A number of bacterial species are capable of degrading the widespread environmental pollutant trichloroethylene (TCE) via aerobic cometabolism, but cytotoxic effects that can debilitate the microorganism often accompany this transformation. In this dissertation the effects of TCE degradation on the well-studied, toluene-oxidizing bacterium *Burkholderia cepacia* G4 were investigated at the physiological and genetic level and compared and contrasted to the effects elicited by several nonhalogenated, short chain alkenes and alkynes. Linear alkynes (C\textsubscript{3}-C\textsubscript{10}) were classified as strong mechanism-based inactivators of toluene 2-monoxygenase activity in *B. cepacia* G4, with 2- and 3-alkynes providing a more potent effect than their 1-alkyne counterparts. The C\textsubscript{2} alkyne, acetylene, was weak inactivator of toluene 2-monoxygenase activity presumably because it does not bind efficiently to this oxygenase. Toluene-grown
cells of *B. cepacia* G4 cells oxidized ethylene and propylene to their respective epoxides with no observable effect on cell culturability or general respiratory activity. In contrast, TCE oxidation was accompanied by a myriad of cytotoxic effects. Accumulation of general cellular damage, manifested as a loss of cell culturability and general respiratory activity, outpaced loss of toluene 2-monooxygenase activity during TCE oxidation. Measures of the culturability of TCE-injured cells varied up to 3 orders of magnitude (depending on the method of assessment), and it was found that TCE-injured cells were ultra sensitive to H$_2$O$_2$ on the surface of agar plates. It was proposed that a toxicity threshold exists for *B. cepacia* G4 during TCE oxidation, and once cells have degraded ≥0.5 μmol of TCE (mg of cells$^{-1}$) the likelihood of recovery decreases significantly. Tn5 mutants of *B. cepacia* G4 with disruptions in genes putatively encoding enzymes involved in DNA repair (including UvrB, RuvB, RecA, and RecG) were ultra susceptible to killing by TCE, as well as the known DNA damaging agents, UV light, mitomycin C, and H$_2$O$_2$. Physiological and genetic analysis of the mutants provided suggestive evidence that nucleotide excision repair and recombinational repair activities are linked to the survivability of TCE-injured *B. cepacia* G4.
Physiological Consequences of Trichloroethylene Degradation by the Toluene-oxidizing Bacterium *Burkholderia cepacia* G4

by

Chris M. Yeager

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented July 24, 2001
Commencement June 2002
Doctor of Philosophy thesis of Chris M. Yeager presented on July 24, 2001

APPROVED:

Redacted for privacy
Co-Major Professor, representing Molecular and Cellular Biology

Redacted for privacy
Co-Major Professor, representing Molecular and Cellular Biology

Redacted for privacy
Director of Molecular and Cellular Biology Program

Redacted for privacy
Dean of Graduate School

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

Redacted for privacy
Chris M. Yeager, Author
ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dan Arp and Peter Bottomley for guidance and support "above and beyond the call" during my time at OSU. Their efforts to develop a simple-minded Wyomingite into an independent scientist are greatly appreciated, even laudable. It was a pleasure fellas.

I would also like to thank Mike Hyman for helping me up the first big hill (i.e., manuscript #1). Mike also showed me that with a Swiss Army knife and a little acetylene anything is possible.

To the Arp lab, I've enjoyed working with all of you. To Luis, Norm, Ryan: Whose idea was hotbiotech.com, anyway? Natsuko and Lisa, thanks for the stimulating conversations, scientific and otherwise. Miriam, Alisa, and Laura thanks for answering all questions biochemical. Kim, I'm jealous. And although Sterling's departure may have added 6 months to my research, his presence made lab a brighter place.

I also had the good fortune of working with members of the Bottomley lab, Rockie, Khrys, Ieda, Sandra, and Traci. Thanks for the invaluable input during lab meetings. Thanks to the MCB faculty and staff for a first-rate education.

Finally, I would like to thank my family. Mom and dad, your encouragement led me down this road. You were my first and best teachers. Shannan, this is as much your accomplishment as mine, and your love and support are deeply cherished.
CONTRIBUTION OF AUTHORS

Daniel J. Arp and Peter J. Bottomley contributed to experimental design and manuscript preparation for chapters 2, 3, and 4. Michael R. Hyman contributed to experimental design and manuscript preparation for chapter 2. All experiments were conducted in the laboratories of Dr. Arp or Dr. Bottomley at Oregon State University.
# TABLE OF CONTENTS

Chapter 1. Introduction ........................................................................................................ 1

1.1. Trichloroethylene – applications, environmental occurrence, and human health effects .......................................................... 1

