AN ABSTRACT OF THE DISSERTATION OF

Anne Elizabeth Taylor for the degree of Doctor of Philosophy in Civil Engineering presented on September 26, 2008.
Title: Factors Controlling Halogenated and Nonhalogenated Alkene Growth Substrate Range of Vinyl Chloride-Utilizing Bacteria

Abstract approved: ___________________________
Lewis Semprini

This thesis explores the factors limiting the alkene substrate range of the vinyl chloride (VC)-utilizing bacteria, and describes a method for measuring VC transformation in situ. Vinyl fluoride (VF) was evaluated as a surrogate for monitoring aerobic VC-transformation utilizing three isolates, *Mycobacterium* EE13a, *Mycobacterium* JS60 and *Nocardoides* JS614. JS614 grew on VF in addition to VC, making it the first bacterium reported to use VF as a sole carbon and energy source. There was little difference among the three strains in $K_s$ or $k_{max}$ for VC or VF. Rates of VF transformation and F$^-$ accumulation were correlated with the rate of VC transformation through a competitive inhibition model, and showed promise for estimating VC rates in situ.

Addition of supplemental ethene oxide (EtO) extended the growth substrate range of JS614 to propene, butene, and vinyl bromide (VB), whereas propene oxide (PrO) and butene oxide had no effect. Despite EtO and PrO being both inducers of *AkMO* and *EaCoMT* and intermediates in the alkene metabolic pathway, they exerted reversible inhibition on growth. Poor growth on propene was likely caused by the low rate of PrO consumption compared to PrO production which caused PrO accumulation to inhibitory levels; and reduced net reductant gain caused by a combination of PrO consumption “bottleneck” and the requirement of a NADPH-dependent CoM-carboxylase for metabolism of $\geq$C$_3$ alkenes. EtO consumption was inhibited by PrO, but overall the combined rate of EtO plus PrO consumption was better than that of PrO alone, perhaps explaining the beneficial effect of EtO as generating reductant to support growth on propene.
EtO-stimulated growth on VB was significantly slower than growth rates on VF and VC, but the maximum rate of VB consumption by Eth-grown cells was actually ~50% greater. Since 30% of the maximum rate of EtO consumption accompanied the maximum rate of VB consumption, it indicated that the “epoxide consumption bottle neck” must also apply to VB-epoxide, and is likely a factor limiting effective VB metabolism. VB was more inhibitory to growth of JS614 both on acetate and during active turnover than either VC and VF.
Factors Controlling Halogenated and Nonhalogenated Alkene Growth
Substrate Range of Vinyl Chloride-Utilizing Bacteria

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Anne Elizabeth Taylor

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APPROVED:

_____________________________________________________________________
Major Professor, representing Civil Engineering

_____________________________________________________________________
Head of the School of Civil and Construction Engineering

_____________________________________________________________________
Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

_____________________________________________________________________
Anne Elizabeth Taylor, Author
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. General Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2. Utilization of vinyl fluoride as a surrogate for</td>
<td>12</td>
</tr>
<tr>
<td>aerobic vinyl chloride transformation</td>
<td></td>
</tr>
<tr>
<td>3. Extending the alkene substrate range of VC-utilizing</td>
<td>33</td>
</tr>
<tr>
<td>Nocardioides JS614 with ethene oxide: insights from a combination of</td>
<td></td>
</tr>
<tr>
<td>molecular, kinetic, and toxicity studies</td>
<td></td>
</tr>
<tr>
<td>4. Application of ethene oxide extends the halogenated</td>
<td>59</td>
</tr>
<tr>
<td>alkene substrate range of Nocardioides JS614</td>
<td></td>
</tr>
<tr>
<td>5. Summary</td>
<td>82</td>
</tr>
<tr>
<td>6. Bibliography</td>
<td>95</td>
</tr>
<tr>
<td>7. Appendix</td>
<td>92</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>CoM-dependent pathway of propylene metabolism utilized by <em>Xanthobacter</em> sp. strain Py2 and <em>R. rhodochrous</em> strain B-276</td>
<td>11</td>
</tr>
<tr>
<td>2.1</td>
<td>Rates of cotransformation by VC-degrading isolates during competition experiments where VC and VF were in the same batch reactor</td>
<td>31</td>
</tr>
<tr>
<td>2.2</td>
<td>VC and VF transformation and Cl- and F- release for each of the VC-degrading isolates</td>
<td>32</td>
</tr>
<tr>
<td>3.1</td>
<td>Growth response of JS614 to alkenes +/- supplemental EtO</td>
<td>50</td>
</tr>
<tr>
<td>3.2</td>
<td>Sensitivity of JS614 to CO₂ deprivation and the CoM analogue bromoethanesulfonate (BES)</td>
<td>52</td>
</tr>
<tr>
<td>3.3</td>
<td>Epoxide-dependent induction of monooxygenase, transferase and PrO-dependent carboxylase activities</td>
<td>53</td>
</tr>
<tr>
<td>3.4</td>
<td>Response of acetate grown JS614 to Prop +/- 440µM EtO</td>
<td>55</td>
</tr>
<tr>
<td>3.5</td>
<td>Growth response of JS614 on Eth in the presence of PrO</td>
<td>56</td>
</tr>
<tr>
<td>3.6</td>
<td>Inhibition of EtO transformation by PrO</td>
<td>57</td>
</tr>
<tr>
<td>3.7</td>
<td>Growth response of JS614 on acetate in the presence and absence of EtO or PrO</td>
<td>58</td>
</tr>
<tr>
<td>4.1</td>
<td>Growth response of acetate-grown JS614 inoculum to halogenated alkenes +/-EtO</td>
<td>75</td>
</tr>
<tr>
<td>4.2</td>
<td>Response of JS614 during growth on VB to different concentrations of supplemental EtO</td>
<td>76</td>
</tr>
<tr>
<td>4.3</td>
<td>Transformation of VB and release of Br⁻ by Eth-grown cells</td>
<td>78</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>4.4</td>
<td>Growth response of JS614 on acetate in the presence of VC and VB.</td>
<td>80</td>
</tr>
<tr>
<td>A.1</td>
<td>Experimental data and results of competitive inhibition model for the three isolates degrading VF or VC individually, and during cotransformation where both substrates were present in batch reactors.</td>
<td>95</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td>Generation times, growth yields ( (Y) ) at 29°C, and transformation capacities ( (T_c) ) for the VC-degrading bacteria when Eth, VC or VF was provided as the sole carbon and energy source</td>
<td>28</td>
</tr>
<tr>
<td>2.2</td>
<td>Kinetic parameter estimates, ( k_{\text{max}} ), ( K_s ) or ( K_c ) obtained from progress curves of three Eth-grown isolates degrading Eth, VF or VC during short-term experiments</td>
<td>29</td>
</tr>
<tr>
<td>2.3</td>
<td>Rates of cotransformation of VF and VC and rates of ( F^- ) and ( Cl^- ) release in batch reactors at equal starting aqueous concentrations of VF and VC (~20μM)</td>
<td>30</td>
</tr>
<tr>
<td>3.1</td>
<td>Oligonucleotides used for RT-PCR</td>
<td>49</td>
</tr>
<tr>
<td>3.2</td>
<td>Expression of mRNA transcript of the first two genes in the alkene metabolic pathway</td>
<td>51</td>
</tr>
<tr>
<td>3.3</td>
<td>Kinetic parameters for substrate utilization by JS614</td>
<td>54</td>
</tr>
<tr>
<td>4.1</td>
<td>Kinetics of halogenated ethene transformation and halide release by JS614</td>
<td>77</td>
</tr>
<tr>
<td>4.2</td>
<td>Rates of VB or EtO transformation ( (V_i) ) in the presence of the other substrate compared to maximum rates ( (V_{\text{max}}) ).</td>
<td>79</td>
</tr>
<tr>
<td>4.3</td>
<td>Short-term growth response of JS614 in the presence and absence of halogenated alkene transformation</td>
<td>81</td>
</tr>
<tr>
<td>A.1</td>
<td>Input parameters for the competitive inhibition modeling analysis of the competition and the halide release experiments shown in Supplemental Figure A.1 and Figure A.2</td>
<td>94</td>
</tr>
</tbody>
</table>
Factors Controlling Halogenated and Nonhalogenated Alkene Growth
Substrate Range of Vinyl Chloride-Utilizing Bacteria

Chapter 1

General Introduction

Short-chained alkenes (C$_2$ – C$_4$) are widely distributed in the environment and
their sources are both natural and anthropogenic. Plants are a major source of the
alkene, ethene (Eth, C$_2$H$_4$). It is one of the five classes of plant growth regulatory
compounds and is produced by ripening fruit as well as shoots, flowers, seeds, leaves
and roots (3). Domestic commercial production of Eth is greater than any other
organic compound (9). Half of the world Eth demand is for the production of
polyethylene for packaging, shopping bags and trash liners. The C$_3$ alkene, propene,
is also a natural product from vegetation, as well as a by-product of petroleum refining
and Eth production. It is an important chemical intermediate for plastics and ranks
second to Eth among chemicals produced domestically (9).

Halogenated alkenes

A special class of short-chained alkenes that is of special concern is the
chlorinated ethenes, perchloroethene (PCE, C$_2$Cl$_4$), and trichloroethene (TCE,
C$_2$H$_3$Cl$_3$). These industrial solvents have been widely utilized in dry cleaning and
metal degreasing applications and their improper disposal has resulted in widespread
groundwater contamination (13). Both PCE and TCE are suspected human
carcinogens and the EPA has set maximum contaminant levels (MCLs) of 5µg/L for
drinking water. PCE and TCE are dense non-aqueous-phase liquids (DNAPLs) that
migrate downwards through non-saturated soils and into saturated zones where they
perch above impervious layers. These entrapped DNAPLs are relatively insoluble and
their slow dissolution results in a long-term source of groundwater contamination.
Other chlorinated alkenes are of concern, including cis-dichloroethene (cDCE,
C$_2$H$_2$Cl$_2$) and vinyl chloride (VC, C$_2$H$_3$Cl$_1$). cDCE and VC are products of anaerobic
PCE and TCE degradation, and VC is synthesized industrially on a large scale for
production of polyvinyl chloride plastics, and accidental release is a cause for environmental concern (16).

It is well recognized that anaerobic reductive dechlorination by bacteria can degrade PCE and TCE completely to Eth, when site conditions and electron donor availability are ideal. It is also a fact that the daughter products, cDCE and VC, can accumulate if redox conditions and electron donor availability are not favorable (13). cDCE and VC are known human carcinogens and their maximum contaminant levels (MCL) in drinking water are 0.07 and 0.002 mg/L, respectively. Furthermore, cDCE and VC are more water soluble and have lower octanol/water partitioning coefficients ($K_{ow}$) than either PCE or TCE, and sorb less strongly to aquifer materials (54). As a result, cDCE and VC can be transported out of an anaerobic zone where reductive processes are favored, and into zones where soluble oxygen is present, potentially allowing aerobic degradative processes to occur. Concentrations of cDCE and VC in aerobic aquifers are often very low (<2μM), and below the effective affinity values that have been reported for reductive dechlorinating consortia (2 – 602μM).

**Bacteria involved in aerobic alkene transformation**

In this context, many aerobic bacteria possess oxygenase enzymes with a broad substrate range that are capable of cometabolically transforming cDCE and VC. Organisms, possessing oxygenases with broad substrate specificity including toluene, methane, propane, and ammonia monooxygenase, are capable of cometabolically degrading VC by oxidizing its double bond (14, 43, 55, 64). The resulting epoxide, chlorooxirane, is unstable and spontaneously degrades yielding a mixture of acetylchloride, glycoaldehyde, chloroacetaldehyde and Cl$^-$ (5, 6). Because the affinity for VC in aerobic cometabolic systems can vary widely (0.6 - 50μM) (1, 65), and can be well above the concentrations found in dilute VOC plumes, aerobic degradative systems might not be very effective under such circumstances. However, VC-transforming bacteria that grow on VC as a sole carbon and energy source have been shown to have a high affinity for VC (~1μM) and have the potential to gain energy from its degradation even at low concentrations (60).
Aerobic VC-transforming bacteria from the genera *Mycobacterium*, *Nocardioides*, *Ochrobactrum*, *Pseudomonas*, and *Ralstonia* have been isolated from contaminated and non-contaminated sites (16). Although phenotypically diverse, the bacteria that use VC as a sole carbon and energy source all utilize Eth as a growth substrate. All have a high affinity (<5 μM) for VC regardless of whether they only cometabolize VC or utilize VC as a carbon and energy source (16, 66, 67). Two VC-utilizing cultures were used in this study. *Mycobacterium* strain JS60, and *Nocardioides* sp. strain JS614 utilize Eth and VC as sole carbon and energy sources (16) and were provided by Dr. Jim Spain (Georgia Tech University, Atlanta, GA). JS60 and JS614 can also utilize acetate and glucose as growth substrates, but under these conditions the alkene metabolic pathway is not expressed (16).

**Physiology of aerobic alkene metabolism**

While our understanding of the complete metabolic pathway of C₂ alkenes is limited, extensive work has been carried out to describe the C₃ propene degradation pathway in *Xanthobacter autotrophicus* Py2, and *Rhodococcus rhodochrous* B-276 (26). They degrade propene to acetoacetate via a four step linear pathway involving five separate enzymes found on a linear mega-plasmid (Figure 1.1). The four steps in Propene utilization in *R. rhodochrous* strain B-276 and *X. autotrophicus* Py2 are (1) reductant-dependent oxidation of the double bond by the action of alkene monooxygenase (AkMO); (2) stabilization and opening of the epoxide by epoxyalkane cofactor M transferase (EaCoMT) to result in 2-R- or 2-S-hydroxypropyl CoM, (3) dehydrogenation by 2-R- or 2-S-hydroxypropyl-CoM Dehydrogenase (HPCDH), and (4) carboxylation by action of NADPH:2-Ketopropyl-CoM Carboxylase/Oxidoreductase (2-KPCC) to produce free CoM and acetoacetate (26). Before identification of the role of CoM in alkene metabolism by *X. autotrophicus* Py2 and *R. rhodochrous* B-276 its only known role was as a cofactor in methanogenesis (26). Evidence points to enzyme products, rather than the substrates per se as major inducers of the alkene degradation pathway (25), but this cannot be known with certainty unless a lacZ reporter strain is constructed where the AkMO promoter
sequence controls β-galactosidase expression rather than AkMO, and has no ability to oxidize substrate (53).

The pathway of C₂ alkene utilization has been investigated in JS60 and JS614 and shows many similarities to the propene utilizing system (18, 45). The alkene degrading genes of strains JS60, JS614, and of a number of other bacteria from several genera which can degrade Eth and VC, are located on linear megaplasmids (20, 45). Similar to the propene system, degradation of Eth and VC begins with the oxidation of the double bond by AkMO and is followed by stabilization with 2-mercaptoethanesulfonic acid (CoM) by the action of EaCoMT.

In the case of strain JS60 the genes coding for AkMO and EaCoMT have been identified and sequenced, and the metabolic intermediates of AkMO, ethene oxide (EtO), and VC-epoxide (VCO) in the cases of Eth and VC respectively, have been identified by mass spectrometry (18). When Eth is the substrate, 2-hydroxyethyl-CoM has been identified as the product of EaCoMT. Because of the similarities to the C₃ system it has been assumed that the next step in metabolism is a dehydrogenation that leads to malonate semialdehyde. While the isolation of other downstream intermediates has not yet been achieved, it may be possible to predict products of metabolism with molecular tools. Genes in proximity to the JS60 EaCoMT gene are similar to CoA transferase and acetyl-CoA synthetase in R. rhodochrous B-276, which may point to acetyl-CoA as the next intermediate. This is supported by some of the earliest work with the Eth-metabolizing bacterium Mycobacterium E20 where acetyl-CoA was the observed product of Eth metabolism (21, 22).

