids. This poses a serious economic and technical challenge for operators (Baluch et al, 2009).

1.5 Impoundment Sites

Impoundments are temporary waste containers that are used to store flowback and produced fluids that are expelled from the geological structure after the fracturing process is complete. These fluids are stored to allow companies to recycle them in the fracturing process over again. The containers are all engineered differently and come in a variety of forms given

that they are constructed by the various operators that use them. “Open-air”, surface impoundments are commonly used to house these fluids. They are not sealed off and lack a covering structure. The use of “open-air” impoundments is becoming quite controversial recently because of their open nature, allowing for the chemicals contained within the fluids to interact with the surrounding environment. This interaction potentially allows for the exposure of hazardous substances to the atmosphere, soil, and ground water.

In the state of Pennsylvania, legislation is currently being discussed in the House of Representatives to ban the use of open air impoundments (or pits) on the Marcellus and Utica Shale formations (White, 2013). These pits are no longer being considered an industry best practice and larger scale operators are beginning to consider the elimination of their use. A recently formed coalition named the Center for Sustainable Shale Development, that includes industry partners such as Shell, Chevron, CONSOL and EQT, is attempting to develop newly formulated industry standards and practices (White, 2013). Smaller-scale operators, on the other hand, will not be bound by these standards and will have the option to continue the use of impoundment structures (White, 2013). State regulators around the country have already begun to create policies that restrict their use. States such as North Dakota have already banned them and other drilling-friendly states, such as Texas, are seeking to discontinue their use as well (White, 2013).

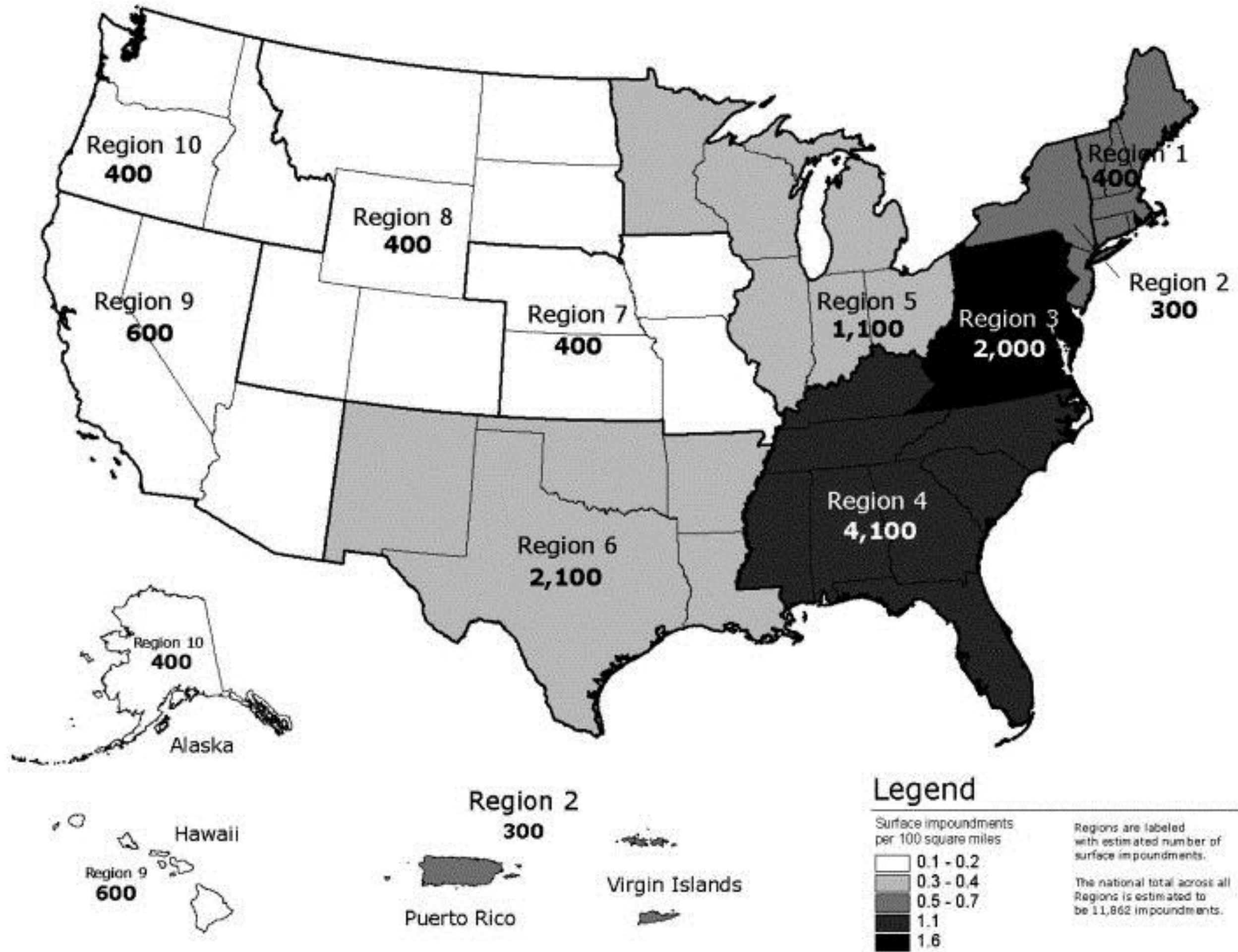
The vulnerability lies in the transportation of their contained substances into the environment. Hypothetically, even if an impoundment is completely secure of leaks, the water has the ability to evaporate promoting chemical emissions into the air (White, 2013). These emissions can potentially pose a serious health hazard for local residents, but despite this threat

provisions under Act 13 permit the use of water impoundments in all zoning districts, including residential areas (White, 2013). This does not give municipalities the authority to regulate them and hinders them from knowing about their construction and use within their communities (White, 2013).

One local case includes a 12,000,000 gallon wastewater impoundment that spilled in Washington County. This impoundment was legal under Act 13 provisions but was unknown to local officials because it was constructed and concealed on top of a hill (White, 2013). The impoundment was originally built to hold freshwater but was converted through a simple permitting process by the Department of Environmental Protection (DEP) to legally store recycled waters (White, 2013). The impoundment permitting process in the state of Pennsylvania is under much review given that they are used very frequently in the region. A geographic analysis of surface impoundments display that Pennsylvania is included in the region that possesses the largest quantity of impoundment in the country at approximately 1.6 per square mile and an overall estimated quantity of 2,000 (Figure 1.1):

Figure 1.1: The Regional distribution of surface impoundments

(Johnson et al., 2003).



(Reprinted with Permission)

As the figure above displays, the use of open-surface impoundments on the Marcellus Shale formation, particularly in the state of Pennsylvania, is quite prevalent and local concern is on the rise.

A study that reviewed 11,900 surface impoundment sites that contained non-hazardous wastewaters showed that 19–46% of facilities still released chemicals of concern into the

environment (Johnson et al., 2003). It was also estimated that mercury was present in 30% of the impoundments' waters and in 66% of their sludge (Johnson et al., 2003). Furthermore, approximately 5950 impoundments that managed non-hazardous wastewaters contained volatile organic compounds (VOCs); these impoundments contained approximately 75% of all wastewaters in the nation (Johnson et al., 2003).

The release of these chemicals can take a variety of transportation routes into the environment through mechanisms that include volatilization, dispersion, and leeching. In the process of volatilization a pollutant becomes an air borne vapor that can contaminate the atmosphere. The dispersion and leeching of a pollutant allows for contaminants to be transported into the surrounding ground water, surface water, and soil. Unfortunately, it is very complicated to determine the interaction of these sites with the surrounding environment making it difficult to supply policy makers with the data that is needed to make informed regulatory decisions.

A six year study was conducted that collected data on groundwater flow and chemistry through observation wells and suction lysimeters around a surface impoundment that was used to hold produced water in the Powder River Basin in Wyoming. The complex geochemistry and groundwater flow paths at the study site confounded the ability to assess the impacts of the surface impoundments on the local water resources. The data displayed that sulfate, bicarbonate, and magnesium were the dominant ions and there was substantial variability in their relative concentrations depending on the site and collected sample (Healy et al, 2011). The study also found that the pH of the surrounding waters varied from less than 3 to more than 9 and the contained total dissolved solids ranged from quantities of less than 5000 to greater than 100,000 mg/L (Healy et al, 2011). Due to all the external variables in the study it was hard to conclusively draw a connection between these erratic conditions to the impoundment itself. Even

so, the possibly unpredictable nature of these impoundment sites makes the organisms that inhabit them fascinating. Many of the species that make up the diverse microbial communities within these fluids possess the ability to adapt to a wide variety of chemical and physical conditions. The versatile metabolic processes of these organisms can be seen as a cause of concern in regards to their possible ability to inhabit environments outside of impoundments given their adaptability to an assortment of conditions. The possible transmission of these microbes into the surrounding environment could potentially impact the biomass and biodiversity of local ecosystems.

1.6 The Microbial Diversity of Shale Waste Waters

The unique composition of the shale waste waters, such as the high levels of total enriched organic matter, biodegradable polymers, metals, metalloids, and large quantities of salts generate a distinctive habitat for a variety of halophilic and/or halotolerant organisms that inhabit them (Struchtemeyer and Elshahed, 2011). The oil and gas industry faces microbial contamination that can drastically affect many of the industry processes (Fitcher et al., 2008). Some of the most common issues that emerge from microbial activity include the altered fate of heavy metals; the formation of odorous and toxic compounds; the biocorrosion of equipment; complications in wastewater management; and the increased overall costs of gas production (Mohan et al., 2013). To aid in mitigating these challenges it is important to distinguish where and how these microbes are making their way into the drilling processes.

There have been multiple avenues identified for the introduction of microorganisms into the drilling process. One possible route is through the water used to make the fracturing mixtures (Fichter et al., 2008), but for this to be a possible the bacteria within the water would have to survive the biocide treatments that are performed prior to the fluid being pumped down well and

then the higher salinities concentrations that would subsequently follow (Fichter et al., 2008). Another potential microbial source is by the drilling mud used for the fracturing wells (Struchtemeyer et al., 2011). Drilling muds are prepared from prepacked powder compounds that could provide carbon and sulfate sources for microorganisms to grow on (Struchtemeyer et al., 2011). After the fracturing is complete, a significant portion of the mud remains in the formation, which could potentially serve as an inoculum of exogenous microorganisms deep in the formation (Struchtemeyer et al., 2011). Additionally, the stimulation caused by the drilling activity itself could promote the growth of indigenous microbial populations within the formation (Eastham, 2012). Temperatures deep within the Marcellus formation range from 35-51ºC (Kargbo et al., 2010), which are suitable temperatures for many species to inhabit.

There has been some research conducted to analyze the microbial communities that are present in samples taken from hydraulic fracturing operations on the Marcellus Shale formation. One study evaluated the microbial populations that developed at different depths of impoundments that were managed using different pretreatment strategies (Mohan et al., 2013). The results revealed that the microbial communities inhabiting the impoundments were particularly diverse; the most prominent organisms being halotolerant species among the α- and γ- subdivisions of the Proteobacteria and Firmicutes. Many of these species have been identified within the produced waters of oil and gas industry (Struchtemeyer and Elshahed, 2011, Mohan et al., 2013). The predominance of halotolerant species suggests that the high levels of salinity in impoundment waters may prohibit the colonization of freshwater species that are introduced from source water or from the environment, such as species that are introduced from meteorological precipitation or from the atmosphere above the waters (Mohan et al., 2013). The composition of these microbial communities additionally varied with depth and management

strategy (untreated, biocide amended, and pretreated and aerated) of the selected impoundment. This denotes that geochemical and ecological factors such as temperature, salinity, oxygen tension, and fluid properties could impact the microbial population diversity (Joshi, 2013).

Members of the α-proteobacteria were found to dominate the surface depths of untreated impoundment sites and within all depths of pretreated and aerated impoundments (Mohan et al., 2013).The genetic sequences found in these samples closely related to members of the genus *Roseovarius*. These species are characterized by their organic iodine oxidation capabilities in the presence of oxygen. The formation of elemental iodine by populations of iodine oxidizing bacteria may produce bactericidal effects and thus limiting the bacterial population diversity in the produced wastewaters from natural gas production facilities (Lim et al. 2011). They could also possibility be the source of bioplugging in the re-injection wells of wastewaters generated from water-dissolved natural gas fields (Sugai et al., 2013). Members of the γ-proteobacteria tended to dominate the middle and bottom depths of untreated impoundments as well as in the surfaces depths of the biocide amended impoundments. A majority of the γ-proteobacteria sequences were most similar to the genus *Marinobacter* that have been isolated from oil fields (Mohan et al., 2013). Some species within this genus can degrade hydrocarbons and other aromatic compounds. They can be strict aerobes (*Marinobacterium georgiense* strain KW-40) or falcultative anaerobes (*M. hydrocarbonoclasticus*) (Mohan et al., 2013). Members of the class Clostridia composed a major portion of the communities that inhabited the biocide amended impoundments. Sequences collected from these facilities are most similar to *Halanaerobium congolense* isolated from oil fields in Africa (Mohan et al., 2013). *Halanaerobium* species are fermentative halophiles that ferment saccarides to H_2, CO_2, and acetate (Mohan et al., 2013).

Additionally, various sulfidogenic taxa were identified in both untreated and biocide amended impoundments that include *H. congolense*, *Thermotogae*, and *Desulfobacter halotolerans* (Mohan et al., 2013). Members within these taxa utilize various sulfur compounds such as sulfates, elemental sulfur, thiosulfate, and sulfite (Mohan et al., 2013). Both treated and untreated impoundments harbored fermentative species such as *Clostridia*, *Thermotogae*, and *Synergistia* (Mohan et al., 2013). The process of fermentation results in the production of organic acids such as acetate. Sulfidogenic or Sulfate-Reducing bacteria (SRB) and acid producing bacteria (APB) create a substantial amount of concern in the industry because of their ability to produce biogenic sulfide, hydrogen sulfide, and iron sulfide (Fitcher et al., 2008, Fitcher et al. 2012). These compounds can create a multitude of operational issues such as microbially influenced corrosion (MIC) and the predegradation of the fracturing fluids (Fitcher et al. 2012). The presence of biogenic sulfide can lead to formation souring, iron sulfide can cause bioplugging (Fitcher et al. 2012), and hydrogen sulfide is a risk to human health and aesthetics (Mohan et al., 2013).

Archea were also detected in untreated and treated impoundments (Mohan et al., 2013). The sequences that were acquired were associated to *Methanoplanus*, *Methanophilus*, *Methanocalculus*, and *Methanolobus*. All of these taxa are composed of halophilic, methylotrophic, and hydrogenotrophic methanogens that have been found in multitude of oil and gas plays (Mohan et al., 2013). It has been discovered that a consortia of methanogenic microbes are degrading organic-matter in deep subsurface reservoirs, such as shale plays, under anaerobic conditions. This organic-matter consists of simple carbon molecules, such as CO_2 and acetate (Schlegel et al., 2013). These substrates are then subsequently metabolized into economic quantities of natural gas in sedimentary basins world-wide (Schlegel et al., 2013). Additionally,

wells that were recently fractured on the Antrim Shale formation, for example, were dominated by species of methylotrophic methanogens that are capable of utilizing high concentrations of methanol stemming from the fracturing fluids (Wuchter et al., 2013).

In 2012, a study was conducted on the impact of commercial natural gas production on the geochemistry and microbiology of the Antrim Shale, an unconventional gas reservoir in Michigan (Kirk et al., 2012). What was found was that CO_2-reducing archea were one of the most abundant groups in the archaeal clones libraries and sulfate-reducing bacteria were the most abundant group in the bacterial clone libraries (Kirk et al., 2012). Due to several different microbial, geochemical, and industrial interactions, they found that, although gas that is commercially being produced due to the reduction CO_2, the concentration of SO_4^{2-} and abundance of SO_4^{2-} reducing bacteria have increased (Kirk et al., 2012). This may ultimately allow for the SO_4^{2-} reducing bacteria to displace the methanogen population (Kirk et al., 2012).

The addition of SO_4^{2-} is also causing other changes in the chemical and isotopic composition of the gas and water within the shale formation (Kirk et al., 2012). In 2013, another study was conducted on the Antrim Shale formation that confirmed some of these changes. The study found that Bacterial sequences greatly outnumbered those of archea in the observation wells that were sampled and that they shared the highest similarity to the mesophiles and moderate halophiles within the Firmicutes, Bacteroidetes, and the δ- and ε-Proteobacteria (Wuchter et al., 2013).

1.7 Halophiles

Waste waters from shale plays are dominated by a wide variety of halophilic microbes that are capable of surviving in high TDS environments (Struchtemeyer and Elshahed, 2011,

Mohan et al., 2013, Kirk et al., 2012). The domains of Archea, Bacteria, and Eukarya all include halophilic species that are capable of inhabiting a multitude of hypersaline habitats (DasSarma and DasSarma, 2012). Halophiles are classified depending on their sodium chloride requirement that range from slightly, moderately, to extremely halophilic (DasSarma and DasSarma, 2012). A halotolerant organism has the ability to grow in saline environments but does not require sodium chloride to grow (DasSarma and DasSarma, 2012). Slight halophiles grow optimally in a NaCl range of 2-5%, moderate halophiles prefer a range of 5-20% NaCl; and extreme halophiles require 20-30% NaCl to grow optimally (DasSarma and DasSarma, 2012).

An environment is deemed hypersaline if they contain NaCl in an excess of that of seawater, which is 3.5% or 35 g/L (DasSarma and DasSarma, 2012). Additionally, hypersaline bodies are sub-classified as either thalassic or athalassic (DasSarma and DasSarma, 2012). Both environments are similar in respect to their high salinities, but thalassic environments contain proportions of salt similar to that of sea water, while athalassic, like the Dead Sea, tend to deviate (DasSarma and DasSarma, 2012). A majority of hypersaline bodies are thalassic; these bodies form due to the evaporation of seawater and contain significant quantities of sea salts (DasSarma and DasSarma, 2012). Thalassic bodies primarily contain sodium and chloride, but they also include high levels of potassium, calcium, magnesium, as well as other metal ions (Edwards et al., 1989 and Stackebrandt et al., 1993). Many halophilic species are from diverse taxa and can inhabit a variety of unique conditions.

