ABSTRACT

SALAS MEZA, LINDA ALEJANDRA. Effect of Propane Grade on Growth and Activity of Gaseous Hydrocarbon-Oxidizing Bacteria (Under the direction of Dr. Michael R. Hyman).

Gaseous hydrocarbon-utilizing bacteria are of great practical value for the bioremediation of small organic pollutants. Propane-oxidizing bacteria are of particular interest as this gas can be easily added to subsurface environments and propane biostimulation has promoted biodegradation of various contaminants in field studies. The overall aim of this project was to determine whether, compared to pure gases, gas mixtures can improve *in situ* bioremediation processes by increasing the diversity of gaseous hydrocarbon-oxidizing bacteria and their non-specific monooxygenase enzymes. The present research has focused on genome-enabled studies of pure cultures of bacteria and is a prelude to future studies of the impacts of gas mixtures on mixed microbial communities in field samples.

Rhodococcus rhodochrous ATCC 21198 expresses two different propane-oxidizing monooxygenases during growth on propane. These enzymes are short chain alkane monooxygenase (SCAM) and propane monooxygenase (PrMO) and both enzymes are thought to contribute to the propane- and contaminant-oxidizing activities of this bacterium. Compared to propane alone, growth of strain 21198 was partially inhibited when consumer grade liquefied propane gas (Blue Rhino HD5) was used as a growth substrate while growth was fully inhibited when custom gas mixtures of either ~5% (Mix 5) or ~10% (Mix 10) (v/v) propylene in propane were used. A similar effect was also observed with several other model strains. These included *R. aetherivorans* BCP1 (strain BCP1) which also expresses SCAM and PrMO during growth on propane and two other strains (*R. rhodochrous* B276 and *R. opacus* PD630) that have PrMO as their sole propane-oxidizing monooxygenase. Two different propylene-metabolizing strains (*Rhodococcus rhodochrous* B276 and *Xanthobacter autotrophicus* Py2) also grew on pure

propylene while strain Py2 also grew on the low concentrations of propylene in the Mix 5 and Mix 10 gas mixtures.

In HD5, ethane is a typical production contaminant while ethanethiol is a deliberately added odorant. Neither gas inhibited growth of strain 21198 on propane when added at concentrations above and below their measured or known concentration in HD5. During growth of strain 21198 on HD5, consumption of propane was accompanied by consumption of propylene; another production contaminant in HD5. Low concentrations of propylene oxide were detected in the culture medium after growth of strain 21198 on defined propane:propylene mixtures and rapid epoxide production was also observed in experiments using high concentrations of resting propane-grown cells. The kinetic constants for propylene oxide production were determined and similar "maximal" rates of propylene oxide production were observed for propane-grown cells of strains 21198, B276 and PD630. Propylene oxide also inhibited growth of strain 21198 to varying degrees when added to cultures growing on propane. Other potential metabolites that could be generated by the oxidation of propylene include 1, 2-propanediol, allyl alcohol and acrylic acid. Although several of the model strains grew on some of these compounds, the effects of these compounds as either carbon or energy sources or as inhibitors of growth on hydrocarbons were generally limited.

Overall, the results suggest presence of low concentrations of propylene (≤1% v/v) in HD5 has a limited inhibitory effect on growth of model gaseous hydrocarbon-oxidizing bacteria but the presence of higher concentrations can be very inhibitory, most likely due propylene oxide production by non-specific propane-oxidizing monooxygenases. The results also indicate some alkene-oxidizing bacteria such as strain Py2 can grow on low levels of propylene in Mix 5 but not HD5. This capability might promote greater microbial and enzymatic diversity as well as

contaminant-degrading capability if gas mixtures similar to Mix 5 were used to as a field-based biostimulant rather than propane alone. Fluorescent activity-based labeling of active monooxygenases is proposed as one potentially promising method for investigating this predicted effect in field samples.

Effect of Propane Grade on Growth and Activity of Gaseous Hydrocarbon-Oxidizing Bacteria

by
Linda Alejandra Salas Meza

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Microbiology

Raleigh, North Carolina
2020

APPROVED BY:

_____	_____
Dr. Michael R. Hyman Committee Chair	Dr. Manuel Kleiner
_____	
Dr. Carlos Goller	

DEDICATION

I dedicate my work to my family and friends who have supported me all these years through their continuous encouragement, motivating me to keep moving forward.

BIOGRAPHY

Linda Alejandra Salas Meza was born in the State of Aguascalientes, Mexico. She spent most of her life in Queretaro (Mexico) where she studied her Bachelor's in Biotechnology Engineering in the Instituto Tecnologico y de Estudios Superiores de Monterrey (ITESM). She pursued her Master's degree in Microbiology on the Department of Microbial and Plant Biology under the direction of Dr. Michael Hyman

ACKNOWLEDGMENTS

I want to express my gratitude to my advisor Dr. Hyman for his guidance during my stay in NC State. Opening the opportunity to work along with his multi-disciplinary research team in a new area of biotechnology to me. I'm grateful to Dr. Manuel Kleiner and Dr. Carlos Goller for their kind mentorship and encouragement. I extend my acknowledgment to Dr. Eric Miller for offering the opportunity to grow at NC State.

I'm thankful to my laboratory mates Alejandra Del Pilar Oyarzun Mejia, Weijue Chen, Amie McElroy, Christy Smith, Sarah Montoya, and Nick Faulkner for their friendship, tea breaks, support in experiments, late-night GC inquiries and advice.

To my past and new friends Madiha Nazir, Clara Tang, Amelia Elis, Bahji Balard, Clara Jones, Morgan Maly, Itahí Angeles Lopez, Colleen Oliver, Lori Townsend, Khuzaima Hameed, Claudia Hernandez, and Azucena Gonzalez. I'm grateful for their optimist mantras, their company, their constructivism and introducing me to new foods, perceptions and board games. I would also like to extend my thanks to my fellow Microbiology Graduate peers for their delightful lunch conversations and generous support.

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CHAPTER 1

Literature Review

Overview of *In situ* Bioremediation Approaches. In soil and groundwater, common pollutants such as volatile organic compounds (VOCs) (*e.g*. gasoline, chlorinated solvents) can often be remediated by methods that involve injection of gases that can promote the activities of native microorganisms. Three prominent examples of *in situ* remediation that involve subsurface gas injection include air sparging, bioventing, and aerobic cometabolic biodegradation (ACB).

Air sparging involves pumping air into contaminated groundwater to promote partitioning of dissolved VOCs into the gas phase and is often operated in conjunction with vacuum-based extraction methods to remove volatilized contaminants (Brusseau & Maier, 2004). Although air sparging is primarily a physical remediation approach, injection of air does promote aerobic biodegradation. Unlike air sparging, which is particularly effective for removing dissolved VOCs such as gasoline, bioventing is an approach that is effective for less volatile pollutants such as diesel range organics and for contaminants that are present in unsaturated soils rather than groundwater. In bioventing, air or oxygen are added to promote aerobic biodegradation of contaminants by native microorganisms. The rates of gas addition are typically much lower than those used for air stripping and bioventing approaches also often involve the addition of nutrients to further promote microbial activity.

In aerobic cometabolic biodegradation (ACB), a benign, non-toxic primary growth substrate and air are co-injected into contaminated groundwater to promote the growth of indigenous aerobic microorganisms (Azubuike *et al*., 2016). The biostimulated microorganisms then degrade the target contaminants through the activity of non-specific enzymes that normally function in the pathway of primary growth substrate catabolism. Like bioventing, ACB depends on the biodiversity, abundance, and location of the microbial communities that enable xenobiotic

degradation (Brusseau & Maier, 2004; Environmental Protection Agency, 2017; Lippincott *et al*., 2015).

Aerobic Cometabolism and Metabolism. In many cases ACB relies on the growth of aerobic bacteria that initiate the catabolism of the primary (growth-supporting) substrate through the activity of non-specific monooxygenase enzymes. These monooxygenases typically catalyze hydroxylation or epoxidation reactions while subsequent enzymes in the catabolic pathway catalyze energy-yielding dehydrogenation reactions. The primary growth substrate is ultimately mineralized through central metabolic pathways and is used as a source of carbon and energy for biosynthesis and growth. An example of the key role of alkane-1-monooxygenase in the catabolism of *n*-alkanes is shown in Figure 1.

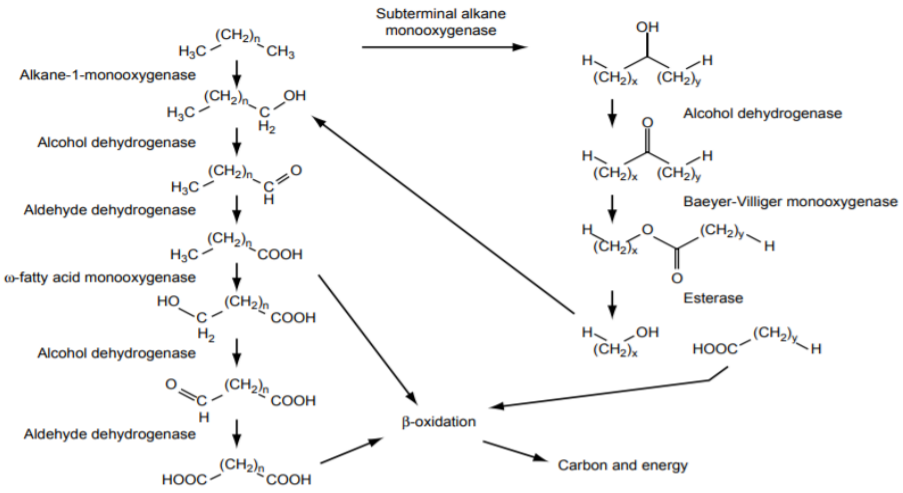


Figure 1. N-Alkane Oxidation Pathway

Oxidation of a generic alkane by an alkane monooxygenase (alkane-1-monooxygenase) generates either a 1^{o} alcohol through terminal oxidation or a 2^{o} alcohol through subterminal oxidation (van Beilen *et al*., 2003)**.**

Monooxygenases often have broad substrate ranges and this is exploited in ACB to degrade organic pollutant co-substrates. As these co-substrates do not independently support growth of the cometabolically-active microorganisms, the primary growth-supporting substrate not only promotes expression of the contaminant-degrading monooxygenases but is also needed to generate sufficient active biomass to achieve useful *in-situ* rates of contaminant biodegradation. The inability of cometabolically-active microorganisms to obtain carbon and energy from co-substrate (contaminant) biodegradation is an often invoked but infrequently tested assumption. While cometabolic degradation of contaminants by pure microbial cultures can lead to accumulation of partially oxidized and potentially toxic metabolites, in mixed cultures multiple microorganisms can potentially contribute to contaminant mineralization (Lontoh & Semrau, 1998; Hazen, 2010).

An especially important feature of ACB is that biomass generation and contaminant degradation are separate processes. This separation of catabolism and anabolism enables ACB processes to degrade contaminants to much lower concentrations than processes based on more conventional metabolic biodegradation in which microorganisms grow directly on the contaminants of concern. For example, some microorganisms can use the chlorinated solvent stabilizer 1,4-dioxane (14D), as a sole source of carbon and energy for growth (Mahendra & Alvarez-Cohen, 2006). In the environment the physical, chemical, and biological properties of 14D often result in the formation of large diffuse dissolved plumes with low 14D concentrations in the ppb (µg/L) range (Chu *et al*., 2018). These concentrations are several orders of magnitude below the minimum concentration needed to maintain the metabolic activity of 14D-metabolizing bacteria. Biodegradation of low ppb concentrations of 14D (≤100 ppb) has therefore been reported for propane-stimulated ACB but not for metabolism-based biodegradation of this compound ((Chu *et al*., 2018; Department of Defense, 2001; Lippincott *et al*., 2015).

Propane-Oxidizing Bacteria and Soluble Diiron Monooxygenases. *In situ* ACB processes require the use of primary substrates that can be easily added to groundwater and have the specificity to promote the growth of bacteria with appropriate contaminant-degrading monooxygenases. In practice, the choice of primary substrate is dictated by the factors listed above but is also influenced by issues of cost, availability, aqueous solubility and toxicity, among others. For many of the reasons mentioned above, propane has emerged as a particularly useful primary substrate for ACB.

Propane can be oxidized by a wide variety of bacterial monooxygenases but current genomic evidence indicates bacteria that can grow on propane as a sole source of energy (propanotrophs) utilize a limited number of monooxygenases that include both membrane-bound, copper-containing monooxygenases (CuMMOs) and cytoplasmic, iron-containing enzymes known as soluble diiron monooxygenases (SDIMOs). The microorganisms examined in the research described in this thesis only express SDIMOs during growth on propane or propylene.

SDIMOs are multicomponent cytoplasmic enzymes that are composed of a reductase, a hydroxylase (2-3 distinct subunits) and a small effector protein. The NAD(P)H-oxidizing flavin-containing reductase transfers reducing equivalents to the di-iron-containing hydroxylase which then activates dioxygen (O_2) and catalyzes the substrate oxidation reaction (Cappelletti *et al*., 2015). These are frequently either hydroxylation or epoxidation reactions in which one atom of oxygen from dioxygen is added a C-H or C=C bond, respectively. In all cases, the second oxygen from dioxygen is reduced to H_2O. The small effector protein serves a regulatory role and contains no prosthetic groups. SDIMOs typically have broader substrate ranges than CuMMOs *(Fasan et al*., 2008; Leahy *et al*., 2003) and are currently classified into six groups according to their operon and holoenzyme structure, and their substrate preference (Leahy *et al*., 2003) (Table 1).

