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Ryan T. Storfa for the degree of Master of Science in Microbiology presented on June 6, 2000. Title: Cooxidation by three Butane-Grown Bacteria and Mechanism-Based Inactivation of Butane Monooxygenase.

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Daniel J. Arp

Butane-grown cells of *Pseudomonas butanovora*, *Nocardioides* sp. CF8 and *Mycobacterium vaccae* JOB5 were tested for their ability to cooxidize methane, ammonia and ethylene. Less than 10 nmol of methane were degraded by each of the bacteria (0.17-0.35 mg protein) in 30 minutes. Hydroxylamine and nitrite accumulated when *Nocardioides* CF8 and *P. butanovora* were incubated with ammonia, while *M. vaccae* JOB5 accumulated only nitrite. The butane monooxygenase (BMO) was implicated in the formation of hydroxylamine and possibly nitrite as the presence of acetylene or butane decreased the production of hydroxylamine and nitrite and the addition of butyrate enhanced hydroxylamine and nitrite production. Oxygen was also required for ammonia oxidation. All three strains oxidized ethylene to ethylene oxide. This reaction was inhibited by

acetylene and enhanced by butyrate. Production of ethylene oxide in *P. butanovora* stopped after 20 minutes, while proceeding at a constant rate for 2 hours in *M. vaccae* and *Nocardioides* CF8. Further tests indicated inactivation of butane oxidizing activity by ethylene oxide in *P. butanovora.*

The characteristics of ethylene oxide inactivation of butane monooxygenase (BMO) *in P. butanovora* were investigated. BMO was found to be irreversibly inactivated by ethylene oxide in a time and concentration dependent manner. Butane protected BMO from inactivation and O_2 was required for inactivation implying turnover was required. Other epoxides were found to inactivate BMO including epoxypropane, 1,2-epoxybutane and 1,2-epoxyhexane. *Cis* and *trans*-2,3-epoxybutane did not inactivate. Other bacterial monooxygenases were tested for sensitivity to ethylene oxide including ammonia monooxygenase in *N. europaea*, toluene-2-monooxygenase in *Burkholderia cepacia* G4 and alkane monooxygenases in *M. vaccae* JOB5, *Nocardioides* CF8 and *Pseudomonas oleovorans*. Of these, only alkane monooxygenases in *Nocardioides* sp. CF8 and *M. vaccae* JOB5 exhibited ethylene oxide sensitivity. The results presented here provide strong evidence that ethylene oxide is a mechanism-based inactivator of BMO in *P. butanovora.*

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Cooxidation by three Butane-Grown Bacteria and Mechanism-Based Inactivation of Butane Monooxygenase

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Ryan T. Storfa

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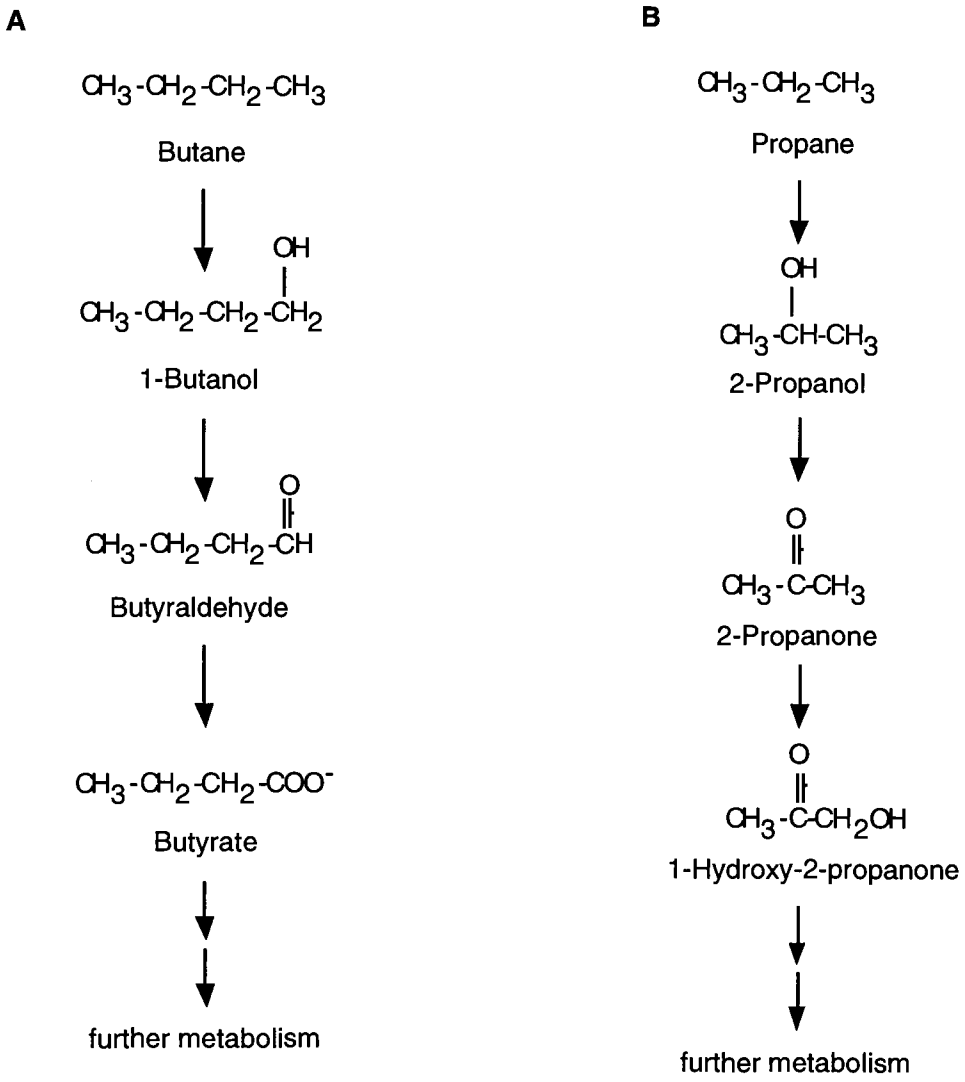
Chapter 1. INTRODUCTION TO THE THESIS

1.1. BUTANE METABOLISM

Alkane-degrading bacteria can generally be divided into three groups depending on the chain length of their carbon substrate. Group 1 consists of methanotrophic bacteria able to grow on methane through the use of particulate or soluble methane monooxygenase. This group has been extensively studied both genetically and physiologically and includes organisms such as *Methylococcus capsulatus* (BATH) and *Methylosinus trichosporium.* A second group of organisms includes those that grow on C_5 to C_{12} liquid alkanes. This group includes the well characterized system of *Pseudomonas oleovorans*, which oxidizes liquid alkanes using an alkane hydroxylase. The final group of alkane oxidizers includes the microorganisms that utilize gaseous C_2 to C_4 *n*-alkanes and includes the bacteria that this research was performed on. This group is comprised mostly of Gram positive bacteria in the *Rhodobacter-Nocardia-Arthrobacter-*

Corynebacterium complex [1-3] although some Gram negative bacteria grouped with the *Pseudomonas* have been found to grow on these gases as well [4]. These microorganisms use monooxygenases to hydroxylate the alkane either terminally, subterminally or both [5-7].

The alkane degradation pathways studied to date are limited to butane metabolism in *Nocardia* TB1 and *Pseudomonas butanovora* and butane and propane degradation in *Mycobacterium vaccae* JOB5. In *P. butanovora*, the pathway for *n*-butane metabolism proceeds through 1-butanol, butyraldehyde and butyrate as shown in scheme1.1A[6]. In *Nocardia* TB1, isocitrate lyase activities and butyrate production strongly suggest the identical pathway for butane degradation. Further evidence involving thiokinase and acetyl-CoA thiolase activities suggests further metabolism of butyrate by beta-oxidation [8]. In *M. vaccae* JOB5, butane assimilation also appears to follow the same pathway through terminal oxidation to 1-butanol [7]. However, propane is primarily oxidized subterminally to 2-propanol in *M. vaccae* JOB5 [2]. 2-Propanol is further metabolized through 2-propanone (acetone) and 1-hydroxy-2-propanone (acetol) as shown in scheme 1.1B, and this three-carbon intermediate is then cleaved to acetate and formaldehyde [9].



Scheme 1.1. Butane (A) and propane (B) degradation pathways for *P. butanovora* and *M. vaccae* JOB5 respectively.

The bacteria utilized in this work were all grown on butane and consisted of *Nocardiodes* sp. CF8, *M. vaccae* JOB5, and *P. butanovora*. *Nocardiodes* sp. CF8

is a Gram positive bacterium originally isolated on butane from aquifer solids from the Hanford Department of Energy site in Washington. This bacterium is able to grow well on C_2 to C_{10} *n*-alkanes, C_2 to C_4 primary alcohols, carboxylic acids and butyraldehyde. Slower growth is observed on C_{11} to C_{16} *n*-alkanes and several sugars. However, it was not able to grow on alkenes or secondary alcohols [10]. Butane oxidation in this organism is inhibited by the addition of allylthiourea (ATU), a known copper chelator [11]. This result suggests a monooxygenase with a copper containing active site such as particulate methane monooxygenase (pMMO) and ammonia monooxygenase (AMO). Additional evidence for similarity between these enzymes comes from acetylene labeling experiments. When *Nitrosomonas europaea* and *Methylococcus capsulatus* (BATH) are exposed to [$U^{14}C$] acetylene, a ca. 30 kDa polypeptide is labeled. Because acetylene is a mechanism-based inactivator of AMO and pMMO, this 30 kDa polypeptide is thought to contain the active site for these two proteins [12, 13]. When *Nocardiodes* CF8 is exposed to [$U^{14}C$] acetylene, a similarly sized 27 kDa polypeptide is labeled [11]. In addition, BMO activity in CF8 is eliminated after exposure to light, which is also observed in pMMO and AMO [14].

Mycobacterium vaccae JOB5 is a Gram positive soil isolate that grows on short chain gaseous alkanes. It primarily oxidizes propane subterminally and butane terminally as mentioned previously, so the possibility of more than one monooxygenase remains. All studies in this research were performed on butane-grown cells. Butane oxidation in these cells is inactivated by acetylene and [$U^{14}C$] acetylene treatment results in two heavily labeled polypeptides of 58 and 30 kDa molecular weight [11]. ATU and copper concentrations do not affect butane oxidation and alkenes, inactivators of cytochrome P-450 type monooxygenases, also do not effect enzyme activity.

Pseudomonas butanovora is a Gram negative monotrichous rod isolated from activated sludge from an oil refinery plant. However, recent work suggests this organism should be reclassified. *P. butanovora* 16s rRNA data shows it is more closely related to members of the *Rhodocycle* group of bacteria in the beta subdivision of the proteobacteria, rather than the *Pseudomonads* in the gamma subdivision [15]. This organism has been found to grow on $C_2 - C_9$ *n*-alkanes, primary alcohols, carboxylic acids, 1,2-propanediol, and 2,3-butanediol but not alkenes, methane, methanol, n-alkanes from $C_{10} - C_{16}$ and sugars [4, 16]. As with *M. vaccae* and *Nocardiodes* CF8, acetylene is a mechanism-based inactivator of

BMO in *P. butanovora* as well. When butane-grown cells are exposed to [$U^{14}C$] acetylene, a 58 kDa polypeptide is primarily labeled. The BMO from this bacterium has yet to be isolated, but copper is not required for activity [11] ruling out a copper centered active site.

1.2. COOXIDATION OF METHANE, AMMONIA AND ETHYLENE

Cooxidation is defined as the fortuitous oxidation of a non-growth substrate by a microorganism [17, 18]. Because cooxidation does not yield electrons for further oxidation, another source must be readily available. The input of electrons can come from further degradation of the substrate or from degradation of endogenously stored compounds such as poly-β-hydroxybutyrate.

