Isolation and Characterization of Benzene Degrading Bacteria from Gasoline Contaminated Water

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ABSTRACT

In the present study, we have isolated AG3 and AG4 bacteria of the *Bacillus* group which are able to grow in aerobic as well as anaerobic conditions. These bacteria were isolated from a location near Rice University, Houston by using BTEX (benzene, toluene, ethylbenzene, and xylene) as the principal carbon source. They are Gram-positive, and rod-shaped. The isolates utilized benzene as a carbon source in both aerobic and anaerobic conditions. Various carbohydrates and aromatic compounds were also tested as substrates for the growth of these bacterial isolates. Their 16S rDNA sequences indicate that they are members of the Bacillaceae; AG3 belongs to *Bacillus cereus* and AG4 belongs to *Bacillus megaterium* bacteria. These bacteria isolates can potentially be utilized in bioremediation of benzene in both aerobic and anaerobic environment.

Keywords: BTEX, Benzene, *Bacillus*, Bacteria

1 INTRODUCTION

Hydrocarbon pollution of water bodies is widely recognized as a serious environmental problem, since it not only gives serious damage on fisheries but also causes adverse effects on the natural environment and ecosystem [1, 2]. Surface oil spills, leaking pipelines and underground fuel storage tanks, improper waste disposal practices, inadvertent spills, and leaching from landfills can lead to subsurface contaminant plumes containing significant amounts of the hazardous aromatic hydrocarbons. BTEX (benzene, toluene, ethylbenzene, and xylene) compounds are the major aromatic hydrocarbon components in many petroleum products that are considered as a significant threat to the health and environment. Some accidental oil spills have activated developments of methods for microbiological degradation of oil. Fortunately, many microbial groups inhabiting contaminated sites have developed interesting metabolic mechanisms for detoxification and degradation of a wide range of aromatic hydrocarbons [3-5].

Among BTEX, benzene is of major concern, because it is soluble, mobile, toxic, carcinogenic and one of the most stable aromatic compounds, especially in ground and surface waters. Microbial degradation of benzene in aerobic environments has been successful, however, benzene is poorly biodegraded in anaerobic conditions [6]. *Pseudomonas* species are common in aerobic bioremediation of benzene [7]. Isolation, identification, and genetic manipulation of a vast number of local bacterial groups for the bioremediation of aromatic hydrocarbons have been the focus of many researchers worldwide [4, 8]. However, there is still a need to isolate more microbial species with novel enzymatic activities that are important for environmental as well as biotechnological applications.

In this study, we describe the isolation and characterization of two isolates. These two bacterial isolates include *Bacillus sp*., which can utilize BTEX as the sole source of carbon and energy. They were isolated from a location near Rice University, Houston. They are also capable of growing on other aromatic hydrocarbons as their sole sources of organic carbon.

2 MATERIAL AND METHODS

2.1 Isolation and cultivation of bacteria

The sample was collected from a location near Rice University, Houston. Sample was mixed with sterile water (1:1 wt.: vol.) on a vortex mixer for 1 min.; 0.1 ml of the resulting supernatant was spread on the surface of solid minimal medium [9]. Aerobic incubations with volatile substrates (BTEX) took place inside glass desiccators with the hydrocarbon supplied as vapor by saturating a piece of filter paper (2 x 2 cm) with 100 µl of pure compound. Plates were incubated at 30°C for 48 and 170 hours, respectively. Of the colonies appearing on each plate, eight were selected, picked and streaked for purification under the same conditions as in the initial incubation. A rapidly growing, visually distinct colony and a separate, morphologically unique isolate were selected for further analysis and purified by repeated plating.

For further studies, mineral medium [10] was used with ATCC trace metal solution and Sigma BME 100X vitamin solution.