Table 1. SDIMO Classification

	Name of monooxygenase	**Hydroxylase structure**	**Co-substrate Examples**
Group 1	**Alkene (AlkMO)** Toluene (T3/4MO)	**$(\alpha\beta y)_2$**	**chloroalkenes**
Group 2	Phenol hydroxylases (PH) Toluene (T2MO)	$(\alpha\beta y)_2$	naphthalene, vinyl chloride
Group 3	Methane (sMMO) Butane (BMO)	$(\alpha\beta y)_2$	chloroethene
Group 4	**Alkene (AMO)**	**$\alpha_2\beta_2$**	**chloroalkenes**
Group 5	**Propane (PrMO)** Tetrahydrofuran (THFMO)	**$\alpha_2\beta_2$**	***N*-nitrosodemethylamine (NDMA)**
Group 6	**Short Chain Alkane (SCAM)**	**$\alpha_2\beta_2$**	**1,4 dioxane**

(Cappelletti et al., 2015; Ensign, Hyman, & Arp, 1992; Leahy et al., 2003; Ryoo, Shim, Canada, Barbieri, & Wood, 2000; Jonathan O. Sharp et al., 2007; Jan B. van Beilen & Funhoff, 2007)

A series of model propane-oxidizing bacteria have been examined in the study described in this thesis and annotated genomes are available for each of these strains. The respective growth substrate ranges and known SDIMO complements of these strains are summarized in Table 2.

Table 2. Bacterial Strains and Their Corresponding Growth Substrates and SDIMOs

Bacterial Strain	Alkane Growth Substrates	Alkene Growth Substrates	SDIMOs
Rhodococcus rhodochrous ATCC 21198	C_2-C_4+	?	SCAM + PrMO
Rhodococcus aetherivorans BCP1	C_2-C_4+	?	SCAM + PrMO
Rhodococcus opacus PD630	C_3 (C_4?)	?	PrMO
Rhodococcus jostii RHA1	C_3 (C_4?)	?	PrMO
Rhodococcus rhodochrous B276	C_3 (C_4?)	C_3	AMO (alkenes) + PrMO (alkanes)
Xanthobacter autotrophicus Py2	None	C_2-C_5	AlkMO (alkenes)

The majority of the research described in this thesis has focused on *Rhodococcus rhodochrous* ATCC 21198. Shotgun proteomic analyses have demonstrated this bacterium expresses all components of a short chain alkane monooxygenase (SCAM) at consistently high levels in cells grown on ethane, *n*-butane, or isobutane (Hyman laboratory, unpublished results). Expression of this enzyme is inducible and it is not detected in dextrose-grown cells while in propane-grown cells it is expressed at lower levels than in cells grown on other gaseous alkanes. In contrast, all components of the other SDIMO, propane monooxygenase (PrMO), are only expressed at high levels in propane-grown cells and are greatly diminished or entirely absent in cells grown on dextrose or other gaseous alkanes. Transcriptomic studies (Cappelletti *et al*., 2015) have demonstrated very similar expression patterns for the gene encoding the α-hydroxylase components of these two enzymes in *Rhodococcus aetherivorans* BCP1, another strain included in this study. The alkene growth substrate range for strains 21198 and BCP1 has not been previously reported but neither strain possesses genes encoding either of the two alkene-oxidizing SDIMOs typically expressed by gaseous alkene-oxidizing bacteria.

Unlike strains 21198 and BCP1 which are versatile alkane-oxidizing strains, *Rhodococcus opacus* and *Rhodococcus jostii* RHA1 have a more limited gaseous alkane-oxidizing ability. The genomes for both strains encode a single SDIMO, propane monooxygenase (PrMO). In strain RHA1, this enzyme is expressed in cells grown on propane (Sharp *et al.*, 2007) and in response to carbon-limitation (Patrauchan *et al*., 2012). Little is known about the expression patterns for this enzyme in strain PD630.

Two other model strains were also examined in this study which are connected by their ability to grow on propylene (Furuhashi *et al*., 1981; Small & Ensign, 1997). The best characterized propylene-metabolizing strain, *Xanthobacter autotrophicus* Py2, uses an alkene monooxygenase to initiate propylene catabolism (Small & Ensign, 1997). The hydroxylase component of this Group 1 SDIMO [$(\alpha\beta y)_2$] is structurally distinct from the Group 4 SDIMO that initiates propylene catabolism in strain B276 [$\alpha_2\beta_2$] (Table 2). A further distinction between these strains is that strain B276 can also grow on both gaseous alkenes and alkanes However, strain B276 grows more substantially on propane compared to other gaseous alkanes (Furuhashi *et al*., 1981). Like strains RHA1 and PD630, the genome of strain B276 encodes a PrMO-like SDIMO.

Activity-based labeling of SDIMOs. The Fe-containing hydroxylase components of SDIMOs contain the active site of these enzymes in which O_2 activation and substrate oxidation occurs. The nucleotide and derived amino acid sequences for the various hydroxylase subunits are sufficiently distinct that molecular tools such as quantitative polymerase chain reactions (qPCR and RTqPCR) have been widely used to specifically quantify the abundance of genes and transcripts for these enzymes in laboratory and field studies (Chu *et al*., 2018; Lippincott *et al*., 2015). However, it is widely recognized that the correlation between gene or transcript abundance and enzyme-specific

activity is often poor and that more accurate assessments of enzyme activity require measurements of protein abundance in environmental samples (Rocca *et al*., 2015). In this study the recently developed technique of activity-based labeling (ABL) has been used to investigate the expression of SDIMOs in axenic and mixed bacterial cultures. This technique was originally developed to study ammonia monooxygenase (AMO) in the autotrophic nitrifying bacterium *Nitrosomonas europaea* (Bennett *et al*., 2016) and involves 2 key steps. Many SDIMOs are commonly inactivated by alkynes through a mechanism-based inactivation process. The initial step in ABL, therefore, involves the *in vivo* addition of a terminal diyne probe such as 1,7-octadiyne to intact cells. The activation of one of the alkyne groups of this probe by SDIMOs results in the formation of a reactive intermediate that then covalently attaches to one or more amino acids in the active site-containing hydroxylase component of the enzyme and results in irreversible loss of enzyme activity. In the second step, the unreacted alkyne group in the now inactive enzyme:probe adduct is reacted *in vitro* with an azide-containing fluor (*e.g.* AlexaFluor 647 azide) using a copper-catalyzed alkyne/azide cycloaddition (click) reaction. The resulting fluorescently labeled polypeptides can then be visualized, quantified, and identified after SDS-PAGE analysis of protein samples using near-infrared scanning.

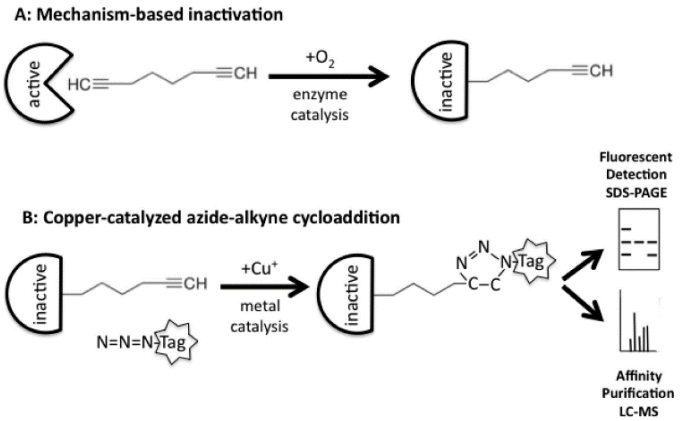


Figure 2. Key steps in activity-based labeling (ABL) of monooxygenases

Use of Propane in Aerobic Cometabolic Bioremediation. As indicated earlier, propane is an inexpensive and reasonably water-soluble hydrocarbon source that several advantages for field applications of ACB (Azadpour-Keeley, 2002). Compared to other gaseous alkanes such as methane and *n*-butane, propane appears to stimulate and maintain the activity of bacteria that can cometabolically degrade a wide range of xenobiotics. For example, in soil microcosm propane biostimulation has been shown to support prolonged degradation of trichloroethylene (TCE) compare to methane biostimulation. Additionally, propanotrophs biodegraded chloroform (CF) and 1,1,1-trichloroethane (111TCA) that were not degraded by methanotrophs (Tovanabootr & Semprini, 1998).

Several studies have described the use of propane in field studies of ACB. In a site in New Jersey contaminated with methyl *tertiary* butyl ether (MTBE)-containing gasoline, air containing <0.2% (v/v) propane was used to promote and sustain the cometabolic MTBE-degrading activity of cultures of *Rhodococcus ruber* ENV425 (ENV425) that were added (bioaugmented) to the contaminated groundwater. This treatment resulted in a <90% decrease in MTBE concentration over 5 months. The concentration of *tertiary* butyl alcohol (TBA), a metabolite of anaerobic MTBE biodegradation, was also reduced and it was also observed that MTBE and TBA degradation ceased when the gas supply was halted. In another study at Port Hueneme (CA), a similar approach involving bioaugmented ENV425 was used to compare the effects of adding air:propane mixtures compared to only air alone. Both treatments achieved 10-fold reductions in MTBE during a 10-month treatment. Microcosm studies revealed propane treatment stimulated propanotrophs with cometabolic MTBE-degrading activities (Azadpour-Keeley, 2002).

Propane-biostimulated ACB in gasoline-impacted environments involves the selective biostimulation of microorganisms to degrade one major recalcitrant compound (MTBE) present in

a background of otherwise very readily biodegradable gasoline hydrocarbons. More recent studies of propane-stimulated ACB have focused on sites contaminated with another recalcitrant ether, 1,4-dioxane (14D). The range of contaminants encountered at 14D-impacted sites is very different than at gasoline-impacted sites and includes not only the ether itself (14D), but also many similarly recalcitrant co-contaminants that are associated with the prior use of 14D as a stabilizer for chlorinated solvents. These co-contaminants often include TCE as well as 111TCA and its various biotic and abiotic degradation products that include, among others, 1,1-dichloroethylene (11DCE) 1,1-dichloroethane (11DCA). Again, propane has been used at these sites because of the recognized ability of propanotrophs to not only cometabolically oxidize 14D but to also oxidize many, but not all, of this ether's common chlorinated co-contaminants. In one field study involving propane biostimulation of a bioaugmented propanotroph (ENV425), the initial concentration of 14D was reduced from >1 ppm to ~2 ppb over 8 months (Lippincott *et al*., 2015). The concentrations of many of the chlorinated co-contaminants was also greatly reduced but this may have involved both biodegradation and physical stripping due to the use of air:propane biosparging. In this study high purity propane (>99.9% v/v) was used as the primary growth substrate. In a separate study involving air:propane mixtures in a recirculation system the 14D concentration was reduced from ~60 ppb to ~2 ppb over a 9-month treatment period. The concentrations of chlorinated co-contaminants such as TCE and 11DCE were also reduced to ≥ 1 ppb (Chu *et al*., 2018). This application of ACB used a consumer grade of propane (HD10) which contains both propane and potentially, small amounts of propylene.

Propane Grades. A summary of the composition of the major different grades of propane that are commercially available (Table 3) indicates that propylene is a consistent contaminant in all major

sources of propane. However, in high purity Research grade propane propylene is typically present at trace (ppm) concentrations, along with small amounts of major air components (N_2 and O_2). In contrast, HD5, the most widely available consumer grade propane (and HD10 which is only available in CA) has specifications that define the minimum amount of propane (≥95% v/v) and the maximum amount of propylene (≤5% v/v). Along with other volatile alkanes, the other important component in consumer grades of propane is an odorant such as ethanethiol. This is a safety feature designed to enable consumers to detect gas leaks at gas concentrations well below the lower explosive limit (LEL) of the gas in air. The type and concentration of these odorants is stipulated by state and federal laws (Department of Transportation, 2012).

Table 3. Summary of commercially available propane grades

Grade	Propane (% v/v)	Contaminants	Use
Research	>99.99%	Propylene, N_2, O_2 and CO_2 ppm (v/v) levels	Research, Industry
Instrument	>99.5%	Propylene, other hydrocarbons, N_2, O_2 and CO_2 at ppm (v/v) levels	Research, Industry
HD5	>90%	Propylene (≤5% v/v), butanes (<2.5% v/v), heavier gases, odorant (ethanethiol)	Domestic appliances, motor fuel
HD10 (Only in California)	>85%	Propylene (<10% v/v), butanes (<5% v/v), heavier gases (<0.5% v/v) odorant (ethanethiol)	Domestic appliances only
Commercial Liquefied Petroleum Gas (LPG)	49-90%	Propylene (<50%),	Forklift fuel

(Ross & White, 2006)

Effects of propane grade on pure and mixed cultures. The likely presence of propylene in the HD10 propane used in field study described by Chu *et al*., (2018) raised intersting questions about the potential effects of propylene on pure cultures of propane-oxidizing bacteria and the potential biostimulation of both propane- and propylene-oxidizing bacteria in the field.

In the case of pure cultures of propane-oxidizing bacteria, it has been recognized for many years that these types of miroorganism usually do not grow on short chain alkenes (C_2-C_5) but can oxidize some or all of these unsaturated gases after growth on propane (Lukins & Foster, 1963). Much of the interest in these biotransformations was associated with the potential use of gaseous hydrocarbon-utilizing bacteria to generate industrially-useful epoxides from plentiful alkene feed stocks. Consequently, the major emphases of the relevant research was to identify highly active epoxide-generating strains and maximal rates of epoxide production. Other factors such as the potential effects of alkene-derived metabolites on propane-dependent growth were not typically considered. For example, small alkyl epoxides such as ethylene and propylene oxides are potent alkylating agents and are widely used as sterilizing agents due to their reactivity with many biomolecules. The high reactivity and resulting toxicity of these epoxides also underlies the diverse mechanisms such as hydrolysis, isomerization and conjugation with thiols (*e.g.* glutathione or Co-enzyme M) that true alkene-metabolizing bacteria have available to sequester and mitigate these compounds. The fact that these epoxide detoxification processes are typically absent from alkane-metabolizing strains has received limited attention with respect to understanding the effects of simple alkyl epoxides but has been an important area of research into understaning the toxic effects of cometabolic degradation of chlorinated alkenes such as TCE by gaseous hydrocarbon-oxidizing bacteria (van Hycklama *et al*.,1997; Chu & Alvarez-Cohen, 1999; Ensign *et al*., 1992; Mattes *et al*., 2010) The possibility that non-epoxide metabolites of alkene-oxidation can also potentially

impact the growth and activity of gaseous alkane-metabolizing bacteria has also not received significant attention.

