ISOLATION OF HALOPHILIC BACTERIA FROM INLAND PETROLEUM-PRODUCING WELLS

MAEDGEN Q. LINDSEY & JENNIFER R. HUDDLESTON*
BIOLOGY DEPARTMENT, ABILENE CHRISTIAN UNIVERSITY, ABILENE, TX 79699

MANUSCRIPT RECEIVED 28 MARCH 2017;
ACCEPTED 20 MAY 2017

ABSTRACT

The goals of this study were to isolate microorganisms from oil well–produced water, identify the microorganisms, and test the microorganisms' salt tolerance. Saltwater collected from two well locations producing from different zones in Jones County, Texas, was spread onto Mannitol Salt Agar (MSA). Isolates showed a 16S rDNA gene sequence identity of 99% with *Idiomarina baltica* and *Marinobacter persicus*. Salt tolerance assays indicated an optimal growth concentration of 10–12.5% NaCl for the *Idiomarina* isolate and a decrease in growth beyond 5% NaCl for the *Marinobacter* isolate. In conclusion, organisms that are phylogenetically similar to marine microorganisms are present in oil well environments, and have variable salt tolerances, which may prove useful in microbial–mediated hydrocarbon bioremediation of high salinity environments.

CORRESPONDING AUTHOR

Jennifer R. Huddleston
Biology Department
Abilene Christian University
ACU Box 27868
Abilene, TX 79699
jennifer.huddleston@acu.edu

KEYWORDS

- *Idiomarina*
- *Marinobacter*
- *Halophile*
- Saltwater
- Petroleum

INTRODUCTION

As of 2015, there were an estimated 1.7 million oil and gas wells currently active in the United States (18). Saltwater is produced as a byproduct of drilling as it is released from the geological formation along with oil and gas. The saltwater was originally sequestered as the respective formations were being laid down at the bottom of shallow oceans in past geological eras (11). In this study, saltwater samples were obtained from the Flippen Limestone and the King Sand formations. The Flippen Limestone is located at a depth of 1850 feet at the drilling location, and is localized to Fisher and Jones County, Texas. The limestone deposition process started in the late Pennsylvanian and continued through the Permian geological time periods (16), approximately 300 million years ago. Sequestered saltwater was released from the limestone upon hydraulic fracturing, a process in which highly pressurized water is used to open fissures within the formation (12). The King Sand was the second formation sampled in this study, and is a Pennsylvanian era channel–sand deposition located in Central Northern Texas (5), and thus slightly older and deeper than the Flippen Limestone, measuring at a depth of 2000 feet at the oil well location. Saltwater produced at these sites is pumped to the surface with oil and gas, where the components are separated and eventually utilized or discarded.

A consequence of the prevalence of oil drilling is hydrocarbon contamination of soils and groundwater. Microbial–mediated bioremediation of hydrocarbon contaminated environments is an effective treatment, however the efficacy is limited by the conditions necessary for the microorganisms to optimally biodegrade hydrocarbons (9). Salinity of the environment, either indigenous or elevated by the contamination event, is an inhibitory condition for microorganisms responsible for the bioremediation (25). Studies of optimum salinities for halophilic, hydrocarbon degrading microorganisms could lead to targeted bioremediation efforts.

Halophilic microorganisms are a type of extremophile which require a high salt content in order to survive. The extent of halotolerance is categorized by the percent salt concentration within the environment required for optimum growth. Slight halophiles exhibit optimum growth at 1–5% NaCl, moderate halophiles at 5–20% NaCl, and extreme halophiles from 20–30% NaCl (10). Halotolerant organisms are resistant to the deleterious effects a high salt content environment poses, but do not require a high salt content for survival. An environment high in salt is inhibitory for most microorganisms, as water from inside a cell will diffuse through the semipermeable cell membrane into the environment *via* osmosis and ultimately result in plasmolysis (3). Halotolerant microorganisms utilize haloadaptation to overcome this challenge, excluding salt from the cytoplasm where possible and biosynthesizing or accumulating organic osmotic solutes to remain isotonic relative to the environment (24).