Halophilic archea of the order *Halocateriales* are extremely halophilic and are found in abundance in hypersaline environments such as the Dead Sea and other soda lakes around the world (Oren, 2002). Species like *Halorhabdus tiamatea* are polyextremophiles in that they have the ability to tolerate high temperatures and extreme salinities (Oren, 2002).

Halophilic bacteria span the taxa of several taxonomic branches that consist of Proteobacteria, Cyanobacteria, Actinobacteria, Spirochetes, and members of the *Flavobacterium Cytophaga* group (Oren, 2002). Many of these halophilic species are fermentative, sulfate-reducing, and methanogentic and these organisms are of a specific interest to the oil and gas industry.

To flourish in hypersaline conditions, halophiles and halotolerant organisms need to establish an osmotic equilibrium across their membrane and regulate the osmotic strength of their cytoplasm so that it is at least equal to the surrounding medium (Galinski and Truper, 1994). Halophiles have developed a variety of osmotic adaptations for highly saline environments (Qurashi and Sabri, 2007). Studies show that an excess of sodium ions are excluded from the cell using Na+/H+ antiporters, although the reason for this transport is unclear (Oren, 2002).

There are two main mechanisms used to maintain osmotic balance. The first mechanism is typically used by the members of Halobacteriaceae. These organisms maintain osmotic pressure by the accumulation of K+ and Cl- ions in concentrations similar to salt concentration in the medium that surrounds them (Galinski and Truper, 1994). Fermenting archea, acetogenic anaerobes (*Haloanaerobium, Acetohalobium*species), and sulfate reducers (*Desulfovibrio halophilus, Desulfohalobium retbaense*) are examples some organisms that utilize this mechanism (Galinski and Truper, 1994). The second means of osmoadaptation involves the accumulation of compatible solutes in the cytoplasm to maintain osmotic balance. This mechanism is wide-spread among eubacterial halophiles (Galinski and Truper, 1994). Compatible solutes used for this purpose range from glycerol; amino acids; to derivatives such as glycine betaine, and ectoine, (Oren, 2002, Galinski and Truper, 1994).

1.8 Microbial Induced Problems of the Oil and Gas Industry

The microbiology within the petroleum industry plays an imperative role in determining the efficiency of the operations that are conducted. Problems that occur in the upstream sector can affect enhanced oil recovery and cause reservoir souring, while issues that happen in the downstream stages can affect product storage and produced water management. Additionally, the presence of some of these microbes could include positive impact potentially through their role in bioremediation (Energy institute, UK).

1.8.1 Microbial Influenced Corrosion and Bioplugging

Iron is a widely used metal in a variety of industrial components such as drilling pads, refineries, gas-fractioning plants, pipeline systems, and exporting terminals (Uchiyama et al., 2010, AlAbbas et al., 2012). The corrosion of iron products can lead to economic damages, which equate to around 276 billion dollars annually in the United States (Mori et al., 2010). Iron corrosion occurs due to pure physicochemical reactions and by microbial metabolic processes, deemed microbiologically influenced corrosion (MIC). MIC is responsible for about 10% of the total damage of metal corrosion (Mori et al., 2010). The process of MIC causes pitting, crevice corrosion, cracking, and dealloying of industrial infrastructure (AlAbbas et al., 2012). It is believed to be responsible for about 75% of total corrosion in productive oil wells and for more than 50% of pipeline system failures (AlAbbas et al., 2012). It was also one of the two major factors for the shutdown of the major Alaska Prudhoe Bay oil field pipeline in 2006 (AlAbbas et al., 2012).

Conventional natural gas fields are not typically at a great risk for MIC problems, but a large amount of treated produced water puts unconventional gas reservoirs at risk like other oil reservoirs (Kuijvenhoven and Wang, 2011). Structures like concrete pipes, stonework, and

cooling towers are also degraded by high concentration of sulfuric acid and low pHs as a result of aerobic growth of microorganisms, such as *Thiobacillus* (Hamilton, 1985). Sulfate reducing bacteria (SRB) are considered a major reason for anaerobic corrosion on iron surfaces (Uchiyama et al., 2010).

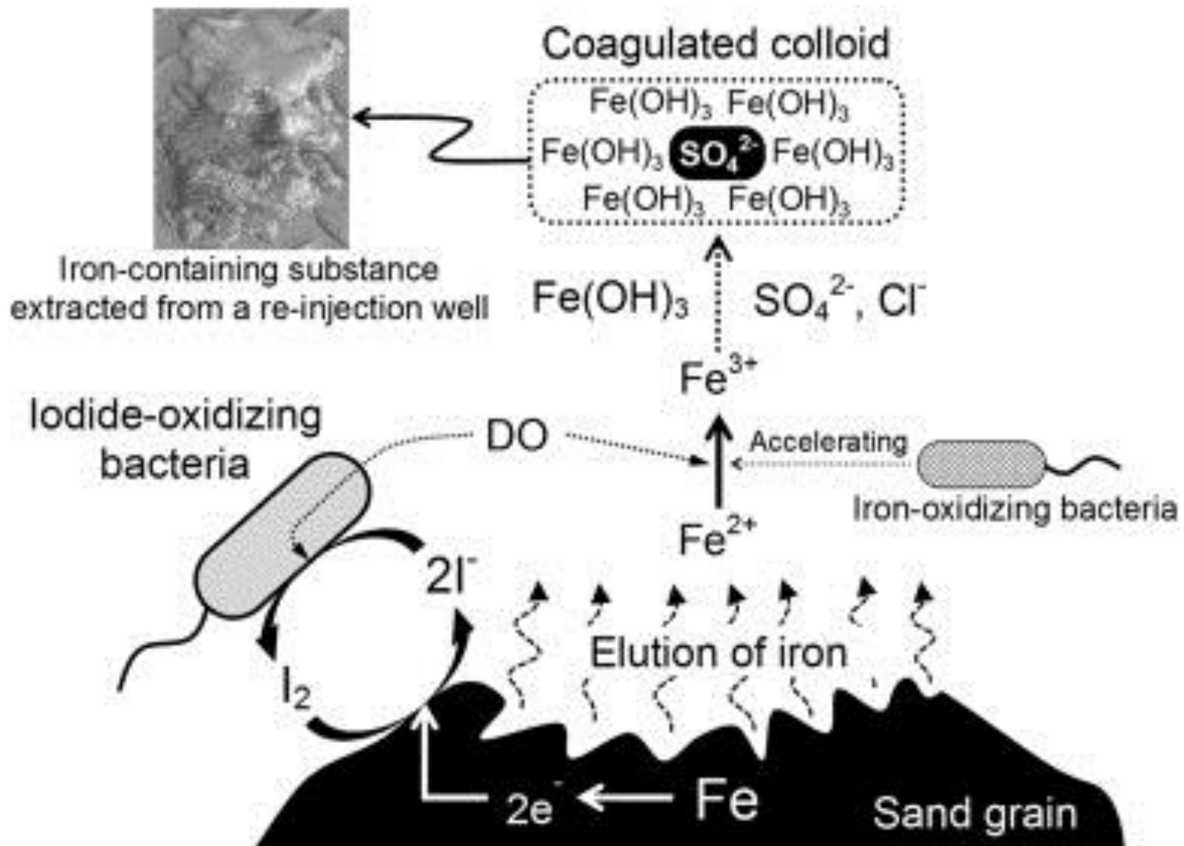
Corrosion is an electrochemical phenomenon and many theories have been offered to describe the mechanisms that are involved in the process. Van Wolzongen Kuhr and Van Der Vlugt (1934) were the first to explain microbiologically induced corrosion influenced by SRB under anaerobic conditions. According to this theory, anode produces $Fe2^{+}$ and electrons, which are consumed at the cathode. Under anaerobic conditions, H^{+} from water is reduced to hydrogen gas at the cathode (Mori et al., 2010). SRB present in biofilms near metal surface consume this hydrogen, facilitating further dissolution of metal, and their metabolic products are deposited as FeS (Mori et al., 2010). In theory, all hydrogen consuming bacteria can promote corrosion, but this still needs to be proven (Mori et al., 2010).

Bioplugging is the blocking of a well due to microbial activity (Alford et al., 1999). These are typically caused by iron related bacteria (IRB), sulfate reducing bacteria (SRB), slime-forming bacteria (SLYM), heterotrophic aerobic bacteria (HAB), denitrifying bacteria (N), coliform bacteria (COLI) (Alford et al., 1999), and now recently investigated iodine-oxidizing bacteria (Sugai et al., 2013). Typically, these bacteria grow in complex consortia within a confined, slimy or concretious structure (Alford et al., 1999). It is important to note that each plug is likely to be composed of a number of biofilms that are part of microbial growth (Alford et al., 1999).

A recent study was conducted on the possibility of microbial clogging in re-injection wells of the waste water generated in a water-dissolved natural gas field. Brine produced from

water-dissolved natural gas reservoirs contains high levels of iodine (Sugai et al., 2013). This iodine is recovered using sulfuric acid and oxidizing agents; these chemicals stimulate microbial activity that may cause clogging (Sugai et al., 2013). Their results showed that the columns that they injected with brine containing indigenous microorganisms, dissolved oxygen, and iodine clogged significantly and were dominated by iodine-reducing bacteria (Sugai et al., 2013). The iodine produced by these microbes corroded the iron in the sand under the presence of dissolved oxygen and the eluted iron that formed ferric hydroxide colloids in the brine causing the clogging of pore spaces (Sugai et al., 2013). A schematic of the clogging mechanism suggested by the study is displayed below (Figure 1.2):

Figure 1.2 Schematic image of the clogging mechanisms
(Sugai et al., 2013)



(Reprinted with Permission)

1.8.2 Biocide Resistance

Biocides are used at multiple stages of the fracturing process. Gultaraldehyde, isothiazolin, tetrakis (hydroxymethyl) phosphonium sulfate (THPS), 2, 2-dibromo-3-nitrilopropionamide (DBNAP), sodium hypochlorite, didecyldimethylammonium chloride, tri-n-butyl tetradecyl phosphonium chloride, and a blend of gutaraldehyde and alkyldimethylbenzylammonium chloride are commonly utilized compounds within the industry (Fichter et al., 2008, Elshahed et al., 2012).

Initially, biocides are added to fracturing fluids (approximately 0.001%) to prevent the degradation of viscosity building polymers. This ensures that the fracturing fluids function properly (Arthur et al., 2009, Elshahed et al., 2012). Flowback water is typically reused multiple times in the unconventional extraction process and so biocides are added continuely at multiple points of its use. This includes the addition to the fluids that are stored in impoundments to prevent establishment of bacterial populations (Mohan et al, 2013). Despite the addition of these chemicals, bacterial communities still establish in industry infrastructure (well heads, formation, pipelines, and impoundments) (Elshahed et al., 2012).

Potential causes for bacterial contamination in produced waters and wells range from the open nature of the impoundments that are exposed to the atmosphere and meteorological precipitation (Fichter et al., 2008) to the reuse of the fracturing fluids that increases the number of opportunities for bacterial contamination and promotes the growth of biocide resistant strains due to increased exposure antimicrobial agents (Mohan et al., 2013). Biodegradation is another possible reason for the reduced effectiveness of biocides in flowback water (Mohan et al., 2013). Biocides used by the industry are less effective in the presence of the high levels of organic content (>10 mg/L) contained within the flowback water (Elshahed et al., 2012). In a study

conducted by Elshahed et al., 2012 indicated that biofilm formation by sulfate-reducing bacteria combined with organic loading rates negatively impacted the efficacy of the used biocides.

The bacterial resistance to biocides also depends on several factors such as temperature, pH of the medium, nutrient limitation and the species of organism (Morton et al., 1998). Two main mechanisms by which bacteria exhibit biocide resistance are intrinsic and acquired resistance (Morton et al., 1998). Acquired resistance is specific to a chemical agent and the selection of a resistant strain from the population. This occurs through plasmid transfer or transposon coding genes in the bacterial cells (Morton et al., 1998). Intrinsic resistance is the inherent structural and functional characteristics that allow for a strain to resist the antimicrobial agents. Intrinsic resistance is considered a more common mechanism in natural environments (Morton et al., 1998).

Morphological factors play a role in the ability for a bacterial strain to be resistant. Gram-positive bacteria such as *Bacillus* and *Clostridium* species form a dormant, resistant morphological form called endospores (Leggett et al., 2012). Bacterial spores are highly resistant to physical and chemical biocide treatments (Leggett et al., 2012, Morton et al., 1998). The resistance is attributed to a combination of structural, chemical, and biochemical makeup of the spores (Leggett et al., 2012). Gram-negative bacteria strains typically display greater resistance to antimicrobial agents due to the outer cellular membrane that acts as a barrier against the entry of the biocides (Morton et al., 1998).

Additionally, the formation of biofilms, which are densely packed multicellular layers of microorganisms that are attached to a surface or an interface, is another means that allows for a microbial community to resist antimicrobial agents (Morikawa, 2005). These biofilms, or pellicles if the layer exists between the air and aqueous interphases, are composed of cells from an individual species or from cells from a diverse bacterial community. Biofilm growth is

promoted by certain environmental factors such as limited nutrient availability and oxygen deficient conditions (Morton et al., 1998 and Morikawa, 2005). They are held together by large amount of exopolysacchride material (EPS) secreted by bacteria (Leggett et al., 2012).

Microorganisms within the biofilm are more resistant to antimicrobial agents as compared to their planktonic counterparts through several mechanisms (Morton et al., 1998). One possible reason is extensive amount of EPS present in biofilms, which may act as a barrier preventing biocide penetration (Morton et al., 1998). This can be contradicted by some studies that suggest that channels inside the biofilm have access to bulk phase fluids, making biocides available (Morton et al., 1998). Another reason is the sessile physiological state of the cells within the biofilms compared to cells in planktonic phase (Morton et al., 1998). Sessile cells live longer resulting in prolonged contact with biocides sensitizing them (Morton et al., 1998). The increased secretion of catabolic enzymes can degrade biocides reducing their effectiveness and the non-living components such as EPS can inactivate antimicrobial agents both chemically and physically (Morton et al., 1998).

A 2013 study, that supports these claims, examined aerobic biofilms that were grown from sediments collected from the Athabasca Watershed (Yergeau et al., 2013). The collected bacterial communities were exposed to increasing amounts of bituminous compounds. The results suggested that biofilms of photosynthetic microbes, such as cyanobacteria, that developed in the presence of higher concentrations of bituminous compounds were less productive and had lower biomass due to inhibitory effects (Yergeau et al., 2013). Other microbial taxa, besides the photosynthetic microbes, were stimulated as were their functional genes by the increased concentrations of the carbon compounds (Yergeau et al., 2013). They were less sensitive to the inhibitory effects and were able to degrade and utilize some bitumen-associated compounds

(Yergeau et al., 2013). The active microbial community that was stimulated by the increased concentrations of bituminous compounds was dominantly composed of the Proteobacteria pylum, mainly the α-proteobacteria, ß-proteobacteria, and γ-proteobacteria classes (Yergeau et al., 2013). These microbes within this watershed have been known to have the ability to degrade bituminous compounds and were previously shown to be positively correlated to various hydrocarbons in field surveys, but it was suspected that an increase in the proportion of bituminous compounds among other carbon sources would reduce their biofilm productivity (Yergeau et al., 2013).

Their results displayed that within the first 8 weeks of biofilm formation, the increased exposure of bituminous compounds overrode any potential increase in carbon and energy (Yergeau et al., 2013). However, the longer certain taxa within the Proteobacteria phylum (α-proteobactria and ß-proteobacteria were observed in this study) were exposed, they began to express hydrocarbon-degrading genes that correlated with the increasing concentration of bituminous compounds (Yergeau et al., 2013). This may have led to an adapted community that was able to form thicker and more productive biofilms that are similar to their counterparts that were exposed to lower concentrations of the same compounds (Yergeau et al., 2013). Essentially, in the short term (<8 week), increased exposure to bituminous compounds reduced the biofilms productivity and biomass, but after 8 weeks, particular microbial taxa were stimulated along with stimulation of some aerobic hydrocarbon-degrading genes (Yergeau et al., 2013). This could mean that longer exposure could lead to the stimulation of already occurring microbes that possess hydrocarbon-degrading genes allowing the organisms to utilize the carbon compounds that are present for growth as well as partially restore productivity if other nutrients are not limiting (Yergeau et al., 2013).

Chapter 2 Specific Aims and Hypotheses

The aim of this study was to isolate a strain of halophilic bacterium that was capable of forming pellicles from a "flowback" or produced water sample. The sample that was selected was a Marcellus Shale waste water impoundment sample that was previously analyzed for enrichment culture data (Eastham, 2012). An isolate, strain LP-1, was obtained through the techniques of serial dilutions and streak plating. Once the culture was pure, an axenic medium was formulated to allow for better physiological characterization. This was accomplished by examining cell growth in various conditions using optical density data from a spectrophotometer. Molecular analysis was performed by sequencing the microbe's 16S rRNA gene. Analysis was done on the strain's metabolic versatility with metal and organics, with special interest in salts, total dissolved solids (TDS), precipitate halides, barium, strontium, electron donors, and electron acceptors that typify the shale-wastewater impoundment environment. These aims are due to the enrichment culture's sample site. Finally, the isolate's cell morphology and mineral precipitate was characterized using transmission electron microscopy (TEM), light microscopy, scanning electron microscopy (SEM), and energy dispersive spectroscopy (EBDS) using whole-mount slides.

Hypothesis: The halophilic bacterium that was isolated from the Marcellus Shale waste water impoundment water sample is capable of forming pellicles, metabolizing organic compounds, and precipitating salts.

Chapter 3 Materials / Methods

These specific aims required an array of methods, including media preparation, aseptic culture techniques, molecular work, physiological characterization methods, and more. The experimental methods for each specific aim fell into the following categories: Medium Reformulation, Isolation Techniques, 16S rRNA gene sequencing, and Growth Experiment and Physiological Characterization Methods.

