Isolation of a Bacterial Culture That Degrades Methyl t-Butyl Ether

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Received 27 December 1993/Accepted 6 May 1994

We have isolated a mixed bacterial culture (BC-1) which is capable of degrading the gasoline oxygenate methyl t-butyl ether (MTBE). BC-1 was developed from seed microorganisms present in a chemical plant biotreater sludge. This enrichment culture has been maintained in continuous culture treating high concentrations of MTBE (120 to 200 mg/liter) as the sole carbon source in a simple feed containing NH₄⁺, PO₄³⁻, Mg²⁺, and Ca²⁺ nutrients. The unit had a stable MTBE removal rate when maintained with a long cell retention time (ca. 80 to 90 days); however, when operated at a ≤50-day cell waste rate, loss of MTBE-degrading activity was observed. The following three noteworthy experimental data show that MTBE is biodegraded extensively by BC-1: (i) the continuous (oxygen-sparged) culture was able to sustain a population of autotrophic ammonia-oxidizing bacteria which could nitrify influent NH₄⁺ concentrations at high rates and obtain CO₂ (sole carbon source for growth) from the metabolism of the alkyl ether, (ii) BC-1 metabolized radiolabeled either (¹⁴CH₂O-MTBE) to ¹⁴CO₂ (40%) and ¹⁴C-labeled cells (40%), and (iii) cell suspensions of the culture were capable of degrading (substrate depletion experiments) MTBE to t-butyl alcohol, a primary metabolite of MTBE. BC-1 is a mixed culture containing several bacterial species and is the first culture of its kind which can completely degrade an alkyl ether.

Alkyl ethers such as methyl t-butyl ether (MTBE) are being used as octane enhancers in the reformulation of low-volatility unleaded gasoline blends and for reducing emissions of volatile organic compounds from engines (6, 11, 19). In general, alkyl ethers are a chemically stable class of compounds and there is little information on their biodegradability in soil, groundwater, and activated-sludge environments. MTBE can persist in groundwater from accidental spills of unleaded gasoline from underground storage tanks. However, no known naturally occurring microbial cultures exist to biotreat groundwater, wastewater, tank bottom wastes, or soils containing this ether.

There are some data in the literature on the metabolism of diphenyl (3, 5) and cyclic (2) ethers (e.g., dioxane and furan) as sole carbon sources used for growth by soil bacteria (Erwinia, Rhodococcus, and Pseudomonas) species. Methoxybenzoate has been shown to be demethylated or demethoxylated under anaerobic conditions by an Acetobacterium organism (13). Alkyl ethers such as symmetric diisopropyl ether have been shown by Modrzakowski and Finnerty (7) to be only partially oxidized by an Acinetobacter strain in which the ether linkage is not cleaved and only the terminal carbons are utilized for growth. Definitive evidence for the growth on or oxidation of dimethyl ether by cells or cell extracts containing methane monooxygenases from methyloths is lacking. Dimethyl ether does not support the growth of Methylococcus capsulatus (Texas) and was marginally oxidized in cell suspensions of the methyloths (9). There are conflicting reports that methane mono-oxygenases of methyloths can oxidize dimethyl ether (14, 17). Dimethyl ether has also been shown to inhibit methane oxidation in methylotrophic cultures (9) and in soils (10).

Recently, van den Wijngaard et al. (18) showed that microbes with dehalogenase activity isolated from saltwater sediments and activated sludge degraded (aerobically) bis(2-chloroethyl) ether and 2-chloroethyl vinyl ether. 2-Chloroethyl vinyl ether was dehalogenated to a hydroxy derivative, and then the ether bond was chemically cleaved to products which were used for growth. Studies on the biodegradability of MTBE in sludges and soils by Fujiwara et al. (4) showed that 100 mg of MTBE or disopropyl ether per liter was not degraded in activated sludge (300 mg of cell solids per liter) in an oxygen uptake assay; also, MTBE did not significantly affect the sludge respiration rate of other gasoline hydrocarbons when blended (12%, wt/vol) with the fuel. Work by Moller and Arvin (8) confirmed that MTBE (10 mg/liter) or t-amyl methyl ether (3 mg/liter) was not degraded in 60 days by microbes in an aquifer soil, topsoil, or activated sludges. In these experiments, MTBE at 200 mg/liter showed a weak inhibitory effect on the biodegradation of aromatic hydrocarbons (3.5 mg of benzene, toluene, ethylbenzene, and xylenes per liter). Recent studies by Sulita and Mormile (16) on the anaerobic decomposition of gasoline oxygenates in landfill aquifer material showed that of several alkyl ethers tested (MTBE, t-amyl methyl ether, ethyl t-butyl ether, disopropyl ether, ethyl ether, and propyl ether), only n-butyl methyl ether was metabolized under methanogenic conditions. The lack of alkyl ether degradation by indigenous microbes in soils and biosludges may be attributed to the very stable and chemically unreactive ether linkage, the inability of these compounds to be transported into cells, and/or the lack of inducible or existing enzyme activities (e.g., oxygenases and hydroxylases) which can attack the ether bond.

