ABSTRACT

KOTTEGODA, W. G. SAMANTHI SUBASHI. Alkene and Alkane Oxidation by the 2-Methylpropene-Metabolizing Strain *Mycobacterium* sp. ELW1. (Under the direction of Dr. Michael R. Hyman).

Microbial degradation of short chain aliphatic alkenes such as ethene and propene has been studied extensively during the last decades. But little is known about the metabolism of other straight chain alkenes and no reports are available on the metabolism of branched chain alkanes and alkenes, especially the simplest ones. The main purpose of this study was to investigate the microbial degradation of the simplest branched-alkene, 2-methylpropene (isobutylene). No organisms have previously been isolated on 2-methylpropene.

Our first study describes the isolation and physiological characterization of a novel alkene-oxidizing bacterium, *Mycobacterium* sp. strain ELW1 that is capable of utilizing 2-methylpropene as a sole source of carbon and energy. The growth of strain ELW1 was largely restricted to 2-methylpropene, and 2-butene was the only other alkene it grew on. We demonstrated that 1,2-epoxy-2-methylpropane, 2-methyl-1,2-propanediol (MPD) and 2-hydroxyisobutyric acid (HIBA) are sequential intermediates in the pathway of 2-methylpropene metabolism suggesting that oxidation of 2-methylpropene, like many other alkenes, proceeds by an epoxidation reaction catalyzed by an alkene monooxygenase. Our results also indicate that the growth of strain ELW1 on 2-methylpropene is cobalt-dependent, a physiological trait found in methyl *tertiary* butyl ether (MTBE)- and *tertiary* butyl alcohol (TBA)-metabolizing organisms as well. These observations are in agreement with our hypothesis about the possible overlap that exists between 2-methylpropene, MTBE and TBA metabolism in terms of enzymes and likely intermediates.

In the second study, we characterized the metabolism of *cis*- and *trans*-2-butene by strain ELW1. We demonstrated that both 2-butene isomers were metabolized by strain ELW1 *via* an initial epoxidation reaction and followed a similar pathway to 2-methylpropene metabolism. Our results also suggest that the same alkene-monooxygenase and epoxide hydrolase enzymes catalyze the initial reactions of metabolism of all three alkenes while the later steps of 2-butene metabolism involve different enzymes than those involved in 2-methylpropene metabolism. The only reported study on *trans*-2-butene metabolism by *Nocardia* sp. strain TB1 has shown that *trans*-2-butene is metabolized *via* an initial hydroxylation at the C_1 position rather than *via* an initial epoxidation reaction.

Finally, we examined the alkane-oxidizing activity of strain ELW1 and the well-characterized alkene-oxidizing bacterium *Xanthobacter* sp. strain Py2 that have not been previously reported to oxidize alkanes. Our results demonstrated that both organisms can slowly oxidize 2-methylpropane (isobutane), the simplest branch alkane, while generating *tertiary* butyl alcohol (TBA) as a substoichiometric product. Both strains also oxidized other *n*-alkanes, albeit slowly. Our results suggest that both strains might use the same alkene-monooxygenase to initiate the oxidation of alkenes as well as alkanes.

Alkene and Alkane Oxidation by the 2-Methylpropene-Metabolizing Strain *Mycobacterium* sp. ELW1

by
W. G. Samanthi Subashi Kottegoda

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

Microbiology

Raleigh, North Carolina

2014

APPROVED BY:

_____	_____
Dr. Michael R. Hyman Chair of Advisory Committee	Dr. Amy M. Grunden
_____	_____
Dr. Hosni M. Hassan	Dr. Eric S. Miller

DEDICATION

I would like to dedicate this work to my parents, my husband and my kids

for their endless love, support and encouragement…

BIOGRAPHY

Samanthi Kottegoda was born and raised in Colombo, Sri Lanka. She earned her Bachelor's degree in Microbiology from University of Kelaniya, Sri Lanka. In 1999, she came to the United States with her husband and had the opportunity to attend University of Illinois at Chicago where she obtained her Master's degree in Chemistry. After moving to North Carolina with her family in 2007, she decided to continue her graduate studies and joined the Department of Microbiology at North Carolina State University in Fall 2008 where she pursued her Ph.D. under the direction of Dr. Michael Hyman.

ACKNOWLEDGMENTS

I am deeply indebted to many people who made this dissertation possible. First and foremost, I would like to thank my advisor, Dr. Michael Hyman, for giving me the opportunity to work in his lab and for his unwavering support and guidance over the past few years. His patience, thoughtfulness and encouragement have been of the utmost importance along the path of completing my degree. My sincerest gratitude to him cannot be adequately put into words.

I would also like to extend my sincere gratitude my committee members, Drs. Amy Grunden, Hosni Hassan and Eric Miller for their invaluable insight, comments and suggestions and most importantly for their willingness to help whenever I may happen to drop by their office. Also, I am very thankful to Dr. Reza Ghiladi, for his willingness to serve on my committee as a Graduate College representative. Many thanks to Dr. Jim Brown for helping me to construct the phylogeny tree for strain ELW1.

I owe a huge amount of appreciation to my lab mates, past and present; Dr. Denise Aslett, Dr. Alan House, Christy Smith, and Kristen Bennett, for providing me advice, support and friendship throughout these years. I would also like thank Cindy Whitehead and Robert Davis, who have always given me a helping hand whenever I needed help. Also I would like to thank T.J. Schneeweis for guiding me through the teaching of general microbiology lab. And, many thanks to the entire Microbiology program for the research advice, support, and for all of the smiles.

Finally, I would like thank my family to whom I owe a huge debt of gratitude. Especially my parents, for their endless love, encouragement and support thoughout my life

and all the prayers and blessings they have sent my way along this journey. And, thank you to my brother for all his support, sacrifices and encouragement. I would also like to thank my son and daughter for being my inspiration. They made me stronger, better and more fulfilled than I could have ever imagined. Last but not least, I would like to thank my husband, Sumith, for his unconditional love and support that carried me through many obstacles. He has sacrificed so much over the past years to let me pursue my dream. Thank you so much for believing in me and being there for me all these years!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: Literature Review

CHAPTER 2: Isolation and characterization of a novel 2-methylpropene-metabolizing strain *Mycobacterium* sp. ELW1

CHAPTER 3: Metabolism of 2-butene by *Mycobacterium* sp. ELW1

CHAPTER 4: Alkane oxidizing activity of *Mycobacterium* sp. ELW1 and *Xanthobacter* sp. Py2

LIST OF ABBREVIATIONS

AMO	alkene monooxygenase
C2B	*cis*-2-butene
CoM	coenzyme M (2-mercaptoethanesulfonate)
EaCoMT	epoxyalkane:CoM transferase
GC	gas chromatography
3HB	3-hydroxybutyric acid
HCM	2-hydroxyisobutyryl-CoA mutase
HIBA	2-hydroxyisobutyric acid
HIBAL	2-hydroxyisobutyraldehye
2M1P	2-methyl-1-propanol
MMO	methane monooxygenase
MPD	2-methyl-1,2-propanediol
MSM	mineral salt medium
MTBE	methyl *tertiary* butyl ether
T2B	*trans*-2-butene
TBA	*tertiary* butyl alcohol
TBF	*tertiary* butyl formate
OD_{600}	optical density at 600 nm

CHAPTER 1

Literature Review

1) Alkenes

1.1 Background and Significance

Alkenes (olefins) are unsaturated hydrocarbons containing carbon-carbon double bonds that make them more reactive than their alkane counterparts. The lower alkenes, ethene, propene, and butene, are colorless gases at room temperature, while alkenes with 5 – 16 carbons are liquids and alkenes with more than 16 carbons are waxy solids. All alkenes with 4 or more carbon atoms show structural isomerism and the presence of double bonds give rise to geometric isomerism (e.g.; *cis*- and *trans*-2-butene). Alkenes are relatively nonpolar and therefore are virtually insoluble in water.

Large quantities of gaseous alkenes are produced annually from biogenic and anthropogenic processes (122, 124, 126). The most predominant of these compounds are the gaseous plant hormone ethene (116), the foliage volatile isoprene (117, 125) and various monoterpenes that are present in plant oils. Many unsaturated hydrocarbons, especially the lower gaseous alkenes ethene, propene, 1, 3-butadiene and butenes are also produced chemically on a large scale and inevitably these compounds are partly released into the environment. The increased atmospheric concentrations of these compounds not only impart a serious risk to human health but also threaten to destabilize ecosystems through a variety of mechanisms. The toxicity of aliphatic alkenes arises from their ability to readily serve as substrates for broad specificity oxygenases such as cytochrome P450 enzymes resulting in the formation of epoxides. It is these epoxides which pose a bigger problem than the alkenes due to their high reactivity. Epoxides, being electrophiles, can react abiotically with cellular nucleophiles such as proteins and DNA and form covalent adducts thereby leading to

mutations and cancer formation (93). Interestingly, some microorganisms are able to grow on aliphatic alkenes and are thought to play an important role in the remineralization of this carbon source (42, 70, 126) (Fig. 1.1).

Organisms use two basic strategies to undermine the potentially toxic effects of epoxides within the cell, 1) transformation of epoxides to less toxic products using detoxification enzymes or 2) productive metabolism of the epoxides formed allowing them to be utilized as a primary carbon and energy source.

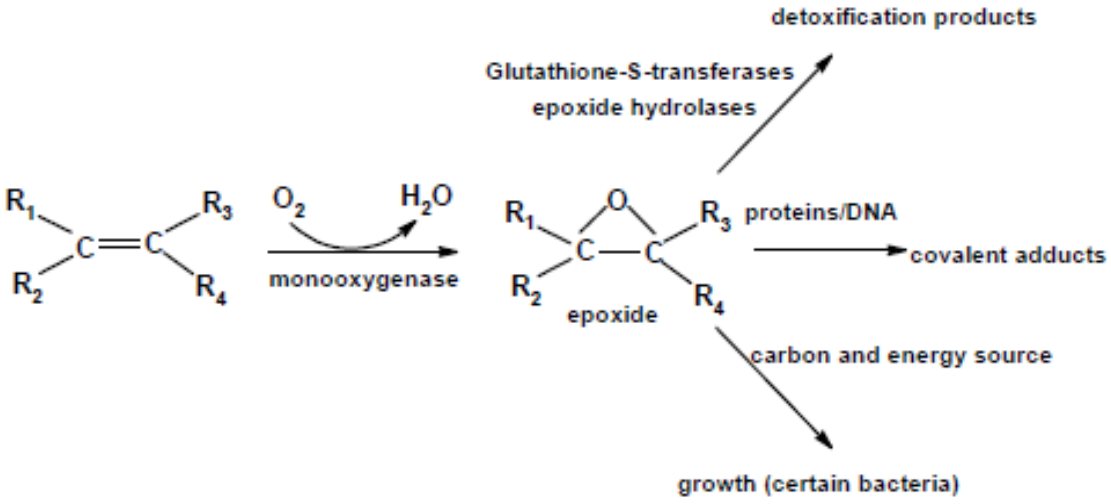


Figure 1-1: Fates of epoxides formed from short chain alkanes.
(Reference: Ensign, S. A.; *Microbial Metabolism of Aliphatic Alkenes*, Biochemistry, 2001. **40**, 5845-53)

Most organisms are not capable of using alkenes or the corresponding epoxides as primary energy sources and instead possess detoxification enzymes that allow the conversion of epoxides to less toxic products, specifically glutathione-S-transferases (GSTs) (114) and epoxide hydrolases (144) that use glutathione or water respectively as nucleophiles to attack and open the oxirane ring (Fig. 1.2a & 1.2b).

Bacteria that are capable of metabolizing epoxides, employ both conventional (hydration or glutathione conjugation) and nonconventional strategies (i.e. isomerization) to convert epoxides to central metabolites. For example, bacteria that grow on epoxypropane or

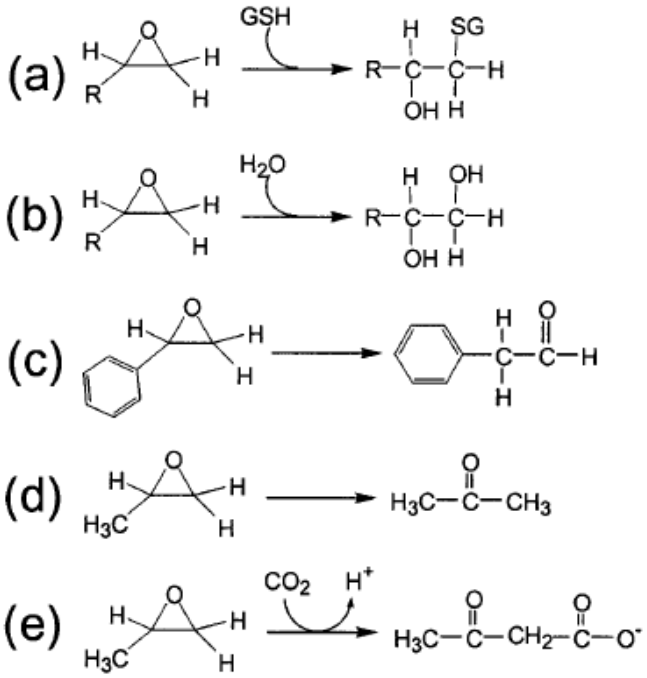


Figure 1-2: Enzymatic transformations of aliphatic and aromatic epoxides: (*a*) glutathione conjugation (*b*) hydration (*c*) isomerization to an aldehyde (*d*) isomerization to a ketone and (*e*) carboxylation to a β-keto acid.
(Reference: Ensign, S. A. and Allen, J. R.*; Aliphatic Epoxide Carboxylation*, Annu. Rev. Biochem, 2003. **72**, 55-76)

epichlorohydrin (3-chloroepoxypropane) use epoxide hydrolases to oxidize epoxides to the corresponding dihydrodiols (37, 86) while bacteria that grow on styrene (phenylethene) metabolize the epoxidation product (phenylepoxyethane) via an isomerization reaction which yields an aldehyde (phenylacetaldehyde) (72, 155) (Fig. 1.2c). It has also been shown that in the absence of CO_2, ketones are formed as products of epoxide degradation in whole-cell suspensions (128) as well as in cell extracts of *Xanthobacter* strain Py2 (161) (Fig. 1.2d). The most unconventional of these methods involves carboxylation of the epoxide to a β-keto acid as for example in *Xanthobacter* strain Py2 (Fig. 1.2e).

Despite the detrimental effects exerted by aliphatic alkenes and epoxides, several bacteria have been identified that are able not only to detoxify these compounds, but are able to convert them to non-reactive central metabolites while fixing a molecule of CO_2 in the process (42). They are capable of growth under aerobic conditions utilizing short-chain aliphatic alkenes (C_2-C_6), ethene, propylene, 1-butene, butadiene, isoprene, vinyl chloride, etc. as the sole source of carbon (42, 70). The majority of the isolates are Gram-positive bacteria belonging to the actinomycete genera *Corynebacterium, Mycobacterium, Nocardia* and *Rhodococcus* (the CMNR group) or strains of the Gram-negative genera *Xanthobacter, Pseudomonas* and *Alcaligenes.* Some alkene oxidizing bacteria are restricted to growth on a single alkene, while others exhibit less selectivity for the growth substrates. For example, in 1976 both Heyer (74) and de Bont (33) reported the isolation of several *Mycobacterium* strains capable of growth on ethene as sole carbon and energy source. Using propene or 1-butene as the carbon source in enrichment cultures, van Ginkel and coworkers (151, 152) in most cases isolated *Xanthobacter* strains. These *Xanthobacter* strains along with *Nocardia*

H8 that was isolated with 1-hexene as a carbon source were all capable of growth on ethene as well. Growth of microorganisms on propene was first reported by Cerniglia et al. (21) and later by de Bont et al. (36). Enrichment cultures with *trans*-2-butene as the carbon source resulted in three bacterial isolates, two strains of the genus *Nocardia* and one strain of *Mycobacterium* (152). Utilization of butadiene has been reported by Watkinson and Somerville who isolated a *Nocardia* sp. strain from enrichments with butadiene as the sole carbon and energy source (160). van Ginkel et al. showed that pure cultures of several *Nocardia* sp. strains were able to use both 1,3-butadiene and isoprene (2-methylbutadiene) as a sole source of carbon and energy (152, 153). Another isoprene-utilizing bacterium, *Rhodococcus* AD45, was isolated from a freshwater- sediment by van Hylckama-Vlieg and coworkers (155). In addition to that, Cleveland and Yavitt have provided strong evidence for a biological sink for atmospheric isoprene in soil (24, 25). Moreover, an early study has shown that vinyl chloride (chloroethene), the simplest chlorinated ethylenic compound, was aerobically utilized by *Mycobacterium* L1 as a sole carbon and energy source (71). It also grew on ethene. More recently, strains of *Mycobacterium* (27, 69), *Nocardioides* (27) and *Pseudomonas* (157, 158) that are capable of growth on both ethene and vinyl chloride have been discovered.

The two best characterized alkene-oxidizing bacteria, Gram-negative *Xanthobacter autotrophicus* strain Py2 (149) and Gram-positive *Rhodococcus rhodochrous* (formerly *Nocardia corallina*) strain B-276 (60), which have been isolated with propene as the source of carbon and energy, are two nutritionally versatile organisms capable of growing with a variety of carbon sources (42). For example, strain Py2 can also utilize ethene, 1-butene, 2-

butene, 1-pentene and 1-hexene (151, 152) as well as several other carbon sources including alcohols, ketones, aldehydes, diols, sugars, organic acids and H_2 plus CO_2 (149).

1.2 Microbial Oxidation of Alkenes

All alkene-oxidizing microorganisms isolated to date are aerobic and initiate alkene oxidation by a monoxygenase reaction. This is the first reaction where the alkene is converted to an epoxide and is carried out by O_2- and reductant-dependent monoxygenases called alkene monoxygenases (AMO).

1.2a Epoxidation of Alkenes

Oxygenases: Oxygenases are ubiquitous in nature and play an important role in the metabolism of a broad range of compounds (19, 145). There are two types of oxygenases namely, monooxygenases and dioxygenases. Monooxygenases catalyze the incorporation of one atom of molecular oxygen (O_2) into the substrate while the other atom is reduced to water. These enzymes generally use NADH or NADPH cofactors to provide reducing potential for the supply of electrons to the substrate and can be metal-, haem- or flavin-dependent (18, 19, 145). In dioxygenase-reactions both atoms from a single molecule of O_2 are incorporated into the organic substrate (13, 16, 19, 145). Dioxygenases include two major classes: haem-dependent iron sulphur dioxygenases and Rieske iron-sulphur non-haem dioxygenases, the majority of which are NADH dependent (19, 159).

1.2b Alkene Monooxygenases (AMO): Alkene monooxygenases (AMO) catalyze the conversion of alkenes to their corresponding epoxides as the initial step of oxidation. The most characterized AMOs are from two propene-oxidizing bacteria: *Rhodococcus rhodochrous* strain B276 (102) and *Xanthobacter autotrophicus* strain Py2 (129). These enzymes are multi-component enzymes that catalyze epoxidation of alkenes in an NADH-dependent fashion as shown in Figure 1.3.

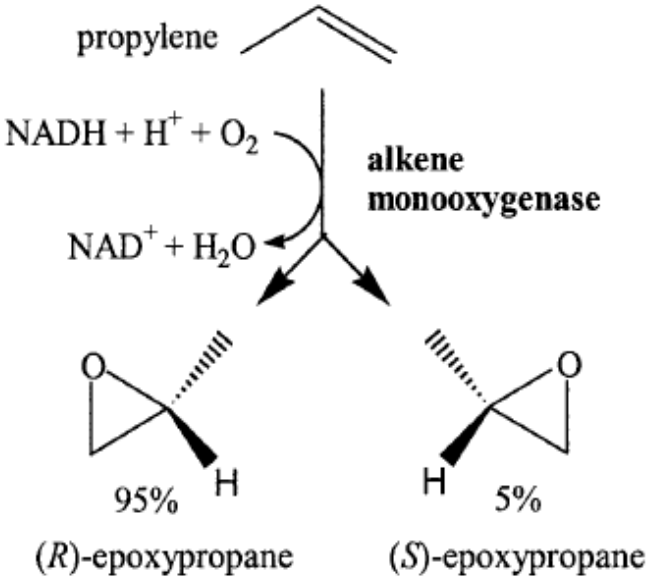


Figure 1-3: NADH-dependent epoxidation of propene by alkene monoxygenase.

(Reference: Ensign, S. A. and Allen, J. R.*; Aliphatic Epoxide Carboxylation*, Annu. Rev. Biochem, 2003. **72**, 55-76)

AMO from *R. rhodochrous* strain B-276 is a three-component enzyme consisting of an epoxygenase, a NADH-reductase and a coupling protein. The 95 kDa epoxygenase is composed of two subunits of 53- and 35-kDa (arranged in an αβ quaternary structure), and contains approximately 2 mol non-haem iron per mol protein. It is considered to be the catalytic component of this enzyme. The reductase is an iron-sulfur flavoprotein of 40 kDa which is responsible for the transfer of reducing equivalents from NADH to the epoxygenase. The coupling protein is a single-subunit protein of 14 kDa not containing any prosthetic groups and appears to function as a regulator of activity (102) (Fig.1.4A). In contrast, AMO from *Xanthobacter* Py2 is composed of four, rather than three components as follows; an epoxygenase, a NADH-reductase, a Rieske-type ferredoxin and an effector protein. The 212 kDa epoxygenase consists of four atoms of non-haem iron and three subunits of 38, 58 and 10 kDa, respectively arranged in a $\alpha_2\beta_2\gamma_2$ quaternary structure, which contains the catalytic center for alkene epoxidation. The 35.5 kDa monomeric reductase is an iron-sulfur flavoprotein (containing FAD and a 2Fe-2S cluster) which provides the reductant for O_2 activation. The 13 kDa homodimeric ferredoxin (containing two Rieske-type 2Fe-2S clusters) is thought to be an intermediate electron carrier between the reductase and terminal catalytic component of the system. And the 11 kDa small protein has no known function (129) (Fig. 1.4B).

The evidence from biochemical and spectroscopic studies show some fundamental differences between these two enzyme systems. For instance, AMO of strain B-276 does not contain an electron-transferring ferredoxin as in AMO of strain Py2. Also, the subunit stoichiometry and quaternary structure of the oxygenase components are very different (two

subunits in *R. rhodochrous* B-276 AMO versus three subunits in *Xanthobacter* Py2 AMO). Although the quaternary structures are different, they both contain diiron catalytic centers. They have therefore been classified into the class of diiron oxygensases (61, 102, 129). The best characterized enzyme of this family is the soluble methane monoxygenase (MMO) which also contains a binuclear iron center in its hydroxylase component (120). The epoxidation reaction of AMO and the hydroxylation reaction of MMO are carried out at the diiron center, where the binding of oxygen and the activation of the organic substrate occurs.

Available evidence on other alkene monoxygenases suggests that they share some similar features with those of *Xanthobacter* strain Py2 and *R. rhodochrous* strain B-276. For example, genetic analysis of isoprene-utilizing *Rhodococcus* sp. strain AD45 shows that it contains a six genes-encoding four-component AMO with predicted high similarity to the four-component AMO of strain Py2 (156) rather than to the three-component AMO of strain B-276 (102). Also, a strong similarity has been observed between the AMOs of vinyl chloride- and ethene-utilizing strain *Mycobacterium* JS60 and strain B-276 in terms of gene sequence and gene organization (29).

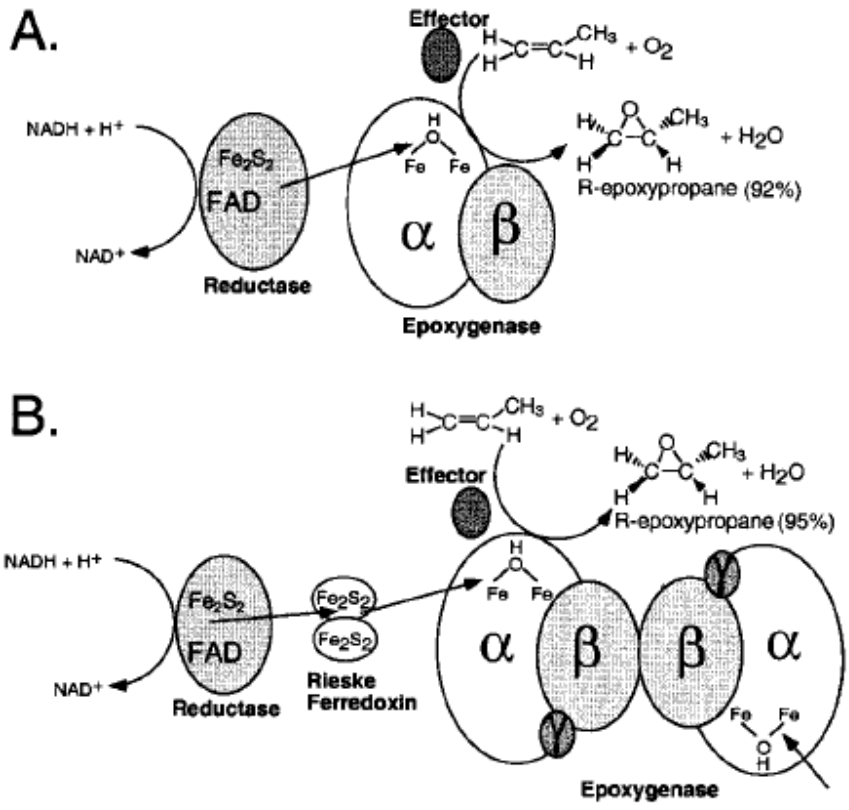


Figure 1-4: Comparison of alkene monooxygenases from (A) *R .rhodochrous* B-276 and (B) *Xanthobacter* sp. strain Py2.
(Reference: Ensign, S. A.; *Microbial Metabolism of Aliphatic Alkenes*, Biochemistry, 2001. **40**, 5845-53)

Xanthobacter Py2 AMO has a broad substrate range for aliphatic alkenes, catalyzing the epoxidation of terminal and internal alkenes varying in chain length from C_2-C_6 (152). Propene-grown *Xanthobacter* cells (strain Py2) also degrade several chlorinated alkenes of environmental concern including trichloroethylene, *cis*- and *trans*-1,2-dichloroethylene, vinyl chloride, 1-chloropropylene, 1,3-dichloropropylene, and 2,3-dichloropropylene (44). AMO

of strain Py2 is an inducible enzyme that is repressed during growth with conventional carbon sources (*i.e.* glucose) and induced upon exposure of cells to a range of aliphatic and chlorinated alkenes and epoxides (41). As opposed to broad-range oxygenases which are not stereoselective, AMOs exhibit a high degree of stereoselectivity for alkene epoxidation; *e.g*. purified AMOs from *Xanthobacter* strain Py2 and *R. rhodochrous* strain B-276 catalyze the oxidation of propene with 95 and 92% yields, respectively, of *R*-epoxypropane (7, 61). These various features make the alkene/epoxide metabolizing system of *Xanthobacter* strain Py2 an attractive one for studying the pathway of aliphatic alkene and epoxide metabolism.

1.2c Metabolism of Epoxides

In contrast to the production of epoxides (epoxyalkanes), little is known about the microbial metabolism of epoxides. As discussed above, an epoxide can be degraded in several different ways such as hydrolysis by an epoxide hydrolase yielding a vicinal diol (47), isomerization into an aldehyde or ketone (in the absence of CO_2) (127, 128) or, alternatively in the presence of CO_2, carboxylating the epoxide leading to the formation of a β-keto acid (3).

Epoxide hydrolase activity has been reported in a few bacteria and according to Ospiran and coworkers, this activity seems to be more associated within the genera *Rhodococcus, Nocardia, Mycobacterium* and *Arthrobacter,* whereas epoxide-isomerization is associated with the *Pseudomonas* family (109). For example, *Nocardia* sp strain A60 grew on 1, 2-epoxypropane with 1, 2-propanediol as an intermediate (37) while a strain of *Pseudomonas putida* catalyzed the hydrolysis of 2-3-epoxysuccinate (8). In studies with

ethene-grown *Mycobacterium* E20 and *Mycobacterium* E44, it was proposed that epoxyethane was converted directly into acetyl-CoA in the presence of NAD^+, CoA and an unknown cofactor (35, 163). But, ethylene glycol was not detected as an intermediate in ethene metabolism.

In general, epoxyalkanes accumulate only when non-growth alkanes are oxidized (152), because the majority of alkene-utilizing bacteria also contain epoxide-degrading enzymes that hydrolyse the product at a much faster rate than the epoxidation step, thus preventing any significant accumulation of epoxyalkanes (65, 149, 151, 152). Meanwhile, epoxide carboxylation appears to be the general strategy for epoxide metabolism in bacteria that grow using aliphatic alkenes as carbon and energy sources.

1.2d Aliphatic Epoxide Carboxylation

One of the most common mechanisms that bacteria employ to metabolize epoxides is carboxylation by utilizing carboxylases (45). Although carboxylases are diverse in terms of their structure, substrate specificity, and cofactor usage, they all have a common feature with regard to their ability to generate a stabilized carbanion for attack on electrophilic CO_2 or activated CO_2 species (91). Different carboxylases use different chemistry to catalyze the carboxylation reaction; *e.g*. Ribulose-1,5-diphosphate carboxylase (RuBisCO) uses general acid-base chemistry along with metal ion catalysis to form the *cis*- enediolate intermediate (23). Metal ion catalysis is also used in the well known enzyme of the glycolysis pathway phosphoenol pyruvate (PEP) carboxylase where the reaction is catalyzed by the formation of the enolate tautamer of pyruvate carbanion when bicarbonate attacks and removes the

phosphate group of PEP (87). Another example of acid-base catalysis is seen in the vitamin K-dependent carboxylases where oxygenation of vitamin K facilitates the formation of a strong base which in turn abstracts a proton from the γ-carbon of glutamate (40). In all these cases it is interesting to note that the substrate itself is a potential nucleophile and the proton abstraction is carried out by the enzyme to generate the carbanion. Contrary to this general scheme, there are carboxylases with unique molecular properties and cofactor requirements which convert aliphatic epoxides to β-keto acids (44, 128, 130).

Alkene metabolism in *X. autotrophicus* strain Py2 and *R. rhodochrous* strain B-276 have been extensively studied with regard to propene metabolism (2, 3, 4, 5, 6, 7, 128). In both organisms, propene metabolism is initiated by the insertion of a single oxygen atom into the olefin bond of propene forming epoxypropane in a stereospecific manner by an alkene monooxygenase (129). The epoxypropane enantiomers are subsequently metabolized by a three-step linear pathway (epoxide-carboxylation) that uses four enzymes and an unusual cofactor, coenzyme M (CoM)/ 2-mercaptoethanesulfonic acid, in the presence of NAD^+, NADPH, and CO_2 to catalyze the net carboxylation of epoxypropane to form the central metabolic acetoacetate (2, 3, 5, 6). This pathway has two interesting features: an unprecedented metabolic conversion of an epoxide to β-ketoacid and the involvement of CoM as a cofactor in the reaction (2). CoM acts as the nucleophile for the epoxide ring opening and as the carrier of hydroxyalkyl- and ketoalkyl-CoM intermediates. Recent studies of ethene- and vinyl chloride-utilizing bacteria have shown that CoM serves as the cofactor for the utilization of these short-chain alkenes as well (28, 29, 99). For these strains, epoxyalkane:CoM transferase (EaCoMT) forms hydroxyethylthioether conjugates which are

believed to undergo subsequent dehydrogenation and conversion to acetyl-CoA rather than carboxylation (29). Other than its role in propene, ethene and vinyl chloride metabolism, CoM was only known previously for its role in methanogenic archaea, where it serves as the methyl group carrier in the terminal reactions in methanogenesis (142, 143, 166).

In contrast to the AMOs of *Xanthobacter* Py2 and *R.rhodochrous* B-276 which differ significantly in composition (Fig. 1.4), the four protein components of epoxide carboxylase, designated I, II, III and IV, are virtually indistinguishable from a biochemical standpoint (*i.e*. subunit composition, molecular weights, cofactors, specific activities, substrate range) in these two bacteria (5, 7).

As shown in Fig.1.5, the first step of epoxide-carboxylation pathway is the transfer of CoM to form a 2-hydroxypropylthioether conjugate. This step is catalyzed by the enzyme epoxypropane: CoM transferase (EaCoMT) (component I) which can use either *R*- or *S*-epoxypropane as a substrate forming the corresponding *R*- or *S*-hydroxypropylthioether (hydroxypropyl-CoM) (2). In the next step, two stereospecific dehydrogenases, *R*- and *S*-hydroxypropyl-CoM dehydrogenases (*R*-HPCDH and *S*-HPCDH) (components III & IV respectively) catalyze the NAD^+-dependant dehydrogenation of *R*- and *S*-hydroxypropyl-CoM (*R*- or *S*-HPC) to a common achiral product, 2-ketopropyl-CoM (2-KPC) (7). *R*-HPCDH and *S*-HPCDH are highly specific for their respective substrates, exhibiting only 0.5 to 1.0% activity with the opposing enantiomer (2). In the final step, 2-ketopropyl carboxylase/reductase (2-KPCC) (component II) catalyzes the NADPH-dependent reduction, cleavage and carboxylation of 2-KPC, forming acetoacetate and CoM. CoM is thus unchanged and recycled for the next round of reactions (22).

Whereas reductive cleavage and carboxylation of the substrate is the primary physiologically relevant reaction catalyzed, in the absence of CO_2, an isomerization of the epoxide occurs resulting in the corresponding ketone. However, these ketones are not further metabolized, suggesting that they are not the physiological products of epoxide conversion. (127,128). The epoxide-converting enzyme is thus both epoxide isomerase and carboxylase, the nature of the reaction depending on the availability of the cosubstrate CO_2.

The biochemical properties and amino acid sequences of the four epoxide carboxylase components (designated components I-IV), together with initial mechanistic studies, have suggested possible roles for the individual components in catalysis and allowed the formulation of a plausible catalytic cycle for the overall reaction. Component I is a homohexameric (42 kDa subunit) zinc-containing protein that is believed to contain the epoxide binding and activation site(s). Component II is a homodimeric (57 kDa subunit) flavoprotein with NADPH: disulfide oxidoreductase activity that is believed to reduce a disulfide on component I, generating a reduced thiol serving as a nucleophile for attacking and opening the epoxide ring. Components III and IV are small (26 and 25.4 kDa subunits, respectively) homodimeric proteins with no detectable organic or inorganic cofactors. The sequences of components III and IV are homologous to NAD^+-dependent dehydrogenases, suggesting that they may be involved in NAD^+ reduction and hydride abstraction from the epoxide substrate (4, 5).

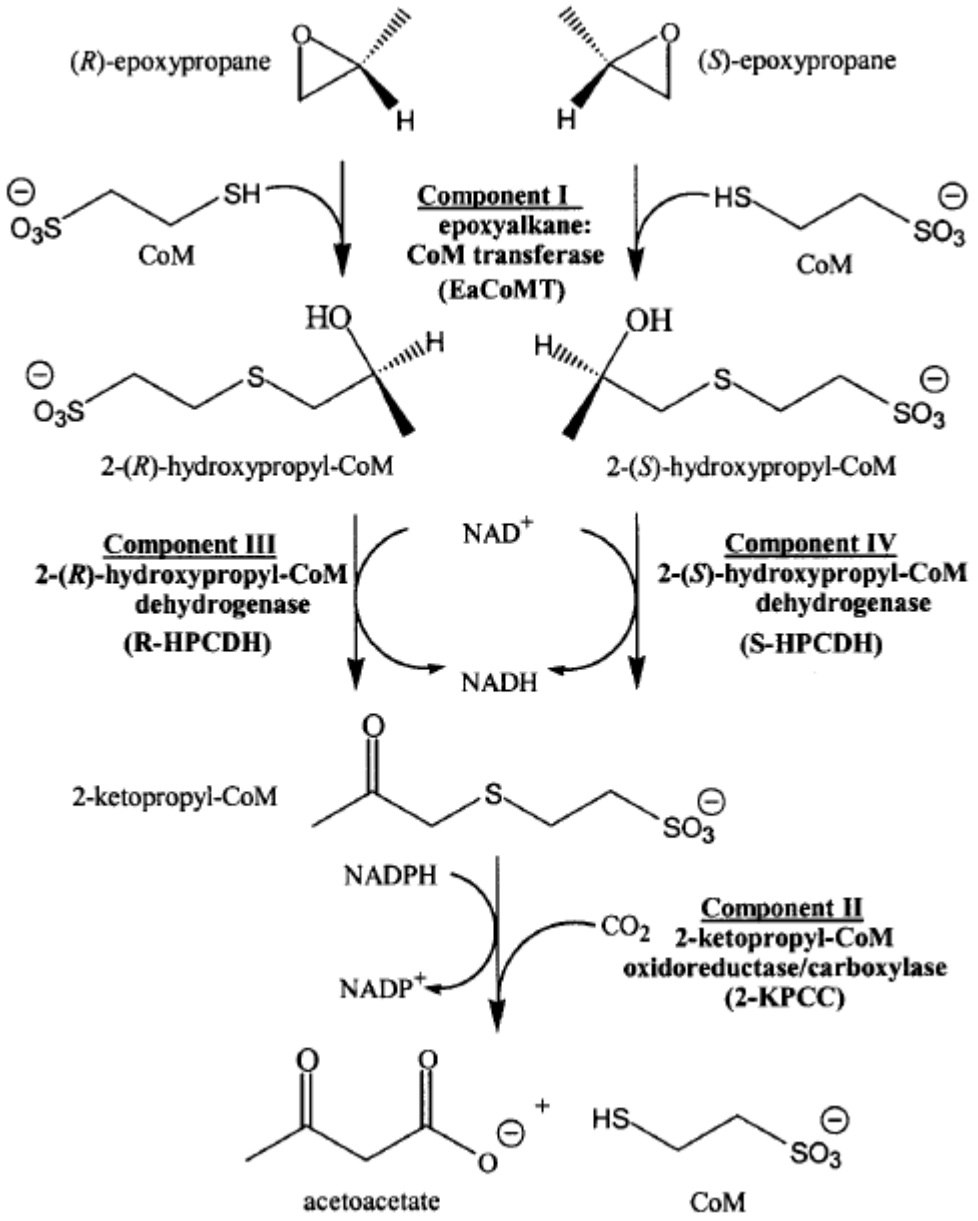


Figure 1-5: Pathway and enzymes of CoM-dependent epoxypropane carboxylation. (Reference: Ensign, S. A. and Allen, J. R.*; Aliphatic Epoxide Carboxylation*, Annu. Rev. Biochem, 2003. **72**, 55-76)

The involvement of stereospecific dehydrogenases in aliphatic epoxide metabolism was first demonstrated by Allen and Ensign (7). They reported that components I and II are required for the utilization of both enantiomers of epoxypropane, while component III and IV confer specificity for utilization of the R- and S-enantiomers, respectively. On the basis of these observations, and sequence analyses of components III and IV, these components are proposed to belong to the family of short-chain dehydrogenases and to impart specificity for abstraction of C2 hydrides from chiral secondary alcohol intermediates formed in the reaction cycle.

1.2e Inhibition of Epoxide-Carboxylase Activity

In an attempt to identify a specific inactivator of the epoxide-carboxylase system for *Xanthobacter* strain Py2, Allen and Ensign (4) recognized 1,2-epoxy-2-methylpropane (isobutylene oxide) as an excellent candidate for such an inactivator. It was used to identify and characterize the active-site-containing component of the system. 1,2-Epoxy-2-methylpropane, which differs from epoxypropane in containing a methyl rather than a hydrogen substituent on the C2 carbon atom, was characterized as a time-dependent, irreversible inactivator of epoxide carboxylase activity in both *Xanthobacter* Py2 (4) and *R.rhodochrous* B-276 (6) strains. It acts as a mechanism-based inactivator of component I (EaCoMT). A catalytic mechanism proposed for epoxide isomerization by Weijers and coworkers, involves a nucleophilic attack of a sulfhydryl on the C1 carbon atom of a terminal epoxide, leading to ring opening and the formation of a β-hydroxythioether, followed by abstraction of a hydride from the C2 carbon, yielding a β-ketothioether intermediate (Fig.

1.6A) (161). In contrast, reaction of 1,2-epoxy-2-methylpropane in a similar fashion would lead to the formation of a methyl-substituted β-hydroxythioether that could not react further due to the presence of the methyl group on the C2 carbon (Fig. 1.6B) (4).

In 2006, Boyd and coworkers have reported that 2-bromoethanesulfonate (BES) was able to inhibit bacterial growth on and metabolism of propene by both *Xanthobacter* strain Py2 and *R. rhodochrous* strain B-276 (15). BES is a structural analog of CoM and a potent inhibitor of methanogenic growth and methanogenesis (11, 12, 64, 135), the only other previously recognized CoM-dependent process. The site of action of BES has recently been shown to be the 2-KPCC component (component II) of the epoxide carboxylase complex from strain Py2 (14).

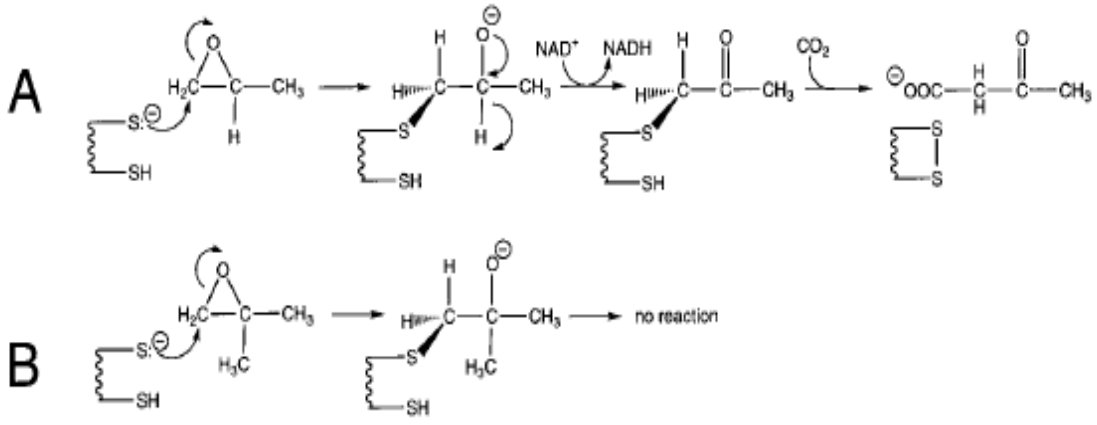


Figure 1-6: Proposed mechanisms for epoxide carboxylase-catalyzed epoxide conversions; (A) epoxypropane, (B) 1,2-epoxy-2-methylpropane

(Reference: Allen J. R and Ensign, S. A.*; Characterization of Three Protein Components Required for Functional Reconstitution of the Epoxide Carboxylase Multienzyme Complex from Xanthobacter Strain Py2*, Journal of Bacteriology, 1997a. **179**, 3110-3115)

1.3 Microbial Oxidation of Alkanes and Alkenes

A major difference between alkane- and alkene- metabolizing bacteria is the substrate specificity towards hydrocarbons of the monooxygenases responsible for their initial oxygenation. In general, resting cells of alkane-grown bacteria are able to hydroxylate alkanes and to epoxidize as well as hydroxylate alkenes (75, 77, 78, 80, 112) whereas the cells of alkene-grown bacteria are only able to epoxidate alkenes (34, 65, 151). In both cases, the oxidation is initiated by the incorporation of one atom from molecular oxygen into the hydrocarbon molecule, a reaction catalyzed by a monooxygenase enzyme. Alkanes are oxidized to the corresponding primary or secondary alcohols while alkenes are oxidized to the corresponding epoxyalkanes/epoxides.

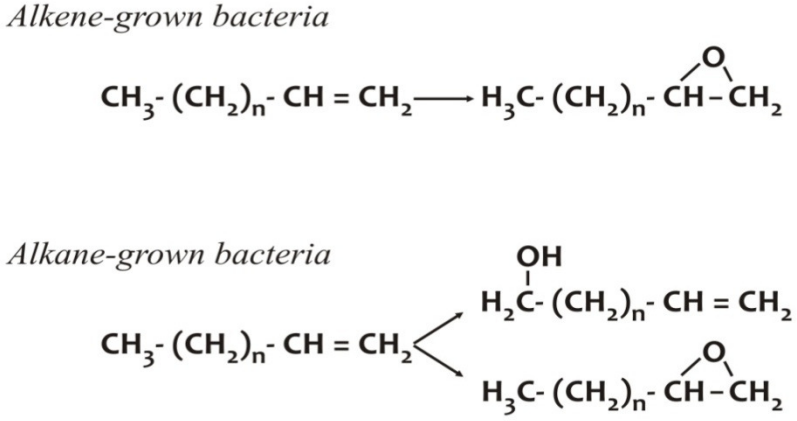


Figure 1-7: General modes of oxidative attack of alkenes by alkene- and alkane-grown bacteria.
(Reference: Hartmans, S., de Bont, J. A. M., Harder, W.*; Microbial Metabolism of short-chain unsaturated hydrocarbons*, FEMS Microbiology Reviews, 1989. **63**, 235-264)

Alkane monooxygenases have broad substrate ranges and can fortuitously catalyze the epoxidation of aliphatic alkenes in an identical manner to the reaction shown for alkene monooxygenase (Fig.1.7) (26, 38, 167). In contrast, alkene monooxygenases have more restricted substrate specificity and are generally incapable of catalyzing the hydroxylation of saturated hydrocarbon substrates and in this respect alkene-utilizing bacteria are exceptional (34).

1.3a Oxidation of Alkenes by Alkane-Utilizers

Alkene oxidation by either alkane- or alkene-grown cells very often resulted in the formation and excretion of epoxides. Epoxide formation by alkane-utilizers is due to the non-specific action of an alkane-hydroxylase that is able to form alcohols from alkanes and epoxides from alkenes. Examples for such organisms are the resting cells of methane- and other alkane-grown bacteria that form epoxides from alkenes as a consequence of either the inability of these bacteria to degrade epoxides (78) or of a negligible oxidation rate of epoxides (112). The epoxidation of 1-alkenes by alkane-grown bacteria was first demonstrated by van der Linden (148) who detected the formation of 1, 2-epoxyoctane from 1-octene by heptane-grown *Pseudomonas aeruginosa*. Several other authors have confirmed this later on by using liquid *n*-alkane-utilizing bacteria. For instance, Cardini and Jurtshuk (20) found that a cell extract of *Corynebacterium* sp. (strain 7E1C) oxidized 1-octene to 1, 2-epoxyoctane in addition to hydroxylating 1-octane to 1-octanol. The epoxidation of 1-octene by whole cells and a purified monooxygenase system of *Pseudomonas oleovorans* grown on *n*-octane have also been reported (1, 100). But, none of these epoxidation systems were

found to be active on gaseous alkenes. Resting cells of bacteria grown on gaseous alkanes (C_2-C_4), however, were able to catalyze the epoxidation of ethene, propene, 1-butene, 1, 3-butadiene and 1-pentene, and epoxide-formation by such bacteria has been observed frequently (80, 112). Methane-utilizing bacteria also catalyzed the epoxidation of gaseous alkenes (75, 76, 77, 139).

1.3b Oxidation of Alkenes by Alkene-Utilizers

The formation of 1, 2-epoxyalkanes has also been studied with alkene-utilizing bacteria. For example, Furuhashi et al. found that *Nocardia corallina* B-276 (*Rhodococcus rhodochrous* B-276) accumulated 1, 2-epoxypropane during growth on propene (60) while Habets-Crützen et al. reported the formation of 1,2-epoxyalkanes from ethene, propene and 1-butene by several strains of genera *Mycobacteria* and *Nocardia.* According to this study, the resting cells of organisms isolated on propene and butene, when grown on these substrates converted ethene quantitatively to epoxyethane. Some, but not all ethene-utilizing strains accumulated 1, 2-epoxypropane or 1, 2-epoxybutane when propene or butene was supplied, although not quantitatively because the epoxides produced were partially further metabolized (65). In contrast, a special situation existed for *Mycobacterium* E20, which grows on both unsaturated and saturated gaseous hydrocarbons. Ethene-grown E20 cells did not yield 1, 2-epoxypropane from propene because the epoxide was further metabolized. Butane-grown E20 cells, however, accumulated the appropriate epoxyalkanes almost quantitatively from ethene and propene, confirming that this organism is not being able to

further metabolize the epoxides, resembling methane-utilizing bacteria and other alkane-utilizers. (65).

Moreover, van Ginkel et al. (152) reported that all 11 strains of alkene-grown bacteria tested (belonging to the genera *Mycobacterium, Nocardia*, and *Xanthobacter*) were able to oxidize the whole range of alkenes (C_2-C_6) used and were also able to accumulate epoxyalkanes from one or more of the alkenes. Eight of these strains oxidized the alkenes at rates of 10-80 nmol/min/mg of protein and the highest rates were found with the alkene on which the bacterium was grown. However, no significant excretion of epoxyalkanes was detected from those alkanes on which the bacteria were grown. Epoxyalkanes also did not accumulate from alkenes, which are potential growth substrates of the bacteria. For instance, 1-hexene utilizing *Nocardia* strain H8 which was able to grow on all tested 1-alkenes (C_2-C_6) (and oxidized them all at the same rate), did not form any 1, 2-epoxyalkane. Nevertheless, few epoxide-accumulations were detected in stoichiometric amounts from the corresponding alkenes, because in most cases epoxides formed were further converted to other compounds like alkanediols. For an example, 1, 2-epoxypropane was hydrolyzed to 1, 2-propanediol by ethene-grown *Mycobacterium* sp. strain E3.

Reports on the production of subterminal epoxides appear to be less prevalent. This may be due to moving the double bond away from the terminal methyl group which has somehow rendered it less susceptible to attack by the oxygenases. For instance, ethene-grown *Micrococcus* sp. M90C was reported to epoxidate *cis*- and *trans*-butenes at significantly lower rates than terminal gaseous alkenes (97). In terms of reactivity, *cis*-2-butene is thought to be more reactive than the *trans* isomer due to its sterically less hindered double bond and

the rotated (and tilted) methyl group which results in more strained structure (9). However, epoxidation of *trans*-2-butene was 2-fold faster than the *cis*-isomer suggesting that the *trans* configuration may be sterically favored for oxidation (97). Moreover, van Ginkel et al (152) demonstrated that *trans*-2-butene-utilizing strain *Nocardia* TB1 grew more abundantly on saturated than on unsaturated gaseous hydrocarbons as well as it oxidized all alkanes tested. Therefore it is thought that these cells contain an alkane-type monooxygenase with broad substrate specificity, and not an alkene-type monooxygenase. In a separate study van Ginkel et al. (153) have shown that *Nocardia* TB1 metabolizes *trans*-2-butene via crotyl alcohol, in which the alkene is metabolized via an initial hydroxylation at the C_1 position rather than going through an initial epoxidation reaction (Fig.1.8).

