BTEX DEGRADATION AT HIGH SALINITY IN ROZEL POINT

By

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Bachelor of Arts/Science in Biology

Ehime University

Matsuyama, Ehime

2005

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 2009

BTEX DEGRADATION AT HIGH SALINITY IN ROZEL POINT

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AKNOWLEDGMENTS

First of all, I wish to show my best appreciation to Dr. Babu Fathepure. I found him to be nice, hospitable and intelligent. He patiently instructed me and taught me many useful things. I wish to thank my other committee members, Dr. Mostafa Elshahed and Dr. Udaya Desilva, for their support, encouragement, and timely counsel. I am grateful to Drs. Xioping and Charuka in Dr. Desilva's lab for their help with microbial diversity experiments. My gratitude extends to Dr. Anand Sukhan for his assistance with molecular works. I am thankful to Laura Christian for collecting Rozel point samples who was my nice colleague.

I have had a great time of working with many of friends in the department of Microbiology and Molecular Genetics, Oklahoma State University. Especially, I would like to thank Timothy King, Sugata Mahapatra, Sumit Pamj, Majed Naster, and Jim Davis. They not only have aided in research but many are good friends. I have enjoyed working along side Shrishti Chhabra and Sarah Panther. I am also deeply grateful to Ms. Connie Budd for her kindness and technical contributions.

Finally, special thanks are due to my friends and my family who encouraged and advised me through my time in the graduate school. I wish to express my appreciation to Chris Reimers who was my roommate and had a nice time together. My parents have given me a lot of cheering and support. My brother, Yu, has advised me for study in abroad. Also, I wish to thank to my sister, Nao.

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ABBREVIATIONS AND SYMBOLS

1. BTEX ..... benzene, toluene, ethylbenzyene, and xylene

2. bp ..... base pairs

4. GC ..... gas chromatograph

5. µ ..... micro

6. ml ..... milliliter

7. mM ..... millimoler

8. MSM ..... mineral salts medium

9. ng ..... nanogram

10. PCR ..... polymerase chain reaction

11. sp ..... specie

12. spp ..... species

13. w/v ..... weight per volume

CHAPTER I

INTRODUCTION

The Great Salt Lake is the fourth largest terminal lakes in the world with salinity as high as 30 % at most locations (Baxter *et al*, 2005). Naturally occurring oil seeps were discovered in the late 1800 in northeastern part of GSL near Rozel Point (Milligan, 2005). In addition, oil companies have attempted to extract oil at Rozel Point for almost 100 years in the past. Such human activities have resulted in many abandoned leaking oil-wells and storage tanks (U.S.EPA, 2005). The co-occurrence of high salinity and petroleum seeps render this site unique. In spite of these unique features at Rozel Point, surprisingly very few studies have been carried out to understand microbial diversity and their dynamics, their capacity to degrade petroleum hydrocarbons, and their role in carbon cycling.

In the present study we explore the degradation potential of non-oxygenated, low molecular weight fuel hydrocarbons including benzene, toluene, ethylbenzene, and xylenes (BTEX) in sediment samples collected from GSL near Rozel Point. We obtained a highly enriched culture from Rozel Point sediment that rapidly degraded BTEX compounds. Using this highly enriched culture, we assessed its BTEX degradation potential under various growth conditions including degradation rate in the presence of different salt concentrations. We also tested the impact of easily utilizable organic substrates on benzene degradation rates. Further we tested BTEX degradation pathways in halophiles by cloning and sequencing key catabolic genes. This is important since not much is known about the phylogenetic diversity and metabolic capabilities of microbes at

Rozel Point. Identification of catabolic genes determines the biodegradation potential of the microbial community and also helps develop rapid and sensitive methods to monitor bioremediation processes.

We have successfully isolated a halophile that degrades benzene as the sole source of carbon. We have studied the isolate's phylogeny, physiology, and capacity to degrade BTEX compounds at various salt concentrations. Phylogentic analysis indicated that our isolate has only 93 % sequence similarity with most closely related organism in the GenBank, *Arhodomonas aquaeolei*. This suggests that perhaps our isolate represents a new species of a new genus in the *Ectothiorhodopriraceae* family of the *Gammaproteobacteria* subclass. We tentatively refer this isolate as strain-Rozel.

CHAPTER II

REVIEW OF LITERATURE

Background

Many hypersaline environments such as natural saline lakes, salt flats, solar salterns, industrial effluents, oil fields, coastal ecosystems, and Sabkhas are often contaminated with high levels of petroleum hydrocarbons. Among the contaminated hypersaline environments, oilfields pose a special problem due to their sheer number all over the world and due their high salinity caused by salty brackish water (produced water) that is generated during oil and natural gas production. For every barrel of oil produced, roughly 10 barrels of produced water are generated. The United States is eleventh in the world with estimated oil reserves of 21.9 billion barrels (Field, 2005). In 2007 USA produced 1,862,441 barrel of crude oil, and state of Oklahoma produced 62,841 barrel of crude oil thus accounting for roughly 3 % of the overall oil production in the United States (Energy Information Administration, 2008). Estimations suggest that the United States produces 20 to 30 billion barrels of produced water every year, and Oklahoma State generated 1.25 billion barrels of produced water as of 2002; at this point, 66 million barrels of crude oil was produced in the state (Veil *et al*, 2004). Produced water contains high levels of salt (0.1 to 400 g/L), petroleum hydrocarbons, heavy metals, and trace elements (Kharaka and Otton, 2003), and it is inevitable that the water contaminates the field around oil production facilities. An important issue is that such contaminated fields are unworthy to use for other activities after oil dries up and wells are

abandoned. This is because components of oil are carcinogenic, stable, and water soluble; the Environmental Protection Agency lists these compounds as priority pollutants.

A variety of physical and/or chemical treatment technologies can be used for the cleanup of hydrocarbon sites, but most of these techniques are costly, ineffective, often damaging to the environment. Bioremediation is a technology that utilizes a variety of microorganisms to degrade toxic pollutants to harmless products such as CO_2, H_2O, and other inorganic compounds and these processes are environmentally safe and cost efficient (Philip *et al*, 2005). For example, it has been reported that roughly 25 % of all petroleum-contaminated land is being remediated using natural attenuation processes thus underscoring the importance of microorganisms in remediation strategies (Holden *et al*, 2002). However, the presence of salt limits the application of this technology since high salinity is detrimental to microbial life. Therefore, for the bioremediation of saline environments, salt-loving (halophilic) organisms are necessary. Halophiles are classified into three groups according to their optimal salt concentration for growth: slightly halophilic (1-3% w/v); moderately halophilic (3-15% w/v); and extremely halophilic (>15 % w/v) (Ventosa and Nieto, 1995).

The majority of halophiles belong to the bacterial and archaeal domains. Halophilic bacteria are diverse and belong to several phyla, while most of halophilic archaea are the members of the family *Halobacteriaceae*. In general, archaea are considered to be the dominant population in high salinity environments, while bacteria prefer lower salinity for optimal growth (Oren, 2002). Nonetheless, there are few examples of bacteria that prefer high salinity environments (Anton *et al*, 2002). For example, recently isolated *Salinibacter ruber* grows in the presence of salt as high as 4 M.

This is a true halophile with optimal. Generally, halophilic bacteria counter their osmotic stress by producing or by accumulating in their cytoplasm large concentrations of organic compounds (compatible solutes) such as proline, glutamine, aspartic acid, glycine, glycine betaine, curnitine, trehalose, and ectoin (Ventosa *et al*, 1998). In contrast, the majority halophilic archaea regulate their osmotic stress by the influx of high concentrations of cations such as K^+ Cl^- into their cytoplasm (Margesin and Schinner, 2001).

Although many microorganisms have been shown to degrade petroleum hydrocarbons and have been successfully used in bioremediation of contaminated environments, our knowledge of halophiles and their ability to degrade hydrocarbons under saline conditions is still limited. Therefore, more studies are needed to understand their diversity, ecology, physiology, and mechanisms of degradation. Such information is significant and can be helpful in designing novel and more efficient strategies for the remediation saline habitats that are contaminated with toxic chemicals. Rozel Point is a unique site because of its high salinity and numerous petroleum seeps. Unfortunately, not much is known about the microbial community of this site and their metabolic capacity to degrade petroleum seeps. Therefore, molecular survey of the microbial diversity and their potential to biodegrade hydrocarbons will help develop biological catalysts to remediate oil-contamination under highly saline environments.

The Great Salt Lake – Rozel Point

The Great Salt Lake (GSL) is located in the northern part of the state of Utah and is the largest salty lake in the US measuring roughly 122 km long and 50 km wide with

an average depth of 4.3 m. The lake is the largest remnant of Lake Bonneville, which covered much of western Utah in prehistoric times. The lake is confined and has no major outlet besides evaporation. The construction of a railroad track in 1955 and 1959 has effectively divided the Great Salt Lake into two parts, the North Arm and the South Arm thus further isolating the North Arm from freshwater input from snow melts in the Wasatch Mountain Range making it highly saline (Baxter *et al*, 2005). The salinity in the South Arm ranges between 5 and 15 %, while it is close to saturation (approximately 30 %) in the North Arm (Gwynn, 2002). Another unique feature of the North Arm is that it contains numerous oil seeps near Rozel Point. These seeps have been known since late 1800 and years of oil-extraction activities of the past have resulted in many abandoned leaking wells and storage tanks (Eardley, 1963).

Although, a few studies have reported the isolation of aerobic and anaerobic halophiles using culture-dependent studies from the Great Salt Lake, molecular approaches are necessary to fully appreciate the diversity and community structure that exist at this unique lake. The first isolate from the South Arm of the Lake was an obligate anaerobic bacterium, *Haloanaerobium praevalens* (Zeikus *et al*, 1983). The organism could thrive in 2-30 % salinity. Since then, several halophilic aerobic/anaerobic microoganisms including bacteria and archaea have been isolated from GSL (Baxter *et al*, 2005; Haws, 2007).

Aerobic degradation of petroleum hydrocarbons by halophiles

The application of bioremediation technology for the treatment of contaminated saline environments has not been tested since normal microorganisms (non-halophiles)

that degrade hydrocarbons can be easily harmed by high salinity. Most microorganisms are sensitive to ionic change caused by high salinity that results in the inhibition of system performance, disrupts normal metabolism, and reduces biological degradation kinetics (Wooland and Irvine, 1995). Fortunately, a vast array of halophiles thrives under saline environments and some require salt to survive (Ventosa and Nieto, 1995). Hence, such organisms can be used for the degradation of petroleum contaminants. The petroleum is mixture of a variety of compounds including aliphatic, cycloaliphatics, mono-aromatic and polyaromatic hydrocarbons and many of these compounds are toxic and carcinogenic (Philip *et al*, 2005). Although, these compounds can be easily degraded under low salinity environments including marine habitats (Swannell *et al*, 1996; Harayama *et al*, 1999; Hua, 2006; DeMello *et al*, 2007; Powell *et al*, 2007), little is known about their fate under moderate to hypersaline conditions (3 to 30% salt). This section reviews recent progress on biodegradation of petroleum compounds by halophiles under moderate to hypersaline conditions. The information is summarized on Table 1.

Crude oil

Crude oil is the original mixture of a variety of petroleum hydrocarbons. Diaz *et al*. (2002) have reported that a bacterial consortium, MPD-M developed from Columbian mangrove roots degraded crude oil in the presence of 0-18 % salt. In addition, several pure culture have been isolated that have the ability to crude oil. For example, *Marinobacter aquaeolei*, *Streptomyces albacialis*, and *Pseudomonas* strains were shown to degrade crude oil in the presence of 0 to 20 % salt (Kuznetsov *et al*, 1992; Huu *et al*, 1999; Kapley *et al*, 1999).

Aliphatic compounds

The first hydrocarbon biodegradation by halophiles was shown using an aliphatic compound, hexadecane (Ward and Brock, 1978). They reported degradation of hexadecane over a wide range of salinity (0 to 20 % salt) by a microbial consortium enriched from water samples obtained from the Great Salt Lake, Utah. Since then, many reports have appeared in the literature on the degradation of a variety of aliphatic compounds by mixed cultures and pure cultures obtained from different hypersaline environments (Table 1). Briefly, a halotolerant bacterium, *Marinobacter aquaeolei*, that degrades n-hexadecane and pristane at 0 to 20 % salinity was isolated from an oil-producing well in southern Vietnam (Huu *et al*, 1999). Fernandez-Linares *et al.* (1996) have reported on the ability of *Marinobacter hydrocarbonoclasticus* to degrade eicosane at various salt concentrations. Unlike previous observations, the biodegradation of eicosane was not affected when the concentration of salt increased from 0.2 to 2.5 M. Bertrand *et al*. (1990) have reported the isolation of an archaeal strain, EH_4 from a salt-marsh. This strain degraded a variety of saturated hydrocarbons at 3.5 M NaCl. To our knowledge this is the only example of archaea that degrades non-oxygenated hydrocarbons. Plotnikova *et al*. (2001) reported degradation of octane at 17 % salinity by *Rhodococcus* sp., *Arthrobacter* sp., and *Bacillus* sp. isolated from sediment samples obtained from chemical- and salt processing plants in Russia.