Unless bioaugmentation strategies are involved, in field applications of propane-supported ACB, it is likely that the presence of other gases such as propylene in propane will have different effects than those observed with pure cultures. For example, given the wide diversity of microorganisms in a typical contaminanted groundwater environment, it is likely that each gas in a gas mixture will eventually support the growth of distinct microbial populations that utilize their respective hydrocarbon growth substrates. Compared to the effects of using high purity propane, the effects of this increased microbial diversity also be expected to lead to an increase in the diversity of the SDIMOs expressed by the total microbial community and consequently an increase in the diversity of contaminants that can be cometabolically transformed by the total microbial community. In addition, in the case of propylene, the presence of a separate propylene-metabolizing community could reasonably be expected to effectively decrease the concentration of propylene to which propane-oxidizing bacteria are exposed and thereby mitigate any potential inhibitory effect this gas might have on the growth and activity of these bacteria.

Project Aims. The overall aim of the research described in this thesis has been to determine which grade of propane is most effective for supporting ACB in the field. The research was divided into two sections that focused on pure and mixed cultures, respectively. The first section aimed to (a) characterize the effect of propane grade and the propylene content of these gases on the growth and activity of genetically-characterized, model propane- and propylene-metabolizing strains and (b) to characterize the effects of known and potential propylene-derived metabolites and other gases on these model strains. This research represents the majority of the results and discussion

presented in this thesis. The second section aimed to explore the effects of these same propane grades on mixed cultures. The goals of this research were (a) to determine the effect of propane grade on the microbial and enzymatic diversity of enrichment cultures and (b) determine whether there was an effect of propane grade on the range and type of contaminants that could be degraded by the respective microbial communities. The research reported in this thesis only addresses these questions in a prelimiary fashion and explores the possibility that activity-based labeling (ABL) could be a useful and innovative approach to address the questions of microbial and monooxygease-diversity in mixed cultures.

MATERIALS AND METHODS

MATERIALS: *Rhodococcus rhodochrous* (ATCC 21198), *Xanthobacter autotrophicus* Py2 (ATCC BAA-1158), and *Rhodococcus rhodochrous* B276 (ATCC 31338) were obtained from the American Type Culture Collection (Manassas VA). *Rhodococcus aetherivorans* BCP1 (DSM 44980), *Rhodococcus jostii* RHA1 (DSM 43269), and *Rhodococcus opacus* PD630 (DSM 44193) were obtained from the German Collection of Microorganisms and Cell Culture (Braunsweig, Germany). Blue Rhino HD5 gas was obtained from a local commercial vendor in Raleigh, North Carolina. Two odorant-free, certified custom mixtures of propylene in propane (Mix 5: [94.96% v/v propane plus 5.04%v/v propylene] and Mix 10 [90.05% v/v propane plus 9.95% v/v propylene]) were obtained from Mesa Specialty Gases (Santa Ana, CA) and were generated using instrument grade gases (>99.99% purity). Propylene (>99.9% purity) was obtained from Linde Specialty Gases (Danbury, CT). Propane (>99.5% purity) was obtained from Airgas Air Liquide Co. (Radnor, PA). Acrylic acid (99% purity), allyl alcohol (99% purity), ethanethiol (97% purity), 1,7-octadiyne (98% purity), propylene oxide (>99% purity), and 1,2-propanediol (>99% purity) were obtained from Millipore-Sigma (St Louis, MO). AlexaFluor 647 azide was obtained from Thermo Scientific, Inc. (Waltham, MA).

METHODS: **Analysis of HD5 gas composition.** The composition of gas discharged from a commercially sourced and newly-filled 20 lb. consumer HD5 gas cylinder was determined by sampling the gas at various times as it was discharged at a high rate (≤10 L/min) while connected to a large gas grill. The discharged gas was combusted in the grill to avoid producing large volumes of potentially explosive gas:air mixtures. The amount of gas discharged from the cylinder was determined over time by measuring changes in the weight of the gas cylinder using a bathroom scale. Gas samples were obtained by puncturing the gas supply tubing to the grill with a needle

and venting the resulting gas stream for 5 minutes through a stoppered glass serum vial fitted with a second needle value to allow gas flow. The composition of the gas was subsequently determined by gas chromatography, as described below.

Maintenance and Growth of Microbial Cultures: All microorganisms were maintained on mineral salts medium (MSM) agar plates incubated inside a borosilicate glass desiccator (7.5 L volume) at room temperature (~25 ◦C). The minerals salts medium contained (per L): NH_4Cl (2.0 g), $MgCl_2.H_2O$ (0.075 g) $(NH_4)_2SO_4$ (0.1g), K_2HPO_4 (0.775 g) NaH_2PO_4, (0.425 g) 0.1g EDTA, $ZnSO_4.7H_2O$ (40 mg), $CaCl_2$ (9 mg), $MnCl_2 \cdot 4H_2O$ (10.12 mg) $FeSO_4 \cdot 7H_2O$ (0.55g), $(NH_4)_2MoO_4 \cdot 4H_2O$ (10 mg), $CuSO_4 \cdot 5H_2O$ (3.01 mg), and $CoCl_2 \cdot 6H_2O$ (3.42 mg). Propane or propylene (120 mL) were added to the desiccator head space through a rubber septum seal using plastic syringes.

Unless otherwise stated, suspended microbial cultures were grown on gaseous substrates in glass serum vials (160 mL) that contained MSM (40 mL). The cultures were inoculated to an initial culture density (OD_{600}) of ~0.02 with cultures previously grown on MSM plates on either propane or propylene (see above). The inoculated culture vials were sealed with sterile butyl rubber stoppers and metal aluminum crimp seals (Wheaton Millville, NJ). Individual gases or gas mixtures were then added to the headspace as an overpressure using sterile plastic syringes (10 mL) fitted with 0.1 µm filters (Durapore PVDF Membrane, Merck Millipore Ltd.). When required, small volumes of gas (≤500 µL) were added with heat-sterilized glass microsyringes. All inoculated cultures were then incubated at 30° C in the dark in an orbital environmental shaker operated at 150 rpm. Subsequent changes in culture density (OD_{600}) were measured using a Shimadzu 1601 UV-Visible spectrophotometer (Kyoto; Japan). At the end of each culture incubation, samples (50 µL) of each culture were streaked on plate count agar (PCA) agar plates

to confirm culture purity. In some experiments, the ability of different strains to grow on non-gaseous substrates was also investigated. In these cases, the microorganisms were cultured as described above except the non-gaseous substrates were added to the MSM directly from sterile aqueous stocks solutions.

Determination of propylene oxide production by resting cells: Production of propylene oxide from oxidation of propylene by resting cells was determined by initially growing each propane-utilizing strain (*R. rhodochrous* ATCC 21198, *R. rhodochrous* B276, and *R. opacus* PD630) separately at 30° C in batch cultures grown in sealed glass serum vials (500 mL) containing MSM (100 mL) and 10% v/v propane (instrument grade) added to the gas phase. Each culture was grown a final culture density (OD_{600}) of ≤0.9 and then harvested by centrifugation (10,000g, 5 min, at 4° C). The cells were resuspended in buffer (50mM NaH_2PO_4, pH7.0) and centrifuged again. The resulting cell pellet was finally resuspended in buffer (1 mL) at a protein concentration of ~10 mg total protein/mL. The ability of each microorganism to generate propylene oxide was then determined in small scale incubations conducted in glass serum vials (10 mL) sealed with butyl rubber stoppers and aluminum crimp seals. Each reaction vial contained buffer (0.4 mL) and varying amounts of propylene (0-40 μmoles) added to the gas phase using glass microsyringes. The reactions were initiated by the addition of harvested washed cells (0.1 mL) and the reaction vials were then incubated at 30° C in a shaking water bath operated at 150 rpm. At the times indicated in each experiment, samples (2 μL) were removed from the reaction vials and were analyzed by gas chromatography for the production of propylene oxide (see below). In some experiments, the ability of resting propane-grown bacteria to degrade propylene oxide was also determined. These experiments were conducted as described above except no propylene was added

to the gas phase and propylene oxide (1 µmole) was added to the reaction medium from an aqueous stock solution (0.1 M). The reactions were initiated by the addition of cells and changes in the concentration of propylene oxide were determined over time by gas chromatography.

Gas Chromatography. The concentrations of propane and propylene in the gas phase were determined by gas chromatography (GC) by analyzing gas samples (10-400 µL) using a Shimadzu GC-14A equipped with a flame ionization detector and a 16ft stainless steel column (16ft x 1/8 inch) packed with a *n*-octane on Res-Sil (Restek, Fisher Scientific Co.) or a 30 m Alumina/Na_2SO4 capillary column (Restek, Fisher Scientific Co.). In both cases the GC was operated with a column temperature of 35° C, a detector temperature of 220° C and an injector temperature of 200° C. Nitrogen was used as the carrier gas at a 150 kPa constant pressure. Unless otherwise stated, calibration standards were set up in sealed glass serum vials (160 mL) containing deionized water (40 mL). Additionally, after every gas addition, the serum vial was left to incubate for 10 min at 30° C in an orbital shaker operated at 150 rpm. For propane calibration standards, instrument grade propane was injected in increments (0.5-5 mL) utilizing 1 mL BD syringes. For propylene and ethane standards, a 4 mL of propane background was injected with a Luer Lock Soft-Ject 12 mL syringe before the addition of each respective gas. Both ethane and propylene were added (0-500 µL) into the serum vials using microsyringes.

The concentration of propylene oxide in the liquid phase was also determined by GC using a Shimadzu GC-8A equipped with a flame ionization detector and a stainless-steel column (6ft x 1/8th inch) packed with 80-100 mesh PorapakQ (Waters, Milford, MA). Aqueous samples (2 µL) were analyzed using a column temperature of 170° C, an injector temperature of 200° C and a detector temperature of 220° C. Nitrogen was used as the carrier gas at a 150 kPa constant pressure.

Pure propylene oxide calibration standards were performed to quantify their experimental concentrations. Aqueous 0.1M propylene oxide standards were prepared fresh by adding propylene oxide in sealed reaction vials (10 mL) that contained deionized water (1 mL). The propylene oxide stocks were left incubating for 5 min in a water bath shaking at 150 rpm at 30^{o} C before utilization. The calibration standards were set up in either 10 mL or 160 mL glass serum vials containing volumes of 0.5 mL or 40 mL deionized water, respectively. Propylene oxide from the aqueous stock was transferred utilizing microsyringes in increment volumes (≤10 µL) into each calibration vial, allowing 5 min incubation periods in between as previously described. 2 µL samples of each concentration were measured utilizing gas chromatography.

Soil Enrichment Cultures: Enrichment cultures were obtained from a surface soil sample obtained from a gasoline spill at a gas station in Durham, North Carolina. The soil sample collected was initially allowed to aerate for 1 month to decrease the residual gasoline concentration. After aeration, samples (1g) of the sol were added to MSM (25 mL) in glass serum vials (160 mL). The vials were sealed with sterile butyl rubber stoppers and metal aluminum crimp seals and each gas or gas mixture was added to the gas phase to an initial concentration of 8.8% (v/v) using plastic syringes fitted with sterile 0.1 µM filters, as described previously. The cultures were incubated in the dark at room temperature on an orbital shaker operated at 100 rpm. After incubation for 3 weeks, a sample (1mL) of the medium was transferred to a new culture vial containing MSM (24mL) and the corresponding gas or gas mixture in the gas phase. Following this first passage, enrichments were passaged every 1-2 weeks maintaining a 1:25 dilution with MSM.

Activity-Based Labeling of Pure and Soil Enrichment Cultures. Activity-based labeling (Bennett *et al*., 2016) was used to investigate the different types of monooxygenase enzyme expressed by the gas-utilizing bacteria included in this study. Axenic bacterial strains and mixed enrichment cultures were grown in MSM as described above except the relevant gases and gas mixtures were added to an initial concentration of 10% v/v gas phase. When the cultures reached an OD_{600}< 0.7, the cultures were harvested by centrifugation, washed, and finally resuspended in buffer, as described earlier. Samples (0.1 mL) of the resting cells were then added to sealed reaction vials (10 mL) that contained buffer (0.9 mL) and 1,7-octadyine (1 mM) added from a stock solution (0.1 M) in dimethylsulfoxide (DMSO). The reactions were incubated for 1 hour at 30^{o} C in a shaking water bath operated at 150 rpm. The cells were then pelleted by brief centrifugation, resuspended in buffer (0.1 mL) and lysed by bead beating using a lysing bead Matrix (MPI, cat no.6540425) and a Bead Miller 4 (Fisher) at 5 m/s for 55 seconds. The lysed cell mixture was then briefly centrifuged to remove the beads and the resulting supernatant containing extracted protein was incubated in a reaction with AlexaFluor 647 azide (8 µM added from a stock solution in DMSO) $CuS0_4$ (2 mM) and sodium ascorbate (11 mM) which were added from freshly prepared aqueous stock solutions. After incubating for 1 hour at room temperature, the reaction was quenched by adding 3-butyn-1-ol (40 mM) added from a stock solution in DMSO. Samples of the resulting protein mixture (10-20 µg) were then mixed with SDS-PAGE sample buffer and were analyzed by SDS-PAGE using 10% gels. The molecular weights and fluorescence of the separated proteins were determined using a prestained NIR Protein Ladder (Thermo Scientific, Waltham, MA) and a LiCor Odyssey Scanner (Lincoln, NE).

Cell Protein Determination: The concentration of protein in all samples was determined by the Biuret method described by Gornall *et al*. 1949 using bovine serum albumin as the standard. In summary, cell suspensions were mixed with an equivalent volume of 3M NaOH and incubated for 30 min at 65° C. The lysed cell suspension was then centrifuged at 10,000 rpm for 5 minutes at room temperature to remove cell debris and unsolubilized protein. The protein content of the supernatant was then used to estimate the overall cell protein content.

RESULTS

Effect of gas composition of growth of model strains: Our initial experiments examined the effects of several different gases on the growth of the model alkane-oxidizing bacterium, *R. rhodochrous* ATCC 21198 (stain 21198) in batch culture (Figure 3). Strain 21198 grew rapidly on propane alone (growth rate = 0.091 OD_{600}/day) and no further change in culture density occurred after 7 days. Statistically significant (t-test matched paired p-value<0.05) slower growth (0.064 OD_{600}/day) was observed when Blue Rhino HD5 was used as the sole carbon and energy source and the maximal culture density was approximately 25% less than that observed for strain 21198 grown on propane alone. In contrast, little or no growth (maximal $OD_{600} \leq 0.05$ was observed after 14 days for strain 21198 using either Mix 5, Mix 10 or propylene as sole sources of carbon and energy.