1.3c Oxidation of Alkanes by Alkene-Utilizers

Only a very limited number of gaseous-alkene utilizing bacteria can grow on alkanes; *e.g*. *Nocardia corallina* (*R. rhodochrous*) starin B-276 (60), *Mycobacterium* strain E20 (36), and other *Mycobacterium* spp. (55). These observations have been confirmed by van Ginkel et al. (152), where only 3 out of 11 strains of alkene-utilizing bacteria tested were able to grow and oxidize the *n*-alkanes (C_1-C_6). Isoprene-grown *Nocardia* strain IP1 oxidized butane, pentane and hexane at rates up to 2 nmol/min/mg of protein. 1-Hexene-grown *Pseudomonas* strain H1 oxidized pentane and hexane at rates of 4-6 nmol/min/mg of protein, whereas the gaseous alkanes were oxidized at negligible rates. *Trans*-2-butene-grown *Nocardia* strain TB1 oxidized all alkanes tested (C_1-C_6) except for methane at rates up to ~2 nmol/min/mg of protein for pentane and hexane and ~4 nmol/min/mg of protein for butane.

Both strains H1 and TB1 grew more abundantly on alkanes than on alkenes and hence can be better described as alkane utilizers (152).

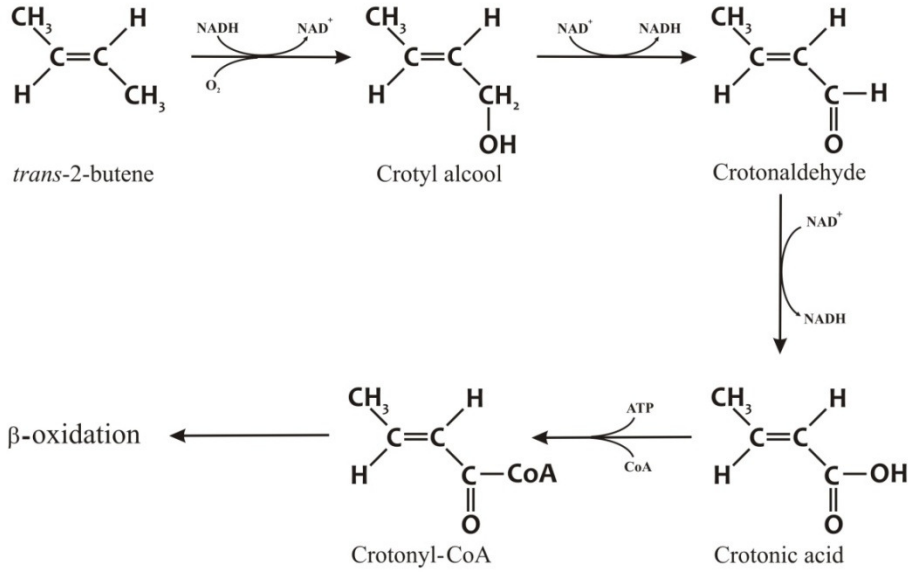


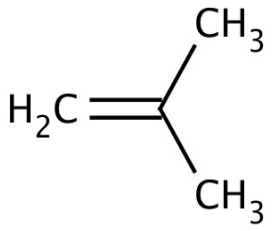
Figure 1-8: Proposed pathway of *trans*-2-butene biodegradation in *Nocardia* TB1. (Reference: van Ginkel C. G., Welten H. G. J., Hartmans S, de Bont J. A. M. Metabolism of *trans*-2-butene and butane in *Nocardia* TB1. J. Gen. Microbiol., 1987a, **133:**1713-1720).

2) 2-Methylpropene (Isobutylene)

2.1 Background and significance

2-Methylpropene (isobutylene), the simplest branched-alkene, is a hydrocarbon of industrial significance. It is one of the four isomers of butenes (C_4H_8), namely 1-butene, *cis*-2-butene, *trans*-2-butene and 2-methylpropene. At standard temperature and pressure, it is a colorless, flammable gas and becomes a liquid at extreme low temperatures. It is insoluble in

water (263 mg/L at 25ºC) and sorbs well to organic matter rather than water (Log K_{ow} = 2.34) (146) (Table 1-1).



Chemical structure of 2-methylpropene. (From http://www.wikipedia.org)

2-Methylpropene is a component of natural gas and crude oil and is used as a feedstock in the production of a variety of products. It is reacted with methanol and ethanol in the manufacture of gasoline oxygenates methyl-*tert*-butyl ether (MTBE) and ethyl-*tert*-butyl ether (ETBE) respectively. Alkylation with butane or dimerization followed by hydrogenation of 2-methylpropene produces isooctane (2, 4, 4-trimethyl-1-pentane), another fuel additive which has an octane number of 100. 2-Methylpropene is also used in the production of methacrolein (isobutenal) as well as the antioxidants, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). Polymerization of isobutylene produces butyl rubber (polyisobutylene) (164).

Although 2-methylpropene has been identified in natural environments, this has traditionally been associated with losses from petrogenic sources resulting from offgassing or venting. Anthropogenic sources of 2-methylpropene can result from combustion of fossil fuels and losses from gas plants and refineries. Even though permitted levels of 2-

methylpropene should not cause adverse health effects, it has been classified as a hazardous material by the U.S. Department of Transportation.

Table 1-1: Physical properties of 2-methylpropene; Taken from a publication prepared for 2-methylpropene by the UNEP (146).

Property	Value	Reference or Comment
Physical state	Gaseous (colorless)	
Molecular weight	56.11 g/ mol	
Boiling point	- 6.9 °C	Lide *et al*., 1997
Melting point	-140.3 °C	Lide *et al*., 1997
Density	0.5879 g/cm^3 (at 25°C)	O'Neil *et al*., 2001
Partition coefficient *n*-octanol/water (log K_{ow} value)	2.34	Hansch *et al*., 1995
Water solubility	263 mg/L (at 25 °C)	
Henry's Law constant (HLC)	63,400 Pa-m3/mole (at 25°C)	HLC calculated using 263 mg/L water solubility, 2,973 hPa vapor pressure, and 56.11 molecular weight.
Vapor pressure	2,973 hPa (2.934 atm) (at 25°C)	EPIWIN, 1999

Why are we interested? Until now a very limited number of gaseous alkenes have been investigated for their degradability and only a few species of bacteria are known as alkene-oxdizers. No studies have been reported on the microbial degradation of branched-chain alkenes besides 2-methyl-1, 3-butadiene (isoprene) degradation. Several bacterial strains that are capable of growing on 2-methyl-1,3-butadiene have been isolated (46, 150, 155) and genes associated with its catabolism by *Rhodococcus* AD45 have also been identified (156).

The pathway of 2-methyl-1,3-butadiene oxidation in strain AD45, initiates by a multicomponent monooxygenase to yield an epoxide (1, 2-epoxy-2-methyl-3-butene) which is then conjugated with glutathione (154, 155). The resulting hydroxylated conjugate is subsequently oxidized to a carboxylic acid by dehydrogenases (156). However, little is known about the microbial degradation and enzymes associated with the oxidation of the simplest branched-alkene, 2-methylpropene.

No organisms have previously been isolated on 2-methylpropene although several alkene-oxidizing strains could co-oxidize this compound. In an early study, Furuhashi and coworkers have examined 2-methylpropene as a potential growth-supporting substrate for *R. rhodochrous* B-276 that grows well on ethene, propene, 1-butene, *n*-butane and 1,3-butadiene. But it did not grow on 2-methylpropene (60). However, many microorganisms have been identified that can generate this gas under aerobic conditions. In the first instance, Fukuda and coworkers have reported the production of 2-methylpropene by 18 strains of *Rhodotorula* yeast (especially *R. minuta* var. *texensis* IFO 1102 at a rate of 16.4 nl/ml/hr) followed by Fujii and coworkers who have confirmed their result (56, 57, 58). Furthermore, the betaproteobacterial strains *Aquincola tertiaricarbonis* L108, *Methylibium petroleiphilum* PM1, and *Methylibium* sp. strain R8, have also been reported to form 2-methylpropene exclusively from MTBE, ETBE, and TBA (123). It can also be produced by several enzymes including cytochrome P450rm (54, 59) diphosphomevalonate decarboxylase (63) and oleate hydratase (98). In contrast to these microbial and enzymatic production routes, little is known about the microbial degradation of this gas.

There are few prior studies of microbial degradation of 2-methylpropene by methanotrophs, propanotrophs and alkene-oxidizers (79, 80, 111). For instance, Hou et al. (79) have reported the epoxidation of 2-methylpropene to 1,2-epoxy-2-methylpropane by resting cell-suspensions of two methanotrophic strains, *Methylococcus capsulatus* CRL MI and *Methylosinus trichosporium* OB3b. The specific activities of these oxidations were 0.46 µmol/h/mg cells and 0.32 µmol/h/mg cells respectively while no further oxidation of the epoxide was observed (79). Moreover, the epoxidation was inhibited by metal-binding and metal-chelating agents and the pattern and magnitude of the inhibition resembled those obtained for methane monooxygenase (77). Neither strain CRL MI nor strain OB3b utilized 2-methylpropene as a growth supporting substrate. In a similar study, Patel and coworkers have demonstrated the oxidation of 2-methylpropene to 1,2-epoxy-2-methylpropane by a soluble-fraction of *Methylobacterium* sp. strain CRL-26 (111). In a separate study, Hou and coworkers showed that propane-grown *Brevibacterium* sp. strain CRL-56 oxidizes 2-methylpropene to 1,2-epoxy-2-methylpropane at the rate of 0.46 µmol/10 min per mg of protein (80).

In the case of *Xanthobacter* strain Py2, propene-grown cells oxidized 2-methylpropene at the rate of 19.4 nmoles/min/mg total protein compared to 22.6 – 26.6 nmoles/min/mg total protein, the rates observed with the oxidation of growth-supporting 1-alkenes, ethene, propene and 1-butene. Moreover, both 2-methylpropene and 1,2-epoxy-2-methylpropane induced alkene oxidizing activity (both alkene monooxygenase and epoxidase activities) in glucose-grown Py2 cells. Interestingly, 1,2-epoxy-2-methylpropane was not degraded by propene-grown Py2 cells to any detectable level (41). Also, 1,2-epoxy-2-

methylpropane has been characterized as a time-dependent, irreversible inactivator of epoxide carboxylase activity in both *Xanthobacter* Py2 (4) and *R. rhodochrous* B-276 (6).

It has also been shown that ethene-grown *Nocardioides* sp. strain JS614 converts 2-methylpropene to 1,2-epoxy-2-methylpropane at the rate ~18.5 nmol/min/mg protein and it was the slowest of all four butenes that were tested (110). Takami et al, (141) reported that 2-methylpropene is converted to 1,2-epoxy-2-methylpropane and 2-methylallyl alcohol by heterologously expressed dimethyl sulfide (DMS) monooxygenase and cumene dioxygenase respectively (degradation rates are not known). The mode of oxidation by the DMS monooxygenase is considered to be similar to the monooxygenation of propene and 1-butene to the corresponding epoxides by *Methylosinus trichosporium* and *Methylococcus capulatus* (140). Furthermore, the oxidation by the cumene dioxygenase is thought to be similar to the monooxygenation of chlorinated propenes to the corresponding chlorinated allyl alcohols by the toluene dioxygenase (TDO) from *Pseudomonas putida* F1 (94). A recent study has demonstrated the catalysis of the epoxidation of 2-methylpropene by an unspecific peroxygenase secreted by the fungus *Agrocybe aegerita*, in which the gas has exclusively been converted to the epoxide (113).

Also we were interested to know to what extent microorganisms that metabolize 2-methylpropene may be evolutionary precursors for organisms that can now metabolize methyl *tertiary* butyl ether (MTBE) and its primary metabolite, *tertiary* butyl alcohol (TBA), frequently encountered gasoline-derived ground water pollutants.

2.2 2-Methylpropene-MTBE pathway overlap

2.2a Background and Biodegradation of MTBE:

MTBE (methyl *tert*-butyl ether) is the most widely used oxygenate in the United States (US) and was originally introduced in late 1970s as an octane-enhancing replacement for lead additives in gasoline (147). Oxygenates are high oxygen containing substances used as blending components in the production of gasoline in order to increase fuel combustion efficiency and decrease exhaust emission. In the 1990s, MTBE use increased in response to the requirements of the Clean Air Act Amendments (1990) for oxygenated fuels. In 1993, production of MTBE exceeded 24 billion gallons, making it the second most manufactured organic chemical in the US (118), and in 1997 its use was greater than 10.5 million gallons per day (89). While there are many different gasoline oxygenates, MTBE was chosen as the preferred oxygenate by gasoline manufacturers based on a few important qualities such as being inexpensive to make (made from two readily available refinery waste products, 2-methylpropene and methanol), blending easily with fuels, and ease of transportation through existing pipelines.

Despite its potential benefits to air quality, MTBE has some properties that can cause serious environmental problems. Its high solubility in water (51 g/L), low adsorption to soil and organic matter (Log_{ow} = 1.2), and underground tank leakage or accidental spills have shown MTBE to be the most commonly detected contaminant in groundwater in the US and Europe. All ether oxygenates were subsequently removed from US gasoline in the mid 2000s. The USEPA has declared it as a potential carcinogen and has issued a drinking-water advisory of 20 - 40 µg/L on the basis of taste and odor thresholds. The environmental fate of

MTBE in gasoline-contaminated ground water is also impacted by its slow rate of biodegradation under both aerobic and anaerobic conditions (49).

TBA is a consistent intermediate of all pathways of aerobic biodegradation of MTBE (82), and it has drawn increased attention as an important ground water contaminant. TBA is fully miscible in water and also does not sorb well to organic matter (Log Kow = 0.35) (85). Consequently, TBA can potentially be found in ground water both in the presence and absence of MTBE and it has similar transport properties that can lead to large plumes in ground water. Although little data is available on human health risks in association with TBA in drinking water, it is thought that TBA may pose a greater environmental risk than MTBE.

MTBE was thought to be non-degradable in soil and groundwater (88) due to high dissociation energy (~ 360 kJ/mol) (162) of the ether bond and the presence of a *tertiary* carbon atom, which make biodegradation enzymatically unfavorable. However, many microorganisms have been isolated that can degrade MTBE as a sole source of carbon and energy. In 1994, Salanitro reported the isolation of an aerobic mixed bacterial culture (BC-1) that was capable of degrading MTBE (121). In 1997, Mo and coworkers reported the isolation of three pure bacterial cultures of genera *Methylobacterium, Rhodococcus*, and *Arthrobacter* that were able to grow, albeit slowly, on MTBE under aerobic conditions (103). Additional studies have been reported recently and MTBE biodegradation under both aerobic and anaerobic conditions became evident. Anaerobic biodegradation of MTBE has been observed under methanogenic (165, 169), sulfate-reducing (134), iron-reducing (52) and nitrate-reducing conditions (17). However, studies have shown (104, 108, 168) that MTBE biodegradation occurs slowly under anaerobic conditions while it is significantly

greater under aerobic conditions. Therefore many studies have been conducted focusing on aerobic biodegradation.

2.2b Aerobic Biodegradation of MTBE:

There are two different metabolic processes that can achieve aerobic biodegradation of MTBE; metabolism and cometabolism**.** Metabolism involves the utilization of a substrate as a sole source of carbon and energy while cometabolism involves the degradation of a compound that does not support growth. In cometabolism, enzymes induced by a primary growth-supporting substrate catalyze the transformation of the second compound (49).

However, strains using MTBE as the sole source of carbon and energy are rarely found. Bacterial isolates capable of aerobic growth on MTBE include the β-proteobacterial strains *Methylibium petroleiphilum* PM1 (39, 67, 105) and *Hydrogenophaga flava* ENV 735 (73, 136), as well as the gram-positive *Mycobacterium austroafricanum* IFP 2012 (53). These three isolates are also capable of metabolizing TBA, the primary metabolite of MTBE oxidation, as a sole carbon and energy source

In contrast, a variety of aerobic microorganisms can degrade MTBE cometabolically after growth on certain hydrocarbons and many of them are also major gasoline components including normal alkanes (62, 95), branched-alkanes (84), aromatics (83, 92) and alicyclic (30) compounds. These organisms include several bacterial genera *i.e. Pseudomonas* (62, 131, 133), *Mycobacteria* (132) and other actinomycetes (95) as well as the filamentous fungus *Graphium sp* (68). The MTBE-oxidizing activity of these organisms has often been attributed to the lack of substrate specificity of monooxygenase enzymes otherwise

responsible for initiating alkane oxidation (68, 131, 132, 137). The cometabolic oxidation of MTBE by these organisms does not follow the same pathway and they differ from each other in numerous ways. For instance, some alkane hydroxylase-expressing organisms such as *Pseudomonas putida* strain GPo1 generate TBA as a dead end product (131) while propane-oxidizing organisms such as *Mycobacterium austroafricanum* JOB5 can further oxidize TBA to 2-methyl-1,2-propanediol (89) and 2-hydroxyisobutyrate and potentially other products (137).

Pathway for Aerobic Biodegradation of MTBE and TBA: Although the pathway of aerobic MTBE degradation has not been fully elucidated, there is agreement on the first steps of oxidation (49, 132, 137) and it is generally thought to proceed according to the diagram shown in Fig. 1.9. Initially, the methoxy carbon of MTBE is hydroxylated by a monooxygenase resulting in an unstable hemiacetal, *tert*-butoxymethanol (68). Monooxygenases involved in this reaction include several forms of alkane hydroxylases (non-haem iron oxygenases) and cytochrome P450s (haem-containing oxygenases). For example, in *M. austroafricanum* JOB5, the initiation of MTBE (and TBA) oxidation is believed to be catalyzed by the alkane-oxidizing monooxygenase, which has not been identified yet. In the case of *Pseudomonas mendocina* KR1 and *Pseudomonas putida* GPo1, several reports suggest that an AlkB-like alkane hydroxylase is responsible for MTBE-oxidation (131, 133, 137). In other studies with *Pseudomonas putida* (ATCC 17453) *Nocardia sp*. ENV425, and *Graphium sp*. results suggested the involvement of a P450-monoxygenase in initiating MTBE oxidation (68, 137).

The unstable hemiacetal can spontaneously decompose to *tert*-butyl alcohol (TBA) and formaldehyde. In *Mycobacteria* and some other strains, this hemiacetal can be enzymatically oxidized to *tert*-butyl formate (TBF) which is thought to be catalyzed by an alcohol dehydrogenase (ADH) (68). It has been shown that TBF is further hydrolyzed to TBA and formate (132). MTBE-oxidizing bacteria can further oxidize TBA to 2-methyl-1, 2-propanediol (MPD) by a reaction catalyzed by a monooxygenase enzyme. In *Mycobacterium* strains that oxidize MTBE cometabolically (*e.g. M. austroafricanum* JOB5, *M. austroafricanum* IFP 2012 and 2015), this reaction is thought to be catalyzed by the same monooxygenase enzyme that is responsible to initiate MTBE (and alkane) oxidation (53, 132). In contrast, in organisms that can metabolize MTBE and TBA (*e.g.* strains PM1 and L108), this reaction is catalyzed by a separate non-haem iron oxygenase (MpdJ) and its associated reductase (MpdK) (81).

The next step of MTBE oxidation is the conversion of MPD to 2-hydroxyisobutyrate (HIBA). In the case of *M. austroafricanum* IFP 2012, it has been proposed that MPD is oxidized to HIBA through 2-hydroxyisobutyraldehyde (HIBAL), which has never been detected as an intermediate (50). This four-electron oxidation is thought to be catalyzed by an alcohol dehydrogenase (MpdB) and an aldehyde dehydrogenase (MpdC) respectively. MpdB is closely related to choline oxidase, a member of the glucose-methanol-choline

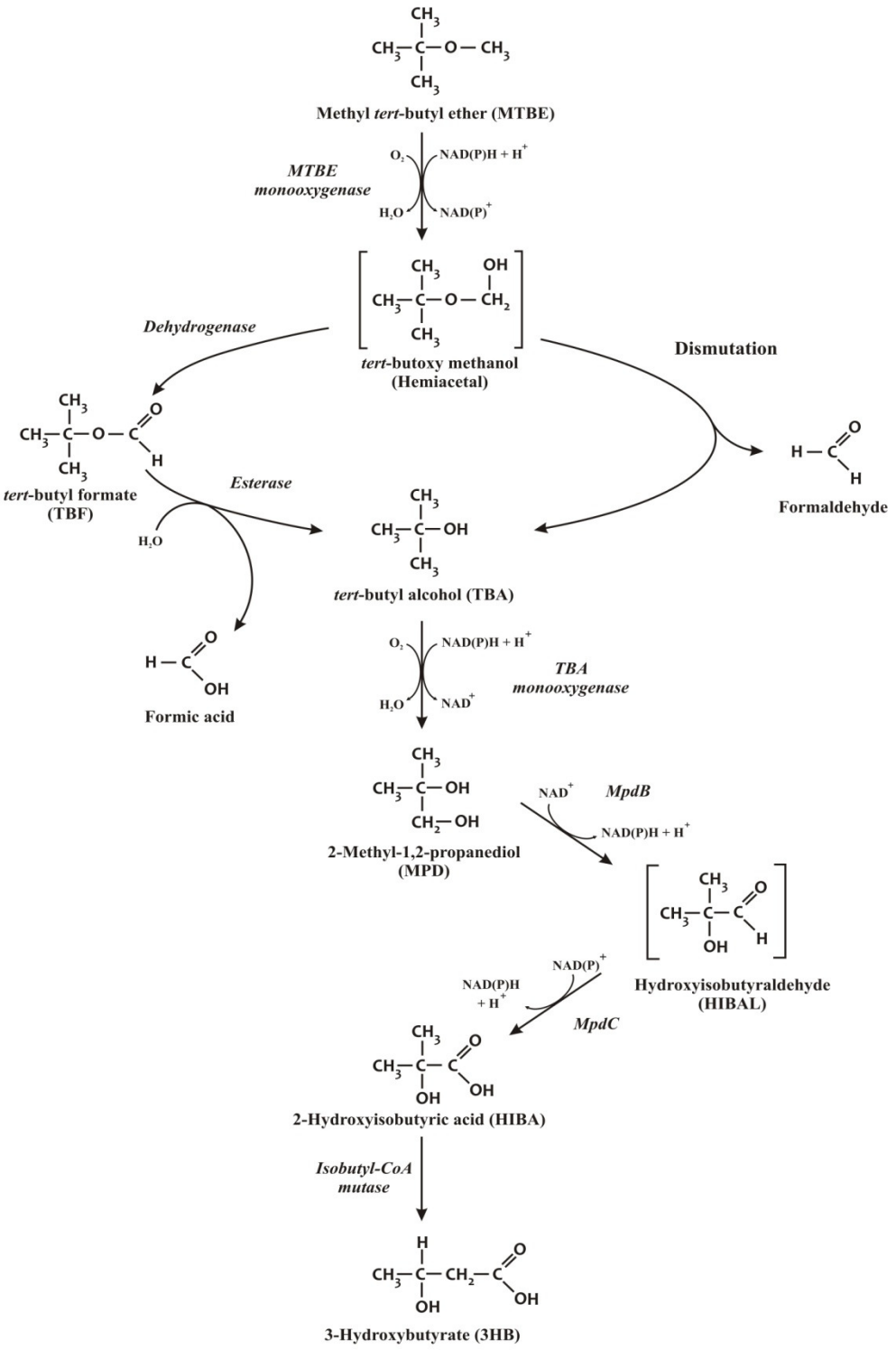


Figure 1-9: The pathways of aerobic MTBE biodegradation.(Lopes Ferreira *et al*., 2000)

oxidoreductase family of enzymes that catalyzes the four-electron oxidation of choline to glycine betaine through an enzyme-bound betaine aldehyde intermediate (48). Several studies have shown that HIBA is further oxidized to 3-hydroxybutyrate (3HB) in the presence of cobalt or cobalamin. For instance, *Burkholderia cepacia* IFP 2003, a strain that is capable of growing on TBA, but not on MTBE, required cobalt ions for growth on TBA (115). François et al (53) has shown that *M. austroafricanum* IFP 2012 also required cobalt for growth on TBA. In another study, Rohwerder and coworkers have reported about three closely related β-proteobacterial strains L108, L10 and CIP I-2052 showed an exceptional nutritional demand for cobalt (or cobalamin) during growth on substrates possessing the *tert*-butyl moiety including HIBA. Also they were able to identify a cobalt-dependent enzyme, isobutyryl-CoA mutase that was shown to convert HIBA to 3HB, which is then further metabolized through central metabolic pathways (119).

The research in this dissertation has focused on the microbial oxidation of alkenes and alkanes by a novel 2-methylpropene-metabolizing strain, *Mycobacterium* sp. ELW1. The first study describes the isolation and physiological characterization of the bacterium and determination of the pathway and enzymes involved in 2-methylpropene oxidation. We hypothesized that the proposed pathway for the oxidation of 2-methylpropene by this new isolate overlaps with the previously established pathway of aerobic degradation of MTBE and TBA. In the second study, a novel pathway for the oxidation of *cis*- and *trans*-2-butene (isomers of 2-butene) was elucidated. This study is of great importance as 2-butene is the only other alkene that the strain ELW1 grows on. Also, the first bacterium isolated on *trans*-

2-butene, *Nocardia* TB1, metabolizes *trans*-2-butene via an initial hydroxylation at the C_1 position rather than via an initial epoxidation reaction similar to what we have proposed for strain ELW1. The third study focuses on the co-oxidation of alkanes by this new isolate although it does not metabolize short-chain alkanes (C_1-C_6) as growth substrates. This feature makes it different from other alkene-utilizers, which generally do not hydroxylate alkanes.

REFERENCES

1. **Abbott BJ, Hou CT.** 1973. Oxidation of 1-alkenes to 1,2-epoxyalkanes by *Pseudomonas oleovorans*. Appl. Microbiol. **26:**86-91.

2. **Allen JR, Clark DD, Krum JG, Ensign SA.** 1999. A role for coenzyme M (2-mercaptoethanesulfonic acid) in a bacterial pathway of aliphatic epoxide carboxylation. Proc. Natl. Acad. Sci. U. S. A. **96:**8432-8437.

3. **Allen JR, Ensign SA.** 1996. Carboxylation of epoxides to beta-keto acids in cell extracts of *Xanthobacter* strain Py2. J. Bacteriol. **178:**1469-1472.

4. **Allen JR, Ensign SA.** 1997a. Characterization of three protein components required for functional reconstitution of the epoxide carboxylase multienzyme complex from *Xanthobacter* strain Py2. J. Bacteriol. **179:**3110-3115.

5. **Allen JR, Ensign SA.** 1997b. Purification to homogeneity and reconstitution of the individual components of the epoxide carboxylase multiprotein enzyme complex from *Xanthobacter* strain Py2. J. Biol. Chem. **272:**32121-32128.

6. **Allen JR, Ensign SA.** 1998. Identification and characterization of epoxide carboxylase activity in cell extracts of *Nocardia corallina* B276. J. Bacteriol. **180:**2072-2078.

7. **Allen JR, Ensign SA.** 1999. Two short-chain dehydrogenases confer stereoselectivity for enantiomers of epoxypropane in the multiprotein epoxide carboxylating systems of *Xanthobacter* strain Py2 and *Nocardia corallina* B276. Biochemistry. **38:**247-256.

8. **Allen RH, Jakoby WB.** 1969. Tartaric acid metabolism. IX. Synthesis with tartrate epoxidase. J. Biol. Chem. **244:**2078-2084.

9. **Allinger NL, Sprague JT.** 1972. Conformational analysis. LXXXIV. Study of the structures and energies of some alkenes and cycloalkenes by the force field method. J. Am. Chem. Soc. **94:**5734-5747.

10. **Armstrong RN.** 1997. Structure, catalytic mechanism, and evolution of the glutathione transferases. Chem. Res. Toxicol. **10:**2-18.

11. **Balch WE, Wolfe RS.** 1979a. Transport of coenzyme M (2-mercaptoethanesulfonic acid) in *Methanobacterium ruminantium*. J. Bacteriol. **137:**264-273.

12. **Balch WE, Wolfe RS.** 1979b. Specificity and biological distribution of coenzyme M (2-mercaptoethanesulfonic acid). J. Bacteriol. **137:**256-263.

13. **Boyd DR, Bugg TD.** 2006. Arene *cis*-dihydrodiol formation: from biology to application. Org. Biomol. Chem. **4:**181-192.

14. **Boyd JM, Clark DD, Kofoed MA, Ensign SA.** 2010. Mechanism of inhibition of aliphatic epoxide carboxylation by the coenzyme M analog 2-bromoethanesulfonate. J. Biol. Chem. **285:**25232-25242.

15. **Boyd JM, Ellsworth A, Ensign SA.** 2006b. Characterization of 2-bromoethanesulfonate as a selective inhibitor of the coenzyme M-dependent pathway and enzymes of bacterial aliphatic epoxide metabolism. J. Bacteriol. **188:**8062-8069.

16. **Boyd DR, Sharma ND, Bowers NI, Dalton H, Garrett MD, Harrison JS, Sheldrake GN.** 2006a. Dioxygenase-catalysed oxidation of disubstituted benzene substrates: benzylic monohydroxylation versus aryl *cis*-dihydroxylation and the meta effect. Org. Biomol. Chem. **4:**3343-3349.

17. **Bradley PM, Chapelle FH, Landmeyer JE.** 2001. Methyl *t*-butyl ether mineralization in surface-water sediment microcosms under denitrifying conditions. Appl. Environ. Microbiol. **67:**1975-1978.

18. **Bugg T.** An Introduction to Enzyme and Coenzyme Chemistry, 1997. Blackwell Science Ltd.

19. **Burton SG.** 2003. Oxidizing enzymes as biocatalysts. Trends. Biotechnol. **21:**543-549.

20. **Cardini G, Jurtshuk P.** 1970. The enzymatic hydroxylation of *n*-octane by *Corynebacterium* sp. strain 7E1C. J. Biol. Chem. **245:**2789-2796.

21. **Cerniglia CE, Blevins WT, Perry JJ.** 1976. Microbial oxidation and assimilation of propylene. Appl. Environ. Microbiol. **32:**764-768.

22. **Clark DD, Allen JR, Ensign SA.** 2000. Characterization of five catalytic activities associated with the NADPH: 2-ketopropyl-coenzyme M [2-(2-ketopropylthio) ethanesulfonate] oxidoreductase/ carboxylase of the *Xanthobacter* strain Py2 epoxide carboxylase system. Biochemistry. **39:**1294-1304.

23. **Cleland WW, Andrews TJ, Gutteridge S, Hartman FC, Lorimer GH.** 1998. Mechanism of Rubisco: The Carbamate as General Base x. Chem. Rev. **98:**549-562.

24. **Cleveland CC, Yavitt JB.** 1997. Consumption of atmospheric isoprene in soil. Geophys. Res. Lett. **24:**2379-2382.

25. **Cleveland CC, Yavitt JB.** 1998. Microbial consumption of atmospheric isoprene in a temperate forest soil. Appl. Environ. Microbiol. **64:**172-177.

26. **Colby J, Stirling DI, Dalton H.** 1977. The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath). Its ability to oxygenate *n*-alkanes, *n*-alkenes, ethers, and alicyclic, aromatic and heterocyclic compounds. Biochem. J. **165:**395-402.

27. **Coleman NV, Mattes TE, Gossett JM, Spain JC.** 2002. Phylogenetic and kinetic diversity of aerobic vinyl chloride-assimilating bacteria from contaminated sites. Appl. Environ. Microbiol. **68:**6162-6171.

28. **Coleman NV, Spain JC.** 2003a. Distribution of the coenzyme M pathway of epoxide metabolism among ethene- and vinyl chloride-degrading *Mycobacterium* strains. Appl. Environ. Microbiol. **69:**6041-6046.

29. **Coleman NV, Spain JC.** 2003b. Epoxyalkane: coenzyme M transferase in the ethene and vinyl chloride biodegradation pathways of *Mycobacterium* strain JS60. J. Bacteriol. **185:**5536-5545.

30. **Corcho D, Watkinson RJ, Lerner DN.** 2000. Cometabolic degradation of MTBE by a cyclohexane-oxidizing bacteria, p. 183–189. *In* G. B. Wickramanayake, A. R. Gavaskar, B. C. Alleman, and V. S. Magar (ed.), Bioremediation and phytoremediation of chlorinated and recalcitrant compounds. Battelle Press, Columbus, OH

31. **Dalton H.** 1979. The fortuitous oxidation and cometabolism of various carbon compounds by whole-cell suspensions of *Methylococcus capsulatus* (Bath). FEMS Microbiol. Lett. **5:**315-318.

32. **Danko AS, Saski CA, Tomkins JP, Freedman DL.** 2006. Involvement of coenzyme M during aerobic biodegradation of vinyl chloride and ethene by *Pseudomonas putida* strain AJ and *Ochrobactrum* sp. strain TD. Appl. Environ. Microbiol. **72:**3756-3758.

33. **de Bont JAM.** 1976. Oxidation of ethylene by soil bacteria. Antonie Van Leeuwenhoek. **42:**59-71.

34. **de Bont JAM, Attwood MM, Primrose SB, Harder W.** 1979. Epoxidation of short chain alkenes in *Mycobacterium* E20: The involvement of a specific mono-oxygenase. FEMS Microbiol. Lett. **6:**183-188.

35. **de Bont JAM, Harder W.** 1978. Metabolism of ethylene by *Mycobacterium* E20. FEMS Microbiol. Lett. **3:**89-93.

36. **de Bont JAM, Primrose SB, Collins MD, Jones D.** 1980. Chemical studies on some bacteria which utilize gaseous unsaturated hydrocarbons. J. Gen. Microbiol. **117:**97-102.

37. **de Bont JAM, Van Dijken JP, Van Ginkel CG.** 1982. The metabolism of 1, 2-propanediol by the propylene oxide utilizing bacterium *Nocardia* A60. Biochim. Biophys. Acta **714:**465-470.

38. **de Smet MJ, Wynberg H, Witholt B.** 1981. Synthesis of 1,2-epoxyoctane by *Pseudomonas oleovorans* during growth in a two-phase system containing high concentrations of 1-octene. Appl. Environ. Microbiol. **42:**811-816.

39. **Deeb RA, Hu H, Hanson JR, Scow KM, Alvarez-Cohen L.** 2001. Substrate interactions in BTEX and MTBE mixtures by an MTBE-degrading isolate. Environ. Sci. Technol. **35:**312-317.

40. **Dowd P, Hershline R, Ham SW, Naganathan S.** 1995. Vitamin K and energy transduction: a base strength amplification mechanism. Science. **269:**1684-1691.

41. **Ensign SA.** 1996. Aliphatic and chlorinated alkenes and epoxides as inducers of alkene monooxygenase and epoxidase activities in *Xanthobacter* strain Py2. Appl. Environ. Microbiol. **62:**61-66

42. **Ensign SA.** 2001. Microbial metabolism of aliphatic alkenes. Biochemistry. **40:**5845-5853.

43. **Ensign SA, Allen JR.** 2003. Aliphatic epoxide carboxylation. Annu. Rev. Biochem. **72:**55-76.

44. **Ensign SA, Hyman MR, Arp DJ.** 1992. Cometabolic degradation of chlorinated alkenes by alkene monooxygenase in a propylene-grown *Xanthobacter* strain. Appl. Environ. Microbiol. **58:**3038-3046.

45. **Ensign SA, Small FJ, Allen JR, Sluis MK.** 1998. New roles for CO_2 in the microbial metabolism of aliphatic epoxides and ketones. Arch. Microbiol. **169:**179-187.

46. **Ewers J, Freier-Schröder D, Knackmuss H.** 1990. Selection of trichloroethene (TCE) degrading bacteria that resist inactivation by TCE. Arch. Microbiol. **154:**410-413.

47. **Faber K, Mischitz M, Kroutil W.** 1996. Microbial epoxide hydrolases. Acta Chem. Scand. **50:**249-258.

48. **Fan F, Germann MW, Gadda G.** 2006. Mechanistic studies of choline oxidase with betaine aldehyde and its isosteric analogue 3, 3-dimethylbutyraldehyde. Biochemistry. **45:**1979-1986.

49. **Fayolle F, Vandecasteele J, Monot F.** 2001. Microbial degradation and fate in the environment of methyl *tert*-butyl ether and related fuel oxygenates. Appl. Microbiol. Biotechnol. **56:**339-349.

50. **Ferreira NL, Maciel H, Mathis H, Monot F, Fayolle-Guichard F, Greer CW.** 2006a. Isolation and characterization of a new *Mycobacterium austroafricanum* strain, IFP 2015, growing on MTBE. Appl. Microbiol. Biotechnol. **70:**358-365.

51. **Ferreira NL, Malandain C, Fayolle-Guichard F.** 2006b. Enzymes and genes involved in the aerobic biodegradation of methyl *tert*-butyl ether (MTBE). Appl. Microbiol. Biotechnol. **72:**252-262.

52. **Finneran KT, Lovley DR.** 2001. Anaerobic degradation of methyl *tert*-butyl ether (MTBE) and *tert*-butyl alcohol (TBA). Environ. Sci. Technol. **35:**1785-1790.

53. **François A, Mathis H, Godefroy D, Piveteau P, Fayolle F, Monot F.** 2002. Biodegradation of methyl *tert*-butyl ether and other fuel oxygenates by a new strain, *Mycobacterium austroafricanum* IFP 2012. Appl. Environ. Microbiol. **68:**2754-2762.

54. **Fujii T, Nakamura K, Shibuya K, Tanase S, Gotoh O, Ogawa T, Fukuda H.** 1997. Structural characterization of the gene and corresponding cDNA for the cytochrome P450rm from *Rhodotorula minuta* which catalyzes formation of isobutene and 4-hydroxylation of benzoate. Mol. Gen. Genet. **256:**115-120.

55. **Fujii T, Ogawa T, Fukuda H.** 1985. A screening system for microbes which produce olefin hydrocarbons. Agric. Biol. Chem. **49:**651-657.

56. **Fujii T, Ogawa T, Fukuda H.** 1987. Isobutene production by *Rhodotorula minuta*. Appl. Microbiol. Biotechnol. **25:**430-433.

57. **Fujii T, Ogawa T, Fukuda H.** 1988. Preparation of a cell-free, isobutene-forming system from *Rhodotorula minuta*. Appl. Environ. Microbiol. **54:**583-584.

58. **Fukuda H, Fujii T, Ogawa T.** 1984. Microbial production of C3-and C4-hydrocarbons under aerobic conditions. Agric. Biol. Chem. **48:**1679-1682.

59. **Fukuda H, Fujii T, Sukita E, Tazaki M, Nagahama S, Ogawa T.** 1994. Reconstitution of the isobutene-forming reaction catalyzed by Cytochrome P450 and P450 reductase from *Rhodotorula minuta*: Decarboxylation with the formation of isobutene. Biochem. Biophys. Res. Commun. **201:**516-522.

60. **Furuhashi K, Taoka A, Uchida S, Karube I, Suzuki S.** 1981. Production of 1, 2-epoxyalkanes from 1-alkenes by *Nocardia corallina* B-276. Eur. J. Appl. Microbiol. Biotechnol. **12:**39-45.

61. **Gallagher SC, Cammack R, Dalton H.** 1997. Alkene monooxygenase from *Nocardia Corallina* B-276 is a member of the class of dinuclear iron proteins capable of stereospecific epoxygenation reactions. Eur. J. Biochem. **247:**635-641.

62. **Garnier P, Auria R, Augur C, Revah S.** 1999. Cometabolic biodegradation of methyl *t*-butyl ether by *Pseudomonas aeruginosa* grown on pentane. Appl. Microbiol. Biotechnol. **51:**498-503.

63. **Gogerty DS, Bobik TA.** 2010. Formation of isobutene from 3-hydroxy-3-methylbutyrate by diphosphomevalonate decarboxylase. Appl. Environ. Microbiol. **76:**8004-8010.

64. **Gunsalus RP, Romesser JA, Wolfe RS.** 1978. Preparation of coenzyme M analogs and their activity in the methyl coenzyme M reductase system of *Methanobacterium thermoautotrophicum*. Biochemistry. **17:**2374-2377.

65. **Habets-Crützen A, Brink L, Van Ginkel C, De Bont J, Tramper J.** 1984. Production of epoxides from gaseous alkenes by resting-cell suspensions and immobilized cells of alkene-utilizing bacteria. Appl. Microbiol. Biotechnol. **20:**245-250.

66. **Hansch C, Leo A, Hoekman D.** 1995. Exploring QSAR: Hydrophobic, Electronic and Steric Constants. ACS Professional Reference Book. American Chemical Society: Washington DC. **269** (p.6).

67. **Hanson JR, Ackerman CE, Scow KM.** 1999. Biodegradation of methyl *tert*-butyl ether by a bacterial pure culture. Appl. Environ. Microbiol. **65:**4788-4792.

68. **Hardison LK, Curry SS, Ciuffetti LM, Hyman MR.** 1997. Metabolism of diethyl ether and cometabolism of methyl *tert*-butyl ether by a filamentous fungus, a *Graphium* sp. Appl. Environ. Microbiol. **63:**3059-3067.

69. **Hartmans S, de Bont JAM.** 1992. Aerobic vinyl chloride metabolism in *Mycobacterium aurum* L1. Appl. Environ. Microbiol. **58:**1220-1226.

70. **Hartmans S, de Bont JAM, Harder W.** 1989a. Microbial metabolism of short-chain unsaturated hydrocarbons. FEMS Microbiol. Lett. **63:**235-264.

71. **Hartmans S, de Bont JAM, Tramper J, Luyben KCA.** 1985. Bacterial degradation of vinyl chloride. Biotechnol. Lett. **7:**383-388.

72. **Hartmans S, Smits JP, van der Werf MJ, Volkering F, de Bont JAM.** 1989b. Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* Strain 124X. Appl. Environ. Microbiol. **55:**2850-2855.

73. **Hatzinger PB, McClay K, Vainberg S, Tugusheva M, Condee CW, Steffan RJ.** 2001. Biodegradation of methyl *tert*-butyl ether by a pure bacterial culture. Appl. Environ. Microbiol. **67:**5601-5607.

74. **Heyer J.** 1976. Mikrobielle Verwertung von Äthylen. Zeitschrift Für Allgemeine Mikrobiologie. **16:**633-637.

75. **Higgins IJ, Best DJ, Hammond RC.** 1980. New findings in methane-utilizing bacteria highlight their importance in the biosphere and their commercial potential. Nature. **286:**561-564.

76. **Higgins IJ, Hammond RC, Sariaslani F, Best D, Davies MM, Tryhorn SE, Taylor F.** 1979. Biotransformation of hydrocarbons and related compounds by whole organism

suspensions of methane-grown *Methylosinus trichosporium* OB3b. Biochem. Biophys. Res. Commun. **89:**671-677.

77. **Hou CT, Patel RN, Laskin AI, Barnabe N.** 1979. Microbial oxidation of gaseous hydrocarbons: epoxidation of C2 to C4 *n*-alkenes by methylotrophic bacteria. Appl. Environ. Microbiol. **38:**127-134.

78. **Hou CT, Patel RN, Laskin AI, Barnabe N.** 1980. Microbial oxidation of gaseous hydrocarbons: Oxidation of lower *n*-alkenes and *n*-alkanes by resting cell suspensions of various methylotrophic bacteria, and the effect of methane metabolites. FEMS Microbiol. Lett. **9:**267-270.

79. **Hou CT, Patel RN, Laskin AI, Barnabe N, Barist I.** 1981. Epoxidation and hydroxylation of C4- and C5- branched-chain alkenes and alkanes by methanotrophs. Ind. Microbiol. **23:** 477-482.

80. **Hou CT, Patel RN, Laskin AI, Barnabe N, Barist I.** 1983. Epoxidation of short-chain alkenes by resting-cell suspensions of propane-grown bacteria. Appl. Environ. Microbiol. **46:**171-177.

81. **Hristova KR, Schmidt R, Chakicherla AY, Legler TC, Wu J, Chain PS, Scow KM, Kane SR.** 2007. Comparative transcriptome analysis of *Methylibium petroleiphilum* PM1 exposed to the fuel oxygenates methyl *tert*-butyl ether and ethanol. Appl. Environ. Microbiol. **73:**7347-7357.

82. **Hyman M.** 2013. Biodegradation of gasoline ether oxygenates. Curr. Opin. Biotechnol. **24:**443-450.

83. **Hyman M, Smith C, O'Reilly K.** 2001. Cometabolism of MTBE by an aromatic hydrocarbon-oxidizing bacterium, p. 145-152. *In* V. S. Magar, J. T. Gibbs, K. T. O'Reilly, M. R. Hyman, and A. Leeson (ed.), Bioremediation of MTBE, alcohols, and ethers. Battelle Press, Columbus, OH

84. **Hyman M, Taylor C, O'Reilly K.** 2000. Cometabolic degradation of MTBE by iso-alkane-utilizing bacteria from gasoline-impacted soils, p. 149-155. *In* G. B. Wickramanayake, A. R. Gavaskar, and B. C. Alleman (ed.), Bioremediation and Phytoremediation of Chlorinated and Recalcitrant Compounds. Battelle Press, Columbus, OH

85. **ITRC** (The Interstate Technology & Regulatory Council). 2005. Overview of groundwater remediation technologies for MTBE and TBA. Available on the Internet at: http://www.itrcweb.org/Guidance/GetDocument?documentID=56

86. **Jacobs MH, Wijngaard AJ, Pentenga M, Janssen DB.** 1991. Characterization of the epoxide hydrolase from an epichlorohydrin-degrading *Pseudomonas* sp. Eur. J. Biochem. **202:**1217-1222.

87. **Janc JW, Urbauer JL, O'Leary MH, Cleland W.** 1992. Mechanistic studies of phosphoenolpyruvate carboxylase from *Zea mays* with (Z)-and (E)-3-fluorophosphoenolpyruvate as substrates. Biochemistry. **31:**6432-6440.

88. **Jensen HM, Arvin E.** 1990. Solubility and degradability of the gasoline additive MTBE, methyl-*tert*-butyl-ether, and gasoline compounds in water, p. 445-448. *In* F. Arendt, M. Hinsenveld, and W. J. van den Brink (ed.), Contaminated Soil. Kluwer Academic Publishers, The Netherlands.

89. **Johnson EL, Smith CA, O'Reilly KT, Hyman MR.** 2004. Induction of methyl *tertiary* butyl ether (MTBE)-oxidizing activity in *Mycobacterium vaccae* JOB5 by MTBE. Appl. Environ. Microbiol. **70:**1023-1030.

90. **Johnson R, Pankow J, Bender D, Price C, Zogorski J.** 2000. MTBE-To what extent will past releases contaminate community water supply wells? Environ. Sci. Technol. **34:**210A-217A.

91. **Knowles JR.** 1989. The mechanism of biotin-dependent enzymes. Annu. Rev. Biochem. **58:**195-221.

92. **Koenigsberg S, Sandefur C, Mahaffey W, Deshusses M, Fortin N.** 1999. Peroxygen mediated bioremediation of MTBE, p. 13-18. *In* B. C. Alleman and A. Leeson (ed.), Proceedings of the Fifth International In Situ and On-Site Bioremediation Symposium, vol. 3. Battelle Press, Columbus, OH

93. **Koskinen M, Plná K.** 2000. Specific DNA adducts induced by some mono-substitued epoxides *in vitro* and *in vivo*. Chem. Biol. Interact. **129:**209-229.

94. **Lange CC, Wackett LP.** 1997. Oxidation of aliphatic olefins by toluene dioxygenase: enzyme rates and product identification. J. Bacteriol. **179:**3858-3865.

95. **Liu CY, Speitel GE, Jr, Georgiou G.** 2001. Kinetics of methyl *t*-butyl ether cometabolism at low concentrations by pure cultures of butane-degrading bacteria. Appl. Environ. Microbiol. **67:**2197-2201.

96. **Lyde DR.** 1997. CRC Handbook of chemistry and physics. 78th Edition (p. 3-289), CRC Press, New York, NY

97. **Mahmoudian M, Michael A.** 1992. Production of chiral epoxides by an ethene-utilising *Micrococcus* sp. J. Ind. Microbiol. **11:**29-35.

98. **Marliere P.** 2011. Method for producing an alkene comprising the step of converting an alcohol by an enzymatic dehydration step. European Patent. EP2336341 A1

99. **Mattes TE, Coleman NV, Spain JC, Gossett JM.** 2005. Physiological and molecular genetic analyses of vinyl chloride and ethene biodegradation in *Nocardioides* sp. strain JS614. Arch. Microbiol. **183:**95-106.

100. **May SW, Schwartz RD, Abbott BJ, Zaborsky OR.** 1975. Structural effects on the reactivity of substrates and inhibitors in the epoxidation system of *Pseudomonas oleovorans*. Biochimica Et Biophysica Acta (BBA)-Enzymology. **403:**245-255.

101. **Meylan W.** 1999. EPIWIN version 3.04. Syracuse Research Corporation, Syracuse, NY, USA.

102. **Miura A, Dalton H.** 1995. Purification and characterization of the alkene monooxygenase from *Nocardia corallina* B-276. Biosci. Biotechnol. Biochem. **59:** 853-859.

103. **Mo K, Lora C, Wanken A, Javanmardian M, Yang X, Kulpa C.** 1997. Biodegradation of methyl *t*-butyl ether by pure bacterial cultures. Appl. Microbiol. Biotechnol. **47:**69-72.

104. **Mormile MR, Liu S, Suflita JM.** 1994. Anaerobic biodegradation of gasoline oxygenates: extrapolation of information to multiple sites and redox conditions. Environ. Sci. Technol. **28:**1727-1732.

105. **Nakatsu CH, Hristova K, Hanada S, Meng XY, Hanson JR, Scow KM, Kamagata Y.** 2006. *Methylibium petroleiphilum* gen. nov., sp. nov., a novel methyl *tert*-butyl ether-degrading methylotroph of the *Betaproteobacteria*. Int. J. Syst. Evol. Microbiol. **56:**983-989.

106. **Nöthe C, Hartmans S.** 1994. Formation and degradation of styrene oxide stereoisomers by different microorganisms. Biocatalysis Biotransformation. **10:**219-225.

107. **O'Neil MJ, Smith A, Heckelman P.** 2001. The Merck Index- An encyclopedia of chemicals, drugs, and biologicals. 13th Edition (p. 1404). Merck Research Laboratories, Merck & Co., Inc. Whitehouse Station, NJ

108. **O'Reilly KT, Moir ME, Taylor CD, Smith CA, Hyman MR.** 2001. Hydrolysis of *tert*-butyl methyl ether (MTBE) in dilute aqueous acid. Environ. Sci. Technol. **35:**3954-3961.

109. **Osprian I, Kroutil W, Mischitz M, Faber K.** 1997. Biocatalytic resolution of 2-methyl-2-(aryl) alkyloxiranes using novel bacterial epoxide hydrolases. Tetrahedron: Asymmetry. **8:**65-71.

110. **Owens CR, Karceski JK, Mattes TE.** 2009. Gaseous alkene biotransformation and enantioselective epoxyalkane formation by *Nocardioides* sp. strain JS614. Appl. Microbiol. Biotechnol. **84:**685-692.