Polyaromatic hydrocarbons (PAHs)

The first halophile able to degrade naphthalene was reported by Ashok *et al.* (1995). The organism was a member in genus *Micrococcus* and degraded naphthalene at 7.5 % salinity. Plotnikva *et al.* (2001) have shown the degradation of naphthalene, phenanthrene, and biphenyl by *Psesudomonas* sp., *Rhodococcus* sp., *Arthrobacter* sp., and *Bacillus* sp. in a medium containing 1 to 1.5 M NaCl. In addition, degradation of acenaphthene, anthracene, phenathrene, and fluorene were also resported at high salinity (Bertrand *et al*, 1990; Ashok *et al*, 1995; Plotnikova *et al*., 2001; Mueini *et al*, 2007).

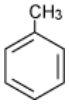
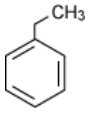
Monoaromatic hydrocarbons

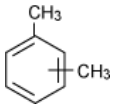
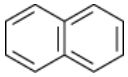
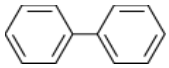
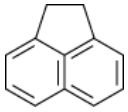
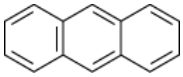
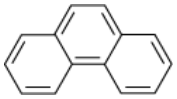
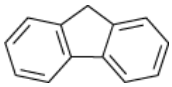
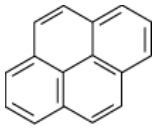
Monocyclic aromatic compounds such as BTEX are common soil and groundwater contaminants and are classified as priority pollutants by the U.S Environmental Protection Agency. Benzene is a category A carcinogen. They are highly water soluble, hence can contaminate a large volume of groundwater. Leakage from underground storage tanks, pipelines, spills, and seepage from surface contaminated sites can cause major BTEX contamination (Philip *et al*, 2005). There are only three published reports about BTEX degradation under hypersaline conditions (Table 2). Nicholson and Fathepure (2004 and 2005) have reported for the first time that BTEX can be degraded by microorganisms present in hypersaline environments. They were able to develop BTEX-degrading enrichment cultures using soil samples collected from oil-production facilities in Oklahoma as well as from sediment collected from the Great Salt Plain, OK. These studies have demonstrated complete mineralization of ^{14}C-benzene to $^{14}CO_2$ by the enrichment cultures in a medium supplemented with 2.5 M NaCl. Microbial community

analysis of the enrichments using DGGE showed that *Marinobacter* spp. were the dominant member of the enrichment community. Later a pure culture of halophile, *Arhodomonas* sp. strain Seminole was isolated from the enrichment cultures. This organism degraded benzene or toluene as the sole carbon source in the presence of 15 to 18 % NaCl. (Nicholson and Fathepure, 2005). Recently, Li *et al.* (2005) have isolated a *Planococcus* sp. using a contaminated soil obtained from a petroleum refinery effluent in China. The organism is a moderate haloalkaliphilic and able to degrade BTEX at 20 % salt. Overall, there is a severe lack of pure cultures of halophiles that degrade petroleum hydrocarbons under hypersaline conditions. Such studies are necessary to discover novel degradation pathways of hydrocarbon degradation. Isolation of novel microbes with superior degradation potential in the presence of various salt concentrations is important for developing biological catalysts and for developing molecular tools for monitoring the presence of such microbes at contaminated hypersaline environments.

TABLE 1

BIODEGRADATION OF PETROLEUM HYDROCARBONS UNDER HYPERSALINE ENVIRONMENTS

Hydrocarbon	Structure	Degrader	NaCl (%)	Reference
Octane	C_8H_{18}	*Rhodococcus* sp. *Arthrobacter* sp. *Bacillus* sp.	17	Plotnikova *et al*, 2001
Tetradecane	$C_{14}H_{30}$	Undefined halophilic Archaea	9-20	Bertrand *et al*, 1990
Pentadecane	$C_{15}H_{32}$	*Actinopolyspora* sp. DPD1	5-25	Mueini *et al*, 2007
	$C_{15}H_{32}$	*Marinobacter aquaeolei* sp	0-20	Huu *et al*, 1999
Hexadecane	$C_{16}H_{34}$	Enrichment from Rozel Point	<20	Ward and Brock, 1978
		Marinobacter aquaeolei sp	0-20	Huu *et al*, 1999
Pristane	$C_{19}H_{40}$	*Halobacterium* sp.	15-31	Bertrand *et al*, 1990
		Marinobacter aquaeolei sp	0-20	Huu *et al*, 1999
Eicosane	$C_{20}H_{42}$	*Actinopolyspora* sp. DPD1	5-25	Mueini *et al*, 2007
		Marinobacter hydrocarbonoclasticus	0-14	Fernandez-Linares *et al*, 1996
Heneicosane	$C_{21}H_{44}$	Undefined halophilic Archaea	9-20	Bertrand *et al*, 1990
Pentacosane	$C_{25}H_{52}$	*Actinopolyspora* sp. DPD1	5-20	Mueini *et al*, 2007
n-alkane	C_{1^oC30}	*Halobacterium* sp.	15-32	Kulichevskaya *et al*, 1991
		Fusarium lateritium *Drechslera* sp. *Papulaspora*	10 10 5	Obuekwe *et al*, 2005
Benzene		Enrichment, oil-brine soil, Oklahoma	14.5	Nicholson and Fathepure, 2004
		Enrichment from Great Salt Plains	0-29	Nicholson and Fathepure, 2005
		Planococcus sp. strain ZD22 pure culture	5-20	Li *et al*, 2006
Toluene		Enrichment from Seminole County, Oklahoma	14.5	Nicholson and Fathepure, 2004
		Enrichment from Great Salt Plains, Oklahoma	0-29	Nicholson and Fathepure, 2005
		Planococcus sp. strain ZD22 pure culture	5-20	Li *et al*, 2006
Ethylbenzene		Enrichment, oil-brine soil, Oklahoma	14.5	Nicholson and Fathepure, 2004
		Planococcus sp. strain ZD22 pure culture	5-20	Li *et al*, 2006

Xylene		Enrichment, oil-brine soil, Oklahoma	14.5	Nicholson and Fathepure, 2004
		Planococcus sp. strain ZD22 pure culture	5-20	Li *et al*, 2006
Naphthalene		*Micrococcus* sp	<7.5	Ashok *et al*, 1995
		Enrichment from chemical- and salt- plant	14.5	Plotnikova *et al*, 2001
		Pseudomonas sp. DN13 (Undefined) *Rhodococcus* sp. *Arthrobacter* sp. *Bacillus* sp.	6	
		Halomonas eurihalina strain H-28	5	Martinez-Checa, *et al*, 2002
Biphenyl		*Rhodococcus* sp. *Arthrobacter* sp.	6	Plotnikova *et al*, 2001
Acenaphthene		Undefined halophilic Archaea	21	Bertrand *et al*, 1990
Anthracene		*Pseudomonas* sp.	<7.5	Ashok *et al*, 1995
		Undefined halophilic Archaea	21	Bertrand *et al*, 1990
Phenanthrene		*Alcaligenes*	<7.5	Ashok *et al*, 1995
		Undefined halophilic Archaea	21	Bertrand *et al*, 1990
		Arthrobacter sp	6	Plotnikova *et al*, 2001
		Halomonas eurihalina strain H-28	5	Martinez-Checa *et al*, 2002
Fluorene		*Actinopolyspora* sp. DPD1	5-20	Mueini *et al*, 2007
		Halomonas eurihalina strain H-28	5	Martinez-Checa *et al*, 2002
Pyrene		*Halomonas eurihalina* strain H-28	5	Martinez-Checa *et al*, 2002
Crude Oil		*Actinopolyspora* sp. DPD1	5-20	Mueini *et al*, 2007
		Marinobacter aquaeolei sp.	0-20	Huu *et al*, 1999
		4 *Pseoudomonas* strains	6	Kapley *et al*, 1999
		Bacterial consortium	0-18	Diaz *et al*, 2002

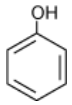
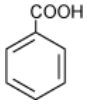
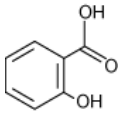
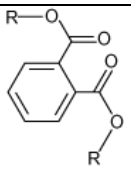
Aromatic compounds (Oxygenated)

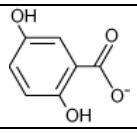
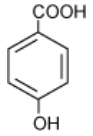
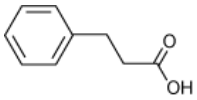
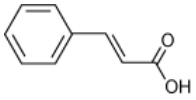
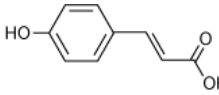
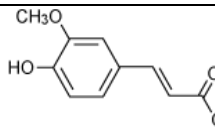
Recent reports about the biodegradation of oxygenated aromatic compounds under moderate to high salinity environments are summarized in Table 2. Industrial effluents generated from many food, dye, pharmaceutical, and chemical processing are characterized by low pH and high salinity and the presence of toxic aromatic compounds including phenolics, benzoates, and hydroxylated aromatics. Many studies have successfully isolated bacteria and archaea that degrade these compounds in saline and hypersaline conditions. Emerson *et al.* (1994) reported benzoate degradation at 1.7 M NaCl using a halophilic archaea, *Haloferax* sp. isolated from an oil-brine soil. In addition, the strain also utilized cinnamate or phenylpropionate as the growth substrate but not benzene or toluene. More recently, Cuadros-Orellana *et al.* (2006) have tested the degradation of *p*-hydroxybenzoate by several archaeal strains isolated from five geographically different saline environments including the Uyuni salt marsh in Bolivia, solar saltern in Chile, solar saltern in Puerto Rico, the Dead Sea, and sabkhas in Saudi Arabia. Garcia *et al.* (2004) have isolated *Halomonas organivorans* that degraded several aromatic compounds including benzoic acid, phenol, salicylic acid, cinnamic acid, ferulic acid, and others. Recently, Garcia *et al.* (2005a) have isolated several strains of *Halomonas* sp. that degraded phenol, benzoate, cinnamate, *p*-hydroxybenzoate, and other aromatic compounds over a wide range of salinity. In addition, salicylate, *o*-phthalate, gentisate, 4-hydroxylbenzoate, and 3-phenylpropionic acid are shown to undergo biodegradation at high salinity (Fu and Oriel, 1999; Plotnikova *et al*., 2001; Fairley *et al*, 2002; Cuadros-Orellana *et al*, 2006; Oie *et al*, 2007). Overall, it appears that *Halomonas*

spp. are common inhabitants of most saline environments and have the ability to degrade mostly oxygenated aromatic compounds.

TABLE 2

BIODEGRADATION OF AROMATIC HYDROCARBONS (OXYGNENATED) UNDER HYPERSALINE ENVIRONMENTS

Hydrocarbon	Structure	Degrader	NaCl (%)	Reference
Phenol		Halophilic isolate	15	Wooland and Irvine, 1995
		Halomonas sp.	1-14	Hinteregger and Streichsbier, 1997
		Candida tropicals	15	Bastos *et al*, 2000
		Alcaligens faecalis	5.6	
		Halomonas organivorans *Halomonas trueperi*	10	Garcia *et al*, 2004 Garcia *et al*, 2005a
		Thelassobacillus devorans sp.	7.5-10	Garcia *et al*, 2005b
		Halomonas campisalis	10	Peytona, 2002
Benzoate		*Pseudomonas halodurans* sp.	15.4	Rosenberg, 1983
		Haloferax sp. D1227	15	Emerson *et al*, 1994
		Halomonas campisalis	5-10	Oie *et al*, 2007
		Halomonas elongate *Halomonas eurihalina* *Marinobacter lipolyticus*	10	Garcia *et al*, 2004 Garcia *et al*, 2005a
		Halomonas sp.	35	Kleinsteuber *et al*, 2001
Salicylate		*Halomonas organivorans* *Salinicoccus. roseus* *Halomonas venusta* *Halomonas alimentaria s*	10	Garcia *et al*, 2004 Garcia *et al*, 2005a
		Halomonas campisalis	5-10	Oie *et al*, 2007
		Pseudomonas sp. DN13 (Undefined) *Rhodococcus* sp. *Arthrobacter* sp. *Bacillus* sp.	0.3-29	Plotnikova *et al*., 2001
o-Phthalate		*Rhodococcus* sp. *Arthrobacter* sp. *Bacillus* sp.		