The effects of the same gases and gas mixtures were also examined for the other model strains. In terms of its complement of SDIMO-encoding genes, *Rhodococcus aetherivorans* BCP1 (strain BCP1) is very similar to strain 21198 and expresses both SCAM and PrMO during growth on propane (Cappelletti *et al*., 2015). Although the inhibitory effect of Blue Rhino HD5 on the growth of strain BCP1 was less than for strain 21198 (Figure 3 & Table 4), neither strain grew on Mix 5, Mix 10 or propylene alone (Table 4). *Rhodococcus jostii* RHA1 (strain RHA1), and *Rhodococcus rhodochrous* B276 (strain B276) are both known to grow on propane *(*Furuhashi *et al*., 1981; Sharp *et al*., 2007) and both possess genes encoding PrMO but not SCAM or any other likely propane-oxidizing monooxygenase *(*Sharp *et al*., 2007; unpublished data Hyman laboratory). However, these two strains are metabolically distinct in that strain B276 can also grow on propylene through the activity of an alkene monooxygenase (Miuran & Dalton, 1995). Under the conditions used in this present study, neither strain RHA1 or B276 grew on either propane

alone nor any of the propane-containing gas mixtures tested although strain B276 did grow on propylene alone (Table 4). The genome for *Xanthobacter autotrophicus* Py2 (strain Py2) does not encode any propane-oxidizing monooxygenases but does encode a Group 1 SDIMO (alkene monooxygenase [AlkMO]) which is distinct from the Group 4 SDIMO alkene monooxygenase (AMO) utilized for alkene oxidation by strain B276. Like strain B276, strain Py2 grew when propylene was the sole source of carbon and energy. In contrast to strain B276, strain Py2 also grew on the lower concentrations of propylene present in Mix 5 and Mix 10 but did not grow on the even lower concentrations of propylene present in Blue Rhino HD5 (Table 4).

Effects of ethane and ethanethiol of growth of strain 21198 on propane: Ethane is present in commercial HD5 formulations as a minor alkane relative to propane. Ethanethiol is also added to HD5 as an odorant which must be detectable by smell by a normal human at gas concentrations that are ≥20% of the lower explosive limit (LEL) of gas in air (LEL for liquefied propane gas [LPG] = 1.9% v/v gas phase). In many cases, ethanethiol is added to LPG at a concentration of 4 ppm (parts per million: 0.0004% v/v gas phase). The growth of strain 21198 on instrument grade propane was unaffected by the presence of either ethane (Figure 4) or ethanethiol (Figure 5) when these gases were added at concentrations that ranged from lower to substantially higher than either the measured concentration of ethane in Blue Rhine HD5 (5 % v/v) (See Appendix A) or the legally-prescribed concentration of ethanethiol in LPG.

Consumption of propylene during growth of strain 21198 on Blue Rhino HD5: The results in Figures 3-5 and Table 4 suggest that the inhibitory effects of Blue Rhino HD5 on the growth of strain 21198 were not due to either the presence of ethane or ethanethiol but could

potentially be associated with the presence, oxidation, and consumption of propylene in this gas mixtures. As growth of strain 21198 was fully inhibited when either ethane- and ethanethiol-free Mix 5 or Mix 10 were used as sole carbon and energy sources (Figure 3 & Table 4), we examined whether propylene was consumed during growth of strain 21198 on Blue Rhino HD5. Using the same culture conditions described in Figure 3, the consumption of propane and propylene was monitored over time by GC analysis of the gas phase while samples of the culture medium were withdrawn to determine changes in the culture density (OD_{600}). The time course of gas utilization demonstrated that, despite being present at very different initial concentrations, propane and propylene where consumed concurrently during growth of strain 21198 on Blue Rhino HD5 (Figure 6A). The results of this experiment (Figure 6A) also demonstrated that the consumption of propylene effectively ceased once the culture entered stationary phase after ~ 4 days. In contrast to the consumption of propylene in the culture grown on Blue Rhino HD5, there was no significant consumption of propylene in uninoculated, abiotic control incubations (Figure 6B).

Production of propylene oxide from propylene by propane-grown strain 21198. Consumption of propylene during growth of strain 21198 on Blue Rhino HD5 (Figure 6A) suggested that metabolites such as propylene oxide could be generated during growth of this strain on this gas mixture. The potential for propylene oxide accumulation from propylene consumption was investigated in an experiment in which strain 21198 was grown for 7 days on propane in the presence of varying amounts of propylene (0-10% v/v of added propane). The concentration of propylene oxide present after growth was determined by GC analysis of the culture medium and compared with the final culture density (OD_{600}). Progressive increases in the concentration of propylene led to progressive decreases in the final culture density and low concentrations of

propylene oxide were detected in some, but not all of these incubations (Figure 7). The effects of propylene concentration on final culture density reiterate our earlier observations (Figure 3, Table 4) that high concentration of propylene (≥5% v/v of propane added) fully inhibit growth of this strain on propane. The results shown in Figure 7 also show that there was little discernable effect of low propylene (≤1% v/v of propane added) while there was a progressively more potent inhibition of growth with higher concentrations of propylene (>1% v/v of propane added). Growth observed at with 2.5% propylene (v/v propane added) was significantly lower (t-test matched pairs, p-value<0.05) than growth observed with 1% propylene (v/v propane added). The amounts of propylene oxide detected in this experiment were generally low and, in some cases, at or below the limit of detection (LOD) for the GC analysis used in this study (10 μM). Despite the low concentrations involved, the results of this experiment suggested that increases in propylene content of the propane: propylene mixtures led to an increase in the amount of propylene oxide generated by this strain during growth on propane.

Production of propylene oxide by resting propane-grown cells: In view of the low concentrations of propylene oxide detected in the growth-based experiment described in Figure 7, the potential for propylene oxide production was also investigated in short term incubations (≤2 h) involving high concentrations of resting, propane-grown cells. Cells of strain 21198 were grown on propane (10 % v/v gas phase), harvested, and then incubated with propylene (10% v/v gas phase), as described in the Methods section. A GC analysis revealed that propylene oxide accumulated in the reaction medium (Figure 8) as the only detected metabolite of propylene oxidation. The rate of production of propylene oxide remained near constant throughout the reaction time course (r^2 = 0.977). The rate of propylene oxide production under these conditions

was estimated at 22.1 nmoles min^{-1} mg total $protein^{-1}$ for this single replicate while the mean of three separate determinations was 20.4 nmoles min^{-1} mg total $protein^{-1}$. The potential for consumption of propylene oxide was also assessed and it was not consumed throughout a 90-minute incubation when resting propane-grown cells were incubated with an initial concentration of 1 mM propylene oxide (Figure 8).

As the results of the experiment described in Figure 8 provided no evidence for rapid consumption of propylene oxide and because propylene oxide appeared to be the sole metabolite generated from propylene oxidation, we attempted to define the kinetic constants for propylene oxidation by propane-grown strain 21198 using propylene oxide as a direct indicator of this activity. In these experiments, the amount of propylene oxide detected in 1-hour incubations was determined as a function of the initial amount of propylene added to the reaction gas phase. The results (Figure 9) indicate that the amount of propylene oxide detected increased with increases in initial propylene concentration but appeared to reach saturation with the highest amounts of added propylene. A computer fit of the data to a hyperbolic, single substrate binding model (Michaelis-Menten) ($r^2 = 0.956$) provided an estimate of the V_{max} and K_s for propylene oxide production of 19.8 ± 1.8 nmoles min^{-1} mg total $protein^{-1}$ and 5. 7 ± 1.1 μmoles of propylene (~1.2% v/v gas phase), respectively.

The ability of the other model propane-utilizing strains used in this study to generate propylene oxide from propylene oxidation was also examined. Cells of strains PD630 and B276 were grown separately on propane (10% v/v), harvested, washed and incubated with propylene (10% v/v gas phase), as described above for strain 21198. The specific rates of propylene oxide production for these strains were determined to be 26 and 32 nmoles propylene oxide min^{-1} mg

total protein^{-1}, respectively. These rates are comparable to the V_{max} determined for strain 21198 under very similar experimental conditions.

Effects of propylene oxide on growth of strain 21198: As strain 21198 and other propane-utilizing strains were capable of generating propylene oxide from propylene (Figure 9), we investigated whether the inhibitory effects of propylene-containing gas mixtures could be observed if propylene oxide was added to cultures grown on propane in the absence of propylene. In one experiment, propylene oxide (1.75 µmoles) was added to cultures of strain 21198 growing on instrument grade propane. Compared to the untreated control which contained only propane, the addition of propylene oxide at t = 0 resulted in a prolonged lag phase of 4 days before culture growth was observed (Figure 10) while the addition of propylene oxide at t = 2 days resulted in a slowing in the rate of growth on propane. After growth for 7 days, the culture density (OD_{600}) in the propylene oxide-treated culture was only ~70% of the density of the culture grown on propane alone. In a separate experiment, the effect of varying initial concentrations of propylene oxide on the final culture density (OD_{600}) was determined for strain 21198. When propylene oxide was added at t =0, there was a progressive decrease in the final culture density (OD_{600}) after growth for 7 days as the initial propylene oxide concentration was increased (Figure 11). The results of this experiment suggest there is a near constant relationship between the initial concentration of propylene oxide added and the final culture density (OD_{600}).

Effects of other potential propylene oxidation metabolites on model bacteria: The results of the experiments described to this point suggest that propylene oxide might be responsible for the inhibitory effects of propylene on the growth of model propane-oxidizing bacteria on

propane: propylene gas mixtures. However, propylene oxidation can potentially also generate 1,2-propanediol as a result of the biological and abiotic hydrolysis of propylene oxide. Similarly, the initial hydroxylation of propylene can also generate allyl alcohol that can then undergo further enzymatic dehydrogenation oxidation to acrylic acid. Initially, the potential growth of each of the model strains on these various metabolites was determined in batch cultures, as described in the Methods section. None of the strains grew on either allyl alcohol or its potential metabolite, acrylic acid (Table 5). In the case of the propane-oxidizing strains, it appeared strain RHA1 exhibited weak growth on propylene oxide and stronger growth on 1,2-propandiol. While it is possible that growth of this strain on propylene oxide simply reflects its ability to grow on the 1,2-propanediol generated from the abiotic hydrolysis of this compound, this seems unlikely as several other strains (21198, BCP1 and B276) also grew well on the diol, but not the epoxide (Table 5). In contrast to the limited growth of strain RHA1 on propylene oxide, the alkene-oxidizing strain Py2 grew on this but not none of the other tested compounds. It is unclear why the other alkene-oxidizing strain, strain B276, did not grow, as expected, on propylene oxide.

The effects of 10-20-fold lower initial concentrations of these potential propylene-derived metabolites was also investigated on the ability of several of the model strain to grow on propane. Both 226 and 446 µM propylene oxide and 1,2-propanediol strongly inhibited the growth of strain 21198 and BCP1 on propane (Table 6). The highest concentrations (446 µM) of allyl alcohol and acrylic acid had limited effects on the growth of strain 21198 on propane but were more effective inhibitors of the growth of strain BCP1. The propylene-utilizing strain, strain Py2, did not grow on any of the mixtures tested and does not grow on propane (Table 6). The effects of the propylene-derived metabolites on the growth of this strain on propylene was not tested in this study.

Activity-based labeling of model bacteria and enrichment cultures: The experiments described to this point have focused on the impacts of propane: propylene mixtures on the growth and activity of axenic strains of model bacteria. Preliminary experiments were also conducted to examine the effects of the same gases and gas mixtures on enrichment cultures generated using a gasoline-impacted soil sample. The aim was to compare the fluorescent protein labeling patterns of enrichment cultures and model bacterial strains using an activity-based labeling approach. The model microorganisms and enrichment cultures were grown with 10% (v/v gas phase) with either propane or propylene alone, harvested, reacted with 1,7-octadiyne and AlexaFluor 647 azide and analyzed by SDS-PAGE, as described in the Methods section. The results of this preliminary analysis show fluorescent labeling of polypeptides was detected for all of the samples and included both model strains and enrichment cultures. In every case, the most intensely labeled polypeptides were in the mass range of 55-65 kDa which corresponds well with the known masses of the hydroxylase components of the gaseous hydrocarbon oxidizing SDIMOs that are known to be present in the model strains examined in this study. The results also suggests that similar enzymes are present in the gaseous hydrocarbon-oxidizing strains organisms present in all of the enrichment cultures.

DISCUSSION

The results of this study demonstrate that mixtures of propane and propylene can have potent inhibitory effects on the growth of axenic cultures of propane-oxidizing bacteria. The results further suggest these effects involve the ability of these bacteria to oxidize propylene to propylene oxide through the activity of propane-oxidizing monooxygenase enzymes. These major conclusions are discussed in more detail in the following sections and are interpreted in terms of their impact on the potential use of gas mixtures as stimulants for *in situ* cometabolic biodegradation processes.

Effect of propane grade on the growth of *R. rhodochrous* ATCC 21198: The effects of Blue Rhino HD5 on the carbon-limited growth of the focal strain of this study, *R. rhodochrous* ATCC 21198, were statistically significant (Figure 3). These inhibitory effects could potentially be attributed to specific effects of an individual components of the HD5 gas mixture or an aggregate effect of multiple components acting simultaneously and possibly through several different mechanisms.

In the Blue Rhino HD5 used in this study, ethane was experimentally determined to represent ~6% v/v of the gas typically discharged from the cylinder. In contrast, the concentration of ethanethiol was not determined as its analysis in complex gas mixtures requires specialized equipment often including non-reactive GC columns and selective flame photometric detectors. However, the likely concentration of this gas is known as it is legally required to be added to commercial liquefied propane gas as a warning odorant at a concentration of ~0.0004% v/v. Neither of these compounds resulted in an inhibition of growth of strain 21198 on instrument propane at concentrations above and below their known or expected concentration in Blue Rhino HD5 (Figures 4 and 5). In the case of ethane, this gas can support the growth of many gaseous

alkane-oxidizing bacteria. Including strain 21198 and BCP1. Consequently, ethane might reasonably have been expected to promote rather than inhibit growth of these strains. In contrast, ethanethiol was considered to be a potential growth inhibitor as it is known to inhibit cytochrome oxidase activity and limit ATP production in mitochondria (Foster *et al*., 1974).

