AN ABSTRACT OF THE THESIS OF

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Chloroform (CF) degradation by three butane-grown bacteria, *Pseudomonas butanovora, Mycobacterium vaccae* JOB5, and an enrichment culture CF8, were studied and the enzymes responsible for this cometabolic process were characterized. All three butane-grown bacteria were able to degrade CF. The degradation rates were comparable to that of methane-grown *Methylosinus trichosporium* OB3b, a known CF degrader. CF degradation was correlated with chloride release, although only about 2 Cl^- were released per chloroform degraded. CF degradation by all four bacteria required O_2. Butane inhibited CF degradation by the butane-grown bacteria. These results suggest that butane monooxygenase is responsible for CF degradation. The addition of exogenous reductant was required for CF degradation by *P. butanovora,* but not for CF8 or *M. vaccae.* The decreased CF degradation rates were observed with time probably due to CF toxicity. CF8 and *P. butanovora* were more sensitive to CF toxicity than *M. trichosporium* or *M. vaccae.* Effects of known monooxygenase inhibitors on CF degradation by three butane-grown bacteria revealed the differences among their monooxygenases. Acetylene irreversibly inactivated CF degradation by all three butane-grown bacteria. The presence of butane protected the cells from inactivation by acetylene. Incubation of butane-grown cells with [^{14}C]acetylene resulted in the labeling

of polypeptides, suggesting that acetylene is a mechanism-based inactivator for butane monooxygenases. *P. butanovora* was also inactivated by ethylene, an inactivator of P-450 monooxygenases. CF8 was strongly inhibited by allylthiourea, a copper selective chelator. The K_s (the apparent K_m observed in intact cells) for butane was estimated as 14.07 ± 8.49, 47.75 ± 4.87, and 17.57 ± 5.43 μM for CF8, *P. butanovora,* and *M. vaccae,* respectively. In addition to CF, CF8 and *P. butanovora* were able to degrade other chlorinated hydrocarbons, including trichloroethylene, 1,2-*cis*-dichloroethylene, and vinyl chloride. CF8 also degraded 1,1,2-trichloroethane. The three butane-grown bacteria examined in this study possess distinct monooxygenases that can degrade a range of chlorinated hydrocarbons.

Chloroform Cometabolism by Butane-grown Bacteria:

Diversity in Butane Monooxygenases

by

Natsuko Hamamura

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Chapter 1

Introduction

This research investigated the potential of using butane as a growth substrate for the aerobic cometabolism of chloroform (CF). CF degradation by a butane-grown enrichment culture, CF8, was compared to that of butane-grown *Pseudomonas butanovora* and *Mycobacterium vaccae* JOB5 and methane-grown *Methylosinus trichosporium* OB3b, which is a known CF-degrader. Potential advantages of butane as a growth substrate are that it is highly soluble in water, inexpensive, and readily available. The diversity of butane monooxygenases in three butane-grown bacteria was further investigated.

1.1 Chloroform biodegradation

Chloroform (CF) is an example of chlorinated aliphatic hydrocarbons (CAHs), which are common environmental contaminants. CF has been used as an industrial solvent and conveyed into the environment by accidental spillage, leaking storage tanks, improper disposal, and landfill leaching. CF is also a common dehalogenation product of carbon tetrachloride in anaerobic aquifers (Egli *et al.*, 1987). CF has been implicated as a potential carcinogen (Infante & Tsongas, 1982).

Under anaerobic conditions, CF is reductively transformed to less-chlorinated intermediates and carbon dioxide (Mikesell & Boyd, 1990; Vogel & McCarty, 1985).

The anaerobic treatment is unacceptable because the reaction intermediates are also toxic and persistent. Aerobic biodegradation of CF by methanotrophic organisms (Alvarez-Cohen & McCarty, 1991; Bowman *et al.*, 1993; Speitel Jr. *et al.*, 1993), ammonia-oxidizing bacteria (Rasche *et al.*, 1991), and toluene-oxidizing bacteria (McClay, 1996) was demonstrated.

1.1.1 Reductive dechlorination of chloroform

Under anaerobic conditions, CF is reductively dechlorinated to dichloromethane (DCM). This reaction is one of the major CF transformation pathways. A high percentage of CF reduction to DCM was reported with two methanol-grown *Methanosarcina* species (Mikesell & Boyd, 1990), fructose-grown *Acetobacterium woodii* (Egli et al., 1987), and *Clostridium* species grown on yeast extract and tryptone (Galli & McCarty, 1989). Several metallocofactors, including cyanocobalamin (vitamin B12), have been shown to catalyze reductive dechlorination of CF and other aliphatic compounds (Krone *et al.*, 1989). Because DCM is also a hazardous compound, the reductive dechlorination treatment of CF is questionable. However, in the absence of CF, DCM can be anaerobically biodegraded to environmentally harmless products. Freedman and Gossett (Freedman & Gossett, 1991) enriched a culture using DCM as a growth substrate. A portion of the DCM was oxidized to CO_2 plus H_2 with the remaining fermented to acetate. The effects of cyanocobalamin on CF degradation were examined by using this DCM-grown enrichment culture (Becker, 1994). In the absence of cyanocobalamin, [^{14}C]CF was transformed into $^{14}CO_2$ and [^{14}C]DCM. More CF was reduced to DCM when CF was biodegraded in the presence of a large amount of DCM. By the addition of cyanocobalamin, the CF biodegradation rate increased approximately 10-fold. The extent of CF oxidation to CO_2 also increased and the accumulation of DCM was eliminated. When cyanocobalamin was present, as much as 10% of the [^{14}C]CF

transformed accumulated as ^{14}C-labeled carbon monoxide, suggesting that the oxidation of CF to CO_2 proceeds via net hydrolysis to CO. These results suggest that the use of cyanocobalamin might be effective for *in situ* anaerobic bioremediation at CF-contaminated sites. However, further study needs to be done before proceeding with field testing.

1.1.2 Aerobic cometabolism of chloroform

Aerobic degradation of CF and trichloroethylene (TCE) in a soil column fed natural gas and air was demonstrated by Wilson and Wilson (Wilson & Wilson, 1985). Since that time there have been many reports of CAH transformation by microbes grown on different cometabolic substrates. Methanotrophs, ammonia oxidizers, and toluene oxidizers have been reported to carry out CF degradation.

1.1.2.1 Methanotroph

The methane-utilizing bacterium *Methylosinus trichosporium* OB3b cometabolizes a number of CAHs, including CF and TCE (Oldenhuis *et al.*, 1991; Speitel Jr. et al., 1993; Tsien *et al.*, 1989). *M. trichosporium* OB3b produces two forms of methane monooxygenases (MMOs) depending on the copper content in the medium. The soluble enzyme (sMMO), which is expressed under conditions of copper limitation, shows a broader substrate range (Stanley *et al.*, 1983). Oldenhuis et al. (Oldenhuis *et al.*, 1989) determined whether the ability of *M. trichosporium* to degrade CAHs was related to a specific type of MMO. Degradation of CAHs was tested with cells of *M. trichosporium* OB3b grown in continuous culture with and without copper in the medium. When added at a concentration of 0.2 mM, CF, TCE, dichloromethane, 1,1-dichloroethane, 1,2-dichloromethane, *trans*-1,2-dichloroethylene, *cis*-1,2-dichloroethylene, and 1,2-dichloropropane were completely degraded by cells grown

under copper limitation. Of these compounds, CF, TCE, dichloromethane, 1,1-dichloroethane, and 1,2-dicloroethane degradation accompanied the release of stoichiometric amounts of Cl^-. Under the same conditions, 1,1,1-trichloroethylene, 1,1-dichloroethylene, and 1,3-dichloropropylene were partially degraded (62, 40, and 85%, respectively). Only 1,2-dichloroethane and *trans*-1,2-dichloroethylene were completely degraded by cells grown with copper in the medium. Oldenhuis et al. (Oldenhuis et al., 1989) proposed that apart from the perchloro compounds, all chlorinated aliphatic hydrocarbons (C_1 to C_3) may be degraded by a methanotroph producing sMMO, which is much more active toward most of these compounds than the pMMO.

The kinetics of CF and TCE degradation by *M. trichosporium* OB3b were determined at relatively low CF and TCE concentrations (100 μg/liter for CF and around 1 mg/liter for TCE) by Speitel et al (Speitel Jr. et al., 1993). These concentrations would be practical for the applications involving ground water contamination or drinking water treatment. Cells were grown under conditions that selected for sMMO production. Their reported pseudo first-order rate constants ranged from 0.2 to 0.4 liter (mg of total suspended solids × day)$^{-1}$ for CF and from 0.5 to 3.3 liters (mg of total suspended solids × day)$^{-1}$ for TCE in the absence of methane. The addition of formate (100 mg/liter) as an electron donor did not improve the CF degradation rate, possibly because of the short duration of the experiments. The presence of methane (0.35 mg/liter) decreased the CF rate constant through the enzyme competition. The decreases in the rate constant were consistent with an enzyme competitive inhibition model. The result from [^{14}C]CF experiment showed that of the chloroform removed, in excess of 90% was converted to carbon dioxide.

Oldenhuis et al. (Oldenhuis et al., 1991) examined the kinetics of the degradation of CAHs including CF and TCE by *M. trichosporium* OB3b at concentrations in the range of 1 mg/liter to 10 mg/liter. Cells were grown under copper stress and thus

expressing sMMO. The initial degradation rates at various concentrations were measured for eight different chlorinated aliphatic compounds, then the data were transformed for the determination of kinetic parameters. CF, *trans*-1,2-dichloroethylene, and TCE, was readily degraded and V_{max} values were 550, 330, and 290 nmol min^{-1} mg of $cells^{-1}$, respectively. Dichloromethane and 1,1-dichloroethylene were poor substrates and showed the lowest K_m values. K_m values for CF and *cis*-1,2-dichloroethylene (35 and 30 μM, respectively) were lower than for methane (90 μM). The first-order rate constant (V_{max}/K_m), shown as k_1, for methane (4 ml min-1 mg of cells-1) was lower than that for dichloromethane, CF, and *cis*-1,2-dichloroethylene. The k_1 varied from 0.1 ml min^{-1} mg of $cells^{-1}$ for 1,1,1-trichloroethane to 16 ml min^{-1} mg of $cells^{-1}$ for CF. This low k_1 value suggested that 1,1,1-trichloroethane was a very poor substrate.

Maximum allowable substrate concentrations were determined for eight different CAHs (Oldenhuis et al., 1991). Below these concentrations, Michaelis-Menten kinetics were found while above the concentrations, a significant deviation occurred because of the toxicity of the chlorinated substrates. Maximum allowable concentrations were calculated for densities of 0.1 mg of cells ml^{-1}. 1,1-Dichloroethylene showed toxicity at very low concentrations (20 to 30 μM), while CF and *cis*-1,2-dichloroethylene did not show toxicity up to high concentrations (185 and 150 μM, respectively). Maximum allowable concentrations for TCE, *trans*-1,2-dichloroethylene, and 1,2-dichloroethane were around 55 to 75 μM. The TCE toxicity during its degradation was studied in detail (Oldenhuis et al., 1991). The TCE degradation rate decreased with time as a result of inactivation of cells or MMO. To determine whether the inactivation of MMO is due to a specific binding of TCE to MMO or to a nonspecific binding of TCE degradation products to cellular components, *M. trichosporium* OB3b cells were incubated with [^{14}C]TCE. When cells were grown under copper limitation thus expressing sMMO, various proteins became radiolabeled, including the α-subunit (54.4 kDa) of the

hydroxylase component of sMMO (Oldenhuis et al., 1991). The β- and γ-subunits of the hydroxylase were not labeled, suggesting that the [^{14}C]TCE binding was nonspecific. None of the proteins were specifically labeled when cells were grown in the presence of copper. From these results, it is shown that the TCE toxicity is mediated by a nonspecific binding of TCE degradation products to cellular components, including the sMMO hydroxylase component.

The toxicity of CF and its transformation products was studied by using a mixed methanotrophic culture (Alvarez-Cohen & McCarty, 1991). The transformation rate and transformation capacity (*Tc*) for CF were 0.34 day^{-1} and 0.0083 mg of CF mg of cells^{-1}, respectively. The addition of formate increased the rate and *Tc* to 1.5 day^{-1} and 0.015 mg of CF mg of cells^{-1}, suggesting that the reductant limitation is one of the factors affecting transformation capability of the cells. In order to determine whether CF itself or its degradation products cause toxicity, cells were treated with acetylene prior to exposure to CF. Acetylene, an inactivator of MMO, inhibits CF degradation without affecting other cell functions. The activity of cells can be evaluated by measuring their formate oxidation because this reaction is catalyzed by formate dehydrogenase which is not inactivated by acetylene. Formate oxidation by acetylene-treated cells was compared to non-acetylene-treated cells with and without prior exposure to CF. The formate oxidation activity of cells exposed to CF without acetylene was lower than that of cells treated with acetylene. This result suggests that CF itself is not toxic to cells and that CF toxicity is caused by its transformation products.

1.1.2.2 Ammonia-oxidizing bacteria

The soil nitrifying bacterium *Nitrosomonas europaea* is known to degrade CF and other CAHs (Rasche *et al.*, 1990b; Rasche et al., 1991). *N. europaea* is an obligate chemolithoautotroph which obtains all of its energy from the oxidation of ammonia to

nitrite. Ammonia monooxygenase (AMO) initiates the ammonia oxidation through reductant-dependent process to produce hydroxylamine. It has been reported that AMO in *N. europaea* is able to catalyze CAHs (Arciero *et al.*, 1989; Rasche et al., 1990b). The molar ratio of chloride iron released to CF degraded was 2.2(Rasche et al., 1991), suggesting incomplete dehalogenation of CF. Rasche et al. (Rasche et al., 1991) observed that *N. europaea* cells lost TCE degradation capability and ammonia-oxidizing activity during TCE degradation. In the presence of allylthiourea, a specific inhibitor of AMO, or under anaerobic conditions, this inactivation did not occur. This result suggests that the TCE-mediated inactivation required AMO activity. Incubation of the cells with [^{14}C]TCE resulted in incorporation of ^{14}C label into a number of cellular proteins.

In addition to TCE, other CAHs were also examined for AMO turnover-dependent inactivation of ammonia oxidation. Rasche et al. (Rasche et al., 1991) proposed that CAHs fall into one of three classes based on their biodegradability and toxicity. Class I compounds were not biodegradable by *N. europaea* and had no toxic effect of the cells, class II compounds were cooxidized by *N. europaea* and had little or no toxic effect on the cells, and class III compounds which were cooxidized and produced a turnover-dependent inactivation of ammonia oxidation by *N. europaea.* Both CF and TCE were class III compounds.

1.1.2.3 Toluene-oxidizing bacteria

Recently, McClay et al. (McClay, 1996) examined CF degradation ability by seven toluene-oxidizing bacterial strains (*Pseudomonas mendocina* KR1, *Burkholderia cepacia* G4, *Pseudomonas putida* F1, *Pseudomonas pickettii* PKO1, *Pseudomonas* sp. strains ENVPC5, ENVBF1, and ENV113). Of these organisms, *P. mendocina* KR1, *Pseudomonas* sp. strain ENVPC5, and ENVBF1 were able to degrade CF. When these three strains were incubated with [^{14}C]CF, ^{14}C was detected from CO_2 (~30 to 57% of

the total products), soluble metabolites (~15%), particulate fraction (~30%), and chloride ions (~75% of the expected yield). The level of ^{14}C activity in the particulate fractions of the culture did not decrease during the prolonged incubation, suggesting that ^{14}C label bound irreversibly to cellular components. The rate of CF degradation was greatest in *Pseudomonas* sp. strain ENVBF1 (1.9 nmol [min x mg of cell protein]$^{-1}$). CF degradation rates for *P. mendocina* KR1 and strain ENVPC5 were 0.48 and 0.49 nmol [min x mg of cell protein]$^{-1}$, respectively. CF was also oxidized by *Escherichia coli* DH510B (pRS202), which contained cloned toluene 4-monooxygenase genes from *P. mendocina* KR1, at rate of 0.16 nmol [min x mg of cell protein]$^{-1}$. It has been shown that toluene monooxygenases in *P. mendocina* KR1 is able to degrade TCE (Winter *et al.*, 1989). CF oxidation by each strain was inhibited in the presence of TCE, and acetylene, a known monooxygenase inhibitor, inhibited TCE oxidation by *P. mendocina* KR1. These results suggest that toluene monooxygenase is responsible for CF degradation.

1.2 Alkane oxidation

Microorganisms utilize a wide range of saturated and unsaturated hydrocarbons as growth substrates. In the case of bacteria, these organisms mainly belong to the genera *Mycobacterium, Nocardia, Corynebacterium, Brevibacterium,* and *Pseudomonas* (Klug & Markovetz, 1971). *n*-Alkanes are oxidized to the corresponding primary or secondary alcohol through either the monoterminal oxidation; biterminal oxidation, or subterminal oxidation pathway. Both terminal oxidations lead to production of carboxylic acids which are further oxidized by fatty acid degradation pathways. In all cases, *n*-alkane oxidation is catalyzed by hydroxylases (monooxygenases), although the enzymes might be different.

