ABSTRACT

Johnson, Erika L. Gasoline oxygenate biodegradation processes in *Mycobacterium vaccae* JOB5 (Under the direction of Dr. Michael R. Hyman).

Ether-based gasoline oxygenates are added to gasoline in the U.S. at concentrations $\leq 15\%$ vol/vol to reduce carbon monoxide, hydrocarbon and particulate automobile emissions. These ether oxygenates, particularly methyl tertiary butyl ether (MTBE), have become a human health concern as they have been widely detected in drinking water sources. This study aimed to investigate the monooxygenase in *Mycobacterium vaccae* JOB5 previously found to cometabolize MTBE, focusing on the regulation and substrate range of this enzyme.

Our first study identified MTBE as an inducer of the monooxygenase enzyme in *M. vaccae* JOB5. In the presence of a nonalkane growth-supporting substrate, including organic acids, MTBE consumption was observed. The expected products of MTBE oxidation, tertiary butyl formate (TBF) and tertiary butyl alcohol (TBA) accumulated in the culture medium. Both the consumption of MTBE and the production of TBF and TBA were inhibited by acetylene, a specific inhibitor of alkane- and MTBE-oxidizing activity and by chloramphenical and rifampicin, transcriptional and translational inhibitors.

Further investigation into the regulation of the monooxygenase in *M. vaccae* JOB5 found that high concentrations of 1-propanol, a product of alkane oxidation by the monooxygenase, leads to inhibition of MTBE-oxidizing activity. The relationship between 1-propanol, MTBE, and the monooxygenase was not fully characterized in this study.
In addition to MTBE, ethyl tertiary butyl ether (ETBE) and tertiary amyl methyl ether (TAME) are alternate ether oxygenates added to gasoline. We found that propane-grown *M. vaccae* JOB5 cells are able to oxidize both ETBE and TAME and their alcohol products, TBA and tertiary amyl alcohol (TAA), respectively. Three lines of evidence suggest that the oxidation of these ethers and alcohols is initiated by the same monooxygenase responsible for MTBE-oxidation: the absence of a lag phase during incubation with propane-grown cells, inhibition of ether-oxidizing activity by acetylene, and the competitive interaction during incubation with propane.

Finally, we investigated the growth-supporting range of *n*-alkanes in the most well characterized alkane-oxidizing system, *P. putida* GPo1. This study led to the expansion of known *n*-alkanes which support growth in this organism to include the gaseous alkanes, *n*-butane and propane. The oxidation of these gaseous *n*-alkanes appears to be induced by the same alkane monooxygenase, AlkB, responsible for oxidation of the previously defined growth-supporting *n*-alkanes (C₅-C₁₂).

These studies have led to a better understanding of monooxygenase activity in both *M. vaccae* JOB5 and *P. putida* GPo1, including expanded knowledge of regulation and substrate ranges of this enzyme.
Gasoline oxygenate biodegradation processes in *Mycobacterium vaccae*

JOB5

by

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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

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PERSONAL BIOGRAPHY

Erika Lynn Johnson was born on September 10, 1978, in Herndon, Virginia. After graduating from Herndon High School, Erika began her undergraduate studies at James Madison University in Harrisonburg, Virginia. During her time at JMU she pursued a B.S. in Biology and worked in the laboratory of Dr. Bruce A. Wiggins. She also studied abroad in Costa Rica where she became especially passionate about the environment. Inspired by her studies in Costa Rica and her work in Dr. Wiggins’ lab, Erika decided to pursue a Ph.D. in Microbiology with an emphasis on the environment. In the fall of 2000, Erika entered Dr. Michael Hyman’s laboratory at North Carolina State University. While at State, Erika met many lifelong friends including her best friend, Rusty and Rusty’s dad, Nathan Borden. She graduated in May of 2005 from North Carolina State University with a Ph.D. in Microbiology.
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CHAPTER 1

Literature Review
Gasoline Oxygenates: Oxygenates are compounds added to reformulated gasoline to increase oxygen content, reduce emissions, and enhance the octane rating of gasoline (1). Oxygenates currently used in reformulated gasoline in the U.S. include ethers such as methyl tertiary butyl ether (MTBE), ethyl tertiary butyl ether (ETBE) and tertiary amyl methyl ether (TAME), and alcohols such as tertiary butyl alcohol (TBA) and ethanol. MTBE was first introduced into gasoline in the 1970s strictly as an octane enhancer after the removal of lead compounds from gasoline led to decreased octane ratings. The use of oxygenates dramatically increased in the 1990s following the passage of the Clean Air Act (CAA) Amendments in 1990 which required areas in the U.S. with the greatest air pollution to add oxygenates to all grades of gasoline (2). The CAA Amendments aimed to minimize carbon monoxide, hydrocarbon and particulate emissions released from automobiles (2). Of the available gasoline oxygenates, MTBE is the most commonly used in the U.S., and is added at concentrations ≤15% (vol/vol). MTBE was chosen as the preferred oxygenate by gasoline manufacturers based on a few important qualities: it is made from two readily available refinery waste products, isobutylene and methanol and it blends well with gasoline formulations making it a convenient additive. Furthermore, pipelines used to transport traditional gasoline can transport MTBE-enhanced gasoline. However, the use of MTBE has come under scrutiny in recent years and has been banned by numerous states including California, New Hampshire, and New York. Recently other oxygenates, particularly ETBE and TAME, have increasingly replaced MTBE, and their use is expected to steadily increase as more states ban MTBE. The structure of these selected oxygenates and some products of their oxidation are represented in Figure 1.1.
Figure 1.1. Structure of Oxygenates and Selected Products. The structure of select ether oxygenates (MTBE, ETBE, TAME, and DIPE) and some of their metabolites including TBA, TAA, TBF, and 2-methyl-2-hydroxy-1-propanol.
Why are we concerned with oxygenates?: As described earlier, oxygenates reduce carbon monoxide, hydrocarbon and particulate emissions (1) released from gasoline combustion. However, these oxygenates pose an environmental threat when gasoline is exposed to the environment in liquid form. Gasoline contaminates the environment through numerous means including gasoline spills in transport or at the gas station, from motorized watercraft (3) and from leaking underground storage tanks (LUSTs) (4). LUSTs are a major contributor to the release of MTBE into the environment. When gasoline leaks into the soil from a LUST it eventually reaches groundwater. Once the gasoline reaches the groundwater a distinct separation occurs as the polar and nonpolar components of gasoline are separated by solubility. Due to its hydrophilic structure, MTBE travels well in groundwater creating a plume that leads it away from the hydrocarbons in gasoline (Figure 1.2). The solubility and extensive dispersion of MTBE in groundwater results in the uptake of MTBE-contaminated water by individual wells. MTBE has a distinctly unpleasant smell and taste that has been described as similar to turpentine. The presence of MTBE in domestic well water led the U.S. Environmental Protection Agency (EPA) to conduct studies on the human health hazards of MTBE consumption. The EPA has declared MTBE a potential carcinogen and issued a drinking water advisory of 20-40 ppb (5).

Environmental fate of MTBE, oxygenates and biodegradation: The environmental fate of MTBE is not fully understood. The plumes created in groundwater contamination sites have led to many studies investigating the anaerobic and aerobic biodegradation of
Figure 1.2. LUST and MTBE plume. A representation of gasoline leaking from an underground storage tank. Once the gasoline reaches the groundwater, a separation occurs between hydrophilic and hydrophobic components of gasoline. MTBE is highly soluble in water and therefore travels farther and faster in the groundwater than gasoline hydrocarbons. As the groundwater flows away from the contamination site, MTBE is carried towards individual wells causing the consumption of MTBE to become a human health concern.
MTBE. Anaerobic degradation of oxygenates is potentially of great significance to the natural attenuation of oxygenates in the environment. The environment of an underground gasoline spill rapidly becomes anaerobic as aerobic microorganisms degrade the gasoline hydrocarbons, consuming the available molecular oxygen and other electron acceptors. However, studies have generally shown (6, 7, 8) that MTBE biodegradation occurs slowly if at all in the absence of O\textsubscript{2}. Slow rates of MTBE biodegradation have been observed under methanogenic (9) or sulfate-, iron- or nitrate-reducing conditions (10, 11, 12). In aerobic conditions, the rate of MTBE biodegradation is significantly greater; therefore many studies have been conducted focusing on aerobic biodegradation.

There are two different metabolic processes that can achieve MTBE biodegradation, metabolism and cometabolism. True metabolism involves the utilization of a substrate as a sole source of carbon and energy. In contrast, cometabolism involves biological transformation of a compound that does not support growth. Cometabolism converts a compound by utilizing one or more non-specific enzymes produced during true metabolism of a growth-supporting substrate (13). It is important to note that the organism typically does not gain energy or carbon from the cometabolized substrate. This process has been identified as a rapid means of degrading environmental pollutants including gasoline oxygenates and chlorinated solvents (13).

Few organisms have been identified that are able to truly metabolize MTBE including *Rubrivivax* sp. strain PM-1, *Mycobacterium austroafricanum* and *Hydrogenophaga flava* ENV 735 (14, 15, 16). Although MTBE is the sole carbon and
energy source for these organisms, they all degrade MTBE slowly and inefficiently. The pathway of metabolic oxidation of MTBE by *Rubrivivax* sp. strain PM-1 has been proposed (15) and described in Figure 1.3. MTBE is initially oxidized by a monooxygenase to an unstable hemiacetal which rapidly dismutates to TBA and formaldehyde. The TBA is further oxidized to 2-methyl-1,2-propanediol, hydroxybutyric acid (HIBA) and finally carbon dioxide. Two distinct enzymes have been identified that are responsible for MTBE oxidation and TBA oxidation (17). The rate limiting steps in this process are currently unknown. The persistence of MTBE plumes suggest that these organisms are inefficient at degrading MTBE at contamination sites, or that they are not widely distributed in these environments.

Many more microorganisms have been identified that biodegrade MTBE through cometabolism. These include, among others, *Pseudomonas mendocina* KR-1, *Pseudomonas putida* GPo1 and *Mycobacterium vaccae* JOB5 (18, 19, 20). The microorganisms responsible for oxidizing MTBE via cometabolism are often hydrocarbon-oxidizing bacteria and propane-and n-pentane-oxidizing microorganisms have been most extensively studied (18, 21, 22). The pathway for MTBE cometabolic oxidation by *Mycobacterium vaccae* JOB5 is represented in Figure 1.3. This pathway differs from *Rubrivivax* sp. strain PM-1 in numerous ways, but the most significant difference is the first observed product of MTBE oxidation, tertiary butyl formate (TBF) in *M. vaccae* JOB5 cells versus TBA in *Rubrivivax* sp. strain PM-1 (18). In propane-grown *M. vaccae* JOB5 cells, MTBE is initially oxidized by an alkane-induced monooxygenase to an unstable hemiacetal. The hemiacetal is likely quickly oxidized by an alcohol dehydrogenase to TBF and formate and TBF is biologically and chemically
Figure 1.3. Comparative pathways of metabolism/cometabolism of MTBE by *Pseudomonas putida* GPo1, *Mycobacterium vaccae* JOB5, and *Rubrivivax* PM1
converted to TBA. TBA oxidation in *M. vaccae* JOB5 is identical to that of *Rubrivivax* sp. strain PM-1, TBA is oxidized to 2-methyl-1,2-propanediol which is oxidized to HIBA eventually releasing carbon dioxide. Unlike *Rubrivivax* sp. strain PM-1, in *M. vaccae* JOB5 MTBE biodegradation, MTBE and TBA are oxidized by the same monooxygenase (18). Another interesting aspect of MTBE cometabolism in *M. vaccae* JOB5 is the inducing effects of MTBE in this organism (23). In this organism, MTBE is oxidized in the presence of both n-alkane and non-alkane carbon sources, although MTBE-oxidation occurs much more rapidly in the presence of n-alkanes.

Although MTBE oxidation by *P. putida* GPo1 is also cometabolic, the pathway of cometabolism is much different than *M. vaccae* JOB5 (Figure 1.3). The cometabolic degradation of MTBE by *P. putida* GPo1 results in the accumulation of TBA and formaldehyde (20). This is an identical initial oxidation reaction to that of *Rubrivivax* sp. strain PM-1, however, cometabolism results in TBA as the final product in *P. putida* GPo1 cells. *P. putida* GPo1 catalyzed MTBE oxidation is an example of true cometabolism. These cells require an n-alkane to stimulate MTBE oxidation and the result is conversion of MTBE by one highly non-specific monooxygenase discussed later in this introduction, AlkB (20).

The biodegradation of MTBE in an aerobic environment can occur by two different metabolic processes, metabolism and cometabolism. Metabolism of MTBE occurs in *Rubrivivax* sp. strain PM-1 while *M. vaccae* JOB5 and *P. putida* GPo1 cometabolize MTBE. Oxidation of MTBE in *M. vaccae* JOB5 is induced by MTBE resulting in an unusual process which lies somewhere between true metabolism and cometabolism, although this organism only productively biodegrades MTBE through
cometabolism. All three of these aerobic MTBE biodegradation examples have one important step in common, the first enzyme required to oxidize MTBE is a monooxygenase.

**Oxygenases:** Almost all hydrocarbon-oxidizing bacteria that have been identified require molecular oxygen for both respiration and oxidation of hydrocarbons by an oxygenase. There are two distinct classifications of oxygenases, monooxygenases and dioxygenases. Both of these enzymes incorporate oxygen from O$_2$ into an aliphatic or aromatic hydrocarbon. Dioxygenases incorporate both oxygen atoms from O$_2$ to oxidize hydrocarbons. Since these enzymes use both oxygen atoms in the hydrocarbon, no outside electron acceptor is required. Dioxygenases commonly act on aromatic hydrocarbons (24). In contrast, monooxygenases incorporate one oxygen atom into the hydrocarbon while reducing the remaining oxygen atom to H$_2$O. To reduce the remaining oxygen atom to water, monooxygenases require NADH (25, 26). Although monooxygenases are capable of oxidizing a hydrocarbon at numerous places on the structure, in the case of aliphatic hydrocarbons, it is most common for a monooxygenase to act on the $\mu$-carbon (27).

**N-Alkane oxidation:** There are a number of alkane-oxidizing enzyme systems known, including methane monooxygenases, cytochrome P450-dependent monooxygenase, AlkB, an alkane hydroxylase found in *Pseudomonas putida* GPo1, AlkM, an alkane hydroxylase found in *Acinetobacter* sp. strain ADP1, and an alkane-induced monooxygenase in *Mycobacterium vaccae* JOB5 (18, 22, 28, 29, 30).
Typically, alkane oxidation involves a series of enzymatic reactions that convert n-alkanes to their corresponding terminal acyl-coenzymeA derivatives. These products then enter the β-oxidation cycle. The oxidation of n-alkanes to their respective acyl-coenzymeA derivatives requires four enzymes: a monooxygenase (alkane hydroxylase), an alcohol dehydrogenase, an aldehyde dehydrogenase and an acyl-coenzymeA synthetase (Figure 1.4). The initial alkane-hydroxylase-catalyzed reaction converts the n-alkane to a primary alcohol. As a monooxygenase, alkane hydroxylase incorporates one atom of molecular oxygen into the alkane as a hydroxyl group while the other oxygen atom is reduced to water in the presence of NADH. The resulting alcohol is then oxidized by an alcohol dehydrogenase producing an aldehyde which is subsequently oxidized by an aldehyde dehydrogenase resulting in a fatty acid. Both the alcohol and aldehyde dehydrogenases require the presence of NAD$^+$ as an electron acceptor. The resulting fatty acid is then converted to its acyl-CoA derivative by the acyl-CoA synthetase. The acyl-CoA derivative then enters the tricarboxylic acid cycle through β-oxidation.

Methanotrophic bacteria produce a methane monooxygenase (MMO) which is responsible for initiating the oxidation of methane to methanol in these organisms. This enzyme is highly non-specific and has been shown to oxidize many other substrates besides methane (28). Two distinct forms of methane monooxygenase occur in methanotrophic bacteria, a particulate methane monooxygenase and a soluble methane monooxygenase. These two forms of the enzyme have marked differences in both their
Figure 1.4. The typical pathway of n-alkane oxidation. The n-alkane is oxidized to a primary alcohol by alkane monooxygenase followed by the oxidation of the primary alcohol to an aldehyde. This reaction is catalyzed by alcohol dehydrogenase. The aldehyde is subsequently oxidized to a fatty acid by an aldehyde dehydrogenase. This fatty acid then enters the TCA cycle following β-oxidation.
specificity and active sites. The soluble form of methane monooxygenase is a highly non-specific enzyme that has been shown to oxidize a range of substrates that do not support growth including carbon monoxide, alkanes, and alkenes (28). The active site for substrate oxidation/reduction in the soluble methane monooxygenase has a binuclear iron center (31). In contrast, the particulate methane monooxygenase does not oxidize the wide range of substrates the soluble methane monooxygenase does. The active site in the particulate methane monooxygenase contains a trinuclear copper cluster (32). However, neither the soluble nor the particulate forms of methane monooxygenase have been found to oxidize MTBE.

Cytochrome P450 monooxygenases have been identified in both eukaryotic and prokaryotic organisms. Cytochrome P450s have been found to be involved in a wide range of biotransformations including detoxification of xenobiotics, such as MTBE, and in secondary metabolic processes. These monooxygenases have been shown to oxidize both long chain hydrocarbons (>C10) in Candida sp. and short chain hydrocarbons (C2-C4) in Graphium sp. in eukaryotes (33, 34, 35). In the Graphium sp. fungal system, MTBE oxidation has been observed following growth on n-alkanes (36).

Cytochrome P450 monooxygenases have also been identified in prokaryotes including Corynebacterium 7EIC and Pseudomonas putida (30, 37). The cytochrome P450cam isolated from camphor-grown P. putida is the paradigm for the structure of cytochrome P450s. The monooxygenase identified in P. putida CAM is a three component enzyme that includes P450cam, putidaredoxin, and putidaredoxin reductase (38). This monooxygenase has been shown to have a large substrate diversity including ethylbenzene, naphthalene, and styrene (39, 40). MTBE oxidation has also been
observed by the \textit{P. putida} P450cam. (22). Regardless of the host, cytochrome P450 monooxygenases contain a b-type heme iron group which is the location of substrate oxidation/reduction (41).

\textbf{\textit{Pseudomonas putida} GPo1 and AlkB:} The most representative non-methane alkane-oxidizing system for bacteria is that of \textit{Pseudomonas putida} GPo1. Much is known about both the enzymes and genetics of the \textit{P. putida} GPo1 system, which includes AlkB, the alkane monooxygenase. All of the enzymes required for alkane oxidation are encoded on the OCT plasmid and are referred to as the \textit{alk} genes (Figure 1.5) (42). The \textit{alk} genes encode the three key components of the alkane hydroxylase complex (\textit{alkB,G,} and \textit{T}) (43), alcohol dehydrogenase (\textit{alkJ}) (44), the aldehyde dehydrogenase (\textit{alkH}) (45) and the acyl-CoA synthetase (\textit{alkK}) (44), all required for n-alkane oxidation. Of the remaining Alk proteins, AlkS has been identified as a transcriptional regulator (46) while the roles of AlkF and AlkL have yet to be fully determined. Studies suggest that \textit{alkL} encodes an outer membrane protein that is homologous to OmpW, a \textit{Vibrio cholerae} outer membrane protein of unknown function (44). The AlkL protein appears to be a non-essential protein for \textit{P. putida} GPo1 growth on n-alkanes (44) and could potentially be a porin increasing the transport of n-alkanes into the cell. The protein encoded by \textit{alkF} has been identified as a rubredoxin similar to \textit{alkG} (44, 45, 46), however, this rubredoxin seems to be non-functional within the alkane oxidizing system of \textit{P. putida} GPo1 (45). All of the \textit{alk} genes are organized into two clusters, \textit{alkBFGHJKL} and \textit{alkST} located on the OCT plasmid. These two gene clusters are separated by \textasciitilde40 kb and are transcribed in opposite directions (47, 48).
Figure 1.5. The arrangement of the alk genes on the OCT plasmid in Pseudomonas putida GPo1. The location of the alk genes. Promoter PalkB encodes the alkane hydroxylase (alkB), non-functional rubredoxin (alkF), rubredoxin (alkG), aldehyde dehydrogenase (alkH), alcohol dehydrogenase (alkJ), and acyl co-A synthetase (alkK) and membrane bound protein (alkL). The PalkS promoter regulates transcription of the transcriptional regulator protein (alkS) and the rubredoxin reductase (alkT). Figure modified from ref 52.
**Transcriptional regulation:** Transcription of these genes is under both catabolite repression and positive transcriptional regulation by the AlkS protein (46, 49, 50, 51). The AlkS protein regulates transcription of the alkBFGHJKL operon as well as its own operon, **alkST**. While the alkBFGHJKL operon contains one promoter region, P_{alkB} (46), the alkST operon has two promoter regions that are 38 bp apart, P_{alkS1} and P_{alkS2} (52). The AlkS protein activates both the P_{alkS2} and P_{alkB} promoter regions (46, 52, 53). The other alkST promoter, P_{alkS1}, is regulated by $^{\text{s}}$, a stationary phase sigma factor (54). By requiring a stationary phase sigma factor, transcription of the AlkS protein is limited during exponential growth when there is a preferred carbon source for the organism to utilize. Minimal transcription of AlkS occurs through this promoter, P_{alkS1}, in the absence of n-alkanes (54). This allows the organism to quickly react to a change in environment should the preferred carbon source be eliminated leaving an n-alkane as the only available carbon source. Positive transcriptional regulation occurs when the binding of AlkS causes repression of the P_{alkS1-$^{\text{s}}$}-dependent promoter and induction of the P_{alkS2} promoter (52). This results in high expression of AlkS in the presence of alkanes and the absence of a preferred carbon source that consequently increases the transcription of the alkBFGHJKL operon.