Due to the range of salinities, temperatures, and possible carbon sources, oil fields and their related infrastructure pose a potential treasure trove of microbial diversity. Previously identified microorganisms isolated from oil well associated environments include *Marinobacter aquaeolei*, which was described after being isolated from the head of an offshore oil rig off the coast of Vung Tau, Vietnam, but which also occurs in the water column of the same area (17). From the same oil field, *Desulfovibri vietnamensis* was isolated from oil storage tanks, as well as oil well produced saltwater (8). Members of the genus *Desulfovibri* have been implicated in the corrosion of oil infrastructure *via* reduction of iron (13). The genus *Petrotoga*, named for its outer toga–like sheath, is associated exclusively with oil production, having been found in offshore wells and inland oil reservoirs (23). The diversity of microorganisms in these salt rich, petroleum associated environments led us to look for variable halophilicity in microbe populations by sampling saltwater produced from different geological formations. In this work, we isolated two microorganisms from saltwater produced as a byproduct of oil drilling, belonging to the genera *Idiomarina* and *Marinobacter*, and characterized their respective salt tolerances.

METHODS

SAMPLING

Permission to sample, access to well sites, and instructions on equipment use were provided by the lease holder, JQL ENERGY, LLC. Saltwater, produced as a byproduct of oil drilling, was collected aseptically from two oil well locations in Jones county, Texas. Locations, shown in Figure 1 were chosen due to contrasting geological characteristics, as the first oil well pumps from a solid limestone formation, while the second pumps from a course-grain sand formation. The Flippen Limestone sample was obtained from a fiberglass saltwater storage tank, while the King Sand sample was obtained from a water knockout, a horizontal tank which separates oil from saltwater based on varying density. Four samples, two from each well site, were collected using 118 mL sterile glass jars and stored overnight at 4°C. The King Sand samples were collected *via* release valve located on the bottom of the water knockout tank. The Flippen Limstone samples were collected *via* an access port on top of the saltwater tank, directly from the separated saltwater stored within. Initial sampling took place in November, 2015. The *Idiomarina* isolate has since been re-isolated, indicating that our method can be replicated.

Figure 1. Map of sampled locations in Jones County, Texas. The Muehlstein lease, located at 32°50'06.29"N, 99°43'51.26 W, elevation 1550 feet, is the location of the sampled saltwater storage tank from which the *Idiomarina* isolate was isolated, and is used to store saltwater pumped from a Flippen Limestone oil field. The Swenson Lease, located at 32°50'48.75" W, elevation 1556 feet, is the location of a sampled water knockout tank from which the *Marinobacter* isolate, and which separates saltwater from oil pumped from a nearby King Sand well.

GROWTH CONDITIONS

Samples of 1.0 ml were plated directly onto Mannitol Salt Agar (MSA) (Hardy Diagnostics, Santa Maria, CA), selected for its 7.5% salt content, and incubated overnight at 37°C. MSA is typically utilized as a selective and differential medium for detection of pathogenic Staphylococci, however it is also suitable for halophilic marine organisms (20). An incubation temperature of 37°C was selected by applying a formula designed to calculate the temperature of a geological formation: Formation Temperature = Surface Temperature + (Temperature Gradient * Vertical Well Depth) (19). The average regional high temperature of Jones County, Texas in November, 2015 was 66 °F, roughly 19°C (6), which when plugged into the formula with the depth of the King Sand well (2000 feet), and a temperature gradient of 0.015 °F/ft., yields a formation temperature of ~36°C. Individual colonies were selected haphazardly and subsequently sub-cultured on MSA.