1.2.1 Methane oxidation

The oxidation of methane to methanol in methanotrophic bacteria is catalyzed by the enzyme methane monooxygenase (MMO). This multicomponent enzyme catalyses the oxidation of a broad range of compounds including aliphatic and aromatic hydrocarbons. MMO is unique in that a single organism can produce soluble (sMMO) and particulate (pMMO) forms of the enzyme. The methanotrophs are divided into three classes depending on the type of MMO produced and metabolic pathways. Type I methanotrophs express only pMMO and utilize the condensation of ribulose 5-phosphate with fomaldehyde to initiate carbon assimilation. Type II and type X methanotrophs are capable of expressing either sMMO or pMMO depending on the copper concentration of the growth medium (Stanley et al., 1983). Type X methanotrophs utilize the same carbon assimilation pathway as type I organisms, while the type II organisms use a pathway based on the addition of formaldehyde to glycine to form serine.

The sMMO complex in the Type II methanotroph, *Methylosinus trichosporium* OB3b, has been characterized. The enzyme consists of three protein components (Fox *et al.*, 1989; Fox *et al.*, 1988). Component A (245 kDa) is composed of three subunits, α, β, and γ (54, 43, and 23 kDa, respectively). This protein is a hydroxylase component of sMMO, containing nonheme iron believed to be the active site of MMO (Fox et al., 1989; Fox et al., 1988). Similar diiron clusters are found in several important enzymes, including ribonucleotide reductase(Nordlund *et al.*, 1990; Petersson *et al.*, 1980) and fatty acid desaturases (Fox *et al.*, 1993). Component B (15.8 kDa) contains no metals or cofactors, acting as an effector of electron transfer in the catalytic mechanism. Component C (39.7 kDa) is a NADH reductase containing FAD and a [Fe_2-S_2] cluster (Fox et al., 1989)). sMMO has a very broad substrate specificity and it also catalyzes the oxidation of a wide range of alkanes, alkenes, ethers, chlorinated aliphatics, some aromatics, and heterocycles (Colby *et al.*, 1977). The structural genes encoding the α,

β, and γ subunits of sMMO protein and the structural gene encoding component B from *M. trichosporium* OB3b have been isolated and sequenced (Cardy *et al.*, 1991).

Particulate MMO is produced under conditions of copper sufficiency. pMMO has not been purified to homogeneity, however the stabilization of particulate enzyme in cell extracts of *M. trichosporium* OB3b has been reported (Tonge *et al.*, 1977). Three components were identified as a soluble CO-binding cytochrome *c* (13 kDa), a copper protein (47 kDa), and a small protein (9,400 Da). Recently, an active preparation of pMMO from *Methylococcus capsulatus* (Bath) was isolated by Zahn and DiSpirito (Zahn & DiSpirito, 1996). This preparation of pMMO consisted of three polypeptides with molecular masses of 47, 27, and 25 kDa, and the active enzyme contained 2.5 iron atoms and 14.6 copper atom. 47 and 27 kDa polypeptides contain one nonheme iron atom, one copper atom and one acid-labile sulfur atom. The functions of each component and their interaction are not well known. pMMO is inhibited by a variety of compounds including acetylene, metal chelators, and electron transport inhibitors (Bédard & Knowles, 1989). The use of radiolabeled [^{14}C]acetylene showed that ^{14}C label bound to 27 kDa polypeptide, indicating the active site of pMMO occurred in this protein.

In spite of different physiologies of organisms, pMMO shares many similarities with AMO. Both enzymes consist of at least two membrane-associated polypeptides (approximately 27 and 45 kDa), share similar substrate ranges, are inhibited by acetylene and thiourea, and contain copper at the active site (Nguyen *et al.*, 1994). Similarity of AMO and pMMO has been shown at genetic level. The genes *pmoA* and *pmoB*, encoding 27 and 45 kDa polypeptides in pMMO, share high sequence identity with *amoA* and *amoB*, encoding similar size polypeptides in AMO (Holmes *et al.*, 1995). Holmes et al. (Holmes et al., 1995) analyzed the predicted amino acid sequences from *pmoA* and *amoA* in a variety of methanotrophic and nitrifying bacteria. Amino acid sequence conservation between AmoA and PmoA showed 40% identity with 65%

similarity. Primary and secondary structure of AmoA and PmoA was strongly conserved. These results suggest that pMMO and AMO are evolutionarily related enzymes.

1.2.2 Butane and propane oxidation

Propane- and butane-grown microorganisms were first isolated for the purpose of biomass production from hydrocarbons. The group of propane-utilizing bacteria belong to the genera of *Mycobacterium* and *Nocardia. Corynebacterium, Arthrobacter* and *Brevibacter* are also major users of aliphatic hydrocarbon compounds in nature. McLee et al. (McLee *et al.*, 1972) isolated fifteen bacterial strains and four molds capable of growth on *n*-butane. The bacteria were mostly *Arthrobacter* sp. and *Brevibacter* sp. *Pseudomonas butanovora* utilized C_1-C_8 alkanes and C_2-C_4 alcohols but did not utilize alkenes or sugars (Takahashi, 1980).

Propane is oxidized either by a subterminal pathway to produce 2-propanol which is further oxidized to acetone (Perry, 1980) or by terminal pathway to produce 1-propanol which is further oxidized to propanoate (Stephens & Dalton, 1986). *Mycobacterium vaccae* JOB5 are able to metabolize propane by both pathways, however subterminal oxidation is dominant. In contrast, *n*-butane is oxidized by *M. vaccae* JOB5 by terminal oxidation pathway (Phillips & Perry, 1974). Apparently, subterminal oxidation in *M. vaccae* was limited to propane. The monooxygenases which catalyze the initial oxidation of butane and propane are not well characterized at the molecular level.

1.2.3 Long-chain alkanes oxidation

In addition to three known *n*-alkane degradation pathways catalyzed by hydroxylases (monooxygenases), Finnerty (Finnerty, 1988) proposed a unique pathway for long-chain *n*-alkane oxidation catalyzed by dioxygenases. In this pathway, *n*-alkanes

are oxidized by a dioxygenase to *n*-alkyl hydroperoxide which is further metabolized to the corresponding aldehyde by oxidation of alkyl peroxide. Finnerty (Finnerty, 1977; Finnerty, 1988) observed that long-chain *n*-alkane oxidation in *Acinetobacter* sp. strain HO1-N required molecular oxygen, cells also oxidized peroxy acid, and *n*-alkane hydroxylase activity was not detected. These results suggest the catalytic activity of dioxygenase, however there is no direct biochemical evidence.

Ho Maeng et al.(Maeng *et al.*, 1996) purified from *Acinetobacter* sp. strain M-1a novel *n*-alkane-oxidizing enzyme that requires only molecular. This strain can utilize long-chain *n*-alkanes ranging from C_{13} to C_{44} (Sakai *et al.*, 1994). The purified enzyme was a homodimeric protein with a molecular mass of 134 kDa, contains FAD, and requires Cu^{2+} for its activity. The enzyme showed broad substrate range including *n*-alkanes (C_{10} to C_{30}), *n*-alkenes (C_{12} to C_{20}), and some aromatic compounds with substituted alkyl groups but branched alkane, alcohol, or aldehyde were not utilized. During *n*-alkane degradation, *n*-alkyl hydroperoxide accumulation was detected, and the addition of oxygen radical scavengers did not affect the enzyme activity. These results suggest that the reaction is probably catalyzed by a dioxygenase to produce the corresponding *n*-alkyl hydroperoxide. This supports the existence of an *n*-alkane oxidation pathway catalyzed by a dioxygenase in *Acinetobacter* sp.

The characterization of monooxygenases which oxidize long-chain alkenes has been focused on the alkane hydroxylase of *Pseudomonas oleovorans.* This organism utilizes C_6-C_{12} *n*-alkanes as a sole source of carbon and energy (Baptist *et al.*, 1963). Long-chain alkanes are oxidized by terminal hydroxylation and dehydrogenation to the resulting alkanol. Inducible enzymes encoded on the OCT plasmid catalyze these initial oxidation steps. The OCT plasmid encodes *alk* system, which consists of at least two separate regions, the *alkBFGHFKL* operon and *alkST.* The *alkB-L* operon encodes for most of the enzymes in the alkane oxidation pathway, and its expression is regulated by

alkS (Eggink, 1987; Eggink, 1988). The alkane hydroxylase system, which oxidizes *n*-alkanes to *n*-alkanols, consists of three peptides (AlkB, AlkG, and AlkT). AlkB is an integral cytoplasmic membrane monooxygenase (alkane hydroxylase) (Fish *et al.*, 1983), AlkG is a rubredoxin, and AlkT is a rubredoxin reductase (Eggink *et al.*, 1990). Rubredoxin reductase transfers electrons supplied by NADH to the membrane hydroxylase via the electron carrier rubredoxin.

Recently, Shanklin et al.(Shanklin *et al.*, 1997) showed that AlkB component in alkane hydroxylase contains a dinuclear-iron cluster which is also found in soluble diiron proteins, including hemerythrin, ribonucleotide reductase, and methane monooxygenase. The diiron enzymes do not share significant overall amino acid homology, however they all contain conserved iron-binding motif containing eight His residues (Shanklin *et al.*, 1994). It is suggested that this conserved His residues serve as ligand for the iron(s) present in the active site. Shanklin et al. (Shanklin et al., 1997) suggested that a diiron active site might be prevalent in a diverse family of integral-membrane enzymes.

1.3 Chloroform biodegradation by butane-grown bacteria

1.3.1 Microcosm study

Kim (Kim, 1996) showed the potential use of butane and propane as cometabolic growth substrates for aerobic transformation of CF. The study was conducted by using mixed cultures in microcosms enriched from the Hanford core materials. The mixed cultures in microcosms were grown on a broad range of substrates, including butane, methane, propane, propene, octane, isoprene, and phenol. The high ratio of the mass of growth substrate to the mass of CF added to the microcosms (150 to 200) was added to stimulate the growth of CF-transforming populations. CF transformation was observed in microcosms grown on ammonia, methane, propane, and butane. The largest amount

of CF was transformed by the butane-grown microcosms. The correlation between CF transformation and butane consumption was observed. CF transformation required oxygen. Carbon tetrachloride, which is not a substrate for aerobic cometabolism, was not transformed. These results suggest that an oxygenase enzyme is involved in CF transformation by butane-grown microcosms.

The transformation yields for CF by butane- and propane-grown microcosms were 0.01 mg of CF/mg of substrate, respectively. When those microcosms were grown in the absence of CF, the transformation yields and rates increased, suggesting CF toxicity affecting the growth of microbes. CF transformation was observed at concentrations as high as 1 mg/liter. At low CF concentrations (about 400 and 700 μg/liter), butane uptake was not inhibited by CF, while at high concentration (about 1200 μg/liter), butane uptake appeared to be inhibited. In contrast, the presence of 2 mg /liter of butane in solution (38 μM) did not strongly inhibit CF transformation. CF transformation was accompanied by stoichiometric release of Cl^-. The molar ratio of Cl^- released to CF degraded increased from 2.1 to 3.1 during the incubation time period, indicating dechlorination was completed over time. Butane-grown microcosms also transformed 1,1-trichloroethane (TCA). The molar ratio of Cl^- released to TCA degraded was 2.1, indicating incomplete dechlorination of TCA.

Kim (Kim, 1996) showed that butane is a promising cometabolic substrate for the transformation of chlorinated methanes, chlorinated ethanes, and potentially chlorinated ethenes. In order to further investigate these cometabolic processes, the study with pure culture of butane-utilizers were pursued. From one of the microcosms which showed CF transforming activity, a highly enriched culture, CF8, was obtained with butane as sole carbon and energy source. The predominant cells in CF8 cultures are coryneform and pleimorphic, typically show aggregation of several cells, and stain gram-

positive. In addition to CF8, two other butane-utilizers from the ATCC culture collections were chosen to test their CAH degradation abilities.

1.3.2 *Pseudomonas butanovora*

P. butanovora was isolated from activated sludge, sampled from an oil refining plant (Takahashi, 1980), for the purpose of biomass production from gaseous hydrocarbons. This organism is gram-negative and classified in the denitrifying group of genus *Pseudomonas* based on biochemical characteristics. *P. butanovora* was screened for the ability to grow on ethane, propane, and *n*-butane as a sole carbon source. During its cellular growth, *P. butanovora* accumulates a large amount of extracellular protein, which is considered to be secreted from the cells as one of the metabolic products. *P. butanovora* utilizes alkanes ranging from C_2 to C_9, primary alcohols and carboxylic acids of C_2, C_3, and C_4, and polyvalent alcohols of C_3 and C_4, while alkanes of C_{10} and more, C_1 compounds, normal alkenes, and sugars are not utilized. CAH degradation by *P. butanovora* has not been reported.

1.3.3 *Mycobacterium vaccae* JOB5

Wackett et al. (Wackett *et al.*, 1989)) reported that propane-grown *M. vaccae* JOB5 cooxidize a number of CAHs, including TCE. Because *M. vaccae* is also capable of growth on butane, this bacterium was included in my study of CF degradation by butane-grown bacteria. *M. vaccae* is capable of growth on C_2 to C_{40} *n*-alkanes, various alkenes, aromatics, and several long chain (C_{16} to C_{18}) monohalogenated alkanes (Murphy & Perry, 1987). Propane is metabolized either by subterminal oxidation or terminal oxidation, while *n*-butane is only oxidized via terminal oxidation (Phillips & Perry, 1974).

M. vaccae showed TCE degradation ability only when it was grown under conditions which induce propane-oxidizing activity. In the presence of propane, TCE degradation was inhibited (Wackett et al., 1989). These results suggested that propane monooxygenase is responsible for TCE degradation by *M. vaccae.* The role of the propane monooxygenase on CAH degradation was further studied by Vanderberg and Perry (Vanderberg, 1994). Glucose-grown cells induced the propane monooxygenase upon exposure to propane (Vestal & Perry, 1969)). Induction of the propane monooxygenase was associated with the ability to degrade 1-chlorobutane, which was oxidized to 2-butanol by the subterminal pathway. *M. vaccae* was able to degrade other chloroalkanes, including monochlorinated alcohols, dichlorinated short chain alkanes, and several multiple-substituted compounds such as TCE.

TCE degradation by propane-grown *M. vaccae* was studied in detail (Vanderberg, 1995). At an initial concentration of 37.5 μM TCE, 53% of added TCE was degraded in 72 h. The maximal transformation was 13.1 μmol mg dry mass cells^{-1}. The degradation rate decreased over time, suggesting that TCE or its degradation products were toxic to cells. 25% of the total TCE degraded was transformed into two chlorinated products, 2,2,2-trichloroethanol and trichloroacetaldehyde. TCE oxidation by methanotrophs have been shown to form trichloroacetaldehyde and 2,2,2-trichloroethanol as minor products (< 10%) (Newman & Wackett, 1991), while trichloroacetaldehyde is the major product of TCE oxidation by mammalian P-450 monooxygenases(Miller & Guengerich, 1982). In the absence of TCE, trichloroethanol (6.7 μM) was completely dehalogenated in 72 h. Four products of trichloroethanol degradation did not accumulate in significant amounts(Vanderberg, 1995). These results suggest that neither trichloroethanol nor its catabolic intermediate are toxic to the cells at that concentration.

In this thesis project, the potential of butane as a cometabolic substrate was examined. CF degradation ability of a butane-grown enrichment culture, CF8 was compared to that of butane-grown pure cultures of *Mycobacterium vaccae* JOB5 and *Pseudomonas butanovora*, and methane-grown *Methylosinus trichosporium* OB3b. CF toxicity, substrate competition, and other CAHs degradation were also studied. Several results indicated butane monooxygenases as catalyzing CF degradation by butane-grown bacteria. Effects of known monooxygenase inhibitors on CF degradation by the three bacteria revealed the differences among the three butane monooxygenases. The diversity in butane monooxygenases in three butane-grown cultures was further characterized.

Chapter 2

Chloroform cometabolism by butane-grown CF8, *Pseudomonas butanovora*, *Mycobacterium vaccae* JOB-5 and methane-grown *Methylosinus trichosporium* OB3b

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2.1 Abstract

Chloroform (CF) degradation by a butane-grown enrichment culture, CF8, was compared to that of butane-grown *Pseudomonas butanovora* and *Mycobacterium vaccae* JOB5, and to a known CF degrader, *Methylosinus trichosporium* OB3b. All three butane-grown bacteria were able to degrade CF at rates comparable to *M. trichosporium.* CF degradation by all four bacteria required O_2. Butane inhibited CF degradation by the butane-grown bacteria, suggesting that butane monooxygenase is responsible for CF degradation. *P. butanovora* required exogenous reductant to degrade CF, while CF8 and *M. vaccae* utilized endogenous reductants. Prolonged incubation with CF resulted in decreased CF degradation. CF8 and *P. butanovora* were more sensitive to CF than either *M. trichosporium* or *M. vaccae*. CF degradation by all three butane-grown bacteria was inactivated by acetylene which is a mechanism-based inhibitor for several monooxygenases. Butane protected all three butane-grown bacteria from the inactivation by acetylene which indicates that the same monooxygenase is responsible for both CF and butane oxidation. CF8 and *P. butanovora* were able to degrade other chlorinated hydrocarbons including trichloroethylene, 1,2-*cis*-dichloroethylene and vinyl chloride. In addition, CF8 degraded 1,1,2-trichloroethane. The results indicate the potential of butane-grown bacteria for chlorinated hydrocarbon transformation.

