

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

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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  $\text{NH}_4^+$ ,  $\text{PO}_4^{3-}$ ,  $\text{Mg}^{2+}$ , and  $\text{Ca}^{2+}$  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  $\leq 50$ -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  $\text{NH}_4^+$  concentrations at high rates and obtain  $\text{CO}_2$  (sole carbon source for growth) from the metabolism of the alkyl ether, (ii) BC-1 metabolized radiolabeled ether ( $^{14}\text{CH}_3\text{O-MTBE}$ ) to  $^{14}\text{CO}_2$  (40%) and  $^{14}\text{C}$ -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 bacterial (*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 dioctyl 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 monoxygenases from methylotrophs is lacking. Dimethyl ether does not support the growth of *Methylococcus capsulatus* (Texas) and was marginally oxidized in cell suspensions of the methylotrophs (9). There are conflicting reports that methane monoxygenases of methylotrophs 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 diisopropyl 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 Suffita 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, diisopropyl 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  $^{14}\text{CH}_3\text{O-MTBE}$  to  $^{14}\text{C}$ -labeled cells and  $^{14}\text{CO}_2$ .

### 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

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TABLE 1. Nitrification and biomass yields in BC-1 continuous culture degrading MTBE<sup>a</sup>

Nitrifying condition	Influent NH <sub>4</sub> <sup>+</sup> (mg/liter)	Effluent NO <sub>3</sub> <sup>-</sup> (mg/liter)	Cell dry wt (mg/liter)	Cell retention time <sup>b</sup> (days)	Avg % MTBE removed	Cell yield (g of cells/g of MTBE utilized)
High NH <sub>4</sub> <sup>+</sup>	120–125	390–450	2,500–2,580	80–90	80–90 <sup>c</sup>	0.21–0.24
Low NH <sub>4</sub> <sup>+</sup>	10–20	50–70	2,020–2,340	80–85	60–65 <sup>d</sup>	0.23–0.28

<sup>a</sup> The results shown are averages of 4 weeks of data for each condition.

<sup>b</sup> Cell waste rates were 1.1 to 1.3 liters/4 weeks. Effluent cell concentrations under both conditions varied from 2 to 12 mg/liter and contributed 25 to 30% of the biomass loss from the unit.

<sup>c</sup> Influent and effluent MTBE concentrations varied from 160 to 210 and 3 to 40 mg/liter, respectively.

<sup>d</sup> Influent and effluent MTBE concentrations varied from 120 to 175 and 50 to 60 mg/liter, respectively.

liter of Sturm solution (15) containing the following minerals (in milligrams per liter) in a 2-liter stirred glass vessel sealed with Viton O rings: KH<sub>2</sub>PO<sub>4</sub>, 17; K<sub>2</sub>HPO<sub>4</sub>, 44; Na<sub>2</sub>HPO<sub>4</sub> · 2H<sub>2</sub>O, 67; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 23; NH<sub>4</sub>Cl, 3.4; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 40; FeCl<sub>3</sub> · 6H<sub>2</sub>O, 1. Cultures were flushed with O<sub>2</sub> for 5 min, and MTBE was added at 30 to 50 mg/liter. A similar vessel used as a control contained 1% NaCN as a microbial respiration inhibitor to monitor any ether loss from volatilization. MTBE is less volatile than benzene (Henry's law constant,  $6 \times 10^{-2}$  kPa/m<sup>3</sup>/mol), has a low octanol-water partition coefficient (0.94 to 1.05), and has high water solubility (45,000 mg/liter at 25°C) (8). All cultures were incubated at 22 to 25°C. In some instances, multiple additions of activated-sludge inocula were made weekly or monthly and these enrichments were more successful in developing an MTBE-degrading culture. After 2 months, one enrichment derived from an industrial chemical biotreater consistently degraded MTBE. This culture was subsequently designated BC-1. Preliminary identification of colonies isolated on a mineral (Sturm solution) agar medium containing 200 mg of MTBE per liter indicated that BC-1 contains at least four or five organisms, including coryneforms, pseudomonads, and achromobacters. However, all of these isolates utilize acetate but none have been shown to grow on MTBE as the sole carbon source.