1.1.1. Applications of trichloroethylene ................................................. 1
1.1.2. Environmental occurrence of TCE ............................................. 2
1.1.3. Health effects of TCE................................................................. 2

1.2. TCE remediation ....................................................................................... 3

1.2.1. Abiotic remediation of TCE .......................................................... 3
1.2.2. General features of TCE bioremediation ....................................... 4
1.2.3. Anaerobic biodegradation of TCE ............................................... 5
1.2.4. Aerobic biodegradation of TCE ...................................................... 7

1.3. Examination of bacterial oxygenases that catalyze the oxidation of TCE .10

1.3.1. Toluene dioxygenase...................................................................... 10
1.3.2. Particulate methane monooxygenase and ammonia monooxygenase ................................................................. 11
1.3.3. Soluble methane monooxygenase .............................................. 11
1.3.4. Toluene 2-monooxygenase............................................................. 13
1.3.5. A potential mechanism for monooxygenase-catalyzed oxidation of TCE ........................................................................... 13

1.4. Mechanism-based inactivators .................................................................. 14

1.4.1. General characteristics of mechanism-based inactivators ............... 14
1.4.2. Alkynes and alkenes as mechanism-based inactivators of oxygenases .................................................................................. 16
1.4.3. Mechanism-based inactivators as experimental tools for TCE biodegradation ................................................................. 18

1.5. Oxygenase inactivation upon TCE transformation ................................... 19

1.5.1. Toluene dioxygenase ...................................................................... 19
1.5.2. Soluble methane monooxygenase .............................................. 20
1.5.3. Toluene 2-monooxygenase ............................................................. 21
1.5.4. Cytochrome P-450 monooxygenases ............................................ 22
TABLE OF CONTENTS (CONTINUED)

1.6. Cytotoxicity and carcinogenicity associated with TCE metabolism in mammalian cells ................................................................. 24
   1.6.1. Peroxisome proliferation and genotoxicity of GSH-TCE conjugates .................................................................................... 25
   1.6.2. Genotoxicity of reactive intermediates of TCE oxidation catalyzed by cytochrome P-450 monooxygenases ............................ 26

1.7. Cytotoxicity associated with TCE oxidation in bacteria .................. 28
   1.7.1. Effect of TCE oxidation on general indices of cell health ....... 29
   1.7.2. [14C]TCE radiolabeling studies ............................................................. 30
   1.7.3. Transformation capacity and TCE oxidation ................................. 31

1.8. Research objectives ....................................................................... 32

Chapter 2. Inactivation of Toluene 2-Monooxygenase in Burkholderia cepacia G4 by Alkynes ......................................................... 34

2.1. Abstract .......................................................................................... 35

2.2. Introduction ..................................................................................... 35

2.3. Materials and Methods .................................................................. 37
   2.3.1. Chemicals and reagents ................................................................. 37
   2.3.2. Bacterial strain and culture conditions ........................................ 38
   2.3.3. Analytical and other methods ..................................................... 38
   2.3.4. Growth inhibition by alkynes ..................................................... 39
   2.3.5. Inhibition of O2 uptake by alkynes ............................................. 40
   2.3.6. Requirement of O2 for inactivation ............................................. 41
   2.3.7. Alkyne and alkene oxidation ........................................................ 42
   2.3.8. Kinetic analysis of propylene and ethylene oxidation ............... 42

2.4. Results ........................................................................................... 43
   2.4.1. Inhibition of growth by alkynes .................................................... 43
   2.4.2. Inactivation of toluene-dependent O2 uptake by alkynes ............ 47


### Table of Contents (continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4.3. Requirement of O₂ for inactivation</td>
<td>52</td>
</tr>
<tr>
<td>2.4.4. Alkene oxidation by <em>B. cepacia</em> G4</td>
<td>53</td>
</tr>
<tr>
<td>2.4.5. Kinetics of ethylene and propylene oxidation</td>
<td>55</td>
</tr>
<tr>
<td>2.5. Discussion</td>
<td>55</td>
</tr>
<tr>
<td>2.6. Acknowledgments</td>
<td>59</td>
</tr>
</tbody>
</table>

Chapter 3. Cytotoxicity Associated with Trichloroethylene Oxidation in *Burkholderia cepacia* G4