2.2 Introduction

Chlorinated aliphatic hydrocarbons (CAHs) cause serious environmental problems through contamination of ground water, drinking water and soil. In situ bioremediation of CAHs by aerobic cometabolism is a promising method for remediating contaminated sites. Degradation of target compounds occurs because of their fortuitous oxidation by enzymes which function physiologically to initiate the oxidation of growth substrates. Methane (Chang & Alvarez-Cohen, 1996; Oldenhuis et al., 1991; Oldenhuis et al., 1989; Speitel Jr. et al., 1993; Tsien et al., 1989), propane (Wackett et al., 1989), ammonia (Rasche *et al.*, 1990a; Vannelli *et al.*, 1990), and toluene (or phenol) (Chang & Alvarez-Cohen, 1995; McClay, 1996; Wackett & Gibson, 1988) are examples of growth substrates which also support the cometabolism of several CAHs. Trichloroethylene (TCE) is an example of a CAH which has received considerable attention and which is cometabolically degraded by a number of bacteria (Chang & Alvarez-Cohen, 1996; Oldenhuis et al., 1991; Oldenhuis et al., 1989; Rasche et al., 1990a; Speitel Jr. et al., 1993; Tsien et al., 1989; Vannelli et al., 1990; Wackett et al., 1989; Wackett & Gibson, 1988). Chloroform (CF), in contrast, is more recalcitrant. CF is used as an industrial solvent and can be produced in drinking water as a result of chlorination. Because of its toxicity, the presence of CF in drinking water is regulated under the Safe Drinking Water Act Amendments of 1986 (Sayre, 1988).

Although recognized as a recalcitrant compound, some bacteria have been shown to dehalogenate CF through aerobic cometabolic processes. The methane-utilizing bacterium *Methylosinus trichosporium* OB3b cometabolizes a number of CAHs including CF and TCE (Chang & Alvarez-Cohen, 1996; Oldenhuis et al., 1991; Oldenhuis et al., 1989; Speitel Jr. et al., 1993; Tsien et al., 1989). *M. trichosporium* OB3b synthesizes two types of methane monooxygenase (MMO): a particulate enzyme

(pMMO) associated with the extensive internal membrane system, and a soluble enzyme (sMMO). sMMO is expressed under conditions of copper limitation (Stanley et al., 1983). Both types of enzymes catalyze dehalogenation (Dispirito, 1992). However, sMMO has a broader substrate specificity and much greater activity towards TCE (Oldenhuis et al., 1991; Tsien et al., 1989). Speitel et al. (Speitel Jr. et al., 1993) studied the kinetics of CF and TCE degradation by *M. trichosporium* OB3b at initial concentrations of around 0.1 mg/l for CF and 1 mg/l for TCE. Their reported pseudo first-order rate constants ranged from 0.2 to 0.4 l(mg total suspended solids × day)$^{-1}$ for CF and from 0.5 to 3.3 l(mg total suspended solids × day)$^{-1}$ for TCE in the absence of methane. The major degradation product from CF was CO_2. Oldenhuis et al. (Oldenhuis et al., 1991) examined the kinetics of the degradation of TCE and other CAHs by *M. trichosporium* OB3b. Compounds that were readily degraded included CF, *trans*-1,1-dichloroethylene and TCE, with V_{max} values of 550, 330, and 290 nmol min^{-1} mg of cells^{-1}, respectively. 1,1-Dichloroethylene was a very poor substrate. The ammonia-oxidizing bacterium *Nitrosomonas europaea* can degrade CF and other CAHs (Rasche et al., 1990a; Vannelli et al., 1990). Rasche et al. (Rasche et al., 1991) proposed that halogenated hydrocarbons fall into one of three classes based on their biodegradability and inactivating potential: (i) compounds which were not biodegradable by *N. europaea* and which had no toxic effect on the cells; (ii) compounds which were cooxidized by *N. europaea* and had little or no toxic effect on the cells; and (iii) compounds which were cooxidized and produced a turnover-dependent inactivation of ammonia oxidation by *N. europaea.* Both CF and TCE were class III compounds. Rasche et al. (Rasche et al., 1991) suggested that ammonia monooxygenase (AMO) catalyzed the dehalogenation. Recently McClay et al. (McClay, 1996) reported CF degradation ability by seven toluene-oxidizing bacterial strains. Three *Pseudomonas* strains, *P. mendocina* KR1, *Pseudomonas* sp. strains ENVPC5 and ENVBF1

mineralized CF to CO_2. The highest rate of CF oxidation was achieved with *Pseudomonas* sp. strain ENVBF1 (1.9 nmol(min × mg of cell protein)$^{-1}$). CF was also oxidized by *P. mendocina* KR1 (0.48 nmol(min × mg of cell protein)$^{-1}$), and strain ENVPC5 (0.49 nmol(min × mg of cell protein)$^{-1}$). CF oxidation by each strain was inhibited by TCE, and acetylene substantially inhibited TCE oxidation by *P. mendocina* KR1.

Kim (Kim, 1996) showed that butane and propane were effective cometabolic substrates to drive the transformation of CF. These studies were performed with mixed cultures in microcosms enriched from aquifer solids from the Hanford DOE site in Washington. The transformation yields (T_y), representing the amount CF transformed in response to growth substrate utilization, were 0.01 mg CF/mg substrate for butane and propane-utilizers. Potential advantages of butane or propane as growth substrates are that they are highly soluble in water, they are inexpensive and they are readily available. From one of these microcosms, we obtained a highly enriched culture, CF8, which grows on butane. The predominant cells in CF8 cultures are coryneform and pleimorphic, typically show aggregation of several cells, and stain Gram-positive. In this work, we compare the CF-degrading ability of CF8 cultures with that of methane-grown copper-limited cultures of *M. trichosporium* OB3b and butane-grown cultures of *Mycobacterium vaccae* JOB5 and *Pseudomonas butanovora.* Wackett et al. (Wackett et al., 1989) reported that propane-grown *Mycobacterium vaccae* JOB5 cooxidized a number of CAHs including TCE. CF was not studied. Because *M. vaccae* is also capable of growth on butane (Phillips & Perry, 1974), we included *M. vaccae* in our study of CF degradation by butane-grown bacteria. *Pseudomonas butanovora* was isolated for its ability to grow on butane although it can also grow on a variety of alkanes (C2 to C9) and alcohols (C2 to C4) but not alkenes or sugars (Takahashi, 1980). CAH degradation by *P. butanovora* has not been reported. In this work, we show that

cultures of all four bacteria degrade CF. We also show that CF8 and *P. butanovora* can degrade other chlorinated ethanes and ethenes. This is the first report of CAH degradation by pure cultures of butane-grown bacteria.

2.3 Materials and methods

2.3.1 Bacterial strains and growth conditions

Methylosinus trichosporium OB3b (ATCC 35070) was cultured at 30°C in a nitrate mineral salts medium (ATCC media 1306) and grown under copper limited conditions. Cultures were grown in sealed vials (150 ml) containing 50 ml liquid medium and 100 ml air with 50 ml of methane gas added as an overpressure. Methane gas (99.99% purity) was purchased from Airco gases, Murray Hill, NJ. The medium was buffered with phosphate (pH 6.8, 2 mM KH_2PO_4 and 2 mM $Na_2HPO_4 \cdot 7H_2O$). *Pseudomonas butanovora* (ATCC 43655) was cultured in 150 ml sealed vials (50 ml medium) with 7 ml *n*-butane and 5 ml CO_2 added as an overpressure. *N*-butane gas (99.0%) was purchased from Airgas, Inc., Randor, PA. The growth medium (50 ml) consisted of 2 mM $MgSO_4 \cdot 7H_2O$, 400 μM $CaCl_2 \cdot 2H_2O$, 0.01% (wt/vol) yeast extract, and the same trace elements as described previously (Wiegant, 1980). The medium was buffered with phosphate (pH 7.1, 60 mM $(NH_4)_2HPO_4$, 7 mM $Na_2HPO_4 \cdot 7H_2O$, and 15 mM KH_2PO_4). *Mycobacterium vaccae* JOB5 (ATCC 29678) was grown in the *Xanthobacter* Py2 medium described previously (Wiegant, 1980) except that NH_4NO_3 replaced $NaNO_3$, yeast extract was removed, and the pH was adjusted to 7.5. Cultures (50 ml medium) were grown in 150 ml sealed vials with 50 ml *n*-butane and 40 ml O_2 added as an overpressure. CF8 was enriched from Hanford core material by growth on *n*-butane as the only reduced carbon source. CF8 was grown in the same media as for *M. vaccae* with 50 ml *n*-butane added as an overpressure. Cell growth was monitored

by removing a portion of the cultures and measuring optical density at 600 nm (OD_{600}). When cells were harvested, typical OD values for *M. trichosporium* OB3b, *P. butanovora,* CF8, and *M. vaccae* were 0.21, 0.60, 0.56, and 1.13, respectively.

2.3.2 Chloroform degradation assay

Cells were harvested from cultures by centrifugation (6000 x *g* for 10 min), washed twice with the same buffer as in the growth medium, and then resuspended to a constant cell density (based on the optical density). Assays were conducted in 10 ml serum vials sealed with Teflon coated butyl rubber stoppers (Alltech Associates, Inc., Deerfield, IL). Chloroform was added as a diluted aqueous solution which was made fresh daily from a chloroform saturated solution at room temperature (approximately 20 °C). The amount of CF added was estimated from solubility tables (Schwille, 1979). The concentrations of CF in the liquid phase were calculated from Henry's law constants (Gossett, 1987). The reaction mixtures (800 μl) containing the same phosphate buffer as in the growth medium and CF solution were equilibrated at 30 °C with constant shaking for at least 30 minutes before starting the assay. The reactions were initiated by the addition of 200 μl of concentrated cell suspensions (approximately 0.35, 0.34, 0.71, and 0.29 mg of protein in *M. trichosporium,* CF8, *P. butanovora,* and *M. vaccae,* respectively). For time course assays, a sample (20μl) of the gas phase was removed for analysis of CF by gas chromatography. The Shimadzu GC-8A gas chromatograph was equipped with an electron capture detector and a stainless steel column (0.25 by 20 inches) packed with Porapak Q (Alltech Associates, Inc., Deerfield, IL) and was run at a column temperature of 135 °C and a detector temperature of 230°C. Experiments were repeated at least three times. Data for each figure were from a typical experiment.

To determine if O_2 was required for CF degradation, the reaction vials were made anaerobic by purging with N_2 gas for 5 minutes prior to addition of the O_2-free CF solution. Sodium dithionite solution (100 μl of an approximately 10 mM solution) was added to a diluted CF stock solution (10 ml) to remove the dissolved O_2. The small amount of dithionite transferred to the reaction mixture was not toxic to the cells. The reaction mixtures included 5 mM formate or 5 mM butyrate as electron donors.

2.3.3 The effects of substrates on chloroform degradation

Various amounts of growth substrates (methane for *M. trichosporium*, butane for CF8, *P. butanovora*, and *M. vaccae*) were added into the reaction vials containing 34.5 nmol CF. Percentages of gas volume to total vial volume (10 ml) are shown (Table 2). The concentrations of methane and butane in the liquid phase were calculated from Bunsen coefficients (Smith & Baresi, 1989) at 30 °C. 1, 5, 25% (v/total v) of methane correspond to liquid phase concentrations of 6.6, 33, 160 μM and 0.01, 0.1, 1, 5% (v/total v) of butane correspond to 0.1, 1, 10, 50 μM.

2.3.4 Chloroform toxicity

To determine if CF degrading ability was lost during CF degradation, cells were allowed to degrade CF (69 nmol) for 60 min then additional CF was added to bring the total amount back to 69 nmol. CF degradation was monitored for another 60 min to compare the degradation patterns before and after respiking. The incubation medium initially contained 5 mM electron donor (formate or butyrate).

To determine if butane degrading ability was lost during CF degradation, butane-dependent O_2 uptake was measured for *P. butanovora* and CF8 cells (200 μl) that had been incubated with 69 nmol CF alone or with 69 nmol CF and 5 mM butyrate for 60 min. As controls, cells were incubated in the absence of CF. Samples of the gas phase

were removed at 0 and 60 min to measure CF by gas chromatography. After 60 min incubation, cells from 1 ml of the assay mixture were harvested and washed twice with phosphate buffer (1 ml) and resuspended in 100 μl of buffer. O_2 uptake rates were measured in the reaction chamber (1.8 ml) of an O_2 electrode (Biological Oxygen Monitor model 5300: Yellow Springs Instrument Company, Incorporated). The chamber was filled with phosphate buffer, then the 100 μl of washed cell suspension was added. O_2 uptake rates were measured after addition of the cells into the chamber, and again after addition of butane saturated solution (120 nmol).

2.3.5 Acetylene inactivation assay

To determine if acetylene inactivated CF degradation, cells were exposed to acetylene prior to addition of CF. Cell suspensions (200 μl) as described previously were incubated for 10 min in sealed 10 ml vials which contained phosphate buffer, 5 mM butyrate and 0.1% (v/total vial v) acetylene, or 0.1% acetylene with 50% butane. 0.05% (v/total vial v) acetylene was used for CF8. Control cells were preincubated in the phosphate buffer and 5 mM butyrate. After preincubation, acetylene and butane were removed from the vials by opening the cap and purging with air for 3 min. The vials were sealed again, then the reactions were initiated by the addition of 34.5 nmol CF to the reaction mixture.

2.3.6 Degradation of chlorinated ethenes and ethanes

Degradation of additional chlorinated hydrocarbons was tested for CF8 and *P. butanovora*. The reaction mixtures (800 μl) contained phosphate buffer and chlorinated aliphatic compounds (amounts used are indicated in table). Assays were initiated by the addition of 200 μl of cell suspensions (described previously) and suspensions were

incubated with shaking for the times indicated in the table. Chlorinated aliphatic compounds were quantified by gas chromatography.

2.3.7 Chloride and protein determinations

Chloride released by cells into the assay medium was measured by using a colorimetric assay (Bergmann & Sanik, 1957). Protein content was determined by using the Biuret assay (Gornall *et al.*, 1949) after the cells were solubilized in 3N NaOH for 30 minutes at 65 °C. Bovine serum albumin was used as the standard.

2.4 Results

Four bacterial cultures, *Methylosinus trichosporium* OB3b, *Pseudomonas butanovora,* CF8, and *Mycobacterium vaccae* JOB5 were grown on appropriate substrates and tested for CF degradation abilities. All four cultures readily degraded CF. After 60 min, the three cultures grown on butane had consumed from 61 to 97% of 34.5 nmol CF initially present (Table 2.1). Therefore CF degradation by butane-grown cells is not limited to CF8. The extent of CF degradation was comparable to *M. trichosporium* which was previously shown to degrade CF (Oldenhuis et al., 1991; Oldenhuis et al., 1989; Speitel Jr. et al., 1993). However, time courses revealed that *M. trichosporium* degraded CF more rapidly than the three butane-grown bacteria (Fig. 2.1). These assays were carried out in the absence of the growth substrate to avoid competition between CF and the growth substrate. The assays were carried out in the presence of an appropriate electron donor (5 mM formate for *M. trichosporium* and 5 mM butyrate for CF8, *P. butanovora* and *M. vaccae*). CF8 and *M. vaccae* exhibited a high rate of endogenous substrate oxidation and, therefore, did not require any exogenous reductant. In the absence of O_2, CF degradation was not detected by any of the bacteria. This result is consistent with the idea that monooxygenases catalyze the degradation of CF.

Table 2.1 Chloroform degradation by methane- and butane-grown organisms

	Growth substrate	Electron donor (5 mM)	Chloroform (nmol)	nmol of CF degraded in 60 min	O_2 Requirement
M.trichosporium	Methane	Formate	34.5	98.8 ± 0.9[a]	Yes
CF8	Butane	NR[b]	34.5	96.9 ± 5.6	Yes
P.butanovora	Butane	Butyrate	34.5	61.0 ± 14.7	Yes
M.vaccae	Butane	NR	34.5	89.6 ± 9.1	Yes

[a] ± Standard deviation.
[b] Not required.

