ABSTRACT

HOUSE, ALAN JAY. Characterizing MTBE Cometabolism and Propane Metabolism by *Mycobacterium austroafricanum* JOB5. (Under the direction of Michael R. Hyman.)

Cometabolic transformations are unable to support cell growth. This process is often catalyzed by, and superimposed upon, enzyme systems expressed to catalyze carbon- or energy-yielding reactions. Biodegradation of the gasoline additive methyl tertiary butyl ether (MTBE) is known to be superimposed upon a propane-oxidizing system in the aerobic bacterium *Mycobacterium austroafricanum* (previously *vaccae*) JOB5. Taking a whole-cell approach, we investigated the physiology of propane metabolism and MTBE cometabolism in this strain.

Multiple major gasoline components are frequent co-contaminants with MTBE in the environment, and we determined the impacts of these hydrocarbons on the cometabolism of both MTBE and its commonly encountered metabolite, tertiary butyl alcohol (TBA). Most of the hydrocarbons tested supported cell growth and concurrent MTBE and TBA oxidation occurred without affecting final culture optical density. Hydrocarbon-grown cells seem to simultaneously express more than one alkane-oxidizing enzyme system.

Nuclear magnetic resonance spectroscopy (NMR) was used to study the pathway of MTBE oxidation in propane-grown cells. We confirmed the existence of predicted intermediates, including a hemiacetal, formate and formaldehyde. Hydroxyisobutyraldehyde, a predicted intermediate in MTBE oxidation by some bacterial strains was not detected, despite attempts
to promote its accumulation. As the pathway of MTBE oxidation progressed, the rate of daughter product oxidation decreased, which may be preventing MTBE-dependent cell growth in strain JOB5.

Propane metabolism was examined using a series of growth experiments and substrate oxidation assays. We observed the simultaneous production, and later consumption of both 1- and 2-propanol during cell growth. This divergent oxidation of propane was apparently followed by the divergent oxidation of propionate and the divergent oxidation of acetone. Our results suggest at least two CO$_2$-fixation steps are involved in propane metabolism in strain JOB5.

Finally, we used NMR to contribute to several studies that characterized the pathway of (i) MTBE oxidation by *Nitrosomonas europaea* or (ii) bacterial oxidation of a fluorinated analog of TBA. The later study identified a compound that may serve as a tracer for TBA degradation *in situ*. 
Characterizing MTBE Cometabolism and Propane Metabolism
by *Mycobacterium austroafricanum* JOB5

by
Alan Jay House

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APPROVED BY:

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Chair of Advisory Committee
BIOGRAPHY

Alan Jay House was born in 1979 to Leland Ralph, from Cincinnati, Ohio, and Fadia Ayoub, from Zahleh, in Lebanon. Alan’s Dad was a plant breeder who focused on hybrid seed development for the semi-arid tropics. As a result, Alan spent his youth in India and Zimbabwe before his Dad retired to the Appalachian Mountains of North Carolina.

In 2001, Alan received his degree in microbiology from North Carolina State University, where he remained to pursue postgraduate work in Dr. Michael Hyman’s environmental microbiology laboratory. Alan concentrated on the biodegradation of two compounds, propane and methyl tertiary butyl ether, a gasoline additive. In addition to microbiology and analytical chemistry training, while in school Alan learned basic scientific glassblowing techniques and assisted in the teaching of both introductory and advanced hands-on laboratory courses.

Alan lives in Cary, North Carolina, with his wife, Nicole Souther, and their son, Ian.
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<td>2-methyl-1,2-propanediol</td>
</tr>
<tr>
<td>HIBA</td>
<td>2-hydroxyisobutyrate</td>
</tr>
<tr>
<td>MTBE</td>
<td>methyl <em>tertiary</em> butyl ether</td>
</tr>
<tr>
<td>SCAM</td>
<td>short-chain alkane monooxygenase</td>
</tr>
<tr>
<td>TBA</td>
<td><em>tertiary</em> butyl alcohol</td>
</tr>
<tr>
<td>TBF</td>
<td><em>tertiary</em> butyl formate</td>
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CHAPTER 1

LITERATURE REVIEW
1.1 Cometabolism and strain JOB5

1.1.a Biodegradation and the cometabolism concept. Antoine Laurent Lavoisier (1743-1794) demonstrated that matter could neither be created nor destroyed, only transformed. Biodegradation denotes the biogenic decomposition of matter. It is a process to which microorganisms are well suited.\textsuperscript{49, 98} In the words of Eugene Madsen: “The small size, ubiquitous distribution, high specific surface area, potentially high rate of metabolic activity, genetic malleability, potentially rapid growth rate, and unrivaled enzymatic and nutritional versatility of microorganisms cast them in the role of recycling agents for the biosphere.”\textsuperscript{98} Some compounds are biodegraded yet yield no carbon or energy benefit to the organism. In these cases, biodegradation is often catalyzed by, and superimposed upon, an enzyme system expressed to catalyze a carbon- or energy-yielding reaction. In this dissertation, we recognize that biodegradation of the gasoline additive methyl tertiary butyl ether (MTBE) is superimposed upon a propane-oxidizing system in \textit{Mycobacterium austroafricanum (vaccae)} JOB5. Therefore, we explore the physiology of propane utilization in this strain, and examine how cells grown on propane (or other short-chain alkanes) use a process termed cometabolism to degrade MTBE.

Not all compounds are biodegraded equally. Biodegradability is in part determined by a compound’s molecular structure,\textsuperscript{124} elemental composition,\textsuperscript{3, 156} and amount of time spent in the environment.\textsuperscript{49} Designing synthetic compounds to resemble natural ones tends to improve biodegradability.\textsuperscript{117} But this is not always desirable, and in some cases (herbicides and insecticides, for example) compounds are designed to persist in the environment.\textsuperscript{3} Biodegradation of these “recalcitrant”\textsuperscript{2, 3} compounds is often slow and incomplete, and the
resulting intermediates may be more benign or more toxic than the substrate.\textsuperscript{1} Incomplete transformations are unable to support cell replication, so a “primary substrate” is required to support the cell population performing this mode of degradation, termed cometabolism.\textsuperscript{33}

Cometabolism describes the microbial transformation of “cosubstrates” that do not support cell-replication.\textsuperscript{64} With such a broad definition, both the meaning\textsuperscript{33,117} and utility\textsuperscript{67} of the word have been debated. The source of debate is best illustrated with the foundational data leading to the cometabolism concept. In 1958,\textsuperscript{87,88,89} and ‘60\textsuperscript{85} Leadbetter and Foster explored the substrate range of the methane-oxidizing (methanotrophic) bacterium \textit{Pseudomonas} (previously \textit{Methylomonas}) \textit{methanica}. This strain was unable to utilize ethane, propane or \textit{n}-butane for growth.\textsuperscript{87} However, when cells were simultaneously incubated with methane (as primary substrate), and either ethane, propane or \textit{n}-butane, oxidation of the three later cosubstrates occurred.\textsuperscript{86} Oxidation of ethane yielded ethanol, acetaldehyde and acetic acid; of propane: \textit{n}-propanol, propionic acid and acetone; of butane: \textit{n}-butanol, \textit{n}-butyric acid and 2-butane.\textsuperscript{85,86} Resting cells were unable to oxidize propane and \textit{n}-butane, although ethanol, \textit{n}-propanol and \textit{n}-butanol were readily transformed to the corresponding carboxylic acid. Resting cells did not receive a carbon-benefit from cosubstrate-transformation since alcohol to acid conversion was stoichiometric, and further degradation of the acid was not observed.\textsuperscript{85,87} In contrast, when cells were simultaneously incubated with methane (as primary substrate) and either \textsuperscript{14}C-ethane or \textsuperscript{14}C-propane (as cosubstrates), a portion of the carbon-14 was not only recovered from the corresponding two or three carbon oxidized products of these cosubstrates, but also from CO\textsubscript{2}, cell material and
extracellular mucoid material. Thus cosubstrate transformation alongside a primary substrate yielded a cosubstrate-derived carbon-benefit in *P. methanica*.

In summary, these results showed that (i) non-growth supporting cosubstrates were transformed; (ii) transformation occurred in the presence or absence of a primary substrate, (iii) cosubstrate oxidation was incomplete resulting in end-product accumulation; and (iv) while cosubstrate transformation did not support cell-replication, transformation products entered biosynthetic pathways and were incorporated into cellular material.

Hulbert and Krawiec argued that the term cometabolism should “be abandoned” as it does not describe a process unique from ordinary metabolism. Cosubstrate transformations are not novel metabolic events. Furthermore, absence of unknown essential nutrients in a culture medium may explain why transformation of a particular compound does not result in cell-replication. Further criticism came when cometabolism was used to describe the transformation of cosubstrates whether or not a primary substrate was simultaneously present. Hulbert and Krawiec, and Perry argued that the prefix “co” implied joint action of cosubstrate and primary substrate. Stirling and Dalton supported this stricter definition of cometabolism. From substrate-specificity studies using methane monooxygenase in the methanotroph *Methylococcus capsulatus* (Bath), they showed that cosubstrate transformation in the absence of a primary substrate (formaldehyde) was physiologically distinct from that when both substrates were present simultaneously. When methane-grown resting cells were incubated with an array of possible cosubstrates in the presence or absence of formaldehyde (a reductant regenerating intermediate during methane catabolism in *M. capsulatus*), the authors found that certain cosubstrates were only transformed in the presence of
formaldehyde, while a smaller number were transformed in its absence. Therefore, Stirling and Dalton concluded that cosubstrate transformation in the absence of a primary substrate was sustained by cosubstrate-derived reductant. The authors suggested the term “fortuitous metabolism” to differentiate this process from cometabolism – where cosubstrate transformation depended on reductant derived from the primary substrate (Figure 1-1). In this context, these authors asserted the utility of the term cometabolism (recognizing Hulbert and Krawiec’s argument) as it organized and described an environmentally important concept.

Despite the formalized differences between fortuitous metabolism and cometabolism, both refer to the biotransformation of a non-growth supporting substrate. It is perhaps for simplicity that current usage of the term cometabolism tends to rely on Horvath’s earlier, less specific definition, which is therefore applied in this dissertation: “usage of co-metabolism refers to any oxidation of [co]substances without utilization of the energy derived from the oxidation to support microbial growth and does not infer presence or absence of [primary] substrate during the oxidation.” Whether or not primary substrate is available, cometabolism is in effect superimposed upon a primary substrate-oxidizing system (Figure 1-1).

Cosubstrate transformations do not generally appear to provide a clear carbon- or energy-benefit for an organism (one exception is carbon-incorporation in *P. methanica*, as discussed above). It is therefore tempting to place cometabolism in an evolutionary context, where such wasteful reductant draining transformations are expected to compromise an organism’s fitness in a competitive landscape (for example, carbon-limited steady-state cultures of *M. capsulatus* (Bath) suffered a decreased cell-yield when a cosubstrate
Primary substrate (methane) metabolism

Fortuitous metabolism

Cometabolism

**Figure 1-1:** Illustration of the importance of reductant source in differentiating fortuitous metabolism from cometabolism. Primary substrate (methane) metabolism is shown to illustrate the superimposition of cometabolism on a primary substrate-oxidizing system. Diagram is based on definitions of these terms outlined by Stirling and Dalton\textsuperscript{139} (MMO: methane monooxygenase).
(propylene) was added to the medium. For a possible evolutionary basis for cometabolism, consider Stephen Jay Gould, writing in Full House: “[A]ny proper theory of explanation in natural history depends upon the distinction of causes and consequences. Darwin’s central theory holds that natural selection acts to increase adaptation to changing local environments.... But many features that become vital to the lives of their bearers may arise as uncaused (or at least indirectly produced) and ‘unintended’ sequelae or side consequences.”

I believe cometabolism is an unintended consequence of enzyme evolution. Indeed, by studying the methane monooxygenase from M. capsulatus (Bath), Green and Dalton suggested that the lack of substrate specificity in this enzyme “may be an evolutionary by-product of the optimization of conditions for methane oxidation.” Is cometabolism vital to the life of its bearer? Probably not. In time, may it become vital to the life of its bearer? It is quite probable that a cometabolic substrate may, in time, become a metabolic one, and Dalton and Stirling endorse this view. It is also possible that by detoxifying a cometabolic substrate an organism has avoided injury and gained an advantage. However, the opposite is also true, and the product of cometabolism may be more toxic than the substrate.

1.1.b A mechanistic basis for cometabolism: metabolic versatility. Enzymes are renowned for their specificity. Yet certain enzymes will attack a wide range of related compounds, though at disparate rates. These enzymes, in large part, make cometabolism possible. The focus of this dissertation is on aerobic MTBE- and propane-oxidation, both monooxygenase-catalyzed reactions. Monooxygenases incorporate one oxygen atom from molecular oxygen into the hydrocarbon substrate while reducing the other atom to water – requiring a reducing agent (such as reduced pyridine nucleotides). Oxidized hydrocarbons are more
soluble and susceptible to further biodegradation.\textsuperscript{117} A brief analysis of the relaxed enzyme specificity for three well characterized monooxygenase systems (MMO, AlkB and cytochrome P-450) will serve to illustrate the potential of these enzymes in cometabolic transformations.

Methane monooxygenase (MMO) is responsible for the conversion of methane to methanol in methanotrophic bacteria. As previously discussed, MMO’s ability to attack a wide substrate range helped establish the cometabolism concept. In addition to methane, MMO attacks \( \text{C}_{2-8} \ n\)-alkanes, terminal and internal \( n\)-alkenes, alicyclics, aromatics, heterocyclics, ethers, carbon monoxide\textsuperscript{24}, haloalkanes\textsuperscript{139} and ammonia.\textsuperscript{32} MMO exists in two forms: a soluble form located in the cytoplasm (sMMO), and a particulate form located in the cell membrane (pMMO). Both forms are well characterized, although more is known about sMMO since it is easier to isolate, purify and study. For instance, in \( M. \ capsulatus \) (Bath), three components have been shown to comprise the sMMO system.\textsuperscript{23} Component C (MMOR) accepts electrons from NADH and shuttles these to component A (MMOH),\textsuperscript{95, 96} which binds and hydroxylates the substrate.\textsuperscript{159} The flow of electrons from MMOR to MMOH is mediated by component B (MMOB), which ensures NADH oxidation is tied to substrate hydroxylation (Figure 1-2A).\textsuperscript{50} For pMMO, the electron donor and electron transfer chain remain to be determined.\textsuperscript{108} However, the structure of pMMO hydroxylase has been solved and is shown to be a trimeric protein\textsuperscript{80} containing copper atoms and non-heme iron.\textsuperscript{11}

The alkane monooxygenase system from \textit{Pseudomonas putida (oleovorans)} GPo1 has also been extensively studied, and is composed of a soluble rubredoxin (AlkG), a soluble rubredoxin reductase (AlkT), and a particulate alkane hydroxylating subunit (AlkB).\textsuperscript{119, 151}
A) Soluble methane monooxygenase (sMMO) system

B) AlkB (ω-hydroxylase) alkane monooxygenase system

C) Cytochrome P-450 alkane monooxygenase systems

Figure 1-2: Enzyme components and electron transfer processes required for substrate hydroxylation by MMO (A), AlkB (B), or cytochrome-P450 (C) alkane monooxygenase systems. MMO illustration is from Green and Dalton\(^\text{50}\) (component C: reductase, component A: hydroxylase). AlkB illustration is from Coon\(^\text{29}\) and cytochrome P450 illustrations are from Fulco.\(^\text{46}\)
Rubredoxin reductase catalyzes the transfer of electrons from the reducing agent (NADH) to rubredoxin. The reduced rubredoxin donates electrons to the hydroxylase, where one oxygen atom is reduced to water, and the other incorporated into the hydrocarbon substrate (Figure 1-2B). The proteins are encoded on a 400-500 kb OCT plasmid. AlkB is a non-heme iron protein that displays six transmembrane domains. It is traditionally known to oxidize C_{5-14} linear and branched chain alkanes, favoring n-octane. Johnson and Hyman have recently shown that propane and n-butane are also substrates. AlkB also catalyzes the oxidation of terminal alcohols, olefins, and the ω-hydroxylation of C_{6-12} fatty acids. Cyclopentane, cyclohexane and methylecyclohexane are oxidized, as are several substituted benzene compounds. AlkB also catalyzes the O-dealkylation of branched alkyl and vinyl ethers, and S-dealkylation of thioethers. The molecular underpinnings of such a relaxed active site are not well known, although initial studies by van Bielen et al. demonstrated an increase in the n-alkane chain length accepted by AlkB upon the substitution of a tryptophan residue in the enzyme’s center with a smaller amino acid, such as serine.

Unlike AlkB, cytochrome P-450 is a hemoprotein. Its name derives from the characteristic light absorption band at 450 nm generated when carbon monoxide interacts with the reduced form of the enzyme. Its designation as a cytochrome has been criticized since the enzyme tends to act more like an oxygenase than a strict electron carrier. Cytochrome P-450s exist is various forms and are not limited to bacteria. For instance, humans contain ~50 distinct P-450s, Arabidopsis may have ~350, and Mycobacterium tuberculosis has ~20. The mode of action of each P-450 distinguishes the enzyme into one
of three groups based on the number of protein components required for activity. Most bacteria and mitochondria contain a three-component system characteristic of P-450cam isolated from *Pseudomonas putida* strain C1. The three components are putidareductase, putidaredoxin and the cytochrome P-450, which binds and hydroxylates the substrate. Putidareductase accepts electrons from NADH and putidaredoxin (an iron-sulfur protein) transfers the electrons from the reductase to the cytochrome P-450 (Figure 1-2C.1). In bacteria, all three components are soluble whereas in mitochondria the hydroxylase component is membrane bound and the electron transfer proteins are soluble. Most eukaryotic cells contain a membrane-bound two-component enzyme system similar to that isolated from rabbit liver microsomes. Here, a reductase component transfers electrons from NADPH to the cytochrome P-450 (Figure 1-2C.2) The third class of cytochrome P-450s is comprised solely of P-450BM-3, a unique soluble one-component system isolated from *Bacillus megatarium* ATCC 14581. The enzyme contains equimolar FAD, FMN and heme. FAD is predicted to receive electrons from NADPH, and these are predicted to flow to the heme moiety via FMN (Figure 1-2C.3). Despite containing both reductase and catalytic domains in a single protein, P-450BM-3 is only functional as a dimer.

The substrate range among P-450s in a given organism differs and may overlap, with some accepting relatively few substrates, whereas others are much more versatile. Cytochrome P-450cam has been shown to oxidize MTBE, and the substrate range for cytochrome P-450s from liver microsomes includes pesticides, solvents and drugs; as well as their natural substrate range including steroids, fatty acids and amino acids. In the words of Minor J. Coon: “The ability of this catalyst to metabolize a multitude of organic compounds
that can now be produced readily by combinatorial techniques but do not occur naturally on this planet indicates that the number and variety of P450 substrates are almost unlimited.\textsuperscript{29} Indeed, recent studies have successfully used rational\textsuperscript{89} or directed evolution\textsuperscript{10} techniques to enhance P-450 substrate binding and turnover rate for target compounds.

Whereas some enzymes have a broad substrate range, the compounds that induce gene expression may be more limited, and some enzyme-encoding genes are only induced by primary substrates.\textsuperscript{30} However, it is impossible to generalize, because enzyme-encoding genes required for cometabolism may also be induced by the cosubstrate itself,\textsuperscript{64, 72, 117} or may be expressed constitutively.\textsuperscript{30} When the enzymes are expressed and presented with a mixture of substrates, a cell may either simultaneously or preferentially transform the primary substrate and/or cosubstrate – a deciding factor being the amount of each substrate present.\textsuperscript{55} For example, Chang and Alvarez-Cohen noticed an inhibition of trichloroethylene-cometabolism by mixed cultures in the presence of high amounts of primary substrate (either methane, propane, toluene or phenol), which likely monopolized enzyme activity and inhibited cosubstrate transformation. Low levels of primary substrate yielded the opposite result, enhancing TCE degradation. Enhancement was likely due to metabolism of the primary substrate which generated reducing power that supported the simultaneous utilization of TCE, the cosubstrate. Indeed methane-utilizing mixed cultures that received a 20 mM formate supplement (an NADH regenerating substrate) degraded twice as much TCE compared to cultures that did not receive a supplement.\textsuperscript{22} Once an exogenous primary substrate is depleted, cosubstrate transformation may be supported by finite intracellular energy stores (such as poly-β-hydroxybutyrate).\textsuperscript{5} The extent to which degradation can occur
under these conditions has been termed transformation capacity.\textsuperscript{4} Transformation of some compounds, such as TCE, may generate toxic products and this will also limit transformation capacity.\textsuperscript{5}

In summary, cometabolism superimposes cosubstrate transformation on a non-specific primary substrate-oxidizing enzyme system. The extent to which enzymes oxidize either the primary substrate or cosubstrate is partly dictated by a complex set of interactions encompassing substrate availability, enzyme kinetics and transformation capacity.\textsuperscript{30}

\textbf{1.1.c Mycobacterium austroafricanum (previously vaccae) JOB5.} The organism we have used to explore the aerobic biodegradation of MTBE and propane is \textit{Mycobacterium austroafricanum} JOB5. Lukins and Foster have described Mycobacteria as “undoubtedly ... the one group of microorganisms best recognized in respect to the capacity to utilize hydrocarbons.” Ooyama and Foster isolated strain JOB5 in 1965 from a soil sample placed on a mineral salts agar surface and incubated for 40 days with 2-methylbutane serving as the sole source of carbon and energy.\textsuperscript{94} Cells are Gram variable, nonmotile and nonsporforming.\textsuperscript{62} Cellular morphology depends on culture conditions, with short “egg shaped” rods observed during growth on dextrose whereas longer, slender, bent rods predominate during hydrocarbon-dependent cell growth. Colonies appear in ~2 to 5 days on nutrient-rich agar incubated at 25 to 30°C and are circular, pinpoint, convex and predominately yellow (personal observations).

In its initial description, strain JOB5 was shown to grow on an unprecedented wide range of hydrocarbon substrates including \( C_{1-22} \) \textit{n}-alkanes and some \( C_{4-7} \) branched chain alkane isomers. Alicyclic-dependent growth was not observed, although resting cells of strain
JOB5 grown on 2-methylbutane oxidized C₅₋₈ alicyclics to their corresponding cycloalkanones.¹¹⁴ Cycloalkanones are readily degraded by some soil microorganisms,¹²⁶ and a comensal relationship between the alicyclic-oxidizing strain JOB5 and the cycloalkanone-utilizing strain CY6 resulted in the complete mineralization of cyclohexane.¹³ Thus partial transformation of cyclohexane via cometabolism led to the complete mineralization of this recalcitrant compound.¹² In addition to cyclohexane, cosubstrates oxidized by propane-grown resting cells of strain JOB5 include (i) benzene, styrene and other aromatics,¹⁸ (ii) dioxane,¹⁸ (iii) halogenated alkanes and alkenes, including TCE;¹⁵² (iv) 2,4,6-trinitrotoluene (TNT),¹⁵³ and (v) MTBE.¹³¹ Oxidation of each compound occurred with no apparent lag, suggesting that cosubstrate transformation was superimposed upon and catalyzed by a versatile propane-induced monooxygenase.

1.2 MTBE: its application and biodegradation

1.2.a History of MTBE use in gasoline in the United States of America. Gasoline combustion within a modern internal combustion engine is a controlled process - releasing pressure and powering the engine. Imprecise burning compromises efficiency and may damage the engine.¹⁵⁷ Keeping combustion precise is partly dictated by the composition of the fuel. An important consideration in fuel composition is that straight chain hydrocarbons burn faster than branched-chain isomers. For instance, if neat n-heptane is ignited, an uncontrolled, rapid explosion occurs. This releases an outburst of pressure from the engine’s cylinder, which is perceived as an audible "ping" or "knock". On the other hand, neat isooctane (2,2,4-trimethylpentane) burns more slowly and predictably.⁹ Following from this, each blend of gasoline is given an octane rating based on how efficiently it burns compared
to pure \(n\)-heptane (with an octane rating of zero), or pure isoctane (with an octane rating of one hundred).  

Certain compounds may be blended into gasoline to enhance the octane rating. A well-known example is tetraethyl lead. Introduced in the early 1920’s, leaded gasoline became widespread despite the availability of alternative anti-knock additives such as benzene and ethanol. Preference for lead was probably due to (i) the low amount (0.1\%) needed to give the same octane rating as blends containing 30\% ethanol or 40\% benzene and (ii) successful marketing campaigns that overlooked lead's known toxicity. However, in 1973, public health concerns about lead poisoning resulted in legislation by the U.S. Environmental Protection Agency (EPA) that required a gradual phase-out of lead from gasoline. The most popular lead substitutes were initially aromatic hydrocarbons. Consequently, urban areas saw increased levels of smog caused in part by tailpipe emissions of carbon monoxide, nitrogen oxides and unburned hydrocarbons. Efforts to reduce smog required the aromatic hydrocarbon content of gasoline to be reduced. The most popular lead substitute then became MTBE. The low-volatility oxygen atom in MTBE (Figure 1-3) also promoted combustion efficiency, providing cleaner burning fuel. MTBE’s dual purpose as an octane booster and gasoline oxygenate would result in it becoming a major gasoline constituent that would meet the demands of the 1990 Clean Air Act Amendments.

The Clean Air Act, passed by U.S. Congress in 1970, authorized the EPA to set national air quality standards. The 1990 amendments addressed air quality hazards overlooked in the original legislation and empowered the EPA to regulate fuel quality. Gasoline refiners were required to sell "reformulated" gasoline in those urban areas most
Figure 1-3: Chemical structure and physical properties of MTBE. Structure is from http://www.wikipedia.org. Henry’s law constant is from Miller and Stuart\textsuperscript{105} and octane rating is from Piel\textsuperscript{116}. All other physical properties are taken from a chemical summary for MTBE prepared by the Office of Pollution Prevention and Toxics at the U.S. Environmental Protection Agency (http://www.epa.gov/opptintr/chemfact/s_mtbe.txt).