Gentisate		*Rhodococcus* sp. *Arthrobacter* sp.		
4-Hydroxylbenzoate		*Haloarcula* sp. strain D1,	17.4	Fairley *et al*, 2002
		Halobacteriaceae	29	Cuadros-Orellana *et al*, 2006
		Halomonas elongate	10	Garcia *et al*, 2005a
Phenylpropionic acid		*Haloferax* sp. D1227	10	Fu and Oriel, 1999
		Halomonas elongata *Halomonas venusta* *N.D.* *Halomonas glaciei* *Halomonas organivorans*	10	Garcia *et al*, 2004 Garcia *et al*, 2005a
Cinnamic acid		*Halomonas salina/Halomonas halophila* *Halomonas organivorans* *Halomonas elongate*		
p-Coumaric acid		*Halomonas organivorans* *Halomonas salina* *Chromohalobacter israelensis*		
Ferulic acid		*Halomonas elongate*		

Aerobic BTEX degradation pathways

Understanding microbial diversity and identifying microorganisms that play a key role in the degradation of pollutants is important for defining new strategies for bioremediation. In order to gain insight into the genetic diversity and metabolic potential of microorganisms involved in the biodegradation of pollutants, analyzing for genes that code for key catabolic steps in the degradation pathways is important. Aerobic degradation pathway and genes involved in the metabolisms of BTEX have been fairly well characterized for many non-halophiles (Figure 1). However, very little information exists about the pathways and enzymes involved in the biodegradation of hydrocarbons in hypersaline environments.

Monooxygenases and dioxygenases initiate the degradation of a wide variety of hydrocarbons under aerobic conditions by the addition of oxygen atom (s) to the alkyl-side chain or aromatic ring (Bouwer and Zehnder, 1993) and converting them to a few central intermediates such as catechols and protocatechuate through convergent pathways. These intermediates are cleaved by *ortho*- and *meta*- cleavage dioxygenases into the Kreb (TCA) cycle intermediates (Figure 1). The primary enzymes involved in the *ortho*- and *meta*-cleavge activity include catechol 1,2-dioxygenase and catechol 2,3-dioxygenase. The genes encoding these enzymes have been characterized for a variety of aerobic microorganisms including several members the genera *Pesudomonas*, *Rhodococcus, Ralstonia,* and *Acinetobacter* (Luz *et al*, 2004).

Toluene degradation can also proceed through another common intermediate, protocatechuate, which is further degraded *via ortho*- or *meta*-cleavge pathways catalyzed by protocatechuate 3,4-dioxygenase or protocatechuate 4,5-dioxygenase

enzymes, respectively. The degradation intermediates are fed into the Kreb cycle (Figure 1).

Recently a few reports have appeared on the pathways and enzymes involved in the degradation of hydrocarbons in halophiles. Hinteregger and Streichsbier (1997) have reported the detection of catechol 1,2-dioxygnase activity in phenol degrading *Halomonas* sp. Garcia *et al.* (2005a) have documented the presence of catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygnase activity in several strains of *Halomonas* isolated from saline habitats in south Spain. *Halomonas campisalis* that grows on benzoate or salicylate was shown to possess catechol 1,2-dioxygenase (Oie *et al* 2007). A recent report by Kim *et al.* (2008) showed the presence of catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase in a benzoate-metabolizing halophilic *Chromohalobacter* sp. Therefore, we hypothesize that hydrocarbon degradation in hypersaline environments proceeds by pathways that are found in non-halophiles. However, more studies are needed to understand the diversity of the key ring-cleaving dioxygenase enzymes and their sequence homology to the enzymes present in non-halophiles.

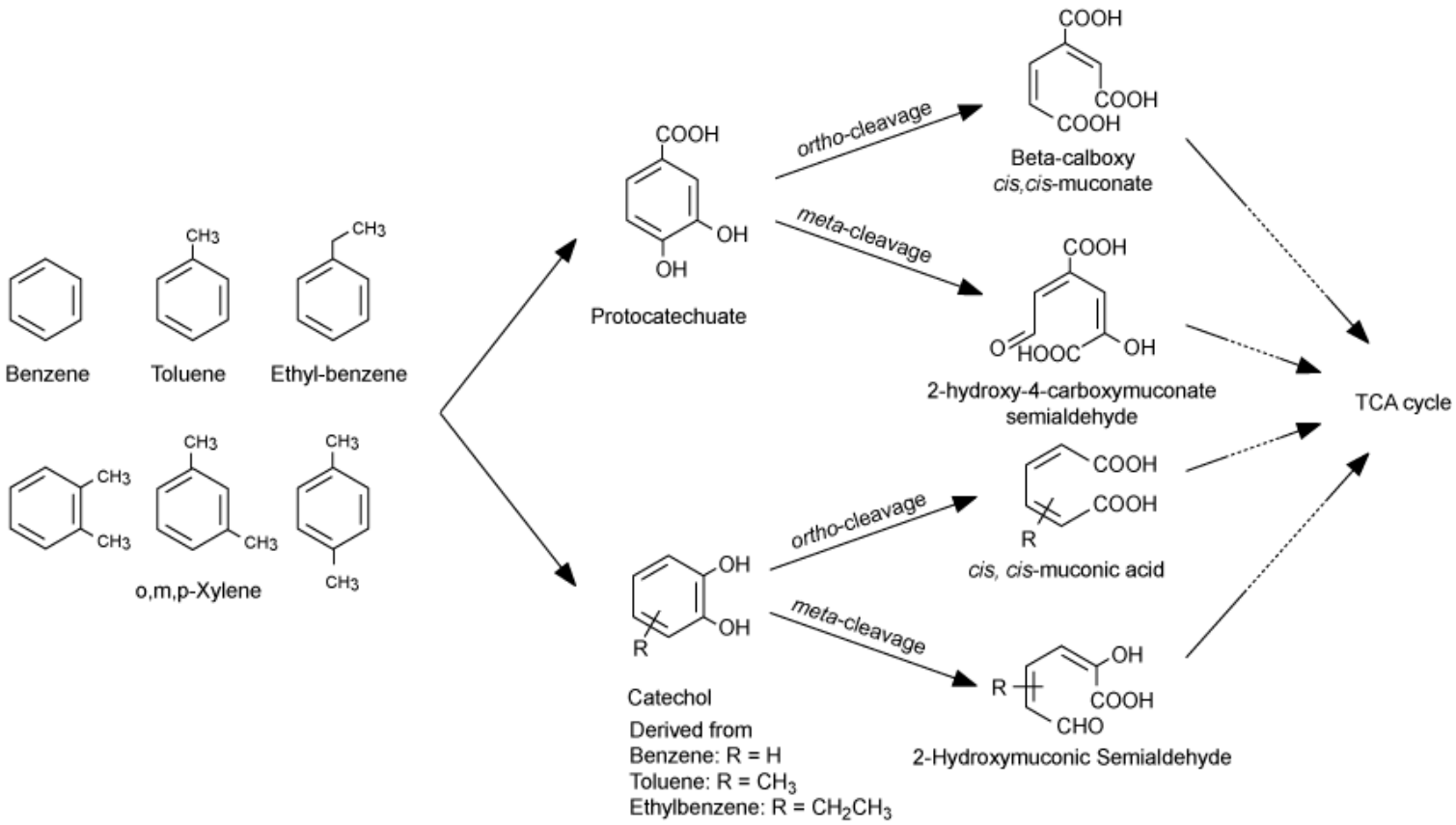


Figure 1. The general aerobic bacterial BTEX degradation pathways modified from; Gross *et al*, 1955; Maier *et al*, 2000; Jindrova *et al*, 2002; Mampel *et al*, 2005.

CHAPTER III

BTEX DEGRADATION IN THE ENRICHMENT FROM ROZEL POINT

Background

The Great Salt Lake is one of the largest terminal lakes in the world with salinity ranging from 5 to 30%. The construction of a railroad track in 1950's has effectively divided the Great Salt Lake in to two parts, the North Arm and the South Arm, thus isolating the North Arm from freshwater input from snow melts in the Wasatch Mountain Range making it a highly saline habitat with salinity reaching as high as 30 % in the North Arm (Baxter *et al*, 2005; Haws, 2007). Numerous, naturally occurring oil seeps are present in the northeastern part of the GSL near Rozel Point. Here, the oil slowly seeps up through the faults and fracture in the bedrock and deposits in the bottom of the lake thus becoming a constant source of contamination at the lake. In addition, extensive of oil-production activities of the past have resulted in many abandoned leaking oil-wells and storage tanks (Baxter *et al*, 2005; Haws, 2007). In spite of high salinity and numerous oil seeps at Rozel Point, little is known about the microbial communities and their role in the degradation of petroleum compounds.

Although most saline habitats harbor a vast array of microorganisms belonging to all domains of life, only a handful of reports exist that describe bacterial degradation of aliphatic, mono- and polyaromatic hydrocarbons in salinity exceeding that of marine habitats (Madigan and Oren, 1999; Ventosa *et al*, 1998). Similarly, reports on archaeal and eukaryal capacity to degrade hydrocarbons in saline habitats are sparse. Only one report documents the apparent role of Archaea in the biodegradation of hydrocarbons

(Bertrand *et al*, 1990). Similarly, a recent report shows fungal utilization of crude oil as a source of carbon at high salinity (Obuekwe *et al*, 2005).

In the present study we explore the biodegradation potential of non-oxygenated, low molecular weight fuel hydrocarbons including benzene, toluene, ethylbenzene, and xylenes (BTEX) in sediment samples collected from GSL near Rozel Point. We developed a highly enriched culture that rapidly degraded monocyclic hydrocarbons. Further we tested if the enrichment culture degraded BTEX using pathways similar to those found in non-halophiles. For this, we screened the enrichment for the presence of key ring-cleaving catabolic genes by using degenerate primers. The ability to rapidly and accurately detect hydrocarbon-degrading organisms and their activity by employing sensitive molecular tools is of major importance for understanding natural attenuation potential and for developing *in-situ* bioremediation technologies.

Materials & Methods

Sediment samples and enrichment culture

Sediment samples were obtained from the surface at Rozel Point (N 41º 26' 7", W 112º 39' 33"). Biodegradation of benzene was evaluated using microcosms prepared in160-ml capacity of serum bottles, 10 g of sediment sample, and 40 ml of mineral salts medium (MSM) containing 2.5 M NaCl. The composition of MSM is described (Nicholson, 2005). Bottles were amended with 2 µl neat benzene as the only source of carbon. Autoclaved bottles prepared as above were used as controls. Bottles were closed with rubber septa and aluminum caps and incubated in the dark in an inverted position at 30 ºC. Air in the headspace served as the source of oxygen.

Enrichment cultures were developed from the bottles that showed significant degradation of added benzene. The bottles were repeatedly spiked with benzene and after 6-7 spikes, a 50 % of the culture was transferred to a fresh MSM containing 2 M NaCl. This was repeated for the next 3-4 months until a sediment-free culture was obtained that consistently degraded benzene within 7 to 10 days. Thus obtained sediment-free mixed culture was further enriched to degrade benzene and toluene at 4 and 5 M NaCl.

Chemicals

Benzene and the mixture of xylene isomers were >99 % pure and were purchased from Fisher Chemical Company (Fair Lawn, NJ). Toluene and ethylbenzene were >99 % pure and were purchased from Aldrich Chemical Company (Milwaukee, WI). All other chemicals were of reagent grade.