3.1 Medium Reformulation

J. Lucas Eastham's 2012 study on the enrichments utilized a laboratory prepared media that mimicked the high total dissolved solid concentrations found in Marcellus Shale produced water, named "Frac Attack" media. This recipe was formulated from the Blauch et al. 2009 study and the DEP flowback averages. The media consisted of the following in 1000 mL dI H_2O: NaCl (60 g), KCl (1.2 g), $CaCl_2$ (8 g), $MgCl_2$ (1.0 g), Ammonium Chloride (1.0 g), KH_2PO_4 (0.5 g), $SrCl_2$ (2.75 g), $BaCl_2$ (150 mg), $FeCl_2$ (10 mg), $NaHCO_3$ (4.2 g), yeast extract (0-1.0 g), 500x vitamin mix (2 mL) and 500x mineral mix (2 mL). The 500x vitamin mix contains the following in one liter: biotin (2 mg), folic acid (2 mg), pyridoxine HCl (10 mg), riboflavin (5 mg), thiamine (5 mg), nicotinic acid (5 mg), pantothenic acid (5 mg), p-aminobenzoic acid (5 mg), thioctic acid (5 mg), and vitamin B12 (0.1 mg). The 500x mineral mix contains the following in one liter: nitriloacetic acid (1.5 g), MgSO4 $\cdot 7H_2O$ (3 g), $MnCl \cdot 4H_2O$ (0.444 g), NaCl (1.0 g), $FeCl_3 \cdot 6H_2O$ (67 mg), $CaCl_2 \cdot 2H_2O$ (100 mg), $CoCl_2 \cdot 6H_2O$(100 mg), $ZnSO_4 \cdot 7H_2O$ (274 mg), $CuSO_4 \cdot 5H_2O$ (10 mg), $AlK(SO_4)_2$ (10 mg), Boric Acid (10 mg), sodium molybdate (25 mg), $NiCl_2 \cdot 6H_2O$ (24 mg), and Na_2WO_4 (25 mg).

This media was utilized during the isolation process of this study. The total dissolved solids within the media made it difficult to get accurate optical density readings (absorbance of

600 nm) from the UV/VIS spectrophotometer and so a medium with less dissolved solids was devised for growth experiments and physiological characterization. This recipe was formulated from Huang et al. 2000 study that characterized the nominotypie subspecies *Salinivibrio costicola* subsp. *vallismortis*. The media consisted of the following in 1000 mL dI H_2O: NaCl (25 g), NH_4Cl (1 g), $MgCl_2.6H_2O$ (7 g), $MgSO_4.7H_2O$ (6 g), $CaCl_2.2H_2O$ (0.5 g), KCl (3.8 g), $K_2HPO_4.3H_20$ (0.4 g), Na_2CO_3 (3 g), 500x trace mineral solution (5 mL), 500x vitamin (5 mL), and yeast extract (1 g).

Once the media was prepared it was filter sterilized using a vacuum-pump. This media was utilized for all experiments with the exception of the studies assessing growth on various carbon sources, growth in anaerobic conditions, and pellicle and precipitate formation.

The carbon source growth experiment utilized the medium with a reduced quantity of yeast extract (0.1 g/L) to evaluate if the isolate was growing on the added carbon sources and not the added yeast extract. The anaerobic growth experiment used medium omitting the yeast extract. The anaerobic medium was prepared, added to Wheaton bottles, degased using 60:40 CO_2:N_2 ratio (with the exception of 4 bottles where the headspace was degased with strictly H_2), autoclaved, and then the vitamin and trace mineral mixes were added via syringe. Finally, cultures grown to examine pellicles and precipitates used a modified medium. In this medium $MgSO_4.7H_2O$ was removed to prevent precipitation and the quantity of $MgCl_2.6H_2O$ was increased to 11.95 g to keep the magnesium concentration the same. $BrCL_2$ (0.15 g), $SrCl_2.6H_2O$ (2.73 g), and $FeCl_3$ (0.1g) were also added to the media to simulate the compounds found in the impoundment water. A comparison of the enrichment medium to the axenic medium is displayed in Table 3.1.

Figure 3.1: Formulas for Frac Attack enrichment medium and Modified Death Valley axenic medium.

*Frac Attack Media (1000 mL) - Enrichments	
NaCl	60 g
KCl	1.2 g
$CaCl_2$	8 g
NH_4 Cl	1 g
$MgCl_2$	1 g
KH_2PO_4	0.5 g
$BaCl_2$	150 mg
$FeCl_2$	100 mg
$SrCl_2$ * $6H_2O$	2.75 g
$NaHCO_3$	4.2g

(Source: Eastham, 2012)

Modified Death Valley Medium (1000 mL) - Axenic	
NaCl	25 g
KCl	3.8 g
$CaCl_2.2H_2O$	0.5 g
NH_4CL	1 g
$MgCl_2.6H_2O$	7 g
$MgSO_4.7H_2O$	6 g
$K_2HPO_4.3H_20$	0.4 g
Na_2CO_3	3 g
Trace Mineral Solution	5 ml
Vitamin Mix	5 ml
Yeast	1 g

(Source: Haung et al, 2000)

3.2 Isolation Techniques

3.2.1 Streak Plating

Streak plating is a technique that was used to isolate the strain from the enrichment culture. Obtaining a pure culture was necessary to identify one species within the LP enrichment's bacterial community. In this procedure, the enrichment culture was diluted utilizing

an inoculating loop by spreading the enrichment over agar plates (Lammert, 2007). To make these plates, agar was added to the “Frac Attack” enrichment medium (Eastham, 2012) and then was poured into petri plates and left to cool and solidify. A single colony was then picked and utilized for liquid, culture dilutions.

3.2.2 Serial Dilutions

Serial Dilutions were also used to accomplish the isolation of the LP-1 strain. In this procedure, the enrichment cultures were diluted in the liquid, “Frac Attack” enrichment medium (Eastham, 2012) in a step-wise fashion. 10% of the inoculated test tube was transferred to inoculate a new autoclaved test tube containing fresh medium; this was done for 5 test tubes. The dilutions were then streaked over agar plates again. The culture was verified as pure through light microscopy to see if the culture of cells was homogenous morphologically and by using a Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI) once the sequencing data was obtained. The sequences were uploaded and a nucleotide BLAST was performed to search the database and see if the matches were closely related to one species.

3.3 Bacterial 16S rRNA Sequencing and Phylogenetic Analysis

3.3.1 DNA Extraction from Pure Culture

The first step in sequencing strain LP-1 16S rRNA gene was the extraction of the isolates DNA using a modified Phenol-Chloroform-IAA extraction protocol (Rutter, 2012). In this procedure, the culture that was grown until late-log phase (~24 hours). Once the culture was at the proper phase of growth, it was spun down in a Coulter Ultra High Speed Centrifuge with the JLA 8.1 rotor for 10 minutes at 7000 rpm. When the pellet was formed, the supernatant was

discarded and the pellet was washed in 1 mL of TE buffer, resuspended, and then transferred to a 1.5 mL tube; this step was repeated twice. The pellet was then resuspended in 494 µL TE buffer by repeated pipetting and vortexing. Once, resuspended, 30 µL of 10% SDS, 72 µL of lysozyme working solution, and 1.2 µL of RNase working solution were added to give the final concentrations of 0.5% SDS, 12 mg/mL lysozyme, and 20 µg/mL of RNase. This was then mixed thoroughly and incubated at 37°C for between 45 minutes to 60 minutes with light shaking on a shaker rack. After, 3 µL of 20 mg/mL of proteinase K was added to achieve a final concentration of 10 µg/mL. This was once again mixed thoroughly and incubated for 30 minutes at 37°C with light shaking.

Phenol (between 0.6 to 0.7 mL) was added to the solution, mixed thoroughly, and spun for 10-15 minutes at 12,000 rpm in the microcentrifuge. The aqueous, viscous supernatant was removed and transferred to a fresh microcentrifuge tube and the interface was left behind. An amount of chloroform (equal to the volume of Phenol) was then added and the step was repeated once more.

After Phenol and Chloroform was added and centrifuged, the supernatant was transferred to a fresh tube. 5M NaCl and 0.6 volumes of isopropanol (with a final concentration of 0.2 M NaCl) were added to precipitate the DNA out of the solution; it should be noted that the amount of NaCl and isopropanol that was added was dependent on the amount of supernatant that remained. The tube was then shaken back and forth by hand at room temperature until the stringy white DNA precipitate was clearly visible. Once it was visible, the tube was centrifuged briefly at 12,000 rpm and at room temperature to pellet the DNA precipitate. The DNA pellet was washed with 1 mL 70% ethanol and then respun for 5 minutes at room temperature to repellet it. The supernatant was removed and the pellet was dried in a speed vacuum at medium heat for

approximately 10 minutes. The pellet was then resuspended in 50 μL of sterile NANOpure water. This resuspension process took some time due to the high molecular weight of the chromosomal DNA. Finally, the nucleic acid concentration was then quantified using an Invitrogen Qubit® spectrofluorometer (Grand Island, NY, USA). An 0.8% agarose gel was also loaded at ~200 ng to confirm RNA-free genomic DNA (Rutter, 2012) and was ran at a constant voltage of 120 volts.

3.3.2 Amplification of the16S rRNA

Before the 16S rRNA gene was sequenced, Polymerase Chain Reactions (PCR) were used to amplify the selected rRNA sequences. When using PCR it is assumed that the gene sequences present in the sample are complimentary to the "universal" primers used for amplification. The primers that were used were universal bacterial primers for 16S rRNA gene sequencing (See Appendex A).

The DNA that was extracted from the pure culture was amplified using the 8F and 1492R 16S primer set. A 100 ng of the DNA extract was added to 40 μL PCR reaction with 5 pmol of each primer and 2x Phoenix Taq Master Mix (Phenix Research Products, Candler, NC). The Polymerase chain reactions were run on a Techne thermocycler (Techne incorporated, Princeton, NJ, USA) under the following conditions: denaturation at 95ºC for 2 mins, followed by 45 cycles of denaturation at 95ºC for 40 seconds, annealing at 55ºC for 30 seconds, extension at 72ºC for 90 seconds and final extension at 72ºC for 5 minutes. After the PCR was run, 10 μL of the amplicon product was run on a 2% agarose gel for an hour at 120v to verify the length of amplified template and to check the relative band intensity to predict concentration.

3.3.3 Litigation and Transformation Reactions

Litigation was accomplished utilizing an Invitrogen™ TOPO® TA Cloning® Kit (Cat. No. 450030) (Grand Island, NY, USA). The fresh PCR product was mixed gently at room temperature with the litigation reaction mix that consisted of 1 µL salt solution, 1 µL pCR4®-TOPO vector, 2 µL PCR product, and 2 uL nuclease-free water to bring the solution up to 6 µL.. Once mixed and incubated, the reaction was then be placed on ice and stored at -20ºC until needed (Invitrogen™).

Following incubation, 2 µL from the litigation reaction was added to a vial of One Shot Chemically Competent *E. coli* mix and shaken gently. This was incubated on ice for 20 minutes. The cells were then heat shocked for 30 seconds at 42 ºC and then immediately transferred back to ice. Following the heat sock, 250 µL of room temperature S.O.C. medium was added to the cells in the tube, capped, and the mix was shaken horizontally for an hour in the 37 ºC. Volumes of 20 µL and 50 µL from each transformation was then spread on separate, pre-warmed LB + [AMP] agar plates to ensure an even colony distribution; the plates were then be incubated over night at 37 ºC .

The next day, a colony PCR was performed by picking colonies off of the agar plates using sterile-toothpicks. The colonies were then resuspended in 50 µL of deionized (DI) water. This was done for 16-20 colonies to ensure there were enough clones to work with. Those colonies were lysed in the thermalcycler at 85-90 ºC for 5 minutes. M13 forward and reverse primer sets were used to amplify the plasmid insert, so that the e-coli 16s rRNA was not amplified instead of the isolates. A volume of 10 µL of lysate was added to 12.5 µL 2x Taq Mastermix (Phenix Research Products, Candler, NC), 3.2 pmol of each forward and reverse M13 primer, and nuclease-free water was added to equal a final volume of 25 µL. This reaction mix was run in the

thermalcycler under the following conditions: initial denaturation at 95^{0}C for 5 mins, followed by 30 cycles of denaturation at 95^{0}C for 30 seconds, annealing at 55 ^{0}C for 30 seconds, extension at 72 ^{0}C for 2 minutes, and final extension at 72 ^{0}C for 10 minutes.

The colony PCR product was then column cleaned using Sephadex G50 (Sigma-Aldrich, Milwaukee, 27 USA). This required 550 µL of autoclaved Sephadex to be added to each well that contained product on the 96-well filter plate. The filter plate was placed on a skirted collection plate and spun on the centrifuge for 3 minutes at 850 x g. The flow through was then discarded and the filtration process was repeated for a second time. The plate was put on a 96-well sequencing plate and the samples were added. This was spun for 4 minutes at 850 x g and then the DNA was quantified using a Qubit spectrofluorometer and a 0.8% agarose gel was run.

3.3.4 16S rRNA Sequencing

The first step in sequencing the amplified product from the colony PCR was to perform a Big Dye Sequencing reaction. In this reaction, 20 ng of the purified PCR product was added to the sequencing reaction. The BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies) was used for this procedure. The reaction required a total volume of 20 µL mix that contain 3 µL of the BigDye Ready Reaction Mix, 4 µL of the BigDye Sequencing Buffer, 1 µL of the primer (3.2 pmol), and DI water to bring the volume up. The reaction was then run on the thermalcycler under the following conditions for 8 hours: denature at 95ºC for 1 minute, followed by 75 cycles of incubation at 95ºC for 30 seconds, 50ºC for 5 seconds, and 60ºC for 4 minutes; then final hold at 4ºC.

The sequencing product was then column cleaned using the Sephadex G50 protocol (Sigma-Aldrich, Milwaukee, 27 USA) and the product was run on 2% agarose gel to check length of amplified template and relative band intensity to predict concentration.

Once cleaned, the sample was covered with a septa mat and denatured at 95°C for 2 minutes in the thermalcycler and then immediately transferred to ice. The plate was then analyzed on the ABI-3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

3.3.5 Phylogenetic Analysis

The sequencing data generated by the ABI-3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA) were initially processed using the GeneMapper software program (Applied Biosystem, Foster City, CA, USA). The sequences were then uploaded to ClustalX2 software program as text files. The reverse complement of each reverse sequence was completed to reorient the strands back to 5' to 3'. The sequences for each individual primer were then aligned and saved as .fasta files. All the sequences were then loaded into the Molecular Evolutionary Genetics Analysis program (MEGA 5), pieced together, and truncated to remove flanking DNA and primer sequences; this formed a consensus sequence.

The consensus sequence was then uploaded as a text file to the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI). A nucleotide BLAST was performed to search the database to see if the isolate's 16S rRNA gene matched any other organisms. From the BLAST, the closet relatives to strain LP-1 were determined based on the percentage of base pair matches. A relative database of the species that most closely match strain LP-1 was then created as a text file in Microsoft NotePad. Each sequence was formatted with .seq to allow compatibility with the ClustalX2 software program.

The consensus sequence was then added to the relative database and it was then loaded into the ClustalX2 and aligned. The output for the alignments (See Appendix B) was a .fasta file. These were then uploaded the MEGA 5 program again and phylogenetic analysis was performed

to construct a maximum likelihood phylogenetic tree that was bootstrapped with 500 replicates for statistical analysis.

3.4 Growth Experiment and Physiological Characterization Methods

3.4.1 Axenic Cultures

The enrichment cultures that were grown in the "Frac Attack" media were serial diluted and streak plated to isolate one strain. Once the culture was pure, 500 um of inoculum was injected into the newly formulated media under aerobic conditions and incubated at 37° C to stimulate growth. The pure culture was monitored for growth. Once growth was observed, the morphology of the inhabiting cells was assessed to determine if the culture was homogeneous.

3.4.2 Gram-Staining

Gram-staining was carried out to determine if the isolate was a gram negative or positive microbe. For the gram stain slide smears were prepared by spreading 2-3 loops of cell culture in a ½" wide area, air drying the slide and then passing the slide over a flame to heat kill and fix the microorganisms to the top of the slide. Smears were then gram stained by covering in crystal violets for twenty seconds, washing in dI H_2O, covering in gram's iodine for one minute, rinsing with 95% ethanol for twenty seconds, followed by safranin for twenty seconds. After staining, the slides were blotted dry and viewed under a light microscope.

3.4.3 Growth Experiments

After initial growth of the isolate was observed, general growth studies were conducted on the newly modified Death Valley media (Table 3.1) to reveal general growth kinetics. All growth experiments were performed in triplicate and measured by optical density. To measure

growth under varying conditions, the turbidity of 1 mL of culture was measured at an optical density of 600 nm on a Perkin-Elmer (Waltham, MA, USA) Lambda2 dual-beam spectrophotometer, at varying time points (Eastham, 2012). All experiments were incubated a 37ºC and the media was kept at 6.0-6.5 pH unless otherwise stated.

Growth in various temperature conditions was examined to determine strain LP-1's optimal temperature and temperature range. Cultures were grown in 10 mL of the axenic, modified Death Valley media (Table 3.1) at temperatures of 4ºC, 25ºC, 37ºC, 50ºC, and 65ºC. Growth was measured in triplicate at 0, 24 and 48 hours. Blank media amendments were also measured at all temperatures and time points as a negative control.

Once the temperature was identified a growth curve was constructed using the axenic media (Table 3.1) to determine how quickly the isolate grew. The cultures were shaken to help stabilize the readings and growth was measured every two hours until the growth curve plateaued. Blank culture amendments were also measured as a negative control. After the growth measurements were obtained the growth rate and mean generation time of strain LP-1 were calculated.

Considering the degree of salinity present within the impoundment samples and within the enrichment media, it was hypothesized that the enrichment community should be able to survive in a range of salinity levels. Cultures were grown in 10 mL cultures of axenic media (Table 3.1) with concentrations of 0%, 2.5%, 5.0%, 7.5%, 10.0%, 12.5%, 15.0%, 17.5%, and 20.0% sodium chloride to determine the salinity tolerance of the isolate. Growth was measured in triplicate at 0, 24 and 48 hours. Blank media amendments were also measured at all salinity values and time points as a negative control.

Strain LP-1 was also grown in a broad range of pH conditions to determine the strain's tolerance. Cultures were grown in 10 mL of axenic medium (Table 3.1) in pHs of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, and 10.5. The pH of the medium was established by adding HCl to acidify and NaOH to alkalize the medium. Growth was measured in triplicate at 0, 24 and 48 hours. Blank media amendments were also measured for all pH increments and time points as a negative control.