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1. Abstract</td>
<td>61</td>
</tr>
<tr>
<td>3.2. Introduction</td>
<td>61</td>
</tr>
<tr>
<td>3.3. Materials and Methods</td>
<td>64</td>
</tr>
<tr>
<td>3.3.1. Chemicals and reagents</td>
<td>64</td>
</tr>
<tr>
<td>3.3.2. Culture conditions</td>
<td>64</td>
</tr>
<tr>
<td>3.3.3. Determining transformation capacities</td>
<td>65</td>
</tr>
<tr>
<td>3.3.4. TCE exposure for toxicity assays</td>
<td>66</td>
</tr>
<tr>
<td>3.3.5. Assays for cell culturability and O₂ uptake rates</td>
<td>66</td>
</tr>
<tr>
<td>3.3.6. Effect of TCE oxidation on surrounding cells</td>
<td>67</td>
</tr>
<tr>
<td>3.3.7. Recovery of growth by <em>B. cepacia</em> G4 cells exposed to TCE</td>
<td>68</td>
</tr>
<tr>
<td>3.3.8. Occurrence of Tol' variants upon TCE oxidation</td>
<td>69</td>
</tr>
<tr>
<td>3.3.9. Analytical and other methods</td>
<td>69</td>
</tr>
<tr>
<td>3.4. Results</td>
<td>70</td>
</tr>
<tr>
<td>3.4.1. Effects of TCE transformation on toluene 2-monoxygenase activity, general respiratory activity and cell viability</td>
<td>70</td>
</tr>
<tr>
<td>3.4.2. Further examination of the culturability of <em>B. cepacia</em> G4 cells following TCE transformation</td>
<td>73</td>
</tr>
<tr>
<td>3.4.3. Recovery of <em>B. cepacia</em> G4 cells following TCE transformation</td>
<td>76</td>
</tr>
<tr>
<td>3.4.4. Excessive TCE damage selects against toluene 2-monoxygenase activity in <em>B. cepacia</em> G4 populations</td>
<td>78</td>
</tr>
<tr>
<td>3.4.5. Nature of the toxic TCE intermediate(s)</td>
<td>81</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (CONTINUED)

3.5. Discussion................................................................................................. 83

3.6. Acknowledgements .................................................................................. 87

Chapter 4. The Requirement of DNA Repair Mechanisms for the Survival of
Burkholderia cepacia G4 upon Degradation of Trichloroethylene. 88

4.1. Abstract...................................................................................................... 89

4.2. Introduction............................................................................................... 89

4.3. Materials and Methods .......................................................................... 91

4.3.1. Bacterial strains and culture conditions ........................................ 91

4.3.2. Transposon mutagenesis ................................................................. 92

4.3.3. Screening for TCE sensitive mutants........................................... 92

4.3.4. Cloning and sequencing DNA flanking the Tn5 inserts.............. 93

4.3.5. Recovery of growth by B. cepacia G4 cells exposed to TCE ....... 94

4.3.6. Chemical and UV sensitivity assays .............................................. 94

4.3.7. Analytical and other methods ......................................................... 95

4.3.8. Nucleotide sequence accession numbers .................................... 95

4.4. Results....................................................................................................... 96

4.4.1. Selection and genetic characterization of TCE sensitive
mutants of B. cepacia G4 ........................................................................... 96

4.4.2. Sequence analysis of the putative uvrB, ruvB, recA, and recG
coding regions from B. cepacia G4 ...................................................... 101

4.4.3. Growth characteristics of TCS strains following TCE exposure .. 102

4.4.4. Sensitivity to TCE, mitomycin C, hydrogen peroxide, and
UV light ...................................................................................................... 104

4.5. Discussion............................................................................................... 104

4.6. Acknowledgement .................................................................................. 110

Chapter 5. Summary .................................................................................... 111
TABLE OF CONTENTS (CONTINUED)

5.1. Inactivation of toluene 2-monooxygenase in *Burkholderia cepacia* G4 by alkynes ................................................................. 111

5.2. Cytotoxicity associated with trichloroethylene oxidation in *Burkholderia cepacia* G4 .............................................................................. 112

5.3. The requirement of DNA repair mechanisms for the survival of *Burkholderia cepacia* G4 upon degradation of trichloroethylene .......... 113