Nocardioides JS614 has been sequenced and genes essential for alkene metabolism, and that have high similarity to those in X. autotrophicus Py2 were identified (45). Peptide mass fingerprinting of JS614 identified seven proteins expressed in response to Eth, VC, and EtO that were not present in acetate grown cells (15). Among these were proteins with high amino acid similarities to essential enzymes in the propene metabolic pathway of X. autotrophicus Py2, including two short-chain dehydrogenases (SDR, Noc4814 and 4841) with similarities to the X.
*X. autotrophicus* Py2 SDR of 46 and 47% respectively, and a FAD-dependent pyridine nucleotide-disulfide oxidoreductase (Noc4827) with a 53% amino acid sequence similarity to the carboxylase in *X. autotrophicus* Py2. The carboxylase in *X. autotrophicus* Py2 is responsible for the transformation of 2-ketopropyl-CoM to acetoacetate and recycling of CoM (26). The presence of a carboxylase-like protein in JS614 suggests the possibility of a carboxylation step recycling CoM during the transformation of Eth and VC.

**Substrate range of alkene-utilizing bacteria**

Some alkene-utilizing bacteria can use a range of alkenes (C₂ to C₆) as growth substrates while others are limited to a single alkene growth substrate (62). For example *X. autotrophicus* Py2 can utilize substrates including Eth, propene, 1-butene, 1-pentene and 1-hexene for growth (26, 62) while many Eth-utilizing bacteria only grow on Eth (62). Monooxygenases are notoriously non-specific and will oxidize substrates besides those that will support growth; therefore AkMO is probably not the limiting step in the utilization of multiple alkenes as growth substrates (60, 62). Physiological limitations that may explain why some alkene-utilizing bacteria cannot utilize multiple alkenes as growth and energy substrates, may be (a) lack of enzymes to carry out the metabolism, (b) lack of induction of the full alkene metabolic pathway, (c) poor downstream enzyme specificity, (d) slow rate of transformation of downstream metabolites, or (e) substrate or product toxicity.

It is unknown what the differences are between Eth-utilizing bacteria that can utilize VC as a growth and energy substrate, versus those that can only utilize Eth. As all Eth-utilizing bacteria can oxidize VC (16), AkMO is probably not the limiting step in the utilization of VC as a growth substrate. The oxidation of VC by AkMO yields an unstable epoxide which spontaneously degrades with a half life of 90s (5, 6). This short half life potentially presents a challenge in the utilization of VC as a carbon and energy source that non-halogenated alkene degrading bacteria do not need to meet because most naturally occurring epoxides have longer half lives. The second enzyme in the metabolic pathway, EaCoMT, needs to have high affinity for the VC-epoxide.
and transform it at a rate sufficiently rapid to capture and utilize it prior to its spontaneous breakdown. Additionally, if monooxygenase products are responsible for the induction of the complete alkene metabolic pathway, the unstable VC-epoxide product of AkMO may not reach a concentration sufficient to promote pathway induction and optimize VC degradation. In *Nocardioides* strain JS614, EtO, the epoxide product of the oxidation of Eth, has been observed to promote growth of cells on VC when they are transferred from acetate growth conditions where the alkene utilizing pathway is repressed, suggesting that epoxide products are important for the expression of the VC metabolic pathway (44).

It is also unknown if bacteria capable of transforming VC are able to transform other halogenated ethenes such as vinyl fluoride (VF) and vinyl bromide (VB). For example, VF is the fluorinated analogue of VC, and as organofluorine compounds use has increased so has interest in their transformation (39). At the start of this research there were no examples in the literature of organisms able to utilize VF or VB as sole growth and energy substrates, and there little work has been carried out to address the question of whether aerobic metabolism of fluorinated or brominated ethenes proceeds as it does for their chlorinated counterparts.

**Evaluating VC transformation in situ**

It is difficult to estimate rates of aerobic VC transformation in situ because the mineralization of VC yields CO₂ and Cl⁻; neither of which can be linked exclusively to VC transformation. Stable carbon isotope fractionation may distinguish between biodegraded and abiologically degraded VC (13), and in lab-based studies can differentiate between aerobic or anaerobic degradation of VC (12, 14), but it has not been utilized to estimate rates of VC degradation. Utilizing a surrogate for VC that is transformed via the same enzyme systems, and yields a discrete analytical response when aerobically metabolized, would potentially allow estimation of VC transformation rates in contaminated aquifers.

In reductive dechlorination systems, trichlorofluoroethene (TCFE) has been used as a surrogate for TCE transformation in situ and in microcosm tests, and 1-
chloro-1-fluoroethene (1,1-CFE) and E-chlorofluoroethene (E-CFE) have served as analogues for VC in microcosm and field push-pull tests (24, 36, 52). In these reductive systems the accumulation of VF was correlated with the formation of ethene from VC (52). VF is a stable molecule in aqueous solution and its aerobic degradation yields fluoride (F\textsuperscript{−}), which would be a unique signature in many aquifers. Work with cytochrome P450-dependent monooxygenases suggests that VF is aerobically degraded in a manner similar to VC; an epoxide is formed from the initial oxidation (10, 19). VF-epoxide is unstable and is expected to yield spontaneous degradation products analogous to VC-epoxide (5, 6). To our knowledge, no bacterium has been reported to oxidize or utilize VF as a sole carbon and energy source, although the similarity of its structure to VC would suggest that the oxidation of the double bond is possible.

**Thesis Objectives:**

1. **Identify a surrogate indicator for VC transformation**

   If the rates of VF transformation and F\textsuperscript{−} accumulation can be correlated with the rate of aerobic degradation of VC, then VF could potentially be used as a surrogate reactive tracer for estimating *in situ* degradation rates of VC. In a VC contaminated aquifer in single-well push-pull tests, VF addition and subsequent co-transformation could be utilized as an indicator of *in situ* VC transformation rates. Although fluorinated surrogates must be considered as toxic as their chlorinated analogues (47), they have been previously utilized *in situ* with reductive dechlorinating systems to estimate rates of VC transformation with the expectation that VF would accumulate (24, 36). In single well push-pull tests, rates of transformation of E-CFE under reduced dechlorinating conditions have been used to estimate rates of VC transformation (24). Because the reduction of E-CFE results in VF formation, its subsequent transformation to F\textsuperscript{−} could be exploited to determine if sequential anaerobic/aerobic processes are transforming TCE and its daughter products to nontoxic end products. As far as is known, VF is not defluorinated under anaerobic
conditions (52), and the accumulation of F\textsuperscript{−} would be a potential indicator that VC is being degraded aerobically.

The objective of research presented in Chapter Two was to evaluate if VF was a suitable surrogate for monitoring aerobic VC utilization or cometabolic transformation. Laboratory experiments were carried out with various oxygenase-containing aerobic bacteria that either cometabolically or catabolically metabolize VC, to evaluate whether (i) rates of VF transformation were similar to those of VC transformation, (ii) VC and VF have similar affinities for the monooxygenase that mediates the initial transformation, (iii) a competitive inhibition kinetic model accurately simulates concurrent VF and VC degradation, and (iv) the rate of F\textsuperscript{−} accumulation can be correlated with that of VC utilization. In addition, the potential of these bacteria to use VF as a carbon and energy source was also evaluated.

(2) Examination of factors influencing the alkene substrate range of *Nocardioides JS614*

*Nocardioides JS614* utilizes the C\textsubscript{2} alkenes Eth, VC and VF as growth substrates (16, 60). Growth yields of JS614 on VC are greater than for other VC-utilizing bacteria and it had been suggested that this might be due to carboxylation of the VC-epoxide intermediate (16). Peptide mass fingerprinting of JS614 identified seven proteins expressed in response to Eth, VC and EtO that were not present in acetate grown cells (15). Among these were proteins with high amino acid similarities to essential enzymes in the propene metabolic pathway of *X. autotrophicus* Py2, including two short-chain dehydrogenases (SDR, Noc4814 and 4841) with similarities to the *X. autotrophicus* Py2 SDR of 46 and 47% respectively, and a FAD-dependent pyridine nucleotide-disulfide oxidoreductase (Noc4827) with an amino acid sequence similarity to the NADPH:2-ketopropyl-CoM carboxylase oxidoreductase in *X. autotrophicus* Py2 of 53%. Although the presence of these genes might suggest that JS614 can utilize propene as a growth substrate, I observed that acetate-grown cultures, not expressing the alkene metabolic pathway, were unable to utilize propene as a sole energy and carbon source. Previously it was demonstrated that supplemental
EtO stimulated growth of JS614 on either Eth or VC after transferring cells from a repressed state, (44). I found that when acetate-grown cells were provided with supplemental EtO that they were able to grow on propene. Research described in Chapter Three, characterizes propene utilization by JS614 and the role of EtO in extending its alkene growth substrate range. The objectives were to determine if (a) lack of growth on propene from a repressed state was due to lack of induction of enzymes in the alkene metabolic pathway, (b) poor kinetics of substrate utilization caused energy restraints that contributed to slow growth, (c) substrate or product toxicity contributed to no/poor growth, and (d) determine the role of EtO in promoting growth.

(3) Evaluate transformation of other halogenated ethenes by JS614.

The oxidation of halogenated C₂ alkenes by AkMO yields unstable epoxides that spontaneously degrade if not quickly transformed (5, 6). Epoxide instability might present a challenge to the utilization of halogenated C₂ alkenes as carbon and energy sources. The second enzyme in the metabolic pathway, EaCoMT, likely needs to have high affinity for the halogenated-epoxide and a rate of transformation sufficiently rapid to capture and transform it before its spontaneous breakdown. Additionally, if monoxygenase products are responsible for the induction of the complete alkene metabolic pathway, the unstable halogenated-epoxide product of AkMO may not reach a concentration sufficient to maximize pathway induction and optimize alkene degradation. In *Nocardioides* strain JS614, EtO was observed to promote growth on ethene when it was transferred from a repressed state, suggesting that stable epoxide products may be important to efficient expression of the complete VC metabolic pathway (44).

Although the mechanism that allows EtO to induce expression of the alkene metabolic pathway is unknown, I tested its stimulatory ability to expand the substrate range of VC-utilizing bacteria for the remediation of groundwater pollutants. If lack of induction is all that prevents some Eth- or VC-utilizing bacteria from transforming
other halogenated C₂ alkene groundwater contaminants, then providing an inducer may promote sustainable transformation. To test the concept, I evaluated the ability of EtO to expand the substrate range of *Nocardioides* JS614 to include the dichloroethenes 1,1-dichloroethene (DCE) and cDCE, and the brominated analogue of VC, vinyl bromide (VB).

During a preliminary survey, I found that growth on VB was induced by EtO. In Chapter Four we evaluated the optimal concentration of EtO required as an inducer of VB transformation, and the toxicity associated with VB transformation. We also used VB transformation to explore the role of EaCoMT in substrate range limitation and compared and contrasted EtO-promoted growth on VB and propene (Chapter 3).
Figure 1.1. CoM-dependent pathway of propylene metabolism utilized by *Xanthobacter* sp. strain Py2 and *R. rhodochrous* strain B-276. From Ensign (26).
Chapter 2

Utilization of Vinyl Fluoride as a Surrogate for Aerobic Vinyl Chloride Transformation

Anne E. Taylor, Mark E. Dolan, Peter J. Bottomley, Lewis Semprini

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ABSTRACT

Vinyl fluoride (VF) is a stable molecule in aqueous solution and its aerobic transformation potentially yields F⁻. This work evaluated if VF is a suitable surrogate for monitoring aerobic vinyl chloride (VC) utilization or cometabolic transformation. Experiments were carried out with three isolates, Mycobacterium strain EE13a, Mycobacterium strain JS60 and Nocardioides strain JS614 to evaluate if their affinities for VF and VC and their rates of transformation were comparable and whether the transformation of VF and F⁻ accumulation could be correlated with VC utilization. JS614 grew on VF in addition to VC, making it the first organism reported to use VF as a sole carbon and energy source. EE13a cometabolized VC and VF, and JS60 catabolized VC and cometabolized VF. There was little difference among the three strains in the $K_s$ or $k_{max}$ values for VC or VF. Competitive inhibition modeled the temporal responses of VF and VC transformations and Cl⁻ and F⁻ release when both substrates were present. Both the rate of VF transformation and rate of F⁻ accumulation could be correlated with the rate of aerobic transformation of VC, and showed promise for estimating VC rates in situ using VF as a reactive surrogate.
INTRODUCTION

Anaerobic reductive dechlorination can reduce the industrial solvents perchloroethene (PCE) and trichloroethene (TCE) completely to ethene (Eth) when site conditions and electron donor availability are ideal, but the daughter products cis-dichloroethene (cDCE) and vinyl chloride (VC) can accumulate if conditions are not favorable (13). VC is more water soluble and has a lower octanol/water partitioning coefficient (K_{ow}) than either PCE or TCE and sorbs less strongly to aquifer materials (54). As a result, VC can be transported out of the anaerobic zone where reductive processes are occurring and into zones where soluble oxygen is present, potentially allowing aerobic processes to occur. Organisms possessing oxygenases with broad substrate specificity including butane, methane, propane, and ammonia monooxygenase are capable of cometabolically transforming VC by oxidizing its double bond (2). The resulting epoxide, chlorooxirane, is unstable and spontaneously degrades yielding a mixture of acetylchloride, glycoaldehyde, chloroacetaldehyde and chloride (Cl\(^-\)) (5, 6). Also widely distributed in the environment are bacteria that can utilize VC as a sole carbon and energy source (16). VC-assimilating bacteria from the genera *Mycobacterium, Nocardioides, Ochrobactrum, Pseudomonas,* and *Ralstonia* have been isolated from contaminated and non-contaminated sites (16, 23). Although phenotypically diverse, the bacteria that mineralize VC all utilize Eth as a growth substrate, and oxidation of Eth and VC is initiated by an alkene monooxygenase (AkMO) (16, 20, 23, 29, 38, 65).

It is difficult to estimate rates of aerobic VC transformation *in situ* because the mineralization of VC yields CO\(_2\) and Cl\(^-\); neither of which can be tied solely to VC transformation. Stable carbon isotope fractionation can distinguish between biodegraded and abiotically degraded VC and can determine whether biodegradation of VC was aerobic or anaerobic, but it has not been utilized to estimate rates of VC degradation (12, 14). Utilizing a surrogate for VC that is transformed via the same enzyme systems, but would yield a discrete analytical response when
aerobically metabolized would allow estimation of VC transformation rates in contaminated aquifers.