Strain LP-1's ability to grow anaerobically was investigated using a variety of electron donors and acceptors to determine the strain's oxygen requirement. Cultures were grown in 28 mL of the modified anaerobic medium (0 g/L yeast). The electron donors that were tested were Na-acetate, Na-acetate with the headspace containing strictly H_2, Na-lactate, Na-formate, and Na-pyruvate. The electron acceptors that were examined were nitrate, sulfate, thiosulfate, and Na-arsenate. Stock solutions of these sources were made at 300 mM with the exception of Na-arsenate, which was made at 150 mM. Once the stock solutions were amended, 1 mL of the stock, 0.05 mL of 500x vitamin mix, and 0.05 mL of the 500x trace mineral mix were added to the 28 mL cultures to make the final concentration of the solution 10mM. Growth was measured in triplicate at 0, 24, 48, 72, 96, and 120 hours. Blank media amendments were also measured at all cultures and time points as a negative control.

Strain LP-1's metabolic capabilities were determined by reducing the amount of yeast extract present in the medium and adding various carbon and energy sources. Cultures were grown in 9.9 mL of a modified medium that contained 0.1 g/L yeast extract. The culture used to inoculate the experiment was grown and transferred three times prior to the experiment in the medium containing less yeast extract to acclimate the strain. The sources that were tested were yeast extract alone (0.1 g/L) as a control, glucose, fructose, arabinose, ribose, xylose, sucrose,

maltose, lactose, trehalose, glycerol, Na-acetate, Na-citrate, casein, Na-lactate, Na-pyruvate, and Na-formate. Stock solutions of these sources were made at 1M. 100µL of the stock was added to the 10 mL cultures to make the final concentration of the culture solution 10mM. Growth was measured in triplicate at 0, 24, 48, 72, and 96 hours. Blank media amendments were measured for all cultures and time points as a negative control. Graph-Pad's Prism statistical software was utilized following the experiment to perform an ANOVA (One Way Analysis of Variance ($P<0.0001$) using the Tuckey Multiple Test algorithm to reveal statistical significance from the 0.1 g/L yeast control.

3.4.4 Microscopy

The cellular morphology of strain LP-1 was determined by light microscopy using Nikon microphot SA phase-contrast microscope (Melville, NY, USA), a JEOL100cxx transmission electron microscope (TEM), and a Hitachi S-3400 scanning electron microscope (SEM).

The morphology and surface characteristics of the bacterium were distinguished by using negative staining and thin section technique with transmission electron microscopy (TEM). The method of negative staining requires the samples to be placed on coated grids that had a stained background. The sample itself is not stained and so the contrast gives for more effective visualization. Previously coated grids were utilized in this process. The coating material on the grids was 0.5% formvar in ethylene dichloride. The grids were pretreated with bacitracin for 1 min. Excess bacitracin was drained from sides using filter paper. A drop of bacterial liquid culture was placed on grid and allowed to stay for 5 minutes. After draining the grid, it was stained with filtered 1% uranyl acetate solution for 1 min. The grids were allowed to air dry and observed using the JEOL 100cxx transmission electron microscope.

Elemental analysis of the formed pellicles was accomplished using a Hitachi S-3400 scanning electron microscope. Prior to the use of the SEM the specimen was prepared by fixing biofilms in 2.5% gluteraldehyde. Ethanol rinses were used for dehydration of the fixed biofilms. A series of ethanol rinses were then applied: 70% ethanol for 10 minutes, 95% ethanol for 15 minutes and 3 times 100% ethanol for 10 minutes each. After dehydration, specimens were chemically dried using HMDS (hexamethyldisilazane). A solution of 2 parts 100% ethanol and 1 part HMDS was used for rinsing specimen for 15 minutes. A rinse of 1 part absolute ethanol and 2 parts HMDS was then done for duration of 15 minutes. The final rinse was done using only HMDS for 15 minutes. The specimens were dried overnight in fume hood. The following day each specimen was attached to a 15 mm stud for SEM analysis. A scan for elemental analysis was executed using a Brucker EBDS that was attached to the SEM. Graphs and images were processed using the QUANTAX program provided by Esprit.

Chapter 4 Results

4.1 Medium Reformulation

The new media utilized was formulated by modifying the recipe presented in Huang et al. 2000 study that characterized the nominotypie subspecie *Salinivibrio costicola* subsp. *vallismortis*, with components used in J. Lucas Eastham's study's "Frac Attack" media that based its formula off of the water assessment done by Blauch et al. 2009. This was done to remove some total suspended solids to prevent precipitation due to changes in temperature, pH, and prolonged periods of time. This gave more accurate readings when using UV/VIS spectrophotometer to measure cell growth in the form of optical density at an absorbance of 600 nm. A comparison of the enrichment medium to the axenic medium is displayed in Table 3.1.

The carbon source growth experiment utilized a medium with a reduced quantity of yeast extract (0.1 g/L) to evaluate if the isolate's ability metabolize organic compounds rather than the yeast extract. The anaerobic growth experiment used a medium containing 0 g/L yeast extract. Additionally, the anaerobic medium was degased using 60:40 CO_2:N_2 ratio (with the exception of 4 bottles where the headspace was degased with strictly H_2). Finally, cultures grown to examine biofilms and precipitate also used a modified medium. In this medium $MgSO_4.7H_2O$ was removed to prevent precipitation and the quantity of $MgCl_2.6H_2O$ was increased to 11.95 g to keep the magnesium concentration the same. $BrCL_2$ (0.15 g), $SrCl_2.6H_2O$ (2.73 g), and $FeCl_3$ (0.1g) were also added to the media to simulate the elements found in impoundment waste waters.

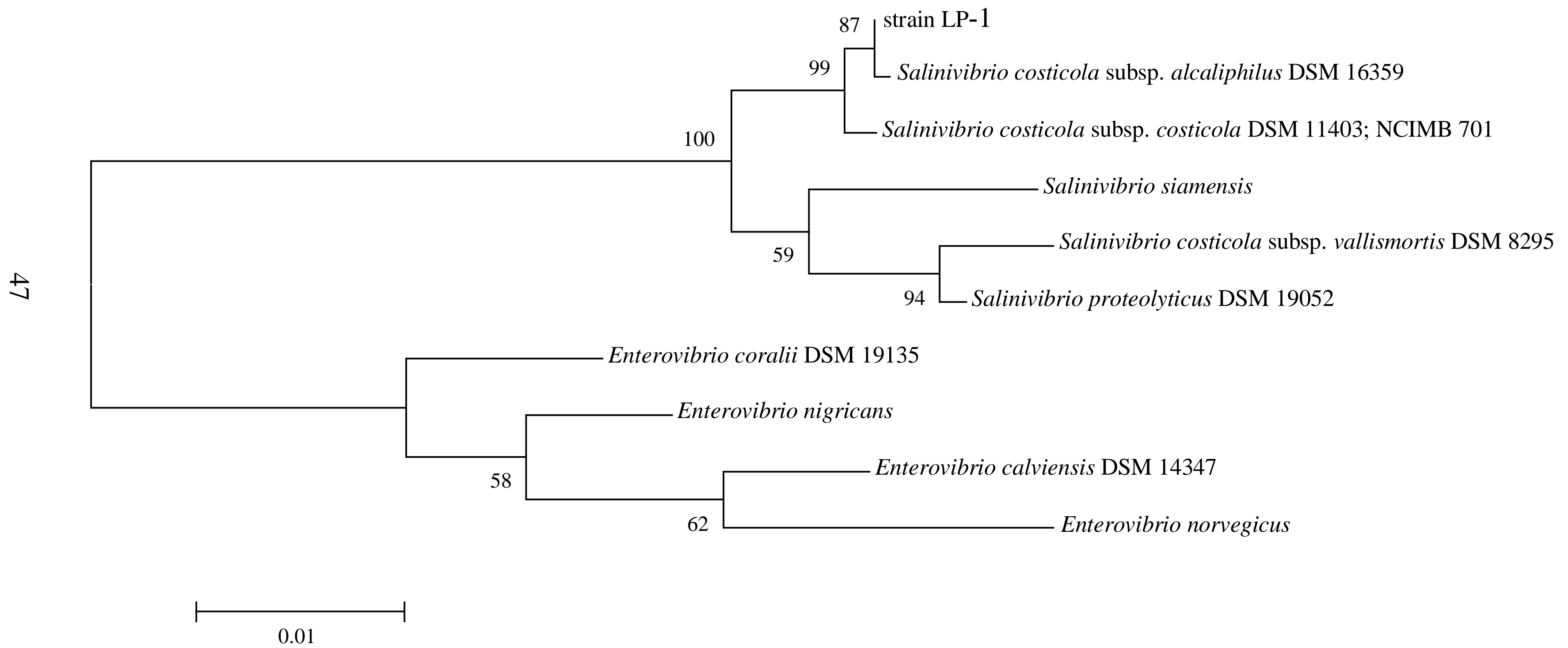
Strain LP-1 growth was recorded in newly formulated and modified mediums. The absorbance readings were more precise and smaller error bars in the growth graphs resulted due to the reduction of precipitation within the medium.

4.2 Bacterial Molecular Profiling

The molecular profiling of the isolate involved amplifying the DNA using 16s primers (See Appendix A), cleaning the DNA product, performing a Big Dye sequencing reaction, and sequencing the DNA on ABI-3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Alignments were accomplished using the ClustalX2 software, and then phylogenetic analysis was completed using the MEGA 5 software. A total of 1,555 base pairs (See Appendix B) were obtained during the sequencing process and a nucleotide BLAST (NCBI) search was ran (See Appendix B). The closest relatives' sequences were selected, aligned (See Appendix B), and a phylogenetic analysis was conducted. The Maximum Likelihood phylogenetic tree constructed (Figure 4.1) was used to identify strain LP-1's closest relatives.

Based on the constructed tree and the nucleotide BLAST search (see Appendix B), strain LP-1 is most closely related to the subspecies of *Salinivibrio costicola* subsp. *costicola* (98%) and subsp *alcaliphilus* (98%). Strain LP-1 utilizes *S. costicola* subsp. *costicola*, *Salinivibrio costicola* subsp. *vallismortis* and *Salinivibrio costicola* subsp. *alcaliphilus* as reference species for comparisons in the discussion of this study.

Figure 4.1: Phylogenetic tree comparing strain LP-1 to reference species.



4.3 Growth Experiment and Physiological Characterization Methods

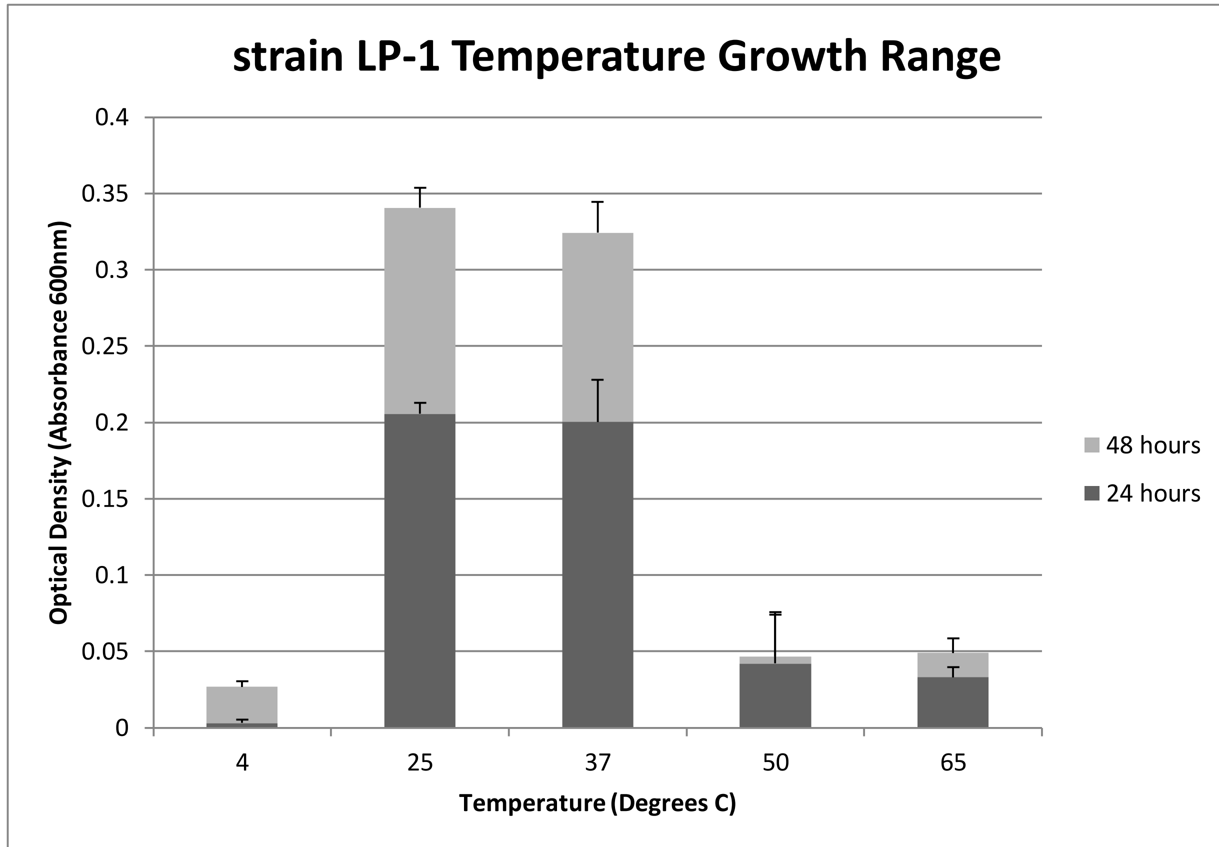
4.3.1 Gram Stain

The result of the performed gram stain exhibited that strain LP-1 is gram negative.

4.3.2 Temperature Growth Range

Strain LP-1 was grown in the following temperatures 4°C, 25°C, 37°C, 50°C, and 65°C to identify its optimal growing temperature and temperature range. Based on the observed readings the temperature growth range for strain LP-1's (Figure 4.2) was between 4°C and 65°C with an optimal growth temperature of 25°C. This is a reasonable find because impoundment waters are typically kept at 30°C. Strain LP-1 can be classified as a mesophile.

Figure 4.2: strain LP-1's growth in various temperatures after 24 and 48 hours.

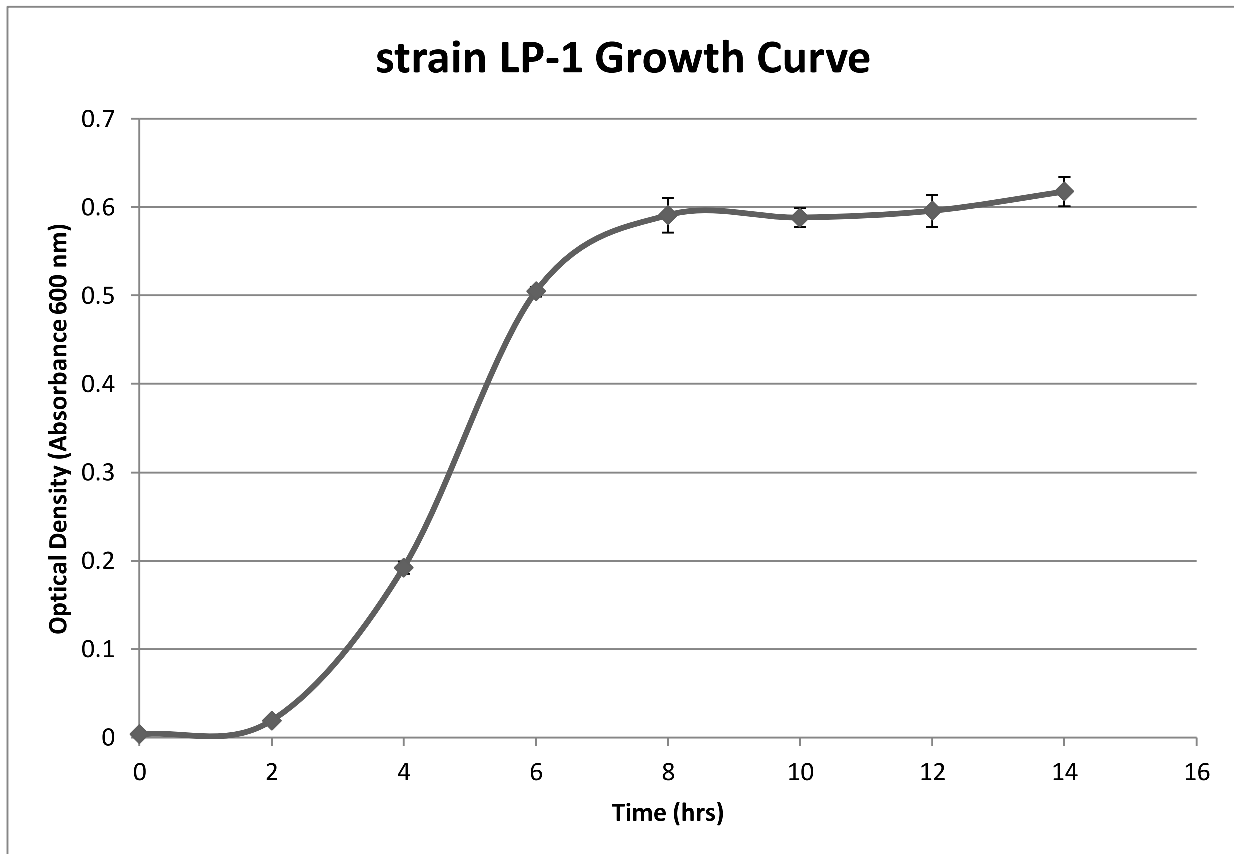


4.3.3 Growth Curve

Once growth temperature data was identified, a grow curve was constructed to illustrate the growth kinetics of strain LP-1 and to establish a sense of time regarding the growth rate of strain LP-1. The growth curve was run in triplicate and accompanied by a blank as a means of a negative control. The cultures were shaken on a shaker rack while being incubated at37ºC to stabilize the growth and give accurate readings. The curve (Figure 4.3) displays how quickly strain LP-1 can grow while being incubated. The isolate's log phase was between 2 and 6 hours.

Strain LP-1 has a growth rate (k) of 0.301 and a mean generation (doubling) time (g) of 25.19 minutes.

Figure 4.3: Growth Curve of strain LP-1.