Monooxygenase reactions require an input of reductant to complete the reduction of O_2. For some bacteria which use a monooxygenase to harvest substrate, the products of the monooxygenase reaction can provide the reductant for the monooxygenase activity. For instance, hydroxylamine (NH_2OH) which is produced from ammonia (NH_3) by ammonia monooxygenase (AMO) is further oxidized to nitrite to provide the reductant for the monooxygenase reaction (Wood, 1986). The products of methane oxidation were tested for their effects on CF degradation by *M. trichosporium.* Addition of methanol and formaldehyde inhibited CF degradation by *M. trichosporium* (data not shown). *M. trichosporium* could degrade CF without an electron donor. However, the presence of formate enhanced CF degradation in the first 20 min (Fig. 2.1). Expected intermediates of butane oxidation, butanol, butyraldehyde, and butyrate were tested for their effects on CF degradation by butane-grown cells. Butanol and butyraldehyde inhibited CF degradation (data not shown), perhaps because of toxicity at high concentration. However, butyrate enhanced CF degradation by *P. butanovora.* The presence of butyrate did not show any effect on CF degradation by CF8 and *M. vaccae.* For *P. butanovora,* the CF degradation rate in the first 20 minutes was comparable to other bacteria, but the degradation stopped after 40 minutes incubation (Fig. 2.1).

Acetylene is a mechanism-based inhibitor for several monooxygenases including MMO and AMO (Prior & Dalton, 1985; BÇdard & Knowles, 1989). Therefore, the effects of acetylene on CF degradation by the three butane-oxidizing bacteria and *M. trichosporium* were tested. When acetylene (0.1% v/total vial v) was added to reaction mixtures along with the electron donor, CF, and the cell suspension, the CF degradation by all four bacteria was completely inhibited (Fig. 2.1). This result further supports the idea that a monooxygenase is responsible for CF degradation in all four bacteria. To distinguish inhibition from inactivation, cells were exposed to acetylene for 10 min, the acetylene was removed by purging with air, then CF degradation was measured (Fig.

Figure 2.1
Time course of chloroform degradation by *M. trichosporium* (A), CF8 (B), *P. butanovora* (C), and *M. vaccae* (D)

Cells were incubated as described in Materials and Methods in the presence of 34.5 nmol CF (▲), CF and 5 mM electron donor (formate for *M. trichosporium* or 5 mM butyrate for CF8, *P. butanovora* and *M. vaccae*) (●), and CF, electron donor, and 0.1% (v/total vial v) acetylene (■).

Figure 2.2
Inactivation of chloroform degradation

Cells of CF8 (A), *P. butanovora* (B), and *M. vaccae* (C) were preincubated for 10 min in vials containing phosphate buffer, 5 mM butyrate and the following additions : none, (●); 0.05% (v/total vial v) acetylene for CF8, or 0.1% for *P. butanovora* and *M. vaccae* (▲); 0.05 or 0.1% acetylene and 50% butane (□). After 10 min, the vials were purged and the time course was initiated by addition of 34.5 nmol CF.

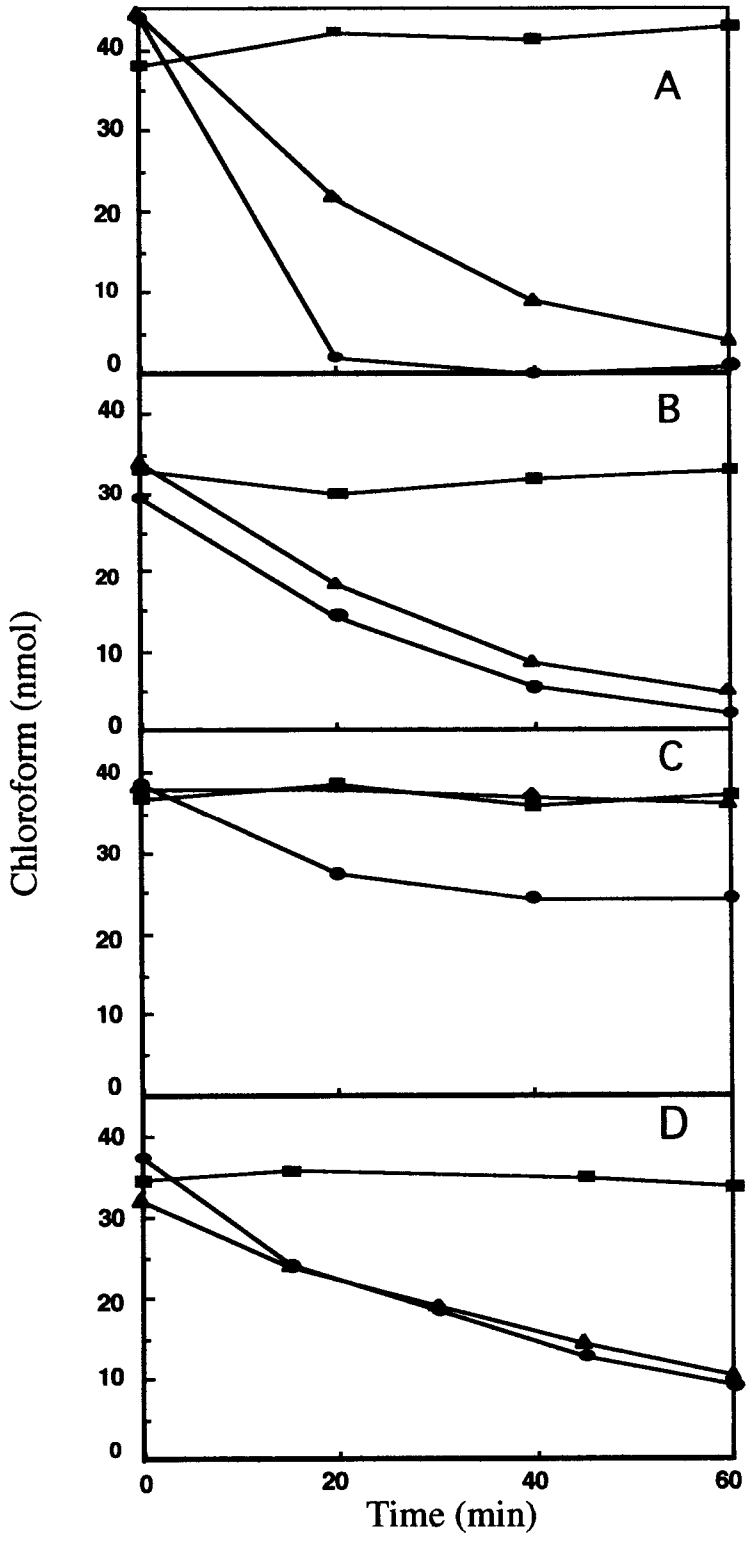

Fig 2.1

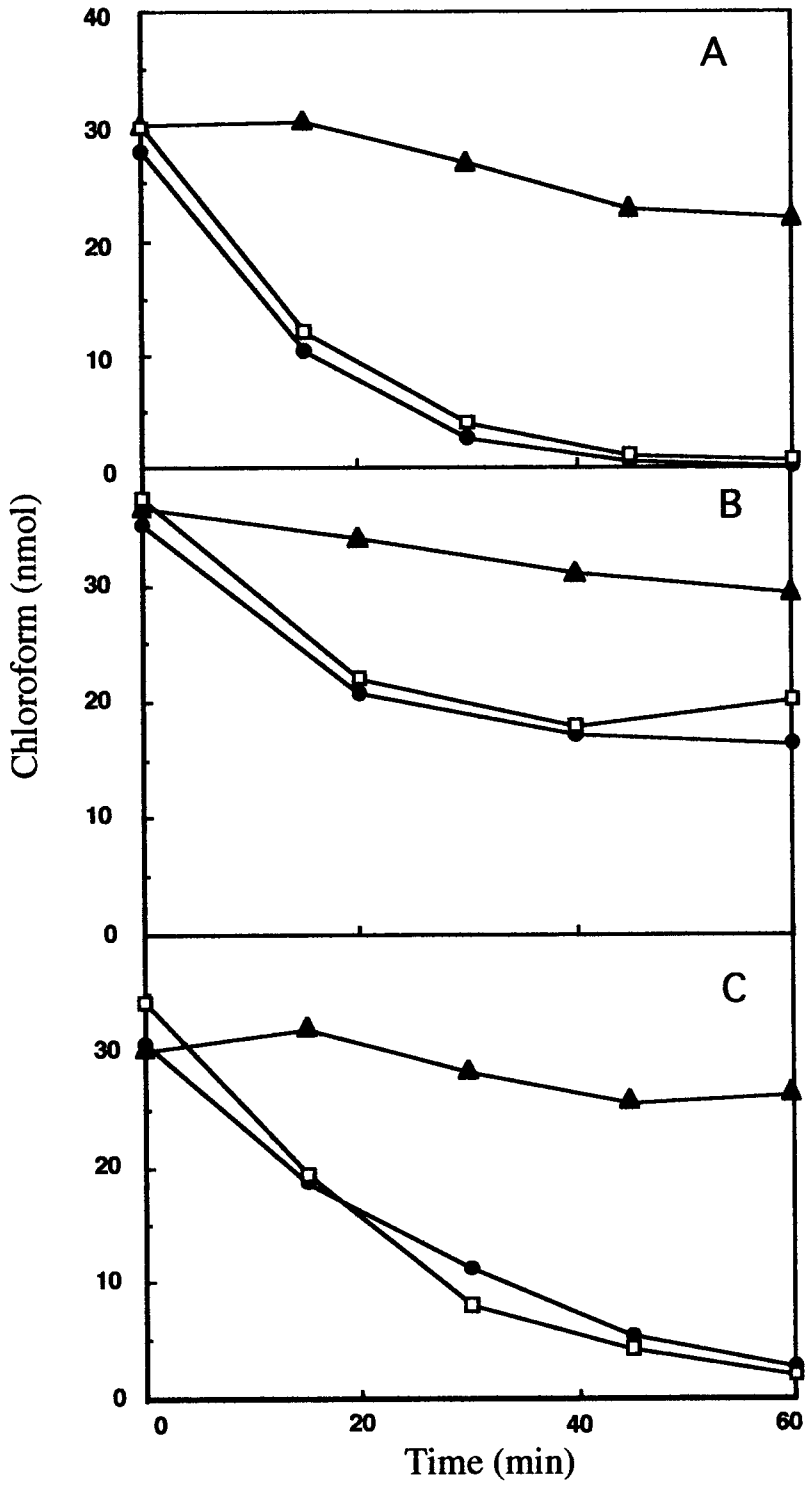

Fig 2.2

2.2). As expected, acetylene irreversibly inactivated CF degradation in all three bacteria. Inclusion of butane during the preincubation protected the cells from losing their CF degrading ability. In CF8, butane oxidation was also inhibited by acetylene (data not shown). Again, this is consistent with acetylene acting as a mechanism-based inactivator of butane monooxygenase.

In cometabolic processes, the presence of the physiological substrate at sufficiently high concentrations is expected to inhibit the degradation of the cosubstrate. To test the effect of the growth substrate on CF degradation by *M. trichosporium, P. butanovora,* CF8, and *M. vaccae*, the cells were incubated with CF in the presence of their growth substrate and an electron donor. The electron donor was included to ensure that reductant depletion did not occur. Three or four substrate concentrations were tested for each bacterium. In all four bacteria, the substrate inhibited CF degradation to some extent (Table 2.2). Less CF was degraded when more substrate was present. The extent to which the substrate inhibited CF degradation varied among the bacteria. With 1% butane, CF degradation by CF8 was completely inhibited, while 5% butane was required to inhibit CF degradation by *P. butanovora* and *M. vaccae* by more than 90%. In contrast, 1 and 5% methane did not inhibit CF degradation ability by *M. trichosporium.* Even 25% methane inhibited CF degradation only partially. Inhibition of CF degradation by the growth substrate is expected if both CF and the growth substrate compete for binding to the same enzyme (the monooxygenase). To confirm this idea, the effect of CF on butane degradation was tested for CF8. Butane oxidation was inhibited in the presence of CF. In the presence of 0.1% butane (1 μM in solution), the rate of butane consumption was inhibited 40% by 94 μM CF.

We determined the amount of CF degraded at different initial CF concentrations. The initial amounts of 34.5, 69, and 103.5 nmol CF (12.9, 25.8, and 38.6 μM) were added to reaction mixtures and the amounts of CF degraded in 20 and 40 minutes were

Table 2.2 Inhibition of chloroform degradation by butane and methane

Organisms	Amount of CF Degraded in 60 min (nmol)					
	none	0.01%	0.1%	1%	5%	25%[a]
M. trichosporium	34.3±0.2	ND[b]	ND	34.3±0.3	32.2±1.2	19.0±2.1
P. butanovora	22.5±2.8	ND	18.1± 3.7	11.9±2.0	2.1±2.9	ND
CF8	33.0±2.5	27.2±7.4	19.2±11.0	BD[c]	ND	ND
M. vaccae	25.8±1.4	25.8±1.9	24.2± 2.6	8.9±1.3	1.4±1.1	ND

[a] Amount of substrate added (v/total vial v). Methane was added to *M. trichosporium,* and butane was added to *P. butanovora,* CF8, and *M. vaccae.* Data are expressed as means ± standard deviation.
[b] Not determined.
[c] Below detection, < 1 nmol CF degraded.

compared (Table 2.3). The assays were carried out in the presence of an appropriate electron donor (5 mM) except for CF8. Within the first 20 min, *M. trichosporium, P. butanovora* and *M. vaccae* degraded more CF at higher CF concentrations. For *M. trichosporium,* the increase in CF degraded was nearly proportional to the increase in CF concentration which indicates the appK_m is well above 38.6 μM. For *P. butanovora* and *M. vaccae,* the increased CF degradation was not proportional to the increase in CF concentration which suggests the appK_m is in the range of the CF concentrations tested. CF8, which showed no increase in CF degradation with increasing CF concentrations, apparently has the appK_m well below 12.9 μM. Between 20 and 40 min, *M. trichosporium* cells degraded CF completely at all three CF levels. In contrast, butane-grown cells were not able to complete the degradation in all but two cases. CF8 and *M. vaccae* consumed 34.5 nmol CF, but were unable to consume either 69 or 103.5 nmol CF. *P. butanovora* consumed only half of the lowest CF concentration. Less degradation of CF from 20-40 min than 0-20 min suggests a toxic effect of CF degradation on the cells.

To determine if the CF degradation capacity of cells decreased with time, CF degradation was monitored over a 2-hr time period. After 60 min, the CF concentration was adjusted to the initial value. An appropriate electron donor (5 mM) was included in the reaction mixture. In the first 60 min, *M. trichosporium,* CF8, *P. butanovora,* and *M. vaccae* degraded 92, 97, 71, and 56%, respectively, of the 69 nmol CF present initially. In the 60 min after respiking, 84, 11, 1, and 24%, respectively, of the CF was degraded (Fig. 2.3). *M. trichosporium* maintained nearly the same rate in CF degradation after the respike. CF8 degraded 69 nmol in the first 60 min, but most of the activity was lost during this time period. Similarly, the CF degradation by *P. butanovora* that occurred during the first 60 min did not occur after the respike. Additional electron donor (5 mM) was added with CF after 60 min to examine whether these losses of CF degradation

Table 2.3 CF degradation at different initial chloroform concentrations

Organisms	Initial CF concn (μM)	Amount of CF (nmol)	Amount of CF degraded (nmol)		
			0-20 min	20-40min	0-40 min
M.trichosporium	12.9	34.5	30.1±2.1	3.3±1.3	33.4±0.8
	25.8	69	61.1±5.4	6.8±3.8	67.8±1.7
	38.6	103.5	86.2±4.9	14.1±2.8	100.3±3.6
P.butanovora	12.9	34.5	13.6±4.6	4.0±1.8	17.6±6.3
	25.8	69	22.3±7.8	6.2±2.9	28.5±5.2
	38.6	103.5	25.8±4.0	9.8±2.4	35.6±2.9
CF8	12.9	34.5	28.5±2.3	6.0±2.3	34.5±0
	25.8	69	36.2±4.9	11.5±0.7	47.6±4.9
	38.6	103.5	31.7±7.2	8.7±6.0	40.4±6.2
M. vaccae	12.9	34.5	23.4±2.1	7.2±1.6	30.7±2.8
	25.8	69	30.6±6.3	15.1±6.3	45.0±11.3
	38.6	103.5	40.9±4.7	23.8±2.9	64.6±3.7

activity resulted from the reductant limitation (data not shown). However, the additional electron donor did not show any effect on CF degradation after respiking suggesting that the decreased CF degradation resulted from CF toxicity. *M. vaccae* retained some activity, though not as much as *M. trichosporium.* These results are consistent with those of Table 2.3 and show that CF8 and *P. butanovora* are more sensitive to CF toxicity than either *M. trichosporium* or *M. vaccae. M. trichosporium* seems to have the most resistance to CF toxicity.

The toxicity of CF to CF8 and *P. butanovora* was further examined by measuring butane-dependent O_2 uptake after exposure to CF. Cells of CF8 and *P. butanovora* were incubated with 69 nmol CF for 60 min either in the presence or absence of butyrate. As a control, cells were also incubated without CF. CF8 cells incubated with 69 nmol CF in the presence and absence of butyrate degraded 79 and 99% of the CF in 60 min, respectively. O_2 uptake by both samples decreased (Fig. 2.4). *P. butanovora* incubated for 60 min with 69 nmol CF in the presence of butyrate degraded 72% of the CF but only 12% of the CF was degraded when cells were incubated in the absence of butyrate. O_2 uptake was similar to the control in the sample not exposed to butyrate but decreased in the sample which included butyrate (Fig. 2.4). Butane-dependent O_2 uptake decreased when cells were incubated under conditions which supported CF degradation.