IDENTIFICATION

To identify the microorganisms, the 16S rDNA genes were amplified and sequenced. DNA was extracted using a Zymo DNA extraction kit (Zymo Research Corporation, Irvine, CA) according to the manufacturer's instructions. Recombinant Vent DNA polymerase (New England BioLabs Inc., Ipswich, MA) was used in PCR to amplify a portion of the 16S rDNA gene. PCR was executed utilizing the primers 5'-AGAGTTTGATCCTGGCTCAG-3' (F'-27m) and 5'-TACCTTGTTACGACTT-3' (R'-1492) (Positions 11–27 and 1489–1506, respectively, according to the *Escherichia coli* 16S rRNA numbering system of the International Union of Biochemistry) (14). Primers were designed to our specifications and synthesized by Invitrogen. The thermocycling conditions for amplification were as follows: initial denaturation: 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute 30 seconds followed by a final extension at 72°C for 10 minutes. The products were then confirmed using gel electrophoresis of a 0.8% agarose (Invitrogen; Carlsbad, CA) gel containing ethidium bromide under a 70 to 90 voltage for 30 to 45 minutes. The resulting amplicons of 1.5 kb were further purified using DNA Clean & Concentrator (Zymo Research Corporation, Irvine, CA) and 16S rDNA sequencing was outsourced to DNA Analysis Facility on Science Hill at Yale University (New Haven, CT). The identification of phylogenetic neighbors and the calculations of pairwise 16S rDNA gene sequence similarities were achieved using NCBI BLASTn Analysis (1). Sequence alignment and creation of phylogeny were performed using Molecular Evolution Genetics Analysis 7.

ASSAY

Salt tolerance assays (Figure 2.) were carried out in Tryptic Soy Broth (TSB) (Becton, Dickinson and Company, Sparks, MD); the *Idiomarina* isolate was tested in 0.5-25% NaCl while the *Marinobacter* isolate was tested in 0.5-12.5% NaCl. Culture tubes containing 2 ml of TSB with concentrations of NaCl from 0.5-25.0% were inoculated with 30 µl of an overnight culture and incubated at 37°C for 48 hours with aeration by a shaker at 200 rpm. The optical density at 600 nm (OD600) of four replicates at each NaCl concentration were measured using a Hewlett Packard 8453 UV-Visible Spectrophotometer. Cultures were diluted 2-fold with sterile water to obtain accurate OD600 readings as necessary.

STATISTICAL ANALYSIS

The error bars in Figure 2 indicate standard deviation of measured OD600 between four replicates. Statistical analysis of the OD600 for both data sets was calculated using Microsoft Excel's Data Analysis add-in. An alpha of 0.05 was used as the cutoff for statistical significance. The *Idiomarina* isolate data set has a *p*-value of 0.43238 and is therefore beyond the cutoff for statistical significance, while the *Marinobacter* isolate data set has a *p-value* of 0.00017 and is therefore statistically significant.

RESULTS

Two *Gram-negative*, mesophilic, rodshaped isolates were obtained from saltwater samples originating in different geological formations utilized in oil and gas drilling. The isolate from the Flippen Limestone showed sequence similarity to the genus *Idiomarina*, while the isolate from the King Sand showed sequence similarity to the genus *Marinobacter*. Figures 3 and 4 illustrate phylogenetic relationships between the isolates and closely related species, chosen based on similar figures in the novel species reports of the isolates' closest 16S rDNA identities, *Idiomarina baltica* and *Marinobacter persicus*, respectively.

The *Idiomarina* isolate showed a 99.7% 16S rDNA sequence identity with *Idiomarina baltica*, originally isolated from the central