5.4. Concluding remarks ..................................................................................... 115

BIBLIOGRAPHY ........................................................................................................ 116
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Proposed mechanism for TCE oxidation by bacterial diiron monooxygenases</td>
<td>15</td>
</tr>
<tr>
<td>2.1</td>
<td>Growth of <em>B. cepacia</em> G4 in the presence of various alkynes</td>
<td>44</td>
</tr>
<tr>
<td>2.2</td>
<td>Effects of 1-butyne on substrate-dependent O2 uptake by <em>B. cepacia</em> G4</td>
<td>48</td>
</tr>
<tr>
<td>2.3</td>
<td>Kinetics of inactivation of toluene-dependent O2 uptake activity by 1-butyne</td>
<td>50</td>
</tr>
<tr>
<td>2.4</td>
<td>Irreversible effects of alkyne exposure on substrate-dependent O2 uptake</td>
<td>51</td>
</tr>
<tr>
<td>2.5</td>
<td>Requirement of O2 for inactivation of toluene-dependent O2 uptake activity by 1-butyne</td>
<td>52</td>
</tr>
<tr>
<td>2.6</td>
<td>Alkene and alkyne consumption by <em>B. cepacia</em> G4</td>
<td>54</td>
</tr>
<tr>
<td>2.7</td>
<td>Kinetic model for a mechanism-based inactivator</td>
<td>55</td>
</tr>
<tr>
<td>3.1</td>
<td>TCE and ethylene consumption by <em>B. cepacia</em> G4</td>
<td>70</td>
</tr>
<tr>
<td>3.2</td>
<td>Effects of TCE transformation on toluene 2-monoxygenase activity, general respiratory activity, and cell culturability</td>
<td>74</td>
</tr>
<tr>
<td>3.3</td>
<td>Recovery of growth by <em>B. cepacia</em> G4 cells exposed to TCE</td>
<td>79</td>
</tr>
<tr>
<td>4.1</td>
<td>Selection of TCE sensitive mutants of <em>B. cepacia</em> G4</td>
<td>97</td>
</tr>
<tr>
<td>4.2</td>
<td>Schematic map of DNA fragments self-cloned from TCS mutants</td>
<td>99</td>
</tr>
<tr>
<td>4.3</td>
<td>Recovery of growth by TCE-treated cells</td>
<td>103</td>
</tr>
<tr>
<td>4.5</td>
<td>Survival of cells upon exposure to TCE, UV light, H2O2, and mitomycin C</td>
<td>105</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Inhibition of toluene-dependent growth of <em>B. cepacia</em> G4 by alkynes</td>
</tr>
<tr>
<td>3.1</td>
<td>Tₜ values of resting cell suspensions of <em>B. cepacia</em> G4 for TCE and ethylene at different cell densities</td>
</tr>
<tr>
<td>3.2</td>
<td>Cell culturability of <em>B. cepacia</em> G4 cells exposed to TCE for selected times</td>
</tr>
<tr>
<td>3.3</td>
<td>Tol⁺ phenotype among <em>B. cepacia</em> G4 cells that survive TCE exposure</td>
</tr>
<tr>
<td>3.4</td>
<td>Diffusible nature of toxic TCE intermediate(s)</td>
</tr>
<tr>
<td>4.1</td>
<td>Blastx analysis of DNA sequences flanking the Tn5-OT182 insertion sites in TCE sensitive mutants of <em>B. cepacia</em> G4</td>
</tr>
</tbody>
</table>
Chapter 1.

Introduction

1.1. Trichloroethylene – applications, environmental occurrence, and human health effects

1.1.1. Applications of trichloroethylene

Trichloroethylene (TCE) is a colorless, volatile liquid that has served as the predominant solvent for degreasing textiles and metals since the introduction of the vapor degreasing process in the early 1930’s (Halogenated Solvents Industry Alliance Inc. 2001). Since that time it has also been used as a component of adhesives, insecticides, paints, cosmetics, drugs, pet foods, lubricants, varnishes and other materials, a solvent in paint-strippers, a caffeine extractant, a component of flame-retardants, and even as a general anesthetic (Agency for Toxic Substances and Disease Registry 1993). Many of these uses have been discontinued due to health concerns. Several properties of TCE are responsible for its versatility as a commercial commodity including: high solvency, low flammability, non-corrosiveness, high stability, low specific heat, low boiling point, and low latent heat of vaporization. Today, TCE is primarily used as a metal cleaning agent (particularly of aluminum, sheet and strip metal, and liquid oxygen and hydrogen tanks) and as an intermediate in the production of hydrofluorocarbon intermediates, such as polyvinyl chloride. The demand for TCE in the United States for 1998 was an estimated 171 million pounds (Halogenated Solvents Industry Alliance Inc. 2001).
1.1.2. Environmental occurrence of TCE

Most TCE, when released purposefully or inadvertently, evaporates quickly and is expected to have a half-life of only 1 to 10 days in the atmosphere because of photooxidative breakdown (Mallinckrodt Chemicals Inc. 2000). However, due to the massive production and ubiquitous use of TCE over the last 70 years coupled with uninformed disposal practices, landfill leachates, and leaking storage containers, TCE has become one of the most widespread and common groundwater and soil contaminants in the United States. According to the Toxics Release Inventory, approximately 100,000 pounds of TCE were released into water and approximately 191,000 pounds into soil from 1987-1993 (EPA Office of Water 2001). It is a frequently detected contaminant in municipal groundwater supplies and is one of the 10 most commonly detected chemicals at hazardous waste sites (Westrick et al. 1984; Abelson 1990). The EPA has established a maximum contaminant level of 5 μg/L and a maximum contaminant level goal of zero for TCE (EPA Office of Water 2001).