Butane-oxidizing bacteria are excellent candidates for cooxidation studies for several reasons. First, alkane monooxygenases generally have wide substrate specificities, perfect for degrading an array of compounds [19]. This ability has not gone unnoticed as alkane-oxidizing bacteria have been the subject of many cooxidation studies [11, 20-23]. Another advantage of gaseous alkane utilizers in *in situ* studies is that their growth substrate will not further pollute the contaminated area. Unlike longer chain alkanes or other liquid substrates that might enter the

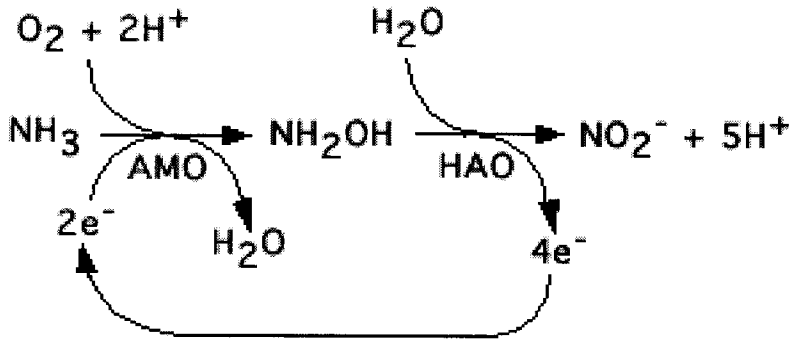
groundwater and create further problems, short chain gaseous alkanes are highly volatile and will escape groundwater contamination. Other advantages of gaseous alkanes include low cost, abundance and availability with a high degree of purity [2].

Use of these organisms for cooxidation also has disadvantages. Bacteria that grow on gaseous alkanes have relatively low growth rates and yields. In addition, growth on highly reduced hydrocarbons requires an abundance of oxygen and the combination of gaseous hydrocarbon and oxygen can sometimes be explosive. High volatility can also be seen as a disadvantage as continuous introduction of substrate will be required to achieve constant bacterial growth [2].

The compounds we analyzed as potential cooxidation substrates were methane, ammonia and ethylene. Methane is normally oxidized by methanotrophs by soluble (sMMO) or particulate methane monooxygenase (pMMO). The pMMO, which can be expressed in the membranes of all methanotrophs isolated to date, is a multi subunit enzyme dependent on copper for activity [24]. The sMMO is only found in some methanotrophs and is produced in times of copper limitation [25, 26]. Fifteen bacterial isolates and four fungi grown on butane were previously studied for their ability to grow on methane [3]. Although degradation of methane

was not determined in this study, none of the isolates were able to grow on methane as their sole source of energy. Ammonia-oxidizing bacteria with ammonia monooxygenase (AMO) are one group of bacteria that have the ability to cooxidize methane [27]. This ability is not surprising considering the similarities between AMO and pMMO, which will be discussed later.

The second cooxidation substrate we studied was ammonia. Chemolithotrophic ammonia-oxidizing bacteria such as *Nitrosomonas europaea* grow on ammonia as their sole energy source and carbon dioxide as their carbon source. The enzymes used in this pathway include AMO, which oxidizes ammonia to hydroxylamine, and hydroxylamine oxidoreductase (HAO) which oxidizes hydroxylamine to nitrite [28]. The oxidation of ammonia by AMO actually requires the input of electrons, which it receives from the further oxidation of hydroxylamine by HAO (Scheme 1.2). AMO has been characterized from several ammonia oxidizers and has been found to be composed of three subunits of 27, 36 and 45 kDa molecular weight with activity dependent on copper [29, 30]. The 27 kDa subunit is labeled with [U^{14}C] acetylene, a mechanism-based inactivator of this enzyme, and is believed to contain the AMO active site [12].



Scheme 1.2. Ammonia oxidation by *N. europaea*.

AMO also shows a high degree of similarity to pMMO of methanotrophs and it has been suggested that these enzymes are related [31]. Both pMMO and AMO require copper for activity and contain subunits of 27 and 45 kDa that show a high degree of sequence similarity to each other [24, 25, 32]. The substrate ranges of these two enzymes also reflect their physiological similarities. Both AMO and pMMO can oxidize aliphatic, aromatic and halogenated molecules, as well as methane and ammonia [27, 33-37].

Nitrification is not limited to chemoautotrophs as some organisms that grow heterotrophically have the ability to nitrify. Some example include the bacteria *Paracoccus denitrificans* [38], *Alcaligenes faecalis* [39, 40], *Pseudomonas putida* [41] and the fungus *Aspergillus flavus* [42]. These organisms obtain little or no energy from nitrification, so it is speculated the oxidation of ammonia in these

organisms may serve as a sink to remove excess electrons or to produce nitrification products that have bactericidal properties [38]. Another possibility is that the process has no function in the cell, but represents a fortuitous degradation by enzymes with low substrate specificity.

Of the few enzymes responsible for heterotrophic nitrification that have been characterized, many are similar to those in autotrophic nitrifiers. Robertson and Kuenen [43] discovered a HAO like enzyme in *Thiosphaera pantotropha* that oxidized hydroxylamine to nitrite and was inhibited by hydrazine and nitrite. Similar activity was observed in a HAO like enzyme in *Arthrobacter globiformis* [44]. *P. denitrificans* contains an AMO that is inhibited by light and chelating agents and is activated by copper, all similarities to AMO from autotrophic nitrifiers [45]. Experiments with cell-free extracts of *T. pantotropha* revealed the presence of a light sensitive AMO that required Mg^{2+} for activity [46]. Perhaps the best example of a heterotrophic nitrifier with similarity to AMO from an autotroph is *Pseudomonas putida.* This organism produces nitrite and nitrate from ammonia. An open reading frame was identified with 39% amino acid similarity to AmoA of *N. europaea* and the deduced hydrophobicity plot was also similar [41].

When studying nitrification *in situ*, researchers generally use various inhibitors and inactivators to separate heterotrophs from autotrophs. For example, heterotrophs studied appear to be resistant to acetylene, which is a very efficient inactivator of AMO in autotrophs [40]. Chlorate, an inhibitor of nitrite oxidation to nitrate in autotrophs, did not affect heterotrophic nitrate production in a forest soil [47]. However, other compounds that inhibit autotrophic nitrification such as thiosulfate, allylthiourea and nitrapyrin, have been shown to also inhibit heterotrophic nitrification in at least one instance [48].

Ethylene is a naturally occurring plant hormone, which is produced by plants, bacteria and fungi. Of the 35×10^6 tons of ethylene produced annually it is estimated that 74% is from biogenic origins, while 26% originates anthropogenically from burning biomass and combustion of fossil fuels [49]. Because ethylene reacts with and destroys ozone in the troposphere, it is important to understand its sources and sinks.

Many bacterial monooxygenases will attack the double bond of ethylene and cytochrome P-450 type monooxygenases are specifically inactivated by ethylene [50]. Bacteria that will oxidize ethylene include methanotrophs [51], ammonia oxidizers [52], alkane utilizers [53] and alkene-utilizing bacteria [54].

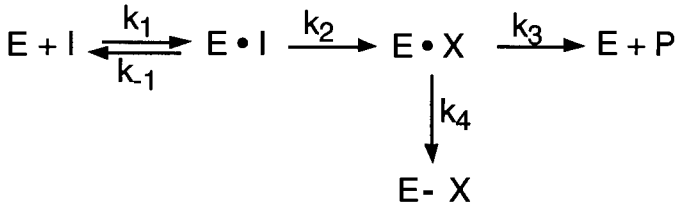
With the exception of the alkene-utilizing bacteria, oxidation of ethylene leads to the accumulation of ethylene oxide. This accumulation can cause problems, as ethylene oxide is a very reactive compound that can damage the cell (discussed further in chapters 2 and 3). Therefore, we sought to characterize the oxidation of ethylene in three butane-oxidizing bacteria.

1.3. MECHANISM-BASED ENZYME INACTIVATION

A mechanism-based inactivator or suicide substrate is a molecule that, through the normal catalytic activity of the target enzyme, is converted to a reactive species. This reactive species can covalently bind to an active site amino acid or prosthetic group, thus rendering the target enzyme inactive. Mechanism-based inactivators usually have structural similarity to the enzyme's normal substrate, but the resulting compound is toxic, hence the designation "suicide substrate". Due to the specificity of these inactivators, they are commonly used for studies on enzyme mechanisms or kinetics, drug design, or enzyme inhibition studies [55, 56].

Mechanism-based inactivation follows the equation in scheme 1.3, where E-X is the inactivated enzyme. An important factor in this type of inactivation is the partition ratio. The partition ratio is defined as the number of times product is

released from the active site of the enzyme relative to the number of times E•X is formed, or k_3/k_4 in terms of scheme 1.3. Therefore, an efficient inactivator has a small partition ratio.



Scheme 1.3. Kinetics of mechanism-based inactivation.

In order to classify a molecule as a mechanism-based inactivator, certain criteria should be met, as outlined by Silverman [55]. The first criterion is that the inactivation reaction should be a time dependent, pseudo first-order process. Because the rate of k_1 is usually much faster than the rate of k_2, the time dependence generally measures the rate of conversion of the inactivator to a reactive intermediate. The second requirement is that the reaction should be independent of inactivator at high concentrations and follow saturation kinetics. This criterion basically states that when the inactivator concentration is high, all of the enzyme will be in the E•I form and additional inactivator will not affect the rate of inactivation. Also, the inactivator must compete with the normal substrate for

the enzyme active site, so substrate should protect the enzyme from inactivation. Next, involvement of a catalytic step must be demonstrated. This requirement can be tested by showing cofactors or other substrates necessary for enzyme turnover (e.g. oxygen in the case of monooxygenases) are required for inactivation. If the reaction is occurring in the active site of the enzyme, then there should also be a 1:1 stoichiometry of radiolabeled inactivator to enzyme active site. The sixth requirement is that inactivation must occur before the activated molecule is released from the active site. A common way to test for this is to look for an absence of a lag time for inactivation or to use nucleophiles such as 2-mercaptoethanol to react with released inactivators, which are usually electrophilic. Lastly, because the enzyme-inactivator reaction is covalent, the reaction is usually irreversible. However, it has been argued that the enzyme-inactivator adduct can undergo a rearrangement to release the inactivator and restore activity [56].

One mechanism-based inactivator used in this work is acetylene (C_2H_2). Acetylene is an inactivator of many monooxygenases including AMO in autotrophic nitrifiers, pMMO and sMMO in methanotrophs and butane monooxygenase in *P. butanovora*, *Nocardiodes* CF8 and *M. vaccae* JOB5 [11, 12, 20, 25]. Because of its specificity, acetylene treated control cells are commonly

used as a negative control for our studies. Another potential mechanism-based inactivator, ethylene oxide, will be discussed in further detail in chapter 3.

Chapter 2. COOXIDATION OF METHANE, AMMONIA AND ETHYLENE BY THREE BUTANE-GROWN BACTERIA

2.1. ABSTRACT

Butane-grown cells of *Pseudomonas butanovora*, *Nocardiodes* sp. CF8 and *Mycobacterium vaccae*JOB5 were tested for their ability to cooxidize methane, ammonia and ethylene. Less than 10 nmol of methane were degraded by each of the bacteria (0.17-0.35 mg protein) in 30 minutes. Hydroxylamine and nitrite accumulated when *Nocardiodes* CF8 and *P. butanovora* were incubated with ammonia, while *M. vaccae* JOB5 accumulated only nitrite. The butane monooxygenase (BMO) was implicated in the formation of hydroxylamine and possibly nitrite as the presence of acetylene or butane decreased the production of hydroxylamine and nitrite and the addition of butyrate enhanced hydroxylamine and nitrite production. Oxygen was also required for ammonia oxidation. All three strains oxidized ethylene to ethylene oxide. This reaction was inhibited by acetylene and enhanced by butyrate. Production of ethylene oxide in *P. butanovora* stopped after 20 minutes, while proceeding at a constant rate for 2 hours in *M. vaccae* and *Nocardiodes* CF8. Further tests indicated inactivation of butane-oxidizing activity by ethylene oxide in *P. butanovora*.

2.2. INTRODUCTION

Cooxidation is defined as the fortuitous oxidation of a non-growth substrate by a microorganism [17, 18]. Alkane-grown bacteria have previously been shown to cooxidize several non-growth substrates including alkenes, aromatics and halogenated compounds [11, 20-23]. This ability is primarily due to the action of low substrate specificity alkane monooxygenases present in these bacteria. In this research, methane, ammonia and ethylene were studied as targets for cooxidation by butane-grown bacteria.

Methane is utilized as a growth substrate by methanotrophic bacteria containing the enzyme methane monooxygenase (MMO). There are two types of MMO in methanotrophs: a particulate form (pMMO) found in all methanotrophs and a soluble form (sMMO) found only in some. The sMMO has been well characterized and consists of a hydroxylase with a diiron-centered active site, a reductase and a regulatory subunit [25, 26]. The pMMO also contains three subunits, but this enzyme's active site includes both iron and copper [24]. In addition to methanotrophs, other bacteria including ammonia-oxidizers will degrade methane. Degradation of methane by alkane-grown bacteria has not been observed.