111. **Patel RN, Hou CT, Laskin AI, Felix A.** 1982. Microbial oxidation of hydrocarbons: properties of a soluble methane monooxygenase from a facultative methane-utilizing organism, *Methylobacterium* sp. Strain CRL-26. Appl. Environ. Microbiol. **44:**1130-1137.

112. **Patel RN, Hou CT, Laskin AI, Felix A, Derelanko P.** 1983. Oxidation of alkanes by organisms grown on C_2-C_4 alkanes. J. Appl. Biochem. **5:**107-120.

113. **Peter S, Kinne M, Ullrich R, Kayser G, Hofrichter M.** 2013. Epoxidation of linear, branched and cyclic alkenes catalyzed by unspecific peroxygenase. Enzyme Microb. Technol. **52:**370-376.

114. **Pickett CB, Lu AY.** 1989. Glutathione S-transferases: gene structure, regulation, and biological function. Annu. Rev. Biochem. **58:**743-764.

115. **Piveteau P, Fayolle F, Vandecasteele J, Monot F.** 2001. Biodegradation of *tert*-butyl alcohol and related xenobiotics by a methylotrophic bacterial isolate. Appl. Microbiol. Biotechnol. **55:**369-373.

116. **Primrose S.** 1979. Ethylene and agriculture: the role of the microbe. J. Appl. Microbiol. **46:**1-25.

117. **Rasmussen RA.** 1970. Isoprene: Identified as a forest-type emission to the atmosphere. Environ. Sci. Technol. **4:**667-671.

118. **Reisch M.** 1994. Top 50 chemicals production rose modestly last year. Chem. Eng. News. **72:**12-15.

119. **Rohwerder T, Breuer U, Benndorf D, Lechner U, Muller RH.** 2006. The alkyl *tert*-butyl ether intermediate 2-hydroxyisobutyrate is degraded via a novel cobalamin-dependent mutase pathway. Appl. Environ. Microbiol. **72:**4128-4135.

120. **Rosenzweig AC, Frederick CA, Lippard SJ, Nordlund P.** 1993. Crystal structure of a bacterial non-haem iron hydroxylase that catalyses the biological oxidation of methane. Nature. **366:** 537-543.

121. **Salanitro JP, Diaz LA, Williams MP, Wisniewski HL.** 1994. Isolation of a bacterial culture that degrades methyl *t*-butyl ether. Appl. Environ. Microbiol. **60:**2593-2596.

122. **Sawada S, Totsuka T.** 1986. Natural and anthropogenic sources and fate of atmospheric ethylene. Atmos. Environ. **20:**821-832.

123. **Schafer F, Muzica L, Schuster J, Treuter N, Rosell M, Harms H, Muller RH, Rohwerder T.** 2011. Formation of alkenes via degradation of *tert*-alkyl ethers and alcohols by *Aquincola tertiaricarbonis* L108 and *Methylibium* spp. Appl. Environ. Microbiol. **77:**5981-5987.

124. **Sexton K, Westberg H.** 1984. Nonmethane hydrocarbon composition of urban and rural atmospheres. Atmos. Environ. **18:**1125-1132.

125. **Sharkey TD.** 1996. Isoprene synthesis by plants and animals. Endeavour. **20:**74-78.

126. **Shennan JL.** 2006. Utilisation of C2–C4 gaseous hydrocarbons and isoprene by microorganisms. J. Chem. Technol. Biotechnol. **81:**237-256.

127. **Sluis MK, Small FJ, Allen JR, Ensign SA.** 1996. Involvement of an ATP-dependent carboxylase in a CO_2-dependent pathway of acetone metabolism by *Xanthobacter* strain Py2. J. Bacteriol. **178:**4020-4026.

128. **Small FJ, Ensign SA.** 1995. Carbon dioxide fixation in the metabolism of propylene and propylene oxide by *Xanthobacter* strain Py2. J. Bacteriol. **177:**6170-6175.

129. **Small FJ, Ensign SA.** 1997. Alkene monooxygenase from *Xanthobacter* strain Py2. Purification and characterization of a four-component system central to the bacterial metabolism of aliphatic alkenes. J. Biol. Chem. **272:**24913-24920.

130. **Small FJ, Tilley JK, Ensign SA.** 1995. Characterization of a new pathway for epichlorohydrin degradation by whole cells of *Xanthobacter* strain Py2. Appl. Environ. Microbiol. **61:**1507-1513.

131. **Smith CA, Hyman MR.** 2004. Oxidation of methyl *tert*-butyl ether by alkane hydroxylase in dicyclopropylketone-induced and *n*-octane-grown *Pseudomonas putida* GPo1. Appl. Environ. Microbiol. **70:**4544-4550.

132. **Smith CA, O'Reilly KT, Hyman MR.** 2003a. Characterization of the initial reactions during the cometabolic oxidation of methyl *tert*-butyl ether by propane-grown *Mycobacterium vaccae* JOB5. Appl. Environ. Microbiol. **69:**796-804.

133. **Smith CA, O'Reilly KT, Hyman MR.** 2003b. Cometabolism of methyl *tertiary* butyl ether and gaseous *n*-alkanes by *Pseudomonas mendocina* KR-1 grown on C5 to C8 *n*-alkanes. Appl. Environ. Microbiol. **69:**7385-7394.

134. **Somsamak P, Cowan RM, Häggblom MM.** 2001. Anaerobic biotransformation of fuel oxygenates under sulfate-reducing conditions. FEMS Microbiol. Ecol. **37:**259-264.

135. **Sparling R, Daniels L.** 1987. The specificity of growth inhibition of methanogenic bacteria by bromoethanesulfonate. Can. J. Microbiol. **33:**1132-1136.

136. **Steffan RJ, Condee C, Quinnan J, Walsh M, Abrams SH, Flanders J.** 2000. In situ application of propane sparging for MTBE bioremediation, p. 157-164. *In* G. B. Wickramanayake, A. R. Gavaskar, B. C. Alleman, and V. S. Magar (ed.), Bioremediation and phytoremediation of chlorinated and recalcitrant compounds. Battelle Press, Columbus, OH

137. **Steffan RJ, McClay K, Vainberg S, Condee CW, Zhang D.** 1997. Biodegradation of the gasoline oxygenates methyl *tert*-butyl ether, ethyl *tert*-butyl ether, and *tert*-amyl methyl ether by propane-oxidizing bacteria. Appl. Environ. Microbiol. **63:**4216-4222.

138. **Steffan RJ, Vainberg S, Condee C, McClay K, Hatzinger P.** 2000. Biotreatment of MTBE with a new bacterial isolate. p. 165-173. *In* G. B. Wickramanayake, A. R. Gavaskar, B. C. Alleman, and V. S. Magar (ed.), Bioremediation and phytoremediation of chlorinated and recalcitrant compounds. Battelle Press, Columbus, OH

139. **Stirling D, Dalton H.** 1979. The fortuitous oxidation and cometabolism of various carbon compounds by whole-cell suspensions of *Methylococcus capsulatus* (Bath). FEMS Microbiol. Lett. **5:**315-318.

140. **Subramanian V.** 1986. Oxidation of propene and 1-butene *byMethylococcus capsulatus* and *Methylosinus trichosporium*. J. Ind. Microbiol. **1:**119-127.

141. **Takami W, Yoshida T, Nojiri H, Yamane H, Omori T.** 1999. Oxidation of chlorinated olefins by *Escherichia coli* transformed with dimethyl sulfide monooxygenase genes or cumene dioxygenase genes. J. Gen. Appl. Microbiol. **45:**69-75.

142. **Taylor CD, Wolfe RS.** 1974. Structure and methylation of coenzyme M ($HSCH_2CH_2SO_3$). J. Biol. Chem. **249:**4879-4885.

143. **Thauer RK.** 1998. Biochemistry of methanogenesis: a tribute to Marjory Stephenson. 1998 Marjory Stephenson Prize Lecture. Microbiology. **144:**2377-2406.

144. **Thomas H, Timms CW, Oesch F.** 1990 *In* Principles, Mechanisms and Biological Consequences of Induction (Ruckpaul, K., and Rein, H., Eds.) p 280-337, Taylor and Francis, New York

145. **Ullrich R, Hofrichter M.** 2007. Enzymatic hydroxylation of aromatic compounds. Cell. Mol. Life Sci. **64:**271-293.

146. **UNEP.** 2003. Isobutylene. CAS No: 115-11-7. Available on the Internet at: http://www.inchem.org/documents/sids/sids/115117.pdf

147. **USEPA.** 1998b. *MTBE Fact Sheet #3: Use and Distribution of MTBE and Ethanol, USEPA Office of Underground Storage Tanks*. EPA 625-K-98-001. January. Available on the Internet at: http://www.epa.gov/swerust1/mtbe/mtbefs3.pdf.

148. **van der Linden A.** 1963. Epoxidation of α-olefins by heptane-grown *Pseudomonas* cells. Biochim. Biophys. Acta. **77:**157-159.

149. **van Ginkel CG, de Bont JAM.** 1986. Isolation and characterization of alkene-utilizing *Xanthobacter* spp. Arch. Microbiol. **145:**403-407.

150. **van Ginkel CG, de Jong E, Tilanus JWR, de Bont JAM.** 1987c. Microbial oxidation of isoprene, a biogenic foliage volatile and of 1, 3-butadiene, an anthropogenic gas. FEMS Microbiol. Lett. **45:**275-279.

151. **van Ginkel CG, Welten HGJ, de Bont JAM.** 1986. Epoxidation of alkenes by alkene-grown *Xanthobacter* spp. Appl. Microbiol. Biotechnol. **24:**334-337.

152. **van Ginkel CG, Welten HGJ, de Bont JAM.** 1987b. Oxidation of gaseous and volatile hydrocarbons by selected alkene-utilizing bacteria. Appl. Environ. Microbiol. **53:**2903-2907.

153. **van Ginkel CG, Welten HGJ, Hartmans S, de Bont JAM.** 1987a. Metabolism of *trans*-2-butene and butane in *Nocardia* TB1. J. Gen. Microbiol. **133:**1713-1720.

154. **van Hylckama Vlieg JET, Kingma J, Kruizinga W, Janssen DB.** 1999. Purification of a glutathione S-transferase and a glutathione conjugate-specific dehydrogenase involved in isoprene metabolism in *Rhodococcus* sp. strain AD45. J. Bacteriol. **181:**2094-2101.

155. **van Hylckama Vlieg JET, Kingma J, van den Wijngaard AJ, Janssen DB.** 1998. A glutathione S-transferase with activity towards *cis*-1, 2-dichloroepoxyethane is involved in isoprene utilization by *Rhodococcus* sp. strain AD45. Appl. Environ. Microbiol. **64:**2800-2805.

156. **van Hylckama Vlieg JET, Leemhuis H, Spelberg JH, Janssen DB.** 2000. Characterization of the gene cluster involved in isoprene metabolism in *Rhodococcus* sp. strain AD45. J. Bacteriol. **182:**1956-1963.

157. **Verce MF, Ulrich RL, Freedman DL.** 2000. Characterization of an isolate that uses vinyl chloride as a growth substrate under aerobic conditions. Appl. Environ. Microbiol. **66:**3535-3542.

158. **Verce MF, Ulrich RL, Freedman DL.** 2001. Transition from cometabolic to growth-linked biodegradation of vinyl chloride by a *Pseudomonas* sp. isolated on ethene. Environ. Sci. Technol. **35:**4242-4251.

159. **Wackett LP, Hershberger CD.** 2001. Biocatalysis and Biodegradation: microbial transformation of organic compounds, 1st Edition. ASM press, Washington, DC

160. **Watkinson RJ, Somerville HJ.** 1976. The microbial utilization of butadiene. *In* Sharply J.M., and J.A.M. Kaplan (eds). Proceedings of the 3rd International Biodeterioration Symposium, Kingston, RI. Applied Science, 35-42

161. **Weijers CAGM, Jongejan H, Franssen MCR, de Groot A, de Bont JAM.** 1995. Dithiol- and NAD-dependent degradation of epoxyalkanes by *Xanthobacter* Py2. Appl. Microbiol. Biotechnol. **42:**775-781.

162. **White GF, Russell NJ, Tidswell EC.** 1996. Bacterial scission of ether bonds. Microbiol. Rev. **60:**216-232.

163. **Wiegant WM, de Bont JAM.** 1980. A new route for ethylene glycol metabolism in *Mycobacterium* E44. J. Gen. Microbiol. **120:**325-331.

164. **Wikipedia.** Isobutylene. (http://en.wikipedia.org/wiki/Isobutylene)

165. **Wilson JT.** 2000. Natural attenuation of MTBE in the subsurface under methanogenic conditions. US Environmental Protection Agency, National Risk Management Research Laboratory, Cincinnati, OH

166. **Wolfe RS.** 1991. My kind of biology. Annu. Rev. Microbiol. **45:**1-36.

167. **Woods N, Murrell J.** 1989. The metabolism of propane in *Rhodococcus rhodochrous* PNKb1. J. Gen. Microbiol. **135:**2335-2344.

168. **Yeh CK, Novak JT.** 1994. Anaerobic biodegradation of gasoline oxygenates in soils. Water Environ. Res. **66:**744-752.

169. **Youngster LK, Somsamak P, Häggblom MM.** 2008. Effects of co-substrates and inhibitors on the anaerobic O-demethylation of methyl *tert*-butyl ether (MTBE). Appl. Microbiol. Biotechnol. **80:**1113-1120.

CHAPTER 2

Isolation and Characterization of an Aerobic 2-Methylpropene (Isobutylene)-Metabolizing Bacterium, *Mycobacterium* sp. ELW1

Samanthi Kottegoda, Elizabeth Waligora and Michael Hyman*

Department of Plant and Microbial Biology
North Carolina State University
Raleigh, NC 27695

Running title: 2-Methylpropene metabolism by *Mycobacterium* sp. ELW1

A manuscript for submission to *Applied and Environmental Microbiology*

*Corresponding author
Contact Information
Dr. Michael Hyman
Department of Plant and Microbial Biology
4545 Thomas Hall
North Carolina State University
Raleigh, NC 27695
Phone: 919-515-78145
Fax: 919-515-7867
E-mail: mrhyman@ncsu.edu

ABSTRACT

We have isolated and characterized an aerobic bacterium (*Mycobacterium* sp. ELW1) that is capable of utilizing 2-methylpropene as a sole source of carbon and energy. It grew well on 2-methylpropene ($\mu = 0.05\ h^{-1}$) but did not grow on any other C_2-C_5 straight chain, branched or chlorinated alkenes tested except 2-butene. It grew on both isomers (*cis* and *trans*) of 2-butene, albeit slowly. It did not grow on short-chain alkanes (C_1-C_6) either. But grew well on *n*-heptane and all other longer chain alkanes tested (C_{10}-C_{18}). Following a similar trend, no growth was observed with smaller branched alkanes (C_4-C_6) but grew well on isoheptane (C_7). It also grew well on all the postulated pathway intermediates of 2-methylpropene-, *cis*-2-butene-, and *trans*-2-butene-oxidation. Resting cells grown on 2-methylpropene consumed ethene, propene and 1-butene without a lag phase and epoxyethane was detected as the only product of ethene oxidation. Both alkene consumption and epoxyethane production were fully inhibited in cells exposed to 1-octyne suggesting that alkene oxidation is initiated by an alkyne-sensitive, epoxide-generating monooxygenase. Kinetic analyses suggest 1,2-epoxy-2-methylpropane is rapidly consumed during 2-methylpropene-degradation while 2-methyl-2-propen-1-ol is not a significant metabolite of 2-methylpropene catabolism. Time course studies demonstrated further metabolism of 1,2-epoxy-2-methylpropane generates 2-methyl-1,2-propanediol and 2-hydroxyisobutyrate, two sequential metabolites previously identified in the microbial metabolism of methyl *tertiary* butyl ether (MTBE) and *tertiary* butyl alcohol (TBA). Growth of strain ELW1 on 2-methylpropene, 1,2-epoxy-2-methylpropane, 2-methyl-1,2-propanediol, and 2-hydroxyisobutyrate (HIBA) was fully inhibited when cobalt ions were excluded from the growth medium while growth on 3-hydroxybutyrate (3HB) and other

substrates was unaffected by the absence of added cobalt ions. These results suggest that, like MTBE- and TBA-metabolizing bacteria, strain ELW1 utilizes a cobalamin-dependent mutase to convert HIBA to 3HB. Our results have been interpreted in terms of their impact on our understanding of the microbial metabolism of alkenes and ether oxygenates.

INTRODUCTION

2-Methylpropene (isobutylene) is one of the four butene (C_4H_8) isomers and is the simplest branched alkene. 2-Methylpropene is produced from cracking of petroleum and worldwide, in excess of 10 million tons of this gas is produced annually (1). 2-Methylpropene is mainly used as feedstock for several important petrochemical products including ether fuel oxygenates such as methyl *tertiary* butyl ether (MTBE), high-octane gasoline blending components such as isooctane (2,2,4-trimethylpentane) and butyl rubber (2). The importance of 2-methylpropene as a feedstock has stimulated interest in its biological production as an alternative to non-renewable petroleum sources. Many microorganisms have been identified that can generate this gas (3, 4), including MTBE-metabolizing bacteria (5). It can also be produced by several enzymes including cytochrome P450rm (6, 7), diphosphomevalonate decarboxylase (8) and oleate hydratase (9). In contrast to these microbial and enzymatic production routes, little is known about the microbial degradation of 2-methylpropene.

From their outset, studies of aerobic microbial alkene metabolism have focused primarily on gaseous short chain linear alkenes such as ethene (10), propene (11-13), 1-butene (14) and 1,3-butadiene (15). With the exception of 2-methyl-1,3-butadiene (isoprene), the microbial metabolism of branched alkenes has been largely ignored. 2-Methyl-1,3-butadiene is a volatile terpenoid that is produced in large quantities by both plants (16) and microorganisms (17). Several bacteria have been isolated that grow on this compound (18-20) and genes associated with its catabolism by *Rhodococcus* AD45 have been identified (21). In strain AD45, 2-methyl-1,3-butadiene is first oxidized to an epoxide (1,2-epoxy-2-

methyl-3-butene) by a multicomponent monooxygenase. The epoxide is then conjugated with glutathione (19, 22) and dehydrogenases subsequently oxidize the resulting hydroxylated conjugate to a carboxylic acid (21).

All gaseous alkene-metabolizing bacteria isolated to date are aerobes and include many *Mycobacterium, Rhodococcus,* and *Nocardia* strains (23). Despite the preponderance of actinobacterial isolates, the best-characterized gaseous alkene-metabolizing strain is a Gram-negative bacterium, *Xanthobacter autotrophicus* Py2 (13). Like all other well-characterized alkene-metabolizing bacteria, this strain initiates oxidation of alkenes using an alkene-inducible monooxygenase (alkene monooxygenase [AMO]) (24, 25). With propene, its best-characterized substrate, AMO generates exclusively epoxypropane as the immediate oxidation product (24, 26). In contrast to the glutathione conjugate involved in isoprene metabolism by *Rhodococcus* AD45, further catabolism of 1,2-epoxypropane and other epoxides by strain Py2 involves their initial conjugation with coenzyme-M (CoM) (27, 28). The epoxyalkane:CoM conjugate is subsequently oxidized and then reductively carboxylated to form readily metabolizable β-ketoacids (29, 30). Collectively, these reactions are catalyzed by an epoxide carboxylase enzyme complex (31) consisting of an epoxyalkane:coenzymeM transferase (EaCoMT) (28), two enantiomer specific 2-hydroxylpropyl-Co-M dehydrogenases (R- and S-HPCDH) (32-34) and NADPH:2-ketopropyl-CoM oxidoreductase/carboxylase (2-KPCC) (35). Like methanogenesis, the only other previously recognized CoM-dependent process, epoxide carboxylation in strain Py2 is irreversibly inactivated by 2-bromoethanesulfonate (BES) (36). The site of action of BES has recently

been shown to be the 2-KPCC component of the epoxide carboxylase complex from strain Py2 (37).

As indicated above, there are few prior studies of microbial degradation of 2-methylpropene. This gas is cometabolically oxidized to 1,2-epoxy-2-methylpropane by methanotrophs (38, 39) and propanotrophs (40). Heterologously expressed dimethylsulfide monooxygenase and cumene dioxygenase oxidize 2-methylpropene to 1,2-epoxy-2-methylpropane and 2-methyl-2-propen-1-ol, respectively (41) while a highly non-specific peroxygenase excreted by the fungus *Agrocybe aegerita* exclusively generates 1,2-epoxy-2-methylpropane from 2-methylpropene (42). Several alkene-metabolizing bacteria have also been reported to oxidize 2-methylpropene (43, 44) but these studies have not reported growth of these bacteria on this gas. With ethene-grown *Nocardiodes* JS614, 2-methylpropene is oxidized at between 42% and 54% of the rate of other butene isomers and 1,2-epoxy-2-methylpropane is the predominant, if not sole product (43). In the case of strain Py2, propene-grown cells oxidize 2-methylpropene at between 73% and 86% of the rate observed with growth-supporting alkenes such as ethene, propene and 1-butene (44). The products of 2-methylpropene oxidation were not characterized in this study although both 2-methylpropene and 1,2-epoxy-2-methylpropane were shown to be potent inducers of both AMO and epoxide carboxylase activities in glucose-grown cells of strain Py2 (44). However, 1,2-epoxy-2-methylpropane is not consumed by propene-grown cells of strain Py2 as this epoxide acts as a potent, time-dependent, irreversible inactivator of EaCoMT activity (31). This effect is also observed in another well-characterized gaseous alkene-metabolizing strain, *Rhodococcus rhodochrous* (*Nocardia corallina)* B-276 that also utilizes CoM conjugation in

epoxide catabolism (45). Since the discovery of the unexpected role for CoM in bacterial epoxide metabolism, several studies have demonstrated that EaCoMT is widely distributed in aerobic alkene-metabolizing bacteria indicating that CoM conjugation is an important, if not predominant, bacterial mechanism for epoxide metabolism (46-50).

Our interest in microbial 2-methylpropene metabolism was prompted by earlier studies of the aerobic microbial degradation of the gasoline oxygenate, MTBE. *Tertiary* butyl alcohol (TBA) is a consistent intermediate in all pathways of aerobic MTBE biodegradation (51). In organisms that can grow on TBA, this alcohol is first oxidized by a monooxygenase to 2-methyl-1,2-propanediol (MPD) which is then oxidized to 2-hydroxyisobutyric acid (HIBA) (51). The final reaction in this pathway before the generation of a readily metabolizable intermediate involves 2-hydroxyisobutyryl CoA mutase (HCM) (52), a cobalamin-dependent enzyme that converts HIBA to 3-hydoxybutyrate (3HB) (53) and expression of this enzyme confers a requirement for cobalt ions or cobalamin in the growth medium of MTBE- and TBA-metabolizing organisms (52, 54).

In view of the facts that (a) alkene-metabolizing bacteria frequently initiate alkene metabolism using monooxygenase enzymes to generate epoxides and (b) 1,2-epoxy-2-methylpropane, the corresponding epoxide for 2-methylpropene, inactivates EaCoMT, it seems likely that a putative 2-methylpropene-metabolizing bacterium would either entirely avoid production of an epoxide intermediate or utilize an EaCoMT-independent pathway of epoxide metabolism. In addition to gluthathione- or CoM-conjugation, isomerization and hydrolysis are two other microbial mechanisms used to overcome the inherent reactivity of epoxides (55). Hydrolysis of 1,2-epoxy-2-methylpropane to MPD occurs in mammalian

systems where epoxide hydrolases are common (56-59). Based on these observations, we hypothesized that bacterial 2-methylpropene metabolism *via* an epoxide intermediate might involve hydrolytic production of MPD from 1,2-epoxy-2-methylpropane and that the pathway and enzymes involved in latter stages of 2-methylpropene catabolism might therefore be similar to those involved in the aerobic bacterial metabolism of MTBE and TBA (Fig. 2.7). The results presented in this study describe the isolation and characterization of a novel 2-methylpropene-utilizing bacterium and studies aimed at elucidating the major enzymatic steps involved in 2-methylpropene catabolism.

MATERIALS AND METHODS

MATERIALS

Bacterial strain and media*:* *Mycobacterium* strain ELW1 was isolated, as described in Methods section. The organism was maintained on mineral salt medium (MSM) agar plates in a glass desiccator containing 2-methylpropene (10% vol/vol, gas phase). The MSM contained (per L of water) 2.0 g NH_4Cl, 0.075 g $MgCl_2 \cdot 6H_2O$, 0.1 g $(NH_4)_2SO_4$ and 2 ml of trace element solution (x 1000 stock). The trace element solution contained (per L of water) 50 g Na-EDTA, 22 g $ZnSO_4 \cdot 7H_2O$, 5.54 g $CaCl_2$, 5.06 g $MnCl_2.4H_2O$, 4.99 g $FeSO_4.SO_4$, 1.1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.57 g $CuSO_4 \cdot 5H_2O$, and 1.61 g $CoCl_2 \cdot 6H_2O$. The medium was buffered (pH 7.0) by the addition of a solution of containing (per L) 1.55 g K_2HPO_4 and 0.85 g NaH_2PO_4. In some experiments the effect of cobalt ions was investigated on the growth of strain ELW1 on 2-methylpropene and other substrates. In these experiments, cells were grown on MSM from which $CoCl_2.6H_2O$ was omitted from the trace element solution. In all other respects this nominally cobalt-free medium (MSM-Co) was identical to MSM.

Chemicals: Acetaldehyde (99% purity), *tertiary* amyl butyl ether (97% purity), benzene (>99.9% purity), 1,3-butadiene (>99% purity), *n*-butane (99% purity), 1,2-butanediol (99% purity), 1,3-butanediol (>99% purity), 1,4-butanediol (>99% purity), *meso*-2,3-butanediol (99 % purity), S,S-2,3-butanediol (97% purity), R,R-2,3-butanediol (97% purity), 1-butanol (99% purity), 2-butanol (99.5% purity), 2-butanone (>99% purity), 1-butene (>99% purity), 2-butene-1-ol; mixture of *cis* & *trans* (96% purity), 3-butene-1-ol (>98% purity), 2-butene; undefined mixture of *cis* & *trans* forms (>99% purity), *cis*-2-butene (>99% purity), *trans*-2-

butene (>99% purity), calcium carbide (~80% purity; for ethyne generation), *n*-decane (>99% purity), 1,1-dichloroethene (99% purity), *cis*-1,2-dichloroethene (97% purity), *trans*-1,2-dichloroethene (98% purity), diisopropyl ether (99% purity), epichlorohydrin (99% purity), 1,2-epoxybutane (>99% purity), *cis*-2,3-epoxybutane (97% purity), 3,4-epoxy-1-butene (98% purity), epoxyethane (>99.5% purity), epoxypropane (99% purity), ethene (>99.5% purity), ethylbenzene (99.8% purity), ethyl *tertiary* butyl ether (99% purity), fructose (>99% purity), galactose (99% purity), glucose (>99.5% purity), *n*-heptane (>99% purity), *n*-hexadecane (99% purity), *n*-hexane (>99% purity), 1-hexyne (98% purity), hydroxyacetone (90% purity), 3-hydroxybutanone (>98% purity), 3-hydroxybutyric acid (95% purity), 2-hydroxyisobutyric acid (99% purity), 2-hydroxy-2-methyl-butyric acid (98% purity), 2-methyl-1,3-butadiene (99% purity), 2-methylbutane (>99% purity), 2-methyl-1-butanol (98% purity), 2-methyl-2-butanol (>99% purity), 3-methyl-1-butanol (98% purity), 3-methyl-2-butanol (98% purity), 2-methyl-1-butene (96% purity), 2-methyl-2-butene (>99% purity), 3-methyl-1-butene (95% purity), 2-methylbutyric acid (98% purity), 3-methylbutyric acid (99% purity), 2-methylhexane (99% purity), 2-methylpentane (>99% purity), 2-methyl-1,3-propanediol (99% purity), 2-methylprop-2-enal (95% purity), 2-methyl-1-propanol (99.5% purity), 2-methyl-2-propanol (99.5% purity), 2-methylpropene (99% purity), 2-methylpropenoic acid (99% purity), 2-methyl-2-propen-1-ol (98% purity), 2-methylpropionaldehyde (>99% purity), 2-methylpropionic acid (>99% purity), methyl *tertiary* butyl ether (99.8% purity), *n*-octadecane (99% purity), *n*-octane (>99% purity), 1-octyne (97% purity), 1-pentanol (>99% purity), 1,2-propanediol (99.5% purity), 1,3-propanediol (98% purity), 1-propanol (99.9% purity), 2-propanol (99.5% purity), 2-propen-1-

ol (99% purity), propyne (98% purity), pyruvic acid (>99% purity), sodium propionate (99% purity), succinic acid (>99% purity), *n*-tetradecane (99% purity), toluene (99.8% purity), trichloroethylene (>99.5% purity), *m*-xylene (>99% purity), *o*-xylene (98% purity), and *p*-xylene (>99% purity) were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI). Acetone (99.5% purity), methanol (99% purity), *n*-pentane (99.5% purity), and sodium acetate (99.5% purity) was obtained from Fisher Scientific (Pittsburgh, PA). 1,2-Ethanediol was obtained from BDH Chemicals (VWR International). *trans-2,*3-Epoxybutane (97% purity) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). Absolute ethanol was obtained from Aaper Alcohol and Chemical Co., (Shelbyville, KY). 1-Butyne (98% purity) was obtained from GFS Chemicals (Columbus, OH). Tetrachloroethylene (98% purity) was obtained from Eastman Kodak Company (Rochester, NY). 1,2-Epoxy-2-methylpropane was obtained from Alfa Aesar (Ward Hill, MA). 2-Methyl-1,2-propanediol [MPD] was a gift from Lyondell Chemical Co. (Houston, TX). Ethane (99% purity) and chloroethene (>99% purity) were supplied by Scott Specialty Gases (Plumsteadville, PA). Methane (CP grade), 2-methylpropane (CP grade), propane (CP grade), propene (CP grade) and compressed gases (H_2, N_2, and air) used for gas chromatography were obtained from local industrial vendors. All other chemicals were of reagent grade or better.

METHODS

Isolation of strain ELW1: Strain ELW1 was isolated by enrichment culture using 2-methylpropene as the sole source of carbon and energy. Enrichment cultures were conducted in glass serum vials (160 ml) sealed with butyl rubber stoppers and aluminum crimp seals

(Wheaton Scientific, Millville, NJ). The enrichment cultures contained MSM (25 ml), 2-methylpropene (~4% vol/vol gas phase) and were seeded with sediment (~1 g) obtained from a stream on the campus of North Carolina State University. The enrichment cultures were incubated at 30°C in the dark in an Innova 4900 environmental shaker (New Brunswick Scientific Co., Inc., Edison, NJ) operated at 150 rpm. After incubation for 14 d, a sample (1 ml) from the culture was transferred to a fresh culture vial that contained MSM (25 ml) and 2-methylpropene (~4% vol/vol gas phase). This procedure was repeated 3 more times every 14 d. A sample (1 ml) of the final enrichment culture was serially diluted in MSM. Aliquots (100 µl) of 10^{-5}, 10^{-6} and 10^{-7} dilutions of these cultures were plated on MSM plates and incubated in a desiccator containing 2-methylpropene (10 % vol/vol, gas phase). Colonies that grew on these plates were picked and restreaked onto MSM plates and incubated with 2-methylpropene in a dessicator until pure cultures were obtained, as determined by visual inspection of colony characteristics and by brightfield optical microscopy and Gram staining. One rapidly growing isolate obtained from these enrichments, strain ELW1 was selected for further study.

Cultivation of strain ELW1: Unless otherwise stated, cultures of strain ELW1 were routinely grown on 2-methylpropene in batch culture in glass media bottles (700 ml) (Wheaton Scientific, Millville, NJ). The bottles contained MSM (100 ml) and were inoculated (initial OD_{600} ~0.02) with a suspension of cells previously grown on MSM plates incubated with 2-methylpropene in a dessicator. The bottles were sealed with open-top caps fitted with butyl rubber septa. 2-Methylpropene (10% vol/vol gas phase) was then added

using sterile plastic syringes fitted with 0.1 µm disposable filters (Millipore Co., Bedford, MA). The bottles were incubated for 4-6 d at 30°C in the dark using a shaker operated at 150 rpm. The final culture density was typically between 0.8 and 1.0 OD_{600}, as determined spectrophotometrically at 600 nm using a Shimadzu 1601 UV/Vis spectrophotometer (Kyoto, Japan). To confirm culture purity after growth, a sample of the culture (50 µl) was routinely streaked onto casein-yeast extract-dextrose (CYD) agar plates (Difco Plate Count Agar; Becton, Dickinson and Co., Sparks, MD).

To determine the range of growth-supporting substrates for strain ELW1, cultures were grown in glass serum vials (160 ml) (Wheaton Scientific, Millville, NJ) sealed with Teflon-lined Mininert valves (Alltech Associates Inc., Deerfield, IL). The vials contained MSM (25 ml) and were inoculated (initial OD_{600} ~ 0.02) using a suspension of cells obtained from cultures previously grown on MSM plates incubated with 2-methylpropene in a dessicator. All solid potential growth substrates were added to the cultures from filter-sterilized aqueous stock solutions (0.1 M) using sterile disposable pipettes. All liquid potential growth substrates were added as neat compounds using glass syringes (Hamilton Company, Reno, NV). All gaseous potential growth substrates were added using sterile plastic disposable syringes fitted with 0.1 µm disposable filters. The culture vials were incubated in the dark at 30°C for up to 56 d in an environmental shaker operated at 150 rpm. The culture densities were determined spectrophotometrically at 600 nm (OD_{600}). For all cultures exhibiting growth, a sample (50 µl) was streaked onto casein-yeast extract-dextrose (CYD) agar plates (Difco Plate Count Agar) to subsequently confirm the purity of the culture.

To examine the effect of cobalt ions on growth of strain ELW1 on various substrates, cultures were grown in glass serum vials (160 ml) containing either 25 ml MSM or MSM-Co. Prior to use the culture vials were soaked overnight in an aqueous solution (10% vol/vol) of Dekasol™ (ICN Biomedicals Inc.; Aurora, OH). The vials were then rinsed three times with deionized water before use. The cultures were inoculated (initial OD_{600} ~ 0.02) with a suspension of cells obtained from cultures previously grown on MSM plates incubated with 2-methylpropene in a dessicator. In these cultures fructose, HIBA, 3HB, or sodium acetate were added from filter-sterilized aqueous stock solutions (0.5 M) using sterile disposable pipettes. Prior to filter-sterilization, the pH of HIBA and 3HB stock solutions was adjusted to 7.0. 1,2-Epoxy-2-methylpropane, MPD, and 2,3-butanediol were added as neat compounds using glass micro syringes. 2-Methylpropene (10% vol/vol gas phase) was added using a sterile plastic syringe fitted with disposable 0.1 µm filter. The culture densities were determined spectrophotometrically at 600 nm (OD_{600}). For all cultures exhibiting growth, a sample (50 µl) was streaked onto casein-yeast extract-dextrose (CYD) agar plates (Difco Plate Count Agar) to subsequently confirm the purity of the culture.

Use of resting cells in small-scale incubations: Cells used for resting cell experiments were grown on individual substrates, as described above. The cells were harvested from the culture medium by centrifugation (10,000 x g for 5 min at 4˚C) and the resulting cell pellet was resuspended in 20 ml of phosphate buffer (50 mM NaH_2PO_4; pH 7.0). The cells were then centrifuged again and the cell pellet was resuspended in phosphate buffer to concentrations ranging from ~2 to 25 mg/ml of total cell protein ml^{-1}. The washed cell suspension was

stored at 4°C and used within 4 h. Unless otherwise stated, all kinetic studies following the consumption of substrates and accumulation of oxidation products were conducted in glass serum vials (10 ml) containing ~900 µl phosphate buffer. The vials were sealed with butyl rubber stoppers and aluminum crimp seals. Substrates or inhibitors were then added from aqueous stock solutions, as neat liquids or as gases using micro syringes. The reaction vials were prepared immediately before use and then incubated at 30^{o} C for 10 min in a shaking water bath (150 rpm) to allow reactants to equilibrate between gas and liquid phases. The reactions were initiated by the addition of an aliquot (up to 100 µl) of concentrated cell suspension to give a final reaction volume of 1 ml and a protein concentration of between ~0.2 to 2.5 mg total cell protein ml^{-1}. The reaction vials were then returned to the shaking water bath and were sampled at the times indicated in each experiment.

Alkyne inactivators of 2-methylpropene oxidation: In all experiments involving the use of alkynes, harvested, 2-methylpropene-grown cells of strain ELW1 were pre-treated with individual alkynes in glass serum vials (25 ml) containing ~7 ml of phosphate buffer. The vials were sealed with butyl rubber stoppers and individual alkynes (90 µmoles) were added either as gases (ethyne and propyne) or as neat liquids. The reactions were initiated by the addition of concentrated washed cells (1 ml) and the reaction vials were incubated at 30˚C in a shaking water bath operated at 150 rpm. After incubation for 1 h, the cells were sedimented by centrifugation (10,000 x g, 5 min) and the resulting cell pellet was resuspended in phosphate buffer (20 ml). The cells were sedimented again and the resulting cell pellet was resuspended in phosphate buffer (1 ml) to a final concentration of 10 to 15 mg total cell

protein ml^{-1}. The alkyne-pretreated cells were stored on ice at 4^{o} C and were used in resting cell assays within 1 h. In all experiments involving alkyne-pretreated cells, untreated control cells were also incubated, washed and resuspended as described above except without exposure to alkynes.

Analytical methods: The concentrations of reactants and products in all experiments were quantified by gas chromatography (GC). For most aqueous phase analytes, samples (2 µl) were taken directly from reaction vials and were immediately injected into a Shimadzu model GC-14A (Kyoto, Japan) gas chromatograph fitted with a flame ionization detector and a stainless steel column (0.3 x 61 cm) filled with Porapak Q (80/100 mesh) (Waters Associates, Framingham, MA). The injection and detector temperatures were 200ºC and 220°C, respectively and nitrogen was used as carrier gas at a flow rate of 15 ml min^{-1}. A column temperature of 100^{o} C was used for the quantification of 1-propanol while the epoxides (1,2-epoxy-2-methylpropane, epoxypropane and 1,2-epoxybutane) were quantified at 120°C. The column temperature was increased to 160º C for the analysis of MPD and HIBA. To quantify the aqueous phase accumulation of epoxyethane, samples (2 µl) were analyzed using a Shimadzu GC-8A gas chromatograph fitted with a flame ionization detector and a stainless steel column (0.3 x 183 cm) filled with Porapak Q (80/100 mesh; Waters Associates, Framingham, MA). The GC was operated with an injector port temperature of 200°C and a detector temperature of 220°C. Nitrogen was used as the carrier gas at a flow rate of 5 ml min^{-1}. In experiments that followed the time courses of gaseous alkene consumption, gas-phase samples (10 µl) were removed directly from reaction vials using gas-

tight micro syringes. The samples were immediately 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 by 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 the carrier gas at a flow rate of 5 ml min^{-1}.

All gas chromatographs were interfaced to Hewlett-Packard HP3395 (Palo Alto, CA) integrators for data collection. Products of 1,2-epoxy-2-methylpropane oxidation (MPD and HIBA) and epoxyethane were identified by co-elution with authentic standards. These compounds were quantified using calibration plots generated by adding known amounts of each compound to reaction vials that containing phosphate buffer (1 ml) but no cells. Each calibration plot contained at least 5 different concentrations of each compound, including at least one concentration above the maximal concentration detected in our experiments. All calibration plots were fitted by linear regression and had final R^2 values of ≥ 0.98.

PCR and Sequencing: Total genomic DNA was extracted from strain ELW1 using the Ultraclean™ Microbial DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to manufacturer's instructions. The quality of extracted DNA was then checked by gel electrophoresis (1% agarose gels stained with ethidium bromide). Polymerase chain reaction (PCR) was performed using purified DNA (3 to 5 ng), primers; 8F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (0.4 μM each), molecular grade PCR water, and an Illustra PuReTaq Ready-To-Go™ PCR

Bead (GE Healthcare Life Sciences) in a final volume of 25 µl. PCR amplification was carried out with a Bio-Rad MJ Mini Personal Thermal cycler using 30 cycles of the following conditions: 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min. Amplified product was purified using the QIAquick PCR Purification Kit (Qiagen, Germantown, MD) and was checked for correct product size and purity by gel electrophoresis. Sterile water (no DNA) was used as a negative control for the reaction. The 16S rRNA amplification product was commercially sequenced in both directions (Eton Bioscience, Inc., San Diego, CA). The resulting nucleotide sequences were compared with the NCBI nucleotide sequence database using the BLASTN sequence alignment tool.

Constants: The saturated aqueous solubilites of ethene, propene, 1-butene and 2-methylpropene were taken as 4.7, 4.8, 3.9 and 4.7 mM, respectively (60). Cell protein concentrations were determined with the Biuret assay (61) after solubilization of cell material for 1 h at 65°C in 3M NaOH and sedimentation of insoluble material by centrifugation (10,000 rpm, 5 min). Bovine serum albumin was used as a standard. Kinetic constants (V_{max} and K_s) for degradation reactions were derived by computer fitting of the data by nonlinear regression to a single substrate-binding model [$Y = V_{max} \cdot X / (K_s + X)$] using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Preliminary characterization: An aerobic 2-methylpropene-utilizing bacterium designated strain ELW1 was isolated from shallow fresh water stream sediment by enrichment culture, as described in the Methods section. The strain grew rapidly (3-5 d at 25^{o} C) on both PCA media plates and MSM agar plates incubated in the presence of 2-methylpropene. The organism formed small (0.5-1.0 mm) yellow circular convex colonies on both plate types. The organism was characterized as urease and catalase positive, non-motile, acid-fast rod (~1-2 µm long and ~1 µm in diameter). PCR amplification and sequencing of the 16S rRNA gene of strain ELW1 was performed as described in the Methods section and the resulting nucleotide sequence was compared with the NCBI nucleotide sequence database. Strain ELW1 was most closely related (>99% sequence similarity) to several other hydrocarbon-utilizing *Mycobacterium* strains including *M. petroleophilum* ATCC 21497 (accession number AF480587.1), *Mycobacterium* sp. TA27 (accession number AB028482) and *Mycobacterium* sp. ATCC 21498 (accession number FJ172312).

Growth on 2-methylpropene and other substrates: The time course of growth of strain ELW1 on 2-methylpropene under carbon-limited conditions was characterized. Changes in culture density (OD_{600}) closely followed 2-methylpropene depletion from the gas phase and no further increases in culture density occurred after depletion of 2-methylpropene (Fig. 2-1). Under the GC conditions used, no intermediates derived from 2-methylpropene oxidation were detected in either the aqueous or gas phases during growth on 2-methylpropene. The

specific growth rate of strain ELW1 on 2-methylpropene from the results described in Fig. 2-1 was estimated at 0.051 h^{-1}.

In addition to 2-methylpropene, we also examined the potential for strain ELW1 to grow on a range of other substrates (Table 2-1). Apart from 2-methylpropene, strain ELW1 did not grow on any of the C_2-C_5 chlorinated, terminal straight chain or branched alkenes tested. However, strain ELW1 did grow slowly on both *cis*- and *trans*-2-butene and an undefined commercial mixture of these two isomers. Strain ELW1 also grew well on 1,2-epoxy-2-methylpropane and both *cis*- and *trans*-2.3-epoxybutane but not on any other epoxides tested. As abiotic hydrolysis may have occurred during the extended culture incubations, we also examined whether the predicted hydrolytic diol products of several epoxides could support growth of strain ELW1. Little or no growth was observed with 1,2-ethanediol, 1,2-propanediol or 1,3-butanediol, the respective products expected from the hydrolysis of epoxyethane, epoxypropane and 1,2-epoxybutane. In contrast, 2-methyl-1,2-propanediol (MPD) and 2,3-butanediol, the respective diols expected from hydrolysis of 1,2-epoxy-2-methylpropane and 2,3-epoxybutane respectively, both supported strong growth. Among the allyl alcohols tested only 2-methyl-2-propen-1-ol, the corresponding allyl alcohol of 2-methylpropene, only supported slow and limited growth. Niether 2-propen-1-ol nor 3-buten-1-ol, the respective allyl alcohols derived from propene and 1-butene respectively, supported growth.

None of the tested aromatic compounds, ether oxygenates or C_1-C_6 *n*-alkanes supported growth while 1-heptane and longer chain *n*-alkanes (C_8 to C_{18}) were effective growth substrates. Following a similar trend, no growth was observed with smaller branched

alkanes such as 2-methylpropane (isobutane) and 2-methylbutane while 2-methylhexane was utilized as a growth substrate. With the exception of methanol, strain ELW1 grew well on all of the normal and branched 1^{o} alcohols tested but not on any tested 2^{o} or 3° alcohols. Neither acetone nor acetaldehyde supported growth while rapid growth was supported by 3-hydroxybutanone (acetoin) and slower growth was supported by 2-methylpropionaldehyde (isobutyraldehyde). Among the organic acids tested, this strain grew well on succinate, pyruvate, acetate, propionate and 3-hydroxybutyrate but growth on a range of branched acids was generally limited. The notable exception to this trend was the rapid growth of this strain on HIBA.

Alkyne inactivators of 2-methylpropene oxidation: Alkynes are well-known mechanism-based inactivators of a number of oxygenase enzymes, including alkene monooxygenase (24). To further characterize the pathway and enzymes involved in 2-methylpropene metabolism, we examined the potential for a range of terminal alkynes to irreversibly inactivate 2-methylpropene-oxidizing activity in strain ELW1. Resting 2-methylpropene-grown cells were incubated individually for 1 h with equivalent amounts (90 µmoles) of five terminal alkynes (ethyne, propyne, 1-butyne, 1-hexyne and 1-octyne) in the absence of 2-methylpropene. Washed, alkyne-pretreated cells were then added to small-scale incubations and subsequent consumption of 2-methylpropene was monitored by analyzing the reaction gas phase by GC. Compared to untreated cells, consumption of 2-methylpropene was largely unaffected by pretreating cells with either ethyne or 1-hexyne (Fig. 2-2). Both propyne- and 1-butyne-pretreated cells were initially strongly inhibited in these assays but this inhibitory

effect decreased with time. In contrast, 1-octyne-treated cells showed no detectable 2-methylpropene-consuming activity over the full time course of the incubation (90 min).

Oxidation of other alkenes and their epoxides: Although strain ELW1 did not grow on any *n*-alkenes tested (Table 2-1), resting 2-methylpropene-grown cells consumed ethene, propene and 1-butene without a lag phase (Fig. 2-3A). When cells were incubated with an equivalent dissolved concentration of each alkene (53 μM), the initial rates of propene and 1-butene oxidation (~48 and ~47 nmoles min^{-1} mg total $protein^{-1}$, respectively) were comparable to the rate observed with 2-methylpropene (~53 nmoles min^{-1} mg total $protein^{-1}$) while ethene was consumed more slowly (~21 nmoles min^{-1} mg total $protein^{-1}$). The oxidation of all of the alkenes tested was fully inhibited in cells pretreated with 1-octyne.

No products were detected in either the aqueous or gas phases for the incubations containing 2-methylpropene, propene or 1-butene. A single product was detected in reactions containing ethene (not shown) that was identified as epoxyethane by co-elution with an authentic standard. In a separate experiment, we examined the kinetics and stoichiometry of concurrent ethene consumption and epoxyethane production by 2-methylpropene-grown cells, as well as the effects of 1-octyne on these processes. The rate of ethene consumption (19.2 nmoles min^{-1} mg total $protein^{-1}$) remained near constant throughout the reaction time course and was accompanied by similar rate of epoxyethane accumulation (12.8 nmoles min^{-1} mg total $protein^{-1}$) (Fig. 2-3B). Like ethene consumption, production of epoxyethane was fully inhibited in cells pretreated with 1-octyne and there was no substantial abiotic degradation of epoxyethane in reactions conducted with heat-killed cells. We also examined

the ability of 2-methylpropene-grown cells to consume epoxides in the absence of alkenes (Table 2-2). Resting 2-methylpropene-grown cells degraded all of the tested epoxides without a lag phase and epoxyethane, epoxypropane and 1,2-epoxybutane were consumed at rates that were ~7%, ~24% and ~33% of the rate of 1,2-epoxy-2-methylpropane degradation, respectively. Rates of epoxyalkane degradation were unaffected by pretreating cells with 1-octyne and abiotic losses of epoxides were minimal (≤ 3 nmoles min-1 mg total protein-1) in control incubations conducted with heat-killed cells. As alkene oxidation could potentially result in alcohol production, we also examined whether 2-methylpropane-grown cells could oxidize 2-propen-1-ol, 3-buten-1-ol and 2-methylprop-2-en-1-ol. All three allyl alcohols were oxidized at similar rates that were ≤15% of the rate of 1,2-epoxy-2-methylpropane degradation. Unlike epoxyalkanes, oxidation of all three allyl alcohols was inhibited substantially (>60%) in cells pretreated with 1-octyne.

Kinetics of 2-methylpropene and 1,2-epoxy-2-methylpropane degradation and metabolite production: The results shown in Fig. 2-3 suggest that, like other alkene-oxidizing bacteria, the enzyme responsible for initiating 2-methylpropene catabolism in strain ELW1 is an epoxide-generating monooxygenase. The results in Table 2-2 also suggest 1,2-epoxy-2-methylpropane can be rapidly consumed and is therefore unlikely to accumulate to detectable levels during 2-methylpropene oxidation by resting cells. A more detailed kinetic analysis of 2-methylpropene and 1,2-epoxy-2-methylpropane consumption by 2-methylpropene-grown cells was conducted to investigate this possibility. Estimates the K_s and V_{max} values for 2-methylpropene oxidation of 11 (SE=1.4) μM and 116 (SE=3.4) nmoles

min^{-1} mg total $protein^{-1}$ were obtained from initial rates measurements (Fig. 2-4A). The corresponding values for 1,2-epoxy-2-methylpropane were 57 (SE=11.8) μM and 244 (SE=9.7) nmoles min^{-1} mg total $protein^{-1}$ respectively (Fig. 2-4B).

To further investigate the pathway of 2-methylpropene metabolism, 2-methylpropene -grown cells of strain ELW1 were incubated with 1,2-epoxy-2-methylpropane (5 mM). The time course of epoxide consumption and metabolite accumulation were determined using GC analysis of the reaction medium and detected metabolites were identified based on coelution with authentic samples. Resting cells rapidly consumed the epoxide within 30 min and stoichiometric amounts of MPD accumulated in the reaction medium (Fig. 2-5). Further oxidation of MPD led to the accumulation and subsequent slower consumption of HIBA during the remainder of the reaction time course. A nearly identical time course of substrate and product conversions was observed using cells pretreated with 1-octyne. No additional metabolites were detected in either reaction type using the GC conditions employed in this experiment.