We measured the amount of Cl^- and CO_2 released during CF degradation. The measurement of chloride release was particularly important to determine if CF degradation (which was determined as a loss of CF) resulted in dechlorination of CF. Cells of *M. trichosporium, P. butanovora,* and CF8 were incubated with CF in the presence of an electron donor for 2 to 3 hours. Chloride ion contents of control treatments containing acetylene were used to correct for the background chloride. The molar ratios of chloride ions released to CF degraded were 2.1 ± 0.2 for *M. trichosporium,* 2.4 ± 0.3 for CF8, and 1.7 ± 0.5 for *P. butanovora.* While chloride

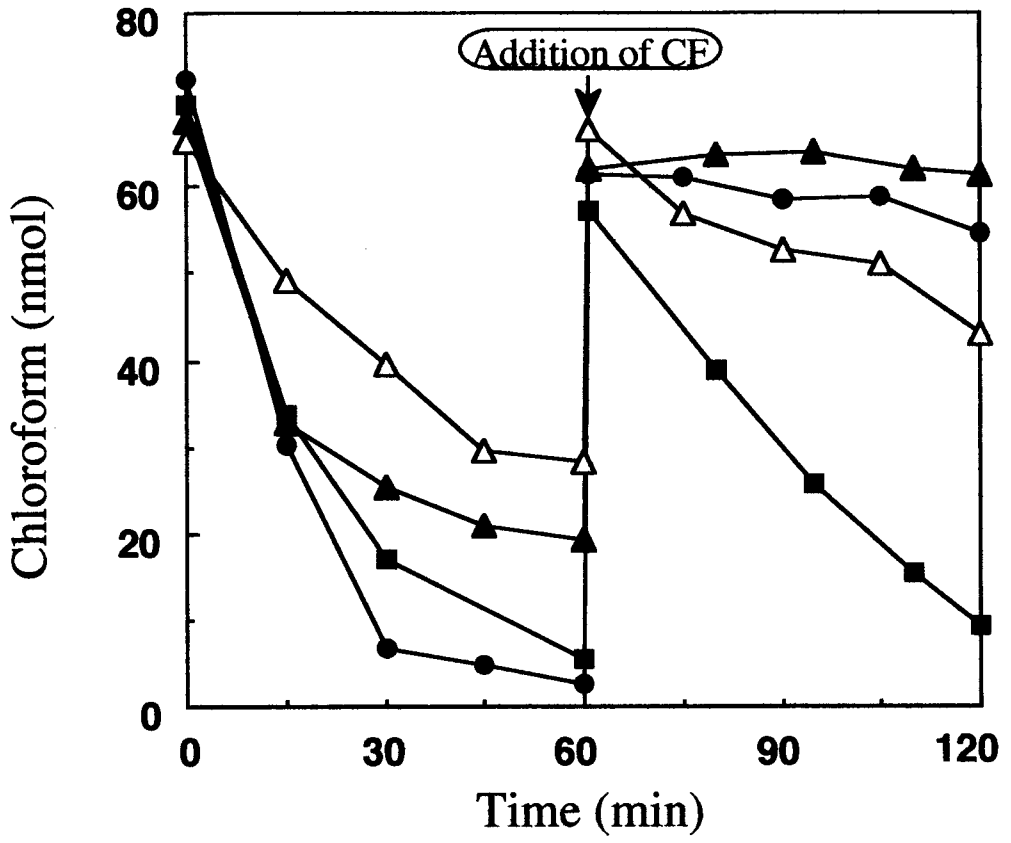

Figure 2.3
Chloroform toxicity

Degradation of CF (69 nmol) in the presence of 5 mM of appropriate electron donor was monitored for 60min, then CF was added to bring the total amount of CF to 69 nmol and CF degradation was monitored for additional 60 min. Symbols: ■, *M. trichosporium*; ●, CF8; ▲, *P. butanovora*; △, *M. vaccae*.

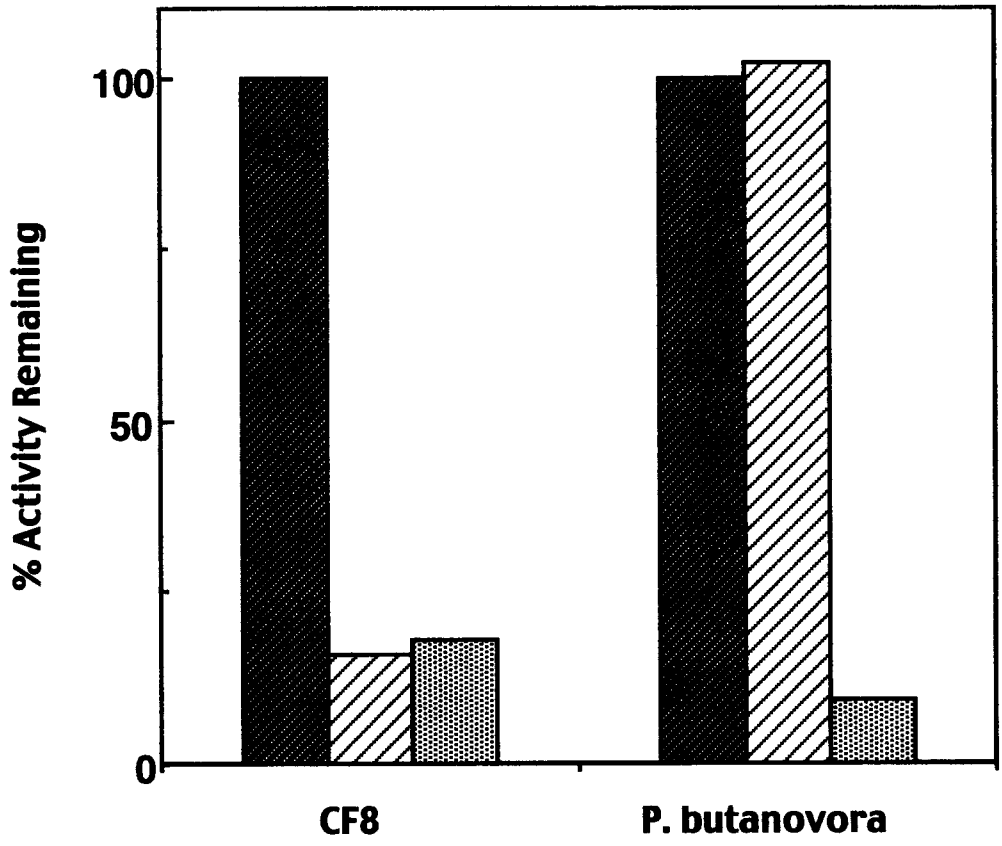

Figure 2.4
O_2 uptake activity remaining in cells of CF8 and *P. butanovora* incubated in the presence or absence of chloroform and an electron donor

Cells were incubated for 60min in phosphate buffer only (■), buffer and 69 nmol CF (▨), and buffer, 69 nmol CF and 5 mM butyrate (▩). The rate of O_2 uptake prior to addition of butane (120 nmol) was subtracted from the O_2 uptake after addition of substrate. Butane-dependent O_2 uptake rates (19.9 and 9.4 nmol O_2/min for CF8 and *P. butanovora*, respectively) by the cells incubated in the absence of CF were considered as 100% activity remaining.

release clearly accompanied CF degradation, complete dechlorination apparently did not occur because the ratios were less than three. This result differs from the butane microcosms (Kim, 1996) where complete dehalogenation was observed. However, at least some portion of the degraded CF is completely dechlorinated because CO_2 is a product. *M. trichosporium, P. butanovora,* or CF8 cells exposed to [^{14}C]chloroform converted 15 to 50 % of the total ^{14}C into CO_2 (data not shown).

It was of interest to determine if CAH degradation by butane-grown bacteria was limited to CF. *M. vaccae* was previously shown to degrade several CAHs (when grown on propane). Therefore, CF8 and *P. butanovora* were further studied for the ability to degrade other chlorinated ethenes and ethanes (Table 2.4). Both CF8 and *P. butanovora* were able to degrade trichloroethylene, 1,2-*cis*-dichloroethylene, and vinyl chloride. In addition, CF8 readily degraded 1,1,2-trichloroethane. 1,1,1-Trichloroethane and 1,2-*trans*-dichloroethylene were not degraded substantially by either CF8 or *P. butanovora.* It is interesting to note that 1,2-*cis*-dichloroethylene was degraded by both bacteria but not 1,2-*trans*-dichloroethylene. CF8 showed higher degradation activity for vinyl chloride and 1,1,2-trichloroethane than for other CAHs. In contrast, *P. butanovora* showed higher degradation activity for 1,2-*cis*-dichloroethylene than for other CAHs.

2.5 Discussion

Kim (Kim, 1996) demonstrated that butane was an effective substrate for cometabolism of CF and 1,1,1-trichloroethane in microcosms with Hanford aquifer core material. In this work, we extend this observation to two bacteria taken from the ATCC culture collection, *M. vaccae* JOB5 and *P. butanovora,* and an enrichment culture CF8 derived from the Hanford microcosms. All three butane-grown microorganisms in this study were able to degrade CF. This is the first report of CF degradation by pure cultures of butane-oxidizing bacteria. In our study, CF degradation by CF8 was

Table 2.4 Degradation of chlorinated ethenes and ethanes by *P. butanovora* and CF8

Compound	*Pseudomonas butanovora*			CF8		
	Amt added (nmol)	Time (min)	Amt degraded (nmol)	Amt added (nmol)	Time (min)	Amt degraded (nmol)
trichloroethylene	89	70	23	10	210	4.9
1,1,1-trichloroethane	29	140	BD[b]	10	180	0.6
1,1,2-trichloroethane	ND[a]	ND	ND	10	80	10
1,2-*cis*-dichloroethylene	106	70	93	83	105	25
1,2-*trans*-dichloroethylene	65	180	BD	70	130	BD
vinyl chloride	100	85	24	100	130	65

[a] Not determined.
[b] Below detection, < 3% of the initial amount consumed.

compared to two butane-oxidizing bacteria obtained from a culture collection and to a known CF degrader, *Methylosinus trichosporium* OB3b.

Our data indicate that these butane-grown bacteria possess a monooxygenase enzyme that initiates the oxidation of butane and CF. In this study, the involvement of butane monooxygenase in CF degradation was indicated by the following results: the requirement of O_2 for CF degradation, inhibition by acetylene, and inhibition by butane. *M. vaccae* is known to produce a propane monooxygenase which also oxidizes butane by subterminal oxidation (Phillips & Perry, 1974; Vestal & Perry, 1969). Most likely the same monooxygenase is produced in propane or butane grown cells.

Competition between the growth substrates (butane or methane) and CF was observed (Table 2.2). With 1% butane (10 μM butane in solution), CF degradation (with 13 μM CF in solution) was decreased to about half of that in the absence of butane for *P. butanovora* and *M. vaccae.* In contrast, CF degradation by CF8 was completely inhibited by 1% butane. In the microcosms from which CF8 was isolated, the presence of 2 mg/l (38 μM) butane in the liquid phase did not inhibit CF transformation (3-6 μM) in microcosms (Kim, 1996). However, at high CF concentration (10 μM), CF transformation appeared to accelerate as butane concentrations decreased, which may indicate butane inhibition of CF transformation. These differences between CF8 and the microcosms may indicate that CF8 was not the dominate butane-oxidizer in the microcosms. Alternatively, the differences may reflect the differences between growth in the presence (microcosms) and absence (CF8) of CF. The effect of methane concentration on CF kinetics of *M. trichosporium* has been studied. Speitel et al. (Speitel Jr. et al., 1993) showed the presence of methane caused significant enzyme competition at methane concentrations as low as 0.35 mg/l (22 μM), resulting in smaller CF rate constants. The rate constant in the presence of 0.35 mg/l methane was only 37% of that observed in the control. Competition between methane and other chlorinated

aliphatics for sMMO has been observed with *M. trichosporium* OB3b (Oldenhuis et al., 1991).

Of the organisms tested, *M. trichosporium* OB3b and *M. vaccae* exhibit potential in continuously degrading a high concentration (38.6 μM) of CF (Table 2.3, Fig. 2.3). In contrast, *P. butanovora* and CF8 rapidly lost their CF degradation potential as a result of CF degradation. The mechanism of CF toxicity is not well known. From the O_2 uptake assay, it is suggested that the cells were damaged as a result of oxidizing CF (Fig. 2.4). Butane-dependent O_2 consumption was not lost unless CF was degraded. Alvarez-Cohen and McCarty (Alvarez-Cohen & McCarty, 1991) studied CF and TCE toxicity with a mixed methane-utilizing culture of resting cells. Toxicity of CF, TCE, and their transformation products to whole cells was evaluated by comparing the formate oxidation activity of acetylene-treated cells to that of non-acetylene-treated cells with and without prior exposure to CF or TCE. The formate oxidation by cells exposed to either CF or TCE without acetylene significantly decreased compared to that with acetylene, suggesting that the solvents themselves were not toxic but their transformation products were. Alvarez-Cohen and McCarty (Alvarez-Cohen & McCarty, 1991) have speculated that phosgene and TCE epoxide are responsible for the product toxicity of CF and TCE, since both compounds have been shown to exhibit irreversible binding to proteins thought to result in toxic behavior in mammalian systems (Bolt & Filser, 1977; Pohl, 1977). A similar reaction may have caused the toxicity we observed with butane-grown bacteria. The results from our chloride release experiments suggested incomplete dehalogenation of CF. Perhaps not all of the CF proceeds through a pathway which leads to complete dehalogenation.

CF degradation by all three butane-grown bacteria and *M. trichosporium* were inactivated by acetylene. Acetylene is known to inactivate a number of monooxygenases including pMMO (Prior & Dalton, 1985) and AMO (Bédard & Knowles, 1989), sMMO

(Prior & Dalton, 1985), and P450 monooxygenases (Ortiz de Montellano & Almira Correia, 1991). Acetylene inactivation required enzyme turnover and the presence of butane protected the enzyme from inactivation (Fig 2.2). These results suggest that acetylene is a mechanism-based inactivator of butane monooxygenase.

Besides CF, a number of other environmentally important chlorinated compounds were degraded by cells of CF8 and *P. butanovora* (Table 2.4). *M. trichosporium* OB3b and *M. vaccae* JOB5 also degrade various chlorinated aliphatic compounds (Oldenhuis et al., 1989; Wackett et al., 1989). *M. trichosporium* degraded 0.2 mM TCE, 1,2-*trans*-dichloroethylene, and 1,2-*cis*-dichloroethylene completely within 24 h. 1,1,1-Trichloroethane was partially degraded (Oldenhuis et al., 1989). In addition, vinyl chloride degradation by *M. trichosporium* was reported (Chang & Alvarez-Cohen, 1996). Propane-grown *M. vaccae* degraded vinyl chloride (14 μM) completely within 2 h, and 1,1-dichloroethylene (20 μM) and 1,2-*cis*-dichloroethylene (17 μM) were degraded to a significant extent. At a starting concentration of 20 μM TCE up to 99% was removed in 24 h. 1,2-*trans*-dichloroethylene was only marginally degraded (Wackett et al., 1989). The ability of CF8 and *P. butanovora* (Table 2.4) to degrade *cis*-dichloroethylene but not *trans*-dichloroethylene is similar to the results with propane-grown *M. vaccae*. This study shows that butane-oxidizing bacteria can degrade chlorinated aliphatic hydrocarbons. Butane-grown bacteria may have potential in the bioremediation of these compounds.

Chapter 3

Diversity in butane-oxidizing bacteria

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3.1 Abstract

A series of inactivators and inhibitors of monooxygenases demonstrated different effects on butane monooxygenases in CF8, *Pseudomonas butanovora,* and *Mycobacterium vaccae* JOB5. All three enzymes were inactivated by acetylene. Ethylene, a known inactivator of P-450 monooxygenases, irreversibly inactivated chloroform degradation by *P. butanovora,* but only inhibited chloroform degradation by CF8 and *M. vaccae.* CF8 was strongly inhibited by allylthiourea, a copper selective chelator. Slight inhibition of chloroform degradation by *M. vaccae* was observed. Treatment of butane-grown cells with [^{14}C]acetylene resulted in the labeling of polypeptides. In *P. butanovora,* at least two polypeptides were labeled, including one heavily labeled polypeptide (ca. 60 kDa). CF8 showed a single labeled polypeptide (ca. 33 kDa). In *M. vaccae,* two polypeptides (ca. 30 and 66 kDa) were radiolabeled to similar intensities. The K_s (the apparent K_m observed in intact cells) for butane was estimated as 14.07 ± 8.49, 47.75 ± 4.87, and 17.57 ± 5.43 μM for CF8, *P. butanovora,* and *M. vaccae,* respectively. The results indicate the diversity in butane monooxygenases in three butane-oxidizing bacteria.