Ammonia-oxidizing bacteria, such as *N. europaea*, grow on ammonia as their sole source of energy. Ammonia is oxidized to hydroxylamine by ammonia monooxygenase (AMO) and hydroxylamine is then oxidized to nitrite by hydroxylamine oxidoreductase (HAO). AMO bears a striking resemblance to pMMO and the two are believed to be evolutionarily related [31]. Some similarities between AMO and pMMO include copper dependent activity and both proteins contain subunits of 27 and 45 kDa that show a high degree of sequence similarity to each other [24, 25, 32]. In addition, methanotrophs readily oxidize ammonia and ammonia oxidizers will degrade methane [27, 35].

While degradation of ammonia by alkane-grown bacteria has not been demonstrated, other bacteria and fungi possess the ability to degrade ammonia when grown heterotrophically on sugars or organic acids [38-42]. Some of these bacteria contain proteins that share characteristics with AMO and pMMO including light inactivation, inhibition by chelating agents and activation by copper [44-46]. One heterotrophic nitrifier, *Pseudomonas putida,* actually contains an open reading frame with amino acid similarity to AmoA, which codes for the hydroxylase subunit of AMO [41].

Ethylene, a naturally occurring plant hormone produced by plants, bacteria and fungi, is degraded by several groups organisms including methanotrophs [51], ammonia oxidizers [57], alkane utilizers [53] and alkene-utilizing bacteria [54]. All of these bacteria contain broad substrate range monooxygenases that attack the double bond of ethylene to produce ethylene oxide. It can also be used as a diagnostic tool to help identify cytochrome P-450 type monooxygenases as they are irreversibly inactivated by ethylene.

Two butane-grown bacteria studied in this lab, *Pseudomonas butanovora* and *Nocardiodes* CF8, have previously been shown to cooxidize C_1 and C_2 chlorinated aliphatics [20]. In addition, *Mycobacterium vaccae* JOB5 will also degrade C_1 to C_6 chlorinated aliphatics, several aromatics, and gasoline oxygenates such as MTBE when grown on butane or propane [20, 21, 23, 58-60]. The enzyme responsible for these cooxidations is a butane monooxygenase (BMO) in all three bacteria. The [U^{14}C] acetylene labeling patterns, inhibition profiles and substrate ranges show different characteristics between BMO for each bacterium. BMO from *Nocardiodes* CF8 requires copper for activity and is inactivated by light and allylthiourea just as is AMO and pMMO [25]. These similarities raise the possibility that this organism can also degrade methane and ammonia. In addition,

all of these organisms degrade the one carbon compound chloroform [20] raising the possibility of methane degradation in *M. vaccae* JOB5 and *P. butanovora* as well. In this work we studied the ability of three butane-grown bacteria, *P. butanovora*, *M. vaccae* JOB5 and *Nocardiodes* CF8 to degrade methane, ammonia and ethylene.

2.3. MATERIALS AND METHODS

2.3.1. Bacterial strains and growth conditions

Pseudomonas butanovora (ATCC 43655) was grown with butane as described previously [20]. *Nocardiodes* sp. CF8 and *Mycobacterium vaccae* JOB5 were grown in *Xanthobacter* Py2 medium as described [61] except that yeast extract was not included and the pH was adjusted to 7.5. Cultures of *Nocardiodes* CF8 and *M. vaccae* were grown in 150 ml vials containing 50 ml of medium. Butane (50 ml) was added as an overpressure to the gas phase, which contained air. Vials for growth of *M. vaccae* also contained additional O_2 (40 ml) added as an overpressure. Prior to methane, ammonia and ethylene degradation assays, cells were harvested by centrifugation (6,000 x *g* for 10 min.), washed twice with buffer and resuspended to a constant cell density (based on OD). For the methane and

ethylene degradation assays, the buffer used was the same as used in the growth medium. For ammonia degradation experiments, the buffer used for growth of *Nocardiodes* CF8 and *M. vaccae* JOB5 was used for all three bacteria.

2.3.2. Methane degradation assays

Experiments were performed in 1 ml Hamilton syringes with washed, butane-grown *P. butanovora* (0.35 mg protein), *M. vaccae* JOB5 (0.17 mg protein) and *Nocardiodes* CF8 (0.17 mg protein). Methane-saturated phosphate buffer was added to a final methane concentration of 124 μM (liquid phase), O_2-saturated phosphate buffer was added to a final O_2 concentration of 1 mM (liquid phase) and sodium butyrate (5 mM) was added as a source of reductant. Acetylene-treated cells were used as a control for the absence of butane-oxidizing activity. Acetylene treatment consisted of acetylene (1% vol/total vial vol) (430 μM liquid phase concentration) for 5 minutes followed by washing by centrifugation. Methane disappearance was measured by gas chromatography on a Shimadzu GC-8A equipped with a flame ionization detector and a 120 cm length by 0.1 cm inner diameter stainless steel column packed with Porapak Q (Alltech Associates, Inc.).

Liquid samples were injected (5 µl) and the gas chromatograph was operated at a column temperature of 25°C and a detector temperature of 220°C.

2.3.3. Ammonia degradation assays

Experiments were performed in 10 ml serum vials containing washed, butane-grown cells (1 ml) with the same protein concentrations as used in the methane degradation experiments. Various treatments such as sodium butyrate (5 or 0.5 mM), acetylene (430 µM), allylthiourea (ATU) (50 µM), butane (130 µM), N_2 gas (100%) were applied at time zero. NH_4Cl was added (10 mM) and the cells were incubated in a water bath (30° C)(150 cycles/minute). At selected times cells were removed by centrifugation and the supernatant was analyzed for hydroxylamine [62] and nitrite formation [63] by spectrophotometry. Experiments were performed to verify that ATU and butyrate did not affect the colorimetric assays.

2.3.4. Ethylene degradation assays

Experiments were performed in 10 ml serum vials containing washed, butane-grown cells (1 ml) with the same protein concentrations as used in the

methane degradation experiments. For acetylene pretreatment, cells were incubated in the presence of 430 μM acetylene (1% vol/total vial volume) for 10 minutes and the vials were purged with nitrogen gas for 2 minutes prior to the reintroduction of O_2 and the addition of ethylene (25% vol/total vol) to the vials. At specified times, a 100 μl headspace sample was analyzed by gas chromatography. A Shimadzu GC-8A equipped with a flame ionization detector and a 120 cm length by 0.1 cm inner diameter stainless steel column packed with Porapak Q (Alltech Associates, Inc.) was used. The gas chromatograph was operated at a column temperature of 130°C and a detector temperature of 220°C.

2.3.5. Protein determinations

Protein was determined using the Biuret assay [64] after the cells were solubilized in 3 N NaOH for 30 min at 65°C. Bovine serum albumin was used as a standard.

2.4. RESULTS

2.4.1. Degradation of methane by butane-grown bacteria

Methane degradation by three butane-grown bacteria was analyzed by gas chromatography. *P. butanovora* (0.35 mg protein) , *M. vaccae* JOB5 (0.17 mg protein) and *Nocardiodes* CF8 (0.17 mg protein) degraded less than 10 nmol of methane in 30 minutes. Typical butane oxidation rates range from 20-40 nmol/(min•mg) for the three bacteria [11]. The addition of sodium butyrate did not enhance methane degradation to a statistically significant rate over that of the control with no cells or acetylene inactivated cells (data not shown).

2.4.2. Degradation of ammonia by butane-grown bacteria

Ammonia was oxidized to hydroxylamine and nitrite by the three butane-grown bacteria (Table 2.1). *Nocardiodes* CF8 accumulated roughly equal amounts of hydroxylamine and nitrite, *M. vaccae* JOB5 accumulated primarily nitrite, while *P. butanovora* accumulated mostly hydroxylamine. Sodium butyrate (5 mM) was added to the samples to act as a source of reductant for BMO. This addition should lead to a stimulation of BMO activity when no other energy producing substrate is present. Activity was stimulated in most samples with the exception of

hydroxylamine accumulation in *Nocardiodes* CF8 and nitrite production in *M. vaccae* JOB5, which declined following addition of butyrate. Vials incubated with butane (640 μM) or butyrate (5 mM) and acetylene (430 μM) did not yield detectable product from ammonia with the exception of *Nocardiodes* CF8, which accumulated less than 30% of the nitrite than when incubated with butyrate alone (Table 2.1). Anaerobic vials purged with nitrogen showed significantly less (at least 50%) product formation from ammonia than aerobically incubated cells. Cells treated with allylthiourea also had lower rates of ammonia oxidation than untreated cells (data not shown).

Time courses of product formation were performed and showed a linear accumulation of hydroxylamine and nitrite by *M. vaccae* JOB5 and *Nocardiodes* CF8 (data not shown) for at least three hours. Nitrite accumulation in *P. butanovora* was also linear for at least 3 hours. The majority of hydroxylamine production, on the other hand, occurred very early in the experiment (Figure 2.1). Additional experiments were performed to determine initial rates of hydroxylamine production by *P. butanovora* and showed the majority of the hydroxylamine was produced in the first 5-10 minutes of the assay, with initial hydroxylamine production rates of over 45 nmol/(min•mg protein) (data not shown).

TABLE 2.1. Ammonia oxidation by butane-grown bacteria

Addition	Oxidation products (nmol)[a]					
	P. butanovora		*Nocardiodes* CF8		*M. vaccae* JOB5	
	NH_2OH	NO_2^-	NH_2OH	NO_2^-	NH_2OH	NO_2^-
None	94 ± 24	1.4 ± 0.5	13.1 ± 2.9	10.5 ± 1.3	<1	18.1 ± 3.2
Butyrate[b]	226 ± 24	8.0 ± 0.2	5.7 ± 2.8	15.0 ± 2.1	<1	4.8 ± 1.0
Acetylene (1%)[c]	<1	<1	<1	3.9 ± 1.0	<1	<1
Butane (10%)[c]	<1	<1	<1	3.8 ± 1.0	<1	<1

[a] Each reaction mixture contained NH_4Cl (10 mM) and the indicated addition. Assays were conducted for 3 hours for *Nocardiodes* CF8 and *M. vaccae* and for 2 hours for *P. butanovora* (approximately 0.17, 0.36, 0.15 mg of protein respectively). Data are expressed as means ± standard deviations.

[b] 5mM sodium butyrate for *M. vaccae* and 0.5 mM for *Nocardiodes* CF8 and *P. butanovora.*

[c] Vol/total vial vol

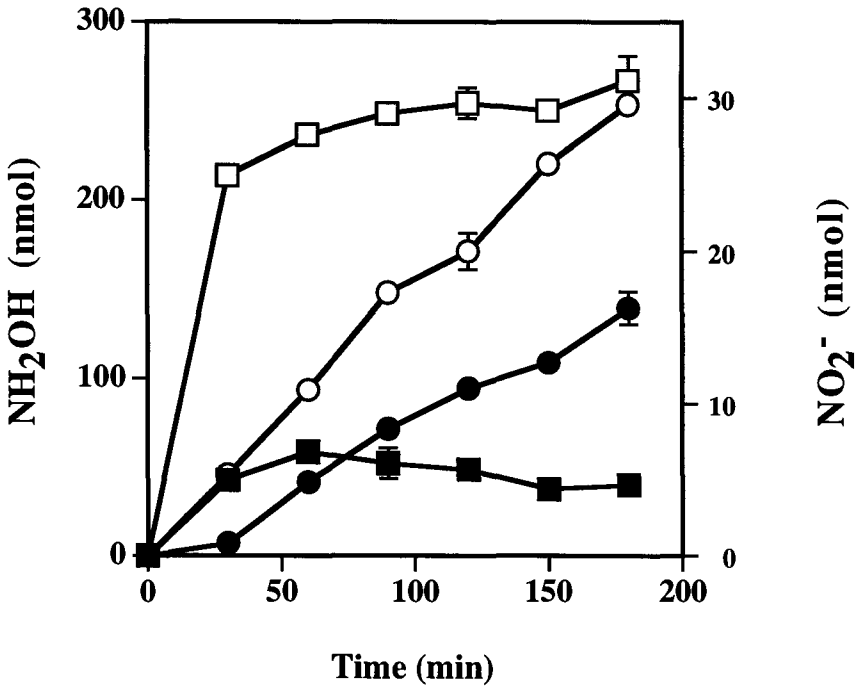


Figure 2.1. NH_2OH and NO_2^- production from NH_4Cl in *P. butanovora*. Butane-grown cells were incubated with 10 mM NH_4Cl and no butyrate (closed symbols) or 0.5 mM butyrate (open symbols). At various time points NH_2OH (squares) and NO_2^- (circles) were quantified. Data points are an average of 3 experiments and error bars represent standard deviations.