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affected by smog. This formulation would contain no less than 2.0 percent oxygen by weight and no more than 1 percent benzene by volume, with a total aromatic hydrocarbon content not exceeding 25 percent by volume. Furthermore, areas containing carbon monoxide in excess of 9.5 ppm were required to sell "oxygenated" gasoline during winter. This formulation would contain no less than 2.7 percent oxygen by weight.\textsuperscript{113} Whereas these programs regulated the amount of oxygen that must be present in particular gasoline formulations, they did not specify how refineries should meet this requirement. Since MTBE was already widely used as an octane booster, refiners simply increased its contribution to gasoline in order to meet the oxygen mandate. Moreover, MTBE (i) is blended into gasoline easier than other natural (methanol, ethanol) and synthetic (ethyl tert-butyl ether, tert-amyl methyl ether) oxygenates,\textsuperscript{101, 116} and (ii) is manufactured by combining isobutylene (a waste product of oil refining) and methanol,\textsuperscript{158} so oil refineries could turn the costs of isobutylene disposal into profits from MTBE sales. An oxygenated fuel requirement of 2.7 percent oxygen by weight meant that gasoline would contain 15 percent MTBE by volume. Thus MTBE became the most abundant single component of gasoline.\textsuperscript{122, 136}

A highly branched structure and an ether bond made MTBE an effective octane enhancer, yet these same properties forecasted its potential to be a persistent environmental contaminant.\textsuperscript{124, 156} The main problem with MTBE in the environment is the contamination of groundwater resulting from leaking underground gasoline storage tanks.\textsuperscript{73} Indeed, MTBE is ~28 times more soluble in water than benzene, the next most soluble gasoline component. Furthermore, MTBE does not sorb readily to soil or organic matter,\textsuperscript{69} and, compared to benzene, MTBE is an order of magnitude less readily volatilized from water (see Figure 1-3
for the physical properties of MTBE). All these factors combine to predict that when gasoline enters groundwater, MTBE will migrate readily in the water table. This problem was made clear in 1996, when significant levels of MTBE (as much as 600 µg/L) forced the Californian city of Santa Monica to close wellfields comprising half of the city's potable water supply. By 2003, 36 States had reported MTBE contamination of ground and surface waters. Concentrations ranged up to 17,800 µg/L, but were generally less than 20 µg/L.

An important part of the MTBE problem is our sensitivity to its taste and smell. We can sense MTBE at levels as low as ~5 µg/L. The distasteful and malodorous qualities of MTBE causes the closure and remediation of tainted water supplies, and may lead the public to perceive the compound as toxic. Unfortunately, by the time MTBE contamination was being detected in public water supplies, there had been no unified scientific voice regarding the toxicity of MTBE. Afflictions associated with acute MTBE exposure, such as headaches, nausea and dizziness, were reported by citizens of Fairbanks, Alaska after pumping fuel containing MTBE. And yet, laboratory-based studies found no significant adverse response after exposing healthy human test groups to ~1 mg/L MTBE vapor for 1 hour. Other lab-based studies chronically exposed rats to high-levels (>1000 mg/L) of MTBE. Males developed renal and testicular tumors whereas females developed lymphoma and leukemia. These results seem alarming, but they fail to recognize the reality of consuming orders of magnitude less MTBE in drinking water. To help clarify MTBE's effect on public health, the EPA Office of Water released a Drinking Water Advisory in 1997 which stated that "most concentrations at which MTBE has been found in drinking water sources are unlikely to cause adverse health effects." Additionally, since maximum allowable
contamination levels of MTBE did not exist, EPA’s document advised State regulatory authorities to set maximum allowable concentrations of MTBE in water in the range of 20 to 40 µg/L, below the taste and odor threshold of most, but not all, people.146 Furthermore, in 1998 a Blue Ribbon Panel was appointed by the EPA to “investigate the air quality benefits and water quality concerns associated with oxygenates in gasoline, and to provide independent advice and recommendations on ways to maintain air quality while protecting water quality.”143 The panel broadly (although not unanimously) advocated a reduction in the amount of MTBE in gasoline, recognizing ethanol as a reasonable replacement.143

Before recommendations of the Blue Ribbon Panel were published, California had taken steps to ban the use of MTBE in gasoline sold within the state. By early 2002, 16 other states had adopted similar policies,147 and by 2005 at least 25 states enacted policies against the use of MTBE in gasoline.37 Although the use of MTBE in gasoline in the United States has subsided, remediating the contaminated water left behind will be costly, with best estimates ranging from $256 to $5099 billion.

1.2.b The biodegradation of MTBE. Biodegradation by native microbes could reduce MTBE-clean up costs. However, early studies failed to demonstrate biodegradation of MTBE or related compounds. In 1984, Fujiwara et al. showed that several tert-butyl ethers resisted biodegradation by an aerobic activated sludge.44 Similarly, samples collected from a sandy aquifer, top soil or activated sludge did not consume 0.1 mM MTBE within 60 days under aerobic conditions at 20°C.70 The anaerobic biodegradation of branched alkyl ethers by polluted methanogenic sediment and groundwater could not be demonstrated.140
Indeed, samples incubated with MTBE generated no methane during a 249 day period. However, as more samples were analyzed, MTBE biodegradation under both aerobic and anaerobic conditions became evident. The biochemistry and genetics underlying aerobic MTBE metabolism and cometabolism have been explored, whereas less is known about anaerobic MTBE biodegradation.

1.2.b.1 Anaerobic biodegradation. A pure culture capable of anaerobic MTBE oxidation has yet to be isolated. Evidence for MTBE consumption has been gained by either monitoring compound disappearance in microcosm based studies predominantly using sediment collected from petroleum-contaminated sites, or monitoring the $^{13}$C:$^{12}$C ratio of MTBE in situ with the understanding that organisms tend to degrade light isotopes first, increasing the isotopic ratio during biodegradation. Anaerobic MTBE oxidation has been observed under methanogenic, denitrifying, sulfate reducing and iron (III) reducing conditions. In some cases, the addition of nutrients such as humic substances or ethanol improved MTBE transformation. Recently, Youngster, Somsamak and Häggblom showed that low levels of aryl O-methyl ethers (such as syringate and 3,4,5-trimethoxybenzoate) stimulated MTBE consumption in a methanogenic sediment enrichment culture. High levels were inhibitory. Moreover, propyl iodide (which inhibits O-demethylation by binding to the methyl-accepting corrinoid) prevented degradation of MTBE and the aryl O-methyl ethers. Together, these results suggest O-demethylating acetogenic bacteria are responsible for MTBE transformation in these methanogenic enrichment cultures.
1.2.b.2 Aerobic metabolism. In 1997, Mo et al. isolated two strains able to grow using MTBE alone, requiring either two or four days, respectively, to show a measurable increase in cell numbers. These two, plus an additional strain, could mineralize MTBE when incubated with 0.02% yeast extract. Since then, several aerobic MTBE-metabolizing strains have been reported, including strain PM1, strains IFP 2012 and IFP 2015, strain ENV 735, and strain L108. MTBE-metabolizing organisms similar to strain PM1 have also been isolated from several contaminated environments.

*Mycobacterium austroafricanum* strains IFP 2012 and IFP 2015 were isolated from an activated sludge sample and an environmental water sample impacted with gasoline containing MTBE, respectively. A common pathway for the degradation of MTBE was observed in both strains. The first two detectable products generated were tertiary butyl formate (TBF) and tertiary butyl alcohol (TBA). MTBE oxidation was catalyzed by a monooxygenase and was predicted to occur at the methoxy-carbon of MTBE, yielding an undetected and unstable hemiacetal. Further oxidation of the hemiacetal likely accounted for TBF-formation. Further transformation of TBF yielded TBA and, predictably, formate. TBA was oxidized to 2-methyl-1,2-propanediol (2M12PD), which was then oxidized to 2-hydroxyisobutyrate (HIBA) (Figure 1-4). The fate of HIBA is unknown, although acetone was detected as a downstream product, and cobalt ions appeared to enhance growth with TBA and HIBA. Intermediates detected during MTBE degradation by strains IFP 2012 and IFP 2015 match those detected during the aerobic cometabolism of MTBE by a filamentous fungus and a propane-oxidizing bacterium. The initial pathway of aerobic MTBE-metabolism in strains IFP 2012 and IFP 2015 is therefore based on the initial pathway of
Figure 1-4: Proposed pathway for the metabolism of MTBE by strains IFP 2012\textsuperscript{22} and IFP 2015\textsuperscript{91} (dotted lines); and the cometabolism of MTBE by propane grown strain ENV425,\textsuperscript{137} (dashed lines) and strain JOB5\textsuperscript{131} and a filamentous fungus \textit{Graphium} species\textsuperscript{56} (solid lines). The hemiacetal is predicted, but not detected, and is therefore enclosed within a dotted box to denote this fact (structures are from http://www.chemfinder.com).
aerobic MTBE comethabolism.\textsuperscript{42}

Aerobic MTBE metabolism in strains IFP 2012 and IFP 2015 resulted in catabolite accumulation. This is atypical of metabolic processes. Typically catabolites are consumed rapidly and escape detection (as during MTBE degradation by strain PM1, for example). Different rates of catabolite consumption between the IFP strains and other MTBE-metabolizing strains (such as strain PM1) suggests that aerobic MTBE-metabolizing organisms may be categorized into those that readily utilize MTBE and those that do not. The distinct culture conditions required for an observable increase in culture optical density (OD) supports this distinction. Cultures of strain IFP 2012 or IFP 2015 in minimal media containing 0.8 or 1.7 mM MTBE, respectively, each took \( \sim 23 \) days to achieve their maximum OD.\textsuperscript{42,91} This value was twice the initial OD\textsubscript{600} of \( \sim 0.1 \) (strain IFP 2012) or \( \sim 0.15 \) (strain IFP 2015). In contrast, strain PM1 in minimal media containing 2.8 mM MTBE took 2 days to achieve an OD\textsubscript{595} of 0.3 from an initial reading of \( \sim 0.02 \).\textsuperscript{66}

Using strain IFP 2012 Lopes-Ferreira \textit{et al.} identified two proteins (and the genes encoding them) responsible for converting 2M12PD to HIBA. The cytosolic protein profiles of organisms grown “in the presence”\textsuperscript{90} of MTBE or glucose were compared, and two proteins (64 kDa and 55 kDa) that were increased in expression in the MTBE-containing sample were selected for further analysis. The sequence of the 64 kDa protein (labeled MpdB) was similar to a putative choline dehydrogenase, the 55 kDa protein (MpdC) was similar to an aldehyde dehydrogenase. Genes encoding these proteins were identified and cloned into a mycobacterial strain incapable of MTBE degradation. The transformed strain converted 2M12PD to HIBA, suggesting MpdB acted as an alcohol dehydrogenase,
oxidizing 2M12PD to an aldehyde that was rapidly dehydrogenated by MpdC to HIBA. The fate of 2M12PD and the aldehyde intermediate in a strain transformed with either MpdB or MpdC alone remains unknown. Orthologs with ~ 50% similarity to mpdB and mpdC have been found in strain PM1, although their expression following growth with MTBE was unchanged, or downregulated, respectively, compared with cells grown with ethanol.

Hanson et al. isolated strain PM1 from biofilter material that had been used to treat MTBE. The strain yielded 0.1 mg of cell protein/mg MTBE. Despite this low yield, when field tested strain PM1 successfully removed MTBE from a contaminated sediment. Based on its growth substrate range and 16S rDNA phylogenetic analysis, strain PM1 was classified as Methylibium petroleiphilum, a novel genus and species of β1-Proteobacteria. Strain PM1 was shown to contain a ~4 Mb circular chromosome and a ~600 kb “megaplasmid”. The megaplasmid was found to be conserved among MTBE-degrading bacteria similar to strain PM1 and strains cured of the plasmid lost their ability to degrade MTBE and TBA. The sequence of the genome of strain PM1 was determined and found to contain an alkB ortholog, mdpA, encoding a protein product (MdpA) with 66% similarity to the prototypical alkane hydroxylase (AlkB) from Pseudomonas putida GPo1 (discussed above in Section 1.1.b.) This enzyme may be the MTBE hydroxylase in strain PM1, and recent inactivation and complementation studies targeting mdpA supported this conclusion. However, a comparative gene expression analysis of strain PM1 grown with MTBE or ethanol found that mdpA was not significantly up-regulated in MTBE grown cells. One gene, up-regulated ~12-fold, was suggested to encode an enzyme responsible for hydroxylating TBA to yield
2M12PD. Another gene also up-regulated ~12-fold likely encodes a dehydrogenase and may be involved in catalyzing the formation of TBF.66

The PM1 genome also seems to contain a gene encoding a propane monooxygenase, and all the putative components required for propane metabolism. Other components required for alkane metabolism (such as alcohol and aldehyde dehydrogenases) and various electron transfer proteins (such as rubredoxins and rubredoxin reductases) are present, in addition to genes required for the aerobic biosynthesis of cobalamin.75

Cobalt or cobalamin was found to be required for MTBE- or TBA-dependent growth in strains L108, L10 and CIP I-2052,121,123 and decreasing the initial concentration of cobalamin in the culture medium resulted in a corresponding increase in the transient accumulation of HIBA. These three β-proteobacterial strains are closely related; strains L108 and L10 are 95.6% identical in 16S rRNA sequence to strain PM1. Strain L108 grows using MTBE whereas strain L10, a mutant of strain L108 that has lost its ability to grow using MTBE, grows using TBA123. CIP I-2052 also grows using TBA.121 Comparing protein profiles of strain L108 grown using MTBE, TBA, HIBA and acetate revealed a small protein present only in MTBE-, TBA- and HIBA-grown cells. Sequence analysis suggested it to be the cobalamin-binding subunit of a two-subunit cobalamin-dependent mutase. Therefore, the authors explored the structural rearrangement of HIBA in a cell-free assay. Extracts of strain L108 cells grown using HIBA generated 3-hydroxybutyrate from HIBA whereas those of cells grown using acetate did not. By providing evidence for the rearrangement of the tert-butyl moiety, Rohwerder et al. demonstrated a pathway by which MTBE catabolism
approaches central carbon metabolism. This pathway may be widespread, as homologs of both mutase subunits are present in the megaplasmid of strain PM1.  

1.2.b.3 Aerobic cometabolism. Efforts to elucidate the pathway of MTBE degradation produced early success with aerobic MTBE-cometabolizing strains. Hardison et al. demonstrated that MTBE could be cometabolized to TBF and TBA by a Graphium (a filamentous fungus) strain grown on either propane or n-butane (Figure 1-4). The same enzyme responsible for n-alkane oxidation, a cytochrome P-450, was shown to oxidize MTBE with a maximum initial rate of 10.5 nmol/h/mg dry weight of mycelia. MTBE oxidation likely yielded an unstable hemiacetal, which was rapidly oxidized to yield TBF. TBF oxidation yielded TBA.  

The biodegradation of MTBE by several bacterial strains (notably Mycobacterium austroafricanum (previously vaccae) JOB5 and an environmental isolate, strain ENV425) following growth with propane was also observed by Steffan et al. Strain ENV425 transformed MTBE to “nearly stoichiometric amounts of” TBA. Formaldehyde and CO$_2$ were generated, probably as a product of the oxidation of the methoxy-carbon of MTBE (Figure 1-4). As with Graphium, a cytochrome P-450 was likely responsible for MTBE oxidation in strain ENV 425. Using propane-grown strain ENV425, Steffan et al. recorded the downstream oxidation of TBA to 2M12PD, which was further oxidized to HIBA (Figure 1-4). The mineralization of HIBA was presumed to occur, albeit slowly since this metabolite accumulates during MTBE degradation. MTBE oxidation was unable to support cell-replication.  

All evidence suggests that MTBE and TBA are oxidized by a monooxygenase, in many cases either a cytochrome P-450 or an alkane monooxygenase, two enzymes
with a wide substrate range as previously discussed in Section 1.1.b. The reactions are catalyzed differently in different strains, and these organisms may be grouped into two dichotomies: (i) those that produce TBF, and those that do not; and (ii) those using the same monooxygenase to oxidize MTBE and TBA and those that do not. How a strain fits into the classification may not necessarily predict its ability to metabolize or cometabolize MTBE, although strains PM1 and ENV 735 (both MTBE metabolizing strains) use a different enzyme to oxidize MTBE and TBA, a characteristic shared by no known cometabolizing strains. The biochemistry of MTBE cometabolism is relatively well characterized for several strains, particularly the metabolically-versatile *M. austroafricanum* strain JOB5.

The capacity of strain JOB5 to degrade a wide range of hydrocarbons has been previously discussed in Section 1.1.c. In 1997, Steffan *et al.* showed that propane-grown cells of strain JOB5 cometabolically transformed MTBE. Smith *et al.* quantified the kinetics of MTBE and TBA oxidation by propane-grown cells of strain JOB5. MTBE was oxidized at a specific rate of ~13 nmol min\(^{-1}\) mg protein\(^{-1}\). The \(K_s\) of MTBE oxidation by whole cells was 1.36 mM with a \(V_{\text{max}}\) of 24.4 nmol min\(^{-1}\) mg protein\(^{-1}\). The \(K_s\) of TBA oxidation was 1.18 mM, with a \(V_{\text{max}}\) of 10.4 nmol min\(^{-1}\) mg protein\(^{-1}\). The ratio \(V_{\text{max}}/K_s\) is a measure of catalytic efficiency, and from this we see that MTBE is the preferred substrate over TBA.

Substrate preference is an especially important determinant of MTBE biodegradation in strain JOB5, since MTBE and TBA are apparently oxidized by the same enzyme, a short-chain alkane monooxygease (SCAM). Several lines of evidence support this conclusion: (i) incubating propane-grown cells with MTBE or TBA in the presence of acetylene (an irreversible inactivator of SCAM activity in strain JOB5) stopped the transformation of
these compounds; (ii) propane inhibited MTBE and TBA oxidation ($K_i$ of 3.3 (MTBE) or 4.4 µM (TBA)), suggesting that the same enzyme catalyzed the oxidation of all three substrates; and (iii) SCAM-expressing cells (grown on propane) degraded MTBE and TBA without a lag, whereas non-SCAM-expressing cells (grown on casein-yeast extract-dextrose media) did not oxidize MTBE or TBA within ~1 hour.\textsuperscript{131}

Furthermore, Johnson \textit{et al.}\textsuperscript{72} have shown that MTBE and TBA induce SCAM gene expression in strain JOB5 grown using non-SCAM inducing substrates such as volatile organic acids, sugars, and tricarboxylic acid cycle intermediates. In all cases acetylene inhibited MTBE- and TBA-oxidizing activity (confirming the role of SCAM) but did not significantly reduce growth with non-SCAM substrates. Resting cells grown with glycerol required ~4 h prior to initiating MTBE degradation. No degradation of MTBE was observed in the presence of rifampin (50 µg ml\textsuperscript{-1}) or chloramphenicol (50 µg ml\textsuperscript{-1}), indicating that transcription and \textit{de novo} protein synthesis were required for MTBE degradation in glycerol-grown cells. Cells incubated with MTBE alone showed no evidence of growth or MTBE transformation to TBA.

Smith \textit{et al.}\textsuperscript{131} determined the reactions leading from MTBE to TBA in propane-grown cells of strain JOB5 (Figure 1-4). The predominant initial product of MTBE degradation was TBF ($\geq 80\%$), although low levels of TBA were also formed. The formation of TBF suggested that SCAM oxidized the methoxy-carbon of MTBE, generating an unstable hemiacetal that either decomposed abiotically to form TBA and formaldehyde, or was further oxidized by an alcohol dehydrogenase to yield TBF. Enzyme catalyzed oxidation to TBF was the dominant process because the hemiacetal is undetectable, suggesting rapid
transformation; and TBF, not TBA, was the predominant initial product of MTBE oxidation. Hydrolysis of TBF by a non-specific esterase led to formate and TBA, and whereas further degradation of TBA was observed, the products of this reaction were not detected.

In all three strains discussed above (Graphium sp., strain JOB5 and strain ENV 425), aerobic MTBE cometabolism was superimposed upon a propane-oxidizing enzyme system. Therefore, in order to put the physiology of MTBE cometabolism into context it is important to understand propane metabolism.

1.3 Microbial propane oxidation

1.3.a Terminal and subterminal hydroxylation of propane. A number of bacterial isolates have the ability to grow on propane as the sole source of carbon and energy. These isolates predominantly belong to the genera Arthrobacter, Mycobacterium, Nocardia, Pseudomonas or Rhodococcus. Strains of Nitrosomonas europea, Methyllococcus capsulatus and Pseudomonas (Methylomonas) methanica have also been found to cometabolize propane. The cometabolism of propane by P. methanica growing with methane generated 1-propanol, propionate and acetone, shedding light on the initial steps of the pathway of propane oxidation. A curious inference from this observation is that two separate pathways operate simultaneously during the oxidation of propane. One pathway is for products arising from the terminal oxidation of propane (propane → 1-propanol → propionaldehyde → propionate) whereas the other is for those arising from subterminal oxidation of propane (propane → 2-propanol → acetone). Therefore it seems that propane may be oxidized at either the terminal- or subterminal-carbon atom to yield a mixture of 1-propanol and 2-propanol. Leadbetter and Foster cautioned against this view, suggesting: “An intermediate
1,2-cyclic oxide, a free radical, or a resonating structure could serve as a common precursor of both types of products. Skepticism about two separate pathways also prompted these authors to test the interconversion of propionate and acetone in \textit{P. methanica}, which they were unable to demonstrate. This suggests that two independent pathways for propane utilization were indeed operating simultaneously in this organism.

In the nearly 50 years following Leadbetter and Foster’s work with \textit{P. methanica}, nearly every isolate shown to oxidize propane has generated both terminal and subterminal oxidation products. Stephens and Dalton categorized these strains into three groups; those that (i) did not grow, (ii) grew slowly or (iii) grew readily using acetone. Those strains belonging to the first two groups excreted acetone into the culture media during growth with propane, and were also unable to oxidize acetone following growth with propane. Those strains that grew readily using acetone gave the opposite result. All strains tested grew readily on 1-propanol or propionate. Following from this, the authors suggested that in the case of the first two groups, only the terminal oxidation pathway supported cell replication, even though propane was oxidized both terminally and subterminally. For the third group, both the terminal and subterminal pathways supported cell replication. Despite expanding our knowledge of the initial step in bacterial propane oxidation, Stephens and Dalton concluded by stating that “The relative importance of the terminal and subterminal oxidation pathways in organisms expressing enzymes associated with both pathways remains obscure.” A statement as true today as it was at the time of its writing, 20 years ago.

So far, the only isolate that does not appear to oxidize propane both terminally and subterminally is a \textit{Gordonia} species, strain TY-5. In this bacterium, propane is oxidized
exclusively subterminally to yield 2-propanol. It appears that propane is oxidized by a putative two-component diiron monooxygenase encoded by *prmA* and *prmC*. Electrons are transferred to the monooxygenase by a putative oxidoreductase (*prmB*); and a fourth component, a coupling protein (*prmD*), may influence the reaction rate. Downstream of the *prmABCD* gene cluster, three genes encoding alcohol dehydrogenases were identified. Each enzyme was shown to play an important role in propane utilization by this strain; likely in the conversion of 2-propanol to acetone. Alcohol dehydrogenases were also studied in *Rhodococcus rhodochrous* PNKb1. This strain generated both 1-propanol and 2-propanol during propane oxidation, and Ashraf and Murrell hypothesized that the number and types of alcohol dehydrogenases expressed may reveal the relative importance of each pathway. Two alcohol dehydrogenase-encoding genes were identified, one for 1-propanol and another for 2-propanol. By disrupting each gene, the authors were able to show that both pathways were important for propane oxidation in this organism. The dehydrogenation of 1-propanol yielded propionaldehyde, which was further oxidized to propionate; whereas the dehydrogenation of 2-propanol yielded acetone.

### 1.3.b The fate of propionate.

The utilization of propionate by bacteria relies predominantly on two pathways: one involving the condensation of propionyl-CoA and CO$_2$ whereas the other combines propionyl-CoA with oxaloacetate, forming 2-methylcitrate (Figure 1-5). These two pathways share a common first step: converting propionate to propionyl-CoA. The ATP-dependent carboxylation of propionyl-CoA yields methylmalonyl-CoA, which is further transformed to succinyl-CoA in an isomerase catalyzed reaction. Succinyl-CoA is hydrolyzed to yield succinate, which enters the
tricarboxylic acid (TCA) cycle.\textsuperscript{41, 97, 132} This pathway has been demonstrated in \textit{Micrococcus denitrificans}\textsuperscript{132} and \textit{Rhodococcus rhodochrous}.\textsuperscript{97} In the latter strain, the rate of propane utilization was shown to correlate directly with the initial amount of CO\textsubscript{2} present.\textsuperscript{97}

The 2-methylcitrate pathway for propionate utilization has been described in \textit{Escherichia coli} strain K12\textsuperscript{142} and in \textit{Salmonella enterica} serovar typhimurium strain LT2.\textsuperscript{63} Here, propionyl-CoA condenses with oxaloacetate to form 2-methylcitrate, which enters a modified form of the TCA cycle. In the first step, 2-methylcitrate is oxidized to 2-methyl-cis-aconitate, which is transformed to 2-methylisocitrate that is cleaved to yield pyruvate and succinate. Succinate regenerates oxaloacetate by the familiar TCA cycle.\textsuperscript{63}

\textbf{1.3.c The fate of acetone.} Several pathways have been proposed for the bacterial utilization of acetone; however, initial transformation may occur by one of three reactions: acetone carboxylase mediated carboxylation to yield acetoacetate; monooxygenase mediated oxidation to yield acetol (1-hydroxyacetone); or a Baeyer-Villager monooxygenase catalyzed reaction to yield methyl acetate (Figure 1-6).

Acetone carboxylase has been extensively studied by Scott Ensign’s laboratory using the aerobe \textit{Xanthobacter autotrophicus} strain Py2,\textsuperscript{128, 129} or \textit{Rhodobacter capsulatus} strain B10 grown under anaerobic photoheterotrophic conditions.\textsuperscript{15, 128} Purified acetone carboxylase from each organism was composed of three subunits with molecular masses ~85-, 78-, and 20-kDa.\textsuperscript{127, 128} Enzyme kinetic metrics and required cofactors were also similar, and the genes encoding each subunit were arranged in similar operons.\textsuperscript{128} Acetone carboxylase-encoding genes were induced by acetone, and harvested cell-suspensions of strain Py2 grown on acetone were unable to consume this substrate in the absence of CO\textsubscript{2}. 