A comprehensive analysis of all of the components of Blue Rhino HD5 was not conducted in this study. However, propylene was detected in this gas mixture at an estimated concentration of ~0.25% v/v (Figure S3). The detection of even relatively low concentrations of propylene in this gas is important for several reasons. First, from a technical perspective, the legal specification for HD5 defines the maximum amount of propylene (≤5% v/v) and the minimum amount of propane (≥95% v/v) allowed in this mixture. However, this specification does not require the presence of propylene in HD5 and consequently even 99.999% v/v propane could be classified and sold as HD5. Second, we observed a much more potent inhibition of growth of strain 21198 when using Mix 5 and Mix 10 as growth substrates compared to Blue Rhino HD5 (Figure 3). Both of these custom gas mixtures contain much higher concentrations of propylene than Blue Rhino HD5. As these custom gas mixtures are synthesized using high purity propane and propylene, they also have much lower concentrations of other contaminating hydrocarbons that might normally be expected in HD5 (*e.g.* C_4-C_6 alkanes). In addition, unlike HD5, neither of the custom gas mixtures contains ethanethiol or any other added odorant. If the mechanism of growth inhibition is the same for Blue Rhino HD5, Mix 5 and Mix 10, these observations collectively suggest that propylene and/or possibly one or more of its metabolites is responsible for the inhibition of growth of model propane-oxidizing bacteria shown in this study (Figure 3, Table 4). Another important observation supporting the role of propylene as source of inhibitory metabolites is that this gas is concurrently consumed with propane during growth of strain 21198 on Blue Rhino HD5 (Figure 6). Concurrent

utilization of two substrates is a common feature of cometabolic transformations that provides supporting evidence that both compounds are substrates for the same enzyme.

Characterization of propylene oxide as a potentially inhibitory metabolite. In this study, two different approaches were used to investigate the potential production of propylene oxide from propylene. The first approach involved analyses of the culture medium after growth of strain 21198 on a range of propane: propylene gas mixtures (Figure 7). The results of this experiment not only confirmed the potent inhibitory effects of high concentrations on propylene (>1% v/v of propane added) on growth of this strain but also provide some evidence that propylene oxide was generated during growth. For example, the amount of propylene oxide detected in the cultures containing propylene up to 1% (v/v of propane added) was at or below the limit of detection (10 µM propylene oxide) of the analytical approach used in this study. However, the amounts of propylene oxide detected in cultures containing 2.5%, 5% or 10% propylene (v/v of propane added) were approximately 20 µM in each case. This represents a conversion of ~18%, ~10% and ~5% of the total available propylene to propylene oxide, respectively. The analysis of low concentrations of highly water-soluble compounds such as propylene oxide in aqueous samples is challenging unless techniques such as "purge and trap" are used to volatilize and concentrate the target analyte before chromatography. In our experiments the inhibitory effects of propylene-derived metabolites also increase with increases in propylene concentration and this effect also limited the amount of propylene oxide that could be generated. Lastly, propylene oxide can also undergo abiotic hydrolysis and has a reported half-life in deionized water of 22 days. Together, these factors suggest that the amount of propylene oxide detected in our growth-based

studies can only be a conservative estimate of the amount of propylene oxide actually generated during growth.

The second approach investigating the potential for propylene oxide production involved shorter (≤2h) reactions using resting cells previously grown on propane alone (Figures 8 and 9). This approach generates much higher concentrations of propylene oxide and avoids many of the analytical problems discussed above. The higher concentrations of metabolites generated in this approach also provides further support for our conclusion that propylene oxide is the predominant, if not sole, product of propylene oxidation by strain 21198 and other propane-oxidizing bacteria examined. The ability of strain 21198 and diverse other propane-oxidizing bacteria to "cometabolically" oxidize propylene to propylene oxide using a similar experimental approach has been previously reported (Hou *et al*., 1983). Although the K_s for propylene oxide production was not previously determined, the maximal rate of propylene oxide production measured in the present study (~20 nmoles min^{-1} mg total $protein^{-1}$) is very similar to the rate previously reported by Hou *et al*., 1983 for this strain.

Beyond the actual production of propylene oxide, two other results in this study support our conclusion that propylene oxide is the propylene-derived metabolite responsible for the inhibition of growth of propane-oxidizing bacteria. First, we demonstrated that the addition of propylene oxide to cultures of strain 21198 growing on high purity propane reproduced some of the inhibitory effects observed during growth of this strain on Blue Rhino HD5 and other gas mixtures (Figures 10 and 11). However, there are considerable differences between experiments in which there is a single addition of exogenous propylene oxide compared to experiments in which this compound is more slowly produced during culture growth. Second, we also observed that other potential propylene-derived metabolites did not either support growth of the model bacteria

examined in this study (Table 5) nor did they consistently inhibit growth of representative strains of propane-oxidizing bacteria (Table 6). The metabolites investigated included 1,2-propanediol which is the hydrolytic product of propylene oxide. We also examined the effects of allyl alcohol which is generated through hydroxylation of the methyl group of propylene and its further oxidation product, acrylic acid. This acid has been reported as a propylene-derived metabolite for one propane-oxidizing bacterium (Cerniglia *et al*., 1976) and is likely generated by the dehydrogenase-catalyzed further oxidation of allyl alcohol and acrolein.

An unresolved question about propylene oxide production in the model strains examined in this study is which of the various propane-oxidizing monooxygenases is responsible for catalyzing the oxidation of propylene. Transcriptomic and proteomic studies have shown that propane monooxygenase (PrMO) and short chain alkane monooxygenase (SCAM) are co-expressed at high levels in propane-grown cells of strains BCP1(Cappelletti *et al.*, 2015) and 21198 (unpublished data Hyman laboratory), respectively. In contrast, strain PD630 and RHA1 only possess genes encoding PrMO and it is assumed, but not yet verified, that this enzyme is expressed in both microorganisms when grown on propane. The fact that these strains all generate propylene oxide at very similar rates might suggest that it is PrMO and not SCAM that is responsible for the propylene-oxidizing activity of these strains.

Potential impacts of gas mixtures on mixed cultures and *in situ* bioremediation. The underlying rationale for this study was to investigate whether the use of propane:propylene gas mixtures rather than pure propane can potentially provide a benefit for *in situ* bioremediation processes as a result of the gas mixture increasing microbial diversity, monooxygenase diversity and consequently a greater range of contaminants that can be biodegraded relative to propane

alone. For example, propane-oxidizing bacteria are inconsistent in their ability to cometabolically oxidize the chlorinated solvent, TCE, while this activity is frequently observed with propylene-oxidizing bacteria. Conversely, propane-oxidizing bacteria frequently oxidize 1,4-dioxane, a chlorinated solvent stabilizer, while propylene-oxidizing bacteria are generally unreactive towards this compound (unpublished data, Hyman laboratory). The use of a propane:propylene gas mixture to stimulate the *in-situ* growth of separate propane- and propylene-oxidizing microbial populations simultaneously could enable both of these frequently co-occurring contaminants to be degraded simultaneously.

Most of the experiments described in this study have made use of low gas concentrations to obtain carbon-limited cultures. One interesting observation made was that the strains that only express PrMO during growth on propane (strains RHA1 and PD630) did not grow in the presence of 3.3% v/v propane alone (Table 4) but did grow when higher (≥10% v/v gas phase) concentrations of propane were used. In contrast, growth was observed with 3.3% v/v propane for strains that express both SCAM and PrMO during growth on propane (strains 21198 and BCP1). This observation could suggest that PrMO has a low affinity for propane as a substrate whereas SCAM has a higher affinity. A low affinity for PrMO for propane would potentially help explain why "PrMO-only" strains often grow slowly on propane, even in the presence of high propane concentrations. If the lack of growth on low propane concentrations we have observed is an intrinsic feature of "PrMO only" strains, the use of low, sub-LEL concentrations of propane in *in situ* biostimulation processes might be a method for actively selecting for bacteria that possess both SCAM and PrMO. This could be highly advantageous because compared to "SCAM plus PrMO" strains, "PrMO-only" strains are only known to cometabolically degrade a very limited range of contaminants. The use of low propane concentrations in *in situ* bioremediation schemes

might therefore be a key step in promoting the activity of versatile contaminant-degrading strains. Conversely, the use of high propane concentrations might promote a greater diversity of propane-oxidizing bacteria as well as a greater relative abundance of "PrMO-only" strains. This in turn might be expected to decrease both the rate and range of contaminants that could be degraded by a propane-biostimulated mixed culture.

Similar considerations also extend to the potential use of propane-propylene gas mixtures. For example, our results (Table 4) suggest that the concentration of propylene in Blue Rhino HD5 (~0.25% v/v of propane added) is too low to support growth of propylene-oxidizing bacteria such as strains B276 and Py2 which each individually express one of the two known types of alkene monooxygenase. Consequently, the *in situ* use of low HD5 concentrations might not be expected to increase either microbial or monooxygenase diversity, nor extend the range of contaminants that can be cometabolically degraded by a suitable biostimulated mixed microbial community. In the case of gas mixtures containing higher levels of propylene (*e.g.* Mix 5 and Mix 10) our results suggest that growth of propylene-oxidizing bacteria can be supported but the activity of propane-oxidizing bacteria is fully inhibited (Table 4). In pure culture studies this is detrimental but if this effect is extrapolated to the field, it is likely that over extended time a robust propylene-utilizing community would be established. The activity of this community would be expected to greatly reduce both the ambient concentration of propylene and the potential production of inhibitory metabolites such as propylene oxide that would otherwise inhibit the growth and activity of propane-oxidizing bacteria. Taken together, these extrapolations suggest that maximal microbial and monooxygenase diversity as well as contaminant-degrading activity could be supported *in situ* using low concentrations of a propane:propylene gas mixture that has a propylene content significantly greater than 1% v/v.

Future Experiments: The results of this study suggest that the next phase of experiments should explore some of the issues raises in the previous section. For example, it would be interesting to determine whether the use of propane:propylene gas mixtures actually does increases microbial diversity compared to propane alone. This could be effectively examined in microcosm studies using soil or water samples. The effects of different gas mixtures on microbial community structure and function could be assessed through metagenomic approaches that focus on 16S rRNA sequences while changes in the abundance of genes encoding specific monooxygenase could be explored by qPCR approaches. Similar experiments could also be used to investigate the effects of individual gases and gas mixtures on the activity and diversity of monooxygenases. For example, differences in monooxygenase activities could be explored by examining the rates of degradation of different contaminants when added to biostimulated microcosms. In these experiments, the rate of degradation of contaminants such as TCE or 1,4-dioxane could be monitored by GC or GC/MS. As shown in our present results (Figure 12), activity-based labeling could also be employed to examine the diversity of active monooxygenases present in biostimulated microcosms. However, rather than focus of SDS-PAGE-based analyses of fluorescent labeling patterns, more informative information could be provided by this technique using on bead-digestion approaches. In summary this would involve biotinylation of enzyme-diyne-probe adducts followed by streptavidin capture and purification. The identities of the capture monooxygenase components could then be determined by mass spectral analysis of peptide fragments generated from the prior tryptic digestion of the captured polypeptides. An alternative mass spectral approach could also include direct protein extraction from microcosms and the quantification of signature peptides for specific monooxygenase enzymes.

FIGURES AND TABLES

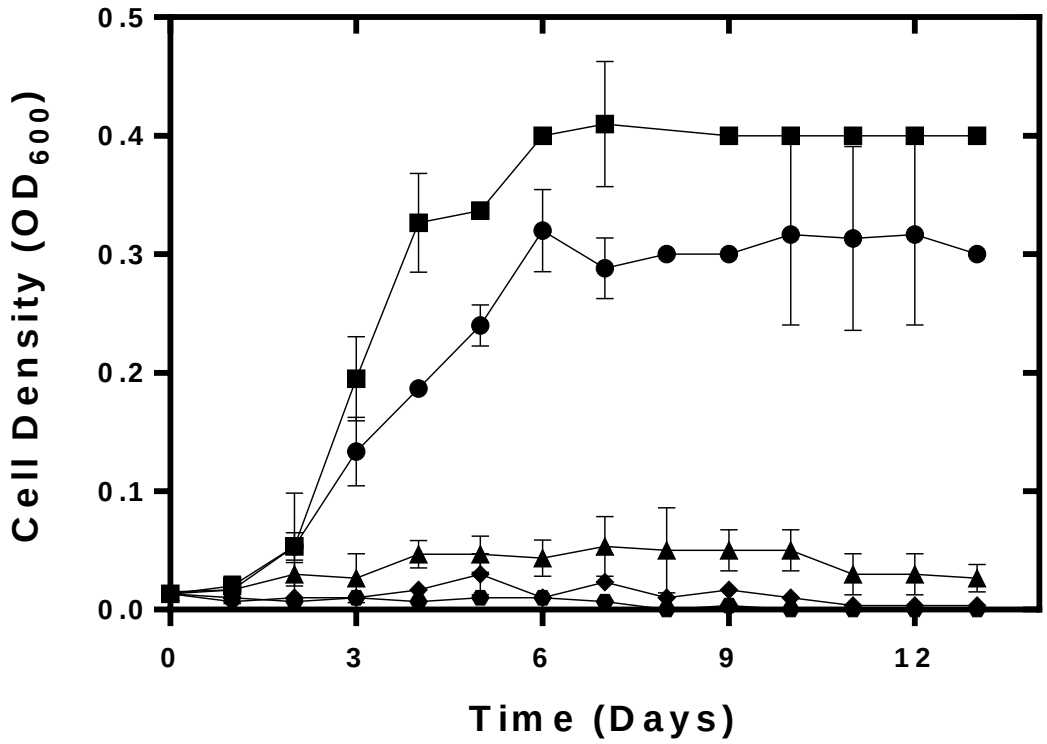


Figure 3. Effect of propane grade on growth of *R. rhodochrous* ATCC 21198. The Figure shows the changes in optical density (OD_{600}) during the time course of growth of strain 21198 in cultures grown on pure gases and gas added to an initial gas phase concentration of 3.3% (v/v) gas phase. The symbols are for (■) instrument grade propane, (●) Blue Rhino HD5, (▲) Mix 5, (◆) Mix 10 and (⬣) propylene. The data presented show the mean and standard deviation of three biological replicates.

