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Effects of gasoline components on MTBE and TBA cometabolism by *Mycobacterium austroafricanum* JOB5

Alan J. House · Michael R. Hyman

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Abstract In this study we have examined the effects of individual gasoline hydrocarbons ($C_{5–10,12,14}$ *n*-alkanes, $C_{5–8}$ isoalkanes, alicyclics [cyclopentane and methylcyclopentane] and BTEX compounds [benzene, toluene, ethylbenzene, *m*-, *o*-, and *p*-xylene]) on cometabolism of methyl *tertiary* butyl ether (MTBE) and *tertiary* butyl alcohol (TBA) by *Mycobacterium austroafricanum* JOB5. All of the alkanes tested supported growth and both MTBE and TBA oxidation. Growth on $C_{5–8}$ *n*-alkanes and isoalkanes was inhibited by acetylene whereas growth on longer chain *n*-alkanes was largely unaffected by this gas. However, oxidation of both MTBE and TBA by resting cells was consistently inhibited by acetylene, irrespective of the alkane used as growth-supporting substrate. A model involving two separate but co-expressed alkane-oxidizing enzyme systems is proposed to account for these observations. Cyclopentane, methylcyclopentane, benzene and ethylbenzene did not support growth but these compounds all inhibited MTBE and TBA oxidation by alkane-grown cells. In the case of benzene, the inhibition was shown to be due to competitive interactions with both MTBE and TBA. Several aromatic compounds (*p*-xylene > toluene > *m*-xylene) did support growth and cells previously grown on these substrates also oxidized MTBE and TBA. Low concentrations of toluene (<10 μM) stimulated MTBE and TBA oxidation by alkane-grown cells whereas higher concentrations were inhibitory. The effects of acetylene suggest strain JOB5 also has two distinct toluene-oxidizing activities. These results have been discussed in terms of their impact on our understanding of MTBE and TBA cometabolism and the enzymes involved in these processes in mycobacteria and other bacteria.

Keywords Cometabolism · MTBE · TBA · Gasoline hydrocarbons · Alkanes · BTEX

A. J. House · M. R. Hyman (✉)
Department of Microbiology, North Carolina State University, Raleigh, NC 27695, USA
e-mail: michael_hyman@ncsu.edu

Introduction

Until recently, methyl *tertiary* butyl ether (MTBE) was frequently added at concentrations up to 15 vol% to gasoline sold in several regions of the United States (Squillace et al. 1996). The principal roles for MTBE in gasoline were to increase fuel octane rating and combustion efficiency and to decrease emissions of air pollutants (Mays 1989; Peil 1989). Contaminated ground water sources of drinking water are a legacy of the extensive use of MTBE in the United States from the early 1980's to the mid 2000's. In many instances this contamination occurred due to leaks of MTBE-containing gasoline from underground storage tanks. While MTBE has some properties such as high aqueous solubility that differentiate it from most other gasoline components (Johnson et al. 2000), a full understanding

of the environmental fate of MTBE and its primary metabolite, TBA, has to adequately consider impacts of other gasoline component co-contaminants frequently encountered with these chemicals. This is particularly true for biodegradation processes as they represent an important mass-reducing process for MTBE and TBA in ground water environments.

A variety of MTBE and TBA biodegradation processes have been described. For example, microcosm studies have shown both MTBE and TBA can biodegrade anaerobically in the presence of many of the electron acceptors that are known to support microbial processes at gasoline-impacted sites (Bradley et al. 2001a, b; Finneran and Lovley 2001; Kuder et al. 2005; Somsamak et al. 2001). Demethylation of MTBE by acetogenic organisms may play an important role in MTBE biodegradation under some anaerobic conditions (Youngster et al. 2008). Aerobic biodegradation has also been shown to be an effective treatment option for both MTBE and TBA (Fortin et al. 2001; Kharoune et al. 2001; Miller et al. 2001; Pruden et al. 2001, 2003; Wilson et al. 2001, 2002). At the organism level, two physiologically distinct aerobic biodegradation processes for these compounds have been described. A number of ß-proteobacteria typified by *Methylibium petroleiphilum* PM1 utilize both MTBE and TBA as sole sources of carbon and energy for growth and TBA is regarded as an obligate MTBE-derived metabolite in these organisms (Hanson et al. 1999; Müller et al. 2008; Nakatsu et al. 2006; Zaitsev et al. 2007). Another more diverse group of bacteria, including pseudomonads (Garnier et al. 1999, 2000; Smith and Hyman 2004; Smith et al. 2003b), mycobacteria (Smith et al. 2003a) and other actinomycetes (Liu et al. 2001) can cometabolize MTBE after growth on propane (Steffan et al. 1997; Smith et al. 2003a), longer chain *n*-alkanes (Garnier et al. 1999; Liu et al. 2001; Smith et al. 2003b, Smith and Hyman 2004) and isoalkanes (Hyman et al. 2000). The MTBE-oxidizing activity of these organisms has often been attributed to the lack of substrate specificity of monooxygenase enzymes otherwise responsible for initiating alkane oxidation (Hardison et al. 1997; Steffan et al. 1997; Smith et al. 2003a; Smith and Hyman 2004). While some MTBE-cometabolizing organisms such as the alkane hydroxylase-expressing *Pseudomonas putida* strain GPo1 generate TBA as a dead end product (Smith and Hyman 2004), propane-oxidizing organisms such as *Mycobacterium austroafricanum* JOB5 can further oxidize TBA to 2-methyl-1,2-propanediol (Johnson et al. 2004) as well as 2-hydroxyisobutyrate and potentially other products (Steffan et al. 1997). The high abundance of alkanes in gasoline and the wide distribution of alkane-oxidizing activities in bacteria (van Beilen et al. 2002, 2003) suggest alkane-dependent MTBE and TBA cometabolism may be frequently encountered in the aerobic portions of the vadose zone at gasoline–impacted sites.

Several previous reports have described the impacts of other gasoline components on aerobic MTBE biodegradation (Fortin et al. 2001; Lin et al. 2007; Pruden et al. 2003; Pruden and Suidan 2004; Raynal and Pruden 2008; Shim et al. 2006; Wang and Deshusses 2007; Zein et al. 2006a, b). However, these studies examined mixed cultures where little was known about the metabolic capabilities of the organisms responsible for MTBE oxidation. Studies of the effects of other gasoline components on pure cultures of MTBE-metabolizing organisms such as *M. petroleiphilum* PM1 (Deeb et al. 2001) and strain UC1 (Pruden and Suidan 2004), as well as MTBE-cometabolizing organisms like *Rhodococcus* strain EH831 (Lee and Cho 2009) have also typically been restricted to considering the impacts of the BTEX components of gasoline on MTBE and TBA biodegradation.