Effects of cobalt ions on growth on 2-methylpropene: As outlined in the Introduction, the microbial metabolism of HIBA has been characterized in MTBE- and TBA-metabolizing strains in which HIBA is converted to 3-hydroxybutyrate (3HB) by a cobalamin-dependent mutase. We therefore examined the growth of strain ELW1 in cobalt supplemented (MSM) and cobalt-deficient (MSM-Co) media. Absence of cobalt ions completely prevented growth on 2-methylpropene (Fig. 2-6A) but had no discernable effect on its growth on 1-propanol (Fig. 2-6B). We also investigated the effects of cobalt ions on a range of growth supporting

substrates including potential metabolites in the pathway of 2-methylpropene metabolism. Growth of strain ELW1 on 3HB and other substrates such as acetate, fructose, 2,3-butanediol, and 2-methyl-1-propanol was largely unaffected when the cells were grown in cobalt-deficient medium (MSM-Co) compared with cobalt-supplemented medium (MSM) (Table 2-3). In contrast, absence of cobalt ions severely inhibited growth on 2-methylpropene, 1,2-epoxy-2-methylpropane, MPD and HIBA.

DISCUSSION

In this study we have described the isolation and physiological characterization of a novel alkene-oxidizing bacterium that grows on 2-methylpropene as a sole source of carbon and energy. This strain utilizes a monooxygenase to initiate 2-methylpropene catabolism and hydrolytically cleaves of the resulting epoxide. The subsequent steps in 2-methylpropene oxidation show overlap with the previously established pathway of bacterial MTBE and TBA catabolism and appear to involve a cobalt-dependent transformation of HIBA. A proposed pathway for 2-methylpropene catabolism is summarized in Fig. 2-7 and the evidence supporting these main conclusions and this pathway are discussed in more detail in the following sections.

***Mycobacterium* ELW1 as a novel isolate:** As far as we are aware, *Mycobacterium* strain ELW1 is the first example of a 2-methylpropene-metabolizing bacterium obtained in pure culture. Although some alkene-metabolizing bacteria can oxidize 2-methylpropene at substantial rates (43, 44), none of these strains have been reported to grow on this gas. The only report we are aware of that examined 2-methylpropene as a potential growth-supporting substrate concerned *R. rhodochrous* B-276 (62) This strain was reported as growing on ethene, propene, 1-butene, *n*-butane and 1,3-butadiene, but not on methane, ethane or 2-methylpropene.

Like many other alkene-oxidizing bacteria, strain ELW1 is a *Mycobacterium* strain. The highest levels of 16S rRNA gene similarity (>99% nucleotide sequence homology) obtained from database searches were with several other hydrocarbon-utilizing mycobacteria

including *M. petroleophilum* ATCC 21497, *Mycobacterium* sp. ATCC 21498, and *Mycobacterium* sp. TA27. The two ATCC strains are physiologically similar *n*-alkane-metabolizing bacteria isolated from samples taken from an oil drilling well. They were both patented for production of single cell protein (63). Strain TA27 is an ethane-metabolizing strain isolated from soil contaminated with tetrachloroethylene (64). This strain can cometabolically oxidize 1,1,1-trichloroethane, trichloroethene, and MTBE (65, 66). While strain ELW1 grows well on some *n*-alkanes (Table 2-1), we are not aware of any reports that any of the three strains phylogenetically closely related to strain ELW1 have alkene-metabolizing capabilities.

Other than 2-methylpropene, the alkene growth substrate range of strain ELW1 is limited and it did not grow on most linear, branched or alkenes tested (Table 2-1). A notable exception to this was its ability to grow, albeit very slowly, on both *cis* and *trans* of 2-butene (Table 2-1). Diverse *n*-alkane- and alkene-metabolizing (14, 43, 44, 67, 68) bacteria can oxidize 2-butene but there are few descriptions of strains that can utilize either the *cis* or *trans* isomers as sole sources of carbon and energy (14, 69). *Nocardia* TB1 can grow on both *trans*-2-butene and butane and is subsequently simultaneously adapted to metabolize both gases (70). This strain initially oxidizes a terminal methyl group of *trans*-2-butene to produce but-2-en-1-ol (crotyl alcohol), but-2-enal (crotonaldehyde) and but-2-enoic acid (crotonic acid) as sequential intermediates. These metabolites reflect a terminal *n*-alkane oxidation pathway rather than one involving initial oxidation of the carbon-carbon double bond to an epoxide. *Mycobacterium vaccae* JOB5 has also been reported to grow on *trans*-2-butene (71) but this capability has not been further characterized.

2-Methylpropene oxidation: Like many other gaseous alkene-metabolizing bacteria (72), strain ELW1 appears to initiate 2-methylpropene catabolism using a monooxygenase to generate an epoxide. Two lines of evidence support this conclusion. First, we observed the non-growth supporting alkenes ethene, propene, and 1-butene were all oxidized by 2-methylpropene-grown cells (Fig. 2-3A). While we did not detect any oxidation products during most of these reactions, epoxyethane was detected and represented a substantial (≤65%) portion of the ethene consumed (Fig. 2-3B). Epoxyethane accumulation appeared to be due to the limited capacity of 2-methylpropene-grown cells to further degrade this epoxide. In contrast, our kinetic studies indicate the V_{max} for 1,2-epoxy-2-methylpropane degradation is 2–fold higher than the V_{max} for 2-methylpropene oxidation while the K_s values for these compounds are both in the low micromolar range (Fig. 2-4A and B). This analysis suggests 1,2-epoxy-2-methylpropane generated during oxidation of 2-methylpropene is unlikely to accumulate due to rapid further degradation. This interpretation agrees with the common observation that epoxides rarely accumulate when resting alkene-grown strains oxidize the alkene on which they were originally grown (14, 26). The relatively high rates of degradation observed for epoxyproane and 1,2-epoxybutane (Table 2-2) also indicates these epoxides can also be consumed rapidly by 2-methylpropene-grown cells and likely also explains why they were also not detected during the oxidation of their corresponding alkenes (Fig. 2-3A). While epoxyethane production from ethene is compatible with a monooxygenase-catalyzed activation of alkenes, it does not exclude the possibility that alkenes are also initially oxidized to unsaturated alcohols. However, compared to 1,2-epoxy-2-methylpropane, 2-methyl-2-propen-1-ol only supported slow and limited growth of strain

ELW1 (Table 2-1) and was also only slowly oxidized by 2-methylpropene-grown cells (Table 2-2). The same trends were observed with 2-propen-1-ol and 3-buten-1-ol, the corresponding allylic alcohols of propene and 1-butene, respectively. The slow oxidation of these alcohols may reflect its further oxidation to the corresponding aldehydes and acids by non-specific dehydrogenases. As oxidation of these alcohols was also consistently partially (~60%) inhibited in 1-octyne-treated cells (Table 2-2), they may also undergo further monooxygenase-catalyzed epoxidation as well. While our results do indicate strain ELW1 can oxidize and productively metabolize 2-methyl-2-propen-1-ol, collectively these reactions appear to be slow and cannot be considered as major components of the pathway of 2-methylpropene-catabolism. However, these reactions may well represent a minor and useful salvage pathway for metabolizing small amounts of 2-methyl-2-propen-1-ol generated during the initial monooxygenase-catalyzed activation of 2-methylpropene.

The second line of evidence is that alkene-oxidizing activity was selectively inactivated by terminal alkynes (Figs. 2-2 and 2-3). Terminal alkynes are mechanism-based inactivators of many bacterial monooxygenases including soluble and particulate methane monooxygenase (73) ammonia monooxygenase (68) toluene-4-monooxygenase (74), and alkane hydroxylase (75). Propyne, is a potent mechanism-based inactivator of the two structurally distinct AMOs in *X. autotrophicus* Py2 (24) and *R. rhodochrous* B-276 (76). As might be expected for mechanism-based inactivators that are activated through the catalytic activity of the target enzyme, the sensitivity of the putative 2-methylpropene-oxidizing monooxygenase in strain ELW1 to terminal alkynes was partly reflected its reactivity towards its alkene substrates. For example, ethyne had limited effects on 2-methylpropene

oxidation (Fig. 2-2) and the corresponding C_2 alkene, ethene, was only slowly oxidized (Fig. 2-3A and B). In contrast, propyne and 1-butyne were much more potent inactivators of 2-methylpropene oxidation (Fig. 2-2) and both propene and 1-butene were rapidly oxidized by 2-methylpropene-grown cells (Fig. 2-3A). The effects of 1-octyne, the most potent alkyne identified in this study, enabled us to demonstrate that 2-methylpropene oxidation and both ethene oxidation and concurrent epoxyethane production were fully inhibited in 2-methylpropene-grown, alkyne-pretreated cells (Fig. 2-3A and B) while 1,2-epoxy-2-methylpropane consumption was unaffected by this compound (Fig. 2-5). There are several examples of longer chain terminal alkynes that inactivate hydrocarbon-oxidizing monooxygenases. For instance, 1-decyne is a potent inactivator of toluene-dependent growth of *Burkholderia cepacia* G4 (74) and 1-octyne is a potent inactivator of ammonia monooxygenase in the nitrifying bacterium *Nitrosomonas europaea* (68).

Degradation of 1,2-epoxy-2-methylpropane: In contrast to the potent effects of alkynes on alkene oxidation, pretreatment of cells with 1-octyne had little effect on theier abilities to degrade various epoxides (Table 2-2) or the time course of 1,2-epoxy-2-methylpropane degradation and subsequent production and consumption of its metabolites, MPD and HIBA (Fig. 2-5). These results suggest the effects of alkynes are specific for the initial conversion of alkenes to epoxides in strain ELW1 and further support our conclusion that MPD and HIBA are legitimate intermediates in the pathway of 2-methylpropene metabolism.

Production of MPD from 1,2-epoxy-2-methylpropane is compatible with a hydrolytic cleavage of the epoxide ring. Epoxide hydrolase-catalyzed hydrolysis is a common epoxide

detoxification mechanism in eukaryotes but has not been frequently implicated in microbial gaseous alkene metabolism. *Nocardia* A60 hydrolyzes epoxypropane to 1,2-propanediol (77) and epoxide hydrolases have been identified in *Agrobacterium radiobacter* (*Pseudomonas*) AD1 (78-80) and *Corynebacterium* N-1074 (81, 82) during growth on epichlorohydrin (1-chloro-2,3-epoxypropane). Despite these limited examples, an early genome survey (83) revealed epoxide hydrolases are widely distributed and putative genes encoding these enzymes were identified in approximately 20% in the 389 sequenced prokaryote genome sequences available at the time the study was conducted. Among all of the major groups of prokaryotes, putative epoxide hydrolase-encoding genes were most frequently detected in mycobacteria and other actinobacteria. Of 30 the actinobacterial genomes analyzed, 18 strains contained putative epoxide hydrolase genes with an average of 5 genes per strain.

A final observation that further supports MPD as the physiologically relevant intermediate in 2-methylpropene catabolism is that the only other growth-supporting diol identified in this study was 2,3-butanediol, the diol expected from the hydrolysis of 2,3-epoxybutane (Table 2-1). In turn, this epoxide is the product expected from the epoxidation of *cis* and *trans*-2-butene, the only other gaseous alkene growth substrates we identified for strain ELW1 (Table 2-1). This raises the possibility that 2-methylpropene and the two 2-butene isomers are degraded through the same initial reactions and that the same enzymes may be involved in the preliminary stages of the catabolism of all three growth-supporting alkenes.

Overlap between 1,2-epoxy-2-methylpropane and MTBE oxidation pathways: The time course of 1,2-epoxy-2-methylpropane degradation (Fig. 2-5) indicates HIBA was the next detected metabolite after MPD. As indicated in the Introduction, MPD and HIBA are also sequential metabolites found in the pathway of aerobic microbial MTBE and TBA degradation (Fig. 2-7). Oxidation of MPD to HIBA is a four-electron oxidation. A conventional two-electron oxidation of MPD catalyzed by a typical alcohol dehydrogenase suggests an aldehyde intermediate, 2-hydroxyisobutyraldehyde (HIBAL), might reasonably be expected prior to HIBA. This putative aldehyde intermediate was not observed in the present study and also has not been observed in any studies of aerobic microbial MTBE or TBA degradation (51). In the TBA-metabolizing strain *Mycobacterium austroafricaunum* IFP 2012, several proteins are differentially expressed in the presence of TBA and genes for two of these proteins have been identified as putative alcohol (*mpdb*) and aldehyde dehydrogenases (*mpdc*) (84). When *mpdb* and *mpdc* are heterologously and functionally co-expressed they confer to host cells the ability to oxidize MPD to HIBA without accumulation of HIBAL. To account for this effect, it was suggested that MPD was first oxidized to HIBAL by MpdA and then rapidly consumed by MpdB so that the aldehyde intermediate does not accumulate to detectable levels. However, this was not confirmed by expressing the genes for these enzymes independently. An alternative is that MPD undergoes a four-electron oxidation to HIBA catalyzed by a single enzyme in a reaction similar to the oxidation of choline to glycine betaine catalyzed by choline dehydrogenase/oxidases (85-87). In this reaction the aldehyde intermediate, betaine aldehyde, remains enzyme-bound (88). The putative alcohol dehydrogenase (MpdB) identified in *M. austroafricanum* IFP is closely

related to the choline dehydrogenase (BetA) in *E. coli* (89), which is known to catalyze the four electron-oxidation of choline to gycine betaine. Further studies will be required to determine what enzymes and reactions are involved in MPD oxidation to HIBA in strain ELW1 and how similar these are to those in MTBE- and TBA-metabolizing strains.

The final metabolite detected during 1,2-epoxy-2-methylpropane degradation (Fig. 2-5) was HIBA. In organisms that grow on MTBE and TBA, HIBA is converted to 3HB by a cobalamin-dependent mutase, 2-hydroxyisobutyryl-CoA mutase (HCM) (51). The key role of this enzyme in MTBE and TBA oxidation places a stringent requirement for cobalt or cobalamin on MTBE- and TBA-metabolizing organisms. Our results indicate growth of strain ELW1 on 2-methylpropene (Fig. 2-6A) was also strictly cobalt-dependent while growth on 1-propanol (Fig. 2-6B), a substrate that is unlikely to require use of HCM, was unaffected by the lack of added cobalt ions. Our results presented in Table 2-3 further suggest that if a pathway of 2-methylpropene catabolism in strain ELW1 involving 2-methylpropene, 1,2-epoxy-2-methylpropane,, MPD, HIBA and 3HB as sequential intermediates is correct (Fig. 2-7), the requirement for cobalt ions can be localized to the terminal step in this pathway involving the enzyme likely responsible for converting HIBA to 3HB (Table 2-3).

HCM was first identified in the MTBE-metabolizing strain *A. tertiaricarbonis* L108 (52). It is a dimeric enzyme consisting of a large substrate-binding component (HcmA) and a smaller B_{12}-binding component (HcmB). The corresponding genes (*hcmA* and *hcmB*) are organized in an operon containing additional genes for an acetyl-CoA synthetase and a chaperonin. The same genes and operon organization occur in MTBE-metabolizing (*M.*

petroleiphilum PM1), photosynthetic (*Rhodobacter sphaeroides*) and marine alkane-metabolizing (*Marinobacter algcola*) bacteria (53). The same genes and similar operon structure also occur in two well-characterized alkene-metabolizing strains; *X. autotrophicus* Py2 and the ethene- and chloroethene-metabolizing strain *Nocardioides* JS614 (53). Although alkene-grown cells of both of these strains oxidize 2-methylpropene, neither strain is known to grow on 2-methylpropene and both strains activate epoxides using EACoMT, which is inactivated by 1,2-epoxy-2-methylpropane. The role of these genes in these strains currently remains unknown. Notably, strain L108 generates small amounts of 2-methylpropene from a dehydration reaction catalyzed by the monooxygenase responsible for oxidizing TBA to 2-methyl-1,2-propanediol (5). It is interesting to speculate that the MTBE- and TBA-degrading activity of strain L108 is connected in some way to its ability to generate and potentially consume 2-methylpropene.

FIGURES AND TABLES

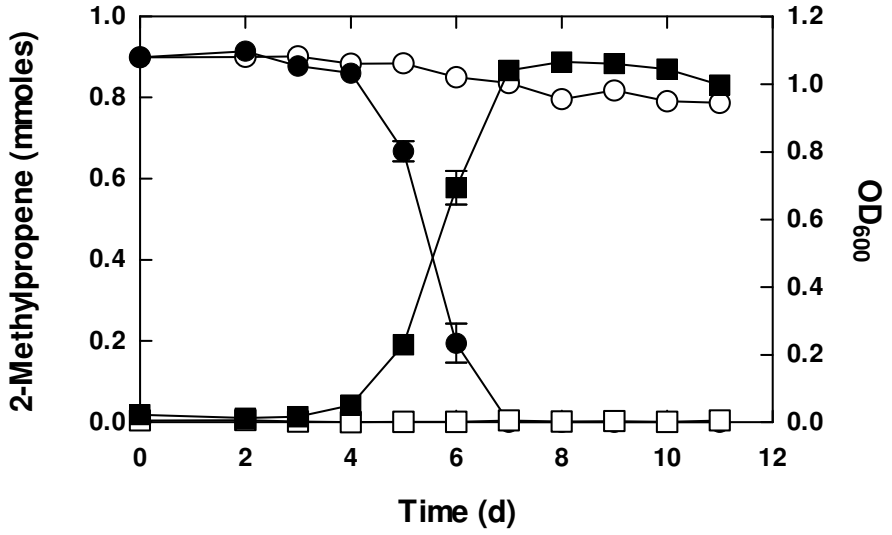


Figure 2-1: Growth of strain ELW1 on 2-methylpropene. Strain ELW1 was grown in batch culture under carbon-limited conditions (3.3% vol/vol 2-methylpropene in gas phase) in sealed glass media bottles (700 ml) containing MSM (100 ml). Changes in culture density (OD_{600}) and the amount of 2-methylpropene in the gas phase were determined over time, as described in the Methods section. The Figure shows the time course for (●,○) 2-methylpropene consumption and (■,□) culture density (OD_{600}) for inoculated (closed symbols) and uninoculated (open symbols) cultures, respectively. Data presented are the means and ranges of values obtained from three separate cultures.

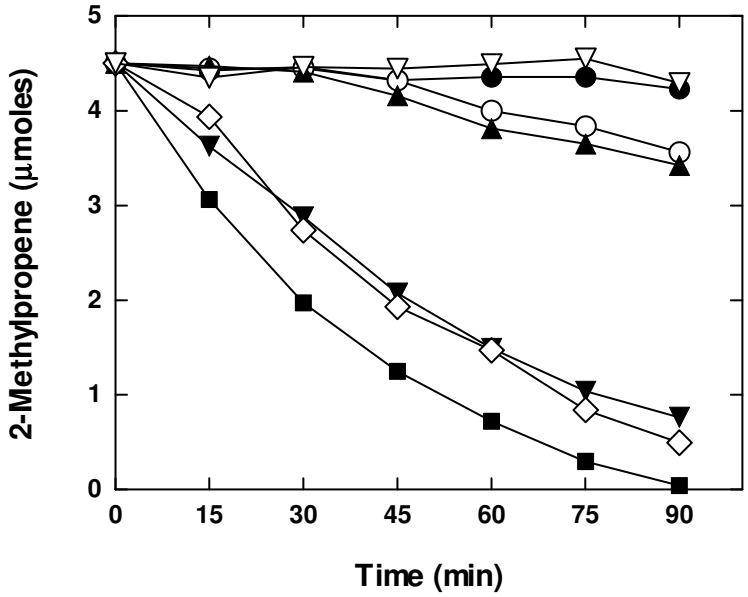


Figure 2-2: Inactivation of 2-methylpropene-oxidizing activity by alkynes. Resting 2-methylpropene-grown cells of strain ELW1 were pretreated in the absence of 2-methylpropene with equal amounts (90 µmoles) of alkynes or without any alkyne for 1 h, as described in the Methods section. Washed, pretreated cells (1.3 mg total protein) were then incubated in buffer in sealed reaction vials (10 ml) with 2-methylpropene (4.5 µmoles). The Figure shows the time course of 2-methylpropene consumption for (●) an abiotic control reaction conducted without cells or reactions containing either (■) cells pretreated without an alkyne or cells pretreated with (▼) ethyne, (○) propyne, (▲) 1-butyne, (◇) 1-hexyne, or (▽) 1-octyne. Data presented are for cells obtained from a single culture and are representative of several similar experiments.

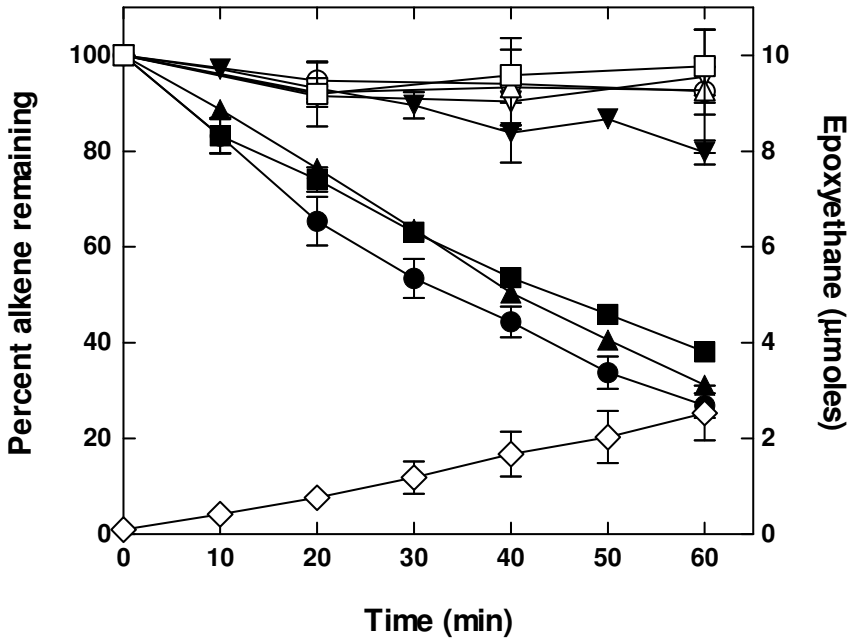


Figure 2-3A: Oxidation of 2-methylpropene and non-growth supporting alkenes. Resting, 2- methylpropene-grown cells of strain ELW1 (1.5 mg of total protein) were incubated in buffer in reactions vials (10 ml) with varying amounts of each alkene to the gas phase to generate an equal initial dissolved concentration (53 µM) of each alkene. Consumption of each gas was monitored over time by GC analysis of the gas phase, as described in the Methods section. The Figure shows the time course of (●,○) 2-methylpropene, (▼, ▽) ethene, (▲,△) propene, and (■,□) 1-butene consumption by cells pretreated either with (open symbols) or without (closed symbols) 1-octyne. The Figure also shows the time course of (◇) epoxyethane accumulation in reactions conducted with ethene. The data are plotted as the mean values obtained for two separate cultures. The error bars show the range of individual data points.

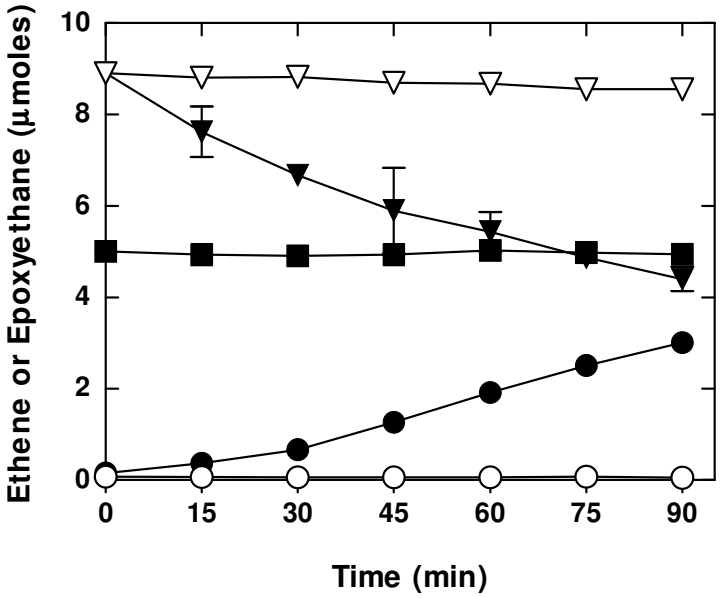


Figure 2-3B: Oxidation of ethene to epoxyethane by strain ELW1. Resting 2-methylpropene-grown cells (2.6 mg of total protein) were incubated in buffer in sealed reaction vials (10 ml) with ethene (9 µmoles) and both the liquid and gas-phases were analyzed by GC at indicated times, as described in the Methods section. The Figure shows the time course for (▼,▽) consumption of ethene from the gas phase, and (●,○) accumulation of epoxyethane in the aqueous phase for cells pretreated with (open symbols) and without (closed symbols) 1-octyne. The Figure also shows (■) the time course of epoxyethane consumption from the aqueous phase in a control reaction that contained epoxyethane (5 µmoles) and cells (2.6 mg total protein) that had been heat-treated at 95ºC for ~15 min. The data are plotted as the mean values obtained for two separate cultures. The error bars show the range of individual data points.

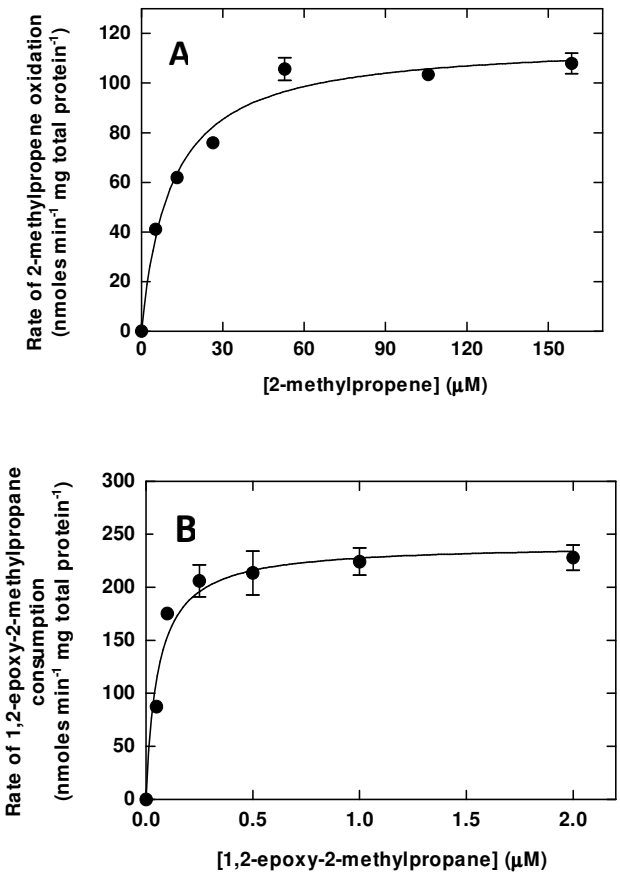


Figure 2-4: Kinetics of 2-methylpropene and 1,2-epoxy-2-methylpropane degradation. The Figure shows plots the effects of substrate concentration of the specific rates of (Panel A) 2-methylpropene and (Panel B) 1,2-epoxy-2-methylpropane degradation by resting 2-methylpropene-grown cells of strain ELW1. **Panel A.** Cells (0.3 mg total protein) were incubated in buffer in sealed reactions vials (10 ml) with varying amounts of 2-methylpropene added to the gas phase to provide the indicated estimated initial dissolved 2-methylpropene concentrations. Consumption of 2-methylpropene was determined over time by GC analysis of the gas phase, as described in the Methods section. The Figure shows a plot of the initial rates of 2-methylpropene consumption (0-20 min) and a computer fit of these data to a hyperbolic single substrate-binding model (R^2 =0.98). **Panel B.** Cells (0.06 mg of total protein) were incubated in sealed reactions vials (10 ml) with a range of initial dissolved 1,2-epoxy-2-methylpropane concentrations. Consumption of 1,2-epoxy-2-methylpropane was determined over time by GC analysis of the reaction aqueous phase, as described in the Methods section. The Figure shows a plot of the initial rates of 1,2-epoxy-2-methylpropane consumption (0-20 min) and a computer fit of these data to a hyperbolic single substrate-binding model (R^2 =0.95). In both Panels the data presented are the means and ranges of values obtained from two separate cultures.

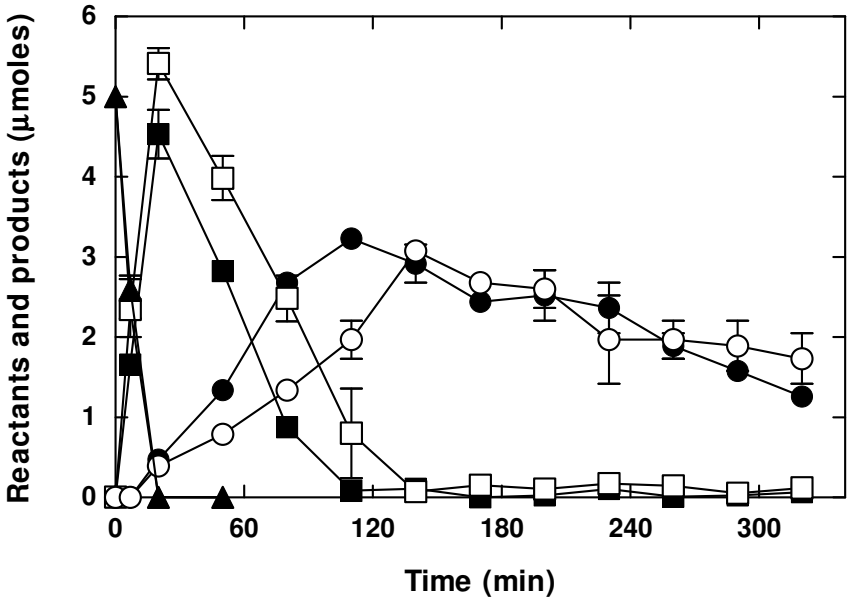


Figure 2-5: Degradation of 1,2-epoxy-2-methylpropane and detection of metabolites. Resting 2-methylpropene-grown cells of strain ELW1 (1 mg total protein) were incubated in buffer in sealed reaction vials (10 ml) with 1,2-epoxy-2-methylpropane (5 µmoles). Changes in amounts of the epoxide and its metabolites were determined by GC analysis of the reaction aqueous phase, as described in the Methods section. The Figure shows the time course of (▲,△) 1,2-epoxy-2-methylpropane consumption and the subsequent production and consumption of (■,□) MPD and (●,○) HIBA by cells either pretreated with (open symbols) or without (closed symbols) 1-octyne. The data presented are the means and ranges of values obtained from two separate cultures.

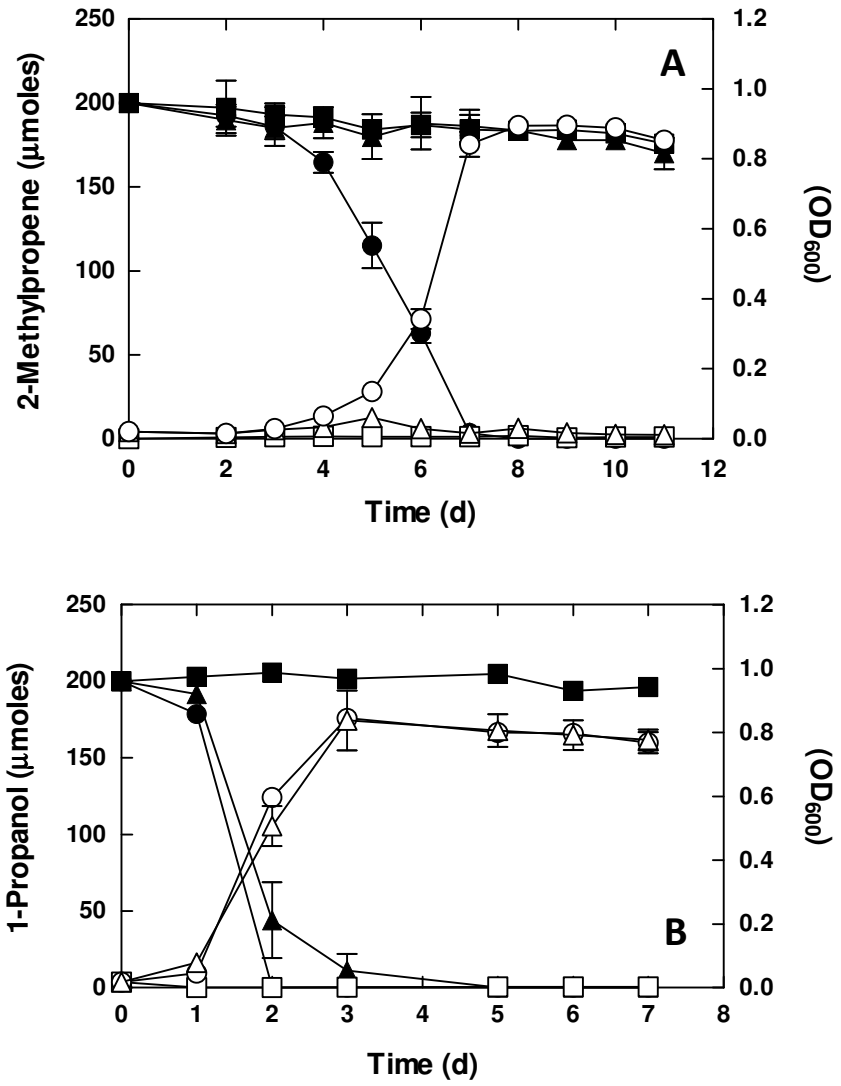
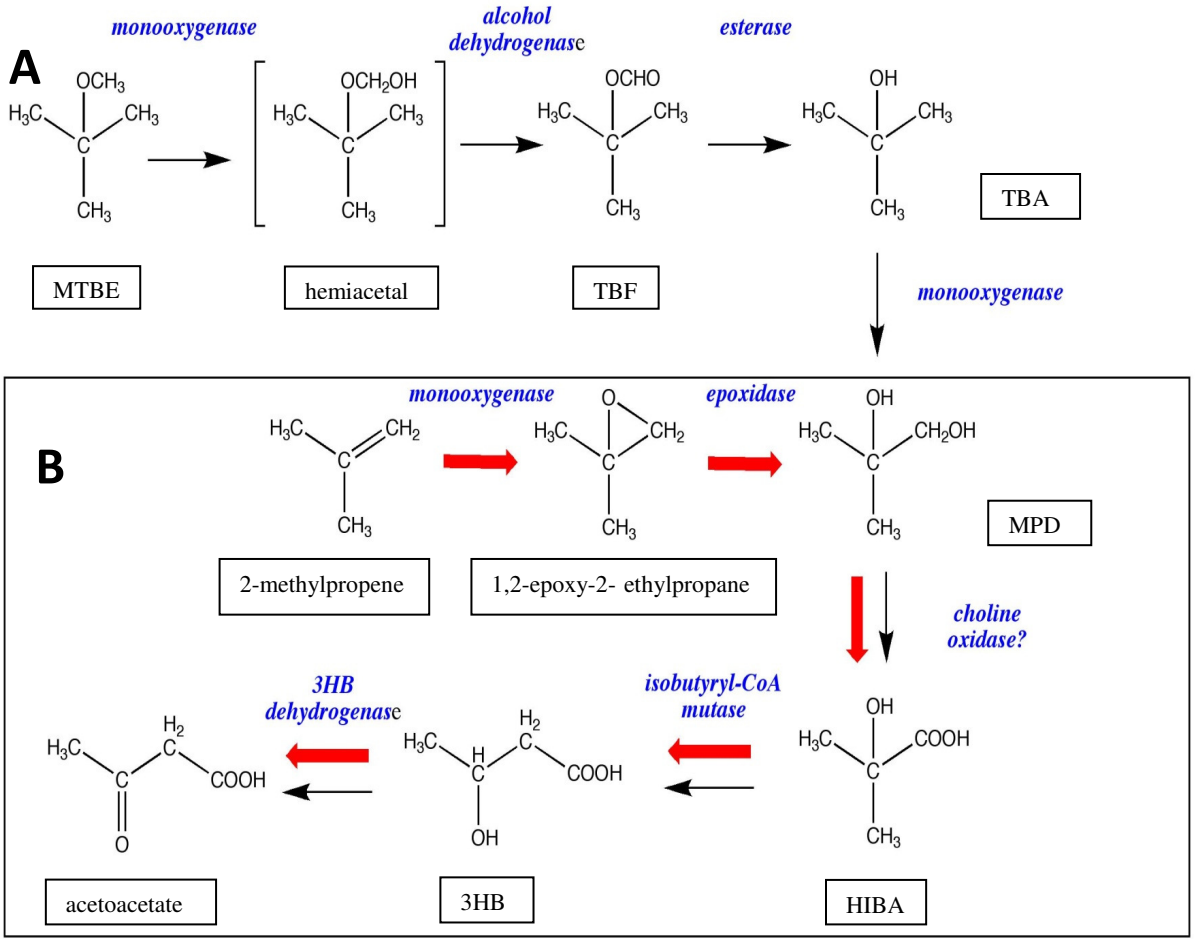


Figure 2-6: Effects of cobalt ions on growth of strain ELW1 on 2-methylpropene and 1-propanol. Cultures of strain ELW1 were grown on (Panel A) 2-methylpropene (3.33% vol/ vol gas phase) and (Panel B) 1-propanol (8 mM) under carbon-limited conditions in either cobalt-sufficient (MSM) or cobalt-depleted (MSM-Co) media, as described in the Methods section. **Panel A:** shows the time course for changes in amount of 2-methylpropene (closed symbols) and culture density (open symbols) for inoculated cultures grown in (●,○) MSM medium, (▲,△) MSM-Co medium and for (■,□) uninoculated MSM medium. **Panel B:** shows the time course for changes in the amount of 1-propanol (closed symbols) and culture density (open symbols) for inoculated cultures grown in (●,○) MSM medium, (▲,△) MSM-Co medium and for (■,□) uninoculated MSM medium. Data presented are the means and ranges of values obtained from three separate cultures.



→ Pathway of MTBE catabolism.

➡ Proposed pathway of 2-methylpropene catabolism by *Mycobacterium* sp. ELW1.

Figure 2-7: Proposed pathway of 2-methylpropene catabolism: The Figure shows a proposed pathway for 2-methylpropene catabolism in *Mycobacterium* sp. ELW1 based on the reactions and metabolites identified in this study. The Figure also shows metabolites previously identified in studies of the aerobic degradation of MTBE (51) and the potential overlap between the pathways of MTBE and 2-methylpropene catabolism. **Panel A:** The panel shows the major intermediates with proposed enzymes in the degradation of MTBE to TBA by aerobic MTBE-oxidizing bacteria. **Panel B:** The panel shows the proposed conversion of 2-methylpropene to 1,2-epoxy-2-methylpropane by *Mycobacterium* sp. ELW1 and the down stream metabolites of 1,2-epoxy-2-methylpropane which are common to both the pathway of MTBE and 2-methylpropene catabolism. The proposed enzymes responsible for each reaction in the two pathways are also indicated.

Table 2-1: Growth substrate range of *Mycobacterium* sp. ELW1.

Substrate [a]	Culture density (OD_{600}) [b]		
	After 7 d	After 14 d	After 28 d
Alkenes			
ethene	≤0.01 (0.00)	≤0.01 (0.00)	0.02 (0.01)
propene	≤0.01 (0.00)	≤0.01 (0.00)	0.01 (0.00)
1-butene	0.01 (0.00)	≤0.01 (0.00)	0.01 (0.00)
2-butene (mixture of *cis* & *trans*)	0.02 (0.00)	0.05 (0.00)	0.53 (0.11)
cis-2-butene	0.02 (0.00)	0.04 (0.00)	0.52 (0.10)
trans-2-butene	0.01 (0.00)	0.03 (0.00)	0.15 (0.06)
2-methylpropene	1.56 (0.00)	1.48 (0.00)	1.09 (0.12)
2-methyl-1-butene	0.01 (0.01)	0.01 (0.14)	0.01 (0.00)
2-methyl-2-butene	0.03 (0.00)	0.01 (0.00)	0.03 (0.00)
3-methyl-1-butene	≤0.01 (0.01)	≤0.01 (0.00)	0.01 (0.01)
1,3-butadiene	≤0.01 (0.00)	0.01 (0.00)	0.01 (0.00)
2-methyl-1,3-butadiene	0.03 (0.00)	0.02 (0.00)	0.04 (0.00)
chloroethene	0.01 (0.02)	0.01 (0.01)	0.01 (0.00)
1,1-dichloroethene	0.01 (0.01)	0.01 (0.00)	0.01 (0.00)
cis-1,2-dichloroethene	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)
trans-1,2-dichloroethene	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)
trichloroethene	0.01 (0.00)	0.01 (0.00)	≤0.01 (0.00)
tetrachloroethene	0.01 (0.00)	0.01 (0.00)	≤0.01 (0.00)
Epoxides			
epoxyethane	≤0.01 (0.00)	≤0.01 (0.00)	≤0.01 (0.00)
epoxypropane	≤0.01 (0.00)	≤0.01 (0.00)	≤0.01 (0.00)
1,2-epoxybutane	≤0.01 (0.00)	≤0.01 (0.00)	≤0.01 (0.00)
3,4-epoxy-1-butene	0.01 (0.00)	≤0.01 (0.00)	0.01 (0.00)
1,2-epoxy-2-methylpropane	0.02 (0.00)	0.79 (0.09)	0.57 (0.08)
cis-2,3-epoxybutane	0.21 (0.13)	0.37 (0.07)	0.51 (0.03)
trans-2,3-epoxybutane	0.13 (0.00)	0.42 (0.01)	0.32 (0.02)
epichlorohydrin	≤0.01 (0.00)	≤0.01 (0.00)	≤0.01 (0.00)
Diols			
1,2-ethanediol	0.01 (0.01)	0.01 (0.00)	0.01 (0.00)
1,2-propanediol	0.01 (0.00)	0.02 (0.00)	0.08 (0.02)
1,2-butanediol	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)
1,3-propanediol	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)
1,3-butanediol	0.01 (0.01)	0.02 (0.01)	0.05 (0.00)
1,4-butanediol	0.01 (0.00)	0.02 (0.00)	0.01 (0.01)
meso-2,3-butanediol	0.48 (0.19)	0.63 (0.12)	0.47 (0.13)
S,S-2,3-butanediol	1.06 (0.09)	0.76 (0.09)	0.58 (0.09)
R,R-2,3-butanediol	0.05 (0.01)	1.01 (0.01)	0.83 (0.00)
2-methyl-1,2-propanediol	0.15 (0.13)	1.13 (0.23)	1.28 (0.00)
2-methyl-1,3-propanediol	0.01 (0.00)	0.04 (0.00)	0.04 (0.02)

Table 2-1: Continued

Substrate [a]	Culture density (OD_{600}) [b]		
	After 7 d	After 14 d	After 28 d
Aromatics			
benzene	≤0.01 (0.00)	0.01 (0.00)	0.01 (0.00)
toluene	≤0.01 (0.00)	0.01 (0.00)	≤0.01 (0.00)
ethylbenzene	0.01 (0.00)	0.01 (0.00)	0.00 (0.00)
o-xylene	0.02 (0.00)	0.01 (0.00)	0.01 (0.00)
m-xylene	≤0.01 (0.00)	0.01 (0.00)	≤0.01 (0.00)
p-xylene	0.01 (0.01)	0.01 (0.00)	≤0.01 (0.00)
Alkanes [c]			
methane	≤0.01 (0.00)	0.01 (0.00)	0.01 (0.00)
ethane	≤0.01 (0.00)	0.01 (0.00)	0.02 (0.00)
propane	≤0.01 (0.00)	0.01 (0.00)	0.05 (0.02)
n-butane	≤0.01 (0.00)	0.01 (0.00)	0.03 (0.02)
n-pentane	≤0.01 (0.00)	0.01 (0.00)	0.01 (0.00)
n-hexane	0.01 (0.01)	0.01 (0.00)	≤0.01 (0.00)
n-heptane	0.08 (0.06)	1.33 (0.08)	1.26 (0.03)
n-octane	0.13 (0.01)	0.13 (0.00)	0.17 (0.00)
n-decane	0.30 (0.11)	0.28 (0.11)	0.42 (0.13)
n-tetradecane	1.00 (0.10)	0.95 (0.13)	1.07 (0.08)
n-hexadecane	0.73 (0.01)	0.76 (0.09)	1.07 (0.23)
n-octadecane	0.08 (0.02)	0.95 (0.03)	0.72 (0.02)
2-methylpropane	≤0.01 (0.00)	0.01 (0.00)	0.01 (0.01)
2-methylbutane	≤0.01 (0.00)	0.01 (0.00)	0.05 (0.00)
2-methylpentane	ND	0.01 (0.00)	0.00 (0.00)
2-methylhexane	ND	0.01 (0.00)	0.58 (0.12)
1^{o} Alcohols			
methanol	0.02 (0.00)	0.01 (0.00)	ND
ethanol	0.39 (0.02)	0.22 (0.08)	ND
1-propanol	0.82 (0.04)	0.72 (0.06)	ND
1-butanol	1.45 (0.14)	1.31 (0.13)	ND
1-pentanol	1.80 (0.12)	1.62 (0.15)	ND
2-methyl-1-propanol	0.11 (0.00)	1.23 (0.04)	ND
2-methyl-1-butanol	0.42 (0.00)	1.03 (0.19)	0.88 (0.16)
3-methyl-1-butanol	0.06 (0.00)	1.16 (0.13)	0.97 (0.10)
2-propen-1-ol	≤0.01 (0.00)	≤0.01 (0.00)	0.00 (0.00)
2-buten-1-ol	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)
3-buten-1-ol	≤0.01 (0.00)	≤0.01 (0.00)	0.02 (0.00)
2-methyl-2-propen-1-ol	0.10 (0.00)	0.36 (0.00)	0.50 (0.02)
2^{o} Alcohols			
2-propanol	0.01 (0.00)	0.03 (0.02)	0.01 (0.00)
2-butanol	≤0.01 (0.00)	0.01 (0.00)	0.01 (0.00)
3-methyl-2-butanol	0.01 (0.00)	0.03 (0.01)	0.01 (0.01)

Table 2-1: Continued

Substrate [a]	Culture density (OD_{600}) [b]		
	After 7 d	After 14 d	After 28 d
3º Alcohols			
2-methyl-2-propanol	≤0.01 (0.00)	0.01 (0.00)	≤0.01 (0.00)
2-methyl-2-butanol	0.01 (0.00)	≤0.01 (0.00)	≤0.01 (0.00)
Aldehydes and Ketones			
acetaldehyde	≤0.01 (0.00)	0.01 (0.00)	≤0.01 (0.00)
2-methylpropionaldehyde	0.03 (0.01)	0.78 (0.07)	0.67 (0.11)
2-methylprop-2-enal	0.01 (0.00)	0.01 (0.00)	0.02 (0.00)
acetone	0.01 (0.00)	0.01 (0.00)	0.01 (0.01)
hydroxyacetone	0.04 (0.00)	0.10 (0.01)	0.12 (0.01)
2-butanone	≤0.01 (0.00)	0.01 (0.00)	0.01 (0.00)
3-hydroxybutanone	0.87 (0.04)	0.81 (0.11)	0.50 (0.04)
Acids			
acetate	0.34 (0.01)	0.29 (0.01)	0.23 (0.01)
propionate	0.77 (0.02)	0.65 (0.01)	0.51 (0.03)
3-hydroxybutyrate	0.51 (0.09)	0.56 (0.09)	ND
2-methylpropionate	0.01 (0.00)	0.53 (0.26)	0.77 (0.07)
2-methyl-2-propenoate	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)
2-methylbutyrate	0.01 (0.00)	0.00 (0.00)	≤0.01 (0.00)
3-methylbutyric acid	0.01 (0.00)	0.01 (0.01)	0.02 (0.01)
2-hydroxyisobutyrate	0.99 (0.01)	0.73 (0.04)	ND
2-hydroxy-2-methyl-butyrate	0.00 (0.00)	0.01 (0.01)	0.01 (0.00)
succinate	0.23 (0.00)	0.18 (0.00)	ND
pyruvate	0.12 (0.00)	0.62 (0.00)	ND
Ether oxygenates			
methyl *tertiary* butyl ether	0.01 (0.01)	0.01 (0.00)	0.02 (0.00)
ethyl *tertiary* butyl ether	0.03 (0.01)	0.01 (0.00)	0.01 (0.00)
tertiary amyl methyl ether	0.00 (0.00)	0.00 (0.00)	0.01 (0.00)
diisopropyl ether	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Sugars			
fructose	1.59 (0.00)	1.26 (0.00)	ND
glucose	0.08 (0.00)	1.78 (0.00)	ND
galactose	0.01 (0.00)	≤0.01 (0.00)	ND

Growth of strain ELW1 was examined on a range of substrates using glass serum vials (160 ml) containing 25 ml MSM and sealed with Teflon-lined mininert valves as described in the Methods section. Cultures were incubated for 14-28 d and culture densities (OD_{600}) were determined after 7, 14 and 28 d of incubation .Data represent the mean and SEM (in parentheses) of two separate cultures.

[a] 10 mM liquid/ solid substrates or 10% (v/v) gaseous substrates were used in cultures. (Filter-sterilized aqueous stock solutions (0.1 M) were used for all solid substrates)

[b] Initial culture $OD_{600} \leq 0.02$

[c] Abiotic controls for $C_{10,12,14,16,18}$ alkanes maintained an $OD_{600} \leq 0.06$ (data not shown).

Table 2-2: Specific rates of degradation of epoxides and allyl alcohols by *Mycobacterium* sp. ELW1 after growth on 2-methylpropene.

Substrate	Specific Activity (nmoles min^{-1} mg total $protein^{-1}$)		Rate of abiotic degradation (nmoles min^{-1})
	without 1-octyne pretreatment	with 1-octyne pretreatment	
epoxyethane	16.1 (5.9)	20.7 (2.6)	2.2 (0.4)
epoxypropane	61.4 (6.8)	59.6 (0.3)	1.1 (0.9)
1,2-epoxybutane	81.2 (10.6)	100.0 (4.8)	3.1 (0.3)
1,2-epoxy-2-methylpropane	246.5 (31.6)	246. 1 (1.6)	2.3 (0.1)
2-propen-1-ol	29.1 (3.1)	9.7 (2.6)	0.6 (0.2)
3-buten-1-ol	30.0 (2.5)	10.8 (0.3)	0.5 (0.3)
2-methyl-2-propen-1-ol	32.3 (0.6)	9.8 (0.7)	0.5 (0.1)

Resting 2-methylpropene-grown cells of strain ELW1 pretreated with or without 1-octyne were incubated in buffer in sealed reaction vials (10 ml) with 2 mM epoxides (0.2 mg total protein) or 2 mM allyl alcohols (0.9 mg total protein), as described in the Methods section. Control incubations were also conducted with heat-killed cells. Consumption of each substrate was monitored over time by GC analysis of the reaction aqueous phase and the specific rates of degradation were calculated. Data represent the mean and SEM (in parentheses) of two separate cultures.