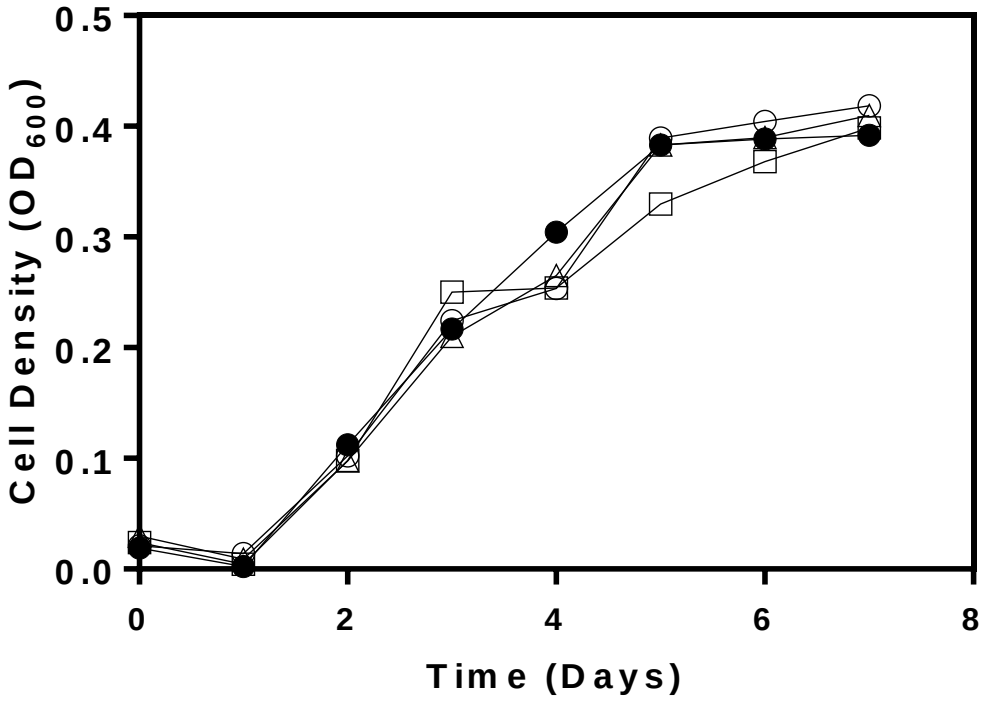


Figure 4. Effect of ethane of growth of *R. rhodochrous* ATCC 21198 on propane. The Figure shows the changes in optical density (OD_{600}) of cultures of strain 21198 during growth on mixtures of instrument grade propane (3.3% v/v propane) and the following initial concentrations of ethane: (●) 0% , (○) 2.5%, (□) 5% and (△) 10% expressed on the basis of v/v of propane in the cultures. The results presented are the mean of two biological replicates.

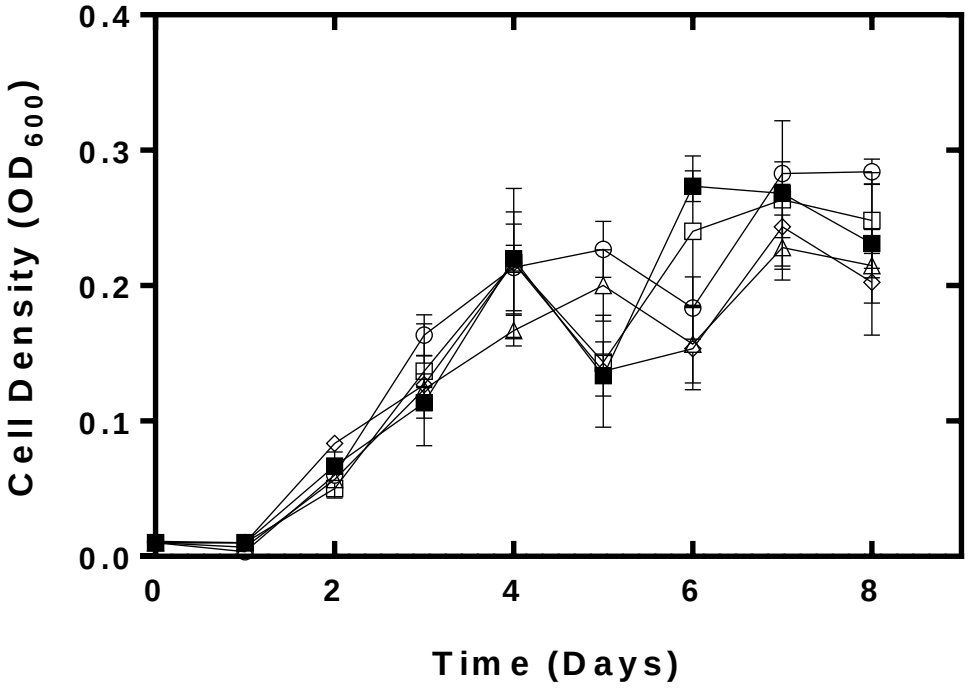
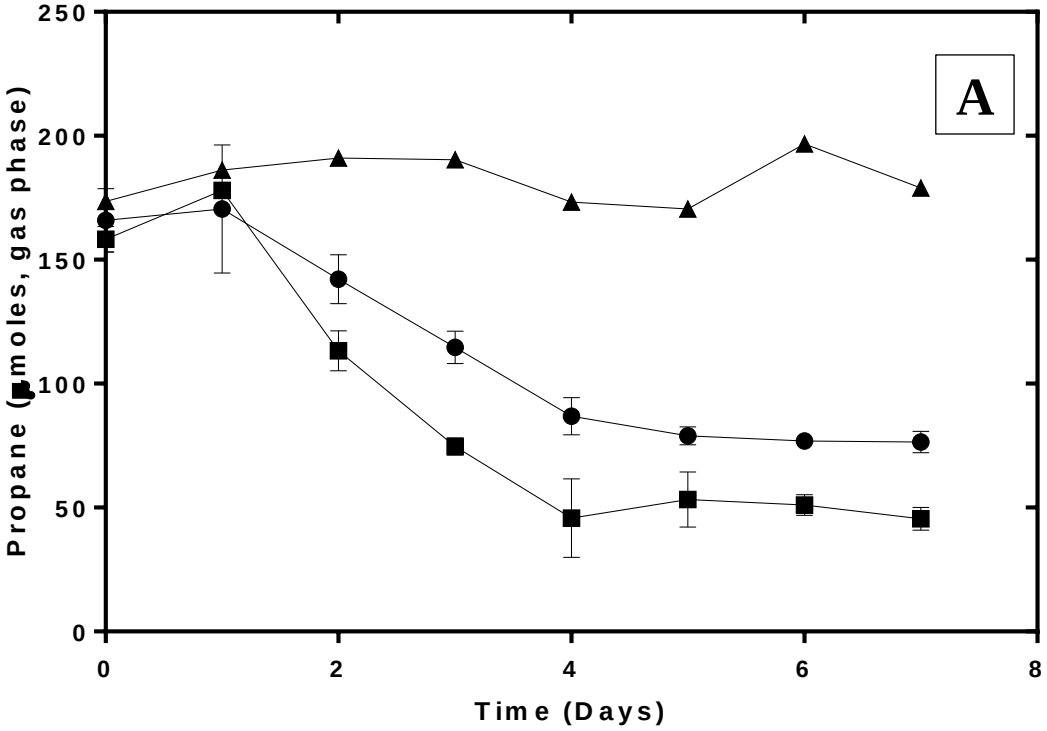


Figure 5. Effect of ethanethiol on growth of *R. rhodochrous* ATCC 21198 on propane. The Figure shows the changes in optical density (OD_{600}) of cultures of strain 21198 during growth on mixtures of instrument grade propane (3.3% v/v propane) and the following initial concentrations of ethanethiol: (□) 0% , (○) 0.01%, (△) 0.02% and (◇) 0.04% and (■) 0.08% expressed on the basis of v/v of propane in the cultures. The results presented are the mean and standard deviation of three biological replicates.

Figure 6A-C. Consumption of propane and propylene during growth of *R. rhodochrous* ATCC 21198 on propane or Blue Rhino HD5. Cells of strain 21198 were grown on either propane or Blue Rhino HD5, as described in the methods section. **Figure 6A** shows changes over time of the amount of propane in the culture headspace for cultures grown on (■) instrument grade propane, (●) Blue Rhino HD5, and (▲) uninoculated control reactions incubated with Blue Rhino HD5 **Figure 6B** show the corresponding changes over time of the amount of propylene in the culture headspace for cultures grown on (●) Blue Rhino HD5 or (■) uninoculated control reactions incubated with Blue Rhino HD5. **Figure 6C** shows the changes in culture density (OD_{600}) for cultures grown on (■) instrument grade propane, or (●) Blue Rhino HD5. In all cases the data presented are the means and standard deviation of three replicate cultures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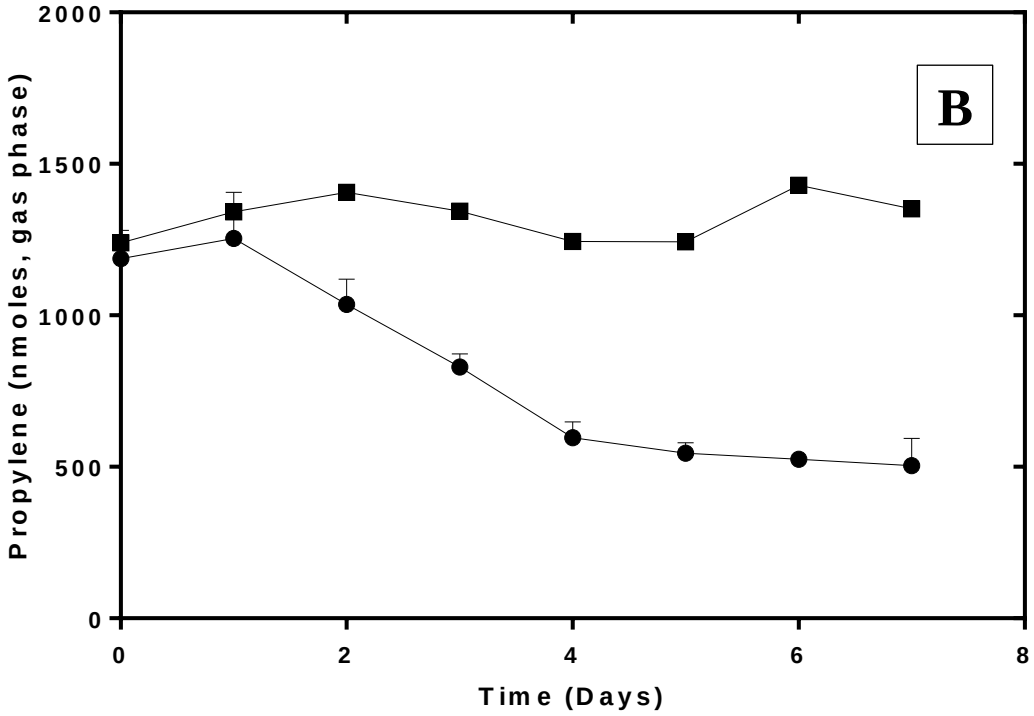
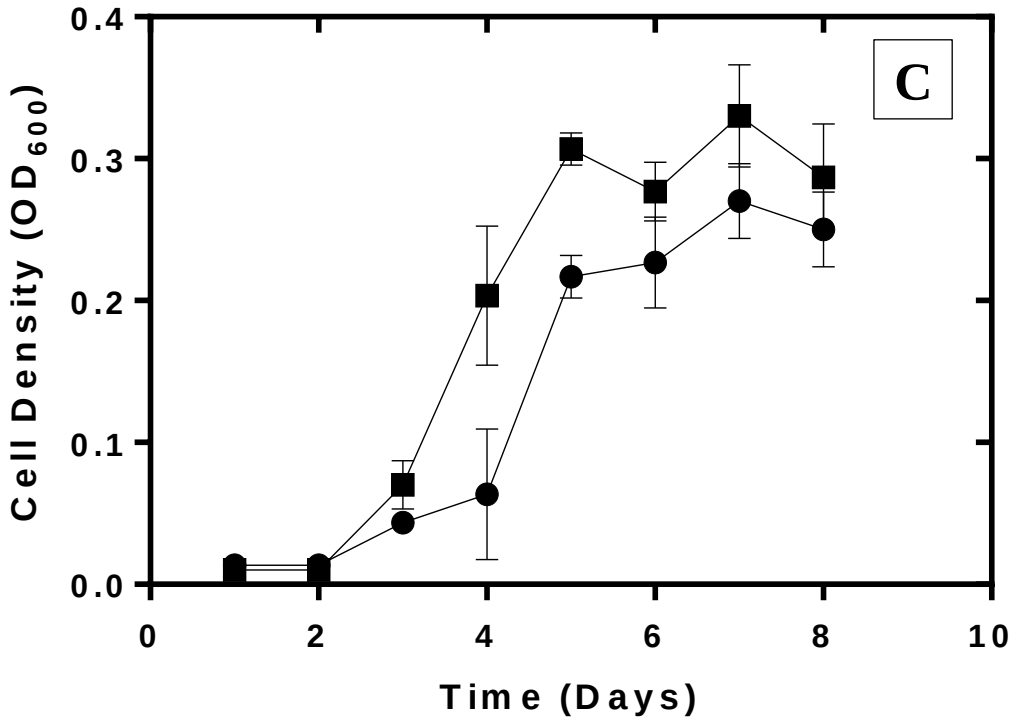
Propane (nmoles, gas phase)
250
200
150
100
50
0
0
2
4
6
8
Time (Days)


B
Propylene (nmoles, gas phase)
2000
1500
1000
500
0
0
2
4
6
8
Time (Days)


C
Cell Density (OD600)
0.0
0.1
0.2
0.3
0.4
0
2
4
6
8
10
Time (Days)