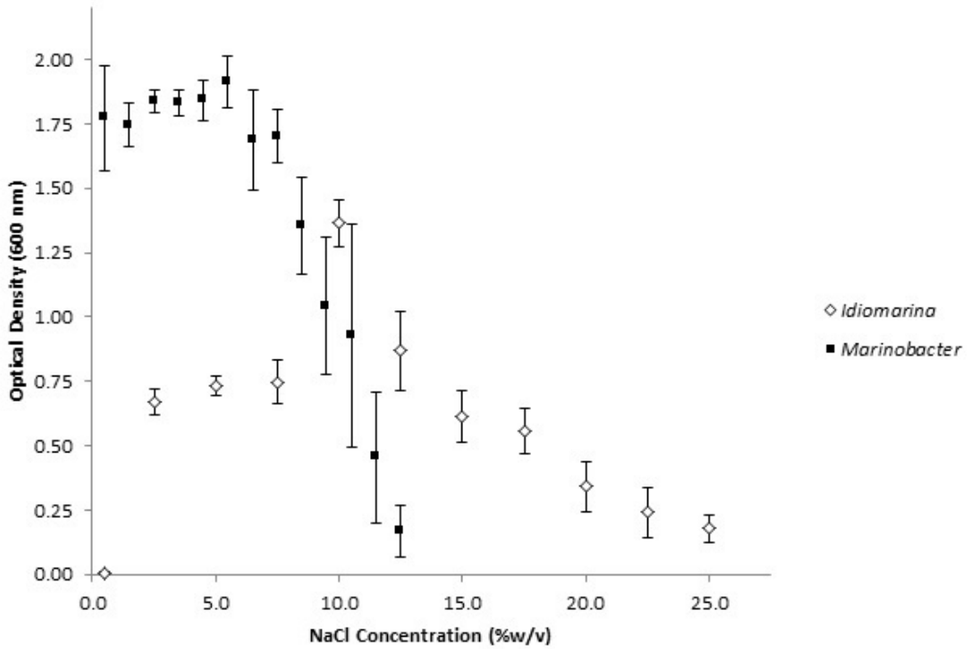

Figure 2. Plot of *Idiomarina* and *Marinobacter* isolate salt tolerance. Tubes containing 2mL TSB with varying concentrations of NaCl were aseptically inoculated and incubated 48 hours at 37 °C with aeration. Optical density at 600 nm was measured using a Hewlett Packard 8453 UV-Visible Spectrophotometer. The y-axis represents the optical density, while the x-axis represents the increasing salt concentration of each tube by weight/volume percent. Error bars indicate standard deviation of the optical densities between four replicates.

Baltic Sea. Morphologically, the isolate produced raised, circular, opaque, mucoid colonies, with white pigmentation that takes on a purple tint as it continues to grow on the MSA. The rod shaped, Gram negative cells were motile by a single polar flagellum. Results from the salt tolerance assay indicate an optimum NaCl concentration between 10-12.5% (Figure 2.), making the isolate a moderate halophile.

The *Marinobacter* isolate showed a 99.1% identity with *Marinobacter persicus*, originally isolated from Lake Aran-Bigdol, a hypersaline lake located in Iran. The isolate grew on MSA overnight incubated at 37°C and produced colonies morphologically characterized as small, convex, circular, mucoid, with a translucent leading edge, and white pigmentation that takes on a purple tint as it continues to grow on MSA. The rod shaped, Gram negative cells were motile by a single polar flagellum. Results from the salt tolerance assay indicated a sharp decrease in growth beyond 5% NaCl (Figure 2.), and the ability to grow in TSB without supplemental salt, making the isolate moderately halotolerant.

DISCUSSION

The identification of the isolates as being moderately halophilic and moderately halotolerant was expected, given the salt-rich environment. However, the close phytological association of the isolates with classically marine bacteria genera was unexpected, as the