Table 2-3: Effects of cobalt ions on growth of *Mycobacterium* sp. ELW1 on 2-methylpropene and other substrates.

Growth Substrate [a]	Culture density (OD_{600})[b] after 7 d		Culture density (OD_{600}) after 14 d	
	MSM	MSM-Co	MSM	MSM-Co
2-methylpropene	1.62 (0.11)	0.03 (0.01)	1.38 (0.11)	0.06 (0.01)
1,2-epoxy-2-methypropane	0.11 (0.01)	0.03 (0.00)	1.02 (0.08)	0.05 (0.00)
2-methyl-1,2-propanediol	0.42 (0.00)	0.03 (0.00)	1.15 (0.00)	0.08 (0.00)
2-hydroxyisobutyrate	0.68 (0.07)	≤0.01 (0.00)	1.21 (0.04)	0.02 (0.00)
3-hydroxybutyrate	0.91 (0.02)	0.87 (0.01)	0.87 (0.05)	0.66 (0.03)
2-methyl-1-propanol	0.28 (0.06)	0.26 (0.01)	1.01 (0.03)	0.98 (0.08)
2,3-butanediol	0.21 (0.02)	0.22 (0.01)	1.15 (0.05)	1.05 (0.02)
fructose	1.54 (0.03)	1.57 (0.01)	1. 38 (0.01)	1.36 (0.05)
acetate	0.38 (0.02)	0.38 (0.01)	0.28 (0.01)	0.29 (0.01)

Cultures of strain ELW1 were grown on a range of growth-supporting substrates including potential metabolites in the pathway of 2-methylpropene metabolism, in sealed glass serum vials (160 ml) containing 25 ml of either cobalt-sufficient (MSM) or cobalt-deficient (MSM-Co) media, as described in the Methods section. Changes in culture density (OD_{600}) were determined after 7 and 14 d of incubation. Data represent the mean and SEM (in parentheses) of duplicate cultures.

[a] 10 mM liquid /solid substrates or 10% (v/v) gaseous substrates were used in cultures. (Filter-sterilized aqueous stock solutions (0.1 M) were used for all solid substrates)

[b] Initial culture $OD_{600} \leq 0.02$

REFERENCES

1. **UNEP** (**United Nations Environment Programme).** 2003. Isobutylene. CAS No: 115-11-7.

2. **van Leeuwen BN, van der Wulp, Albertus M, Duijnstee I, van Maris AJ, Straathof AJ.** 2012. Fermentative production of isobutene. Appl. Microbiol. Biotechnol. **93:**1377-1387.

3. **Fukuda H, Fujii T, Ogawa T.** 1984. Microbial production of C_3-and C_4-hydrocarbons under aerobic conditions. Agric. Biol. Chem. **48:** 1679-1682.

4. **Fujii T, Ogawa T, Fukuda H.** 1988. Preparation of a cell-free, isobutene-forming system from *Rhodotorula minuta*. Appl. Environ. Microbiol. **54:**583-584.

5. **Schäfer F, Muzica L, Schuster J, Treuter N, Rosell M, Harms H, Müller RH, Rohwerder T.** 2011. Formation of alkenes via degradation of *tert*-alkyl ethers and alcohols by *Aquincola tertiaricarbonis* L108 and *Methylibium* spp. Appl. Environ. Microbiol. **77:**5981-5987.

6. **Fujii T, Nakamura K, Shibuya K, Tanase S, Gotoh O, Ogawa T, Fukuda H.** 1997. Structural characterization of the gene and corresponding cDNA for the cytochrome P450rm from *Rhodotorula minuta* which catalyzes formation of isobutene and 4-hydroxylation of benzoate. Mol. Gen. Genet. **256:**115-120.

7. **Fukuda H, Fujii T, Sukita E, Tazaki M, Nagahama S, Ogawa T.** 1994. Reconstitution of the isobutene-forming reaction catalyzed by cytochrome P450 and P450 Reductase from *Rhodotorula minuta*: decarboxylation with the formation of isobutene. Biochem. Biophys. Res. Commun. **201:**516-522.

8. **Gogerty DS, Bobik TA.** 2010. Formation of isobutene from 3-hydroxy-3-methylbutyrate by diphosphomevalonate decarboxylase. Appl. Environ. Microbiol. **76:**8004-8010.

9. **Marliere P.** 2011. Method for producing an alkene comprising the step of converting an alcohol by an enzymatic dehydration step. European Patent. EP2336341 A1.

10. **de Bont JAM.** 1976. Oxidation of ethylene by soil bacteria. Antonie Van Leeuwenhoek. **42:**59-71.

11. **Cerniglia C, Blevins W, Perry J.** 1976. Microbial oxidation and assimilation of propylene. Appl. Environ. Microbiol. **32:**764-768.

12. **de Bont JAM, Primrose SB, Collins MD, Jones D.** 1980. Chemical studies on some bacteria which utilize gaseous unsaturated hydrocarbons. J. Gen. Microbiol. **117:**97-102.

13. **van Ginkel CG, de Bont JAM.** 1986. Isolation and characterization of alkene-utilizing *Xanthobaeter* spp. Arch Microbiol. **145:**403-407.

14. **van Ginkel CG, Welten HG, de Bont JAM.** 1987. Oxidation of gaseous and volatile hydrocarbons by selected alkene-utilizing bacteria. Appl. Environ. Microbiol. **53:**2903-2907.

15. **Watkinson RJ, Somerville HJ.** 1976. The microbial utilization of butadiene. In: Sharply J.M., and J.A.M. Kaplan (eds). Proceedings of the 3rd International Biodeterioration Symposium, Kingston, RI. Applied Science, 35-42

16. **Sharkey TD.** 1996. Isoprene synthesis by plants and animals. Endeavour. **20:**74-78.

17. **Fall R, Copley SD.** 2000. Bacterial sources and sinks of isoprene, a reactive atmospheric hydrocarbon. Environ. Microbiol. **2:**123-130.

18. **Ewers J, Freier-Schröder D, Knackmuss H.** 1990. Selection of trichloroethene (TCE) degrading bacteria that resist inactivation by TCE. Arch. Microbiol. **154:**410-413.

19. **van Hylckama Vlieg, Johan ET, Kingma J, van den Wijngaard, Arjan J, Janssen DB.** 1998. A glutathione S-transferase with activity towards *cis*-1, 2-dichloroepoxyethane is involved in isoprene utilization by *Rhodococcus* sp. strain AD45. Appl. Environ. Microbiol. **64:**2800-2805.

20. **van Ginkel CG, de Jong E, Tilanus JWR, de Bont JAM.** 1987. Microbial oxidation of isoprene, a biogenic foliage volatile and of 1, 3-butadiene, an anthropogenic gas. FEMS Microbiol. Lett. **45:**275-279.

21. **van Hylckama Vlieg, Johan ET, Leemhuis H, Spelberg JHL, Janssen DB.** 2000. Characterization of the gene cluster involved in isoprene metabolism in *Rhodococcus* sp. strain AD45. J. Bacteriol. **182:**1956-1963.

22. **van Hylckama Vlieg, Johan ET, Kingma J, Kruizinga W, Janssen DB.** 1999. Purification of a glutathione S-transferase and a glutathione conjugate-specific dehydrogenase involved in isoprene metabolism in *Rhodococcus* sp. Strain AD45. J. Bacteriol. **181:**2094-2101.

23. **Shennan JL.** 2006. Utilisation of C_2–C_4 gaseous hydrocarbons and isoprene by microorganisms. J. Chem. Technol. Biotechnol. **81:**237-256.

24. **Small FJ, Ensign SA.** 1997. Alkene monooxygenase from *Xanthobacter* strain Py2. Purification and characterization of a four-component system central to the bacterial metabolism of aliphatic alkenes. J. Biol. Chem. **272:**24913-24920.

25. **Zhou N, Jenkins A, Chion, Chan K. N. Chan Kwo, Leak DJ.** 1999. The alkene monooxygenase from *Xanthobacter* Strain Py2 is closely related to aromatic monooxygenases and catalyzes aromatic monohydroxylation of benzene, toluene, and phenol. Appl. Environ. Microbiol. **65:**1589-1595.

26. **van Ginkel CG, Welten HG, de Bont JAM.** 1986. Epoxidation of alkenes by alkene-grown *Xanthobacter* spp. Appl. Microbiol. Biotechnol. **24:**334-337.

27. **Allen JR, Clark DD, Krum JG, Ensign SA.** 1999. A role for coenzyme M (2-mercaptoethanesulfonic acid) in a bacterial pathway of aliphatic epoxide carboxylation. Proc. Natl. Acad. Sci. U. S. A. **96:**8432-8437.

28. **Krum JG, Ellsworth H, Sargeant RR, Rich G, Ensign SA.** 2002. Kinetic and microcalorimetric analysis of substrate and cofactor interactions in epoxyalkane: CoM transferase, a zinc-dependent epoxidase. Biochemistry 41:5005-5014.

29. **Allen JR, Ensign SA.** 1996. Carboxylation of epoxides to β-keto acids in cell extracts of *Xanthobacter* strain Py2. J. Bacteriol. **178:**1469-1472.

30. **Ensign SA.** 2001. Microbial metabolism of aliphatic alkenes. Biochemistry **40:**5845-5853.

31. **Allen JR, Ensign SA.** 1997. Purification to homogeneity and reconstitution of the individual components of the epoxide carboxylase multiprotein enzyme complex from *Xanthobacter* strain Py2. J. Biol. Chem. **272:**32121-32128.

32. **Allen JR, Ensign SA.** 1999. Two short-chain dehydrogenases confer stereoselectivity for enantiomers of epoxypropane in the multiprotein epoxide carboxylating systems of *Xanthobacter* strain Py2 and *Nocardia corallina* B276. Biochemistry **38:**247-256.

33. **Sliwa DA, Krishnakumar AM, Peters JW, Ensign SA.** 2010. Molecular basis for enantioselectivity in the (R)- and (S)-hydroxypropylthioethanesulfonate dehydrogenases, a unique pair of stereoselective short-chain dehydrogenases/reductases involved in aliphatic epoxide carboxylation. Biochemistry **49:**3487-3498.

34. **Krishnakumar AM, Nocek BP, Clark DD, Ensign SA, Peters JW.** 2006. Structural basis for stereoselectivity in the (R)- and (S)-hydroxypropylthioethanesulfonate dehydrogenases. Biochemistry **45:**8831-8840.

35. **Nocek B, Jang SB, Jeong MS, Clark DD, Ensign SA, Peters JW.** 2002. Structural basis for CO_2 fixation by a novel member of the disulfide oxidoreductase family of enzymes, 2-ketopropyl-coenzyme M oxidoreductase/carboxylase. Biochemistry **41:**12907-12913.

36. **Boyd JM, Ellsworth A, Ensign SA.** 2006. Characterization of 2-bromoethanesulfonate as a selective inhibitor of the coenzyme M-dependent pathway and enzymes of bacterial aliphatic epoxide metabolism. J. Bacteriol. **188:**8062-8069.

37. **Boyd JM, Clark DD, Kofoed MA, Ensign SA.** 2010. Mechanism of inhibition of aliphatic epoxide carboxylation by the coenzyme M analog 2-bromoethanesulfonate. J. Biol. Chem. **285:**25232-25242.

38. **Patel RN, Hou CT, Laskin AI, Felix A.** 1982. Microbial oxidation of hydrocarbons: properties of a soluble methane monooxygenase from a facultative methane-utilizing organism, *Methylobacterium* sp. strain CRL-26. Appl. Environ. Microbiol. **44:**1130-1137.

39. **Hou CT, Patel RN, Laskin AI, Barnabe N, Barist I.** 1981. Epoxidation and hydroxylation of C_4- and C_5-branched-chain alkenes and alkanes by methanotrophs. J. Ind. Microbiol. **23:**477-482.

40. **Hou CT, Patel RN, Laskin AI, Barnabe N, Barist I.** 1983. Epoxidation of short-chain alkenes by resting-cell suspensions of propane-grown bacteria. Appl. Environ. Microbiol. **46:**171-177.

41. **Takami W, Yoshida T, Nojiri H, Yamane H, Omori T.** 1999. Oxidation of chlorinated olefins by *Escherichia coli* transformed with dimethyl sulfide monooxygenase genes or cumene dioxygenase genes. J. Gen. Appl. Microbiol. **45:**69-75.

42. **Peter S, Kinne M, Ullrich R, Kayser G, Hofrichter M.** 2013. Epoxidation of linear, branched and cyclic alkenes catalyzed by unspecific peroxygenase. Enzyme Microb. Technol. **52:**370-376.

43. **Owens CR, Karceski JK, Mattes TE.** 2009. Gaseous alkene biotransformation and enantioselective epoxyalkane formation by *Nocardioides* sp. strain JS614. Appl. Microbiol. Biotechnol. **84:**685-692.

44. **Ensign SA.** 1996. Aliphatic and chlorinated alkenes and epoxides as inducers of alkene monooxygenase and epoxidase activities in *Xanthobacter* Strain Py2. Appl. Environ. Microbiol. **62:**61-66.

45. **Allen JR, Ensign SA.** 1998. Identification and characterization of epoxide carboxylase activity in cell extracts of *Nocardia corallina* B276. J. Bacteriol. **180:**2072-2078.

46. **Coleman NV, Spain JC.** 2003. Distribution of the coenzyme M pathway of epoxide metabolism among ethene- and vinyl chloride-degrading *Mycobacterium* strains. Appl. Environ. Microbiol. **69:**6041-6046.

47. **Coleman NV, Spain JC.** 2003. Epoxyalkane:coenzyme M transferase in the ethene and vinyl chloride biodegradation pathways of *Mycobacterium* Strain JS60. J. Bacteriol. **185:**5536-5545.

48. **Chuang AS, Mattes TE.** 2007. Identification of polypeptides expressed in response to vinyl chloride, ethene, and epoxyethane in *Nocardioides* sp. strain JS614 by using peptide mass fingerprinting. Appl. Environ. Microbiol. **73:**4368-4372.

49. **Danko AS, Saski CA, Tomkins JP, Freedman DL.** 2006. Involvement of coenzyme M during aerobic biodegradation of vinyl chloride and ethene by *Pseudomonas putida* strain AJ and *Ochrobactrum* sp. strain TD. Appl. Environ. Microbiol. **72:**3756-3758.

50. **Chuang AS, Jin YO, Schmidt LS, Li Y, Fogel S, Smoler D, Mattes TE.** 2010. Proteomic analysis of ethene-enriched groundwater microcosms from a vinyl chloride-contaminated site. Environ. Sci. Technol. **44:**1594-1601.

51. **Hyman M.** 2013. Biodegradation of gasoline ether oxygenates. Curr. Opin. Biotechnol. **24:**443-450.

52. **Rohwerder T, Breuer U, Benndorf D, Lechner U, Müller RH.** 2006. The alkyl *tert*-butyl ether intermediate 2-hydroxyisobutyrate is degraded via a novel cobalamin-dependent mutase pathway. Appl. Environ. Microbiol. **72:**4128-4135.

53. **Yaneva N, Schuster J, Schäfer F, Lede V, Przybylski D, Paproth T, Harms H, Müller RH, Rohwerder T.** 2012. Bacterial acyl-CoA mutase specifically catalyzes coenzyme B_{12}-dependent isomerization of 2-hydroxyisobutyryl-CoA and (S)-3-hydroxybutyryl-CoA. J. Biol. Chem. **287:**15502-15511.

54. **Piveteau P, Fayolle F, Vandecasteele J, Monot F.** 2001. Biodegradation of *tert*-butyl alcohol and related xenobiotics by a methylotrophic bacterial isolate. Appl. Microbiol. Biotechnol. **55:**369-373.

55. **Ensign SA, Small FJ, Allen JR, Sluis MK.** 1998. New roles for CO_2 in the microbial metabolism of aliphatic epoxides and ketones. Arch. Microbiol. **169:**179-187.

56. **Cornet M, Sonck W, Callaerts A, Csanády G, Vercruysse A, Laib RJ, Rogiers V.** 1991. In vitro biotransformation of 2-methylpropene (isobutene): epoxide formation in mice liver. Arch. Toxicol. **65:**263-267.

57. **Cornet M, Callaerts A, Jorritsma U, Bolt H, Vercruysse A, Rogiers V.** 1995. Species-dependent differences in biotransformation pathways of 2-methylpropene (isobutene). Chem. Res. Toxicol. **8:**987-992.

58. **Cornet M, Rogiers V.** 1997. Metabolism and toxicity of 2-methylpropene (isobutene) -a review. Crit. Rev. Toxicol. **27:**223-232.

59. **Decker M, Arand M, Cronin A.** 2009. Mammalian epoxide hydrolases in xenobiotic metabolism and signalling. Arch. Toxicol. **83:**297-318.

60. **McAuliffe C.** 1966. Solubility in water of paraffin, cycloparaffin, olefin, acetylene, cycloolefin, and aromatic hydrocarbons. J. Phys. Chem. **70:**1267-1275.

61. **Gornall AG, Bardawill CJ, David MM.** 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177:**751-766.

62. **Furuhashi K, Taoka A, Uchida S, Karube l, Suzuki S.** 1981. Production of 1,2-epoxyalkanes from 1-alkenes by *Nocardia corallina* B-276. Eur. J. Appl Microbiol Biotechnol. **12:**39-45.

63. **Iizuka H, Sakayanagi S, Seto N.** 1975. Method of recovering microbial cells containing protein. US Patent US3888736A.

64. **Yagi O, Hashimoto A, Iwasaki K, Nakajima M.** 1999. Aerobic degradation of 1, 1, 1-trichloroethane by *Mycobacterium* spp. isolated from soil. Appl. Environ. Microbiol. **65:**4693-4696.

65. **Hashimoto A, Iwasaki K, Nakasugi N, Nakajima M, Yagi O.** 2002. Degradation pathways of trichloroethylene and 1, 1, 1-trichloroethane by *Mycobacterium* sp. TA27. Biosci. Biotechnol. Biochem. **66:**385-390.

66. **Ohkubo N, Hashimoto AH, Iwasaki K, Yagi O.** 2009. Biodegradation of methyl *tert*-butyl ether by *Mycobacterium* spp. J. Environ. Biotechnol. **9:**113-122.

67. **Higgins I, Hammond R, Sariaslani F, Best D, Davies M, Tryhorn S, Taylor F.** 1979. Biotransformation of hydrocarbons and related compounds by whole organism suspensions of methane-grown *Methylosinus trichosporium* OB3b. Biochem. Biophys. Res. Commun. **89:**671-677.

68. **Hyman MR, Murton IB, Arp DJ.** 1988. Interaction of ammonia monooxygenase from *Nitrosomonas europaea* with alkanes, alkenes, and alkynes. Appl. Environ. Microbiol. **54:**3187-3190.

69. **Fujii T, Ogawa T, Fukuda H.** 1985. A screening system for microbes which produce olefin hydrocarbons. Agric. Biol. Chem. **49:**651-657.

70. **van Ginkel CG, Welten HG, Hartmans S, de Bont JAM.** 1987. Metabolism of *trans*-2-butene and butane in *Nocardia* TBl. J. Gen. Microbiol. **133:**1713-1720.

71. **Weijers CAGM, van Ginkel CG, de Bont JAM.** 1988. Enantiomeric composition of lower epoxyalkanes produced by methane-, alkane-and alkene-utilizing bacteria. Enzyme Microb. Technol. **10:**214-218.

72. **Habets-Crützen AQ, Brink LE, van Ginkel CG, de Bont JAM, Tramper J.** 1984. Production of epoxides from gaseous alkenes by resting-cell suspensions and immobilized cells of alkene-utilizing bacteria. Appl. Microbiol. Biotechnol. **20:**245-250.

73. **Prior S, Dalton H.** 1985. Acetylene as a suicide substrate and active site probe for methane monooxygenase from *Methylococcus capsulatus* (Bath). FEMS Microbiol. Lett. **29:**105-109.

74. **Yeager CM, Bottomley PJ, Arp DJ, Hyman MR.** 1999. Inactivation of toluene 2-monooxygenase in *Burkholderia cepacia* G4 by alkynes. Appl. Environ. Microbiol. **65:**632-639.

75. **May SW, Katopodis AG.** 1986. Oxygenation of alcohol and sulphide substrates by a prototypical non-haem iron monooxygenase: catalysis and biotechnological potential. Enzyme Microb. Technol. **8:**17-21.

76. **Fosdike WL, Smith TJ, Dalton H.** 2005. Adventitious reactions of alkene monooxygenase reveal common reaction pathways and component interactions among bacterial hydrocarbon oxygenases. FEBS Journal. **272:**2661-2669.

77. **de Bont JAM, van Dijken JP, van Ginkel CG.** 1982. The metabolism of 1, 2-propanediol by the propylene oxide utilizing bacterium *Nocardia* A60. Biochim. Biophys. Acta **714:**465-470.

78. **van Den Wijngaard, Arjan J, Janssen DB, Witholt B.** 1989. Degradation of epichlorohydrin and halohydrins by bacterial cultures isolated from freshwater sediment. J. Gen. Microbiol. **135:**2199-2208.

79. **Jacobs MH, Wijngaard AJ, Pentenga M, Janssen DB.** 1991. Characterization of the epoxide hydrolase from an epichlorohydrin-degrading *Pseudomonas* sp. Eur. J. Biochem. **202:**1217-1222.

80. **Nardini M, Ridder IS, Rozeboom HJ, Kalk KH, Rink R, Janssen DB, Dijkstra BW.** 1999. The X-ray Structure of epoxide hydrolase from *Agrobacterium radiobacter* AD1. An enzyme to detoxify harmful epoxides. J. Biol. Chem. **274:**14579-14586.

81. **Nakamura T, Nagasawa T, Yu F, Watanabe I, Yamada H.** 1992. Resolution and some properties of enzymes involved in enantioselective transformation of 1, 3-dichloro-2-propanol to (R)-3-chloro-1, 2-propanediol by *Corynebacterium* sp. strain N-1074. J. Bacteriol. **174:**7613-7619.

82. **Nakamura T, Nagasawa T, Yu F, Watanabe I, Yamada H.** 1994. Purification and characterization of two epoxide hydrolases from *Corynebacterium* sp. strain N-1074. Appl. Environ. Microbiol. **60:**4630-4633.

83. **van Loo B, Kingma J, Arand M, Wubbolts MG, Janssen DB.** 2006. Diversity and biocatalytic potential of epoxide hydrolases identified by genome analysis. Appl. Environ. Microbiol. **72:**2905-2917.

84. **Ferreira NL, Labbé D, Monot F, Fayolle-Guichard F, Greer CW.** 2006. Genes involved in the methyl *tert*-butyl ether (MTBE) metabolic pathway of *Mycobacterium austroafricanum* IFP 2012. Microbiology **152:**1361-1374.

85. **Landfald B, Strøm AR.** 1986. Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. J. Bacteriol. **165:**849-855.

86. **Fan F, Ghanem M, Gadda G.** 2004. Cloning, sequence analysis, and purification of choline oxidase from *Arthrobacter globiformis*: a bacterial enzyme involved in osmotic stress tolerance. Arch. Biochem. Biophys. **421:**149-158.

87. **Ikuta S, Imamura S, Misaki H, Horiuti Y.** 1977. Purification and characterization of choline oxidase from *Arthrobacter globiformis*. J. Biochem. **82:**1741-1749.

88. **Fan F, Germann MW, Gadda G.** 2006. Mechanistic studies of choline oxidase with betaine aldehyde and its isosteric analogue 3, 3-dimethylbutyraldehyde. Biochemistry **45:**1979-1986.

89. **Lamark T, Kaasen I, Eshoo M, Falkenberg P, McDougall J, Strøm A.** 1991. DNA sequence and analysis of the *bet* genes encoding the osmoregulatory choline-glycine betaine pathway of *Escherichia coli*. Mol. Microbiol. **5:**1049-1064.

CHAPTER 3

Metabolism of 2-butene by *Mycobacterium* sp. ELW1

Samanthi Kottegoda and Michael Hyman*

Department of Plant and Microbial Biology
North Carolina State University
Raleigh, NC 27695

A manuscript for submission to *Applied and Environmental Microbiology*

Key words: alkene monooxygenase, epoxide hydrolase, 2,3-butanediol, acetoin dehydrogenase

*Corresponding author
Contact Information
Dr. Michael Hyman
Department of Plant and Microbial Biology
4545 Thomas Hall
North Carolina State University
Raleigh, NC 27695
Phone: 919-515-78145
Fax: 919-515-7867
E-mail: mrhyman@ncsu.edu

ABSTRACT

We have characterized the metabolism of 2-butene by a newly described 2-methylpropene-metabolizing bacterium *Mycobacterium* sp. ELW1. The maximal growth rate on both *cis*- (C2B) and *trans*- (T2B) 2-butene isomers (0.008 h^{-1}) was much slower than growth on 2-methylpropene (0.05 h^{-1}). Growth studies and reactions with resting cells suggest cells grown on C2B and T2B initially oxidize the 2-butene isomers to their corresponding 2,3-epoxyalkanes and that these epoxides are then hydrolyzed to their corresponding diols. These initial reactions are substantially different to the terminal oxidation pathway of 2-butene-metabolism proposed for *Nocardia* TB1, the only previously characterized 2-butene-metabolizing bacterium. Physiological and kinetic evidence is presented that suggests cells of strain ELW1 grown on both 2-butene isomers sequentially use the same alkene-oxidizing monoooxygenase and epoxide hydrolase previously implicated in the initial steps in 2-methylpropene metabolism by this strain. The slow rate of epoxide hydrolysis is proposed as the enzymatic step that dictates the slow rate of growth of strain ELW1 on 2-butene. The degradation of *trans*-2,3-epoxybutane was also strongly enantioselective towards the (2S-3S)-enantiomer. Our results also suggest the later steps in 2-butene metabolism involve different enzymes than those involved in 2-methylpropene metabolism. The 2,3-butanediols generated from epoxide hydrolysis appear to be first oxidized to 3-hydroxy-2-butanone (acetoin), which is then further oxidized to acetaldehyde and acetyl-CoA by an acetoin dehydrogenase-like activity. Our results have been interpreted in terms of their impact on our understanding of the bacterial metabolism of 2-alkenes and the role of enantioselective epoxide hydrolysis in bacterial alkene metabolism.

INTRODUCTION

2-Butene is the simplest alkene exhibiting geometric isomerism and consists of two stereoisomers, *cis*- (C2B) and *trans*-2-butene (T2B). Industrially, 2-butene is produced by catalytic cracking of crude oil and it is used for the production of butadiene, *sec*-butanol, butanone and gasoline alkylates such as 2,2,4-trimethylpentane. In 2001, production of 2-butene in the United States was approximately 9 million tonnes. Although some bacteria and fungi produce small amounts of both 2-butene isomers (1), the low levels of this gas found in the environment are thought to be mainly associated with industrial processes, natural gas seeps and automobile exhaust (2).

A variety of aerobic bacteria can oxidize 2-butene and these organisms can be separated based on their abilities to either cometabolically oxidize alkenes or to fully metabolize alkenes as sole sources of carbon and energy for growth. Among the former microorganisms, the ammonia-oxidizing bacterium *Nitrosomonas europaea* oxidizes both C2B and T2B through the activity of ammonia monooxygenase (AMO) (3). 2-Buten-1-ol represents ≥80% of the detected products while *cis*- and *trans* 2,3-epoxybutane are the other minor products. Both 2-butene isomers are also oxidized by methane-oxidizing bacteria through the activity of methane monooxygenase (MMO) (4-6). The soluble form of MMO (sMMO) from *Methylococcus capsulatus* (Bath) oxidizes C2B to nearly equivalent amounts of 2-buten-1-ol and *cis*-2,3-epoxybutane, while 2-buten-1-ol is the predominant (≥70%) product of T2B oxidation (7, 8). Solubilized particulate MMO (pMMO) from *M. capsulatus* (Bath) oxidizes C2B exclusively to *cis* 2,3-epoxybutane while T2B is oxidized predominantly (~60%) to 2-buten-1-ol (9). Oxidation of 2-butene by microorganisms

expressing other alkene-oxidizing enzymes such as toluene-2- and toluene-4-monooxygenase (10), a soluble form of propane monoooxygenase (11), and a fungal peroxygenase (12), all suggest 2-butene is oxidized exclusively to 2,3-epoxybutanes and not a mixtures of epoxides and alcohols.

The ability to oxidize 2-butene has also been reported for many gaseous alkene-metabolizing bacteria including ethene-, propene-, 1-butene- and 1,3-butadiene-metabolizing isolates (13-15). In most cases these organisms were originally isolated based on their ability to metabolize 1-alkenes. Unlike many of the alkane-oxidizing enzymes and organisms described earlier, alkene-metabolizing organisms appear to only generate 2,3-epoxybutanes from the initial oxidation of 2-butene. In the case of *Xanthobacter autotrophicus* Py2, one of the best characterized alkene-metabolizing bacteria, propene-grown cells oxidized propene at ~80 nmoles min. mg total protein^{-1} and C2B and T2B were oxidized at 84 and 76% of this rate, respectively. The rate of accumulation of the *cis*- and *trans*-2,3-epoxybutane was 24% and 68% of the rate of C2B and T2B consumption, respectively (16).

In addition to demonstrating C2B- and T2B-oxidizing activity in 1-alkene-metabolizing isolates, van Ginkel *et al*. (13) also described three strains that grew slowly on T2B. One of these strains, *Nocardia* TB1, was shown to also grow on C_3-C_6 *n*-alkanes. A subsequent and more detailed study of strain TB1 demonstrated that this strain also grows slowly on C2B and that cells grown on T2B and *n*-butane have the same abilities to oxidize *n*-alkanes, 1-alkenes, 2-alkenes and alcohols (17). A pathway of T2B oxidation was proposed in which T2B is initially terminally oxidized to 2-buten-1-ol and it was suggested that T2B metabolism in strain TB1 is a manifestation of its terminal *n*-butane oxidation pathway rather

than an epoxide-generating route found in all other well characterized gaseous alkene-metabolizing strains (17). The versatile alkane-metabolizing strain *Mycobacterium vaccae* JOB5 has also been reported to grow on T2B (18) although the pathway of T2B oxidation by this strain has not been determined. Similarly, little is known about alkene metabolism by strains H-131 and M-141, the only other 2-butene-metabolizing strains we are aware of that have been described in the literature (19).

In this study we have characterized the 2-butene-metabolizing activity of the recently described 2-methylpropene-utilizing strain, *Mycobacterium* sp. ELW1. This strain does not grow on 1-alkenes and, apart from 2-methylpropene, C2B and T2B are the only other gaseous alkenes that support growth of this strain (20). Our previous study suggested that, like all other well-characterized alkene-metabolizing strains, 2-methylpropene is initially oxidized by a monooxygenase. The resulting epoxide, 1,2-epoxy-2-methylpropane, is then rapidly hydrolyzed to 2-methyl-1,2-propanediol by an epoxide hydrolase. A later metabolite, 2-hydroxyisobutyrate, is then converted to 3-hydroxybutyrate through the activity of a cobalt-dependent mutase. Our previous study also demonstrated that strain ELW1 can grow on both 1,2-epoxy-2-methylpropane, *cis* and *trans* 2,3-epoxybutane and both 2-methyl-1,2-propanediol and 2,3-butanediol, but not on other epoxides and diols. These observations suggested that a similar pathway and potentially the same enzymes are involved in the initial steps in the metabolism of 2-methylpropene and both C2B and T2B. In this study we have characterized the various reactions and intermediates involved in C2B and T2B metabolism by strain ELW1 and have investigated the enantioselectivity associated with some of these reactions.

MATERIALS AND METHODS

Materials: *Mycobacterium* strain ELW1 was isolated and maintained as previously described (20). *Xanthobacter autotrophicus* strain Py2 (ATCC BAA-1158) was obtained from the American Type Culture Collection (Manassas, VA.) and maintained on casein-yeast extract-dextrose (CYD) agar plates (Difco Plate Count Agar; Becton, Dickinson and Co., Sparks, MD). Acetaldehyde (99% purity), (2S,3S)-2,3-butanediol (97% purity), (2R,3R)-2,3-butanediol (97% purity), *meso*-2,3-butanediol (99 % purity), *cis*-2-butene (>99% purity), *trans*-2-butene (>99% purity), *cis*-2,3-epoxybutane (97% purity), fructose (>99% purity), 3-hydroxy-2-butanone (>98% purity), 2-hydroxyisobutyric acid (99% purity), 2-hydroxy-3-pentanone (95% purity), 2-methylpropene (99% purity), and 1-octyne (97% purity) were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI). Acetone (99.5% purity) and sodium acetate (99.5% purity) were obtained from Fisher Scientific (Pittsburgh, PA). Racemic *trans*-2,3-epoxybutane (97% purity) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). Absolute ethanol was obtained from Aaper Alcohol and Chemical Co., (Shelbyville, KY). 1,2-Epoxy-2-methylpropane (>99% purity) was obtained from Alfa Aesar (Ward Hill, MA). Propene (CP grade) and compressed gases (H_2, N_2, and air) used for gas chromatography were obtained from local industrial vendors.

Cell growth: *Mycobacterium* strain ELW1 was grown in batch culture using either 2-methylpropene, C2B or T2B as the sole source of carbon and energy. Cultures were grown in glass media bottles (700 ml; Wheaton Scientific, Millville, NJ) containing mineral salt medium (MSM; 100 ml) (20). The medium was inoculated (initial OD_{600} ~0.02) with cells

grown on MSM agar plates that had been incubated in air containing ~5% (v/v gas phase) 2-methylpropene. The inoculated bottles were sealed with screw caps fitted with butyl rubber septa and gaseous alkene (60 ml) was added using plastic syringes fitted with Millex sterile disposable filters (0.1 µm; Millipore Co., Bedford, MA). Cultures were incubated at 30°C in the dark in an Innova 4900 environmental shaker (New Brunswick Scientific Co., Inc., Edison, NJ) operated at 150 rpm. Culture purity after growth was confirmed by streaking a culture sample (20 µl) onto PCA plates. Cells of *X. autotrophicus* Py2 were grown on propene using the same procedure described above except that the cultures were inoculated with cells previously grown on plate count agar (PCA) plates and propene (60 ml) was used as the sole source of carbon and energy.

Growth of strain ELW1 on C2B and T2B was also examined in cobalt-deficient medium. These cultures were grown in glass serum vials (160 ml) containing MSM (25 ml) from which $CoCl_2.6H_2O$ had been omitted from the trace elements. Prior to use, the culture serum vials were soaked overnight in an aqueous solution (10% v/v) of Dekasol™ (ICN Biomedicals Inc.; Aurora, OH) to remove cobalt adventitiously bound to the glassware. The vials were then rinsed three times with deionized water before cobalt-deficient MSM (MSM-Co) was added. The cultures were inoculated (initial OD_{600} ~ 0.02) with a suspension of cells obtained from cultures previously grown on MSM plates incubated in the presence of 2-methylpropene. The vials were then sealed with butyl rubber stoppers and aluminum crimps seals (Wheaton Scientific, Millville, NJ) and C2B or T2B (20% v/v gas phase) were added using a sterile plastic syringe fitted with sterile disposable 0.1 µm filters. A sample (50 µl) of

the fully grown cultures was streaked onto casein-yeast extract-dextrose (CYD) agar plates (Difco Plate Count Agar) to confirm the purity of the culture.

The effect of gas concentration on the growth of strain ELW1 on 2-methylpropene, C2B and T2B was examined in glass serum vials (160 ml; Wheaton Scientific, Millville, NJ) that contained MSM (25 ml). Vials were inoculated (initial OD_{600} ~ 0.02) with a liquid suspension of cells previously grown on MSM agar plates incubated in the presence of 2-methylpropene. The vials were then sealed with butyl rubber stoppers and aluminum crimp seals and varying amounts of 2-methylpropene, C2B or T2B equivalent to 1.25, 2.5, 5, 10 and 20% [v/v] of the initial gas phase (135 ml) were added to sealed vials using plastic syringes fitted with Millex disposable filters (0.1 µm; Millipore Co., Bedford, MA). The cultures were incubated in the dark at 30°C in an Innova 4900 environmental shaker operated at 150 rpm. Samples (600 µl) were aseptically withdrawn from sealed vials throughout the experiment to monitor cell growth. Growth was determined by measurements of the changes in absorbance at 600 nm (OD_{600}) using a Shimadzu 1601 UV/Vis spectrophotometer (Shimadzu, Kyoto, Japan). Growth of strain ELW1 on the isomers of 2,3-butanediol was also determined in cultures grown in glass serum vials (160 ml) and MSM (25 ml). The cultures were prepared as described above except the vials were sealed with Teflon-lined Mininert valves (Alltech Associates Inc., Deerfield, IL) and 2,3-butanediol (10 mM initial concentration) was added to the vials from aqueous stock solutions using sterile microsyringes.

Initial rate and time course studies with resting cells: Cells were harvested from the culture medium (final OD_{600} ~ 0.8) by centrifugation (10,000 x g for 5 min at 4˚C). The resulting cell pellet was washed in phosphate buffer (20 ml; 50 mM sodium phosphate; pH 7.0) and centrifuged again. After repeating the washing step, the sedimented cells were resuspended in phosphate buffer (~1.0 ml) to a final concentration of ~10 mg of total cell protein ml^{-1}. The cell suspension was stored at 4°C and used within 4 hrs.

All experiments involving initial rate determinations or reaction time courses using resting cells were conducted in glass serum vials (10 ml). The reaction vials contained sodium phosphate buffer (50 mM, pH 7.0) (~900 µl) and were sealed with butyl rubber stoppers and aluminum crimp seals. Substrates were added directly to the sealed vials either as gases or from aqueous stock solutions using microsyringes. The vials were placed in a shaking water bath (30°C, 150 rpm) for ~10 min to allow equilibration of the reactants between the gas and liquid phases. The reactions were initiated by the addition of the appropriate resting cell suspension (100 µl; ~0.5 to 2.0 mg of total protein) to give a final aqueous reaction volume of 1 ml. The reaction vials were then returned to the shaking water bath and were sampled at the times indicated in each experiment.

In some experiments cells were pretreated with 1-octyne to inactivate alkene-oxidizing activity. In these experiments cells were harvested from a single culture and the resulting resting cell suspension was divided in to two equal aliquots (~500 µl). Each aliquot was added to phosphate buffer (6.5 ml) in glass serum vials (25 ml) sealed with butyl rubber stoppers and aluminum crimp seals. 1-octyne (90 µmoles) was then added as a neat compound to one vial and both vials were incubated in a shaking water bath operated at 30^{o}

C and 150 rpm. After 1 h, cells from both incubations were sedimented by centrifugation (10,000 x g for 5 min at 4˚C). The resulting cell pellets were washed in phosphate buffer (20 ml) and centrifuged again. After repeating the washing step, the sedimented cells were finally resuspended in phosphate buffer (500 µl) to a final concentration of ~10 mg total cell protein ml^{-1}. The cell suspensions were stored at 4°C and used within 4 hrs. The 1-octyne-pretreated cells generated from this procedure were used in all experiments that describe the use of this inactivator. In these experiments, all other cells used were the cells pre-incubated without 1-octyne.

In one experiment we also made use of propene-grown cells of *X. autotrophicus* Py2 that had been either heat-treated or exposed to methylepoxypropane to inactivate their epoxide-metabolizing activity. These cells were prepared as follows. A culture of strain Py2 was grown on propene, as described above. The cells were harvested by centrifugation (10,000 x g for 5 min at 4˚C). The resulting cell pellet was washed in phosphate buffer (20 ml; 50 mM sodium phosphate; pH 7.0) and centrifuged again. After repeating the washing step, the sedimented cells were resuspended in phosphate buffer (1.5 ml) to a final concentration of ~30 mg of total cell protein ml^{-1}. This cell suspension was then divided into three equal aliquots (500 µl each). One aliquot was heat-inactivated by sealing the cells in a microfuge tube which was then placed for 15 min in a heating block operated at 95° C. The two remaining aliquots were then added to two separate glass serum vials (25 ml) that contained phosphate buffer (6.5 ml). The serum vials were sealed with butyl rubber stoppers and aluminum crimp seals. 1,2-Epoxy-2-methylpropane (70 µmoles) was added to one of the vials and both vials were incubated in a shaking water bath operated at 30^{o} C and 150 rpm.

After 1 h incubation, the cells from both incubations and the heat-treated cells were separately harvested by centrifugation (10,000 x g for 5 min at 4˚C). The resulting cell pellets were washed in phosphate buffer (20 ml) and centrifuged again. After repeating the washing step, the sedimented cells were finally resuspended in phosphate buffer (0.5 ml) to a final concentration of ~30 mg of total cell protein ml^{-1}. The cell suspensions were stored at 4°C and used within 4 hrs.

Analytical methods: In all experiments substrate consumption and product formation were determined by gas chromatography (GC). The analyses for all compounds other than gaseous substrates (2-methylpropene, C2B and T2B) involved aqueous phase samples and two Shimadzu GC-14A chromatographs (Kyoto, Japan), each equipped with a flame ionization detector and stainless steel columns (0.3 x 61 cm or 0.3 x 183 cm) filled with Porapak Q (80/100 mesh) (Water Associates, Framingham, MA). The gas chromatographs were both operated with an injection temperature of 200°C, detector temperature of 220° C and column temperatures of 110-140° C. Nitrogen was used as a carrier gas at a flow rate of 15 ml/min. In all cases, the aqueous phase samples (2 µl) were taken from the various reaction vials and were injected directly into the gas chromatographs without any additional sample preparation.

The analyses for gaseous substrates (2-methylpropene, C2B and T2B) involved gas phase samples and a Shimadzu GC-14A gas chromatograph fitted with a flame ionization detector and a DB-MTBE capillary column [30 m x 0.45 mm (internal diameter), 2.55-µm film; J & W Scientific, Folsom, CA). The analyses were 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 the carrier gas at a flow rate of 5 ml/min. In all cases, the gas phase samples (10 µl) were taken from the various reaction vials and were injected directly into the gas chromatograph without any additional sample preparation.

All gas chromatographs were interfaced to Hewlett-Packard HP3395 (Palo Alto, CA) integrators for data collection. All reaction products were identified by co-elution to authentic standards. These compounds were quantified using calibration plots generated by adding known amounts of each compound to reactions vials that containing phosphate buffer (1 ml) but no cells. Each calibration plot contained at least 5 different concentrations of each compound, including at least one concentration above the maximal concentration detected in our experiments. All calibration plots were fitted to a straight line by linear regression with a final R^2 value of ≥ 0.98.

Cell protein concentrations were determined with the Biuret protein assay (21) after solubilization of cell material for 1 h at 65°C in 3 N NaOH and sedimentation of insoluble material by centrifugation (10,000 rpm, 5 min). Bovine serum albumin was used as a standard. The saturated aqueous phase solubility of C2B and T2B at 25^{o} C was taken as 6.2 mM (2) Kinetic constants (V_{max} and K_s) were derived by computer fitting of the data by nonlinear regression to a single substrate-binding model [$Y = V_{max} \cdot X / (K_s + X)$] using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego,CA).

RESULTS

Growth on 2-butene isomers: Our initial report describing slow growth of strain ELW1 on both C2B and T2B only examined a single culture condition (10%, v/v gas phase) (20). In this present study, we first examined whether the growth rate on 2-butene was impacted by 2-butene concentration. Cells were grown in batch cultures containing a range of concentrations (0-20%, v/v gas phase) of C2B, T2B, or 2-methylpropene (Fig. 3-1). There was no discernable effect of substrate concentration for cultures grown on 2-methypropene (Fig. 3-1A), irrespective of whether they were nominally carbon- (1.25% and 2.5% v/v gas phase) or oxygen-limited (10% and 20% v/v gas phase) conditions. The growth rate on 2-methylpropane was consistently estimated at 0.053 h^{-1} (Fig. 3-1D). With C2B, growth was initiated within 5 d (Fig. 3-1B) while with T2B there was a lag phase of ~15 d before growth was detected (Fig. 3-1C). Once growth was initiated, there was a distinct and similar effect of substrate concentration on growth rate for both 2-butene isomers. The growth rates increased from ~0.003 h^{-1} to ~0.008 h^{-1} with increases in 2-butene concentration from 1.25 to 20% v/v gas phase (Fig. 3-1D).

While growth of strain ELW1 was always consistent on 2-methylpropene, some variability was observed in the slow growth on 2-butene isomers, especially when T2B-grown cells were transferred directly to new T2B-containing medium. To investigate possible causes of this effect, cultures containing C2B or T2B (20% v/v gas phase) were inoculated with 2-methylpropene-grown cells and accumulation of alkene-derived metabolites during growth was determined by GC analysis of the culture medium. During growth on C2B, low levels of *cis*-2,3-epoxybutane were detected after 30 d (Fig. 3-S1). The maximum amount of

epoxide (~30 µmoles) accumulated after 40 d, the point at which growth on C2B was complete and the culture entered stationary phase. *Trans*-2,3-epoxybutane was also detected in the medium of T2B-grown cells but the accumulation pattern was substantially different to *cis*-2,3-epoxybutane. For example, *trans*-2,3-epoxybutane was detected earlier in the culture period than *cis*-2,3-epoxybutane (15 d). The maximum amount of *trans*-2,3-epoxybutane (≤180 µmoles) was as much as 6-fold higher than the maximum amount of *cis*-2,3-epoxybutane detected in C2B-grown cultures. Lastly, the amount of *trans*-2,3-epoxybutane decreased rapidly after the culture growth ceased while the lower amount of *cis*-2,3-epoxybutane detected remained essentially constant after growth on C2B ceased. Other than *cis* and *trans*-2,3-epoxybutane, no other potential metabolites of C2B and T2B were detected in the culture medium in these cultures.

Oxidation of C2B and T2B by resting cells: Both 2-butene isomers were oxidized without a lag phase by 2-methylpropane-grown cells and initial rates were used to estimate the kinetic constants for C2B and T2B oxidation. The constants for both isomers were comparable with V_{max} values ranging from 29 to 51 nmoles min^{-1} mg total $protein^{-1}$ and K_s values ranging from 13 to 19 µM (Fig. 3-S2). The V_{max} values were 2 to 3-fold lower than our previous estimate for 2-methylpropene, while the K_s values for both 2-butene isomers were comparable to our previous estimate of 11 µM for 2-methylpropene (20).

We also compared the metabolites generated from C2B oxidation by resting cells previously grown on 2-methylpropene, C2B and T2B. Cells grown on C2B oxidized C2B without a lag phase (Fig. 3-2A) and *cis*-2,3-epoxybutane accumulated and was also

consumed during the reaction. The maximal amount of the epoxide (1.6 µmoles) was observed after 3 h when ~90% of the initial C2B (9 µmoles) had been consumed. No other metabolites were detected. Cells pretreated with 1-octyne oxidized C2B at less than 5% of rate of untreated cells and the losses of C2B from abiotic incubations without cells were minimal over the reaction time course. With 2-methylpropene-grown cells, C2B was also oxidized without a lag phase. *cis*-2,3-epoxybutane accumulated in the reaction medium and was also consumed (Fig. 3-2B). The maximum amount of the epoxide (1.6 µmoles) was detected after 1.5 h when ~6.5 µmoles of the initial C2B had been consumed. Two additional metabolites, 2,3-butanediol and 3-hydroxy-2-butanone, were also detected at lower concentrations. These metabolites initially accumulated at a near constant rate over the first 5 h of the reaction although the rate of 3-hydroxy-2-butanone accumulation was ~5-fold slower than 2,3-butanediol. The maximal concentration of both metabolites was observed at the same time (5 h) when all of the produced *cis*-2,3-epoxybutane had been consumed. After this, both 2,3-butanediol and 3-hydroxy-2-butanone were slowly consumed during the remainder of the reaction. 2-Methylpropene-grown cells pretreated with 1-octyne only slowly oxidized C2B and *cis*-2,3-epoxybutane, 2,3-butanediol and 3-hydroxy-2-butanone did not accumulate in the reaction medium.

Qualitatively similar results were obtained when we compared T2B oxidation by T2B- and 2-methylpropene-grown cells (Fig. 3-3). Resting T2B-grown cells oxidized T2B and *trans*-2,3-epoxybutane accumulated in the reaction medium (Fig. 3-3A). The maximal amount of *trans*-2,3-epoxybutane (~2 µmoles) was detected once all T2B had been consumed. However, unlike *cis*-2,3-epoxybutane (Fig, 3-2A), the accumulated *trans*-2,3-

epoxybutane was only very slowly degraded. A similar effect was also observed during T2B oxidation by 2-methypropene-grown cells (Fig. 3-3B). In this case, T2B was initially oxidized without a lag phase and *trans*-2,3-epoxybutane, 2,3-butanediol and 3-hydroxy-2-butanone all accumulated in the reaction medium. The maximal amounts of 2,3-butanediol (400 nmoles) and 3-hydroxy-2-butanone (175 nmoles) (Fig. 3-3B) were ~3-fold lower than those observed during C2B oxidation (Fig. 3-2B) and both metabolites were fully consumed during the reaction time course. Conversely, the maximal amount of *trans*-2,3-epoxybutane (~4 µmoles) detected during T2B oxidation (Fig. 3-3B) was ~3-fold greater than the amount of *cis*-2,3-epoxybutane detected during C2B oxidation (Fig. 3-2B). The levels of *trans*-2,3-epoxybutane also remained effectively constant after T2B had been fully consumed.

Initial rates of alkene and epoxide consumption were also determined for resting cells previously grown on 2-methylpropene, C2B and T2B. Cells grown on each alkene behaved very similarly towards these substrates (Table 3-1). Cells grown on each alkene oxidized 2-methylpropene at substantial rates (24-56 nmoles min^{-1} mg total $protein^{-1}$) and in all cases this rate was strongly inhibited in cells pretreated with 1-octyne. The initial rates of C2B (26-50 nmoles min^{-1} mg total $protein^{-1}$) and T2B (31-57 nmoles min^{-1} mg total $protein^{-1}$) oxidation were also comparable for all cell types and were similarly strongly inhibited in 1-octyne-pretreated cells. All cell types also rapidly oxidized methylepoxypropane while *cis*- and *trans*-2-3-epoxybutane were consistently consumed at much lower rates (<10 nmoles min^{-1} mg total $protein^{-1}$). There was little or no effect of 1-octyne pretreatment on any of the rates of epoxide oxidation.

To investigate the possible role of 2-buten-1-ol in 2-butene metabolism, rates of oxidation for this alcohol were also determined for resting cells previously grown on 2-methylpropene, C2B, or T2B (Fig. 3-S3). 2-Butene-1-ol was oxidized by each cell type at similar low rates (9-14 nmoles min^{-1} mg total $protein^{-1}$) that were comparable to the initial rates of 2,3-epoxybutane oxidation measured for each cell type (Table 3-1).