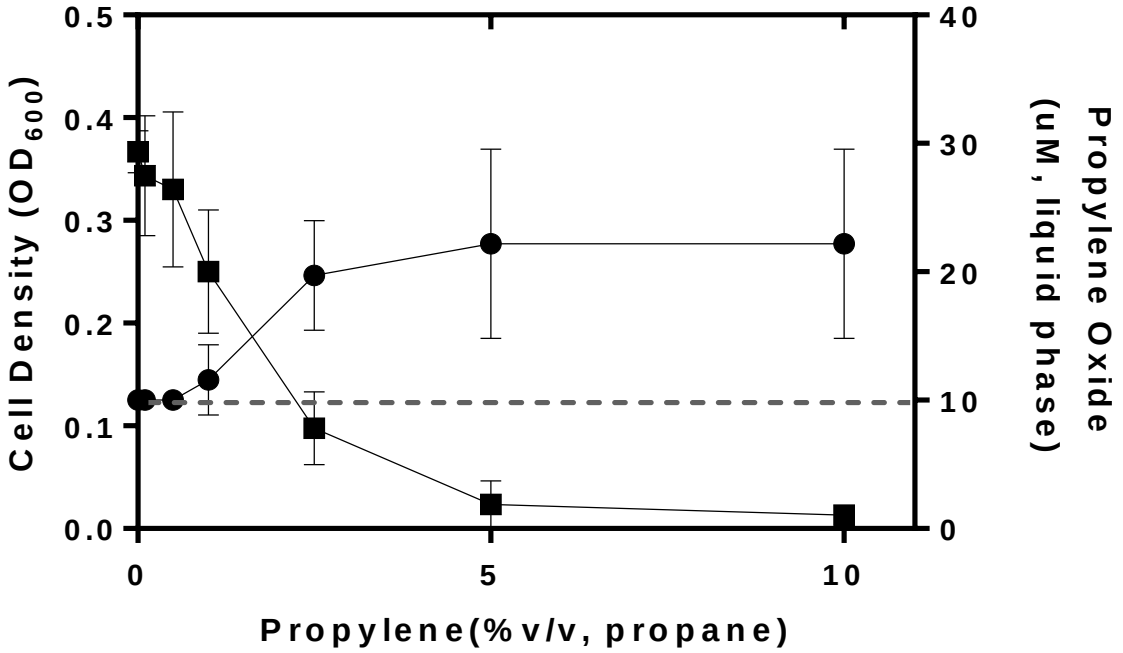


Figure 7. Production of propylene oxide during growth of *R. rhodochrous* ATCC 21198 on propane:propylene mixtures. The Figure shows a plot of the (●) amount of propylene oxide and (■) the final culture density (OD_{600}) for cultures of strain 21198 after growth for 7 days on propane that contained the indicated amounts of propylene added as a % of the volume of the initial amount (3.3 % v/v of total gas phase) of propane added in the cultures. The results presents are the mean and standard deviation of three biological replicates. The limit of detection of 10 μM of propylene oxide in the liquid phase is denoted by the grey dotted line.

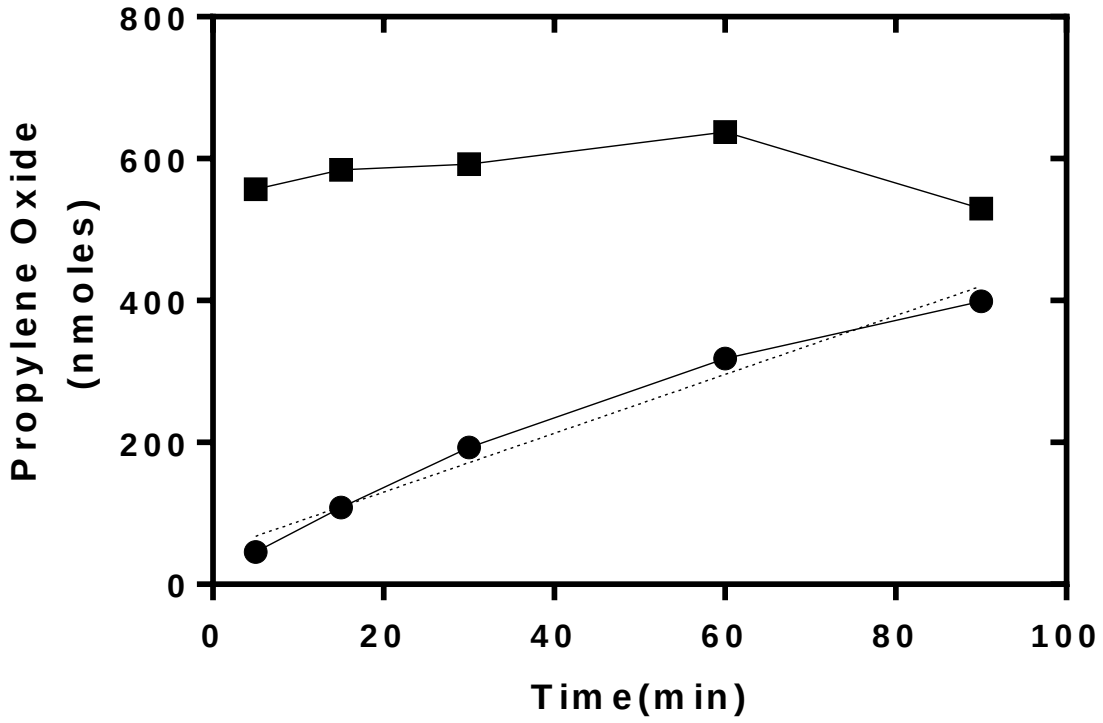


Figure 8. Production and consumption of propylene oxide by propane-grown resting cells of *R. rhodochrous* ATCC 21198. The Figure shows a representative time course for the (●) production and (■) consumption of propylene oxide by resting, propane-grown cells of strain 21198. The cells (~ 1 mg total protein) were grown, harvested and exposed to either propylene (10% v/v gas phase) or propylene oxide (1 mM initial concentration), as described in the Methods section.

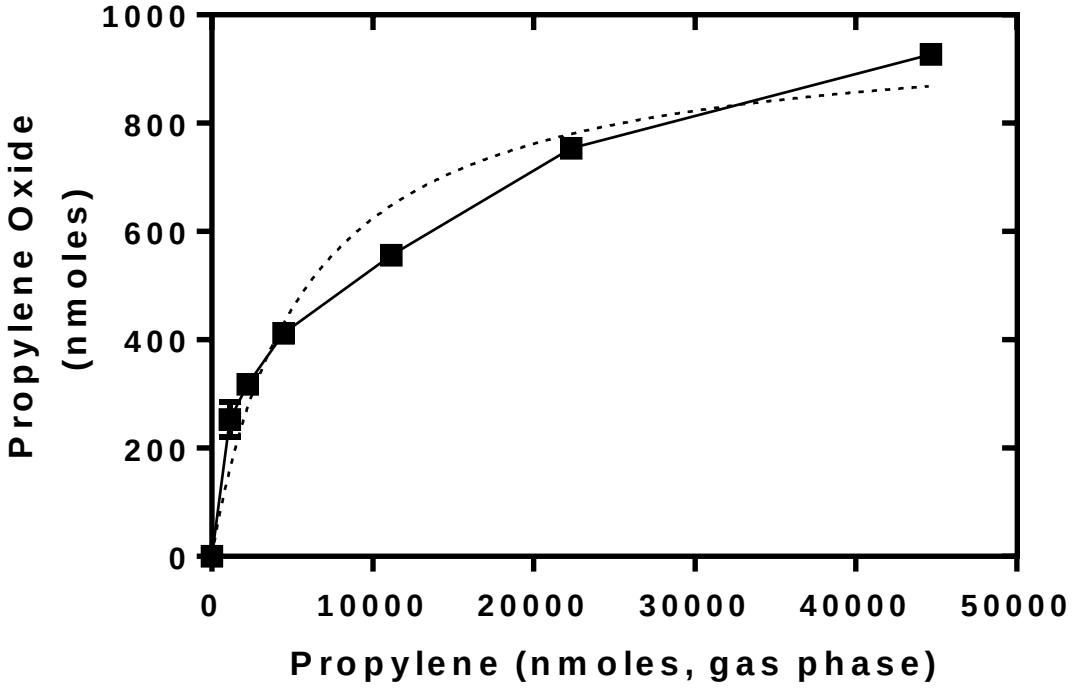


Figure 9. Effect of propylene concentration on the rate of propylene oxide production by propane-grown, resting cells of *R. rhodochrous* ATCC 21198. The Figure shows the amount of propylene oxide detected by GC analysis of the reaction medium after resting, propane-grown cells of strain 21198 (~0.85 mg total protein) were incubated for 60 minutes with the indicated amounts of propylene added to the reaction gas phase. The cells were grown, harvested and exposed to propylene, as described in the Methods section. The results presented show the mean and range of two biological replicates.

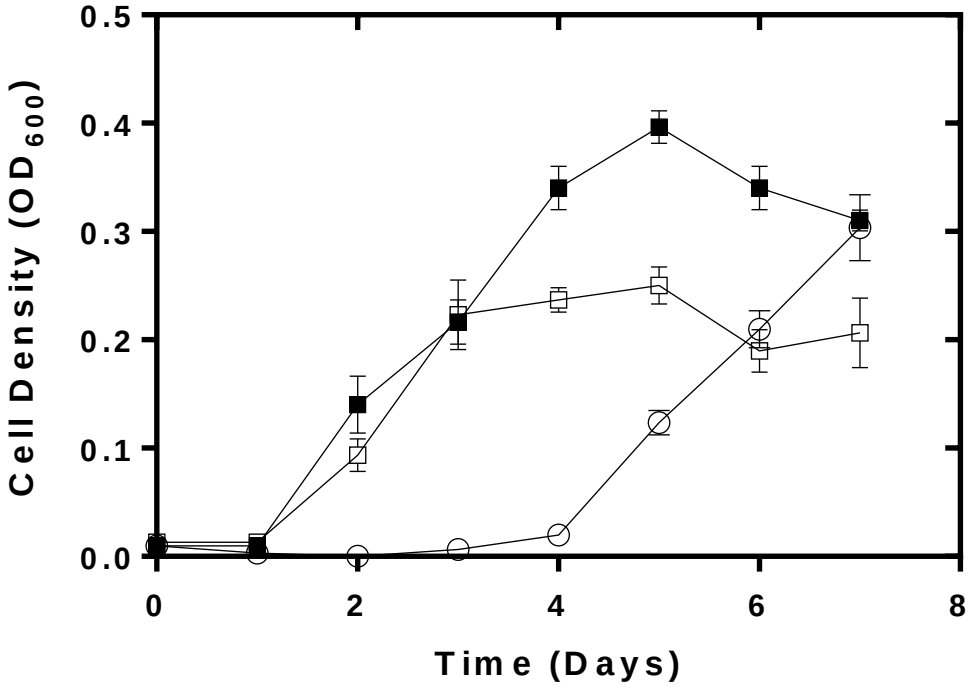


Figure 10. Effect of propylene oxide on time course of growth of *R. rhodochrous* 21198 on propane. The Figure shows the effects of propylene oxide on the growth of strain 21198 on instrument grade propane. The cultures were constructed and incubated, as described in the Methods section. The symbols are for the changes in culture density (OD_{600}) for (■) control cultures without propylene oxide or for cultures in which propylene oxide (1.75 µmoles) was added either at (○) the start of the incubation, or (□**)** 2 days after the cultures were initiated. The data presented are the mean and standard deviation of three biological replicates.

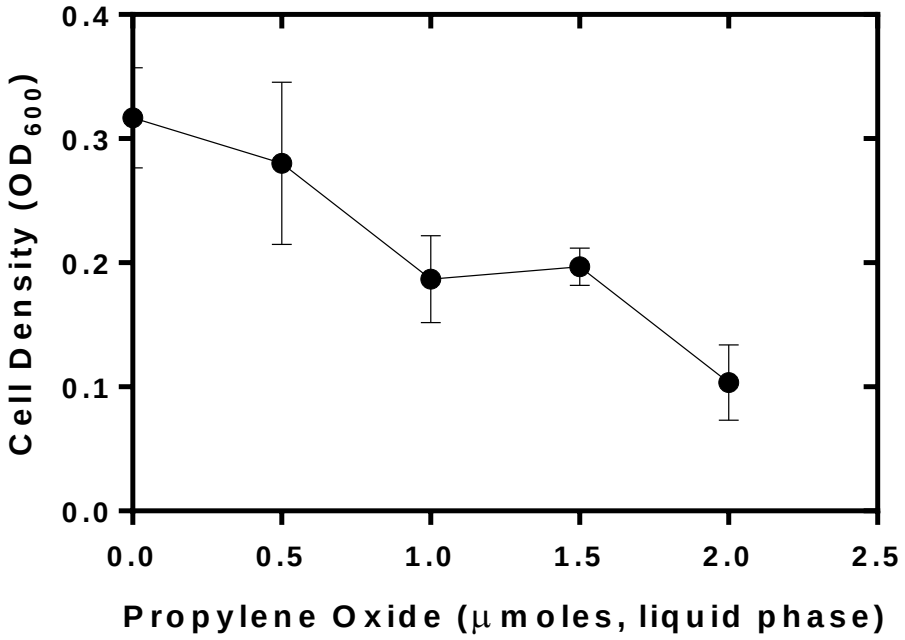


Figure 11. Effects of propylene oxide concentration on growth of *R. rhodochrous* ATCC 21198 on propane. The Figure shows the final culture density for cultures of strain 21198 grown on instrument grade propane (3.3 % v/v gas phase) in the presence of the indicated initial amounts of propylene oxide. The date presented are the means and standard deviation for three biological replicates.