In reductive dechlorination systems, trichlorofluoroethene (TCFE) has been used as a surrogate for TCE transformation *in situ* and in microcosm tests, and 1-chloro-1-fluoroethene (1,1-CFE) and E-chlorofluoroethene (E-CFE) have served as analogues for VC in microcosm and field push-pull tests (24, 36, 52). In these reductive systems using a fluorinated surrogate, the accumulation of vinyl fluoride (VF) was correlated with the formation of ethene from VC (52). VF is a stable molecule in aqueous solution and its aerobic degradation yields fluoride (F⁻), which is a unique signature in most aquifers. Work with cytochrome P450-dependent monooxygenases suggests that VF is aerobically degraded in a manner similar to VC; an epoxide is formed from the initial oxidation (10, 19). VF-epoxide is unstable and is expected to yield spontaneous degradation products analogous to VC-epoxide (5, 6). To our knowledge, no bacterium has been reported to oxidize or utilize VF as a sole carbon and energy source, although the similarity of its structure to VC would suggest that the oxidation of the double bond is possible.

The objective of this work was to evaluate if VF is a suitable surrogate for monitoring aerobic VC utilization or cometabolic transformation. Laboratory experiments were carried out with various oxygenase-containing aerobic bacteria that either cometabolically or catabolically metabolize VC, to evaluate if (i) rates of VF transformation are similar to those of VC transformation, (ii) VC and VF have similar affinities for the monooxygenase that mediates the initial transformation, (iii) a competitive inhibition kinetic model accurately simulates concurrent VF and VC degradation, and (iv) the rate of F⁻ accumulation can be correlated with that of VC utilization. In addition the potential for bacteria to use VF as a carbon and energy source was evaluated.
MATERIALS AND METHODS

Chemicals and medium. VC (99.5%), TCE (99.9%), acetylene, and propyne (97%) were obtained from Aldrich, (Milwaukee, WI), VF (98%) from SynQuest (Alachua, Fl.), and Eth (C.P. grade) from Airgas (Radnor, PA). cDCE (97%) was purchased from Acros (Fisher Scientific, Pittsburgh, PA). Cells were grown in minimal salts medium (MSM) (16). One-tenth-strength tryptic soy agar (TSA) was used as a non-selective media and contained 3g Bacto tryptic soy powder (Difco), 10g glucose and 15g agar per liter.

Cultures and maintenance. Strain EE13A was isolated from chloroethene-contaminated groundwater collected from well LC191, Ft. Lewis, WA. The groundwater was contaminated with both cDCE and TCE and contained dissolved oxygen (4). Isolation was achieved by streaking an Eth enrichment culture incubated at room temperature (23°C) on TSA plates and subculturing individual colonies on MSM with Eth provided as sole carbon source. Repeated streaking and culturing eventually yielded a strain that utilizes Eth as a growth substrate and cometabolically degrades VC and VF. Identification of strain EE13a was done by partial sequencing of amplified 16S rDNA at the Center for Genome Research and Biocomputing at Oregon State University on an ABI Prism 3730 Genetic Analyzer, Applied Biosystems (Foster City, CA), and the 16S rDNA gene sequence was deposited in GenBank under the accession number EF405863. The partial 16S rDNA of strain EE13a (900 bp) shared 99% sequence identity with Mycobacterium sp. O228YA isolated from a dioxin contaminated landfill in Japan (68) and 96% similarity with Mycobacterium strain JS60. Mycobacterium strain JS60, and Nocardioides sp. strain JS614 utilize Eth and VC as sole carbon and energy sources (16) and were provided by Dr. Jim Spain (Georgia Tech University, Atlanta, GA). All cultures were grown in 500 ml glass Wheaton bottles sealed with phenolic caps and gray butyl rubber septa, in 200 ml MSM and either 4% (vol/vol) headspace Eth, VF or VC added as the sole carbon and energy source. Cultures were shaken, inverted, at 120 RPM in a Brunswick controlled environment shaker at 29±1°C. Growth was followed by
monitoring the increase in optical density (OD) at 600nm. For experimental use, cells were harvested at mid-log phase by centrifugation. Cells were pelleted and washed twice in 4mM potassium phosphate buffer at pH 7.0. Cell pellets were held at room temperature (<15min) and resuspended to dense concentration (40-60 mg protein/ml) in phosphate buffer immediately before initiating an experiment.

**Analytical Methods.** A microbiuret assay was used to determine the protein content of cells in the batch reactors used in kinetic tests (31). Progress curves of Eth, VC and VF disappearance were constructed by analyzing headspace samples (100 or 500μl) by gas chromatography (GC) with flame-ionization detection (FID) using a Shimadzu (Kyoto, Japan) GC-14A gas chromatograph fitted with an Alltech (Deerfield, IL) AT-1 capillary column (0.53mm x 15m). The FID and injector temperatures were adjusted to 200°C, and the column to 70°C. The mass of substrate remaining during kinetic tests was calculated from the concentration in the headspace samples using Henry’s Law.

Concentrations of Cl⁻ and F⁻ were determined using ion chromatography analysis. Aqueous samples were withdrawn through the gray butyl septa during the short-term kinetic studies, filtered through 0.45μm pore size cellulose acetate membrane filters into 2ml auto sampler vials sealed with crimp caps, and amended with 5% propyne. Samples were incubated with propyne for 1h to inhibit any residual activity by cells that may have passed through the membrane filter and were then transferred to microcentrifuge tubes and stored at 4°C until analysis. Cl⁻ and F⁻ concentrations were determined using a Dionex DX-500 Ion Chromatograph (Sunnyvale, CA) equipped with an electrical conductivity detector and a Dionex AS14 column.

**Determination of kinetic parameters.** Kinetic parameters were obtained from batch kinetic tests using resting cells grown and harvested as described above. VC and VF disappearance and halide release batch tests were carried out in 155ml serum bottles sealed with phenolic caps and gray butyl rubber septa and containing 100ml of 4mM phosphate buffer, pH 7.0. Specific amounts of gases were added to achieve desired
aqueous concentrations based on Henry’s Law, and were incubated with shaking at 150 RPM at room temperature, 23±2°C, to achieve phase equilibrium. Experiments were initiated by adding aliquots of a dense cell suspension (typically 100 μl) to the bottles. The initial concentrations of substrates were significantly greater than $K_s$ or $K_c$ values (half-velocity coefficients for a growth and nongrowth substrates respectively, μM) and transformation was followed to completion. Eth, VF or VC utilization or transformation progress curves were fitted using a weighted nonlinear least-squares analysis of the integrated Monod equation to estimate the kinetic parameters $k_{max}$, (maximum specific substrate utilization rate, nmol/min/mg protein), and $K_{s/c}$, (57). All experiments were carried out in triplicate and $k_{max}$ and $K_{s/c}$ were estimated from each progress curve and then averaged. The generation time was determined by monitoring the optical density at 600nm. Dry weight was determined by filtering a volume of cell culture on a 0.2 μm membrane, and then dried at 65°C, and weighed. This dry weight was correlated with cell culture optical density at 600 nm for the yield determination. Transformation capacity ($T_c$) values for the nongrowth substrates were determined by incubating Eth-grown cultures with nongrowth substrates and transformation monitored until it ceased. Abiotic controls showed minimal losses of substrate over the time course of the experiments (<120 min).

The possibility of mass transfer limitations between the aqueous and gas phases was evaluated by inoculating bottles prepared as previously described with 1X and 2X cell concentration and comparing the rates of substrate transformation. Rates of degradation in the bottles with 2X cells were twice the rates of the bottles with 1X cells (data not shown), indicating mass transfer did not limit the rate of biological reactions under the experimental conditions. To eliminate concerns that oxygen limited rates of transformation, a second aliquot of gas substrate was added to the batch tests after the completion of a progress curve and transformation followed. Rates of transformation for the second progress curve were similar to those of the first (data not shown), indicating that oxygen did not limit rates.
Modeling experimental data. The kinetic parameters from the nonlinear least-squares analysis were used to model progress curves in the presence of two substrates in order to identify the inhibition type. The model incorporated partitioning between gas and liquid compartments (55 and 100ml respectively) using dimensionless Henry’s coefficients of 7.24 for Eth, 0.99 for VC, and 2.66 for VF determined at room temperature (23±2°C) (23, 32, 52), and the assumption was made that halide release occurred instantaneously with transformation. Stoichiometry of halide release was determined and incorporated into the model. The non-steady-state, non-linear equations were solved simultaneously using STELLA 8.1.1 (isee systems, Lebanon, NH). The assumption was made that cell concentration did not change due to cell growth over the course of the short experiments (<120min), because these bacteria are slow growing (generation time >24h at 23°C, data not shown), and that cell death did not occur since the amounts of nongrowth substrates transformed were well below Tc. Competitive, noncompetitive and uncompetitive inhibition models were used to model experimental data (46) (Supplemental Information, eqs. S1-6). The independently determined kinetic parameters, $k_{\text{max}}$ and $K_{s/c}$, and initial concentration of substrate were held constant while solving two equations simultaneously for VC as substrate and VF as an inhibitor, and visa versa. The inhibition constant $K_i$, was adjusted to refine the fit of the model to the experimental data. The experimental data were plotted against the model output and $r^2$ was calculated by linear least-squares regression to determine the goodness of fit. The sum of squares error (SSE) between the experimental data and the model output was also calculated to evaluate how well the model simulated the experimental data.
RESULTS

Early in the research, growth-linked transformation of VF was observed for JS614 (Table 2.1). The discovery that this bacterium utilized VF for growth and energy provided the opportunity to test our experimental objectives against a suite of bacteria with three distinct phenotypes. While doubling times and growth yields ($Y$, g protein/mol substrate) on Eth were similar for all three strains, the doubling time for JS60 on VC was longer than that for JS614 (27.4 vs. 17.5h), and JS614 had a proportionally greater growth yield during growth on VC. With VF as the sole carbon and energy source, JS614 produced a yield and generation time similar to those for VC. Growth yield for JS60 when VC was the substrate was 47% of the yield when Eth was the growth substrate. Decreases in yield for JS614 on the halogenated ethenes in comparison with Eth were less dramatic than those for JS60, and the doubling times on VC and VF were very similar to those for Eth. Yields expressed in terms of COD illustrate that JS614 is more efficient than JS60 at incorporating VC-carbon into biomass, or better able to overcome toxic effects of VC-epoxide. Lower yields for JS60 and JS614 than those reported by Coleman et. al. (16) are possibly due to the indirect method Coleman employed to estimate protein yield. The $T_c$ of VC and VF during cometabolic transformation by EE13a were similar, and were comparable to the $T_c$ of JS60 when VF was transformed (Table 2.1).

Despite the fact that the three VC-degrading isolates responded differently to Eth, VC and VF as growth substrates, there were no differences between the $K_{sc}$ or $k_{max}$ values for VF and VC of any individual isolate, and there was little difference between the three isolates in their rates of transformation or affinity for the halogenated substrates (Table 2.2). Additionally, rates of maximum VC and VF transformation or utilization were similar for all three isolates, indicating that the presence of the smaller F atom did not affect the AkMO’s ability to accept VF as a substrate. Values of $k_{max}$ were different that those previously reported for JS614 and JS60 (16), but could be attributed to growth condition differences and the use of Eth-grown cells in this work while VC and Eth-grown cells were used formerly.
Competition experiments were performed to evaluate how VF was transformed in the presence of VC. For these tests the initial aqueous concentration of VC was held constant at a concentration above the $K_s$ (~20μM), and VF concentrations were varied (0, and ~20, 40, 60 and 80μM). When initial aqueous concentrations of VF and VC were both ~20μM, the cotransformation rates of VF by EE13a, JS60 and JS614 were 58, 65, and 37% of the rate of the VF-only control, while the rates of cotransformation for VC were 50, 43 and 66% of the VC-only control (Figure 2.1). As initial aqueous concentrations of VF were increased, and the initial aqueous VC concentration was held constant, inhibition of VF transformation by VC was overcome and rates of VF cotransformation by EE13a and JS60 increased to rates comparable to those achieved when VC was not present, indicating inhibition was competitive for these two organisms (Figure 2.1A). In contrast, although cotransformation rates of VF by JS614 also increased with VF concentration, the maximum cotransformation rate was only 57% of that achieved when VF was the sole substrate (Figure 2.1A), and a slightly higher rate of residual VC cotransformation was retained (Figure 2.1B).

The independently determined $K_{s/c}$ and $k_{max}$ in Table 2.2 could be utilized to model temporal responses when VF and VC were both present using a competitive inhibition model, but values for $K_i$ were unknown. The initial assumption was made that $K_i$ was equal to the measured $K_{s/c}$ values. This assumption was a good approximation for VC and 1,1-CFE in a reductive system where they inhibited each other (52), but in an aerobic mixed culture of butane-utilizing bacteria the $K_i$ equals $K_s$ assumption was not always valid when modeling concurrent utilization of growth substrate and transformation of nongrowth substrates 1,1,1-trichloroethane, 1,1-dichloroethane and 1,1-dichloroethene (42).

Competitive inhibition was modeled for the specific case where the initial aqueous concentrations of VC and VF were equal (~20μM) and $X$, $k_{max}$ and $K_{s/c}$ were held constant, and $K_i$ was heuristically varied from the measured $K_{s/c}$ value to provide better fits to the experimental data (Results are presented in Appendix, Table S1, Figure S1). Making changes in $K_i$ improved the SSE for all three isolates by an order
of magnitude. These kinetic parameters also successfully modeled competitive inhibition as the VF concentrations increased (Appendix Figure S1). Because the VF cotransformation rates of JS614 showed a lesser response to increases in VF concentration in the presence of VC, experimental data were also modeled with uncompetitive and noncompetitive models. These models produced SSE an order of magnitude greater than the competitive inhibition model, and for the uncompetitive and noncompetitive models to approach a fit equal to the competitive inhibition model a 10-fold increase in $k_{\text{max}}$ was required (data not shown).

In a separate experiment we also determined if it was possible to monitor VC transformation by modeling the rates of $F^-$ accumulation. During cotransformation, the initial rates of halide release matched that of substrate transformed for each VC-degrading isolate (Table 2.3), and in separate experiments where the degradation of individual substrates was followed, halide release ceased as soon as substrate transformation was complete. This demonstrated that there was no further halide release from halogenated products that might have been formed during cometabolism (data not shown). For VC, mass balance of the substrate transformed and halide released were nearly stoichiometric regardless of whether it was a growth or nongrowth substrate and averaged 0.98 ($\pm$0.11) on a mole fraction basis. There was a trend for stoichiometric release of $F^-$ during transformation of VF by JS614 (1.0 $\pm$0.16 mole percent), while $F^-$ released during cometabolic transformation of VF by EE13a and JS60 averaged 0.84 ($\pm$0.04) mole percent. Competitive inhibition between substrates, halide release rates equivalent to substrate transformation rates, a known mole fraction of halide released, and previously determined $X$, $k_{\text{max}}$ and $K_{x/c}$ values were incorporated into the model. This model accurately estimated the accumulation of halide in the batch reactors using heuristically fit $K_i$ values (Table 2.2, Figure 2.2). $K_i$ values can deviate between experiments, as they did in our case (41). In the case of EE13a, $K_i$ values for VF and VC, neither of which are growth substrates, were the same in both the competition experiment (Appendix, Table S1, Figure S1) and the
halide release experiment (Figure 2.2), but this was not the case for JS60 and JS614, which can utilize one or both VC and VF as growth substrates.
DISSCUSSION

To our knowledge JS614 is the first microorganism reported to grow aerobically on VF. Similar growth yields for JS614 when utilizing VF or VC, along with similar kinetics of utilization suggest that the same metabolic pathway is utilized for both substrates. It will be interesting to determine what steps in the metabolism of VC by JS60 differ from those in JS614 and thereby prevent growth of the former on VF. The proposed pathway for VC metabolism by JS614 and JS60 show Cl⁻ release as a spontaneous reaction (18, 45); but it is unknown if F⁻ release would utilize the same mechanism as Cl⁻ release. Differences between JS614 and JS60 have previously been noted; Y is much greater for JS614 than for JS60 when VC is the sole carbon and energy source (16) (Table 2.1). That observation, plus new observations from this work, (i) the ability of JS614 to grow on VF with a yield similar to that for VC, and (ii) different response to competing substrates (Figure 2.1), indicate that JS614’s VC metabolic pathway may differ from JS60’s.