2.4.3. Degradation of ethylene by butane-grown bacteria

All three bacteria oxidized ethylene to ethylene oxide, which then accumulated and was measured by gas chromatography. No other products of ethylene oxidation were observed by gas chromatography during the experiments. Over 30 minutes, *M. vaccae* JOB5 produced the most ethylene oxide followed by *P. butanovora* and *Nocardiodes* CF8 respectively (Table 2.2). The addition of sodium butyrate as a source of reductant substantially increased ethylene oxide production in all three cases. Cells pretreated with 1% acetylene (430 μM) then incubated with butyrate and 25% ethylene (vol/total vial vol) produced less than 2% of the ethylene oxide of the same sample with untreated cells (Table 2.2).

Time courses of ethylene oxide production were performed and showed a linear accumulation of ethylene oxide by *M. vaccae* JOB5 and *Nocardiodes* CF8 for over 120 minutes (Figure 2.2). However, ethylene oxide production by *P. butanovora* stopped after 20 minutes of incubation with 25% ethylene. When *P. butanovora* was incubated in the presence of only 0.5% ethylene (vol/total vial vol), ethylene oxide production stopped even earlier (Figure 2.3).

TABLE 2.2. Oxidation of ethylene to ethylene oxide by butane-grown bacteria.

Addition	Ethylene oxide produced (nmol/mg of protein) in 30 minutes[a]		
	P. butanovora	*Nocardiodes* CF8	*M. vaccae* JOB5
None	151 ± 14	50.4 ± 3.9	566 ± 88
Butyrate (5mM)	525 ± 88	359 ± 21	1301 ± 311
Acetylene (1%)[b]	9.5 ± 1.6	< 1.3	17 ± 14

[a] Each reaction mixture contained ethylene (25% vol/total vial vol) and the indicated addition. Data are expressed as means ± standard deviations.
[b] Vol/total vial vol.

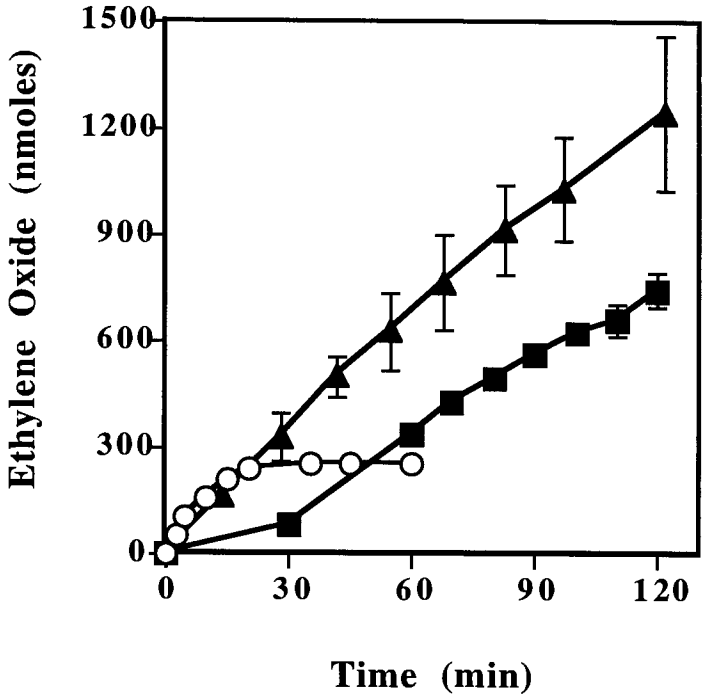


Figure 2.2. Time course of ethylene oxide production from ethylene by *P. butanovora*, *M. vaccae* JOB5 and *Nocardiodes* CF8. Concentrated cell suspensions of *P. butanovora* (○)(0.35 mg protein/ml), *M. vaccae* JOB5 (▲)(.17 mg protein/ml) and *Nocardiodes* CF8 (■)(0.17 mg protein/ml) were incubated in the presence of 25% ethylene (vol/total vial vol) and 5 mM sodium butyrate. The accumulation of ethylene oxide was measured by gas chromatography. Data shown for *M. vaccae* and *Nocardiodes* CF8 represent the average of 3 experiments and error bars represent standard deviations. Data for *P. butanovora* is from a single experiment.

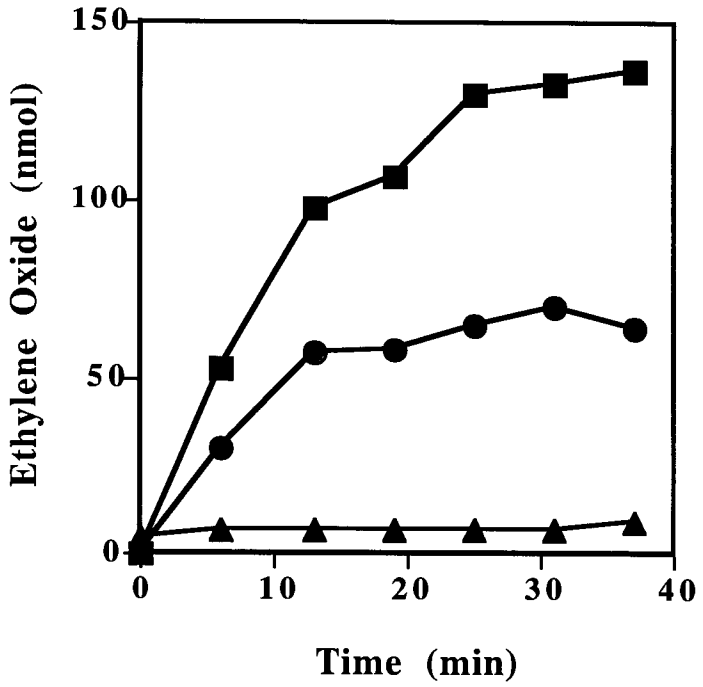


Figure 2.3. Ethylene oxide production from ethylene by whole cells of *P. butanovora*. Cells were incubated in the presence of butyrate and 25% (■) or 0.5% (●) ethylene. Control cells (▲) were preincubated with 430 μM acetylene, washed and incubated in the presence of butyrate and 25% ethylene. Ethylene oxide production was monitored by gas chromatography.

2.5. DISCUSSION

While methanotrophs will cooxidize other compounds such as alkenes and alkanes [36, 65], oxidation of methane by organisms other than methanotrophs or ammonia oxidizers has yet not been demonstrated. Mclee et al. studied 15 butane-degrading bacterial isolates and four fungi for their ability to grow on methane. Although degradation of methane was not determined in this study, none of the isolates were able to grow on methane as their sole source of energy [3]. Currently, ammonia-oxidizing bacteria are the only other group that has been shown to degrade methane. The extensive similarities between pMMO and AMO such as copper dependent activity, light inactivation, and inhibition by chelating agents as well as amino acid sequence similarity have led to the hypothesis that pMMO and AMO are evolutionarily related enzymes [24, 25, 31, 32]. Therefore, it is not surprising that AMO from autotrophic nitrifiers will oxidize methane and methanotrophs will oxidize ammonia [27, 35, 37].

In this study, three butane-grown bacteria were tested for their ability to cooxidize methane. *Nocardiodes* CF8 contains a BMO with similarities to AMO and pMMO including copper dependent activity, light inactivation, inhibition by chelating agents such as ATU and similar subunit sizes as determined by [U^{14}C]

acetylene labeling experiments [10, 11, 20]. Even with a source of exogenous reductant for BMO, no significant methane degradation was observed. However, it is important to note that the detection limit of the assay was 10 nmol over 30 minutes due to abiotic loss of methane from the liquid phase in these assays. It is possible that the organisms were degrading methane at a low level that was undetectable in this assay.

No alkane-grown bacterium has been shown to nitrify and given the broad substrate range of the alkane monooxygenase, we decided to study the capability of alkane-grown bacteria to degrade ammonia. The three butane-grown bacteria, *P. butanovora*, *M. vaccae* JOB5 and *Nocardiodes* CF8 are all able to produce various amounts of hydroxylamine and/or nitrite from ammonia (Table 2.1). BMO appears to be at least partially responsible for this oxidation as the presence of butane decreased product formation to almost undetectable levels in all samples and the presence of butyrate as an exogenous source of reductant for BMO, increased product formation in three of five cases. In addition, O_2 enhanced product formation and acetylene, a specific inactivator of BMO, reduced product formation to almost undetectable levels. Ammonia oxidation to nitrite in autotrophic nitrifiers requires the activity of AMO and HAO. Therefore, the production of nitrite

indicates the possibility of an HAO like enzyme in *P. butanovora*, *M. vaccae*, JOB5 and *Nocardiodes* CF8 as well. It is also possible that BMO catalyzes both the oxidation of nitrite and hydroxylamine, in which case, the inactivation or inhibition of BMO would also lead to the formation of less nitrite from ammonia.

In addition to the three examples in this research, methanotrophs and some heterotrophs will also oxidize ammonia. Methanotrophs will oxidize ammonia to hydroxylamine and nitrite. The oxidation of ammonia to hydroxylamine has been shown to be carried out by both sMMO and pMMO [25]. In *Methylococcus capsulatus* (BATH) the oxidation of hydroxylamine to nitrite is not affected by the addition of MMO inactivators such as acetylene and 8-hydroxyquinoline or by the addition of methanol, the substrate for methanol dehydrogenase [35]. In addition, the methanol dehydrogenase and hydroxylamine oxidase activities of *Methylococcus thermophilus* were separated by ion-exchange chromatography [66], indicating hydroxylamine oxidation is not carried out by MMO or methanol dehydrogenase and in at least two cases involves a separate hydroxylamine oxidase.

Other bacteria and fungi that grow heterotrophically also have the ability to degrade ammonia to hydroxylamine and nitrite. Some of these organisms have

proteins that show similarities to AMO and HAO of autotrophic nitrifiers. Robertson and Kuenen [43] discovered a HAO like enzyme in *Thiosphaera pantotropha* that oxidizes hydroxylamine to nitrite and is inhibited by hydrazine and nitrite. Similar activity is observed in a HAO like enzyme in *Arthrobacter globiformis* [44]. *P. denitrificans* contains an AMO that is inhibited by light and chelating agents and is activated by copper, [45], and a light sensitive AMO was found in cell free extracts of *T. pantotropha* [46]. Perhaps the best example of a heterotrophic nitrifier with similarity to AMO from an autotroph is *Pseudomonas putida.*, which produces nitrite and nitrate from ammonia. An open reading frame was identified with 39% amino acid similarity to AmoA of *N. europaea* and the deduced hydrophobicity plot was also similar [41].

The calculated nitrification rates of *M. vaccae* JOB5 and *Nocardiodes* CF8 are roughly 0.5 nmol nitrite produced/(min•mg protein). In comparison, the heterotrophic nitrifier *T. pantotropha* consumes ammonia at a rate of 35 nmol/(min•mg protein) [67], methanotrophs nitrify at 0.5-17 nmol/(min•mg protein) and the autotrophic nitrifiers such as *N. europaea* will produce nitrite at a rate of over 1 μmol/(min•mg protein) [25]. *P. butanovora* has an initial rate of hydroxylamine production of over 45 nmol/(min•mg protein), which compares

favorably with other heterotrophic nitrifiers and methanotrophs. However, this initial rate slows very quickly as the organisms produced over 200 μM hydroxylamine. Because hydroxylamine is a very toxic intermediate in ammonia oxidation, it is possible that the accumulation of this compound is damaging the cell or inhibiting the enzyme in some way.

Ethylene oxidation by other monooxygenases has previously been demonstrated in methanotrophs, nitrifiers, alkene and alkane-utilizing bacteria [51] [57] [54] [53]. *N. europaea*, an ammonia oxidizer, produced 1.54 μmol ethylene oxide/mg protein in 1 hour when incubated with ammonia (10 mM) and ethylene (30 μmol). However, this should not be looked at as a maximal rate due to the presence of ammonia as reductant and the competitive nature of ethylene and ammonia binding to AMO [57]. *Xanthobacter* Py2 grows on alkenes and will oxidize ethylene at a rate of 26 nmol/(min•mg protein) [54]. Hou et al. tested 27 isolates grown on propane for their ability to degrade various alkenes. All of the isolates produced from 0.07 - 2.60 μmol ethylene oxide/mg protein when incubated with ethylene for 1 hour [53]. In comparison, *P. butanovora*, *Nocardiodes* CF8, and *M. vaccae* JOB5 produced 0.53, 0.36, and 1.30 μmol ethylene oxide/mg protein in 30 minutes.

