ABSTRACT

LAN, RENNY SHANG-LUN. Pathway and Enzymology of Cyclic Ether Biodegradation by Mycobacterium vaccae JOB5. (Under the direction of Michael Hyman.)

In this study we investigated the cometabolic oxidation of tetrahydrofruran (THF), 1,4-dioxane (14D) and other cyclic ethers by alkane-grown Mycobacterium vaccae JOB5. An initial screening demonstrated this strain oxidized all six ethers tested after growth on propane, n-butane, n-pentane, isobutane and isopentane. Ether-degrading activity was limited in dextrose-grown cells or in alkane-grown cells incubated with ethers and acetylene. Oxidation of THF and 14D was further characterized using propane-grown cells. Propane competitively inhibited THF oxidation and γ-butyrolactone (γBL) accumulated and was also consumed during THF oxidation. In contrast, no products were detected during 14D oxidation. Oxidation of γBL and the 3-hydroxy homolog of its unstable but presumed precursor, 2-hydroxytetrahydrofruran, were largely unaffected by acetylene. This suggested other enzymes co-expressed with the acetylene-sensitive, propane-oxidizing monooxygenase contributed to THF oxidation. Although strain JOB5 did not grow on any cyclic ethers tested, slight growth was observed with several lactones while vigorous growth was supported by 4-hydroxybutyrate, the expected immediate product of γBL degradation. The ability of strain JOB5 to assimilate THF metabolites during growth on n-alkanes was examined in carbon-limited batch cultures. Relative to growth on n-pentane alone, the final culture density and protein concentration were doubled in the presence of THF while no stimulation was observed with 14D. Our results are discussed in terms of the overlap between the enzymes and pathway involved in alkane and ether oxidation, their significance to our understanding of cometabolism and their potential impact on approaches for cyclic ether biodegradation in the environment.
BIOGRAPHY

Before I came to North Carolina State University, I had been working as a graduate student in the lab, as a technician in the teaching hospital and as a science teacher in the senior high school. The research I did in the past was focused on heat shock proteins of the food poisoning pathogen, *Vibrio parahaemolyticus*. However, in NC state I found another interesting and practical microbial topic—bioremediation.

Most of the experiments in my research were GC analyses. By using this conventional but powerful technique, we conducted our experiments of predicting the substrates consumption and products generation in degradation of cyclic ethers. While I was in the graduate program in microbiology department, I also taught biology and general microbiology labs for over 500 students. With the experiences of research, teaching, taking courses and brainstorming with my advisor, Dr. Hyman, we are about ready to publish a paper of our extraordinary finding in cometabolism.
ACKNOWLEDGEMENTS

This has been a long way, but it gave me so many lessons to learn.

I want to thank the Lord for giving me these tasks as well as enough strength and grace to keep persevering. Also, I want to thank a lot of people. Without them, I wouldn’t have this thesis done today.

I want to thank my parents, my sister, Sherry, and my girl friend, Ping-Ching. Their prayers and encouragements were not cut off by distance. No matter what happened, they were always there. They made me find myself again.

I want to thank my committee: Dr. de los Reyes, Dr. Grunden, Dr. Olson and last but not the least, my advisor, Dr. Hyman. Their patience and conscientious instructions made me progress in this program. Especially Dr. Hyman, he still helped me one disappointment after another. I will always keep his teaching and admonishment in my mind.

Also, I want to thank my lab mates who took good care of me during these years. Especially, Alan, who helped me a lot in NMR experiment and thesis correction, Christy, who provided everything I needed in my lab work and has being a good company at night (and Roland too); Danise, who was my excellent partner in BIT course. In addition, I want to thank TJ, who gave me complete instructions in teaching the Micro lab and gave me lots of help in job hunting; Cindy, who endured lots of trouble that I made but were still willing to help me in this graduate program. All these people are like my family in Raleigh and gave me so many pleasant memories before I leave.

I also want to thank the Biology program for supporting my study as well as my daily life. Particularly Dr. Patterson, whom I know I can always talk to and gain some valuable suggestions from. Teaching really improved my language skills.

So many people I have to thank, I will thank God instead. God bless every one of you.
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CHAPTER 1

INTRODUCTION

A wide range of ether-bonded compounds either occur as natural products or are generated in large quantities by the chemical industry. In this study I have examined the biodegradation of a number of simple cyclic ethers by the alkane-utilizing bacterium *Mycobacterium vaccae* JOB5. The names and structures of the compounds examined in this study and thesis are shown in Fig. 1.

**Industrial uses and environmental impacts of cyclic ethers:** Tetrahydrofuran (THF) is widely used as a solvent in polyvinyl chloride (PVC) synthesis and the printing industry. A major use for 1,4-dioxane (14D) is as a solvent for fumigants, coolants, and textiles (32). It is also generated as a byproduct during the polymerization of ethylene terephthalate (50) ethylene glycol and ethylene oxide which are widely used in manufacture of shampoos, detergents, and cosmetic emulsifiers (1, 10). Due to its solvent characteristics and its inhibitory effect on cytochrome-P450 enzyme, THF may cause human health problems (21). The US EPA has classified THF as a potential human carcinogen. Based on long term study on rodents, 14D has been shown to increase the incidence of renal, hepatic and nasal turbinate tumors (19). The United State Environmental Protection Agency classifies 14D as weak human carcinogen.

Approximately 324 tons of 14D were released into the environment in 1997 (35). Despite this 14D has recently been described as an “emerging” contaminant as a result of its
<table>
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<td>2-hydroxytetrahydrofuran (2HTHF)</td>
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<td>3-hydroxytetrahydrofuran (3-HTHF)</td>
<td>4-hydroxybutyric acid (4HBA)</td>
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Fig 1. Structures, names and abbreviations of compounds examined in this study
frequent use as a stabilizer for chlorinated solvents, such as 1,1,2-trichloroethylene (TCE) and 1,1,1-trichloroethane (TCA) (63). Because of the cyclic structure and the high bond energy of C-O (360 Kj mol⁻¹), cyclic ethers such as THF and 14D are difficult to biodegrade and remain stable in the environment. Due to their high aqueous solubility, low Henry’s Law constants (THF = 7.05 × 10⁻⁵ atm m³/mol, 14D = 4.88 × 10⁻⁶ atm m³/mol) and low log K_{ow} (THF = 0.46 14D = -0.27) (31), these compounds move rapidly in ground water and can generate large plumes that threaten drinking water supplies often far from the source zone (36).

**Biodegradation of THF:** Among the cyclic ethers examined in this project the biodegradation of THF has been the most thoroughly characterized process. Early studies with activated sludge from different sources suggested THF is not readily biodegradable (18). However, several organisms have been isolated that can grow on THF as sole carbon and energy source. These include *Rhodococcus ruber* strain 219 (9), *Pseudonocardia* strain K1 (41), *Pseudonocardia dioxanivorans* strain CB1190 (44, 48) and *Pseudonocardia sp.* strain ENV478 (63).

*Rhodococcus ruber* strain 219 can utilize THF, γ-butyrolactone (γBL), 14D and tetrahydropyran (THP) as sole carbon and energy sources. The attack on THF is thought to be at C-2 position based on the fact that a potential subsequent intermediate, γBL, was not detected since γBL is oxidized faster than THF in this strain. *Pseudonocardia sp.* strain K1, a more recently isolated strain, can grow on up to 60 mM THF but does not grow on 14D. This strain also grows on 3-hydroxytetrahydrofuran (3-HTHF), γBL, 4-hydroxybutyric acid.
(4HBA), and 1,4-butanediol (14DB). Unlike *Rhodococcus* strain 219, strain K1 does not grow on 14D or THP (9).