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Propionate-carboxylation pathway

2-Methylcitrate pathway

Figure 1-5: Proposed pathways for the utilization of propionate. Propionate-carboxylation pathway is modeled after the diagram by MacMichael and Brown. 2-Methylcitrate pathway is taken from Horswill and Escalante-Semerena.
Figure 1-6: Proposed pathways for the utilization of acetone. Acetone-carboxylation (A) is modeled after diagrams by Ensign et al. Methylglyoxal (B) and hydroxymethyleneacetate (C) routes of acetol metabolism are modeled after diagrams by Taylor et al. and Hartmans and de Bont, respectively. The methylacetate (D) pathway is modeled after the diagram by Kotani et al. (Note: poly-β-hydroxybutyrate is abbreviated PHB.)
Cell extracts of acetone-grown cells carboxylated acetone to form acetoacetate in a CO$_2$-, ATP-, and Mn$^{2+}$-dependent manner.$^{129}$ When cells were grown using acetone in a nitrogen-deficient medium containing NaH$^{13}$CO$_3$, the $^{13}$C label was recovered in the carbon storage polymer poly-$\beta$-hydroxybutyrate (PHB).$^{129}$ PHB may be formed from the 3-hydroxybutyrate that results from the direct reduction of acetoacetate. Another possible fate of acetoacetate is activation by Coenzyme-A, yielding acetoacetyl-CoA, which is cleaved to form two molecules of acetyl-CoA that can enter the TCA cycle.$^{38}$

The oxidation of acetone to yield acetol has been described for several isolates, including *Mycobacterium smegmatis* strain 422,$^{93}$ *Mycobacterium* strain Py1,$^{57}$ and four soil isolates that appear to be *Corynebacterium* species.$^{141}$ The monooxygenase responsible for acetone oxidation is unknown; but in strain Py1, the suite of effective reaction inhibitors suggests the role of a cytochrome P-450 monooxygenase.$^{57}$ In strain Py1, acetol was oxidized to yield an unstable hydroxymethyleneacetate intermediate that spontaneously decomposed to form acetate and formaldehyde.$^{57}$ In contrast, oxidation of acetol by the *Corynebacterium* species was by dehydrogenation of the hydroxyl group to yield methylglyoxal, that was further oxidized to pyruvate.$^{141}$ From this we see how acetol can be converted to acetate or pyruvate, two common central carbon metabolites.

The Baeyer-Villager monooxygenase catalyzed transformation of acetone has been characterized in *Gordonia* strain TY-5. A Baeyer-Villager oxidation of ketones describes the insertion of an oxygen atom between the carbonyl carbon and an adjoining carbon, giving rise to an ester.$^{20}$ In this case, acetone was oxidized to yield methyl acetate. Two-dimensional gel electrophoresis of cell extracts of acetone-grown strain TY-5 revealed two substantially
induced proteins. These were identified as a Baeyer-Villager monooxygenase and an esterase. The esterase was shown to cleave methyl acetate, yielding acetate and methanol. Whereas acetate is used in central carbon metabolism, the fate of methanol is unknown. 83

1.3.d Propane utilization in strain JOB5. Jerome Perry’s laboratory characterized propane oxidation in strain JOB5 from the late 1960’s to the mid 1980’s. During that time, new data, and a fresh look at old data, caused them to substantially alter their view on propane utilization in this strain. Initial data suggested that propane was utilized via the subterminal oxidation pathway only. Cells grown on propane or 2-propanol expressed isocitrate lyase; whereas those grown on 1-propanol or propionate did not. 154 This suggested that propane and 1-propanol were metabolized by non-overlapping pathways, and 1-propanol was therefore not a product of propane oxidation. Three other lines of evidence helped solidify this view: (i) 1-propanol and propionate were shown to be metabolized via the carboxylation of propionyl-CoA (as described in section 1.3.c above) but no evidence of this pathway was detected during propane-metabolism; 154 (ii) a secondary alcohol dehydrogenase appeared to be essential for propane metabolism, and this enzyme was expressed when cells were grown on propane or 2-propanol, but not 1-propanol or propionate; 27 and (iii) the only product detected during propane metabolism was acetone, despite efforts to promote the accumulation of many suspected metabolites, including 1-propanol. 154 Propane-grown strain JOB5 was shown to further oxidize acetone to acetal. It was then postulated that a series of steps would result in the cleavage of acetal into acetate and a C1 product, 154 most likely formaldehyde. 26 As an initiator of the glyoxalate shunt, acetate production would explain the expression of isocitrate lyase in propane- and 2-propanol-grown cells. 154
However, work by Dunlap and Perry, predating the isocitrate lyase data, showed that propane- and acetate-grown cells had substantially different fatty acid profiles, with the fatty acids of the former containing more odd numbers of carbons than the latter.\textsuperscript{35} This work was extended to include propionate and 2-propanol, in which fatty acids from propane-grown cells most resembled those of cells grown using propionate.\textsuperscript{155} These results suggested that strain JOB5 could oxidize either the terminal- or subterminal-carbon of propane. Coleman compared the fatty acid chain lengths of cells grown with propane, 1-propanol, propionate, 2-propanol, acetone and acetate, showing that those of propane-grown cells lay intermediate between those of 2-propanol, acetone and acetate on one hand, and 1-propanol and propionate on the other.\textsuperscript{25} Coleman concluded by writing that in strain JOB5, “propane metabolism is ‘1-propanol-like’ in some respects and ‘2-propanol-like’ in other respects.\textsuperscript{25}”

1.4 Investigations in this dissertation

The ability of propane-grown cells of strain JOB5 to degrade MTBE via aerobic cometabolism using a non-specific SCAM has been established.\textsuperscript{131} Primary substrates for SCAM are components of gasoline, and may therefore support MTBE-cometabolism in gasoline-impacted environments. In Chapter 2, we explore how a diverse array of gasoline hydrocarbons impact MTBE and TBA cometabolism in strain JOB5. In Chapter 3, we extend the known pathway of MTBE transformation in propane-grown strain JOB5 by primarily using \textsuperscript{13}C-nuclear magnetic resonance spectroscopy. And, recognizing that the process of MTBE cometabolism is superimposed on a propane-oxidizing system in strain JOB5, in Chapter 4 we seek to clarify the process of propane catabolism and propane and acetone metabolism in this strain. In Chapter 5, we identify the products excreted during the
oxidation of a fluorinated analog of TBA by bacterial strains that are able to use TBA as a sole source of carbon and energy. Finally, in Chapter 6 we identify products generated during MTBE oxidation by the non-specific ammonia monooxygenase in *Nitrosomonas europaea*. 
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CHAPTER 2

EFFECTS OF GASOLINE CONSTITUENTS ON MTBE AND TBA COMETABOLISM BY MYCOBACTERIUM AUSTROAFRICANUM JOB5†

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† Preliminary findings from this study have been previously reported.
The ability of individual gasoline hydrocarbons (C_{5-10, 12, 14} n-alkanes, C_{5-8} isoalkanes, alicyclics [cyclopentane and methylcyclopentane] and the six BTEX compounds [benzene, toluene, ethylbenzene, m-, o-, and p-xylene]) to support the cometabolism of methyl tertiary butyl ether (MTBE) and tertiary butyl alcohol (TBA) was assayed using Mycobacterium austroafricanum (previously vaccae) JOB5. Most of the tested compounds supported cell growth and concurrent MTBE and TBA oxidation occurred in these cultures without affecting final culture optical density. Growth on C_{5-8} n-alkanes and isoalkanes and concurrent MTBE and TBA oxidation were all inhibited by acetylene whereas an alternate acetylene-insensitive enzyme system appeared to be involved in oxidation of longer chain n-alkanes. A model involving two separate but co-expressed alkane-oxidizing systems is proposed to account for these observations. Cyclopentane, methylcyclopentane, benzene and ethylbenzene did not support growth of this bacterium but all of these compounds inhibited MTBE and TBA oxidation as alternate, competitive substrates for the short-chain alkane monooxygenase (SCAM) thought to be responsible for MTBE and TBA oxidation. Several aromatic compounds (p-xylene>toluene>m-xylene) also supported growth of strain JOB5 and cells grown on these substrates also oxidized MTBE and TBA. The effects of acetylene suggest there are also two distinct toluene-oxidizing activities, a cometabolic process catalyzed by SCAM and another process catalyzed by an undefined system. Low concentrations of toluene were shown to stimulate MTBE and TBA oxidation whereas higher concentrations were inhibitory. $K_s$ values for benzene (6.9 to 23.9 µM) and toluene (15.8 to
20.8 μM) were derived from competitive inhibition experiments and these values were compatible with the effects of these compounds on MTBE oxidation when studied in time course experiments. The results of this study suggest a number of different enzymes are co-expressed in response to gasoline hydrocarbons. 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.
INTRODUCTION

Until recently, methyl tertiary butyl ether (MTBE) was frequently added at concentrations up to 15% (vol/vol) to gasoline sold in regions of the United States. The principal roles for MTBE in gasoline were to increase fuel octane rating and combustion efficiency and to decrease emissions of air pollutants (25, 32). Contamination of ground water sources of drinking water is an important 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 as a result of 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 (16), a full understanding of the environmental fate of MTBE has to adequately consider impacts of other gasoline component co-contaminants frequently encountered with this chemical. This is particularly true for biodegradation processes as they represent the single most important mass-reducing process for MTBE in ground water environments.

A variety of MTBE biodegradation processes have been described. For example, MTBE has been shown to degrade under most of the anaerobic conditions that are often encountered at gasoline-impacted sites (1, 2, 5, 19, 46). Demethylation of MTBE by acetogenic organisms may play an important role in MTBE biodegradation under anaerobic conditions (62) but our understanding of this process is still limited as no pure cultures of anaerobic MTBE-degrading organisms are currently available. Aerobic processes have been shown to be
effective treatment options for MTBE (6, 18, 27, 34, 36, 58, 59). At the organism level, two metabolically distinct aerobic biodegradation processes have been described. A number of β-proteobacteria typified by *Methylibium petroleiphilum* PM1 utilize MTBE as a sole source of carbon and energy for growth (12, 29, 30, 63). Another more diverse group of bacteria, including pseudomonads (7, 8, 41, 43), mycobacteria (42) and actinomycetes (22) can cometabolize MTBE after growth on compounds such as propane, as well as longer chain *n*-alkanes (47) and isoalkanes (14). The abundance of alkanes in gasoline and the wide distribution of alkane-oxidizing activities in bacteria (50, 51) suggest alkane-dependent MTBE cometabolism may be frequently encountered at gasoline–impacted sites. Alkane-dependent MTBE cometabolism could be expected to be especially important when treatment processes involving extensive aeration such as soil vapor extraction and air sparging are employed to simultaneously remove both MTBE and other gasoline co-contaminants from spill site source areas.

Several previous reports have described the impacts of other gasoline components on aerobic MTBE biodegradation (6, 21, 34, 35, 37, 40, 56, 64, 65). However, these studies have typically examined mixed cultures where little is known about the metabolic capabilities of the individual 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 (4) and the closely related strain UC1 (35) have also been restricted to considering the impacts of the aromatic components of gasoline on MTBE oxidation. These
aromatics typically include benzene, toluene, ethylbenzene and \( \alpha-, m- \) and \( p- \) xylene; a group commonly known as the BTEX fraction of gasoline.

Despite the potential importance of cometabolic MTBE biodegradation in aerobic MTBE bioremediation processes, little attention has been focused on the effects of other gasoline components on this process. For example, a recent study of MTBE oxidation by \( n- \) hexane-grown \textit{Rhodococcus} strain EH831 (20) again focused on BTEX compounds and did not address the impacts of other major \( n- \) alkanes, isoalkanes and alicyclics that collectively exceed the total mass of BTEX in gasoline (38). In the present study we have characterized the impacts of multiple major gasoline components on the biodegradation of both MTBE and its commonly encountered metabolite, tertiary butyl alcohol (TBA), by the versatile soil bacterium \textit{Mycobacterium austroafricanum} (previously \textit{vaccae}) JOB5. This organism was originally isolated from an enrichment culture using isopentane (2-methylbutane) as a sole source of carbon and energy (31). Strain JOB5 grows on a wide range of \( n- \) alkanes and isoalkanes (31) but it is best known for its ability to cometabolically oxidize diverse organic pollutants after growth on propane (3, 10, 39, 54, 55). 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 these compounds as mutually exclusive substrates (42). We have also observed that both MTBE and TBA can induce the \textit{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 (15).

The results of this present study demonstrate that cells of strain JOB5 have the ability to oxidize MTBE and TBA after growth on an extensive range of gasoline components including $n$-alkanes, isoalkanes and BTEX compounds including toluene and $p$-xylene. We have characterized both the inhibitory and stimulatory effects of diverse gasoline components on MTBE and TBA oxidation. The results of this study 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), tertiary butyl formate (TBF) (99% 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, Wis.). *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, Tex.). Compressed gases, H₂, N₂, and air, used for gas chromatography (GC) were obtained from local industrial vendors.

Determination of cell growth and MTBE or TBA cometabolism. The ability of individual gasoline hydrocarbons to support growth of strain JOB5 was examined in glass serum vials (160 ml; Wheaton Scientific, Millville, N.J.) containing mineral salts medium (25 ml) (57) and sealed with Teflon-lined Mininert Valves (Alltech Associates Inc., Deerfield, Ill.). Neat
liquid substrates (MTBE, TBA, gasoline hydrocarbons; 5 µl each) were added to sealed vials using a sterile microsyringe. Acetylene gas was added to requisite vials 5% (vol/vol) with a plastic syringe coupled to a Millex disposable filter (0.1 µm; Millipore Co., Bedford, Ma.). To equilibrate reactants between gas and liquid phases, vials were incubated for 18 to 24 h at 30°C in the dark in an Innova 4900 environmental shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) operated at 150 rpm. MTBE and TBA were quantified by removing an aqueous sample (0.2 ml) from the sealed vial using a sterile disposable plastic syringe. This sample was transferred to a flattrip polypropylene microcentrifuge tube (0.5 ml), and 2 µl of the aqueous phase immediately analyzed by GC (see Analytical Methods below for GC parameters). Vials were inoculated (initial OD₆₀₀ ≤0.01) with a liquid suspension of cells (grown on CYD agar plates) in minimal media. After incubating (as above) for 7 days (14 days for BTEX), the amount of MTBE or TBA remaining was quantified by GC, and culture optical density (OD₆₀₀) was determined using a Shimadzu 1601 UV/Vis spectrophotometer (Kyoto, Japan). In some cultures, growth was visibly particulate; therefore all OD₆₀₀ measurements were made following sonication (10 min) of all cultures in a Model 75T Aquasonic water bath (VWR International, Inc., West Chester, Pa.) to help disperse particles.

**Preparation of harvested cells for short-term experiments.** Harvested cells used in all short-term (<8 h) degradation assays, and kinetic constant determinations, were from cultures grown in screw-cap glass media bottles (750 ml; Wheaton Scientific) containing minimal media (100 ml; as above) and sealed with open-top caps fitted with butyl rubber septa. Media was inoculated (initial OD₆₀₀ ~0.01) with cells grown on CYD agar plates. Individual
hydrocarbons 0.05% (vol/vol) served as the sole source of carbon and energy. Cultures were incubated for at least 4 days (see incubation conditions above). Purity was determined by streaking a culture sample (20 µl) onto CYD 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 to 10 mg of total cell protein ml$^{-1}$. The cell suspension was stored at 4°C and used within 4 h.

**Degradation assays and apparent inhibitory constant determinations.** Experiments were conducted in an aqueous reaction volume of 1 ml, in glass serum vials (15 ml) sealed with Teflon-lined Mininert valves. Vials contained phosphate buffer (50 mM; pH 7) and substrates or inhibitors were added directly to sealed vials from aqueous stock solutions or as neat liquids or gases. 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 upon the addition of cells. MTBE or TBA were quantified by GC using aqueous samples (2 µl) taken directly from the reaction medium.

**Analytical Methods.** To quantify MTBE or TBA in growth studies and during short-term degradation assays, aqueous phase samples (2 µl) were analyzed on a Shimadzu GC-14A gas chromatograph (Kyoto, Japan) fitted with a 0.3 × 183 cm stainless steel column filled with Proapak Q (60/80 mesh; Waters Associates, Framingham, Mass.) and held at 180°C. In inhibitory constant determination assays, a shorter (0.3 by 61 cm; held at 160°C) column was
used to improve analysis time and prevent compound coelution with 2-methyl-1,2-propanediol, added as a reductant-regenerating substrate in the reaction medium (as explained in the Results). Kinetic constants were derived by fitting the data to a single substrate-binding model \( y = V_{\text{max}} \cdot \frac{x}{K_s + x} \) using Graph-Pad Prism version 3.0a for Macintosh (Graphpad Software, San Diego, Calif.). Short-term BTEX degradation assays were quantified by introducing a headspace sample 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, Calif.). GC oven temperature varied according to the BTEX compound being analyzed. For benzene and toluene the oven was set at 55°C, for ethylbenzene, 85°C, and for \( m-, o-, \) and \( p-\) xylene, 115°C. For benzene or toluene time course experiments with MTBE, all reactants were quantified from headspace samples introduced to the DB-MTBE capillary column held at 35°C.

All GCs used \( \text{N}_2 \) (flow rate of 15 ml min\(^{-1}\)) as carrier gas and had direct injector ports held at 200°C. All columns were attached to flame ionization detectors operated at 220°C. The GCs were interfaced to Hewlett Packard HP3395 integrators (Palo Alto, Calif.) for data collection.

Cell protein concentrations were determined using the Biuret assay (9) 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, 5.58, 1.43,
1.52, 1.65 and 1.74 mM respectively (26, 33, 60). 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 (24, 28).
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 potential growth-supporting substrates for strain JOB5 in carbon-limited 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 2.1). 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) under the conditions used in these experiments.

When MTBE was added with each growth-supporting substrate at a 1:1 vol% ratio, there was generally little (\leq 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 2.1). In these instances, MTBE decreased the final culture density obtained by between \sim 35 to \sim 85\% compared to cultures grown in the absence of MTBE. In contrast to MTBE, TBA (1:1 vol% ratio) had more limited effects and only inhibited growth by \geq 25\% in cultures containing n-tetradecane and toluene. Neither MTBE nor TBA supported growth of strain JOB5 when present as the sole organics in the culture medium.
With the exception of \textit{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 was \textasciitilde40\% and the highest levels (\textgtr=50\%) were observed in cultures grown on \textit{n}-pentane, \textit{n}-hexane, 2-methylbutane, 2-methylhexane and \textit{p}-xylene. The levels of TBA consumption in all cultures typically exceeded those observed with MTBE. The average level of TBA oxidation among all growth substrates was \textasciitilde51\% and MTBE consumption only exceeded TBA consumption in cultures grown on \textit{n}-hexane, \textit{n}-tetradecane, toluene, and \textit{p}-xylene.

We also tested the effects of acetylene on growth of strain JOB5 on hydrocarbons. Growth on C\textsubscript{5-9} \textit{n}-alkanes and all branched alkanes was fully inhibited by acetylene (Table 2.1) but variable results were observed with other compounds. For example, in the presence of acetylene, growth on \textit{n}-tetradecane (C\textsubscript{14}) and \textit{m}-xylene was unaffected, while growth on toluene or \textit{p}-xylene was only partially inhibited and growth on \textit{n}-dodecane (C\textsubscript{12}) was stimulated relative to cultures grown in the absence of this gas. When culture growth was observed in the presence of acetylene, this gas also 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 \textit{n}-dodecane-grown cultures.

**MTBE and TBA oxidation by resting cells.** The results presented in Table 2.1 suggest strain JOB5 can oxidize both MTBE and TBA during growth on a wide range of
hydrocarbons. In subsequent experiments, cells were grown on each of the growth-supporting substrates identified in Table 2.1 and initial specific rates of both MTBE and TBA oxidation were then determined for resting cells that had been washed to remove residual growth-supporting hydrocarbons. For cells grown on n-alkanes and isoalkanes, the specific rates of MTBE generally decreased with increases in alkane chain length although other trends could be observed (Table 2.2). For example, cells grown on either C₅ and C₆ n-alkanes or isoalkanes oxidized MTBE at similar rates (mean = 40 nmol min⁻¹ mg total protein⁻¹), as did cells grown on C₇ alkanes; n-heptane and 2-methylhexane (mean = 25 nmol min⁻¹ mg total protein⁻¹). Cells grown on n-alkanes longer than C₈ also oxidized MTBE at a generally consistent rate that was between 4- to 6-fold lower than the maximal rate observed with cells grown on n-pentane. With cells grown on isoalkanes, a similar fold decrease in specific activity was observed between cells grown on 2-methylbutane compared to cells grown on 2-methylheptane. The specific rates of TBA oxidation also generally decreased with increases in n-alkane chain length. Cells grown on C₅₆ n-alkanes oxidized TBA at roughly similar rates (mean = 20 nmol min⁻¹ mg total protein⁻¹), whereas those grown on C₁₀₋₁₄ n-alkanes showed virtually no TBA-oxidizing activity (mean = 1 nmol min⁻¹ mg total protein⁻¹). The most rapid rate of TBA oxidation (42 nmol min⁻¹ mg total protein⁻¹) was observed for 2-methylbutane grown cells, and an ~4-fold decrease in this rate was observed for cells grown on all longer chain isoalkanes.

The specific rates of both MTBE and TBA oxidation by cells grown on aromatic compounds (toluene, m- and p-xylene) were within the range observed with cells grown on n-alkanes and
isoalkanes. However, substantial variability in the rates was observed with cells grown on toluene and \textit{m}-xylene.

**Inhibition of MTBE and TBA oxidation.** Short term incubations (≤1 h) with resting cells previously grown on either \textit{n}-pentane or \textit{2-methyl}butane were used to examine potential inhibitory effects of each growth-supporting hydrocarbon on MTBE and TBA oxidation. The rationale for these experiments was that compounds that could be oxidized by the same enzyme responsible for MTBE and TBA oxidation should act as mutually exclusive competitive inhibitors. In these experiments substantially similar effects were observed with both \textit{n}-pentane and \textit{2-methyl}butane-grown cells (Table 2.3). With \textit{n}-alkanes, shorter chain compounds (C\textsubscript{5-8}) consistently and strongly (≥70\%) inhibited both MTBE and TBA oxidation and the inhibitory effects then tended to decrease with further increases in carbon chain length.

All of the isoalkanes and alicyclic compounds tested were potent inhibitors of both MTBE and TBA oxidation by both \textit{n}-pentane and \textit{2-methyl}butane-grown cells (Table 2.3). With the exception of TBA oxidation by \textit{2-methyl}butane-grown cells, both MTBE and TBA oxidation were inhibited ≥70\% in all cases. All of the aromatic compounds tested also inhibited MTBE and TBA oxidation by \textit{n}-pentane and \textit{2-methyl}butane-grown cells but these aromatics were consistently less inhibitory than any of the isoalkanes or alicyclic compounds tested. Toluene was the most consistently inhibitory aromatic compound tested (67-88\%) while \textit{p}-xylene was consistently the least inhibitory compound (28-55\%).
The inhibitory effects of several important gasoline components (n-pentane, 2-methylbutane, benzene and toluene) on MTBE and TBA oxidation by n-pentane-grown cells were quantified by determining the effect of varying concentrations of each compound on the initial rate of oxidation of a fixed amount of MTBE or TBA. When the tested compound was also a growth-supporting substrate (n-pentane, 2-methylbutane and toluene), low concentrations (≤10 µM) consistently stimulated both MTBE and TBA oxidation while inhibitory effects were observed with higher concentrations (Figure 2.1 A, B and D inset). This stimulatory effect was eliminated when an alternate source of reductant (2-methyl-1,2-propanediol) was included in the reaction mixture. Good fits ($r^2 \geq 0.92$) of these inhibition data were obtained for all tested compounds using a hyperbolic, single substrate competitive binding model. The apparent $K_i$ ($K_i^{app}$) values derived from these analyses (Table 2.4) were converted to true $K_i$ values ($K_i^{true}$) using the following equation $K_i^{true} = K_i^{app} / (1 + [MTBE \text{ or } TBA] / K_s$) using the previously determined $K_s$ values for MTBE (1.36 mM) and TBA (1.18 mM) for propane-grown cells of strain JOB5 (42). 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 2.4). 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) while the value for benzene as an inhibitor of TBA oxidation was ~2-fold lower (~7 µM).
Oxidation of aromatic compounds: In view of the competitive nature of the inhibitory effects of benzene and toluene on MTBE and TBA oxidation (Tables 2.3 and 2.4), we investigated the potential oxidation of aromatic compounds by 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 2.5). Benzene consumption was also fully (>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 fully inhibited (≥90%) when heat-killed cells were used indicating that abiotic losses in our experimental system were negligible.

The effects of benzene and toluene on the oxidation of MTBE and TBA by n-pentane-grown cells were also examined in time course experiments. n-Pentane-grown cells rapidly oxidized benzene (250 nmoles) or MTBE (1 µmoles) without a lag phase when these compounds were added individually and oxidation of both compounds was strongly inhibited by acetylene (Figure 2.2A). 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 strongly inhibited by the presence benzene although a slow but progressively increasing rate of MTBE oxidation developed after 4 h as the residual benzene (≤50 µM) was consumed. In comparable experiments conducted with toluene and MTBE, the rate of toluene oxidation was ~8-fold faster than the rate of benzene oxidation when these compounds were added individually while the rate of MTBE oxidation as a sole substrate was substantially similar. Like the effect observed with mixtures of benzene and MTBE, 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 (Figure 2.2B). Unlike the effect of acetylene on benzene and MTBE oxidation, toluene oxidation was only partially inhibited by this gas.
DISCUSSION

The results of this study demonstrate gasoline hydrocarbons have diverse effects on MTBE and TBA oxidation by strain JOB5. The following sections individually discuss the effects of alkanes (n-alkanes and isoalkanes), and then alicyclics and aromatics. The broader implications of our findings are addressed in the final section of this discussion.

Effects of \textit{n-alkanes and isoalkanes}. Our results confirm the broad alkane-metabolizing activity of strain JOB5 (31) and indicate that cells grown on many of the major normal and branched alkane components of gasoline (C$_5$-C$_{10}$) consistently oxidize both MTBE and TBA in carbon-limited batch cultures (Table 2.1). Our results also show acetylene consistently inhibited both MTBE and TBA oxidation while this inhibitor had variable effects on alkane-dependent growth. For instance, growth on shorter chain \textit{n}-alkanes (C$_5$-C$_9$) and all isoalkanes tested was strongly inhibited by this gas, while growth on longer chain (≥C$_{10}$) \textit{n}-alkanes was largely unaffected.