Ethylene oxide production in *P. butanovora* stopped after 20 minutes of incubation with 25% ethylene. When *P. butanovora* was incubated with 0.5% ethylene, ethylene oxide production stopped even earlier (Figure 2.3) indicating the high concentrations of ethylene are preserving enzyme activity in some way. Further research showed that the ethylene oxide rather than ethylene was inactivating BMO. This will be discussed further in chapter 3.

Chapter 3. ETHYLENE OXIDE INACTIVATION OF BUTANE MONOOXYGENASE IN *Pseudomonas butanovora*

3.1. ABSTRACT

The characteristics of ethylene oxide inactivation of butane monooxygenase (BMO) *in Pseudomonas butanovora* were investigated. BMO was found to be irreversibly inactivated by ethylene oxide in a time and concentration dependent manner. Butane protected BMO from inactivation and O_2 was required for inactivation implying turnover was required. Other epoxides were found to inactivate BMO including epoxypropane, 1,2-epoxybutane and 1,2-epoxyhexane. *Cis* and *trans*-2,3-epoxybutane did not inactivate. Other bacterial monooxygenases were tested for sensitivity to ethylene oxide including ammonia monooxygenase in *N. europaea*, toluene-2-monooxygenase in *Burkholderia cepacia* G4 and alkane monooxygenases in *Mycobacterium vaccae* JOB5, *Nocardiodes* CF8 and *Pseudomonas oleovorans*. Of these, only alkane monooxygenases in *Nocardiodes* sp. CF8 and *M. vaccae* JOB5 exhibited ethylene oxide sensitivity. The results presented here provide strong evidence that ethylene oxide is a mechanism-based inactivator of BMO in *P. butanovora*.

3.2. INTRODUCTION

Ethylene oxide has long been known to have deleterious effects on biological molecules. It indiscriminately alkylates highly nucleophilic molecules such as DNA base pairs and certain amino acids in proteins. This alkylation leads to DNA mutations and nonfunctional proteins, which is why ethylene oxide has toxic effects on mammals, insects, plants and microorganisms. Because of its toxicity, it is used as a fumigant in the sterilization of items sensitive to the high temperatures of autoclaving such as foodstuffs and medical equipment [68] [69] [70].

In addition to general damage through non-specific alkylation, some bacterial monooxygenases appear to be specifically damaged by epoxides. For example, van Hylckama Vlieg et al. [71] found *Rhodococcus* AD45 unable to grow on isoprene in the presence of 1,2-epoxyhexane. However, it is unclear as to whether this is due to inactivation of a monooxygenase or to sequestering of an epoxide scavenging enzyme. In addition, Habets-Crutzen and de Bont studied the toxicity of propylene oxide in *Mycobacterium* E20 [72]. The authors believe this organism contains an alkene and alkane monooxygenase, both of which appear to be inactivated by propylene oxide. Ethanol degradation, which is not catalyzed by

either monooxygenase, is not affected by propylene oxide. Ethylene grown *Mycobacterium* E3 containing an alkene monooxygenase is also irreversibly inactivated by propylene oxide, ethylene oxide and 1,2-epoxybutane [72]. These results are intriguing because the degradation pathway of ethylene and propene leads through an epoxide intermediate [19].

Epoxide inactivation of methane monooxygenase has also been studied [71, 73, 74]. Soluble methane monooxygenase (sMMO) activity in *Methylosinus trichosporium* OB3b is eliminated after treatment with propylene oxide [72] and cis 1,2-dichloroethylene epoxide [73]. Particulate methane monooxygenase (pMMO) in *Methylococcus capsulatus* (BATH) is irreversibly inactivated by propylene oxide [74]. The authors suggested propylene oxide was a mechanism based inactivator of pMMO based on several lines of evidence.

In this work we show that a butane monooxygenase (BMO) in *Pseudomonas butanovora* is specifically inactivated by low concentrations of ethylene oxide. This enzyme, as with pMMO in *M. capsulatus* (BATH), also appears to be inactivated by certain epoxides in a mechanism-based fashion. This paper characterizes the reaction between *P. butanovora* BMO and epoxides, most

notably ethylene oxide. We also look at the sensitivity of other monooxygenases to ethylene oxide to determine the rarity of the ethylene oxide:BMO reaction

3.3. MATERIALS AND METHODS

3.3.1. Bacterial strains and growth conditions

Pseudomonas butanovora (ATCC 43655) was grown with butane as described previously [20]. *Nocardiodes* sp. CF8 and *Mycobacterium vaccae* JOB5 were grown in *Xanthobacter* Py2 medium as described [61] except that yeast extract was not included and the pH was adjusted to 7.5. Cultures of *Nocardiodes* CF8 and *M. vaccae* were grown in150 ml vials containing 50 ml of medium. Butane (50 ml) was added as an overpressure to the gas phase, which contained air. Vials for growth of *M. vaccae* also contained additional O_2 (40 ml) added as an overpressure. *Burkholderia cepacia* G4 was grown with toluene as described [75]. *Nitrosomonas europaea* was grown with ammonium as described [34]. *Pseudomonas oleovorans* (ATCC 29347) was grown in 150 ml vials containing 50 ml of *P. butanovora* medium to which filter sterilized octane was added to a final concentration of 10 mM.

3.3.2. Measurement of cell activities that require BMO activity

Cell activities that require BMO activity were assayed to provide an indication of the level of BMO activity in intact cells. Butane consumption or ethylene oxide production from ethylene (an alternative substrate for BMO) by butane-grown cells of *P. butanovora, Nocardiodes* CF8 and *M. vaccae* JOB5 was determined. Cells were first harvested by centrifugation (10 min. at 11,000 x *g*, 4°C), washed twice with the same buffer used in the growth medium and resuspended to a constant cell density (see protein concentrations below) based on optical density (600 nm).

Butane consumption assays were performed as described [11] with *P. butanovora* (0.35 mg protein) and *Nocardiodes* CF8 (0.29 mg protein). Experiments were carried out in a water bath (23°C)(150 cycles/minute) in 2 ml vials completely filled with 0.5 ml cell suspension (0.29-0.35 mg protein), 1.2 ml O_2-saturated solution (720 μM) and 0.3 ml butane-saturated solution (360 μM). The vials were sealed with screw caps and Teflon coated rubber liners (Alltech Associates, Inc., Deerfield, IL) and glass beads were added to provide agitation. Butane consumption in the vials was monitored by gas chromatography on a Shimadzu GC-8A equipped with a flame ionization detector and a 60 cm length by

0.1 cm inner diameter stainless steel column packed with Porapak Q (Alltech Associates, Inc.). Liquid samples were injected (4 µl). The gas chromatograph was operated at a column temperature of 90°C and a detector temperature of 220°C. Acetylene-treated cells were used as a control for the absence of butane consumption activity.

Production of ethylene oxide was assayed in 10 ml serum vials with a 1 ml cell suspension of *P. butanovora* (0.35 mg protein) and *M. vaccae* JOB5 (0.17 mg protein). Sodium butyrate (5 mM) was added as a source of exogenous reductant and the vials were sealed with butyl rubber stoppers and aluminum crimp seals (Wheaton Scientific, Millville NJ). Ethylene was then added to the vials at a final concentration of 25% (vol/total vial vol) and the vials were constantly shaken in a water bath (23°C)(150 cycles/minute). Ethylene oxide production was monitored by gas chromatography on a Shimadzu GC-8A equipped with a flame ionization detector and a 120 cm length by 0.1 cm inner diameter stainless steel column packed with Porapak Q (Alltech Associates, Inc.). The gas chromatograph was operated at a column temperature of 130°C and a detector temperature of 220°C. Previous studies [11] showed that BMO catalyzes the oxidation of ethylene to ethylene oxide and ethylene oxide production remained linear for at least 10

minutes under the conditions of the assay. Because this assay was much more sensitive than the butane consumption assay, it was used to determine BMO activity in most experiments.

3.3.3. 1-Butanol degradation assay

Butane-grown *P. butanovora* was washed twice as described. Experiments were performed in 10 ml sealed serum vials with 1 ml of a concentrated cell suspension (0.35 mg protein). 1-Butanol was added to a final concentration of 2 mM. The vials were constantly shaken in a water bath (23°C)(150 cycles/minute). Consumption of 1-butanol was determined by gas chromatography on a Shimadzu GC-8A equipped with a flame ionization detector and a 60 cm length by 0.1 cm inner diameter stainless steel column packed with Porapak Q (Alltech Associates, Inc.). The gas chromatograph was operated at a column temperature of 160°C and a detector temperature of 220°C.

3.3.4. Oxygen consumption assays

Octane-dependent O_2 consumption experiments were performed with washed cells of octane-grown *P. oleovorans*. The experiments were performed on

a Clark style O_2 electrode at 23°C under constant stirring. Buffer and washed cells (0.8 mg protein) were added to the 2 ml reaction chamber and after an initial rate of endogenous O_2 uptake was obtained, a mixture of *N,N*-dimethylformamide (DMF) and octane was added to achieve a final concentration of 1.5 mM octane. The rate of octane-dependent O_2 uptake was taken as the octane stimulated rate minus the endogenous rate.

Ammonia-dependent O_2 uptake experiments with ammonia-grown *N. europaea* were performed in the same manner except that 1 mg of protein was used and NH_4Cl (10 mM final concentration) was added instead of octane.

3.3.5. Toluene degradation assay

Toluene-2-monooxygenase activity in toluene-grown *B. cepacia* G4 was measured by observing toluene degradation. Toluene-grown *B. cepacia* G4 cells were washed and resuspended in the same phosphate buffer used in the growth media. Experiments were performed in sealed 10 ml serum vials containing cell suspension (1 ml, 0.8 mg protein). A toluene:dimethylformamide (DMF) mixture was added to a final toluene concentration (liquid phase) of 250 μM and its degradation was measured using a Shimadzu GC-8A gas chromatograph equipped

with a 15 m x 0.35 mm bonded FSOT capillary column with polydimethylsiloxane. Column temperature was 120°C and the detector temperature was 220°C. Vials were shaken in a water bath (30°C)(150 cycles/minute) for the duration of the experiment. Control experiments were performed to verify that DMF alone was not affecting T2MO activity.

3.3.6. Epoxide inactivation assays

Ethylene oxide inactivation experiments were performed on butane-grown *P. butanovora*, *Nocardiodes* CF8, and *M. vaccae* JOB5; toluene-grown *B. cepacia* G4; octane-grown *P. oleovorans*; and ammonia-grown *N. europaea*. Inactivation experiments with other epoxides were performed on *P. butanovora* only. The experiments were performed in sealed 10 ml serum vials with 1 ml of washed, concentrated cell suspensions containing the same protein concentration as in the activity assay for those bacteria. The cells were exposed to the epoxide for 6 minutes at 23°C with constant shaking (150 cycles/minute). Vials were made anaerobic by repeated cycles of evacuation and purging with N_2 on the vacuum manifold. Exogenous reductant was also supplied to all samples during this period to facilitate monooxygenase turnover and consisted of sodium butyrate (2.5mM)

for *P. butanovora*, *Nocardiodes* CF8 and *M. vaccae* JOB5; 1-octanol (1 mM) for *P. oleovorans*; 3-methylcatechol (0.5 mM) for *B. cepacia* G4; and hydroxylamine (0.5 mM) for *N. europaea*. The cells were then washed twice by centrifugation and resuspended for use in one of the activity assays mentioned previously.

3.3.7. Solubility calculations

Headspace and liquid phase gas chromatography were used to calculate an Ostwald coefficient ($[C_L]/[C_G]$) of 137 for ethylene oxide. The Ostwald coefficients for propylene oxide [76], acetylene [77], ethylene and butane [78] at 23°C were calculated to be 1259, 0.947, 0.116, 0.0259 respectively. At 30°C, the Ostwald coefficient for toluene was calculated to be 2.915 [79]. In the absence of information regarding solubility of 1,2-epoxybutane, *cis* and *trans*-2,3-epoxybutane,1,2-epoxyhexane, and 2-hexyne our calculations assumed that all of the epoxide was in the liquid phase.

3.3.8. Cell-free extract experiments

Cells of butane-grown *P. butanovora* were harvested by centrifugation as described above. The cell suspension was resuspended in phosphate buffer to

nearly 35 mg protein/ml, twice passed through a French pressure cell and then subjected to centrifugation for 15 minutes at 11,000 x *g* at 4°C to remove unbroken cells and cell debris. The cell-free extract was then subjected to centrifugation for 1 hour at 200,000 x *g* at 4°C, and the resulting supernatant was obtained as the soluble fraction. Activity measurements were performed using the ethylene oxide production assay as described above except NADH (5 mM) was added in place of sodium butyrate as a source of reductant. Also, to remove the ethylene oxide prior to the activity assay, the vials were repeatedly evacuated and flushed with nitrogen using a vacuum manifold.