BTEX degradation assay

Biodegradation of BTEX compounds was analyzed using a Hewlett Packard 6890 gas chromatograph (GC) equipped with a flame ionization detector (FID) and HP-1 capillary column, 30 m long x 0.25 µm film thickness (Agilent Technologies). N_2 (ultra pure) served as both carrier and make up gas at flow rates of 10 and 20 ml/min, respectively. The flow rates of H_2 and air were at 40 and 450 ml/min, respectively. The GC was operated using the following conditions: Oven temperature, 70 ºC for 4 min; inlet temperature, 200 ºC; and detector temperature, 250 ºC. Approximately, 100 µl of headspace gas from microcosms was injected into the GC using a 1 ml disposable syringe attached to a 25G 1½ needle (Becton Dickinson). The GC response for each compound tested was calibrated to give the total mass of that compound in the bottle. The total mass in the bottle was determined using standards. Standards were prepared in 160 ml of serum bottles containing 50 ml of 2.5 M NaCl solution and closed with rubber septa and aluminum caps. After equilibration (after 12 to 24 h) at room temperature, the GC response for a range of mass (µmol/bottle) of each compound tested was plotted and the slopes were used to calculate the unknown. Since salt is known to impact the partitioning of BTEX compounds in the headspace, separate sets of standards were prepared as above for each salt concentration tested. After equilibration for 12 – 24 h at room temperature, the GC responses were plotted and the respective slopes were used to quantify the unknown. Fresh standards were prepared on bimonthly basis.

Benzene degradation at different salt concentrations

To study the degradation of benzene at different salt concentrations, 160 ml serum bottles containing 45 ml of MSM supplemented with various concentrations of NaCl ranging from 0 to 5 M were inoculated with 5 ml of the Rozel Point-enrichment culture. Bottles were incubated upside-down in the dark at 30 ºC. Headspace samples were analyzed periodically by the GC.

Molecular microbial community analysis

Genomic DNA was isolated from the enrichment culture maintained on benzene as the sole carbon source in MSM containing 4 M NaCl (23 % salt) using UltraClean™ Soil DNA Kit (MoBio Laboratories, Inc, Carlsbad, CA) according to the manufacturer's protocol. 16S rRNA-genes were amplified with PCR and the universal primers, 515f (5'-GTG CCA GCM GCC GCG GTA A-3') and 1391r (5'-GAC GGG CGG TGW GTR CA-3'). The amplification was performed using a Bio-Rad Cycler (Hercules, CA) in a 25 µl reaction volume containing 20 ng of genomic DNA, 150 µM dNTPs, 2 µM (each) primer, 2.5 mM $MgCl_2$, 1 U of *Taq* polymerase in 1 x PCR buffer (Promega, Madison, WI) and 1 µg of bovine serum albumin. The PCR was carried out using the following condition: one cycle of initial denaturation at 95 ºC for 10 minutes, 25 cycles of denaturation at 94 ºC for 1 minute, annealing at 60 ºC for 45 seconds, extension at 72 ºC for 2 minutes, followed by one cycle of 8 minutes at 72 ºC. The PCR products were pooled from three cycling and amplicons were separated on 1.5 % agarose gel, excised, and purified using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. The purified PCR fragments were ligated into the pCR

2.1 plasmid vector and transformed into One Shot® chemically competent TOP10 *Escherichia coli* cells using the manufacturer's protocol (Invitrogen, Carlsbad, CA). The transformed cells were plated onto Luria-Bertani (LB) agar plates containing ampicillin (50 µg/ml). Colonies were randomly picked using sterilized toothpicks and grown in 96-plate deep wells filled with 1 ml of LB broth containing ampicillin (50 µg/ml). The plates were incubated overnight on a shaker at 37 ºC. Clones were checked for inserts by PCR with the M13 (-20) f primer using the above PCR conditions, except the annealing temperature was 45 ºC. Plasmid DNAs were isolated and sequenced by the use of a 96-well alkaline lysis procedure (Sambrook, 2001). The sequencing was done using the ABI PRISM 3700 DNA analyzer (Applied Biosystems). The nucleotide sequences determined in this study were compared with existing sequences in GenBank by performing a BLASTn search.

Detection and identification of key ring-cleaving dioxygenase genes using PCR, cloning of PCR fragments, and sequencing

Genomic DNA from the enrichment grown on benzene in MSM at 4 M NaCl (23 % salt) and also from a reference strain, *Arhodomonas* sp. was isolated using the method described above. The reference strain, *Arhodomonas* sp. degrades BTEX under saline conditions and its genome contains major ring-cleaving catabolic genes (unpublished results). The isolated DNA from the enrichment culture and the reference strain was screened for the presence of key ring-cleaving genes including catechol 1,2-dioxygenase (1,2-CTD), catechol 2,3-dioxygenase (2,3-CTD), protocatechuate 3,4-dioxygenase (3,4-PCA), and protocatechuate 4,5-dioxygenase (4,5-PCA). The

following degenerate primers were used to amplified the above genes using the PCR method described in the corresponding reference: 1,2-CTDf (5'-ACC ATC GAR GGY CCS CTS TAY-3'), 1,2-CTDr (5'-GTT RAT CTG GGT GGT SAG-3'); 2,3-CTDf (5'-GAR CTS TAY GCS GAY AAG GAR-3'), 2,3-CTDr (5'-RCC GCT SGG RTC GAA GAA RTA-3) (Garcia, *et al*, 2005a); 3,4-PCAf (5'-GAG RTS TGG CAR GCS AAY-3'), 3,4-PCAr (5'-CCG YSS AGC ACG ATG TC-3') (Azhari *et al*, 2007); 4,5-PCAf (5'-GCC CGG CAC CAT CAT TTT-3'), 4,5-PCAr (5'-AGT TGC CTT CCT GGT TCT GGT-3') (Mampel *et al*, 2005). The expected sizes of the PCR products of all four catabolic genes were about 400 bp long.

To validate that the correct PCR fragments had been amplified, suspected bands were cloned into One Shot® chemically competent TOP10 *Escherichia coli* cells as described above. The transformed cells were plated on Luria-Bertani (LB) agar plates containing ampicillin (50 µg/ml). Colonies were randomly picked using sterilized toothpicks, and directly amplified by PCR using the M13 (-20) f primer using PCR conditions described above. The amplified clone DNAs were purified using the ExoSAP-IT® enzyme (USB Corporation, Cleveland, OH) according the method described by the manufacturer. The purified DNA was sequenced using the ABI PRISM 3700 DNA analyzer (Applied Biosystems). The nucleotide sequences determined in this study were compared with existing sequences in GenBank by performing a BLASTn search.

Result & Discussion

In spite of high salinity and numerous petroleum seeps in GSL's Rozel Point sediment, surprisingly very few studies have been carried out to understand microbial diversity and their capacity to degrade petroleum hydrocarbons. In this study, we focus on the biodegradation of non-oxygenated aromatic compounds (BTEX) using microcosms established with sediment samples obtained form Rozel Point.

Figure 2 shows the evidence of benzene degradation in bottles established with Rozel Point-enrichment at 2.5 M NaCl (14.5 % salinity). Approximately, 20-25 μmole of initially added benzene was repeatedly degraded within 7–10 days with no apparent lag time. No degradation was seen in autoclaved bottles. These results clearly demonstrate the ability of microbial communities at Rozel Point to metabolize benzene as the sole carbon source.

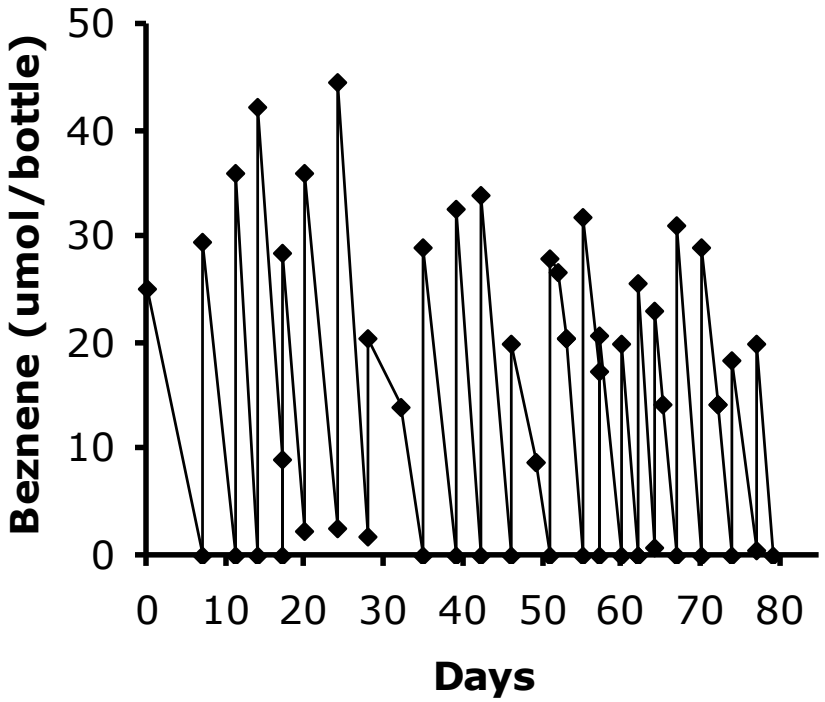


Figure 2. Benzene degradation by the Rozel Point sediment in 2.5 M NaCl
Repeated use of benzene (◆) as the sole carbon and energy source in the presence of 2.5 M NaCl in the Rozel Point enrichment. The enrichments were maintained in 160-ml capacity bottles containing 40 ml of MSM-NaCl at 30ºC. The data was taken from triplicate enrichments which behaved similarly. Every after several spike, a 50 % of the culture was transferred to a 25 ml of MSM-NaCl until the sediment was removed completely. Enriching was continued even after 80 days.

To assess the robustness of our enrichment, we studied degradation of benzene (Figure 3) in the presence of various concentrations of salt ranging from 0 to 5 M NaCl (0 to 29 % salt). Degradation occurred at all the tested NaCl concentrations. Benzene was completely degraded within 2 weeks in bottles with 1 or 2 M NaCl, while it took roughly 3 weeks in bottles containing 0, 3, or 4 M NaCl. More than 6 weeks were needed to degrade 20-25 μmol benzene at the highest salinity tested (5 M NaCl). These results clearly reveal the natural attenuation potential of hydrocarbons in wide ranging salinities at Rozel Point. This can be attributed to the adaptation of microorganism to temporal and spatial salt concentrations at Rozel Point. Considering many high salinity environments around the world and the apparent inhibitory effects of high salt concentration on biodegradation kinetics, this ability of Rozel Point-microbes to degrade benzene and toluene at high salinity is noteworthy and furthermore it suggests that oil seeps are a major source of carbon for microorganisms at this location.

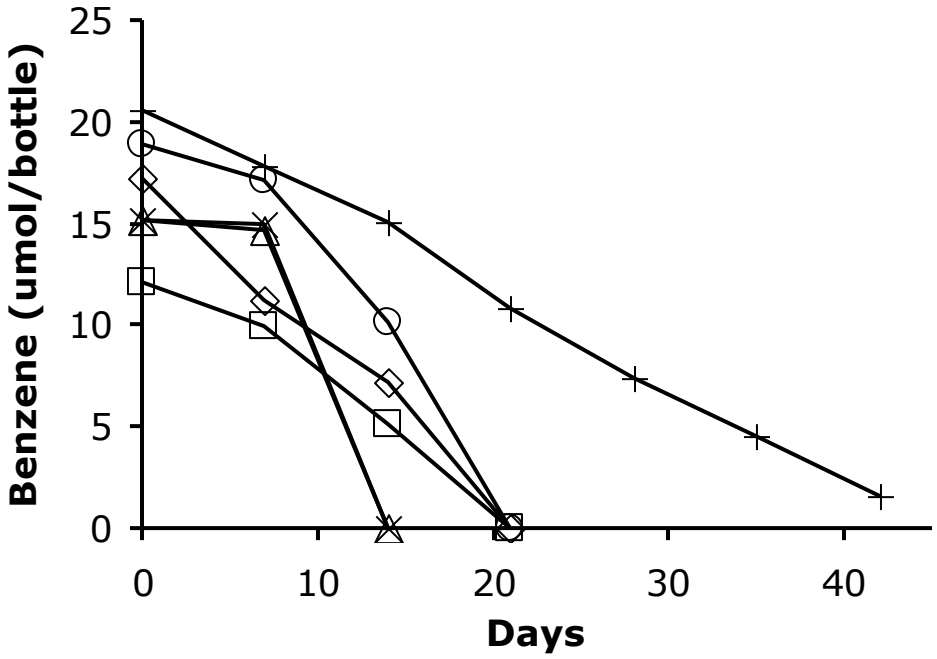


Figure 3. Benzene degradation by the Rozel Point enrichment in the presence of various NaCl concentrations

Microcosms were established in 160 ml capacity serum bottles containing 45 ml MSM and inoculate with 5 ml of inoculum grown in 2.5M NaCl. All microcosms were spiked with ~25 µmole benzene and amended with various concentaraions of NaCl. Symbols: 0 M (□); 1 M(△); 2 M (×); 3 M (◇); 4 M (○); 5 M (+) NaCl. The results are means for triplicate active microcosms.