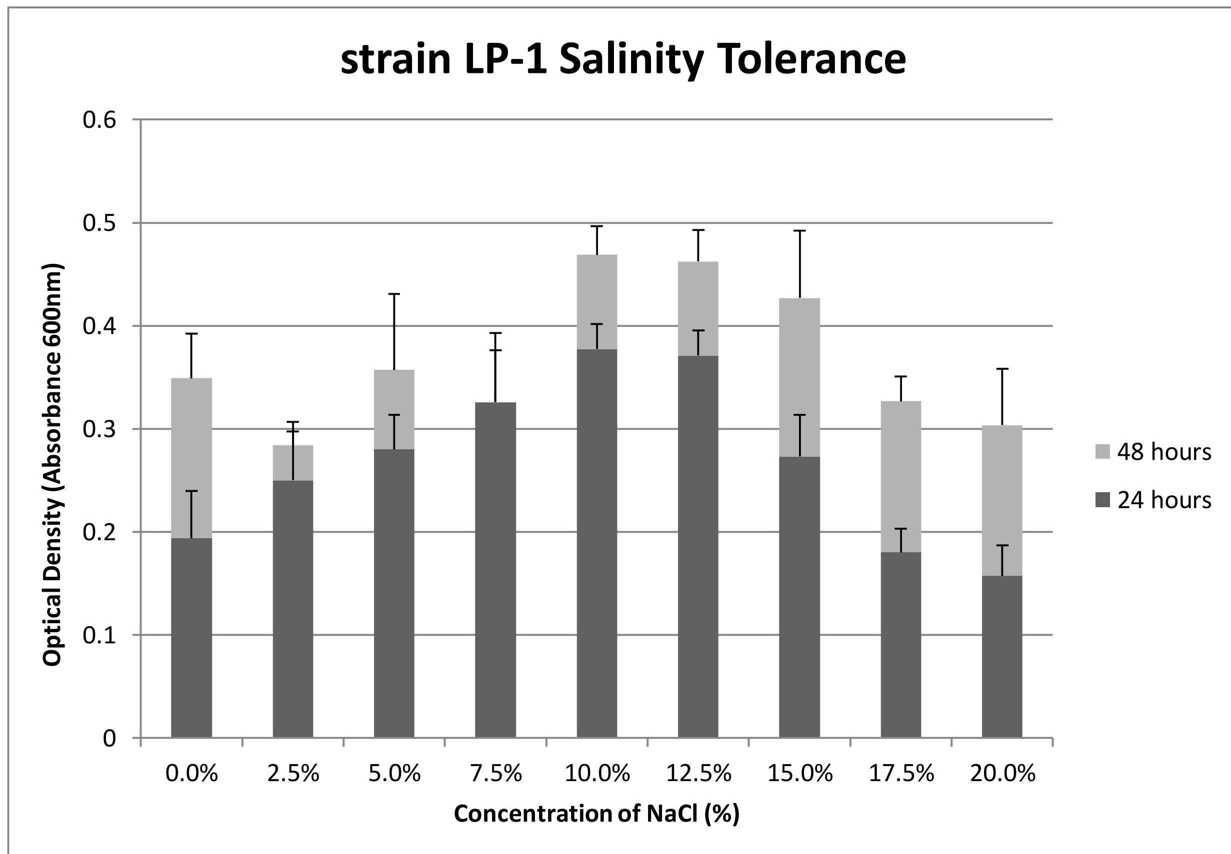
4.3.4 Salinity Range

The salinity tolerance of strain LP-1 was of particular interest during the characterizing process. A distinguishing attribute of *Salinivibrio costicola* is its tolerance of a wide array of NaCl concentrations. Additionally, because the strain was isolated from an impoundment that contained fracturing waste waters that are associated with high and variable salinity concentrations. The cultures were grown in 10 mL of the axenic medium (Table 3.1) with

concentrations of 0.0%, 2.5%, 5.0%, 7.5%, 10.0%, 12.5%, 15.0%, 17.5%, and 20.0% sodium chloride.

The recorded measurements display that strain LP-1 has a wide salinity tolerance range (Figure 4.4). Strain LP-1 had a salinity requirement of 0.0% NaCl and a salinity tolerance of 20.0%. Its optimal growth salinity concentration was 10.0%. This wide tolerance of NaCl concentrations warrants the strain's classification as a euryhalic species.

Figure 4.4: strain LP-1 growth on various NaCl concentrations after 24 and 48 hours.

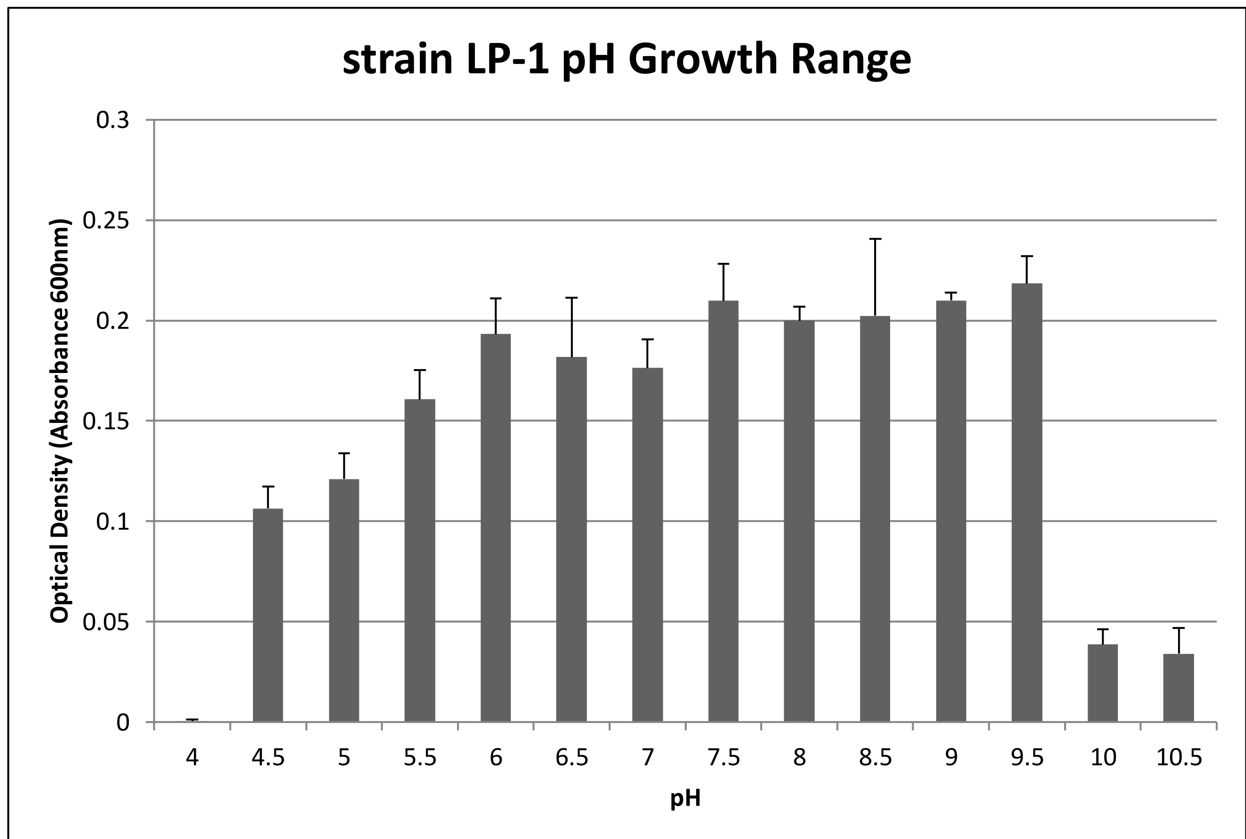


4.3.5 pH Range

As with salinity, strain LP-1 grew in a broad spectrum of pHs. It is important to note that after the pH of 8.0 the precipitation of suspended solids began to occur within the media. This did not confound results because the precipitate settled at the bottom of the test tube. This can be confirmed since the optical density readings fell after a pH of 9.5.

In this growth experiment strain LP-1 was grown in 10 mL of axenic medium in pHs of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, and 10.5 for 24 hours. The growth range (Figure 4.5) of strain LP-1 is from 4.5 to 10.5 pH units with an optimal growth pH of 7.5. These results are typical for a *Salinivibrio costicola* strain.

Figure 4.5: pH Growth Curve for strain LP-1 after 24 hours.

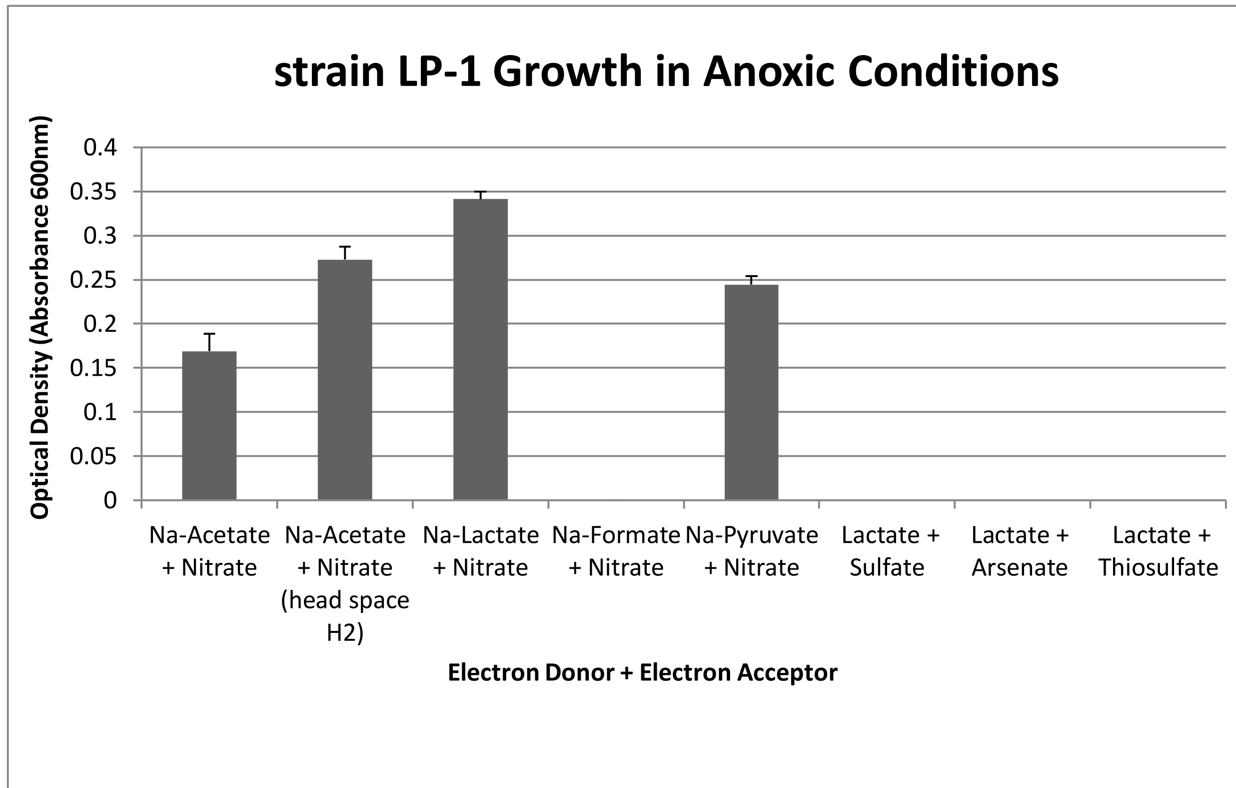


4.3.6 Growth in Anaerobic Conditions

An interesting variable to define in the characterization process was the oxygen requirement of strain LP-1. This is because of the limited quantity of oxygen in the geologic subsurface structures that drilling activities take place in. The isolate was grown using a variation of electron donor and electron acceptor pairings to determine if the strain can respiration anaerobically. To test this, cultures were grown in 28 mL of anaerobic, axenic medium (0 g/L yeast). The electron donors that used were Na-acetate, Na-acetate with the headspace containing strictly H_2, Na-lactate, Na-formate, and Na-pyruvate. The medium contain acetate was used twice but once with the headspace containing strictly H_2 to determine if hydrogen, as an electron donor, could contribute or expedite cell growth. The electron acceptors that were used were Na-nitrate, Na-sulfate, thiosulfate, and Na-arsenate. The final concentration of the culture solutions were 10mM.

A preliminary experiment was run to determine the optimal electron donor and acceptor, which was lactate and nitrate. Once that was determined, nitrate was paired with all the electron donors and lactate was paired with all the electron acceptors. Growth was measured in triplicate at 0, 24, 48, 72, 96, and 120 hours. After 120 hours in anoxic conditions (Figure 4.6), strain LP-1 grew on donors Na-Acetate, H_2, Na-Lactate, and Na-Pyruvate. It grew exclusively on nitrate as the acceptor. The isolate can be characterized as a facultative anaerobe given the slower growth in anaerobic conditions.

Figure 4.6: Growth of strain LP-1 in anoxic conditions utilizing a variety of electron acceptors and donors.



4.3.7 Growth on Various Carbon Sources

The metabolic capabilities of strain LP-1 were examined utilizing a variety of carbon sources to determine the strain's preferred energy sources. To accomplish this, cultures were grown in 10 mL of axenic medium (Table 3.1) with a reduced quantity of yeast extract (0.1 g/L).The sources that were tested were yeast extract alone (0.1 g/L) as the control, glucose, fructose, arabinose, ribose, xylose, sucrose, maltose, lactose, trehalose, glycerol, Na-acetate, Na-citrate, casein, Na-lactate, Na-pyruvate, and Na-formate. The final concentration of the solutions were 10mM.

After the specified growth periods, it was determined that strain LP-1 grew significantly on all sources with the exception of lactose and Na-formate (Figure 4.7). An ANOVA (One Way Analysis of Variance ($P<0.0001$) using the Tuckey Multiple Test algorithm was run using Graph-Pad's Prism statistical software to identify statistical significance from the 0.1 g/L yeast control. This statistical analysis is displayed in Table 4.8. It was also noted that the presence of these organic compounds, with the exception of Lactose and Na-formate, significantly enhanced the growth rate of strain LP-1.

Figure 4.7: strain LP-1's growth on various carbon sources during the 0, 24, 48, 72, and 96 hours.

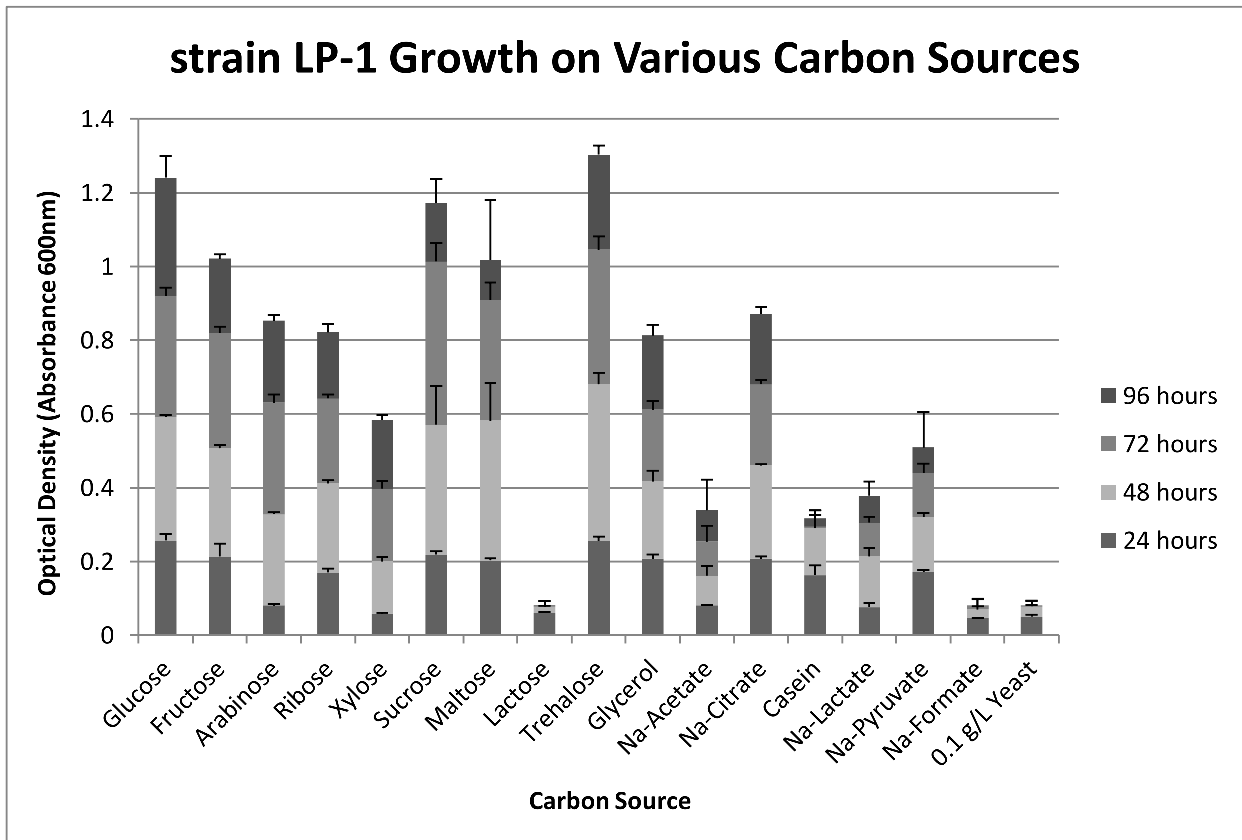


Table 4.1: One Way Analysis of Variance ($P<0.0001$) of strain LP-1's Growth on Organics Compared to Yeast Extract (0.1 g/L) Control

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Glucose vs 0.1 g/L Yeast	1.16	28.77	Yes	***	0.9469 to 1.374
Fructose vs 0.1 g/L Yeast	0.941	23.33	Yes	***	0.7275 to 1.154
Arabinose vs 0.1 g/L Yeast	0.7727	19.16	Yes	***	0.5592 to 0.9861
Ribose vs 0.1 g/L Yeast	0.7413	18.38	Yes	***	0.5279 to 0.9548
Xylose vs 0.1 g/L Yeast	0.5037	12.49	Yes	***	0.2902 to 0.7171
Sucrose vs 0.1 g/L Yeast	1.092	27.07	Yes	***	0.8782 to 1.305
Maltose vs 0.1 g/L Yeast	0.9373	23.24	Yes	***	0.7239 to 1.151
Lactose vs 0.1 g/L Yeast	0.002333	0.05786	No	ns	-0.2111 to 0.2158
Trehalose vs Glycerol	0.49	12.15	Yes	***	0.2765 to 0.7035
Trehalose vs 0.1 g/L Yeast	1.223	30.33	Yes	***	1.010 to 1.436
Glycerol vs Na-Acetate	0.4745	10.52	Yes	***	0.2358 to 0.7132
Glycerol vs 0.1 g/L Yeast	0.733	18.18	Yes	***	0.5195 to 0.9465
Na-Acetate vs 0.1 g/L Yeast	0.2585	5.733	Yes	*	0.01983 to 0.4972
Na-Citrate vs 0.1 g/L Yeast	0.7913	19.62	Yes	***	0.5779 to 1.005
Casein vs 0.1 g/L Yeast	0.237	5.877	Yes	*	0.02352 to 0.4505
Na-Lactate vs 0.1 g/L Yeast	0.2977	7.381	Yes	**	0.08419 to 0.5111
Na-Pyruvate vs 0.1 g/L Yeast	0.4293	10.65	Yes	***	0.2159 to 0.6428
Na-Formate vs 0.1 g/L Yeast	0.0003333	0.008266	No	ns	-0.2131 to 0.2138

4.3.8 Microscopy

The scanning electron microscope micrographs displayed curved rods that occurred in single, paired, s-shaped formations (Figure 4.8). The bacteria appeared to sit on top of the solid precipitate matrix but at higher magnification displays that the bacteria are linked together in groups and the pellicles appear to be porous (Figure 4.9). The size of the pores is roughly the equivalent to the size of the strain's cells. This formation could be due to the bacteria forming pores to serve as a means of a gas exchange from the air to the medium (Eastham 2012).