**Maintenance and analysis of BC-1 in a cell recycle continuous culture.** The BC-1 culture was placed into a 4-liter glass vessel for continuous culture maintenance. A similar cell solids recycle apparatus with an aerator (4 liters) and an external clarifier (1 liter) has been described (12), except that pure oxygen was used in place of air to provide aerobic conditions. Dissolved oxygen was monitored with a Leeds and Northrup 7932 meter and probe and maintained at 4 to 7 mg/liter with an O<sub>2</sub> flow rate of 10 ml/min. MTBE (2% solution) was added continuously at a rate of 30 to 40 ml/day (150 to 200 mg/liter) with a Watson-Marlow 101U peristaltic pump. The pH was kept at 7.2 to 7.5 by infusion of 2 M NaOH solution from a Masterflex peristaltic pump. The culture was also fed a mineral solution (4 liters/day) consisting of NaCl (1,000 mg/liter), NH<sub>4</sub>Cl (380 mg/liter), KH<sub>2</sub>PO<sub>4</sub> (350 pmg/liter), and MgSO<sub>4</sub> · 7H<sub>2</sub>O (30 mg/liter). The ether-degrading culture developed a stable nitrifying population with high (380 mg/liter)- or low (65 mg/liter)-level NH<sub>4</sub><sup>+</sup>Cl addition. Suspended culture solids removed from the unit included 35 to 40 ml/day from the aerator (intentionally wasted) and 8 to 48 mg/day from the effluent. This waste rate was equivalent to an 80- to 90-day cell residence time. Less than 5% of the influent MTBE concentration was removed by volatilization in the closed bioreactor.

Influent and effluent samples from the continuous culture were analyzed for cell dry weight as outlined in reference 1. NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> ions were estimated by routine Dionex liquid chromatography.

**Batch substrate removal experiments.** Utilization of MTBE and *t*-butyl alcohol (TBA), a possible major metabolite of MTBE, was assessed in batch removal assays with BC-1. Individual compounds were added (120 to 130 mg/liter) to 1 liter of BC-1 culture in a 1.5-liter vessel. The culture was flushed with sterile 100% O<sub>2</sub> 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.** <sup>14</sup>CH<sub>3</sub>O-MTBE was custom synthesized by Amersham Corp. (Arlington Heights, Ill.), had a specific activity of 1.19 μCi/μmol, and was 99.3% pure by radiochromatography. Cultures were centrifuged, washed and resuspended in the same volume of sterile phosphate-buffered saline (PBS, 0.85% NaCl, 0.03 M Na<sub>2</sub>HPO<sub>4</sub>, 0.05 M KH<sub>2</sub>PO<sub>4</sub>, pH 7.2), and placed in 125-ml serum bottles sealed with Teflon-lined septa. <sup>14</sup>CH<sub>3</sub>O-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 <sup>14</sup>CO<sub>2</sub> 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 N<sub>2</sub> into three gas washing bottles containing 100 ml of 0.1 M Ba(OH)<sub>2</sub>. The Ba<sup>14</sup>CO<sub>3</sub> precipitate (formed after coprecipitation with 0.1 M Na<sub>2</sub>CO<sub>3</sub>) was collected on 0.45-μm-pore-size Millipore filters, washed with PBS, and dried, and the radioactivity was counted. After removal of <sup>14</sup>CO<sub>2</sub>, the culture was filtered with a 0.22-μm-pore-size Millipore filter, washed with PBS, and dried, and the <sup>14</sup>C activity incorporated into the biomass (cells) was counted. The remaining radioactivity in the filtrate represented undegraded <sup>14</sup>CH<sub>3</sub>O-MTBE and/or <sup>14</sup>C-labeled metabolites. The efficiency of <sup>14</sup>CO<sub>2</sub> trapping by the method described was confirmed in separate experiments in which NaH<sup>14</sup>CO<sub>3</sub> was added (0.06 μCi with CO<sub>2</sub> at 70 ppm) to PBS or azide-inhibited cultures, acidified (pH ≤2), and flushed into Ba(OH)<sub>2</sub> traps. Recovery of H<sup>14</sup>CO<sub>3</sub><sup>-</sup> as Ba<sup>14</sup>CO<sub>3</sub> was 95 to 100% of the applied radioactivity. The <sup>14</sup>C radioactivity was determined by placing 1-ml amounts of culture fluid (total initial <sup>14</sup>C) and filtrates or filters containing Ba<sup>14</sup>CO<sub>3</sub> 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)