For the three aerobic VC-degrading isolates studied, both the rates of VF transformation and F⁻ accumulation could be correlated with the rate of aerobic degradation of VC. VF therefore has the potential to be used as a surrogate reactive tracer for estimating rates of VC degradation in situ. In a VC contaminated aquifer, for example, in single-well push-pull tests, VF addition and subsequent cotransformation could be utilized as an indicator of in situ VC transformation rates. Although fluorinated surrogates must be considered as toxic as their chlorinated analogues (48) they have been previously utilized in-situ in reductive dechlorinating systems to estimate rates of VC transformation with the expectation that VF would accumulate (24, 36). In single well push-pull tests, rates of transformation of the analogue for VC under dechlorinating conditions, E-CFE, has been used to estimate rates of VC transformation (24). Because the reduction of E-CFE results in VF, its formation and subsequent transformation and accumulation of F⁻ could be exploited to determine if sequential anaerobic/aerobic processes are transforming TCE and its daughter products to non-toxic end products. As far as is known, VF is not
transformed in anaerobic systems (52) and the accumulation of F⁻ would be a potential indicator that VC is being degraded aerobically.

During injection for a push-pull test, a solution with a low concentration of VF would mix with native ground water and VC and VF may both be present. This would require that competitive inhibition of the two substrates be taken into consideration when estimating rates of VC transformation. The average inhibition of rates of VF transformation at equal VC and VF concentrations was 53 (±14)% (Figure 2.1). When this average is applied to the F⁻ release data in Table 2.3, estimates of VC transformation rates are 113, 99 and 88% of $k_{\text{max}}$ values reported in Table 2.2 for EE13a, JS60, and JS614 respectively. Furthermore, there is the potential for the detection of very low rates of F⁻ release. The ion chromatographic method used in this work had a detection limit of approximately 7 μM, but detection of F⁻ with an ion selective electrode has been reported in the range of 0.01 μM (61).

VF was transformed by VC-degrading bacteria with a significant and measurable release of halide. F⁻ release during aerobic transformation has been reported from fluorophenols by an activated sludge, fluorosulfonates by *Pseudomonas* sp. strain D2, and fluorinated anilines by *Pseudomonas fluorescens* 26-K (11, 40, 61). F⁻ release has also been observed during the aerobic cometabolism of a variety of chlorofluorocarbons by the methanotroph *Methylosinus trichosporium* OB3b and the propane utilizing *Mycobacterium vaccae* JOB5 (59). Ours is the first report of release of F⁻ release during aerobic VF transformation by VC-degrading bacteria. The release of F⁻ during the cometabolic transformation of VF by EE13a and JS60 implies that an unstable epoxide was formed during the initial transformation by a bacterial monooxygenase and is consistent with the observations made during transformation by Cytochrome P450-dependent monooxygenases (10, 19).

Aerobic VC-degrading bacteria like those in this study with high affinity for VC ($K_{s/c} \sim 1$ μM) and high transformation capacities can be important in the remediation of VC contaminated aquifers. Ethene formed during the slow cometabolic reductive dechlorination of VC could support VC-utilizing bacteria in the aerobic fringe of the
anaerobic zone. The complementing limitations and capabilities of anaerobic (higher affinity for TCE and cDCE) and aerobic (higher affinity for VC) microorganisms illustrate the importance of understanding both processes in the detoxification of chlorinated solvent contamination.
ACKNOWLEDGMENTS

We wish to thank Dr. Jim Spain for providing the JS60 and JS614 cultures, Mohammad Azizian for valuable analytical support, and Stanford C. Taylor for laboratory help. Funding was provided by the NSF-IGERT program, and by a research grant from the U.S. Environmental Protection Agency-sponsored Western Region Hazardous Substance Research Center under agreement R-828772. This article has not been reviewed by the agency, and no official endorsement should be inferred.
Table 2.1. Generation times, growth yields ($Y$) at 29°C, and transformation capacities ($T_c$) for the VC-degrading bacteria when Eth, VC or VF was provided as the sole carbon and energy source. Substrates that did not support growth are indicated by ‘no growth’ and there are no values for generation time or yield. $T_c$ represents cometabolic transformation capacity and there are no values for growth substrates. Standard deviations of three replicates are in parentheses.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Substrate</th>
<th>Generation time (h)</th>
<th>$Y_{g protein}$/g biomass</th>
<th>$Y_{g biomass COD}$/μmol/mol substrate</th>
<th>$Y_{g substrate COD}$/mg dry weight</th>
<th>$T_c$ (μmol/mg dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE13a</td>
<td>Eth</td>
<td>18.3 (2.9)</td>
<td>7.4 (0.5)</td>
<td>0.22</td>
<td>-</td>
<td>43.3 (1.5)</td>
</tr>
<tr>
<td></td>
<td>VC</td>
<td>no growth</td>
<td>-</td>
<td>-</td>
<td>40.7 (2.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VF</td>
<td>no growth</td>
<td>-</td>
<td>-</td>
<td>36.6 (3.0)</td>
<td></td>
</tr>
<tr>
<td>JS60</td>
<td>Eth</td>
<td>15.6 (0.5)</td>
<td>9.1 (1.3)</td>
<td>0.27</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VC</td>
<td>27.4 (0.5)</td>
<td>4.3 (0.3)</td>
<td>0.14</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VF</td>
<td>no growth</td>
<td>-</td>
<td>-</td>
<td>36.6 (3.0)</td>
<td></td>
</tr>
<tr>
<td>JS614</td>
<td>Eth</td>
<td>17.8 (2.3)</td>
<td>10.3 (1.8)</td>
<td>0.30</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VC</td>
<td>17.5 (0.9)</td>
<td>7.3 (0.2)</td>
<td>0.24</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VF</td>
<td>18.5 (0.5)</td>
<td>6.2 (0.5)</td>
<td>0.20</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2. Kinetic parameter estimates, $k_{\text{max}}$, $K_s$ or $K_c$, obtained from progress curves of three Eth-grown isolates degrading Eth, VF or VC during short-term experiments. The estimates were obtained using a non-linear least squares method (57). The standard deviations of three replicates are in parentheses. $K_i$ values gave the best fit for the experimental data in Figure 2.2. ND= not determined.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Substrate</th>
<th>$k_{\text{max}}$ (nmol/(min-mg protein))</th>
<th>$K_{s/c}$ (μM)</th>
<th>$K_i$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE13a</td>
<td>Eth</td>
<td>31.1 (0.8)</td>
<td>1.5 (0.1)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VF</td>
<td>34.9 (9.4)</td>
<td>1.4 (0.8)</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>VC</td>
<td>30.0 (11.6)</td>
<td>0.8 (0.3)</td>
<td>2.9</td>
</tr>
<tr>
<td>JS60</td>
<td>Eth</td>
<td>23.8 (2.8)</td>
<td>1.0 (0.4)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VF</td>
<td>38.2 (6.6)</td>
<td>1.3 (0.4)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>VC</td>
<td>36.0 (6.1)</td>
<td>0.9 (0.4)</td>
<td>0.1</td>
</tr>
<tr>
<td>JS614</td>
<td>Eth</td>
<td>41.1 (2.6)</td>
<td>1.4 (0.2)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VF</td>
<td>31.4 (6.8)</td>
<td>1.0 (0.6)</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>VC</td>
<td>29.8 (2.6)</td>
<td>1.4 (0.1)</td>
<td>3.5</td>
</tr>
</tbody>
</table>
Table 2.3. Rates of cotransformation of VF and VC and rates of F⁻ and Cl⁻ release in batch reactors at equal starting aqueous concentrations of VF and VC (~20μM). The standard deviations of three replicates are in parentheses.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Substrate</th>
<th>Transformation Rate</th>
<th>Halide Release Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nmol/(min-mg protein)</td>
<td></td>
</tr>
<tr>
<td>EE13a</td>
<td>VF</td>
<td>22.3 (0.1)</td>
<td>18.0 (1.3)</td>
</tr>
<tr>
<td></td>
<td>VC</td>
<td>9.6 (0.3)</td>
<td>8.2 (1.8)</td>
</tr>
<tr>
<td>JS60</td>
<td>VF</td>
<td>22.0 (3.3)</td>
<td>19.0 (2.9)</td>
</tr>
<tr>
<td></td>
<td>VC</td>
<td>6.4 (0.7)</td>
<td>6.9 (0.3)</td>
</tr>
<tr>
<td>JS614</td>
<td>VF</td>
<td>11.6 (1.5)</td>
<td>13.9 (1.2)</td>
</tr>
<tr>
<td></td>
<td>VC</td>
<td>18.2 (0.2)</td>
<td>20.6 (3.6)</td>
</tr>
</tbody>
</table>
**Figure 2.1.** Rates of cotransformation by VC-degrading isolates during competition experiments where VC and VF were in the same batch reactor. VC aqueous concentrations were initially at ~20μM and VF aqueous concentrations initially set at 0, and ~20, 40, 60 and 80μM. A) Response of the VF cotransformation rate to increasing VF concentration and fixed VC initial concentration (~20μM). Maximum rates of VF transformation in the absence of VC were determined at 20μM and were 40, 41 and 35nmol/min/mg protein for EE13a, JS60 and JS614 respectively. B) Response of the VC cotransformation rate to increasing VF concentration and VC concentration (~20μM).
Figure 2.2. VC and VF transformation and Cl\(^-\) and F\(^-\) release (symbols) for each of the VC-degrading isolates. Error bars represent the standard deviation of three replicates. The solid lines represent the competitive inhibition modeling of substrate transformation and halide release.
Chapter 3

Extending the alkene substrate range of VC-utilizing *Nocardioides* JS614 with ethene oxide: insights from a combination of molecular, kinetic, and toxicity studies

Anne E. Taylor, Daniel J. Arp, Peter J. Bottomley, and Lewis Semprini

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ABSTRACT

*Nocardioides* JS614 has been reported to grow on the C₂ alkenes ethene (Eth), vinyl chloride (VC), and vinyl fluoride (VF). The addition of ethene oxide (EtO) to an acetate-grown inoculum extended the growth substrate range to the C₃ and C₄ alkenes, propene (Prop) and butene (But). Propene oxide (PrO) and butene oxide (BuO) however, had no growth inducing effects. Although these data indicate that the alkene metabolizing pathway in JS614 might have a specific requirement for EtO as a pathway inducer, both Prop and PrO were found to be just as effective as Eth and EtO in inducing expression of genes encoding the alkene monooxygenase alpha subunit (AkMO) and epoxynethane CoM transferase (EaCoMT). The activities of AkMO, EaCoMT, and a PrO-dependent carboxylase were also detected in EtO and PrO induced cells, with the first two enzymes being more active in EtO than in PrO induced cells, while the rates of EtO transformation were induced to similar levels by both epoxides. Although Prop was consumed by Eth-grown JS614 at about one half the rate of Eth, PrO was consumed at < one quarter of the rate it was generated, and accumulated in spent growth medium to ~0.9 mM during EtO induced growth on Prop. Despite both EtO and PrO being inducers and key intermediates in the alkene metabolic pathway of JS614, they exerted pronounced inhibitory effects on growth of JS614 on acetate. The duration of inhibition was concentration dependent, and the inhibitory effect of PrO was sustained for a longer time than the equivalent amount of EtO, presumably because PrO was transformed by the acetate-grown cells more slowly than EtO. In both cases, acetate-dependent growth commenced after the epoxides were transformed. The apparent limited alkene substrate range, poor growth of JS614 on Prop, and the stimulatory effect of EtO seem to be caused by a combination of effects that include: (a) a narrow substrate range of EaCoMT activity that ineffectively consumes ≥C₃ epoxides, which results in epoxide accumulation to growth inhibitory levels; (b) a reduced net reductant gain from Prop catabolism caused by reductant consumption by the broad substrate range AkMO and the CoM
carboxylase required for metabolism of \( \geq C3 \) alkenes, compounded by reduced reductant gain caused by the bottle neck where only a fraction of the PrO produced from Prop makes its way into downstream metabolism. (c) The incomplete inhibition of EtO transformation by PrO, allowing some EtO transformation to occur in addition to PrO transformation, giving a better overall flow of epoxides through EaCoMT and greater net reductant production.
INTRODUCTION

Bacteria that utilize short-chain alkenes as carbon and energy sources are phenotypically diverse, but have characteristics in common in their alkene metabolism. The oxidation of alkenes is initiated by a soluble diiron alkene monooxygenase (AkMO), resulting in an epoxide which is stabilized by the addition of cofactor M (CoM) by action of epoxy-alkane cofactor M transferase (EaCoMT) (45). The alkene metabolic pathway has been described in detail in the propene (Prop) utilizing bacterium, *Xanthobacter autotrophicus* Py2, where the stabilized epoxide intermediate is carboxylated, eventually yielding acetoacetate (26). Although alkene-utilizing bacteria collectively use a range of different chain length alkenes as growth substrates, it has also been found that the substrate range of individual strains is often quite limited, with many ethene (Eth)-utilizing bacteria, in particular, only growing on Eth (62). Because monooxygenases are usually quite non-specific for alkenes, and will oxidize substrates besides those that will support growth, AkMO is probably not the limiting factor in the range of alkenes used as growth substrates (60, 62). Indeed, it was observed 20y ago that alkene utilizing bacteria accumulate alkene epoxides when oxidizing nongrowth alkenes, suggesting that lack of, or narrow substrate range of critical downstream enzymes, might be important factors (62). Other possible reasons for a limited growth substrate range include inability of the alkene substrates to induce the alkene metabolic pathway (25), or product toxicity. In the latter case, however, a general dogma exists that alkene utilizing bacteria are insensitive to the epoxide products of alkene oxidation, despite their well known antimicrobial activities (62).

*Nocardioides* JS614 utilizes the C2 alkenes Eth, vinyl chloride (VC) and vinyl fluoride (VF) as growth substrates (16, 60). Peptide mass fingerprinting of JS614 identified seven proteins expressed in response to incubation with Eth, VC and ethene oxide (EtO) that were not present in acetate grown cells (15). Among these were proteins with high amino acid similarities to essential enzymes in the Prop metabolic pathway of *X. autotrophicus* Py2, including two enantiomeric specific short-chain
dehydrogenases (SDR, Noc4814 and 4841) with similarities to the *X. autotrophicus* Py2 SDRs of 46 and 47%, respectively, and a FAD-dependent pyridine nucleotide-disulfide oxidoreductase (Noc4827) with high amino acid sequence similarity (53%) to the NADPH:2-ketopropyl-CoM carboxylase oxidoreductase of *X. autotrophicus* Py2. The latter enzyme was characterized in *X. autotrophicus* Py2 and shown to play a critical role during growth on ≥C3 alkenes (27). Although the latter observation suggests that JS614 may be able to utilize Prop and butene (But) as growth substrates, we could not induce acetate-grown JS614 to grow on Prop or But as sole energy and carbon sources. Previously, it had been demonstrated that supplemental EtO accelerated Eth-dependent growth of JS614 when coming out of a repressed state (44). Therefore, we explored the possibility that EtO might extend the growth range of JS614 to Prop and But. When preliminary data were obtained showing that EtO would promote growth of JS614 on Prop and But, objectives were formulated to identify the problems associated with inducing growth on Prop, and to determine the role of EtO in promoting growth.
MATERIALS AND METHODS

Chemicals and medium. Butene (99+%), Prop (99%), propyne (97%), butene oxide (BuO, 99+%), ethene oxide (99.5%), and sodium 2-mercaptoethanesulfonate (CoM) were obtained from Sigma Aldrich, (Milwaukee, WI). Eth (C.P. grade) was purchased from Airgas (Radnor, PA), 2-bromoethanesulfonic acid, sodium salt (BES, 98%) and propene oxide (PrO, 99%) were purchased from Alfa Aesar (Ward Hill, MA). Cells were grown in minimal salts medium (MSM) with either acetate or Eth as the growth substrate unless stated otherwise (60). One-tenth-strength tryptic soy agar (TSA) was used as a non-selective medium and contained 3g Bacto tryptic soy powder (Difco), 10g glucose and 15g agar per liter.