The surface of the pellicles were analyzed for their elemental composition using a Brucker EBDS in concert with the SEM. The spectral scanner detected the presence of magnesium, phosphorus, calcium, strontium, and barium (Figure 4.10).

The TEM negative stain imaging result (Figure 4.11) displays a curved-rod shaped bacterium with a polar flagellum. This morphology is typical for a strain of *Salinivibrio costicola.*

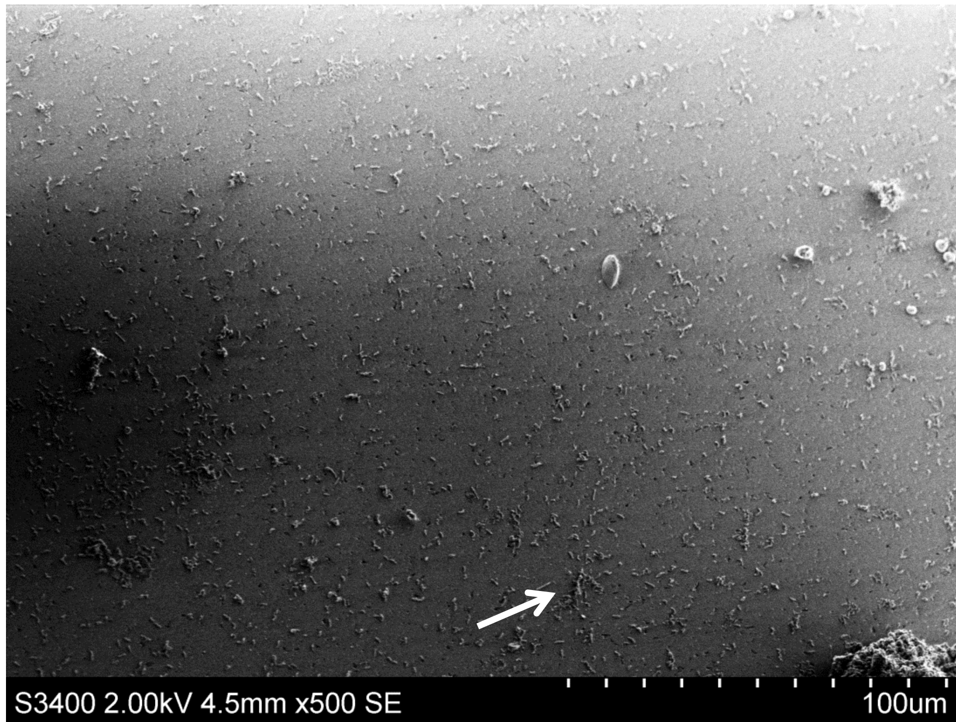


Figure 4.8: Scanning Electron micrograph of strain LP-1 colonies (100 µm). Arrow – Rod-shaped bacteria in groups on surface of the pellicle.

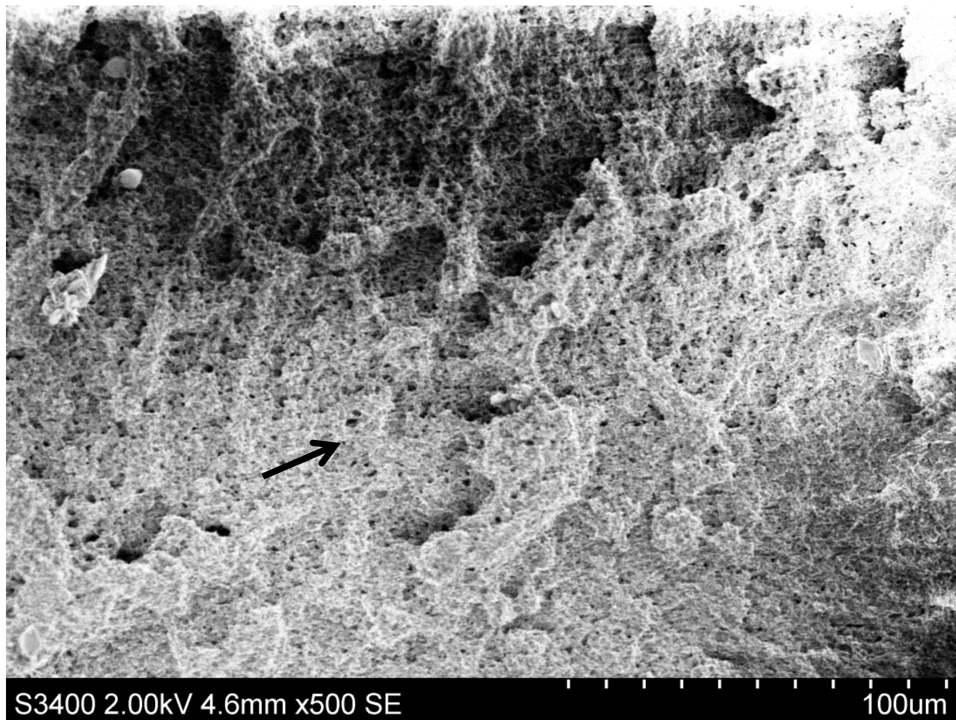
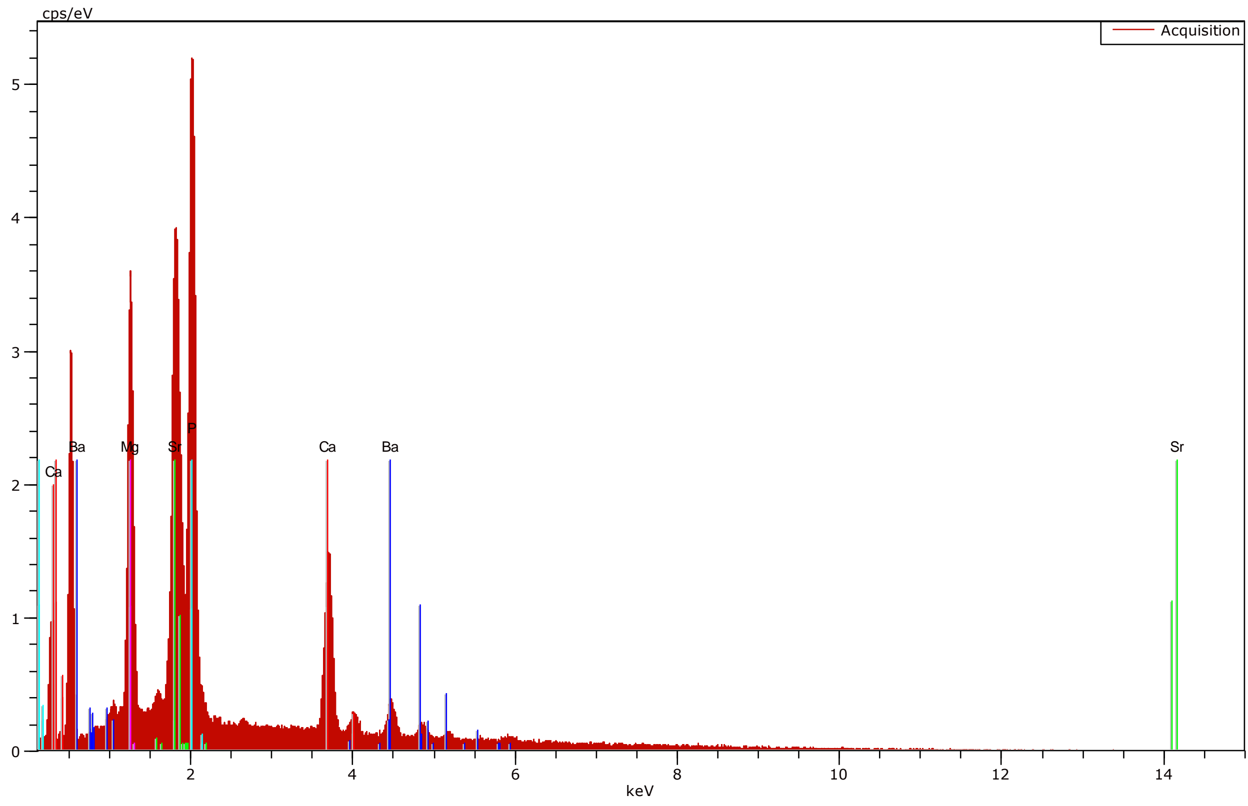


Figure 4.9: Scanning Electron micrograph of strain LP-1 produced biofilm (100 µm). Arrow – Voids left where Bacteria bore.



Spectrum: Acquisition

El	AN	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]	Error (1 Sigma) [wt.%]
Mg	12	K-series	7.72	12.42	22.62	0.44
P	15	K-series	18.00	28.98	41.42	0.72
Ca	20	K-series	8.20	13.21	14.59	0.28
Sr	38	L-series	22.90	36.87	18.62	0.96
Ba	56	L-series	5.29	8.52	2.75	0.20
		Total:	62.11	100.00	100.00	

Figure 4.10: Elemental spectral scan of strain LP-1 biofilm

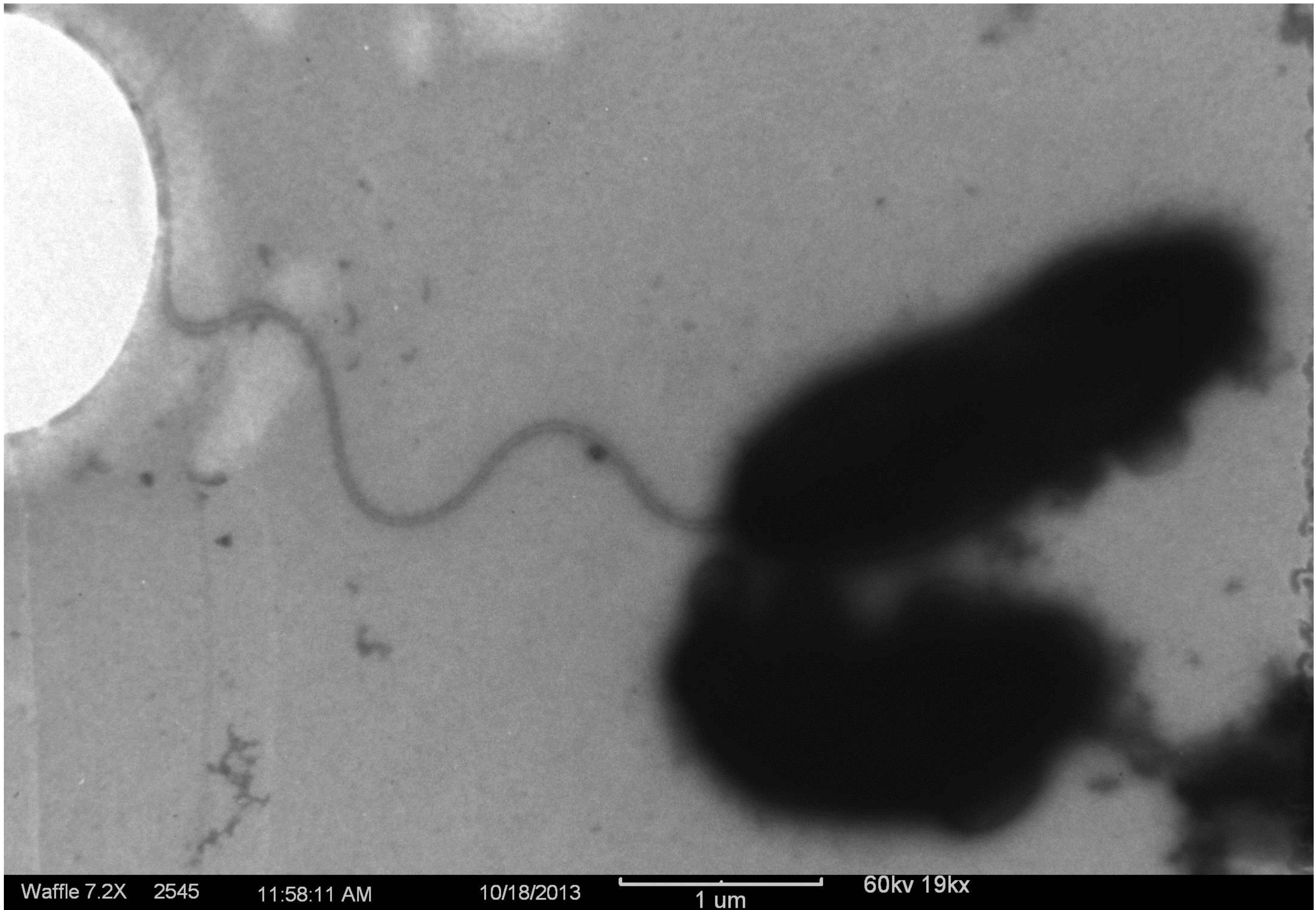


Figure 4.11: TEM image of a negatively stained strain LP-1.

Chapter 5 Discussion

5.1 Medium Reformulation

The modification of the enrichment medium used during the course of this study was important for multiple reasons. It allowed for the cultures to remain viable, while limiting the quantity of total dissolved solids within the media and preventing precipitation that was hindering the ability to collect optical density results during the growth experiments and physiological characterization. Previously, the solids would precipitate out after elongated periods of time, slight changes in temperature, and shifts in pH, making the enrichment media sensitive to work with. When preparing the samples the precipitate would become suspended within the medium making the solutions turbid. These solids were being detected by the UV/VIS spectrophotometer and adding statistical error to the results by making it hard to differentiate between cell growth counts and solids that were suspended in solution.

The combination of components from J. Lucas Eastham's "Frac Attack" media (Table 3,1) based off of the Blauch et al. 2009 paper and the quantities of ingredients formulated for the Huang et al. 2000 study, allowed for the ability to have the elements that would be characteristic of the impoundment water while giving increased clarity when measuring the growth data with the UV/VIS spectrophotometer.

5.2 Bacterial Molecular Profiling

The initial aim of this study was to isolate a strain of halophilic bacterium that inhabited a sample of fluid that was associated with the gas extraction process. Through the use of molecular profiling it was established that strain LP-1 was isolated into a pure culture due to the sequences results from the molecular work (See Appendix B). Additionally, phylogenetic analysis allowed us to distinguish strain LP-1 halophilic relatives, namely the nominotypie subspecies of

Salinivibrio costicola. subsp. *costicola* and *alcaliphilus*; both subspecies were 98% similar to the isolate according to the nucleotide BLAST (See Appendix B). The 16S gene sequence and phylogenetic analysis was imperative for the identifying strain LP-1's identity.

The molecular identification is consistent with J. Lucas Eastham's analysis of the Lone Pine enrichment cultures that strain LP-1 was isolated from. The 16S rDNA sequencing in that study showed that one of the clones from the Lone Pine sample was within the family *Vibrionaceae* and closely resembled *Salinivibrio costicola* (Eastham, 2012).

5.3 Growth Experiment and Physiological Characterization

The majority of this study's efforts were aimed at characterizing an isolated strain that inhabited the impoundment sample. The growth experiments utilized a UV/VIS spectrophotometer to collect optical density readings at an absorbance of 600 nm in a various different conditions to determine the strain's temperature preference, growth kinetics, salinity tolerance, pH range, oxygen requirement, and metabolic capabilities. Based on the results strain LP-1 can be characterized as a mesophilic, euryhalic, gram-negative, facultatively anaerobe that grew on a large spectrum of pH conditions. It is curved-rod in shape and is able to utilize a range of carbon sources for sustenance. Strain LP-1 was then to compared to the characteristics of the closely related reference species to determine strain LP-1's phenotypical similarities and differences to its congeners.

The reference species' data was acquired from Table 1 in Romano et al. 2005 paper on the isolated subspecies *Salinivibrio costicola* subsp. *alcaliphilus*,. A modified table was constructed (Table 5.1) to include and compare strain LP-1 to *Salinivibrio costicola* subsp. *alcaliphilus*, *Salinivibrio costicola* subsp. *costicola*, and *Salinivibrio costicola* subsp. *vallismortis*. These reference species were isolated from a variety of saline environments that

include a saltish spring with algal mats; salted foods; a hypersaline pond in Death Valley, CA; as well as other hypersaline environments.