TABLE 2. Metabolism of  $^{14}\text{CH}_3\text{O}$ -MTBE by BC-1

Condition	% of applied $^{14}\text{CH}_3\text{O}$ -MTBE <sup>a</sup> in:			% Recovery
	CO <sub>2</sub>	Cells	MTBE and/or metabolites	
Control (no cells)	0.2	4.1	13.7	18
BC-1 <sup>b</sup> + 2% azide	0.9	5.1	17.1	23.1
BC-1	39.0	42.1	17.8	98.9

<sup>a</sup> Means of duplicate cultures did not differ by more than 10%.

<sup>b</sup> Cell dry weight was 2,440 mg/liter.

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 N<sub>2</sub> 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 NH<sub>4</sub><sup>+</sup> (120 mg/liter) at high rates (ca. 400 mg/liter of effluent NO<sub>3</sub><sup>-</sup>). This initial observation suggested that the mixed culture in BC-1 utilizing pure O<sub>2</sub> degraded MTBE at least partly to CO<sub>2</sub> to sustain autotrophic ammonia-oxidizing organisms.

When influent NH<sub>4</sub><sup>+</sup> and PO<sub>4</sub><sup>3-</sup> levels were reduced to about 20 mg/liter (low-NH<sub>4</sub><sup>+</sup> condition), nitrification continued and NO<sub>3</sub><sup>-</sup> 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  $^{14}\text{CH}_3\text{O}$ -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  $^{14}\text{CO}_2$  and  $^{14}\text{C}$ -labeled cells, respectively, in the

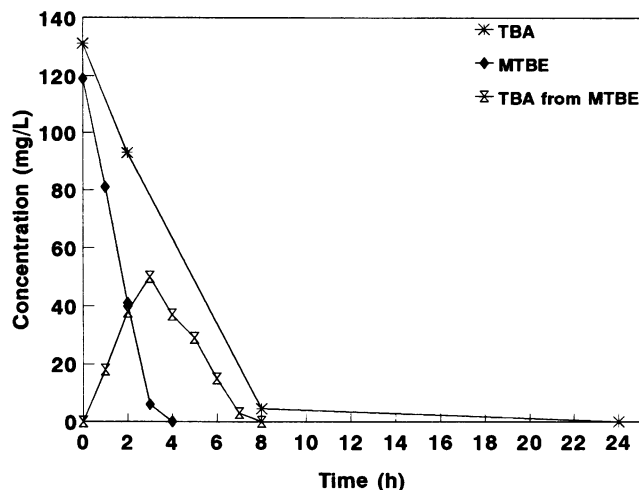


FIG. 1. Substrate depletion curves for the utilization of MTBE, TBA alone, and TBA formed from MTBE biodegradation by the BC-1 mixed culture.

abiotic (no-culture) control and cultures containing the respiration inhibitor sodium azide (2%). About 80% of the  $^{14}\text{CH}_3\text{O}$ -MTBE was incorporated into CO<sub>2</sub> and cells, and the remainder (ca. 15%) was undegraded ether and/or  $^{14}\text{C}$ -labeled metabolites. Addition of 100 mg of NH<sub>4</sub><sup>+</sup> 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 NH<sub>3</sub>-oxygenase enzymes of these autotrophs. Little or no isotope was metabolized to CO<sub>2</sub> or cellular material (similar to the azide-inhibited control) when BC-1 cultures were incubated with 20 mg of  $^{14}\text{CH}_3\text{O}$ -MTBE per liter (data not shown), suggesting that at certain concentrations, MTBE may be more 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.

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