One interpretation of our observations is that while individual alkane growth substrates potentially impacted the type of alkane-oxidizing enzymes expressed by strain JOB5, these effects were obscured by the previously described ability of both MTBE and TBA to induce the expression of the enzyme system required for their own biodegradation (15). However, this interpretation seems unlikely as resting cells previously grown on alkanes in the absence of MTBE or TBA all had varying but typically substantial (≥10 nmoles min$^{-1}$ mg protein$^{-1}$)
levels of MTBE-oxidizing activity (Table 2.2). An alternative and more likely explanation of these results (Table 2.1 and 2.2) is that strain JOB5 can simultaneously express more than one alkane-oxidizing enzyme system while only one of these enzymes is responsible for oxidizing MTBE and TBA. In support of this model we observed high levels of expression of an acetylene-sensitive alkane-oxidizing system during growth on shorter chain \(n\)-alkanes (\(C_5-C_8\)) (Tables 2.1 and 2.2). We suggest this activity is catalyzed by a short-chain alkane monooxygenase (SCAM) and that this is the same enzyme system we have previously characterized during our studies of MTBE and TBA oxidation by propane-grown cells of this bacterium (42). We further suggest that this is the only enzyme that is responsible for MTBE and TBA oxidation. In cells grown on longer chain \(n\)-alkanes (\(\geq C_9\)), our model suggests this SCAM system is still expressed, albeit at lower levels than those expressed during growth on shorter chain \(n\)-alkanes (\(\leq C_8\)) (Table 2.2). This model also suggests strain JOB5 progressively responds to longer \(n\)-alkanes (\(\geq C_9\)) by expressing a second, acetylene-insensitive long-chain alkane-oxidizing system (LCAM) that initiates alkane catabolism but does not oxidize MTBE or TBA.

Further support for this two-enzyme model can be seen in our experiments examining \(n\)-alkanes as potential inhibitors of MTBE and TBA oxidation (Table 2.3). Shorter chain \(n\)-alkanes (\(\leq C_8\)) were generally equally effective as inhibitors but inhibition by longer chain \(n\)-alkanes rapidly decreased with further increases in carbon chain length. \textit{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 accompanying decrease in aqueous
solubility. In contrast, the step-like decrease in inhibition we observed likely reflects that C₅ to C₈ n-alkanes are effective substrates for SCAM whereas longer chain n-alkanes (≥C₈) would be expected to be weaker inhibitors due to their diminished ability to bind to an enzyme seemingly adapted to the oxidation of shorter chain n-alkanes. The same trends observed in our inhibition studies (Table 2.3) were also seen in our specific activity measurements (Table 2.2) and growth data (Table 2.1). All of these approaches consistently point to a significant alteration in enzyme activities that occurs during the transition from growth on shorter chain n-alkanes (≤C₈) to growth on longer chain n-alkanes (≥C₁₀).

While we recognize the model outlined above is based only on culture and inhibition studies, a number of molecular studies have also indicated that multiple and sometime diverse alkane-oxidizing enzyme systems are common in alkane-oxidizing bacteria exhibiting broad alkane growth substrate ranges (48). A currently extreme example includes various Rhodococcus erythropolis isolates that contain as many as five genes encoding non-heme iron alkane hydroxylases and two genes encoding heme-containing alkane-oxidizing cytochrome P450s (49). A particularly relevant study involves the n-butane-metabolizing bacterium, Nocardioïdes CF8 (11). This organism grows on a similar range of n-alkanes (C₂-C₁₆) to those examined in this study and the diversity of n-alkane-oxidizing systems in this strain was also initially revealed through the use of selective inhibitors such as allylthiourea, a copper selective chelator, and 1-hexyne, a proposed mechanism-based inactivator similar to acetylene. This study concluded that n-butane-grown cells simultaneously express two
different alkane-oxidizing enzyme systems with different but overlapping substrate specificities.

**Effects of alicyclics and aromatics:** The two alicyclic compounds examined in this study, cyclopentane and methylcyclopentane, did not support growth of strain JOB5 (Table 2.1) but were both potent inhibitors of MTBE and TBA oxidation by \( n \)-pentane- and 2-methylbutane-grown cells (Table 2.3). Numerous alicyclics, including both cyclopentane and methylcyclopentane, are oxidized to the corresponding cycloketones by strain JOB5 after growth on 2-methylbutane (31). Although we did not confirm the cometabolic oxidation of these compounds, our results and these earlier observations suggest cyclopentane and methylcyclopentane are likely to inhibit MTBE and TBA oxidation primarily through competitive interactions at the active site of SCAM.

A broad range of effects was observed with the six aromatic compounds tested in this study. It was previously reported that benzene does not support growth of strain JOB5 but that it is cometabolically oxidized by propane-grown cells (3). Most observations in this present study are compatible with, or extend these earlier observations. These include culture growth data (Table 2.1), the complete inhibition of benzene oxidation by \( n \)-pentane-grown cells using acetylene (Table 2.5 and Figure 2.2A), and the behavior of benzene as a competitive inhibitor of both MTBE (Figure 2.1C and 2.2A) and TBA (Figure 2.2A) oxidation by \( n \)-pentane-grown cells. We have also confirmed benzene oxidation generates phenol and hydroquinol as oxidation products (data not shown). Collectively, these observations suggest that benzene,
like the alicyclic compounds discussed above, most likely inhibits MTBE and TBA oxidation through competitive interactions at the active site of SCAM.

The $K_i$ values derived for benzene as an inhibitor of MTBE (~24 µM) and TBA (~7 µM) oxidation (Table 2.4) also provide two estimates of the $K_i$ value for this compound as an independent SCAM substrate. Both estimates are considerably lower than the $K_i$ for MTBE (~1.4 mM) in propane-grown cells of strain JOB5 (42). Assuming this $K_i$ for MTBE is a consistent feature of SCAM, irrespective of the alkane used to support cell growth, it is not surprising that MTBE (1 µmole) had little discernable effect on the rate of oxidation of benzene (250 nmoles) when n-pentane-grown cells were simultaneously exposed to both compounds (Figure 2.2A). Conversely, it is also not surprising that MTBE-oxidizing activity was strongly inhibited in the presence of benzene, and MTBE oxidation was only observed once the initial benzene concentration had been reduced by ~80% (Figure 2.2A).

Although more limited, our results for ethylbenzene also agree with the earlier findings of Burback and Perry (3) and suggest the effects of this non-growth-supporting aromatic on MTBE and TBA oxidation can also be primarily accounted for through competition for cometabolic oxidation by SCAM. Burback and Perry (3) also noted both benzene and ethylbenzene are not oxidized by propane-grown cells of strain JOB5 when added at high initial concentrations (0.1 g l$^{-1}$). This suggests these compounds, like many organics, may indirectly inhibit MTBE and TBA oxidation through non-specific toxic effects. This possibility was not explored in this study.
Toluene was also confirmed as a growth-supporting substrate for strain JOB5 (3) but unlike this earlier study, our results suggest there are two distinct toluene-oxidizing activities. For example, several experiments (Figure 2.1D and Figure 2.2B) indicate toluene competently interacts with MTBE while in other experiments the limited growth on toluene (Table 2.1) and the rapid oxidation of toluene by n-pentane-grown cells (Table 2.5 and Figure 2.2B) were both only partially inhibited by acetylene. Taken together, these results suggest acetylene-sensitive toluene oxidation is most likely due to SCAM activity while the second toluene-oxidizing activity could be reasonably attributed to another enzyme that, like toluene 2-monooxygenase (61), is essentially unresponsive to the concentrations of acetylene used in this study.

The $K_{i}^{true}$ and hence $K_i$ values determined for the competitive inhibition of SCAM-dependent MTBE and TBA oxidation (Figure 2.1D) indicate toluene is similar to benzene in its effectiveness as an inhibitor of MTBE and TBA oxidation (Table 2.4). Like benzene, time course experiments confirmed toluene strongly inhibited MTBE oxidation until the initial concentration of toluene had been substantially reduced (Figure 2.2B). However, unlike benzene, low concentrations of toluene also stimulated both MTBE and TBA oxidation (Figure 2.1D inset). This effect was also observed with low concentrations of n-pentane and 2-methylbutane and in all cases the stimulation effect was eliminated using an alternate reductant source (2-methyl-1,2-propanediol) (Figure 2.1 A, B). These results suggest that only compounds that can be substantially catabolized as growth-supporting and hence
reductant-generating substrates can stimulate SCAM-catalyzed reactions. This interpretation again suggests that alkane-grown cells have two toluene-oxidizing activities; a SCAM-catalyzed reaction that would be expected to generate a hydroxylated toluene derivative that may or may not be further oxidized, as well as a toluene-metabolizing system that can generate reducing power that can be utilized in SCAM-catalyzed reactions. A stimulating effect of toluene has also previously been described for trichloroethylene biodegradation of propane-grown cells of strain JOB5 (52). Like our present results, this earlier observation suggests co-expression of both a toluene-cometabolizing activity and a toluene-metabolizing activity in cells of strain JOB5 grown under conditions where SCAM is the predominantly expressed alkane-oxidizing enzyme.

Our observation that p-xylene, and to a lesser extent m-xylene, are also growth-supporting substrates for strain JOB5 is a novel observation that extends the recognized hydrocarbon-oxidizing capabilities of this versatile bacterium. Growth on p-xylene was comparable to some longer chain n-alkanes (Table 2.1) and p-xylene-grown cells readily oxidized MTBE both in carbon-limited cultures (Table 2.1) and after growth in MTBE-free media (Table 2.2). Like toluene, oxidation of p-xylene by n-pentane-grown cells was only partially inhibited by acetylene (Table 2.5). Our ongoing studies with this compound have shown p-xylene-grown cells rapidly oxidize toluene and that toluene-grown cells can also rapidly oxidize p-xylene (data not shown). These observations suggest there may be a common enzyme system involved in the catabolism of these aromatic compounds. However, further studies are needed to determine whether MTBE oxidation by p-xylene-grown cells is due to a co-
expressed alkane-oxidizing enzyme like SCAM or whether this is an unusual example of MTBE oxidation catalyzed by an enzyme involved in aromatic catabolism.

**Broader implications.** An important general observation made in this study is that the inhibitory and stimulatory effects observed for MTBE oxidation have typically also equally impacted TBA oxidation. This supports our earlier suggestion that MTBE and TBA are oxidized by the same enzyme in cells of strain JOB5 expressing SCAM activity (42). However, some exceptions were noted. For example, cells grown on longer chain (>C<sub>10</sub>) n-alkanes had consistently higher mean specific rates of MTBE oxidation than TBA oxidation (Table 2.2). This disparity may be meaningful but could also reflect the large variability we encountered in determining low MTBE and TBA oxidation rates. Another example is the 4-fold difference in the \( K_{i, \text{true}} \) values determined for benzene inhibition of MTBE and TBA oxidation (Table 2.4). The difference in \( K_{i, \text{true}} \) values may again reflect variability or possibly a secondary effect of the phenol that we confirmed is generated and further oxidized to hydroquinol during benzene oxidation.

While we examined a wide range of gasoline components, there are also several major gasoline fractions such as alkenes and multibranched alkanes that were not examined in this study. Isooctane (2,2,4-trimethylpentane) is a slowly biodegradable component (45) but 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 (45) and this strain is known to cometabolically oxidize MTBE (44). As we have shown in
this study of strain JOB5, this MTBE-oxidizing activity may be related to the ability of these organisms to catabolize branched hydrocarbon structures.

A limitation of this study is that key enzymes were not identified at the molecular level. However, the trends and models presented are providing the framework for current genome-enabled proteomic studies with strain JOB5 that aim to further explore the enzymology of alkane, MTBE, and TBA oxidation and co-expression of alkane- and aromatic-oxidizing enzyme systems in this organism. The molecular nature of the enzyme responsible for initiating TBA oxidation in strain JOB5 has also been investigated by others interested in TBA biodegradation in various mycobacteria (23). A closely-related alkane hydroxylase has consistently been shown to be present in the genomes of TBA-oxidizing mycobacteria that can grow on diverse \textit{n}-alkanes from C\textsubscript{2} to C\textsubscript{16}. From physiological and molecular studies it was concluded that this enzyme is responsible for initiating TBA oxidation in these strains. However, TBA-oxidizing activity was determined in resting cells exposed to TBA for 24 h; conditions we have previously shown can lead to autoinduction of both MTBE- and TBA-oxidizing activity (15). Furthermore, in studies of the alkane hydroxylase variant in \textit{Pseudomonas putida} GPo1 we have shown this enzyme does not oxidize TBA and has a very high $K_s$ value for MTBE (41), and we have subsequently established this enzyme is not inhibited by acetylene. The alkane hydroxylase variant from strain GPo1 is one of a limited number of examples of this enzyme which oxidize shorter chain \textit{n}-alkanes ($<C_{10}$) while most variants have substrate ranges restricted to longer chain \textit{n}-alkanes ($\geq C_{10}$). Based on these observations, and our evidence presented here that strain JOB5 can simultaneously express
more than one alkane-oxidizing enzyme after growth on alkane growth substrates, it seems likely that these studies have identified an alkane-oxidizing enzyme common to TBA-oxidizing mycobacteria, but not one that is responsible for either TBA or MTBE oxidation. By the same argument, our evidence presented here that alkane-grown cells of strain JOB5 can co-express not only different alkane-oxidizing enzymes but apparently also different toluene-oxidizing enzymes raises concerns about the previous assignment of diverse cometabolic oxidation reactions to the propane-oxidizing enzyme system in strain JOB5 (3, 53, 54).

A final broader implication concerns a recent molecular characterization of another alkane-hydroxylase-like enzyme in the MTBE-metabolizing bacterium *M. petroleiphilum* PM1. This enzyme has been argued to initiate MTBE oxidation and has also been suggested to enable this organism to initiate alkane catabolism although no data has been presented to support this second physiological capability (13, 17). 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 components can be expected to inhibit MTBE oxidation by this organism.
ACKNOWLEDGMENTS

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<table>
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<th>Final OD$_{600}$$^c$</th>
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<td>(0.00)</td>
</tr>
<tr>
<td>Aromatics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>benzene</td>
<td>0.12 -</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>3.50</td>
<td>(0.00)</td>
<td>(0.00)</td>
</tr>
<tr>
<td>toluene</td>
<td>2.73 -</td>
<td>0.09</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>21.80</td>
<td>(0.01)</td>
<td>(0.01)</td>
</tr>
<tr>
<td>ethylbenzene</td>
<td>0.36 -</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>2.86</td>
<td>(0.00)</td>
<td>(0.00)</td>
</tr>
<tr>
<td>o-xylene</td>
<td>0.68 -</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>2.86</td>
<td>(0.00)</td>
<td>(0.00)</td>
</tr>
<tr>
<td>m-xylene</td>
<td>1.77 -</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>3.87</td>
<td>(0.00)</td>
<td>(0.00)</td>
</tr>
<tr>
<td>p-xylene</td>
<td>0.77 -</td>
<td>0.17</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>1.58</td>
<td>(0.01)</td>
<td>(0.03)</td>
</tr>
</tbody>
</table>
Table 2.1 continued

" Cultures were incubated for 7 days (14 days for BTEX). Data represent the mean and SEM (in parentheses) of at least duplicate samples. Abiotic controls maintained an OD$_{600}$ ≤ 0.01, and showed no loss of MTBE or TBA (data not shown).

b Gasoline composition reported by Riser-Roberts (38).

c Initial culture OD$_{600}$ ≤ 0.01.

d Percent consumed relative to initial amount of either MTBE or TBA.

e ND: not determined.
**Table 2.2.** Specific activity of MTBE or TBA oxidation

<table>
<thead>
<tr>
<th>Growth supporting substrate</th>
<th>Specific activity (nmol min⁻¹ mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTBE</td>
</tr>
<tr>
<td><strong>n-alkanes</strong></td>
<td></td>
</tr>
<tr>
<td>n-pentane</td>
<td>46 (2.5)</td>
</tr>
<tr>
<td>n-hexane</td>
<td>41 (4)</td>
</tr>
<tr>
<td>n-heptane</td>
<td>24 (6)</td>
</tr>
<tr>
<td>n-octane</td>
<td>23 (2.5)</td>
</tr>
<tr>
<td>n-nonane</td>
<td>11 (10)</td>
</tr>
<tr>
<td>n-decane</td>
<td>11 (6.5)</td>
</tr>
<tr>
<td>n-dodecane</td>
<td>12 (2.5)</td>
</tr>
<tr>
<td>n-tetradecane</td>
<td>8 (6.5)</td>
</tr>
<tr>
<td><strong>isoalkanes</strong></td>
<td></td>
</tr>
<tr>
<td>2-methylbutane</td>
<td>34 (1.5)</td>
</tr>
<tr>
<td>2-methylpentane</td>
<td>37 (7)</td>
</tr>
<tr>
<td>2-methylhexane</td>
<td>25 (3)</td>
</tr>
<tr>
<td>2-methylheptane</td>
<td>6 (5)</td>
</tr>
<tr>
<td><strong>aromatics</strong></td>
<td></td>
</tr>
<tr>
<td>toluene</td>
<td>5 (4)</td>
</tr>
<tr>
<td>m-xylene</td>
<td>19 (10.5)</td>
</tr>
<tr>
<td>p-xylene</td>
<td>20 (7)</td>
</tr>
</tbody>
</table>

* Data represent the mean and SEM (in parentheses) of at least duplicate samples.
* Harvested cells (0.12-0.57 mg of total protein) incubated with 2.5 µmoles of either MTBE or TBA.
Table 2.3. Effect of gasoline hydrocarbons on either MTBE or TBA oxidation

<table>
<thead>
<tr>
<th>Hydrocarbon added</th>
<th>Percent inhibition&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MTBE</th>
<th>TBA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Harvested cells grown on</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n-Pentane</td>
<td>2-Methylbutane</td>
<td>n-Pentane</td>
</tr>
<tr>
<td>n-alkanes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-pentane</td>
<td>90 (10)</td>
<td>87 (1)</td>
<td>98 (8)</td>
</tr>
<tr>
<td>n-hexane</td>
<td>80 (6)</td>
<td>113 (3)</td>
<td>84 (6)</td>
</tr>
<tr>
<td>n-heptane</td>
<td>91 (5)</td>
<td>84 (8)</td>
<td>97 (2)</td>
</tr>
<tr>
<td>n-octane</td>
<td>89 (6)</td>
<td>88 (4)</td>
<td>71 (4)</td>
</tr>
<tr>
<td>n-nonane</td>
<td>40 (4)</td>
<td>45 (5)</td>
<td>48 (9)</td>
</tr>
<tr>
<td>n-decane</td>
<td>14 (3)</td>
<td>23 (3)</td>
<td>8 (4)</td>
</tr>
<tr>
<td>n-dodecane</td>
<td>≤1 (7)</td>
<td>5 (9)</td>
<td>≤1 (8)</td>
</tr>
<tr>
<td>n-tetradecane</td>
<td>≤1 (7)</td>
<td>2 (2)</td>
<td>≤1 (1)</td>
</tr>
<tr>
<td>isoalkanes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-methylbutane</td>
<td>75 (8)</td>
<td>90 (2)</td>
<td>92 (8)</td>
</tr>
<tr>
<td>2-methylpentane</td>
<td>101 (5)</td>
<td>99 (0)</td>
<td>95 (3)</td>
</tr>
<tr>
<td>2-methylhexane</td>
<td>78 (2)</td>
<td>88 (7)</td>
<td>92 (6)</td>
</tr>
<tr>
<td>2-methylheptane</td>
<td>71 (5)</td>
<td>56 (2)</td>
<td>96 (1)</td>
</tr>
<tr>
<td>alicyclics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyclopentane</td>
<td>100 (3)</td>
<td>98 (7)</td>
<td>102 (2)</td>
</tr>
<tr>
<td>methylcyclopentane</td>
<td>99 (3)</td>
<td>98 (0)</td>
<td>95 (7)</td>
</tr>
<tr>
<td>BTEX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>benzene</td>
<td>41 (2)</td>
<td>34 (6)</td>
<td>57 (4)</td>
</tr>
<tr>
<td>toluene</td>
<td>75 (10)</td>
<td>74 (6)</td>
<td>88 (1)</td>
</tr>
<tr>
<td>ethylbenzene</td>
<td>63 (9)</td>
<td>71 (9)</td>
<td>71 (6)</td>
</tr>
<tr>
<td>o-xylene</td>
<td>49 (2)</td>
<td>71 (3)</td>
<td>84 (0)</td>
</tr>
<tr>
<td>m-xylene</td>
<td>23 (2)</td>
<td>64 (8)</td>
<td>43 (7)</td>
</tr>
<tr>
<td>p-xylene</td>
<td>30 (6)</td>
<td>28 (6)</td>
<td>28 (5)</td>
</tr>
</tbody>
</table>

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

<sup>b</sup> Percent inhibition reports the degree to which MTBE or TBA oxidation was inhibited by each hydrocarbon added relative to reactions incubated in the presence of either MTBE or TBA alone.

<sup>c</sup> Greater than 100% inhibition indicates an increase in the amount of MTBE or TBA detected during the experiment, and is likely a sampling artifact.
Figure 2.1. Inhibition of MTBE and TBA oxidation by major gasoline components: \textit{n}-pentane (A), 2-methylbutane (B), benzene (C) and toluene (D). \textit{n}-Pentane grown cells were incubated with either MTBE (0.9 mM dissolved concentration) or TBA (1 mM dissolved concentration), increasing amounts of inhibitor, and, in parts A, B and D, a reductant regenerating substrate (2-methyl-1,2-propanediol; as explained in the Results section). The inset to parts A, B and D shows results obtained in the absence of the reductant regenerating substrate. Data points represent percent inhibition of either MTBE (○) or TBA (△) oxidation at each inhibitor concentration relative to cells incubated in the absence of inhibitor. Results were computer fitted to a hyperbola by nonlinear regression and used to derive apparent constants of inhibition listed in Table 2.4.
Table 2.4. Apparent and true inhibition constants

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i^{\text{app}}$ (µM)</th>
<th>$K_i^{\text{true}}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTBE</td>
<td>TBA</td>
</tr>
<tr>
<td>n-pentane</td>
<td>9.4 (2.0)</td>
<td>10.1 (2.9)</td>
</tr>
<tr>
<td>2-methylbutane</td>
<td>5.9 (1.8)</td>
<td>3.0 (0.8)</td>
</tr>
<tr>
<td>benzene</td>
<td>39.6 (10.1)</td>
<td>12.8 (2.6)</td>
</tr>
<tr>
<td>toluene</td>
<td>34.6 (11.0)</td>
<td>29.3 (5.5)</td>
</tr>
</tbody>
</table>

Each experiment used harvested cells (grown on n-pentane). Data represent the mean and SEM (in parentheses) of duplicate samples. In all cases, good fits ($r^2 \geq 0.92$) between data points and hyperbolic regression curves were obtained (graphs are shown in Figure 2.1).

$K_i^{\text{true}}$ values were calculated from the $K_i^{\text{app}}$ value, as explained in the Results section.
Table 2.5. Consumption of BTEX by SCAM induced cells$^a$

<table>
<thead>
<tr>
<th>BTEX added</th>
<th>Percent consumed</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Active cells</td>
<td>Acetylene treated cells</td>
<td>Heat treated cells</td>
<td></td>
</tr>
<tr>
<td>benzene</td>
<td>$&gt;99$ (0)</td>
<td>8 (0)</td>
<td>$\leq 1$ (1)</td>
<td></td>
</tr>
<tr>
<td>toluene</td>
<td>$&gt;99$ (0)</td>
<td>50 (2)</td>
<td>$\leq 1$ (1)</td>
<td></td>
</tr>
<tr>
<td>ethylbenzene</td>
<td>$&gt;99$ (0)</td>
<td>39 (5)</td>
<td>$\leq 1$ (6)</td>
<td></td>
</tr>
<tr>
<td>$o$-xylene</td>
<td>50 (4)</td>
<td>29 (4)</td>
<td>$\leq 1$ (0)</td>
<td></td>
</tr>
<tr>
<td>$m$-xylene</td>
<td>$&gt;99$ (0)</td>
<td>51 (3)</td>
<td>$\leq 1$ (6)</td>
<td></td>
</tr>
<tr>
<td>$p$-xylene</td>
<td>$&gt;99$ (0)</td>
<td>51 (2)</td>
<td>7 (11)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Harvested cells (grown on $n$-pentane) were incubated with each BTEX compound (250 nmoles) for 3 h. Data represent the mean and SEM (in parentheses) of duplicate samples that contained either 0.5 or 0.4 mg of total cell protein, respectively.
Figure 2.2. Degradation of either benzene (A) or toluene (B) (250 nmoles each) and MTBE (1000 nmoles) by n-pentane grown cells (0.9 mg (A) or 0.4 mg (B) total cell protein). Reaction vials contained (i) benzene or toluene alone (▲), (ii) MTBE alone (●), (iii) mixtures of either benzene or toluene (△) and MTBE (○), and (iv) mixtures of either benzene or toluene (+) and MTBE (✗) in the presence of acetylene (10% [vol/vol], gas phase).
REFERENCES


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CHAPTER 3

NUCLEAR MAGNETIC RESONANCE STUDIES OF METHYL TERTIARY BUTYL ETHER COMETABOLISM BY PROPANE-GROWN MYCOBACTERIUM AUSTROAFRICANUM JOB5

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Running title: NMR studies of MTBE cometabolism by strain JOB5

Prepared for submission to Applied and Environmental Microbiology

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Cometabolic oxidation of the gasoline oxygenate methyl tertiary butyl ether (MTBE) by *Mycobacterium austroafricanum* (previously *vaccae*) JOB5 is known to sequentially generate tertiary butyl formate (TBF), tertiary butyl alcohol (TBA), 2-methyl-1,2-propanediol and 2-hydroxyisobutyrate (HIBA). In this study we used $^{19}$F- and $^{13}$C-nuclear magnetic resonance spectroscopy (NMR), and either $^{19}$F- or universally-$^{13}$C-labeled substrates, respectively, to extend the known pathway of MTBE degradation, and confirm the existence of predicted intermediates, including a hemiacetal, formate, formaldehyde, and hydroxyisobutyraldehyde (HIBAL). Both C$_1$-products were detected during the early stages of MTBE degradation, and formaldehyde oxidation yielded formate, which appeared to accumulate extracellularly. The hemiacetal was not detected, although its existence was indirectly confirmed using pyrazole, an alcohol dehydrogenase inhibitor that disrupted the hemiacetal’s normal fate. HIBAL was also not detected, despite attempts to promote its accumulation by assaying the degradation of a fluorinated analog of TBA. This study also provides an initial characterization of the fate of HIBA in strain JOB5. HIBA consumption by propane- or casein-yeast extract-dextrose-grown cells was initially slow (~40% consumed in ~30 h), before suddenly increasing to consume the remaining HIBA in ≤10 h. This second, relatively rapid oxidation phase was absent in cells treated with chloramphenicol, suggesting a novel enzyme system(s) was required to efficiently degrade HIBA. HIBA was mineralized to yield bicarbonate although intervening steps remain unresolved. The initial linear rate of HIBA oxidation (0.25 nmoles min$^{-1}$ mg protein$^{-1}$) was ~50 times slower than corresponding
rates of MTBE, TBF or TBA oxidation – suggesting this step may prevent MTBE-dependent cell growth of strain JOB5.
INTRODUCTION

Methyl tertiary butyl ether (MTBE) increases the combustion efficiency of gasoline and leads to cleaner burning fuel. Its use in the United States has subsided in an effort to preserve public water supplies threatened by the relative ease with which MTBE migrates in groundwater when underground gasoline storage tanks leak. MTBE biodegradation can be a cheap and non-invasive approach to remediate tainted water supplies. However, degradation pathways must be understood in order to assess efficacy and determine if pathway intermediates pose a public health risk. Little is known about anaerobic MTBE oxidation, although it has been observed under methanogenic (43, 44), denitrifying (1), sulfate reducing (37) and iron (III) reducing (3) conditions. Compared to the aerobic reaction, the rate of anaerobic MTBE transformation is slow, although humic substances (3), ethanol (24), or low amounts of aryl O-methyl ethers (44) have a stimulatory effect. The mineralization of MTBE has been observed under both denitrifying (1) and iron (III) reducing conditions (3), but tertiary butyl alcohol (TBA) has been the only pathway intermediate detected (1, 24, 25). A pure culture capable of anaerobic MTBE transformation remains to be isolated. However, a recent study found that O-demethylating acetogenic bacteria were responsible for MTBE transformation in methanogenic enrichment cultures (44).

