Metagenomic evaluation of a Utah tar sand microbiota suggests the predominant hydrocarbonoclastic role of Actinobacteria [version 1; peer review: 1 approved]

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Abstract

Background: Occurrences of tar sands have been reported in 22 states in the United States; however, the largest deposit is located in southwestern Utah. It has been suggested that tar sands were created by the microbial degradation of immobile subsurface oil over several million years; however, little is known about the indigenous microbial communities in the bituminous tar sands. Methods: This study identified Utah tar sand microbiota using next-generation sequencing technology and characterized the functional diversity using community-level physiological profile (CLPP). Results: Microbiota identified in these tar sands are mainly affiliated with the Gram-positive Actinobacteria and representatives of genera that have also been previously shown to degrade aromatic hydrocarbons, including Arthrobacter, Dietzia, Janibacter, Nocardioides, Microbacterium, Agrococcus and Salinibacterium, suggesting that these microbes likely play roles in the biodegradation of oil-hydrocarbons. CLPP analysis revealed less than 24 h was needed for the first color development in the microplate wells containing the polymers, whereas the duration of the lag phase of the carboxylic acids was prolonged. Conclusions: The quick utilization of the polymers suggests that the indigenous microbial community, especially the actinomycetes in the tar sand habitat, are poised and primed to degrade these recalcitrant compounds.

Keywords
Tar, Sand, Microbiota
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Introduction

'Tar sands' is a term used to describe a combination of bituminous sand, sandstone, oil-impregnated rock, oil sand and rock asphalt. The tar sands is a rapidly developing source of unconventional petroleum. The bitumen, an oil-rich residue, can be extracted from the tar sands and refined into crude oil. In the United States, occurrences of tar sands have been reported in 22 states; however, one of the largest deposits is located in southwestern Utah, with estimated recoverable oil ranging from 12 to 20 barrels.

It has been suggested that tar sands were created by the microbial degradation of immobile subsurface oil over several million years. Little is known about the origin of the microorganisms in the tar sands of Utah, although the indigenous microbial communities exist in extreme conditions in the bituminous tar sands and are limited by harsh conditions such as low moisture and oxygen, recalcitrant hydrocarbons and a high concentrations of toxic metals. However, a paucity of data exists on the indigenous microbial communities of the tar sands of Utah.

Traditionally, the diversity of the bacterial communities in the tar sands has been investigated using the isolation and cultivation approaches. However, only 1.5% of bacteria in the soil can be readily cultured. Most cultural conditions cannot mimic the specific microhabitats that many prokaryotes thrive within. Therefore, new methods for the analysis of whole microbial community structure and metabolic function in the bituminous tar sands have been developed. One of the methods developed to provide a dynamic tool for the assessment of microbial community structure and function is the BIOLOG™ Community Level Physiological Profile (CLPP). This method is based upon the preferential metabolism of 31 different carbon sources on a microtiter plate. These carbon sources include a wide range of chemical classes, such as carbohydrates, esters, polymers, carboxylic acids, alcohols, amines, phosphorylated chemicals, amino acids, aromatic chemicals, and amines. Each well on the BIOLOG™ Ecolate contains a unique carbon source, peptone, and a 2,3,5-triphenyltetrazolium chloride (TTC) dye. NADH is produced from the respiration of the specific carbon sources. The final electron acceptor TTC is irreversibly reduced to formazan, a red pigment that can be quantified visually by use of a microplate reader. The intensity of the color change correlates to the amount of metabolism of the carbon source in that well. The net intensity of the color change is calculated by subtracting the absorbance of the non-carbon-source control well. The oxidation of the carbon substrate oxidized by the microbe can be considered to be its metabolic fingerprint. However, Windig reported that the BIOLOG™ system excludes strict anaerobes and bacteria that lack certain electron transport enzymes. Yao et al. advocated that CLPP is selective and favors microbes that grow quickly or those with a high inoculum density in the initial sample. Hence, culture-independent analysis is the method of choice for the investigation of the bituminous tar sands.

The objective of this study was to identify and characterize the indigenous bacterial communities of the Utah tar sands using next-generation sequencing technology and to assess the functional diversity using the CLPP system. The overall goal of this project is significant because studies have demonstrated that naturally occurring microbes can be harnessed for the degradation of recalcitrant polyaromatic hydrocarbons, heavy metals, and naphthenic acids. The combination of the functional diversity and the characterization of the indigenous microbiota will advance our understanding of the fate of tar-associated potentially toxic compounds by environmental microbiota.