Epoxide degradation reactions: The results in Figs. 3-2 & 3-3 suggested *cis*-2,3-epoxybutane was more readily degraded than the *trans* form of this epoxide. While *cis*-2,3-epoxybutane is achiral, *trans*-2,3-epoxybutane has two enantiomers. Commercial sources of this compound are racemic and contain equal amounts of the (2S,3S)- and (2R,3R)-enantiomers. Resting T2B-grown cells consumed racemic *trans*-2,3-epoxybutane without a lag phase but this reaction abruptly slowed once ~50% of the initial epoxide had been degraded (Fig. 3-4A). No additional oxidation products were detected in the reaction medium. 2-Methylpropene-grown cells also rapidly consumed racemeic *trans*-2,3-epoxybutane until ~50% of the initial epoxide remained (Fig. 3-4B). Both 2,3-butanediol and 3-hydroxy-2-butanone were also generated. The maximal amount of the diol was observed at the time when the initial rapid phase of epoxide consumption was completed. When comparable incubations were conducted C2B-grown cells and commercially supplied *cis*-2-3-epoxybutane (Fig. 3-S4A), the epoxide was rapidly and completely consumed and no metabolites were detected in the reaction medium. 2-Methylpropane-grown cells also rapidly and completely oxidized *cis*-2-3-epoxybutane at comparable rates to C2B-grown cells. Low

levels of 2,3-butanediol and 3-hydroxy-2-butanone also accumulated and were subsequently consumed during this reaction time course (Fig. 3-S4B).

The biphasic degradation of *trans*-2,3-epoxybutane (Fig. 3-4) suggested that both 2-methylpropene- and T2B-grown cells catalyze an enantioselective degradation of *trans*-2-3-epoxybutane with one enantiomer being degraded much faster than the other. To estimate the relative rates for these two reactions, varying amounts of T2B- or 2-methylpropene-grown cells were incubated with a fixed initial concentration of racemeic *trans*-2-3-epoxybutane. The rates of both phases of the epoxide degradation reaction were estimated from the linear portions of each reaction time course. With T2B-grown cells, the two rates of epoxide consumption were both directly proportional to the amount of cells added to the reaction (Fig. 3-5A). The same effect was also observed with 2-methylpropane-grown cells. A plot of the initial and secondary rate of epoxide consumption for both cell types suggested these reactions were indistinguishable (Fig. 3-5B) and that the initial rate of epoxide consumption was ~25-fold faster than the secondary rate.

Propene-grown *X. autotrophicus* Py2 only degrades the 2S,3S-enantiomer of *trans*-2,3-epoxybutane (22). The initial step in epoxyalkane degradation in this strain involves epoxyalkane:Coenzyme M transferase [EaCoMT]; an enzyme that is irreversibly inactivated by exposing cells to methylepoxypropane (23). We exploited the known enantioselectivity of strain Py2 and the sensitivity of its EaCoMT to 1,2-epoxy-2-methylpropane to determine which enantiomer of *trans*-2,3-epoxybutane was preferentially degraded by strain ELW1. 2-Methylpropene-grown cells rapidly degraded ~50% of racemic *trans*-2-3-epoxybutane within 1 h and only slowly further degraded the residual epoxide during the next 3 h of the reaction

(Fig. 3-6). After 4 h, propylene-grown cells of strain Py2 were added to this reaction. No increase in the rate of degradation of the residual epoxide was observed over the following 4 h. In a separate reaction, there was also no degradation of racemic *trans*-2-3-epoxybutane over 4 h in the absence of cells of strain ELW1. However, rapid degradation of ~50% of the epoxide occurred when cells of strain Py2 were added to this reaction after 4 h. In contrast, no degradation of the epoxide was observed in comparable initially abiotic reactions when either boiled or 1,2-epoxy-2-methylpropane-pretreated cells of strain Py2 were added after the first 4 h of the reaction. These results indicate that strain ELW1, like strain Py2, preferentially degrades the (2S,3S)- enantiomer of *trans*-2,3-epoxybutane.

Degradation of 2,3-butanediol and 3-hydroxy-2-butanone: We have previously reported that strain ELW1 grows on both 2,3-butanediol and 3-hydroxy-2-butanone (20). The enantioselective degradation of *trans*-2-3-epoxybutane (Fig. 3-6) suggested some degree of enantioselectivity might also occur during the subsequent degradation of 2,3-butanediol, the expected hydrolysis product of this epoxide. The growth rate of strain ELW1 was therefore determined in batch cultures grown on all three 2,3-butanediol stereoisomers (Fig. 3-7). The growth rates on the (2S,3S)-enantiomer (0.039 h^{-1}) and *meso* isomer (0.038 h^{-1}) were very similar while the slowest growth rate (0.022 h^{-1}) was observed with the (2R,3R)-enantiomer.

In aerobic bacteria such as *Pseudomonas putida,* 2,3-butanediol is initially oxidized to 3-hydroxy-2-butanone by 2,3-butanediol dehydrogenase and 3-hydroxy-2-butanone is then oxidatively cleaved to equimolar amounts of acetaldehyde and acetyl-CoA by acetoin dehydrogenase (24). We determined the initial rates of oxidation of the three 2,3-butanediol

stereoisomers and potential metabolites of 3-hydroxy-2-butanone cleavage by cells grown on 2-methylpropene, C2B and T2B (Table 3-2). While all cell types oxidized all of the 2,3-butanediol stereoisomers at rates >10 nmoles min^{-1} mg total $protein^{-1}$, cells grown on T2B consistently exhibited higher rates of diol consumption than either cells grown on C2B or 2-methylpropene. Cells grown on the 2-butene isomers also showed as much as 10-fold higher initial rates of oxidation of 3-hydroxy-2-butanone and acetaldehyde compared to 2-methylpropene-grown cells. In addition to cleaving 3-hydroxy-2-butanone, acetoin dehydrogenase can also cleave 2-hydroxy-3-pentanone to equimolar amounts of acetone and acetyl-CoA (25). C2B- and T2B-grown cells slowly oxidized 2-hydroxy-3-pentanone while this activity was absent in 2-methylpropane-grown cells. None of the cell types oxidized acetone at any substantial rate (≤1 nmoles min^{-1} mg total $protein^{-1}$) (Table 3-2). The time course of 2-hydroxy-3-pentanone degradation was also examined for C2B-, T2B- and 2-methylpropene-grown cells (Fig. 3-8). 2-Hydroxy-3-pentanone was not degraded by 2-methylpropene-grown cells but was slowly degraded by both C2B- and T2B-grown cells. In reactions containing C2B- and T2B-grown cells, acetone accumulated in the reaction mixture and was consistently detected at concentrations equivalent to 50% of the amount of 2-hydroxy-3-pentanone degraded.

2-Hydroxyisobutyrate (HIBA) is a key metabolite generated during 2-methylpropene metabolism by strain ELW1 (20). We have also previously shown that growth on 2-methylpropene and all subsequent metabolites up to and including HIBA is strictly dependent on the presence of cobalt in the growth medium (20). To investigate whether 2-butene metabolism had a similar cobalt requirement, we examined growth of strain ELW1 on C2B

and T2B in the presence and absence of cobalt under the same culture conditions previously used to demonstrate the complete inhibition of growth on 2-methylpropene in cobalt-deficient media. Our results (Table 3-3) show there was no effect of cobalt on the growth of strain ELW1 on either 2-butene isomer.

DISCUSSION

The aim of this study was to characterize the previously reported ability of the 2-methylpropene-metabolizing *Mycobacterium* strain ELW1 to grow on 2-butene. Although the 2-butene isomers were both similarly poor growth substrates, we conclude the pathway of 2-butene metabolism in strain ELW1 is substantially different to the terminal oxidation pathway previously proposed for *Nocardia* strain TB1 (17). Specifically, our results suggest that both 2-butene isomers are initially oxidized to epoxides that are then hydrolyzed to their corresponding diols. These are the same reactions proposed for the initial steps in 2-methylpropene metabolism and we provide physiological evidence that they are likely catalyzed by the same enzymes in 2-butene- and 2-methylpropene-grown cells. We also conclude that later steps in 2-butene metabolism are enzymatically distinct from 2-methypropene metabolism and exhibit preferential degradation of S-enantiomers of *trans*-2,3-epoxybutane, 2,3-butanediol and possibly 3-hydroxy-2-butanone. These main conclusions and their supporting evidence are discussed more fully in the following sections. The pathways we have previously proposed for 2-methylpropene metabolism and now propose for C2B and T2B metabolism are summarized in Fig. 3-9.

Initial steps in 2-butene metabolism: With the notable exception of the 2-butene-metabolizing strain *Nocardia* strain TB1 (17), all sufficiently well characterized aerobic gaseous alkene-metabolizing bacteria use alkene monooxygenases to initiate alkene catabolism and generate epoxides as the immediate and sole product of alkene oxidation (26). In this study we observed that 2-methylpropene-, C2B-, and T2B-grown cells were able to

oxidize all three of these alkenes (Table 3-1), and the first, and in some cases, the only detected products of C2B and T2B oxidation were 2,3-epoxybutanes (Figs 3-2 & 3-3). This suggests an alkene-oxidizing monooxygenase is also responsible for the initial step of 2-butene metabolism in strain ELW1. This conclusion is further supported by our observation that oxidation of both C2B and T2B was strongly inhibited in C2B-, T2B-, and 2-methylpropane-grown cells pretreated with 1-octyne (Figs. 3-2 & 3-3). The effects of 1-octyne are specific for the initial monooxygenase-catalyzed step in 2-methylpropene metabolism (20) and 1-octyne appears to act as a mechanism-based inactivator of this alkene-oxidizing monooxygenase. Our observations that the K_s values for all three alkenes for 2-methylpropene-grown cells are very similar (Fig. 3-S2) also supports our conclusion that strain ELW1 uses the same monooxygenase to initiate the oxidation of all three alkenes. While confirmation of this is beyond the scope of this present study and will require identification of this enzyme at the molecular level, use of the same monooxygenase to initiate the catabolism of several structurally related substrates is common among both alkane- and alkene-metabolizing bacteria (26).

In *Nocardia* strain TB1, 2-buten-1-ol is proposed to be the immediate oxidation product generated from T2B by an *n*-butane- and 2-butene-oxidizing alkane monooxygenase (17). Although C2B-, T2B- and 2-methylpropene grown cells could all oxidize 2-buten-1-ol at comparable rates (Fig. 3-S3), the rates of oxidation were similar to those determined for *cis* and *trans*-2-3-epoxybutane consumption (Table 3-1). It therefore seems unlikely that we would have only detected accumulation of 2,3-epoxybutane during C2B and T2B oxidation if 2-buten-1-ol were a substantial portion of the initial products of 2-butene oxidation. While

we cannot exclude limited production of this alcohol, we conclude 2-buten-1-ol is not a significant or physiologically relevant product of C2B or T2B oxidation. This conclusion is further supported by our earlier observations that strain ELW1 does not grow on this compound but does grow on both *cis* and *trans*-2,3-epoxybutane (20). Our conclusion is also supported by the general observation that while alkane-oxidizing monooxygenases are well known to catalyze both hydroxylation and epoxidation reactions, there are few examples of alkene monooxygenase-catalyzed hydroxylation reactions and none of these involve alkenes (27).

Like 2-methylpropene metabolism, the subsequent step in the 2-butene metabolism appears to involve epoxide hydrolysis. During growth of strain ELW1 on 2-methylpropene, this alkene is first oxidized to 1,2-epoxy-2-methylpropane which is then hydrolyzed to 2-methyl-1,2-propanediol (Fig. 3-9). This 1-octyne-insensitive hydrolysis reaction occurs sufficiently rapidly that the epoxide is not detected during 2-methylpropene oxidation by resting 2-methylpropene-grown cells. The substrate range of the putative epoxide hydrolase responsible for this reaction also likely includes both 1,2-epoxypropane and 1,2-epoxybutane as these epoxides are also not detected during rapid propene and 1-butene oxidation by 2-methylpropene-grown cells (20). In this study, cells grown on C2B, T2B or 2-methylpropene all exhibited similarly high specific rates of 1-octyne insensitive 1,2-epoxy-2-methylpropane degradation and similarly low specific rates of 1-octyne insensitive 2,3-epoxybutane degradation (Table 3-1). While this result establishes that C2B- and T2B-grown cells have a vigorous epoxide-hydrolyzing capability, it does not explain why transient accumulation of 2,3-butanediol, the expected product of 2,3-epoxybutane hydrolysis, was only observed in

incubations using 2-methylpropene-grown cells and not with C2B- or T2B-grown cells. A likely explanation of this is provided by the results presented in Table 3-2. Irrespective of the 2,3-butanediol isomers tested, the specific rates of 2,3-butanediol oxidation by 2-methylpropene-grown cells were similar to the rates of 2,3-epoxybutane degradation measured for these cells (Table 3-1). In contrast, the enantiomer-specific rates of 2,3-butanediol oxidation exhibited by T2B-grown cells were all as much as 9-fold higher than both the equivalent rates exhibited by 2-methylpropene-grown cells (Table 3-2) and consistently more than 5-fold higher than the rates of *trans*-2,3-epoxybutane degradation exhibited by T2B-grown cells (Table 3-1). This difference in activities would enable rapid 2,3-butanediol degradation by T2B-grown cells and limit accumulation of this metabolite as it is generated from *trans*-2,3-epoxide hydrolysis. This effect would not be expected in 2-methylpropene-grown cells. The differences between these various rates was not as evident with C2B-grown cells but our results do indicate the rate of (2S,3S)-butanediol oxidation was ~2-fold higher than both the rate in 2-methylpropene-grown cells (Table 3-2) and the rate of *cis*-2,3-epoxybutane degradation measured for C2B-grown cells.

Based on the points above, we conclude that it is also very likely that cells of strain ELW1 grown on C2B, T2B and 2-methylpropene utilize the same epoxide hydrolase to further degrade the epoxides generated during the metabolism of these alkenes. While confirmation of this possibility will also require further studies at the molecular level, this would not be unique as other alkene-oxidizing bacteria such as strain Py2 utilize the same epoxide carboxylase system to metabolize epoxides generated from ethene, propene and longer chain 1-alkenes (26). It is important to note that if this conclusion is correct, the slow

rate of hydrolysis of 2,3-epoxybutanes by an epoxide hydrolase (Table 3-1) may be the principal factor determining the slow growth rate of strain ELW1 on both C2B and T2B. The faster rates of T2B and C2B oxidation and 2,3-butanediol degradation compared to epoxide hydrolysis, as well as the relatively fast rates of growth on 2,3-butanediol (Fig. 3-7), further point to epoxide hydrolysis as the likely growth rate-limiting step in 2-butene metabolism.

Enantioselective epoxide degradation reactions: Another factor that may specifically impact growth of strain ELW1 on T2B rather than C2B is the enantioselectivity associated with the hydrolysis of *trans*-2,3-epoxybutane. Like *X. autotrophicus* strain Py2 (22), our results (Fig. 3-6) indicate strain ELW1 preferentially degrades the (2S,3S) enantiomer. The same enantioselectivity is also exhibited by ethene-grown *Nocardiodes* JS614 (14), ethene-grown *Micrococcus* M90C (15), and propene-grown *Nocardia* H8 (28). The cause of this enantioselectivity is not known but in strain Py2 propene metabolism involves two enantioselective processes. Propene oxidation itself generates predominantly (≥95%) (1R,2R) –epoxypropane (29). Both 1,2-epoxypropane enantiomers are then transformed in several steps by an epoxide carboxylase enzyme system. After conjugation with CoM by epoxyalkane: CoM transferase (EaCoMT), the two resulting chiral conjugates, 2-(R)- and 2-(S)-hydroxypropyl-CoM, are oxidized to achiral 2-ketopropyl-CoM by two separate enantiospecific 2-hydroxypropyl-CoM dehydrogenases (*R*- and *S*-HPCDH). 2-ketopropyl-CoM is then carboxylated by NADPH:2-ketopropyl-CoM oxidoreductase/carboxylase (2-KPCC) to generate acetoacetate and regenerate free CoM. As HPCDH catalyzes the only enantioselective reaction in the epoxide carboxylase system, the preference of strain Py2 for

trans-(2S,3S*)*-epoxybutane may result from an inability of HPCDH to oxidize the CoM conjugate of *trans*-(2R-3R*)*-epoxybutane. The enantioselectivity of this enzyme is known to be strongly impacted by interactions between C-terminal arginine residues and the sulfonate group of CoM (30).

Beyond EaCoMT-utilizing bacteria, there are few reports describing the enantioselectivity associated with either the bacterial production or degradation of *trans*-2-3-epoxybutane. Methanotrophs and other alkane-metabolizing bacteria that cometabolically oxidize propene, 1-butene and T2B generate essentially racemic mixtures of epoxide enantiomers (18). As these organisms typically lack enzymes to specifically further degrade epoxides, these results imply the alkane-oxidizing monooxygenases in these organisms lack stereospecificity with alkene substrates. In contrast, alkene-metabolizing bacteria typified by strain Py2, all preferentially generate R-enantiomers from propene, 1-butene and T2B (18). In whole cell systems, this reflects not only the stereospecificity of the initial alkene epoxidation reaction but also the potential enantioselective degradation of (1S,2S)-enantiomers through the activity of a CoM-dependent epoxide carboxylase system.

We can estimate the stereospecificity of an alkene-oxidizing monooxygenase in strain ELW1 towards T2B from our short-term incubations that followed T2B oxidation by either T2B- (Fig. 3-3A) or 2-methylpropene-grown cells (Fig. 3-3B). In these reactions we observed complete consumption of T2B and the stable accumulation of *trans*-2,3-epoxybutane. In view of the rapid rate of degradation of the S-enantiomer (Fig. 3-6), the residual epoxide detected in these reactions was likely to be almost exclusively *trans*-(2R-3R*)*-epoxybutane. Our results therefore imply that the initial epoxidation of T2B can generate

as much as 45% *trans*-(2R-3R)-epoxybutane (Fig 3-3B). This level of R-enantiomer production is comparable to those seen with T2B oxidation by rat and mouse liver micrososmes (31). Although this initial estimate requires confirmation using both an epoxide hydrolase-free source of alkene monooxygenase and more suitable analytical approaches such as chiral GC analysis, this apparently substantial production of a slowly degradable enantiomer likely explains why much higher amounts of *trans*-2,3-epoxybutane and lower amounts of later metabolites such as 2,3-butanediol and 3-hydroxy-2-butanone were detected during T2B oxidation (Fig. 3-3B) compared to the oxidation of C2B (Fig. 3-2B). A high level of *trans*-(2R,3R)-epoxybutane production during T2B oxidation coupled with a slow rate of degradation of this enantiomer also likely explains why much higher levels of *trans*-2,3-epoxybutane accumulation occurred during growth of strain ELW1 on T2B compared to *cis*-2,3-epoxybutane accumulation during growth on C2B (Fig 3-S1). The accumulation of *trans*-2,3-epoxybutane during growth may also explain why variability was sometimes encountered during growth on T2B and when T2B-grown cells were used as an inoculum for fresh T2B-containing cultures.

In eukaryotic systems, epoxide hydrolase in rat liver microsomes preferentially hydrolyzes the (2S-3S)-enantiomer of *trans*-2,3-epoxybutane (32). Selective hydrolysis of *trans*-(2S,3S)-epoxybutane by the yeast *Rhodotorula glutinis* also results in accumulation of (2R-3R)-enantiomer although this was the slowest reaction out of 15 epoxides tested (33). Although many enantioselective bacterial epoxide hydrolases have been identified and characterized (34), there are no reports we are aware of that describe the enantioselectivity of these enzymes towards *trans*-2,3-epoxybutane. This may reflect a lack of research into this

process as many alkene-metabolizing bacteria utilize a CoM-dependent process for epoxide metabolism rather than epoxide hydrolases. However, it may also point to a scarcity of epoxide hydrolases with this activity. For example, van Loo *et al*. (35) compared the reactivity of twelve known and putative bacterial epoxide hydrolases that were cloned and overexpressed after their identification in a genome-based survey. These enzymes were all screened for their reactivity toward 25 aromatic, cyclic and aliphatic epoxides. None of the eight enzymes with confirmed activity hydrolyzed racemic *trans*-2-3-epoxybutane and *cis*-2,3-epoxybutane was also only slowly hydrolyzed by two of these enzymes.

While discussing microbial epoxide hydrolases, it is particularly noteworthy that subsequent to the characterization of its ability to metabolize T2B (17), *Nocardia* strain TB1 (*Rhodococcus ruber* DSM 44539) has been shown to exhibit high levels of epoxide hydrolase activity towards diverse epoxides (36-40). This activity has been characterized for cells grown on a peptone- and glucose-containing medium and neither *cis* or *trans*-2,3-epoxybutane have been reported as substrates for this organism. In the original report describing T2B metabolism by strain TB1 it was observed that 2,3-epoxybutane did not accumulate during T2B oxidation by T2B-grown cells although 1,2-epoxyalkanes were generated during oxidation of ethene, propene and 1-butene (13). This observation was part of the evidence used to suggest that T2B is not oxidized to an epoxide and is terminally oxidized to 2-buten-1-ol. However, most resting alkene-metabolizing bacteria do not excrete an epoxide when they are exposed to the alkene on which they were previously grown. In light of more recent studies, the lack of epoxide accumulation during T2B oxidation could have been due to rapid degradation of this metabolite by epoxide hydrolase activity. Other

observations in this previous study also suggest that T2B metabolism by strain TB1 may actually involve an epoxide. For example, 2,3-epoxybutane stimulated oxygen uptake in 2-butene-grown cells of strain TB1 and *trans*-2,3-epoxybutane was consumed by cells grown on both *n*-butane and T2B (13). In view of the high levels of non-specific epoxide hydrolase activity this organism is now known to be capable of exhibiting, the metabolism of 2,3-epoxybutanes by 2-butene-grown cells of strain TB1 deserves reexamination and the role of terminal oxidation as the sole route of 2-butene metabolism in this organisms should be reassessed.

Later steps in 2-butene metabolism: Our results shown in Figs. 3-4 and 3-S4 support our earlier suggestion that the further metabolism of both *cis*- and trans-2,3-epoxybutane involves hydrolysis to 2,3-butanediol. We did not detect 2,3-butanediol accumulation during 2,3-epoxybutane degradation by either C2B- (Fig. 3-S4A) or T2B-grown cells (Fig. 3-4A), but accumulation and degradation of this diol was observed during both *cis*- and *trans*-2,3-epoxybutane degradation by 2-methylpropene-grown cells (Figs. 3-S4B and 3-4B). These different diol accumulation and degradation patterns likely represent enzymatic differences imposed by respective growth substrates. For example, these results can be accounted for if 2-methylpropene- and 2-butene-grown cells use common enzymes to oxidize alkenes and hydrolyse the resulting epoxides but then use different enzymes to further metabolize the hydrolysis products. Consequently, 2-methylpropene-grown cells would be expected to oxidize the products of *cis*- and *trans*-2,3-epoxybutane hydrolysis more slowly than C2B- and T2B-grown cells as these cells require these later enzymes systems to be able to grow on

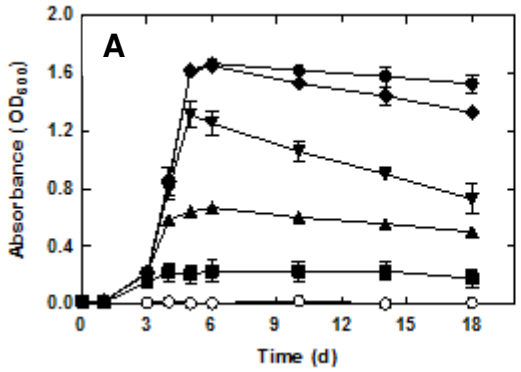
2-butene. This interpretation is supported by several of our results. For example, we demonstrated that growth on 2-butene was unaffected by depletion of cobalt from the growth medium (Table 3-3). In contrast, growth on 2-methylpropene and all intermediates including and preceding 2-hydroxyisobutyrate (Fig. 3-9) is fully inhibited in this strain in the absence of cobalt (20). More specifically, we demonstrated that T2B-grown cells oxidized all three 2,3-butanediol stereoisomers much faster than 2-methylpropene grown cells (Table 3-2). The same difference in rates was also observed for the oxidation of 3-hydroxy-2-butanone, the anticipated and observed product of 2,3-butanediol oxidation. A large difference between T2B- and 2-methylpropene-grown cells was also observed for the oxidation rates for acetaldehyde and acetate, the products expected from the oxidative cleavage of 3-hydroxy-2-butanone. We also demonstrated stable acetone accumulation during 3-hydroxy-2-pentanone degradation by C2B- and T2B-grown cells. This strongly suggests that 3-hydroxy-2-butanone is further oxidatively cleaved to acetaldehyde and acetyl-CoA by an acetoin dehydrogenase (Table 3-2 & Fig 3-8).

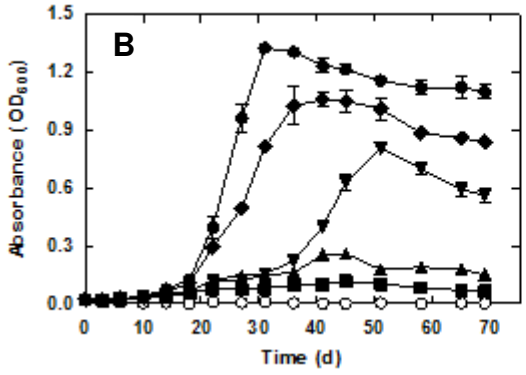
We have not characterized the enantiomeric composition of either the 2,3-butanediol or 3-hydroxy-2-butanone detected during C2B and T2B oxidation. However, we did observe some degree of enantioselectivity towards the (2S,3S)-enantiomer of 2,3-butanediol both in terms of resting cell oxidation rates exhibited by C2B- and T2B-grown cells (Table 3-2) and growth rates on these three stereoisomers (Fig. 3-7). While these differences were less dramatic than the strongly enantioselective degradation of *trans*-(2S,3S)-epoxybutane, these two processes are potentially connected if an enantioselective degradation in one step in a pathway leads to enantioselectivity in subsequent enzymatic steps. For example, the

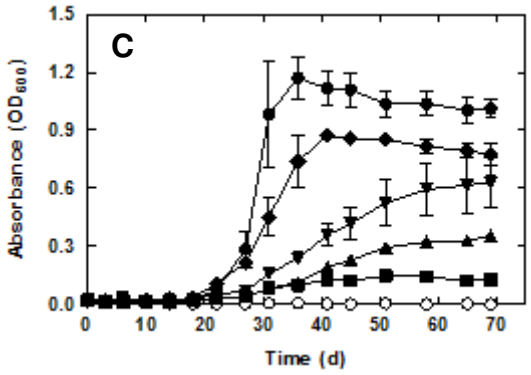
enantioselective hydrolysis of the S-enantiomer of *trans*-2,3-epoxybutane might be expected to lead to a subsequent bias towards the degradation of the S-enantiomers of 2,3-butanediol and possibly the S-enantiomer of 3-hydroxy-2-butanone. The 2,3-butanediol- and 3-hydroxy-2-butanone-oxidizing enzymes in 2-butene-grown cells of strain ELW1 will require further studies to elucidate their potential enantioselectivity.

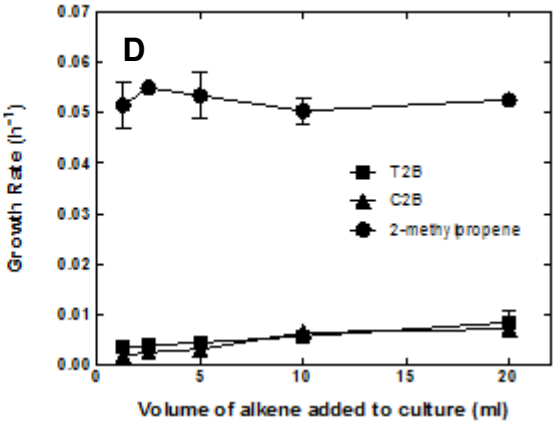
FIGURES AND TABLES

Figure 3-1: Effect of gas concentration on the growth of strain ELW1 on 2-methylpropene, C2B and T2B. A series of batch cultures containing (**Panel A**) 2-methylpropene, (**Panel B**) C2B, and (**Panel C**) T2B were inoculated with cells previously grown on 2-methylpropene and incubated, as described in the Methods section. The Figure shows the changes in culture density (OD_{600}) over time for cultures grown with (○) 0, (■) 1.25, (▲) 2.5, (▼) 5.0, (◆) 10.0, and (●) 20.0 % (vol/vol gas phase) of each alkene. **Panel D** shows the effect of gas concentration on the growth rate of strain ELW1 on all three alkenes. In all cases the plotted data show the mean and ranges of values from three separate cultures.


A
Absorbance (OD_{600})
Time (d)


B
Absorbance (OD_{600})
Time (d)


C
Absorbance (OD_{600})
Time (d)


D
Growth Rate (h^{-1})
T2B
C2B
2-methylpropene
Volume of alkene added to culture (ml)

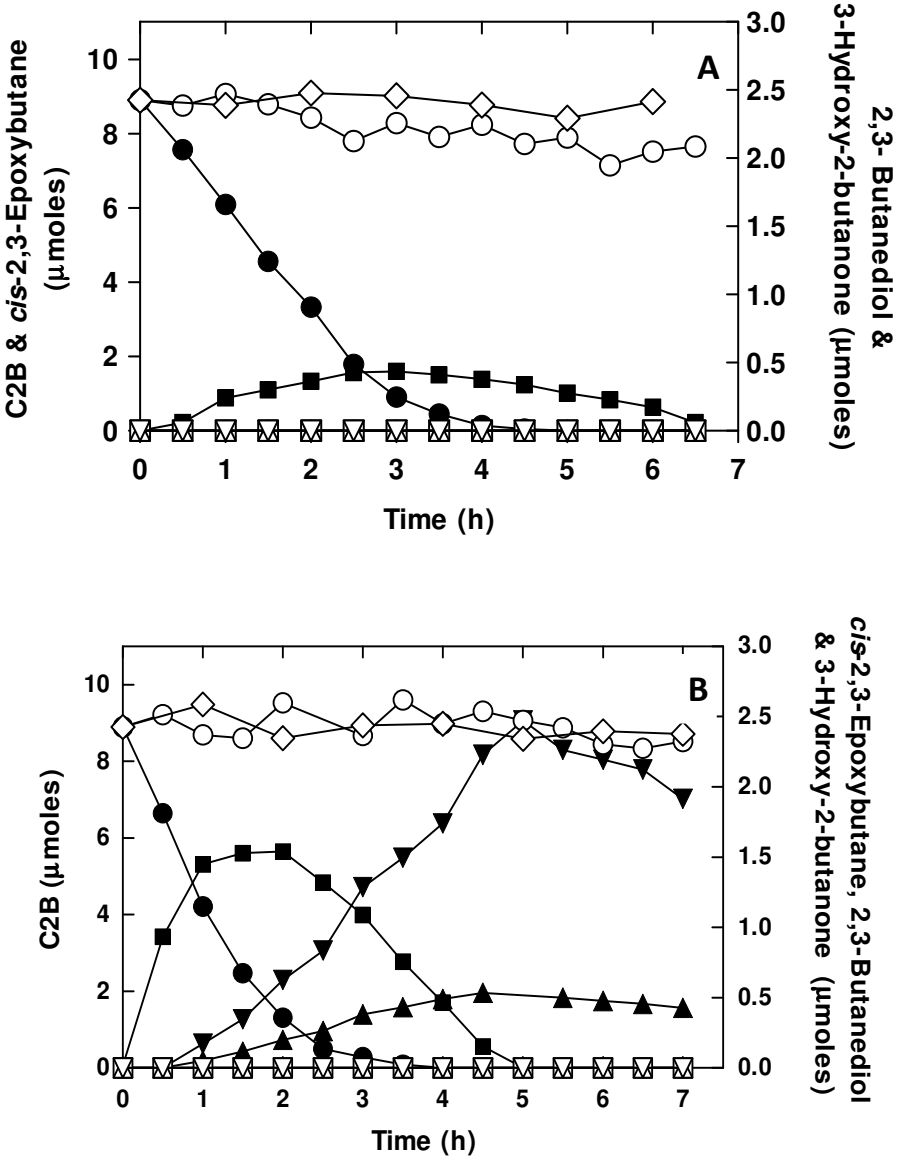


Figure 3-2: Oxidation of C2B by C2B-, and 2-methylpropene-grown cells. Panel A: C2B- (1.6 mg total protein) or **Panel B:** 2-methylpropene-grown cells (4.5 mg total protein), were incubated with C2B (200 µl) in glass serum vials (10 ml) and the consumption of C2B and the production of metabolites was determined by GC analysis, as described in the Methods section. When required**,** cells were also pretreated with 1-octyne, as described in the Methods section. The symbols indicate (●,○) C2B, (■,□) *cis*-2,3-epoxybutane, (▼,▽), 2,3-butanediol, and (▲,△) 3-hydroxy-2-butanone. The open symbols indicate 1-octyne-pretreated cells and the closed symbols indicate untreated cells. The Figure also shows (◇) C2B remaining in a reaction vial incubated without cells.

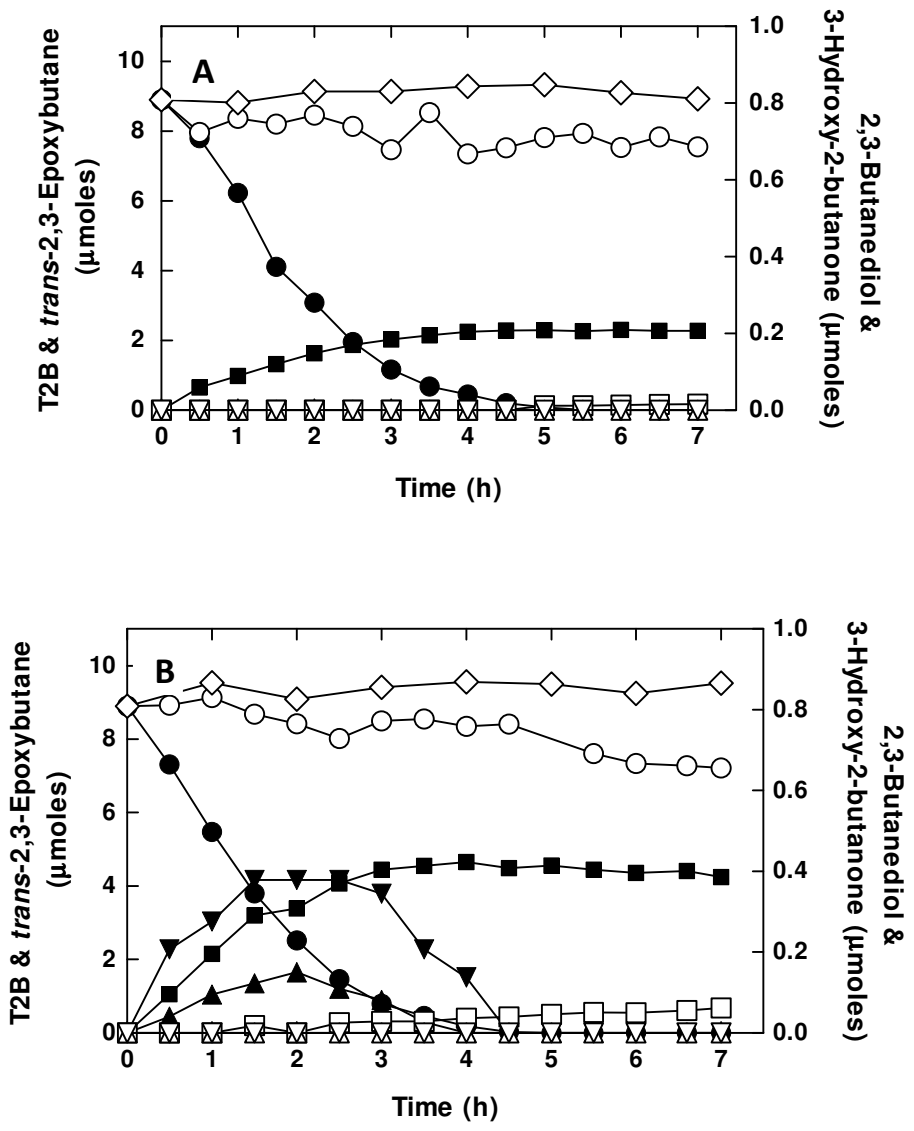


Figure 3-3: Oxidation of T2B by T2B-, and 2-methylpropene-grown cells. Panel A: T2B- (2.2 mg total protein) or **Panel B:** 2-methylpropene-grown cells (3.2 mg total protein), were incubated with T2B (200 µl) in glass serum vials (10 ml) and the consumption of T2B and the production of metabolites was determined by GC analysis, as described in the Methods section. When required**,** cells were also pretreated with 1-octyne, as described in the Methods section. The symbols indicate (●,○) T2B, (■,□) *trans*-2,3-epoxybutane, (▼,▽), 2,3-butanediol, and (▲,△) 3-hydroxy-2-butanone. The open symbols indicate 1-octyne-pretreated cells and the closed symbols indicate untreated cells. The Figure also shows (◇) T2B remaining in a reaction vial incubated without cells.

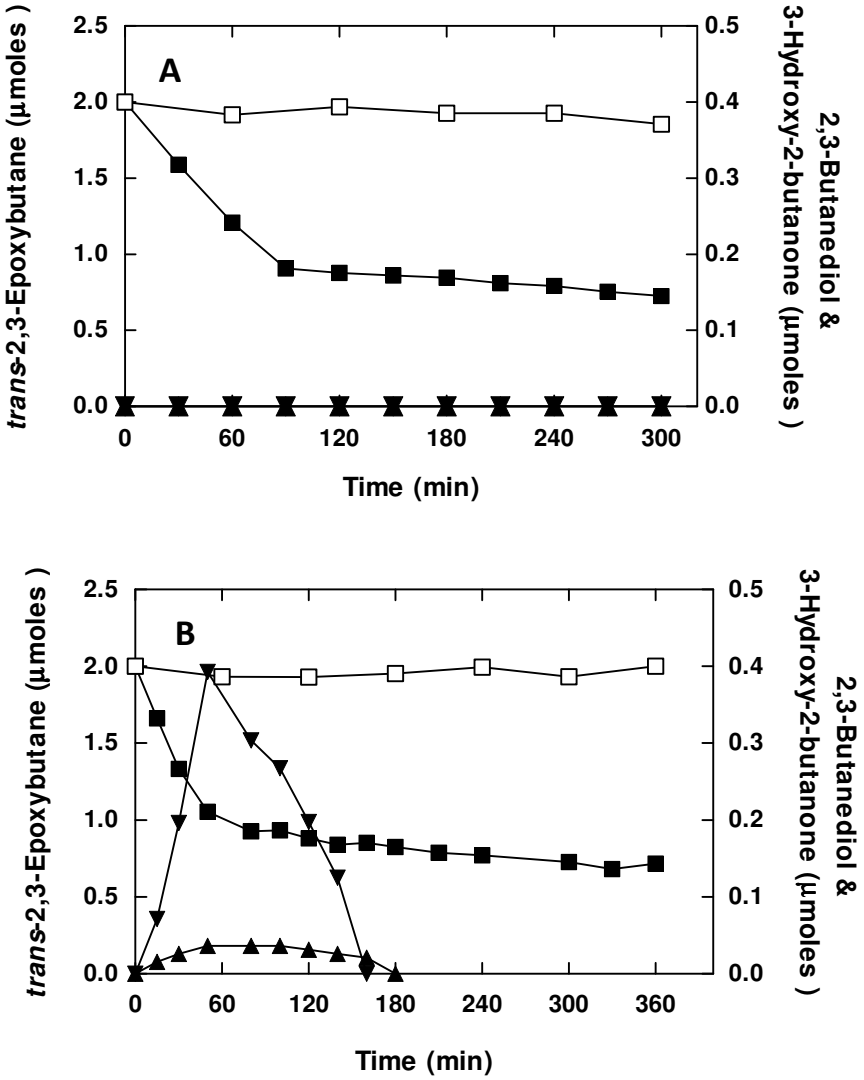


Figure 3-4: ***Trans*****-2,3-epoxybutane degradation by T2B- and 2-methylpropene-grown cells. Panel A:** T2B- (2.0 mg total protein) and **Panel B:** 2-methylpropene-grown cells (2.7 mg total protein) were incubated with racemic *trans*-2,3-epoxybutane (2 mM) and the consumption of the epoxide and the production of metabolites was determined by GC analysis, as described in the Methods section. The symbols indicate (■) *trans*-2,3-epoxybutane, (▼) 2,3-butanediol, and (▲) 3-hydroxy-2-butanone. Both Panels also show the time course of (□) *trans*-2,3-epoxybutane consumption in reactions conducted without added cells.

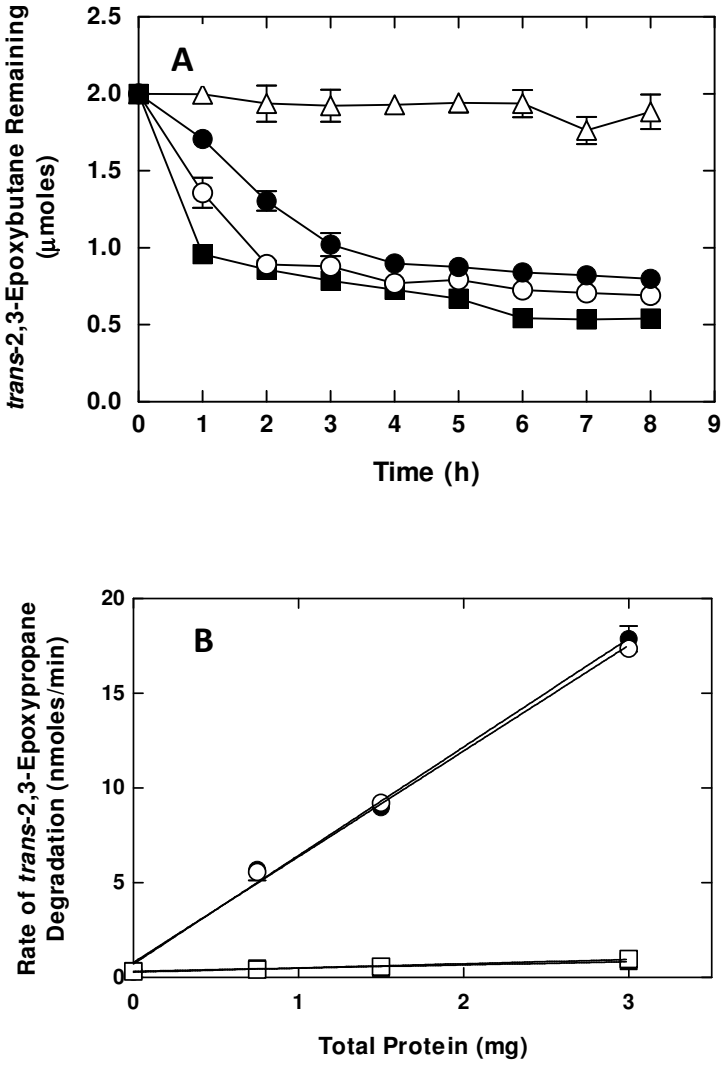


Figure 3-5: Effect of cell concentration on *trans*-2,3-epoxybutane degradation. Varying amounts of cells of strain ELW1 grown on T2B or 2-methylpropene were incubated with racemic *trans*-2,3-epoxybutane (2 µmoles) in glass serum vials (10 ml) and consumption of the epoxide was determined by GC analysis of the reaction mixture, as described in Methods section. **Panel A:** The Figure shows the time course for consumption of *trans*-2,3-epoxybutane by T2B-grown cells in reactions containing either (△) no cells or (●) 0.75 mg, (○) 1.5 mg, and (■) 3 mg total protein. **Panel B:** The Figure shows the effect of protein concentration on (●,○) the rapid initial and (■,□) slower secondary rates of *trans*-2,3-epoxybutane degradation by cells grown on T2B (open symbols) and 2-methylpropene (closed symbols). In both Panels the error bars indicate the standard error of values obtained from reactions catalyzed by cells from two separate cultures.

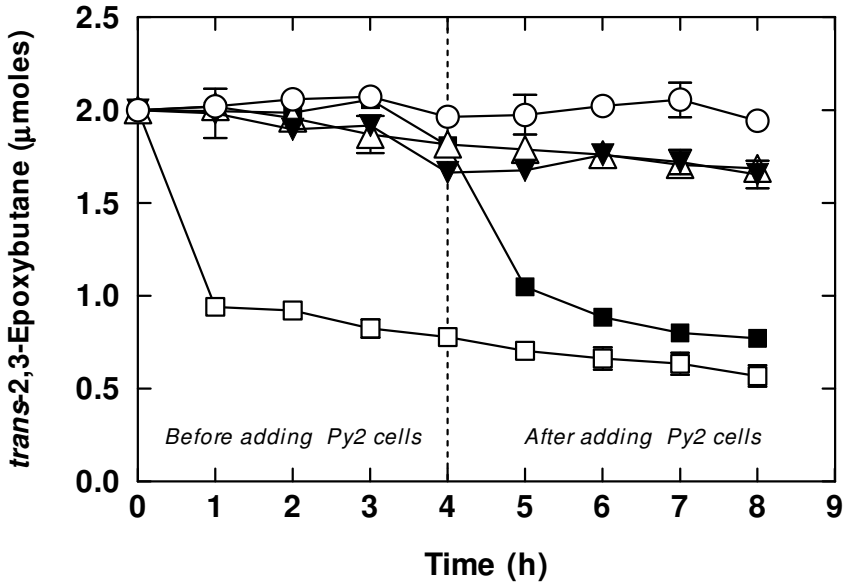


Figure 3-6: Degradation of racemeic *trans*-2,3-epoxybutane by *Mycobacterium* ELW1 and *X. autotrophicus* Py2. Five reaction vials (10 ml) were prepared that contained racemic *trans*-2,3-epoxybutane (2 µmoles) in phosphate buffer, as described in the Methods section. At the start of the reaction, resting 2-methylpropene-grown cells (1.5 mg total cell protein) were added to one reaction. No cells were added to the remaining four reactions. All five reactions vials then were incubated in at 30° C in a shaking water bath (150 rpm) and each reaction was regularly sampled and analyzed by GC to determine the residual amount of epoxide. After 4h, cells of propene-grown X. *autotrophicus* Py2 (1.5 mg total protein) were added to several of the reactions. The reaction vials were returned to the shaking water bath and continued to be regularly sampled and analyzed by GC to determine the residual amount of *trans*-2,3-epoxybutane. The Figure shows the time course of degradation for racemic *trans*-2,3-epoxybutane under the following reaction conditions. (□) 2-methylpropene-grown cells of strain ELW1 added at t = 0h and propene-grown cells of strain Py2 added at t = 4h, (■) propene-grown cells of strain Py2 added at t = 4h, (▼) heat-inactivated, propene-grown cells of strain Py2 added at t = 4h, (△) methylepoxypropane-treated propene-grown cells of strain Py2 added at t = 4h, and (○) no addition at either t = 0h or t = 4h. The data presented are the means and standard errors for reactions conducted with two separate cultures of strain ELW1 and Py2.

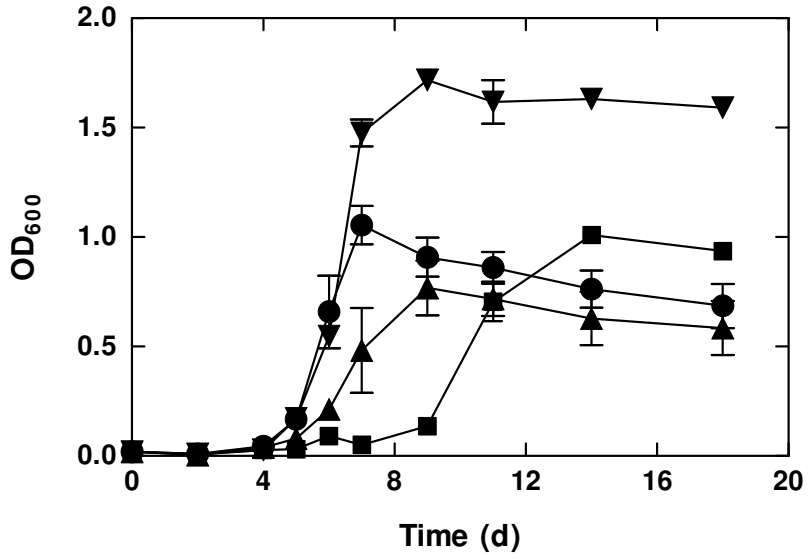


Figure 3-7: Growth of strain ELW1 on 2,3-butanediol stereoisomers. The Figure shows the time course of growth of strain ELW1 on (▼) 2-methylpropene (10% v/v gas phase), and (●) (2S,3S)-butanediol, (■) (2R,3R)-butanediol and (▲) *meso*-butanediol (each at 10 mM initial concentration). The cultures were grown in sealed glass serum vials (160 ml) containing 25 ml of MSM, as described in the Methods section. The data plotted are the means and ranges of values obtained from two separate cultures grown on each compound.

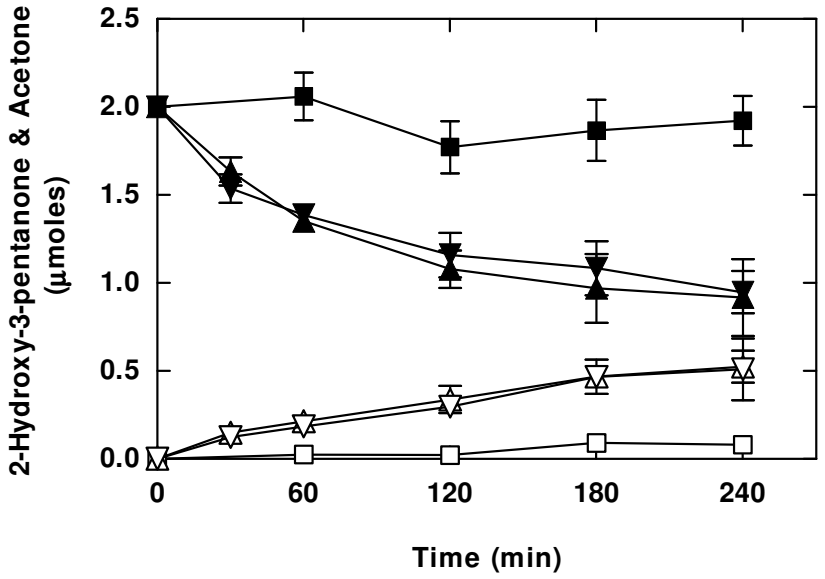


Figure 3-8: Oxidation of 2-hydroxy-3-pentanone by 2-methylpropene-, C2B-, and T2B-grown cells. Reaction vials (10 ml) containing 2-hydroxy-3-pentanone (2 µmoles) in phosphate buffer were incubated with resting cells. The degradation of 2-hydroxy-3-pentanone and the accumulation of metabolites were determined by GC analysis of the reaction medium, as described in the Methods section. The Figure shows the time course of 2-hydroxy-3-pentanone degradation (closed symbols) and the accumulation of acetone (open symbols) for reactions containing resting cells previously grown on (■,□) 2-methylpropene (1.3 mg total protein), (▲,△) C2B (1.4 mg total protein) and (▼,▽) T2B (1.5 mg total protein). The data presented show the means and standard error of values obtained at reactions conducted with cells obtained from two separate cultures.