Considerable progress has been made in determining the enzymes and pathways involved in THF degradation in *Pseudonocardia* strain K1. The activity of NADH-dependent cytochrome *c* oxidoreductase containing an Fe-S cluster with a covalently bound flavin cofactor (FAD) was detected during growth on THF (61). A gene cluster involved in the utilization of THF was subsequently cloned and sequenced using a specific primer derived from the N-terminal amino acid sequence of this reductase (Thm D) (61) and the upstream and downstream regions were obtained from the gene library (60). The gene cluster, *thmADBC*, encodes the components of a putative binuclear iron-containing multicomponent THF monooxygenase including THF-monooxygenase α- and β-subunits, a NADH:acceptor oxidoreductase. An additional ORF (*sad*) which encodes succinate semialdehyde dehydrogenase, is located upstream of *thm*. Both *thm* and *sad* gene can be induced during the growth on THF. Other additional ORF (*aldH*) encoding an aldehyde dehydrogenase, which can be induced during the growth on 14BD but not THF. The functions of other ORFs were not identified (60). Although *Pseudonocardia sp.* strain K1 does not lose the ability to grow on THF after repeated transfer to other carbon sources (41), the *thm* gene cluster seems to be located on a large size plasmid determined by Southern hybridization. Based on the gene expression induced during growth on THF, the role of various enzymes of the degradation pathway was proposed by Thiemer *et al.* (Fig. 2) (60).
The most recently isolated THF-utilizing strain is *Pseudonocardia* sp. strain ENV478. This organism is closely related to strain K1 and cells of this strain grown on THF cometabolically oxidize 14D, 1,3-dioxolane (13DO), bis-2-chloroethylether, and methyl tertiary butyl ether (MTBE) (63). A product, 2-hydroxyethoxyacetic acid (2HEAA), was shown by HPLC analysis of the reaction medium to accumulate during 14D oxidation. It was suggested that the inability of *Pseudonocardia* strain ENV478 or other bacteria to grow on 14D is due to its inability to metabolize 2HEAA (63).

**14D biodegradation:** Non-biological treatments such as carbon adsorption, air stripping, distillation, chlorination, precipitation-coagulation, hydrogen peroxide and ozone treatment and UV photooxidation are either not effective for 14D-contaminated groundwater or are too expensive or generate more toxic compounds (2, 71). Like THF, 14D has not been found to be biodegradable under anaerobic conditions. The majority of the biodegradation research
has therefore focused on aerobic processes. Although the available data suggests that 14D is not rapidly degraded by conventional biological treatment systems, some studies have reported that 14D can be metabolized by assimilative degradation or cometabolism by isolated organisms (39, 44, 46, 60, 71).

Early studies of mammalian toxicology of 14D revealed the primary metabolite of 14D degradation is 1,4-dioxane-2-one (69). By pretreatment of the rats with mixed-function-oxidase inducers, 14D degradation rate was significantly increased. Apart from \( \text{R. ruber} \ 219 \) discussed above, the only other aerobic 14D-utilizing strain currently known is a nocardioform actinomycete (strain CB1190), which utilizes a broad range of cyclic ethers, including 14D and THF, as sole carbon and energy sources. This organism was isolated from 14D-contaminated sludge from an enrichment culture supplied with THF (48). This bacterium has recently been reclassified as \( \text{Pseudonocardia dioxanivorans} \) strain CB1190 (44). This strain grows on other nonether substrates such as 2-propanol, 1-butanol, 2-butanol, glucose, and is capable of aerobic autotrophic growth on \( \text{H}_2 \) and \( \text{CO}_2 \) (48). Based on substrate utilization, THF is a more potent inducer of 14D-degrading activity than 14D itself. This has been taken to suggest strain CB1190 may be a regulatory mutant of a THF-oxidizing strain or a mutant with expanded capability to oxidize 14D and its downstream intermediates. This strain has also been used in bioaugmentation studies that examine 14D mineralization in soils (39). Besides bacteria, THF and 14D can also be metabolized by fungi. \( \text{Cordyceps sinensis} \), utilizes 14D as a sole carbon and energy source and was isolated from soil. Ethylene glycol is the major detectable metabolite of 14D degradation and was identified by gas chromatography-mass spectrometry (46). \( \text{C. sinensis} \) also degrades ary
cyclic ethers and recent studies revealed a new degradation pathway for dioxins by this organism (47).

**Cometabolism:** During early studies of the substrate range of hydrocarbon-utilizing bacteria, Foster observed that during growth of some strains on methane, select organisms were able to oxidize other hydrocarbons such as propane that could not support the growth of the same strains when supplied as sole sources of carbon and energy (22). The process was termed “co-oxidation”. Jensen subsequently noted that a strain of *Pseudomonas* that grew on monochloracetate was able to dehalogenate trichloroacetate but not use the later compound as a carbon source for growth (37). Alexander first termed the process “cometabolism” to allow the basic principle of co-oxidation to be expanded beyond oxidation and included other reactions, such as dehalogenations. The definition of this cometabolism was “*The transformation of an organic compound by a microorganism that is unable to use the substrate as a source of energy of one of its constituent elements*” (4). Also, the obligate requirement for presence of a growth substrate was dropped in this definition due to the fact that cell suspensions grown on the growth supporting substrates (the resting cells) were able to oxidize non-growth supporting substrates (37). Later, Stirling and Dalton dressed this definition by demonstrating that resting cells of *Methylococcus capsulatus* (Bath) can oxidize two classes of non-growth supporting substrates. The first class of compounds which can be oxidized without co-substrates due to the non-specific catalysis of monooxygenase and do not constitute a new metabolic event was named “fortuitous oxidation”. The product of fortuitous oxidation usually provides energy for further metabolism, which is similar to the co-substrate degradation. The second class of compounds can be oxidized in presence of co-
substrates was named “cometabolism” whose products are not subject to further oxidation and is accumulated (58).

Due to the controversial definition of cometabolism, the most integrated definition was stated by Horvath as “Although cometabolism does imply the concomitant oxidation of a non-growth substrate during growth of a microorganism on a utilizable carbon and energy source, it also describes oxidation of non-utilizable substrates by resting cell suspensions grown at the expense of substances capable of supporting microbial growth. Therefore, usage of cometabolism refers to any oxidation of substances without utilization of the energy derived from the oxidation to support microbial growth and does not infer presence or absence of growth substrate during the oxidation” (30).

Several explanations of why bacteria can oxidize certain compounds but not use them as sole growth-supporting substrates have been proposed. The two most significant are first, the initial enzyme or enzymes generate products that are not further oxidized due to the lack of appropriate enzymes. Therefore, the intermediate cannot be used for biosynthesis or energy production and accumulates extracellularly (58, 59). Second, the initial substrate is oxidized to toxic products that suppress the growth of the organism. For example, toluene-grown Pseudomonas putida cometabolize chlorobenzene to 3-chlorocatechol, but the latter compound is not degraded due to the inhibition of the further enzymes (40). A significant number of environmental pollutants can be cometabolically degraded using pure cultures. These include compounds such as cyclohexane, polychlorinated biphenyls, chlorophenols,
1,3,5-trinitrobenzene and others (3). In situ cometabolic processes have also been successfully used at a number of contaminated sites (51).