3.2 Introduction

In chapter 2, the CF degradation ability by butane-grown CF8, *Pseudomonas butanovora,* and *Mycobacterium vaccae* JOB5 was shown. The results suggested that butane monooxygenase is responsible for CF degradation. CF degradation by all three butane-grown bacteria was inactivated by acetylene, which is a mechanism-based inactivator of several monooxygenases. In order to further characterize the butane monooxygenases in each of the three butane-grown bacterium, the responses of the cells to two additional diagnostic inhibitors/inactivators, ethylene and allylthiourea (ATU), will be examined in this chapter.

Acetylene and larger alkynes have been shown to inactivate a number of monooxygenases including MMO (Prior & Dalton, 1985), AMO (Hyman & Wood, 1985), and P-450 monooxygenases (Ortiz de Montellano & Almira Correia, 1991). Acetylene inhibition was demonstrated for both the soluble and particulate forms of MMO in *Methylococcus capsulatus* (Bath) (Prior & Dalton, 1985). Inhibition was dependent on the presence of both NADH and O_2, suggesting that acetylene inhibition required enzyme turnover. The possibility that acetylene or a product of acetylene oxidation causing inhibition by irreversible binding to the enzyme was investigated using [^{14}C]acetylene (Prior & Dalton, 1985). When the soluble fraction of cell extracts of *M. capsulatus* (Bath) expressing sMMO was incubated with [^{14}C]acetylene, ^{14}C label bound to a single polypeptide (54 kDa). This polypeptide corresponds to the α-subunit of protein A of sMMO. The cells grown under conditions where they expressed pMMO activity showed that [^{14}C]acetylene bound to a single polypeptide (26 kDa) in the particulate fraction of cell extracts. This corresponds to one of three proteins induced when cells are expressing pMMO. Acetylene bound to specific polypeptides which might be the active site of methane oxidation.

Ammonia monooxygenase (AMO) in *Nitrosomonas europaea* is also inactivated by acetylene (Hyman & Wood, 1985). Inactivation by acetylene requires O_2, and the inactivation rate follows first-order kinetics. In the presence of thiourea, a reversible non-competitive inhibitor, AMO is protected from acetylene inactivation. These results suggest that acetylene is a suicide substrate for AMO. Incubation of cells with [^{14}C]acetylene resulted in labeling of a single membrane polypeptide of M_r 28 000. The property of suicide substrates to bind specifically to an active site of the enzyme can be used to obtain information on the enzyme structure and the mechanism of the catalytic process.

The cytochrome P-450 monooxygenase of hepatic microsomes is known to be inhibited by a wide variety of acetylenic compounds (Ortiz de Montellano & Kunze, 1980). Inactivation of hepatic cytochrome P-450 by acetylenic compounds requires NADPH and O_2 and results in production of an inactive green pigment which contains *N*-alkylated heme (Kunze et al., 1983). A suicide mechanism is thought to be involved. Certain P-450 inhibitors, such as β-diethylamino ethyldiphenylpropyl acetate, have been shown to inhibit CH_4 and NH_4^+ oxidation by AMO and MMO, although high concentrations are required (Bédard & Knowles, 1989).

Ethylene serves as a substrate for alkane monooxygenases (Hyman *et al.*, 1988). Therefore, inhibition of butane monooxygenase by ethylene might be a result of substrate competition. However, it has been known that ethylene irreversibly inactivates the monooxygenase containing a P-450 prosthetic group by alkylating the heme group (Ortiz de Montellano & Reich, 1986). Spectroscopic studies showed that the alkylated porphyrin contains a porphyrin nitrogen bound to the terminal carbon of the double bond and an oxygen to the internal carbon(Ortiz de Montellano & Correia, 1983). This oxygen in the ethylene adducts is a catalytically activated oxygen derived from molecular oxygen (Ortiz de Montellano & Correia, 1983). Ethylene epoxide does not inactivate the

enzyme. These results suggest that P-450 monooxygenase is inactivated by transfer of catalytic oxygen to the double bond but not by the epoxide metabolite.

Alkane oxidation by a bacterial cytochrome P-450 has been reported in octane-grown *Corynebacterium* 7E1C (Cardini & Jurtshuk, 1970). The hydroxylation of *n*-octane to 1-octanol and octanoic acid by the cell-free extract of *Corynebacterium* 7E1C required NADH and O_2. The ^{18}O experiment showed that molecular oxygen was incorporated into *n*-octane during its conversion to 1-octanol. *n*-Octane hydroxylation was inhibited by CO (Cardini & Jurtshuk, 1970), but not by SKF-525 which is known to inhibit cytochrome P-450 in the rat liver microsomes (Sladek & Mannering, 1966). Spectral studies showed that a broad absorption peak at 450 mμ appeared by the addition of CO to the reduced preparation, indicating the presence of cytochrome P-450. Comparative studies with enzymes from *n*-octane-grown cells and acetate-grown cells indicated that cytochrome P-450 is an inducible hemoprotein, and its concentration was correlated with hydroxylating activity (Cardini & Jurtshuk, 1970).

Allylthiourea (ATU), a copper selective chelator, reversibly inhibits pMMO and AMO (Bédard & Knowles, 1989). Copper plays an important role in both ammonia and methane oxidation. The presence of copper in AMO was suggested by the fact that metal chelating agents inhibited ammonia oxidation in *Nitrosomonas europaea* (Hooper & Terry, 1973). A proposed catalytic cycle for AMO involves oxidation and reduction of the copper site (Lerch, 1981). AMO transforms among the three states, reduced (deoxy), oxygenated (oxy), and oxidized (met). O_2 reversibly attacks the deoxy form and binds as peroxide (O_2^{2-}), then oxidizes the Cu(I) to Cu(II). This oxy monooxygenase inserts one atom of oxygen into the substrate and reduces the other one to water. The enzyme, now in the met state, can be reduced to the deoxy state by transferring two electrons (Shears & Wood, 1985). Metal chelating agents, such as thiourea, bind to deoxy form of AMO and prevent it from oxidizing to the oxy state.

The concentration of copper in the medium is the main factor to differentiate the monooxygenase expressed in methanotrophs. In both *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b, sMMO expression dominates when copper is present in limiting amounts, while pMMO dominates when copper is nonlimited in the medium (Burrows *et al.*, 1984; Dalton *et al.*, 1984). pMMO requires copper for its activity (Patel *et al.*, 1982).

In this chapter, the butane monooxygenases in three butane-grown bacteria, CF8, *Pseudomonas butanovora,* and *Mycobacterium vaccae* JOB5, are further characterized. The responses of the cells to three diagnostic inhibitors/inactivators are investigated. The mechanism-based inactivator, acetylene, is used to identify the active-site containing polypeptides in butane-grown bacteria. The K_s values (the apparent K_m observed in intact cells) for butane in intact cells of each of the three bacteria are determined. This work provides another level of discrimination among the butane monooxygenases.

3.3 Materials and methods

3.3.1 Bacterial strains and growth conditions

CF8, *Pseudomonas butanovora,* and *Mycobacterium vaccae* JOB5 were grown as described in Chapter 2.

3.3.2 Inhibition and inactivation assay

As inhibitors, 0.1 or 0.05% (v/total vial v) acetylene, 2% ethylene, and 200 μM allylthiourea were added to reaction vials, which contained the same phosphate buffer as in the growth medium, CF (34.5 nmol), and butyrate (5 mM). After equilibration of the gas and liquid phases, the reactions were initiated by the addition of 200 μl of concentrated cell suspensions (prepared as described in Chapter 2). CF degradation was

measured as described previously (Chapter 2). For time course assays, a sample (20 μl) of the gas phase was removed for analysis of CF by gas chromatography.

To determine if acetylene or ethylene inactivated CF degradation, cells were exposed to each gas prior to the addition of CF. Cell suspensions (200 μl) were preincubated for 10 min in sealed 10-ml vials which contained phosphate buffer, butyrate (5 mM), and either 0.1% (v/total vial v) acetylene, 0.1% acetylene with 50% butane, or 2% ethylene. For *P. butanovora,* cells were also preincubated in the vials which contained 2% ethylene with 10% or 50% butane. Control cells were preincubated in phosphate buffer and butyrate (5 mM). After preincubation, acetylene, ethylene, and butane were removed from the vials by opening the cap and purging with air for 3 min. The vials were sealed again, and then the reactions were initiated by the addition of 34.5 nmol CF to the reaction mixture. Experiments were repeated at least three times. Data for each figure were from a typical experiment.

3.3.3 ^{14}C acetylene labeling of cellular proteins

Cells of three butane-grown bacteria were treated with [^{14}C]acetylene synthesized from $Ba^{14}CO_3$ as described previously (Hyman & Arp, 1990). Concentrated cell suspensions (200 μl) were incubated for 10 min at 30 °C with constant shaking in 10-ml sealed vials containing phosphate buffer (800 μl), butyrate (5 mM), and $^{14}C_2H_2$. As controls, cells were incubated with $^{14}C_2H_2$ in the presence of 10% or 50% (v/total vial v) of butane, or preincubated for 10 min with 2% ethylene for *P. butanovora* or 200 μM allylthiourea for CF8 prior to addition of $^{14}C_2H_2$. After incubation, cells were harvested and washed twice with phosphate buffer (1 ml), then resuspended in 100 μl loading buffer for SDS-PAGE. Protein samples (50 μg per lane) were separated on a 10% polyacrylamide gel at a constant voltage of 15 mA. After the gel was stained with coomassie-blue, it was dried onto filter paper. Radioactive polypeptides were visualized

by Phosphorimage analysis of the dried gels. The gel was exposed to the phosphorimage cassette for 2 to 10 days.

3.3.4 Butane degradation assay

Assays were conducted in serum vials (7.8 ml) sealed with butyl rubber stoppers. Butane was added into the gas phase without overpressure. The concentrations of butane in the liquid phase were calculated from Bunsen coefficients (Smith & Baresi, 1989). At 30 °C, 0.01, 0.1, 1, and 5% (v/v of gas phase) of butane correspond to 0.1, 1, 10, and 50 μM in solution. Vials containing the same phosphate buffer as in the growth medium (800 μl) and butane in the gas phase were equilibrated at 30 °C with constant shaking for at least 30 minutes before starting the assay. The reactions were initiated by the addition of 200 μl of concentrated cell suspensions (described previously). For time course assays, samples (20 μl) of the gas phase were removed for analysis of CF by gas chromatography. The Shimadzu GC-8A gas chromatograph was equipped with a flame-ionization detector and a stainless-steel column (0.25 by 48 in.) packed with Porapak Q (Alltech Associates, Inc., Deerfield, IL) and was run at a column temperature of 150 °C and a detector temperature of 200°C. For the determination of K_s (the apparent K_m observed in intact cells), five to seven different initial concentrations of butane between 0.1 and 200 μM were used. Butane degradation was monitored for 2 h. K_s was estimated by fitting the data to the Michaelis-Menten model.

3.4 Results

The effects of ethylene and ATU on CF degradation by the butane-grown bacteria were compared to acetylene-treated and untreated samples (Fig. 3.1). In the presence of acetylene (0.1 or 0.05%) or ethylene (2%), CF degradation by all three bacteria was

Figure 3.1
Effects of inhibitors on chloroform degradation by butane-grown bacteria

CF8 (A), *P. butanovora* (B), and *M. vaccae* (C) cells were incubated as described in Materials and Methods in the presence of 34.5 nmol CF, butyrate (5 mM), and the following additions: none, (●); 0.1% (v/total vial v) acetylene, (■); 2% ethylene, (▲); ATU (200 μM), (△).

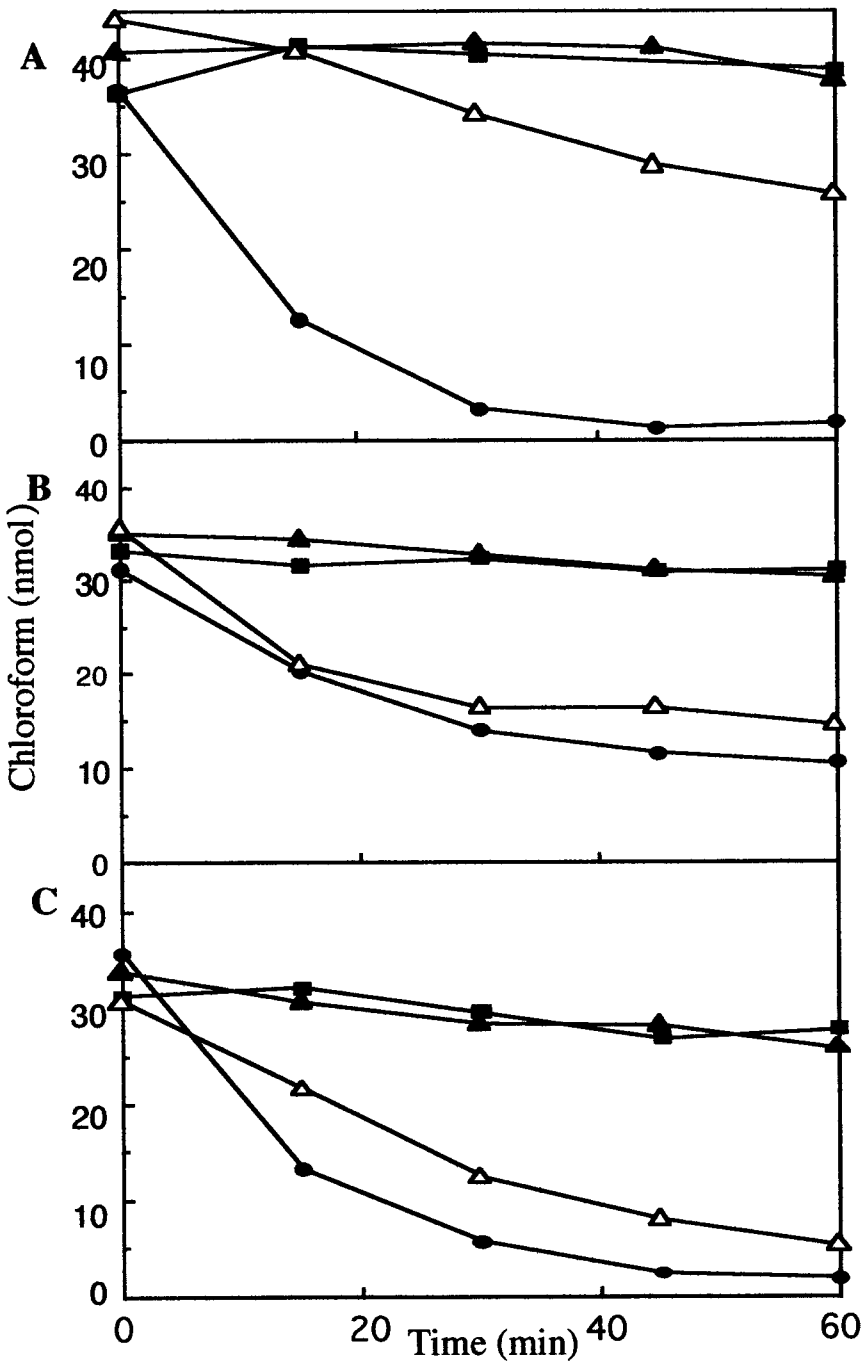


Figure 3.1

inhibited completely. ATU strongly inhibited CF degradation by CF8 (Fig 3.1). CF degradation by *M. vaccae* was only slightly inhibited by ATU (~20% less CF degraded when ATU was present). ATU did not inhibit CF degradation by *P. butanovora.*

To distinguish between inhibition and inactivation, cells were exposed to ethylene for 10 min, the ethylene was removed by purging with air, then CF degradation was monitored. Acetylene, a proposed mechanism-based inactivator for monooxygenases, was also examined (Fig. 3.2). Acetylene irreversibly inactivated CF degradation in all three bacteria. However, after pretreatment with ethylene, CF8 and *M. vaccae* recovered the ability to degrade CF, indicating that ethylene inhibits but does not inactivate the monooxygenases in these bacteria (Fig. 3.2, A and C). In contrast, *P. butanovora* did not recover its CF degradation activity (Fig. 3.2, B). The presence of butane during the preincubation protected the *P. butanovora* cells from inactivation by ethylene (Fig. 3.3). 50% butane protected the enzyme completely, while 10% butane protected only partially. These results suggest that ethylene irreversibly inactivates the monooxygenase in *P. butanovora* and also support the idea that the same monooxygenase catalyzes both butane and CF degradation. The results of these inhibition and inactivation experiments are summarized in Table 3.1. Remaining CF degradation activities were shown as the percentages of amount of CF degraded compared to controls. Controls for inactivation assays were preincubated with butyrate (5 mM) in the absence of inhibitors. CF degradation by three butane-grown bacteria can be distinguished based on their responses to ethylene and ATU.