Salinity limits microbial degradation of hydrocarbons in hypersaline environments. To our knowledge there is only one report that documents the potential of GSL microorganisms to degrade hydrocarbons (Ward and Brock, 1978). These investigators tested the biodegradation of mineral oil and hexadecane at salinities ranging from 3 to 28 % in water samples collected from GSL. Results show that the rate of degradation decreased as salinity increased and negligible degradation occurred at salinity >20 % salt. In addition, attempts to enrich mineral oil-degrading organisms from GSL at high salinity (>20 % salt) were not successful suggesting extreme salinity maybe a natural barrier to hydrocarbon metabolism. Similar observations on the degradation of non-oxygenated hydrocarbons were reported in samples obtained from other hypersaline environments (Mille *et al*, 1991; Rhykerd, *et al*, 1995; Diaz *et al*, 2002; Riis *et al*. 2003; Nicholson and Fathepure, 2004; Nicholson and Fathepure, 2005; Abed *et al*, 2006)

We also tested the ability of the enrichment to degrade other monocyclic hydrocarbons including toluene, ethylbenzene, and xylenes (*o*, *m*, and *p*-xylenes) at high salinity such as 4 and 5 M NaCl (Table 3). Benzene and toluene were degraded within 15 days at a rate of 1.7 and 1.9 µmol/bottle/day, respectively in bottles containing 4 M NaCl, while both compounds degraded at slower rates (0.80 µmol/bottle/day) and needed almost 6 weeks at 5 M NaCl. No degradation of ethylbenzene or xylenes occurred at 4 or 5 M NaCl when added as a sole carbon source even after incubating for more than 2 months. However, ethylbenzene was degraded (0.8 µmol/bottle/day) at 4 M NaCl in 2 months when benzene was added as the primary carbon source, thus suggesting co-metabolic degradation of ethylbenzene. Xylenes did not degrade even in the presence

of benzene as a primary carbon source. The lack of xylene degradation could be due to the lack of requisite microorganisms in our enrichment and this does not necessarily indicate that this activity is missing at Rozel Point.

TABLE 3
BTEX DEGRADATION AT 4 AND 5 M NaCl

	Salt Concentration	
	4 M	5 M
Benzene	+	+
Toluene	+	+
Ethylbenzene	+*	-
Xylene (*o*-, *m*-, and *p*-)	-	-

*Degraded co-metabolically in the presence of benzene

The enrichment was supplemented with a 20-25 μmole/bottle of benzene, toluene, ethylbenzene and xylene mixture separately. The degradation was analyzed by a GC. "+" denotes degradation, "-" denotes no degradation by the Rozel Point enrichment

Microbial community structure and dynamics of the Rozel Point enrichment

We investigated shifts in microbial community structure of the enrichment during active benzene metabolism (Figure 4). The community structure was analyzed using 16S rRNA-gene clone libraries generated at two time points such as after first spike (in the early stages of degradation) and after 8 consecutive spikes of benzene (25 µmole x 8) to the enrichment culture. Members of the *Gammaproteobacteria* (*Arhodomonas* spp., *Spingomonas* spp. and *Halomonas* spp.) and *Bacteriodetes* were the dominant groups accounting for > 80 and 12 % of the total clones, respectively in the initial stages of benzene metabolism. However, with repeated metabolism of added benzene, the microbial composition significantly shifted where the *Bacteriodetes* increased from 12 to 46 %, while the members of the *Gammaproteobacteria,* specially the *Arhodomonans* group decreased from 68 to 43 %. Similarly, *Halomonas* spp. decreased from 11 to 5 %. On the other hand, archaeal population increased from non-detect to 5 % of total clones analyzed. Although it is not possible to correctly predict the physiological capabilities of individual phylotypes, members of the *Bacteriodetes* are shown to be specialized in the metabolism of complex organic polymers and other organic carbon released from other microbes (Bauer *et al*, 2006). Therefore, it is reasonable assume that *Bacteriodetes* maybe involved in the metabolism of degradation products of benzene generated by the members of the *Gammaproteobacteria* such as *Arhodomonas* spp., *Halomonas* spp. and *Spingomonas* spp. Recently, we have successfully isolated a *Arhodomonas* sp. that degrades BTEX at high salinity (unpublished results) from an oilfield (unpublished results). The *Halomonas* spp. were shown to degrade phenol, benzoate, and hydroxylated benzoate derivatives (Hinteregger and Streichsbier, 1997; Garcia *et al*,

2005a), though not much is known about their ability to degrade BTEX compounds. Similarly, *Spingomonas* spp. have been shown to degrade a variety of hydrocarbons (Shi *et al*, 2001). Though our results point out that *Arhodomonas* spp. are the dominant ring-degrading populations in the enrichment, their relative abundance in GSL near Rozel Point is not known. Therefore, further studies using specific primers are needed to assess their prevalence in GSL.

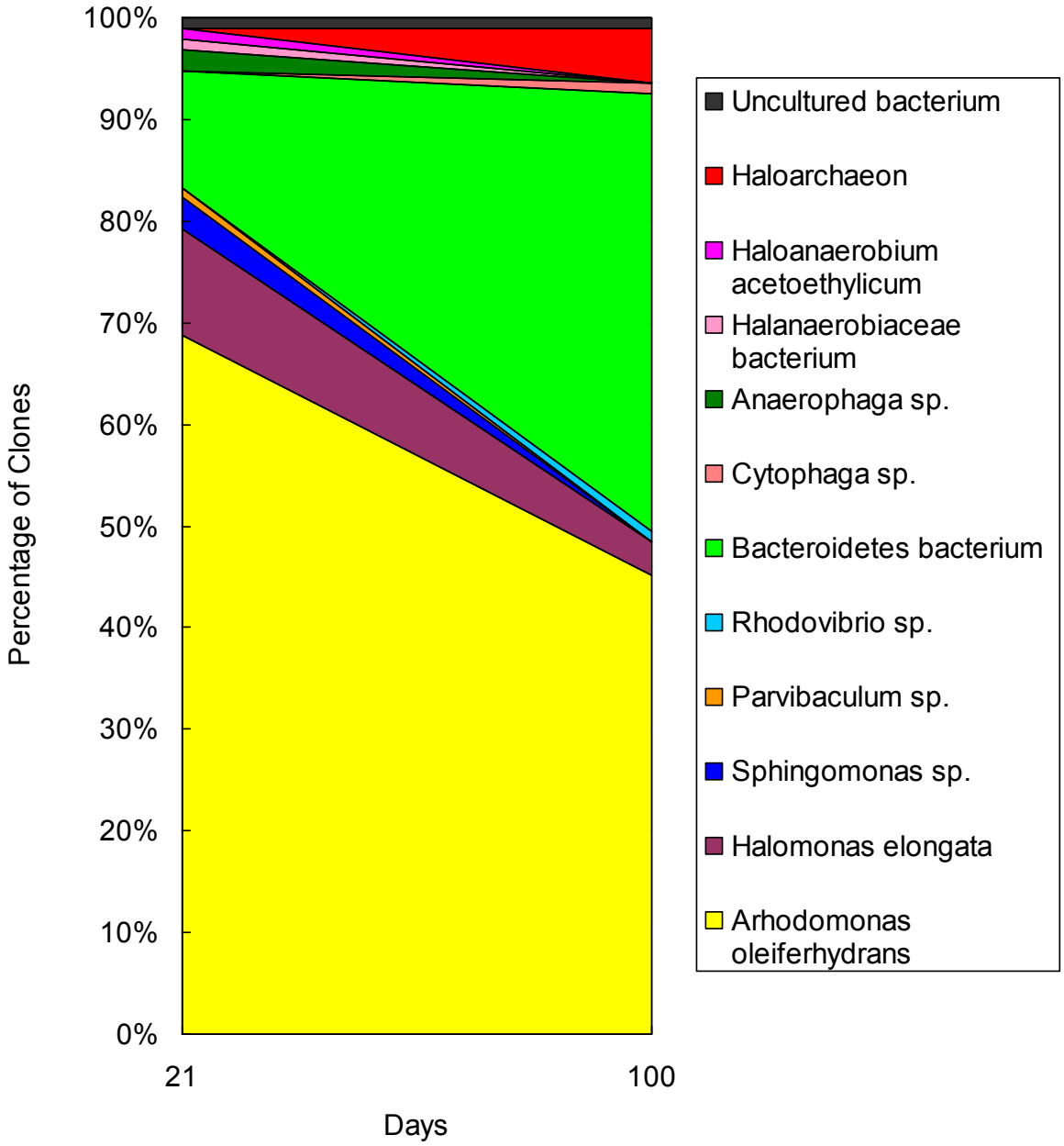


Figure 4. Microbial community structure and dynamics of the Rozel Point enrichment Microbial community structure in the enrichment was analyzed with 16S rDNA sequences. Cultures were corrected in every spike, and the first sample (Day 21) and the final sample (day 100) were selected for this experiment. DNA from the samples was amplified with an universal primer set (Univ515f-1391r), and the PCR products were cloned and sequenced. Obtained sequences were crosschecked by BLAST.

BTEX degradation pathways under hypersaline conditions

A decisive step in the degradation of aromatic compounds is the cleavage of benzene ring. Under aerobic conditions, microorganisms use mono- and dioxygenase enzymes to hydroxylate a variety of ring compounds to a few key intermediates including catechol and protocatechuate that are subsequently broken down by *ortho*- or *meta*-cleavage dioxygenases leading to the formation of Kreb cycle intermediates (Powlowski and Shingler, 1994; Harwood and Parales, 1996; Andreoni and Gianfreda, 2007). Studies have used genes that code for the ring-cleavage enzymes as molecular markers to detect aromatic compounds-degrading microorganisms in a wide variety of contaminated environments including saline habitats (Garcia *et al*, 2005a; Kasuga *et al*, 2007).

We investigated whether the degradation of benzene and toluene by our enrichment proceeds *via* the known pathways that involves the formation of catechol or substituted catechols found in most non-halophiles (Jindrova *et al*, 2002; Garcia *et al*, 2005a) and some halophiles belongoing to *Halomonas* group (Gibson and Subramanian, 1984). Our results (Figure 5A and B) clearly show the presence of 2,3-CTD genes in the DNA isolated from the benzene or toluene-grown enrichment culture suggesting that biodegradation proceeds primarily *via* catechol using *meta*- cleavage pathway (for the sequence, see Appendix A). It is important to note that the detection of 2,3-CTD genes in the PCR assay does not necessarily indicate the expression of the genes. We did not detect other genes that code for 1,2-CTD, 3,4-PCA and 4,5-PCA in the DNA extracted from the enrichment indicating that perhaps *meta*-cleavage is the dominant degradation pathway for BTEX compounds at Rozel Point. However, we caution that the degenerate primers used in

this study were designed using conserved sequences from non-halophiles (see Azahari *et al*, 2007; Garcia *et al*, 2005a, Mempel *et al*, 2005). Therefore, it is possible that some of the primers used in this study may not be suitable for the detection of ring-cleaving genes in all halophiles and hence a new set of degenerate primers need to be designed, preferably using halophiles to accurately determine the identity and pathways of BTEX degradation under saline conditions.

A

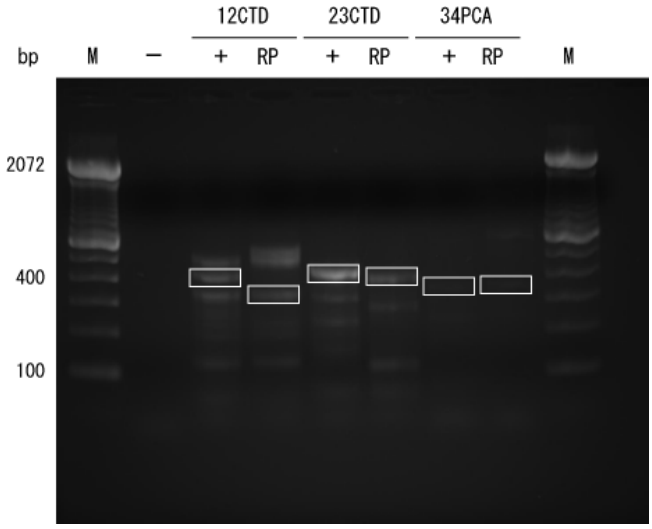


Figure 5A. PCR detection of the genes for ring-cleavage enzymes in the Rozel Point enrichment. Symbols: (M), 100 bp ladder; (-), negative control; *Arhodomonas* sp. strain SEM-2 as positive control (+): (RP), Rozel Point enrichment. Boxes represent a 400 bp-size expected PCR product. For identification, each PCR product was cloned in *E. coli*.