3.3.9. [U^{14}C] acetylene labeling experiments

Concentrated cell suspensions (2 mg/ml protein) and cell-free extracts (8 mg/ml protein) of butane-grown *P. butanovora* were pretreated with varying concentrations of ethylene oxide and butane. Cell suspensions of butane-grown *P. butanovora* were incubated at 30°C with constant shaking in 10 ml serum vials containing sodium butyrate (5 mM) and [U^{14}C] acetylene (0.4 mmol of [U^{14}C] acetylene (0.005 mCi/μmol)). [U^{14}C] acetylene was made from $Ba^{14}CO_3$ as described previously [80]. Labeling of cell free extracts was performed in the same

manner as the whole cell suspensions except NADH (5 mM) was added in place of sodium butyrate. After 30 minutes, the cells were washed twice with phosphate buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide gel) [81]. Gels were stained with Coomassie blue and dried onto filter paper and radioactive polypeptides were visualized by exposure on a storage phosphor screen (Molecular Dynamics, Sunnyvale, CA) for 3 days. Densitometry was conducted with Image Quant software (Molecular Dynamics) to quantify [^{14}C] labeling intensities.

3.3.10. Protein determinations

Protein concentrations were determined using the Biuret assay [64] after the cells were solubilized in 3 N NaOH for 30 min at 65°C. Bovine serum albumin was used as a standard.

3.4. RESULTS

3.4.1. Characteristics of ethylene oxide inactivation

In previous studies [11], we determined that treatment of *P. butanovora* cells with ethylene oxide resulted in a loss of butane consumption activity. We

conducted a series of tests to determine if ethylene oxide is a mechanism-based inactivator. First, we tested the effects of exposure time and concentration of ethylene oxide on the loss of butane consumption activity. Butane-grown cells were treated with 1.6 μM or 14.6 μM ethylene oxide for 0 to 4 minutes. The cells were then washed and tested for ethylene oxide production activity (Table 3.1). Note that the sample indicated at 0 second was actually exposed to ethylene oxide, but immediately after its introduction the cell washing period was started. The loss in ethylene oxide production activity was greatest when exposed to the higher ethylene oxide concentration for longer times, and the extent of inactivation shows a clear dependence on exposure time and inactivator concentration. A strict adherence to an exponential decay of activity, as expected of a mechanism-based inactivator, was not demonstrated. Given the extreme sensitivity of the cells to ethylene oxide, more detailed kinetic experiments were not feasible.

We next determined if catalytic activity was required and if natural BMO substrates protected the enzyme from inactivation. To determine if turnover was required for enzyme inactivation, we tested the effect of ethylene oxide exposure under anaerobic conditions on butane consumption. Cells treated aerobically with acetylene, a mechanism-based inactivator of BMO [11], served as a positive control

TABLE 3.1. Effect of time and concentration of ethylene oxide on BMO activity in *P. butanovora*.

Ethylene Oxide Conc. (μM)	BMO activity[a] recovered from washed cells after incubations in the presence of ethylene oxide for the following times.					
	No exposure	0 sec.[b]	30 sec.	60 sec.	120 sec.	240 sec.
1.6	17.90	12.06	10.63	7.39	8.44	6.45
14.6	17.90	4.91	3.67	1.68	1.22	1.14

[a]Activity (nmol/min • mg) was measured using the ethylene oxide production assay.
[b]The 0 second exposure was actually exposed to ethylene oxide and then immediately washed.

for loss of activity. When the cells were incubated anaerobically in the presence of ethylene oxide, full activity was retained (Figure 3.1). Under the same conditions in the presence of O_2, butane-oxidizing activity was reduced to a level similar to acetylene-treated cells.

To determine if a non-inactivating substrate could protect the enzyme from inactivation, butane was added to the reaction vials. In the presence of 50% butane (vol/total vial vol), full activity was retained, while the identical sample with no butane present lost almost all butane-oxidizing activity (Figure 3.1). Another substrate for BMO is ethylene, which is oxidized to ethylene oxide. When *P. butanovora* was incubated with low concentrations of ethylene (0.5% vol/total vial vol), ethylene oxide production stopped earlier and at lower concentrations than in samples containing 50% ethylene (Figure 3.2). Ethylene concentrations, as measured by gas chromatography, did not decrease in any of the sample vials (data not shown). Acetylene-treated control cells incubated in the presence of 25% ethylene produced no ethylene oxide.

Experiments were also performed to determine activity recovery after inactivation. After ethylene oxide exposure, the cells were shaken in the presence of butane with or without chloramphenicol. Cells incubated with butane alone

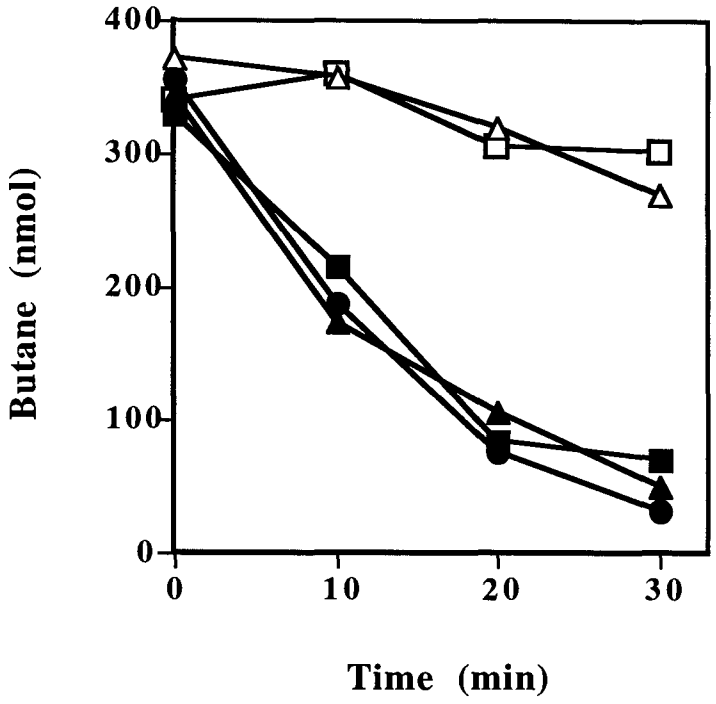


Figure 3.1. Butane degradation by *P. butanovora* exposed to various pretreatments including 10% butane (vol/total vol) (●), 380 μM ethylene oxide (△), 380 μM ethylene oxide + 10% butane (▲), 380 μM ethylene oxide + no O_2 (■), 430 μM acetylene (□). Following the pretreatments, the cells were washed and assayed for their ability to degrade butane by gas chromatography. The data shown represents the average of five experiments. The average standard deviation for the data points is ± 33.5 nmol.

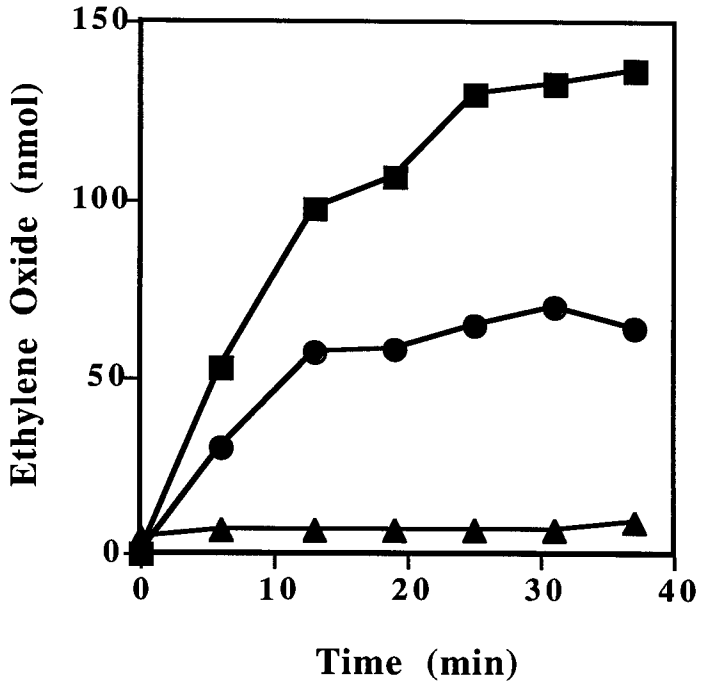


Figure 3.2. Ethylene oxide production from ethylene by whole cells of *P. butanovora*. Cells were incubated in the presence of butyrate and 25% (■) or 0.5% (●) ethylene. Control cells (▲) were preincubated with 430 μM acetylene, washed and incubated in the presence of butyrate and 25% ethylene. Ethylene oxide production was monitored by gas chromatography.

recovered 23% of the original activity over 5 hours, while chloramphenicol treated samples showed no recovery (data not shown). Thus ethylene oxide inactivation appears to be irreversible because recovery required protein synthesis.

3.4.2. Specificity of ethylene oxide towards BMO

Loss of butane degradation activity could result from a loss of BMO activity from damage to another component required for activity (e.g. downstream enzymes). To determine if downstream components or processes were affected, cells were preincubated in the presence of ethylene oxide, washed, and then 1-butanol degradation was determined. Neither ethylene oxide nor acetylene affected 1-butanol oxidation rates (Figure 3.3). Because butyraldehyde consumption is slower than its production from 1-butanol, butyraldehyde accumulated in these samples. No significant difference in butyraldehyde accumulation between the samples was observed (data not shown). Since1-butanol consumption and butyraldehyde accumulation were not affected, we can deduce that butyraldehyde consumption was not affected as well.

To determine if general cellular damage was taking place that could be attributed to ethylene oxide, the cells were grown on either lactate or butane in the

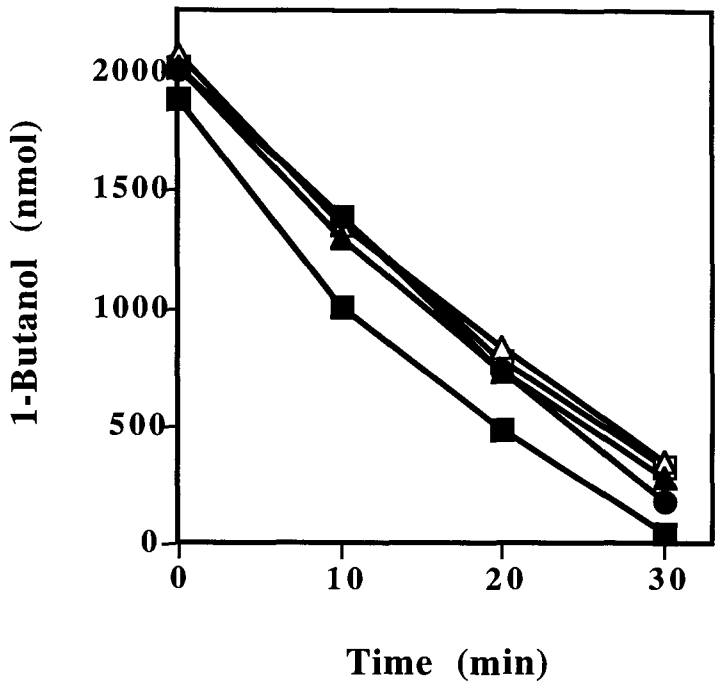


Figure 3.3. 1-Butanol degradation by *P. butanovora* exposed to various pretreatments including 10% butane (vol/total vol) (●), 380 μM ethylene oxide (△), 380 μM ethylene oxide + 10% butane (▲), 380 μM ethylene oxide + no O_2 (■), 430 μM acetylene (□). Following the pretreatments, the cells were washed and assayed for their ability to degrade 1-butanol by gas chromatography. The data shown represents the average of five experiments. The average standard deviation for the data points is ± 98.7 nmol.

presence or absence of ethylene oxide and growth was measured as an increase in optical density (Figure 3.4). In the presence of 1 mM ethylene oxide, *P. butanovora* growth on lactate was unaffected, while growth on butane was completely inhibited over 48 hours. When no epoxide was added, the cells grew normally reaching an OD of over 0.65 within 48 hours.