Pure strains capable of aerobic MTBE oxidation have been isolated, and these differ in their propensity to utilize MTBE as a sole source of carbon and energy. For instance, strain L108 (31) and Methylibium petroleiphilum PM1 (10) readily grow on MTBE, and metabolic
pathway intermediates are consumed too rapidly to be detected. In other strains, such as *Mycobacterium austroafricanum* strains IFP 2012 (5) and IFP 2015 (23), and *Hydrogenophaga flava* strain ENV735 (12), MTBE can be metabolized, but slowly, and some pathway intermediates transiently accumulate to detectable levels. Last are those strains that cometabolize MTBE after growth on various substrates. These include strain ENV 425 grown on propane (38), *Pseudomonas mendocina* KR-1 grown on C₅ to C₈ n-alkanes (36), *Pseudomonas putida* GPo1 grown on n-octane (34), a *Graphium* (filamentous fungus) strain grown on either propane or n-butane (11), and *Mycobacterium austroafricanum* (previously *vaccae*) JOB5 grown on either propane (35) or diverse nonalkane substrates (17). Cometabolic reactions are either too slow to support cell growth, or pathway intermediates do not participate in cellular biosynthetic reactions. The process is therefore amenable to elucidating oxidation pathways since intermediates tend to accumulate relative to reactions that support cell growth (13). Indeed, early evidence collected using strain ENV 425 (38) and the *Graphium* strain (11) served as the basis for the currently accepted MTBE oxidation pathway model, presumed to operate in strains L108 (31) and PM1 (14), and partly shown to operate in strains IFP 2012 (5), IFP 2015 (23), and JOB5 (35).

In this study, we analyzed the cometabolic oxidation of MTBE by propane-grown cells of strain JOB5 in order to address unresolved issues in the currently accepted MTBE oxidation pathway model. The biochemistry of MTBE cometabolism in this strain is well characterized allowing us to ask specific questions. Both MTBE- and TBA-oxidizing activity in this strain have been linked to the same short-chain alkane monooxygenase responsible for catalyzing
propane-oxidation (35). The product of MTBE oxidation was predicted to be an unstable hemiacetal with two fates: A major reaction involving alcohol dehydrogenase catalyzed oxidation to yield tertiary butyl formate (TBF), and a minor reaction involving abiotic-cleavage to yield TBA and predictably, formaldehyde. TBF was shown to undergo combined abiotic and esterase-mediated cleavage to yield TBA and predictably, formate (35). Further work (17) using glycerol-grown cells showed that TBA oxidation generated 2-methyl-1,2-propanediol (2M12PD), and, in a separate study (15), n-pentane grown cells were shown to oxidize 2M12PD to yield 2-hydroxyisobutyrate (HIBA). The fate of HIBA in this strain is unknown. Steffan et al. (38) showed that propane-grown strain ENV 425 mineralized HIBA and proposed several intermediates for the pathway of HIBA degradation. Acetone was one such intermediate, and this was detected during TBA degradation by strain IFP 2015 (23). In contrast, in strain L108, HIBA transformation was shown to be a mutase catalyzed reaction. This structurally rearranged the tertiary butyl moiety to yield 3-hydroxybutyrate (31), and provides a path by which MTBE oxidation moves towards central metabolism. This path may be widespread, as homologs for the mutase subunits have been identified in the megaplasmid of strain PM1 (18, 31). Cobalt is an essential cofactor for HIBA degradation in strain L108 (31) and also in strain IFP 2012 (5).

The goals of this study were: First, to detect compounds whose role in MTBE cometabolism has remained presumptive. These include the hemiacetal, formate, formaldehyde, and hydroxyisobutyraldehyde (HIBAL), which is a predicted intermediate in the conversion of 2M12PD to HIBA (22). Formaldehyde has been detected during MTBE oxidation by
propane-grown strain ENV 425 (38), but it has not been detected in strain JOB5. Our second goal was to characterize the fate of HIBA. And third, we aimed to quantify the initial rates of degradation for MTBE and its oxidation pathway intermediates. We used $^{13}$C- or $^{19}$F-labeled substrates and either $^{13}$C- or $^{19}$F-nuclear magnetic resonance spectroscopy (NMR), respectively, to detect intermediates in the MTBE oxidation pathway. NMR is a comprehensive analytical tool that detects all labeled compounds without requiring chemical modification of the sample. Furthermore, compound detection is not biased by chromatographic techniques or selective detectors. Because the $^{13}$C-isotope is only ~1% naturally abundant, we used universally $^{13}$C-labeled substrates to determine the fate of each carbon atom in the molecule. Also, because some compounds are expected to be rapidly consumed (such as HIBAL (22)), we attempted to promote compound accumulation by assaying the oxidation of 2-trifluoromethyl-2-hydroxypropane (TFMP), a fluorinated analog of TBA. Replacing a methyl group with a trifluoromethyl group is expected to promote the accumulation of pathway intermediates since the higher carbon-fluorine bond energy inhibits enzyme catalysis (2, 40). For instance, fluorotoluenes have been used to effect the accumulation of intermediates that would have otherwise been rapidly consumed in several species of toluene-metabolizing fungi (30).

Evidence confirming the existence of all predicted compounds was obtained, except for HIBAL. HIBA was oxidized to bicarbonate, although intervening steps remain unknown. Considering the initial rates of oxidation observed for MTBE and each of its daughter
products, we suggest that slow rates, rather than incomplete metabolism, prevented strain JOB5 from using MTBE as a sole source of carbon and energy.
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.). Universally \(^{13}\)C-labeled compounds (\(^{13}\)C\(_5\)-MTBE (99% purity), \(^{13}\)C\(_4\)-TBA (99% purity), \(^{13}\)C\(_5\)-HIBA (98% purity), \(^{13}\)C-formaldehyde (~20% [wt/vol] solution in water) and sodium \(^{13}\)C-formate (99% purity) were obtained from ISOTEC (Miamisburg, Ohio). Unlabeled MTBE (99.8% purity), TBA (>99.3% purity), TBF (99% purity), HIBA (99% purity), formaldehyde (~37% [wt/vol] solution in water), 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (trade name: Purpald; ≥99% purity), pyrazole (98% purity), chloramphenicol, and deuterium oxide (\(^2\)H\(_2\)O) (99.9% purity) were obtained from Sigma-Aldrich (Milwaukee, Wis.). TFMP (98% purity) was obtained from Lancaster Synthesis (Pelham, NH). 2M12PD was a gift from Lyondell Chemical Co. (Houston, Tex.). Propane (instrument grade) was obtained from Matheson Gas Products, Inc. (Montgomeryville, Pa.). Compressed gases, H\(_2\), He, N\(_2\), and air, used for gas chromatography (GC) were obtained from local industrial vendors.

Cell growth and harvesting. Cells of strain JOB5 were grown in screw-cap glass media bottles (750 ml; Wheaton Scientific, Millville, NJ) containing either mineral salts medium (MSM) (42) or CYD-broth (100 ml). Bottles were sealed with open-top caps fitted with butyl rubber septa. Media was inoculated (to an initial OD\(_{600}\) ~0.01) with cells grown on CYD-agar plates. When required, propane (40 ml) was added to sealed vials using a plastic syringe.
fitted with a Millex disposable filter (0.1 µm; Millipore Co., Bedford, Ma.). Cultures were incubated at 30°C in the dark for either 3 days (CYD-broth) or 5 days (MSM) in an Innova 4900 environmental shaker (New Brunswick Scientific Co., Inc., Edison, NJ) operated at 150 rpm. The purity of the culture was determined by streaking a sample (20 µl) of each culture onto CYD-agar plates. Cells were then harvested from the culture medium (final OD₆₀₀ ~0.7 to 1.4) 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 ~6 to 12 mg total cell protein ml⁻¹. The cell suspension was stored at 4°C and used within 4 h.

**Incubation conditions.** Reactions were conducted in glass serum vials (15 ml or 25 ml) sealed with Teflon-lined Mininert valves (Alltech Associates Inc., Deerfield, Ill.). The vials contained phosphate buffer (up to 5.5 ml) and substrates or inhibitors were added directly to sealed vials from either aqueous stock solutions or as neat liquids. Vials were incubated in the dark in a shaking water bath (30°C; 150 rpm) for at least 15 min to allow reactants to equilibrate between gas and liquid phases. All reactions were initiated by adding cells to the equilibrated reaction vials, after which the vials were returned to the shaking water bath. Samples (≤700 µl) were removed from the reaction medium at indicated times and the rates of substrate consumption and reaction product identification were determined as described below.
In some incubations examining HIBA degradation carbon dioxide was removed from the reaction vial using the method described by Sluis et al. (33). Briefly, a center well was created in a glass serum vial (15 ml) by attaching a smaller glass sample vial (2 ml) to the inner base of the larger vial using a silicone-based adhesive. An aqueous solution of potassium hydroxide (6 M; 375 µl) was added to the center well and the reaction medium was added to the remaining section of the serum vial. The vials were sealed with butyl rubber stoppers and aluminum crimp seals and incubated in a shaking water bath (30°C; 150 rpm) overnight to trap ambient CO₂. Reactions were then initiated by adding cells, as described above.

**Gas Chromatography.** Initial rates of MTBE, TBA, TBF and 2M12PD oxidation, and the impacts of pyrazole on the pathway of MTBE oxidation, were determined by GC using aqueous phase samples (2 µl) taken directly from the reaction mixture using glass microsyringes. The analyses were conducted using a Shimadzu GC-14A gas chromatograph (Kyoto, Japan) fitted to a flame ionization detector and a stainless steel column (0.3 x 183 cm) filled with Proapak Q (80/100 mesh; Waters Associates, Framingham, Mass.). The GC was operated with an injection port temperature of 200°C, and a detector temperature of 220°C. The column was operated at 140°C for the quantification of MTBE and TBA and at 160°C for the quantification of TBF and 2M12PD. Data was collected using a Hewlett Packard HP3395 integrator (Palo Alto, Calif.).
**High-pressure liquid chromatography:** Pyrazole degradation and the rate of HIBA oxidation were determined using HPLC. For the rate of HIBA oxidation, a sample (150 µl) of the reaction solution was withdrawn and the cells were sedimented, as described above. The resulting supernatant was acidified using 8 N H$_2$SO$_4$ (2 µl) and an aliquot (50 µl) of the acidified supernatant was analyzed using a Shimadzu LC-10AT liquid chromatograph (Kyoto, Japan). The chromatograph was fitted with a 4.6 x 150 mm 5 µm Alltech Hypersil BDS C$_{18}$ column (Deerfield, Ill.) and a Shimadzu SPD-10A UV-visible detector operated at 210 nm. An aqueous solution of sulfuric acid (0.01 N) was used as the mobile phase at a flow rate of 0.5 ml min$^{-1}$. Degradation of pyrazole was monitored using the same sample collection and HPLC procedure described above, except the cell-free supernatant was not acidified, and the mobile phase consisted of a 25% acetonitrile solution in deionized water.

**Determination of formaldehyde:** The rate of formaldehyde oxidation was determined colorimetrically, as described by Jacobsen and Dickinson (16). At select time points, samples (50 µl) of the reaction mixture were withdrawn with a glass microsyringe and transferred to plastic microfuge tubes (0.5 ml). The cells were then sedimented by centrifugation using an Eppendorf microfuge (14,000 × g for 5 min). An aliquot (25 µl) of the supernatant was immediately transferred to a test tube containing 975 µl of a freshly prepared aqueous solution of 1% Purpald (wt/vol) dissolved in 1N NaOH. The mixture was then incubated and shaken at room temperature (23°C) using a test-tube holder mounted to a Fisher Scientific Vortex Genie 2 (Pittsburgh, Pa.). After 1 h the sample absorbance was determined at 549 nm using a Shimadzu 1601 UV/Vis spectrophotometer (Kyoto, Japan). The rate of formaldehyde
consumption in active cells was calculated by subtracting the rate of formaldehyde consumption observed in duplicate incubations using cells that had been heat-treated (100°C for 30 min).

**Analysis of metabolites using $^{13}$C- and $^{19}$F-NMR.** Samples (≤700 µl) of the reaction solution were withdrawn using a disposable plastic syringe and the cells were removed by centrifugation, as described above. For $^{13}$C-NMR analyses, an aliquot (510 µl) of reaction supernatant was transferred to a 5 mm NMR tube. $^2$H$_2$O (75 µl) and neat methanol (2 µl) were added to provide a magnetic field lock and an internal standard and reference ($\delta = 49.15$ relative to tetramethylsilane), respectively. For qualitative experiments, proton decoupled $^{13}$C-NMR spectra were collected using a Varian Mercury 300 spectrometer operated at 75 MHz with a spectral width of 18.8 kHz and a pulse width of 8.7 µs (corresponding to a flip angle of 43.5°) with no delay between pulses. A total of 1024 scans, with 68K data points in the time domain, were collected for Fourier transformation. For quantitative experiments, proton decoupling occurred only during data acquisition (to minimize the nuclear Overhauser effect). Spectra were collected using a Varian Innova 300 spectrometer operated at 75 MHz with a spectral width of 16.5 kHz and a pulse width of 9.2 µs (corresponding to a 90° flip angle), with a 25 s delay between pulses. A total of 2048 scans, with 60K data points in the time domain, were collected for Fourier transformation.

For $^{19}$F-NMR analysis, samples (550 µl) of cell-free supernatant were transferred to a 5 mm NMR tube. $^2$H$_2$O (75 µl) and hexafluoroacetone (5 µl of a 0.2% [vol/vol] solution) were
added to provide a magnetic field lock and an internal standard and reference (δ = -84.0 relative to fluorotrichloromethane), respectively. Proton decoupled $^{19}$F-NMR spectra were collected using a Varian Mercury 300 spectrometer operated at 282 MHz with a spectral width of 51 kHz, a pulse width of 5 µs (corresponding to a flip angle of 20°), and a 1 s delay between pulses. A total of 128 scans, with 30K data points in the time domain, were collected for Fourier transformation.

In both quantitative $^{13}$C- and $^{19}$F-NMR the relative amounts of each compound detected at a given time point was determined by integrating each resonance detected in the NMR spectrum, and summing those integrals associated with each compound. Results are reported in terms of signal intensity expressed as a percentage of all compounds detected in the sample. Using this approach, a full accounting of all $^{13}$C- or $^{19}$F-nuclei is achieved if the integration total attributed to an introduced standard (methanol or hexafluoroacetone) remains constant among samples.

**Gas chromatography/mass spectrometry:** Fluorinated products detected by $^{19}$F-NMR were identified using gas chromatography/mass spectrometry (GC/MS). The samples analyzed by GC/MS were the same as those analyzed by $^{19}$F-NMR. In each instance, half of the original cell-free supernatant (250 µl) was acidified to pH 2 with 8 µl of 8N H$_2$SO$_4$ while the other half of the sample was left untreated. The acidified and non-acidified solutions were then extracted three times with ethyl acetate (200 µl). The organic phases from each extraction were pooled and then evaporated under a stream of He gas at room temperature until ~10 µl
remained. From this residue, a sample (1 µl) was analyzed by GC/MS using a Shimadzu GC-17A, GCMS-QP5000 (Kyoto, Japan). The GC/MS was fitted with a DB-5ms capillary column (30 m x 0.25 mm, 0.25 µm film thickness; Agilent Technologies, Palo Alto, CA) and was operated in splitless mode using an injection port temperature of 200°C and a GC-MS interface temperature of 250°C. He was used as carrier gas. Following sample injection, the GC/MS was operated at 35°C for 4.8 min. The column temperature was then increased to 60°C for 30 s at 5°C min⁻¹, and then further increased to 160°C for 2 min at 40°C min⁻¹. Eluting compounds were ionized by electron impact (70eV). Data were collected and further analyzed using Shimadzu Class 5K software.

Cell protein concentrations were determined using the Biuret assay (8) after cell material was solubilized (65°C, 1 h, 3 M NaOH), and insoluble portions sedimented by centrifugation using an Eppendorf microfuge (14,000 × g for 5 min). Bovine serum albumin was used as a standard.
RESULTS

Products detected during MTBE oxidation. Samples collected during the 27 h time course of $^{13}$C$_5$-MTBE oxidation by propane-grown cells were analyzed using quantitative $^{13}$C-NMR (Figure 3.1 A, B). Based on chemical shift comparisons to authentic standards and on $^{13}$C-$^{13}$C coupling patterns, the products detected within the first hour of $^{13}$C$_5$-MTBE degradation were identified as TBF, TBA, formate and bicarbonate, while later products included 2M12PD and HIBA (Figure 3.1 B). We did not detect HIBAL, which is a predicted intermediate in the conversion of 2M12PD to HIBA in strain IFP 2012 (22) and strain PM1 (14). Chemical shifts resulting from each compound were quantified as described in the Materials and Methods section. The value attributed to the introduced internal standard (methanol) remained constant among samples, indicating all $^{13}$C-atoms originating from $^{13}$C$_5$-MTBE were accounted for as the time course progressed. The disappearance of MTBE and TBF coincided with the point (5 h) when TBA consumption outpaced production and formate production plateaued at ~9-10% relative peak area. Apparent consumption of 2M12PD coincided with the disappearance of TBA (9 h), and the process was slower than the consumption of MTBE, TBF or TBA. Production of HIBA was also slow, but this experiment concluded before the fate of HIBA was determined. HIBA consumption was therefore tested in separate experiments (discussed below).

Fate of formate and formaldehyde. The formate generated during MTBE oxidation by propane-grown cells was either slowly transformed, or was an apparent dead-end product.
Continued formate production during MTBE oxidation may have masked formate consumption, but this seems unlikely since a separate experiment failed to demonstrate formate production downstream of TBA (data not shown). We explicitly tested the ability of propane-grown cells of strain JOB5 to oxidize formate. MTBE transformation in strain JOB5 is expected to yield formate via either esterase mediated cleavage of TBF (yielding formate and TBA) or spontaneous cleavage of the hemiacetal (yielding formaldehyde and TBA, with further oxidation of the formaldehyde yielding formate) (35) (Figure 3.7). To confirm the latter source of formate, we tested whether cells could generate formate via formaldehyde oxidation. Qualitative $^{13}$C-NMR was used to analyze samples collected during a 1 h time course, and at the 24 h time point of $^{13}$C-formate and $^{13}$C-formaldehyde oxidation. While no formate-oxidizing activity was observed, formaldehyde oxidation generated formate (Figure 3.2).

**Effect of pyrazole.** The short-chain alkane monooxygenase-catalyzed oxidation of MTBE in strain JOB5 is expected to generate an unstable hemiacetal (35). This compound may undergo abiotic cleavage to yield formaldehyde and TBA, or oxidation by an alcohol dehydrogenase, to yield TBF (35) (Figure 3.7). The hemiacetal has not been detected, and its role in MTBE oxidation by several microbial isolates (5, 11, 38) remains presumptive. We used pyrazole, a recognized inhibitor of alcohol dehydrogenases (21), to help confirm the role of the hemiacetal in MTBE oxidation and TBF formation. Pyrazole’s ability to disrupt alcohol dehydrogenation in strain JOB5 was verified by observing the inhibition of 2-propanol consumption in propane-grown cells treated with pyrazole (data not shown).
Propane-grown cells were incubated with MTBE in the presence or absence of pyrazole for ~3 h, and the reactions were monitored using GC (Figure 3.3A). MTBE oxidation in the absence of pyrazole yielded both TBF and TBA, whereas cells incubated with both pyrazole and MTBE generated TBA but not TBF. Mass balances for each reaction (with or without pyrazole) were nearly constant, suggesting reaction stoichiometry was preserved despite the inhibition of TBF production by pyrazole.

A more complete analysis of the impact of pyrazole on the initial pathway of MTBE oxidation was gained using $^{13}$C$_5$-MTBE and qualitative $^{13}$C-NMR. After 80 min of degradation, TBF, TBA and formate were detected from cells incubated with $^{13}$C$_5$-MTBE alone, while TBA, formaldehyde, formate, and only a trace amount of TBF were detected from cells incubated with $^{13}$C$_5$-MTBE in the presence of pyrazole (Figure 3.3B).

**Determining specific activities.** Initial linear rates of degradation for MTBE and all daughter products identified in this study were determined using propane-grown cells (Table 3.1). Comparable rates were observed for MTBE, TBA, TBF and formaldehyde oxidation (~14 nmoles min$^{-1}$ mg total protein$^{-1}$), but 2M12PD was oxidized at approximately half this rate. The initial linear rate of HIBA oxidation was much slower: 0.25 nmoles min$^{-1}$ mg protein$^{-1}$. This rate evidently reflected biological activity since neither heat-inactivated cells nor an abiotic control consumed HIBA during a 72 h incubation period (data not shown).
Degradation of a fluorinated analog of TBA. HIBAL, which is a predicted intermediate in the conversion of 2M12PD to HIBA (14, 22), was not detected during $^{13}$C$_5$-MTBE degradation. A rapid oxidation rate may explain why this compound not detected (22), therefore, we attempted to promote HIBAL accumulation by assaying the oxidation of 2-trifluoromethyl-2-hydroxypropane (TFMP), a fluorinated analog of TBA. Replacing a methyl group with a trifluoromethyl group is expected to promote the accumulation of metabolic intermediates since the higher carbon-fluorine bond energy inhibits enzyme catalysis (2, 40).

Samples collected during the 50 h time course of TFMP oxidation were analyzed using quantitative $^{19}$F-NMR. This technique requires no sample preparation, detects all fluorinated compounds, and detects low analyte amounts since $^{19}$F is naturally NMR-sensitive. Propane-grown strain JOB5 consumed 93% of the TFMP in 10 h and sequentially generated two daughter products (Figure 3.4). The value attributed to the introduced internal standard (hexafluoroacetone) remained constant among samples, indicating all $^{19}$F-atoms originating from TFMP were accounted for as the time course progressed. The transformation of TFMP and its two daughter products was noticeably slower compared to the transformation of TBA and its two daughter products: 2M12PD and HIBA.

Fluorinated TFMP oxidation products detected by $^{19}$F-NMR were identified using GC/MS. Sequential generation of the two fluorinated daughter products allowed us to separately analyze each product in select samples. Authentic standards for these fluorinated products were not available; therefore, peak retention times and major mass fragments ($\geq 5\%$ intensity
relative to the base peak) were compared to authentic standards for the non-fluorinated analogs. Results obtained from authentic TFMP and TBA served as a benchmark to gauge the accuracy of this comparison.

Sample acidification was required to detect the HIBA authentic standard in the GC/MS, and also the compound present in the 50 h time point of TFMP oxidation. These compounds had identical retention times of 11.9 min. In contrast, acidification did not influence detection of the 2M12PD standard, nor the compound present in the 10 h time point. These compounds eluted at 8.0 and 7.2 min respectively (data not shown); a difference that may reflect fluorine’s influence on compound elution at the relatively low oven-temperature in this time range. The retention times for TBA and TFMP, which also eluted at a low oven-temperature, were 2.25 and 2.1 min, respectively (temperature program for the GC is described in the Materials and Methods section). Comparable mass fragment patterns were observed between HIBA and the compound present at 50 h, or 2M12PD and the compound present at 10 h (Figure S3.1). Some shared fragments were observed between authentic standards and fluorinated analogs. We also observed a mass shift between some fragments that corresponded to the difference between harboring a C–H bond or a C–F bond (Table S3.1). Chemical structures were assigned to each major mass fragment arising from all fluorinated compounds showing how these masses may have been derived from their predicted parent-compounds (Table S3.2). In summary, results from our GC/MS analysis suggested 2-trifluoromethyl-1,2-propanediol and 2-trifluoromethyl-2-hydroxypropionic acid, fluorinated
analogs of 2M12PD and HIBA, respectively, were the major products of TFMP oxidation. A fluorinated analog of HIBAL was not detected.

**Degradation of HIBA.** HIBA production was evident during the time course of $^{13}$C$_5$-MTBE degradation (Figure 3.1A), but that experiment concluded before the fate of HIBA could be determined. We tested the ability of propane- and CYD-grown cells to degrade HIBA in the presence or absence of either chloramphenicol or a CO$_2$-trap. Samples collected during the 48 h time course of $^{13}$C$_5$-HIBA oxidation were analyzed using HPLC to measure HIBA and qualitative $^{13}$C-NMR to detect products. In all cases, degradation proceeded slowly during the first 24 h, but after 30 h the rate increased for both propane- and CYD-grown cells incubated in either the presence or absence of a CO$_2$-trap; whereas cells treated with chloramphenicol lost HIBA-oxidizing activity after 30 h (Figure 3.5). Bicarbonate was the sole product of HIBA oxidation, and was detected only in supernatant samples from cells incubated with HIBA alone. Acetone was not detected despite the inhibitory effects of a CO$_2$-trap on acetone-oxidizing activity in propane-grown strain JOB5 (data not shown). No intracellular products were detected whether or not cells were disrupted using ultra-sonication and resuspended in either water or boiling chloroform (to solubilize PHB (20)) (data not shown). When $^{13}$C$_5$-HIBA oxidation by propane-grown cells was monitored using quantitative-$^{13}$C-NMR (Figure 3.6) bicarbonate was again the sole product detected. However, the value attributed to the introduced internal standard (methanol) did not remain constant among samples, indicating some of the $^{13}$C-nuclei from $^{13}$C$_5$-HIBA were distributed in compound(s) that were not detected.
DISCUSSION

In this study we used $^{13}$C- and $^{19}$F-NMR to expand the known pathway (15, 17, 35) of MTBE-biodegradation in propane-grown strain JOB5. Our results indirectly confirmed that a hemiacetal is the immediate product of MTBE oxidation, and we showed that both formate and formaldehyde were produced upstream of TBA during MTBE transformation. HIBAL, a predicted (14, 22) intermediate in the conversion of 2M12PD to HIBA was not detected, despite efforts to promote its accumulation. HIBA was transformed to bicarbonate, but not stoichiometrically, and the intermediates in this transformation remain unknown. Efficient HIBA oxidation by either propane- or CYD-grown cells apparently required the expression of a novel enzyme system(s). In the end, only formate and bicarbonate accumulated in the reaction medium following MTBE degradation. Initial rates of MTBE and daughter product oxidation suggested slow rates were primarily responsible for impeding MTBE-dependent cell replication in strain JOB5.