**Mycobacterium vaccae JOB5:** The organism studied in this project was *M. vaccae* JOB5. This strain was first isolated on 2-methylbutane and has known to grow on straight chain alkanes from C$_2$ to C$_{22}$. This organism is of interest for two unusual features that both relate to the propane-oxidizing activity of this strain. One unusual feature is this strain is believed to oxidize propane through both a terminal and a sub-terminal pathway (Fig. 3). In these two concurrently operating pathways propane is initially oxidized by a single monooxygenase to yield a mixture of 1- and 2-propanol. In the terminal oxidation pathway 1-propanol is subsequently oxidized to propionaldehyde and propionate by dehydrogenases. Propionate is then thought to be carboxylated to yield methylmalonate that is then mutated to yield succinate. In the subterminal pathway 2-propanol is oxidized to acetone and this simple ketone is further oxidized by another monooxygenase to yield acetol (hydroxyacetone). The subsequent steps remain poorly characterized but are believed to involve further oxidation of acetol to yield acetate and formaldehyde. The presence of a subterminal pathway is supported by studies of isocitrate lyase activity that show high activities of this enzyme in propane-, isopropanol-, acetate-grown cells but low activities in 1-propanol- and propionate-grown cells (19) Radioisotope studies using $^{14}$C-labeled substrates have also supported the role of subterminal oxidation in this organism (11, 16, 28, 56). It is notable that unlike propane oxidation, the pathway for *n*-butane metabolism appears to only involve terminal oxidation in this strain (49).
Fig 3. Potential pathways of propane oxidation in *M. vaccae* JOB5
The other unusual feature of propane-grown cells of *M. vaccae* JOB5 is this organism has been shown to oxidize a wide range of non-growth supporting organic substrates. Many of these compounds are important environmental pollutants and include, among others, recalcitrant gasoline components (*e.g.* cyclohexane (7) and methyl tertiary butyl ether (MTBE) (52, 55)), energetics (*e.g.* 2,4,6-trinitrotoluene (65)), and a range of halogenated hydrocarbons (*e.g.* trichloroethylene (67), chloroform (26), and methyl bromide (59)). This substrate range is summarized in Table 2. Previous studies with cyclic ethers have demonstrated THF is oxidized to γBL and 14D is oxidized to undetermined products.

Our own recent studies with this organism have focused on defining the pathway of oxidation of ether-bonded compounds by propane-grown cells of this bacterium (38, 52). Although this strain does not grow on the gasoline oxygenate MTBE, the cometabolic oxidation of this branched ether-bonded compound is unusual as MTBE undergoes extensive, if not complete, oxidation by this organism. In contrast to many other cometabolic degradation processes that only involve single enzyme-catalyzed reactions, MTBE oxidation involves several enzyme activities that are co-expressed in propane-grown cells. In sequence, the initial steps in MTBE degradation are believed to involve a monooxygenase-catalyzed oxidation of MTBE to a hemiacetal, which is then rapidly oxidized to an ester (*tertiary* butyl formate [TBF]) by an alcohol dehydrogenase. During growth on propane this monooxygenase and alcohol dehydrogenase activities most likely function to sequentially terminally oxidize propane to 1-propanol and propionaldehyde. An esterase-catalyzed hydrolysis of TBF to *tertiary* butyl alcohol (TBA) and formate is followed by a further monooxygenase-catalyzed oxidation of TBA to 2-methy-1,2-propanediol. This diol is further oxidized to 2-hydroxyisobutyric acid
through sequential alcohol and aldehyde dehydrogenase activities (42). The latter enzymes are again reasonably expected to be directly associated with the alkane-oxidizing activity of this strain.

A comparison of the various steps involved in both MTBE and THF oxidation described above suggests there is potentially significant enzymatic overlap between the oxidation of these ether-bonded compounds and growth-supporting alkanes such as propane. In this study we have examined the oxidation of a number of cyclic ethers by *M. vaccae* JOB5 and have characterized several of the major steps in the THF oxidation pathway. Our results show cyclic ether-degrading activity can be supported by propane as well as a number of other simple linear and branched alkanes. Our results also demonstrate THF oxidation can provide a metabolic benefit to cells grown on n-pentane. Our results are discussed in terms of their impact on our understanding of cyclic ether oxidation, potential avenues for cyclic ether bioremediation and our understanding of cometabolism.
<table>
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<th>Cometabolized Substrates</th>
<th>Identified Major Products</th>
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CHAPTER 2

MATERIALS AND METHODS

Materials: Mycobacterium vaccae (austroafricanum) JOB5 (ATCC 29648) was obtained from the American Type Culture Collection (Manassas, VA). The following chemicals (with manufacturers specified purity) were obtained from Sigma-Aldrich Chemical Co. Inc. (Milwaukee, WI): n-butane (99%), 1,4-butanediol (14BD) (99+%), γBL (99+%), calcium carbide (80% technical grade for acetylene generation), ε-caprolactone (99%), 1,3-dioxane [13D] (97%), 1,4-dioxane [14D] (99.9%), 1,3-dioxolane [13DO] (99.8%), 4HBA (Na salt) ((99%), 3-hydroxytetrahydrofuran (99%), 2-methylbutane (≥99%), 2-methylpropane (99%) 1,5-pentanediol (96%), 1,3-propanediol (98%), β-propiolactone (90%), THF (99.9%), tetrahydropyran [THP] (99% anhydrous), trimethylene oxide [TMO] (99%), and δ-valerolactone (≤ 25% polymer). Propane (Instrument grade) was supplied by National Specialty Products and Chemicals Inc. (Allentown, PA). N-pentane (99.5%), 1-propanol (99.9%) and 2-propanol (99.9%) were obtained from Fisher Scientific (Pittsburgh, PA.). All gases for gas chromatography (H₂, N₂ and compressed air) were obtained from local vendors.

Growth of M. vaccae JOB5: Plate cultures of M. vaccae JOB5 were maintained at room temperature (22°C) on DIFCO Plate Count Agar (PCA) (Becton, Dickinson and Co., Franklin Lakes, NJ). Cells of M. vaccae JOB5 used in resting cell biodegradation assays were grown in batch culture in glass bottles (650 ml) sealed with screw caps fitted with butyl rubber septa (Wheaton Science Products, Millville, NJ). The bottles contained mineral salts medium (100 ml) (70) and were inoculated to an initial optical density at 600nm [OD₆₀₀]
\[ \leq 0.02 \] with a suspension of cells obtained from a PCA plate. Gaseous growth substrates (120 ml) were injected into the sealed bottles using plastic syringes (60 ml) fitted with 0.2 µm Acrodisk filters (Pall Corp., Ann Arbor, MI). Unless otherwise stated, liquid growth substrates (25 µl) were added to the sealed bottles as neat compounds using sterile glass microsyringes (Hamilton Co. Reno, NV). All cultures were grown in the dark in a temperature-controlled (30°C) orbital shaker (150 rpm). After 4 d the residual growth substrates (hydrocarbons and O\(_2\)) were replenished by removing the culture bottle screw caps under sterile conditions in a laminar flow hood. After 5 min, the culture bottles were resealed and the appropriate hydrocarbon was reintroduced into the culture bottle to the initial concentration described above. The cultures were then returned to the shaker and incubated for a maximum of another 16 hours.

For some experiments cells of *M. vaccae* JOB5 were also grown in a dextrose-containing medium (DIFCO, Becton, Dickinson and Co., Franklin Lakes, NJ) consisting of (g l\(^{-1}\) distilled water); pancreatic digest of casein (5.0 g), yeast extract (2.5 g), dextrose (1.0 g). The same culture method was used for these cells as described above except without a re-aeration step. Cells grown on either hydrocarbons or dextrose-containing media were harvested from their culture medium by centrifugation (10,000 x g for 10 min at 4°C). The supernatant was discarded and the cell pellet was resuspended in buffer (10 ml, 50mM NaH\(_2\)PO\(_4\), pH 7.0). The resuspended cells were sedimented again by centrifugation and the washed cell pellet was resuspended in buffer (1 ml) to a final protein concentration of \[ \leq 10 \text{ mg/ml} \]. The washed cells were placed on ice and were used in experiments within 6 hours of harvesting.
**Cultures containing two substrates:** In some experiments, cells of *M. vaccae* JOB5 were grown in the combined presence of n-pentane and either THF or 14D. These cells were grown in batch culture in glass serum vials (125 ml) sealed with Teflon-lined Mininert valves (Alltech Associates Inc., Deerfield, IL). The vials contained a mineral salts medium (25 ml) and were inoculated to an initial density [OD$_{600}$] ≤ 0.01 with a suspension of cells obtained from axenic cultures previously grown on PCA plates. The liquid growth substrates were added to the sealed vials as neat compounds (5 µl; 0.02%, vol/vol liquid phase) using sterile glass microsyringes (Hamilton Co. Reno, NV). All cultures were grown in the dark in a temperature-controlled (30°C) orbital shaker (150 rpm). At selected times aqueous samples (700 µl) were removed from the culture vials using disposable sterile plastic syringes and were used to determine culture growth (OD$_{600}$) using a Shimadzu 1601 UV/Vis spectrophotometer (Kyoto, Japan). At the end of each growth experiment a sample (50 µl) of the residual culture medium was streaked on PCA plates to determine and confirm the purity of the culture.