Table 5.1: Phenotypic properties of strain LP-1 and selected reference organisms

Property:	**strain LP**	***Salinivibrio costicola* subsp. *costicola***	***Salinivibrio costicola* subsp. *vallismortis***	***Salinivibrio costicola* subsp. *alcaliphilus***
Site sampling	Impoundment used for shale waste water storage in Washington County, PA, USA.	Hypersaline habitats; salted food.	Hypersaline pond in Death Valley, California, USA.	Saltish spring with algal mat in Campania, South Italy.
Cell morphology	Curved rods	Curved rods	Short, curved rods	Curved rods
Colony color	Cream-colored	Cream colored	Cream-white	Cream-pink
Oxygen Requirement	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe	Aerobe
Spore formation	-	-	-	-
Motility	+	+	+	+
Gram reaction	-	-	-	-
NaCl Requirement	0	0.5	0	2.0
NaCl tolerance (%)	20.0	20.0	12.5	25.0
NaCl optimum (%)	10.0	10.0	2.5	10.0
Temperature range (°C)	4.0-65.0	5.0-45.0	20-50	10-40
Optimal growth temperature (°C)	25.0	37.0	37.0	30.0
pH range	4-10.5	5.0-10.0	5.5-8.2	7.0-10.5
Optimal pH	7.5	7.5	7.3	9.0

It is apparent how phenotypically similar strain LP-1 is to the *Salinivibrio costicola* reference species. Based on the molecular and physiological data it can be determined that strain LP-1 is a strain of the *S. costicola* species, and more specifically related to the subspecies *costicola* and *alcaliphilus*. From this it can be suggested that waste waters from natural gas extraction activities are becoming habitats to adaptable, halophilic species that are found in other hypersaline environments. This can be concerning from a conservation standpoint given wide range of conditions that these organisms can survive in, especially when considering that the impoundments housing these organisms are open-air in nature. Fluids that are contained in these impoundments are readily exposed to the environment, potentially providing the opportunity for impoundment-inhabiting species to be transported into the environment surrounding them. Organisms like strain LP-1 do not require a salinity concentration to survive and could possibly survive in an environment outside of an impoundment site. This could potentially interrupt the biomass and biodiversity of regional ecosystems that surround shale plays.

Additionally, strain LP-1 can metabolize a variety of organic compounds. The data from the carbon source growth experiment was compared to the same reference species (Table 5.2). The presence of these organic compounds increased the strains growth rate significantly. Once again, the availability of these compounds may allow for this strain to survive in habitats other than impoundments.

Table 5.2: Utilization of organic compounds by strain LP and *Salinivibrio costicola* subsp. *costicola*; *Salinivibrio costicola* subsp. *vallismortis*; and *Salinivibrio costicola* subsp. *alcaliphilus*.

Carbon Source	strain LP	*Salinivibrio costicola* subsp. *costicola*	*Salinivibrio costicola* subsp. *vallismortis*	*Salinivibrio costicola* subsp. *alcaliphilus*
Glucose	+	+	+	+
Galactose	NR	-	-	+
Fructose	+	-	NR	+
Mannose	NR	-	+	+
Arabinose	+	-	-	-
Sorbose	NR	NR	NR	-
Ribose	+	-	NR	-
Xylose	+	+	+/-	-
Sucrose	+	+	+	+
Maltose	+	-	-	+
Lactose	-	-	-	+
Trehalose	+	+	+	+
Glycerol	+	+	NR	+
Na-acetate	+	+	NR	+
Na-citrate	+	-	-	-
Casein	+	NR	NR	-
Na-lactate	+	NR	NR	NR
Pyruvic Acid	+	NR	NR	NR
Formate	-	NR	NR	NR

5.4 Microscopy

The microscopy images reveal cell morphology and groupings that are consistent with what would be characteristic of a *Salinivibrio costicola* strain. An aim of this study was to isolate a strain that was capable of forming pellicles and that could potential precipitate out metals. Based on the analysis, this aim was accomplished. The elemental spectral scan (Figure 4.10) displays that strain LP-1 produced pellicles that contained barium and strontium.

As stated earlier, the production of biofilms are thought to be a cause for bioplugging and corrosion in industrial equipment used in unconventional drilling operations (Fichter et al., 2008). This is a large scale industry problem. Biocides are used as a remedy to prevent the establishment of these microbial communities to reduce the degradation of industry infrastructure, but it appears that the formation of microbial biofilms may reduce the effectiveness of biocides in performing their designated purpose (Morton et al., 1998).

Furthermore, in the Yergeau et al., 2013 study, biofilms provide certain taxa, such as the Proteobacteria phylum, with increased exposure to the compounds that surrounded them. Exposure to these compounds to cells for a duration of greater than 8 weeks, potentially allowed for the establishment of adapted communities (Yergeau et al., 2013). Additionally, the increased presence of carbon compounds promoted growth for these organisms due to the stimulated expression of hydrocarbon-degrading genes, allowing microbes to utilize the carbon compounds present for growth, if other nutrients are not limiting (Yergeau et al., 2013).

This is of particular interest considering strain LP-1 is closely related to *Salinivibrio costicola*, which is a member of the Proteobacteria phylum, specifically the γ-proteobacteria class. γ-proteobacteria members tend to dominate the middle and bottom depths of untreated

impoundments as well as in the surfaces depths of the biocide amended impoundments (Mohan et al., 2013).

Potentially, the pellicles produced by strain LP-1 are allowing cells exposure to surrounding elements, giving them the ability to form adapted communities to those conditions. This is an important concept that may allow for the increased efficiency and effectiveness of industry practices that involve biocide treatment by targeting microbial defense mechanisms. It also raises the question of whether the exposures to hydrocarbons, which are prevalent in the oil and gas industry, are stimulating hydrocarbon-degrading genes within strain LP-1. These are important queries that should be pursued in future studies.

5.5 The Species *Salinivibrio costicola*

Salinivibrio costicola is a common inhabitant of produced waters within impoundments (Bergey, 2001). The genus *Salinivibrio* is composed of gram negative curved rods that are approximately 0.5-0.6 x 1.0-3.2 μm in size and can appear singly, in pairs, or occasionally united by an S-shapes or spirals. Cells are motile by a one polar flagellum and are non-spore forming. Their colonies are circular, convex, opaque, smooth, and cream colored (Bergey, 2001).

S. costicola are moderately halophilic with an optimum NaCl concentration for growth between 2.5% and 10% at 37° C. These bacteria are known for their ability to grow in a broad salinity growth range, between 0% and 20% NaCl. The species grow in temperatures that range between 5-50° C with an optimum temperature of 37 ^{o} C. Additionally, they have a broad pH growth range that is between 5-10 with an optimum pH of 7.3-7.5 (Bergey, 2001).

Salinivibrios are facultatively anaerobic, chemoorganotrophs (Bergey, 2001). They produce catalase and oxidase and acid is produced from the hydrolization of D-glucose and gelatin. They test positive when examined using the Voges-Proskauer. Additionally, *S. costicola*

tests negatively when assessed using arginine decarboxylase tests and in the Indole, β-galactosidase, lysine, and ornithine decarboxylase tests (Bergey, 2001).

Molecularly, the mol% G + C of DNA is 49.4-50.5 (Smith et al., 1938; emend. Haung et al, 2000). The genome size sizes of six strains ranged from 2100 to 2600 Kb (Mellado et al., 1997) based on investigations using pulsed field electrophoresis. Several plasmids and one megaplasmid have been reported (Fernandez-Castillo et al., 1992; Mellado et al., 1997). Conjugation is the only means of genetic transfer that has been described for this genus. A bacteriophage, phage UTAK, that infected and lysed *S. costicola* was isolated from salterns in Alicante, Spain and propagated optimally at 1-2 M NaCl (Goel et al., 1996), but the genetic transfer by transduction has yet to be studied (Beregy, 2001).

Although, more sub-species are being identified, two subspecies *S. costicola* subsp. *costicola* and *S. costicola* subsp. *vallismortis*, currently, represent the species. The nominotypie subspecies *costicola* was isolated from hypersaline environments (salterns, saline soils) and from salted food (Garcia et al., 1987b; mellado et al. 1996). Recently, Haung et al. (2000) isolated a single strain from a hypersaline pond in Death Valley, California, USA, which was phenotypically and genotypically similar to subsp. *costicola* with a mean sequence similarity of 97.7% based on 16S rDNA gene sequence comparison and a high degree of relatedness based on DNA-DNA hybridization studies (93%). Due to phenotypical differences the subspecies designation was proposed for the *vallismortis* strain creating two subspecies (Huang et al. 2000).

The distinct characteristic of *S. costicola* is its ability to grow in environments that contain high salinity concentrations, distinguishing it as a moderate halophile. With respect to its physiology and biochemistry, *S. costicola* is considered a model representative of the

physiological group. Several studies show that *S. costicola* has a specific requirement for Na+, but with the addition of high concentrations of glucose and glycerol the NaCl requirement can be lowered to 0.5 M to 0.3 M NaCl (Adams, et al., 1987). Temperature and medium composition can also affect the NaCl requirement (Kushner 1987; Adams and Russel, 1992). Complex media can stimulate growth at high salinities. This effect may be due to the presence in the medium of compatible solutes or their precursors, or other growth factors that may be synthesized more slowly under high salt conditions (Ventosa et al., 1998).

Strains of *S. costicola* demonstrate a heterogeneous response to antibiotics. They are very sensitive to chloramphenicol and rifampin (Garcia et al., 1987a). The salt concentrations may influence the susceptibility of *S. costicola* to antimicrobial compounds. However, *S. costicola* displayed a high sensitivity to rifampin and trimethoprim regardless of the salt concentration (Corondo et al., 1995). Additionally, the susceptibility of *S. costicola* to heavy metals have been studied and all 58 strains that were examined were susceptible to cadmium, copper, silver, zinc, and mercury, but showed tolerance to lead (Garcia et al., 1987a).

S. costicola has been found in salted foods as well as hypersaline habitats within nature such as salt lakes and salterns; these environments contain concentrations of 10-25% salts. In habitats that contain less than 15% salt, *S. costicola* is identified to be one of the predominant bacteria in the community (Rodriguez-Valera et. al., 1985; Marquez et al., 1987). They tend to be less evident in hypersaline soils (Quesada et al., 1992).

Due to the fact that *S. costicola* has served as a model organism in studies involving moderately halophilic bacteria, there is a substantial amount of information available concerning their ecology, physiology, and biochemistry (Bergey, 2001). The genus *Salinivibrio* can be

differentiated from other related genera, with the exception of *Vibrio*, based on morphology, since they are curved-rods in shape. Typical characteristics of this genus are their optimal growth medium containing 10.0% NaCl and its ability to produce arginine decarboxylase. Additionally, *Salinivibrio* can be can be differentiated from the genus *Vibrio* and other genera of the family *Vibrionaceae* by comparative analysis of 16S rRNA gene sequences. When compared to genus *Vibrio*, *Salinivibrio costicola* has two unique helical sequences and secondary structures at positions 178-197 and 197-219 of the 16S rRNA (*E. coli* 16S rRNA gene sequence numbering) (Huang et. al.,2000).

A majority of these characteristics that describe *Salinivibrio costicola* are consistent with this study's description of strain LP-1.

Chapter 6 Conclusion and Future Direction

This study's aim was to isolate and characterize a strain from an enrichment culture that was associated with Marcellus Shale waste waters; this was accomplished. Molecular analysis indicated that the microorganism belongs to the genus *Salinivibrio* and most closely related to the species *Salinivibrio costicola* susp. c*osticola* and *alcaliphilus*; these strains that have been found in numerous hypersaline habitats. Additionally, the hypothesis of the halophilic bacterium, strain LP-1, that was isolated from the Marcellus Shale waste water impoundment water sample was capable of forming pellicles, metabolizing organic compounds, and precipitating salts, has been confirmed through the results of this study.

Further investigation should be conducted in the future to identify how significant of a component strains of *Salinivibrio costicola* are within different sites involved in the Marcellus Shale natural gas extraction processes. This can be accomplished by reviewing Eastham's 2012

Ribosomal Intergenic Spacer Analysis (RISA) and Automated Ribosomal Intergenic Spacer Analysis (ARISA) data of the studied enrichments that were sampled from a variety of Marcellus Shale sites. If the banding pattern that is consistent with *Salinivibrio costicola* strains is present on a majority of the RISA gel's lanes as well as present on the ARISA heatmaps, then it can be determined how prevalent this species is within Marcellus Shale sites.

Further investigation should also include the hydrocarbon degradation potential of the *Salinivibrio costicola* strain LP-1 as well as the determinatione if stain LP-1 possesses hydrocarbon-degrading genes. These metabolic processes could have an effect not only within the impoundment community that the strain was isolated from, but also could have impacts within the formation considering the amount of organic matter that is present. The potential positive aspect of this strain metabolic ability, including the isolate's ability to form pellicles and precipitates, could possibly serve as a means for bioremediation in areas such as the treatment of produced water.

Investigation of the pellicles and precipitate forming potential of this strain could be conducted to determine if they have any impact on industrial infrastructure and have the ability to hinder industrial productivity through bioplugging, microbial induced corrosion, and the reduced effectiveness biocide treatment. Biocide resistance through the formation of pellicles could be studied to determine the most effective biocide composition and optimum concentration to be utilized for microbial control. Conversely, this could also lead to opportunities in aquifer bioremediation treatment for immobilization of heavy metals and co-precipitation. The isolation and characterization of EPS from strain LP-1 may offer an insight into the development of a natural flocculating agent for the removal of suspended solids from produced water. Data produced from these efforts could help provide insight for industry partners to create more

effective practices and efficient processes that could save them time, money, and materials. Finally, the investigation of fracturing fluid and produced water transport from open-air impoundments into the environment could provide insight into the possible spread of this species into local ecosystems and the affect that they may have on their biomass and biodiversity. This would also give insight how to improve shale waste water containment.

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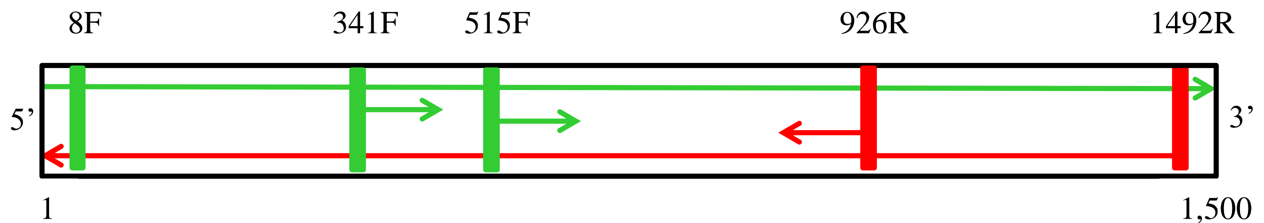
Appendix A: 16S rRNA Universal Bacterial Primers and Gene Map

16S rRNA Universal Primers:

Primer* Sequence (5'-3')	Target Group	Reference
8F – AGAGTTTGATCATGGCTCAG	Universal	Turner et al., 1999; Wuyts et al., 2002
341F – CCTACGGGRSGCAGCAG	Universal	Hanson et al., 1998; Baker et al., 2003
515F – GTGCCAGCMGCCGCGGTAA	Universal	Reysenbach et al., 1995; Baker et al., 2003
926R – CCGTCAATTCCTTTRAGTTT	Universal	Reysenbach et al., 1995; Baker et al., 2003
1492R – GGTTACCTTGTTACGACTT	Universal	Turner et al., 1999; Youssef et al., 2009

16S rRNA Gene Map with Universal Primers:



Appendix B: Sequencing Data

16S rRNA Gene Sequence of the strain LP-1 isolate:

ATTAGCTTTGAGGGCCGGGCAAGCTACACAAATGACAAGTCGAGCGGAACGGCAA
GTATTGAAAGCTTCGGTGGATTTACTGGACGTCGAGCGGCGGACGGGTGAGTAACG
GCTGGGAACCTGCCCTGACGAGGGGGATAACCGTTGGAAACGACGGCTAATACCGC
ATAATGTCTTAGTTCATTACGAGCTGGGACCAAAGGTGGCCTCTACATGTAAGCTAT
CGCGTTGGGATGGGCCCAGTTAGGAGTTAGCTAGTTGGTAAGGTAATGGCTTACCAA
GGCAACGATCCTTAGCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGGAATATTGCACAATGGGGGGAGAC
CCTGATGCAGCCATGCCGCGTGTGTGAAGAANGCCTTCGGGTTGTAAAGCACTTTTC
AGCAGTGAGGAAGGTCGTGTACTTAATACGTGCACGGCTTGACGTTAGCTGCAGAA
GAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTT
AATCGGAATTACTGGGCGTAAAGCGCATGCAGGCGGTTTGTTAAGTCAGATGTGAA
AGCCCGGGGCTCAACCTCGGAACCGCATTTGAAACTGGCAGGCTAGAGTCTTGTAG
AGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACC
AGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGATGCGAAAGCGTGGGT
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGCTGTCTACTTGGAG
GTTGAGGTTTTAGACTTTGGCTTTCGGCGCTAACGCATTAAGTAGACCGCCTGGGGG
AGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAG
CGAAGCCTTTAGAGATAGAGGCGTGCCTTTCGGGAGCGCTGAGACAGGTGCTGCAT
GGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTATCCTTGTTTGCCAGCACATAATGGTGGGAACTCCAGGGAGACTGCCGGTGATA
AACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTA
CACACGTGCTACAATGGCAGATACAGAGGGCAGCGAGACAGCGATGTTTAGCGAAT
CCCTTAAAGTTTGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGG
AATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTAC
ACACCGCCCGTCACACCATGGGAGTGGGCTGCACCAGAAGTAGATAGCTTAACCTC
GGGAGGGCGTTACCACGGTGGGTTCCAAGAAAGGGACCTTGATCCCCCAAGTTAAT
TTTCCCCCTAGAGAAGGGGTCTTTGTCCTGCAGGTAAAAAACTCCCCCTTTATTTCCT
CACCTTTCTCTTT

strain LP-1 16S rRNA BLAST Results (NCBI):