B

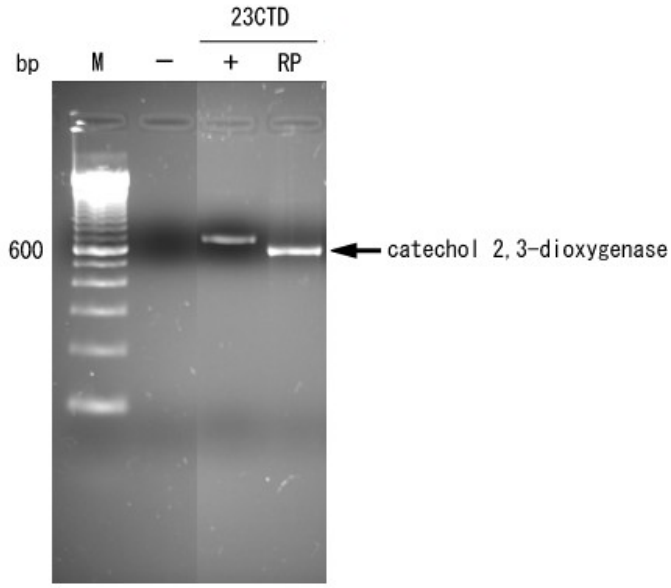


Figure 5B. The PCR products of the genes were cloned in *E. coli*. Symbols: (M), 100 bp ladder; (-), negative control; (+), *Arhodomonas* sp. as positive control; (RP), Rozel Point enrichment.

Overall, our study has demonstrated for the first time the ability of microorganisms enriched Rozel Point to degrade low molecular weight fuel hydrocarbons over a broad salinity range suggesting the natural attenuation potential of hydrocarbons at Rozel Point. Also, the study identifies that BTEX are degraded primarily *via* the *meta*-pathway using the 2,3-CTD under hypersaline condition. These observations are important since 2,3-CTD is a functional gene that has been shown to play an important role in the degradation of BTEX compounds. Hence this gene can be used as a suitable molecular marker for the *in-situ* detection of microbial communities and their metabolic potential at Rozel Point.

Conclusion

This study has conclusively demonstrated that the enrichment from Rozel Point sediment has the ability to degrade low molecular weight petroleum compounds such as benzene and toluene as the sole carbon source at high salinity. The enrichment degraded benzene and toluene in the presence of NaCl ranging from 0 to 5 M. However, degradation occurred at maximum rate in the presence of 1 or 2 M NaCl. These results are significant because many high salinity environments such as the Great Salt Lake, UT., Great Salt Plain, OK., salt marshes, salterns, and industrial effluents are often contaminated with a variety of hydrocarbons and require remediation.

The Assessment of the microbial community composition of the enrichment revealed that the *Gammaproteobacteria* and *Bacteroidetes* formed the major microbial groups. The members of *Gammaproteobacteria* including *Arhodomonas* spp., *Halomonas* spp. and *Spingomonas* spp. were reported to have the ability to degrade aromatic compounds in saline environments, while the *Bacteroidetes* are known to utilize polysaccharides and other organic compounds. Our analysis showed a remarkable shift in microbial composition during the course of benzene metabolism. The members of the *Gammaproteobacteria* decreased significantly, while the *Bacteroidetes* population increased proportionally.

Our cloning and sequencing of key genes that encode ring-cleavage enzymes showed that BTEX are mainly degraded *via* catechol and *meta*-ring cleavage pathway by catechol 2,3-dioxygenase enzyme.

CHAPTER IV

ISOLATION AND CHARACTERIZATION OF HALOPHILIC HYDROCARBON-DEGRADING BACTERIUM FROM THE ROZEL POINT ENRICHMENT

Background

Molecular studies have increasing used to study microbial identity and their metabolic capacity in natural settings without cultivating in the laboratory. However, there are clear advantages to successfully isolating and growing pure cultures compared to molecular techniques to demonstrate their presence in environmental samples. Firstly, isolates can be examined in detail to elucidate links between physiology and metabolic potential. Second, some isolates that degrade hydrocarbons may not contain genes with high homology to known genes implicated in the degradation of aromatic compounds. These isolates can then be studied as the source of highly divergent or novel hydrocarbon-degrading enzymes. Third, studies with pure cultures will help understand requirement for specific growth nutrients that can be added to the contaminated sites to stimulate their growth for *in situ* biostimulation. Also, large numbers of pure cultures can be grown in the laboratory and added to the contaminated matrix to achieve rapid biodegradation. In addition, specific molecular probes can be developed for detection and monitoring of the added strains in the subsurface.

Previous studies have isolated many strains of *Halomonas* sp. that utilize oxygenated aromatic compounds such as benzoate, phenol, salicylate, cinnamate, and other hydroxylated and carboxylated derivates of aromatic compounds (see Table 2). Also, a few studies report on the isolation of pure cultures that metabolize *n*-alkane and

PAHs. However, to the best of our knowledge, there are only two reports of isolation of halophiles degrading BTEX. The first isolate capable of degrading BTEX compounds as the carbon source was reported in 2005 by Nicholson and Fathepure. They reported on the isolation an *Arhodomonas* sp. from an oil production facility in Seminole County, Oklahoma. The *Arhodomonas* sp. is a gram-negative and rod-shape aerobic bacterium capable of degrading BTEX compounds as the growth substrate in the presence of 6-17 % NaCl (optimal growth at 2 M salt). No growth occurs in the absence of NaCl. The second isolate was reported in 2006 by Li *et al*. The isolate is a psychrotolerant and moderately haloalkaliphilic Gram positive bacterium belonged to the genus *Planococcus* capable of utilizing BTEX and other compounds between 5 and 20 % salinity. Although this organism couldn't grow on *m*- and *p*-xylene, it oxidized *m*- and *p*-xylene compounds into 2,4-dimethylresorcinol and 2,5-dimethylhydroquinone, respectively. More importantly, this organism can degrade fairly high concentrations of benzene ranging from 1 to 6 mM.

Here, we report the isolation of third organism capable of degrading benzene and toluene as the source of carbon at high salinity (6-23 %). The new organism was isolated from a highly enriched microbial consortium developed from a sediment sample collected from the GSL near Rozel Point (Chapter III). We have studied the isolate's phylogeny, physiology, and capacity to degrade BTEX compounds at various salt concentrations.

Material & Method

Isolation of Benzene-utilizing Bacterium

Benzene-degrading halphilic bacterium was isolated on agar plates prepared with MSM containing 1 M NaCl with 1.5 % agar. First, a 0.1 ml of 10-fold serial dilution of the enrichment was plated onto agar plates prepared with MSM containing 1 M NaCl. The plates were incubated at 30 ºC. Tiny colonies first appeared after a month. The colonies appeared smooth, round, and measured less than 1 mm in diameter. Subsequently, single colonies were aseptically transferred to 160-ml serum bottles containing 50 ml of sterile MSM-2 M NaCl, 0.02 % yeast extract, and 25 μmoles of benzene. Benzene degradation was monitored using GC. The injection volume was 0.1 ml of headspace samples (please refer to Chapter III for microcosm set up and analysis of hydrocarbons). Purity of the culture was confirmed by routine microscopic observations.

Phylogenetic analysis

Phylogenetic identification of the isolate was enabled by means of sequence analysis of the 16S rRNA gene. A 3 ml sample of the pure culture was centrifuged at 12,000 rpm for 15 minutes. The supernatant was decanted and the cells were washed twice with 1 ml of nano-pure water. The cells were resuspended in 0.5 ml of nano-pure water. Then, genomic DNA was extracted from the pure culture using a MoBio UltraClean Soil DNA Kit (MO BIO, Carlsbad, CA) as described in Chapter III. A primer set [Bact27f (5'-AGA GTT TGA TC(A/C) TGG CTC AG-3') and Bact1098r (5'-AAG GGT TGC GCT CGT TGC G-3') (Chang *et al*, 2000)] was used for PCR amplification.

Theoretically, amplification with the primer set should yield 1108 bp. For sequence, see Appendix B.

PCR amplification was performed in a total volume of 25 µl in Bio-Rad My Cycler (Hercules, CA). Each PCR mixture was composed of 15 ng of template DNA, 2 µM of each primer, 2.5 mM $MgCl_2$, 150 µM of dNTPs, 0.04 µg/µl of Bovine Serum Albumin (BSA), and 1 U of *Taq* polymerase in Buffer A (M1865, Promega Chemicals, Madison, WI). Amplification of 16S rRNA gene using the primer set consisted of an initial denaturation of the genomic DNA at 94 ºC for 3 minutes, followed by 30 cycles of denaturation at 94 ºC for 1 minute, annealing at 53 ºC for 45 seconds, and extension at 72 ºC for 2 minutes. This was followed by a 8-minute extension to the final cycle at 72 ºC. PCR products were checked for expected size on 1.5 % agarose gels. The PCR product was purified ExoSAP-IT® enzyme (USB Corporation, Clevland, OH). After purification, a sample of the PCR product was sequenced in both directions using the ABI PRISM 3700 DNA analyzer (Applied Biosystems).

The nucleotide sequences determined in this study were entered for BLAST searching into the Web site of NCBI (http://www.ncbi.nlm.nih.gov/blast/). Sequences were aligned using the integrated Clustal-W (MEGA 4.0). The phylogenetic tree was constructed using neighbor-joining algorithm and *p*-distance estimation method implemented in MEGA, version 4.0 (Kumar *et al*, 2004). The confidence for individual brances of the resulting tree was estimated by performing 1,000 bootstrap replciates. *Planococcus* spp. and *Bacteroidetes bacterium* were used as outgroups.

Optimum salt concentration for benzene degradation

The isolate's ability to degrade benzene at different initial concentrations was studied in 160-ml serum bottles containing 45 ml of MSM amended with 0, 1, 2, 3, 4, or 5 M NaCl. The bottles were inoculated with 5 ml of the culture of the isolate culture. A 25 µmoles of benzene and toluene were added as the sole source of carbon and electron donor. The microcosms were incubated upside down at 30 ºC in the dark. Periodically the amount of benzene and toluene in the head space was analyzed by GC through the same method described in chapter III. The total protein of the samples was analyzed as described by Lowry *et al*. (1951).

Effect of other carbon sources to benzene degradation

The isolate's ability to degrade benzene in the presence of other carbon sources was studied. Bottles were setup with MSM-3 M NaCl and 5 mM of glucose, lactic acid, pyruvate, or sodium acetate to determine the effects of these carbon substrates on benzene degradation.

Detection and identification of key ring-cleaving dioxygenase genes using PCR, cloning of PCR fragments, and sequencing

Genomic DNA from the isolate was screened for the presence of key ring-cleaving genes including catechol 1,2-dioxygenase (1,2-CTD), catechol 2,3-dioxygenase (2,3-CTD), protocatechuate 3,4-dioxygenase (3,4-PCA), and protocatechuate 4,5-dioxygenase (4,5-PCA). The detail method and PCR conditions were described in Chapter III.

Result & Discussion

Identification of the isolate from Rozel Point

A halophilic bacterium that degrades benzene or toluene as the sole source of carbon was isolated from the Rozel Point-enrichment. The isolate is a gram-negative, rod-shape halophilic aerobic bacterium. The isolate grew on agar plates on benzene vapors within 3-4 weeks. The colonies were round, smooth, transparent and grew to < 1 mm in size. Attempt to culture on rich media such as 1 % nutrient agar and trypticase soy agar plates did not yield colonies. Comparison of the 16S rRNA gene sequence (> 1000 bp) of the isolate with the sequences in GenBank showed 93 % sequence similarity with the most closely related organism, *Arhodomonas aquaeolei;* 94 % similarity to an uncultured bacterium clone from a gypsum-precipitating environmental sample, and 87 % similarity to an *Ectothiorhodopriraceae* clone obtained from the Hawaiian archipelago microbial community (NCBI). Based on the 16S rRNA sequence data, it appears that the isolate may represent a new species of a new genus in the *Ectothiorhodopriraceae* family of the *Gammaproteobacteria* subclass. Therefore, we tentatively refer this isolate as strain-Rozel.