The protein content of cultures containing both n-pentane and cyclic ethers was estimated from the relationship between final culture density (OD$_{600}$) and the protein content of cultures grown with C-limiting amounts of n-pentane. Cells were grown in glass serum vials (125 ml) containing medium (25 ml) and varying amounts of n-pentane (0-16 µl; 0- 0.064% vol/vol liquid phase) as described above. The consumption of n-pentane was determined by GC analysis of the culture headspace. Once the n-pentane was exhausted in all cultures (≤ 4 d) the final culture density was determined spectrophotometrically and the entire culture (25 ml) was centrifuged (10,000 x g for 10 min at 4°C). The supernatant was discarded and the
cell pellet was resuspended in buffer (1 ml). The washed cells centrifuged again (14,000 × g, 5 min) in a microfuge and the final cell pellet was resuspended with buffer (150 µl). The protein concentration was then determined using the Biuret protein assay with BSA as standard (24) after solubilizing cell material for 30 min at 65°C in 3N NaOH and sedimentation of insoluble material by centrifugation (14,000 × g, 5 min). Each culture was grown in triplicate and a plot of final culture density versus final culture protein concentration was linear (r² 0.93) over the range of n-pentane concentrations tested (8 data points total).

**Reaction conditions:** Reactions following the degradation of THF, γBL 14D and other mono- or di-cyclic ethers and reactions examining the effects of propane on THF degradation were all conducted in glass serum vials (10 ml). Buffer (≥ 900 µl) was added to the reaction vials that were then sealed with butyl rubber stoppers and aluminum crimp seals (Wheaton Science Products, Millville, NJ). Liquid substrates were added to the sealed vials from aqueous stock solutions using glass microsyringes. The sealed reactions vials were then incubated for 5 min in a shaking water bath (30°C and 150 rpm) to allow equilibration of substrates between the gas and liquid phases. The reactions were initiated by the addition of an aliquot (100 µl) of a concentrated cell suspension to give a final reaction volume of 1 ml and a total cell protein content of ~1 mg. The reaction vials were then returned to the shaking water bath and were analyzed according to the demands of each experiment. In some experiments acetylene (1 ml, 10% vol./vol. gas phase) was added to the reactions vials to inactivate the n-alkane-oxidizing activity of *M. vaccae* JOB5 (26, 38, 52). In other experiments propane (≤ 2.5 ml) was added as a competitive substrate to inhibit THF
oxidation by propane-grown cells. In these experiments propane was added to the sealed vials and the excess pressure was released by briefly inserting a syringe needle (2.5 cm, 22 gauge) into the stopper. The final concentration of gaseous n-alkane in the reaction vial was then determined by analysis of the gas phase by gas chromatography (see below). The aqueous phase concentration of propane was subsequently calculated assuming a total gas (air + propane) pressure of 1 atm.

**Analytical methods:** In most experiments substrate consumption, product accumulation and product identification were determined by gas chromatography using a Shimadzu GC-14A chromatograph (Kyoto, Japan) equipped with a flame ionization detector and a stainless steel column (1/8” by 6’) packed with Porapak Q (60-80 mesh) (Water Associates, Framingham, MA). The gas chromatograph was operated with an injection temperature of 200°C, a detector temperature of 220°C and a column temperature of 120–150°C (depending on the substrate). Nitrogen was used as a carrier gas at a flow rate of 20 ml min⁻¹. All products and substrates were analyzed using aqueous phase samples (2 µl) with the exception of n-pentane and propane that were analyzed using gas phase samples (25 µl). The gas chromatograph was interfaced to an HP3395 integrator (Hewlett Packed, Palo Alto, CA) for data collection and analysis. Products were identified by co-elution and quantification of each compound was based on calibration plots established by known concentration of the authentic compound.

**Constants:** The aqueous solubility of propane at 1 atm and 30°C was taken as 1.41 mM (68). Kinetic constants were derived by computer fitting data by non-linear regression to a
hyperbolic single substrate-binding model \[ Y = \frac{V_{\text{max}}}{K_s + X} \] using GraphPad Prism version 3.0a for Macintosh (GraphPad Software, San Diego CA).

**RESULTS**

**Cometabolic oxidation of cyclic ethers by resting cells:** We initially surveyed a range of cyclic ethers as potential substrates for oxidation by *M. vaccae* JOB5 using resting cells grown on a number of simple linear and branched C<sub>3</sub> to C<sub>5</sub> alkanes. The ethers tested included the C<sub>3</sub>, C<sub>4</sub>, and C<sub>5</sub> homologs containing a single ether bond (TMO, THF, and THP) as well as one C<sub>3</sub> (13DO) and two C<sub>4</sub> (13D and 14D) ethers containing two ether bonds. Cells grown on all of the alkanes tested oxidized all of these ethers and in most instances this activity was strongly inhibited (> 90%) by the presence of acetylene (Table 1). This effect of acetylene suggested the ethers were all oxidized by an alkane-inducible monooxygenase activity, an activity that has been most frequently characterized in propane-grown cells of this organism. The role of monooxygenase activity in ether oxidation is further supported by the much lower levels of ether-oxidizing activity in cells grown on dextrose-containing medium (Table 1). Based on overall levels of ether consumption throughout the survey, TMO and THP were consistently the least effective substrates for alkane-grown cells while THF and 13DO were the most effective.