Evidence of the hemiacetal. Smith et al. (35) have shown that propane-grown cells of strain JOB5 oxidize MTBE using the same short-chain alkane monoxygenase responsible for catalyzing propane oxidation in this strain. The immediate product of MTBE oxidation was predicted to be an unstable hemiacetal. This compound has not been detected although it has been the proposed precursor to TBF not only in strain JOB5 (35), but also in strains IFP 2012 (5) and IFP 2015 (23), and in a fungal Graphium species (11). The current model of MTBE oxidation suggests TBF is produced by the alcohol dehydrogenase catalyzed oxidation of the
hemiacetal. A reaction which must be sufficiently rapid (35) to outpace the spontaneous cleavage of the hemiacetal (41) that would otherwise occur to yield TBA and formaldehyde. We proposed to gain indirect proof of the hemiacetal by inhibiting the alcohol dehydrogenase, which should prevent TBF formation. This result was observed when cells were incubated with MTBE in the presence of pyrazole (Figure 3.3), a wholesale alcohol dehydrogenase inhibitor (21). By preventing TBF formation, TBA and formaldehyde became the only primary daughter products detected during MTBE oxidation.

Cells treated with pyrazole oxidized MTBE and generated TBA more efficiently than cells incubated with MTBE alone (Figure 3.3A). TBA formation may be stimulated since pyrazole-treated cells generated TBA directly from the hemiacetal, whereas untreated cells primarily (35) generated TBF from the hemiacetal as a precursor to TBA (Figure 3.7). MTBE-oxidation in pyrazole-treated cells may have been stimulated by the excess reducing power that likely resulted from the oxidation of pyrazole evident in propane-grown strain JOB5 (data not shown). Reducing power resulting from the conversion of formaldehyde to formate (Figure 3.2) may have also stimulated MTBE oxidation, since the addition of pyrazole to the reaction medium increased the amount of formaldehyde detected (Figure 3.3B). Alternatively, by not generating TBF, pyrazole-treated cells may support enhanced MTBE-oxidizing activity by avoiding the inhibitory effects of TBF evident in strain IFP 2012 (4). Our data is insufficient to prove whether or not TBF moderated MTBE-oxidizing activity in strain JOB5. However, in strain IFP 2012 0.17 mM TBF decreased the initial rate of MTBE oxidation by 43% (4), whereas in strain JOB5 neither 0.5 mM (35) nor 6 mM (this
study, Figure 3.1, quantified by GC) TBF substantially altered MTBE-oxidizing activity. Likewise, challenging a *Graphium* species with ≤ 7.5 mM TBF did not impact MTBE-degradation (32), suggesting TBF is not a general regulator of MTBE-oxidizing activity.

**Detecting C₁-products.** Formaldehyde and formate are predicted (35) C₁-products resulting from hydrolysis of either the hemiacetal or TBF, respectively, to yield TBA (Figure 3.7). Using ^13^C-NMR, we detected both formate (Figure 3.1) and formaldehyde (Figure 3.3) production upstream of TBA. Formaldehyde was oxidized to yield formate whereas formate oxidation was not detected (Figure 3.2). Formate is recognized as an unfavorable substrate in strain JOB5 because it does not support cell-growth (35), and is only weakly oxidized by propane-grown cells (28). This weak oxidizing-activity likely accounts for the trace amount of bicarbonate detected during the first hour of ^13^C₅-MTBE degradation (Figure 3.1). Formate accumulation evident during MTBE transformation (Figure 3.1) suggests strain JOB5 failed to realize an important and anticipated (26) energy benefit resulting from the reductant-regenerating formate dehydrogenase-catalyzed oxidation of formate.

**Failure to detect HIBAL.** Our ^13^C-NMR analysis showed the sequential progression of, and stoichiometric relationship between MTBE, TBF, TBA, 2M12PD and HIBA. We did not detect HIBAL, which is a predicted intermediate in the conversion of 2M12PD to HIBA in strain PM1 (14) and IFP 2012 (22). Rapid oxidation of HIBAL could explain the difficulty in detecting this compound (22), although as the pathway of MTBE transformation progressed in strain JOB5, the rate of oxidation for each successive pathway intermediate decreased.
(Table 3.1), suggesting enzyme specificity increased as substrates approached central metabolism. HIBAL oxidation in strain JOB5 may still be rapid. Therefore, we attempted to generate a fluorinated analog of HIBAL by assaying the oxidation of TFMP, a fluorinated analog of TBA. Oxidation of a fluorinated analog of HIBAL should be slow (2, 40), allowing the compound to accumulate. TFMP oxidation yielded two sequential daughter products (Figure 3.4) which were identified as fluorinated analogs of 2M12PD and HIBA, sequentially. TFMP and its daughter products were oxidized at a slower rate relative to their respective native analogs, but a fluorinated analog of HIBAL was still not detected, and the role of HIBAL in the MTBE oxidation pathway remains presumptive. Indeed, it remains to be determined whether the gene products suspected of oxidizing 2M12PD to yield HIBAL (MdpH or Mpdb in strain PM1 (14) or IFP 2012 (22), respectively), or of oxidizing HIBAL to yield HIBA (MpeA0361 or Mpdb in strain PM1 or IFP 2012, respectively) function as suspected.

In strain IFP 2012, Mpdb was related to choline dehydrogenase (22), an enzyme that, in the bacteria Escherichia coli K10 (19) and Halomonas elongata (7), has been shown to catalyze the oxidation of both choline and its aldehyde product (betaine-aldehyde) to yield glycine-betaine. This suggests that Mpdb may not be required for the oxidation of 2M12PD to HIBA. Furthermore, in H. elongata, choline dehydrogenase is also known to function as an oxidase (7). Choline oxidase catalyzes the four-electron oxidation of the hydroxyl group in choline to generate the carboxyl group in glycine-betaine. The intermediate betaine-aldehyde is generated, but remains enzyme-bound (6). This raises the possibility that, rather than being
released as a stable product of 2M12PD oxidation, HIBAL may remain enzyme bound during HIBA production. Lopes Ferreira et. al (22) have shown that a mycobacterial strain gained the ability to transform 2M12PD to HIBA while harboring a plasmid containing both mpdB and mpdC. Whether this strain containing mpdB alone would have generated either HIBAL or HIBA from 2M12PD remains unknown. In strain PM1, the roles of MdpH and MpeA0361 were based on attempting to fit these proteins to the currently accepted MTBE oxidation pathway model (14); which has been largely based on evidence collected using MTBE cometabolic systems (11, 35, 38) and strain IFP 2012 (5, 22) and IFP 2015 (23). These proteins were not homologous to MpdB or MpdC in strain IFP 2012, and the evidence linking mpeA0361 to HIBAL dehydrogenase was tenuous (14). Based on our evidence, and the above discussion, it seems plausible that this enzyme may not be required for MTBE oxidation, and it appears that more work is needed before HIBAL is generally accepted as an intermediate in the MTBE oxidation pathway model.

**HIBA: production and consumption.** $^{13}$C$_5$-HIBA degradation was assayed in the presence or absence of either chloramphenicol (to prevent *de novo* protein synthesis) or a CO$_2$-trap (which inhibits acetone consumption in strain JOB5 (data not shown)) by resting cells grown on either propane or CYD-broth (Figure 3.5). In all cases, degradation was relatively slow for the first 24 h. However, after 30 h the rate increased dramatically for cells incubated in the absence of chloramphenicol whereas chloramphenicol-treated cells lost HIBA-degrading activity. These results suggest efficient HIBA degradation required a protein(s) not expressed in either propane- or CYD-grown cells. Novel protein expression is also expected to be
required for HIBA degradation in propane-grown strain ENV425 (38) and in strains L108 and L10 following growth on various substrates (31). Cobalt is an essential cofactor for HIBA degradation in strain L108 (31) and also in strain IFP 2012 (5). Indeed, the rate of HIBA degradation and the efficiency of HIBA-dependent cell growth are dictated by cobalt availability (1 mg l\(^{-1}\) cobalt for strain IFP 2012, and 50 µg l\(^{-1}\) for strain L108). In contrast, ~3 mg l\(^{-1}\) cobalt failed to enhance the rate of HIBA degradation in strain JOB5, and failed to stimulate MTBE-, TBA-, or HIBA-dependent cell growth (data not shown). These results suggest strain JOB5 may use a different, cobalt independent mechanism for HIBA degradation.

Bicarbonate was the sole extracellular product detected (via \(^{13}\)C-NMR) during \(^{13}\)C\(_5\)-HIBA degradation; and no intracellular-products were detected. Evidence from quantitative \(^{13}\)C-NMR (Figure 3.6) suggests that the amount of bicarbonate detected does not provide a full accounting of all \(^{13}\)C-HIBA atoms present at the beginning of the reaction. If products of HIBA oxidation were shunted into cellular biosynthetic pathways, then these “missing” \(^{13}\)C-atoms may be incorporated into diverse cellular materials, effectively diluting signal intensity, and making it impossible to detect daughter products of the parent \(^{13}\)C\(_5\)-HIBA against background noise. Future experiments may test this speculation by comparing \(^{13}\)C-levels present in cellular material following \(^{13}\)C\(_5\)-HIBA degradation with the amount detected following natural abundance-\(^{13}\)C-HIBA degradation.
Degradation rates. Substrate utilization rates must eclipse cell maintenance needs in order to support cell growth (29). Indeed, Müller et al. theorized that the balance between MTBE degradation and a cell’s maintenance needs determines the MTBE-dependent growth yield, with slow degradation rates unable to support cell growth (26). Initial oxidation rates for MTBE and each of its daughter products – save formate, essentially a dead-end product (see above) – were collected for resting cells grown on propane (Table 3.1). MTBE, TBF, TBA and formaldehyde were all oxidized at ~14 nmoles min\(^{-1}\) mg protein\(^{-1}\), while 2M12PD and HIBA were oxidized ~2 and 50 times slower.

The initial rate of MTBE degradation by propane-grown cells of strain JOB5 was slower than nearly all other aerobic strains that use MTBE to support cell growth (5, 10, 12, 27, 34, 38). This relatively slow rate may prevent MTBE-dependent cell growth in strain JOB5. However, the much slower rate of 2M12PD and HIBA oxidation would impede catabolism and is the most likely limitation to growth. In the propane-oxidizing and MTBE-cometabolizing strain ENV425, HIBA oxidation was also slow and possibly growth-limiting (38). In contrast, in the MTBE-metabolizing strain L108, the rate of daughter product oxidation increased as the MTBE degradation pathway progressed (27). The slow initial rate of HIBA oxidation in propane- or CYD-grown strain JOB5 evidently improved following presumed expression of a novel enzyme system(s) (Figure 3.5). This echoes a recent finding by Rohwerder et al. for strain L108 where cells grown on substrates that did not induce the HIBA-degrading mutase oxidized HIBA at <1 nmol min\(^{-1}\) mg biomass\(^{-1}\); whereas those grown on mutase-inducing substrates supported a rate of ~10 to 40 nmol min\(^{-1}\) mg biomass\(^{-1}\)
Similarly, inoculating a culture with cells expressing the rapid HIBA-oxidizing enzyme may permit HIBA- or even MTBE-dependent cell growth in strain JOB5.

An important outcome of this study is evidence suggesting TFMP may be an attractive tracer to estimate the extent of TBA-degradation occurring in situ. The pathway of TFMP-oxidation mimicked that of TBA-oxidation (Figure 3.7), and daughter products of TFMP resisted degradation (Figure 3.4). Since compounds containing C-F bonds are rare in nature (40), the dead-end fluorinated products of TFMP-degradation will be characteristic indicators of an environment’s ability to support TBA-biodegradation. More work is needed to prove TFMP’s value as a tracer for TBA, although fluorinated analogs are known to serve this role. For instance, a fluorinated analog of trichloroethene has been shown to serve as a tracer to monitor the degradation of trichloroethene in situ, where consumption of the fluorinated analog was measured, and characteristic dead-end products (39) were detected (9).
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Figure 3.1. Time course of 5mM $^{13}$C$_5$-MTBE oxidation quantified using $^{13}$C-NMR. Propane-grown cells (19 mg total cell protein) were incubated in a 25 ml reaction vial containing a 7 ml aqueous phase. Part A shows the time course of MTBE consumption (●) and progression of the resulting daughter products: TBF (▼), TBA (▲), formate (▼), bicarbonate (★), 2M12PD (▲) and HIBA (□). Methanol served as the internal standard (+) and reference. Part B shows two spectra selected from the time course to show each chemical shift detected. Letters above each peak correspond to atoms labeled in the pathway diagram (Figure 3.7). Compounds were identified via appreciation of $^{13}$C-$^{13}$C coupling patterns and chemical shift comparisons to non-$^{13}$C-enriched authentic standards.
Figure 3.2. Time course of 2 mM $^{13}$C-formaldehyde (A) and 2 mM $^{13}$C-formate (B) oxidation monitored using qualitative $^{13}$C-NMR. Propane-grown cells (3.4 mg total cell protein) were incubated in 25 ml reaction vials containing a 5 ml aqueous phase. Methanol served as the internal reference.
Figure 3.3. Impact of pyrazole on MTBE-oxidation. Part A shows the mass balance (◇,●) reflecting the degradation of MTBE (◇,●) and production of TBF (▽,▼) or TBA (△,▲) in the presence (open symbols) or absence (closed symbols) of 1 mM pyrazole (monitored by GC). Duplicate suspensions of propane-grown cells (0.7 and 0.8 mg total cell protein) were incubated in 15 ml reaction vials containing a 1 ml aqueous phase. Error bars represent the standard error of the mean. Part B shows products detected via qualitative $^{13}$C-NMR after 80 min of 5 mM $^{13}$C$_5$-MTBE degradation in the absence (upper spectrum) or presence (lower spectrum) of 1 mM pyrazole. Propane-grown cells (2.2 mg total cell protein) were incubated in 15 ml reaction vials containing a 1.5 ml aqueous phase. Letters above each peak correspond to atoms labeled in the pathway diagram (Figure 3.7).
Table 3.1. Initial linear degradation rates for MTBE and its oxidation pathway intermediates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (nmoles min(^{-1}) mg protein(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTBE</td>
<td>14.41 (0.97)</td>
</tr>
<tr>
<td>TBF</td>
<td>11.89 (1.16)</td>
</tr>
<tr>
<td>TBA</td>
<td>15.19 (0.51)</td>
</tr>
<tr>
<td>2M12PD</td>
<td>5.68 (0.11)</td>
</tr>
<tr>
<td>HIBA</td>
<td>0.25 (0.02)</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>14.35 (1.1)</td>
</tr>
</tbody>
</table>

\(^{a}\) Data represent the mean and SEM (in parentheses) of duplicate samples. All linear rates consisted of at least 4 data points collected in ≤2 h of degradation, save HIBA (8 h).
Figure 3.4. Time course of 5 mM TFMP oxidation monitored using $^{19}$F-NMR spectroscopy. Propane-grown cells (13 mg total cell protein) were incubated in a 25 ml reaction vial containing a 6 ml aqueous phase. Part A plots the NMR data shown in part B, showing the oxidation of TFMP (○) and formation of 2-trifluoromethyl-1,2-propanediol (△) and 2-trifluoromethyl-2-hydroxypropionic acid (□). Hexafluoroacetone served as the internal standard (+) and reference. Peaks detected using $^{19}$F-NMR were identified using GC/MS, as described in the Results. Full-length NMR-spectra (~180 ppm) were collected for each sample. All chemical shifts detected fell within a 10 ppm range. Only this range is shown in order to assist data presentation.
**Figure 3.5.** Time course of HIBA degradation monitored using HPLC. Propane- (closed symbols) or CYD- (open symbols) grown cells (5.6 or 2.1 mg total cell protein, respectively) were incubated with either 2 mM $^{13}$C$_{5}$-HIBA alone (■,□), or in the presence of either a CO$_2$-trap (▲,△) or 62.5 µg ml$^{-1}$ chloramphenicol (●,○). Reactions were conducted in 25 ml reaction vials containing a 4 ml aqueous phase. The data shown is representative of replicate experiments.
Figure 3.6. Time course of 2 mM $^{13}$C$_5$-HIBA oxidation quantified using $^{13}$C-NMR. Propane-grown cells (4.5 mg total cell protein) were incubated in a 25 ml reaction vial containing a 4 ml aqueous phase. The figure shows the consumption of HIBA (□) and resulting production of bicarbonate (＊). Methanol served as the internal standard (+) and reference. The data shown is representative of replicate experiments.
Figure 3.7. Proposed pathway of MTBE oxidation in propane-grown cells of strain JOB5. Pathway reflects $^{13}$C- and $^{19}$F-NMR data. Carbon atoms labeled with superscript letters correspond to peaks labeled in Figures 3.1B and 3.3B. Pyrazole is schematically shown to inhibit tert-butyl formate production (evident in Figure 3.3) presumably via alcohol dehydrogenase (ADH) inhibition. The proposed pathway of TFMP oxidation is not shown explicitly. However, a dashed box surrounds the portion of the MTBE oxidation pathway supported by $^{19}$F-NMR and GC/MS data collected during the time course of TFMP-oxidation.
Figure S3.1. Mass spectra observed during the fragmentation of authentic standards (TBA, TFMP, 2M12PD and HIBA) and compounds present at the 10 h (labeled: 2-trifluoromethyl-1,2-propanediol) and 50 h (labeled: 2-trifluoromethyl-2-hydroxypropionic acid) time points of TFMP oxidation by propane-grown cells of strain JOB5.
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Shared</th>
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<th>Convertible</th>
<th>Convertible</th>
<th>Unique</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>H→F (+18 Da)</td>
<td>H₂→F₂ (+36 Da)</td>
<td>H₃→F₃ (+54 Da)</td>
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<tr>
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<td>95, 113</td>
<td>20, 28, 32, 33, 42, 44, 46, 51, 69, 94</td>
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<tr>
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<td>113</td>
<td>114</td>
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<tr>
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</table>

**Table S3.1.** Mass fragments for authentic standards (TBA, TFMP, 2M12PD, and HIBA) compared with mass fragments for the two products generated during TFMP oxidation (labeled, 2-trifluoromethyl-1,2-propanediol (2TFM12PD) from the 10 h time point, and 2-trifluoromethyl-2-hydroxypropionic acid (2TFM2HP) from the 50 h time point). For mass fragment patterns, refer to Figure S3.1. For chemical structures of each fragment, please refer to the fragment key (Table S3.2).
<table>
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<tr>
<th>m/z</th>
<th>Structural assignment</th>
<th>m/z</th>
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<tr>
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<td>&quot;C≡&quot;O CH₂=+CH₂</td>
<td>46</td>
<td>HOCH=&quot;O</td>
<td>73</td>
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<td>(CFH₂)CH=&quot;OH</td>
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**Table S3.2.** Chemical structure assignments for each major mass fragment (≥5% intensity relative to the base peak) derived from the fragmentation of TFMP and the two fluorinated products of TFMP oxidation, identified as 2-trifluoromethyl-1,2-propanediol and 2-trifluoromethyl-2-hydroxypropionic acid.
REFERENCES


   Second European Conference on MTBE. Barcelona, Spain.


CHAPTER 4

PROPANE CATABOLISM, AND IMPACTS OF CO₂ ON PROPANE AND ACETONE METABOLISM BY Mycobacterium Austroafricanum JOB5

The work in this chapter describes contributions from both Christy Smith and the author. This work will become part of a larger manuscript to include experiments that are ongoing in Michael Hyman’s laboratory. The authors of this manuscript will include Christy Smith, Alan House and Michael Hyman. The author wishes to recognize José Bruno-Bárcena for helpful discussions focused on interpreting the growth curve data.
ABSTRACT

Propane metabolism in *Mycobacterium austroafricanum* (previously *vaccae*) JOB5 was examined in a series of growth experiments and substrate oxidation assays. Propane oxidation yielded 1-propanol and 2-propanol, which were oxidized to yield propionate and acetone, respectively. The curve of propane-dependent culture growth under ambient CO$_2$ concentrations consisted of two exponential phases separated by a transition phase. This transition phase did not appear to reflect the time required for the culture to transition between sequential utilization of 1-propanol and 2-propanol since both products were simultaneously generated and then consumed within the first exponential growth phase. Rather, we suggest this lag phase was triggered by toxicity derived from propionate. The addition of bicarbonate to the culture medium removed the intermediary lag phase and roughly halved the culture’s doubling time during the second exponential phase. This suggests the CO$_2$-dependent methylmalonyl-CoA pathway for propionate utilization was expressed to increase propionate turnover and reduce toxicity. Results obtained during 1-propanol-dependent culture growth support the conclusion that propionate was utilized by two separate pathways acting sequentially, with the methylmalonyl-CoA pathway appearing secondary to an unresolved primary pathway, likely the 2-methylcitrate pathway. The addition of bicarbonate also (i) stimulated 2-propanol-dependent culture growth, (ii) was required for growth on acetone, and (iii) improved the acetone oxidation rate, whereas a CO$_2$-trap had the opposite effect. These results suggest strain JOB5 may encode an acetone
carboxylase. Testing the impact of bicarbonate on cell growth for a range of substrates confirmed that this compound fulfilled a specific, not a fundamental physiological need.
INTRODUCTION

The biodegradation of persistent environmental contaminants is often slow, incomplete and unable to support cell growth. This process, termed cometabolism, is often catalyzed by, and superimposed upon, enzyme systems expressed to catalyze carbon- or energy-yielding reactions. Propane-grown cells of *Mycobacterium austroafricanum* (previously *vacca*), strain JOB5 are recognized for their ability to degrade a variety of environmental pollutants via aerobic cometabolism. These include dioxane (4), methyl tertiary butyl ether (28), 2,4,5-trinitrotoluene (37) and trichloroethylene (36). Each compound is degraded without a lag and this activity has been shown (28) to be inhibited by acetylene (a specific inactivator of the short-chain alkane monooxygenase (10) (SCAM) responsible for catalyzing propane oxidation in this strain (28)) suggesting propane-grown cells are equipped for cometabolism. The sparging of contaminated environments with propane and oxygen has been shown to stimulate and support the *in situ* aerobic cometabolism of groundwater pollutants (15, 30). Hence, engineered bioremediation strategies may be improved by understanding the physiology of propane metabolism in strain JOB5.

Propane metabolism in strain JOB5 was characterized by J.J. Perry’s laboratory from the late 1960s to the mid 1980s. During this time, the initial view that propane was oxidized via subterminal oxidation only (38) was challenged by the fatty acid pattern of propane-grown cells, which indicated that both terminal (propane → 1-propanol → propionaldehyde → propionate) and subterminal (propane → 2-propanol → acetone) propane oxidation products
participated in the biosynthetic reactions of the cell (5, 39). Biochemical evidence suggesting both pathways contribute to propane-dependent cell growth is lacking. And, more evidence is required to substantiate the view that subterminal oxidation is the preferred pathway (6, 23).

Bacterial strains that grow on propane are known to metabolize this substrate either by terminal (20) or subterminal (16) oxidation alone, or by the combination of both pathways (42). When both pathways are used, an organism’s preference for each remains unknown (1, 31). The most extensive studies to determine relative substrate preference have been performed in *Rhodococcus rhodochrous* PNKb1. By assaying enzyme activities in cell-free extracts (42), and using biochemical, genetic and immunological approaches, Ashraf, Woods and Murrell were able to demonstrate that two separate alcohol dehydrogenase systems catalyzed the oxidation of 1-propanol and 2-propanol, although the relative importance of each pathway remained unresolved (2).

The two pathways of propane oxidation may be met by a diversity of propionate and acetone catabolic pathways. In aerobic bacteria, propionate is converted to propionyl-CoA (8, 13, 20, 29, 33) which may enter central metabolism via either the methylmalonyl-CoA or 2-methylcitrate pathways. The methylmalonyl-CoA pathway involves condensation of propionyl-CoA and CO₂ (8, 20, 29) to yield methylmalonyl-CoA,(8) which is converted to succinyl-CoA (3) and then succinate, which enters the tricarboxylic acid (TCA) cycle (8, 20, 29). In the 2-methylcitrate pathway, propionyl-CoA condenses with oxaloacetate to yield 2-methylcitrate, which enters a modified form of the TCA cycle. In the first step, 2-
methylcitrate is oxidized to 2-methyl-cis-aconitate, which is transformed to 2-methylisocitrate which is then cleaved to yield pyruvate and succinate. Succinate regenerates oxaloacetate per the familiar TCA cycle (13). Propionate metabolites are known to inhibit bacterial growth. For instance, the accumulation of propionyl-CoA was toxic to *Rhodopseudomonas sphaeroides* (21) whereas 2-methylcitrate accumulation caused toxicity in *Salmonella enterica* (12) and *Mycobacterium smegmatis* (34).

The aerobic utilization of acetone may be initiated by one of three mechanisms: acetone carboxylase mediated carboxylation to yield acetoacetate (26), monooxygenase mediated oxidation to yield acetol (1-hydroxyacetone) (11, 32, 38), or a Baeyer-Villager monooxygenase catalyzed reaction to yield methyl acetate (17). All three compounds may enter central metabolism after conversion to acetyl-CoA (11, 17, 27, 38), and acetol may additionally be transformed to yield pyruvate (32).