In the present study we have characterized the effects of multiple major gasoline components on the biodegradation of MTBE by the versatile soil bacterium *Mycobacterium austroafricanum* JOB5. These include not only BTEX compounds but also major *n*-alkanes, isoalkanes and alicyclics that can collectively exceed the total mass of aromatics in gasoline (Riser-Roberts 1998). In addition to considering MTBE oxidation, we have also examined the effects of these compounds on TBA oxidation. The organism used in this study, previously known as *M. vaccae* JOB5, was originally isolated from soil by enrichment culture using isopentane (2-methylbutane) as a sole source of carbon and energy (Ooyama and Foster 1965). Strain JOB5 grows on a wide range of *n*-alkanes and isoalkanes (Ooyama and Foster 1965) but it is perhaps best known for its ability to cometabolically oxidize diverse organic pollutants after growth on propane. These compounds include chlorinated solvents (Hamamura et al. 1997; Wackett et al. 1989) *N*-nitrosodimethylamine (Sharp et al. 2005), 2,4,6-trinitrotoluene (Vanderberg et al. 1995b), 1,4-dioxane (Mahendra and Alvarez-Cohen 2006), as well as some BTEX compounds (Burback and

Perry 1993). Our own studies of MTBE oxidation by propane-grown cells of strain JOB5 indicate MTBE and TBA are both substrates for the same monooxygenase responsible for initiating the oxidation of propane. Evidence for these multiple activities of this as yet uncharacterized enzyme include the irreversible inactivating effects of acetylene on propane, MTBE and TBA oxidation, as well as competitive interactions between propane, MTBE and TBA as mutually exclusive substrates (Smith et al. 2003a). Both MTBE and TBA can also induce the de novo synthesis of the enzyme system required for their own oxidation in cells previously grown on substrates such as fatty acids that do not initially have either alkane-, MTBE- or TBA-oxidizing activities (Johnson et al. 2004).

The results of this present study demonstrate that strain JOB5 has the ability to oxidize both MTBE and TBA after growth on a wide range of gasoline components including *n*-alkanes, isoalkanes and various BTEX compounds. We have characterized the inhibitory and stimulatory effects of diverse gasoline components on both MTBE and TBA oxidation. Our results have been interpreted in terms of their impact on our understanding of MTBE and TBA cometabolism and hydrocarbon oxidation by strain JOB5 and the potential significance of these findings to our general understanding of aerobic MTBE and TBA biodegradation processes.

Materials and methods

Materials

M. austroafricanum JOB5 (ATCC 29678) was obtained from the American type culture collection (Manassas, VA) and maintained on casein-yeast extract-dextrose (CYD) agar plates (Difco Plate Count Agar; Becton, Dickinson and Co., Sparks, MD). Benzene (>99.9% purity), cyclopentane (>99% purity), *n*-decane (>99% purity), *n*-dodecane (99% purity), ethylbenzene (99.8% purity), *n*-heptane (>99% purity), *n*-hexane (>99% purity), 2-methylbutane (>99.5% purity), 2-methylheptane (99% purity), 2-methylhexane (99% purity), MTBE (99.8% purity), *n*-nonane (99% purity), *n*-octane (>99% purity), TBA (>99.3% purity), *n*-tetradecane (99% purity), toluene (99.8% purity), *m*-xylene (>99% purity), *o*-xylene (98% purity), *p*-xylene (>99% purity) and calcium carbide pieces (~80% purity; for acetylene generation) were obtained from Sigma–Aldrich Chemical Co. (Milwaukee, WI). *n*-Pentane (99.5% purity) was obtained from Fisher Scientific (Pittsburgh, PA). Methylcyclopentane and 2-methylpentane (99.9% purity) were obtained from Chemical Samples Co. (Columbus, OH). 2-methyl-1,2-propanediol was a gift from Lyondell Chemical Co. (Houston, TX). Compressed gases (H_2, N_2, and air) were obtained from local industrial vendors.

Determination of cell growth and MTBE or TBA cometabolism

Growth of strain JOB5 on individual gasoline components was examined in glass serum vials (160 ml; Wheaton Scientific, Millville, NJ) that contained mineral salts medium (MSM; 25 ml; Wiegant and deBont 1980). Vials were inoculated (initial $OD_{600} \leq 0.01$) with a liquid suspension of cells (grown on CYD agar plates) in MSM and then sealed with Teflon-lined Mininert Valves (Alltech Associates Inc., Deerfield, IL). Neat liquid substrates (5 µl each) were added to sealed vials using a sterile microsyringe. Acetylene gas (5% v/v gas phase) was added to culture vials as required using a plastic syringe fitted with a Millex disposable filter (0.1 µm; Millipore Co., Bedford, MA). The cultures were incubated at 30°C in the dark in an Innova 4900 environmental shaker (New Brunswick Scientific Co., Inc., Edison, NJ) operated at 150 rpm. After 7 days (14 days for BTEX compounds), the amount of MTBE or TBA remaining in each culture vial was quantified by gas chromatography (see below), and culture optical density (OD_{600}) was determined using a Shimadzu 1601 UV/Vis spectrophotometer (Kyoto, Japan). In some cultures, growth was visibly particulate. All optical density measurements were therefore made following dispersion of aggregates using a Model 75T Aquasonic water bath (VWR International, Inc., West Chester, PA). Culture purity after growth was determined by streaking a culture sample (20 µl) onto CYD agar plates.

Preparation of harvested cells for short-term experiments

Cells used in all short-term (<8 h) biodegradation assays and kinetic constant determinations were from

cultures grown in glass media bottles (750 ml; Wheaton Scientific) containing MSM (100 ml). The medium was inoculated (initial OD_{600} ~0.01) with cells grown on CYD agar plates and the bottles were sealed with open-top caps fitted with butyl rubber septa. Individual hydrocarbons (0.05% v/v, liquid phase) were added to sealed vials using a sterile microsyringe. Cultures were incubated for at least 4 days (see incubation conditions above). Culture purity after growth was determined by streaking a culture sample (20 µl) onto CYD agar plates. Cells were harvested from the culture medium (final OD_{600} ~0.7) by centrifugation (10,000*g*; 10 min). The resulting cell pellet was washed in phosphate buffer (20 ml; 50 mM sodium phosphate; pH 7) and centrifuged again. The sedimented cells were resuspended in buffer (~1.5 ml) to a final concentration of ~1–10 mg of total cell protein ml^{-1}. The cell suspension was stored at 4°C and used within 4 h.

Degradation assays and determination of inhibition constants

Degradation experiments using resting cells were conducted in glass serum vials (15 ml) containing buffer (~900 µl; 50 mM Na phosphate, pH 7). The vials were sealed with Teflon-lined Mininert valves and substrates or inhibitors were then added from aqueous stock solutions or as neat liquids or gases using microsyringes. Vials were placed in a shaking water bath (30°C; 150 rpm) for at least 10 min to allow reactants to equilibrate between gas and liquid phases. Unless otherwise stated, experiments were initiated by the addition of a concentrated cell suspension (100 µl) to give a final reaction volume of 1 ml and a protein concentration of between ~0.1 and 1.0 mg total protein ml^{-1}. Substrate consumption in these incubations was determined by gas chromatography (see below). Initial specific rates of MTBE or TBA oxidation were determined from the slope of a linear regression analysis of five data points collected at 15 min intervals during the first hour of either MTBE or TBA oxidation.

Analytical methods

To quantify MTBE or TBA biodegradation in growth studies and during short-term degradation assays, aqueous phase samples (2 µl) were injected into a Shimadzu GC-14A gas chromatograph (Kyoto, Japan) fitted with a 0.3 × 183 cm stainless steel column containing Porapak Q (60/80 mesh; Waters Associates, Framingham, MA) operated at 180°C. In some experiments a shorter (0.3 by 61 cm) version of the same column operated at 160°C was used to quantify MTBE and TBA degradation in the presence of 2-methyl-1,2-propanediol. To quantify BTEX biodegradation, headspace samples (25 µl) were injected into a Shimadzu GC-14A gas chromatograph fitted with a DB-MTBE capillary column (30 m by 0.45 mm (internal diameter), 2.55 µm film; J and W Scientific, Folsom, CA). The column was operated at 55°C for analysis of benzene and toluene, 85°C for analysis of ethylbenzene, and at 115°C for analysis of *m*-, *o*-, and *p*-xylene. All gas chromatographs were fitted with flame ionization detectors operated at 220°C. In all cases the injection ports were operated at 200°C and N_2 was used as carrier gas at a flow rate of 15 ml min^{-1}. All gas chromatographs were interfaced to Hewlett Packard HP3395 integrators (Palo Alto, CA) for data collection.