Mechanism-based inactivators are expected to covalently bind to the target enzyme. To determine if acetylene forms a covalent adduct with cellular proteins of butane-grown bacteria, cells were treated with [^{14}C] radiolabeled acetylene. Figure 3.4 (A) shows a coomassie-blue stained SDS-PAGE gel showing the different polypeptides for each butane-grown bacterium. CF8, *P. butanovora* and *M. vaccae* cells treated with

Figure 3.2
Inactivation of chloroform degradation by butane-grown bacteria

CF8 (A), *P. butanovora* (B) and *M. vaccae* (C) cells were preincubated for 10 min in the vials containing phosphate buffer, butyrate (5 mM), and the following additions: none (●); 0.05% (v/total vial v) (A) or 0.1% (B and C) acetylene (°); 0.05% or 0.1% (v/total vial v) acetylene and 50% butane (■); 2% ethylene (▲). After 10 min, the vials were purged with air and the time course was initiated by the addition of 34.5 nmol CF.

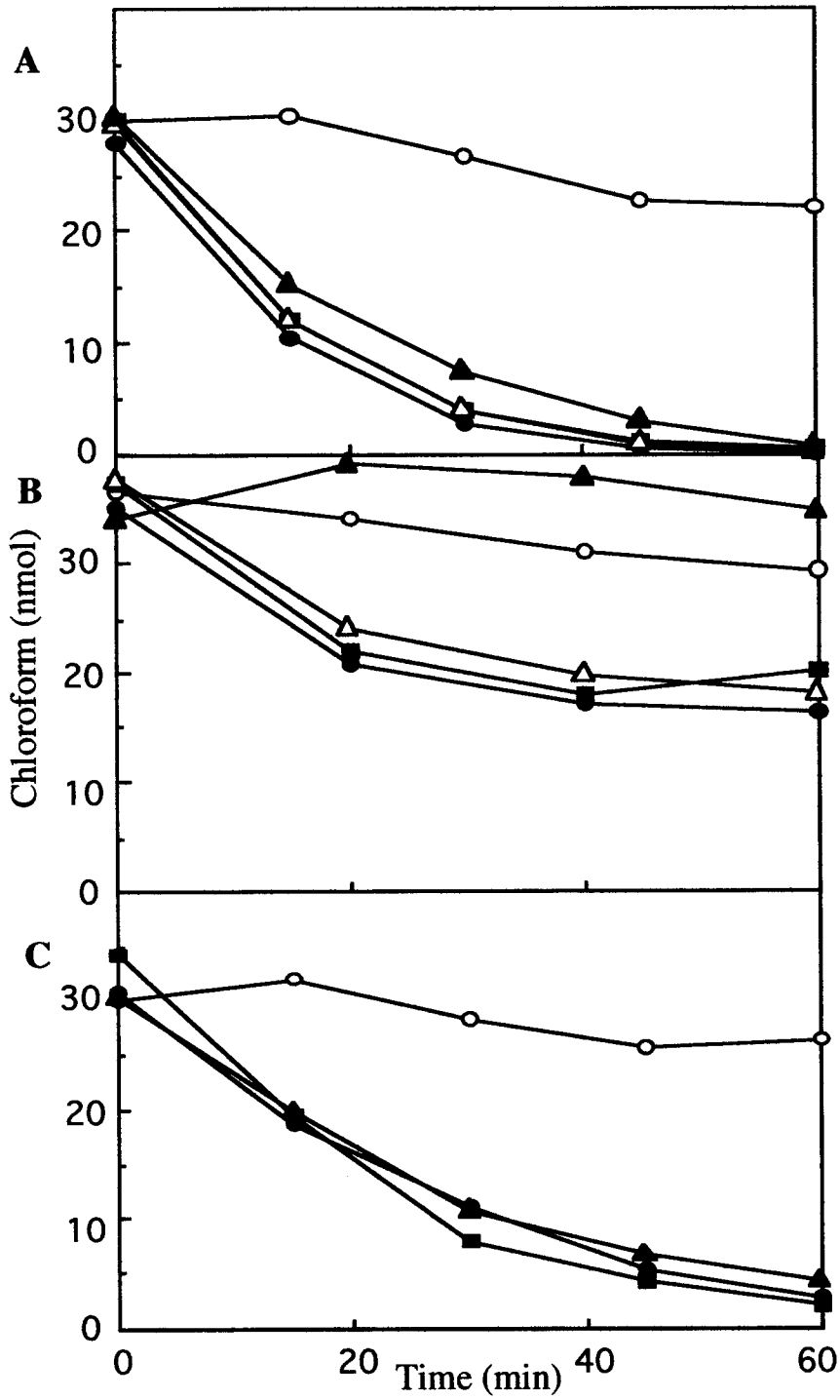


Figure 3.2

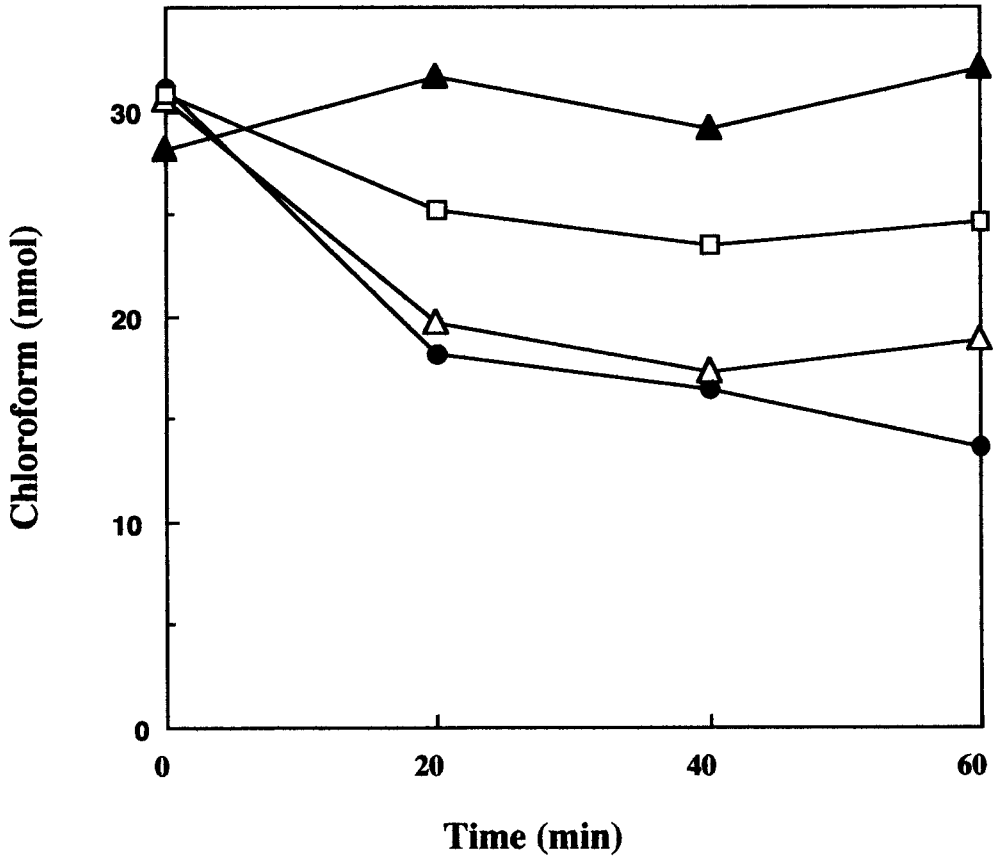


Figure 3.3
Inactivation of *P. butanovora* by ethylene

P. butanovora cells were preincubated for 10 min in the vials containing phosphate buffer, butyrate (5 mM), and the following additions: none (●); 2% (v/total vial v) ethylene (▲); 2% ethylene and 50% butane (△); 2% ethylene and 10% butane (□). After 10 min, the vials were purged with air and the time course was initiated by the addition of 34.5 nmol CF.

Table 3.1 Inhibition and inactivation assays

Organisms	% Activity Remaining					
	Inhibition[a]			Inactivation		
	Acetylene	Ethylene	ATU	Acetylene	Acetylene + 50% Butane	Ethylene
CF8	1.6±1.2[b]	1.2±1.7	17.4±8.1	10.9±11.5	99.2±1.2	100±0
P. butanovora	0	4.7±8.1	97.4±4.5	11.2±11.2	92.8±5.4	0
M. vaccae	11.9±5.9	6.6±10.5	62.7±26.8	15.4±13.2	90.9±12.2	100±0

[a] Cells were incubated with 34.5 nmol CF in the presence of following inhibitors; 0.1% (v/total vial v) acetylene for *P. butanovora* and *M. vaccae*, 1% acetylene for CF8, 2% ethylene, or 200 μM ATU.
[b] Remaining CF degradation activities were showed as percentages of amount of CF degraded compared to controls. Controls for inactivation assays were prencubated with 5 mM butyrate in the absence of inhibitors.

[^{14}C] acetylene incorporated the radiolabel into cellular proteins which were visualized by Phosphorimager analysis (Fig. 3.4; lane 4, 6 and 8 for CF8, *P. butanovora,* and *M. vaccae,* respectively). In addition to polypeptides, heavily labeled bands were observed within the well in the stacking gel. The differences in the intensity of radiolabeling among the three bacteria probably reflect the amount of protein in the samples. In *P. butanovora,* at least two separate radio-labeled polypeptides could be identified, including one major band with a molecular mass of approximately 60 kDa. In addition to proteins, small polypeptide molecules that migrated with the dye front were also labeled with ^{14}C. In CF8, one polypeptide with a molecular mass of approximately 33 kDa was identified. In *M. vaccae,* two polypeptides with apparent molecular masses of 30 and 66 kDa were identified and were radiolabeled to a similar extent.

Butane should protect the monooxygenase enzymes from [^{14}C] acetylene radiolabeling by competing with acetylene for the active site of the monooxygenase. When cells were incubated with [^{14}C]acetylene in the presence of 50% butane, ^{14}C label was not incorporated into polypeptides (Fig. 3.4; lane 5, 7 and 9). 10% butane only partially protected the [^{14}C] acetylene radiolabeling in *P. butanovora* (Fig. 3.5; lane 7). Ethylene is an inactivator of CF degradation by *P. butanovora,* therefore cells pre-treated with ethylene should not be able to interact with acetylene. As expected, no radiolabel was detected in the *P. butanovora* cells pre-treated with ethylene (Fig 3.5; lane 8). When CF8 cells were pre-treated with ATU (200 μM) (Fig. 3.5; lane 4), ^{14}C label incorporation into polypeptide was decreased to 67% of the cells treated only with [^{14}C] acetylene (Fig. 3.5; lane 1). Compared to the remaining enzyme activity after inhibition by ATU (17%) shown by the inhibition assay in Table 1, the incorporated radiolabel was higher than expected. It is probably because of the reversibility of ATU inhibition mechanism.

Figure 3.4
Incorporation of ^{14}C from $[^{14}C]C_2H_2$ into cellular proteins of butane-grown bacteria

The figure shows the protein banding pattern on SDS-PAGE (A) and the phosphorimage (B). Lanes; 1, CF8; 2, *P. butanovora*; 3, *M. vaccae*;. 4, phosphorimage of CF8 cells incubated with $[^{14}C]C_2H_2$; 5, CF8 cells incubated with $[^{14}C]C_2H_2$ and 50% (v/total vial v) butane; 6, *P. butanovora* cells incubated with $[^{14}C]C_2H_2$; 7, *P. butanovora* cells incubated with $[^{14}C]C_2H_2$ and 50% butane; 8, *M. vaccae* cells incubated with $[^{14}C]C_2H_2$; 9, *M. vaccae* cells incubated with $[^{14}C]C_2H_2$ and 50% butane.

Figure 3.5
Incorporation of ^{14}C from $[^{14}C]C_2H_2$ into cellular proteins of CF8 (A) and *P. butanovora* (B)

The figure shows the phosphorimage of the cells incubated with $[^{14}C]C_2H_2$. CF8 cells incubated with $[^{14}C]C_2H_2$ (lane 1), in the presence of 50% (v/total vial v) butane (lane 2), 10% butane (lane 3), preincubated with ATU (200 μM) (lane 4). *P. butanovora* cells incubated with $[^{14}C]C_2H_2$ (lane 5), in the presence of 50% butane (lane 6), 10% butane (lane 7), preincubated with 2% ethylene (lane 8).

Figure 3.4

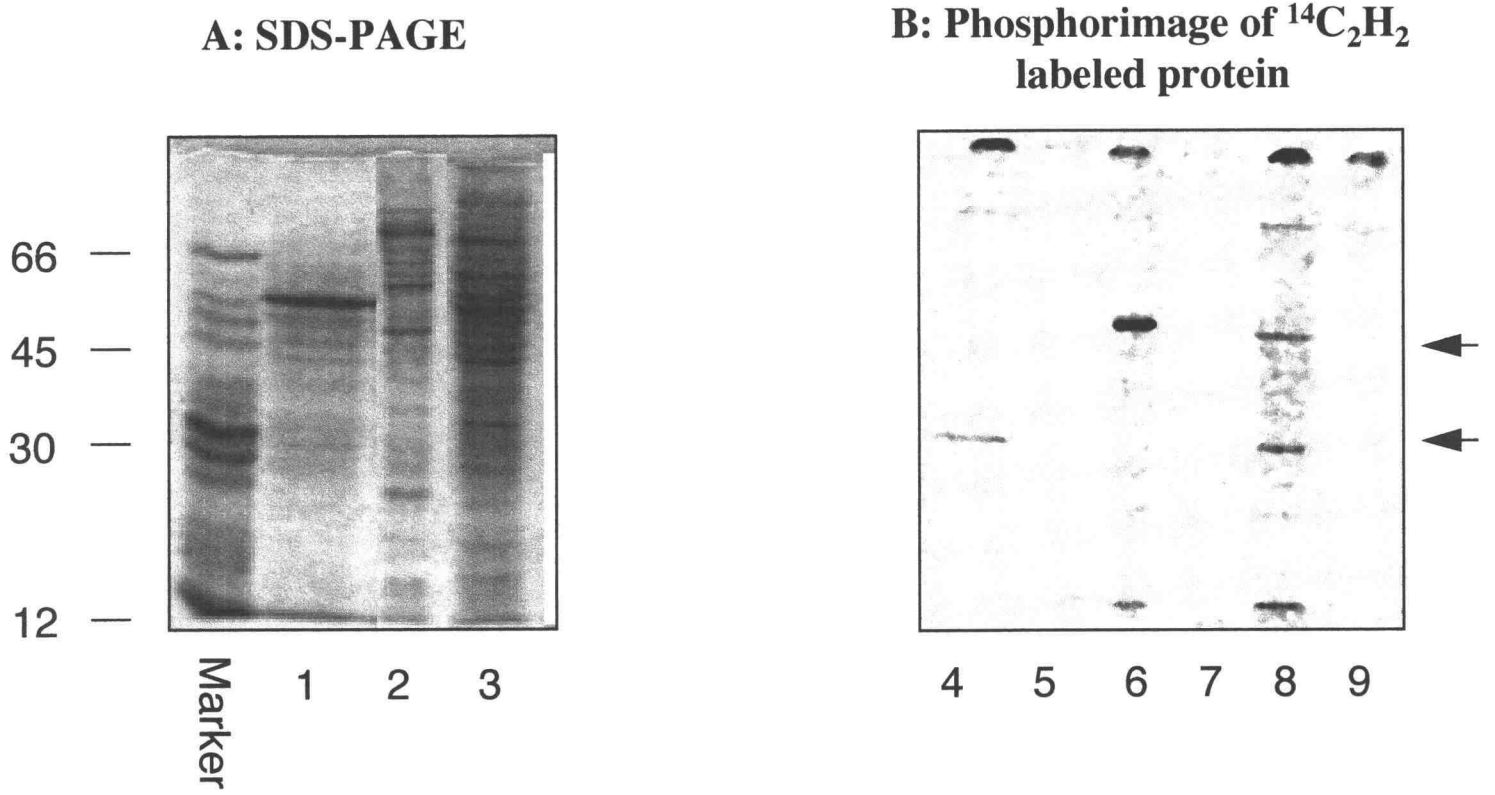
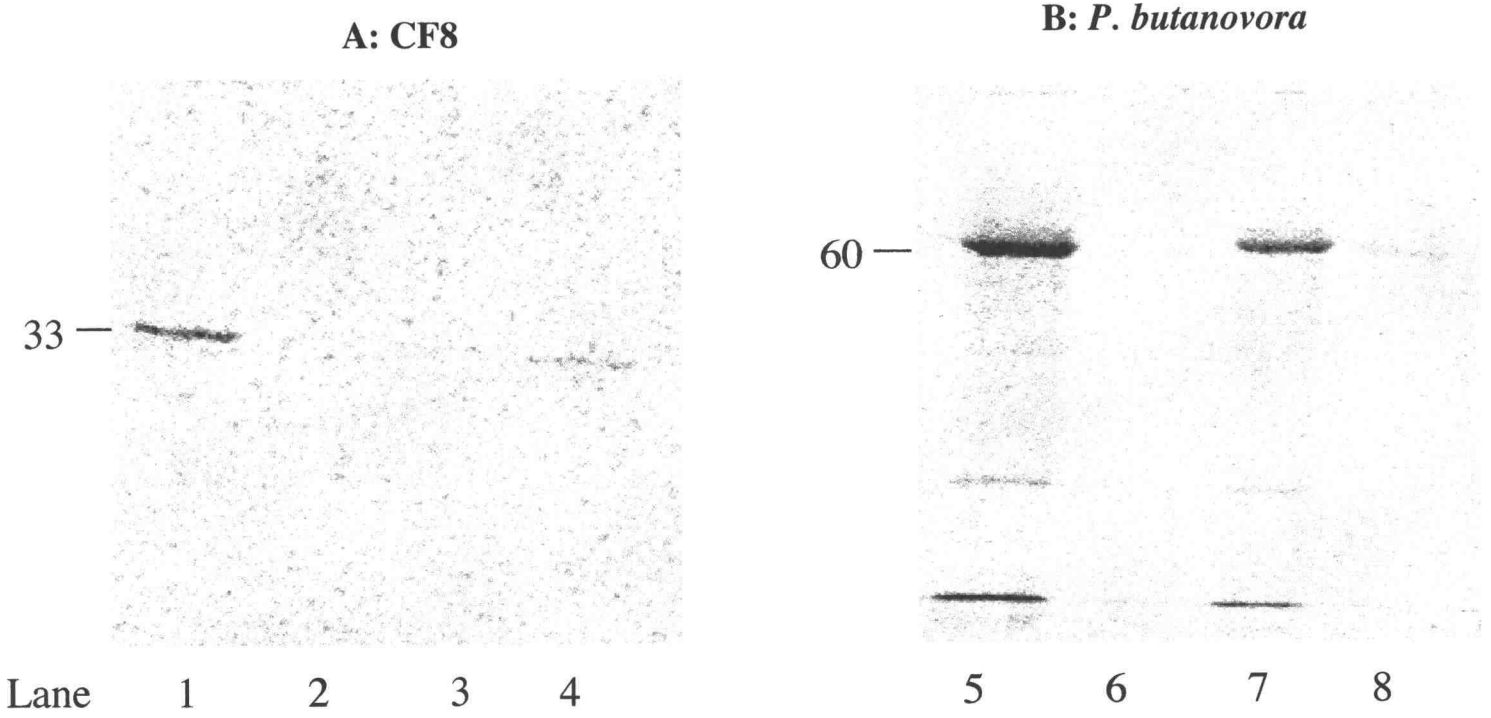