Figure 6 shows the phylogenetic relationship of the strain Rozel with other organisms that degrade oxygenated and non-oxygenated hydrocarbons that have been isolated from various saline environments. The phylogenetic tree clearly shows that member of the *Halomonas* spp., *Marinobacter* spp. and *Planococcus* spp. formed distinct clusters from the *Arhodomonas* cluster (Figure 2). This phylogenetic clustering is to some extent reflected in their physiology and ecology. For example, *Halomonas* spp. have been isolated from a variety of saline habitats and shown to degrade primarily

oxygenated aromatic compounds such as phenol, benzoate, and other hydroxylated derivatives of aromatic compounds (Hinteregger and Streichsbier, 1997; Kleinsteuber *et al*, 2001; Peyton *et al*, 2002; Garcia *et al*, 2005a; Oie *et al*, 2007). The *Marinobacter* spp. have been isolated mainly from marine environments and shown to degrade *n*-alkanes and PAHs. (Fernandez-Linares *et al*, 1996; Huu *et al*, 1999; Golyshin *et al*, 2003; Garcia *et al*, 2005a). The *Planococcus* spp. belong to the phylum, Firmicute and have the ability to degrade *n*-alkanes, crude oil, BTEX (Engelhardt *et al*, 2001). Similarly, *Arhodomonas aquaeolei*, strain-Seminole, and strain-Rozel formed a distinct clade. All three organisms have been isolated from similar ecologies, petroleum-impacted hypersaline habitats and have similar metabolic capabilities, degrade BTEX at high salinity.

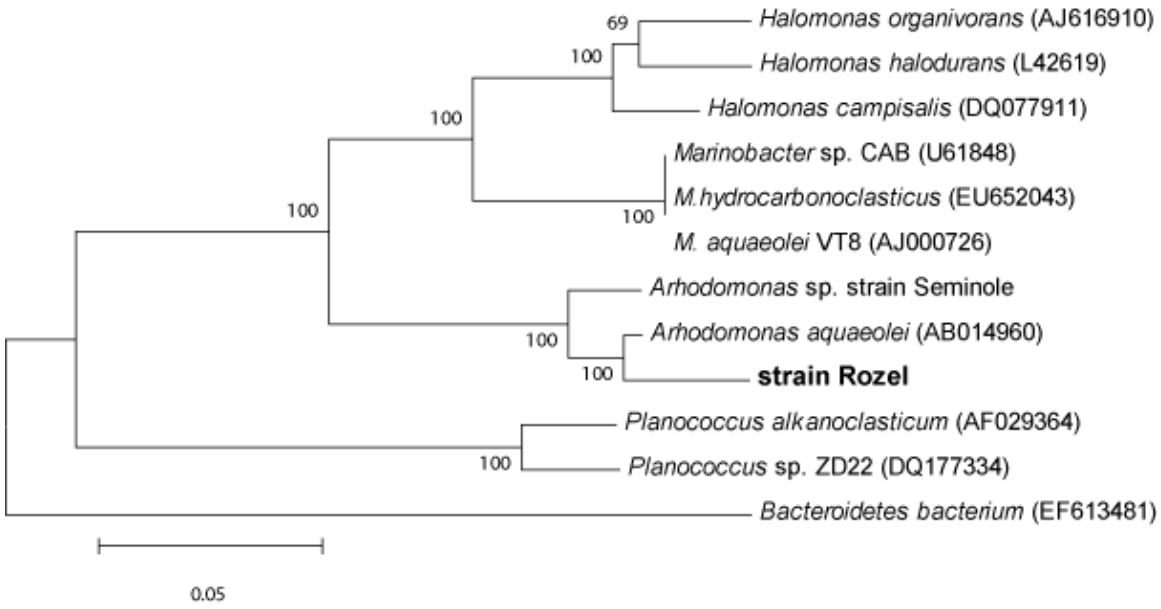


Figure 6. Phylogenetic tree based on 16SrRNA gene sequences showing the relationship of the strain Rozel to other halotolerant/halophilic organisms.
Accession numbers of the sequences are shown in parentheses after the strain designation. Numbers at nodes are estimate confidence levels in percentages and were determined by bootstrap analysis with 1,000 replicates. Bar denotes 5 % sequence diverge.

Data in Figure 7 shows biodegradation capacity of the isolate. The organisms degraded 20-25 µmole of benzene and toluene within 5 days at a rate of roughly 4-5 µmol/day in MSM supplemented with 3 M NaCl. Degradation of benzene and toluene was coupled to a corresponding increase in biomass protein suggesting that the organism utilizes the substrates as the carbon sources. No such removal occurred in autoclaved control bottles suggesting biological degradation of the added benzene and toluene.

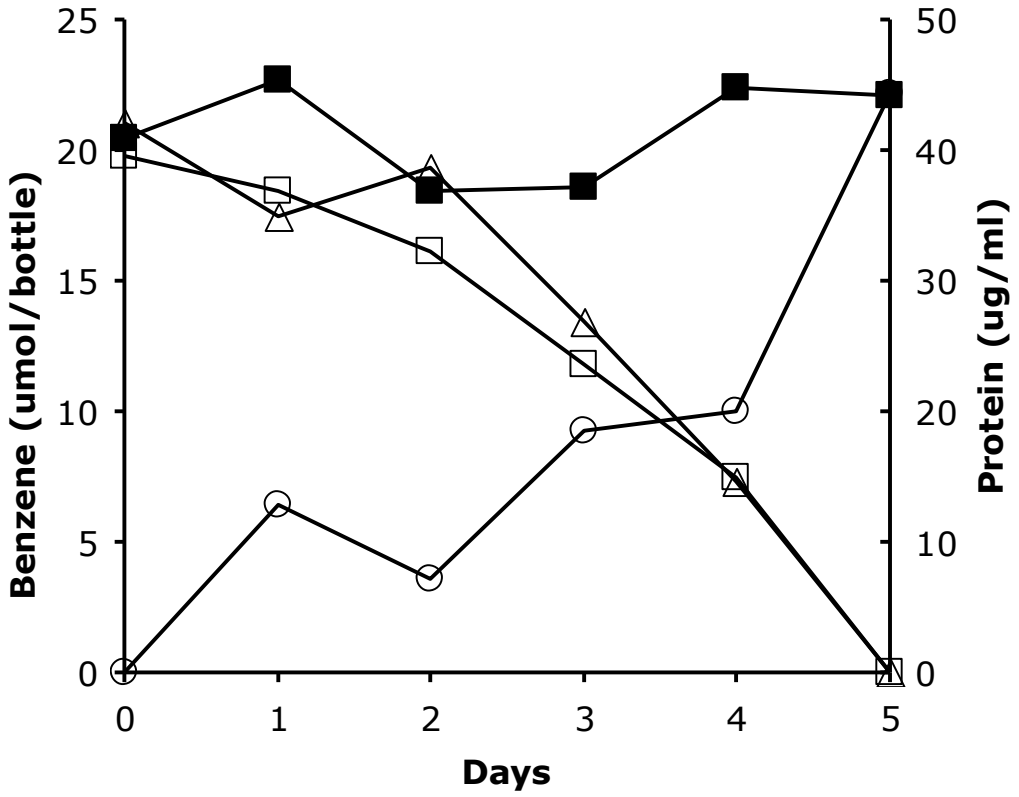


Figure 7. Benzene degradation coupled to growth.
The culture of the isolate 5 ml in 2 M MSM was transferred to fresh 45 ml of MSM containing its optimum salinity in 160 ml serum bottles. Benzene (~25 µmol) was added as the sole source of carbon and electron donor. At every benzene analysis, 1.5 ml of the culture was collected to estimate the amount of the protein. Symbol; protein amount (○); benzene amount in active (□); toluene amount in active (△); benzene amount in Control (autoclaved bottles) (■). Toluene amount in control had same property to benzene control (data not shown).

Assessment of degradation pathway

There is a growing interest in understanding and optimizing natural attenuation processes for hydrocarbon degradation in saline and hypersaline environments. To detect and monitor for microbial communities involved in the natural degradation processes, molecular methods are needed. Under aerobic conditions, aromatic compounds are primarily oxidized by mono- and dioxygenases into a few central intermediates such as catechol and protocatechuate, and then these intermediates undergo further ring cleavage at *ortho*- or *meta* position by catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, protocatechuate 3,4-dioxygenase, and protocatechuate 4,5-dioxygenase (van der Meer *et al*, 1992). For pathways, see Chapter III. Although, these pathways and enzymes have been studied well in non-halophiles (Gibson and Subramanian, 1984; van der Meer *et al*, 1992; Harwood and Parales, 1996), little is known about these enzymes and their diversity in halophiles.

Recently, Garacia *et al*. (2005) have reported the existence of these catabolic pathways by using degenerate primers in aromatic hydrocarbon-degrading *Halomoans* spp. isolated from a variety of saline habitats. In addition, cell extracts from the *Halomonas organivorans* showed catechol 1,2-dioxygenase activities, which was determined spectrophotometrically by measuring production of 2-hydroxy-muconic semialdehyde (2HMSA), when the bacterium was grown with benzoic acid. Also protocatechuate 3,4-dioxygenase activities was determined by measuring oxidation products of protocatechuate in cells grown with *p*-hydroxybenzoic acid, *p*-coumaric acid, and ferulic acid. More recently, Oie *et al*. (2007) detected catechol 1,2-dioxygenase in

benzoate-grown *Halomonas campisalis*. These investigators measured the formation of *cis, cis*-muconate, which is the ring-cleavage product of catechol by *ortho*-pathway.

These studies suggest that perhaps similar genes and pathways are present in our newly isolated organism that degrades benzene and toluene at high salinity. To ascertain our assumption, we PCR amplified the key ring-cleaving genes that code for the above mentioned four dioxygenase enzymes using degenerate primers. PCR amplification resulted in the expected 400 bp size PCR product from every primer set, except for the protocatechuate 4,5-dioxygenase gene (Figure 8). To validate the PCR products, we cloned and sequenced respective PCR fragments in *E. coli.* The clone sequence of each dioxygenase gene was BLASTed to compare its sequence identity and homology with corresponding sequences in GenBank. Our result showed that only catechol 2,3-dioxygenase clone sequence was matched albeit with low similarity suggesting that the isolate degrades benzene and toluene primarily *via meta*-pathway using a highly divergent or novel catechol 2,3-dioxygenase gene (for the sequence, see Appendix C). However, additional studies are needed to confirm our hypothesis.

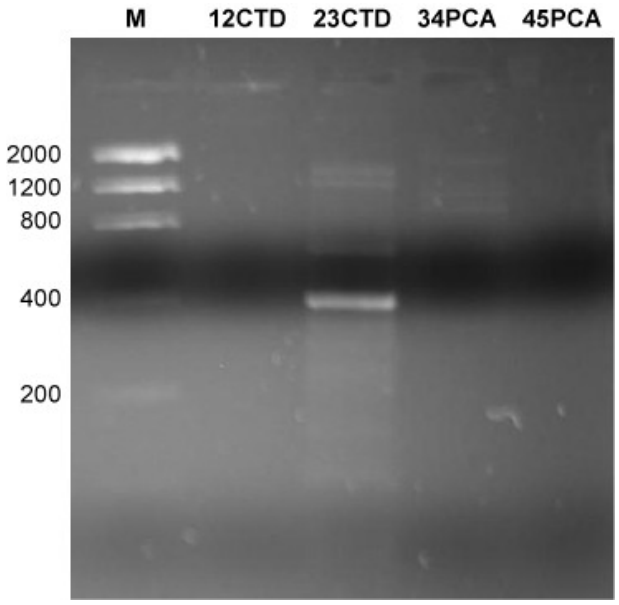


Figure 8. Result of PCR amplification with primer sets for 12-CTD, 23-CTD and 34-PCA.
Arhodomonas sp. strain Seminole was used as the positive control (+). Other symbols: M, 100 bp ladder; -, the negative control; RP, the Rozel Point isolate. Predicted size of every product was approximately 400 bp. Bands around 400 bp were cut, extracted, and sequenced. Since there was no band in the reaction with primers for 4,5-PCA, the data is not shown.

Optimum salt concentration for benzene degradation

In natural environments salinity can rapidly vary spatially and temporally. For effective bioremediation, microorganisms that withstand high and low salinity are important for the remediation of saline habitats. Therefore, we tested the growth of our isolate on benzene at varied salt concentrations ranging from 0 to 5 M NaCl. (0-29 % NaCl). Results (Figure 9) showed that benzene was rapidly degraded at salt concentrations between 1 to 4 M NaCl (6 to 23%) with a maximal rate at 3 M NaCl. Benzene was completely degraded in < 7 days in the presence of 1, 2, and 3 M NaCl. It needed > 2 weeks to completely degrade benzene at 4 M NaCl. No degradation occurred in bottles with 0 and 5 M NaCl thus suggesting that the isolate is a strict halophile. This ability of the organism to degrade at both low and high salinity is relevant for its survival in the field where salinity fluctuates considerably.