1.1.3. Health effects of TCE

Acute exposure to high levels of TCE (10,000 ppm) can cause vomiting, abdominal pain, respiratory inflammation, headache, dizziness, and drowsiness. It can also lead to cardiac arrest, unconsciousness and death under extraordinary circumstances. Chronic overexposure to TCE (greater that the maximum contaminant level) can cause liver and kidney damage, and has been linked to cancer. Additionally, TCE exposure has been associated with a potentially lethal immune disorder, systemic sclerosis or scleroderma-like syndrome (Pumford et al. 1997).

Rodents exposed to TCE have a higher incidence of lung, liver, and testicular tumors than control animals (Moore and Harrington-Brock 2000). There is some epidemiological evidence that TCE acts as a carcinogen in humans, but hard conclusions are impossible to draw from the available data (Wartenberg et al. 2000). Currently, the International Agency for Research on Cancer classifies TCE as a probable carcinogen, the National Toxicology Program classifies it as “reasonably
anticipated to be a human carcinogen", and the EPA and National Center for Environmental Assessment are currently conducting a thorough evaluation and risk assessment of TCE, formerly considered a suspected human carcinogen by these organizations (Moore and Harrington-Brock 2000; Halogenated Solvents Industry Alliance Inc. 2001).

1.2. TCE remediation

One of the functional characteristics of chlorinated aliphatic hydrocarbons (CAHs), including TCE, is their relatively stable nature. Indeed, these compounds were designed to resist breakdown at high temperatures and pressures in order to serve as safe industrial solvents. However, the stability of these compounds acts as a double-edged sword in that they are beneficial in terms of commercial applications, but once released into certain environments (subsurface soils and aquifers, aquatic sediments, and some surface waters) they can be remarkably recalcitrant to breakdown and can persist for years (Martin 1994). Because of the health risks associated with TCE exposure and the recalcitrant nature of this molecule, considerable effort has been put forth to develop appropriate remediation techniques.

1.2.1. Abiotic remediation of TCE

A number of physical, chemical, and thermal technologies have been used to treat TCE-contaminated sites (Wickramanayake and Hinchee 1998). For example, TCE-contaminated soils can be sequestered in situ or removed to other storage facilities to prevent human exposure or ecosystem damage. Alternatively, TCE can be removed from contaminated soils by air-purging, vacuum extraction, thermal technologies, solvent extraction or by simple incineration. Traditionally, TCE-contaminated groundwater has been treated by pumping the groundwater to the surface and air-stripping TCE into the atmosphere or sorbing it onto activated carbon (Love and Eilers 1982).
Although all of these technologies can successfully treat certain TCE-contaminated environments, they all have drawbacks. For example, soil removal may be problematic due to disposal regulations and aesthetic considerations. Also, many of the aforementioned technologies do not actually convert TCE into other benign molecules, but simply removes it to another location or medium (i.e. the atmosphere). Most importantly however, the costs associated with many of these technologies can be enormous. Incineration offers almost 100% removal of many organic contaminants, but costs can be overwhelming with large volumes (Noyes 1991). The timeline for TCE removal from aquifers using existing pump and treat methodology has been estimated at decades to centuries and again, the costs would be prohibitive (National Academy of Sciences 1994).

1.2.2. General features of TCE bioremediation

Although not a panacea, the use of biological methodologies for TCE remediation has blossomed over the last several decades. Advantages of biological treatment technologies include: cost effectiveness, complete mineralization, potentially low maintenance, and they can be utilized in diverse environments (sewage-treatment systems, sediments, soils, aquifers, and surface water). It has been estimated that bioremediation of TCE-contaminated soils can be up to ten times less expensive than traditional excavation technologies, and bioremediation of groundwater containing TCE can result in savings of as much as 50 to 75% (Agency for Toxic Substances and Disease Registry 1993). Additionally, bioremediation technologies generally attain high levels of public acceptance. For example, phytoremediation (utilizing plants or microbes associated with plant roots to uptake or transform pollutants) is aesthetically pleasing and it can be coupled to ecological restoration during TCE remediation efforts (Newman et al. 1999).