2.2 Phenotypic testing

Isolates were Gram stained by using Sigma Gram staining kit. Furthermore, oxidase test (Fluka) and microscopy for shape were conducted. The growth of isolates was tested at a temperature of 30°C. The absorbance at 600 nm for the growth was measured with spectrophotometer. The following compounds were tested for growth in aerobic and anaerobic conditions as a carbon source by HIMEDIA carbohydrate kit: L-arabinose, fructose, glucose, dextrose sucrose, and glycerol. The following aromatic compounds were also tested as the sole carbon sources for growth in aerobic and anaerobic conditions: 100µl of benzene, ethylbenzene, toluene and xylene each on the solid plate, and 250 ppm of phenol and Na-benzoate in the liquid medium. Isolates growth were scored as positive or negative by comparing the turbidity of the liquid medium and growth on solid plates after one month of incubation at 30^0C temperature.

2.3 Identification of bacteria by 16S rDNA sequencing

16S rDNA sequence was used to identify the isolates. Genomic DNA was prepared with a DNA extraction kit (ultra clean microbial DNA isolation kit, MO BIO Laboratories). The 16S rDNA were amplified by PCR with primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACGACTT-3') [11]. The temperature parameters used were as follows: hot start at 94^0C for 5 min, 30 cycles of denaturation at 94^0C for 30 s, annealing at 58^0C for 30 s, and extension at 72^0C for 30 s followed by one cycle at 72^0C for 7 min to complete extension. The PCR assays were examined in 1.0% agarose gels. The PCR fragments obtained were purified by using a Qiagen QIAquick gel extraction kit according to the manufacturer's instructions. The amplified DNA was cloned by using a TA cloning kit (Invitrogen USA). The cloned genes were then sequenced with an automated sequencer (ABI Prism Genetic Analyzer, Applied Biosystems). The sequences were compared with the data in GenBank using the basic local alignment search tool (BLAST) from the National Center for Biotechnology Information (NCBI).

3 RESULTS AND DISCUSSION

3.1 Phenotypic characteristics

Two bacteria (AG3 and AG4) were isolated from the above mentioned location which were able to grow well using BTEX as the sole source of carbon. Physiological characteristics of the two isolates were examined. In general, both isolates show identical results in the phenotypic tests. Both bacterial isolates were straw yellow in color; rod-shaped, aerobic/anaerobic, gram-positive and oxidase-positive [Table 1 and Fig. 1].

The following aromatic hydrocarbons and carbohydrate were utilized as sole sources of carbon by AG3 and AG4 in mineral medium: benzene, ethylbenzene, toluene, xylene, Na-benzoate, phenol, L-arabinose, fructose, glucose, dextrose sucrose, and glycerol. Growth was not observed for AG3 in sucrose and L-arabinose. AG4 utilized all tested carbohydrates as a carbon source. For aromatic compounds both isolates were able to grow in both aerobic and anaerobic conditions on benzene, xylene, Na-benzoate and phenol. AG3 was unable to grow in aerobic/anaerobic conditions in toluene and ethylbenzene. AG4 was able to grow in aerobic condition in toluene but unable to grow in anaerobic condition. For ethylebenzene, AG4 did not show any growth in both aerobic and anaerobic conditions.

3.2 Amplification and 16S rDNA sequencing

PCR amplification of 16S rDNA from strains AG3 and AG4 resulted in 1.5 kb PCR fragments on the agarose gel [Fig. 2]. The results were consistent with the results of the BLASTN search and suggest high affiliation with genera of the family Bacillaceae. Based on a BLASTN search of GenBank, the closest matches to strain AG3 was *Bacillus cereus* (96% sequence similarity) and AG4 *Bacillus megaterium* (96% sequence similarity).

Many *Bacillus* strains for degradation of polycyclic aromatic hydrocarbon compounds and other chemical classes of petroleum origin have been previously reported [12, 13]. It seems likely that members of the Bacillaceae family dominate in oil-contaminated soils or water.