Methods

Microbial community analysis by genomic DNA extraction and 454 pyrosequencing

The University of Alberta Geotechnical Centre, Canada, kindly provided the raw bacterial communities of the Utah tar sands using the isolation and cultivation approaches. However, only 1.5% of bacteria in the soil can be readily cultured. Most cultural conditions cannot mimic the specific microhabitats that many prokaryotes thrive within. Therefore, new methods for the analysis of whole microbial community structure and metabolic function in the bituminous tar sands have been developed. One of the methods developed to provide a dynamic tool for the assessment of microbial community structure and function is the BIOLOG™ Community Level Physiological Profile (CLPP). This method is based upon the preferential metabolism of 31 different carbon sources on a microtiter plate. These carbon sources include a wide range of chemical classes, such as carbohydrates, esters, polymers, carboxylic acids, alcohols, amines, phosphorylated chemicals, amino acids, aromatic chemicals, and amines. Each well on the BIOLOG™ Ecolate contains a unique carbon source, peptone, and a 2,3,5-triphenyltetrazolium chloride (TTC) dye. NADH is produced from the respiration of the specific carbon sources. The final electron acceptor TTC is irreversibly reduced to formazan, a red pigment that can be quantified visually by use of a microplate reader. The intensity of the color change correlates to the amount of metabolism of the carbon source in that well. The net intensity of the color change is calculated by subtracting the absorbance of the non-carbon-source control well. The oxidation of the carbon substrate oxidized by the microbe can be considered to be its metabolic fingerprint. However, Windig reported that the BIOLOG™ system excludes strict anaerobes and bacteria that lack certain electron transport enzymes. Yao et al. advocated that CLPP is selective and favors microbes that grow quickly or those with a high inoculum density in the initial sample. Hence, culture-independent analysis is the method of choice for the investigation of the bituminous tar sands.

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In this study the 31 carbon sources were organized into groups 1–5 as described by Zak et al. Carbon sources originally grouped as miscellaneous by Zak et al. or those new to the BIOLOG™ Ecolate, were grouped into one of the other five categories - carbohydrates, carboxy acids, amino acids, esters and...
polymers. Grouping the data into 5 guilds compresses a 31-dimensional data set into 5 dimensions, significantly reducing the complexity of the data and subsequent interpretation\(^6\).

The average well color development (AWCD) was used to assess the microbial response for all the carbon sources. The AWCD was determined as follows:

\[
AWCD = \frac{\sum OD_i}{31}
\]

where OD\(_i\) is optical density value from each well, corrected subtracting the blank well (inoculated with water) values from each plate well\(^8\). The 96-h data was used for statistical analysis of CLPPs.

The diversity of substrate utilization was calculated using the Shannon’s diversity index (\(H\)) and evenness (\(E\))\(^9\). The Shannon-Weaver index is a measure of the capacity of the bacterial community to degrade the carbon sources in the well and can be considered as an index of the physiological diversity of the bacterial community in the tar sands. Higher values of \(H\) indicate the ability of the microbial community to degrade the substrates with a high efficiency\(^16\):

\[
H = -\sum p_i \ln p_i
\]

where \(p_i = (\text{OD reading of well I})/(\text{sum of all wells})\), based on the OD in the Ecoplates.

The Shannon evenness (\(E\)) is a measure of how dissimilar the abundances of the species in a community are from each other\(^17\), and was calculated as:

\[
E = H/\ln S
\]

where \(S\) (Shannon richness) is the number of substrates used by the microbial communities. Further, the cluster analysis of the substrate utilization pattern was constructed using the nearest neighbor method with Euclidian distance to form linkage dendrogram.

**Results and discussion**

**Microbial community analysis of tar sands**

In this study, the compositions of bacterial community of the tar sands was investigated by a pyrosequencing-based analysis of the 16S rRNA gene sequences. The gram-positive Actinobacteria (50\%) were dominant in the tar sand followed by Betaproteobacteria (27\%), Alphaproteobacteria (7\%), Gammaproteobacteria (7\%) and Acidobacteria (2\%) (Figure 1).

![Figure 1. Taxonomic distribution of the obtained metagenome sequences shown at the phylum level. The gram-positive Actinobacteria were dominant in the tar sand followed by Betaproteobacteria, Alphaproteobacteria, Gammaproteobacteria and Acidobacteria.](image-url)
Actinobacteria are known for their role in the biodegradation of a variety of different pollutants including petroleum hydrocarbons\textsuperscript{18,19}. The predominant genus identified in these tar sands was *Arthrobacter*, followed by *Dietzia, Janibacter, Nocardiooides, Microbacterium, Agrococcus* and *Salinibacterium* (Figure 2). Most of these genera have also been previously shown to degrade aromatic hydrocarbons, indicating that tar sands are a very active repository of hydrocarbonoclastic microorganisms. When the taxonomic affiliation of the obtained metagenomic sequences were investigated, we found that the gram-positive *Arthrobacter* spp. from the Actinobacteria phyla were predominant, which comprised approximately half of the total microbial community assemblage in this particular Utah tar sand. *Arthrobacter* species possess a significant hydrocarbonoclastic potential as demonstrated by previous studies and we recommend that Actinobacteria, native to the tar sand habitats, should be targeted for future research on this area.