Cultures and maintenance. Nocardioides sp. strain JS614 utilizes Eth and VC as sole carbon and energy sources and was provided by Dr. Jim Spain (Georgia Tech University, Atlanta, GA). Cultures were routinely grown in 500 ml glass Wheaton bottles sealed with phenolic caps and gray butyl rubber septa, in 100 ml MSM and 4% (vol/vol) headspace Eth. Acetate grown cultures were grown in 500ml Erlenmeyer flasks with 100ml MSM and 20mM sodium acetate. Cultures were shaken at 120 RPM in a Brunswick controlled environment shaker at 29±1°C. Growth was followed by monitoring the increase in optical density (OD) at 600nm.

Growth experiments. Alkenes. Acetate grown cells were harvested at mid-log phase and concentrated by centrifugation. Pelleted cells were washed and resuspended in 50 ml MSM to an OD of 0.05 – 0.13 in 150ml bottles with phenolic caps and gray butyl rubber septa. Gaseous growth substrates (5ml, ~0.2 mMoles) were injected and cultures were shaken at 120 RPM in a Brunswick controlled environment shaker at 29±1°C. When required, supplemental epoxides were added as indicated for specific experiments. Growth was routinely followed by monitoring the increase in OD at 600nm. Potassium hydroxide (KOH) traps were utilized in growth experiments to evaluate CO2 effects on growth. These consisted of a central well containing KOH pellets to trap ambient and respired CO2. Acetate. Acetate grown cells were harvested at mid-log phase and back transferred into 50ml MSM at OD<0.01 in 150ml
bottles. Sodium acetate (15mM) was provided as growth substrate and supplemental epoxides were supplied as indicated.

**Gene expression.** Genomic DNA for primer validation and quantitative PCR (q-PCR) calibration was extracted from JS614 after a lysozyme treatment using the DNeasy® Tissue Kit (Quiagen) following the manufacturer’s protocol. Total RNA was extracted after an initial incubation with lysozyme. Lysed cells were treated with Trizol (Invitrogen), and total RNA extracted using the RNeasy® Mini Kit (Quiagen), including an optional on-column DNase digest using the RNase-Free DNase Set (Quiagen). cDNA was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad). DNA, RNA and cDNA were quantified with a NanoDrop ND-1000 UV-Vis spectrophotometer. q-PCR was performed on an iCycler iQ single color Real-Time PCR detection system with iQ SYBER Green Supermix (Bio-Rad). Primers for q-PCR were designed using Primer3 software through Biocomputing and Bioinformatics of the Center for Genome Research and Biocomputing (CGRB) at Oregon State University (Table 3.1).

**PrO dependent 14CO2 incorporation.** PrO-dependent carboxylase activity was assayed essentially as described in Boyd et. al. (8). Acetate grown cells were incubated with either 440µM EtO, or PrO for 24h, centrifuged, washed twice, and resuspended in 50mM phosphate buffer, pH 7.0 to ~2µg protein ml⁻¹. Reaction mixtures were constructed in 10ml vials in a total volume of 1.5 ml that contained 50mM phosphate buffer, pH 6.8, (1.2 ml), 5mM CoM (0.1ml), 0.3 µCi 14C in 50 mM NaHCO₃ (0.1ml, 0.06 µCi umol⁻¹). PrO (1.8µmol) was added to the vials to evaluate the PrO-dependent 14CO₂ incorporation. Control vials did not receive a PrO amendment. An aliquot of a suspension of cells (~1mg protein) was added to initiate the assay, and vials were incubated with shaking at 150 RPM at room temperature, 23±2°C. After a 2h incubation, an aliquot of cells (0.7ml) was withdrawn from each vial, pelleted by microcentrifugation, and washed twice with ice-cold 50mM NaHCO₃ in 50mM phosphate buffer, pH 6.8. The cell pellets were resuspended in 0.3ml Econolume scintillation fluid (ICN) and added to 3.5ml of Econolume in a scintillation
vial. Vials were left on the bench overnight to complete cell lysis, and $^{14}$C incorporation into cells was measured on a Beckman 5600 multi-purpose scintillation counter.

**Determination of kinetic parameters.** The generation time was determined by monitoring the optical density at 600nm. Kinetic parameters including $k_{\text{max}}$, (maximum specific substrate utilization rate, nmol/(min-mg protein)), and $K_s$ (the half-velocity coefficient of the substrate, $\mu$M), of alkene and epoxide consumption were obtained from batch kinetic tests using Eth-grown cells. Cells were harvested at mid-log phase by centrifugation, and washed twice in 4mM potassium phosphate buffer (pH 7.0). Cell pellets were held at room temperature (<15 min) and resuspended phosphate buffer immediately to a dense concentration (20 mg protein/ml) before initiating an experiment. Eth and Prop batch kinetic tests were performed in 155ml sealed serum bottles containing 100ml of 4mM phosphate buffer (pH 7.0). EtO and PrO consumption rates were determined in sealed 10ml vials containing 1ml of phosphate buffer. Specific amounts of alkenes or epoxides were added to achieve desired aqueous concentrations based on Henry’s Law, and were incubated at 23±2°C with shaking at 150 RPM to achieve phase equilibrium. Henry’s constants of 7.64, 8.0, 0.006, and 0.0028 were used for Eth, Prop, EtO and PrO, respectively. The initial concentrations of substrates were significantly greater than $K_s$ values. Experiments were initiated by adding 100 $\mu$L aliquots of cell suspension (~2mg protein) to the bottles or vials, and transformation was followed to completion. To evaluate if AkMO was contributing to epoxide consumption under these assay conditions, incubations were also conducted in the presence of 1% propyne to inactivate AkMO. The difference between the rates of consumption in the presence and absence of propyne were assumed to be due to AkMO dependent epoxide consumption. Consumption progress curves were fitted using a weighted nonlinear least-squares analysis of the integrated Monod equation to estimate the kinetic parameters $k_{\text{max}}$, and $K_s$ (57). All experiments were carried out in triplicate and $k_{\text{max}}$ and $K_s$ were estimated from each progress curve and then averaged. Abiotic controls showed minimal losses of substrate
over the time course of the experiments. The possibility of mass transfer limitations between the aqueous and gas phases were evaluated as described previously (60), and shown to be minimal.

**Analytical Methods.** A microbiuret assay was used to determine the protein content of cells used in kinetic tests (31). Progress curves of Eth, Prop, EtO and PrO consumption were constructed by analyzing headspace samples using gas chromatography (GC) with flame-ionization detection (FID). A Shimadzu (Kyoto, Japan) GC-8A gas chromatograph fitted with an Alltech (Deerfield, IL) 4ft PorapakQ column was used. The FID and injector temperatures were adjusted to 230°C, and the column to 165°C. The method was calibrated using external standards. The total mass of substrate remaining during batch reactions was calculated from the headspace concentration in using Henry’s Law and the liquid and gas volumes. To determine the aqueous concentrations of epoxides, the samples were processed in a HP 7695 purge-and-trap system equipped with a Vocarb-3000 trap as described previously (4).

**Cell viability.** To evaluate the effect of epoxides on cell viability, acetate-grown cells were incubated with 15mM acetate, 180µM EtO or PrO for 24h and increase in OD monitored. Cells were harvested by centrifugation, washed twice with phosphate buffer and resuspended to OD 0.1 (1 x 10^8 cells/ml). Serial dilutions were made to achieve desired cell concentrations (1 x 10^4 cells/ml) and 25µl spread on tryptic soy plus glucose plates, in triplicate. Plates were incubated at room temperature (25°C±2) for 5 - 7 days and then colonies counted, and normalized to OD of inoculating suspension.
RESULTS

An inoculum of acetate-grown JS614 commenced growth after a long lag (~100h) upon transfer to MSM containing Eth as the sole carbon and energy source (Figure 3.1a). In contrast, no growth was observed when acetate-grown cells were transferred into MSM containing Prop or But as carbon sources (Figures 3.1b and c). As observed previously, supplemental EtO significantly shortened the lag phase for growth on Eth, at concentrations as low as 25 $\mu$M. Supplemental EtO also promoted growth of JS614 on Prop and But (Figure 3.1b and c), but, in contrast to Eth, significantly higher concentrations of EtO were required to maximize growth on Prop (440 $\mu$M) and But (880 $\mu$M). Furthermore, the properties of EtO-induced growth on Prop and But differed from growth on Eth. Whereas growth of JS614 on Eth became exponential for a period of time with a generation time of ~17h, growth on Prop was slow, and never achieved an exponential phase. Growth on But only occurred for a short period of time (~50h), and the final cell density was lower than achieved on either Eth or Prop. To determine if the stimulatory effect of supplemental EtO could be produced by the epoxide derivatives of Prop and But, acetate grown JS614 was incubated with a combination of either But or Prop and a range of concentrations (25-600 $\mu$M) of either PrO or BuO (data not shown). No growth was observed at any epoxide concentration, suggesting that EtO might have unique growth inducing properties.

The expression of mRNA transcripts for the alpha subunit of AkMO and of EaCoMT were compared after acetate grown cells were incubated for 2-8h in the presence of initial aqueous concentrations (C_w) of either Prop or Eth (240 $\mu$M), PrO or EtO (180 $\mu$M) (Table 3.2). Whereas mRNA transcripts of AkMO and EaCoMT were undetected in acetate-grown cells, after 2h of incubation in the presence of EtO or PrO, AkMO and EaCoMT were both induced and expressed at levels that were somewhat higher than the expression found in Eth and Prop treatments. By 8h,
however, all compounds had promoted gene expression to levels similar to those found in Eth-grown cells (1.8% and 1.1% for *AkMO* and *EaCoMT*, respectively).

Since the Prop/But growth phenotype was not correlated with the inability of JS614 to induce *AkMO* or *EaCoMT* expression in response to substrate and/or product presence, the corresponding enzyme activities were measured. In addition to AkMO and EaCoMT activities PrO-dependent CO$_2$ incorporation was measured, since growth at the expense of Prop was CO$_2$ dependent and BES sensitive, while growth on Eth was not (Figure 3.2). Eth and EtO consumption, and PrO dependent CO$_2$ incorporation were not detected in cells incubated in the absence of Prop and EtO or PrO. Enzyme activities were detected in cells exposed to Prop plus EtO or PrO for 24h, with EtO-exposed cells expressing higher levels of Eth consumption and PrO-dependent carboxylase activities than PrO exposed cells (Figure 3.3). EaCoMT activities were similar in both treatments. These data show that active enzymes critical for metabolism and growth on Prop can be expressed in response to the presence of Prop/PrO.

The kinetics of Prop, Eth, EtO and PrO consumption were compared in Eth-grown cells to determine if poor growth on Prop relative to Eth might be due to kinetic differences in the rates of alkene and epoxide consumption (Table 3.3). The maximum rate of Prop consumption was about one half that of Eth, and AkMO had similar affinities ($K_s$) for Prop and Eth. In contrast, both the rate of PrO consumption and the affinity for PrO were lower than for EtO, suggesting that PrO was a poorer substrate for EaCoMT than EtO. EtO transformation in propyne treated (AkMO inactivated) cells was no different than untreated cells, but PrO transformation was only 57% of the rate of untreated cells, indicating that PrO can be transformed by AkMO, at least in the absence of Prop. Furthermore, while the rates of consumption of Eth and EtO are approximately the same, the rate of PrO consumption is about 40% that of Prop, and after correcting for propyne sensitive PrO consumption, the rate is reduced to 24%.
The difference between the rates of Prop and PrO consumption indicated that PrO would likely accumulate during growth on Prop and experiments were performed to test this possibility. Acetate-grown cells were washed and resuspended in MSM with Prop and 440µM EtO. Growth became apparent in the Prop with EtO treatments after 48h, while no OD increase occurred in the Prop only treatment (Figure 3.4a). EtO was completely consumed by 48h in the Prop with EtO treatment, and measurable Prop transformation began at the same time (Figure 3.4b). PrO accumulated (up to 900µM) in the Prop with EtO treatment until the Prop was essentially depleted (Figure 3.4b). The calculated rates of Prop transformation and PrO accumulation (before 200h) were about 20.7 and 14.4 nmoles/(min-mg protein) which was similar to the PrO consumption rate reported in Table 3.3. After Prop had been consumed, the rate of PrO transformation increased to 9.7 nmoles/(min-mg protein).

Given the fact that PrO accumulated during Prop dependent growth, the possibility was considered that PrO might be inhibitory to the cells and thereby contribute to the slow growth on Prop. To evaluate this, first the effects of PrO on growth of JS614 on Eth were evaluated (Figure 3.5). Induction of Eth-dependent growth by acetate-grown JS614 was substantially delayed by 100µM PrO, and 180µM completely prevented growth on Eth (Figure 3.5). To evaluate what effects the presence of PrO might have on EtO transformation, competition between the two substrates was evaluated (Figure 3.6). While EaCoMT had greater affinity for EtO than PrO (Table 3.3), PrO inhibited EtO consumption in a concentration-dependent manner. When the EtO:PrO ratio was 5:1, EtO transformation rates fell to ~65% of \( k_{max} \), but the presence of EtO did not change the rate of PrO transformation. Rates of EtO transformation decreased further as PrO concentrations increased, with no change in PrO transformation rates. To evaluate if PrO inhibition was limited to alkene metabolism, JS614 was grown on acetate in the presence of PrO and compared to growth on acetate in the presence of EtO (Figure 3.7a and b). Both EtO and PrO inhibited growth on acetate, with the effects of PrO persisting for a longer time than EtO. This likely resulted from PrO being transformed at a slower rate than EtO under
these experimental conditions (Figure 3.7c). Acetate-dependent growth resumed after the epoxides had been consumed suggesting the inhibition was reversible. Reversibility of inhibition was also evaluated by determining cell viability of JS614 after 24h incubation with 15mM acetate and 180µM EtO or Pro. The numbers of colony-forming cells per ml were similar in acetate control cultures and those exposed to either EtO or PrO (data not shown).
**DISCUSSION**

I hypothesized that the most logical explanation of the EtO effect on substrate range was that Prop and But, and their corresponding epoxide products, are unable to induce expression of the genes in the alkene metabolic pathway. Yet, because the levels of Prop and PrO induced transcription of \textit{AkMO} and \textit{EaCoMT} are as high as those found in Eth-grown cells, the reasons for the inability of JS614 to initiate growth and to grow effectively on Prop must lie elsewhere. For example, if the pathway of Prop utilization in JS614 follows that described in \textit{X. autotrophicus} Py2, there is less net energy gain than occurs during growth on Eth (26) because the first step in Prop transformation to PrO involves reductant dependent oxidation by AkMO, which occurs at least 2x faster than the subsequent transformation of PrO mediated by EaCoMT (Figure 3.3, Table 3.3). Furthermore, if the mechanism of PrO and BuO carboxylation is reductant consuming, as described in \textit{X. autotrophicus} Py2 (26), then additional reductant debt is accrued during Prop and But metabolism, but not in Eth metabolism. Along with measuring PrO dependent CO$_2$ fixation in JS614, we showed that Prop dependent growth is CO$_2$ dependent and is inhibited by BES, a specific inhibitor of CoM carboxylase (8) (Figure 3.2), while growth on Eth is unaffected by both. Although a dogma has existed for many years that epoxides do not accumulate when alkene-utilizing bacteria oxidize alkene substrates that support growth (62), whereas epoxides often accumulate from oxidation of non-growth supporting alkenes (35), to the authors’ knowledge detailed stoichiometric analyses have not yet been carried out. The well studied Prop-utilizing bacterium, \textit{X. autotrophicus} Py2, accumulates EtO almost stoichiometrically with the rate of Eth oxidation and is reported to grow very poorly on Eth, implying that EaCoMT of that organism has a strong preference for C$_3$ over C$_2$ epoxides (62). Interestingly, the situation in JS614 is just the opposite. Further work is needed to elucidate what properties of the EaCoMT of these two bacteria account for such a high degree of chain length specificity.