Cell protein concentrations were determined using the Biuret assay (Gornall et al. 1949) after cell material was solubilized (65°C, 1 h, 3 M NaOH), and insoluble portions sedimented by centrifugation (Eppendorf microfuge; 10,000 rpm, 5 min). Bovine serum albumin was used as the standard. The concentrations of MTBE, benzene, toluene, ethylbenzene, *m*-, *o*- and *p*-xylene in saturated aqueous solutions at room temperature (23°C) were taken as 544, 22.8, 5.6, 1.4, 1.5, 1.7 and 1.7 mM respectively (McAuliffe 1966; Polak and Lu 1973; Windholz et al. 1983). The dimensionless Henry's constant for MTBE, benzene, toluene, *n*-pentane and 2-methylbutane at 30°C were taken as 0.0255, 0.2241, 0.2600, 49.6 and 54.7 respectively (Mackay and Shiu 1981; Miller and Stuart 2000).

Kinetic constants were derived by fitting the data to a single substrate-binding model $\left[y = V_{\max} \cdot x/K_s + x\right]$ using Graph-Pad Prism version 4.0a for Macintosh (Graphpad Software, San Diego, CA).

Results

Hydrocarbon-dependent growth and concurrent MTBE and TBA oxidation

A range of hydrocarbons including $C_{5–10,12,14}$ *n*-alkanes, $C_{5–8}$ isoalkanes, alicyclics [cyclopentane

and methylcyclopentane] and six aromatics [benzene, toluene, ethylbenzene, *m*-, *o*-, and *p*-xylene] were examined as potenial growth-supporting substrates for strain JOB5 in batch cultures. Growth (final $OD_{600} \geq 0.02$) was observed with all of the tested compounds except cyclopentane, methylcyclopentane, benzene, ethylbenzene and *o*-xylene (Table 1). Overall, growth on *n*-alkanes (mean final $OD_{600} = 0.21$) and isoalkanes (mean final $OD_{600} = 0.22$) was more robust than growth on the aromatic compounds (mean final $OD_{600} = 0.11$). Among the aromatic compounds, growth on toluene was the most limited while growth on *p*-xylene was comparable to that observed with some *n*-alkanes.

When MTBE was added with each growth-supporting substrate at an initial 1:1 ratio (v/v) (molar ratios ranged from 1:0.5 [MTBE:*n*-tetradecane] to 1:1.13[MTBE:benzene]), there was generally little (≤25%) or no effect on the final culture density, except in cultures grown on *n*-octane, 2-methylbutane, 2-methylhexane, 2-methylheptane and toluene (Table 1). In these instances, MTBE decreased the final culture density by between ~35 and ~85% compared to cultures grown in the absence of MTBE. When TBA was added at an initial 1:1 ratio (v/v) (molar ratios ranged from 1:0.4 [TBA:*n*-tetradecane] to 1:1.11[TBA:benzene]), the effects on growth were more limited and TBA only inhibited growth by ≥25% in cultures grown on *n*-tetradecane and toluene. Neither MTBE nor TBA supported growth of strain JOB5 when present as the sole organics in the culture medium (Table 1).

With the exception of *m*-xylene, in every case where growth was observed in cultures containing either MTBE or TBA, substantial depletion of these two compounds was also observed. The average level of MTBE consumption among all cultures was ~40% (~17 μmoles) and the highest levels (≥50%) were observed in cultures grown on *n*-pentane, *n*-hexane, 2-methylbutane, 2-methylhexane and *p*-xylene. The levels of TBA consumption in all cultures typically exceeded those observed with MTBE. The average level of TBA consumption among all growth substrates was ~51% (~27 μmoles) and MTBE consumption only exceeded TBA consumption in cultures grown on *n*-hexane, *n*-tetradecane, toluene, and *p*-xylene.

We also tested the effects of acetylene on growth of strain JOB5 on hydrocarbons. Growth on $C_{5–9}$ *n*-alkanes and all branched alkanes was fully inhibited by this gas (Table 1) but variable results were observed with other growth-supporting hydrocarbons. For example, growth on *n*-tetradecane and *m*-xylene was unaffected by this gas, while growth on toluene or *p*-xylene was only partially inhibited and growth on *n*-dodecane was stimulated relative to cultures grown in the absence of acetylene. When culture growth was observed in the presence of acetylene (*n*-dodecane > *n*-tetradecane > *n*-decane > *m*-xylene > toluene > *p*-xylene), acetylene generally strongly inhibited the associated oxidation of MTBE and TBA. However, incomplete inhibition of MTBE oxidation by acetylene was observed in toluene-grown cultures and incomplete inhibition of TBA oxidation was observed in *n*-dodecane-grown cultures.

MTBE and TBA oxidation by resting cells

Cells were grown in the absence of both MTBE and TBA using each of the growth-supporting substrates identified in Table 1. Initial specific rates of both MTBE and TBA oxidation were then determined for harvested resting cells. For cells grown on *n*-alkanes and isoalkanes, the specific rates of MTBE tended to decrease with increases in the carbon chain length of the growth substrate although other trends could be observed (Table 2). For example, cells grown on either C_5 and C_6 *n*-alkanes or C_5 and C_6 isoalkanes oxidized MTBE at similar rates (mean = 40 nmoles min^{-1} mg total $protein^{-1}$), as did cells grown on C_7 alkanes: *n*-heptane and 2-methylhexane (mean = 25 nmoles min^{-1} mg total $protein^{-1}$). Although the rates were less consistent than those observed with cells grown on shorter chain *n*-alkanes and isoalkanes, cells grown on *n*-alkanes $>C_8$ or isoalkanes $>C_5$ consistently oxidized MTBE ~fivefold slower than the maximal rate observed with cells grown on *n*-pentane or 2-methylbutane, respectively. Specific rates of TBA oxidation also generally decreased with increases in the carbon chain length of alkane growth substrates. Cells grown on $C_{5–8}$ *n*-alkanes oxidized TBA at similar rates (mean = 20 nmoles min^{-1} mg total $protein^{-1}$), whereas cells grown on $C_{10–14}$ *n*-alkanes exhibited variable but generally low TBA-oxidizing activities (mean = 1 nmole min^{-1} mg total $protein^{-1}$). The most rapid rate of TBA oxidation (42 moles min^{-1} mg total $protein^{-1}$) was observed for 2-methylbutane grown cells, and an ~fourfold decrease in this rate was observed for cells grown on