**Pseudomonas putida GPo1 alkane hydroxylase structure:** The first enzyme required for alkane oxidation is the most complex enzyme, a three-component alkane hydroxylase (25, 55). It consists of a 41kDa hydroxylase (AlkB), a 19kDa rubredoxin (AlkG) (26), and a 54kDa rubredoxin reductase (AlkT) (56). The hydroxylase is a cytoplasmic membrane protein while the rubredoxin and the rubredoxin reductase are both
cytoplasmic proteins (Figure 1.6) (44, 57, 58). The rubredoxin is a simple iron-sulfur redox active protein that shuttles electrons from the rubredoxin reductase to the alkane hydroxylase (25). The alkane hydroxylase is a di-iron protein and has been studied extensively for its potential use as a biocatalyst for production of alcohols, fatty acids, and epoxides (59). There is a significant increase in the amount of iron required by the cell when it switches from a preferred carbon source to growth on alkanes because of the iron required by the rubredoxin and the alkane hydroxylase (60).

The alkane hydroxylase contains nine hydrophobic regions (eight of which are long enough to span the membrane) and five hydrophilic regions (46). Data has suggested that there are six transmembrane segments of the alkane hydroxylase while the amino terminus, the carboxyl-terminal domain and two loops are exposed to the cytoplasm (61).

Interestingly, the three components of the alkane hydroxylase are not expressed in stoichiometric amounts. In fact, the molar proportions of the AlkB, AlkG, and AlkT proteins have been projected at 50:10:1 in vivo (62). In addition, alkane-grown P. putida GPo1 cells have been found to exhibit MTBE-oxidizing activity (20).

**Acinetobacter sp. strain ADP1 and AlkM:** Acinetobacter sp. strain ADP1 is able to metabolize long chain hydrocarbons of >12 carbons in length. The process of alkane metabolism in Acinetobacter sp. strain ADP1 requires at least five essential genes
Figure 1.6. The arrangement of the Alk proteins within the cell of Pseudomonas putida GPo1. The location of the Alk proteins during growth on alkanes including the alkane hydroxylase (AlkB), rubredoxin (AlkG), rubredoxin reductase (AlkT), alcohol dehydrogenase (AlkJ), aldehyde dehydrogenase (AlkH), acyl co-A synthetase (AlkK), transcriptional regulator protein (AlkS), AlkL is located in the outer membrane but the function is unknown. Figure modified from ref 77.
including a rubredoxin, rubredoxin reductase (rubAB), alkane hydroxylase (alkM), transcriptional regulator protein (alkR), and a protein responsible for the general secretory pathway (xcpR) (29, 63, 64). Unlike the highly organized alkane-oxidizing genes in *P. putida* GPo1, these genes are not centrally located on a plasmid, nor are they organized in one region on the chromosome. Instead, these genes appear to be dispersed throughout the chromosome in no apparent order (65).

Other aspects of alkane oxidation are similar between *Acinetobacter* sp. strain ADP1 and *P. putida* GPo1. The critical step of alkane oxidation in both of these organisms is the conversion of an n-alkane to a primary alcohol. In *Acinetobacter* sp. strain ADP1, this first step requires an alkane hydroxylase (AlkM), rubredoxin (RubA), and a rubredoxin reductase (RubB) (29, 63). These are the same components of the alkane monooxygenase in *P. putida* GPo1 and the roles of these components are likely the same. Due to their evident disorder throughout the chromosome, the transcriptional regulation of these genes is clearly different from *P. putida* GPo1. However, similar to the AlkS regulation of alkB transcription described in *P. putida* GPo1, the transcription of alkM in strain ADP1 is regulated by AlkR (66). Transcription of alkR is induced by a number of various chain length n-alkanes and in the absence of these n-alkanes, is transcribed in low levels (66). The remaining essential genes for alkane metabolism, rubAB and xcpR, are constitutively expressed (63, 64).

*Mycobacterium vaccae* JOB5 and an alkane monooxygenase: *Mycobacterium vaccae* JOB5 was originally isolated on 2-methyl butane, but has since been shown to grow on straight chain n-alkanes, from C$_2$-C$_{22}$ in length (67). The monooxygenase produced
during growth on these short chain n-alkanes (C₂-C₈) is highly non-specific enabling the enzyme to degrade many unusual substrates including trichloroethylene, vinyl chloride, benzene, ethyl benzene, cyclohexane, HCFCs, and 1-chlorobutane (67, 68, 69, 70, 71, 72). Due to this non-specific enzyme and the range of substrates it is able to cometabolize, *M. vaccae* JOB5 has become a model organism in the understanding of cometabolism.

Another unusual characteristic of the alkane-induced monooxygenase in *Mycobacterium vaccae* JOB5 is the atypical location of hydroxylation on the n-alkane. As previously mentioned, most monooxygenases oxidize the primary carbon. In contrast, the alkane-induced monooxygenase in *M. vaccae* JOB5 has been shown to oxidize the secondary carbon of an n-alkane resulting in a secondary alcohol (Figure 1.7). This subterminal oxidation in *Mycobacterium vaccae* JOB5 was first identified by Vestal and Perry (73) who discovered this phenomenon by identifying levels of isocitrate lyase activity in propane-grown cells. Isocitrate lyase functions by producing glucose through the glyoxylate shunt pathway from a two carbon source such as acetyl-CoA from beta-oxidation of fatty acids. Vestal and Perry (73) identified the presence of isocitrate lyase in propane-grown but not propionic acid-grown *M. vaccae* JOB5 cells. The subterminal oxidation argument was strengthened when Coleman and Perry (74) further suggested, through ¹⁴C-labeled acetone, that propane is oxidized to 2-propanol, 2-propanol is further oxidized to acetone then to acetol. Acetate, the result of acetol cleavage, is metabolized by the cell while the remaining C₁ fragment was involved in amino acid synthesis (and other cellular constituents). This atypical hydroxylation may enhance the organism’s ability to oxidize the wide range of cometabolized substrates.
Figure 1.7. Terminal vs. subterminal oxidation in *Mycobacterium vaccae* JOB5. The first enzyme that acts on propane is a short chain alkane monooxygenase (SCAM) followed by a primary or secondary alcohol dehydrogenase (ADH). In terminal oxidation, an aldehyde dehydrogenase (ALDH) oxidizes the aldehyde to a fatty acid. In subterminal oxidation, the ketone is hydroxylated by alkane monooxygenase (AMO) resulting in hydroxyacetone.
Having discussed subterminal oxidation in *M. vaccae* JOB5, it is important to note that only terminal oxidation has been identified by *n*-butane-grown *M. vaccae* JOB5 (75). In addition to this information, further studies (76) of propane-grown *M. vaccae* JOB5 have discovered both terminal and subterminal oxidation of propane, resulting in both 1-propanol and 2-propanol. The presence of both terminal and subterminal oxidation in *M. vaccae* JOB5 could be explained by the particularly non-specific alkane-induced monooxygenase in this organism.

Cometabolism of gasoline ether oxygenates have been studied primarily on hydrocarbon-grown microorganisms (18, 21, 22). However, some effort has been made to determine if cometabolism of ethers occurs when microbes are grown on alcohols or acids, downstream products of *n*-alkane oxidation. Steffan, et. al found that the rate of degradation of these oxygenates depended on the growth substrate. For example, in 24 hour incubations, propane-grown cells of *M. vaccae* JOB5 consumed 100% of the initial concentrations of MTBE, ETBE, and TAME, and 55% of TBA. However, during this time, 2-propanol-grown cells consumed 93% MTBE, 80% ETBE, 100% TAME, and 11% TBA. Strain ENV was also shown to more readily degrade ethers following growth on propane. In 24 hours, propane-grown ENV cells almost always resulted in increased rates of oxygenate (MTBE, ETBE, TAME, and TBA) consumption compared to 2-propanol-, ethanol-, acetone-, and propionic acid-grown cells (22).

This dissertation focuses on the alkane monooxygenase of *M. vaccae* JOB5. We investigated the range of substrates this enzyme oxidizes including ETBE, TAME, DIPE, and tertiary alcohol products, TAA and TBA. We have also studied regulation of the
alkane monooxygenase in *M. vaccae* JOB5, expanding on current knowledge of MTBE-oxidizing activities in this organism. We discovered that MTBE induces MTBE-oxidizing activity in *M. vaccae* JOB5. Lastly, we discovered that the range of growth-supporting *n*-alkanes in *Pseudomonas putida* GPo1 includes the gaseous alkanes propane and *n*-butane. In addition, these gaseous alkanes appear to be oxidized by the same monooxygenase present during growth on C₅-C₁₀.
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CHAPTER 2

Induction of Methyl Tertiary Butyl Ether (MTBE)-Oxidizing Activity in
Mycobacterium vaccae JOB5 by MTBE

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ABSTRACT

Alkane-grown cells of Mycobacterium vaccae JOB5 cometabolically degrade the gasoline oxygenate methyl tertiary butyl ether (MTBE) through the activities of an alkane-inducible monooxygenase and other enzymes in the alkane oxidation pathway. In this study we examined the effects of MTBE on the MTBE-oxidizing activity of M. vaccae JOB5 grown on diverse nonalkane substrates. Carbon-limited cultures were grown on glycerol, lactate, several sugars, and tricarboxylic acid cycle intermediates, both in the presence and absence of MTBE. In all MTBE-containing cultures, MTBE consumption occurred and tertiary butyl alcohol (TBA) and tertiarybutyl formate accumulated in the culture medium. Acetylene, a specific inactivator of alkane- and MTBE-oxidizing activities, fully inhibited MTBE consumption and product accumulation but had no other apparent effects on culture growth. The MTBE-dependent stimulation of MTBE-oxidizing activity in fructose- and glycerol-grown cells was saturable with respect to MTBE concentration (50% saturation level = 2.4 to 2.75 mM), and the onset of MTBE oxidation in glycerol-grown cells was inhibited by both rifampin and chloramphenicol. Other oxygenates (TBA and tertiary amyl methyl ether) also induced the enzyme activity required for their own degradation in glycerol-grown cells. Presence of MTBE also promoted MTBE oxidation in cells grown on organic acids, compounds that are often found in anaerobic, gasoline-contaminated environments. Experiments with acid-grown cells suggested induction of MTBE-oxidizing activity by MTBE is subject to catabolite repression. The results of this study are discussed in terms of their potential implications towards our understanding of the role of cometabolism in MTBE and TBA biodegradation in gasoline-contaminated environments.
INTRODUCTION

Methyl tertiary butyl ether (MTBE) is an oxygenating compound that is currently added to gasoline to reduce automobile emissions of carbon monoxide and smog-related air pollutants. Approximately 30% of the gasoline sold in the United States contains MTBE, and its widespread use has led to concerns over the human health effects resulting from chronic exposure to this compound through gasoline contamination of drinking water supplies (19, 35). The U.S. Environmental Protection Agency currently classifies MTBE as a possible human carcinogen and has issued a drinking water advisory for MTBE of 20 to 40 ppb (37). Several recent studies have shown MTBE can be biodegraded under anaerobic conditions (2, 3, 9, 34, 40). However, like most other gasoline components, the fastest rates of MTBE biodegradation are observed under aerobic conditions (4, 11, 15, 33, 36). Several aerobic bacteria have been isolated that can use MTBE as a sole source of carbon and energy for growth (10, 13, 15, 26). Various other aerobic MTBE-degrading organisms have also been identified that are unable to grow on MTBE but can cometabolically degrade this compound after growth on a variety of hydrocarbons. Like MTBE, some of these hydrocarbons are also present at high concentrations in gasoline and include alkanes (11, 14, 23, 31, 33, 36), aromatics (18, 20), and alicyclics (5, 31). Cometabolic degradation of MTBE has been most extensively studied in propane- (33, 36) and \( n \)-pentane-oxidizing bacteria (11). In the case of propane-grown cells of *Mycobacterium vaccae* JOB5, MTBE is initially oxidized to tertiary butyl formate (TBF) through the sequential activities of an alkane-inducible alkane monooxygenase and a putative hemiacetal-oxidizing alcohol dehydrogenase (33). The subsequent abiotic and biotic hydrolysis of TBF yields tertiary butyl alcohol (TBA), which is then further
oxidized by the same monooxygenase responsible for initiating MTBE oxidation. Further steps in the oxidation of MTBE have been proposed but have not been extensively characterized (36). The role of cometabolism in the environmental fate of MTBE is currently unclear. For instance, addition of both propane and oxygen to gasoline-contaminated groundwater has been shown to promote MTBE oxidation (1). However, it is not known whether cometabolic processes supported by gasoline hydrocarbon cocontaminants represent an important natural attenuation process for MTBE under aerobic conditions. Recent field studies have shown MTBE biodegradation can be stimulated when anaerobic, gasoline-impacted ground water is oxygenated, either through engineered approaches (30, 41) or through natural ground water transport mechanisms (4, 21). However, as the currently recognized growth substrates thought to be required for cometabolic MTBE biodegradation are often reported to be absent from these environments, these effects of oxygenation have been generally interpreted in terms of a stimulation of growth-related microbial metabolism of MTBE. Nonetheless, a recent report (17) noted that MTBE biodegradation occurred in samples taken from oxygenated environments, both in the absence as well as the presence of organisms similar to the MTBE-metabolizing strain PM-1. Studies of microbial cometabolic degradation processes for important pollutants such as trichloroethylene (TCE) and MTBE have often focused on identifying substrates that support high rates of biodegradation of these compounds. These substrates are of interest because they not only support microbial growth but also lead to high levels of the key catabolic enzyme activities required for cosubstrate (e.g., TCE and MTBE) degradation. However, as studies of cometabolic TCE degradation have repeatedly demonstrated (8, 16, 22, 24, 29, 32), it is also important to
recognize the potential inducing effects of the target pollutant on the expression of enzymes required for its own biodegradation. In the present study we have examined the effect of MTBE on the MTBE-oxidizing activity of \textit{M. vaccae} JOB5 during carbon-limited growth on diverse nonalkane substrates. Our results demonstrate that cells grown on a wide range of substrates in the presence of MTBE and other oxygenates express the enzyme activities required for the degradation of these gasoline additives. The results of this study have been interpreted in terms of their potential impact on our understanding of the underlying physiology of MTBE cometabolism and the potential role of cometabolism in the environmental fate of MTBE.
MATERIALS AND METHODS

Materials. *M. vaccae* JOB5 (ATCC 29678) was obtained from the American Type Culture Collection (Manassas, Va.). Galactose (99% purity), glucose (99.5% purity), fructose (99% purity), pyruvic acid (99% purity), succinic acid (99% purity), lactic acid (98% purity), glycerol (99% purity), sodium propionate (99% purity), sodium butyrate (98% purity), valeric acid (99% purity), caproic acid (99.5% purity), heptanoic acid (99% purity), isovaleric acid (99% purity), 2-methylbutyric acid (98% purity), 2-methylvaleric acid (98% purity), 3-methylvaleric acid (97% purity), 2-methylhexanoic acid (99% purity), TBF (97% purity), TBA (99% purity), *tertiary* amyl alcohol (TAA; 99%), MTBE (99.8% purity), ethyl *tertiary* butyl ether (ETBE; 99% purity), *tertiary* amyl methyl ether (TAME; 97% purity), 1-propanol (99.5% purity), 2-propanol (99.5% purity), and rifampin, chloramphenicol, and calcium carbide (technical grade, ~80% purity; for acetylene generation) were obtained from Sigma Aldrich Chemical Co., Inc. (Milwaukee, Wis.). Sodium acetate (99.5% purity) was obtained from Fisher Scientific (Pittsburgh, Pa.). 2-Methyl-1,2-propanediol (2M12PD) was a gift from Lyondell Chemical Co. (Houston, Tex.). Compressed gases used for gas chromatography (GC) (H₂, N₂, and air) were obtained from local industrial vendors.

Growth experiments. Most of the experiments described in this study used cells of *M. vaccae* JOB5 grown in batch culture in glass serum vials (160 ml) sealed with Teflon-lined Mininert valves (Alltech Associates Inc., Deerfield, Ill.). The vials contained mineral salts medium (25 ml) (39), and unless otherwise stated all growth substrates were added from filter-sterilized aqueous solutions to give an initial concentration of 2.5 mM. The culture vials were inoculated (initial optical density at 600 nm [OD₆₀₀] of ≤0.02) with
a suspension of cells obtained from axenic cultures of *M. vaccae* JOB5 previously grown on casein-yeast extract-dextrose (CYD) agar plates (Difco plate count agar). When required, MTBE, ETBE, TAME, TBA, or TAA was added to the sealed vials from a saturated aqueous solution using sterile glass microsyringes. Acetylene (5 ml) was added to the sealed vials as required using sterile disposable plastic syringes fitted with sterile Acrodisc 0.1-μm filters (Pall Corp., Ann Arbor, Mich.). The culture vials were incubated at 30°C in the dark in an Innova 4900 environmental shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) operated at 150 rpm. Losses of MTBE from abiotic control incubations containing no added cells were less than 3% over 7 days under these conditions. Culture growth was measured by determining the OD$_{600}$ with a Shimadzu 1601 UV/Vis spectrophotometer (Kyoto, Japan). In every experiment, a sample (50 μl) was streaked on CYD plates to subsequently confirm the purity of the culture. In some experiments, concentrated washed cells were used. In these cases the cells were grown in batch culture with the required substrate, as described above. The cells were then harvested from the culture medium by centrifugation (10,000 × g; 10 min), and the resulting cell pellet was resuspended in buffer (10 ml; 50 mM sodium phosphate; pH 7). The washed cells were sedimented again by centrifugation (as above), and the resulting cell pellet was finally resuspended with buffer (1.0 ml, as above) to a final protein concentration of ~2.5 mg of total cell protein ml$^{-1}$.