In this study, we use strain JOB5 and take a whole-cell physiology approach to assay substrate preference during propane-dependent culture growth. Furthermore, since the diversity of propionate and acetone catabolic pathways each contain a candidate CO$_2$ fixation step, we determine the biochemical and physiological impact of different CO$_2$ concentrations on substrate oxidation and cell growth. Our results show that propane metabolism in strain JOB5 simultaneously uses both terminal and subterminal oxidation pathways to support cell growth. This divergent metabolism of propane is apparently followed by the divergent metabolism of propionate (2-methylcitrate and methylmalonyl-CoA pathways) and the
divergent oxidation of acetone (acetol and acetoacetate pathways). Acetone carboxylation was required for growth on this substrate, and the addition of bicarbonate improved the acetone oxidation rate, whereas a CO$_2$-trap had the opposite effect.
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.). *n*-Butane (99% purity), 1-butanol (99% purity), sodium butyrate (98% purity), isobutane (99% purity), *n*-hexane (>99% purity), propionaldehyde (97% purity), 1-propanol (99.9% purity), sodium propionate (99% purity), chloramphenicol, deuterium oxide (\(\text{D}_2\)O) (99.9% purity), and calcium carbide (~80% purity; for acetylene generation) were obtained from Sigma-Aldrich (Milwaukee, Wis.). *n*-Pentane (99.5% purity) and 2-propanol (99.9% purity) were obtained from Fisher Scientific (Pittsburgh, Pa.). Acetol (> 97% purity) and 2-butanol (> 99% purity) were obtained from Fluka (Buchs, Switzerland). Acetone (99.8% purity) and sodium bicarbonate (100% purity) were obtained from Mallinckrodt (Hazelwood, Missouri). Universally \(^{13}\)C-labeled acetone (\(^{13}\)C\(_3\)-acetone) (99.2% purity) was obtained from ISOTEC (Miamisburg, Ohio). 2-Butanone (> 99% purity) was obtained from Acros Organics (Morris Plains, NJ). Propane (instrument grade) was obtained from Matheson Gas Products, Inc. (Montgomeryville, Pa.). Compressed gases, H\(_2\), N\(_2\), and air, used for gas chromatography (GC) were obtained from local industrial vendors.

Cell growth and harvesting. Experiments designed to measure the growth of strain JOB5 used batch cultures in glass serum vials (160 ml; Wheaton Scientific, Millville, N.J.) containing either mineral salts medium (MSM) (41) or CYD-broth (25 ml). Vials were sealed
with Teflon-lined Mininert Valves (Alltech Associates Inc., Deerfield, Ill.). Growth substrates were either added from filter-sterilized aqueous stock solutions, or as neat liquids or filter-sterilized gases. Vials were incubated overnight in the dark in an Innova 4900 environmental shaker (30°C; 150 rpm) (New Brunswick Scientific Co., Inc., Edison, N.J.) to allow growth substrates to equilibrate between gas and liquid phases. Media was inoculated (initial OD$_{600} \leq 0.01$) with cells that had been taken from CYD-agar plates and suspended in MSM. Culture growth was determined by measuring optical density (OD$_{600}$) using a Shimadzu 1601 UV/Vis spectrophotometer (Kyoto, Japan). Culture purity was determined at regular intervals by streaking a sample (20 µl) of the culture medium onto CYD-agar plates.

Some experiments described in this study used harvested cell suspensions. In these cases, cells were grown in screw-cap glass media bottles (750 ml; Wheaton Scientific, Millville, NJ) containing MSM (100 ml) and either propane (40 ml) or both acetone and bicarbonate (to achieve an initial concentration of 10 mM each) were added to the culture bottles as described above. Bottles were sealed with either open-top caps fitted with butyl rubber septa (propane) or Teflon-lined caps (acetone and bicarbonate). Media was inoculated (to an initial OD$_{600}$ ~0.01) with cells grown on CYD-agar plates. Cultures were incubated (as described above) for at least 4 days, and purity was determined (as described above) prior to harvesting cells. Cells were harvested from the culture medium by centrifugation (10,000 × g; 10 min), and the resulting cell pellet was washed in phosphate buffer (20 ml; 50 mM sodium phosphate; pH 7) and centrifuged again. Sedimented cells were resuspended in buffer (~1 ml)
to a final protein concentration of ~4 to 18 mg of total cell protein ml⁻¹. The cell suspension was stored at 4°C and used within 4 h.

**Analytical methods.** In some experiments the concentration of propane and its aqueous-phase oxidation products was determined during cell growth. Propane was measured by using a gas-tight microsyringe with a dry heat-treated needle (1 min at 350°C). A sample (5 µl) of the culture vial headspace was directly injected into a Shimadzu GC-14A (Kyoto, Japan) gas chromatograph (GC) fitted with a DB-MTBE capillary column (30 m by 0.45 mm (internal diameter), 2.55 µm film; J and W Scientific, Folsom, Calif.) and a flame ionization detector. The GC was operated with an injection port temperature of 200°C, a detector temperature of 220°C, and an oven temperature of 35°C. The Henry’s constant for propane at 1 atm pressure and 25°C was taken as 71.6 (kPa•m³)/mol (19). The aqueous-phase products of propane oxidation were measured by removing samples (~0.7 ml) of the culture medium using sterile plastic syringes (1 ml) and needles. A portion of this sample (0.5 ml) was diluted in buffer (0.5 ml) and used to determine OD₆₀₀ (as described above) while the remainder was transferred to a flattop polypropylene microcentrifuge tube. Using a glass microsyringe, an aqueous sample (2 µl) from the microcentrifuge tube was immediately injected into a Shimadzu GC-8A fitted with a stainless steel column (0.3 x 183 cm) filled with Proapak Q (80/100 mesh; Waters Associates, Framingham, Mass.) and a flame ionization detector. The GC was operated with an injection port temperature of 200°C, a detector temperature of 220°C, and an oven temperature of 105°C. Data from both GC-14A and GC-8A were
collected using a Hewlett Packard HP3395 integrator (Palo Alto, Calif.). Products of propane oxidation were identified by coelution to authentic standards.

Some experiments used harvested cell suspensions to monitor substrate degradation in short-term assays. Reactions were conducted in glass serum vials (15 ml) that contained phosphate buffer (up to 1.5 ml) and were sealed with butyl rubber stoppers and aluminum crimp seals. When required, CO₂ was removed from the reaction vial using the method described by Sluis et al. (26). Briefly, a center well was created in the serum vial by attaching a smaller glass sample vial (2 ml) to the inner base of the larger vial using a silicone-based adhesive. An aqueous solution of potassium hydroxide (6 M; 375 µl) was added to the center well and the reaction medium was added to the remaining section of the serum vial. The vials were sealed (as described above) and incubated in a shaking water bath (30°C; 150 rpm) for at least 12 h to trap ambient CO₂. Following this step, the remainder of the reaction vials were prepared. Cells and acetylene gas (1.5 ml) were added to requisite reaction vials, and all vials were then incubated in a shaking water bath (as above) for at least 5 min to permit short-chain alkane monooxygenase inactivation in those vials containing acetylene, and to allow any CO₂ introduced upon the addition of cells to be trapped in those vials containing a CO₂-trap. Experiments were then initiated by adding the degradation assay substrate to an initial concentration of 5 mM. Reactions vials were incubated in the shaking water bath for the duration of the experiment, and aqueous phase samples (2 µl) were taken directly from the reaction mixture using glass microsyringes at the indicated times. Samples were directly injected into a Shimadzu GC-8A (described above) operated at the following oven
temperatures: 105°C for 2-propanol, 160°C for 1-propanol, and 180°C for acetone and acetol degradation experiments.

\[ ^{13}\text{C} \]–Nuclear magnetic resonance spectroscopy (\(^{13}\text{C}–\text{NMR}\)) was used to detect the diversity of oxidation products generated during \(^{13}\text{C}_3\)-acetone degradation by a harvested cell suspension. Reaction vials (as described above) were incubated for 90 min and samples (~700 µl) from each reaction solution were removed at both the start and end of the incubation time period. Cells were removed by high-speed centrifugation (14,000 × g for 5 min) and an aliquot (510 µl) of reaction supernatant was transferred to a 5 mm NMR tube. \(^2\text{H}_2\text{O} (75 \mu l)\) and neat methanol (2 µl) were added to provide a magnetic field lock and an internal reference (δ = 49.15 relative to tetramethylsilane), respectively. Proton decoupled \(^{13}\text{C}–\text{NMR}\) spectra were collected using a Varian Mercury 300 spectrometer operated at 75 MHz. A total of 1024 scans were collected, with no delay between pulses. To monitor the fate of each \(^{13}\text{C}\)-atom, a spectral width of 18.8 kHz was used with a pulse width of 8.7 µs (corresponding to a flip angle of 43.5°), collecting 68K data points. To achieve higher resolution spectra for methyl-\(^{13}\text{C}\)-atoms only, a spectral width of 1.1 kHz was used with a pulse width of 16 µs (corresponding to a flip angle of 80°), collecting 15K data points in the time domain for Fourier transformation.

Cell protein concentrations were determined using the Biuret assay (9) after cell material was solubilized (65°C, 1 h, 3 M NaOH), and insoluble portions sedimented by centrifugation.
using an Eppendorf microfuge (14,000 \times g for 5 min). Bovine serum albumin was used as a standard.
RESULTS

Propane utilization. The profile of propane-dependent growth and product evolution was determined using batch cultures containing 2.25, 5 or 15 mM propane (corresponding to 5, 12.5 or 40% [vol/vol] propane in the culture vial respectively). By increasing the initial amount of propane, we expected to promote the accumulation of metabolic intermediates. In all three cultures, propane consumption resulted in the simultaneous and sub-stoichiometric production of both 1-propanol and 2-propanol. Acetone production occurred later, coincident with 1-propanol and 2-propanol consumption (Figure 4.1). A higher initial amount of propane promoted the accumulation of 1-propanol and acetone, but also resulted in the accumulation of ~20% of the initial amount of propane provided; whereas the 2.25 mM culture consumed propane to undetectable levels.

The growth curve of all three cultures consisted of two exponential phases separated by an intermediary lag phase. The first exponential phase was dependent on propane consumption, and the beginning of the intermediary lag phase coincided with the height of 1-propanol and 2-propanol accumulation. Propane consumption was not evident during the second exponential phase, and propionate was not detected in either culture, although this may be due to the GC parameters employed, which were not optimized to detect propionate.

Impacts of bicarbonate on propane, 1-propanol and 2-propanol utilization. Cultures incubated under ambient CO₂ concentrations readily utilized either propane or 1-propanol,
whereas a five-day lag phase preceded 2-propanol-dependent culture growth (Figure 4.2). Growth on each substrate used two exponential phases separated by an intermediary transition phase. The addition of bicarbonate to the culture medium increased the maximum biomass yield obtained in all three cases. Two exponential phases separated by an intermediary transition phase were still evident during 1-propanol-dependent growth, but the addition of bicarbonate roughly halved the doubling time for the second exponential phase (Table 4.1). Propane was similar, although no intermediary phase was observed during culture growth in the presence of bicarbonate. The addition of bicarbonate had the most dramatic impact on 2-propanol-dependent culture growth, decreasing the initial lag phase by four days, and absolving the second exponential phase altogether. This finding suggests acetone utilization in strain JOB5 is CO$_2$-dependent.

Batch cultures were established with a fixed amount of acetone (10 mM) and increasing amounts of bicarbonate (0 to 50 mM). When $\leq$10 mM bicarbonate was used, the maximum optical density obtained in each culture correlated with the amount of bicarbonate available (Figure 4.3). Greater amounts did not impact growth. Therefore, future experiments requiring a bicarbonate addition received 10 mM bicarbonate; resulting in a pH of 6.9 (culture media) or 7 (phosphate buffer).

**Oxidation of propane-catabolites.** The impacts of CO$_2$ on either 1-propanol, 2-propanol or acetone oxidation was further explored in a series of short-term degradation assays using harvested cells grown on propane. Cells were incubated with each of these substrates in the
presence or absence of either bicarbonate, a CO$_2$-trap, or acetylene. Neither of these incubation conditions impacted the oxidation of 1-propanol or its two daughter products: propionaldehyde and propionate (Figure 4.4 A, B). Similarly, 2-propanol oxidation was not influenced by the different incubation conditions, although the acetone generated during this reaction was more readily consumed when bicarbonate was added to the reaction medium compared to ambient CO$_2$ concentrations. A CO$_2$-trap essentially inhibited the consumption of the acetone generated during 2-propanol oxidation. Acetylene treatment appeared to have a negative impact on acetone oxidation considering the amount of acetone remaining at the end of the time course relative to the amount remaining in reactions containing untreated cells (Figure 4.4 C, D). When propane-grown cells were initially incubated with acetone, their acetone-oxidizing activity also correlated with the amount of CO$_2$ and bicarbonate available, and acetylene treatment also slowed this reaction. Complete inhibition of acetone-oxidizing activity was only observed when cells were incubated with both acetylene and a CO$_2$-trap. Acetone oxidation in the absence of acetylene resulted in the transient accumulation of acetol (Figure 4.4 E) – which was further consumed by propane-grown cells, albeit relatively slowly (Figure 4.4 G).

Next we assayed the impact of bicarbonate on the acetone- and acetol-oxidizing activities of harvested cells grown on acetone and bicarbonate. Again, acetone oxidation was enhanced by the addition of bicarbonate to the reaction medium. However, acetylene had no effect on acetone oxidation, although a CO$_2$-trap fully inhibited this reaction (Figure 4.4 F). Products of acetone oxidation were not detected, and acetone and bicarbonate-grown cells did not
transform acetol (Figure 4.4 H).

**Products of acetone oxidation.** The transient accumulation of acetol during acetone oxidation by propane-grown cells was observed using GC (Figure 4.4 E). Experiments to identify the diversity of oxidation products generated under different incubation conditions may benefit from a less selective technique. Thus, propane-grown cells were incubated with $^{13}$C$_3$-acetone in the presence of (i) ambient CO$_2$, (ii) bicarbonate, (iii) a CO$_2$-trap, (iv) acetylene, and (v) both acetylene and a CO$_2$-trap together in the same reaction vial. At the beginning and end of a 90 min incubation period, a sample from each vial was collected for $^{13}$C-NMR analysis. $^{13}$C-NMR is a comprehensive analytical tool that detects all labeled compounds without requiring chemical modification of the sample. Because the $^{13}$C-isotope is only ~1% naturally abundant, we used universally $^{13}$C-labeled acetone to determine the fate of each carbon atom in the molecule. The only products observed were acetol and 1,2-propanediol. Similar to the results reported above, acetone-oxidizing activity was most active in cells that received bicarbonate, whereas a CO$_2$-trap and acetylene were inhibitory. Acetol was detected in cells incubated with a CO$_2$-trap, but not in cells incubated with acetylene (Figure 4.5). Since acetol is a hydroxylated derivative of acetone, and since acetol production was inhibited by acetylene, we suggest acetol and 1,2-propanediol result from a monooxygenase catalyzed reaction with acetone. Thus, products indicative of acetone carboxylase activity were not detected. Attempts to detect both intra- and extracellular products of $^{13}$C$_3$-acetone oxidation by acetone and bicarbonate grown cells were also unsuccessful (data not shown).
**Impacts of CO$_2$ on short-chain (oxy)alkane-mediated growth.** Elevated CO$_2$-concentrations improved propane-, 1-propanol-, 2-propanol- and acetone-dependent cell growth (Figures 4.2, 4.3). The impact of CO$_2$ on culture growth dependent on homologous compounds was explored using cultures supplied with C$_{3-6}$ alkanes and oxygenated derivatives of propane and $n$-butane. Cultures were incubated under either ambient CO$_2$ concentrations, or in MSM containing bicarbonate (Table 4.2), or in vials containing a CO$_2$-trap. Initial culture optical densities (day 0) were collected and compared to those obtained on the third, seventh and fourteenth days of growth. The addition of bicarbonate increased culture optical densities when cells were grown using propane, 1-propanol, 2-propanol and acetone – supporting the data presented in Figures 4.2 and 4.3. The addition of bicarbonate also enhanced growth with propionaldehyde and propionate, but did not affect strain JOB5’s inability to utilize acetol. Growth with $n$-hexane, $n$-butane, 1-butanol and butyrate were also not affected. However, the addition of bicarbonate was required for growth with 2-butanol and 2-butanone, and also slightly improved the culture optical densities obtained when cells were grown using isobutane or $n$-pentane.

Cultures containing a CO$_2$-trap showed essentially no signs of growth (data not shown). Yet rather than reflecting a specific CO$_2$ requirement during short-chain (oxy)alkane-dependent growth; this result may reflect a bacterium’s fundamental need for CO$_2$ when cells are grown in minimal media (18, 22, 35, 40). When strain JOB5 was grown in CYD-broth rather than MSM, a CO$_2$-trap did not hinder growth (data not shown). Other experimental controls showed that neither CO$_2$ alone nor CO$_2$ alongside H$_2$ (20 mM) supported culture growth (data
not shown).
DISCUSSION

Propane-dependent culture growth consisted of two exponential phases separated by a transition phase. However, rather than reflecting the sequential utilization of terminal and subterminal oxidation products, our data suggests each pathway was utilized simultaneously, with the transition phase reflecting a period of growth cessation due to product toxicity. The addition of bicarbonate presumably helped alleviate this toxicity by facilitating propionate metabolism, and generally improved cell growth on propane, its catabolic intermediates, and homologous substrates. The results of our study suggest strain JOB5 uses multiple pathways to oxidize propane, propionate and acetone.

**Propane metabolism.** The curve of propane-dependent culture growth under ambient CO$_2$ concentrations consisted of two exponential phases separated by an intermediary lag phase (Figure 4.1). This lag phase did not appear to reflect the time required for the culture to transition between the sequential utilization of either the terminal or subterminal oxidation pathways since both 1-propanol and 2-propanol were simultaneously generated and then consumed entirely within the first exponential growth phase (Figure 4.1). Thus, the relative importance of terminal versus subterminal oxidation during cell growth remains unknown. More 1-propanol accumulated in the culture medium during growth, but this could be interpreted as either suggesting a greater flux through the terminal-oxidation pathway, or a greater preference for 2-propanol utilization.
The first exponential growth phase ended at the height of 1-propanol and 2-propanol accumulation (Figure 4.1), suggesting the intermediary lag phase that followed may have been caused by toxicity from products of propane metabolism. Based on previous studies, the toxic products were likely downstream metabolites of propionate, including propionyl-CoA (21) and 2-methylcitrate (12, 34). Propane consumption was not evident during the second exponential phase suggesting the propane-oxidizing short-chain alkane monooxygenase in strain JOB5 had been irreversibly feedback inhibited by a downstream product of propane oxidation, such as propionate. Doughty et al. recently characterized the inactivation of butane monooxygenase by propionate in propane- or n-butane-grown *Pseudomonas butanovora* (7). The physiological significance of this feedback inhibition could be to mitigate toxicity derived from propane oxidation products. The metabolism of at least two of these products, propionate and acetone, is known to be CO\(_2\)-dependent in several bacterial strains (20, 26, 29). And, the addition of bicarbonate to the culture media removed the intermediary lag phase from the growth curve, and roughly halved the doubling time during the second exponential phase (Table 4.1), suggesting the addition of bicarbonate had a dramatic impact on propane-dependent growth by increasing product (propionate and acetone) turnover (Figure 4.2).

**1-Propanol oxidation and metabolism.** Carbon dioxide did not appear to impact either 1-propanol, propionaldehyde or propionate oxidation (Figure 4.4 A, B), but the addition of bicarbonate increased the final biomass density obtained during growth on all three substrates (Table 4.2). This finding suggests CO\(_2\) impacts 1-propanol metabolism downstream of
propionate, implicating the well-recognized methylmalonyl-CoA pathway for propionate utilization (Figure 4.6).

For cultures growing on 1-propanol, the addition of bicarbonate to the culture medium did not impact the first exponential phase of growth, but roughly halved the doubling time during the second exponential phase (Figure 4.2, Table 4.1). And the presence of an intermediary lag phase was evident in either the presence or absence of bicarbonate. Taken together, these results suggest at least two pathways operated sequentially during 1-propanol metabolism, and the less preferred pathway was likely the CO$_2$-dependent methylmalonyl-CoA pathway. The preferred pathway for propionate metabolism may have been the 2-methylcitrate pathway (Figure 4.6). The transition phase separating the first and second exponential phases may have been triggered by toxicity from either propionyl-CoA (21) or 2-methylcitrate (12, 34). And, this transition phase may reflect the time required for cells to express the proteins required for the methylmalonyl-CoA pathway. This could alleviate toxicity by increasing propionyl-CoA turnover. Indeed, the implied activity of the methylmalonyl-CoA pathway in *R. sphaeroides* has been shown to alleviate propionyl-CoA derived toxicity in these cells by halving the intracellular level of propionyl-CoA (21). Vestal and Perry have shown that the methylmalonyl-CoA pathway operates during propionate metabolism in strain JOB5 (38), but more work is required to determine if the 2-methylcitrate pathway also plays a role. That both pathways operate is not without precedent. A recent study found *Mycobacterium tuberculosis* utilizes either the 2-methylcitrate or methylmalonyl-CoA pathway depending on experimental conditions (25).
2-Propanol and acetone, oxidation and metabolism. Carbon dioxide did not appear to impact 2-propanol oxidation, but had a dramatic effect on the oxidation of acetone (Figures 4.4 C, D). Indeed, our evidence (Figure 4.3, Table 4.2) suggests that acetone metabolism in strain JOB5 is dependent on the addition of bicarbonate to the culture media. A 1:1 molar ratio of acetone:bicarbonate allowed the culture to achieve its maximum optical density (Figure 4.3). The condensation of acetone and CO$_2$ in bacteria is catalyzed by an acetone carboxylase, and has been extensively studied by Scott Ensign’s laboratory using the aerobe *Xanthobacter autotrophicus* strain Py2. Acetone metabolism in both strain JOB5 (Figure 4.3) and strain Py2 (26) is CO$_2$-dependent; and, resting cells grown on acetone do not oxidize this substrate if CO$_2$ is trapped out of the reaction medium (Figure 4.4 F) (26). The similarity in this physiological evidence suggests strain JOB5 encodes an acetone carboxylase. Molecular work to confirm this conclusion is ongoing in our laboratory.

For cultures growing on 2-propanol, the addition of bicarbonate to the culture medium reduced the initial lag phase preceding cell replication from 5 days to 1 day (Figure 4.2). Since cultures were incubated in airtight vials, the 5 day lag phase evident under ambient CO$_2$ concentrations may have reflected the time required for the inoculum to increase the CO$_2$ concentration in the vial (e.g. through maintenance-based metabolism) in order to support acetone carboxylation and cell growth. Indeed, the time required for the concentration of CO$_2$ in the culture vial to reach an amount required for growth has been previously shown to be one of the factors determining the lag phase (40). Cultures growing
on 2-propanol under ambient CO$_2$ concentrations used two exponential phases, although no intermediary transition phase was detected (Figure 4.2). The addition of bicarbonate resulted in only one exponential phase of growth, with a doubling time equal to the first exponential phase evident in cultures incubated under ambient CO$_2$ concentrations. This finding suggests that, after the prolonged lag phase, sufficient amounts of CO$_2$ were present in the culture vial incubated under initial ambient CO$_2$ concentrations to sustain growth at the maximum rate for ~1 day. After this time, perhaps the amount of CO$_2$ in these cultures was insufficient to keep up with the amount of acetone generated during 2-propanol metabolism. This would have resulted in the longer doubling time evident during the second exponential phase of growth in cultures incubated under initial ambient CO$_2$ concentrations.

The products of acetone carboxylation are unlikely to cause toxicity. Neither GC nor $^{13}$C-NMR revealed products of $^{13}$C$_3$-acetone consumption by acetone and bicarbonate-grown cells (data not shown), suggesting the products of this reaction were rapidly consumed. Acetone-grown strain Py2 carboxylates acetone to form acetoacetate which appears to have two metabolic fates: reduction to β-hydroxybutyrate followed by incorporation into the carbon storage polymer poly-β-hydroxybutyrate (26) or activation by Coenzyme-A, yielding acetoacetate-CoA which is cleaved to form two molecules of acetyl-CoA (27) (Figure 4.6).

Cells grown on propane generated acetol and 1,2-propanediol during acetone oxidation (Figure 4.5), suggesting acetone and bicarbonate-grown cells consume acetone differently from propane-grown cells. Based on the inhibitory effects of acetylene and a CO$_2$-trap, our
results suggest that the catabolism of acetone in propane-grown cells is catalyzed by both SCAM and acetone carboxylase, whereas in acetone and bicarbonate grown cells, acetone carboxylase is entirely responsible for this reaction. The primary product of acetone oxidation catalyzed by SCAM was acetol (Figure 4.4 E, Figure 4.5), which was not a growth-supporting substrate in strain JOB5 (Table 4.2). This ability of SCAM to catalyze the oxidation of substrates to products that do support cell growth is well known (4, 28, 36, 37) and reflects the relatively loose substrate-specificity of SCAM. Vestal and Perry’s earlier assessment of propane and 2-propanol metabolism in strain JOB5 presumed that cell growth occurred via the C_2+C_1 cleavage of acetol, which would yield acetate (38). This reaction was presumed to occur in order to provide an initiator (acetate) of the glyoxylate shunt that would account for the isocitrate lyase expression observed during both propane and 2-propanol metabolism. However, acetate is a growth-supporting substrate for strain JOB5 (38), but acetol is not (Table 4.2). An alternative initiator of the glyoxylate shunt may be the acetyl-CoA generated via acetone carboxylation (27). Metabolism of β-hydroxybutyrate (another possible product of acetone carboxylation (26)) has also been shown to induce isocitrate lyase expression in strain JOB5 (24). This suggests the fate of acetol and 1,2-propanediol in strain JOB5 remains unresolved. 1,2-Propanediol may be oxidized back to acetol, as has been suggested to occur in strain JOB5 (23). And, since the time course of acetol oxidation by propane-grown cells mimicked the results obtained during acetone oxidation (Figure 4.4 G), acetol may slowly be reduced back to acetone, its parent substrate.
**Broader role for bicarbonate during short chain (oxy)alkane-mediated growth.** The dramatic impact of bicarbonate on culture optical densities for cells grown using propane and its oxidation products was not shared across the board by cells grown using homologous substrates (Table 4.2). This suggests bicarbonate fulfilled a specific, not a fundamental physiological need. *n*-Butane-metabolism in strain JOB5 is known to proceed via terminal oxidation exclusively (24), and a bicarbonate addition did not improve culture optical densities for either *n*-butane-, 1-butanol- or butyrate-grown cells. The opposite result was evident for 2-butanol- and 2-butanone-grown cells, which are known to be metabolized via propionate and the methylmalonyl-CoA pathway in strain JOB5 (24).