**Time course of THF and 14D oxidation by propane-grown cells:** As THF was readily oxidized by alkane-grown cells (Table 1), the oxidation of this compound was examined in more detail. When propane-grown cells were incubated with THF the ether was rapidly consumed without a lag phase (Fig. 1). The initial rate of THF oxidation (0-40 min) was
estimated at \( \sim 21 \text{ nmoles min}^{-1} \text{ mg total protein}^{-1} \). In these experiments THF (~2 mM) was completely consumed within 2 h. During THF degradation a single oxidation product was detected in the reaction medium and was identified as \( \gamma \text{BL} \) by co-elution with the authentic compound and by its hydrolysis and dehydration in response to increased and decreased pH (15). The production of \( \gamma \text{BL} \) closely followed the time course of THF oxidation and the maximum level of \( \gamma \text{BL} \) accumulation was observed after \( \sim 100 \) min when the majority (\( \geq 90\% \)) of the THF had been consumed. \( \gamma \text{BL} \) was also degraded at an equivalent rate to THF once THF had been fully consumed. Only slight THF consumption (\( \leq 10\% \)) was observed over 3 h with propane-grown cells incubated with THF and acetylene and no \( \gamma \text{BL} \) was detected in the reaction medium (Fig. 1). The THF-oxidizing activities of cells grown on 1-propanol, 2-propanol and dextrose-containing medium were also determined. For cells grown on these substrates and incubated with THF (1 mM) the specific rates of THF oxidation were \(~6, \sim 1 \) and \(~2 \) nmoles min\(^{-1} \) mg total protein\(^{-1} \), respectively. In addition to the effects of acetylene (Fig. 1), we also examined the effects of propane on THF oxidation. Propane inhibited THF oxidation in a saturable manner (Fig. 2). The inhibition data fitted well to a hyperbolic single substrate-binding model \( (r^2=0.94) \) and an apparent \( K_i \) \( (K_{i_{app}}) \) of 10.1 \( \mu \text{M} \) (SD.1.6 \( \mu \text{M} \)) for propane was derived from these data. Propane-grown cells also rapidly consumed 14D (1 mM) without a lag phase (Fig. 3). The initial rate of 14D oxidation (\(~6 \) nmoles min\(^{-1} \) mg total protein\(^{-1} \)) was 3- to 4-fold slower than for THF above. Oxidation of 14D was fully (\( \geq 95\% \)) inhibited by acetylene but unlike THF oxidation, no products from 14D oxidation were detected in the reaction medium using the GC conditions used in this study.
Oxidation of 3-hydroxytetrahydrofuran by propane-grown cells: As indicated in the Introduction, 2-HTHF is believed to be common intermediate in all currently recognized pathways of THF oxidation. However, this unstable hemiacetal is not commercially available and has not been unequivocally identified or independently evaluated in any studies of THF oxidation. To determine whether propane-grown cells can oxidize a structural analog of this compound, we examined the oxidation of 3-hydroxytetrahydrafuran (3HTHF). Propane-grown cells rapidly degraded 3HTHF without a lag phase, both in the presence and absence of acetylene (Fig. 4). The initial rates (0-30 min) were $\sim$10 and $\sim$6 nmoles min$^{-1}$ mg total protein$^{-1}$ in the presence and absence of this inhibitor, respectively. In the absence of acetylene no products from 3HTHF oxidation were detected using the GC conditions used in this study. However, in the presence of acetylene a single major oxidation product was detected. This product was not further identified or quantified but eluted slightly later than 3HTHF. After exhaustion of 3HTHF in the presence of acetylene this unidentified product generated a comparable FID detector response to the 3HTHF present at the start of the reaction.

The degradation of $\gamma$-butyrolactone by propane-grown cells: Propane-grown cells were also incubated with $\gamma$BL (1 mM) in the presence and absence of acetylene (Fig. 5). The cells rapidly consumed $\gamma$BL (initial rate [0-40 min] $\sim$39 nmoles min$^{-1}$ mg total protein$^{-1}$) in the absence of acetylene and $\sim$55% slower than this in the presence of this inhibitor. The acetylene-insensitive consumption of $\gamma$BL was not due to either abiotic reactions (e.g. hydrolysis) or non-specific absorption by cell material as no $\gamma$BL consumption was observed over 3 h in reactions containing either no cells or cells that had been heat-treated (15 min at
No products of γBL degradation were observed to accumulate either in the presence or absence of acetylene using the GC conditions used throughout this study. Specific rates of γBL degradation were also determined for cells grown on 1-propanol-, 2-propanol- and dextrose-containing media. These rates were ~7, ~11 and ~3 nmoles min\(^{-1}\) mg protein\(^{-1}\), respectively.

**Growth of *M. vaccae* JOB5 on cyclic ethers and their putative oxidation products:** The apparent lack of accumulation of downstream oxidation products during the rapid oxidation of γBL (Fig. 5) suggested this compound and its metabolites might be fully oxidizable and could potentially serve as sole carbon and energy sources for growth. A range of cyclic ethers and their known or postulated metabolites were therefore examined as potential growth-supporting substrates for strain JOB5. None of the three cyclic ethers examined supported growth of this strain, even in extended incubations for 14 d (Table 2). Similar results were also obtained with the three terminal diols tested. In contrast, slight to modest growth after 14 d was observed with all of the lactones tested. In general, the level of growth increased with increasing carbon number. The most rapid and abundant growth was achieved with 4HBA.

**Cometabolism of THF and 14D during growth on n-pentane:** The ability of strain JOB5 to grow on 4HBA and possibly γBL (Table 2) suggested to us that oxidation of THF during growth of strain JOB5 on alkanes could potentially result in higher levels of growth than those achieved with n-alkanes alone. To examine this we followed the time course of substrate oxidation and culture density when strain JOB5 was grown under C-limiting conditions on n-pentane (44 μmoles) alone or n-pentane plus THF (44 + 61 μmoles). In the
absence of THF, n-pentane was rapidly consumed within 3 d (Fig. 6A). There was also a concurrent increase in culture density that reached a maximum (OD$_{600} = 0.15$) at the time the n-pentane was exhausted (Fig. 6B). After this the culture density gradually decreased by ~30% over the subsequent 10 d. When cells were grown on n-pentane plus THF the initiation of n-pentane consumption was markedly delayed and only ~15% of the n-pentane was consumed within 3 d. Over the next 6 d n-pentane was progressively more rapidly consumed and was eventually exhausted after 9 d. Consumption of THF closely followed the time course of n-pentane consumption and was also fully consumed after 9 d. In these cultures the final culture density was ~2-fold higher (34 µg ml$^{-1}$) than observed in cultures with n-pentane alone (18 µg ml$^{-1}$). This maximum was achieved after 10 d, 7 d later than for cells grown with n-pentane alone. During growth on n-pentane plus THF γBL was detected in the culture medium. After 6 d ~50% (~30 µmoles) of THF had been consumed and close to stoichiometric (≥95%) amounts of γBL relative the total THF consumed were detected in the culture medium. In control incubations no THF consumption, γBL production, or cell growth was observed in cultures containing THF alone or in uninoculated control cultures containing n-pentane plus THF. The same experiment described was also conducted with cells grown on n-pentane (44 µmoles) in the presence and absence of 14D (59 µmoles) No substrate consumption or growth was observed in cultures containing either 14D alone or uninoculated cultures containing n-pentane plus 14D (Fig. 6C). For inoculated cultures containing n-pentane alone the time course of n-pentane consumption and changes in culture density were equivalent to those shown in Fig. 6 A&B. In the cultures containing n-pentane plus 14D there was a 2 d delay in the onset of n-pentane consumption relative to those containing n-pentane alone. However, after this lag, both n-pentane and 14D were consumed concurrently and
~60% of the 14D was consumed by the time n-pentane had been exhausted (5 d). No further detectable 14D consumption was observed over the following 5 d. Unlike THF, the consumption of 14D did not impact the final culture density obtained with n-pentane (data not shown).

**DISCUSSION**

The results of this study demonstrate *M. vaccae* JOB5 can oxidize a number of simple cyclic ethers through the activity of an alkane-inducible monooxygenase. Our results also demonstrate that THF is extensively oxidized by alkane-grown cells although it does not serve as a sole carbon and energy source for growth. Despite this, concurrent oxidation of n-alkanes and THF results in an increase in cell growth. The following sections discuss these findings and their impacts on our understanding of the pathways of cyclic ether biodegradation, the concept of cometabolism and potential approaches for cyclic ether bioremediation.

**Pathway of THF biodegradation:** The results of this study (Table 1) demonstrate *M. vaccae* JOB5 can oxidize a wide range of simple cyclic ethers. In all cases oxidation of these compounds was strongly inhibited by acetylene and, like alkane-oxidizing activity, was effectively absent in cells grown on dextrose-containing medium. These observations suggest that like THF-metabolizing organisms (60) the key enzyme responsible for initiating ether oxidation is an alkane-inducible monooxygenase. Further support for this was provided by the competitive inhibitory effects of propane on THF oxidation by propane-grown cells (Fig. 2). We have previously estimated the $K_s$ for propane in propane-grown cells at ~4µM (52).
As propane and THF appear to be mutually competitive substrates an estimate a $K_s$ value for THF of $\sim 400 \mu M$ can be derived from the following equation $K_{s-(propane)} = K_{s-(app)}(propane)/(1 + [THF]/K_{s(THF)})$ (52).