**Analytical methods.** In some experiments the concentrations of MTBE and its oxidation products (TBA and TBF) were determined by GC using aqueous samples (2 μl) taken directly from the culture vessels. In experiments that followed the time course of organic
acid consumption as well as MTBE oxidation, aqueous samples (0.5 ml) were taken from
the sealed culture vials at the indicated times using disposable sterile plastic syringes (1
ml) and needles. The samples were transferred to flat-top polypropylene microcentrifuge
tubes (1.5 ml), and aqueous samples (2 ml) of the media were then immediately injected
into a gas chromatograph. In all experiments the samples were analyzed using Shimadzu
GC-8A or GC-14A gas chromatographs fitted with flame ionization detectors and
stainless steel columns (0.3 by 183 cm) filled with Porapak Q (60 to 80 mesh; Waters
Associates, Framingham, Mass.). The analysis of MTBE, TBF, TBA, ETBE, TAME,
TAA, and 2M12PD was conducted at 160C, while the quantification of valeric acid in the
presence of MTBE, TBF, and TBA was conducted at 150C. In both analyses the injection
and detector temperatures were 200C and 220C, and nitrogen was used as the carrier gas
at a flow rate of 15 ml/min. The gas chromatographs were interfaced to Hewlett Packard
HP3395 integrators (Palo Alto, Calif.) for data collection. The minimum detection limits
for TBF and TBA were ~20 and ~3 nmol ml\(^{-1}\), respectively. Cell protein concentrations
were determined using the Biuret assay (12) after solubilizing cell material for 30 min at
65C in 3 N NaOH and sedimentation of insoluble material by centrifugation (14,000 \(\times\)
5 min). Bovine serum albumin was used as the standard. The concentration of MTBE in
saturated aqueous solution at room temperature (23C) was taken as 0.544 M (33). The
dimensionless Henry’s constant (He) for MTBE at 30C was taken as 0.0255 (25). The
kinetic constants were derived by computer fitting of the data by nonlinear regression to a
single substrate-binding model \([Y = V_{\text{max}} \cdot X/(K_s + X)]\) using GraphPad Prism version 3.0a
for Macintosh (GraphPad Software, San Diego, Calif.).
RESULTS

Effects of nonhydrocarbon growth substrates on MTBE oxidizing activity. Cells of *M. vaccae* JOB5 were grown under carbon-limited conditions on a variety of sugars, tricarboxylic acid cycle intermediates, lactate, and glycerol either without MTBE, with MTBE (14 μmol [~450 μM dissolved MTBE]), or with MTBE (14 μmol) and acetylene (3.7% [vol/vol] gas phase), a potent irreversible inactivator of MTBE-oxidizing activity in this organism (33). After 7 days, culture growth was determined (OD₆₀₀) and the culture medium was analyzed by GC to determine the extent of MTBE consumption and the accumulation of TBA and TBF. No detectable growth or MTBE consumption occurred when cells were incubated with MTBE alone (data not shown). In contrast, the organism grew, to varying degrees, on all of the other substrates tested, and neither MTBE nor acetylene had any consistent effect on the final culture density (OD₆₀₀) for any of these substrates (Fig. 2.1A). The GC analysis (Fig. 2.1B) revealed variable but often extensive consumption of MTBE had occurred in all of the cultures that contained MTBE alone, whereas little or no MTBE consumption had occurred in cultures grown in the presence of both MTBE and acetylene. For instance, ≥70% of the added MTBE (~10 μmol) was consumed by cells grown on either glucose, pyruvate, or fructose in the presence of MTBE. In contrast, ≤7% (1 μmol) of the MTBE was consumed when cells were grown on the same substrates in the presence of acetylene. In all cultures where MTBE consumption was observed, both TBA and TBF were also detected. With the exception of cells grown on succinate, the molar ratio of MTBE consumed to total products (TBA plus TBF) detected was low (1:0.21) but close to constant (standard deviation [SD] = 4.4%).
**Effect of MTBE concentration on MTBE-oxidizing activity.** Two growth substrates characterized in Fig. 2.1, glycerol and fructose, were used to investigate the effect of MTBE concentration on the level of MTBE oxidation. Cells were grown for 7 days on either glycerol (7.5 mM) or fructose (2.5 mM) in the presence of various amounts of MTBE (0 to ~140 μmol; 0 to ~5 mM in solution). A plot of the total MTBE consumed versus initial dissolved MTBE concentration appeared to be saturable for both growth substrates (Fig. 2.2). These data were fitted to a hyperbolic, single substrate-binding curve, and good fits ($r^2 = 0.99$) were obtained in both cases. Half-saturation values ($S_{50}$) for glycerol (2.4 mM; standard error [SE] = 0.45) and fructose (2.75 mM; SE = 0.53) were obtained from these analyses. Both TBF and TBA were detected in the culture medium, and these products accounted for ~20% of the MTBE consumed in each culture. A plot of total products (TBA and TBF) detected versus initial dissolved MTBE concentration (Fig. 2.2 inset) also provided comparable $S_{50}$ values of 3.2 mM ($r^2 = 0.98$; SE = 0.88) and 2.8 mM ($r^2 = 0.98$; SE = 0.38) for cells grown on glycerol and fructose, respectively. The results described in Fig. 2.1 and 2.2 suggest that the presence of MTBE during growth on diverse nonalkane substrates led to the production of enzyme systems capable of degrading MTBE. However, these results did not address the possibility that MTBE-oxidizing activity was also present in cells grown in the absence of MTBE. We conducted two experiments to investigate this possibility. First, we attempted to determine the specific MTBE-oxidizing activity of concentrated cell suspensions grown on either glycerol (7.5 mM) or fructose (2.5 mM) in the presence and absence of MTBE (initially ~2 mM in solution). After growth for 7 days, the cells were harvested by
centrifugation, washed, and finally resuspended at a protein concentration of ~5 mg of total protein ml⁻¹. Samples of the concentrated cell suspension (0.2 ml) were incubated at 30°C in buffer (0.8 ml; 50 mM sodium phosphate [pH 7.0]) in stoppered glass serum vials (10 ml) in the presence of MTBE (1 µmol). After 2 h, the reaction media were analyzed by GC to quantify accumulation of TBA and TBF. We did not detect MTBE consumption or TBA- or TBF-generating activity for cells grown either in the presence or absence of MTBE in these short-term assays. In the second experiment, cells were initially grown on glycerol (35 mM) and then harvested and concentrated by centrifugation. These cells were then incubated with MTBE (~2 mM dissolved MTBE) and a low concentration (1 mM) of glycerol as an energy source. The time course of TBA and TBF production was then determined by GC analysis of the reaction medium. The results (Fig. 2.3) showed there was a lag phase of 4 h before TBA was first detected in the reaction medium. Although TBF also accumulated during the reaction time course, the concentration of detected TBF never exceeded 20% of the total TBA detected (data not shown). Over the next 6 to 8 h there was a progressive increase in the rate of TBA accumulation, after which the rate of TBA accumulation remained almost constant. When cells were incubated with MTBE in the presence of either chloramphenicol or rifampin (50 µg ml⁻¹ each), the production of both TBA and TBF (data not shown) was strongly or completely inhibited relative to that in the incubation containing MTBE alone. Complete inhibition of both TBA and TBF accumulation was also observed when cells were incubated with MTBE and acetylene (10% [vol/vol] gas phase).

**Effects of other ethers and tertiary alcohols.** We also examined whether other ether oxygenates and their tertiary alcohol oxidation products behaved similarly to MTBE.
Cells were grown on glycerol (7.5 mM) in the presence of either TAME, ETBE, TAA, or TBA. Additional cultures were also grown using the same growth substrate and oxygenate-alcohol combinations in the presence of acetylene (3.7% [vol/vol] gas phase). After growth for 5 days, the reaction media were analyzed by GC to determine the extent of oxygenate consumption and product accumulation. The results (Table 2.1) showed that substantial consumption of TAME but not ETBE occurred during growth on glycerol. TAA (~1 µmol) was detected as a product of TAME oxidation, although TAA accumulation only represented ~10% of the TAME consumed. Consumption of TAA (~6 µmol) also occurred when cells were grown in the presence of TAA, and both TAME and TAA consumption was inhibited by acetylene. These results suggest that the low recovery of TAA in the cultures grown with TAME was most likely due to concurrent oxidation of both TAME and TAA. Similar results were also observed for cultures grown in the presence of TBA. Approximately 50% (~23 µmol) of the added TBA was consumed during growth on glycerol, and a single high-boiling-point product that coeluted with 2M12PD was detected. This accounted for ~50% (~12 µmol) of the TBA consumed by glycerol-grown cells. Both the consumption of TBA and the production of 2M12PD were inhibited by the presence of acetylene.

**Effects using organic acids as growth substrates.** In addition to conventional growth substrates described above, we also examined whether MTBE-oxidizing activity was stimulated by MTBE in cells grown on more environmentally relevant compounds. Volatile organic acids were chosen for study because they are frequently found in gasoline-impacted ground water environments as products of anaerobic biodegradation of
gasoline hydrocarbons (6, 7). In one experiment, cells were grown in the presence of MTBE (14 mol) using carbon-limiting concentrations (2.5 mM) of several branched acids (isovaleric, 2-methylbutyric, 2-methylvaleric, 3-methylvaleric, and 2-methylhexanoic acids). After growth for 7 days, both TBA and TBF were detected in the incubations containing acids and MTBE, whereas neither product was observed in the same incubations conducted in the presence of acetylene (3.7% [vol/vol] gas phase). The average consumption of MTBE in these cultures was 7.4 mol (SD = 2.1), and the average molar yield of TBF and TBA combined was 68% of the MTBE consumed. We were unable to quantify cell growth in these and subsequent acid-grown cultures described later, due to clumping of cells. Cells were also grown under carbon-limited conditions on equimolar concentrations (2.5 mM) of a series (C₂ to C₇) of straight-chain acids, either in the presence of MTBE (14 mol) or MTBE (14 mol) plus acetylene (3.7% [vol/vol] gas phase). The time course of TBA and TBF production was then determined by GC analysis of the culture medium. In all cultures containing MTBE alone, neither product was detected until at least 24 h after the culture was initiated (Fig. 2.4). The chain length of the acid substrate had two distinct effects. First, in general the longer the acid carbon chain length, the longer the lag phase before MTBE oxidation products (TBA and TBF) were observed. For example, products were observed with acetate- grown cells after 24 h, while cells grown on heptanoic acid did not show product accumulation until ~50 h. Second, in general the longer the acid chain length, the greater the amounts of MTBE oxidation products that were observed. For example, acetate-grown cells generated <2 mol of products, while caproic acid-grown cells generated ~4
mol of products. The average molar ratio of MTBE consumed to total products (TBA plus TBF) detected for the range of acids tested was 1:0.62. However, this ratio progressively decreased from 1:0.84 with acetate-grown cells to 1:0.37 with cells grown on heptanoic acid. In a duplicate series of incubations, acetylene fully inhibited the production of both TBA and TBF in all cases (data not shown).

**Effect of acid concentration on MTBE-oxidizing activity.** The results described in Fig. 2.4 have features that might be expected if the expression of the enzymes involved in MTBE oxidation were subject to catabolite repression. To investigate this further we quantified the time course of TBA and TBF production in relation to both MTBE and valeric acid consumption for cells grown either with or without MTBE (7.5 mol) in the presence of two different initial amounts of valeric acid (70 and 35 mol). These culture conditions were also duplicated in incubations containing acetylene (3.7% [vol/vol] gas phase). In the cultures containing the lower initial concentration of valeric acid, the acid was fully consumed within 40 h (Fig. 2.5). The consumption of MTBE and the production of both TBA and TBF were first detected in the growth medium when ~10 mol of valeric acid remained. The time course of both TBA and TBF accumulation continued to reflect MTBE consumption over the next 40 h. However, the MTBE oxidation reaction was not sustainable, and after ~80 h the rates of both MTBE consumption and product accumulation steadily declined to close to zero. After 120 h the molar ratio of MTBE consumed to total products (TBF plus TBA) detected was 1:0.72. The corresponding control experiment (valeric acid plus MTBE plus acetylene) showed acetylene had no discernible effect on the rate of valeric acid consumption but completely
inhibited both MTBE consumption and production of both TBA and TBF over the entire reaction time course. A substantially similar pattern of biodegradation was observed when the experiment was repeated with twofold-higher initial amounts of valeric acid. The onset of both MTBE consumption and production of TBA and TBF was delayed by ~20 h relative to that for the cultures grown with lower initial amounts of valeric acid. However, the onset of both of these activities still occurred when ~10 μmol of valeric acid remained in the culture medium. The total amounts of MTBE degraded and TBA and TBF generated were greater in these cultures than in those with lower initial amounts of valeric acid. However, the molar ratio of MTBE consumed to total products detected (TBA plus TBF) after 120 h was 1:0.80 and was comparable to the results obtained with the lower valeric acid concentration. As a final component of this study, we also conducted a similar experiment to that described in Table 2.1 using valeric acid rather than glycerol as a growth substrate. Substantially similar activities to those observed with glycerol were observed and included the oxidation of TBA, TAA, and TAME but not ETBE by valeric acid-grown cells (data not shown).
DISCUSSION

The results of this study provide strong and consistent evidence showing MTBE oxidation occurs during growth of \textit{M. vaccae} JOB5 on a wide range of nonalkane substrates (Fig. 2.1, 2.3, and 2.4). We have also shown acetylene inhibits the production of both TBA and TBF (Fig. 2.1B and 2.5) from MTBE, as well as consumption of MTBE (Fig. 2.5). Based on these observations, we conclude that the enzyme responsible for MTBE oxidation in this study is the same alkane-inducible, acetylene-sensitive, MTBE- and TBA-oxidizing monooxygenase we have previously characterized in propane-grown cells of this bacterium (33). The remaining sections of the Discussion expand on this conclusion and the broader implications of our findings.

\textbf{Evidence for an inductive effect of MTBE.} Three lines of evidence suggest the MTBE-oxidizing activity characterized in this study is due to a specific inducing effect of MTBE on the expression of alkane monooxygenase, rather than due to constitutive, low-level expression of this enzyme during growth on nonhydrocarbon substrates. These lines of evidence are as follows. First, MTBE-oxidizing activity was not detected immediately after glycerol-grown cells were harvested (Fig. 2.3). However, these cells showed a time-dependent acquisition of MTBE-oxidizing activity after exposure to MTBE. The appearance of this activity was strongly inhibited by both chloramphenicol and rifampin, evidence that indicates this response involves both transcription and de novo protein synthesis. Our failure to detect MTBE-oxidizing activity in cells previously grown for 7 days in the presence of MTBE probably reflects the consistent postinduction decline in MTBE-oxidizing activity we observed in several experiments (Fig. 2.4 and 2.5) conducted over an equivalent period of time. Second, cells grown on valeric acid did not
show a progressive increase in MTBE oxidation rate throughout the time course of acid consumption (Fig. 2.5), an effect that would be predicted if MTBE-oxidizing activity was constitutive and increased consistently with increases in cell density. In contrast, these cells only showed evidence for both MTBE consumption and TBA and TBF production once the residual valeric acid had been depleted to ~10 μmol (Fig. 2.5). This effect occurred with two different acid concentrations. The apparent need for cells to deplete the acid concentration to below a threshold value is strongly suggestive of a catabolite repression effect, a feature that again supports a model involving MTBE-dependent gene induction. This is consistent with a previous study of the growth substrate range of *M. vaccae* JOB5 that indicated cells grown on acetate, propionate, and butyrate did not have detectable *n*-alkane (C_1 to C_8) or primary alcohol (C_2 to C_8) oxidizing activity (28). Third, cells grown on either glycerol (Table 2.1) or valeric acid oxidized both TBA and TAME, but not ETBE. All of these compounds are oxidized by propane-grown cells of *M. vaccae* JOB5 (33, 36; C. A. Smith and M. R. Hyman, unpublished results). However, our observation that only two of these compounds were oxidized during growth on nonalkane substrates (Table 2.1) suggests that the oxidation process is determined by features of these compounds as inducers rather than by the substrate range of a constitutively expressed enzyme.

**Inductive effects of cometabolites in other organisms.** If the MTBE-oxidizing activity described in this study is due to an inductive effect controlled by catabolite repression, it is important to recognize that these effects would be expected to be most apparent in cultures grown under the carbon-limited conditions used in this study. It is also important to recognize that it is not uncommon for bacterial monooxygenase gene expression to be
induced by substrates for these enzymes that themselves do not support cell growth. For instance, alkane hydroxylase activity in *Pseudomonas aeruginosa* is strongly induced by diethoxymethane and dicyclopropylmethanol. Neither of these compounds supports cell growth, although both compounds are oxidized by induced cells (38). Another relevant example is given by the strong effect of TCE on the diverse organisms that cometabolically degrade this compound. Diverse toluene-oxidizing oxygenases (16, 22, 24, 29, 32) are all induced to various degrees by the presence of TCE. In the case of *Pseudomonas mendocina* KR-1, the induction of toluene-4-monooxyganease activity in cells grown on glutamate with TCE is ~86% of the level of activity observed with toluene-grown cells (24). The inducing effect of TCE in toluene-oxidizing organisms has been proposed to reflect the structural similarity between the carbon-carbon double bond in TCE and the carbon-carbon bond within the aromatic ring of toluene. A wide range of chlorinated alkenes, including TCE, also induce propylene monooxygenase activity in *Xanthobacter* sp. strain Py2 (8). In this case there is an even stronger structural resemblance between the growth substrates for this organism and TCE. It is notable that *M. vaccae* JOB5 was originally isolated from a 2-methyl butane enrichment culture and grows on a wide range of branched alkanes (27). The inductive effects of MTBE may therefore be a reflection of the ability of this organism to respond to more metabolizable branched alkanes that structurally resemble MTBE and other oxygenates. The inductive effect of MTBE on the MTBE-oxidizing activity of *M. vaccae* JOB5 appears to be considerably weaker than the inductive effects of TCE described above. For example, the best estimate of the rate of MTBE oxidation we can derive from our data is from the experiment described in Fig. 2.3. The maximal rate of TBA production after induction
was 0.35 nmol min\(^{-1}\) mg of total protein\(^{-1}\). This is close to the rate we have previously described for MTBE oxidation by 1-propanol-grown cells and is only ~1\% of the estimated \(V_{\text{max}}\) value for propane-grown cells (33). However, in this study we have also shown that the molar ratio of products detected to MTBE consumed varies widely depending on which substrate is used to support growth. The rate estimate given above therefore most likely underestimates the true rate of MTBE oxidation, which could be as much as fivefold higher if the combined production of TBA and TBF represents only 20\% of the consumed MTBE (Fig. 2.1 and 2.2). It should also be recognized that these cells were exposed to concentrations of MTBE below the \(S_{50}\) for MTBE (~2.5 mM) (Fig. 2.3) and only slightly higher than the \(K_s\) for MTBE (1.3 mM) (33). These factors suggest the maximal level of induction that can be achieved under appropriate conditions is likely to be considerably higher than 5\% of the maximal activity of propane-grown cells.

**Significance to understanding of MTBE cometabolism.** Our results also provide several other interesting observations relevant to our understanding of MTBE oxidation by this organism. For example, our results with acid-grown cells (Fig. 2.4 and 2.5) showed MTBE oxidation was unsustainable (Fig. 2.5). It may be that a delicate balance exists between the maximum concentration of growth substrate that allows for induction of MTBE-oxidizing activity and the minimum concentration of growth substrate needed to supply the anabolic demands for de novo protein synthesis and reductant supply to the newly synthesized MTBE-oxidizing monooxygenase. While these are interesting questions for future studies, our present results certainly add further weight to our previous report (33) that *M. vaccae* JOB5 does not grow on MTBE when it is supplied as a sole carbon and energy source to this organism. Another interesting observation was the
accumulation of 2M12PD when cells were grown on glycerol in the presence of TBA (Table 2.1). Oxidation of TBA by propane-grown M. vaccae JOB5 is catalyzed by the same alkane monooxygenase responsible for MTBE oxidation (33, 36), and 2M12PD is the predicted product of this reaction (36). We have not previously observed accumulation of 2M12PD during MTBE oxidation by propane-grown cells. This may reflect our previous focus on oxidation of low MTBE concentrations and the likelihood this product is rapidly further oxidized by alcohol and aldehyde dehydrogenase activities coinduced with alkane monooxygenase activity in alkane-grown cells. Accumulation of 2M12PD in both glycerol-grown and valeric acid-grown cells exposed to TBA may indicate that the effects of TBA lead to the induction of alkane monooxygenase without extensive concurrent coinduction of alcohol dehydrogenase activity. Again, this interpretation is compatible with a previous study of substrate utilization patterns by M. vaccae JOB5 that reported cells grown on fatty acids do not have detectable alcohol-oxidizing activity (28).

**Implications for the environmental fate of MTBE and TBA.**

Our results also have potential impacts on our understanding of the role of cometabolism in the environmental fate of MTBE. As indicated in the introduction, several studies have demonstrated that oxygenation of anaerobic, gasoline-impacted environments can promote MTBE biodegradation. These studies have typically been conducted in environments that do not contain gasoline-derived alkanes or other substrates that could be argued to support “conventional” cometabolic degradation processes. However, our results with organic acids suggest cometabolic processes could have an unforeseen role in these environments. Organic acids accumulate in gasoline-impacted environments as a
result of anaerobic degradation of gasoline hydrocarbons (6, 7). Our present results therefore suggest that if low concentrations of acids were present with MTBE in environments undergoing oxygenation, the physiological conditions could be met for a cometabolic degradation process to occur. These conditions include organic acids as a growth substrate, MTBE as an inducer, and oxygen as both a terminal electron acceptor and a substrate for monooxygenase activity. Our results (Table 2.1) also suggest a similar effect can be expected with TBA, a compound that is often regarded as an indicator of MTBE biodegradation. Future studies are clearly needed to determine whether the effects described in this study are specific for *M. vaccae* JOB5 or are generally applicable to organisms capable of MTBE cometabolism. Future studies are also clearly needed to address the possibility that MTBE degradation in oxygenated environments is not solely due to bacterial metabolism of MTBE.