Microorganisms have thus far served as the workhorse for bioremediation applications primarily because of their vast biochemical armament and the near ubiquity of these organisms throughout the biosphere. Although microorganisms are able to metabolize an enormous diversity of organic molecules, certain xenobiotics
can be relatively resistant to microbial degradation. For example, the half-life of TCE was determined to be 300 days in one aquifer (Wackett et al. 1989), and in another study it was found that of 43 soil and water samples screened only one contained indigenous microorganisms capable of significant rates of TCE degradation (Nelson et al. 1986). Despite the apparent recalcitrance of TCE in situ, microorganisms are known to catalyze TCE degradation under both aerobic and anaerobic conditions.

1.2.3. Anaerobic biodegradation of TCE

Chlorinated ethenes can be degraded under anaerobic conditions via a reaction sequence known as reductive dehalogenation (PCE → TCE → DCEs → vinyl chloride → ethylene). Reductive dehalogenation can be separated into two distinct processes. The first process is essentially a cometabolic process in which reduced metal cofactors such as corrinoids, hemes, and cofactor F_{430}, found in abundance in certain methanogens, acetogens, and sulfate-reducing bacteria, act to reductively dechlorinate chlorinated ethenes (Vogel and McCarty 1985; Gantzer and Wackett 1991). These organisms utilize terminal electron acceptors such as CO₂, acetate, and sulfate for growth, whereas the chlorinated ethenes are fortuitously reduced by enzymes containing the metal cofactors listed above. Reductive dehalogenation carried out in this fashion is a slow process. Isolated strains of methanogens and sulfate-reducing bacteria reduced PCE to TCE at a rate of <0.001 μmol of PCE h⁻¹ mg cells⁻¹ (dry weight) utilizing this process (Sharma and McCarty 1996).

In a second type of reductive dehalogenation, microorganisms utilize chloroethenes (or chloroaromatics) as terminal electron acceptors for growth, a process termed dehalorespiration. Dehalorespiration essentially couples ATP synthesis via a chemiosmotic mechanism to reductive dechlorination. About ten eubacterial strains have been isolated and shown to grow via this unique anaerobic process. All are strict anaerobes, except for the facultative anaerobe, Enterobacter strain MS-1 (Sharma and McCarty 1996). Other dehalorespirers cluster with sulfate- or sulfur-reducing organisms of the ε and γ branches of the Proteobacteria or the low G+C, gram-positive bacteria (Hollinger et al. 1999). One dehalorespirer,
Dehalococcoides ethenogenes, is possibly related to the green nonsulfur bacteria (Magnuson et al. 2000). The enzymes that catalyze reductive dehalogenation in dehalorespiring bacteria (reductive dehalogenases) have been isolated and characterized from most of the known strains of this group. Four of the five reductive dehalogenases examined to date are membrane bound, while the other may be associated with the membrane via an anchoring protein in vivo (Hollinger et al. 1999; Magnuson et al. 2000). The 3-chlorobenzoate reductive halogenase from Desulfomonile tiedjei is thought to contain a heme prosthetic group, while all other known reductive dehalogenases contain cobalamin and iron-sulfur clusters as cofactors (Hollinger et al. 1999). Electron donors capable of supporting dehalorespiration include H2 and formate, and possibly, pyruvate, acetate, and tryptophan (it is difficult to prove unambiguously that dehalorespiration is occurring with electron donors other than H2 or formate). Dehalorespirers have been reported to transform PCE at rates of approximately 0.5 – 1.5 μmol h⁻¹ mg cells⁻¹ (dry weight) (Sharma and McCarty 1996).

Anaerobic bioremediation of chlorinated ethenes, such as TCE, is advantageous in that many of the subsurface and sediment environments contaminated with these compounds are already highly anaerobic or are driven such by the addition of nutrients for microbial growth or activity. Furthermore, reductive dehalogenation of PCE and TCE appears to occur naturally at many contaminated sites, giving hope that intrinsic remediation can be employed to remediate these areas (Wickramanayake and Hinchee 1998). However, reductive dehalogenation proceeds at a relatively slow rate in the absence of dehalorespiring organisms and complete mineralization of the target molecule (i.e. TCE or PCE) is oft times not achieved. In most cases, reductive dehalogenation of TCE or PCE only proceeds as far as a DCE isomer or vinyl chloride (Bouwer and McCarty 1983; Vogel and McCarty 1985), and this can be problematic since vinyl chloride, a known human carcinogen, is more toxic than either PCE or TCE (Infante and Tsongas 1987).
1.2.4. Aerobic biodegradation of TCE

Under aerobic conditions microorganisms degrade TCE with monooxygenases or dioxygenases that exhibit broad substrate specificities. In all cases described to date, TCE oxidation cannot support growth of the transforming microorganism; therefore, a separate, growth-supporting substrate is required to maintain an active population of TCE-degrading cells. Additionally, an inducing substrate is usually required to stimulate synthesis of the monooxygenase(s) and/or dioxygenase(s) responsible for TCE oxidation in a given bacterial strain. Although it has been shown that TCE, itself, can serve as an inducer, physiological substrates for the TCE-transforming oxygenase(s) provide the highest levels of induction (Heald and Jenkins 1994; McClay et al. 1995; Leahy et al. 1996). Therefore, physiological substrates for various oxygenases support TCE degradation in a dual capacity, as growth-supporting substrates and as inducers of the TCE-transforming enzymes.