Table 1 Culture growth and MTBE and TBA consumption in batch cultures

Growth supporting substrate	Wt% in gasoline[a]	Final OD_{600}^{b}						Percent consumed[c]			
		Substrate			Substrate + acetylene			−Acetylene		+Acetylene	
		Alone	+MTBE	+TBA	Alone	+MTBE	+TBA	MTBE	TBA	MTBE	TBA
n-Alkanes											
n-Pentane	5.75–10.92	0.21 (0.01)	0.20 (0.02)	0.22 (0.02)	NG[e]	ND		50 (5)	86 (12)	ND	
n-Hexane	0.24–3.50	0.26 (0.02)	0.27 (0.02)	0.25 (0.00)	NG			71 (1)	56 (4)		
n-Heptane	0.31–1.96	0.24 (0.01)	0.20 (0.00)	0.23 (0.01)	0.03 (0.00)			38 (3)	83 (5)		
n-Octane	0.36–1.43	0.14 (0.02)	0.09 (0.00)	0.16 (0.02)	NG			25 (1)	40 (6)		
n-Nonane	0.07–0.83	0.21 (0.00)	0.19 (0.00)	0.23 (0.00)	0.02 (0.00)			41 (8)	53 (10)		
n-Decane	0.04–0.50	0.23 (0.02)	0.22 (0.03)	0.24 (0.02)	0.11 (0.06)	0.04 (0.03)	NG	42 (5)	60 (6)	≤1 (0)	≤1 (1)
n-Dodecane	0.04–0.09	0.18 (0.01)	0.19 (0.01)	0.14 (0.01)	0.29 (0.01)	0.28 (0.05)	0.28 (0.02)	28 (7)	72 (8)	≤1 (4)	14 (25)
n-Tetradecane	ND[d]	0.23 (0.04)	0.19 (0.01)	0.17 (0.01)	0.21 (0.01)	0.20 (0.02)	0.21 (0.04)	21 (12)	15 (8)	2 (8)	≤1 (2)
Isoalkanes											
2-Methyl butane	6.07–10.17	0.21 (0.03)	0.14 (0.02)	0.16 (0.01)	NG	ND		57 (2)	79 (11)	ND	
2-Methyl pentane	2.91–3.85	0.18 (0.03)	0.20 (0.00)	0.23 (0.01)	NG			44 (5)	66 (11)		
2-Methyl hexane	0.36–1.48	0.26 (0.02)	0.13 (0.00)	0.20 (0.03)	NG			58 (6)	69 (2)		
2-Methyl heptane	0.48–1.05	0.23 (0.00)	0.03 (0.00)	0.19 (0.00)	NG			29 (1)	47 (12)		
Alicyclics											
Cyclopentane	0.19–0.58	NG	NG	NG	NG	ND					
Methyl cyclopentane	ND	NG	NG	NG	NG						
Aromatics											
Benzene	0.12–3.50	NG	NG	NG	NG	ND					
Toluene	2.73–21.80	0.09 (0.01)	0.06 (0.01)	0.06 (0.01)	0.04 (0.01)	0.05 (0.03)	NG	36 (2)	15 (4)	9 (2)	≤1 (1)
Ethylbenzene	0.36–2.86	NG	NG	NG	NG	ND					
o-Xylene	0.68–2.86	NG	NG	NG	NG						
m-Xylene	1.77–3.87	0.08 (0.00)	0.09 (0.00)	0.09 (0.00)	0.08 (0.01)	0.08 (0.00)	0.08 (0.01)	≤1 (6)	8 (1)	≤1	≤1 (2)
p-Xylene	0.77–1.58	0.17 (0.01)	0.16 (0.03)	0.15 (0.06)	NG	0.11 (0.00)	0.21	63 (15)	19 (5)	≤1 (10)	≤1

Table 1 continued

Growth supporting substrate	Wt% in gasoline[a]	Final OD_{600}^{b}						Percent consumed[c]			
		Substrate			Substrate + acetylene			−Acetylene		+Acetylene	
		Alone	+MTBE	+TBA	Alone	+MTBE	+TBA	MTBE	TBA	MTBE	TBA
Oxygenates											
MTBE or TBA		NG	ND		ND			≤1 (0)	≤1 (0)	ND	

Cultures were grown for 7 days except for cultures grown on BTEX compounds (14 days). Data presented the mean and SEM (in parentheses) of at least duplicate cultures. Abiotic controls maintained an $OD_{600} \leq 0.01$, and showed no loss of MTBE or TBA (data not shown)

[a] Gasoline composition reported by Riser-Roberts (1998)

[b] Initial culture $OD_{600} \leq 0.01$

[c] Percent consumed relative to initial amount of either MTBE or TBA

[d] *ND* not determined

[e] *NG* no growth (final $OD_{600} \leq 0.02$)

Table 2 Initial specific rate of MTBE or TBA oxidation

Growth-supporting substrate	Initial specific rate nmol min^{-1} mg $protein^{-1}$ [a]	
	MTBE	TBA
n-Alkanes		
n-Pentane	46 (2.5)	18 (4)
n-Hexane	41 (4)	15 (3.5)
n-Heptane	24 (6)	22 (4.5)
n-Octane	23 (2.5)	24 (6.4)
n-Nonane	11 (10)	36 (8)
n-Decane	11 (6.5)	1 (9.6)
n-Dodecane	12 (2.5)	1 (3)
n-Tetradecane	8 (6.5)	2 (7.5)
Isoalkanes		
2-Methylbutane	34 (1.5)	42 (1)
2-Methylpentane	37 (7)	12 (1.8)
2-Methylhexane	25 (3)	7 (3.5)
2-Methylheptane	6 (5)	10 (0.5)
Aromatics		
Toluene	5 (4)	14 (6)
m-Xylene	19 (10.5)	21 (14)
p-Xylene	20 (7)	31 (4.5)

Data presented are the mean and SEM (in parentheses) of at least duplicate samples

[a] Harvested cells (0.1–0.6 mg of total protein) were incubated with either MTBE or TBA (2.5 μmoles), as described in the "Methods" section

all longer chain isoalkanes. Despite some increased variability, the specific rates of both MTBE and TBA oxidation by cells grown on toluene, *m*- and *p*-xylene were within the range observed for cells grown on *n*-alkanes and isoalkanes (Table 2).

Inhibition of MTBE and TBA oxidation

Collectively, the highest combined rates of MTBE and TBA oxidation in the experiments described in Table 2 were observed with cells grown on *n*-pentane and 2-methylbutane. Cells grown on these substrates were then used in short term incubations (1 h) to examine potential inhibitory effects of hydrocarbons on MTBE and TBA oxidation. Shorter chain *n*-alkanes (C_{5-8}) consistently and strongly (≥70%) inhibited both MTBE and TBA oxidation (Table 3). These inhibitory effects then tended to decrease with further increases in carbon chain length. Similar effects were observed for both *n*-pentane- and 2-methylbutane-grown cells and

all of the isoalkanes and alicylic compounds tested were also potent inhibitors of both MTBE and TBA oxidation (Table 3). With the exception of TBA oxidation by 2-methylbutane-grown cells, both MTBE and TBA oxidation were inhibited ≥70% in all cases. All of the aromatic compounds tested also inhibited both MTBE and TBA oxidation by *n*-pentane- and 2-methylbutane-grown cells. However, the aromatic compounds tested were generally less inhibitory than the isoalkanes or alicyclic compounds tested.

The inhibitory effects of *n*-pentane, 2-methylbutane, benzene and toluene on MTBE and TBA oxidation by *n*-pentane-grown cells were further quantified by determining the effect of varying dissolved concentrations of each hydrocarbon (≤250 μM) on the initial rate of oxidation of a fixed initial amount of dissolved MTBE (0.9 mM) or TBA (1 mM). When the tested compound was also a growth-supporting substrate (*n*-pentane, 2-methylbutane and toluene), low concentrations (≤10 μM) of each hydrocarbon stimulated both MTBE and TBA oxidation by as much as 85% relative to the rate of oxidation observed without the growth substrate (Fig. 1 inserts in Panels A, B and D). However, higher concentrations of these growth-supporting substrates all inhibited both MTBE and TBA oxidation. This stimulating effect was not observed when an alternate source of reductant (2-methyl-1,2-propanediol) was included in the reaction mixture. In reactions conducted in the presence of 2-methyl-1,2-propanediol, good fits ($r^2 \geq 0.92$) were obtained for each inhibition data set using a hyperbolic, single