**ACKNOWLEDGMENTS**
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Figure 2.1. Growth of *M. vaccae* JOB5 on nonalkane substrates and concurrent oxidation of MTBE. Cultures of *M. vaccae* JOB5 were grown for 7 days under carbon-limited conditions in batch culture on the indicated substrates, as described in Materials and Methods. All substrates other than glycerol (7.5 mM) were added to an initial concentration of 2.5 mM. Acetylene (3.75% [vol/vol] gas phase) and MTBE (14 μmol) were added to the cultures as required. (A) Average final culture density (OD$_{600}$) for three replicate cultures grown under the indicated conditions. The error bars indicate the range of values for all three cultures. (B) Average amounts of MTBE consumed (white bars) and TBA (black bars) and TBF (gray bars) generated after 7 days for the three replicate cultures. The error bars indicate the range of values for MTBE, TBA, and TBF for all three cultures.
Figure 2.2. Effect of MTBE concentration on MTBE oxidation and production of TBA and TBF. Cultures of *M. vaccae* JOB5 were grown for 7 days under carbon-limited conditions in batch culture on either glycerol (7.5 mM [■]) or fructose (2.5 mM [▲]) in the presence of a range of initial MTBE concentrations (0 to 140 μmol; 0 to 4.9 mM dissolved MTBE), as described in Materials and Methods. The amount of MTBE consumed in each culture after 7 days was plotted versus the initial amount of MTBE added to each culture. Inset: combined amounts of TBA and TBF detected after 7 days for the same cultures. In all cases the curves drawn are the computer fits to a single substrate-binding model, as described in Materials and Methods.
Figure 2.3. Effects of chloramphenicol, rifampin, and acetylene on MTBE-oxidizing activity in glycerol-grown cells of *M. vaccae* JOB5. Cells of *M. vaccae* JOB5 were grown in batch culture for 5 days on glycerol (35 mM) in the absence of MTBE. The cells were harvested, washed, and stored at 4C, as described in Materials and Methods. The reactions were conducted in glass serum vials (10 ml) sealed with butyl rubber stoppers and aluminum crimp seals. The reaction vials contained buffer (50 mM sodium phosphate; pH 7; ~900 µl), MTBE (2.8 µmol), and glycerol (1 µmol). The reactions were initiated by the addition of an aliquot (100 µl) of concentrated cell suspension (0.21 mg of total protein), and the vials were incubated at 30C in a shaking water bath (150 rpm). At the indicated times, aqueous samples (2 µl) were removed and analyzed by GC for the accumulation of TBA and TBF, as described in Materials and Methods. The time course for TBA accumulation is shown for cells incubated with MTBE alone (■), MTBE plus acetylene (10% [vol/vol] gas phase) (□), MTBE plus chloramphenicol (50 µg ml⁻¹) (●), and MTBE plus rifampin (50 µg ml⁻¹) (○).
Figure 2.4. Time course of TBA production by cells of *M. vaccae* JOB5 during growth on linear organic acids. A series of cultures of *M. vaccae* JOB5 were grown on linear organic acids (C₂ to C₇) (initial concentration = 2.5 mM) in the presence of MTBE (14 μmol), as described in Materials and Methods. The time course of TBA accumulation is shown for cultures grown on acetic (▲), propionic (△), butyric (■), valeric (□), caproic (●), and heptanoic (○) acids. The symbols represent the average for two replicate cultures, and the error bars show the range of values for both cultures combined.
### TABLE 2.1. Oxygenate consumption and product accumulation by cells grown on glycerol

<table>
<thead>
<tr>
<th>Oxygenate (µmol added)</th>
<th>Amt of oxygenate consumed (µmol)</th>
<th>Product detected, −C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Amt of product generated (µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Abiotic&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>+C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>TBA (50)</td>
<td>0.3</td>
<td>22.9 (2.1)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2M12PD</td>
</tr>
<tr>
<td>ETBE (20)</td>
<td>≤0.1</td>
<td>0.5 (0.3)</td>
<td>ND</td>
</tr>
<tr>
<td>TAME (25)</td>
<td>1.4</td>
<td>9.5 (0.5)</td>
<td>TAA</td>
</tr>
<tr>
<td>TAA (50)</td>
<td>0.6</td>
<td>6.5 (1.05)</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Glycerol was supplied as the growth substrate at an initial concentration of 7.5 mM.

<sup>b</sup> Abiotic control incubations contained all substrates but no cells. Losses of each substrate in abiotic controls were indistinguishable from cultures containing all substrates, cells, and acetylene (3.75% [vol/vol] gas phase).

<sup>c</sup> The values reported are the averages of two replicate cultures for each condition. The values in parentheses indicate the range of values around the mean for the combined data for both cultures.

<sup>d</sup> ND = none detected
Figure 2.5. Time course of MTBE and valeric acid oxidation during growth of *M. vaccae* JOB5. Cultures of *M. vaccae* JOB5 were grown in batch culture on valeric acid in the presence of MTBE (7.5 μmol), as described in Materials and Methods. The time course for valeric acid consumption is shown for cultures grown with 35 μmol (squares) or 70 μmol (circles) of valeric acid, either in the presence (open symbols) or absence (closed symbols) of acetylene (3.75% [vol/vol] gas phase). Also shown is the corresponding time course for MTBE consumption for cultures grown with 35 μmol (inverted triangles) or 70 μmol (upright triangles) of valeric acid, either in the presence (open symbols) or absence (closed symbols) of acetylene (3.75% [vol/vol] gas phase). In addition, the combined amount of TBA and TBF generated from MTBE is shown for cultures grown with 35 μmol (asterisks) or 70 μmol (filled diamonds) of valeric acid. No TBF or TBA was observed for cultures grown in the presence of acetylene (3.75% [vol/vol] gas phase), and these data were not plotted, to aid in the clarity of the figure. The data plotted are the averages of two replicates for cultures grown in the absence of acetylene and a single replicate for all cultures that contained acetylene. The error bars show the range of values obtained for the replicate cultures.
REFERENCES


CHAPTER 3

Oxidation of Ethyl *Tertiary* Butyl Ether (ETBE), *Tertiary* Amyl Methyl Ether (TAME) and Their Products, *Tertiary* Butyl Alcohol (TBA) and *Tertiary* Amyl Alcohol (TAA) by *Mycobacterium vaccae* JOB5

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ABSTRACT

Methyl tertiary butyl ether (MTBE) is the most common oxygenate added to gasoline in the United States. However, other ether-based oxygenates including ethyl tertiary butyl ether (ETBE), tertiary amyl methyl ether (TAME), and di-isopropyl ether (DIPE) are also used to a lesser but growing extent. In this study we have characterized features of the cometabolic degradation of ETBE, TAME and DIPE by Mycobacterium vaccae JOB5. Oxidation of both ETBE and TAME occurred without a lag phase with propane-grown cells while no oxidation of either compound was observed with dextrose-grown cells. Acetylene, a mechanism based inhibitor of the propane-induced alkane-oxidizing monooxygenase, fully inhibited these two reactions. In addition, the oxidation of both ETBE and TAME was inhibited by propane. M. vaccae JOB5 cells grown on n-butane, n-pentane or isopentane also rapidly oxidized ETBE and TAME. In contrast to ETBE and TAME, we did not observe detectable rates of DIPE oxidation under any of the conditions tested. The major products of ETBE and TAME oxidation were identified as tertiary butyl alcohol (TBA) and tertiary amyl alcohol (TAA), respectively. These initial alcohol products of ether biodegradation were oxidized at comparable rates by propane-grown cells and were not consumed by cells grown on dextrose-containing medium. The oxidation of both TAA and TBA was also fully inhibited by acetylene and responded similarly to competitive inhibition by propane. The results described in this study are consistent with the pathways and enzymes we have previously identified with MTBE degradation by propane-grown M. vaccae JOB5.
INTRODUCTION

The United States and several other countries include several alkyl ethers such as methyl tertiary butyl ether (MTBE), ethyl tertiary butyl ether (ETBE), and tertiary amyl methyl ether (TAME) in gasoline as octane enhancers and as oxygenates. In the U.S., the main ether-based oxygenate is MTBE. This compound is inexpensive to manufacture from petroleum refining byproducts (isobutylene and methanol) and blends well with gasoline. In France, ETBE is the most commonly used oxygenate. This choice reflects the use of ethanol generated from renewable resources.

The use of MTBE has become a human health concern in the U.S. because of gasoline-contaminated drinking water supplies (1,2). The U.S. Environmental Protection Agency has classified MTBE as a possible carcinogen and has set a drinking water advisory of 20 to 40 ppb (3). Although the U.S. EPA has not yet banned use of MTBE in gasoline, its detection in groundwater has prompted numerous states to independently phase out use of this compound. One consequence of the recent changes in oxygenate use patterns has been an increase in use of ethanol as a fuel oxygenate and octane enhancer. Use of other ether-based oxygenates such as ETBE and TAME has also increased and understanding the environmental fate of these ether-based oxygenates has emerged as an important environmental issue.

Previous studies have examined the biodegradation of ETBE and TAME by microcosms, mixed cultures, and pure cultures. Microcosm studies have demonstrated biodegradation of MTBE, ETBE, and TBA under sulfate-reducing, methanogenic, and denitrifying conditions (4). Although all three oxygenates were shown to be biodegraded in this study, a further study showed that soils with ETBE-degrading activity were
unreactive towards MTBE (5). These results suggested ETBE and MTBE may be biodegraded through different mechanisms or by different microorganisms.

This apparent discrimination between biodegradation of methoxy (MTBE) and ethoxy (ETBE) ethers is reiterated in studies conducted with mixed and pure cultures. For example, Kharoune et al. (6) reported a microbial consortium obtained from gasoline-polluted soil degraded MTBE, ETBE, TAME, and TBA. An aerobic study by Fayolle et al. resulted in ETBE degradation but no TAME or MTBE degradation (7). Microcosm studies suggest the degradation of MTBE and TAME could be isolated from ETBE oxidation.

Another means of substrate degradation in mixed cultures is cometabolism. Cometabolism converts a compound by utilizing one or more non-specific enzymes produced during true metabolism of a growth-supporting substrate. During cometabolism, the organism does not obtain energy from the cometabolized substrate. Since the first and critical step in ether oxygenate degradation requires a monooxygenase, the study of hydrocarbon-oxidizing organisms have been the focus of ether oxygenate cometabolism. Steffan et al. conducted an important study of ether oxygenate cometabolism by propane-grown cells and found that propane-grown cells of ENV425 and M. vaccae JOB5 degraded MTBE, ETBE, TAME, TBA, and TAA. Experiments in this study ran at a minimum of eight hours (8-24h) raising the possibility of enzyme induction by the ether oxygenates themselves, an effect we have recently characterized for M. vaccae JOB5 with MTBE (8). In addition, this study did not investigate the oxidizing effects on di-isopropyl ether (DIPE), another important ether oxygenate. An independent investigation of ether oxygenate cometabolism showed G. terrae IFP-2001,
previously shown to metabolize ETBE, was found to cometabolize MTBE and TAME in the presence of ethanol (9).

Interestingly, microorganisms that metabolize ETBE or TAME in pure culture often fail to oxidize the other oxygenate. For example, both *Rhodococcus equi* and *Gordona terrae* strain IFP 2001, organisms isolated on ETBE from the previously described aerobic bacterial consortium, were able to degrade ETBE but are unable to degrade MTBE or TAME (7). The opposite of this also occurs, where organisms such as *Mycobacterium austroafricanum* IFP 2012 are capable of metabolizing MTBE and TAME but only weakly degrades ETBE (10). However, the metabolism of both MTBE and TAME appears to be a generally consistent trait although two strains isolated from gasoline-polluted soil, strains E₁ and E₂, metabolically degraded MTBE, TAME, ETBE, TBF, and TBA (11).

In this study we investigated the degradation rates of ETBE, TAME, and DIPE and their expected products, TBA and TAA by propane-grown *Mycobacterium vaccae* JOB5. Our results suggest that propane-grown *M. vaccae* JOB5 oxidizes ETBE at a slower rate than TAME and MTBE oxidation and that DIPE oxidation occurs slowly if at all. We also determined kinetic inhibition of propane on ETBE, TAME, TAA, and TBA oxidation. Finally, our results strongly suggest that the enzyme responsible for the oxidation of ETBE, TAME, TAA, and TBA is the same alkane monooxygenase we have previously shown to oxidize MTBE.
MATERIALS AND METHODS

Materials. *Mycobacterium vaccae* JOB5 (ATCC 29678) was obtained from the American Type Culture Collection (Manassas, Va). Calcium carbide (~80% technical grade for acetylene production), n-butane (99% purity), 2-methyl-2-propanol [tertiary butyl alcohol (TBA)] (99.3% purity), methyl tertiary butyl ether [MTBE] (99.8% purity), isopentane (99.5% purity), tertiary amyl alcohol [TAA] (99+% purity), tertiary butyl ethyl ether [ETBE] (99% purity) and tertiary amyl methyl ether [TAME] (97% purity) were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO). Isopropyl ether [DIPE] (99.9+% purity) and n-pentane (HPLC grade) were obtained from Fisher Scientific Co. (Pittsburgh, PA). 2-methyl-1,2-propanediol (min 92% purity) was a gift from Lyondell Chemical Co. (Houston, TX). Propane (instrument grade) was obtained from Matheson Gas Products, Inc. (Montgomeryville, PA). Other compressed gases used for gas chromatography (H₂, N₂, and air) were obtained from local industrial vendors.

Cell Growth. Most of the experiments described in this study used cells of *M. vaccae* JOB5 grown in batch culture at 30°C by using propane as the sole source of carbon and energy. Cells were grown in glass serum vials (750 ml) in a mineral salts medium (100 ml) (12) and sealed with screw caps fitted with butyl rubber septa (Wheaton Scientific, Millville, NJ). The vials were inoculated (initial *A*₆₀₀ = ~0.02) with a suspension of cells obtained from axenic cultures of *M. vaccae* JOB5 previously grown on casein-yeast extract-dextrose (CYD) agar plates (Difco Plate Count Agar). Propane (180 ml) was added to the sealed vials by using plastic syringes fitted with sterile filters (0.25 μm). *M. vaccae* JOB5 cells used in Table 1 were grown as described above with butane (180 ml),
pentane (50 ml) or isopentane (50 ml) as the sole source of carbon and energy. After growth for ~5 days (final $A_{600} = \sim0.6$), the cells were harvested by centrifugation (10,000 x g; 10 min) and the resulting pellet was resuspended in buffer (10 ml, 50 mM sodium phosphate, pH 7). The washed cells were sedimented again by centrifugation (as above) and the resulting cell pellet was finally resuspended with buffer (~1.0 ml, as above) to a final protein concentration of 3-15 mg total cell protein ml$^{-1}$. The cells were stored at 4°C and were used within 4 h. In some experiments (see Figure 3.1), cells were also grown as described above on dextrose-containing Difco Plate Count medium (DPC) (Becton Dickinson and Company, Sparks, Md). This medium contained (in grams per liter) dextrose (1.0), pancreatic digest of casein (5.0), and yeast extract (2.5). Cells grown in DPC liquid media were also cultivated and harvested, as described above. In all cases before harvesting a sample (50 ml) of the culture was plated on CYD plates to subsequently confirm the purity of the cultures.

**Reaction conditions.** Reactions were all conducted in glass serum vials (15 ml) prepared by adding buffer (50 mM sodium phosphate, pH 7) (~800 ml or ~900 ml) after which the vials were sealed with Teflon-lined Mininert valves (Alltech Associates Inc., Deerfield, Ill.). Saturated aqueous solutions of MTBE, ETBE, TAME and DIPE and stock solutions of TAA, TBA, butyl methyl ether and sec-butyl ether were added directly to the sealed vial using sterile glass microsyringes. Reactions were initiated by the addition of concentrated cells (~100-200 ml) to give a final reaction volume of 1.0 ml.
Analytical Methods. In all experiments concentrations of reactants and products were determined by gas chromatography (GC). For analyses of TBA or TAA, aqueous samples (2 ml) were taken directly from reaction vials and were injected into a Shimadzu GC-14A gas chromatograph (Kyoto, Japan) fitted with a flame ionization detector and a stainless steel column (0.3 x 183 cm) filled with Porapak Q (60-80 mesh) (Waters Associates, Framingham, MA). The analysis was conducted using a column temperature of 160°C, injection port temperature of 200°C and a detector temperature of 220°C. Nitrogen was used as carrier gas at a flow rate of 15 ml min⁻¹. In experiments that followed only ether consumption gas phase samples (25 ml) were removed using gas tight syringes. The samples were directly injected into a Shimadzu GC-14A gas chromatograph fitted with a flame ionization detector and a DB-MTBE capillary column (30 m x 0.45 mm I.D. [2.55 μm film]) (J & W Scientific, Folsom, CA). The analysis was conducted using a column temperature of 35°C, an injection port temperature of 200°C and a detector temperature of 220°C. Nitrogen was used as carrier gas at a flow rate of 5 ml min⁻¹. The gas chromatograph was interfaced to a Hewlett Packard HP3395 (Palo Alto, CA) integrator for data collection.

Cell protein concentrations were determined using the Biuret assay (13) after solubilization of cell material for 30 min at 65°C in 3N NaOH and sedimentation of insoluble material by centrifugation in an Eppendorf microfuge (14,000 rpm for 5 min). Bovine serum albumin was used as the standard. The concentrations of MTBE, ETBE, TAME and DIPE in saturated aqueous solution at room temperature (23°C) were taken as 0.544, 0.117, 0.117 and 0.02 M, respectively (14, 15, 16.). The dimensionless Henry’s
constant for MTBE, DIPE, ETBE, TAME at 30C were taken as 0.0255, 0.0932, 0.0972, 0.0776, respectively (17).
RESULTS

Relative rates of MTBE, ETBE, TAME and DIPE degradation. The rates of degradation of MTBE, ETBE, TAME and DIPE at equimolar dissolved aqueous phase concentrations (1 mM) were determined for propane-grown cells of *M. vaccae* JOB5 (Figure 3.1). The oxidation of MTBE, ETBE and TAME was initiated without a lag phase although different rates of ether consumption were observed (Figure 3.1). For example, the initial rates of MTBE and TAME consumption were substantially similar whereas ETBE was oxidized at ~30% of the initial rate of the methoxy-based ethers. Unlike MTBE, ETBE, and TAME there was no evidence for DIPE oxidation. In contrast to propane-grown cells, we observed no oxidation of ETBE, TAME or DIPE by dextrose-grown cells. There was a slow loss of MTBE by dextrose-grown cells after ~120 minutes. This effect is most likely accounted for by the induction of MTBE-oxidizing activity we have previously reported for this organism (8).

We also examined whether these ethers (MTBE, TAME, ETBE and DIPE) were oxidized by cells of *M. vaccae* JOB5 grown on other n-alkanes including n-pentane and isopentane, two of the most abundant alkanes found in gasoline. Concentrated alkane-grown or dextrose-grown cells *M. vaccae* JOB5 cells were incubated with either MTBE, ETBE, TAME or DIPE (1 mM initial dissolved aqueous phase concentration) and the reactions were sampled by gas phase GC after 60 minutes to determine the amount of ether consumed. The results of these studies (Table 3.1) reiterate the trends shown above in Figure 1. For example, none of the ethers were consumed by dextrose-grown cells whereas MTBE, ETBE and TAME were all readily consumed by cells grown on each of the alkanes tested while there was no apparent consumption of DIPE under any of the
conditions tested. In general the fastest rates of MTBE, ETBE and TAME degradation were observed with cells grown on \(n\)-pentane and isopentane. Likewise, the rate of ETBE degradation was typically the slowest ether degradation reaction while the rates of MTBE and TAME consumption were close to equivalent.

**Production of TBA and TAA production during ETBE and TAME oxidation.** The experiments described above focused on gas-phase sampling to determine the rates of ether oxygenate consumption. We subsequently examined the time course of ETBE and TAME degradation using liquid phase sampling to identify the initial degradation products of these compounds. Concentrated cells of propane-grown cells of *M. vaccae* JOB5 were incubated with either ETBE or TAME (1.1 \(\mu\)moles) either in the presence or absence of acetylene (10% v/v gas phase) (Figure 3.2). Acetylene is a potent mechanism-based inactivator of the short-chain alkane monooxygenase responsible for initiating alkane, MTBE and TBA oxidation. Under these conditions ETBE and TAME were oxidized at comparable rates and complete consumption of these ethers occurred within 150 min. The consumption of both compounds was also strongly inhibited (90%) by the presence of acetylene (10% v/v gas phase). Consumption of ETBE was associated with the accumulation of TBA as the sole detected oxidation product. The maximal concentration of TBA detected (150 \(\mu\)M) during this reaction was observed after 30 min and the TBA concentration remained essentially constant until \(\geq\)90% of the initial ETBE had been consumed (120 min). After this time, the concentration of TBA slowly declined. No TBA production was observed when propane-grown cells were incubated with both ETBE and acetylene (10% v/v gas phase).
During the degradation of TAME, two oxidation products were observed. One product was identified as TAA based on co-elution with an authentic standard. The second minor and slowly-eluting compound was not identified or quantified. However, this product exhibited a strong tailing effect during the GC analysis, an effect commonly observed with acidic compounds. The maximal concentration of TAA (~300 µM) was observed after 100 min when ~90% of the initial TAME had been consumed. Our results also suggests that like TBA derived from ETBE, the concentration of TAA slowly declined once the added TAME had been fully (>95%) consumed. Again, like TBA generation from ETBE, no TAA production was observed when propane-grown cells were incubated with TAME and acetylene (10% v/v gas phase). The presence of acetylene also fully inhibited the production of the second uncharacterized product of TAME oxidation (data not shown).