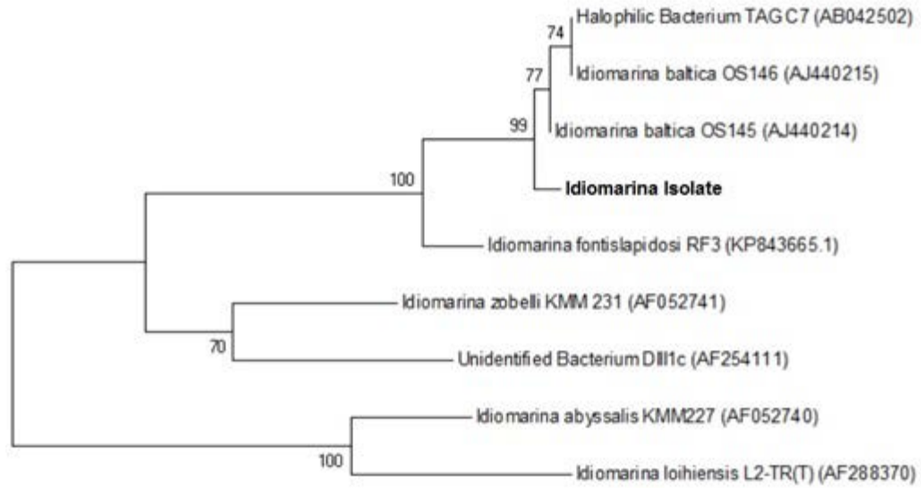

Figure 3. Unrooted phylogenetic tree generated from 16S rDNA sequences of members of the genus *Idiomarina*. Phylogenetic tree illustrates an inferred evolutionary relationships with selected species. Species were selected within the genus *Idiomarina* based on relation to *I. baltica*, our *Idiomarina* isolate's closest 16S rDNA identity match. A neighbor-joining algorithm with 500 bootstrap replications was used to produce the following topology. Alignment and phylogeny preformed using Molecular Evolution Genetics Analysis 7.

oil wells sampled are hundreds of miles inland and draw from reservoirs thousands of feet deep. Given the origin of the saltwater present in these formations, perhaps it should not be unexpected, as a likely explanation is that the seawater was sequestered from ancient seas as the strata of the geological formations were being deposited approximately 300 million years ago. Other less likely possibilities are that the oil wells could have become contaminated from fluid or equipment used to drill the well initially. Drilling rigs and associated equipment move from site to site, increasing chance of cross-contamination. Hydraulic fracturing, which used municipal water from Albany, Texas in both well sites, could have potentially introduced contaminants deep into the formations, and may change the indigenous chloride concentration, as the water is treated with 2% potassium chloride before use.

A contemporary study of two Appalachian Basin shales also found *Idiomarina* and *Marinobacter* members in low abundance during their 328 day metagenomics and metabolic analysis study. Findings from the study include niche differentiation among closely related strains, and interconnected metabolisms of microorganisms persisting in hydraulically fractured shales. A mechanism for aerobic oxidation of alkanes and respiration of sugars *via* nitrate and oxygen electron acceptors is proposed to be utilized by members of the genus *Marinobacter* present in the studied environment (7).

The salt tolerance assay shown in Figure 2. utilized varying optical densities with NaCl concentration as an indicator of microbial growth. As the bacteria have their growth inhibited with changing salt concentration, the optical density decreased as there were less cells present to scatter light from the spectrophotometer. This allowed us to chart the isolates' growth from NaCl concentrations of 0.5% w/v, which is TSB with no supplemental

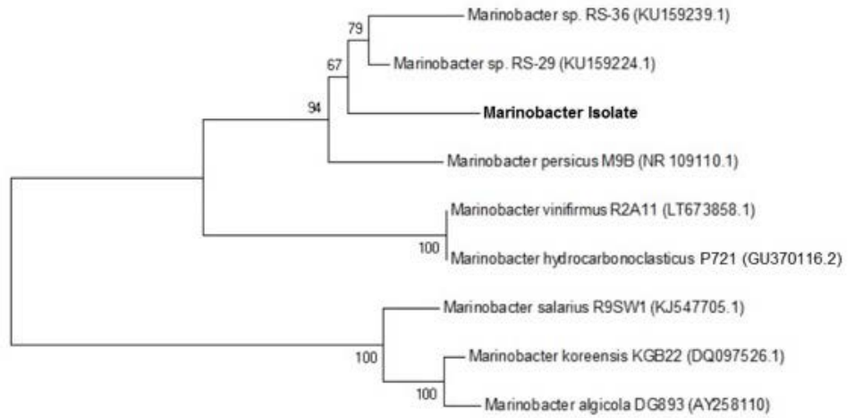

Figure 4. Unrooted phylogenetic tree generated from 16S rDNA sequences of members of the genus *Marinobacter*. Phylogenetic tree illustrates an inferred evolutionary relationships with selected species. Species were selected within the genus *Marinobacter*, based on relation to *M. perscicus*, our *Marinobacter* isolate's closest 16S rDNA identity match.

salt, to a supplemental NaCl concentration of 25% w/v. The results of the experiment indicate decreased growth beyond 5% NaCl for the *Marinobacter* isolate, but the ability to grow with only 0.5% NaCl, indicating that it was moderately halotolerant. The *Idiomarina* isolate could not grow at 0.5% NaCl, and could grow optimally at 10–12.5% NaCl, indicating that it was moderately halophilic.