Table 3 Effect of gasoline hydrocarbons on either MTBE or TBA oxidation

Hydrocarbon added	Percent inhibition[a]			
	MTBE		TBA	
	Harvested cells grown on		Harvested cells grown on	
	n-Pentane	2-Methylbutane	*n*-Pentane	2-Methylbutane
n-Alkanes				
n-Pentane	90 (10)	87 (1)	98 (8)	100 (3)
n-Hexane	80 (6)	100 (3)	84 (6)	100 (3)
n-Heptane	91 (5)	84 (8)	97 (2)	98 (1)
n-Octane	89 (6)	88 (4)	71 (4)	92 (7)
n-Nonane	40 (4)	45 (5)	48 (9)	47 (3)
n-Decane	14 (3)	23 (3)	8 (4)	37 (4)
n-Dodecane	≤1 (7)	5 (9)	≤1 (8)	≤1 (7)
n-Tetradecane	≤1 (7)	2 (2)	≤1 (1)	13 (7)
Isoalkanes				
2-Methylbutane	75 (8)	90 (2)	92 (8)	93 (2)
2-Methylpentane	101 (5)	99 (0)	95 (3)	95 (3)
2-Methylhexane	78 (2)	88 (7)	92 (6)	90 (8)
2-Methylheptane	71 (5)	56 (2)	96 (1)	78 (6)
Alicyclics				
Cyclopentane	100 (3)	98 (7)	100 (2)	87 (6)
Methylcyclopentane	99 (3)	98 (0)	95 (7)	96 (1)
Aromatics				
Benzene	41 (2)	34 (6)	57 (4)	75 (2)
Toluene	75 (10)	74 (6)	88 (1)	67 (2)
Ethylbenzene	63 (9)	71 (9)	71 (6)	87 (5)
o-Xylene	49 (2)	71 (3)	84 (0)	85 (8)
m-Xylene	23 (2)	64 (8)	43 (7)	91 (3)
p-Xylene	30 (6)	28 (6)	28 (5)	55 (2)

Harvested cells incubated with either MTBE or TBA (1 μmole) and each alkane (2.5 μl neat), or 250 nmoles of each BTEX compound, in a 1 ml aqueous reaction volume. Data presented the mean and SEM (in parentheses) of at least duplicate samples

[a] Percent inhibition data describe the extent to which MTBE or TBA oxidation was inhibited by the presence of each hydrocarbon, relative to reactions conducted with either MTBE or TBA alone

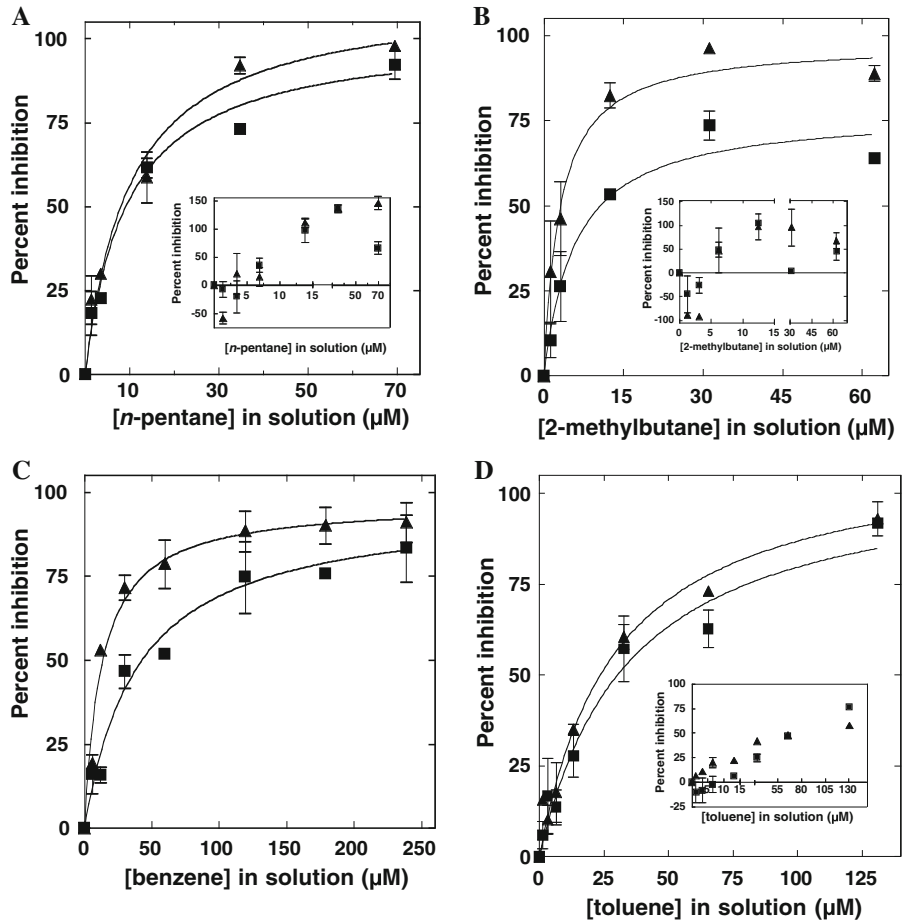

Fig. 1 Competitive inhibition of MTBE and TBA oxidation by gasoline components. *n*-Pentane-grown cells were incubated with 2-methy-1,2-propanediol ([Panels A, B and D] 10 mM), MTBE (0.9 mM dissolved concentration) or TBA (1 mM dissolved concentration) and the indicated dissolved concentrations of (*Panel A*) *n*-pentane, (*Panel B*) 2-methylbutane, (*Panel C*) benzene and (*Panel D*) toluene, as described in the "Methods" section. The *Figures* show the data for inhibition of (*filled square*) MTBE and (*filled triangle*) TBA oxidation relative to the amount consumed in the absence of each test compound. The data were fitted to a hyperbola using a single substrate binding model, as described in the "Methods" section. The *insets* for *Panels A*, *B* and *D* show the effects of each test compound on the rate of (*filled square*) MTBE and (*filled triangle*) TBA oxidation observed in the absence of 2-methyl-1,2-propanediol

substrate binding model. These analyses suggest that all of the tested compounds (*n*-pentane, 2-methylbutane, toluene and benzene) act as competitive inhibitors of both MTBE and TBA oxidation by *n*-pentane-grown cells. The apparent K_i (K_i^{app}) values derived from these analyses (Table 4) were converted to true K_i values (K_i^{true}) using the following equation $K_i^{\text{true}} = K_i^{\text{app}} / \left(1 + [\text{MTBE or TBA}] / K_{s[\text{MTBE or TBA}]}\right)$ and the previously determined K_s values for MTBE (1.36 mM) and TBA (1.18 mM) for propane-grown cells of strain JOB5 (Smith et al. 2003a). With the exception of benzene, the calculated K_i^{true} values for each test compound were similar for inhibition of MTBE or TBA oxidation, and ranged from the low micromolar range (~2–4 μM) for 2-methylbutane to an order of magnitude larger values for toluene (~16–24 μM; Table 4). For benzene, the K_i^{true} value estimated for the inhibition of MTBE oxidation (~24 μM) was similar to the value estimated for toluene (~21 μM). In contrast, the value for benzene as an inhibitor of TBA oxidation (~7 μM) was ~twofold lower than the value determined for toluene inhibition of TBA oxidation.

Oxidation of aromatic compounds

In view of the competitive nature of the inhibitory effects of benzene and toluene on MTBE and TBA oxidation (Tables 3, 4), we investigated the potential oxidation of aromatic compounds by resting *n*-pentane grown cells. In short term incubations (3 h), *n*-pentane-grown cells rapidly consumed each of the aromatic compounds tested although *o*-xylene was more slowly consumed than the other five aromatics (Table 5). Benzene consumption was also strongly (>90%) inhibited by acetylene while oxidation of all of the other aromatics was only partially inhibited (≤50%) by this gas. Consumption of each aromatic was minimal (≤10%) when heat-killed cells were used indicating that abiotic losses and sorption of organics to cell materials were negligible in this experimental system.