This report describes the isolation of an enrichment culture (mixed bacterial population) growing on MTBE as the sole carbon and energy source and its ability to metabolize ¹⁴CH₃-O-MTBE to ¹⁴C-labeled cells and ¹⁴CO₂.

MATERIALS AND METHODS

Derivation of enrichment culture BC-1. Activated sludges from municipal, refinery, and chemical plant biotreaters were also used as microbial sources for isolating MTBE-degrading enrichments. Biosludge solids (100 to 200 ml) were added to 1
were analyzed and was This (intentionally NH4'Cl mg/liter)-level vessel for at 7.2 to 9.0 PMTBE was added to a culture that was incubated with sterile 100% O2 for 2 to 5 min to achieve a dissolved-oxygen level of 20 mg/liter. The reaction vessel was stirred continuously at 22 to 25°C, and depletion of substrates was monitored by sampling the culture (2 to 3 ml) over a 24-h period. MTBE and TBA were analyzed by methods described below.

**Radiolabeled-MTBE experiments.** 14CH3O-MTBE was custom synthesized by Amersham Corp. (Arlington Heights, Ill.), and had a specific activity of 1.19 μCi/μmol, and was 99.3% pure by radiocromatography. Cultures were centrifuged, washed, and resuspended in the same volume of sterile phosphate-buffered saline (PBS, 0.85% NaCl, 0.03 M NaH2PO4, 0.05 M K2HPO4, pH 7.2), and placed in 250-ml serum bottles sealed with Teflon-lined septa. 14CH3O-MTBE was added to an initial concentration of 0.08 μCi/ml with MTBE at 2 ppm. Cultures were incubated at 30°C on a rotary shaker (150 to 200 rpm) for 7 days. The amount of 14CO2 formed was determined by placing a 10-ml aliquot of the culture in a similar serum bottle, adjusting the pH to ≤2 with 6 N HCl, and then flushing the bottle for 1 h with a steady stream of N2 into three gas washing bottles containing 100 ml of 0.1 M Ba(OH)2. The Ba14CO3 precipitate (formed after coprecipitation with 0.1 M Na2CO3) was collected on 0.45-μm-pore-size Millipore filters, washed with PBS, and dried, and the 14C activity incorporated into the biomass (cells) was counted. The remaining radioactivity in the filtrate represented undegraded 14CH3O-MTBE and/or 14C-labeled metabolites. The efficiency of 14CO2 trapping by the method described was confirmed in separate experiments in which NaH14CO3 was added (0.06 μCi with CO2 at 70 ppm) to PBS or azide-inhibited cultures, acidified (pH ≤2), and flushed into Ba(OH)2 traps. Recovery of H14CO3- as Ba14CO3 was 95 to 100% of the applied radioactivity. The 14C radioactivity was determined by placing 1-ml amounts of culture fluid (total initial 14C) and filtrates or filters containing Ba14CO3 precipitates into glass scintillation vials containing 15 ml of Aquasol-2 Universal 2SC Cocktail (NEN Dupont Research Products, Boston, Mass.). Vials were counted in a Tri-Carb 2500 TR liquid scintillation analyzer (Packard Instrument Co., Meriden, Conn.).

**Analysis of MTBE and TBA.** Culture samples were analyzed for MTBE and TBA with a Hewlett-Packard 280 gas chromatography-flame ionization detection system. Compounds were separated on a Quadrex methyl silicone (1-μm-thick film)
capillary column 25 m long with a 0.025-mm inside diameter (Alltech/Applied Science Labs, State College, Pa.). The column temperature was initially 30°C for 3 min and was programmed to rise to 70°C at 20°C/min. The carrier gas consisted of helium (30 ml/min) and an N₂ makeup gas. One-microliter split samples were analyzed. The retention times of TBA and MTBE were 3 and 3.8 min, respectively.

**Chemicals.** Common laboratory chemicals, e.g., salts, bases, acids, and alcohols, used were purchased from Mallinckrodt or Sigma Chemical Co. MTBE and TBA was obtained as ≥98% pure material from Chem Service Inc., West Chester, Pa.

