

# Isolation of Methyl-tert Butyl Ether (MTBE) Degrading Bacteria from Environmental Samples

Nicholas Strasser

Faculty Sponsor: Bonnie Jo Bratina, Department of Microbiology

## ABSTRACT

Methyl tertiary butyl-ether (MTBE), a gasoline additive, is a widely recognized environmental contaminant which has infiltrated many of our nation's groundwater supplies. Once dissolved in water, bioremediation appears to be the best option for removal since MTBE is difficult to strip using mechanical and chemical techniques. We have isolated a MTBE-degrading bacterium from the gravel of an Ohio gas station parking lot. The isolate was a gram-negative rod (1  $\mu\text{m}$  x 2  $\mu\text{m}$ ) and grew at room temperature on mineral salts - Noble agar supplemented with 25  $\mu\text{g/ml}$  MTBE. The isolate was highly motile and oxidase and catalase positive. Although MTBE-degrading bacteria have been previously isolated, the characteristics of our isolate differ from those in the literature. Phylogenetic analysis using a fragment of the 16S ribosomal RNA gene indicates that the isolate is likely a *Pseudomonas putida* strain.

## INTRODUCTION

Methyl tertiary-butyl ether (MTBE) was used extensively in the early 1990's as an octane booster for automobile gasoline. The use of MTBE peaked shortly after the Clean Air Act Amendments of 1990. In an attempt to lower air pollution, these amendments made it mandatory for several large cities in the U.S. to add octane boosters, mainly MTBE, to their gasoline. The use of MTBE increased dramatically because it was cheaper to produce than other additives, and, in 1992, it became the second most produced organic compound at a rate of 1.8 billion gallons/year.

Leaky underground gas tanks, shipping spills, and partially burned gasoline contributed to a large release of MTBE into the environment (Fig. 1) (2). One by one, sites heavily contaminated with MTBE were identified, which eventually lead to an increased awareness of the MTBE problem. The Environmental Protection Agency has issued secondary standards for drinking water containing MTBE (2). These standards include taste and odor warnings and are not testable standards.

Researchers began to investigate MTBE contaminated sites and we now know much more about how MTBE interacts in our environment. Once released in soil, MTBE quickly seeps into surface water and groundwater supplies. Since both air stripping and chemical treatment have proven to be ineffective in removing MTBE from large amounts of contaminated soil or water, researchers are investigating the possibility of using bacteria capable of MTBE degradation for removal via bioremediation (3).



FIG. 1. MTBE leaking from gas station storage tanks infiltrates ground water supplies which eventually interact with surface waters. Once in surface waters, evaporation allows MTBE to travel through the atmosphere associated with various forms of precipitation. The most dangerous consequence of groundwater contamination is poisoning of fresh water supplies.

## MATERIALS AND METHODS

**Sample Collection.** Environmental samples were collected from sites likely to be contaminated with MTBE. Soil, water or sludge samples from sites such as marinas, industrial corridor drainage ditches, and gas station storm drains/parking lots were used to inoculate either mineral salts Noble agar plates supplemented with MTBE or a mineral salts broth supplemented with MTBE. In both cases, MTBE (25  $\mu\text{g/ml}$ ) was the sole carbon and energy source. Plates were incubated at room temperature until pinpoint colonies were seen. Broths were incubated at room temperature on a rotary shaker.

**16S ribosomal RNA (rRNA) sequencing and phylogenetic analysis.** Isolated colonies were picked from Noble agar - MTBE plates and streaked to TSA plates for purity. Colonies were picked from the TSA plates after 48 hours and lysed using Lyse-N-Go PCR reagent (Pierce, Rockford, IL). The 16S rRNA gene was amplified using 8F and 1492R primers (1). The PCR product was purified using a spin column prior to sequencing. Sequence reactions were performed using 8F, 338R and 690R primers and sent to the University of Wisconsin - Madison for sequence analysis. Phylogenetic analysis was performed by comparison to sequences in the Ribosomal Database Project (RDP).

**Growth studies.** Isolates were tested for their ability to grow using MTBE as the sole carbon and energy source by plating to agarose plates with and without MTBE amendments. An agarose extract was prepared by hydrating agarose (1.5%) in distilled water overnight. The suspension was centrifuged and the supernatant was filter sterilized. Aliquots of 1, 2 and 5 ml agarose extract were added to culture tubes containing 5 ml of mineral salts broth. MTBE (final concentration 110  $\mu\text{g/ml}$ ), was added as the sole carbon and energy source to each agarose-amended culture tube. DS1 and DL1 were added to the various tubes individually and as a consortia. The tubes were incubated at room temperature on a rotary shaker. Catalase and oxidize reactions were tested as described (4).

## RESULTS

Samples were taken from a number of different sites likely to be contaminated with MTBE. These sites included gas station parking lots, gas station storm drains, industrial corridor ditches, and river marinas. For each sample, an enrichment was set up using mineral salts broth supplemented with MTBE. Any enrichments showing growth were tested for the ability to degrade MTBE (Fig. 2).

Preliminary results show that isolated bacteria from an Ohio gas station parking lot may have been degrading MTBE (Fig. 3). These bacteria were capable of growing on Noble agar supplemented with MTBE. When streaked to Nobel agar without MTBE, the bacteria were incapable of growing. On general media, the isolates formed two distinct colony sizes. The resulting small colonies, designated strain DS1, and large colony formers, designated DL1, were streaked for purity on general media and used to inoculate Noble agar plates + MTBE.