Our results also demonstrate THF and several of its established metabolites are rapidly and substantially oxidized by resting propane-grown cells and the pathway of THF oxidation appears to be very similar if not identical to the pathway in true THF-utilizing organisms (9, 41). For example, THF oxidation by propane-grown cells involves the production and subsequent consumption of $\gamma$BL (Fig. 1). In THF-utilizing bacteria (9, 41), $\gamma$BL is believed to generated by an alcohol dehydrogenase-catalyzed oxidation of the immediate product of THF oxidation, 2HTHF. Although 2HTHF is unstable and commercially unavailable, we observed rapid 3HTHF oxidation (Fig. 4), an observation that indicates propane-grown cells can at least oxidize structurally similar hydroxylated cyclic ethers. A further interesting observation was the effect of acetylene on 3HTHF oxidation. In the absence of acetylene no products of 3HTHF oxidation were observed while an unidentified but stable product accumulated to high concentrations in the presence of this inhibitor. Our interpretation of these results is that 3HTHF is initially oxidized by an alcohol dehydrogenase, and the product of this reaction itself was then further oxidized by the alkane-inducible, acetylene-sensitive monooxygenase. Like 2HTHF and $\gamma$BL, the immediate product of 3HTHF oxidation is expected to be a ketone while a further monooxygenase-catalyzed oxidation would most likely involve subsequent hydroxylation of the C4 or C5 position. As acetylene inhibited the rate of 3HTHF oxidation by $\sim 50\%$ (Fig. 4) it is also likely that 3HTHF can also serve as a substrate for propane monooxygenase even before the proposed dehydrogenation of the initial hydroxy group.
While some uncertainty may remain about 2HTHF as an intermediate, we clearly demonstrated that γBL is a near stoichiometric product of THF oxidation (Figs 1 & 6) and that this compound is also rapidly oxidized by propane-grown cells (Figs. 1 & 5). Despite using several different approaches, we were unable to detect the previously reported product of γBL degradation, 4HBA (9, 41, 48, 63). However, this polar, high boiling point compound is difficult to quantify by GC and is normally estimated by this technique after acid-catalyzed conversion to γBL (15). Another factor potentially impacting our ability to detect 4HBA is that *M. vaccae* JOB5 grows well on this compound (Table 2) and can therefore be expected to rapidly metabolize this compound under certain conditions such as growth on n-alkanes like n-pentane. In this study we observed 2-fold increases in both culture density and culture protein content when cells were grown on n-pentane plus THF compared to growth on n-pentane alone (Fig. 6). As *M. vaccae* JOB5 did not grow on THF (Table 2) and grew very poorly, if at all, on γBL, the simplest explanation of this result is the organism grew on 4HBA generated from the γBL accumulated during THF oxidation. It should be noted 4HBA and γBL exist in a pH-dependent equilibrium (15) and this may account for the limited growth we observed with γBL alone. However, our experiments with γBL and propane-grown cells indicate no detectable loss of γBL over 3 h in abiotic incubations or control incubations containing boiled cells (Fig. 5). This suggests γBL degradation is predominantly a biological process during long-term incubations such as those described in Fig. 6. It should also be noted that acetylene inhibited the initial rate of γBL degradation by ~50% (Fig. 6). This effect suggests that like 3HTHF, γBL may also be oxidized by the alkane-induced
monooxygenase activity and the rate of γBL consumption in the absence of this inhibitor represents the sum of a monooxygenase and an esterase-catalyzed activities.

If *M. vaccae* JOB5 can grow on 4HBA generated during cometabolic THF oxidation one wonders why this organism apparently cannot also grow on THF as a sole source of carbon and energy? One possible explanation is THF is an ineffective inducer of the enzymes required for THF oxidation. A second possibility is the rates of reactions involved in THF oxidation occur too slowly to support growth. A final possibility is the enzymes required for 4HBA utilization are not co-expressed with n-alkane-oxidizing activity and the enhanced growth observed in cells grown on THF and n-pentane is a result of a form of diauxie. In this model growth on n-pentane allows for expression of the key reductant-requiring monooxygenase, reductant-generating dehydrogenase and esterase that catalyze the energetically neutral conversion of THF to 4HBA. A second suite of enzymes may then be needed to metabolize 4HBA and the energy needed for the synthesis of these enzymes also needs to be provided by another substrate such as n-pentane. Similar questions and possibilities have also been raised about the degradation of another ether-bonded pollutant, MTBE, by *M. vaccae* JOB5. As indicated in the Introduction, propane-grown cells substantially oxidize MTBE through the activities of several enzymes co-expressed in alkane-grown cells (52, 55). We have also shown MTBE and TBA induce the expression of several of these enzyme activities (38). Finally, studies with a closely related strain, *M. austroafricanum* IFP 2102 (23) have used incubations with high cell densities (OD$_{600} \geq$ 0.1) to show a final 2-fold change in culture density only occurs once MTBE has been substantially oxidized to 2-hydroxyisobutyric acid and consumption of this acid has been
initiated. The small changes in culture density seen with *M. austroafricanum* IFP 2012 under these conditions may simply reflect impacts of polyhydroxybutyrate accumulation or consumption on light scattering by resting cells rather than cell division and growth. However, in other propane-oxidizing bacteria the slow rate of 2HIBA oxidation has been suggested to be the rate-limiting step in MTBE oxidation and the key reaction that prevents growth of propane-oxidizers on MTBE (55).

**Potential Impacts on 14D bioremediation**: As indicated in the Introduction, interest in cyclic ether biodegradation has recently been stimulated following the recognition that 14D is a widespread contaminant associated with chlorinated solvents (36, 45, 71). As 14D is reportedly stable under anaerobic conditions (71) and infrequently used a bacterial carbon and energy source under aerobic conditions, the most recent research emphasis on this compound has focused on aerobic cometabolic processes. Several reports have shown 14D can be cometabolically oxidized by organisms grown on THF, methane and toluene (43, 63) and similar results have also been obtained with *M. vaccae* JOB5 as a model propane-oxidizing bacterium (43). In the present study we observed all six cyclic ethers we examined were rapidly oxidized by this organism after growth on propane, n-butane, n-pentane and simple branched alkanes such as isobutane and isopentane. While chloroform and MTBE oxidation have been previously reported for cells of *M. vaccae* JOB5 grown on alkanes other than propane (26, 33), our present observations greatly expand the number of substrates that can support cometabolic cyclic ether biodegradation. Our results also potentially impact cometabolic approaches for *in situ* remediation of 14D. For example, branched hydrocarbons are generally less readily biodegradable than n-alkanes (54) and use of these types of
compounds may provide a more selective and reliable approach for stimulating *in situ* cyclic ether biodegradation than the use of propane and other n-alkanes (51). n-Pentane and isopentane are also two of the most abundant non-aromatic components of gasoline (54). Our results (Table 1 and Fig. 6) suggest environments containing both gasoline and 14D might be susceptible to remediation through aerobic treatments designed primarily to remove gasoline components. Even if 14D and C\textsubscript{5}-alkanes do not coexist in the environment, our results also suggest the addition of n-pentane can lead to the oxidation of high concentrations of 14D with little or no evidence for toxic effects of 14D described in some recent studies of 14D cometabolism (43, 63). This may be a reflection of this particular organism’s general insensitivity to toxic effects in cometabolic processes (25, 43). Alternatively, it may simply reflect differences between experiments conducted with actively growing versus resting cells.