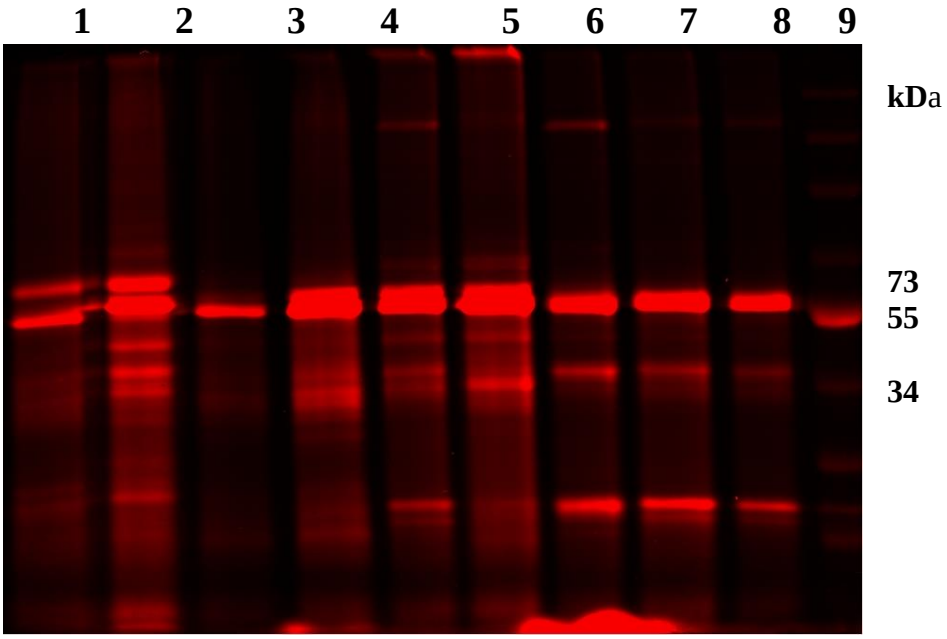


Figure 12. Activity based protein labelling of model cultures and enrichments. The Figure shows the NIR fluorescence pattern for protein samples (20 µg) after activity-based labeling of the following axenic strains or samples from enrichment cultures and their corresponding growth substrates (in parentheses). Lane 1: strain Py2 (propylene); Lane 2: strain RHA1 (propane +0.01 wt % yeast extract); Lane 3: strain BCP1 (propane); Lane 4: strain 21198 (propane); Lane 5: strain B276 (propane); Lane 6: Mix 10 enrichment; Lane 7: Mix 5 enrichment; Lane 8: Blue Rhino enrichment; Lane 9: Propane enrichment; Lane 10: NIR Ladder.

Table 4. Effect of gas composition on growth of model strains

Strain	Propane	Blue Rhino	Mix 5	Mix 10	Propylene
21198	0.41 (0.005)	0.29 (0.03)	0.05 (0.03)	0.02 (0.001)	0.01 (0.01)
BCP1	0.3 (0.005)	0.28 (0.03)	0.03 (0.03)	0.01 (0.001)	0.01 (0.01)
RHA1[*a]	0.03 (0.015)	0.06 (0.03)	0.03 (0.006)	0.05 (0.01)	0.04 (0.005)
B276	0.01 (0.01)	0.01 (0.01)	0.02 (0.02)	0.04 (0.03)	0.4 (0.02)
PY2	0.01 (0)	0.01 (0)	0.09 (0.02)	0.13 (0.03)	0.38 (0.02)

All data presented are the mean of three biological replicates (standard deviation).

*a This microorganism required 0.01 wt % yeast extract as a nutritional supplement

All propane grades were supplied at 3.3% (v/v headspace).

Table 5. Growth of model microorganisms on potential metabolites of propylene oxidation

Strain	Propylene oxide	Allyl alcohol	1,2-Propanediol	Acrylic acid
21198	0.01 (0.005)	0.01 (0.005)	0.2(0.13)	0.01 (0.004)
BCP1	0.01 (0.04)	0.02 (0.005)	0.8(0.004)	0.02 (0.006)
RHA1[*a]	0.14 (0.005)	0.01(0.00)	0.54(0.06)	0.05 (0.01)
B276	0.01 (0.004)	0.0 (0.005)	0.6(0.28)	0.01 (0.005)
PY2	0.25 (0.044)	0.01 (0.001)	0.02(0.01)	0 (0.004)

All strains were incubated with 5 mM of each compound as potential growth substrates.

All data presented are the mean of three biological replicates (standard deviation).

*a This strain required 0.01% yeast extract as a nutritional supplement.

Table 6. Effect of potential propylene oxidation metabolites on growth on propane

Strain	223µM Propylene oxide	223µM Allyl alcohol	223µM 1,2-Propanediol	223µM Acrylic acid
21198	0.01(0)	0.4 (0.02)	0.01 (0.00)	0.26 (0.05)
BCP1	0 (0.0)	0 (0.00)	0.08 (0.005)	0.07 (0.05)
Py2	0.01 (0.005)	0.01 (0.004)	0 (0.0)	0.01 (0.007)
Strain	446µM Propylene oxide	446µM All yl alcohol	446µM 1,2-Propanediol	446µM Acrylic acid
21198	0.01 (0.005)	0.48 (0.07)	0.01 (0.0)	0.27 (0.08)
BCP1	0.0 (0.005)	0 (0.00)	0.08 (0.0)	0.105 (0.02)
Py2	0.03 (0.01)	0.0 (0.00)	0.03 (0.01)	0.03 (0.01)

All strains were grown with a 3.3% (v/v headspace) propane.

All data presented are the means of three biological replicates (standard deviation).

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APENDIX

APPENDIX A

Analysis of changes in composition of Blue Rhino HD5 during cylinder discharge: The composition of the gas discharged from a newly-filled 20 lb cylinder of commercial (Blue Rhino brand) HD5 was determined by obtaining a gas sample from the transfer hose between the cylinder and a large gas-fired grill. The grill was used to combust the gas to avoid exceeding the lower explosive limit for liquified propane gas. The composition of the gas samples was determined by GC analysis, as described in the Methods section. Changes in the composition of the gas with discharge volume were determined by taking additional gas samples as the cylinder was discharged. The mass of gas released from the cylinder at any given time was determined from changes in the weight of the cylinder, as determined using a bathroom scale.

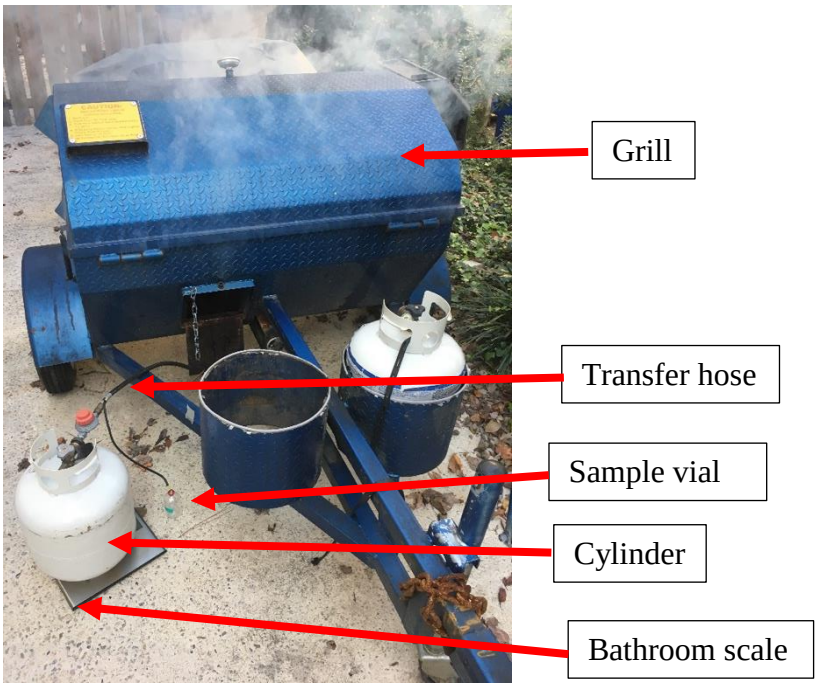

Figure S1. Cylinder discharge analysis. The Figure shows the system used to monitor and sample the gas released from a consumer-grade HD5 (Blue Rhino) cylinder

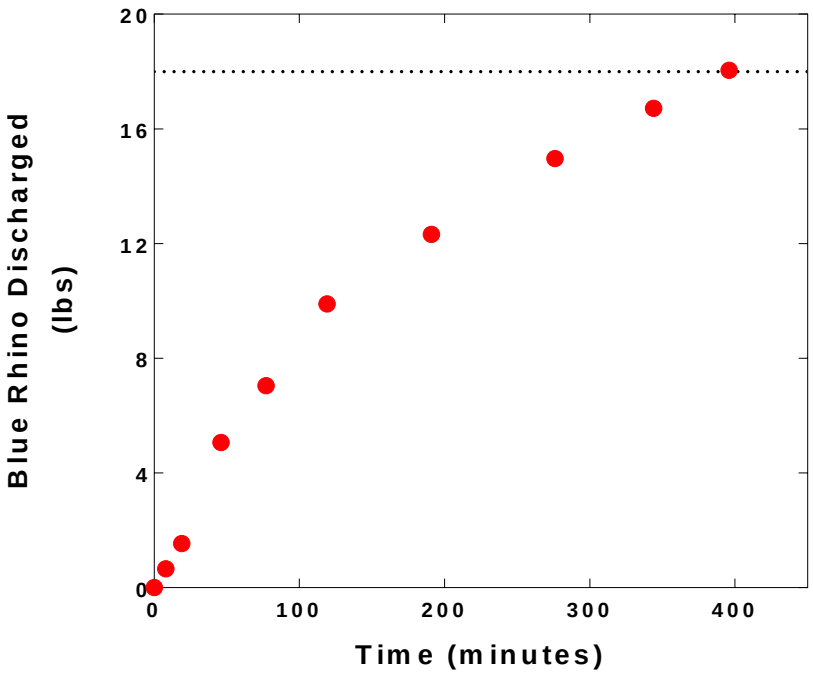

Figure S2. Time course of cylinder discharge. The Figure shows the changes in cylinder weight over time. The cylinder was initially discharged at a rate of ~10 L of gas per minute.

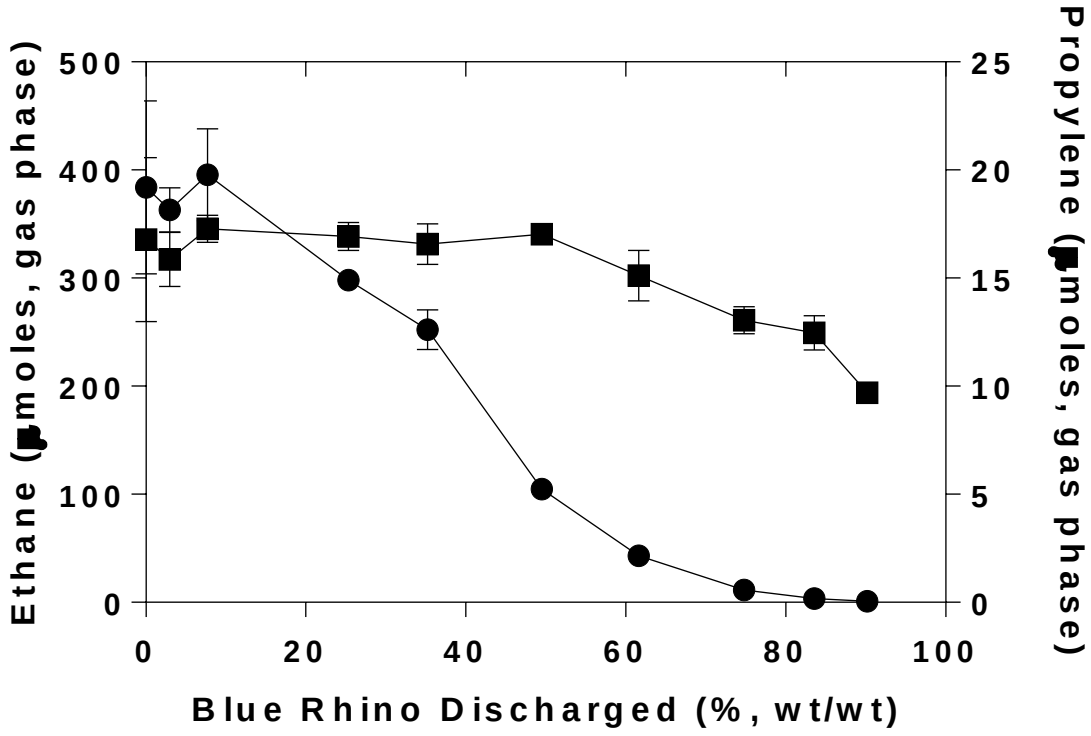

Figure S3. Changes in concentration of propylene and ethane during cylinder discharge. The Figure shows the changes in the concentration of (●) ethane and (■) propylene during the cylinder discharge study described in Figure S2.

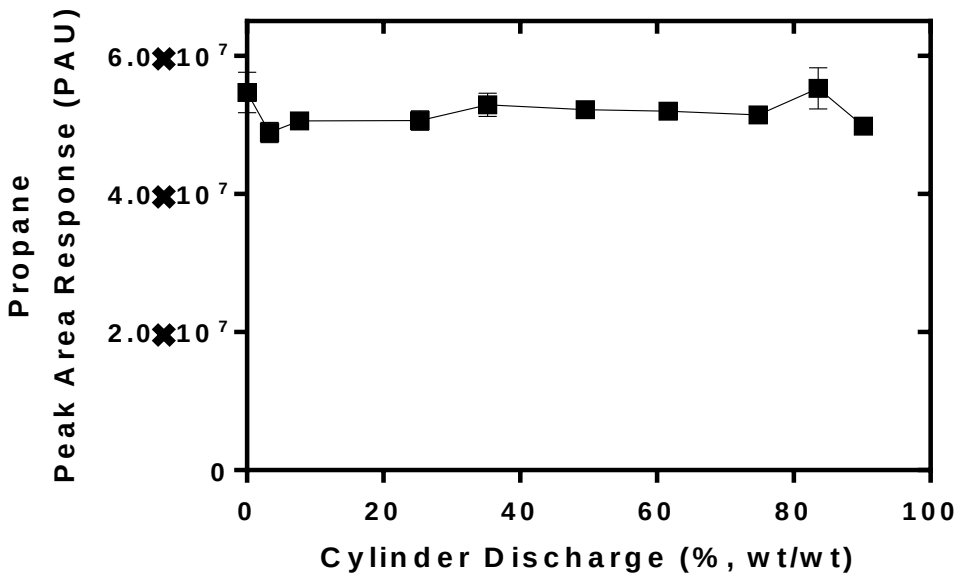

Figure S4. Changes in propane concentration during cylinder discharge. The Figure shows the concentration of propane during the cylinder discharge study described in Figure S2.