3.4.3. Reaction of P. butanovora *cell-free extracts to ethylene oxide*

To further localize the effects of ethylene oxide on BMO and to determine if the effects observed *in vivo* were similar *in vitro*, experiments were carried out with cell extracts. Membranes, unbroken cells and cell debris were removed from disrupted cells. The resulting supernatant was then tested for ethylene oxide production in the presence of 0.5% and 25% ethylene (Figure 3.5). The results were similar to those obtained with whole cells (Figure 3.2) except epoxide production proceeded for a longer period of time and to higher concentrations before stopping. In fact, the sample with 25% ethylene did not lose activity during the entire sampling period. The 0.5% ethylene sample stopped at 250 nmol of ethylene oxide produced. This amount is significantly more than in the same

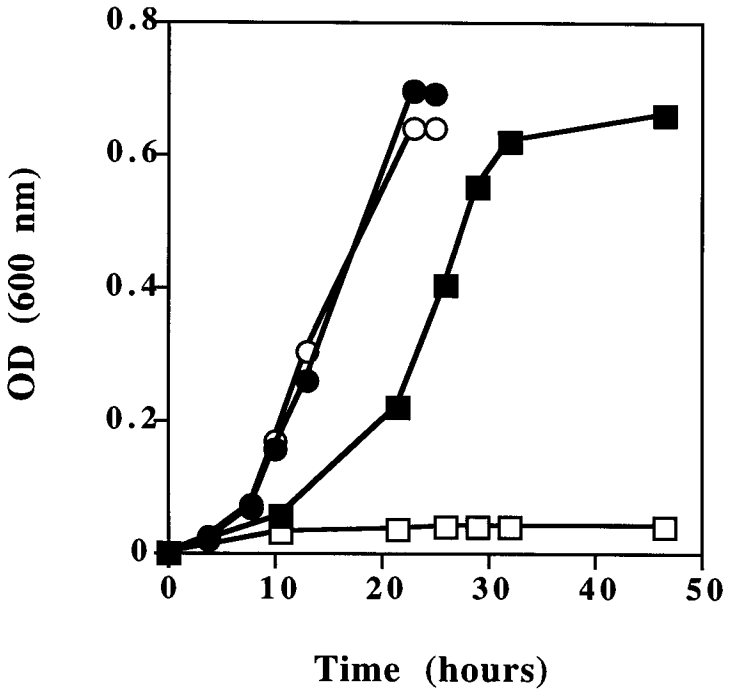


Figure 3.4. Growth of *P. butanovora* in the presence of ethylene oxide. *P. butanovora* was grown on butane (squares) or lactate (circles) in the absence of epoxide (closed symbols) or in the presence of 1 mM ethylene oxide (open symbols). Growth was monitored spectrophotometrically by optical density (600 nm).

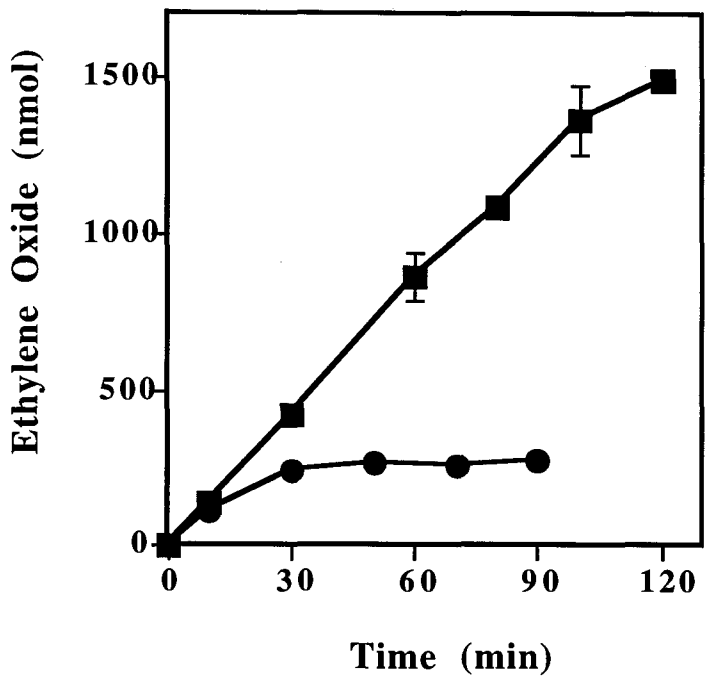


Figure 3.5. Ethylene oxide production from ethylene by cell-free extracts of butane-grown *P. butanovora.* Cells were incubated in the presence of NADH and 25% (■) or 0.5% (●) ethylene. Ethylene oxide production was monitored by gas chromatography. The data shown represents the average of two experiments and error bars represent the standard deviations.

experiment with whole cells, where the cells incubated with 0.5% ethylene produced less than 75 nmol of ethylene oxide (Figure 3.2).

3.4.4. [U^{14}C] Acetylene labeling of P. butanovora *whole cells and cell free extracts pretreated with ethylene oxide*

Acetylene is a mechanism-based inactivator of many monooxygenases including BMO in *P. butanovora* [11] and previous studies have shown [U^{14}C] acetylene labeling to be an effective way to quantify the active BMO in the cell. Autoradiograms of SDS gels reveal a heavily labeled band for *P. butanovora* that correlates to a 58 kDa polypeptide believed to contain the active site of the monooxygenase [11, 20]. Whole cells and cell free extracts were labeled with [U^{14}C] acetylene following a preincubation in the presence of various concentrations of ethylene oxide (Figure 3.6). The 58 kDa band was quantified for each lane and the results are shown below the gel. Label intensities are based on a 100% value for the cells not exposed to ethylene oxide. Because acetylene labeling requires enzyme turnover to catalytically activate acetylene, less labeling of the 58 kDa band correlates with less active enzyme in the cell. *P. butanovora* whole cells treated with higher concentrations of ethylene oxide incorporated less label, indicating there is less active BMO in the cells exposed to higher epoxide

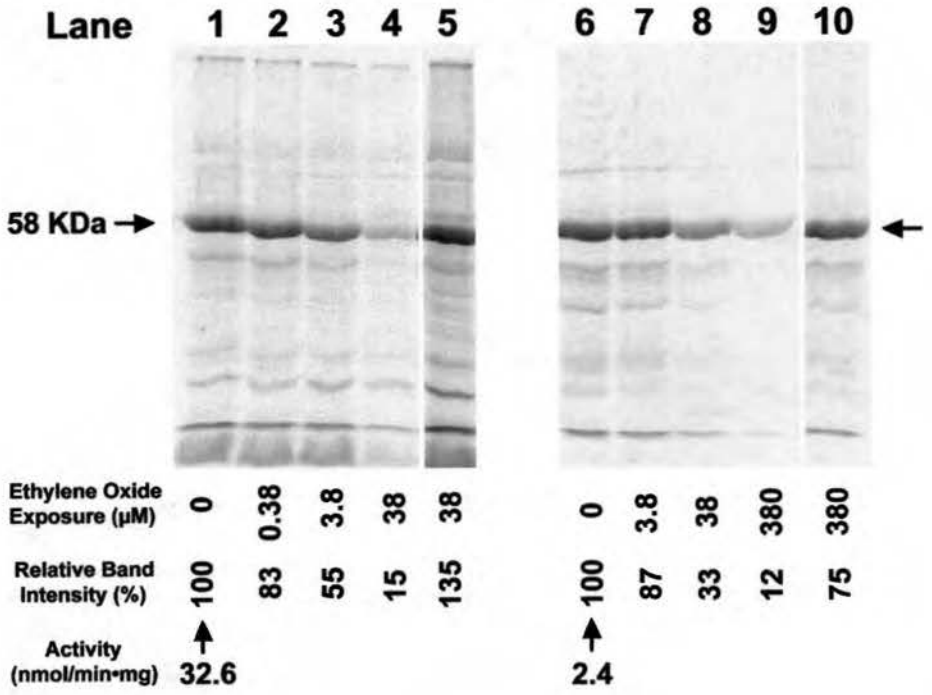


Figure 3.6. [U^{14}C] acetylene labeling of *P. butanovora* cellular proteins from whole cells and cell free extracts. *P. butanovora* whole cells (lanes 1-5) and cell-free extracts (lanes 6-10) were pretreated with ethylene oxide at the concentrations shown. Lanes 5 and 10 were also pretreated with 50% butane (vol/total vol) in addition to ethylene oxide. Following the pretreatment, the cells were washed and exposed to [U^{14}C] acetylene and incorporation of ^{14}C into polypeptides was measured by SDS-PAGE and visualized by a phosphorimager. Lanes 5 and 10 were performed on separate gels than lanes 1-4 and 6-9, but were from the same cell suspension. It is important to note that approximately the same amount of soluble protein was loaded into each lane.

concentrations. The cell free extracts followed the same pattern, but required roughly 10 fold more ethylene oxide for the same extent of inactivation. This result is consistent with the information from the ethylene oxide production experiments (Figures 3.2 and 3.5). When 50% (vol/total vol) butane was included with ethylene oxide during thepreincubation, BMO was fully labeled by [U^{14}C] acetylene. This result was consistent with the observation that butane protects BMO from inactivation.

Material derived from about the same amount of cells was loaded in the whole cell and extract lanes. In the samples not exposed to ethylene oxide (lanes 1 and 6), the labeling intensity was similar between the whole cells and cell-free extracts. However, the rate of ethylene oxide formation in whole cells was 13.6 times higher than that from cell free extracts with a similar amount of cell material. These results indicate that the amount of active enzyme remaining after cell breakage is similar to that in whole cells, but the specific activity is diminished in the extracts.

3.4.5. Effect of various epoxides on ethylene oxide production in P. butanovora

The effects of various epoxides on ethylene oxide production were tested for butane-grown *P. butanovora.* Cells were exposed to each epoxide for six minutes, washed and an ethylene oxide activity assay was performed. The activity of cells incubated for six minutes with no addition of epoxide or butane was taken as 100% activity (Table 3.2). When the cells were incubated in the presence of 10% butane, activity was 19% higher than the samples that had neither butane nor epoxide, which indicated that the untreated sample had lost some activity under the oxidizing conditions. Cells incubated anaerobically also showed greater BMO activity than those incubated in air (data not shown).

With regard to the different epoxides, ethylene oxide and epoxypropane were the most effective inactivators, followed by 1,2-epoxybutane and 1,2-epoxyhexane. *Cis*-2,3-epoxybutane did not decrease BMO activity unless very high concentrations (380 mM) were used (Table 3.2). *Trans*-2,3-epoxybutane treated cells actually had an activity greater than the 100% control (no epoxide or butane), which raises the possibility that this compound may have helped to protect BMO from auto-inactivation in a manner similar to butane.

TABLE 3.2. Effect of various epoxides on BMO activity in butane-grown *P. butanovora.*

Epoxide	% BMO activity recovered[a] from washed cells after 6 minute incubations in the presence of epoxide				
	0.38 μM	38 μM	3.8 mM	380 mM	50% Butane
Ethylene Oxide	48.0 ± 2.9	3.9 ± 1.3			
Epoxypropane	54.6 ± 3.2	16.8 ± 0.2			
1,2-Epoxybutane[b]		42.8 ± 1.4	28.9 ± 5.7		
1,2-Epoxyhexane[b]		78.9 ± 5.1	48.3 ± 3.6		
Cis-2,3-epoxybutane[b]			100.0 ± 12.9	9.0 ± 3.6	123.4 ± 21.7
Trans-2,3-epoxybutane[b]			118.8 ± 19.7	111.3 ± 25.3	123.4 ± 21.7

[a] Activity was measured using the ethylene oxide production assay. The 100% control consisted of cells and butyrate with no butane where as the 50% butane column represents those samples which had 50% (vol/total vol) butane present during the pretreatment. Data are expressed as means ± standard deviations.

[b] The epoxide concentration shown represents the assumption that all of the epoxide is in the liquid phase.

3.4.6. Effect of ethylene oxide on other monooxygenase-containing bacteria

Additional bacteria that produce monooxygenases to initiate the degradation of growth substrates were tested for their sensitivity to ethylene oxide. The bacteria consisted of butane-grown *Nocardiodes* CF8 and *Mycobacterium vaccae* JOB5, which expressed butane monooxygenase; octane-grown *Pseudomonas oleovorans*, which expressed alkane hydroxylase; toluene-grown *Burkholderia cepacia* G4, which expressed toluene-2-monooxygenase; and ammonia-grown *Nitrosomonas europaea,* which expressed ammonia monooxygenase. Cells were grown under specific conditions to express each monooxygenase and then exposed to ethylene oxide at concentrations up to 3.8 mM. During this pretreatment, a source of reductant was included to support enzyme turnover. The cells were then washed and the rate of consumption of the growth substrate or growth substrate dependent O_2 uptake was determined (Table 3.3). Consumption of the growth substrate requires the activity of the monooxygenases indicated above. The rates of growth substrate consumption or O_2 uptake by *B. cepacia* G4, *N. europaea*, and *P. oleovorans* were not affected by 3.8 mM ethylene oxide, which indicates that the monooxygenases were not inactivated. In *Nocardiodes* CF8 and *M. vaccae* JOB5 the rates of butane consumption (*Nocardiodes* CF8) or ethylene oxide production

TABLE 3.3. Effect of ethylene oxide on other monooxygenases[a].