The effects of benzene and toluene on the oxidation of MTBE and TBA by resting *n*-pentane-grown cells were also examined in time course experiments. *n*-Pentane-grown cells oxidized benzene (250 nmoles) or MTBE (1 μmole) without a lag phase when these compounds were added individually and oxidation of both compounds was strongly inhibited by acetylene (Fig. 2a). When both MTBE and benzene were added simultaneously, the time course of benzene oxidation was largely unaffected by the presence of MTBE. In contrast, MTBE oxidation was initially inhibited by the presence of benzene although a slow but progressively increasing rate of MTBE oxidation developed after 4 h as the residual benzene (≤50 μM) was consumed. A comparable experiment was conducted with toluene and MTBE. When these compounds were added individually (Fig. 2b), the time course of MTBE oxidation was substantially similar to the experiment described in Fig. 2a. However, after accounting for differences in cell protein, the rate of toluene oxidation (Fig. 2b) was ~fourfold faster than the rate of benzene oxidation (Fig. 2a). Like the effect observed with mixtures of benzene and MTBE (Fig. 2a), the rate of MTBE oxidation in reactions containing both MTBE and toluene was initially (0–1 h) slow but increased once the toluene concentration had been reduced to low concentrations (Fig. 2b). Unlike the effect of acetylene on benzene and MTBE oxidation (Fig. 2a), toluene oxidation was only partially inhibited by this gas (Fig. 2b).

Table 4 Inhibition constants determined for *n*-pentane-grown cells

Inhibitor	K_i^{app} (μM)		K_i^{true} (μM)[b]	
	MTBE	TBA	MTBE	TBA
n-Pentane	9.4 (2.0)[a]	10.1 (2.9)	5.7	5.5
2-Methylbutane	5.9 (1.8)	3.0 (0.8)	3.6	1.7
Benzene	39.6 (10.1)	12.8 (2.6)	23.9	6.9
Toluene	34.6 (11.0)	29.3 (5.5)	20.8	15.8

[a] Data represent the mean and SEM (in parentheses) of duplicate samples

[b] K_i^{true} values were calculated from the mean K_i^{app} value, as explained in the "Results" section

Table 5 Consumption of BTEX by *n*-pentane-grown cells

BTEX added	Percent consumed[a]		
	Active cells	Acetylene treated cells	Heat treated cells
Benzene	>99 (0)	8 (0)	≤1 (1)
Toluene	>99 (0)	50 (2)	≤1 (1)
Ethylbenzene	>99 (0)	39 (5)	≤1 (6)
o-Xylene	50 (4)	29 (4)	≤1 (0)
m-Xylene	>99 (0)	51 (3)	≤1 (6)
p-Xylene	>99 (0)	51 (2)	7 (11)

Harvested cells were incubated with each BTEX compound (250 nmoles) for 3 h, as described in the "Methods" section

[a] Data presented are the mean and SEM (in parentheses) of duplicate samples that contained ~0.5 mg of total cell protein

Discussion

Effects of *n*-alkanes and isoalkanes

Our results confirm the broad alkane-metabolizing activity of strain JOB5 (Ooyama and Foster 1965) and demonstrate cells grown on the major normal and branched alkane components of gasoline (C_{5-10}) consistently oxidized both MTBE and TBA in batch cultures (Table 1). Acetylene also consistently inhibited both MTBE and TBA oxidation but had variable effects on alkane-dependent growth. For instance, growth on shorter chain *n*-alkanes (≤C_9) and all isoalkanes tested was strongly inhibited by this gas, while growth on longer chain (≥C_{10}) *n*-alkanes was largely unaffected (Table 1).

One potential interpretation of these results (Table 1) is that while individual alkane growth substrates impacted the type of alkane-oxidizing

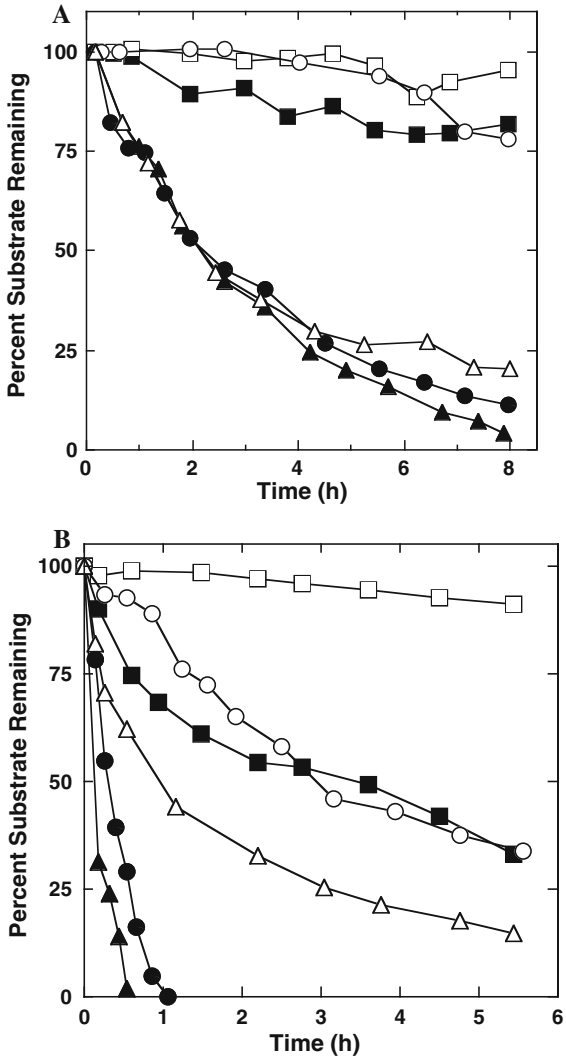

Fig. 2 Effects of benzene and toluene on MTBE oxidation by *n*-pentane-grown cells. The *Figure* shows the effects of (*Panel A*) benzene (250 nmoles) and (*Panel B*) toluene (250 nmoles) on the oxidation of MTBE (1 μmole) by *n*-pentane-grown cells of strain JOB5. In both *Panels* the *Figure* shows the time course for oxidation of (*filled triangle*) the aromatic compound alone, (*open triangle*) MTBE alone, (*filled circle*) the aromatic in MTBE/aromatic mixture, (*open circle*) MTBE in MTBE/aromatic mixture, (*filled square*) the aromatic in MTBE/aromatic/acetylene mixture and (*open square*) MTBE in MTBE/aromatic/acetylene mixture The reactions described in *Panel A* and *B* contained cells at a concentration of 0.4 and 0.9 mg total protein ml^{-1}, respectively. When applicable, acetylene was used at concentration of 10% (v/v gas phase)

enzymes expressed by strain JOB5 in these batch cultures, these effects were obscured by the previously described ability of both MTBE and TBA to induce the expression of the enzymes required for their own biodegradation (Johnson et al. 2004). However, an explanation based on an inducing effect does not account for our observation that cells grown on alkanes in the absence of MTBE all had varying but typically substantial (≥10 nmoles min^{-1} mg $protein^{-1}$) levels of MTBE-oxidizing activity (Table 2). An alternative explanation is strain JOB5 can simultaneously express more than one alkane-oxidizing monooxygenase in response to different individual alkane substrates, while only one of these enzymes is responsible for oxidizing MTBE and TBA. Based on this model we suggest strain JOB5 can express an acetylene-sensitive, MTBE- and TBA-oxidizing, short chain alkane monooxygenase (SCAM) that is primarily responsible for initiating oxidation and catabolism of shorter chain alkanes ($<C_9$). Conversely, this organism can also express a long chain alkane monooxygenase (LCAM) that is insensitive to acetylene, does not oxidize MTBE and TBA, and is primarily responsible for initiating oxidation and catabolism of longer chain *n*-alkanes ($>C_{10}$). The ability of cells to grow on long chain *n*-alkanes ($>C_{10}$) in the presence of acetylene and yet still catalyze acetylene-sensitive MTBE and TBA oxidation (Tables 1, 2) would be accounted for by co-expression of both SCAM and LCAM. The higher specific activities for MTBE oxidation in cells grown on shorter chain alkanes compared to longer chain alkanes (Table 2) suggests expression of SCAM is maximal during growth on short chain alkanes and is decreased but not eliminated when cells are grown on longer chain alkanes.