Another aspect of this work that needs discussion is the requirement for a higher concentration of EtO to induce growth on Prop and But than to stimulate
growth on Eth. The evaluation of the kinetics of EtO and PrO uptake by JS614 provided some insight. PrO was found to be both a poor substrate for EaCoMT relative to EtO (Table 3.3), and an inhibitor of EtO consumption (Figure 3.6). Nonetheless, the total flux of epoxide consumption in the presence of EtO and PrO combinations was greater than by PrO alone. Therefore, it is possible that EtO might serve as a supplementary electron donor during metabolism of Prop and But that partially compensates for the reductant deficiencies described above. The higher EtO concentration requirement is simply to enable it to compete with PrO and BuO. Alternately, it is possible that downstream genes may require higher concentrations of EtO for effective induction and even more so in the presence of competing levels of PrO. Resolution of these issues will require additional experimentation.

Although the acute toxicity of unstable epoxides of halogenated ethenes has been extensively documented as a major factor limiting the bioremediatory potential of alkane oxidizing bacteria (37, 63), little attention has been given to the toxic effects of epoxides on alkene utilizing bacteria. It is well documented that EtO and PrO are effective alkylating agents of DNA and proteins (49, 58), and since both exhibit negative effects on acetate-growing JS614 (Figure 3.7), the inhibitory effects are not necessarily targeted at the alkene metabolic pathway. Furthermore, since JS614 cells maintained their viability after exposure to growth inhibiting concentrations of EtO and PrO, they must be able to repair or replace DNA and proteins and initiate cell growth. Clearly, because EtO and PrO were transformed in the presence of acetate (Figure 3.7C), the epoxide transforming system must be induced by EtO and PrO in the presence of a substrate (acetate) that normally represses the alkene metabolizing system. This observation agrees with previous work by Ensign (25), who showed that Prop oxidation and PrO consumption could be induced in X. autotrophicus Py2 by exposing a glucose-growing culture to Prop. However, in the case of JS614, a point to note is that the activity of EtO consumption was induced to similar levels by EtO and PrO (Figure 3.3), implying that the genes and proteins are to some extent insensitive to damage, even though EtO and PrO seriously inhibit growth until the epoxides are
consumed (Figure 3.7). More work is needed to examine if the alkene metabolizing pathway is involved in the detoxification of the epoxides, and why epoxide consumption is uninhibited while cell growth is substantially slowed.

In summary, the data clearly reveals that JS614 has a broader alkene substrate range than previously reported. Although the alkene degradative pathway is induced by a range of substrates and epoxides, the narrower specificity of EaCoMT relative to AkMO creates a series of negative consequences that include accumulation of epoxides to levels that are growth inhibitory. In addition, the difference in the rates of Prop and PrO transformation, results in a “semi-cometabolic situation” which is particularly important in metabolism of $\geq C_3$ alkenes where an additional reductant-consuming carboxylation step is required.
### Table 3.1. Oligonucleotides used for RT-PCR.

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<th>Expected product size (bp)</th>
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<td>NocR0032</td>
<td>147</td>
<td>this study</td>
</tr>
<tr>
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<td>NocR0032</td>
<td>147</td>
<td>this study</td>
</tr>
<tr>
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<td>CGTCGATCTGAATGATGTCG</td>
<td>Noc4810</td>
<td>150</td>
<td>this study</td>
</tr>
</tbody>
</table>

\(^a\)Gene from the JS614 genome sequence used to design the oligonucleotides.
Figure 3.1. Growth response of JS614 to alkenes +/- supplemental EtO. A) Eth, B) Prop, C) But, D) EtO controls.
Table 3.2. Expression of mRNA transcripts of the first two genes in the alkene metabolic pathway. Acetate grown cells were washed and resuspended in MSM with Eth or Prop ($C_w = 240 \mu M$), EtO or PrO ($C_w = 180 \mu M$) and incubated for 2h. The mRNA transcripts of *AkMO* and *EaCoMT* are expressed as a percentage of the amount of 16s rRNA.

<table>
<thead>
<tr>
<th></th>
<th><em>AkMO</em></th>
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<th><em>EaCoMT</em></th>
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<td></td>
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Figure 3.2. Sensitivity of JS614 to CO₂ deprivation and the CoM analogue bromoethanesulfonate (BES). Growth response of acetate grown JS614 on Prop (A) or Eth (B) plus supplemental EtO to the presence and absence of CO₂. CO₂ was trapped in a well with KOH in the minus CO₂ treatments, while ambient and respired CO₂ were present in the plus CO₂ treatments. Growth response on Prop (C) or Eth (D) in the presence of BES and with ambient and respired CO₂ present. Acetate grown cells were harvested and incubated with alkene, supplemental EtO, and +/- BES as a selective inhibitor of carboxylation.
Figure 3.3. Epoxide-dependent induction of monooxygenase, transferase and PrO-dependent carboxylase activities. Acetate-grown cells were incubated with Prop and EtO or PrO (440µM) for 24h. Cells were harvested and AkMO (A) and EaCoMT (B) activities evaluated by Eth and EtO transformation, respectively, and carboxylase activity (C) by PrO-dependent $^{14}$CO$_2$ incorporation during a 2h incubation.
Table 3.3. Kinetic parameters for substrate utilization by JS614. Eth-grown cells were washed and resuspended in phosphate buffer for short term assays. Propyne treated cells were incubated in the presence of 1% propyne for 1h and then washed twice before determining $k_{max}$. To determine generation times, Eth-grown cells were washed and added to MSM with Eth or Prop ($C_w = 240\mu M$). *$K_s$ for Eto is an upper range estimate due to the lower limits of accurate detection of EtO by gas chromatography. ND=not determined.

<table>
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<th>$k_{max}$</th>
<th>$K_s$</th>
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<tr>
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<td>101.9 (10.8)</td>
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<td>PrO + propyne</td>
<td>4.6 (0.2)</td>
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Figure 3.4. Response of acetate grown JS614 to Prop +/- 440µM EtO. A) Growth response as measured by increase in OD. B) Transformation of EtO and Prop and the accumulation of PrO in the Prop +EtO treatment.
Figure 3.5. Growth response of JS614 on Eth in the presence of PrO. Acetate grown cells were harvested and resuspended in MSM with Eth provided as the growth substrate and supplemental PrO as indicated.
Figure 3.6. Inhibition of EtO transformation by PrO. $V_i/V_{max}$ (ratio of the rate of EtO or PrO transformation observed for indicated experimental conditions/maximum rate of transformation without a competing substrate, Table 3.2).
Figure 3.7. Growth response of JS614 on acetate in the presence and absence of (A) EtO, or (B) PrO, transformation of EtO or PrO in the presence of acetate (C).
Chapter Four

Application of ethene oxide extends the halogenated alkene substrate range of *Nocardioides JS614*

Anne E. Taylor, Daniel J. Arp, Peter J. Bottomley, and Lewis Semprini

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ABSTRACT

An acetate-grown inoculum of Nocardioides JS614 grows readily in a mineral salts medium with either vinyl chloride (VC) or vinyl fluoride (VF) as the sole carbon and energy source, but does not grow solely on vinyl bromide (VB). The addition of ethene oxide (EtO) concentrations as low as 25 \( \mu \)M stimulated growth on VB, yet, 180\( \mu \)M EtO was required to support optimal growth, albeit with a relatively long generation time of 57.5±5.1h, and higher concentrations of EtO (440\( \mu \)M) caused growth inhibition. The addition of EtO did not stimulate growth on 1,1-dichloroethene (1,1-DCE) and 1,2-cis-dichloroethene (cDCE). While the growth rate on VB was significantly slower than on VF and VC, the maximum rate of VB consumption by Eth-grown cells was actually ~50% greater than VC and VF. This indicates that a “bottle neck” in VB metabolism downstream of the alkene monooxygenase (AkMO) was responsible for the slower growth rate on VB.

Assuming that the high turnover of VB implies a high rate of production of VB-epoxide (VBO), EtO consumption still occurred at 30% of its maximum rate in the presence of sufficient VB concentrations to support maximum VB oxidation. Although this circumstantial evidence suggests that VBO is an inhibitor of EtO consumption, the fact that some EtO continued to be consumed during maximum VB oxidation indicates that VBO is not an good substrate for EaCoMT, and that some fraction of the VBO probably degrades spontaneously before being processed by EaCoMT. This phenomenon will place a reductant consumption/production imbalance on the cell and probably contributes to the lower growth rate. The observed transformation of residual EtO in the presence of VB transformation might provide reductant to support growth on VB. VB was much more inhibitory to JS614 grown on acetate than either VF or VC. Some of the growth inhibition by VB was turnover dependent, since propyne treated (AkMO inactivated) cells grew better on acetate in the presence of VB than untreated cells. Poor growth of JS614 on VB was probably the result of a combination of reductant imbalance caused by the bottleneck in epoxide
processing, accompanied by inhibitory effects of VB \textit{per se} and of VB oxidation products, such as VBO.
INTRODUCTION

Traditionally the approach for degradation of the pervasive ground water contaminant vinyl chloride (VC) under aerobic conditions focused on cometabolism. Reasons for this have been two-fold. Limited numbers of organisms were available for evaluation that could metabolize and gain energy from VC, and those that could utilize VC as a growth and energy substrate, did so at a relatively slow rate (38). In contrast, many bacteria that cannot utilize VC as a growth substrate, but have broad-substrate-specificity oxygenases, including toluene, methane, propane and ammonia monooxygenase, cometabolized VC more rapidly than known aerobic VC-utilizers (14, 43, 55, 64). The resulting epoxide, chlorooxirane, is unstable and spontaneously degrades yielding a mixture of acetyl chloride, glycoaldehyde, chloroacetaldehyde and chloride ion (Cl⁻) (5, 6).

Cometabolism of VC is not without its own challenges. Cometabolic systems require a cosubstrate to serve as a growth and energy source supporting the transformation of the contaminant. Delivery of the substrate to subsurface aquifers can be difficult, and supplying a constant source can be cost prohibitive. The cosubstrate often competes with VC for the monooxygenase active site. In the case of VC, the effective affinity in aerobic cometabolic systems can vary widely (0.6 - 50μM) (1, 65), and tends to be above the concentrations found in contaminant plumes (~2μM), often making it likely that VC would not be transformed efficiently. Additionally, the oxidation of VC and the spontaneous degradation of its epoxide inflicts inhibitory or toxic effects, often inactivating the oxygenase (28), and making cometabolism unsustainable even if a cosubstrate is provided to sustain growth.

In recent years more ethene (Eth)- and VC-utilizing bacteria have been isolated and brought into culture and their properties of VC utilization explored (16, 23, 30, 56, 66, 67). It is not known why some Eth-utilizing bacteria cannot utilize VC as a growth and energy substrate, but possibilities include lack of induction of the metabolic pathway or absence of essential proteins to carry out the metabolism. Like other oxygenase-containing bacteria, Eth-utilizing bacteria can transform VC to
chlorooxirane, and in general have higher affinity for VC (<5\mu M) than the previously mentioned non-alkene-growing oxygenase-containing bacteria (16, 60, 67).

The VC-utilizing isolate that has received the most experimental scrutiny is *Nocardioides* JS614 because of its rapid rate of VC transformation and faster growth on VC (16). JS614 exhibits an unusually long lag before growth on alkenes when transferred from growth conditions where alkene metabolism was repressed (44). It has been observed that ethene oxide (EtO), the product of Eth oxidation, shortens this lag, suggesting that product induction might be important (44). In fact, in Chapter Three supplemental EtO was shown to induce JS614 to grow on propene. But the first two enzymes in the metabolic pathway, alkene monooxygenase (AkMO) and epoxy-alkane cofactor M transferase (EaCoMT), were effectively induced by the substrate Prop and its oxidative product, propene oxide (PrO), indicating that the expression of the full metabolic pathway is under a regulation that is currently not understood.

Although the mechanism that allows EtO to induce expression of the alkene metabolic pathway is unknown, we can test the ability of EtO to expand the substrate range of VC-utilizing bacteria for the remediation of groundwater pollutants. If lack of induction is all that prevents some Eth- or VC-utilizing bacteria from transforming other groundwater contaminants, then providing an inducer may promote sustainable transformation. To test this concept, we evaluated the ability of EtO to expand the substrate range of *Nocardioides* JS614 to include the dichloroethenes 1,1, dichloroethene (1,1-DCE) and 1,2-cis-dichloroethene (cDCE), and vinyl bromide (VB), the halogenated analogue of VC. VB and VF are not pervasive environmental contaminants, but evaluating the transformation of other vinyl alkenes might help identify factors that limit substrate range in other Eth-utilizing bacteria.
MATERIALS AND METHODS

Chemicals and medium. VC (99.5%), VB (99%), and propyne (97%) were obtained from Aldrich, (Milwaukee, WI), VF (98%) from SynQuest (Alachua, Fl.). EtO (99.5%), was obtained from Sigma Aldrich, (Milwaukee, WI), and Eth (C.P. grade) was purchased from Airgas (Radnor, PA). Cells were grown in minimal salts medium (MSM) with either acetate or Eth as the growth substrate unless stated otherwise (60). One-tenth-strength tryptic soy agar (TSA) was used as a non-selective medium and contained 3g Bacto tryptic soy powder (Difco), 10g glucose and 15g agar per liter.

Cultures and maintenance. *Nocardioides* sp. strain JS614 utilizes Eth, VC and VF as sole carbon and energy sources and was provided by Dr. Jim Spain (Georgia Tech University, Atlanta, GA). Cultures were routinely grown in 500ml glass Wheaton bottles sealed with phenolic caps and gray butyl rubber septa, in 100ml MSM and 4% (vol/vol) headspace Eth. Acetate-grown cultures were grown in 500ml Erlenmeyer flasks with 100ml MSM and 20mM sodium acetate. Cultures were shaken at 120 RPM in a Brunswick controlled environment shaker at 29±1°C. Growth was followed by monitoring the increase in optical density (OD) at 600nm.