**Impacts on cometabolism:** There remains debate about whether cometabolism is a meaningful term in microbial physiology (66). One of the earlier dialogs about this process focused on the question of why organisms would retain the ability to oxidize compounds that they apparently receive no energy or carbon benefit from (17, 29, 57, 58). In recent years considerable attention has been focused on the cometabolic degradation of chlorinated solvents such as TCE and the physiological penalty that occurs due to the toxic effects of reactive intermediates generated during the degradation process (6). In contrast, far fewer studies have examined the possibility that cometabolism can actually be a beneficial process for the active organisms. Recently several studies have demonstrated that organisms such as methane- and alkane-oxidizing bacteria (27, 53) can obtain a metabolic benefit from the cometabolic oxidation of compounds such as chloromethane and propane that are structural
analogs of the substrate used to grow the active organism. In the present study we have demonstrated that cells of *M. vaccae* JOB5 grown on n-pentane can obtain a substantial benefit from the oxidation of a structural unrelated compound. Our current studies with this organism are examining whether similar benefits can be obtained with the cometabolic oxidation of other compounds.
CHAPTER 3

CONCLUSIONS

The results presented in this study demonstrate that *M. vaccae* JOB5 can oxidize a wide range of cyclic ethers after growth on a range of linear and branched alkanes. The results of this study also suggest the oxidation of THF, and most likely 14D, not only involves the activity of the alkane-inducible monooxygenase but also other co-expressed enzymes that normally serve to efficiently oxidize growth-supporting alkane substrates. These co-expressed enzymes include dehydrogenases and hydrolytic enzymes such as esterases.

While the presence of dehydrogenases in alkane-oxidizing pathways are well established, the role of hydrolytic enzymes such as esterases or more specific lactonases are less clearly defined. One example where a lactonase activity is integral to hydrocarbon oxidation is in the catabolism of another cyclic compound, cyclohexane. The pathway of cyclohexane degradation has been deduced (Fig 7) (5, 62) and the genes induced by cyclohexane have also been identified in two clusters in a *Brachymonas* strain (12). One gene cluster encodes the multicomponent hydroxylase that oxidizes cyclohexane into cyclohexanol (MONO1). Another gene cluster encodes all the genes involved in cyclohexanol oxidation (12). The induced enzymes these genes encode for including an alcohol dehydrogenase (ADH1) that oxidizes cyclohexanol to cyclohexanone, a second monooxygenase that further oxidizes cyclohexanone to ε-caprolactone (MONO2), a lactonase that hydrolytically cleaves ε-caprolactone to 6-hydroxyhexanoate. Another alcohol dehydrogenase (ADH2) subsequently oxidizes 6-hydroxyhexanaote to 6-oxohexanoate, and an aldehyde dehydrogenase (ALDH)
finally oxidizes 6-hydroxyhexanaote to adipic acid which then enters the central metabolic pathway.

![Pathway of bacterial cyclohexanone oxidation](image)

**Figure 7: Pathway of bacterial cyclohexanone oxidation**

It should be noted that cyclohexane is also cometabolically oxidized to cyclohexanone by propane-grown *M. vaccae* JOB5 (8). However, this organism does not possess a Baeyer-Villiger-type monooxygenase activity required to oxidize cyclohexanone to ε-caprolactone. Despite this, Beam and Perry (8) demonstrated a mixed culture of *M. vaccae* JOB5 and a cyclohexanone-degrading *Pseudomonas* can completely mineralize cyclohexane, although neither strains can fully oxidize cyclohexane alone (8). Based on the enzymatic and pathway similarities between THF and cyclohexane oxidation it would be interesting to examine the potential growth of cyclohexane-degraders on THF and *vice versa*.

**FUTURE RESEARCH**

Although this study has demonstrated that *M. vaccae* JOB5 can oxidize a number of cyclic ethers, it is also clear that some gaps remain in identifying the various dead-end products and transient intermediates generated during the oxidation of THF and compounds such as 14D. As indicated before, some of the intermediates such as 2HTHF are unstable and have not been detected in any studies of THF degradation. Other compounds such as 4HBA, 2HEAA
and related hydroxyacids also represent an analytical challenge as they are high boiling point polar compounds that can readily relactonize.

A more complete picture of THF and cyclic ether degradation could potentially emerge if we were able to easily detect all of the metabolites generated and consumed by this organism. One approach investigated during this study was the use of proton-nuclear magnetic resonance spectroscopy (\(^1\)H-NMR) (14, 20). The low sensitivity of this technique is offset by the fact that it can detect any dissolved compound without sample work up. The \(^1\)H-NMR spectra shown in Fig. 8 were obtained using authentic compounds.

![Fig 8. \(^1\)H NMR spectra of \(\gamma\)BL and 4HBA](image)
In one experiment, propane-grown cells of *M. vaccae* JOB5 were incubated with γBL and acrylic acid, a potent inhibitor of organic acid metabolism. The potential accumulation of 4HBA was then examined using $^1$H-NMR. The results of this experiment (Fig. 9) show low levels of a compound with resonances that match 4HBA were detected in the reaction medium.

![Gamma-butyrolactone and Gamma-Hydroxybutyric acid](image)

**Fig. 9:** $^1$H NMR spectra of γ-butyrolactone degradation by propane-grown *M. vaccae* JOB5. Propane-grown cells were incubated in presence of acrylic acid (10 mM) and γBL (40 mM) Panel (A) $^1$H NMR spectra of supernatant collected from cell reaction at time 0. Panel (B) $^1$H NMR spectra of supernatant collected from cell reaction after 96h. The resonances marked d, e, and f correspond with resonances for 4HBA (Fig. 9).
$^1$H NMR was also used to attempt to identify the intermediates generated during 14D degradation by propane-grown cells (Fig. 10).

**Fig 10.** $^1$H NMR spectra of 14D degradation by propane-grown *M. vaccae* JOB5. Propane-grown cells were incubated in presence of 14D (50mM). The Figure shows the $^1$H NMR spectra of the supernatant collected from the reaction after 0, 24, 48 and 96 h, respectively.

At least 3 groups of new resonance peaks distinct from the peak of 14D were detected during 14D degradation. Based on the chemical shift and coupling of these new peaks, the products of 14D degradation by *M. vaccae* JOB5 are not ethylene glycol, glycolic acid, or oxalic acid. These are the major metabolites of 14D catabolism by the ether-degrading fungus *Cordyceps sinensis* (46, 47). We hypothesize that the product could be 2-hydroxyethoxyacetic acid (2-HEAA) that was recently identified by HPLC during 14D degradation by *Pseudonocardia* sp. strain ENV478 (63). Future experiments should compare the results of our experiment with the resonances obtained from a commercial standard of 2-HEAA.
Other alternative approaches could also be applied to examine the biodegradation of THF and other cyclic ethers. For example, $^{13}$C-labeled substrates could be used to enable $^{13}$C-NMR to be utilized as an analytical approach. Likewise, $^{14}$C-labeled substrates could also be used to determine the level of mineralization (i.e. $^{14}$CO$_2$ production) from THF and other ethers. However, isotopically labeled compounds are frequently expensive and generally unavailable unless custom synthesized.

In addition to alternative analytical procedures, future experiments should also address other specific questions raised by the present study. For example, it would be interesting to know if growth of *M. vaccae* JOB5 on n-alkanes can also be stimulated by other cyclic ethers such as THP and hexamethylene oxide (HMO). This seems likely as this bacterium grew on the corresponding lactones for these compounds. Additional experiments should also address whether *M. vaccae* JOB5 grows on the corresponding hydroxy acids derived from these lactones. From a more practical approach, it would also be interesting to determine the full range of alkanes that can support the cometabolic degradation of cyclic ethers. Current experiments conducted in the Hyman laboratory suggest *M. vaccae* uses the same alkane-inducible monooxygenase to oxidize n-alkanes from C$_2$ to C$_8$ and this enzyme is also involved in the oxidation of a number of branched alkanes. Longer chain alkanes appear to be oxidized by different monooxygenase that has a much more restricted cosubstrate range. We are particularly interested in the determining which alkanes control expression of ether-oxidizing activity as highly branched alkanes have considerable potential as specific stimulants for *in situ* remediation of cyclic ethers (71).
Table 1: Degradation of cyclic ethers by resting cells of *M. vaccae* JOB5 after growth on alkanes or dextrose-containing medium