The process whereby microbes transform non-growth-supporting substrates, such as TCE, (usually in the presence of a growth-supporting substrate) is widely known as cometabolism. Even in the presence of a growth substrate however, a cell can experience a net loss of reductant during TCE cometabolism. For example, monooxygenase reactions require a source of reductant to effect the reduction of one atom of oxygen from \( \text{O}_2 \) to \( \text{H}_2\text{O} \). With a primary growth substrate, such as toluene or methane, this reductant is supplied by further metabolism of the initial oxidation products. TCE, on the other hand, undergoes a single step oxidation, which does not return reductant to the organism. Additionally, competition between a physiological growth substrate and cometabolite (TCE) for the active site of an oxygenase will limit metabolism of the growth substrate, thus limiting reductant regeneration. Therefore, the efficiency of TCE cometabolism can depend largely upon the concentration of growth substrate available to the transforming microorganisms relative to the concentration of TCE. Because of the energetic limitations associated with TCE cometabolism, considerable effort has been aimed at engineering or isolating microorganisms that are capable of expressing CAH-degrading oxygenases in the
presence of growth-supporting substrates that do not compete for the active site of the transforming oxygenase (Wackett et al. 1994; Saeki et al. 1999; Takami et al. 1999).

A broad spectrum of taxonomic diversity is represented among microorganisms that are known to degrade TCE aerobically including alpha, beta, and gamma proteobacteria, high GC gram (+) bacteria, and some fungi (Ensley 1991; Arp et al. 2001). Bacteria known to aerobically degrade TCE are often categorized according to the primary growth substrate that supports TCE cometabolism, which include: methane, propane, butane, propylene, ammonia, phenol, toluene, 2,4-dichlorophenoxyacetate, and isopropylbenzene (Arp et al. 2001). Among these bacteria, the methanotrophs and toluene/phenol oxidizers have received the most attention in regards to bioremediation of CAHs.

The widespread nature of methanotrophs, along with the low cost and availability of methane, make these organisms an ideal choice for TCE bioremediation applications. Methanotrophs, which are grouped within the beta and gamma subdivisions of the proteobacteria, harvest methane via methane monoxygenase. Two forms of methane monoxygenase have been identified, a particulate, membrane-bound form (pMMO) and a soluble form that is located in the cytoplasmic fraction (sMMO). All methanotrophs can express pMMO in the presence of copper, whereas sMMO, which is expressed under copper-limited conditions, is found in a limited number of strains (Hanson and Hanson 1996). sMMO has a broader substrate range than pMMO and in most instances sMMO exhibits higher rates of catalytic turnover. For instance, TCE is oxidized by whole cells of *Methylosinus trichosporium* OB3b (this microbe contains both pMMO and sMMO) with reported \( V_{\text{max}} \) values ranging between 16-995 nmol min\(^{-1}\) mg of total cell protein\(^{-1}\) for cells expressing sMMO and 0.2-4.1 nmol min\(^{-1}\) mg of total cell protein\(^{-1}\) for cells expressing pMMO (Lontoh and Semrau 1998; Arp et al. 2001).

Many bacteria degrade CAHs when grown on toluene as a carbon and energy source. The initial oxidation of toluene as a growth substrate is known to proceed via five distinct pathways in bacteria. Several toluene-oxidizing pseudomonads, including *Pseudomonas putida* mt-2, harbor the TOL plasmid. The pathway conferred by this
plasmid involves an initial oxidation of the alkyl group by xylene monoxygenase to form benzyl alcohol (Worsey and Williams 1975). In the four remaining pathways, toluene oxidation is initiated by a direct attack on the aromatic ring by an oxygenase. *Pseudomonas putida* F1 utilizes the chromosomally encoded *tod* pathway (Zylstra et al. 1988), in which toluene 2,3 dioxygenase forms cis-toluene-2,3-dihydriodiol from toluene. *B. cepacia* G4 hydroxylates the aromatic ring at the *ortho* position and further hydroxylates the resulting *o*-cresol to 3-methylcatechol (Shields et al. 1989). Both steps are catalyzed by toluene 2-monoxygenase, which is encoded by the TOM plasmid (Shields et al. 1995). *Ralstonia pickettii* PKO1 transforms toluene to *m*-cresol by a toluene 3-monoxygenase, which is encoded by genes in the *thu* cluster (Olsen et al. 1994). Lastly, *Pseudomonas mendocina* KR1 converts toluene to *p*-cresol with a toluene 4-monoxygenase. This pathway is chromosomally encoded by the *tmo* genes (Yen et al. 1991; Wright and Olsen 1994).