Bacterium (enzyme studied)	% Monooxygenase activity recovered[b] from washed cells after 6 minute incubations in the presence of exogenous reductant and the following concentrations of inactivators.					
	Ethylene Oxide (0.38 μM)	Ethylene Oxide (38 μM)	Ethylene Oxide (3.8 mM)	2-Hexyne (4.4 mM[c])	Acetylene (430 μM)	Heat Killed
P. butanovora (BMO)	48.0 ± 2.9	3.9 ± 1.3			5.7 ± 1.4	
Nocardiodes sp. CF8 (BMO)			61.0 ± 3.3		< 1.0	
Mycobacterium vaccae JOB5 (BMO)			51.5 ± 2.0		9.9 ± 2.4	
Pseudomonas oleovorans (AlkS)			105.1 ± 1.9			2.8 ± 0.7
Burkholderia cepacia G4 (T2MO)			93.27 ± 5.5	19.8 ± 1.8		
Nitrosomonas europaea (AMO)			90.1 ± 1.4		9.3 ± 3.8	

[a] Bacteria were grown to express a certain monooxygenase (in parentheses), which was then tested for its resistance to ethylene oxide. A negative control was included for each system studied.

[b] Activity was tested by oxygen uptake or gas chromatography (see methods). 100% represents the activity obtained in cells not exposed to epoxide. Data are expressed as means ± standard deviations.

[c] Concentration represents the assumption that all 2-hexyne is in the liquid phase.

(*M. vaccae* JOB5) were reduced 39% and 50% respectively after exposure to 3.8 mM ethylene oxide. These results are in stark contrast to *P. butanovora*, which lost half of its ethylene oxide production activity after being exposed to only 0.38 μM ethylene oxide (Table 3.3).

3.5. DISCUSSION

Butane oxidation by *P. butanovora* is remarkably sensitive to inactivation by ethylene oxide. This effect appears to be specifically directed to the butane monooxygenase as based on several lines of evidence. 1) Ethylene oxide irreversibly inactivates butane-oxidizing activity in *P. butanovora* whole cells (Figures 3.1, 3.6) with no effect on 1-butanol degradation (Figure 3.3) or butyraldehyde consumption (data not shown). Because 1-butanol is the product of butane oxidation by butane monooxygenase [6], this result indicates that metabolism steps downstream of butane high concentrations (25% vol/total vol), lead to production of more ethylene oxide for a longer period of time than the identical sample with less ethylene (Figure 3.2). This difference was not due to a shortage of ethylene in the 0.5% sample because the ethylene oxide produced represents less than 3.5 percent of the original ethylene in the vial and ethylene concentrations did not decrease in any of the vials (data not shown). Again,

substrate protection of the active site could explain the extended activity in the samples with 50% ethylene.

Another criterion for mechanism based inactivators is the requirement for catalytic turnover. When O_2 was omitted from the reaction mixture, ethylene oxide did not affect butane oxidation (Figure 3.1), which indicates that catalytic turnover leading to transformation of ethylene oxide was required for inactivation. The inactivation should also be irreversible. Simply removing the ethylene oxide did not restore butane-oxidizing activity. While activity was eventually recovered, this recovery required protein synthesis, so the criterion of irreversibility was met.

Two more requirements for mechanism-based inactivation are a time dependence on the extent of inactivation, and a dependence of the rate of inactivation on the concentration of the inactivating molecule. The data in table 3.1 clearly show the extent of BMO inactivation to be dependent on the exposure time. Because the inactivation was rapid relative to the exposure time required to remove ethylene oxide from the cells (5 minute wash cycle), it was difficult to determine a kinetic mechanism for the loss of activity. The loss of BMO activity was also affected by the concentration of ethylene oxide to which the cells were exposed. While not all criteria for a mechanism-based inactivator were tested, those we examined were met.

Stanley et al. found pMMO activity from *Methylococcus capsulatus* (BATH) to be irreversibly inactivated by propylene oxide [74]. They found whole cell propylene oxidizing activity (pMMO oxidizes propylene to propylene oxide) was irreversibly inactivated after addition of propylene oxide and a source of reductant, while ammonium chloride, a substrate for pMMO preserved propylene oxidizing activity. In addition, they found the inactivation to be dependent on the exposure time to the inactivator. The authors suggested mechanism-based inactivation of pMMO by propylene oxide.

Inactivation of cellular activities that require a monooxygenase is not unique to *P. butanovora*. In addition to the previously mentioned inactivation of pMMO in *M. capsulatus* (BATH), propylene oxidizing activity in *Methylosinus trichosporium* whole cells and cell free extracts was reduced 50% after exposure to 5 mM propylene oxide [72]. *Mycobacterium* E20, presumed to contain alkene and alkane monooxygenases, was also affected by epoxypropane. When exposed to 90 μM epoxypropane for 15 minutes, the cells lost 50% of their propene oxidizing activity and 85% of their ethane oxidizing activity. In contrast, *Mycobacterium* E3 grown on ethene lost only half of its activity when exposed to 10 mM ethylene oxide for 15 minutes [72].

Several additional bacteria were tested in this work for their sensitivity of their growth-substrate monooxygenases to ethylene oxide. These bacteria contained a wide array of monooxygenases including alkane, ammonia, and toluene-2-monooxygenases. Among these, *Nocardiodes* CF8 [11] and *N. europaea* [29] are thought to contain copper in their active sites, and *P. oleovorans* [82] and *B. cepacia* G4 [83] contain non-heme diiron centers. Among the systems tested, only BMO in *M vaccae* JOB5 and *Nocardiodes* CF8 exhibited substantial sensitivity to ethylene oxide as they lost 50% and 39% of their activity respectively after exposure to 3.8 mM epoxide (Table 3.3). In contrast to *M. vaccae* JOB5, *Nocardiodes* CF8 and the examples found in the literature, BMO activity in *P. butanovora* is much more sensitive to epoxides. Butane oxidation is reduced 50% after a 6 minute exposure to only 380 nM ethylene oxide (Table 3.2), which is a concentration that is more than three orders of magnitude less than is required to inactivate the monooxygenase system in *Mycobacterium* E20.

Ethylene-grown *Mycobacterium* E3 was monitored after exposure to ethylene oxide, 1,2-epoxypropane and 1,2-epoxybutane. Ethylene degradation activity was found to be more susceptible to attack by smaller epoxides [72]. When BMO in *P. butanovora* was analyzed for sensitivity to other epoxides, a similar trend was observed. The extent of inactivation depended on the chain length and

location of the epoxide. Interestingly, only the 1,2-epoxides appeared to decrease BMO activity and the size of the epoxide was inversely proportional to the amount of inactivation observed (Table 3.2). Perhaps the shorter chain length and terminal position allows the epoxide easier access to the active site of the enzyme. *Trans*-2,3-epoxybutane treatment, like butane treatment, actually resulted in activities higher than those observed in controls with no additions. Apparently, these compounds protected the monooxygenase from the autooxidative damage that occurs in the presence of O_2 but absence of a substrate.

Previous studies have shown [$U^{14}C$] acetylene labeling to be an effective way of monitoring active monooxygenase levels in *P. butanovora, M. vaccae, Nocardiodes* CF8 [11, 20], *N. europaea* [12], and *Methylococcus capsulatus* (BATH) [13]. Acetylene is a mechanism-based inactivator of BMO that binds to a 58 kDa polypeptide [11, 20]. This 58 kDa band is thought to contain the active site of BMO and because labeling requires enzyme turnover, less labeling can be translated to less active enzyme in the cell. Ethylene oxide exposure decreased the amount of [$U^{14}C$] labeling in the 58 kDa band in whole cells and cell-free extracts (Figure 5). However, cell-free extracts required ten-fold more ethylene oxide for the same inactivation as seen in whole cells. During the cell breakage process, it is possible that a vital component of the enzyme is disrupted. This could lead to

decreased enzyme activity, while the total amount of active enzyme per cell remains unchanged. The lower enzyme activity would decrease ethylene oxide turnover, which would lead to fewer inactivation events in a given time period. This explanation is supported by the fact that the same amount of BMO was loaded into each lane and the total amount of label (e.g. active enzyme) was the same between whole cells and cell-free extracts (Figure 5, lane 1 and 6). However, *in vivo* activity was reduced by 95% after cell breakage and centrifugation.

The data presented in this paper provide substantial support for the hypothesis that ethylene oxide is a mechanism-based inactivator of butane monooxygenase from *P. butanovora*. The increasing number of cases of monooxygenase inactivation by an epoxide suggests this occurrence may be widespread. However the *P. butanovora*:BMO system is considerably more sensitive to epoxides than any other bacterium we have tested or found in the literature.

Chapter 4. CONCLUSION TO THESIS

The ability of microorganisms to cometabolize non-growth compounds has received considerable attention for the possible degradation of environmental pollutants and elemental cycling throughout the environment. Specifically, the degradation of methane and ethylene is important because both compounds are greenhouse gases that can contribute to global warming. The degradation of methane by methanotrophic organisms has been well studied, however methane cooxidation by non-methanotrophs other than autotrophic ammonia-oxidizing bacteria [27] has not previously been demonstrated. This work also failed to demonstrate significant methane oxidation by three butane-grown bacteria.

Ethylene, on the other hand, is a compound commonly degraded by methane, ammonia, alkene and alkane-oxidizing bacteria [50-54]. In this work, three butane-grown bacteria expressing butane monooxygenase degraded ethylene to ethylene oxide. Ethylene concentrations used in these experiments were over 100 μM. Environmental concentrations of ethylene are generally less than 50 nM [19], so it is unknown if these butane-grown organisms would degrade ethylene under *in situ* conditions.

The oxidation of ethylene to ethylene oxide lead to inactivation of butane monooxygenase in *P. butanovora*. Further work implicated ethylene oxide as a possible mechanism-based inactivator of butane monooxygenase based on several lines of evidence such as irreversibility, time and concentration dependence, substrate protection and the requirement of oxygen. These results appeared similar to the inactivation of pMMO in *M. capsulatus* (BATH) [74], which is irreversibly inactivated by propylene oxide. However, the *P. butanovora*:BMO system is much more sensitive to lower epoxide concentrations than the *M. capsulatus*:pMMO system. Also intriguing is the observation that small, terminal epoxides are the most efficient inactivators of BMO in *P. butanovora*. This effect has been observed in one other instance (*Mycobacterium* E3 [72]. This could be due to the manner in which the epoxide can associate with the active site of the monooxygenase or selective cellular uptake of short terminal epoxides. The cometabolism of ammonia by three butane-grown bacteria was also studied. It was found that *Nocardiodes* CF8 and *P. butanovora* produced both hydroxylamine and nitrite from ammonia and *M. vaccae* JOB5 accumulated only nitrite. The presence of butane and acetylene in the reaction mixtures inhibited product formation in all cases, indicating butane monooxygenase was responsible for the oxidation of ammonia. Hydroxylamine production in *P. butanovora* reached initial rates of over

50 nmol/min•mg protein. This rate is comparable to hydroxylamine production by methanotrophs and some heterotrophic nitrifiers, however ammonia-oxidizing bacteria, such as *N. europaea* will produce hydroxylamine at rates of over 1 μmol/min•mg [25, 26, 67].

After 5-10 minutes, hydroxylamine production in P. butanovora slowed considerably. Hydroxylamine concentrations over 200 μM were obtained, so it is possible this was due to the toxic effects of hydroxylamine, which is a known mutagen. Hydroxylamine concentrations of 100μM are sufficient to inhibit ammonia oxidation in methanotrophs, while concentrations of over 1 mM are required to inhibit autotrophic ammonia-oxidizing bacteria [26].

It is unknown if the three butane-grown bacteria tested will effectively nitrify under *in situ* conditions, given the experiments were run under more ideal laboratory settings. However, if these bacteria are representative of other monooxygenase containing soil isolates, their contribution to soil nitrification rates would likely not be insignificant.

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