<table>
<thead>
<tr>
<th>Cyclic Ether</th>
<th>Growth Substrate</th>
<th>propane</th>
<th>n-butane</th>
<th>n-pentane</th>
<th>isobutane</th>
<th>isopentane</th>
<th>dextrose</th>
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<tbody>
<tr>
<td>trimethylene oxide</td>
<td>-</td>
<td>24.9a</td>
<td>≤1.0</td>
<td>39.6b</td>
<td>38.3</td>
<td>36.6</td>
<td>≤1.0</td>
</tr>
<tr>
<td>tetrahydrofuran</td>
<td>+</td>
<td>3.5</td>
<td>100.0</td>
<td>3.5</td>
<td>5.8</td>
<td>17.3</td>
<td>4.8</td>
</tr>
<tr>
<td>tetrahydropyran</td>
<td>-</td>
<td>92.0a</td>
<td>≤1.0</td>
<td>89.0</td>
<td>100.0</td>
<td>99.2</td>
<td>≤1.0</td>
</tr>
<tr>
<td>1,3-dioxolane</td>
<td>+</td>
<td>2.6</td>
<td>100.0</td>
<td>9.9</td>
<td>4.6</td>
<td>5.9</td>
<td>4.8</td>
</tr>
<tr>
<td>1,3-dioxane</td>
<td>-</td>
<td>50.3</td>
<td>≤1.0</td>
<td>51.9b</td>
<td>83.1</td>
<td>67.4</td>
<td>14.2</td>
</tr>
<tr>
<td>1,4-dioxane</td>
<td>+</td>
<td>8.6</td>
<td>57.2b</td>
<td>15.3b</td>
<td>1.1</td>
<td>3.5</td>
<td>13.4</td>
</tr>
</tbody>
</table>

C: amount of substrate remaining after incubation for 2 h.

b: std deviation >10%

c: std deviation >20%


Fig 1. Time course of tetrahydrofuran degradation by propane-grown *Mycobacterium vaccae* JOB5.

Propane-grown cells (~1 mg total protein) were incubated in the presence of THF (~2 mM) in reaction vials (10 ml), as described in the Methods section. At the indicated times, samples (2 µl) were removed from the reaction vials and were analyzed by GC. The Figure shows the time course for THF consumption (circles) and production of γBL (squares) for cells incubated in the presence (open symbols) and absence (solid symbols) of acetylene (10% vol./vol. gas phase). The plotted data are the mean and the range for 2 replicate experiments.
Fig 2. Effect of propane on THF oxidation by propane-grown *M. vaccae* JOB5.

Reaction vials (10 ml) containing THF (1 mM) and varying amounts of propane were prepared as described in the Methods section. The reactions were initiated by the addition of cells (~1.1 mg total protein) and after 20 min the remaining THF in each reaction was determined by GC analysis. The Figure plots show the percent inhibition of THF oxidation as a function of dissolved propane concentration. The data plotted are the mean and range from 2 separate experiments. The data were computer-fitted to a single site binding model as described in the Methods section.
Fig 3. Time course of 1,4-dioxane degradation by propane-grown *M. vaccae* JOB5.

Propane-grown cells of *M. vaccae* (~1.2 mg total protein) were incubated with 14D (1 mM) in reaction vials (10 ml), as described in the Methods section. At the indicated times, samples (2 µl) were removed from the reaction vials and were analyzed by GC. The Figure shows the mean and range of data for cells from 2 replicate cultures incubated with 14D in the (▲) presence and (■) absence of acetylene (10% vol./vol. gas phase).
Fig 4. Time course of 3-hydroxytetrahydrofuran (3-HTFT) degradation by propane-grown *M. vaccae* JOB5.

Propane-grown cells of *M. vaccae* JOB5 (~0.8 mg total protein) were incubated with 3HTHF (1 mM) in reactions vials (10 ml), as described in the Methods section. The Figure shows the time course for 3HTHF consumption for cells incubated in the (■) presence and (□) absence of acetylene (10% vol./vol. gas phase. As indicated in the text, an unidentified product was detected in reactions containing acetylene. The Figure shows a plot of (●) integrator peak height of this product versus time. The data presented in the Figure are the mean and range of for cells from 2 replicate cultures.
Fig 5. Time course of γ-butyrolactone degradation by propane-grown *Mycobacterium vaccae* JOB5.

Propane-grown cells of *M. vaccae* JOB5 (~0.48 mg total protein) were incubated in the presence of γBL (1mM) in reactions vials (10 ml), as described in the Methods section. At the indicated times, samples (2 µl) were analyzed for residual γBL by GC. The Figure shows the time course for γBL consumption by cells incubated in the (□) presence and (■) absence of acetylene (10% vol./vol. gas phase) and for (●) heated treated cells (~95°C for 15 min) and (○) reaction vials containing no cells. The data plotted in the Figure show the mean and range for cells from 2 replicate cultures.
Fig 6. Effect of tetrahydrofuran and 1,4-dioxane on growth of *M. vaccae* JOB5 on n-pentane.
Cultures of *M. vaccae* JOB5 were grown on n-pentane in the presence of absence of THF or 14D, as described in the Methods section. **Panel A:** the Figure shows the time course of substrate consumption for cultures grown on n-pentane in the presence or absence of THF. The square symbols represent the percent of initial n-pentane remaining in cultures incubated (□) with and (■) without THF. The circular symbols represent the percent initial THF remaining in cultures incubated (●) with and (○) without n-pentane. The diamond symbol (◆) represents the ratio of γBL generated to THF consumed for cultures containing both n-pentane and THF. The triangular symbols represent the percent initial (▲) THF and (△) n-pentane in uninoculated control cultures. **Panel B:** the Figure shows the corresponding changes in optical density (OD$_{600}$) for the cultures described in Panel A: (○) cultures with n-pentane alone, (■) cultures with n-pentane and THF, (♦) cultures with THF alone and (■) uninoculated control cultures with n-pentane plus THF. **Panel C:** the Figure shows the time course of substrate consumption for cultures grown on n-pentane in the presence of absence of 14D. The Figure shows the percent of initial n-pentane remaining in cultures incubated (◆) with and (■) without 14D and the percent of initial 14D remaining in cultures incubated (♦) with and (●) without n-pentane. The Figure also shows the percent initial (○) 14D and (□) n-pentane in uninoculated control cultures. In all cases the data presented represent the mean and SEM of 3 replicate cultures.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Culture density (OD$_{600}$)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>at 7d</td>
<td>at 14d</td>
</tr>
<tr>
<td>trimethylene oxide</td>
<td>≤0.01</td>
<td>≤0.01</td>
</tr>
<tr>
<td>tetrahydrofuran</td>
<td>≤0.01</td>
<td>≤0.01</td>
</tr>
<tr>
<td>tetrahydropyran</td>
<td>≤0.01</td>
<td>≤0.01</td>
</tr>
<tr>
<td>propiolactone</td>
<td>≤0.01</td>
<td>&lt;0.1 (0.02)</td>
</tr>
<tr>
<td>γ-butyrolactone</td>
<td>&lt;0.01</td>
<td>&lt;0.1 (0.01)</td>
</tr>
<tr>
<td>valerolactone</td>
<td>&lt;0.1 (0.01)</td>
<td>0.21 (0.07)</td>
</tr>
<tr>
<td>caprolactone</td>
<td>0.15 (0.02)</td>
<td>0.27 (0.04)</td>
</tr>
<tr>
<td>4-hydroxybutyric acid</td>
<td>0.41 (0.01)</td>
<td>0.22 (0.05)</td>
</tr>
<tr>
<td>1,3-propanediol</td>
<td>≤0.01</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>1,4-butanediol</td>
<td>≤0.01</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>1,5-pentanediol</td>
<td>≤0.01</td>
<td>&lt;0.1</td>
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</tbody>
</table>
REFERENCES