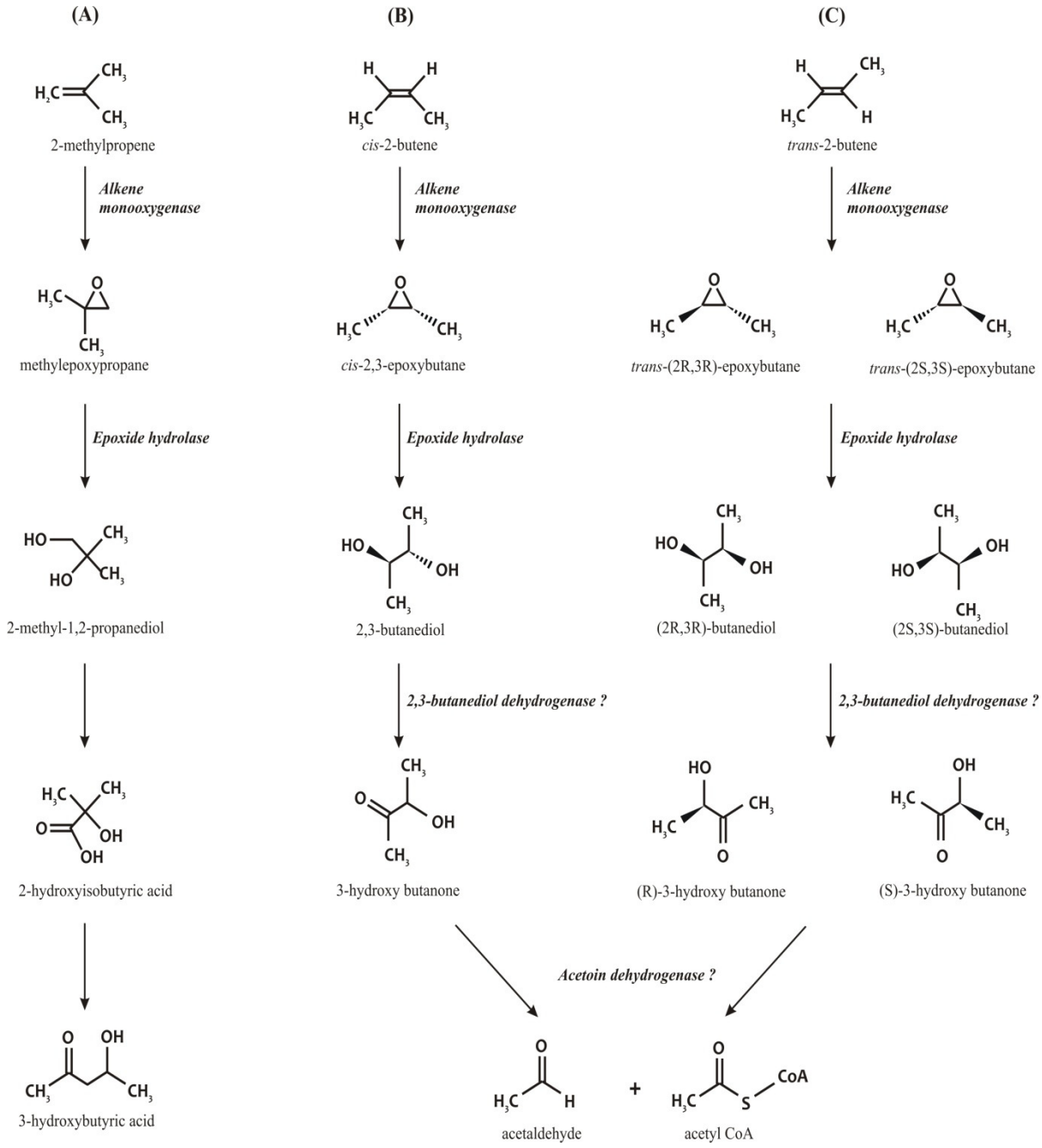


Figure 3-9: Proposed pathways of (A) 2-methylpropene (B) *cis*-2-butene, and (C) *trans*-2-butene in strain ELW1

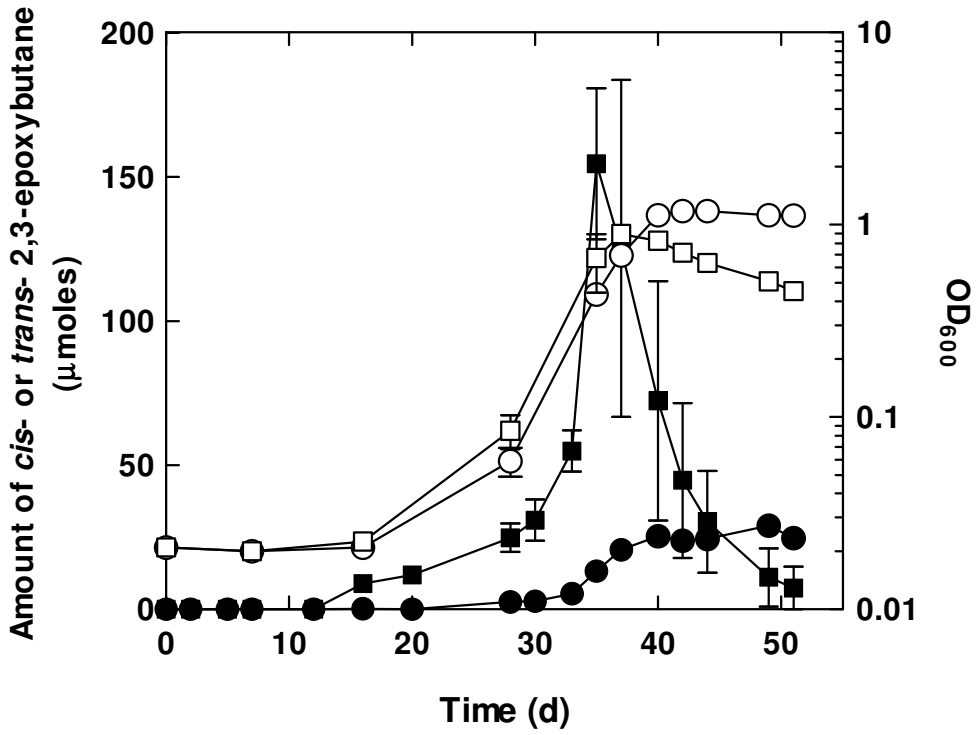


Figure 3-S1: Production of epoxides during growth of strain ELW1 on C2B or T2B. Cells of strain ELW1 were grown in sealed glass serum vials (160 ml) containing 25ml of MSM and 20% (v/v gas phase) of C2B or T2B gas. The cultures were incubated for over 8 weeks and the production of corresponding epoxides (*cis*-2,3-epoxybutane or *trans*-2,3-epoxybutane) and culture density (OD_{600}) were determined over time as described in the Methods section. The Figure shows the time course for (●) *cis*-2,3-epoxybutane production and (○) culture density in C2B cultures and (■) *trans*-2,3-epoxybutane production and (□) culture density in T2B cultures. The data plotted show the averages and ranges of values obtained from two separate cultures.

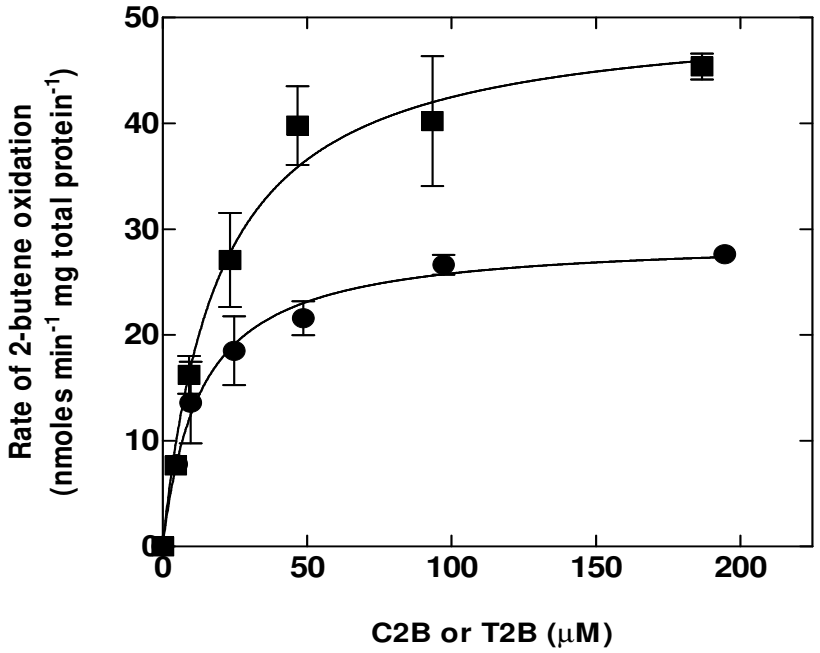


Figure 3-S2: Kinetics of C2B and T2B oxidation by strain ELW1. The figure shows the plots of the specific rates of (●) C2B oxidation and (■) T2B oxidation for cells (~0.4 mg total protein) of strain ELW1 grown on 2-methylpropene. A range of dissolve concentrations of C2B (0 to ~195 μM) and T2B (0 to ~190 μM) were incubated with 2-methylpropene-grown ELW1 cells and the consumption of C2B or T2B determined over time as described in the Methods section. The Figure shows the resulting curves when the results for each reaction were computer fitted to a single substrate-binding model described in the Methods section. The error bars represent the range of values obtained from two different cultures of ELW1 cells grown on 2-methylpropene.

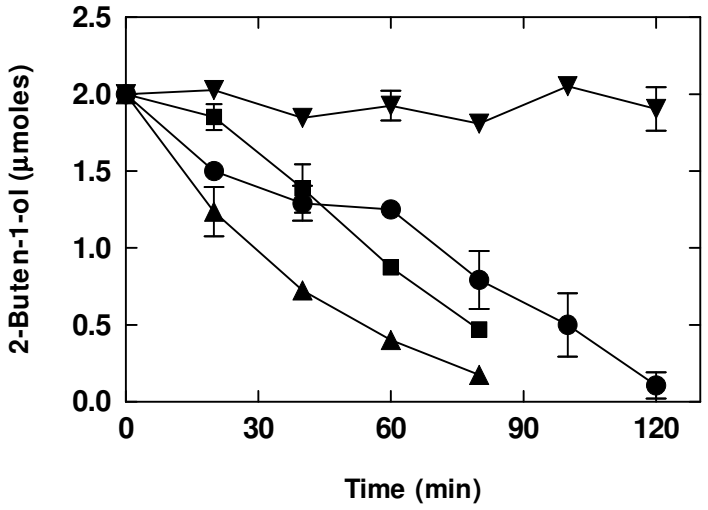


Figure 3-S3: Oxidation of 2-buten-1-ol by 2-methylpropene-, C2B-, and T2B-grown ELW1 cells. Relative rates of 2-buten-1-ol (2 µmoles) oxidation were determined using cells of strain ELW1 grown on 2-methylpropene, C2B and T2B (~1.6 total cell protein in each case). The figure shows the time course of degradation for 2-butene-1-ol consumption with cells grown on (▲) 2-methylpropene, (●) C2B, and (■) T2B. The figure also shows the time course of an (▼) abiotic reaction containing 2 µmoles of 2-buten-1-ol. Substrate consumption in each reaction was determined over time by GC as described in the Methods section. The error bars indicate the standard error of values obtained from two separate cultures.

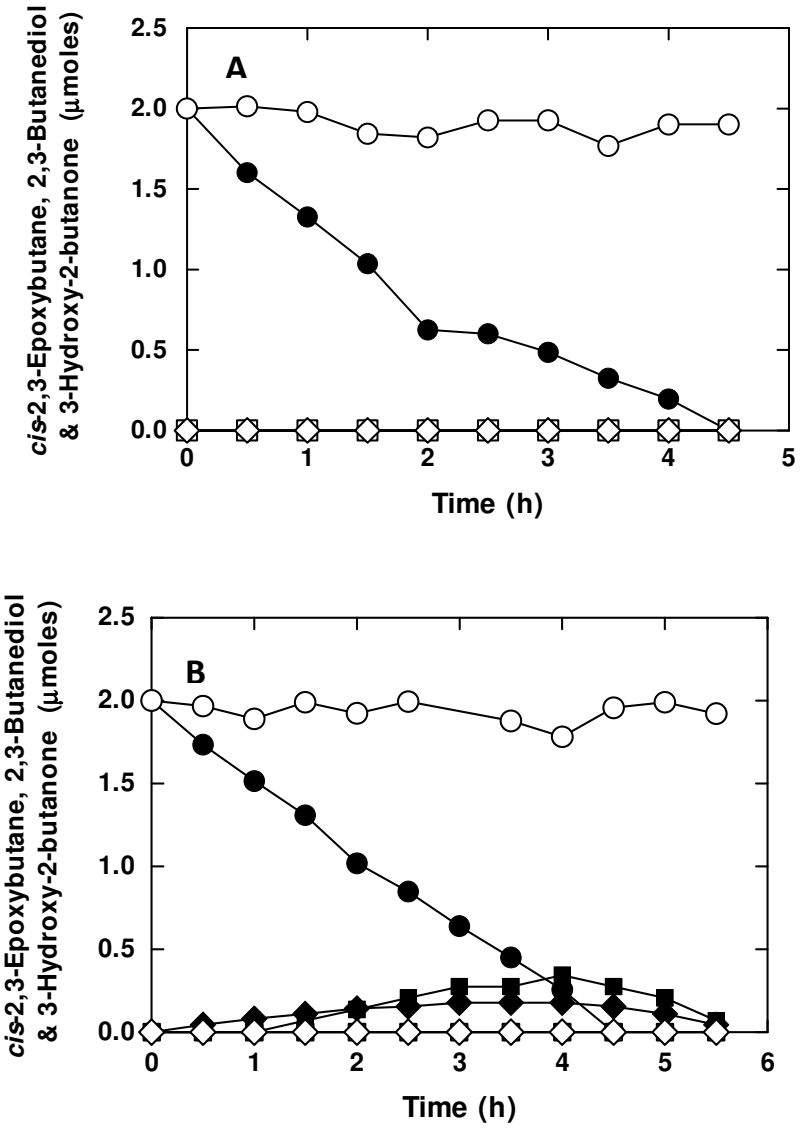


Figure 3-S4: ***cis*****-2,3-epoxybutane degradation by C2B- and 2-methylpropene-grown cells. Panel A:** C2B- (1.3 mg total protein) and **Panel B:** 2-methylpropene-grown cells (1.6 mg total protein) were incubated with racemic *trans*-2,3-epoxybutane (2 mM) in glass serum vials (10 ml) and the consumption of the epoxide and the production of metabolites was determined by GC analysis, as described in the Methods section. The symbols indicate (●,○) *cis*-2,3-epoxybutane, (■,□) 2,3-butanediol, and (◆,◇) 3-hydroxy-2-butanone. The closed symbols indicate the reaction with cells and the open symbols indicate the reaction without cells.

Table 3-1: Initial rates of alkene and epoxide consumption by 2-methylpropene-, C2B- and T2B-grown cells.

Substrate	Initial specific rate (nmoles min^{-1} mg $protein^{-1}$)					
	2-methylpropene-grown cells		C2B-grown cells		T2B-grown cells	
	- octyne	+ octyne	- octyne	+ octyne	- octyne	+ octyne
2-methylpropene	56.4 (6.7)	2.9 (0.8)	24.8 (3.2)	6.9 (2.5)	33.4 (4.2)	6.4 (1.1)
cis-2-butene	43.1 (5.0)	1.6 (0.5)	50.4 (10.2)	8.3 (2.1)	26.0 (2.1)	7.6 (4.6)
trans-2-butene	30.7 (1.5)	1.1 (0.5)	43.6 (4.9)	6.7 (5.1)	57.3 (8.7)	1.0 0.3)
1,2-epoxy-2-methylpropane	285.4 (19.4)	273.1 (20.5)	360.0 (28.7)	379.2 (47.0)	259.6 (0)	189.3 (0)
cis-2,3-epoxybutane	5.5 (0.7)	4.1 (0.5)	5.8 (0.8)	4.6 (0.3)	6.6 (1.7)	6.0 (3.6)
trans-2,3-epoxybutane	6.4 (1.2)	7.1 (0.6)	8.7 (0.6)	7.7 (0.5)	5.5 (0.3)	3.7 (0.3)

Resting cells of strain ELW1 grown on 2-methylpropene, C2B and T2B (0.3–1.5 mg of total protein) were incubated in buffer in reactions vials (10 ml) with varying amounts of alkenes (2-methylpropene, C2B, T2B) to the gas phase to generate an equal initial dissolved concentration (53 μM) of each alkene and their corresponding epoxides (2 mM), as described in the Methods section. Consumption of each alkene and epoxide was monitored over time by GC analysis of the reaction gas phase and aqueous phase, respectively. Data presented are the mean and SEM (in parentheses) of two separate cultures.

Table 3-2: Initial rates of oxidation of 2,3-butanediol stereoisomers and their putative metabolites by 2-methylpropene-, C2B-, and T2B-grown cells.

Substrate	Initial specific rate (nmoles min^{-1} mg $protein^{-1}$)[a]		
	2-methylpropene-grown cells	C2B-grown cells	T2B-grown cells
S,S-2,3-butanediol	4.7 (1.5)	9.4 (0.6)	33.1 (1.5)
R,R-2,3-butanediol	6.8 (1.1)	4.1 (0.2)	25.4 (0.6)
meso-2,3-butanediol	3.4 (0.7)	5.7 (0.2)	32.7 (1.6)
3-hydroxy-2-butanone	3.9 (0.0)	48.0 (4.9)	37.6 (6.8)
2-hydroxy-3-pentanone	0.7 (0.3)	6.5 (0.7)	8.0 (1.2)
acetone	0.4 (0.1)	0.4 (0.1)	0.6 (0.4)
acetate	20.9 (4.6)	51.2 (16.6)	71.9 (10.9)
acetaldehyde	45.3 (4.2)	465.8 (93.7)	273.4 (39.7)

Resting cells of strain ELW1 grown on 2-methylpropene, C2B and T2B (0.3–1.7 mg of total protein) were incubated in buffer in reactions vials (10 ml) with 2 mM 2,3-butanediol stereoisomers and their putative metabolites as described in the Methods section. Consumption of each substrate was monitored over time by GC analysis of the reaction aqueous phase. Data presented are the mean and SEM (in parentheses) of two separate cultures.

Table 3-3: Effects of colbalt ions on growth of strain ELW1 on C2B and T2B.

Substrate (+/- cobalt)	OD_{600} after 21d	OD_{600} after 28d	OD_{600} after 35d	OD_{600} after 40d
C2B + cobalt	0.93 (0.1)	1.08 (0.0)	0.98 (0.0)	0.94 (0.03)
C2B - cobalt	0.92 (0.0)	1.02 (0.0)	0.94 (0.0)	0.94 (0.01)
T2B + cobalt	0.18 (0.0)	0.56 (0.1)	0.88 (0.2)	0.80 (0.2)
T2B - cobalt	0.38 (0.1)	1.04 (0.0)	0.88 (0.0)	0.78 (0.1)

Cells of strain ELW1 (previously grown on 2-methylpropene) were incubated with C2B or T2B, in sealed glass serum vials (160 ml) containing either cobalt-sufficient (MSM) or cobalt-deficient (MSM-Co) media (25 ml), as described in the Methods section. Determination of culture density (OD_{600}) was started after 21d of incubation and continued up until 40d. Data represent the mean and SEM (in parentheses) of two separate cultures.

REFERENCES

1. **Fukuda H, Fujii T, Ogawa T.** 1984. Microbial production of C3-and C4-hydrocarbons under aerobic conditions. Agric. Biol. Chem. **48:**1679-1682.

2. **Texas Commission on Environmental Quality (TCEQ)**. 2-Butene (*Cis* and *Trans*); CAS Registry Number: 107-01-7. Development Support Document; Final, April 15, 2008. (http://www.tceq.texas.gov/assets/public/implementation/tox/dsd/final/butene,_2-_107-01-7_final_4-15-08.pdf)

3. **Hyman MR, Murton IB, Arp DJ.** 1988. Interaction of ammonia monooxygenase from *Nitrosomonas europaea* with alkanes, alkenes, and alkynes. Appl. Environ. Microbiol. **54:**3187-3190.

4. **Higgins I, Hammond R, Sariaslani F, Best D, Davies M, Tryhorn S, Taylor F.** 1979. Biotransformation of hydrocarbons and related compounds by whole organism suspensions of methane-grown *Methylosinus trichosporium* OB3b. Biochem. Biophys. Res. Commun. **89:**671-677.

5. **Patel RN, Hou CT, Laskin AI, Felix A.** 1982. Microbial oxidation of hydrocarbons: properties of a soluble methane monooxygenase from a facultative methane-utilizing organism, *Methylobacterium* sp. strain CRL-26. Appl. Environ. Microbiol. **44:**1130-1137.

6. **Subramanian V.** 1986. Oxidation of propene and 1-butene by *Methylococcus capsulatus* and *Methylosinus trichosporium*. J. Ind. Microbiol. **1:**119-127.

7. **Colby J, Stirling DI, Dalton H.** 1977. The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath). Its ability to oxygenate *n*-alkanes, *n*-alkenes, ethers, and alicyclic, aromatic and heterocyclic compounds. Biochem. J. **165:**395-402.

8. **Green J, Dalton H.** 1989. Substrate specificity of soluble methane monooxygenase. J. Biol. Chem. **264:** 17698-17703.

9. **Smith DDS, Dalton H.** 1989. Solubilisation of methane monooxygenase from *Methylococcus capsulatus* (Bath). Eur. J. Biochem. **182:**667-671.

10. **McClay K, Fox BG, Steffan RJ.** 2000. Toluene monooxygenase-catalyzed epoxidation of alkenes. Appl. Environ. Microbiol. **66:**1877-1882.

11. **Hou CT, Patel R, Laskin AI, Barnabe N, Barist I.** 1983. Epoxidation of short-chain alkenes by resting-cell suspensions of propane-grown bacteria. Appl. Environ. Microbiol. **46:**171-177.

12. **Peter S, Kinne M, Ullrich R, Kayser G, Hofrichter M.** 2013. Epoxidation of linear, branched and cyclic alkenes catalyzed by unspecific peroxygenase. Enzyme Microb. Technol. **52:**370-376

13. **van Ginkel CG, Welten HG, de Bont JAM.** 1987. Oxidation of gaseous and volatile hydrocarbons by selected alkene-utilizing bacteria. Appl. Environ. Microbiol. **53:**2903-2907.

14. **Owens CR, Karceski JK, Mattes TE.** 2009. Gaseous alkene biotransformation and enantioselective epoxyalkane formation by *Nocardioides* sp. strain JS614. Appl Microbiol Biotechnol. **84:**685-692.

15. **Mahmoudian M, Michael A.** 1992. Production of chiral epoxides by an ethene-utilising *Micrococcus* sp. J. Ind. Microbiol. **11:**29-35.

16. **van Ginkel CG, Welten HG, de Bont JAM.** 1986. Epoxidation of alkenes by alkene-grown *Xanthobacter* spp. Appl. Microbiol. Biotechnol. **24:**334-337.

17. **van Ginkel CG, Welten HG, Hartmans S, de Bont JAM.** 1987. Metabolism of trans-2-butene and butane in *Nocardia* TBl. J. Gen. Microbiol. **133:**1713-1720.

18. **Weijers CAGM, van Ginkel CG, De Bont JAM.** 1988. Enantiomeric composition of lower epoxyalkanes produced by methane-, alkane-, and alkene-utilizing bacteria. Enzyme Microb. Technol. **10:**214-218.

19. **Fujii T, Ogawa T, Fukuda H.** 1985. A screening system for microbes which produce olefin hydrocarbons. Agric. Biol. Chem. **49:**651-657.

20. **Kottegoda S, Waligora E, Hyman M.** Isolation and characterization of a novel 2-methylpropene-metabolizing strain, *Mycobacterium* sp. ELW1. To be submitted for publication in *Applied and Environmental Microbiology* (manuscript in preparation).

21. **Gornall AG, Bardawill CJ, David MM.** 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177:**751-766.

22. **Weijers CAGM, de Haan A, de Bont JAM.** 1988. Chiral resolution of 2, 3-epoxyalkanes by *Xanthobacter* Py2. Appl. Microbiol. Biotechnol. **27:**337-340.

23. **Allen JR, Ensign SA.** 1997. Characterization of three protein components required for functional reconstitution of the epoxide carboxylase multienzyme complex from *Xanthobacter* strain Py2. J. Bacteriol. **179:**3110-3115.

24. **Huang M, Oppermann FB, Steinbüchel A.** 1994. Molecular characterization of the *Pseudomonas putida* 2, 3-butanediol catabolic pathway. FEMS Microbiol. Lett. **124:**141-150.

25. **López JM, Thomas B, Rehbein H.** 1975. Acetoin degradation in *Bacillus subtilis* by direct oxidative cleavage. Eur. J. Biochem. **57:**425-430.

26. **Shennan JL.** 2006. Utilisation of C2–C4 gaseous hydrocarbons and isoprene by microorganisms. J. Chem. Technol. Biotechnol. **81:**237-256.

27. **Zhou N, Jenkins A, Chion, Chan K. N. Chan Kwo, Leak DJ.** 1999. The alkene monooxygenase from *Xanthobacter* strain py2 is closely related to aromatic monooxygenases and catalyzes aromatic monohydroxylation of benzene, toluene, and phenol. Appl. Environ. Microbiol. **65:**1589-1595.

28. **Weijers CAGM, de Bont JAM.** 1991. Enantioselective degradation of 1, 2-epoxyalkanes by *Nocardia* H8. Enzyme Microb. Technol. **13:**306-308.

29. **Habets-Crützen A, Carlier S, de Bont J, Wistuba D, Schurig V, Hartmans S, Tramper J.** 1985. Stereospecific formation of 1, 2-epoxypropane, 1, 2-epoxybutane and 1-chloro-2, 3-epoxypropane by alkene-utilizing bacteria. Enzyme Microb. Technol. **7:**17-21.

30. **Clark DD, Boyd JM, Ensign SA.** 2004. The stereoselectivity and catalytic properties of *Xanthobacter autotrophicus* 2-[(R)-2-hydroxypropylthio]ethanesulfonate dehydrogenase are controlled by interactions between C-terminal arginine residues and the sulfonate of coenzyme M. Biochemistry **43:**6763-6771.

31. **Wistuba D, Nowotny H, Träger O, Schurig V.** 1989. Cytochrome P-450-catalyzed asymmetric epoxidation of simple prochiral and chiral aliphatic alkenes: species dependence and effect of enzyme induction on enantioselective oxirane formation. Chirality. **1:**127-136.

32. **Wistuba D, Schurig V.** 1986. Complementary epoxide hydrolase vs glutathione s-transferase-catalyzed kinetic resolution of simple aliphatic oxiranes–complete regio-and enantioselective hydrolysis of *cis*-2-ethyl-3-methyloxirane. Angew. Chem., Int. Ed. Engl. **25:**1032-1034.

33. **Weijers CAGM.** 1997. Enantioselective hydrolysis of aryl, alicyclic and aliphatic epoxides by *Rhodotorula glutinis*. Tetrahedron: Asymmetry. **8:**639-647.

34. **Orru RV, Faber K.** 1999. Stereoselectivities of microbial epoxide hydrolases. Curr. Opin. Chem. Biol. **3:**16-21.

35. **van Loo B, Kingma J, Heyman G, Wittenaar A, Lutje Spelberg JH, Sonke T, Janssen DB.** 2009. Improved enantioselective conversion of styrene epoxides and *meso*-epoxides through epoxide hydrolases with a mutated nucleophile-flanking residue. Enzyme Microb. Technol. **44:**145-153.

36. **Osprian I, Kroutil W, Mischitz M, Faber K.** 1997. Biocatalytic resolution of 2-methyl-2-(aryl) alkyloxiranes using novel bacterial epoxide hydrolases. Tetrahedron: Asymmetry. **8:**65-71.

37. **Osprian I, Stampfer W, Faber K.** 2000. Selectivity enhancement of epoxide hydrolase catalyzed resolution of 2, 2-disubstituted oxiranes by substrate modification. J. Chem. Soc. Perkin Trans.1. 3779-3785.

38. **Steinreiber A, Osprian I, Mayer SF, Orru RV, Faber K.** 2000. Enantioselective hydrolysis of functionalized 2, 2-disubstituted oxiranes with bacterial epoxide hydrolases. Eur. J. Org. Chem. **2000:**3703-3711.

39. **Funar-Timofei S, Suzuki T, Paier JA, Steinreiber A, Faber K, Fabian WM.** 2003. Quantitative structure-activity relationships for the enantioselectivity of oxirane ring-opening catalyzed by epoxide hydrolases. J. Chem. Inf. Comput. Sci. **43:**934-940.

40. **Paier J, Stockner T, Steinreiber A, Faber K, Fabian WM.** 2003. Enantioselectivity of epoxide hydrolase catalysed oxirane ring opening: a 3D QSAR Study. J. Comput. Aided Mol. Des. **17:**1-11.

CHAPTER 4

Alkane-oxidizing activity of *Mycobacterium* sp. ELW1 and *Xanthobacter* sp. Py2

Samanthi Kottegoda, Chrisy Smith and Michael Hyman*

Department of Plant and Microbial Biology
North Carolina State University
Raleigh, NC 27695

*Corresponding author
Contact Information
Dr. Michael Hyman
Department of Plant and Microbial Biology
4545 Thomas Hall
North Carolina State University
Raleigh, NC 27695
Phone: 919-515-78145
Fax: 919-515-7867
E-mail: mrhyman@ncsu.edu

Prepared for submission to *FEMS Microbiology Letters.*

ABSTRACT

Alkane-oxidizing activities of the newly isolated 2-methylpropene metabolizing strain *Mycobacterium* sp. ELW1 and a well-characterized alkene-oxidizing bacterium, *Xanthobacter* sp. Py2, were investigated. Neither smaller branched alkanes (C_1-C_6) nor 2-methylpropane supported growth of strain ELW1. Resting cells of 2-methylpropene-grown strain ELW1 consumed ethane, propane, and *n*-butane without a lag phase and 2-propanol and 2-butanol were detected as the only products of propane- and *n*-butane-oxidation, respectively. But it did not oxidize methane. Propane and *n*-butane were also degraded rapidly by propene-grown strain Py2 and did not consume either methane or ethane. 2-Methylpropene-grown cells of strain ELW1 also oxidized 2-methylpropane and generated ~70% *tertiary* butyl alcohol (TBA), the sub-terminal oxidation product of 2-methylpropene oxidation, as detected product while 2-methyl-1-propanol (2M1P), the terminal oxidation product, was not detected. Moreover, strain ELW1 did not oxidize TBA, but rapidly oxidized 2M1P. Oxidation of 2-methylpropane was fully inhibited by 1-octyne. We observed the same results with propene-grown Py2 cells and the oxidation of 2-methylpropane in Py2 cells was fully inhibited by 1-propyne. Several lines of evidence suggest that strain ELW1 possesses an alkene monooxygenase that has a broad substrate-specificity and is responsible for initiating both alkene- and alkane-metabolism. First, 2-methylpropene-grown cells of strain ELW1 degraded alkanes rapidly without a lag phase. Second, alkane oxidation was completely inhibited in ELW1 cells pretreated with 1-octyne, the potent inhibitor of 2-methylpropene oxidation. Third, 2-methylpropene acts as a competitive inhibitor of 2-methylpropane oxidation.

INTRODUCTION

The biological formation of epoxides from aliphatic alkenes has been studied in many aerobic microorganisms and many of these studies involved alkane-utilizing bacteria. For example, methane-utilizing bacteria (19, 21, 34), propane-and butane-utilizing bacteria (20, 32), heptane-utilizing *Pseudomonas* (35), octane-utilizing *Pseudomonas oleovorans* (1, 10, 11) and octane-utilizing *Corynebacterium* sp. (5) are all known to oxidize 1-alkenes. Although alkane-utilizing bacteria are generally not able to metabolize and grow on alkenes, they nevertheless are able to oxidize these compounds either by the hydroxylation of a terminal methyl group to form an alcohol or more commonly, by the epoxidation of the double bond to form an epoxide. The epoxidation of alkenes by these alkane-grown bacteria is due to the activity of non-specific alkane-oxidizing monooxygenases.

The formation of epoxides has also been studied with many aerobic alkene-utilizing bacteria including strains isolated on ethene (9, 18), propene and 1-butene (6, 8, 36), and 1,3-butadiene (39). Alkene-utilizing bacteria also possess alkene-oxidizing monooxygenases with broad substrate specificity, but enzymes from these organisms typically do not hydroxylate either alkenes or alkanes and are generally thought to be restricted to alkene epoxidation reactions (17, 38). The well characterized propene-metabolizing strain, *Xanthobacter autotrophicus* Py2 can grow on several 1-alkenes (C_2-C_6) and can cometabolically oxidize a number of chlorinated ethenes including trichloroethene, vinyl chloride and *cis*- and *trans*-1,2-dichloroethene. All of the initial monooxygenase-catalyzed reactions involving these substrates are thought to involve epoxidation rather than hydroxylation reactions. Propene-grown cells of strain Py2 can also oxidize benzene, toluene

and phenol (40) but this strain has not been reported to oxidize alkanes. A small number of alkene-metabolizing bacteria have also been shown to have both alkene- and alkane-metabolizing activity. These strains include *Mycobacterium* E20 (8), *Nocardia* TB1 (37) and *Rhodococcus rhodocrous* B276 (14). In strain E20 and B276 the ability of these organisms to metabolize alkanes has been attributed to alkane-oxidizing monooxygenases that are distinct from of the alkene-oxidizing enzymes used to initiate alkene catabolism. In the case of strain TB1, the ability of this organism to metabolize 2-butene is attributed to the terminal oxidation of *trans*-2-butene by an *n*-butane-oxidizing alkane monooxygenase.

In this study, we have explored the ability of the newly isolated 2-methylpropene-metabolizing strain *Mycobacterium* ELW1 to oxidize alkanes. This organism does not grow on 1-alkenes (C_2-C_4) and only grows on 2-methylpropene and both 2-butene isomers. This strain also does not grow on any of the short-chain n-alkanes (C_2-C_6) but grew readily on 1-heptane and all other longer-chain alkanes (C_8, C_{10}, C_{12}, C_{14}, C_{16} and C_{18}) tested (26). In this previous study we also proposed that the metabolism of 2-methylpropene involves several steps that are very similar to those involved in the aerobic biodegradation of methyl *tertiary* butyl ether (MTBE) and its primary metabolite, *tertiary* butyl alcohol (TBA). The pathway of TBA metabolism involves the initial monooxygenase-catalyzed oxidation to 2-methyl-1,2-propanediol (MPD) and further oxidation of this diol to 2-hydroxyisobutyrate (HIBA). This hydroxyacid is then transformed to 3-hydroxybutyrate (3HB) through the activity of a cobalamin-dependent mutase. In the case of 2-methylpropene metabolism by strain ELW1, the same sequence of reactions is believed to occur except that MPD is initially generated as

a hydrolysis product derived from 1,2-epoxy-2-methylpropane, the immediate epoxide product generated from 2-methylpropene oxidation.

In view of the enzymatic overlap between MTBE/TBA metabolism and 2-methylpropene metabolism in strain ELW1, we were interested to characterize the ability of this strain to oxidize 2-methylpropane, the alkane analog of 2-methylpropene. This investigation was prompted by the fact that several aerobic alkane-oxidizing bacteria have been shown to oxidize 2-methylpropane to mixtures of 2M1P and TBA and the further oxidation of TBA to MPD has also been shown to be catalyzed by several alkane-oxidizing monooxygenases. The results of this study demonstrate that 2-methylpropene-grown cells rapidly oxidize 2-methylpropane to mixtures of TBA and 2M1P and that this activity can be attributed to the alkene-oxidizing monooxygenase present in alkene-grown cells. Our results also demonstrate that this alkane-oxidizing activity extends to several *n*-alkanes and that both 2-methylpropane and propane-oxidizing activity can also be observed with propene-grown *X. autotrophicus* Py2, a strain that has no alternative *n*-alkane-oxidizing enzyme encoded in its genome. Our results have been interpreted in terms of their impact on our understanding of the substrate ranges of alkene-oxidizing monoooxygenases.

MATERIALS AND METHODS

Materials: *Mycobacterium* strain ELW1 was isolated and maintained as previously described (26). *Xanthobacter* strain Py2 (ATCC BAA-1158) was obtained from the American Type Culture Collection (Manassas, VA.) and maintained on casein-yeast extract-dextrose (CYD) agar plates (Difco Plate Count Agar; Becton, Dickinson and Co., Sparks, MD). *n*-butane (99% purity), 1-butanol (99% purity), 2-butanol (99.5% purity), *cis*-2-butene (>99% purity), *trans*-2-butene (>99% purity), fructose (>99% purity), glucose (>99.5% purity), 2-methylpropane (99% purity), 2-methyl-1-propanol (99.5% purity), 2-methyl-2-propanol [*tertiary* butyl alcohol] (99.5% purity), 2-methylpropene (99% purity), 2-methylpropionic acid (>99% purity), 1-octyne (97% purity), 1-propanol (99.9% purity), 2-propanol (99.5% purity), propyne (98% purity) and sodium propionate (99% purity) were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI). Acetone (99.5% purity), methanol (99% purity) and sodium acetate (99.5% purity) were obtained from Fisher Scientific (Pittsburgh, PA). Absolute ethanol was obtained from Aaper Alcohol and Chemical Co., (Shelbyville, KY). Ethane was supplied by Scott Specialty Gases (Plumsteadville, PA). Methane (CP grade), propane (research grade), propene (CP grade) and compressed gases (H_2, N_2, and air) used for gas chromatography were obtained from local industrial vendors.

Cell growth and preparation of harvested cells for short-term experiments: Cells for all of the short-term experiments in this study were grown in batch cultures using glass bottles (700 ml; Wheaton Scientific, Millville, NJ) containing 100 ml of mineral salt medium

(MSM) (26) and sealed with screw-caps fitted with butyl rubber septa. 10% (v/v) gas phase of either 2-methylpropene (strain ELW1) or propene (strain Py2) were used as the sole source of carbon and energy. The bottles were inoculated (initial OD_{600} ~0.02) with cells obtained from axenic cultures previously grown on MSM plates with 10% 2-methylpropene (strain ELW1) or on casein-yeast extract-dextrose (CYD) agar plates (Difco Plate Count Agar) (strain Py2). 2-Methylpropene or propene (60 ml) was added to the sealed bottles as an overpressure by using plastic syringes fitted with sterile filters (0.25 µm). Cultures were incubated in the dark at 30°C for 4-6 days in an Innova 4990 environmental shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) operated at 150 rpm. After incubation, a sample (~20 µl) of the culture was plated on CYD plates to subsequently confirm the purity. Cells were harvested by centrifugation (10,000 x g, 5 min at 4°C) and the sedimented cells were resuspended in buffer (50 mM sodium phosphate, pH 7.0). The cells were centrifuged again and resuspended in buffer as described above at a final protein concentration of between 10 and 20 mg/ml. Cell suspensions were stored on ice and used within 4 h.

In experiments requiring cell-inactivation, harvested cells were split into two and one half of cells were incubated with a potent inhibitor while the other half was incubated without an inhibitor. 1-Octyne was used to inactivate strain ELW1 while 1-propyne was used to inactivate strain Py2. Incubations were conducted in sealed serum vials (25 ml) containing ~6.5 ml of phosphate buffer and 90 µmoles of either 1-octyne (dissolved concentration; ~1.5 µmoles) or 1-propyne (10% v/v gas phase) for 1 hr at 30ºC with shaking prior to the experiment. After incubation, the cells were harvested, washed twice and resuspended in

phosphate buffer as described above. Cell suspensions were stored at 4°C and used within 4 h.

Growth curves: The effect of 2-methylpropane on growth of strain ELW1 on 2-methylpropene was examined using glass serum vials (160 ml; Wheaton Scientific, Millville, NJ) that contained 25 ml of MSM. Vials were inoculated (initial OD_{600} ~ 0.02) with a liquid suspension of cells grown on MSM agar plates supplemented with 2-methylpropene and then sealed with Teflon-lined mininert valves (Alltech Associates Inc., Deerfield, IL). The cells were grown under limited-carbon conditions using 2.5 ml of 2-methylpropane and/or 2.5 ml of 2-methylpropene as the sole source of carbon and energy. Gases were added to sealed vials using plastic syringes fitted with Millex disposable filters (0.1 µm; Millipore Co., Bedford, MA). Four different reactions were set up in triplicates as follows (i) abiotic; containing 2.5 ml 2-methylpropene only (ii) biotic; containing cells plus 2.5 ml 2-methylpropene (iii) biotic; containing cells plus 2.5 ml 2-methylpropane (iv) biotic; containing cells plus 2.5 ml of 2-methylpropene and 2.5 ml of 2-methylpropane. The cultures were incubated in the dark at 30°C in an Innova 4900 environmental shaker (New Brunswick Scientific Co., Inc., Edison, NJ) operated at 150 rpm. Samples (~0.6 ml) were aseptically withdrawn from sealed vials throughout the experiment to monitor cell growth, as determined by measurements of optical density at 600 nm (OD_{600}) with a Shimadzu 1601 UV/Vis spectrophotometer (Shimadzu, Kyoto, Japan). Consumption of substrate(s) (2-methylpropene and /or 2-methylpropane) and generation of products (TBA and 2M1P) were determined by gas phase- and liquid phase-GC analysis respectively, as described below.

Degradation assays: All degradation assays and kinetic constant determinations performed in this study were carried out in glass serum vials (10 ml) containing ~900 ml buffer (50 mM sodium phosphate, pH 7.0). The vials were sealed with butyl rubber stoppers and aluminum crimp seals. Substrates were then added from aqueous stock solutions or gases using microsyringes. In all experiments, the reaction vials were prepared immediately before use and then incubated for ~10 min in a shaking water bath (30°C; 150 rpm) to allow reactants to equilibrate between gas and liquid phases. Reactions were initiated by the addition of an aliquot (100 µl) of the concentrated cell suspension to give a final reaction volume of 1 ml and a protein concentration of between ~1.0 to 2.0 mg of total protein. Then the reactions were returned to the shaking water bath and were sampled at the times indicated in each experiment. In short-term degradation assays, substrate consumption or product formation was determined by gas chromatography (GC) using liquid samples (2 µl) or headspace samples (10 µl) taken directly from the reaction vials. In some experiments 1-octyne-treated cells were used as controls to determine enzyme inactivation while in most cases an abiotic reaction was conducted to detect abiotic loses.

O_2 uptake rate determination: Substrate-specific rates of O_2 uptake for cells grown on 2-methylpropene were measured using a Clark -style O_2 electrode (Hansatech Instruments Ltd, Norfolk, England). The elecrtrode was mounted in a glass water-jacketed reaction vessel maintained at 30°C. In all assays, phosphate buffer (2 ml; 50 mM sodium phosphate; pH 7.0) was added to the reaction chamber and allowed to equilibrate. Once the electrode output had stabilized, an aliquot (50 µl) of concentrated cell-suspension was added and the endogenous

basal rate of O_2 uptake was initially determined for 3-5 min in the absence of exogenous substrates. Substrates were then added at an initial concentration of 5 mM for liquids and 300 μM of dissolved concentration for gases, into the O_2 electrode chamber in which the buffer and cells had been pre-equilibrate for ~5 min. The substrate-dependent O_2 uptake rates were determined by subtracting the basal rate of cellular respiration from substrate-induced respiration rates.

Analytical methods: In all experiments, the concentrations of reactants and products were determined by GC analysis. For the analysis of 1° and 2° alcohols, aqueous samples (2 μl) of reaction media or calibration standards were directly injected into a gas chromatograph (Shimadzu model GC-14A, Kyoto, Japan) fitted with a flame ionization detector and a stainless steel column filled with Porapak Q (80/100 mesh) (Waters Associates, Framingham, MA). Two different columns; short (0.3 x 61 cm) and long (0.3 x 183 cm), conducted at a temperature of 90 – 140 °C, were used to analyze liquid-phase samples. The injector and detector temperatures were set at 200ºC and 220°C respectively. Nitrogen was used as carrier gas at a flow rate of 15 ml/min. In experiments that followed the consumption of gaseous substrates, samples (10 μl) were removed by using gas-tight syringes with dry heat-treated needles (~1 min at 350°C). 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 (internal diameter), 2.55-μm film; J & W Scientific, Folsom, CA]. The analysis was conducted by 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 the carrier gas at a flow

rate of 5 ml/min. Substrate consumption or product formation was determined by peak area quantification with an HP3395 integrator (Palo Alto, CA) and then by comparing them to standard curves constructed from known amounts of the authentic compounds.

Cell protein concentrations were determined using the Biuret assay (15) after solubilization of cell material for 1 hr at 65°C in 3M NaOH and sedimentation of insoluble material by centrifugation (10,000 rpm, 5 min). Bovine serum albumin was used as a standard. The aqueous solubilities of methane, ethane, propane, and *n*-butane at 1 atm at 30°C were taken as 1.41, 1.81, 1.44, and 1.01 mM, respectively (28). The aqueous solubility of 2-methylpropane and 2-methylpropene at 1 atm and 30°C were taken as 0.92 and 4.76 mM respectively (28). Kinetic constants (V_{max} and K_s) were derived by computer fitting of the data by nonlinear regression to a single substrate-binding model [$Y = V_{max} \cdot X / (K_s + X)$] using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Oxidation of 2-methylpropane and its metabolites by strain ELW1: When resting, 2-methylpropene-grown cells of strain ELW1 were incubated with 2-methylpropane (4.5 μmoles), the gas was consumed and TBA was accumulated in the reaction medium as the only detected product (Fig. 4-1). The rates of 2-methylpropane consumption and TBA production were essentially constant throughout the reaction and the amount of TBA generated was equivalent to ~70% of the 2-methylpropane consumed. The oxidation of 2-methylpropane and accumulation of TBA were both fully inhibited in cells that had been pretreated with 1-octyne to inactivate alkene monooxygense activity. Cells incubated with TBA alone did not consume TBA while cells incubated with 2M1P rapidly consumed this compound. No abiotic losses of either TBA or 2M1P were observed when these compounds were incubated in the absence of cells.

The oxidation of 2-methylpropane to TBA was also examined for resting cells grown on a variety of other substrates (Table 4-1). The initial rate of TBA production by cells grown 2-methylpropene was ~8 nmoles min^{-1} mg total $protein^{-1}$ while the cells grown on *cis*- and *trans*-2-butene exhibited slower but similar rates (~3 nmoles min^{-1} mg total $protein^{-1}$). In contrast, low rates (< 0.5 nmoles min^{-1} mg total $protein^{-1}$) were observed with cells grown on 1° alcohols (ethanol, 1-propanol and 1-butanol), and acids (acetate, propionate and 2-methylpropionic acid). Glucose-grown cells exhibited slow 2-methylpropane oxidizing activity (~1.9 nmoles min^{-1} mg total $protein^{-1}$) while there was no detectable activity with fructose-grown cells. In all cases, when 2-methylpropane-oxidation was observed, this activity was fully inhibited when cells were pretreated with 1-octyne.

Kinetics of 2-methylpropane oxidation by ELW1: The results present in Fig. 4-1 suggest 2-methylpropene-grown cells oxidized 2-methylpropane to predominantly TBA through the activity of alkene monooxygenase. There was no evidence that TBA underwent further oxidation. In contrast, the other likely hydroxylation product, 2M1P, was not detected and our results suggest that this product could have been rapidly degraded during 2-methylpropane oxidation. To further characterize the kinetics of this reaction we used the stable accumulation of TBA to determine the K_s and V_{max} values for or 2-methylpropane oxidation by 2-methylpropene-grown cells. The initial rates of TBA production were determined for a range of 2-methylpropane concentrations (0 - 250 μM). The rate of 2-methylpropane oxidation to TBA was saturable and fitted well (R^2 = 0.96) to a hyperbolic single substrate-binding model. This analysis provided an estimate for the K_s for 2-methylpropane of 19.2 μM (SE = 3.5 μM) and a V_{max} value of 6.5 (SE = 0.32) nmoles min^{-1} mg of $protein^{-1}$ for 2-methylpropane oxidation (Fig. 4-2). We also used the stability of TBA to investigate whether 2-methylpropane and 2-methylpropene were mutually competitive substrates. 2-methylpropene-grown cells were incubated with a fixed concentration of 2-methylpropane and varying concentrations of 2-methylpropene. The effect of 2-methylpropene on TBA production was then determined by GC analysis of the reaction medium. Our results (Fig. 4-3) demonstrate that 2-methylpropene was a potent inhibitor of TBA production. The effects of 2-methylpropene were saturable and fitted well (R^2 = 0.95) to a single substrate-binding model well.

Effects of 2-methylpropane on growth of ELW1 on 2-methylpropene: Strain ELW1 does not grow on either 2-methylpropane or TBA but grows readily grows readily on 2M1P (26). As oxidation of 2-methylpropane by 2-methylpropene-grown cells potentially generates appreciable quantities of 2M1P, we examined whether the cooxidation of 2-methylpropane during growth of strain ELW1 on 2-methylpropene resulted in a benefit to in terms of increased culture growth and biomass production resulting from 2M1P production and consumption. Three different carbon-limited cultures were prepared that contained 2-methylpropene alone, 2-methylpropane alone, or 2-methylpropane plus 2-methylpropene. All three cultures were inoculated with 2-methylpropene-grown cells. A fourth uninoculated control culture was also prepared that contained 2-methylpropene plus 2-methylpropane. All four cultures were incubated and regularly analyzed for culture growth. The consumption of gaseous substrates and production of TBA and 2M1P were also determined by GC analysis. In the cultures that contained 2-methylpropane alone, there was no growth or production of TBA or 2M1P over the entire culture period (14 d). The consumption of 2-methylpropane in these cultures was equivalent to the losses observed in the uninoculated control culture. In the cultures that contained 2-methylpropene alone, consumption of this gas was completed within 6 d and was accompanied by cell growth that ceased once the alkene had been consumed. As expected, there was no production of TBA or 2M1P over the entire culture period. A very similar pattern of 2-methylpropene consumption and culture growth was also observed for the cultures that contained 2-methylpropene plus 2-methylpropane. In these cultures, 2-methylpropane consumption was only initiated once the majority of the 2-methylpropene had been consumed. The time course of 2-methylpropane consumption was

reflected by a similar time course of TBA accumulation. 2M1P was not detected in these cultures and no increase in culture density was observed compared to cultures grown with 2-methylpropene alone (Fig. 4-4).