4 CONCLUSIONS

Two strains, AG3 and AG4 were isolated and identified as *Bacillus cereus* and *Bacillus megaterium* respectively. These isolates are able to utilize benzene as a carbon source in aerobic and anaerobic conditions. These isolates were also capable of utilizing other aromatic compounds in both environments. Further studies are needed to clarify their exact taxonomic position and to explore their biodegradation potential to other polycyclic aromatic hydrocarbon.

5 ACKNOWLEDGEMENTS

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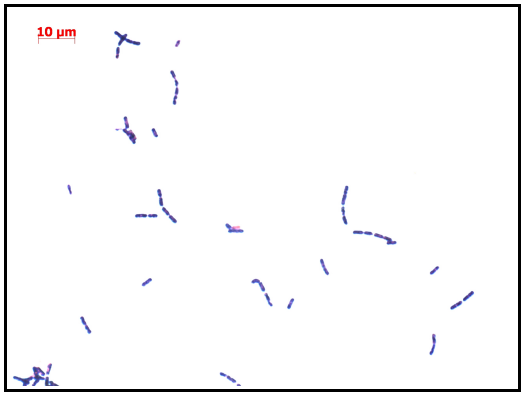
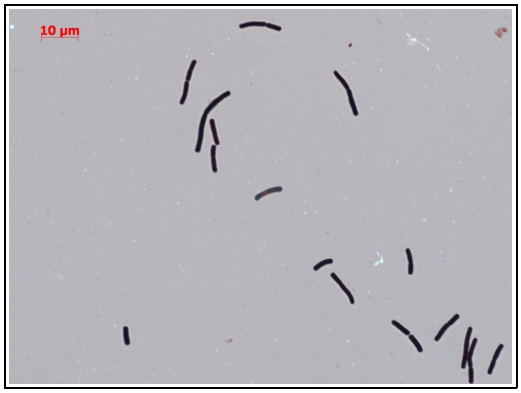
6 TABLE AND FIGURES



(A)



(B)

Figure 1: Picture of bacterial isolates under light microscopy (A) AG3 bacterial isolates and (B) AG4 bacterial isolates.

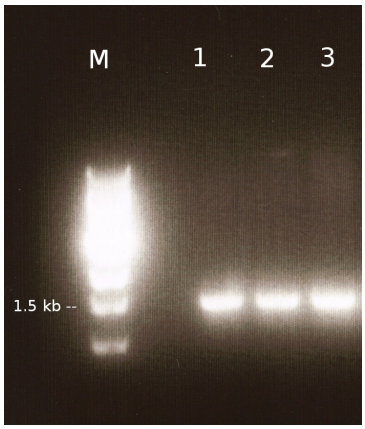


Figure 2: Amplification of 16S rDNA gene using specific primers 27F and 1492R from bacterial isolates AG3 (lane 1) and AG4 (lane 2), lane 3 is the control and M is 1.0 kb DNA ladder marker.

Table 1: Biochemical and physiological characteristics of AG3 and AG4 bacteria:

Characteristics	AG3	AG4
Color of bacterial colonies	Straw yellow	Straw yellow
Morphology	Rod	Rod
Gram staining	+	+
Aerobic growth	+	+
Anaerobic growth	+	+
Oxidase reaction	+	+
Carbohydrate utilization		
Glucose	+	+
Sucrose	-	+
Dextrose	+	+
L-Arabinose	-	+
Glycerol	+	+
Aromatic compounds utilization		
Benzene	+ve in aerobic / anaerobic	+ve in aerobic / anaerobic
Xylene	+ve in aerobic / anaerobic	+ve in aerobic / anaerobic
Toulene	- ve in aerobic / anaerobic	+ve in aerobic, -ve in anaerobic
Ethylebenzene	-ve in aerobic / anaerobic	-ve in aerobic / anaerobic
Na-benzoate	+ve in aerobic / anaerobic	+ve in aerobic / anaerobic
Phenol	+ve in aerobic / anaerobic	+ve in aerobic / anaerobic

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