**RESULTS AND DISCUSSION**

**Growth characteristics of BC-1 in a continuous cell recycle unit.** Data on the growth and metabolism of the BC-1 culture in a solids recycle culture are given in Table 1. During the first 7 weeks, the culture oxidized most of the influent NH₄⁺ (120 mg/liter) at high rates (ca. 400 mg/liter of effluent NO₃⁻). This initial observation suggested that the mixed culture in BC-1 utilizing pure O₂ degraded MTBE at least partly to CO₂ to sustain autotrophic ammonia-oxidizing organisms.

When influent NH₄⁺ and PO₄³⁻ levels were reduced to about 20 mg/liter (low-NH₄⁺ condition), nitrification continued and NO₃⁻ concentrations stabilized at 65 to 70 mg/liter. MTBE removal rates during and after this step change in nutrient addition decreased from 80 to 90% to 60 to 65%, suggesting that nitrifier populations may directly or indirectly stabilize MTBE degradation in BC-1. The cell retention time during the first 15 weeks was maintained at 80 to 90 days. When the cell retention time was decreased to <50 days, MTBE removal declined significantly to only 30% of the influent concentration. This loss in MTBE removal indicates that the growth of the MTBE-oxidizing organism(s) is very slow (<0.01/day).

Biomass yields based on cell accumulation for the BC-1 culture calculated during phases of high and low nitrification were 0.21 to 0.28 g of dry weight of cells per g of MTBE utilized (Table 1). These low cell yields appear to be comparable to those of anaerobic fermenting bacteria and autotrophs (0.03 to 0.20 g/g) and are somewhat less than those of aerobic-oxidizing cultures (0.3 to 0.6 g/g) growing on sugars, alcohols, and fatty acids (1a). These results suggest that the low growth rates and cell yields in BC-1 may be due to the slow metabolism of a rate-limiting intermediate(s) and/or MTBE may also be a metabolic or electron transport inhibitor or uncoupler of ATP formation. Additional studies are required to confirm these potential inhibitory effects on growth and metabolism.

**Metabolism of ¹⁴CH₃O-MTBE.** Results of the biodegradation of radiolabeled ether (2 mg/liter) by BC-1 are given in Table 2. Less than 1 and 5% of the applied isotope was recovered as ¹⁴CO₂ and ¹⁴C-labeled cells, respectively, in the

<table>
<thead>
<tr>
<th>Condition</th>
<th>% of applied ¹⁴CH₃O-MTBE* in:</th>
<th>% Recovery</th>
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<tr>
<td></td>
<td>CO₂ Cells MTBE and/or</td>
<td></td>
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<tr>
<td>Control (no cells)</td>
<td>0.2  4.1  13.7  18</td>
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<tr>
<td>BC-1 + 2% azide</td>
<td>0.9  5.1  17.1  23.1</td>
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<tr>
<td>BC-1</td>
<td>39.0 42.1 17.8  98.9</td>
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* Means of duplicate cultures did not differ by more than 10%.

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abiotic (no-culture) control and cultures containing the respiration inhibitor sodium azide (2%). About 80% of the ¹⁴CH₃O-MTBE was incorporated into CO₂ and cells, and the remainder (ca. 15%) was degraded ether and/or ¹⁴C-labeled metabolites. Addition of 100 mg of NH₄⁺ per liter to metabolizing cultures had no effect on stimulating or inhibiting MTBE biotransformation (data not shown). These results indicate that although nitrifiers are present in BC-1, the MTBE-oxidizing system is apparently different from the NH₃-oxygenase enzymes of these autotrophs. Little or no isotopic was metabolized to CO₂ or cellular material (similar to the azide-inhibited control) when BC-1 cultures were incubated with 20 mg of ¹⁴CH₃O-MTBE per liter (data not shown), suggesting that at certain concentrations, MTBE may be more than an inhibitor than a substrate.

**Substrate removal experiments.** Results of batch substrate depletion assays with BC-1 in the presence of MTBE and TBA are shown in Fig. 1. MTBE (120 mg/liter) was rapidly degraded, within 4 h at a rate of 34 mg/g of cells per h. TBA was formed as a transient metabolic product of MTBE breakdown. The highest levels of TBA were reached after MTBE was completely utilized. TBA alone and TBA formed from MTBE declined at a slower rate (14 mg/g of cells per h) than did MTBE. These results provide additional evidence that BC-1 degrades MTBE to TBA as a primary intermediate. Detailed mechanistic studies are required to determine the metabolic pathway in BC-1 beyond TBA, as well as the nature of the initial oxidative attack on MTBE.

**ACKNOWLEDGMENTS**

We thank Shell Oil Company and other members of the Petroleum Environmental Research Forum (Amoco, ARCO, British Petroleum, Chevron, Exxon, Mobil, and Phillips) for support of this work.

**REFERENCES**