An optimum salt concentration of 3–6% was initially reported for *Idiomarina baltica*, compared to the *Idiomarina* isolate's 10–12.5% reported here, classifying this strain as moderately halophilic (Figure 2). Morphologically, the two are similar with *Idiomarina baltica* producing colonies characterized as circular, smooth, opaque, and with a slight yellow pigmentation on marine agar. The most similar sequence reported for *Idiomarina baltica* type strain was a 95–96% affiliation with *Idiomarina* zobelli, while our *Idiomarina* isolate showed a closer association with 98% identity to the same species (4). Our *Idiomarina* isolate also showed a 99.2% identity to *Idiomarina Fontislapidosi*, which is reported to grow optimally at 3–5% salt concentration, and is remarkable for being the first member of the genus *Idiomarina* isolated from hypersaline soil rather than water (22). The isolate clusters closely with *Idiomarina Fontislapidosi* and strains OS145 and OS146 of *Idiomarina baltica* (Figure 3.), but has a much higher optimum salt concentration.

An optimum salt concentration of 7.5–10% was previously reported for *Marinobacter persicus*, making it moderately halophilic, while our *Marinobacter* isolate has decreased growth beyond 5% supplemental salt. The two are morphologically different as well, with *Marinobacter persicus* producing colonies characterized as raised, punctiform, contoured, and with a yellow-orange pigmentation when grown on HM agar. At the time of *Marinobacter persicus*'s novel species report, M. hydrocarbonoclasticus was the closest relative with an identity of 97.7%, while our *Marinobacter* isolate shows a 98.2% identity with the same species (2). The isolate also clusters closely with *Marinobacter* sp. RS-29, and *Marinobacter* RS-36 (Figure 4.), isolated from Yuncheng Salt Lake in China (21). Given the sharp decrease in the *Marinobacter* isolate's growth beyond 5% NaCl, it seems likely that the Yuncheng Salt Lake isolates have a higher salt tolerance due to the lake's inherent salt richness (27).

Hydrocarbon degradation abilities notwithstanding, results from the salt tolerance assays (Figure 2.) suggest that the isolates would be suitable for a range of bioremediation efforts that might otherwise be inhibited by a high environmental salt content. The *Idiomarina* isolate could prove useful in scenarios where separation of oil and saltwater does not occur before the contamination event, such as well site contamination due to leaking lines or storage tank failure, which ultimately results in the contaminated area being saltier than a typical oil spill. The *Marinobacter* isolate grows well at a concentration of 3.5% salt, ocean level salinity, and would be well suited to cleaning up hydrocarbon contamination on beaches and marine environments.

In conclusion, halophilic and halotolerant microorganisms phylogenetically associated with microorganisms present in saline and hypersaline bodies of water, such as the ocean and salt lakes, can be found in the saltwater produced as a byproduct of drilling for oil and gas. By sampling different geological formations, we were able to successfully isolate and characterize microorganisms with differing levels of halotolerance. Results indicate the *Idiomarina* isolate was moderately halophilic with an optimal NaCl concentration of 10–12.5%, while the *Marinobacter* isolate, was moderately halotolerant with decreased growth beyond 5% NaCl. Future studies will investigate hydrocarbon degradation by the new isolates, as the related *Idiomarina xiamenensis* is known to act in a hydrocarbon degrading consortium with other marine bacteria (26), and the genus *Marinobacter* has multiple members reported as capable of utilizing hydrocarbons (15).

ACKNOWLEDGMENTS

We would like to thank Abilene Christian University's Office of Undergraduate Research for funding this research, Abilene Christian University's Department of Chemistry and Biochemistry for use of its spectrophotometer, and JQL ENERGY, LLC for permission to sample and instruction on equipment use. This research was done in compliance with federal regulations and institutional policies relating to recombinant DNA and infectious agents.

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