Growth experiments. Alkenes. Acetate-grown cells were harvested at mid-log phase and concentrated by centrifugation. Pelleted cells were washed and resuspended in 50ml MSM to an OD of 0.05 – 0.13 in 150ml bottles with phenolic caps and gray butyl rubber septa. Gaseous growth substrates (5ml, ~0.2 m mol) were injected and cultures were shaken at 120 RPM in a Brunswick controlled environment shaker at 29±1°C. When required, supplemental epoxides were added as indicated for specific experiments. Growth was followed routinely by monitoring the increase in OD at 600nm. Acetate. Acetate-grown cells were sampled at mid-log phase and back transferred into 50ml MSM at OD<0.01 in 150ml bottles, with 15mM sodium acetate and 5ml (~0.2m mol) alkene.

Determination of kinetic parameters. Kinetic parameters of alkene and epoxide consumption were obtained from batch kinetic tests using Eth-grown cells, as previously described (60). Briefly, specific amounts of alkenes or epoxides were
added to achieve desired aqueous concentrations based on Henry’s Law, and were incubated with shaking at 150 RPM at 23±2°C, to achieve phase equilibrium. Dimensionless Henry’s coefficients of 0.006, 7.24, 0.49, 0.99, and 2.66 for EtO, Eth, VB, VC and VF respectively, were determined at 23°C (23, 32, 52). Experiments were initiated by adding 100µL aliquots of cell suspension (~2mg protein) to the bottles. The initial concentrations of substrates were significantly greater than $K_v$ values (half-velocity coefficients for growth, $\mu$M) and transformation was followed to completion. To evaluate if AkMO was contributing to epoxide consumption under these assay conditions, incubations were also set up with cells treated with propyne to inactivate AkMO. Cell suspensions were treated with 1% propyne and 0.5% Eth (to promote turnover), incubated for 1h, then pelleted and washed twice in phosphate buffer before being utilized experimentally. The difference between rates of consumption in the presence and absence of propyne were assumed to be due to AkMO dependent epoxide consumption.

Growth inhibition experiments. To evaluate the effect of halogenated alkenes on cell viability, acetate-grown cells were incubated with 15mM acetate, 5ml (~0.2mMoles) alkene +/-1% propyne for 24h and increase in OD monitored. Cells were harvested and concentrated by centrifugation, washed twice with phosphate buffer and resuspended to OD 0.1 (1 x 10^8 cells/ml). Serial dilutions were made to achieve desired cell concentrations (1 x 10^4 cells/ml) and 25µl was spread on tryptic soy plus glucose plates, in triplicate. Plates were incubated at room temperature (25°C±2) for 5 - 7 days and then colonies counted.

Analytical Methods. A microbiuret assay was used to determine the protein content of cells used in the kinetic tests (31). Progress curves of VB, VC and VF consumption were constructed by analyzing headspace samples by gas chromatography (GC) with flame-ionization detection (FID). A Shimadzu (Kyoto, Japan) GC-8A gas chromatograph fitted with an Alltech (Deerfield, IL) 4ft PorapakQ column was used. The FID and injector temperatures were adjusted to 230°C, and the column to 165°C. The mass of substrate remaining during kinetic tests was calculated from the
concentration in the headspace samples, using Henry’s Law and volumes of gas and liquid phases. The GC method was calibrated with known standards of VB, VC, VF or EtO to produce calibrations curves. Br⁻, Cl⁻ and F⁻ concentrations were determined using a Dionex DX-500 Ion Chromatograph (Sunnyvale, CA) equipped with an electrical conductivity detector and a Dionex AS14 column. The method was calibrated with external standards of KBr, NaCl or NaF as described previously (60).
RESULTS

The ability of JS614 to grow on the halogenated ethenes VB, 1,1-DCE and cDCE with and without EtO was compared with growth characteristics on VC and VF. When provided with VC or VF as a sole carbon and energy source, an inoculum of acetate-grown JS614 grew after a lag of 80-100h (Figure 4.1a and b), and the only effect of supplemental EtO was to shorten the lag phase to ~60h. In contrast, acetate-grown JS614 did not grow on VB as the sole carbon and energy source unless supplemental EtO was provided (Figure 4.1c). In comparison to growth on VC and VF, growth on VB was slow, about 3x longer, with a generation time (doubling of OD) of 101±19h, while generation times on VC and VF were ~37h. Additionally, the OD increase on VB was nearly linear while growth on VC and VF had a period of rapid exponential growth. EtO did not stimulate growth of JS614 on either DCE tested (data not shown).

EtO concentrations as low as 25μM were effective in stimulating growth on VB (data not shown), with 180μM EtO supporting fastest growth with a generation time of 57.5±5.1h and achieving a final OD = 0.22 (Figure 4.2). High concentrations of EtO were detrimental to growth on VB when provided either as one application (400μM), or when 180μM was added at time-zero followed by incremental additions of 100μM at 24h intervals (Figure 4.2).

The putative VB oxidation product, VB epoxide (VBO), is unstable and cannot be directly measured. Therefore, potential for transformation by EaCoMT was indirectly evaluated. VB is a good substrate for AkMO and is rapidly transformed at a rate ~50% greater than either VC or VF (Table 4.1). Affinity ($K_s$) for all three substrates by AkMO is <5μM. In contrast to VC and VF, only a fraction of bromine is released as Br− (0.66), while VC and VF produce almost stoichiometric release of Cl− and F−. One possible interpretation for the incomplete Br− release is that EaCoMT cannot effectively transform all of the VBO that is formed by AkMO and allows some to spontaneously degrade into both Br− and putative brominated organic products. Throughout the assay, the rate of Br− release was constant at ~65% of the rate of VB
transformation (Figure 4.3), indicating that non-stoichiometric release of Br⁻ is probably characteristic of limited transformation by EaCoMT, and not the result of turnover-dependent inactivation of EaCoMT as transformation continues at a constant rate. Organohalide accumulation in the growth media was also considered as a possible cause for poor growth on VB, but a new inoculum of JS614 added to spent growth media (after growth on VB) showed similar growth characteristics as in fresh medium (data not shown).

Another experiment was carried out to indirectly evaluate the effectiveness of VBO transformation by evaluating EtO transformation in the presence of VBO (Table 4.2). Control experiments showed that the rate of VB transformation in the presence of EtO was nearly the same as when EtO was not present, and that the rate of EtO transformation by propyne treated (AkMO inactivated) cells was not different than untreated cells. Both of these data indicate that EtO is not transformed by AkMO to a great extent. When VB was being maximally transformed by AkMO, however, EtO transformation occurred at a fraction (0.30) of its maximum rate. These data imply (a) that VBO produced by AkMO is an inhibitor of EaCoMT, and (b) because EtO is still being consumed during VB turnover it also implies that VBO cannot saturate EaCoMT despite being produced at a rate faster than VC or VF is transformed. The data infer that supplemental EtO will be transformed by EaCoMT during VB turnover and could be generating supplementary reductant for sustaining VB dependent growth and making up for the reductant consumption/production imbalance.

Other data indicate that VB and/or the formation of its epoxide are inhibitory to growth of JS614. This was observed by comparing the toxicity of VC and VB on JS614 cultures growing on acetate (Figure 4.4a). The generation time on acetate alone was 10.2±0.8h. VC was slightly inhibitory to growth on acetate with a generation time of 13.1±0.3h, while VB showed a much greater inhibition of growth on acetate (24.1±4.3h). There was significant transformation of VC and VB during growth on acetate (Figure 4.4b), indicating that AkMO was induced upon exposure to VC or VB. Much more VC was transformed than VB, perhaps because of the greater biomass
produced in the presence of VC, relative to VB. This indicates that slow growth on VB is not just due to inefficient utilization of VBO, but is also due to toxic or inhibitory effects produced by VB or VBO that are much greater than inhibition by VC.

To determine if the inhibitory effect of VB was due to transformation by AkMO, acetate-grown cells of JS614 were incubated with acetate and combinations of either VB, VC, or VF, +/-propyne to prevent transformation by AkMO, and to distinguish between substrate or product toxicity (Table 4.3). Less growth occurred in the VB treatments than either VC or VF treatments, in the presence or absence of propyne. VB cells that were treated with propyne showed a greater increase in OD than untreated cells, indicating that although VB per se was inhibitory to growth, much of the growth inhibition was associated with VB turnover. In contrast, cells incubated with VC or VF had similar changes in OD +/- propyne, indicating that transformation of these substrates did not promote growth inhibition.

We also evaluated the effect of VB, VC and VF toxicity on OD increase of Eth-grown cells already expressing the alkene metabolic pathway. While cultures exposed to VC or VF had OD increases of ~1.5-fold, there was no evidence for growth during a 24h incubation of Eth-grown cells in VB, further demonstrating the toxicity of VB (data not shown).
DISCUSSION

In the previous chapter, evidence was presented that the alkene substrate range of JS614 could be extended to propene and butene if relatively high concentrations of supplemental EtO (440 and 880 µM respectively), were provided in the growth medium. The inability of JS614 to grow on these intermediary chain length alkenes seemed to be due primarily to the inability of EaCoMT to consume the epoxide products propene oxide (PrO) and butene oxide (BuO) at rates equivalent to their rates of formation by AkMO. This resulted in the stable epoxides accumulating to levels that inhibited growth and the cells became reductant depleted. A possible beneficial role of high EtO concentrations was to compete effectively with the alternate epoxides for EaCoMT, and to be processed via downstream metabolism for reductant generation. In this chapter, attempts were made to extend the EtO effect to a range of halogenated ethenes, some of which are priority pollutants, and that generate unstable and reactive epoxide products upon oxidation (6, 7, 34). Success was limited to VB, and its growth response to EtO was different from that seen with propene, in that concentrations of EtO as low as 25 µM could stimulate growth. Furthermore, growth stimulation became growth inhibition when EtO was raised to a concentration that had been previously shown to be effective in supporting propene-dependent growth.

Because the oxidation of halogenated alkenes can yield unstable epoxides that abiotically transform if not quickly utilized (5, 6), serious challenges are faced by bacteria attempting to use them as carbon and energy sources. For example, the second enzyme in the metabolic pathway, EaCoMT, should have high affinity for the halogenated-epoxide and turn it over rapidly before its spontaneous breakdown. Additionally, if monooxygenase products are responsible for the induction of the complete alkene metabolic pathway, an unstable halo-epoxide product of AkMO may not reach a concentration sufficient to maximize pathway induction and optimize alkene degradation. Previous work with JS614 showed that EtO promoted growth on an alkene growth substrate when transferred from growth conditions where the alkene metabolism pathway is repressed, suggesting that epoxide products might be important
in the expression of the complete alkene metabolic pathway (44). This implies that 1) substrates may not induce the complete alkene metabolic pathway, and 2) if epoxides are unstable they might not effectively induce the metabolic pathway. This potentially explains why some Eth-utilizing bacteria cannot utilize VC. In the previous chapter it was shown that the substrates Eth and propene, as well as their epoxide products EtO and PrO, could induce expression of \textit{AkMO} and \textit{EaCoMT}, challenging the hypothesis that alkene metabolism is only product induced. But still, supplemental EtO is required for growth on propene and VB, indicating that either 1) supplemental EtO is an initial energy source to support alkene metabolism because reductant yield on propene and VB is so poor, or 2) that expression of metabolic enzymes downstream of \textit{EaCoMT} have a specific requirement for induction by EtO.

Intriguingly, in this study, VB which cannot stimulate growth of JS614 without supplemental EtO, was found to promote alkene transformation in the presence of 15mM acetate within 24h (Table 4.3), while growth on VC or VF does not begin until after 80h (Figure 4.1). In contrast, earlier work showed that 1mM supplemental acetate delayed transformation of VC rather than promote it (44). The VB transformation evident at 24h in the presence of 15mM acetate was propyne sensitive (Table 4.3) inferring that AkMO was responsible for the transformation. In addition, in the previous chapter EtO and PrO transformation was observed in the presence of 15mM acetate within 24h (Figure 3.7C) indicating that \textit{EaCoMT} was expressed. Previous results indicating that EtO might be essential for the expression of the full metabolic pathway, and evidence that AkMO and EaCoMT can be substrate induced may suggest that the first two enzymes in the metabolic pathway might function in a detoxification role when growth is supported by a growth substrate such as acetate. Further work needs to be done to evaluate if the transformation of VB in the presence of growth supporting amounts of acetate leads to growth enhancement or if it is cometabolic in nature.

Results presented in the previous chapter showed that although the rate of propene oxidation was significantly lower than that of Eth, PrO still accumulated, thus
indicating it was a poor substrate of EaCoMT relative to propene as a substrate for AkMO. In the case of VB, the latter situation is even more exaggerated because the initial rate of VB transformation by AkMO is substantially higher than VC or VF and rivals the transformation rate of Eth. This likely places an even higher initial reductant burden on the cell than for other substrates, particularly if EaCoMT cannot effectively transform all of the VB epoxide before it degrades and potentially reacts with cytoplasmic constituents (6, 7, 34). It is well established that the reactive epoxide products of VB, 2-bromoacetaldehyde, and bromoacetic acid, are not only mutagenic, but will attack and inactivate heme in cytochrome P-450 enzymes (47, 50, 58). Although the instability of VBO prevents the direct measurement of its transformation, circumstantial evidence indicates that VBO transformation via EaCoMT might not be able to keep up with its rate of formation. While the stoichiometric release of Cl\(^-\) and F\(^-\) during VC and VF transformation provides indirect evidence that VC and VF epoxides are efficiently metabolized by JS614, Br\(^-\) release accounted for less bromine than the amount of VB transformed, suggesting that the products of VB transformation and/or fate of VBO differ from VC and VF. Since VB transformation reduced, but did not stop EtO consumption, it is possible that despite the high rate of VBO formation, it could not completely inhibit EaCoMT utilization of EtO. Thus EtO utilization resulted in the generation of reductant, and growth enhancement on VB. This work, and that of others (62), has established that there is a limitation in the substrate range of EaCoMT in alkene-utilizing bacteria as indicated by the slow rate of PrO transformation and its accumulation during growth on propene (Chapter 3), and the near stoichiometric accumulation of EtO during Eth transformation by X. autotrophicus Py2 (62). Therefore, many Eth-utilizing bacteria that are unable to use VC as a growth and energy substrate may have similar inability to effectively deal with the unstable VC-epoxide (17). Additionally, since the energy yielding steps in alkene metabolism are downstream of EaCoMT, the reductant deficit created by the EaCoMT “bottleneck” will lead at best to a poor growth scenario, or at worst, an unsustainable cometabolic transformation.
In the previous chapter the stimulatory effect of EtO on propene growth was explained as possibly due to its use as a supplementary reductant source to compensate for the low rates of PrO or BuO transformation relative to their reductant dependent formation. However, in the case of VB, a negative effect of EtO was manifested above a critical EtO concentration of 180µM, a concentration that was not inhibitory for growth on propene. In the previous chapter it was established that EtO per se had some growth inhibitory properties, and in this work it was shown that VB was also growth inhibitory even when its transformation was inhibited by propyne. VBO inhibits transformation of EtO (Table 4.2), but is unknown how EtO might inhibit VBO transformation. The inhibitory effects of higher EtO concentrations during growth on VB might be due to EtO preventing VBO transformation and subsequent negative effects of VBO abiotic decay. As shown in Table 4.3 the bulk of VB inhibition is due to turnover, while there is no additional VC and VF inhibition due to turnover, presumably because those epoxides are efficiently transformed by EaCoMT. EtO competing for the EaCoMT active site with VBO would only increase the inhibitory effects caused by VB transformation.