**Degradation of TBA and TAA.** We have previously demonstrated that TBA is readily oxidized by propane-grown cells of *M. vaccae* JOB5 (18). Our evidence from our earlier experiments (see above) suggested a similar reaction occurred with TAA. To more clearly evaluate the potential for TAA oxidation in the complete absence of TAME we incubated propane-grown cells of *M. vaccae* JOB5 with equimolar dissolved concentrations of either TAA or TBA. These reactions were also conducted in the presence or absence of acetylene (10% v/v gas phase). In the absence of acetylene we observed both alcohols were consumed at essentially identical rates and in both cases these reactions were fully inhibited by acetylene (Figure 3.3).
Effects of propane on ETBE, TBA, TAME and TAA oxidation. The results of our physiological and inhibitor experiments presented above suggest that alkane-grown cells of *M. vaccae* JOB5 oxidize ETBE and TAME and their corresponding alcohol products (TBA and TAA, respectively) through the activity of an alkane-inducible monooxygenase. This suggests that all of these ether and alcohol oxidation reactions should be susceptible to competitive inhibition by an alkane substrate for this enzyme. To test this, initial rates (0 to 30 min) of ETBE (0.23 mM) and TAME (0.47 mM) oxidation by propane-grown cells of *M. vaccae* JOB5 was determined in the presence of varying concentrations of propane added to the gas phase (Figure 3.4). The aqueous concentrations of propane (0--80 µM) were estimated by the partial pressure of propane added to the gas phase of the reaction vials by assuming that the small volumes of gas added (≤5% of gas phase) had a negligible effect on the total gas pressure within the reaction vial. The level of inhibition of either ETBE or TAME oxidation at each concentration of propane was plotted versus the dissolved propane concentration and these data were computer fitted to a hyperbola by nonlinear regression to determine the apparent $K_i (K_i^{app})$ values for propane. Good fits were obtained for both these analyses, resulting in $r^2$ values of 0.979 for ETBE and 0.979 for TAME. *K_i^{app}* values of 5.0 and 22.0 µM were determined for ETBE and TAME, respectively.

Similar experiments were also conducted to determine the effects of propane on the oxidation of TBA and TAA (Figure 3.5). Propane-grown *M. vaccae* JOB5 cells were incubated with fixed initial concentrations of TBA (0.5 mM) or TAA (0.5 mM) in the presence of varying concentrations of dissolved propane (0 to ~80 µM). Good fits were obtained for both these analyses, resulting in $r^2$ values of 0.984 for TBA and 0.944 for
TAA. Apparent $K_i (K_i^{app})$ values of 19 and 21 µM were determined for TBA and TAA, respectively.
DISCUSSION

The results of this study provide evidence that alkane-grown cells of *M. vaccae* JOB5 are able to cometabolically oxidize both ETBE and TAME as well as their initial respective oxidation products, TBA and TAA. Our evidence also suggests that these reactions are all catalyzed by the same alkane-inducible monooxygenase enzyme previously implicated in the cometabolic degradation of MTBE by this bacterium. The following sections discuss our evidence for these conclusions and the possible implications of our observations on our understanding of the environmental fate of ether oxygenates.

**Evidence for the role of short chain alkane monooxygenase in ether and alcohol oxidation:**

The results of this study provide several strong lines of evidence that suggest a short chain alkane monooxygenase (SCAM) is responsible for the oxidation of both ETBE and TAME, as well as their corresponding oxidation products, TBA and TAA. This evidence includes our observations that ether and alcohol oxidation occurred without a lag phase in cells grown on propane (Figures 3.1, 3.2 and 3.3) and other alkanes (Table 3.1) whereas this activity was absent from cells grown in dextrose-containing medium. Further evidence includes our observations that both ether and alcohol oxidation were fully inhibited by acetylene (Figures 3.2 & 3.3) while propane acted as an apparently potent competitive inhibitor (Figures 3.4 & 3.5). Finally, the identified products of ETBE and TAME oxidation were both tertiary alcohols. These are the products expected from monooxygenase-catalyzed o-dealkylation reactions directed at the methoxy and ethoxy groups on these branched ether-bonded compounds.
The physiological results we have summarized above are very similar to those we have previously reported for the cometabolic degradation of MTBE by this bacterium. Although the oxidation of ETBE, TAME, TBA and TAA have been previously reported for propane-oxidizing bacteria, the results we present here provide a more detailed comparison of the relative rates of oxidation of these compounds and highlight some significant differences between the processes involved in MTBE oxidation and the oxidation of other ether oxygenates. At the mechanistic level we have previously identified tertiary butyl formate (TBF) as a key intermediate in MTBE degradation by *M. vaccae* JOB5. This ester is believed to be generated by an alcohol dehydrogenase-catalyzed oxidation of a transient unstable hemiacetal generated during the initial monooxygenation of the methoxy group of MTBE. In this study we did not observe the accumulation of an equivalent ester during the oxidation of either ETBE or TAME. In the case of ETBE the lack of ester production may reflect the fact that an o-dealkylation reaction would need to involve oxidation of the carbon atom closest to the ether oxygen. This hemiacetal may be inaccessible to the alcohol dehydrogenases present in propane-grown cells. In this case the hemiacetal would decompose directly to TBA and acetaldehyde. Although we detected TBA accumulation during ETBE degradation, we did not observe acetaldehyde accumulation even though this compound is readily detectable by FID-GC. However, as *n*-alkane-oxidizing bacteria are required to metabolize aldehydes during alkane oxidation, a lack of accumulation of acetaldehyde would therefore not be particularly surprising especially as the rate of ether oxygenate degradation occurs at a small fraction of the rates of substrate turnover during alkane oxidation.
In contrast to the aldehyde intermediate expected from the oxidation of an ethoxy compound, it seems more reasonable to expect the accumulation of an ester during the oxidation of TAME, another methoxy group-containing compound. In this study we observed TAME oxidation generated both TAA and a second as yet unidentified product. As indicated in the Results section, this unidentified compound had chromatographic properties suggesting it is likely to be an organic acid. If this is correct and an ester is not generated during TAME oxidation, it is likely that a hemiacetal generated from oxidation of the methoxy group spontaneously decomposes to TAA and formaldehyde, a C₁ compound that is not detected by FID-GC analyses. Additional studies are clearly needed to further characterize the intermediates generated during TAME and ETBE degradation. These studies should focus on the production and fate of the C₁ and C₂ products generated during the ether dealkylation reactions. These studies should also examine the possibility of oxidation reactions that do not result in the immediate dealkylation of these compounds. These would include initial reactions directed at the tertiary amyl group of TAME and reactions targeted at the ethoxy group of ETBE. This last possibility seems particularly appropriate as the stoichiometry of alcohol production to ETBE consumption were low (Figure 3.2) compared to the corresponding reaction reported here for TAME oxidation and our previous observations made with MTBE. Although it has not been a major focus of the present study, future work should also be directed at identification of the products generated during TAA oxidation in the absence of TAME. Mammalian studies have previously identified a number of TAA oxidation metabolites but nothing is currently known about the products of TAA oxidation by microorganisms.
The results of this study also provide some indication of the relative affinity of SCAM towards the various ethers and alcohols we have examined. For example, our results suggest that this enzyme system has comparable affinities for TBA and TAA. This conclusion is supported by our observations that these compounds were consumed at equivalent rates by propane-grown cells (Figure 3.3) and that propane was equally effective as a competitive inhibitor of these reactions. A comparison of the relative rates of ETBE, TAME, and MTBE oxidation is less clear-cut with our existing data. For example, our studies following the rates of degradation of each ether at equimolar dissolve aqueous phase concentrations (Figure 3.1) suggested ETBE is oxidized at a significantly slower rate than TAME or MTBE. However, when fixed amounts of these compounds were added to reactions, the rate of ETBE oxidation appeared to be only slightly slower than TAME oxidation. This observation could be due to the difference in solubility between the two ethers and the impact of their respective distribution patterns between the gas and liquid phases, as described by their respective Henry’s constant values. Support for the suggestion that TAME is a superior substrate (lower $K_s$ and or higher $V_{max}$) is given by our observation that propane was a more effective inhibitor of ETBE oxidation than TAME oxidation (Figure 3.5). Our observation that TBA accumulation during ETBE oxidation was lower than that observed with either MTBE or TAME could also suggest that TBA oxidation by SCAM during ETBE oxidation occurs at a faster rate than with the other oxygenates because ETBE is less effective than the other ethers at competitively displacing this SCAM substrate.
**Potential Environmental Significance.** The results of this study provide some indication of the potential environmental fate of the various ethers and alcohols we have examined. For example, one key observation we have made is the apparent lack of reactivity of *M. vaccae* JOB5 towards DIPE (Figure 3.1 and Table 3.1). To date there has been little or no information published about the biodegradation of DIPE by pure cultures of bacteria. This lack of information may reflect difficulties associated with reporting negative results but they may also reflect a general lack of reactivity of recognized MTBE-, ETBE- and TAME-degrading organisms towards this compound. It should be noted that SCAM in *M. vaccae* JOB5 is a remarkably non-specific enzyme and has been implicated in the oxidation of a wide range of compounds including ether-bonded, chlorinated and cyclic compounds. It should also be noted that the expected products of DIPE oxidation are 2-propanol and acetone, two compounds that are readily oxidized by this organism. Given the apparent lack of reactivity of propane-grown *M. vaccae* JOB5 towards DIPE it seems likely that we need to look to alternative enzyme systems and organisms to identify potential routes for DIPE degradation. If use of DIPE continues to expand as use of MTBE diminishes, identification of DIPE-degrading organisms is expected to emerge as a pressing issue for environmental microbiologists.

In contrast to DIPE, our present results are more encouraging with respect to ETBE and TAME. Although ETBE appears to be more slowly biodegraded than either TAME or MTBE, our results do suggest that the lack of general cross reactivity between the degradation of methoxy- and ethoxy-bonded alkyl ethers seen in previous studies (see Introduction) does not occur with *M. vaccae* JOB5. Our results also confirm that the tertiary alcohol products (TBA and TAA) derived from ETBE, MTBE and TAME are
both degraded at similar rates. As indicated above, this encouraging observation has to be qualified by the need to more clearly characterize and quantify the products of TAA oxidation by *M. vaccae* JOB5 and other TAME degrading organisms.
Figure 3.1. Degradation of MTBE, ETBE, TAME, and DIPE by propane-grown and dextrose-grown *M. vaccae* JOB5. Cultures of *M. vaccae* JOB5 were grown in batch culture on propane (closed symbols) or dextrose (open symbols) and harvested as described in Materials and Methods. The time course for MTBE (squares), ETBE (upright triangles), TAME (inverted triangles), and DIPE (circles) (initial concentration of each ether = 1 mM dissolved) consumption by either propane- or dextrose-grown cells is shown for 280 minutes. Cells were incubated with MTBE, ETBE, TAME, or DIPE and an analysis was conducted using gas phase gas chromatography.
Table 3.1. The nmoles of ether oxygenate consumed/min/mg of protein by *M. vaccae* JOB5 cells grown on various substrates.

<table>
<thead>
<tr>
<th>MTBE</th>
<th>Growth Substrate</th>
<th>nmoles MTBE consumed/min/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Butane</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>Pentane</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>Isopentane</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>Dextrose</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ETBE</th>
<th>Growth Substrate</th>
<th>nmoles ETBE consumed/min/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Butane</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Pentane</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>Isopentane</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>Dextrose</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TAME</th>
<th>Growth Substrate</th>
<th>nmoles TAME consumed/min/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Butane</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>Pentane</td>
<td>15.6</td>
</tr>
<tr>
<td></td>
<td>Isopentane</td>
<td>8.6</td>
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<tr>
<td></td>
<td>Dextrose</td>
<td>&lt;0.1</td>
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<table>
<thead>
<tr>
<th>DIPE</th>
<th>Growth Substrate</th>
<th>nmoles DIPE consumed/min/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Butane</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td>Pentane</td>
<td>&lt;0.1</td>
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<tr>
<td></td>
<td>Isopentane</td>
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<td></td>
<td>Dextrose</td>
<td>&lt;0.1</td>
</tr>
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</table>
Figure 3.2. Time course of ETBE and TAME oxidation by propane-grown *M. vaccae* JOB5 cells in the presence and absence of acetylene. Cultures of *M. vaccae* JOB5 were grown in batch culture on propane and harvested as described in Materials and Methods. The time course for ETBE (squares) and TAME (circles) consumption and TBA (upright triangles) and TAA (inverted triangles) production by propane-grown *M. vaccae* JOB5 cells is shown for 120 (TAME) or 150 (ETBE) minute incubations. Experimental vials were incubated with concentrated propane-grown *M. vaccae* JOB5 cells and ETBE or TAME (~1100 nmoles) in the presence (open symbols) or absence (closed symbols) of acetylene (10% vol/vol) for three replicate cultures and analyzed in the liquid phase by gas chromatography. The error bars indicate the range of values for ETBE, TAME, TBA and TAA for all three cultures.
Figure 3.3. Time course of TBA and TAA oxidation by propane-grown *M. vaccae* JOB5 cells in the presence and absence of acetylene. A culture of *M. vaccae* JOB5 was grown in one batch culture on propane and harvested as described in Materials and Methods. The time course for TBA (squares) and TAA (circles) consumption by propane-grown *M. vaccae* JOB5 cells is shown over 120 minute incubations. Cells were incubated with TBA or TAA (~115 nmoles) in the presence or absence of acetylene (10% vol/vol) and a liquid phase analysis was conducted by gas chromatography.
Figure 3.4. Propane inhibition of ETBE and TAME oxidation by propane-grown *M. vaccae* JOB5. The figure shows the effect of various concentrations of propane on ETBE and TAME oxidation. Propane grown cells of *M. vaccae* JOB5 were incubated for 30 min. with a fixed concentration of either ETBE (0.23 mM) or TAME (0.47 mM) in the presence of the indicated aqueous concentrations of propane. The aqueous concentrations of propane were estimated from the partial pressure of propane added to the gas phase of the reaction vials by assuming that the small volumes of gas added (≤5% of gas phase) had a negligible effect on the total gas pressure within the reaction vial. The plot shows the percent inhibition of ETBE (squares) and TAME (triangles) consumption at each propane concentration, compared to the amount of each substrate consumed in the absence of propane. The figure shows the resulting curves generated when the values for percent inhibition were fitted by computer, as described in Materials and Methods.
Figure 3.5. Propane inhibition of TBA and TAA oxidation by propane-grown *M. vaccae* JOB5. The figure shows the effect of various concentrations of propane on TBA and TAA oxidation. Propane grown cells of *M. vaccae* JOB5 were incubated for 30 min. with a fixed concentration of either TBA (0.5 mM) or TAA (0.5 mM) in the presence of the indicated aqueous concentrations of propane. The aqueous concentrations of propane were estimated from the partial pressure of propane added to the gas phase of the reaction vials by assuming that the small volumes of gas added (≤5% of gas phase) had a negligible effect on the total gas pressure within the reaction vial. The plot shows the percent inhibition of TBA (squares) and TAA (triangles) consumption at each propane concentration, compared to the amount of each substrate consumed in the absence of propane. The figure shows the resulting curves generated when the values for percent inhibition were fitted by computer, as described in Materials and Methods.
REFERENCES


CHAPTER 4

Inhibitory effects of 1-propanol on Methyl Tertiary Butyl Ether (MTBE)-
oxidizing activity in Mycobacterium vaccae JOB5

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ABSTRACT

We have investigated the effect of 1-propanol on MTBE oxidation in propane-grown Mycobacterium vaccae JOB5 cells. When propane-grown cells were incubated with 1-propanol (20 mM) and MTBE (1 mM), MTBE oxidation was strongly inhibited until the 1-propanol concentration was reduced to <2 mM. The presence of MTBE did not affect the rate of 1-propanol consumption or the concentration of products produced during 1-propanol oxidation. When 1-propanol concentrations decreased to <2 mM and MTBE oxidation commenced, the rate of MTBE oxidation was slower than when cells were never exposed to 1-propanol. In addition, increased 1-propanol concentrations led to increased inhibition of MTBE-oxidizing activity. Analysis of the kinetics of ethylene oxidation in the presence or absence of 1-propanol (5 mM) resulted in an increase in $K_s$ for ethylene from 13.7 to 47.1 µM and a decrease in $V_{max}$ (from 106.3 to 18.6 nmoles min$^{-1}$ mg total protein$^{-1}$) in the presence of 1-propanol. Other primary alcohols and secondary alcohols were also inhibited by ethylene oxidation whereas propionic acid and two propanediols tested did not inhibit ethylene-oxidizing activity. Fully understanding the inhibition of MTBE-oxidizing activity by 1-propanol requires additional studies to determine the exact relationship between these two substrates in propane-grown M. vaccae JOB5 cells. However, our data suggests that the interaction between 1-propanol and MTBE is not a metabolic regulation but could potentially be due to competitive interactions between substrates for oxidation by short chain alkane monooxygenase.
INTRODUCTION

Methyl tertiary butyl ether (MTBE) is an alkyl ether compound that is currently added to gasoline in the United States and several other countries. Although originally added to increase octane levels in the late 1970s, MTBE is now frequently added to gasoline as an oxygenate to reduce automobile emissions including carbon monoxide and other smog-related air pollutants. The use of ether-based oxygenates has increased dramatically in the US following passage of the 1990 Clean Air Act Amendments (1). The widespread use of MTBE in the United States and the detection of MTBE in groundwater supplies (2) have led to increasing human health concerns. This also prompted the U.S. Environmental Protection Agency to investigate the human effects of chronic exposure to MTBE in drinking water (3). The US EPA currently classifies MTBE as a possible carcinogen and has set a drinking water advisory of 20 to 40 ppb (4).

Biodegradation of MTBE has been investigated in both anaerobic and aerobic environments. Although anaerobic biodegradation of MTBE is the most probable occurrence of biodegradation of MTBE in the environment, MTBE degradation in anaerobic environments occurs slowly if at all (5, 6, 7, 8). The rate of MTBE oxidation is greatly enhanced in aerobic environments. In the presence of molecular oxygen, MTBE biodegradation occurs by both metabolic and cometabolic processes. True metabolism of MTBE has been identified by numerous microorganisms, including *Rubrivivax* sp. strain PM-1, *Mycobacterium austroafricanum*, and *Hydrogenophaga flava* ENV 735, which use MTBE as a sole source of carbon and energy for growth (5, 6, 7). MTBE metabolism by these organisms has consistently shown to be slow, leading to low biomass accumulation.

A more efficient biodegradation of MTBE by aerobic microorganisms occurs through cometabolism. Cometabolism of MTBE involves growth of organisms on substrates that
promote the expression of enzyme systems that allow these organisms to fortuitously oxidize MTBE. Hydrocarbons including normal and branched alkanes (9, 10, 11), aromatics (12, 13), and alicyclic (14) compounds have been identified as growth-supporting substrates for MTBE cometabolizing bacteria. The environmental application of cometabolism in MTBE biodegradation is clearly relevant due to the presence of both MTBE and hydrocarbons in gasoline-impacted environments.

We have previously examined the regulation of MTBE-oxidizing activity in *M. vaccae* JOB5 and have shown MTBE induces production of enzyme systems required for its own oxidation (15). This inducing effect occurs even though this organism apparently does not gain a carbon or energetic benefit from MTBE oxidation. In this study, we examined the potential regulatory effects of propane oxidation metabolites on the MTBE-oxidizing activity of propane-grown cells of *M. vaccae* JOB5.
MATERIALS AND METHODS

Materials. *M. vaccae* JOB5 (ATCC 29678) was obtained from the American Type Culture Collection (Manassas, Va.). 1-butanol (99% purity), 2-butanol (99.5% purity), ethylene (99.5+% purity), ethylene oxide (99.5+% purity), 2-hexanol (99% purity), methyl tertiary butyl ether (MTBE) (99.8% purity), 1-pentanol (99+% purity), 2-pentanol (98% purity), 1,2-propanediol (99.5+% purity), 1,3-propanediol (98% purity), 1-propanol (99.5% purity), 2-propanol (99.5% purity), propionaldehyde (97% purity) and sodium propionate (99% purity) were obtained from Sigma Aldrich Chemical Co., Inc. (Milwaukee, Wis.). Propane (instrument grade) was obtained from Matheson Gas Products, Inc. (Montgomeryville, PA). Compressed gases used for gas chromatography (GC) (H₂, N₂, and air) were obtained from local industrial vendors.

Growth experiments. All of the experiments described in this study used cells of *M. vaccae* JOB5 grown in batch culture at 30°C by using propane as the sole source of carbon and energy. Cells were grown in glass serum vials (750 ml) in a mineral salts medium (100 ml) (16) and sealed with screw caps fitted with butyl rubber septa (Wheaton Scientific, Millville, NJ). The vials were inoculated (initial *A*₆₀₀ = ~0.02) with a suspension of cells obtained from axenic cultures of *M. vaccae* JOB5 previously grown on casein-yeast extract-dextrose (CYD) agar plates (Difco Plate Count Agar). Propane (180 ml) was added to the sealed vials by using plastic syringes fitted with sterile filters (0.25 μm). After growth for ~5 days (final *A*₆₀₀ = ~0.6), a sample (50 ml) of the culture was plated on CYD plates to subsequently confirm the purity of the cultures. The remaining cells were harvested by centrifugation (10,000 x g; 10 min) and the resulting pellet was resuspended in buffer (10 ml, 50 mM sodium phosphate, pH 7). The washed cells were sedimented again by centrifugation (as above) and the resulting cell pellet was finally
resuspended with buffer (~1.0 ml, as above) to a final protein concentration of 4-14 mg total cell protein ml\(^{-1}\). The cells were stored at 4°C and were used within 4h.