A: SDS-PAGE
B: Phosphorimage of $^{14}C_2H_2$ labeled protein
66
45
30
12
Marker
1
2
3
4
5
6
7
8
9

Fig 3.5



Degradation of [^{14}C]CF by CF8 or *P. butanovora* cells resulted in incorporation of 8 to 10% of the total added ^{14}C into the cellular fraction (data not shown). The cellular polypeptides were separated by SDS-PAGE and analyzed for radiolabel incorporation by Phosphorimage analysis (data not shown). In the case of *P. butanovora,* several polypeptides were very weakly labeled but most of the total ^{14}C migrated with the dye front (data not shown). The polypeptides weakly labeled by [^{14}C]CF corresponded to the polypeptides labeled by [^{14}C]acetylene. Similar experiments with CF8 demonstrated that one polypeptide (ca. 60 kDa) was heavily labeled by [^{14}C]CF and there was noticeable background labeling through the lane (data not shown). The pattern of the [^{14}C]CF labeling was comparable to the protein pattern on coomassie-stained SDS-PAGE, suggesting that the ^{14}C label from [^{14}C]CF bound non-specifically to the abundant cellular proteins. The proposed CF transformation intermediate, phosgene, has been shown to exhibit irreversible binding to protein (Bolt & Filser, 1977). The non-specific [^{14}C]CF binding might be due to phosgene from the incomplete dehalogenation of CF.

Butane degradation was measured by using the resting cells of three butane-grown bacteria. The degradation rates at different initial concentrations of butane were measured, and the kinetic parameter was estimated by fitting the data to Michaelis-Menten model. The K_s (the apparent K_m observed in intact cells) for butane was 14.07 ± 8.49, 47.57 ± 4.87, and 17.57 ± 5.43 μM for the intact cells of CF8, *P. butanovora,* and *M. vaccae* JOB5, respectively. *P. butanovora* had the highest K_s, which was about 8 times higher than that of CF8. CF8 and *M. vaccae* JOB5 showed very similar K_s values for butane.

3.4 Discussion

Three butane-grown bacteria were shown to possess a monooxygenase enzyme that catalyzes the oxidation of both butane and CF. Butane monooxygenases in three butane-grown bacteria were further characterized in this chapter. CF8, *P. butanovora,* and *M. vaccae* JOB5 seem to have enzymes which can be distinguished by their inhibitor and inactivator profiles. As shown in chapter 2, CF degradation by all three butane-grown bacteria were inactivated by acetylene, which was suggested as a mechanism-based inactivator of butane monooxygenase.

In contrast to acetylene, the responses to ethylene and ATU were different for the three butane-grown bacteria. Ethylene irreversibly inactivated CF degradation by *P. butanovora,* but only inhibited CF degradation by CF8 and *M. vaccae.* Ethylene serves as a substrate for alkane monooxygenases, therefore it may competitively inhibit CF degradation. The presence of butane protected the cells of *P. butanovora* from inactivation by ethylene, suggesting that ethylene worked as a mechanism-based inactivator. Ethylene inactivation implies the presence of the P-450 prosthetic group in butane monooxygenase of *P. butanovora.*

The effect of ATU further discriminated among the three monooxygenases. ATU strongly inhibited CF degradation by CF8, suggesting the presence of copper in its butane monooxygenase. Similarly, the inhibition of AMO and pMMO by ATU was used to support a role for copper in these enzymes as well (Bédard and Knowles, 1989). CF degradation by *M. vaccae* was slightly inhibited by ATU. This weak inhibition might be due to ineffective binding of ATU to a copper site or to non-specific binding of ATU to metal prosthetic groups other than copper. The presence of iron prosthetic group might be one possibility since other monooxygenases, such as sMMO and alkane hydroxylase in *P. oleovorans,* have been shown to contain a diiron prosthetic group (Nordlund *et al.*,

1992; Shanklin et al., 1994). However, the differences between CF8 and *M. vaccae* were not clearly shown by this inhibitor/inactivator assay.

Mechanism-based inactivators, such as acetylene, covalently bind to the polypeptides which are most likely in the active site of the enzyme. Treatment of butane-grown cells with [^{14}C]acetylene resulted in the labeling of polypeptides (Fig 3.4). In *P. butanovora,* a single polypeptide with a molecular mass around 60 kDa was heavily labeled. From inhibitor/inactivator profile, *P. butanovora* was proposed to contain P-450 prosthetic group. Acetylene inactivates the P-450 monooxygenases by alkylation of the prosthetic heme group (Ortiz de Montellano, 1991), while some alkynes attack proteins in addition to heme (Ortiz de Montellano & Almira Correia, 1991). If acetylene adducts bound to the heme, the ^{14}C label should have not been identified on the polypeptides because the heme dissociates from the polypeptides during SDS treatment. Our data suggest that acetylene reacts with the protein in the butane monooxygenase of *P. butanovora.*

In *M. vaccae,* two ^{14}C labeled polypeptides with almost same intensities and molecular masses of 30 and 66 kDa were identified. One possible explanations for this result is that the labeled polypeptides are components of two different monooxygenases, both of which are inactivated by acetylene. This might not be too unrealistic because some methanotrophs are known to produce two forms of MMOs depending upon the copper concentration in the growth media(Stanley et al., 1983). Both pMMO and sMMO are inactivated by acetylene. The 54 kDa subunit of component A of sMMO and the 27 kDa subunit of pMMO become labeled with ^{14}C upon inactivation of the monooxygenase activity with [^{14}C]acetylene (Prior & Dalton, 1985). Another explanation is that the activated acetylene species traveled from the active site to react with closely located polypeptides. In this case, the active site and the polypeptides have to be in the specific configuration to incorporate the label with the same intensities. It is also possible that

one polypeptide was degraded into two components during SDS-PAGE or sample preparation. However, this is unlikely since both of the components contained almost equal amount of ^{14}C label.

CF8 showed labeling of a single polypeptide with a molecular mass around 30 kDa. Interestingly, the polypeptides which have close molecular mass (27 kDa) were shown to labeled with ^{14}C from [^{14}C]acetylene in both pMMO and AMO (Hyman & Wood, 1985; Prior & Dalton, 1985). This ^{14}C labeled 27 kDa polypeptide in AMO has shown to aggregate by heating in the presence of SDS-PAGE sample buffer (Hyman & Arp, 1993). The aggregated sample cannot enter the stacking gel, and the labeled band appears near the well in the stacking gel. This aggregation reaction is also observed with $^{14}C_2H_2$-labeled polypeptides in other species of autotrophic nitrifiers and in methanotrophs expressing pMMO. It is suggested that strongly hydrophobic amino acid sequences present in AMO are responsible for the aggregation reaction (Hyman & Arp, 1993). A similar phenomenon was observed with CF8 during the sample preparation for SDS-PAGE. When CF8 cells containing $^{14}C_2H_2$-labeled polypeptides were solubilized and heated at 95 °C for 5 min in the sample buffer containing SDS and β-mercaptoethanol and then electrophoresed, ^{14}C-labeled 30 kDa polypeptide disappeared and the labeled band appeared within the sample well in the stacking gel (data not shown). This observation further supports the similarity between butane monooxygenase in CF8 and the copper-containing monooxygenases, AMO and pMMO.

The K_s for butane provides another level of discrimination among three butane-grown bacteria. *P. butanovora* showed a higher K_s than CF8 and *M. vaccae.* The high affinity of CF8 and *M. vaccae* cells for butane is consistent with the observation that low concentrations of butane inhibited their CF degradation (Chapter 2, Table 2.2). From the results in Chapter 2 (Table 2.3), it was suggested that *P. butanovora* and *M. vaccae* may have an apparent K_m for CF in the range of 13 to 39 μM, while the apparent *Km* for CF

of CF8 is well below 13 μM. The kinetic constants with methane and chlorinated aliphatics by *Methylosinus trichosporium* OB3b have been studied . Oldenhuis et al. (Oldenhuis et al., 1991) reported that the K_m for methane was 92 ± 30 μM when cells were expressing only the sMMO. The kinetic constants found with chlorinated aliphatics were in the same order of magnitude as with methane. For instance, *Km* for CF and TCE were 34 ± 6 and 145 ± 61 μM, respectively (Oldenhuis et al., 1991).

This study showed a remarkable level of diversity among the monooxygenases in three butane-grown bacteria. The results suggested the presence of different prosthetic groups in each butane monooxygenase; a cytochrome P-450 in *P. butanovora*, a copper prosthetic group in the enrichment culture, CF8, and a diiron center in *M. vaccae*. Bacterial oxygenases are known to utilize metal ions such as iron, copper, and manganese as cofactor to bind dioxygen (Harayama *et al.*, 1992). The diverse cofactors have been found in alkane-oxidizing monooxygenases; non-heme iron in alkane hydroxylase from *P. oleovorans* (Ruettinger *et al.*, 1974) and xylene monooxygenase from *P. putida* (Harayama et al., 1992). P-450 in octane hydroxylase from *Corynebacterium* 7E1C, binuclear iron clusters in sMMO from *M. trichosporium* OB3b (Fox et al., 1989), and both iron and copper in pMMO from *M. capsulatus* (Bath) (Zahn & DiSpirito, 1996). Among these, butane monooxygenases are significant in that various cofactors are utilized to oxidize a single substrate by three different bacteria. In order to further confirm this diversity, the study needs to be extended to biochemical and molecular biological characterization of butane monooxygenases.

Chapter 4

Summary and Conclusions

In this study, chloroform (CF) degradation by three butane-grown bacteria, an enrichment culture CF8 derived from the Hanford microcosms and two bacteria from the ATCC culture collection, *Mycobacterium vaccae* JOB5 and *Pseudomonas butanovora* was demonstrated. This is the first report of CF degradation by pure cultures of butane-oxidizing bacteria.

All three butane-grown bacteria were able to degrade CF at rates comparable to that of a known CF degrader, *Methylosinus trichosporium* OB3b. CF degradation by all four bacteria required O_2 and was inhibited by acetylene, a known monooxygenase inhibitor. The presence of growth substrate, inhibited CF degradation. These results suggest that a monooxygenase is responsible for both CF and growth substrate degradation.

P. butanovora required exogenous reductant to degrade CF, while CF8 and *M. vaccae* utilized endogenous reductants. *M. trichosporium* was able to degrade CF without an exogenous electron donor, however, the presence of formate enhanced CF degradation. The amounts of CF degraded at different initial CF concentrations (ranging from 13 to 39 μM) were determined. In the case of *M. trichosporium,* the increase in the amount of CF degraded was proportional to the increase in initial CF concentrations, indicating that the apparent K_m (appK_m) is well above 39 μM (the highest initial CF concentration used in this assay). In contrast, *P. butanovora* and *M. vaccae* did not degrade CF proportional to the increase in CF concentration which suggests that the app K_m is in the range of the CF concentrations tested (between 13 and 39 μM). CF8, which

showed no increase in CF degradation with increasing CF concentrations, probably has an app K_m of below 13 μM.

The CF degradation rates decreased with time of incubation, probably due to CF toxicity to the cells. CF8 and *P. butanovora* were more sensitive to CF than either *M. trichosporium* or *M. vaccae*. The CF toxicity on CF8 and *P. butanovora* was further examined by measuring butane-dependent O_2 uptake after exposure to CF. CF8 cells incubated with CF in either the presence or absence of butyrate degraded CF. In contrast, *P. butanovora* cells degraded CF only when cells were incubated with CF in the presence of butyrate. Decreased butane-dependent O_2 uptake was correlated with CF degradation activity of the cells. This result suggests that CF toxicity is due to its reaction with the enzyme.

CF8 and *P. butanovora* were able to degrade other chlorinated aliphatic hydrocarbons (CAHs), including trichloroethylene, 1,2-*cis*-dichloroethylene, and vinyl chloride. In addition, CF8 degraded 1,1,2-trichloroethane. 1,1,1-Trichloroethane and 1,2-*trans*-dichloroethylene were not degraded at detectable amounts by either CF8 or *P. butanovora*. The same enzyme which catalyzes CF degradation is most likely responsible for degradation of other CAHs. The butane monooxygenases in CF8 and *P. butanovora* showed a broad substrate specificity, which makes these organisms useful in bioremediation of environmental contaminants.

Known monooxygenase inhibitors were examined for their effects on CF degradation by butane-grown bacteria. Acetylene irreversibly inactivated CF degradation by all three butane-grown bacteria, and the presence of butane protected the enzyme from being inactivated. This result suggests that acetylene works as a mechanism-based inactivator of butane monooxygenase. In contrast, the responses to ethylene and allylthiourea (ATU) were different among the three butane-grown bacteria. Ethylene irreversibly inactivated CF degradation by *P. butanovora,* but only inhibited CF

degradation by CF8 and *M. vaccae.* ATU strongly inhibited CF degradation by CF8 but only slightly inhibited CF degradation by *M. vaccae.* These differences could be due to the presence of different prosthetic groups in their butane monooxygenases. Ethylene inactivation of *P. butanovora* indicates the presence of a P-450 moiety. Strong inhibition of CF8 by ATU, a copper selective chelator, is suggestive of a copper prosthetic group. The prosthetic group in *M. vaccae* cannot be specified by this inhibitor and inactivator assay. However, it is possible that the weak inhibition by ATU was due to the non-specific interaction of ATU with metal prosthetic group other than copper, such as iron since this is common cofactor among known monooxygenases.

Treatment of butane-grown cells with [^{14}C]acetylene resulted in the covalent binding of radiolabel to polypeptides. In *P. butanovora,* at least two separate radiolabeled polypeptides were identified, including one heavily labeled polypeptide with a molecular mass of approximately 60 kDa. In *M. vaccae,* two polypeptides with molecular masses of 30 and 66 kDa were identified and those were radiolabeled to a similar extent. CF8 showed labeling of a single polypeptide with molecular mass of approximately 33 kDa. The polypeptides heavily labeled with [^{14}C]acetylene are likely to contain the active site of butane monooxygenase.

In order to further investigate the differences among the three butane monooxygenases, the kinetic parameter was determined. Butane degradation rates by the resting cells of three butane-grown bacteria were measured at different initial concentrations of butane. The K_s (the apparent K_m in intact cells) for butane was estimated as 14, 48, and 18 μM for CF8, *P. butanovora,* and *M. vaccae,* respectively. This provides another level of discrimination among the three butane-grown bacteria.

This study demonstrated the potential use of butane as a cometabolic substrate for CAH degradation. Because of their broad substrate range, butane-oxidizing bacteria may be effective in bioremediation. The results from the inhibitor and inactivator assays,

[^{14}C]acetylene labeled polypeptides patterns, and the kinetic measurements have indicated differences among the butane monooxygenases in three butane-grown bacteria examined in this study. These differences may be due to the presence of different prosthetic groups in these butane monooxygenases. Characterization of these butane monooxygenases at the biochemical and molecular level is required to confirm the observed diversity.

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