Oxidation of *n*-alkanes and alcohols by strain ELW1*:* We also examined the ability of 2-methylpropene-grown cells to oxidize other short-chain alkanes. In one experiment, 2-methylpropene-grown cells were incubated with an equal dissolved concentration of methane, ethane, propane, *n*-butane and 2-methylpropane. The consumption of each alkane and the accumulation of oxidation products were determined by GC analysis. Methane consumption was not observed in these reactions but slow consumption of ethane, propane, *n*-butane and 2-methylpropane was observed. During these reactions 2-propanol, 2-butanol and TBA were detected as products of propane, *n*-butane and 2-methylpropane oxidation respectively while no product was observed in the reactions containing ethane (Fig. 4-5). We subsequently examined the relative rates of 1^{o} and 2^{o} alcohol degradation by 2-methylpropene-grown cells. With the exception of methanol and ethanol, the corresponding 1^{o} alcohols (1-propanol and 1-butanol) were more rapidly consumed than their corresponding 2° alcohols (2-propanol and 2-butanol) (Fig. 4-S1). As reported in an earlier experiment, 2M1P was rapidly degraded while TBA was not consumed. The abiotic losses of each alcohol were also examined and all alcohols were stable over the entire reaction time course (data not shown). The results of these time course studies (Fig. 4-S1) were also reflected in substrate-specific rates of O_2 uptake measured for 2-methylpropene-grown cells (Table 4-2). Cells grown on 2-methylpropene, exhibited high rates of O_2 uptake (28 to 73 nmoles min^{-1}

mg total protein^{-1}) with several 1° alcohols (1-propanol, 1-butanol, and 2M1P). In contrast, methanol, ethanol and both 2-propanol and 2-butanol had little or no effect on O_2 uptake rates. None of the alkanes other than 2-methylpropane stimulated O_2 uptake and in all cases but one, the rates of O_2 uptake measured for cells exposed to alkanes and their potential or detected metabolites were substantially lower than the rates observed for 2-methylpropane and its metabolites.

Oxidation of 2-methylpropane and propane by *Xanthobacter* Py2: The results presented to this point suggest that alkene-grown cells of strain ELW1 can oxidize several alkanes, albeit at slow rates. As strain ELW1 can also grow on alkanes it is possible that the low rates of alkane oxidation we observed were due to low levels of activity of an alkane-oxidizing monooxygenase. We therefore examined whether similar alkane-oxidizing activities could be observed in propene-grown cells of *X. autotrophicus* Py2. The genome of this organism does not encode an alkane-oxidizing monooxyganase and the organism has not been reported to grow on any alkanes. Propene-grown cells of strain Py2 were incubated with 2-methylpropane under the same conditions described earlier for strain ELW1 (see Fig. 4-1). The results of this experiment (Fig. 4-6) demonstrate that 2-methylpropane was steadily oxidized throughout the reaction time course (~3.4 nmoles min^{-1} mg total protein^{-1}) and was accompanied by accumulation of TBA (~2.5 nmoles min^{-1} mg total protein^{-1}). Neither of these processes was observed in cells that had been previously treated with 1-propyne to inactivate alkene monooxygenase activity. Similar to strain ELW1, TBA was not consumed by propene-grown cells of strain Py2 when it was added as a sole substrate rather than as a

product of 2-methylpropane oxidation. Although not detected as a product, 2M1P was rapidly oxidized by propene-grown Py2 cells (not shown).

In a subsequent experiment we examined whether strain Py2 could also oxidize propane, the alkane analog of propene. When propene-grown cells were incubated with propane a slow degradation of the gas was observed but no oxidation products accumulated in the reaction medium (Fig. 4-7A). Like 2-methylpropane oxidation, the consumption of propane was fully inhibited in cells pretreated with propyne. To further characterize the possible fates of 1- and 2-propanol as likely propane oxidation products we examined the degradation of these compounds by propene-grown cells of strain Py2. Propene-grown cells rapidly oxidized both 1- and 2-propanol (26.5 and 49.8 nmoles min^{-1} mg total $protein^{-1}$, respectively). 2-Propanol was stoichiometrically converted to acetone which was not oxidized further (Fig. 4-7B).

DISCUSSION

In this study we examined *Mycobacterium* strain ELW1 and the well-characterized alkene-oxidizing bacterium strain Py2, for their abilities to oxidize several gaseous alkanes. Our results indicate both bacteria slowly oxidize 2-methylpropane and generate TBA as a substoichiometric product of this reaction. Our results suggest that in both cases these reactions are catalyzed by the alkene monoooxygenases that are normally responsible for the epoxidation reaction used to initiate alkene metabolism. Our results also suggest that both organisms can also slowly oxidize other *n*-alkanes. The major conclusions are discussed in more detail in the following sections.

Oxidation of 2-methylpropane*:* In this study we have demonstrated that cells of strain ELW1 grown on 2-methylpropene can slowly oxidize 2-methylpropane to TBA. Several lines of evidence suggest that this reaction involves the alkene-oxidizing monooxygenase responsible for initiating alkene catabolism in this strain. First, 2-methylpropane oxidation occurred without a lag phase with 2-methylpropene-grown cells (Fig. 4-1). This indicates that the reaction was catalyzed by an existing enzyme in these cells and did not require *de novo* protein synthesis in response to 2-methylpropane. Second, 2-methylpropane oxidation was inhibited in cells pretreated with 1-octyne (Fig. 4-1). 1-octyne is thought to act as a mechanism-based inactivator of the alkene-oxidizing monooxygenase in strain ELW1 and inhibits alkene oxidation but has no effect on the subsequent degradation of epoxides and other intermediates (25, 26). Third, 2-methylpropane-oxidizing activity was competitively inhibited by 2-methylpropene (Fig. 4-3). This suggests both 2-methylpropane and 2-

methylpropene bind in a mutually exclusive fashion to the enzyme responsible for their oxidation. Lastly, the highest rates of 2-methylpropane oxidation were observed with cells grown on alkenes, 2-methylpropene and both 2-butene-isomers, while lower activities were observed with cells grown on non-alkene substrates (Table 4-1). Substantially similar results were also obtained with propene-grown cells of *X. autotrophicus* PY2. For example, the oxidation of 2-methylpropane to TBA was occurred without a lag phase (Fig. 4-6) and was inhibited in cells pretreated with propyne, a known mechanism-based inactivator of alkene monooxygenase activity in this strain (13).

The facts that strain Py2 does not grow on gaseous alkanes (38) and does not have another alkane-oxidizing monooxygenase encoded in its genome strongly supports our conclusion that the 2-methylpropane-oxidizing activity we have described for both strains examined in this study can be attributed to an alkene rather than an alkane monooxygenase. Additional similarities between strain ELW1 and Py2 further support this important conclusion. For example, both strains oxidized 2-methylpropane at similar rates. Strain ELW1 oxidized 2-methylpropane at a rate of ~3.6 nmoles min^{-1} mg total $protein^{-1}$ (Fig. 4-1) while the rate with strain Py2 was ~2.5 nmoles min^{-1} mg total $protein^{-1}$ under the same experimental conditions (Fig. 4-6). Both organisms also generated substoichiometric amounts of TBA during 2-methylpropane oxidation. In the case of strain ELW1, TBA accounted for ~70% of the 2-methylpropane consumed (Fig. 4-1) while with strain Py2, this value was ~53% (Fig. 4-6). Furthermore, in both strains there was also no evidence for the further oxidation of TBA (Figs 4-1 & 4-6).

As TBA production from 2-methylpropane involves a hydroxylation reaction and was substoichiometric for both strains ELW1 and Py2, we also conclude that 2M1P likely represents the other immediate products of 2-methylpropane oxidation in both cases. We did not detect accumulation of this alcohol in any of our experiments reported here. We also demonstrated that 2-methylpropene-grown cells rapidly oxidize this alcohol at rates that would likely have prevented its detection during 2-methylpropane oxidation (Fig. 4-1). In view of this ability to rapidly oxidize 2M1P and the substantially amounts of this alcohol that were likely generated, it was surprising that cooxidation of 2-methylpropane during growth on 2-methylpropene-did not result in a stimulation of growth of strain ELW1 (Fig. 4-4). One possible explanation of this is that while 2M1P could likely be oxidized by 2-methylpropene-grown cells, they may have lacked the enzyme systems necessary to fully mineralize this compound and a more highly oxidized and polar metabolite may have accumulated but gone undetected by the GC analysis used in this study.

There are a number of other reports of microbial oxidation of 2-methylpropane. Several actinomycetes have been described that can grow on this compound (4, 27, 29, 30, 33) but accumulation of TBA has not been reported for any of these organisms. In contrast, TBA production has been reported for both whole cells and cell-free systems of methanothrophic bacteria. Whole cells of *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* strain CRLM1 both slowly oxidize 2-methylpropane and the latter generates both TBA and 2M1P as metabolites. While 2M1P is further oxidized to 2-methylpropionaldehyde, TBA is not further oxidized (22). The thermophilic methanotroph strain H-2 has also been reported to have similar product profile when oxidizing 2-

methylpropane (24). Cell-free methane monooxygenase (MMO) preparations from *Methylobacterium* strain CRL26 generate TBA and 2M1P in approximately equimolar concentrations (31) while purified soluble MMO from *M. capsulatus* (Bath) generates TBA as 70% of the products of 2-methylpropane oxidation (16). In contrast, purified butane monooxygenase from *Pseudomonas butanovora* also slowly oxidizes 2-methylpropane but TBA only reprsents ~5% of the oxidation products (12). The oxidation of 2-methylpropane we have characterized in this study is therefore substantially similar to previous reports which indicate that 2-methylpropane can be both terminal oxidized to 2M1P and subterminally oxidized to TBA. While there is some variability in the ratios of products, the activity we have observed with alkene-oxidizing strains is very similar to the 2-methylpropane-oxidizing activity previously reported for methanotrophs with soluble methane monooxygenase activity.

Oxidation of *n*-alkanes*:* Our results in this study also indicate that alkene-grown cells of strains ELW1 and Py2 can also terminally and subterminally oxidize short chain *n*-alkanes. With strain ELW1 we observed the accumulation of 2-propanol and 2-butanol production during the slow oxidation of propane and *n*-butane (Fig. 4-5). We also observed that 2-methylpropene grown cells could oxidize both 1^o and 2^o alcohols but generally 2^o alcohols were more slowly degraded than 1^o alcohols (Fig 4-S1 and Table 4-2). It is therefore possible that the oxidation of both propane and *n*-butane by strain ELW1 generated mixtures of 1^o and 2^o alcohols but only 2^o alcohols accumulated due to the faster degradation of 1^o alcohols. It is also possible that only 2^o alcohols were generated. While further studies will be required to

determine the relative ratios of 1^{o} and 2^{o} alcohols, the propane oxidation catalyzed by strain Py2 appears to be significantly different. With this organism we did not observe propanol accumulation during propane oxidation by propene-grown cells (Fig. 4-7A). While propene-grown cells could rapidly oxidize both 1-propanol and 2-propanol, we also demonstrated 2-propanol is quantitatively oxidized to acetone which is not further degraded during the time course of these reactions (Fig. 4-7B). As we did not detect acetone accumulation during propane oxidation by propene-grown cells of strain Py2 (Fig. 4-7A), we conclude that propane oxidation by this strain exclusively generates 1-propanol as an immediate oxidation product.

There are several pathways for the oxidation of propane by bacteria that can grow on this alkane (2). These include the terminal oxidation pathway of *Mycobacterium austroafricanum* (formerly *M. vaccae*) JOB5 (7), both the terminal and sub-terminal oxidation pathways of *P. fluorescens* NRRL-B-1244 (20), and the sub-terminal oxidation pathway of *R. rhodochrous* PNKb1 (3). Many of these pathways have been proposed on the basis of the properties of the alcohol dehydrogenases that participate in 1-or 2-propanol oxidation. Perhaps more relevant to this study, propane can also be cometabolically oxidized by a number of bacteria that do not grow on this compound. For example, the ammonia-oxidizing bacterium *Nitrosomonas europaea* oxidizes propane to mixtures of 1-propanol (~13%) and 2-propanol (~87%) (23). 2-propanol is also the predominant product of propane oxidation by both soluble and particulate forms of methane monooxygenase whereas 1-propanol is the dominant product (95%) of propane oxidation by purified butane monooxygenase.

Significance of alkane-oxidizing activity in alkene-oxidizing bacteria*:* The findings in this study are significant for two main reasons. First, these results test the long-standing assumption that alkene-metabolizing bacteria do not normally oxidize alkanes through the activity of alkene monooxygenases. Propene-grown cells of strain Py2 have been previously shown to oxidize several chlorinated alkenes but these reactions likely involve epoxidation rather than hydroxylation reactions (40). Strain Py2 can also oxidize several aromatic compounds including benzene, phenol and toluene but there are no previous reports of alkane oxidation by this organism or its alkene monooxygenase. It is not clear how widely distributed 2-methylpropane-oxidizing activity is among alkene-metabolizing bacteria but in view of the stability of TBA and its lack of further oxidation, it is possible that the oxidation of 2-methylpropane to TBA may be a useful assay for alkene-oxidizing monooxygenase in whole cells where other alcohols generated from alkane oxidation or epoxides generated from alkene oxidation may be quickly consumed.

The second reason why the results of this study are significant is that they demonstrate that 2-methylpropane, a common and typical significant component of gasoline, can be oxidized to TBA. The accumulation of TBA in gasoline-impacted environments is often taken as an indication of MTBE biodegradation. The ability of organisms such as strain ELW1 or Py2 to oxidize another gasoline component to TBA may question the underlying assumption that TBA accumulation is suitable evidence for underlying degradation of MTBE.

FIGURES AND TABLES

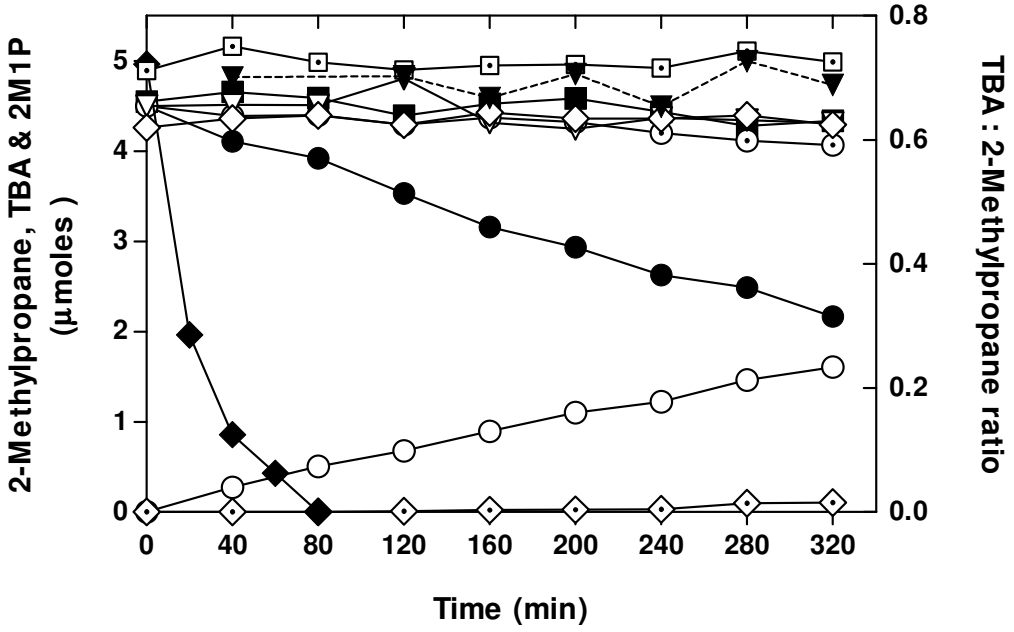


Figure 4-1: Oxidation of 2-methylpropane and its putative metabolites by strain ELW1. 2-Methylpropane and its potential metabolites (TBA and 2M1P) were incubated with 2-methylpropene-grown ELW1 cells (1.9 mg of total protein) that were pre-treated with or without 1-octyne in 10 ml glass serum vials and changes in reactant concentrations were determined over time by GC analysis, as described in the Methods section. The Figure shows the time course for the consumption of (●) 2-methylpropane, (■) TBA, and (◆) 2M1P, and the (○) production of TBA (from 2-methylpropane oxidation), with the cells pretreated with 1-octyne. The Figure also shows the time course for the (⊙) consumption of 2-methylpropane and (◈) production of TBA with the cells pretreated without 1-octyne. Abiotic reactions were also conducted for (▽) 2-methylpropane, (⊡) TBA and (◇) 2M1P. Each reaction contained an initial substrate concentration of 4.5 µmoles. Conversion ratio of TBA: 2-methylpropane (--▼--) for each datum point is also shown here.

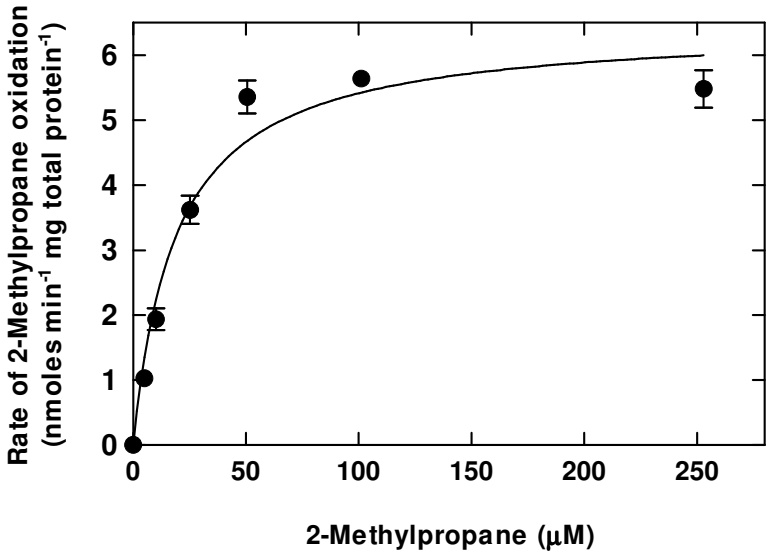


Figure 4-2: Kinetics of 2-methylpropane oxidation by strain ELW1. Cells of strain ELW1 grown on 2-methylpropene (1.8 mg of total protein) were incubated in buffer in sealed reactions vials (10 ml) with varying amounts of 2-methylpropane added to the gas phase to provide the indicated estimated initial dissolved 2-methylpropane concentrations (0 -250 μM). After 1h incubation, the accumulation of TBA in each reaction was determined. The Figure shows a plot of the initial rates of 2-methylpropane consumption (in terms of TBA production) and a computer fit of these data to a hyperbolic single substrate-binding model (R^2 =0.96). The data presented are the means and ranges of values obtained from two separate cultures.

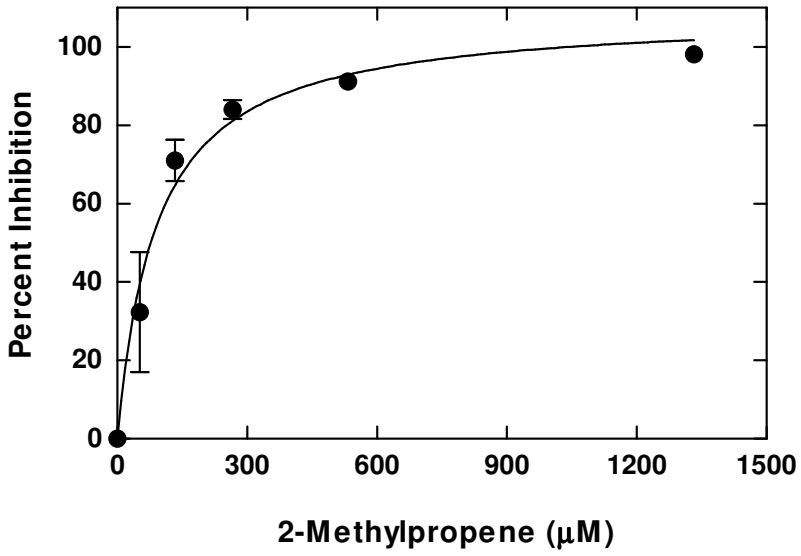


Figure 4-3: Effect of 2-methylpropene on 2-methylpropane oxidation. 2-Methylpropene-grown cells (1.0 mg total protein) were incubated in buffer in sealed reactions vials (10 ml) with a fixed initial concentration (133 μM) of 2-methylpropane and varying amounts of 2-methylpropene added to the gas phase to provide the indicated estimated initial dissolved 2-methylpropene concentrations (0-1350 μM). Consumption of 2-methylpropane was determined over time by GC analysis of the gas phase, as described in the Methods section. The Figure shows a plot of the initial rates of 2-methylpropene consumption (0-30 min) (in the presence of 2-methylpropene) and a computer fit of these data to a hyperbolic single substrate-binding model (R^2 =0.95). The data presented are the means and ranges of values obtained from two separate cultures.

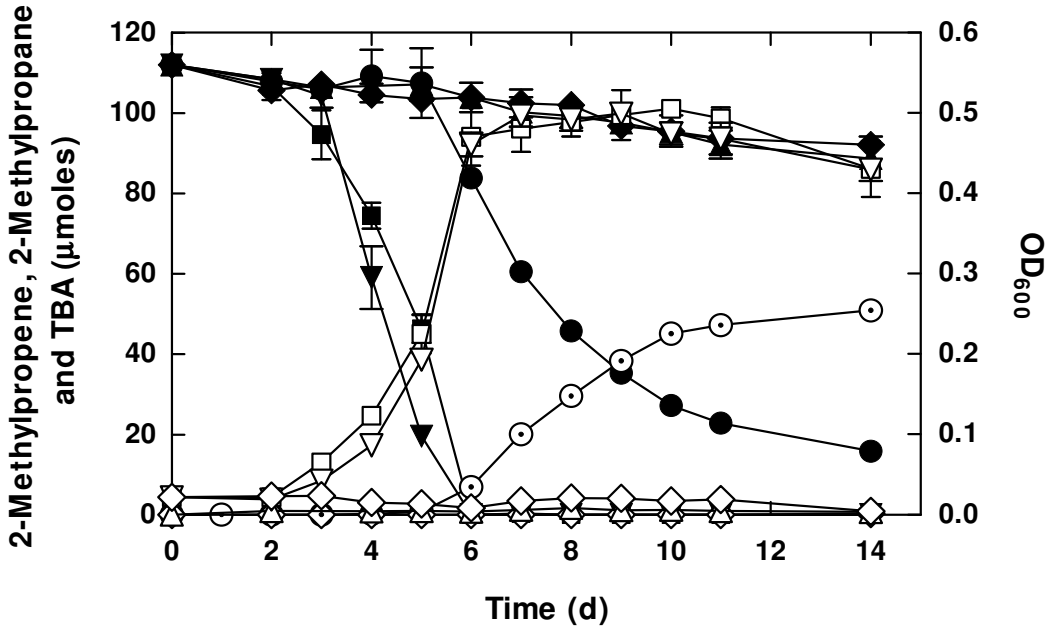


Figure 4-4: Effects of 2-methylpropane on growth of strain ELW1 on 2-methylpropene. Cells of strain ELW1 (previously grown on 2-methylpropene) were grown on limiting-C amounts of 2-methylpropene (1.85% v/v gas phase) and/or 2-methylpropane (1.85% v/v gas phase) in sealed glass serum vials (160 ml) containing 25 ml of MSM as described in the Methods section. The Figure shows the time course for changes in culture density (OD_{600}) (open symbols), amount of 2-methylpropene or 2-methylpropane (closed symbols), and the production of TBA from 2-methylpropane (symbols with a centered dot). The symbols represent; (□,■) culture density and 2-methylpropene consumption in cultures containing only 2-methylpropene, (▽,▼) culture density and 2-methylpropene consumption in cultures containing both 2-methylpropene and 2-methylpropane, (●,⊙) 2-methylpropane consumption and TBA production in cultures containing both 2-methylpropene and 2-methylpropane, (◇,◆,⟐) culture density, 2-methylpropane consumption and TBA production in cultures containing only 2-methylpropane, (△,▲) culture density and 2-methylpropene consumption in abiotic (uninoculated) rcultures containing only 2-methylpropene. Data presented are the means and ranges of values obtained from three separate cultures.

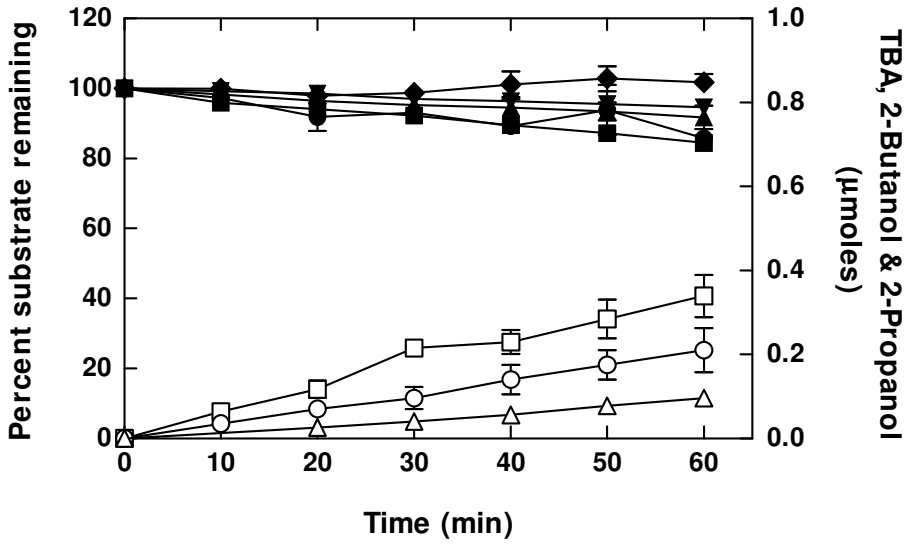


Figure 4-5: Oxidation of non growth-supporting alkanes by strain ELW1. 2-Methylpropene-grown cells of strain ELW1 (1.6 mg total protein) were incubated in buffer in sealed reactions vials (10 ml) with an equal dissolved concentration of each alkane (C_1-C_4) as described in the Methods section. The Figure shows the time course for the consumption of (◆) methane, (▼) ethane, (▲) propane, (●) *n*-butane, (■) 2-methylpropane and the formation of corresponding alcohols; (△) 2-propanol from propane, (○) 2-butanol from *n*-butane and (□) TBA from 2-methylpropane. The symbols represent the average for two replicate cultures, and the error bars show the range of values for both cultures combined. Data presented are the means and ranges of values obtained from two separate cultures.

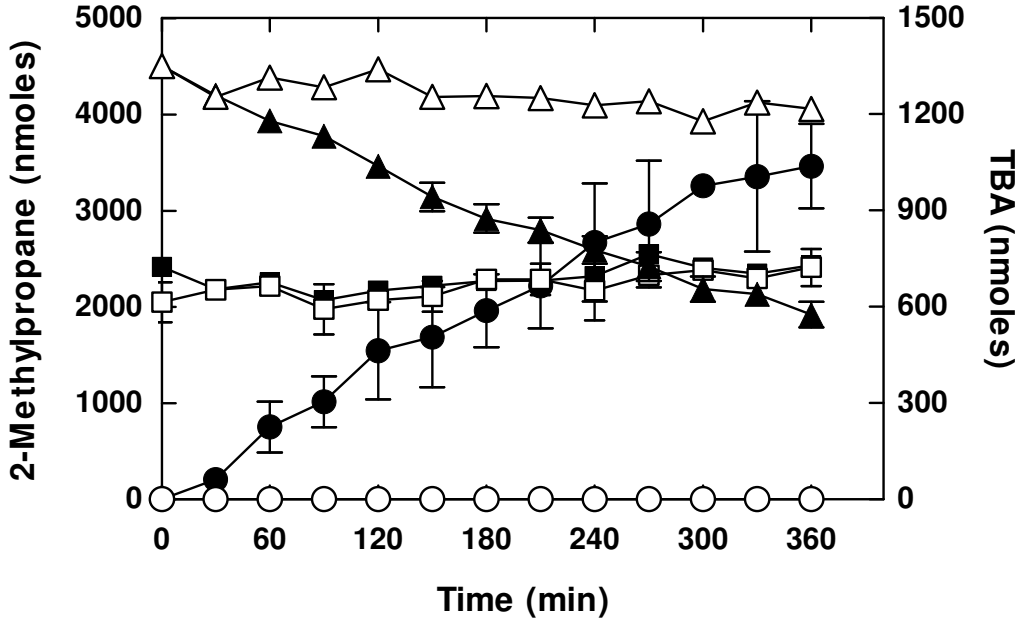


Figure 4-6: Oxidation of 2-methylpropane and its putative metabolites by strain Py2. Propene-grown cells of strain Py2 (2.8 mg total protein) were incubated in buffer in sealed glass serum vials (10 ml) with 100 µl (4.5 µmoles) of 2-methylpropane and the changes in reactant and product concentrations were determined over time by GC. Cells were pre-treated with (open symbols) or without (closed symbols) 1-propyne, as described in the Methods section. The Figure shows the time course for the (△,▲) consumption of 2-methylpropane, (○,●) production of TBA and (□,■) consumption of TBA. The data presented are the means and standard errors for reactions conducted with two separate cultures of strain Py2.

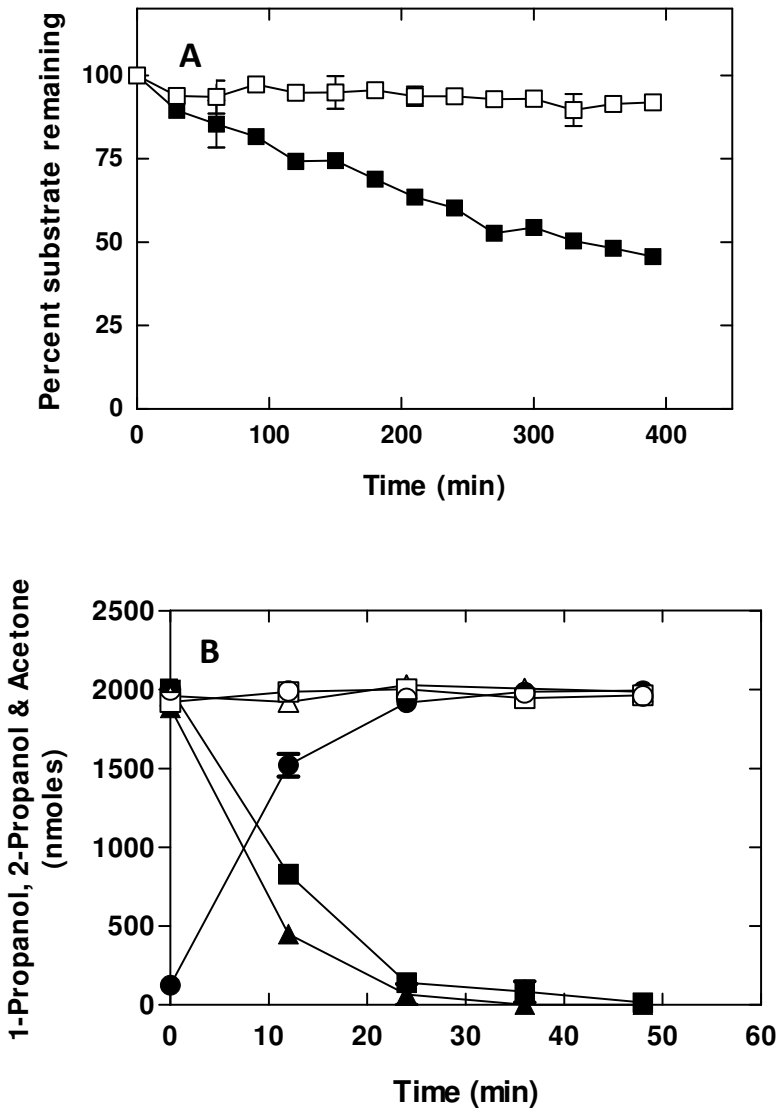


Figure 4-7: Oxidation of propane and its putative metabolites by strain Py2. Panel A Shows the time course for oxidation of propane (100 µl) by propene-grown *Xanthobacter* Py2 cells pre-treated (□) with or (■) without 1-propyne. **Panel B** shows the time course for the oxidation of puatative metabolites of propane oxidation; (○,●) 1-propanol, (△,▲) 2-propanol, and (◇,◆) acetone in abiotic (open symbols) and biotic (closed symbols) incubations. All reactions were conducted in 10 ml glass serum vials and the cells used in the reactions described in *panel A* contained 4.0 mg total protein while cells used in reactions described in *panel B* contained 2.4 and 3.7 mg total protein in 2-propanol and 1-propanol reactions respectively. In each case, the data presented are the means and standard errors for reactions conducted with two separate cultures of strain Py2.

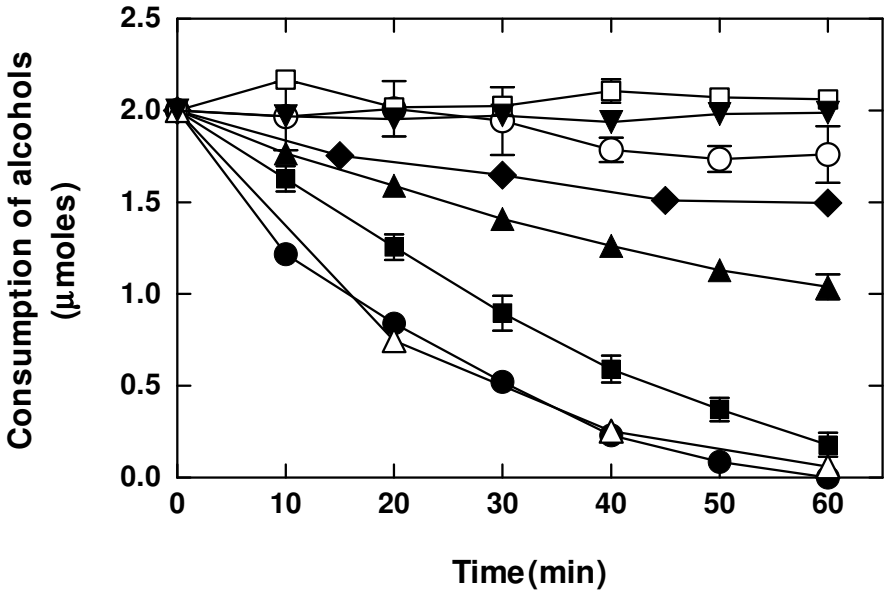


Figure 4-S1: Oxidation of alcohols by strain ELW1. 2-Methylpropene-grown cells of strain ELW1 (2.0 mg total protein) were incubated in buffer with 2 μmoles of each alcohol in sealed glass serum vials (10 ml) for 60 min and the changes in reactants were determined over time by GC as described in the Methods section. The Figure shows the time course for the consumption of (□) methanol, (○) ethanol, (■) 1-propanol, (▲) 2-propanol, (●) 1-butanol, (◆) 2-butanol, (△) 2M1P and (▼) TBA. The data presented are the means and standard errors for reactions conducted with two separate cultures.

Table 4-1: 2-Methylpropane-oxidizing activity after growth on alkene and non-alkene substrates.

Substrate	Rate of TBA production (nmoles min^{-1} mg total $protein^{-1}$)	
	without 1-octyne pretreated	with 1-octyne pretreated
2-methylpropene	7.92 (0.7)	0.310 (0.07)
cis-2-butene	3.05 (0.1)	0
trans-2-butene	3.04 (0.6)	0
ethanol	0.52 (0.1)	0
1-propanol	0.33 (0.0)	0
1-butanol	0	0
acetate	0.41 (0.2)	0
propionate	0.36 (0.0)	0
2-methylpropionic acid	0	0
fructose	0	0
glucose	1.85 (0.2)	0

Resting cells of strain ELW1 grown on a variety of substrates (1 to 2 mg of total protein) were incubated in buffer in sealed glass serum vials (10 ml) with 1 ml (~45 μmoles) of 2-methylpropane for 60 min and the production of TBA was determined by GC analysis of the reaction aqueous phase, as described in the Methods section. Data presented are the mean and SEM (in parentheses) of two separate cultures of strain ELW1 grown on different substrates as indicated.

Table 4-2: O_2 uptake rates with key substrates.

Substrate	Specific rate of O_2 uptake (nmoles min^{-1} mg total $protein^{-1}$)
Endogenous rate	16.9 (0.9)
1-propanol	44.9 (1.0)
2-propanol	28.7 (0.2)
1-butanol	89.4 (2.8)
2-butanol	21.7 (3.8)
methanol	14.4 (0.5)
2-methyl-1-propanol	82.3 (13.0)
tert-butyl alcohol	14.0 (1.8)
propane	16.7
butane	14.6 (2.7)
methane	14.5
2-methylpropane	23.8 (7.2)
Endogenous rate	20.9 (3.5)
2-methylpropene	167.3 (24.0)
1,2-epoxy-2-methylpropane	132.7 (1.8)
2-hydroxyisobutyrare	72.1 (7.2)
Endogenous rate	19.2 (3.9)
fructose	25.8 (3.2)
acetate	32.6 (1.8)
ethanol	21.7 (3.5)
2-methyl-1,3-propanediol	28.4 (6.8)

Substrate-specific rates of O_2 uptake for cells grown on 2-methylpropene were measured using a Clark-style O_2 electrode in the presence as described in the Methods section. In each case, an aliquot (50 µl) of concentrated cell-suspension was added into the O_2 electrode chamber and the endogenous basal rate of O_2 uptake was initially determined for 3-5 min in the absence of exogenous substrates. Substrates were then added at an initial concentration of 5 mM for liquids and 300 µM of dissolved concentration for gases, into the O_2 electrode chamber in which the buffer and cells had been pre-equilibrated for ~5 min. The substrate-dependent O_2 uptake rates were determined by subtracting the basal rate of cellular respiration from substrate-induced respiration rates. Data presented are the mean and SEM (in parentheses) of two separate cultures.

REFERENCES

1. **Abbott BJ, Hou CT.** 1973. Oxidation of 1-alkenes to 1,2-epoxyalkanes by *Pseudomonas oleovorans*. Appl. Microbiol. **26:**86-91.

2. **Ashraf W, Mihdhir A, Colin Murrell J.** 1994. Bacterial oxidation of propane. FEMS Microbiol. Lett. **122:**1-6.

3. **Ashraf W, Murrell JC.** 1990. Purification and characterization of a NAD-dependent secondary alcohol dehydrogenase from propane-grown *Rhodococcus rhodochrous* PNKb1. Arch. Microbiol. **153:**163-168.

4. **Babu JP, Brown LR.** 1984. New type of oxygenase involved in the metabolism of propane and isobutane. Appl. Environ. Microbiol. **48:**260-264.

5. **Cardini G, Jurtshuk P.** 1970. The enzymatic hydroxylation of *n*-octane by *Corynebacterium* sp. strain 7E1C. J. Biol. Chem. **245:**2789-2796.

6. **Cerniglia C, Blevins W, Perry J.** 1976. Microbial oxidation and assimilation of propylene. Appl. Environ. Microbiol. **32:**764-768.

7. **Coleman JP, Perry JJ.** 1985. Purification and characterization of the secondary alcohol dehydrogenase from propane-utilizing *Mycobacterium vaccae* strain JOB-5. J. Gen. Microbiol. **131:**2901-2907.

8. **de Bont JAM, Primrose SB, Collins MD, Jones D.** 1980. Chemical studies on some bacteria which utilize gaseous unsaturated hydrocarbons. J. Gen. Microbiol. **117:**97-102.

9. **de Bont J.** 1976. Oxidation of ethylene by soil bacteria. Antonie Van Leeuwenhoek. **42:**59-71.

10. **de Smet M, Kingma J, Wynberg H, Witholt B.** 1983. *Pseudomonas oleovorans* as a tool in bioconversions of hydrocarbons: growth, morphology and conversion characteristics in different two-phase systems. Enzyme Microb. Technol. **5:**352-360.

11. **de Smet MJ, Wynberg H, Witholt B.** 1981. Synthesis of 1,2-epoxyoctane by *Pseudomonas oleovorans* during growth in a two-phase system containing high concentrations of 1-octene. Appl. Environ. Microbiol. **42:**811-816.

12. **Dubbels BL, Sayavedra-Soto LA, Arp DJ.** 2007. Butane monooxygenase of *'Pseudomonas butanovora*': purification and biochemical characterization of a terminal-alkane hydroxylating diiron monooxygenase. Microbiology. **153:**1808-1816.

13. **Ensign SA, Hyman MR, Arp DJ.** 1992. Cometabolic degradation of chlorinated alkenes by alkene monooxygenase in a propylene-grown *Xanthobacte*r strain. Appl. Environ. Microbiol. **58:**3038-3046.

14. **Furuhashi K, Taoka A, Uchida S, Karube l, Suzuki S.** 1981. Production of 1,2-epoxyalkanes from 1-alkenes by *Nocardia corallina* B-276. European J Appl Microbiol Biotechnol. **12:**39-45.

15. **Gornall AG, Bardawill CJ, David MM.** 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177:**751-766.

16. **Green J, Dalton H.** 1989. Substrate specificity of soluble methane monooxygenase. mechanistic implications. J. Biol. Chem. **264:**17698-17703.

17. **Habets-Crützen A, Brink L, van Ginkel CG, de Bont JAM, Tramper J.** 1984. Production of epoxides from gaseous alkenes by resting-cell suspensions and immobilized cells of alkene-utilizing bacteria. Appl. Microbiol. Biotechnol. **20:**245-250.

18. **Heyer J.** 1976. Mikrobielle Verwertung von Äthylen. Zeitschrift Für Allgemeine Mikrobiologie. **16:**633-637.

19. **Higgins I, Hammond R, Sariaslani F, Best D, Davies M, Tryhorn S, Taylor F.** 1979. Biotransformation of hydrocarbons and related compounds by whole organism suspensions of methane-grown *Methylosinus trichosporium* OB3b. Biochem. Biophys. Res. Commun. **89:**671-677.

20. **Hou CT, Patel R, Laskin AI, Barnabe N, Barist I.** 1983. Epoxidation of short-chain alkenes by resting-cell suspensions of propane-grown bacteria. Applied Appl. Environ. Microbiol. **46 (1):**171-177.

21. **Hou CT, Patel R, Laskin AI, Barnabe N.** 1979. Microbial oxidation of gaseous hydrocarbons: epoxidation of C2 to C4 *n*-alkenes by methylotrophic bacteria. Appl. Environ. Microbiol. **38:**127-134.

22. **Hou CT, Patel RN, Laskin AI, Barist I, Barnabe N.** 1983. Thermostable NAD-linked secondary alcohol dehydrogenase from propane-grown *Pseudomonas fluorescens* NRRL B-1244. Appl. Environ. Microbiol. **46:**98-105.

23. **Hyman MR, Murton IB, Arp DJ.** 1988. Interaction of ammonia monooxygenase from *Nitrosomonas europaea* with alkanes, alkenes, and alkynes. Appl. Environ. Microbiol. **54:**3187-3190.

24. **Imai T, Takigawa H, Nakagawa S, Shen GJ, Kodama T, Minoda Y.** 1986. Microbial oxidation of hydrocarbons and related compounds by whole-cell suspensions of the methane-oxidizing bacterium H-2. Appl. Environ. Microbiol. **52:**1403-1406.

25. **Kottegoda S, Hyman M.** Metabolism of 2-butene by Mycobacterium sp. ELW1. To be submitted for publication in *Applied and Environmental Microbiology* (manuscript in preparation).

26. **Kottegoda S, Waligora E, Hyman M.** Isolation and characterization of a novel 2-methylpropene-metabolizing strain, *Mycobacterium* ELW1. To be submitted for publication in *Applied and Environmental Microbiology* (manuscript in preparation).

27. **Lukins HB.** 1962. On the utilization of hydrocarbons, methyl ketones and hydrogen by Mycobacteria. Ph.D. Dissertation, The University of Texas.

28. **McAuliffe C.** 1966. Solubility in water of paraffin, cycloparaffin, olefin, acetylene, cycloolefin, and aromatic hydrocarbons. J. Phys. Chem. **70:**1267-1275.

29. **McLee A, Kormendy AC, Wayman M.** 1972. Isolation and characterization of *n*-butane-utilizing microorganisms. Can. J. Microbiol. **18:**1191-1195.

30. **O'Brien WE, Brown LR.** 1968. The catabolism of isobutane and other alkanes by a member of the genus *Mycobacterium*. Dev. Ind. Microbiol. **9:** 389-393.

31. **Patel RN, Hou CT, Laskin AI, Felix A.** 1982. Microbial oxidation of hydrocarbons: properties of a soluble methane monooxygenase from a facultative methane-utilizing organism, *Methylobacterium* sp. strain CRL-26. Appl. Environ. Microbiol. **44:**1130-1137.

32. **Patel R, Hou C, Laskin A, Felix A, Derelanko P.** 1983. Epoxidation of *n*-alkenes by organisms grown on gaseous alkanes. J. Appl. Biochem. **5:**121-131.

33. **Solano-Serena F, Marchal R, Casare´gola S, Vasnier C, Lebeault J, Vandecasteele J.** 2000. A *Mycobacterium* strain with extended capacities for degradation of gasoline hydrocarbons. Appl. Environ. Microbiol. **66:**2392.

34. **Stirling D, Dalton H.** 1979. The fortuitous oxidation and cometabolism of various carbon compounds by whole-cell suspensions of *Methylococcus capsulatus* (Bath). FEMS Microbiol. Lett. **5:**315-318.

35. **van der Linden A.** 1963. Epoxidation of α-olefins by heptane-grown *Pseudomonas* cells. Biochim. Biophys. Acta. **77:**157-159.

36. **van Ginkel CG, de Bont JAM.** 1986. Isolation and characterization of alkene-utilizing *Xanthobaeter* spp. Arch. Microbiol. **145:**403-407.

37. **van Ginkel CG, Welten HG, de Bont JAM.** 1987. Oxidation of gaseous and volatile hydrocarbons by selected alkene-utilizing bacteria. Appl. Environ. Microbiol. **53 (12):**2903-2907.

38. **van Ginkel CG, Welten HG, de Bont JAM.** 1986. Epoxidation of alkenes by alkene-grown *Xanthobacter* spp. Appl. Microbiol. Biotechnol. **24:**334-337.

39. **Watkinson RJ, Somerville HJ.** 1976. The microbial utilization of butadiene. In: Sharply J.M., and J.A.M. Kaplan (eds) Proceedings of the 3rd International Biodeterioration Symposium, Kingston, RI. Applied Science, 35-42

40. **Zhou N, Jenkins A, Chion, Chan K. N. Chan Kwo, Leak DJ.** 1999. The alkene monooxygenase from *Xanthobacter* strain py2 is closely related to aromatic monooxygenases and catalyzes aromatic monohydroxylation of benzene, toluene, and phenol. Appl. Environ. Microbiol. **65:**1589-1595.

CONCLUDING REMARKS

In this study, we have described the isolation and physiological characterization of a novel aerobic alkene-oxidizing bacterium, *Mycobacterium* sp. ELW1, which utilizes 2-methylpropene as a sole source of carbon and energy. No other organism has previously been isolated on 2-methylpropene although several alkane-metabolizing strains can cometabolize this compound. 2-Butene (both *cis*- and *trans*-isomers) is the only other alkene it grew on. Strain ELW1 also grew well on the potential epoxide and diol products of 2-methylpropene- and the two isomers of 2-butene-oxidation. This observation led us to assume that 2-methylpropene and the two 2-butene isomers are metabolized through the same initial reactions and that the same enzymes may be involved in overlapping stages of the catabolism of all three growth supporting gases. Like many other gaseous alkene-metabolizing bacteria, strain ELW1 appears to initiate 2-methylpropene catabolism using a monooxygenase to generate an epoxide. Our results further suggest that 1,2-epoxy-2-methylpropane, MPD and HIBA are sequential intermediates in the pathway of 2-methylpropene metabolism. In organisms that grow on MTBE and TBA, HIBA is converted to 3HB by a cobalamin-dependent mutase, which has a stringent requirement for cobalt. The ability to metabolize 2-methylpropene in strain ELW1 also appears to be a cobalt-dependent physiological trait and the requirement for cobalt ions is localized to the terminal step in the pathway, the conversion of HIBA to 3HB. Based on these observations, we suggest that the pathway and enzymes involved in latter stages of 2-methylpropene catabolism might be similar to those involved in the aerobic bacterial metabolism of MTBE and TBA. Most alkene-oxidizing bacteria metabolize epoxides via carboxylation reaction rather than using

epoxide hydroylases. However, 1,2-epoxy-2-methylpropane irreversibly inactivates EaCoMT (epoxyalkane:coenzymeM transferase), one of the enzymes that involves in carboxylation of epoxides. This eliminates the possibility that strain ELW1 metabolizes 1,2-epoxy-2-methylpropane *via* carboxylation.

We have also demonstrated that strain ELW1 metabolizes the two isomers of 2-butene *via* an initial epoxidation reaction and follows a pathway similar to 2-metylpropene metabolism. Moreover, our postulated pathway is substantially different from the pathway described for T2B oxidation by *Nocardia* TB1, the only previously characterized *trans*-2-butene (T2B) metabolizing bacterium. According to our results, 2,3-epoxybutane, 2,3-butanediol and 3-hydroxy-2-butanone are the sequential intermediates of both *cis*- and *trans*-2-butene oxidation. Degradation of *trans*-2,3-epoxybutane, the epoxide of T2B, by strain ELW1 was strongly enantioselective towards the S-isomer of *trans*-2,3-epoxybutane.

We also demonstrated the alkane-oxidizing ability of 2-methylpropene-grown strain ELW1. Although not utilized as growth substrates, short-chain alkanes (C_1-C_6) were cometabolized by strain ELW1. This indicates that the alkene monooxygenase in this strain has a broad substrate specificity in contrast to most of other alkene monooxygenases that are not able to hydroxylate alkanes. It also oxidized 2-methylpropane and generated ~70% TBA as the only detectable product. We also demonstrated that 2-mehylpropane acts as a competitive inhibitor of 2-methylpropene oxidation. Moreover, the oxidation of alkanes was fully inhibited by 1-octyne, the potent inhibitor of 2-methylpropene oxidation. These data suggest that the same monooxygenase enzyme is involved in both alkene- and alkane-oxidation in strain ELW1.

The research described in this dissertation contributes to the knowledge of alkene-biodegradation with an identification of a novel 2-methylpropene-utilizing bacterium as well as the characterization of a potential pathway of 2-methylpropene oxidation in that strain. Furthermore, we characterized a novel pathway for the metabolism of T2B by this novel bacterium. Although this study has demonstrated possible pathways for the oxidation of these three alkenes, it is also clear that some gaps remain in identifying and/or verifying the intermediate or dead-end products. For example, 1,2-epoxy-2-methylpropane, which is very reactive and unstable, has not been detected. Other compounds such as HIBA and 3HB represent an analytical challenge for identifying. Identification of these intermediates has to be confirmed by a better analytical tool such as HPLC. In the case of *trans*-2,3-epoxybutane enantiomers and their downstream metabolites, it is in need of determine the enatiomeric excess of these compounds. Also, it is of great importance to identify the alkene-oxidizing monooxygenase and epoxide hydrolase in strain ELW1 at the molecular level.