**Reaction conditions.** Reactions following the oxidation of MTBE, 1-propanol, and ethylene were all conducted in glass serum vials (10 ml). The reaction vials were prepared by adding buffer (50 mM sodium phosphate, pH 7) (~800 ml) and concentrated cells (~200 ml), after which the vials were sealed with butyl rubber stoppers and aluminum crimp seals (Wheaton Scientific, Millville, NJ). Saturated aqueous solutions of MTBE and stock solutions of 1-propanol, 1-butanol, 1-pentanol, 2-propanol, 2-butanol, 2-pentanol, 2-hexanol, 1,2-propanediol, 1,3-propanediol, propionaldehyde, and propionic acid were added directly to the sealed vial using sterile glass microsyringes. Ethylene gas was added to sealed vials using sterile glass microsyringes.

**Analytical Methods.** In all experiments the concentrations of MTBE, 1-propanol, propionaldehyde, propionic acid, ethylene and ethylene oxide were determined by gas chromatography. For the analysis of MTBE, 1-propanol, propionaldehyde and propionic acid, aqueous samples (2 ml) were taken directly from the reaction vials and were injected into a Shimadzu GC-14A gas chromatograph (Kyoto, Japan) fitted with a flame ionization detector and a stainless steel column (0.3 x 183 cm) filled with Porapak Q (60-80 mesh) (Waters Associates, Framingham, MA). The analysis was conducted using a column temperature of 160°C, injection port temperature of 200°C and a detector temperature of 220°C. Nitrogen was used as carrier gas at a flow rate of 15 ml min\(^{-1}\). In experiments that followed only ethylene oxidation, gas phase samples (25 ml) were removed from reaction vials using gas tight syringes. The samples were directly injected into a Shimadzu GC-14A gas chromatograph fitted with a flame ionization detector.
detector and a DB-MTBE capillary column (30 m x 0.45 mm I.D. [2.55 μm film]) (J & W Scientific, Folsom, CA). The analysis was conducted using a column temperature of 35°C, an injection port temperature of 200°C and a detector temperature of 220°C. Nitrogen was used as carrier gas at a flow rate of 5 ml min⁻¹. The gas chromatograph was interfaced to a Hewlett Packard HP3395 (Palo Alto, CA) integrator for data collection. Cell protein concentrations were determined using the Biuret assay (17) after solubilization of cell material for 30 min at 65°C in 3N NaOH and sedimentation of insoluble material by centrifugation in a microfuge (14,000 rpm for 5 min). Bovine serum albumin was used as the standard. The concentration of MTBE in saturated aqueous solution at room temperature (23°C) was taken as 0.544M (18). The dimensionless Henry’s constant for MTBE at 30°C was taken as 0.0255 (19).
RESULTS

Effect of 1-propanol on MTBE-oxidizing activity. Propane-grown cells of *M. vaccae* JOB5 were incubated with 1-propanol alone (20 mM), MTBE alone (~1 mM) or 1-propanol (20 mM) plus MTBE (~1 mM) (Figure 4.1). The oxidation of 1-propanol (regardless of whether or not MTBE was present) occurred over 120 minutes and both propionaldehyde and propionic acid were detected as products of 1-propanol oxidation. When propane-grown *M. vaccae* JOB5 cells were incubated with MTBE alone, >50% of the MTBE was consumed in the first 40 minutes. However, the presence of 20 mM 1-propanol strongly inhibited MTBE-oxidizing activity and no MTBE consumption was observed for 90 min. Oxidation of MTBE commenced once the concentration of 1-propanol had declined to ~2 mM and the propionaldehyde generated from 1-propanol oxidation had decreased to <2.5 mM. The maximal rate of MTBE oxidation established after the effects of 1-propanol and propionaldehyde had been consumed was lower than cells that had not been exposed to 1-propanol. In contrast, the presence of MTBE did not appear to affect the rate of 1-propanol consumption or the stoichiometry of propanol oxidation products produced.

Effect of 1-propanol concentration of MTBE-oxidizing activity. We subsequently investigated the effects of varying concentrations of 1-propanol on MTBE oxidation. When propane-grown cells of *M. vaccae* JOB5 were incubated with MTBE (1 mM) and varying initial concentrations of 1-propanol from 0 to 20 mM we observed a strong effect of 1-propanol concentration on the initial rate of MTBE oxidation (0 to 30 min) (Figure 4.2). These data were computer-fitted to a hyperbola by nonlinear regression (data in triplicate, $r^2=0.955$) and an apparent $K_i (K_i^{app})$ for 1-propanol of 8.2 mM was determined from this curve.
**Effects of other alkane-oxidation metabolites on MTBE-oxidizing activity.** Initially, we aimed to examine the products of 1-propanol oxidation to determine if the inhibition of MTBE-oxidizing activity described above was due directly to the presence of 1-propanol or whether similar effects were also observed with the products of 1-propanol oxidation, propionaldehyde or propionic acid. Propionaldehyde was especially of interest since the oxidation of MTBE did not occur until the propionaldehyde produced by 1-propanol oxidation was <2.5 mM (Figure 4.1). Our data show that propionaldehyde (5 mM) resulted in a strong inhibition of MTBE oxidation (Table 4.1). However, this observation is equivocal as significant amounts of 1-propanol were generated during this reaction due to alcohol dehydrogenase activity in propane-grown cells. In contrast, there was no inhibitory effect of propionic acid (5 mM) on the initial rate of MTBE oxidation (0-60 min).

We also examined a variety of other alcohols (primary and secondary) to observe if these substrates resulted in the same inhibition of MTBE oxidation as 1-propanol. Among the 1° alcohols tested (all at 5 mM initial concentration), 1-butanol and 1-pentanol caused complete inhibition of MTBE oxidation, whereas 2° alcohols (2-propanol, 2-butanol, 2-pentanol or 2-hexanol) there was only a slight inhibition of MTBE oxidizing activity. The inhibitory effect of the 2° alcohols increased as the carbon chain length increased. For example, 2-propanol caused a ~10% inhibition of MTBE oxidation while the presence of 2-hexanol resulted in 100% inhibition of MTBE oxidation. Lastly, the presence of 1,2-propanediol and 1,3-propanediol, the predicted products of 1-propanol oxidation by the alkane monooxygenase, had a stimulating effect on MTBE oxidation.
Effect of ethylene concentration on ethylene-oxidizing activity in the presence of 1-propanol. We also examined whether the effects of 1-propanol were specific for MTBE or whether they could be observed with other substrates for the short chain alkane monooxygenase in *M. vaccae* JOB5. We therefore investigated the effects of a varying concentrations of ethylene on propane-grown cells incubated in the presence of a fixed initial concentration of 1-propanol (5 mM). Ethylene was chosen as a suitable alternative substrate for these experiments as it is oxidized to a readily detectable water-soluble stable product (ethylene oxide) and because ethylene has a substantially lower $K_s$ value than MTBE.

Our results show oxidation of ethylene to ethylene oxide was strongly inhibited by 1-propanol, irrespective of the initial ethylene concentration (Figure 4.3). As determined by computer-fitted data, fit to a hyperbola by nonlinear regression, the $K_s$ and $V_{max}$ for ethylene oxidation in the absence of 1-propanol by propane-grown *M. vaccae* JOB5 were 13.7 µM and 47.1 nmoles ethylene oxidized min$^{-1}$ mg total protein$^{-1}$, respectively. The corresponding $K_s$ and $V_{max}$ values for ethylene oxidation in the presence of 5 mM 1-propanol were 106.3 µM and 18.6 nmoles ethylene oxidized min$^{-1}$ mg total protein$^{-1}$, respectively.
DISCUSSION

The results of this study provide evidence that MTBE oxidation is inhibited by 1-propanol during incubation with propane-grown cells of *M. vaccae* JOB5 (Figure 4.1). Our original hypothesis to account for this effect was that it was the result of a form of metabolic regulation. Bacterial alkane-oxidizing systems usually consist of an initial reductant (NADH)-utilizing monooxygenase enzyme followed by reductant (NADH)-generating enzymes such as an alcohol and aldehyde dehydrogenase. Given that cells have to carefully maintain their intracellular ratio of NAD\(^+\) to NADH (*aka* metabolic energy charge) we hypothesized that alkane-oxidizing bacteria are most likely compelled to metabolically regulate the activity of the initial alkane oxidation step so they are able to maximize the efficiency of recovery of reductant during the downstream dehydrogenase-catalyzed oxidation steps. Without a form of regulation it appeared likely that the reductant invested in converting alkanes to alcohols could be lost if the rate of alkane oxidation exceeded the capacity of cells to oxidize alcohols. We also argued a simple method to achieve regulation in this type of system would be to have a direct influence of the NADH/NAD\(^+\) ratio on activity of the initial monooxygenase. As the ratio increased, the activity of the monooxygenase would be forced to decrease to oxidize the accumulated alcohols and aldehydes. As the ratio fell, the activity of the monooxygenase would be allowed to increase to allow the NADH/NAD\(^+\) ratio to reach homeostasis. Additional support for this hypothesis comes from the fact that many hydrocarbon-oxidizing enzymes systems contain a small regulatory protein that controls the levels and types of activity catalyzed by these enzymes.

The results presented in Figure 4.1 are in many ways compatible with the model presented above. This experiment demonstrates that the activity of a reductant-utilizing monooxygenase (MTBE oxidation) was strongly but transiently suppressed by the presence of 1-
propanol and propionaldehyde. This experiment also shows this inhibitory effect was rapidly eliminated once the concentration of 1-propanol and propionaldehyde had been significantly decreased. This would be compatible with a return to reductant homeostasis and a relief of a regulatory effect on the MTBE-oxidizing monooxygenase. It is also important to note propionaldehyde and propionic acid were produced at substantially similar rates and concentrations regardless of whether or not MTBE was present. This suggests that MTBE does not affect the metabolism of 1-propanol but 1-propanol clearly affects the consumption of MTBE.

While we also observed that the effect of 1-propanol is saturable \((K_i = 8.2\ \text{mM})\) and that a similar inhibitory effect occurs with other readily metabolizable 1° alcohols (Table 4.1) our experiments with ethylene suggested to us that the impacts of 1-propanol and other alkane oxidation metabolites might have a simpler explanation than the metabolic regulation model outlined above. Our main reasons for choosing ethylene for these experiments was because it has a substantially lower \(K_s\) value than MTBE and it produced a single oxidation product (ethylene oxide) that did not co-elute with 1-propanol (and its oxidation products) during our GC-based reaction analysis. In combination, these features enabled us to demonstrate that the effects of 1-propanol on ethylene oxidation had some characteristics of a competitive interaction between ethylene and 1-propanol as substrates for the same enzyme. For example, 1-propanol decreased the \(V_{max}\) for ethylene oxidation and increased the \(K_s\) for ethylene. Although a truly competitive interaction would be expected to only produce a decrease in the \(K_s\) value without an effect on \(V_{max}\), we concluded the ambiguity of the effects of 1-propanol were not worth further examination. This decision was further impacted by the fact that an obvious prediction of a model based on a competitive interaction between ethylene and 1-propanol as substrates for
oxidation would predict 1-propanol is most likely oxidized by the alkane-oxidizing monooxygenase to either propionaldehyde or propan-1,2-diol. Both of these compounds are readily oxidized by alkane-grown *M. vaccae* JOB5 and it would be extremely difficult to demonstrate further oxidation of 1-propanol through a monooxygenase-catalyzed process.
Figure 4.1. The effect of 20 mM 1-propanol on MTBE oxidation by propane-grown *M. vaccae* JOB5 cells. Propane-grown *M. vaccae* JOB5 were grown in batch culture and harvested as described in Materials and Methods. Concentrated cells were incubated with MTBE (1 mM) (open symbols), 1-propanol (20 mM) (open symbols), or MTBE (1 mM) and 1-propanol (20 mM) (closed symbols). Concentrations of 1-propanol (squares), propionaldehyde (upright triangles), propionic acid (inverted triangles), and MTBE (circles) are shown during a time course incubation with concentrated propane-grown *M. vaccae* JOB5 cells.
Figure 4.2. The effect of varying concentrations of 1-propanol on MTBE oxidation in the presence of 5/10 mM MTBE and propane-grown *M. vaccae* JOB5 cells. Propane-grown *M. vaccae* JOB5 cells were grown in batch culture and harvested as described in Materials and Methods. Concentrated cells were incubated with MTBE (1.0 mM) and varying concentrations of 1-propanol (0-20 mM) for 30 min for three replicate cultures. The percent of MTBE-oxidizing activity inhibited by the presence of 1-propanol is shown. The error bars indicate the range of values for percent inhibition of MTBE-oxidizing activity during incubation with a defined concentration of 1-propanol (0-20 mM) for all three cultures.
Table 4.1. Additional reductants (5mM) and their effect on ethylene oxidation following 60 minute incubation with substrate and propane-grown *M. vaccae* JOB5 cells.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-butanol</td>
<td>Inhibits</td>
</tr>
<tr>
<td>1-pentanol</td>
<td>Inhibits</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>Inhibits&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Propionic Acid</td>
<td>No inhibition</td>
</tr>
<tr>
<td>2-propanol</td>
<td>11% inhibition</td>
</tr>
<tr>
<td>2-butanol</td>
<td>44% inhibition</td>
</tr>
<tr>
<td>2-pentanol</td>
<td>85% inhibition</td>
</tr>
<tr>
<td>2-hexanol</td>
<td>Complete inhibition</td>
</tr>
<tr>
<td>1,2-propanediol</td>
<td>Increases oxidation rate</td>
</tr>
<tr>
<td>1,3-propanediol</td>
<td>Increases oxidation rate</td>
</tr>
</tbody>
</table>

<sup>a</sup> The production of 1-propanol was observed during incubation of propane-grown *M. vaccae* JOB5 cells with propionaldehyde. Unable to determine if the inhibition of ethylene oxidation is due to propionaldehyde or 1-propanol.
Figure 4.3. The effects of increasing concentrations of ethylene on the rate of ethylene oxidation in the presence of 5mM 1-propanol during incubation with propane-grown *M. vaccae* JOB5 cells. Propane-grown *M. vaccae* JOB5 cells were grown in batch cultures and harvested as described in Materials and Methods. Concentrated cells were incubated with varying concentrations of ethylene (0-400 µl) (squares) or varying concentrations of ethylene (0-400 µl) and 5 mM 1-propanol (triangles). The amount of ethylene oxide produced following a 30 minute incubation is shown.
REFERENCES


CHAPTER 5

Propane and n-Butane Oxidation by *Pseudomonas putida* GPo1

Submitted by

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Running Title: Gaseous alkane oxidation by *Pseudomonas putida* GPo1

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Catabolism of $C_5$ to $C_{10}$ n-alkane growth substrates in *Pseudomonas putida* GPo1 is initiated by alkane hydroxylase activity. We previously reported propane and $n$-butane inhibit the alkane hydroxylase-dependent oxidation of methyl tertiary butyl ether (MTBE) by strain GPo1 and we suggested these gases might be unrecognized substrates for this enzyme. We have now further characterized the effects of these gases on this bacterium. Propane and $n$-butane, but not ethane or methane, were co-oxidized during growth of strain Gpo1 on C-limiting amounts of n-octane. Propane and $n$-butane co-oxidation during growth on $C_5$ to $C_9$ n-alkanes consistently increased final culture density and biomass production. These results suggest the products of gaseous n-alkane oxidation were assimilated by n-alkane-grown cells. Strain GPo1 also grew on both $n$-butane and propane as sole carbon and energy sources. Growth on these gases exhibited a lag phase relative to $n$-octane although the lag phase was impacted by the concentration of the gases added to cultures. An immunoblot analysis of total cell protein from propane-, $n$-butane- or $n$-octane-grown cells demonstrated the alkB component of alkane hydroxylase was present at high levels but was largely absent from cells grown on $1^o$ alcohols. Cells grown on propane-, $n$-butane-, and $n$-octane also all rapidly oxidized MTBE (17-38 nmoles min$^{-1}$ mg total protein$^{-1}$) while no activity (<0.1 nmoles min$^{-1}$ mg total protein$^{-1}$) was observed in cells grown on 1-propanol and 1-butanol. Our results suggest propane and $n$-butane were co-oxidized and metabolized through the same enzyme activities involved in the metabolism of n-alkanes such as $n$-octane.
INTRODUCTION

The \textit{n}-alkane-oxidizing system of \textit{Pseudomonas putida} GPo1 has been extensively studied and it is viewed as a model for the bacterial oxidation of \textit{n}-alkanes other than methane (27). In \textit{P. putida} Gpo1 four sequential enzymes are required to convert \textit{n}-alkanes to their corresponding terminal acyl-coenzymeA derivatives: alkane hydroxylase, alcohol dehydrogenase, aldehyde dehydrogenase and acyl-coenzymeA synthetase (27). In \textit{P. putida} Gpo1 all of these enzymes are encoded in genes on the OCT plasmid, so named to reflect \textit{n}-octane is a highly effective growth substrate for this organism (3). The alkane hydroxylase enzyme responsible for initiating \textit{n}-alkane oxidation in this strain is a three-component monooxygenase. A 41 kDa membrane-bound hydroxylase component is responsible for substrate (\textit{n}-alkane and \textit{O}_2) binding and activation (27). This component contains a binuclear iron cluster (20) and is encoded by the \textit{alkB} gene. Two soluble proteins, a Fe-S–containing rubredoxin and a flavin-containing rubredoxin reductase, mediate electron transfer to the hydroxylase from NADH. These proteins are encoded by the \textit{alkG} and \textit{alkT} genes, respectively (27).

The first description of the organism now known as \textit{P. putida} GPo1 was provided by Baptist \textit{et al.} (1) and a taxonomic description of this strain and its progenitors was recently outlined van Beilen \textit{et al.} (29). The \textit{n}-alkane growth substrate range of strain GPo1 is known to extend from C\textsubscript{5} to C\textsubscript{12} although the growth substrate range appears more limited when cells are grown on mineral salts plates in the presence of alkane vapors (1, 7, 16) as opposed to shake flash cultures (19). In cell-free systems the GPo1 alkane hydroxylase will also slowly oxidize \textit{n}-alkanes up to tetradecane (C\textsubscript{14}) (26). Although the distinction between the \textit{n}-alkane substrate range of alkane hydroxylase and the growth substrate range for strain GPo1 is sometimes


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overlooked, little attention appears to have been given to the possibility that either of these ranges extends to \(n\)-alkanes smaller than \(C_5\).

In a recent study we characterized the ability of cells of strain GPo1 to oxidize the gasoline oxygenate MTBE to tertiary butyl alcohol (TBA) and formaldehyde (23). Various lines of evidence were presented to indicate this activity is catalyzed by alkane hydroxylase. For example, MTBE-oxidizing activity was observed in cells grown on n-octane as well as in cells in which alkane-oxidizing activity had been stimulated by the gratuitous inducer, dicyclopynylketone (DCPK). Conversely, no MTBE-oxidizing activity was observed in DCPK-treated cells of strain GPo12, a strain cured of the OCT plasmid (27). Oxidation of MTBE in strain GPo1 was also fully inhibited by a putative mechanism-based inactivator of alkane hydroxylase, 1,7-octadiyne (11, 15) while partial inhibition of MTBE oxidation was also observed with \(C_5\) to \(C_{12}\) \(n\)-alkanes. The inhibitory effect of \(n\)-alkanes on MTBE oxidation was interpreted in terms of a competitive interaction between MTBE and \(n\)-alkanes for binding and oxidation by alkane hydroxylase. In this study we observed that propane and \(n\)-butane also strongly inhibited MTBE oxidation (23). The effects of these gaseous \(n\)-alkanes on MTBE oxidation was also interpreted in terms of a mutually exclusive competitive interaction between alkane hydroxylase substrates. However, no evidence for \(n\)-butane or propane oxidation by strain GPo1 was presented in our earlier study and neither propane nor \(n\)-butane are recognized substrates for alkane hydroxylase in this organism.