We have previously shown propane-grown cells of strain JOB5 oxidize MTBE and TBA at rates as high as 24 and 10 nmoles min^{-1} mg total $protein^{-1}$, respectively. These activities, like propane oxidation itself, are also fully inhibited by acetylene (Smith et al. 2003a). Our current results examining growth (Table 1), the effects of acetylene (Table 1) and specific MTBE- and TBA-oxidizing activities (Table 2) all suggest the same enzyme system found in propane-grown cells is also expressed in cells grown on longer chain *n*-alkanes ($>C_3 \leq C_9$). This suggests SCAM is most strongly expressed by strain JOB5 during growth on C_3–C_9 *n*-alkanes, with maximal activity observed in cells grown on C_5 and C_6 alkanes. A similar trend also appears to apply to isoalkanes. This suggests SCAM is maximally expressed by strain JOB5 after growth on both of

the two most abundant alkanes (*n*-pentane and 2-methylbutane) in gasoline (Table 1). This further suggests that cells grown on these alkanes are also likely to support higher rates of cometabolic oxidation of the diverse compounds already known to be oxidized by propane-grown cells (Hamamura et al. 1997; Mahendra and Alvarez-Cohen 2006; Sharp et al. 2005; Vanderberg et al. 1995a, b; Wackett et al. 1989).

Further support for a two enzyme model and the similarities between cells grown on *n*-pentane and 2-methylbutane can also be seen in our experiments examining alkanes as potential inhibitors of MTBE and TBA oxidation (Table 3). Shorter chain *n*-alkanes ($<C_8$) were generally equally effective potent inhibitors of both MTBE and TBA oxidation but inhibition by longer chain *n*-alkanes decreased with increases in carbon chain length. A priori the potency of *n*-alkanes as potential competitive inhibitors might be expected to decrease progressively with increases in carbon chain length due to the progressive decrease in aqueous solubility. However, the step-like decrease in inhibition we observed might simply reflect that $\leq C_9$ *n*-alkanes are substrates for SCAM while longer chain *n*-alkanes ($>C_{10}$) are weaker inhibitors due to their limited ability to bind in the active site of an enzyme adapted to oxidize shorter chain *n*-alkanes.

While we recognize this model is only based on culture and inhibition studies, molecular studies indicate multiple and sometime diverse alkane-oxidizing enzyme systems are common in alkane-oxidizing bacteria (van Beilen and Funhoff 2007). For example, *Rhodococcus erythropolis* isolates contain as many as five genes encoding non-heme alkane hydroxylases and two genes encoding heme-containing alkane-oxidizing cytochrome P450s (van Beilen et al. 2006). Other actinomycetes including *Rhodococcus* RHA1 (Sharp et al. 2007) as well as *Mycobacterium* TY-6 and *Pseudonocardia* TY-7 (Kotani et al. 2006) can express both propane monoooxygenases and alkane hydroxylases with separate *n*-alkane substrate profiles. Perhaps the most relevant example is the *n*-butane-metabolizing bacterium, *Nocardioides* CF8, which grows on a similar range of *n*-alkanes (C_2–C_{16}) to strain JOB5. The diversity of *n*-alkane-oxidizing systems in strain CF8 was initially revealed through use of inhibitors such as 1-hexyne, a putative mechanism-based inactivator similar to acetylene (Hamamura et al. 2001). This study demonstrated this organism can simultaneously express two different alkane-oxidizing enzyme systems with different but partially overlapping substrate specificities.

Effects of alicyclics and aromatics

Cyclopentane and methylcyclopentane did not support growth of strain JOB5 (Table 1) but both inhibited MTBE and TBA oxidation (Table 3). Diverse alicyclics, including cyclopentane and methylcyclopentane, are oxidized to their corresponding cycloketones by strain JOB5 after growth on 2-methylbutane (Ooyama and Foster 1965). Although we did not confirm this reaction, our results and these earlier observations suggest these compounds most likely inhibit MTBE and TBA oxidation through competitive interactions at the active site of SCAM.

A broad range of effects was observed with the six aromatic compounds. Benzene does not support growth of strain JOB5 but is cometabolically oxidized by propane-grown cells. Like MTBE and TBA oxidation, benzene oxidation by propane-grown cells appears to involve two sequential monooxygenation reactions leading to the formation of phenol and then hydroquinone (Burback and Perry 1993). Most of our observations are compatible with, or extend these earlier observations. These include culture growth data (Table 1), the inhibition of benzene oxidation by acetylene (Table 5; Fig. 2a), and the behavior of benzene as a competitive inhibitor of both MTBE (Figs. 1c, 2a) and TBA (Fig. 2a) oxidation. We have confirmed benzene oxidation by *n*-pentane-grown cells sequentially generates both phenol and hydroquinone (data not shown). Collectively, these observations suggest benzene also most likely inhibits MTBE oxidation through competitive interactions at the active site of SCAM.

As the K_i value of a competitive substrate is equal to its K_s value (Cornish-Bowden 1979), the K_i^{true} values we have derived for benzene as an inhibitor of MTBE ($\sim$24 μM) and TBA ($\sim$7 μM) oxidation (Table 4) provide two estimates of the affinity for this compound as a SCAM substrate. Both estimates are considerably lower than the K_s for MTBE ($\sim$1.4 mM) derived for propane-grown cells of strain JOB5 (Smith et al. 2003a). It is therefore unsurprising MTBE had little effect on benzene oxidation by *n*-pentane-grown cells and that MTBE oxidation was initially strongly inhibited by benzene (Fig. 2a).

Although more limited, our results for ethylbenzene also agree with the earlier findings of Burback and Perry (1993) that this compound is oxidized by propane-grown cells of strain JOB5. This suggests the effects of this non-growth-supporting aromatic on MTBE and TBA oxidation can again most likely be accounted for through competition for cometabolic oxidation by SCAM.

The K_i^{true} and hence K_s values determined for the competitive inhibition of MTBE and TBA oxidation by toluene (Fig. 1d) indicate toluene is similar to benzene in its potency as an inhibitor of MTBE and TBA oxidation (Table 4). Our time course experiments confirmed toluene strongly inhibited MTBE oxidation (Fig. 2b). However, unlike benzene, low concentrations of toluene also stimulated both MTBE and TBA oxidation (Fig. 1d inset). As this effect was also observed with two other growth-supporting substrates (*n*-pentane and 2-methylbutane), this stimulation may reflect an increased supply of reductant to SCAM when these growth-supporting compounds are catabolized at low concentrations that are otherwise insufficient to substantially inhibit MTBE and TBA oxidation through competitive interactions. One implication of our observation that 2-methyl-1,2-propanediol apparently alleviated reductant limitation in our experiments (Fig. 1 a, b, d) is that this compound is a metabolite of MTBE and TBA oxidation in strain JOB5 (Johnson et al. 2004). Even though strain JOB5 does not grow on either MTBE or TBA (Table 1), our results (Fig. 1) suggest oxidation of this diol may be a source of reductant to help sustain MTBE oxidation by this organism.