**Microbial community metabolic analyses**

Biolog\textregistered Ecoplates were used to evaluate the functional diversity of the microbial community of the tar sands. The AWCD revealed sigmoidal relationships between the OD\textsubscript{590} and time for all carbon sources (polymers, carbohydrates, amino acids, esters) except the carboxylic acids (Figure 3). This pattern
for the polymers, carbohydrates, amino acids, and esters is fundamentally similar to the bacterial growth curve. Yao et al.\textsuperscript{20} suggested that color development reflected species metabolic activity and the ability of the bacterial community to respond to substrates. The AWCD for all the substrates in the Biolog\textregistered{} Eco-microplates was 0.25. Less than 24h was needed for the first color development the microplate wells containing the polymers whereas the duration of the lag phase of the carboxylic acids was prolonged. The first color development of the carboxylic acids (AWCD\textsubscript{0.2}) was recorded at 48 h (Figure 3). The highest utilization was observed in the microplate wells containing the polymers (AWCD\textsubscript{0.78}) after 96 hours. The observed differences in the substrate utilization pattern of five major carbon groups (Figure 3) might be due to the presence of different functional groups, such as carbohydrates R-C=O), amino acids (-NH\textsubscript{2} and -COOH), carboxylic acid (-COOH), esters, amines and phosphorylated acids (-COOR\textsuperscript{2}, -NH\textsubscript{3}), and polymers (-{(CH\textsubscript{2}-CH\textsubscript{2})}\textsubscript{n}). The high utilization of the recalcitrant polymers suggest that the polymers could be more easily utilized by the indigenous microbial community in the tar sands. After 96 h, there was a decrease in the rate of AWCD; thus, the analysis of functional diversity of microbial community of tar sands was completed at 96 h. The decreased rate may be due to the change of metabolic activity of active microbial communities utilizing the C-substrates. Cluster analysis of the substrates revealed a systematic grouping of the substrates based on their utilization pattern (Figure 4).

The Shannon-Wiener index is an indication of the spread or distribution of carbon source utilization by the microbial community\textsuperscript{31}. Typical values of the Shannon-Wiener Index usually lies between 1.5 and 3.5, and rarely exceed 4.0\textsuperscript{22}. In this study, the Shannon-Weiner index, \(H\), of the microbial community in the tar sands was 3.072 and the Shannon evenness, \(E\), was 1.009. These values indicate a more diverse microbial community and an even distribution of the species within the tar sands.

**Conclusion**

This study investigated the indigenous microbial communities of the tar sands of Utah in an effort to understand the structure and the functional diversity of the microbial community. The substrate utilization patterns of resident

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**Figure 4.** Substrate Utilization Pattern shown by Utah tar sand soils.
microbial communities and the identification of the hydrocarbon-degrading microorganisms in the tar sands provide valuable baseline information than can be used for hydrocarbon bioremediation and for devising biotechnological approaches to tar sands bioremediation efforts.

Data availability
Dataset 1. Raw data obtained from the community-level physiological profile, 10.5256/f1000research.16126.d218909.

The DNA sequences from this metagenomic project are available from the Sequence Read Archive-European Nucleotide Archive, accession number SRR1699470.

References


Grant information
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This manuscript documented microbial communities in Utah tar sand. The methods are well described and results are clearly presented. The performance of these communities to consume 31 different carbon sources was compared. Conceptually, I like the idea to compare the degradation rate of 31 different carbon sources using Utah tar sand indigenous microbial communities at micro plate. However, I believe the microbial community characterization, especially the high-throughput data, are not vigorously examined. I have a few minor concerns as follows.

Major concerns:

1. As you mentioned in the Introduction part, the BIOLOG system excludes strict anaerobes and bacteria that lack certain electron transport enzymes. That means the indigenous microbes of Utah sand were selected after inoculation and cultivation at 96-well plates. Some of the Utah sand anaerobes and other microbes were inhibited. It is possible that those inhibited Utah sand anaerobes are efficient for the degradation of other recalcitrant compounds.

2. It is necessary to mention the primer pairs and the regions of the 16S rRNA genes, because it is essential to ensure full reproducibility by others.

3. All raw pyrosequencing data that was obtained from this study should be deposited to the NCBI Sequence Read Archive, and the accession no. should be provide in the method.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes
If applicable, is the statistical analysis and its interpretation appropriate? Yes

Are all the source data underlying the results available to ensure full reproducibility? Yes

Are the conclusions drawn adequately supported by the results? Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biological wastewater treatment, biofilms, oil sands process-affected water (OSPW)

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