In the present study we have further examined the effects of propane and \(n\)-butane on the \(n\)-alkane-oxidizing activity of strain GPo1. Our results confirm our previous suggestion that these gases are substrates for \(n\)-alkane grown cells of this bacterium and they further demonstrate these gases can serve as independent growth-supporting substrates for this organism.
MATERIALS AND METHODS

Materials: The bacterial strains used in this study were *P. putida* GPo1 [ATCC 29347] (American Type Culture Collection, Manassas, VA) and *P. putida* GPo12, a derivative of strain GPo1 cured of the alkane hydroxylase-encoding OCT plasmid [kindly supplied by Dr. J. van Beilen, ETH Hönggerberg, Zürich, Switzerland]. 1-Butanol (99% purity), *n*-decane (99% purity), *n*-dodecane (99% purity), *n*-heptane (99% purity), *n*-hexane (99% purity), 2-methyl-2-propanol [*tertiary* butyl alcohol (TBA)] (99.3% purity), methyl *tertiary* butyl ether [MTBE] (99.8% purity), *n*-nonane (99% purity), *n*-octane (99% purity), 1-pentanol (99% purity), 1-propanol (99.9% purity) and *n*-undecane (99% purity) were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO).  N-pentane (99.5% purity) was obtained from Fisher Scientific (Pittsburgh, PA).  Absolute ethanol was obtained from Aaper Alcohol and Chemical Co., (Shelbyville, KY).  1-octanol (99% purity) was obtained from Lancaster Synthesis Inc., (Windham, NH).  Methane and ethane (Chemically Pure [CP] grade) were supplied by National Specialty Products, (Durham, NC).  *N*-butane (99% purity) was also obtained from Sigma Aldrich Chemical Co. (St. Louis, MO). The hydrocarbon contaminants listed for this batch by the supplier were as follows (ppm: µl/l) ethane (15), propane (69), isobutane (1650), isopentane (15), 2,2-dimethylpropane (1400), and 1-butene (203).  Propane (Instrument grade) was supplied by Air Products and Chemicals Inc., (Allentown, PA).  The hydrocarbon contaminants listed by the supplier were as follows (ppm; µl/l) *n*-butane (<500) C₄H₁₀ total (<3000). Compressed gases used for gas chromatography (GC) (H₂, N₂, and air) were obtained locally.

Cell Growth: Most of the experiments in this study used cells of *P. putida* GPo1 grown in batch culture in glass serum vials (160 ml) sealed with butyl rubber stoppers and aluminum crimp seals
(Wheaton Scientific, Millville, NJ). The vials contained a mineral salts medium (25 ml) (22) and were inoculated (initial OD_{600}>0.01) with a suspension of cells obtained from axenic cultures of \textit{P. putida} GPo1 previously grown on agar plates containing mineral salts medium with lactate (20 mM) as the sole carbon and energy source. Unless otherwise stated, all potential liquid growth substrates were added to the sealed vial as the pure compound using sterile glass microsyringes. Gaseous n-alkanes (methane, ethane, propane and n-butane) were added to the sealed culture vials using plastic syringes fitted with sterile needles and disposable filters (0.1\(\mu\)m) (Gelman Laboratory, Ann Arbor, MI). These additions created an overpressure in the culture vial and the concentrations of gases are therefore expressed as approximate values calculated in terms of the volume of gaseous n-alkane added/ total gas volume in the sealed vials (\textit{e.g.} 10 ml n-alkane added to 135 ml gas phase expressed as \(~7.5\%\) v/v gas phase). Unless otherwise noted, the culture vials were incubated at 30°C in the dark for 5d in an Innova 4900 (New Brunswick Scientific Co., Inc. Edison, NJ), environmental shaker operated at 150 rpm. Culture growth was determined by measuring optical density at 600 nm (OD_{600}) using a Shimadzu 1601 UV/Vis spectrophotometer (Kyoto, Japan). When required, cells were also grown as described above on dextrose-containing Difco Plate Count Medium (Becton, Dickinson and Company, Sparks, MD). This medium contained (g/l) dextrose (1.0), pancreatic digest of casein (5.0) and yeast extract (2.5). For every culture, a sample (50\(\mu\)l) was streaked on mineral salts/lactate plates to subsequently confirm the purity of the culture.

In experiments requiring concentrated suspensions of cells for enzyme activity measurements, cultures were grown in glass bottles (700 ml) sealed with screw caps and butyl rubber septa (Wheaton Scientific, Millville, NJ). The culture bottles contained mineral salts medium (100 ml)
and all n-alkanes were added to the sealed, inoculated culture bottles, as described above. N-octane was added to give an initial concentration of 0.04% (v/v liquid phase). Propane (180 ml) and n-butane (40 ml) were added to the gas phase to create an overpressure. The concentrations of gaseous n-alkanes were expressed as approximate values, as described above. Depending on the growth substrate, these cultures were incubated for 3-7 days in the dark at 30°C in the environmental shaker described above. Approximately 24 h before experimental use the culture bottles were opened for 5 min in a laminar flow hood to allow re-aeration of the culture vessel. The culture vessel was then resealed and the n-alkanes were replenished to their initial concentration used at the beginning of the incubation. After further incubation for 24 h the cells were harvested from the culture medium by centrifugation (10,000 x g; 10 min) and the resulting cell pellet was resuspended in buffer (10 ml, 50 mM sodium phosphate, pH 7). The cells were sedinted again by centrifugation (as above) and the resulting washed cell pellet was finally resuspended with buffer (1.0 ml, as above) to a final protein concentration of 3-15 mg total cell protein ml⁻¹. The cells were stored at 4°C and were used within 4h.

**Reaction Conditions:** Reactions following the degradation of MTBE and 1° alcohols were all conducted in glass serum vials (10 ml). The reaction vials were prepared by adding concentrated cells (~200 μl: 0.6 to 3 mg total protein) and buffer (50 mM sodium phosphate, pH 7) (~800 μl). The vials were then sealed with butyl rubber stoppers and aluminum crimp seals and were briefly (1 min) incubated at 30°C in a shaking water bath (150 rpm) to equilibrate the reaction medium temperature. The reactions were initiated by adding exogenous substrates to the sealed vial from aqueous stock solutions using microsyringes. The reaction vials were then returned to the shaking water bath and were sampled as required for each experiment.
**Analytical Methods:** In all experiments the concentrations of substrates and products were determined by gas chromatography (GC). For the analysis of MTBE, TBA and all 1º alcohols, aqueous samples (2 ml) were taken directly from the reaction vials (see above) and were injected into a Shimadzu GC-14A GC (Kyoto, Japan) fitted with a stainless steel column (0.3 x 183 cm) filled with Porapak Q (60-80 mesh) (Waters Associates, Framingham, MA) and a flame ionization detector. The analysis was usually conducted using a column temperature of 160C, injection port temperature of 200C and a detector temperature of 220C. In the case of 1-propanol, the column temperature was operated at 120C. In all cases \( \text{N}_2 \) was used as carrier gas at a flow rate of 15 ml min\(^{-1}\). In experiments that followed the time course of n-alkane consumption during culture growth, gas phase samples (25 ml) were removed using gas tight syringes with dry heat-treated needles (45 s at 350C). The samples were directly injected into a Shimadzu GC-14A GC fitted with a DB-MTBE capillary column (30 m x 0.45 mm I.D. [2.55 \( \mu \)m film]) (J & W Scientific, Folsom, CA) and a flame ionization detector. The analysis was conducted using a column temperature of 35C, an injection port temperature of 200C and a detector temperature of 220C. The carrier gas (\( \text{N}_2 \)) was used at a flow rate of 5ml min\(^{-1}\). Both GCs were interfaced to Hewlett Packard HP3395 (Palo Alto, CA) integrators for data collection.

Cell protein concentrations were determined using the Biuret assay (6) after solubilizing cell material for 30 min at 65C in 3N NaOH and sedimentation of insoluble material by centrifugation in a microfuge (10,000 x \( g \) for 5 min). Bovine serum albumin was used as the standard.
**SDS-PAGE and Immunoblot Analysis:** Samples of total cell protein were solubilized at room temperature in sample buffer containing SDS (1% w/v), glycerol (10% v/v) Tris/HCl (62.5 mM, pH 6.8), β-mercaptoethanol (5% v/v) and bromophenol blue (0.01% w/v). Aliquots of cell protein (50µg) and pre-stained low-range markers (Bio-Rad, Hercules, CA) were analyzed by SDS-PAGE using 12% polyacrylamide slab gels (16 x 16 cm). The gels were fixed in methanol/acetic aid /water (45:9:46 v/v) and stained with Coomassie Brilliant Blue. Immunoblot analyses were also conducted using total cell protein samples (50 µg) prepared and electrophoresed as described above. After electrophoresis the separated polypeptides were electroblotted onto a nitrocellulose membrane (16 x 8 cm) (MSI Hybridization Transfer Membrane, Westboro, MA) using a BioRad semi-dry blotter. The transfer was conducted at a constant current of 100 mA for 1 hour. The nitrocellulose membrane was stored in dH₂O. The remainder of the immunoblot analysis was performed using a WesternBreeze Chemiluminescent Immunodetection system (anti-rabbit) (Invitrogen Life Technologies Carlsbad, CA). The immunoblot was conducted as described by the manufacturer. The primary antibody, rabbit anti-AlkB (generously supplied by Dr. J. van Beilen, ETH Hönggerberg, Zürich, Switzerland), was used at a dilution of 1:2500. To eliminate non-specific binding the primary antibody solution was preincubated for one hour with a nitrocellulose membrane blotted with protein samples from dextrose-grown cells of *Pseudomonas putida* strain GPo12. The chemiluminescence was detected using X-MAT AR Film (Kodak, Rochester, NY).
RESULTS

Co-oxidation of propane and n-butane: Our initial experiments aimed to address the question raised by our earlier study (23) as to whether cells of strain GPo1 grown on recognized n-alkane growth substrates such as n-octane can also oxidize gaseous n-alkanes (C$_1$-C$_4$). To examine this cultures of strain GPo1 were grown on C-limiting amounts of n-octane alone or n-octane with each gaseous n-alkane (C$_1$-C$_4$) added individually to the culture gas phase (~7.5% v/v gas phase). Consumption of all n-alkanes was determined over time by analysis of the culture gas phase by GC. The time course of n-octane consumption under each culture condition was almost identical and ≥ 95% of this substrate was consumed within 140 h (Figure 5.1). In abiotic control incubations ≤ 20% of the added n-octane was lost over the same time period. In cultures grown on n-octane in the presence of either methane or ethane, no consumption of the gaseous n-alkanes was observed. In contrast, approximately 20% and 60% of the initial propane and n-butane were consumed over the 140 h incubation, respectively. In abiotic control incubations there was minimal (≤ 3%) consumption of gaseous n-alkanes over the entire incubation period.

The results shown in Fig. 5.1 suggested n-butane and propane, but not ethane or methane, were co-oxidized during growth on n-octane. We investigated whether this co-oxidation process could also involve further catabolism of metabolites of propane and n-butane oxidation that would be manifested by increases in culture density and biomass production above those generated by cells grown on n-octane alone. The final culture density and culture protein content of cultures grown for 5 d on C-limiting amounts of n-octane alone were determined and these values were compared to cultures grown under the same conditions in the presence of each gaseous n-alkane (~7.5% v/v gas phase). Complete (≥ 97%) consumption of n-octane after 5 d was confirmed for each culture by analysis of the culture gas phase by GC. After 5 d there was
no detected effect of either methane or ethane on the final culture density (OD$_{600}$) (Figure 5.2) whereas substantial increases were observed with both propane (+95%) and $n$-butane (+390%). The average final protein content for the cultures shown in Fig 5.2 grown on n-octane alone, n–octane + propane and n-octane + $n$-butane were 46, 62 and 148 µg ml$^{-1}$, respectively.

The stimulating effect of propane and $n$-butane on growth of strain GPo1 on other non-gaseous $n$-alkanes was also examined. Cells were grown in cultures containing C-limiting amounts of each $n$–alkane from C$_5$ to C$_{12}$ either with or without propane or $n$-butane (~7.5 % v/v gas phase). The culture density was determined after 5d, irrespective of the final level of utilization of the C$_5$-C$_{12}$ $n$-alkanes. Vigorous growth was observed for cultures containing C$_5$ to C$_9$ $n$-alkanes, but not in cultures containing C$_{10}$ to C$_{12}$ $n$-alkanes (Figure 5.3). In the cultures grown on C$_5$ to C$_9$ $n$-alkanes, the presence of either propane or $n$-butane consistently increased the final culture density. For propane this stimulation ranged from +34% to +95% (mean +62%) above the density observed in cultures grown without this gas. For $n$-butane, much greater increases were observed. These ranged from +202 to +526% (mean +323%) above the culture density observed in cultures grown without this gas.

**Growth on $n$-butane and propane:** The growth-stimulating effect of propane and $n$-butane observed with cultures grown on C$_5$ to C$_9$ $n$-alkanes (Figure 5.3) were substantially similar to our earlier observations with $n$-octane-grown cultures (Figure 5.2). However, our results obtained with cultures incubated with C$_{10}$ to C$_{12}$ $n$-alkanes were unusual for two reasons. First, all of these longer chain $n$-alkanes are recognized growth substrates for strain GPo1 and no growth was observed within 5 d under the conditions used for the shorter $n$-alkanes (C$_5$-C$_9$). We subsequently tested higher (1% v/v) non-C-limiting concentrations of C$_{10}$ to C$_{12}$ $n$-alkanes and
observed vigorous growth on \( n \)-decane, but not on \( n \)-undecane or \( n \)-dodecane (data not shown). This result suggests the lack of growth of strain GPo1 on \( n \)-decane under the conditions used in our experiment (Figure 5.3) reflects the combined effects of the low aqueous solubility and low concentrations on \( n \)-decane used in this C-limited experiment.

The second unusual observation in the cultures containing \( C_{10} \) to \( C_{12} \) \( n \)-alkanes (Figure 5.3) was low levels of growth (\( \leq 0.1 \ OD_{600} \)) were observed in all of the \( n \)-butane-containing cultures, irrespective of whether the longer chain \( n \)-alkanes were (\( C_{10} \)) or were not (\( C_{11} \) and \( C_{12} \)) used as growth-supporting substrates at high (1\% \ v/v liquid phase) initial concentrations, as described above. We hypothesized this effect might reflect slow growth of the cultures on \( n \)-butane, an effect that could be more apparent if cultures were incubated for longer periods of time beyond 5 d. To test this, cultures were established that contained propane, \( n \)-butane (both at ~7.5\% \ v/v gas phase), \( n \)-pentane or \( n \)-octane (both at 0.04 \% \ v/v liquid phase) added individually as sole carbon and energy sources. The time course of culture growth was determined spectrophotometrically (\( OD_{600} \)), while consumption of \( n \)-alkanes was determined by GC analysis of the gas phase. The time courses of \( n \)-pentane and \( n \)-octane consumption were almost identical and \( \geq 90\% \) of these substrates were consumed within 3d without a detectable lag phase (Figure 5.4). In abiotic control incubations, \( \leq 15\% \) of these substrates were lost over the same period of time. The increase in culture density (\( OD_{600} \)) of \( n \)-pentane- and \( n \)-octane-grown cultures also closely reflected the time course of \( n \)-alkane consumption. The maximum culture density (0.50 and 0.67 for \( n \)-pentane and \( n \)-octane, respectively) was also observed after 3 d once the majority (\( \leq 90\% \)) of the \( n \)-alkane growth substrates had been consumed. In contrast, the cultures incubated with either propane or \( n \)-butane did not show either significant (\( \geq 10\% \)) \( n \)-alkane consumption or increases in culture density over the initial 4-5 d of the incubation (Figure 5.4). However, after
this lag phase, \(n\)-butane consumption became apparent and accelerated to a final rate comparable to that observed for \(n\)-pentane and \(n\)-octane. The onset of rapid \(n\)-butane consumption was accompanied by a corresponding increase in culture density. After 9 d ≥70% of the initial \(n\)-butane had been consumed and the culture density had increased to a maximum value of 0.75. After this no further \(n\)-butane consumption and no further increases in culture density were observed. In the propane-containing cultures, no gas consumption or increase in culture density was observed over the entire 10 d incubation. In the abiotic control incubation ≤5% of the added \(n\)-butane and propane were lost over the 10 d incubation period. The \(n\)-butane used in this study contained several contaminants (see Materials and Methods). We examined whether the main reported contaminant (isobutane) and an unreported contaminant (\(n\)-pentane) had any effect on the lag phase and final culture density of cultures grown on \(n\)-butane. There was no detected effect on either of these parameters for cultures grown on \(n\)-butane (~7.5% v/v gas phase) in the presence of ~0.7% (~7000 ppm)(v/v gas phase) isobutane or ~0.0035% (~35 ppm) (v/v gas phase) \(n\)-pentane (data not shown).

As no growth of strain GPo1 on propane (~7.5% v/v gas phase) was observed in the experiment described in Figure 5.4, we examined the effects of increased propane concentrations. Cultures were established using initial concentrations of propane of 0, ~15, ~30, and ~45% (v/v gas phase) and culture growth was monitored by spectrophotometrically over time. We observed no growth of the culture containing ~15% (v/v gas phase) propane over 7d (Figure 5.5). However, low levels of growth (~0.1 OD\textsubscript{600}) were observed after 4-5 d in cultures containing ~30% (v/v gas phase) propane and abundant growth (>0.6 OD\textsubscript{600}) was observed over the same time in cultures containing the highest propane concentration tested (~45% v/v gas phase). Strong effects of \(n\)-butane concentration on the time course of culture growth were also
observed. For example, substantial culture growth (~0.25 OD<sub>600</sub>) was observed after 2 d in cultures containing ~45% (v/v gas phase) n-butane (Figure 5.5). As shown in the previous experiment (Figure 5.4), this culture density was only achieved after 6-7 d in cultures containing lower (~7.5 % v/v gas phase) concentrations of this gas. No growth on propane or n-butane was observed over 10 d when the OCT plasmid-deficient strain GPo12 was incubated in cultures containing the highest concentration of each n-alkane (~45% v/v gas phase) tested (data not shown).

Growth of strain GPo1 on propane and n-butane led us to consider whether the same enzymes and activities involved in catabolism of the longer chain n-alkanes such as n-octane were also present in cells grown on these gases. Initially, we examined the possibility that alkane hydroxylase could be responsible for initiating the oxidation of propane and n-butane. To test this we determined the specific alkane hydroxylase- and MTBE-dependent, TBA-generating activity of cells of strain GPo1 and strain GPo12 grown on dextrose-containing media and media containing selected n-alkanes and 1° alcohols. These activities were then compared with results of a SDS-PAGE / immunoblot analysis conducted on total protein extracts from these cells using rabbit anti-GPo1 AlkB antiserum. The specific TBA-generating activities for cells of strain GPo1 grown on propane, n-butane, and n-octane were 17.9, 35.1 and 37.8 nmoles min<sup>-1</sup> mg total protein<sup>-1</sup>, respectively. Low activity (2.7 nmoles min<sup>-1</sup> mg total protein<sup>-1</sup>) was observed for cells of strain GPo1 grown on 1-octanol while no activity (≤ 0.1 nmoles min<sup>-1</sup> mg total protein<sup>-1</sup>) was observed for cells grown on 1-propanol, 1-butanol or in dextrose-containing medium. Similarly, no TBA-generating activity was observed in cells of strain GPo12 grown on either 1-octanol or dextrose-containing medium. The results of these physiological assays correlated well with the results of the immunoblot analysis (Figure 5.6). A strong cross-reaction was observed to a ~40
kDa polypeptide in cells of strain GPo1 grown on \( n \)-octane, \( n \)-butane and propane. A much lower level of cross-reaction was observed in cells grown on 1° alcohols and no cross-reaction was observed cells grown on dextrose-containing medium. No cross-reaction was observed for cells of strain GPo12, irrespective of growth substrate.

As the initial oxidation products on \( n \)-alkane oxidation by alkane hydroxylase are 1° alcohols (27) we also examined the alcohol-utilizing activities of strain GPo1. The bacterium grew readily when incubated for 5 d with all of the 1° alcohols tested (\( C_1 \) to \( C_6 \), 0.02% v/v liquid phase) other than methanol. The final culture densities (OD\( _{600} \)) for duplicate cultures were 0.16 (± 0.01), 0.28 (± 0.02), 0.30 (± 0.01), 0.35 (± 0.02), and 0.33 (± 0.04), for cultures grown on ethanol, \( n \)-propanol, \( n \)-butanol, \( n \)-pentanol and \( n \)-hexanol, respectively. The corresponding values for strain GPo12 grown under the same culture conditions were 0.13 (± 0.01), 0.17 (± 0.01), 0.22 (± 0.02), 0.28 (± 0.01) and, 0.28 (± 0.01), respectively. No growth (final OD\( _{600} \) ≤0.01) of either strain was observed when 2-propanol, 2-butanol or 2-hexanol were supplied as potential growth substrates. We also examined the rates of 1° alcohol oxidation for cells of strain GPo1 grown on propane, \( n \)-butane and \( n \)-octane. Harvested and washed cells grown on each \( n \)-alkane oxidized 1-propanol, 1-butanol and 1-octanol at comparable high rates (42-99 nmoles min\(^{-1} \) mg total protein\(^{-1} \)) when these compounds were supplied individually (10 mM).
DISCUSSION

The original aim of this study was to test the hypothesis that propane and \( n \)-butane are substrates for alkane hydroxylase in \( n \)-alkane-grown cells of \( P. \) putida GPo1. Overall, the results we have presented here confirm this activity. We have also shown these short chain \( n \)-alkanes are not only co-oxidized by cells grown on recognized \( n \)-alkane substrates (\( C_5 \) to \( C_{10} \)) for strain GPo1 but can also serve as independent growth substrates. In the following section we separately discuss gaseous alkane co-oxidation and metabolism by strain GPo1. The final section examines the impact of our observations on our current understanding of the enzymology of bacterial gaseous \( n \)-alkanes oxidation.