It should be noted that a similar stimulating effect of toluene has also previously been described for cometabolitc trichloroethylene oxidation by propane-grown cells of strain JOB5 (Vanderberg et al. 1995a). Our observations that growth on toluene (Table 1) and the oxidation of toluene by alkane-grown cells (Fig. 2b; Table 5) are both only partially inhibited by acetylene suggests strain JOB may also have two distinct toluene-oxidizing activities. In *n*-pentane-grown cells these appear to include an acetylene-sensitive, SCAM-catalyzed cometabolic oxidation of toluene and an acetylene-insensitive SCAM-independent activity. If the second of these activities underlies the ability of this organism to actually grow albeit poorly, on toluene (Table 1), it is this activity that would most likely lead to substantial catabolism of toluene and hence the production of reductant that can be used by SCAM.

The strong growth on *p*-xylene (Table 1) is a novel observation that extends the recognized hydrocarbon-oxidizing capabilities of strain JOB5. Growth on *p*-xylene was comparable to some longer chain *n*-alkanes (Table 1) and *p*-xylene-grown cells readily oxidized MTBE both in batch cultures (Table 1) and after growth in MTBE-free media (Table 2). Like toluene, *p*-xylene oxidation by *n*-pentane-grown cells was only partially inhibited by acetylene (Table 5). Our ongoing studies have shown *p*-xylene-grown cells rapidly oxidize toluene and vice versa (data not shown) and suggest there may be a common enzyme system involved in initiating catabolism of these aromatics. However, further studies are needed to determine whether MTBE oxidation by *p*-xylene-grown cells is due to a co-expressed enzyme like SCAM or whether this reflects MTBE oxidation catalyzed by an enzyme involved directly in aerobic catabolism of aromatics.

Broader implications

Strain JOB5 has been noted for the sustainability of its cometabolic oxidation reactions and to be unusually resistant to toxic effects associated with some of these reactions (Hamamura et al. 1997; Mahendra and Alvarez-Cohen 2006; Wackett et al. 1989). The high ($\sim$150 ppm) concentrations of cosubstrates (MTBE and TBA) we have used and the substantial amount of biodegradation we have observed (Table 1) suggest that MTBE and TBA cometabolism is a robust process in cells of strain JOB5 grown on diverse gasoline hydrocarbons. The relative concentrations (1:1 v/v ratio) of MTBE and gasoline hydrocarbons used in this study may approximate the concentrations an organism may encounter in a gasoline impacted site (Table 1). The sustainability of the cometabolic degradation observed with MTBE and TBA may reflect the fact that reductant can be generated during further oxidation of MTBE-derived metabolites, an outcome that is potentially rooted in the ability of strain JOB5 to grow on branched alkane structures like 2-methylbutane (Ooyama and Foster 1965; Table 1). It should be noted that several gasoline fractions such as alkenes and multibranched alkanes were not examined in this study. While alkenes are relatively minor gasoline components, isooctane (2,2,4-trimethylpentane) can

be present at substantial concentrations, especially in high octane gasoline. *Mycobacterium austroafricanum* IFP 2173 is one of the few currently known isooctane-oxidizing bacteria (Solano-Serena et al. 2004) and this strain can cometabolically oxidize MTBE (Solano-Serena et al. 2000). As shown in this study of strain JOB5, this MTBE-oxidizing activity may again be related to the ability of these organisms to catabolize branched hydrocarbons.

Another important general observation in this study is that the inhibitory and stimulatory effects observed for MTBE oxidation were typically also observed with TBA oxidation. As TBA is an important ground water pollutant in its own right, the observations may provide insights into possible natural fates for TBA in gasoline-impacted environments or engineered approaches that could be used to remediate TBA. The similarity between the effects on MTBE and TBA also supports our earlier suggestion that MTBE and TBA are oxidized by the same enzyme in cells of strain JOB5 expressing SCAM activity (Smith et al. 2003a). However, some exceptions were noted. For example, cells grown on longer chain ($>C_{10}$) *n*-alkanes had consistently higher mean specific rates of MTBE oxidation than TBA oxidation (Table 2). This disparity may be meaningful but could also reflect the large variability we encountered in determining the generally low MTBE and TBA oxidation rates exhibited by these cells. Another example is the fourfold difference in the K_i^{true} values determined for benzene inhibition of MTBE and TBA oxidation (Table 4). In this case the difference in K_i^{true} values may possibly reflect a secondary effect of phenol that is generated and further oxidized to hydroquinone during benzene oxidation.

A limitation of this study is that key enzymes were not identified at the molecular level. Recent studies in MTBE-cometabolizing and metabolizing organisms have focused on the possible involvement of *alkB*-encoded alkane hydroxylases as key enzymes in these processes. Our own studies with the well characterized alkane hydroxylase in *Pseudomonas putida* GPo1 have shown this enzyme does not oxidize TBA and has a very high K_s value (>20 mM) for MTBE (Smith and Hyman 2004). We have also determined this enzyme is not inhibited by acetylene at the concentrations used in this current study (data not shown). The alkane hydroxylase variant from strain GPo1 is also one of a limited number of examples of this enzyme which oxidize shorter chain *n*-alkanes ($<C_{10}$) while most variants have substrate ranges restricted to longer chain *n*-alkanes ($\geq C_{10}$; van Beilen and Funhoff 2007).

Lopes Ferreira et al. (2007) identified an alkane hydroxylase in the genomes of several TBA- and alkane-oxidizing mycobacteria, including strain JOB5. From physiological and molecular evidence it was concluded that this enzyme is responsible for initiating TBA oxidation in these strains. However, TBA-oxidizing activity was determined in resting alkane-grown cells that were initially exposed to TBA for 24 h. We have previously shown similar conditions lead to induction of both MTBE- and TBA-oxidizing activity in strain JOB5 (Johnson et al. 2004). Based on these observations, and our evidence presented here that strain JOB5 can apparently simultaneously express more than one alkane-oxidizing enzyme after growth on some alkanes, it seems likely that Lopes Ferreira et al. (2007) identified an alkane-oxidizing enzyme that is common to TBA-oxidizing mycobacteria, but probably not one that is responsible for either TBA or MTBE oxidation in strain JOB5. Our evidence presented here that alkane-grown cells of strain JOB5 can apparently co-express different alkane- and aromatic-oxidizing enzymes also raises concerns about the previous assignment of diverse cometabolic oxidation reactions to the propane-oxidizing enzyme system (SCAM) in strain JOB5 (Burback and Perry 1993; Vanderberg and Perry 1994; Vanderberg et al. 1995a, b).

An alkane hydroxylase has also been suggested to initiate MTBE oxidation in the MTBE-metabolizing bacterium, *M. petroleiphilum* PM1 (Schmidt et al. 2008). This enzyme has also been suggested to enable this organism to initiate alkane catabolism although no data has been presented to support this second physiological capability (Hristova et al. 2007; Kane et al. 2007). Given the wide range of effects of *n*-alkanes and isoalkanes shown here for strain JOB5, it can be anticipated that if the same enzyme is responsible for initiating oxidation of both alkanes and MTBE in strain PM1, many gasoline alkanes might be expected to impact MTBE oxidation by this organism.

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