In conclusion, the divergent metabolism of propane (1-propanol and 2-propanol routes) is apparently followed by the divergent metabolism of propionate (2-methylcitrate and methylmalonyl-CoA routes) and the divergent oxidation of acetone (acetol and acetoacetate routes) (Figure 4.6). Confirming these routes, and determining which are dominant under which conditions may require the integration of genomic, transcriptomic, proteomic and metabolomic data. Bicarbonate concentrations played an important role in determining or enhancing cell growth on propane and its oxidation products. A logical outcome of this work is the realization that elevated CO₂ concentrations in the environment may help support pollutant biodegradation processes that rely on propane-oxidizing bacteria, which execute many such transformations via aerobic cometabolism.
Figure 4.1. Profile of propane consumption (■), culture growth (□) (upper panel), and products (1-propanol (●), 2-propanol (○) and acetone (△)) detected during growth (lower panel) for cultures provided three different initial amounts of propane. Note the propane-scale changes to accommodate the different amounts. The data shown is representative of duplicate experiments.
Figure 4.2. Propane- (~2.25 mM), 1-propanol- (10 mM), or 2-propanol- (10 mM) dependent cell growth for cultures incubated under either ambient CO$_2$ concentrations (O) or in the additional presence of 10 mM bicarbonate (●). Data points represent the mean of triplicate samples while error bars demonstrate the standard error of the mean. Data points for the stationary/death phase of propane- and 1-propanol dependent cell growth are from one culture – the other two were sacrificed for experiments not reported here. Doubling times are quantified in Table 4.1.
Table 4.1. Impact of bicarbonate (10 mM) on the doubling times of strain JOB5$^a$

<table>
<thead>
<tr>
<th>Growth supporting substrate</th>
<th>Mean doubling time (h)</th>
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<tbody>
<tr>
<td></td>
<td>first exponential phase</td>
<td>second exponential phase</td>
<td></td>
</tr>
<tr>
<td>propane</td>
<td></td>
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<tr>
<td>ambient-CO$_2$</td>
<td>9 (2)</td>
<td>14 (3)</td>
<td></td>
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<tr>
<td>plus bicarbonate</td>
<td>9.5 (0.3)</td>
<td>6 (0.4)</td>
<td></td>
</tr>
<tr>
<td>1-propanol</td>
<td></td>
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<td></td>
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<tr>
<td>ambient-CO$_2$</td>
<td>11 (1.5)</td>
<td>11 (1)</td>
<td></td>
</tr>
<tr>
<td>plus bicarbonate</td>
<td>16 (2)</td>
<td>6 (0.5)</td>
<td></td>
</tr>
<tr>
<td>2-propanol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ambient-CO$_2$</td>
<td>10 (2)</td>
<td>26 (5)</td>
<td></td>
</tr>
<tr>
<td>plus bicarbonate</td>
<td>11 (0.3)</td>
<td>ND$^b$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Quantifying data shown in Figure 4.2. Data represent the average of triplicate samples with the standard error in parentheses.

$^b$ ND, none detected.
Figure 4.3. Effect of increasing amounts of bicarbonate on the maximum optical density obtained by cultures supplied with acetone (10 mM). Data points represent the mean of duplicate samples while error bars demonstrate the standard error of the mean.
Figure 4.4. All data collected by Christy Smith. Figure legend continued on next page.
Figure 4.4. continued.
Impact of CO$_2$ and/or acetylene on the transformation of intermediates in the propane-catabolic pathway of strain JOB5. All substrates were added to an initial concentration of 5 mM. The time courses for 1-propanol (A) and 2-propanol (C) oxidation by propane-grown cells were extended (B and D, respectively) in order to determine the fate of products generated during substrate oxidation. We also compared the fate of acetone and acetol in propane-grown cells (E and G, respectively) with that obtained in acetone plus bicarbonate-grown cells (F and H, respectively). Note the axes change scale to accommodate each time course. Closed or open symbols represent the parent substrate or daughter product(s) respectively under either: ambient CO$_2$ (■,□), plus 10 mM bicarbonate (●,○), CO$_2$-trap (▼,▼), acetylene (1.5 mL) (▲,△), CO$_2$-trap plus acetylene (1.5 mL) (◆,◇), or abiotic (×) conditions. Products of 1-propanol oxidation were propionaldehyde (dotted line) and propionate (solid line); of 2-propanol, acetone; and of acetone, acetol. Each vial contained: (A) 0.31, (B) 0.59, (C) 0.18, (D) 0.36, (E) 1.8, (F) 0.41, (G) 1.2, (H) 1.1 mg of total cell protein.
Figure 4.5. Products of 5 mM $^{13}$C$_3$-acetone oxidation by propane-grown cells incubated under different conditions (as indicated in the figure). $^{13}$C-NMR spectra reflect the compounds present after a 90 min incubation. Full-length spectra (~250 ppm) were collected for each sample, and each compound detected contained methyl-carbon atoms. Therefore, to aid data presentation, each spectrum was collected again but only the methyl-carbon region (~15 ppm) was analyzed, and a 3-Hz line broadening function was applied to all spectra.
Table 4.2. Impact of bicarbonate (10 mM) on culture OD$_{600}^a$.

<table>
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<tr>
<th>Growth supporting substrate$^b$</th>
<th>OD$_{600}$</th>
<th>ambient-CO$_2$</th>
<th>day 3</th>
<th>day 7</th>
<th>plus bicarbonate</th>
<th>day 3</th>
<th>day 7</th>
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<td>propane</td>
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<td>(0.02)</td>
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<td><strong>propane-pathway intermediates</strong></td>
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$^a$ Data represent the mean and standard error (in parentheses) of at least triplicate samples. Initial OD$_{600} \leqslant 0.01$. Measurements collected on day 14 were $\leq$ data on day 7, and therefore excluded from the table (unless otherwise noted).

$^b$ Cultures contained 10 mM of each substrate except each alkane (2.5 mM) and propionaldehyde (5 mM, since 10 mM did not support growth – perhaps due to toxicity).

$^c$ OD$_{600}$ on day 14: 0.39 (0.10)
Figure 4.6. Proposed pathway of propane catabolism in strain JOB5 leading to central metabolic intermediates. Pathways following the asterisk (*) marks were inferred from physiological evidence rather than direct detection of the intermediates shown. Dotted arrows symbolize reactions requiring multiple steps. Fates of methylmalonyl-CoA, 2-methylcitrate and acetoacetate are taken from Flavin and Ochoa (8), Horswill and Escalante-Semerena (14) and Small and Ensign (27), respectively.
REFERENCES


CHAPTER 5

PRODUCTS GENERATED DURING BACTERIAL OXIDATION OF A FLUORINATED ANALOG OF TERTIARY BUTYL ALCOHOL,
A $^{19}$F-NMR APPROACH

The work in this chapter describes the author’s contribution to a series of manuscripts that characterize the metabolism of tertiary butyl alcohol by bacterial strains PM1, S1B1 and G2B2. The authors of these manuscripts will include Christy Smith, Kimberly Golart, Alan House and Michael Hyman.
ABSTRACT

Fluorinated substrate analogs participate in the same biochemical reactions as native substrates, though at slower rates, promoting the accumulation of intermediates and facilitating the determination of metabolic pathways. In this study we use $^{19}$F-nuclear magnetic resonance spectroscopy to detect products excreted during 2-trifluoromethyl-2-hydroxypropane (TFMP) oxidation by bacterial isolates capable of utilizing tertiary butyl alcohol (TBA) as a sole source of carbon and energy. The studies to which these data contribute show that TFMP is a fluorinated substrate analog of TBA in MTBE- and TBA-grown *Methylibium petroleiphilum* PM1, and two TBA-metabolizing isolates: *Aquincola* strain S1B1 and *Hydrogenophaga* strain G2B2. In each case TFMP was oxidized to yield the same two products that were identified as fluorinated analogs of 2-methyl-1,2-propanediol and 2-hydroxyisobutyric acid. These compounds are recognized TBA degradation products in bacterial isolates that either slowly metabolize or cometabolize MTBE or TBA under aerobic conditions. These results show that at least two intermediates are common to the pathway of aerobic TBA catabolism in all organisms studied to date.
INTRODUCTION

*Methylibium petroleiphilum* PM1 is studied for its ability to utilize methyl tertiary butyl ether (MTBE) as its sole source of carbon and energy (3). This organism is also capable of growing with tertiary butyl alcohol (TBA), a generally accepted primary metabolite of MTBE oxidation. The pathway of MTBE and TBA catabolism in strain PM1 is unknown since intermediates are consumed too rapidly to be detected. Our laboratory has also isolated two strains, *Aquincola* strain S1B1 and *Hydrogenophaga* strain G2B2, which use TBA, but not MTBE, as a sole source of carbon and energy. Intermediates of TBA catabolism in these organisms are also undetected. In this study we attempted to promote the accumulation of TBA catabolites in strains PM1, S1B1 and G2B2 by assaying the oxidation of 2-trifluoromethyl-2-hydroxypropane (TFMP), a fluorinated analog of TBA. Replacing a methyl group with a trifluoromethyl group is expected to promote the accumulation of pathway intermediates since the higher carbon-fluorine bond energy inhibits enzyme catalysis (1, 9). For instance, fluorotoluenes have been used to effect the accumulation of intermediates that would have otherwise been rapidly consumed in several species of toluene-metabolizing fungi (7). The suitability of TFMP to serve as a substrate analog of TBA has been demonstrated in Dr. Hyman’s laboratory by Christy Smith and Kimberly Golart. The author’s contribution to this study has been to use $^{19}$F-NMR spectroscopy to detect fluorinated products generated by these bacteria when exposed to TFMP.
ANALYTICAL METHODS

NMR Spectroscopy. Products formed during TFMP oxidation were identified using $^{19}$F-NMR. For NMR analysis, samples (550 µl) of cell-free supernatant (see below) were transferred to a 5 mm NMR tube. $^2$H$_2$O (75 µl) and hexafluoroacetone (5 µl of a 0.2% [vol/vol] solution) were added to provide a magnetic field lock and an internal standard and reference ($\delta = -84.0$ relative to fluorotrichloromethane), respectively. Proton decoupled $^{19}$F-NMR spectra were collected using a Varian Mercury 300 spectrometer operated at 282 MHz with a spectral width of 51 kHz, a pulse width of 5 µs (corresponding to a flip angle of 20°) and a 1 s delay between pulses. A total of 128 scans, with 30K data points in the time domain, were collected for Fourier transformation. The relative amounts of each compound detected at a given time point was determined by integrating each resonance detected in the NMR spectrum. Results are reported in terms of signal intensity expressed as a percentage of all compounds detected in the sample. Using this approach, a full accounting of all $^{19}$F-nuclei is achieved if the integral attributed to the introduced standard (hexafluoroacetone) remains constant among samples.
RESULTS AND DISCUSSION

$^{19}$F-NMR detects all $^{19}$F nuclei and separates each nucleus based on its local chemical environment. The three $^{19}$F nuclei in TFMP are chemically and magnetically equivalent, such that all three nuclei contribute to one signal in the NMR spectrum. Similarly, the trifluoromethylated products of TFMP oxidation are also predicted to present one distinct signal each. $^{19}$F-NMR is a powerful analytical tool used to elucidate catabolic pathways because (i) all fluorinated compounds are detected, avoiding biases arising from chromatographic techniques or selective detectors; (ii) $^{19}$F is naturally NMR-sensitive, facilitating the detection of low analyte amounts, and (iii) no sample preparation is required, and here we attempt to detect extracellular products by analyzing cell-free supernatant.

Using gas chromatography, we monitored the time course of TFMP oxidation by resting cells of strain PM1 (grown on either MTBE or TBA), or strains S1B1 or G2B2 (each grown on TBA). At select time points, aqueous samples (~700 µl) were removed from the reaction medium and the cells were sedimented by centrifugation. The resulting supernatant was stored at 4°C until analysis, as described in the Methods section. Substantial (>70%) oxidation of TFMP occurred in all strains tested (Figure 5.1), and in each case, the same two distinct signals arising from products of TFMP oxidation were detected (Figure 5.2). The value attributed to the introduced internal standard (hexafluoroacetone) remained constant among samples, indicating all $^{19}$F-atoms originating from TFMP were accounted for as the time course progressed. The two unique signals detected were identical to those we had
previously (see Chapter 3 of this dissertation) observed during TFMP oxidation by propane-grown *Mycobacterium austroafricanum* strain JOB5, an organism that cometabolizes MTBE and TBA. Using gas chromatography/mass spectrometry, the products of TFMP oxidation in strain JOB5 were identified as 2-trifluoromethyl-1,2-propanediol and 2-trifluoromethyl-2-hydroxypropionic acid, fluorinated analogs of 2-methyl-1,2-propanediol (2M12PD) and 2-hydroxyisobutyric acid (HIBA), respectively (Figure 5.3). These compounds are recognized TBA degradation products in several bacterial strains that either slowly metabolize (2, 6) or cometabolize (8) MTBE or TBA under aerobic conditions. These results show that at least two intermediates are common to the pathway of aerobic TBA catabolism in all organisms studied to date, whether or not these organisms are capable of efficiently utilizing TBA as a sole source of carbon and energy.

The currently accepted model of MTBE catabolism (4, 5) suggests that the oxidation of 2M12PD to HIBA occurs through a stable aldehyde intermediate. Evidence for the aldehyde was not obtained in either this, or previous (4, 5) studies. And in Chapter 3 of this dissertation we argue that more work is required before generally accepting the aldehyde as a stable product of 2M12PD oxidation. However, in the time course of TFMP oxidation by TBA-grown cells of strain G2B2, a unique low intensity third signal was detected in addition to the signals corresponding to the fluorinated analogs of 2M12PD and HIBA. Attempts to reproduce this finding failed (Figure 5.2), although the production of the fluorinated analogs of 2M12PD and HIBA were reproduced during TFMP oxidation in MTBE- or TBA-grown strain PM1, TBA-grown strain S1B1 and TBA-grown strain G2B2 (data not shown).
Our results showed that each strain tested oxidized TFMP. In MTBE-grown strain PM1 and TBA-grown strain S1B1, both products of this reaction accumulated in the culture medium; whereas for TBA-grown strains PM1 and G2B2, the fluorinated analog of 2M12PD was consumed, while the fluorinated analog of HIBA accumulated (Figure 5.1). Because the pathway of TFMP oxidation mimicked that of TBA oxidation (Figure 5.3), and was similar in all MTBE- and TBA-metabolizing strains tested; and because daughter products of TFMP resisted degradation in these strains, our results support the suggestion that TFMP may be an attractive tracer to estimate the extent of TBA degradation occurring in situ, as described in Chapter 3 of this dissertation.
Figure 5.1. Time course of 5 mM TFMP (○) oxidation and resulting formation of 2-trifluoromethyl-1,2-propanediol (△) and 2-trifluoromethyl-2-hydroxypropionic acid (□), quantified using $^{19}$F-NMR. A signal arising from an unidentified compound (▽) was also detected during TFMP-oxidation in strain G2B2. Hexafluoroacetone served as the internal standard (⋆) and reference.
Figure 5.2. $^{19}$F-NMR spectra showing chemical shifts observed during the time course of TFMP oxidation by all strains tested in this study. Spectrum for strain JOB5 is taken from Chapter 3 of this dissertation, and shown here for comparison to assist data interpretation. Arrow in spectrum collected for strain G2B2 points to a product observed during the time course that was not seen in subsequent, replicate experiments.
Figure 5.3. Proposed pathway of TFMP oxidation. A recognized (2, 6, 8) pathway of TBA oxidation is shown for comparison.
REFERENCES


CHAPTER 6

PRODUCTS GENERATED DURING METHYL TERTIARY BUTYL ETHER OXIDATION BY NITROSOMONAS EUROPAEA, A $^{13}$C-NMR APPROACH

The work in this chapter describes the author’s contribution to a manuscript entitled “Oxidation of methyl tertiary butyl ether (MTBE) by Nitrosomonas europaea” to be submitted for publication in Biodegradation. The authors of this manuscript will be Denise Aslett, Christy Smith, Alan House and Michael Hyman.
ABSTRACT

$^{13}$C-Nuclear magnetic resonance spectroscopy ($^{13}$C-NMR) detects all $^{13}$C-nuclei in a given sample, making it a powerful and non-selective analytical tool used to elucidate catabolic pathways. Here, this technique has been applied to characterize the products of methyl tertiary butyl ether (MTBE) degradation by the chemolithoautotrophic ammonia-oxidizing bacterium, *Nitrosomonas europaea*. Two products, tertiary butyl alcohol and formate were detected after the oxidation of universally $^{13}$C-labeled MTBE ($^{13}$C$_{5}$-MTBE). The overall results of the study to which these data contribute suggest MTBE oxidation is catalyzed by ammonia monooxygenase. Therefore, the pathway of MTBE oxidation is proposed to involve an initial monooxygenation reaction at the methoxy carbon atom. This reaction would yield an unstable hemiacetal that spontaneously decomposes to yield formaldehyde and tertiary butyl alcohol. Formaldehyde appears to be further oxidized to yield formate.
INTRODUCTION

*Nitrosomonas europaea* utilizes CO$_2$ as its carbon source and derives its energy for growth from the oxidation of ammonia to nitrite. Ammonia oxidation is initiated by ammonia monooxygenase (AMO), which catalyzes the transformation of ammonia to hydroxylamine (5) according to the following reaction (9): \( \text{NH}_3 + \text{O}_2 + 2[\text{H}] \rightarrow \text{NH}_2\text{OH} + \text{H}_2\text{O} \). Like other monooxygenase enzymes (2, 17), AMO oxidizes a wide range of compounds including \( n \)-alkanes (up to C$_8$), \( n \)-terminal alkenes (up to C$_5$) (7), the groundwater pollutant trichloroethylene (8), and the halogenated fumigants methyl bromide, 1,2-dichloropropane and 1,2-dibromo-3-chloropropane (12). These compounds are transformed via cometabolism and are considered unlikely to yield either a carbon or energy benefit to the organism as alternate carbon catabolic pathways are scarce in *N. europaea* (1). The role of ammonia-oxidizing bacteria in methyl tertiary butyl ether (MTBE)-oxidizing consortia is unresolved. For example, in 1994 Salanitro *et al.* isolated a mixed culture capable of degrading MTBE and oxidizing ammonia (13). The methoxy carbon of at least 40% of MTBE molecules was mineralized to CO$_2$, and this process sustained the activity of the mixed culture. Increasing the ammonia content of the culture medium did not impact MTBE transformation, which suggested to the authors that MTBE and ammonia were oxidized by different enzymes (13). The effects of MTBE on *N. europaea* have recently been characterized in Dr. Hyman’s laboratory by Denise Aslett and Christy Smith. The author’s contribution to this study has been to use $^{13}$C-NMR to establish the diversity of oxidation products generated by this bacterium when exposed to MTBE.
ANALYTICAL METHODS

NMR Spectroscopy. Products formed during $^{13}$C$_2$-MTBE oxidation were identified using $^{13}$C-NMR. For NMR analysis, samples (510 µl) of thawed supernatant (see below) were transferred to a 5 mm NMR tube. $^2$H$_2$O (75 µl) and methanol (2 µl, neat) were added to provide a magnetic field lock and an internal reference ($\delta = 49.15$ relative to tetramethylsilane), respectively. Proton decoupled $^{13}$C-NMR spectra were collected using a Varian Mercury 300 spectrometer operated at 75 MHz with a spectral width of 18.8 kHz and a pulse width of 8.7 µs (corresponding to a flip angle of 43.5°), with no delay between pulses. A total of 1024 scans, with 68K data points in the time domain, were collected for Fourier transformation.
RESULTS AND DISCUSSION

Initial experiments designed to characterize MTBE oxidation by *N. europaea* used gas chromatography to detect predicted organic oxidation products such as tertiary butyl alcohol (TBA). However, gas chromatography is often selective and favors the detection of uncharged, volatile, and relatively non-polar compounds. Different detectors further impact the selectivity and sensitivity of this technique. For example, the most commonly used detector, the flame ionization detector (FID), responds poorly to methanol and is unable to detect either formaldehyde or formate. Consequently, a more inclusive analytical technique is appropriate when trying to determine the full range of metabolites generated during a biodegradation process. In the present study we have used $^{13}$C-NMR to investigate the diversity of oxidation products generated by *N. europaea* during the oxidation of MTBE. This technique detects all $^{13}$C nuclei and separates each nucleus based on its local chemical environment. Because the $^{13}$C isotope is only ~ 1% naturally abundant, we used a universally $^{13}$C-labeled form of MTBE ($^{13}$C$_5$-MTBE) to determine the fate of each carbon atom in the molecule.

The time course of $^{13}$C$_5$-MTBE oxidation by *N. europaea* was monitored by gas chromatography and at select time points aqueous samples (~700 µL) were removed from the reaction medium. The cells were sedimented by centrifugation and the resulting supernatant stored at -20°C until analysis, as described in the Methods section. $^{13}$C-signals arising from each compound were relatively low in intensity, but were sufficient for the qualitative aims
of this study (Figure 6.1). Comparing spectra obtained with experimental samples to those obtained with pure $^{13}$C-labeled compounds showed that only two organic products, TBA and formate, were detected after incubation of $N. europaea$ with $^{13}$C$_5$-MTBE (Figure 6.1).

Whole-cell biochemical assays, including the specific inactivation of AMO by acetylene (10), indicate MTBE oxidation in $N. europaea$ is catalyzed by AMO. An analysis of our $^{13}$C-NMR data in light of this finding suggests AMO oxidizes MTBE at the methoxy carbon. This monooxygenation reaction is predicted to generate a hemiacetal (18) which decomposes to yield TBA and formaldehyde, the latter being further oxidized to formate (Figure 6.2).

In a filamentous fungus $Graphium$ species (4), and several strains of $Mycobacterium austroafricanum$ (strains IFP 2012 (3), IFP 2015 (11) and JOB5 (14)) MTBE oxidation is also a monooxygenase catalyzed reaction, but generates tertiary butyl formate (TBF) as an initial stable oxidation product. In these organisms it is thought the initial hemiacetal generated during MTBE oxidation is further oxidized to TBF by an alcohol dehydrogenase. The lack of TBF production by $N. europaea$ in the presence of MTBE most likely reflects the lack of an alcohol dehydrogenase activity in this organism. In contrast, abiotic cleavage of the hemiacetal to yield TBA and formaldehyde has been observed in $n$-alkane grown $Pseudomonas mendocina$ strain KR-1 (15) and the propane-oxidizing bacterium strain ENV 425 (16).
The fate of the formaldehyde generated during either aerobic MTBE metabolism or cometabolism has not been studied extensively. We have previously suggested that the inability of MTBE-degrading organisms to further metabolize and detoxify formaldehyde may contribute to the limited ability to isolate MTBE-metabolizing organisms in pure culture (6). In the present study we did not observe formaldehyde in the reaction supernatant while trace levels of formate were detected. From these limited experiments it appears that *N. europaea* has some ability to further oxidize formaldehyde to formate although evidence for \(^{13}\text{CO}_2\) accumulation, an indicator of the further oxidation of formate, was not observed. Likewise, no evidence for the further oxidation of TBA to 2-methyl-1,2-propanediol was obtained in this study. Future experiments with *N. europaea* using \(^{13}\text{C}\)-methoxy-labeled MTBE are warranted and will examine the possibility that the lack of formaldehyde detection is due to indiscriminate alkylation of cell material by this reactive compound. These experiments will also examine whether any \(\text{CO}_2\) released during formate oxidation is assimilated through the normal carbon assimilation pathway of this autotrophic bacterium.
Figure 6.1. Products detected by $^{13}$C-NMR from samples selected during the time course of $^{13}$C$_5$-MTBE oxidation by *N. europaea*. Pathway reflects data collected. Letters above each peak correspond to atoms labeled in the pathway diagram. Signals arising from time course samples were compared to those obtained with authentic standards for $^{13}$C$_4$-TBA, $^{13}$C-formate and $^{13}$C-formaldehyde. Dashed boxes surround the area of each spectrum that has been magnified to assist data interpretation.
Figure 6.2. Proposed pathway of MTBE oxidation by *Nitrosomonas europaea*. Dashed boxes surround suspected intermediates which were not detected. Also shown is the route to tertiary butyl formate, a recognized product of MTBE oxidation which was not detected in this study (indicated by the crossed-out arrow). AMO, ammonia monooxygenase.
REFERENCES


CONCLUDING STATEMENT

The work described in this dissertation explores methyl tertiary butyl ether (MTBE) cometabolism and propane metabolism at the whole-cell level using the aerobic hydrocarbon-oxidizing bacterium Mycobacterium austroafricanum (previously vaccae) JOB5. Our results show that several major gasoline components, which are frequent co-contaminants encountered with MTBE in the environment, are capable of supporting the cometabolism of both MTBE and its commonly encountered metabolite, tertiary butyl alcohol (TBA). Cultures grown with only one hydrocarbon in the medium simultaneously expressed more than one distinct hydrocarbon-oxidizing enzyme, and we showed that cometabolic transformations were catalyzed by an enzyme system(s) distinct from that required for cell growth. This contrasts with the prevailing model of cometabolism where cometabolic transformations are generally believed to be catalyzed by the same enzyme system required for cell growth. In a separate project, we obtained a more complete understanding of the MTBE oxidation pathway that may serve to inform molecular studies aiming to fit genes and proteins to a generally accepted pathway model. We also observed that the initial rate of degradation for MTBE and each of its pathway intermediates decreased as the pathway progressed towards central metabolism, suggesting that slow oxidation rates, rather than incomplete metabolism, prevents MTBE-dependent cell growth in strain JOB5.

Contributions were made to a series of projects by using nuclear magnetic resonance spectroscopy (NMR) to identify pathway intermediates. TBA and formate were identified
during MTBE oxidation by *Nitrosomonas europaea*, and this result contributes to a study suggesting that MTBE oxidation is catalyzed by ammonia monooxygenase in this bacterium.

NMR was also used to contribute to studies characterizing the oxidation of a fluorinated substrate analog of TBA in several bacterial strains. Results from these studies suggest that the fluorinated analog of TBA may be an attractive tracer to estimate the extent of TBA degradation occurring *in situ*.

Propane-grown cells of strain JOB5 are known to cometabolically oxidize a variety of groundwater contaminants. This organism appears to simultaneously use both terminal and subterminal propane oxidation pathways to support growth. This divergent metabolism of propane is apparently followed by the divergent metabolism of propionate (2-methylcitrate and methylmalonyl-CoA pathways) and the divergent oxidation of acetone (acetol and acetoacetate pathways). Adding bicarbonate to the culture media improved cell growth on propane and its oxidation products, suggesting that elevated CO$_2$ concentrations *in situ* may stimulate pollutant biodegradation processes that rely on propane-oxidizing bacteria.

This dissertation has provided exciting hypotheses for future experimenters to test both the relevance of our conclusions *in situ*, and the molecular biology underpinning the physiology we infer from *in vitro* whole-cell analyses. It is the author’s sincere hope that, in the words of Eugene Madsen, “...these hypotheses will be tested…so that the knowledge of environmental microbiology will constantly grow, be refined, and asymptotically approach truth.” [Madsen, E. L. 1998. Environ. Sci. Technol. 32:429-439.]