The bacteria are slow growers and do not grow well in mineral salts broth. Since we were able to culture these organisms on Agarose plates + MTBE, we made an agarose extract by hydrating 1.5% agarose in distilled water. The agarose-amended broth, however, did not allow the bacteria to grow to high enough densities for degradation assays. We are continuing to modify the broth composition by adding vitamins and trace elements.

Initial characterization of DS1 and DL1 showed both isolates to be motile, gram-negative rods ( $1 \mu\text{m} \times 2 \mu\text{m}$ ). DL1 was oxidase and catalase positive. Phylogenetic analysis of the 16S rRNA gene sequence places the isolate DL1 in the genus *Pseudomonas* and most closely related to *P. putida* strain R1 (Fig. 4). No further analysis was performed on DS1.

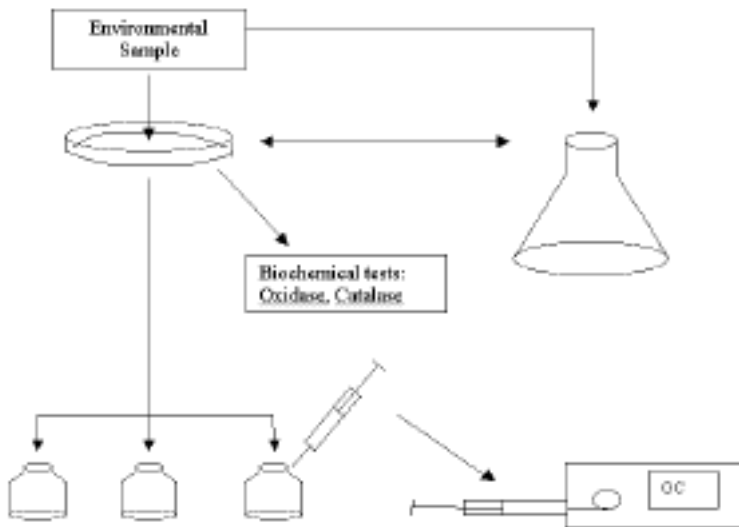


FIG. 2. Environmental samples were used to inoculate Noble agar plates and mineral salts broth. MTBE was added as the sole carbon and energy source in both cases. New colonies from plates were used to inoculate serum vials and for biochemical tests. Headspace samples were taken from serum vials for GC analysis. Broths were used to test for the ability of isolated colonies to grow in liquid media.

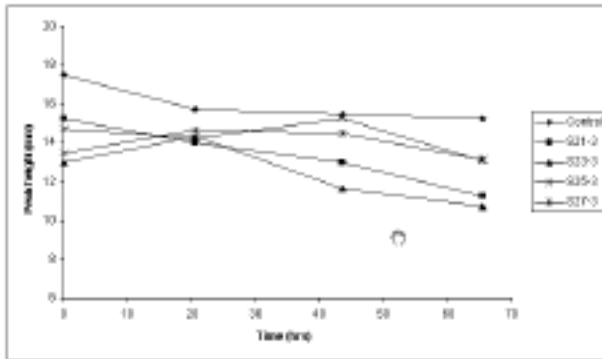


FIG. 3. Four isolates were assayed for the ability to degrade MTBE as determined by the decrease in peak height (as measured by GC) over time. Sites of inoculum were - S21-3: Oxford, OH gas station, S23-3: Chicago industrial corridor dirt, S25-3: Ditch between Chicago land fill and steel mill, and S27-3: Biosolids processing plant ditch. The control contained no inoculum.

Phylogenetic analysis of DL1

Bacteria

Proteobacteria

Gamma subdivision

Pseudomonas and relatives

*P. putida* subgroup

DL1

Percent similarity between DL1 and closely related *Pseudomonas*

|                                      |        |
|--------------------------------------|--------|
| Strain R1                            | 97.59% |
| <i>Pseudomonas putida</i> str. mt-2  | 96.39% |
| <i>Pseudomonas putida</i> str. F1    | 96.39% |
| <i>Pseudomonas putida</i> str. K23-1 | 96.29% |

FIG. 4. Phylogenetic analysis of isolate DL1 based on 16S rRNA partial gene sequence.

ACKNOWLEDGEMENTS

Thank you to the Dean’s Distinguished Summer Fellowship committee and Dean Nelson for the funding of this undergraduate research. Thank you to all the local businesses that allowed me to sample and to those who helped with the collection process, especially Chad Galer. A special thank you to Dr. Bonnie Jo Bratina for advising me with the research.

## REFERENCES

1. Eden, P.A., T.M. Schmidt, R.P. Blakemore, and N.R. Pace. 1991. Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction amplified 16S rRNA-specific DNA. *Int. J. Syst. Bacteriol.* **41**:324-325.
2. Environmental Protection Agency. URL><http://www.epa.gov/swerust1/mtbe/> MTBE and underground storage tanks. Accessed 25 June 2001.
3. Steffan, R.J., K. McClay, S. Vainberg, C.W. Condee, and D. Zhang. 1997. Biodegradation of the gasoline oxygenates methyl *tert*-butyl ether, ethyl *tert*-butyl ether, and *tert*-amyl methyl ether by propane-oxidizing bacteria. *Appl. Environ. Microbiol.* **63**:4216-4222.
4. Winfrey, M.R., M.A. Rott, S. Anglehart, and R.M. Burns. 1998. Laboratory manual for introductory microbiology, p.20-1 – 20-5. Department of Microbiology, University of Wisconsin-La Crosse.