The observation that JS614 will utilize VB, grow, and liberate bromide when the pathway is properly induced, might have some practical significance for site remediation. VB is produced industrially for the manufacture of polymers used in flame retardant and in fibers for carpet-backing. VB was identified as one of the recalcitrant daughter products of the biologically mediated anaerobic transformation of tribromoethane (TriBE) in a groundwater plume (51). While dehalogenation of VB in methanogenic reductive microcosm experiments has been reported (33), VB-oxidizing bacteria may prove to be effective in remediating VB in aerobic zones adjacent to reductively debrominating environments.

In Chapter Two VF was demonstrated to be a surrogate indicator of VC transforming activity in aerobic groundwater systems (60). Because Br⁻ has had a long history of use as a nonreactive tracer in groundwater flow studies (24, 36), it would make intuitive sense to evaluate Br⁻ release from VB as a surrogate indicator of in situ
aerobic VC transforming activity. Rates of VF transformation and subsequent \( \text{F}^- \) release can be correlated with VC transformation (60), while \( \text{Br}^- \) release is non-stoichiometric with VB consumption, and the kinetics of VB consumption do not match that of VC utilization, making VF a better choice as a surrogate indicator of VC activity. Additionally, because VB oxidation is more growth inhibitory than VC, a push-pull test with VB that requires a residence time of several days (4) might exert serious inhibitory effects on a native VC-transforming bacterial community.

In summary, poor growth on VB can be attributed to inhibitory effects of VB itself and also its transformation. VBO is a poor substrate for EaCoMT resulting in increased inhibition by VBO, plus poor net reductant generation. Future work should focus on identifying the mechanism by which EtO promotes growth on VB, and how EtO becomes growth inhibitory when present in concentrations high enough to promote growth on propene.
Figure 4.1. Growth response of acetate-grown JS614 inoculum to halogenated alkenes +/-EtO. Acetate-grown JS614 was exposed to VB, VC, VF, 1-1,DCE, and cDCE +/- 800μM supplemental EtO. Growth of JS614 is represented by the change in OD over the EtO only control (Δ OD A_{600}). There was no increase in OD when DCEs were provided as the sole carbon and energy source. Growth response to VC (A), VF (B), and VB (C).
Figure 4.2. Response of JS614 during growth on VB to different concentrations of supplemental EtO. A) Growth response to none, 180, and 440µM initial supplemental EtO. The incremental EtO treatment received an initial 180µM EtO and then 100 µM EtO additions as indicated by arrows at 24, 48, and 80h. B) Corresponding transformation of VB. Loss of VB from controls has been taken into account here.
Table 4.1. Kinetics of halogenated ethene transformation and halide release by JS614. Halide release is expressed as a fraction of halide released/substrate transformed.

<table>
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<tr>
<th>Substrate</th>
<th>$k_{max}$ nmol/(min-mg protein)</th>
<th>$K_s$ μM</th>
<th>Halide release</th>
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<td>VB</td>
<td>46.0 (1.7)</td>
<td>4.2 (0.4)</td>
<td>0.66 (0.02)</td>
</tr>
<tr>
<td>VC</td>
<td>29.8 (2.6)</td>
<td>1.4 (0.1)</td>
<td>0.98 (0.11)</td>
</tr>
<tr>
<td>VF</td>
<td>31.4 (6.8)</td>
<td>1.0 (0.6)</td>
<td>1.0 (0.16)</td>
</tr>
</tbody>
</table>
Figure 4.3. Transformation of VB and release of Br⁻ by Eth-grown cells.
**Table 4.2.** Rates of VB or EtO transformation ($V_i$) in the presence of the other substrate compared to maximum rates ($V_{\text{max}}$). $V_i/V_{\text{max}} =$ Rate of substrate transformation in the presence of indicated molecule (in parenthesis)/maximum rate of substrate transformation. Standard deviation is in parentheses. Initial $C_w \text{ VB} = 85\mu\text{M}$, and $C_w \text{ EtO} = 440\mu\text{M}$.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_i/V_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>VB (+EtO)</td>
<td>0.89 (0.01)</td>
</tr>
<tr>
<td>EtO (+VB) propyne treated cells</td>
<td>0.89 (0.12)</td>
</tr>
<tr>
<td>EtO (+VB)</td>
<td>0.30 (0.14)</td>
</tr>
</tbody>
</table>
Figure 4.4. Growth response of JS614 on acetate in the presence of VC and VB. Acetate-grown cells were transferred into fresh MSM with 15mM acetate and ~200µmoles of alkene. A) Increase in OD. B) Transformation of VC and VB.
Table 4.3. Short-term growth response of JS614 in the presence and absence of halogenated alkene transformation. Change in OD evaluated during 24h incubation of acetate-grown JS614 with acetate and alkene +/- propyne. Acetate-grown cells were inoculated into fresh MSM with 15mM acetate and ~200µmoles halogenated alkene. Initial OD ~0.1. 1% propyne (vol/vol) included in propyne treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ΔOD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-propyne</td>
</tr>
<tr>
<td>Ace +VB</td>
<td>0.18</td>
</tr>
<tr>
<td>Ace +VC</td>
<td>0.38</td>
</tr>
<tr>
<td>Ace +VF</td>
<td>0.42</td>
</tr>
<tr>
<td>Ace</td>
<td>0.51</td>
</tr>
</tbody>
</table>
Chapter Five

Summary

Compared with anaerobic reductive dechlorinating bacteria that use vinyl chloride (VC) as an electron acceptor, relatively little is understood about bacteria that utilize VC as a growth and energy substrate under aerobic conditions. Because VC-utilizing bacteria, which can also grow on ethene (Eth), have high affinity for VC, they possess the potential to gain energy from its degradation at low aqueous concentrations. Thus they are of interest for \textit{in situ} bioremediation of contaminated aquifers. A better understanding of the conditions under which VC-utilizing bacteria are effective is needed. Tools for evaluating VC-transformation ability \textit{in situ} are also required to determining whether intrinsic transformation is occurring and to evaluate the effectiveness of enhanced remediation.

In this thesis three strains of VC-utilizing bacteria were evaluated to determine if vinyl fluoride (VF) would serve as a reactive surrogate to estimate VC-transformation \textit{in situ}. Utilization or transformation of VF yields F\textsuperscript{−}, which would be a unique signal in many aquifers. The rates of VF and VC transformation were similar for all three isolates whether the process was cometabolic or growth-linked. VF transformation and F\textsuperscript{−} accumulation were correlated with VC transformation through a competitive inhibition model. The latter observation has particular engineering significance because the identification of the correct inhibition model allows an estimation of VC transformation rates when both VF and VC are present in a contaminated aquifer.

One of the VC-utilizing isolates, \textit{Nocardioides} JS614, was able to use VF as a sole carbon and energy source, making it the first bacterial isolate demonstrated to grow on this fluorinated compound. Propene, considered to be a cometabolic substrate for JS614, was also found to be a growth substrate (albeit a poor one) for acetate grown cells (not expressing the alkene metabolic pathway) when they were initially supplied with supplemental ethene oxide (EtO), the product of oxidation of
ethene (Eth) by alkene monooxygenase (AkMO). Lack of growth without supplemental EtO was not due to lack of induction of the alkene metabolic pathway since propene/propene oxide (PrO) induced AkMO and epoxyalkane cofactor M transferase (EaCoMT) as well as did Eth/EtO. An examination of the propene growth phenotype revealed that EaCoMT has an effective substrate range that is more limited than that of AkMO, as shown by poor kinetics of PrO transformation and PrO accumulation. Poor growth on propene could be explained by poor net reductant yield and growth inhibition caused by PrO accumulation due to the EaCoMT “bottleneck”, where rates of production of PrO by AkMO are faster than the rate of transformation by EaCoMT.

JS614 also grew (poorly) on vinyl bromide (VB) when provided with initial supplemental EtO. VB was able to induce expression of AkMO and EaCoMT in the presence of acetate, and VB was a good substrate for AkMO, eliminating lack of induction and challenged kinetics of VB transformation as reasons for no/poor growth on VB. VB, VF, and VC oxidations yield unstable epoxides. Indirect evidence suggests that VC- and VF-epoxides are good substrates for EaCoMT, including the stoichiometric release of F⁻ and Cl⁻, and growth rates and yields are comparable to Eth. VB-epoxide appears to be a poorer substrate with less than stoichiometric amounts of Br⁻ release, and inability to completely prevent EtO transformation during maximum VB transformation. Poor growth on VB can largely be attributed to the inability of EaCoMT to efficiently transform VB-epoxide, resulting in growth inhibitory effects due to the reactive epoxide, and poor net reductant gain due to loss of metabolic intermediates before the energy investment from the initial utilization steps is recouped.

In addition to the engineering significance already noted, these finding as a whole have scientific merit because they increase the body of knowledge about VC-utilizing bacteria. Evidence was presented that demonstrated the role of EaCoMT as a potentially limiting factor in substrate range utilization by JS614. The substrate range of the JS614 EaCoMT was sensitive to both carbon-chain length of the alkene
and to the specific halogen size associated with the halogenated ethenes. This observation may explain why some Eth-utilizing bacteria cannot also utilize VC as a growth and energy substrate. This observation is supported by early work with the propene-utilizing bacteria *Xanthobacter autotrophicus* Py2 which can effectively utilize propene as a growth substrate, but grows poorly on Eth while almost stoichiometric amounts of EtO accumulate (62).

Moreover, EtO and PrO, intermediates in Eth and propene metabolism respectively, were shown to have negative effects on alkene-utilizing bacteria. While the antimicrobial properties of EtO and PrO are well known, a dogma has existed for a long time that alkene-utilizing bacteria are somewhat immune to their effects. Although the effects of EtO and PrO were inhibitory to growth on acetate, there was no loss in cell viability and normal growth resumed after EtO or PrO had been transformed. How JS614 is able to mediate the toxicity of EtO or PrO is an interesting scientific question.

The observation that EtO has inhibitory effects on growth of JS614 is particularly intriguing because EtO also seems to play a unique role in shortening the lag phase for cultures grown on alkenes after growth under repressed conditions, and will also stimulate growth on substrates that would not normally promote growth. Understanding the mechanism by which EtO stimulates growth is a question that should be further pursued. While JS614 could not be stimulated with EtO to grow on 1,1-dichloroethene (1,1-DCE) and 1,2-cis-dichloroethene (cDCE), there may be other bacteria in contaminated aquifers that would utilize DCEs once stimulated. This could be an important engineering application, since utilizing an inducer that would effectively stimulate expression of the alkene metabolic pathway of native aquifer bacteria has the potential to enhance remediation of cDCE and 1,1-DCE contamination.
Bibliography


9. **C&EN.** 2006. Production: Growth is the Norm. Chemical and Engineering News **84:**59-69.


Appendix
Supporting Information

The inhibition of substrate S by inhibitor I may be modeled by the following expressions describing different mechanisms of inhibition (46)

Competitive
\[
\frac{dS}{dt} = \left(\frac{k_{\text{max}} [S]X}{[S] + K_s (1 + [I]/K_i)}\right)
\] (S1)

Noncompetitive
\[
\frac{dS}{dt} = \left(\frac{k_{\text{max}}(1 + [I]/K_i)[S]X}{[S] + K_s}\right)
\] (S2)

Uncompetitive
\[
\frac{dS}{dt} = \left(\frac{k_{\text{max}}(1 + [I]/K_i)[S]X}{[S] + K_s/(1 + [I]/K_i)}\right)
\] (S3)

where \(dS/dt\) is the reaction velocity (nmol/min/mg protein), S is the substrate concentration (\(\mu\text{M}\)), X is the concentration of biomass (mg protein/L), I is the inhibitor concentration (\(\mu\text{M}\)) and \(K_i\) the half-velocity constant of the inhibitor (\(\mu\text{M}\)).

The inhibition of a nongrowth substrate C by inhibitor I is modeled by equations 4-6.

Competitive
\[
\frac{dC}{dt} = \left(\frac{k_{\text{max}} [C]X}{[C] + K_c (1 + [I]/K_i)}\right)
\] (S4)

Noncompetitive
\[
\frac{dC}{dt} = \left(\frac{k_{\text{max}}(1 + [I]/K_i)[C]X}{[C] + K_c}\right)
\] (S5)

Uncompetitive
\[
\frac{dC}{dt} = \left(\frac{k_{\text{max}}(1 + [I]/K_i)[C]X}{[S] + K_c/(1 + [I]/K_i)}\right)
\] (S6)

Where \(dC/dt\) is the reaction velocity (nmol/min/mg protein) and \(K_c\) is the half-velocity of the nongrowth substrate.
Table A.1. Input parameters for the competitive inhibition modeling analysis of the competition and the halide release experiments shown in Supplemental Figure S1 and Figure 2.

<table>
<thead>
<tr>
<th>Isolate Substrate</th>
<th>EE13a</th>
<th>JS60</th>
<th>JS614</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>FE</td>
<td>VC</td>
<td>FE</td>
</tr>
<tr>
<td>$K_s$ (μM)</td>
<td>1.4</td>
<td>0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>$k_{\text{max}}$</td>
<td>35</td>
<td>30</td>
<td>38</td>
</tr>
<tr>
<td>% Halide release</td>
<td>84</td>
<td>100</td>
<td>84</td>
</tr>
<tr>
<td>Kinetic Parameters</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Competition Experiment (Supplemental Information Figure S1)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>$K_i$ (μM)</td>
<td>0.5</td>
<td>2.9</td>
<td>0.5</td>
</tr>
<tr>
<td>X (mg protein)</td>
<td>4.6</td>
<td>4.6</td>
<td>4.6</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.994-0.999</td>
<td>0.974-0.996</td>
<td>0.785-0.996</td>
</tr>
<tr>
<td>SSE $^b$</td>
<td>2.00E-05</td>
<td>1.80E-07</td>
<td>7.90E-06</td>
</tr>
<tr>
<td>Halide Release Experiment (Figure 2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_i$ (μM)</td>
<td>0.5</td>
<td>2.9</td>
<td>0.15</td>
</tr>
<tr>
<td>X (mg protein)</td>
<td>2.2</td>
<td>2.2</td>
<td>5.1</td>
</tr>
<tr>
<td>$r^2$ $^c$</td>
<td>0.979</td>
<td>0.99</td>
<td>0.994</td>
</tr>
<tr>
<td>SSE $^c$</td>
<td>3.40E-07</td>
<td>1.20E-07</td>
<td>9.00E-07</td>
</tr>
</tbody>
</table>

$^a$ Rates given in nmol/min/mg protein. $^b$ SSE for the competition experiment in Supplemental Figure S2 are averages from all concentrations. $^c$ $r^2$ and SSE for the halide release experiment represent the fit for the average release curve for the model output.
Figure A.1. Experimental data (symbols) and results of competitive inhibition model (lines) for the three isolates degrading FE or VC individually, and during cotransformation where both substrates were present in batch reactors. Initial aqueous concentrations of FE were 0, and approximately 20, 40, 60 and 80μM while VC concentration was ~20μM.