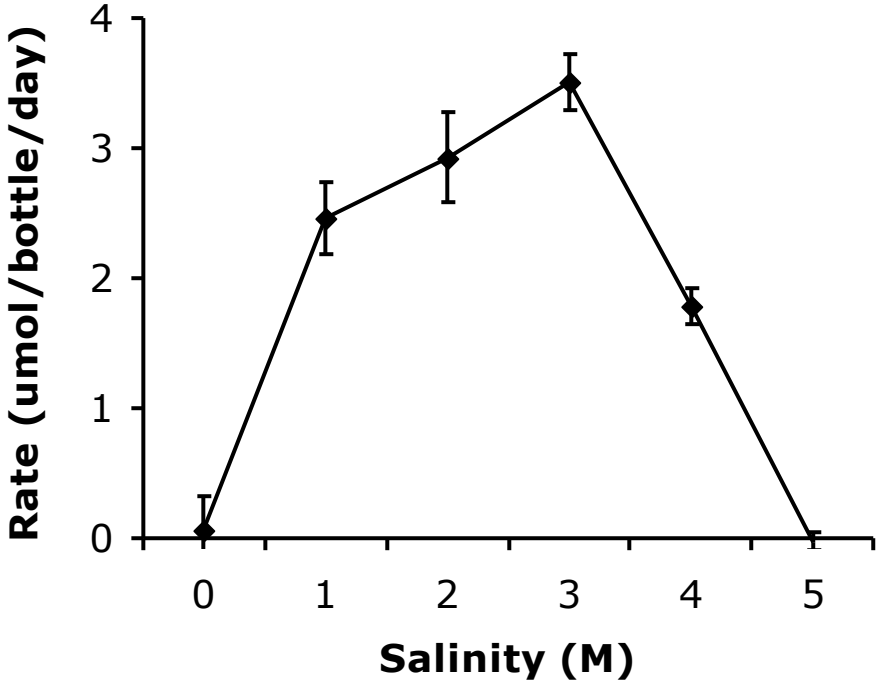


Figure 9. Degradation rate of the isolate in different NaCl concentrations.
Bottles containing 45 ml of MSM, 20-25 μmole/bottle of Benzene, and different concenytations of NaCl were inoculated with 5 ml of strain-Rozel. Benzene degradation was analyzed on weekly basis. The data points are average of three bottles and the error bars indicate ± standard deviation.

Benzene degradation in the presence of other carbon substrates

To study the isolate's ability to degrade hydrocarbons in the presence of other organic substrates, we tested benzene degradation in the presence of easily metabolizable and common substrates such as glucose, acetate, lactate, and pyruvate. The results in Table 4 show that degradation proceeded in the presence of all the tested substrates, except lactate. Results also show that turbidity was much higher in bottles containing glucose, acetate and pyruvate than in bottles with benzene alone. No turbidity was seen in lactate bottles suggesting inhibition of growth and degradation. Data also shows that rate of benzene degradation was almost similar in bottle with or without glucose compared to acetate or pyruvate. These results clearly show that the isolate was able to utilize both benzene and other substrates simultaneously suggesting that perhaps degradation was catalyzed by consititive enzymes. This ability of the organism is important for its field application.

TABLE 4
BENZENE DEGRADATION IN THE PRESENCE OF OTHER CARBON SUBSTARATES

Substrate	Rate (μmol/bottle/day)
Benzene alone	3.95 ± 0.42
Benzene + Acetate	1.93 ± 0.25
Benzene + Pyruvate	1.85 ± 0.10
Benzene + Lactic Acid	-0.10 ± 0.23
Benzene + Glucose	3.95 ± 0.42

Microcosms were set up 45 ml of MSM supplemented with 3 M NaCl. Bottles were amended with benzene and/or 5 mM other substrate. The bottles were inoculated with 5 ml of benzene-grwon culture. The symbol, "+" denotes benzene degradation and "-" denotes no degradation.

Conclusion

A halophilic benzene degrading bacterium (strain-Rozel) was successfully isolated from the Rozel Point enrichment. Phylogenetic analysis revealed that the organism is closely related to *Arhodomonas* spp. in the *Ectothiorhodopriraceae* family of the *Gammaproteobacteria* subclass. The bacterium degraded benzene at 1-4 M salinity with optimal degradation at 3 M NaCl. No degradation occurred in the absence of salt thus suggesting that the strain is a strict halophile. Analysis showed that the isolate was able to degrade benzene in the presence of easily utilizable compounds such as glucose, pyruvate, and acetate. However, growth and degradation of benzene was inhibited in the presence of lactic acid. The reason for this inhibition is not known. These observations are important since contaminated sites often contain a variety of easily utilizable substrates. The assessment of benzene degradation pathway using degenerate primers for the detection of key catabolic genes in the DNA isolated from the pure culture showed that the isolate degrades benzene *via* catechol and *meta*-cleavage pathway. These observations are important since they help develop specific probes for the detection of similar phylotypes in variety of saline habitats.

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APPENDIX A

SEQUENCE OF CATECHOL 2,3-DIOXYEGNASE GENE OF ROZEL POINT ENRICHMENT

```
0001 GCCGCTGGGG TCGAAGAAGT ATTCGCAGGA GACCGCGAAG CATTCGCCGG
0051 GATGGCTGGC GGCATAGTCG TCGATGGGCG TGGCCTCGCC GCGTGCGAGG
0101 CGTGCCTGAA GATCGTCCCA GACGGCGGAG AAGGTGCGGT GCCATTCCTG
0151 GGGGGCCATG TCGCCCGAGA GCGGCGGATA GCCGTCGGCA TCCAGGGAGT
0201 TGCCCATGTC CAGCTTGTGG GACAACTCGT GGATCAGCAC ATTGAAGTCG
0251 TTCAGGCCAC CGCTGGCCAT CAGGTCCGGG TAGGCGACCA CCACCGGCCC
0301 CTGGTGCGGG GTCTCGCCGG CGCGTTCGTC GTCGTACTCA TGCATCACGC
0351 CGAATTCATC TACTTCTTCG ACCCGAGCGG TTA
```

APPENDIX B

SEQUENCE OF 16S RIBOSOME DNA OF ROZEL POINT ISOLATE

```
0001 TGGCTCGCTG  CGGCATGCCT  AAACATGCAG  TCGAGCGGCA  GCAGCTCCTT
0051 CGGGAGGCTG  GCGAGCGGCG  GACGGGTGAG  TAACGCGTGG  GAATCTGCCC
0101 TTTGGTGGGG  GATAGCCCGG  GGAAACTCGG  ATTAATACCG  CATACGCCCT
0151 GCGGGGCAAA  GTGGCCCTCT  GAATATGGTC  ACGCCGAAGG  ATGAGCCCGC
0201 GTCCGATTAG  CTTGTTGGTA  AGGTAAAGGC  CTACCCAGGC  AACGATCGGT
0251 AGCTGGTCTT  AGCGGACGAT  CAACCACACC  GGGACTGAAA  CACGGCCCGG
0301 ACTCCTACGG  GAGGCAGCAG  TGGGGAATAT  TGGACAATGG  GGGAAACCCT
0351 GATCCAGCAC  TGCCGCGTGG  GTGAAGAAGG  CTTGCGGGTT  GTAAAGCCCT
0401 TTCACCCGGG  AGGAAAATCA  TTCGGTTAAT  ACCCGAATGT  GTTGACGTTA
0451 CCGGCAAAGC  AAGCACCGGC  TAACTCCGTG  CCCTTTCCGC  GGTAATACGG
0501 AGGGTGCAAG  CGTTAATCGG  AATTACTGGG  CGTAAAGCGC  GCGTAGGCGG
0551 TCGGATAAGT  CGGGTGTGAA  AGCCCCGGGC  TCATCCTGGG  AATTGCATTC
0601 GATACTGTCT  GGCTAGAGTC  TGGCTAAGGG  AGGTGGAATT  TCCGGTGTAG
0651 CTGTGAAATG  CTTACATATC  GGAAGGAACA  CTCGTGGCGA  AGGCAACCTC
0701 CTGGGCCAAG  ACTGACGCTT  GAGGTGCGAA  AGCGTGTGCA  GCAAACAGGA
0751 TTAGTCCGGT  GGTAGTCCAC  GCCGTAGACT  ATGAGAACTA  GCCCTTGGAC
0801 CAGCTTATGA  TGCTCGTGGC  GCAGCGCACG  CGACCAGTTC  ACCGCCTGGG
0851 GAGAACCGCC  CTCATGCTTA  AGCTTAAACG  AATTGAGAGG  GGCCCTTTCA
0901 CCCGGAGGAG  CATTCGGTTA  ATTTCCTGCT  CGCGCAGACT  ATCACCTGCC
0951 ATGGCCTCAT  CCCACTCGTC  ACACATCAGT  CGGCGCTTCC  CGAACTCAGT
1001 GACGAGCTGC  TGCATGCTTG  ACTTGCATGT  GTGTGCTTAG  CATGCTAGCG
1051 TTACAGTCTG  AAACAGATCA  AACCTCTA
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APPENDIX C

SEQUENCE OF CATECHOL 2,3-DIOXYEGNASE GENE OF ROZEL POINT ISOLATE

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0001 GCGGCATGGG CGCGCACGCC CACACCGTAC AACTTGAGGT CTTGATATTG
0051 AAAAATCCCT CCTTATCGAG CGTTCTGTAT CCGGGACTTC TATACTTGCG
0101 CGGCGAGGTT TGGAATCTTT CAGATGTGGC CTGACGAAGT TTTCGATTTC
0151 GGAAACAGCT TCCTCGATCC TCTGGGTAAG AGGGTTCCCT TCCCTGCGAG
0201 CAGCAGACAG ATTTGCCCTA ATTTCTTTGC CAAAAGCAGG ATCACACCGG
0251 AAATCCTCGC CGCTACTCTG TTCATTGTTT AAAAACGAGT CAGTTGAACC
0301 ATTCAGCTCC CTAACGCAAT CTTTTAACTT TTCGGCTCGT CCTTTTACTT
0351 CTTCGACCCC AGCGGCAA
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VITA

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Master of Science or Arts

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Scope and Method of Study: Rozel Point is a unique habitat since it is highly saline and also contaminated with high levels crude oil from natural seeps. Unfortunately, very few studies were carried out to understand microbial diversity and their capacity to degrade petroleum compounds at Rozel Point. While halophiles have been shown to degrade aliphatic, aromatic, and poly aromatic compounds, little is known about the fate of benzene, toluene, ethylbenzene, and xylenes (BTEX) in saline environments. Therefore, our study explores bacterial degradation of BTEX in Rozel Point sediment. We obtained a highly enriched microbial consortium from a sediment sample from Rozel Point and assessed its BTEX degradation ability at various salt concentrations. We also identified the microbial community composition of the enrichment and determined major pathway of benzene metabolism using cloning and sequencing techniques. These studies are important for understanding natural attenuation potential of hydrocarbons at Rozel Point and also for designing *in-situ* bioremediation techniques for contaminated saline habitats.

Findings and Conclusions: The enrichment degraded benzene and toluene across a wide rang of salinities including 0 to 5 M NaCl. Very few studies have shown such ability, though many contaminated habitats are hypersaline. Our studies have shown that benzene and toluene are primarily degraded *via* catechol and *meta*-cleavage pathway. Molecular analysis of microbial community composition of the enrichment revealed that the *Gammaproteobacter* and *Bacteriodetes* formed the dominant groups. We also successfully isolated a pure culture that degrades benzene or toluene as the sole source of carbon. Phylogenetic analysis shows that the isolate's 16S rRNA-gene sequence had only 93% sequence identity to *Arhodomonas aquaeolei* suggesting that perhaps the isolate is a novel species in the *Gammaproteobacteri.* The organism degrades benzene in the presence of 1 to 4 M NaCl with an optimal degradation at 3 M salt. Also, the isolate degrades benzene in the presence of easily utilizable substrates including glucose, pyruvate, or acetate. This is important since most contaminated sites also contain a variety of organic compounds and inhibition of degradation often occurs in the presence of easily utilizable substrates. In conclusion, very few pure cultures capable of degrading BTEX at high salinities have been isolated. Isolation of pure cultures is important for clear understanding of ecology, physiology, mechanism of degradation and how to enhance biodegradation of hydrocarbons.

ADVISER'S APPROVAL: Babu Z. Fathepure