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Salinivibrio costicola subsp. costicola strain ATCC 33508 16S ribosomal RNA gene, partial sequence	2571	2571	93%	0.0	98%	NR_027590.1
☐	Salinivibrio costicola subsp. alcaliphilus strain 18AG 16S ribosomal RNA gene, partial sequence	2573	2573	94%	0.0	98%	NR_042255.1
☐	Salinivibrio costicola subsp. vallismortis strain DV 16S ribosomal RNA gene, complete sequence	2370	2370	94%	0.0	96%	NR_028703.1
☐	Salinivibrio proteolyticus strain AF-2004 16S ribosomal RNA gene, partial sequence	2362	2362	94%	0.0	96%	NR_043536.1
☐	Salinivibrio siamensis strain ND1-1 16S ribosomal RNA gene, partial sequence	2355	2355	94%	0.0	96%	NR_041552.1
☐	Enterovibrio nigricans strain DAl 1-1-5 16S ribosomal RNA gene, partial sequence	1958	1958	90%	0.0	92%	NR_042699.1
☐	Enterovibrio calviensis strain RE35F/12 16S ribosomal RNA gene, partial sequence	1934	1934	90%	0.0	92%	NR_041741.1
☐	Photobacterium profundum strain DSJ4 16S ribosomal RNA gene, partial sequence	1903	1903	90%	0.0	91%	NR_036943.1
☐	Enterovibrio norvegicus strain LMG 19839 16S ribosomal RNA gene, complete sequence	1973	1973	94%	0.0	91%	NR_042082.1
☐	Enterovibrio coralii strain CC17 16S ribosomal RNA gene, partial sequence	1973	1973	94%	0.0	91%	NR_042342.1
☐	Photobacterium frigidiphilum strain SL13 16S ribosomal RNA gene, complete sequence	1869	1869	90%	0.0	91%	NR_042964.1
☐	Photobacterium indicum strain MBIC3157 16S ribosomal RNA gene, partial sequence	1864	1864	90%	0.0	91%	NR_028002.1
☐	Vibrio penaeicida strain DSM 14398 16S ribosomal RNA gene, complete sequence	1857	1857	90%	0.0	91%	NR_042121.1
☐	Photobacterium kishitanii strain pjapo.1.1 16S ribosomal RNA gene, partial sequence	1855	1855	90%	0.0	91%	NR_042852.1
☐	Photobacterium lipolyticum strain M37 16S ribosomal RNA gene, partial sequence	1853	1853	90%	0.0	91%	NR_025813.1

16S rRNA Alignments Comparing strain LP-1 to Reference Species:

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                                                                                                                                                                                                        ********************** ****************    * *
strain_LP-1.seq                                 --------------ATTAGCTTTGAGGGCCGG--GCAAGCTACACAAATGACAAGTCGAGCGGGAACGGCAAGTATTGAAAGCTTCGGTGGATTTACTGGACGTCGAGCGGCGGACGGGTGAGTAACGGCTGGGAACCTGCCCTGACGAG   150
Salinivibrio_costicola_subsp._costicola.seq     -GAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATG-CAAGTCGAGCGGAAACGGCA-GTATTGAA-GCTTCGGTGGATTTACTGGACGTCGAGCGGCGGACGGGTGAGTAACGGCTGGGAACCTGCCCTGACGAG   150
Salinivibrio_costicola_subsp._alcaliphilus.seq  ----------------TCAGATTGAACGCTGGCGGCAGGCCTAACACATG-CAAGTCGAGCGGAAACGGCA-GTATTGAA-GCTTCGGTGGATTTACTGGACGTCGAGCGGCGGACGGGTGAGTAACGGCTGGGAACCTGCCCTGACGAG   150
Salinivibrio_costicola_subsp._vallismortis.seq  AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATG-CAAGTCGAGCGGAAACGGCA-GTATTGAA-GCTTCGGTGGATTTACTGGACGTCGAGCGGCGGACGGGTGAGTAACGGCTGGGAACCTGCCCTGACGAG   150
Salinivibrio_proteolyticus.seq                  --------------------ATTGAACGCTGGCGGCAGGCCTAACACATG-CAAGTCGAGCGGAAACGGCA-GTATTGAA-GCTTCGGTGGATTTACTGGACGTCGAGCGGCGGACGGGTGAGTAACGGCTGGGAACCTGCCCTGACGAG   150
Salinivibrio_siamensis.seq                      TGAGTTTGATCCTGGCTCAGATTGAACGCCGGCGGCAGGCCTAACACATG-CAAGTCGAGCGGAAACGGCA-GTATTGAA-GCTTCGGTGGATTTACTGGACGTCGAGCGGCGGACGGGTGAGTAACGGCTGGGAACCTGCCCTGACGAG   150
Enterovibrio_nigricans.seq                      --GTTTGAATTATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATG-CAAGTCGAGCGGTAACATTT-CAA---AA-GCTT-----GCTTTTGAAGATGACGAGCGGCGGACGGGTGAGTAATGGCTGGGAACCTGCCCTGATGTG   150
Enterovibrio_calviensis.seq                     --------------------------------------------------------------------------------------------------------CGAGCGGCGGACGGGTGAGTAATGGCTGGGAACCTGCCCTGACGTG   150
Enterovibrio_coralii.seq                        --------------------ATTGAACGCTGGCGGCAGGCCTAACACATG-CAAGTCGAGCGGCAGCGACT-TAACTGAA-CCTTCGGGGGAYGTTAAGGGCGGCGAGCGGCGGACGGGTGAGTAATGGCTGGGAACCTGCCCGATAGAG   150
Enterovibrio_norvegicus.seq                     ---------------------------------GGCAGGCCTAACACATG-CAAGTCGAGCGGCAGCGACA-ACAAMGAT-TCTTCGGAKGATTTGTTGGGCGGCGAGCGGCGGACGGGTGAGTAATGGCTGGGAACCTGCCCTGACGTG   150
                                                1.......10........20........30........40........50........60........70........80........90.......100.......110.......120.......130.......140.......150

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strain_LP-1.seq                                 GGGGATAACCGTTGGAAACGACGGCTAATACCGCATAATGTCTTAG-TTCATTACGAGCTGGGACCAAAGGTGGCCTCTACATGTAAGCTATCGCGTTGGGATGGGCCCAGTTAGGAGTTAGCTAGTTGGTAAGGTAATGGCTTACCAAG   300
Salinivibrio_costicola_subsp._costicola.seq     GGGGATAACCGTTGGAAACGACGGCTAATACCGCATAATGTCTTAGATTCATTA-GAGCTGGGACCAAAGGTGGCCTCTACATGTAAGCTATCGCGTTGGGATGGGCCCAGTTAGGA-TTAGCTAGTTGGTAAGGTAATGGCTTACCAAG   300
Salinivibrio_costicola_subsp._alcaliphilus.seq  GGGGATAACCGTTGGAAACGACGGCTAATACCGCATAATGTCTTGA-TTCGTTA-GAGTCAGGACCAAAGGTGGCCTCTACATGTAAGCTATCGCGTTGGGATGGGCCCAGTTAGGA-TTAGCTAGTTGGTAAGGTAATGGCTTACCAAG   300
Salinivibrio_costicola_subsp._vallismortis.seq  GGGGATAACCGTTGGAAACGACGGCTAATACCGCATAATGTC-TAC---------------GGACCAAAGGTGGCCTCTACATGTAAGCTATCGCGTTGGGATGGGCCCAGTTAGGA-TTAGCTAGTTGGTAAGGTAACGGCTTACCAAG   300
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                                                .......160.......170.......180.......190.......200.......210.......220.......230.......240.......250.......260.......270.......280.......290.......300

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Salinivibrio_costicola_subsp._vallismortis.seq  GCGACGATCCTTAGCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG-AATATTGCACAATGGGGG-AGACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCG   450
Salinivibrio_proteolyticus.seq                  GCGACGATCCTTAGCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG-AATATTGCACAATGGGGG-AGACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCG   450
Salinivibrio_siamensis.seq                      GCGACGATCCTTAGCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG-AATATTCCACAATGGGGG-AGACCCTGATGCAGCCATGCGGCGTGTGTGAAGAAGGCCTTCG   450
Enterovibrio_nigricans.seq                      GCGACGATCCCTAGCTGGTTTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG-AATATTGCACAATGGGCG-CAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCG   450
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Enterovibrio_norvegicus.seq                     GCGACGATCCCTAGCTGGTTTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG-AATATTGCACAATGGGCG-CAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCG   450
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Salinivibrio_costicola_subsp._alcaliphilus.seq  GGTTGTAAAGCACTTT-CAGCAGTGAGGAAGGTCGTGTACTTAATACGTGCACGGCTTGACGTTAGCTGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTA   600
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Salinivibrio_siamensis.seq                      GGTTGTAAANCACTTT-CAGCAGTGAGGAAGGTGGTGTACTTAATACNTGCATGGCTTGACGTTAGCTGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTNCGAGCGTTAATCGGAATTACTGGGCGTA   600
Enterovibrio_nigricans.seq                      GGTTGTAAAGCACTTT-CAGCAGTGAGGAAGGTTACGTAGTTAATACCTGCGTGATTTGACGTTAGCTGCAGAAGAAGGACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTCCGAGCGTTAATCGGAATTACTGGGCGTA   600
Enterovibrio_calviensis.seq                     GGTTGTAAAGCACTTT-CAGCAGTGAGGAAGGTTGGTGCGTTAATAGCGTATCAATTTGACGTTAGCTGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTA   600
Enterovibrio_coralii.seq                        GGTTGTAAAGCACTTT-CAGCAGTGAGGAAGGTTACGTAGTTAATACCTGCGTRATTTGACGTTAGCTGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTA   600
Enterovibrio_norvegicus.seq                     GGTTGTAAAGCACTTT-CAGCAGTGAGGAAGGGTRATGAGTTAATACCTCATTATCTTGACGTTAGCTGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTA   600
                                                .......460.......470.......480.......490.......500.......510.......520.......530.......540.......550.......560.......570.......580.......590.......600

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strain_LP-1.seq                                 AAGCGCATGCAGGCGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAACCGCATTTGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCAG   750
Salinivibrio_costicola_subsp._costicola.seq     AAGCGCATGCAGGCGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAACCGCATTTGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCAG   750
Salinivibrio_costicola_subsp._alcaliphilus.seq  AAGCGCATGCAGGCGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAACCGCATTTGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCAG   750
Salinivibrio_costicola_subsp._vallismortis.seq  AAGCGCATGCAGGCGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAACCGCATTTGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCAG   750
Salinivibrio_proteolyticus.seq                  AAGCGCATGCAGGCGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAACCGCATTTGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCAG   750
Salinivibrio_siamensis.seq                      AAGCGCATGCAGGCGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAACCGCATTTGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCAG   750
Enterovibrio_nigricans.seq                      AAGCGCATGCAGGCGGTTTGTTAAGCAAGATGTGAAAGCCCCGGGCTTAACCTGGGAACCGCATTTTGAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGG   750
Enterovibrio_calviensis.seq                     AAGCGCATGCAGGCGGTTTGTTAAGCAAGATGTGAAAGCCCCGGGCTCAACCTGGGAACCGCATTTTGAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGG   750
Enterovibrio_coralii.seq                        AAGCGCATGCAGGCGGTTTGTTAAGCAAGATGTGAAAGCCCCGGGCTTAACCTGGGAACAGCATTTTGAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGG   750
Enterovibrio_norvegicus.seq                     AAGCGCATGCAGGCGGTTTGTTAAGCAAGATGTGAAAGCCCCGGGCTCAACCTGGGAACCGCATTTTGAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGG   750
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strain_LP-1.seq                                      TGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGATGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGCTGTCTACTTGGAGGTTGAGGTTTTAGACTTTGGCTTTCGGCGCTAACGCATTA  900
Salinivibrio_costicola_subsp._costicola.seq          TGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGATGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGCTGTCTACTTGGAGGTTGAGGTTTTAGACTTTGGCTTTCGGAGCTAACGCATTA  900
Salinivibrio_costicola_subsp._alcaliphilus.seq       TGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGATGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGCTGTCTACTTGGAGGTTGAGGTTTTAGACTTTGGCTTTCGGCGCTAACGCATTA  900
Salinivibrio_costicola_subsp._vallismortis.seq       TGGCGAAAGCGGCCCCCTGGACAAAGACTGACGCTCAGATGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGCTGAGGTTTAAGACTTTGGCTTTCGGAGCTAACGCATTA  900
Salinivibrio_proteolyticus.seq                       TGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGATGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGCTGAGGTTTTAGACTTTGGCTTTCGGAGCTAACGCATTA  900
Salinivibrio_siamensis.seq                           TGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGATGAGAAAGCGTGGGTAGCAAACAGAATTAGATACCCTGGTAGTCCACGCAGTAAACGCTGTCTACTTGGAGGTTGAGGTTT-AGACTTTGGCTTTCGGCGCTAACGCATTA  900
Enterovibrio_nigricans.seq                           TGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCTACTTGGAGGTTGTTCTCTAGAGGAGTGGCTTTCGGAGCTAACGCGTTA  900
Enterovibrio_calviensis.seq                          TGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCTACTTGGAGGTTGTGACCTARAGTCGTGGCTTTCGGAGCTAACGCGTTA  900
Enterovibrio_coralii.seq                             TGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCTACTTGGAGGTTGTGACCTAGAGTCGTGGCTTTCGGAGCTAACGCGTTA  900
Enterovibrio_norvegicus.seq                          TGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCTACTTGGAGGTTGCGGTCTTGAACTGTGGCTTTCGGAGCTAACGCGTTA  900
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strain_LP-1.seq                                      AGTAGACCGCCTGGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGCGAAGCCTTTAGAGATAGAG  1050
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Salinivibrio_costicola_subsp._alcaliphilus.seq       AGTAGACCGCCTGGGG-AGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGCGAAGCCTTTAGAGATAGAG  1050
Salinivibrio_costicola_subsp._vallismortis.seq       AGTAGACCGCCTGGGG-AGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATCTTCCAGAGATGGAG  1050
Salinivibrio_proteolyticus.seq                       AGTAGACCGCCTGGGG-AGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGAT  1050
Salinivibrio_siamensis.seq                           AGTAGACCGCCTGGGG-AGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGAT  1050
Enterovibrio_nigricans.seq                           AGTAGACCGCCTGGGG-AGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAAGCCGAAAGAGATTTTG  1050
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Enterovibrio_norvegicus.seq                          AGTAGACCGCCTGGGG-AGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTYGCTAGAGATAGCT  1050
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strain_LP-1.seq                                      GCGTGCCTTTCGGGAGCGCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCACATAATGGTGGGAACTCCAGGGAGACTGCCGGTGATA  1200
Salinivibrio_costicola_subsp._costicola.seq          GTGTGCCTT-CGGGAGCGCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCACNTAATGGTGGGAACTCCAGGGAGACTGCCGGTGATA  1200
Salinivibrio_costicola_subsp._alcaliphilus.seq       GCGTGCCTT-CGGGAGCGCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCACRTAATGGTGGGAACTCCAGGGAGACTGCCGGTGATA  1200
Salinivibrio_costicola_subsp._vallismortis.seq       GAGTGCCTT-CGGGAGCTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCAAGTAATGTCGGGAACTCCAGGGAGACTGCCGGTGATA  1200
Salinivibrio_proteolyticus.seq                       TGGTGCCTT-CGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCACGTAAAGGTGGGAACTCCAGGGAGACTGCCGGTGATA  1200
Salinivibrio_siamensis.seq                           TGGTGCCTT-CGGGAGCTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCACGTCATGGTGGGAACTCCAGGGAGACTGCCGGTGATA  1200
Enterovibrio_nigricans.seq                           GTGTGCCTT-CGGGAGCTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCACATAATGGTGGGAACTCCAGGGAGACTGCCGGTGATA  1200
Enterovibrio_calviensis.seq                          GTGTGCCTT-CGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCGAGTAATGTCGGGAACTCCAGGGAGACTGCCGGTGATA  1200
Enterovibrio_coralii.seq                             GTGTGCCTT-CGGGARCTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCACGTAATGGTGGGAACTCCAGGGAGACTGCCGGTGATA  1200
Enterovibrio_norvegicus.seq                          TAGTGCCTT-CGGGAGCTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCGAGTAATGTCGGGAACTCCAGGGAGACTGCCGGTGATA  1200
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strain_LP-1.seq                                      AACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCAGATACAGAGGGCAGCGAGACAGCGATGTTTAGCGAATCCCTTAAAGTTTGTC-GTAGTCCGGATTGGAGTCTGCA  1350
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Salinivibrio_proteolyticus.seq                       AACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCAGATACAGAGGGCAGCGAGACAGCGATGTTAAGCGAATCCCTTAAAGTTTGTCTGTAGTCCGGATTGGAGTCTGCA  1350
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Enterovibrio_nigricans.seq                           AACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAGAGGGCAGCGAGGCCGCGAGGTGGAGCGAATCCCAGAAAGTATGTC-GTAGTCCGGATCGGAGTCTGCA  1350
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Enterovibrio_norvegicus.seq                          AACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAGAGGGCAGCGAGGCCGCGAGGTGGAGCGAATCCCAGAAAGTATGTC-GTAGTCCGGATTGGAGTCTGCA  1350
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strain_LP-1.seq                                      ACTCGACTCCATGAAGT-CGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGCTGCACCAGAAGTAGATAGCTTAACCT-CGGGAGGGCGTT-ACC  1500
Salinivibrio_costicola_subsp._costicola.seq          ACTCGACTCCATGAAGT-CGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGCTGCACCAGAAGTAGATAGCTTAACCTTCGGGAGGGCGTT-TA-  1500
Salinivibrio_costicola_subsp._alcaliphilus.seq       ACTCGACTCCATGAAGT-CGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGCTGCACCAGAAGTAGATAGCTTAACCTTCGGGAGGGCGTTTACC  1500
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Enterovibrio_norvegicus.seq                          ACTCGACTCCATGAAGT-CGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGCTGCACCAGAAGTAGATAGCTTAACCTTCGGGAGGGCGTTTACC  1500
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Salinivibrio_costicola_subsp._alcaliphilus.seq     --ACGGTGTGGTTCATG-ACTGGGGTGAAGTCGTAACAAGGTAG-----CCCTAGGGGAACCTGCGGCTGGATCAC--- 1579
Salinivibrio_costicola_subsp._vallismortis.seq     --ACGGTGTGGTTCATG-ACTGGGGTGAAGTCGTAACAAGGTAGCCCTAGGGGAACCTGGGGCTGGATCACCTCCTTA- 1579
Salinivibrio_proteolyticus.seq                     --ACGGTGTGGTACCTGCGGCTGGATCACCTCCTTA------------------------------------------- 1579
Salinivibrio_siamensis.seq                         CAACGGTGTGGTTC----------------------------------------------------------------- 1579
Enterovibrio_nigricans.seq                         --ACGGTGTGGTTCATG-ACTGGGGTGAAGTCGTA-------------------------------------------- 1579
Enterovibrio_calviensis.seq                        --ACGGTGTGGTTC----------------------------------------------------------------- 1579
Enterovibrio_coralii.seq                           --ACGGTGTGGTTCATG-ACTGGGGTGAAGTCGTAACAAGGTAGCCCTAGGGGAACC---------------------- 1579
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