Co-oxidation of propane and \( n \)-butane: Our recent studies with \( n \)-alkane-oxidizing strains \( P. \) mendocina strain KR-1 (22) and \( P. \) putida strain GPo1 (23) focused on the previously unrecognized ability of these organisms to cometabolically oxidize MTBE. These two earlier studies used similar experimental approaches to those used in this report and showed several important physiological similarities between these organisms that are relevant to the present study. In summary, we have shown strain KR-1 and GPo1 both dealkylate MTBE to generate TBA and formaldehyde. Strain KR-1only catalyzes this reaction after growth on \( C_5 \) to \( C_{10} \) \( n \)-alkanes, the same \( n \)-alkane growth substrate range reported for strain GPo1. Propane and \( n \)-butane act as apparent competitive inhibitors of MTBE oxidation by \( n \)-pentane-grown cells of strain KR-1 while methane and ethane have no significant inhibitory effect on this activity. We have not observed growth of strain KR-1 on gaseous \( n \)-alkane (\( C_1 \) to \( C_4 \)). However, propane and \( n \)-butane (but not ethane or methane) are both consumed during growth of strain KR-1 on \( n \)-pentane and this co-oxidation process leads to increases in culture density and biomass.
production. We concluded this stimulating effect is the result of the further metabolism of the products of propane and \(n\)-butane oxidation by the non-specific enzymes expressed by strain KR-1 during growth on \(n\)-alkanes.

Although we have not previously observed propane or \(n\)-butane oxidation by \(n\)-alkane-grown cells of strain GPo1, like strain KR-1 (22), we have observed propane and \(n\)-butane appear to act as competitive inhibitors of the alkane hydroxylase-catalyzed MTBE oxidation. In contrast, neither methane nor ethane has any detectable effect on this reaction. The experimental approaches have enabled us to show both propane and \(n\)-butane (but not ethane and methane) were consumed by strain GPo1 during growth on C-limiting amounts of the well-characterized \(n\)-alkane growth substrate, \(n\)-octane (Figure 5.1). We also demonstrated this process resulted in increases in both the final culture density and biomass production in \(n\)-octane-grown cells (Figure 5.2) and that similar impacts were observed with the majority (\(C_5\) to \(C_9\)) of the currently recognized \(n\)-alkane growth substrates for this organism (Figure 5.3). As was the case for strain KR-1, we conclude that an intrinsic lack of specificity of the alkane-oxidizing enzyme systems in strain GPo1 is the underlying cause of this bacterium’s ability to productively co-oxidize gaseous \(n\)-alkanes and to obtain a metabolic benefit from this process. It is likely this lack of specificity has to occur at two levels, the hydroxylase responsible for initiating \(n\)-alkane oxidation and the enzymes responsible for the further oxidation of the presumed \(1^\circ\) alcohol products of gaseous \(n\)-alkane hydroxylation. The literature suggests the hydroxylation of propane and \(n\)-butane by strain GPo1 should not necessarily be a surprising observation. For example, nearly 30 years ago it was reported \(n\)-octane-grown cells of strain GPo1 rapidly hydroxylate rather than epoxidize propylene and 1-butene, the alkene analogs of
propane and n-butane (14). This enzyme is also known to oxidize another C₄ compound, methyl propyl sulfide to its corresponding sulfoxide. (12)

The broad substrate specificities of the enzymes involved in alcohol, aldehyde and â-oxidation are also well recognized and to some extent it would be remarkable if these secondary enzymes systems did not oxidize the presumed 1° alcohol products of gaseous n-alkane oxidation. Our growth studies demonstrated this capability exists in this bacterium and showed strains GPo1 and GPo12 both grew well on 1° alcohols (C₂-C₆). Furthermore, cells grown on gaseous n-alkanes and n-octane were also all shown to have substantial 1-propanol and 1-butanol-oxidizing activities. Another well characterized n-alkane-utilizing pseudomonad (P. aureginosa strain 423) that grows on the same range of n-alkanes as strain GPo1 has also previous been shown to rapidly oxidize propanol and propionate after growth on n-hexane (25).

**Use of propane and n-butane as independent growth substrates:** If initial hydroxylation of propane and n-butane and the further metabolism of the initial products of these reactions are individually unsurprising reactions, actual growth of this strain on propane and n-butane should perhaps be seen more as an anticipated rather than unexpected physiological trait. What is possibly more surprising about this metabolic capability is that it has previously gone unnoticed. As indicated in the Introduction, several often-cited descriptions of the n-alkane-growth substrate range of strain GPo1 involved cultures grown on plates using vapors of liquid n-alkanes as carbon and energy sources (1, 7, 16). These studies all tended to underreport the n-alkane growth substrate range of strain GPo1 and it seems likely that even if gaseous n-alkanes were considered as potential growth substrates they too would have been overlooked by this approach.
Alternatively, organisms such as strain GPo1 often emerge as model strains because they grow particularly rapidly on certain classes of substrates. Our present results show growth on \( n \)-butane and particularly propane is initiated very slowly relative to \( n \)-pentane and \( n \)-octane (Figure 5.5) and growth is highly dependent on gaseous \( n \)-alkane concentration. If \( n \)-butane and propane have been previously evaluated as growth substrates it may be these analyses were either not conducted for long enough or made use of low concentrations of these gases.

While the lag phase observed during growth on \( n \)-butane and propane may explain why these compounds have not been previously identified as \( n \)-alkane growth substrates for strain GPo1, the underlying cause of the lag phase is not currently known. The results of our concurrent MTBE oxidation assays and immunoblot analysis (Figure 5.6) certainly suggest alkane hydroxylase is responsible for initiating the catabolism of propane and \( n \)-butane. Conversely, the lack of MTBE-oxidizing activity and an anti-alkB cross-reacting polypeptide in cells grown on propanol and \( 1 \)-butanol (Figure 5.6) suggests that the lag phase cannot be explained by the need to accumulate \( n \)-alkane oxidation products such as \( 1^\circ \) alcohols to a critical concentration for these metabolites to act as independent inducers of alkane hydroxylase activity. The lack of an inducing effect of \( 1 \)-propanol and \( 1 \)-butanol also agrees with previous studies of the regulation of alkane hydroxylase activity (7).

Although the concentration of \( n \)-butane and particularly propane has an impact on the initiation of growth on these compounds (Figure 5.5) it is difficult from our present results to determine whether this is the result of concentration effects of these gases as inducers of the alkane-oxidizing activity or the result of an intrinsic kinetic feature of gaseous \( n \)-alkanes as alkane hydroxylase substrates. From a regulation perspective, a strong effect of carbon chain length has been observed for the inductive effects of non-growth supporting \( n \)-alkanes (\( C_6-C_{11} \))
on a chromosomally-located alkM::lacZ fusion in Acinetobacter ADP1, another well-characterized strain that grows on C_{12} to C_{18} n-alkanes (18). N-butane has also been reported as a potent inducer of the alkane hydroxylase system in *P. aureginosa* strain 423 (30), an organism with an alkane hydroxylase that is highly homologous to the enzyme found in strain GPo1 (28).

From a kinetic perspective, our studies suggest propane is inferior to *n*-butane as an alkane hydroxylase substrate. For example, propane consumption was 3-fold slower than *n*-butane consumption during growth of strain GPo1 on *n*-octane (Figure 5.1). This effect occurred despite both gases being present at equal initial gas phase concentrations (~7.5% v/v) and propane having a higher aqueous solubility (1.44 mM) than *n*-butane (1.01 mM) at 1 atm (31). We also observed that propane stimulated growth on longer chain *n*-alkanes less effectively than *n*-butane, again under conditions where the dissolved propane concentration exceeded the dissolved *n*-butane concentration. While these arguments could obviously be impacted by the effects of other alkane hydroxylase substrates in these growth-based experiments, our interpretation is consistent with our previous observation that *n*-butane (*K_i* = 13 µM) is also a more potent inhibitor of alkane hydroxylase-catalyzed MTBE oxidation than propane (*K_i* = 66 µM) by strain GPo1. It should be noted that *K_i* values for inhibitors that act as competitive substrates are the equivalent of the *K_s* values for these compounds as independent substrates (5).

Although we have not determined the *V_{max}* values for the oxidation of any gaseous *n*-alkanes by strain GPo1, van Beilen *et al.* (26) have shown both *in vivo* rates of *n*-alkane consumption in DCPK-treated, alcohol-dehydrogenase-deficient derivatives of *P. putida* GPo1 and *in vitro* alkane hydroxylase activity steadily decrease from *n*-nonane towards *n*-pentane. Taken together, these results suggest progressive decreases in the *n*-alkane carbon chain length are likely to result in progressively increasing *K_s* values and concurrently decreasing *V_{max}* values for *n*-alkane
oxidation. Our present study suggests this trend continues through \textit{n}-butane and propane but eventually the combined effects of these trends causes the substrate range of alkane hydroxylase to terminate with ethane. This conclusion is supported by our observations that ethane was not consumed by \textit{n}-octane-grown cells (Figure 5.1) over extended time periods and does not inhibit MTBE oxidation by \textit{n}-alkane grown cells (23). Ethane also did not stimulate growth on \textit{n}-octane (Figure 5.2) even though the bacterium can utilize ethanol, the predicted product of ethane oxidation, as an independent growth substrate.

\textbf{Relevance to current understanding of bacterial gaseous \textit{n}-alkane metabolism}. A limited number of bacterial gaseous \textit{n}-alkane-oxidizing monooxygenases have been identified at the molecular level. Ammonia-oxidizing bacteria (10) and methanotrophs (2, 4) cometabolically oxidize propane, and \textit{n}-butane and longer chain \textit{n}-alkanes through the activity of ammonia monooxygenase and soluble (sMMO) and particulate (pMMO) methane monooxygenase, respectively. A propane-specific monooxygenase has been recently characterized in a \textit{Gordonia} strain TY-5 (13) and this enzyme appears to be very similar to the tetrahydrofuran monooxygenase in \textit{Pseudonocardia} K1 (24). Arp and coworkers have characterized several \textit{n}-butane-oxidizing monooxygenases in \textit{n}-butane-utilizing bacteria (8). These include an sMMO-like enzyme in \textit{Pseudomonas butanovora} (21) and an as yet uncharacterized but well documented promiscuous short chain alkane monooxygenase in \textit{Mycobacterium vaccae} JOB5 (17). Inhibitor studies with a third organism, \textit{Nocardiodes} CF8, suggest this bacterium has 2 different monooxygenases capable of initiating \textit{n}-butane metabolism (9). A putative copper-containing enzyme catalyzes the majority ($\geq 95\%$) of \textit{n}-butane oxidation in \textit{n}-butane-grown cells. This enzyme is also present in cells grown on longer chain \textit{n}-alkanes up to C\textsubscript{10} and contributes
significantly (≥70%) to the \( n \)-butane-oxidizing activity of resting cells grown on these substrates. The remaining \( n \)-butane-oxidizing activity in resting cells grown on \( C_6 \) to \( C_{10} \) \( n \)-alkanes is attributed to a putative binuclear-iron-containing alkane hydroxylase-rubredoxin fusion enzyme. This enzyme has significant amino acid sequence homology to the alkane hydroxylase in strain GPo1. These observations suggest that gaseous \( n \)-alkane-oxidizing activity may be a relatively common feature among alkane hydroxylases that are structurally similar to the enzyme in strain GPo1. Our current research is addressing whether this similarity extends to other \( n \)-alkane-utilizing pseudomonads including \textit{P. mendocina} KR-1.
Figure 5.1. Time course of \( n \)-octane and gaseous \( n \)-alkane oxidation during growth of \( P. \) putida GPo1. Cultures of \( P. \) putida GPo1 were grown in batch culture on \( n \)-octane (0.04%, vol/vol) in the presence or absence of methane, ethane, propane or \( n \)-butane (7.5%, vol/vol of each compound), as described in Materials and Methods. The time course for \( n \)-octane and gaseous \( n \)-alkane consumption is shown for cultures grown with \( n \)-octane alone (squares), \( n \)-octane and methane (triangles), \( n \)-octane and ethane (inverted triangles), \( n \)-octane and propane (diamonds) and \( n \)-octane and \( n \)-butane (circles). The percent of \( n \)-octane remaining in each culture is designated by open symbols and closed symbols represent the percent of gaseous \( n \)-alkane remaining in the culture. In addition, the combined percent of methane, ethane, propane and \( n \)-butane is shown for abiotic cultures (asterisks) and abiotic cultures containing \( n \)-octane alone (Xs). The data plotted are the averages of two replicates for cultures grown in the presence of \( P. \) putida GPo1 cells and a single replicate for all abiotic cultures. The error bars show the range of values obtained for the replicate cultures.
Figure 5.2. Effects of gaseous $n$-alkanes on growth yield of cells grown on $n$-octane. The figure shows the effects of gaseous $n$-alkanes on the final-culture density of *P. putida* GPo1 cells grown under carbon-limited conditions on $n$-octane. A series of cultures of *P. putida* GPo1 were prepared in glass serum vials (125 ml) as described in Materials and Methods. The cultures contained either $n$-octane alone (0.02%, vol/vol) or (in sequence from left to right) $n$-octane (0.02%, vol/vol) with either methane, ethane, propane or $n$-butane (7.5%, vol/vol of each compound). The cultures were grown for 5 days, and each culture condition was replicated three times. The figure shows the average final optical density ($OD_{600}$) for all of the cultures. The error bars show the range of values of the final optical densities ($OD_{600}$).
Figure 5.3. Effects of cosubstrates on growth yield of cells grown on liquid n-alkanes. The figure shows the effects of propane and n-butane on the final-culture density of *P. putida* GPO1 cells grown under carbon-limited conditions on C₅ to C₁₂ n-alkanes. A series of cultures of *P. putida* GPO1 were prepared in glass serum vials (125 ml) as described in Materials and Methods. The cultures contained either an n-alkane growth substrate alone (0.02%, vol/vol) or (in sequence from left to right) each n-alkane growth substrate (0.02%, vol/vol) with either propane or n-butane (7.5%, vol/vol of each compound). The cultures were grown for 5 days, and each culture condition was replicated three times. The figure shows the average final optical density (OD₆₀₀) for all of the cultures. The error bars show the range of values of the final optical densities.
Figure 5.4. Time course of $n$-butane, $n$-pentane and $n$-octane oxidation during growth of *P. putida* GPo1. Cultures of *P. putida* GPo1 were grown in batch culture on propane (7.5%, vol/vol), $n$-butane (7.5%, vol/vol), $n$-pentane or $n$-octane (0.04%, vol/vol), as described in Materials and Methods. The time course for $n$-alkane consumption is shown for cultures grown with propane (inverted triangles), $n$-butane (squares), $n$-pentane (triangles) or $n$-octane (circles). Open symbols represent OD$_{600}$ and closed symbols signify percent of $n$-alkane remaining. Abiotic controls are shown for propane (†), $n$-butane (X), $n$-pentane (+), and $n$-octane (asterisks). The data plotted are the averages of two replicates for cultures grown in the presence of *P. putida* GPo1 cells and a single replicate for all abiotic cultures. The error bars show the range of values obtained for the replicate cultures.
Figure 5.5. Time course of culture density (OD$_{600}$) during growth of *P. putida* GPo1 on various concentrations of propane or *n*-butane. Cultures of *P. putida* GPo1 were grown in batch culture on propane (closed symbols) or *n*-butane (open symbols) as described in Materials and Methods. The time course for culture density (OD$_{600}$) is shown for cultures grown with 15% vol/vol propane or *n*-butane (circles), 30% vol/vol propane or *n*-butane (diamonds), and 45% vol/vol propane or *n*-butane (triangles). The data plotted are the averages of three replicates for cultures grown in the presence of *P. putida* GPo1 cells. The error bars show the range of values obtained for the replicate cultures.
Figure 5.6. Immunoblot assay of *P. putida* GPo1 and *P. putida* GPo12 cells grown on various substrates using anti-AlkB antiserum. Lanes in sequence from left to right with specific activity (nmoles TBA produced min$^{-1}$ mg of total protein$^{-1}$) in parentheses: dextrose-grown *P. putida* GPo1 (<0.1), 1-propanol-grown *P. putida* GPo1 (<0.1), propane-grown *P. putida* GPo1 (17.9), 1-butanol-grown *P. putida* GPo1 (<0.1), *n*-butane-grown *P. putida* GPo1 (35.1), 1-octanol-grown *P. putida* GPo1 (2.7), *n*-octane-grown *P. putida* GPo1 (37.8), dextrose-grown *P. putida* GPo12 (<0.1), and 1-octanol-grown *P. putida* GPo12 (<0.1).
REFERENCES


Conclusion and Summary

In conclusion, the research in this dissertation greatly expands on the current knowledge of alkane monooxygenase activity. These studies focused on two alkane monooxygenase model organisms, *Pseudomonas putida* GPo1 and *Mycobacterium vaccae* JOB5. *M. vaccae* JOB5 was one of the first organisms investigated for cometabolic capabilities and the alkane hydroxylase in *P. putida* GPo1, AlkB, is the most well characterized non-methane alkane monooxygenase system known to date.

MTBE-oxidizing activity in propane-grown *M. vaccae* JOB5 cells has previously been characterized in our lab. This study led us to consider the possibility of MTBE inducing MTBE-oxidizing activity in these cells. The first study presented in this dissertation strongly suggests MTBE does induce the production of enzymes responsible for MTBE-oxidizing activity in *M. vaccae* JOB5 cells, despite the inability of this compound to support growth. This finding has significance to MTBE bioremediation at contamination sites.

Our next investigation of *M. vaccae* JOB5 cells focused on the capability of alkane monooxygenase produced during growth on short chain alkanes to oxidize other common ether oxygenates and their alcohol products. We investigated ETBE, TAME, and DIPE and the alcohol product of TAME degradation, TAA. Our results show that TAME, ETBE, and TAA are cometabolized by propane-grown *M. vaccae* JOB5 cells. However, we found that DIPE was not oxidized at a substantial rate by these cells. This study also suggests the initial oxidation of all three of the degraded oxygenates, ETBE, TAME, and TAA, is catalyzed by the short chain alkane monooxygenase produced
during growth on propane. This study is important for the future gasoline-contaminated sites as the use of alternative oxygenates in reformulated gasoline increases and LUSTs begin to leach these ethers.

The final aspect of alkane-induced monooxygenase activity in *M. vaccae* JOB5 cells in this dissertation is the inhibitory effect of 1-propanol on MTBE oxidation. Although incomplete, this investigation clearly shows that high concentrations of 1-propanol lead to complete inhibition of MTBE-oxidizing activity. This study does not conclusively define the relationship between these two substrates, but suggests that this is a competitive or uncompetitive interaction.

Lastly, we investigated the acknowledged *n*-alkanes capable of supporting growth in *P. putida* GPo1. This study identified both propane and *n*-butane as growth-supporting alkanes for *P. putida* GPo1. Previous investigations into AlkB, the alkane hydroxylase produced by this organism during growth on *n*-alkanes, had not explored the possibility of either of these alkanes to support growth. Since this is a model organism and a model system for bacterial non-methane alkane monooxygenases, it is of importance to clearly understand the capabilities of this enzyme.

The unifying concept throughout the studies presented in this dissertation is an alkane monooxygenase. The investigations included in this dissertation examined the capabilities of this enzyme in both metabolic and cometabolic systems.

Although the research presented in this study greatly improves our knowledge of alkane monooxygenases present in different bacteria, there are clearly areas where additional research is required. One important exploration would expand the knowledge of alkane monooxygenase oxidizing activity in organisms other than *M. vaccae* JOB5 and
*Pseudomonas putida* GPo1. These studies could expand to monooxygenase systems such as the prokaryotic P450 systems among others.

Further exploration into the regulation of ETBE and TAME oxidizing activity in *Mycobacterium vaccae* JOB5 is another area where research should be extended. This includes determining if ETBE and TAME induce ETBE- and TAME-oxidizing activity similar to the one we discovered with MTBE and MTBE-oxidizing activity. As the use of ETBE and TAME increases, it will become increasingly important to understand the environmental fate of these substrates. Additional studies should be conducted on the pathway of degradation of both of these ethers and their primary products, TBA and TAA.

In general, understanding more about the short chain alkane monooxygenase, subsequent enzymes in ether degradation, and the genes that encode these enzymes in *M. vaccae* JOB5 would be beneficial to understanding cometabolism in this organism. This knowledge could enhance our ability to bioremediate environmental sites of oxygenate contamination.