As with other TCE-degrading bacteria, the relaxed substrate specificity of the monoxygenase or dioxygenase that initiates oxidation of the primary growth substrates makes toluene-oxidizing bacteria useful for bioremediation applications. Substrates for the various toluene oxygenases include an impressive list of pollutants: phenol, toluene, cresols, xylene, benzene, styrene, biphenyl, chlorobenzene, anthracene, ethyl-, methyl-, and chloro-substituted toluene, dichloroethylenes, chloroform, and TCE (Wackett and Gibson 1988; Abril et al. 1989; Zylstra et al. 1989). With the exception of the TOL-encoded xylene monoxygenase, each of the toluene oxygenases described above is capable of co-oxidizing TCE (Nelson et al. 1987; Wackett and Gibson 1988; Winter et al. 1989; Leahy et al. 1996). The rates of TCE oxidation observed with whole cells of these bacteria range from 0.5 to 20 nmol min$^{-1}$ mg of total cell protein$^{-1}$ and the $K_v$ values for TCE range from 3-10 µM (Arp et al. 2001).

Toluene oxidizing bacteria appear to be widespread, and data from field studies indicate that many of these microorganisms have the potential to degrade TCE. For example, TCE was successfully degraded upon stimulation of the indigenous microbial population with toluene at Moffett Federal Airfield, Mountain View, CA
(Hopkins and McCarty 1995; Fries et al. 1997). From 273 phenol- and toluene-degrading isolates from the Moffett Field aquifer, 63 distinct strains were identified of which 60% were capable of cometabolizing TCE (Fries et al. 1997). Furthermore, 55% of these strains hybridized to a probe for the toluene 2-monoxygenase (TOM) gene. Of considerable interest, TOM-positive isolates exhibited similar rates of toluene degradation under the conditions examined, but varied significantly among their ability to degrade TCE. Initial rates of TCE degradation were often rapid, followed by a gradual cessation of activity.

1.3. Examination of bacterial oxygenases that catalyze the oxidation of TCE

TCE oxidation by bacterial oxygenases has been studied with purified forms of three enzymes: toluene dioxygenase (Fox et al. 1990), sMMO (Li and Wackett 1992), and toluene 2-monoxygenase (Newman and Wackett 1997). Additionally, considerable information concerning TCE oxidation by other bacterial oxygenases, particularly pMMO and ammonia monooxygenase, has been obtained through careful studies utilizing whole cells and cell extracts.

1.3.1. Toluene dioxygenase

Toluene dioxygenase consists of three components (Subramanian et al. 1981; Subramanian et al. 1985; Zylstra and Gibson 1989). The first component, reductase_{TOL}, is a flavoprotein that transfers electrons from NADH to the second component of the complex, ferredoxin_{TOL}. Ferredoxin_{TOL}, in turn, shuttles electrons to the dioxygenase component, ISP_{TOL}, which contains a Rieske-type [2Fe-2S] center and a mononuclear iron center. The rate of TCE oxidation by toluene dioxygenase purified from recombinant E. coli strains containing tod genes from P. putida F1 was 12 nmol min^{-1} mg of iron-sulfur protein_{TOL}^{-1} (Li and Wackett 1992). Non-volatile products accounted for 70% of the TCE oxidized by the purified enzyme, with formic acid accounting for 47% and glyoxylic acid accounting for 17% of the products. The identity of the reaction products indicate that monooxygenation of TCE is not
occurring at significant levels. Furthermore, the absence of phosgene and/or CO₂ argues against release of TCE-dioxetene from the active site of toluene dioxygenase. From the experimental observations, the authors suggested that a Fe-bound deoxygenated intermediate could rearrange within the active site to release formyl chloride or glyoxyl chloride, which would hydrolyze to formate and glyoxylate, respectively.

1.3.2. Particulate methane monooxygenase and ammonia monooxygenase

Particulate methane monooxygenase has recently been purified from the membrane fractions of *M. capsulatus* (Bath) and consists of three polypeptides of 23, 26, and 45 kDa (Zahn and DiSpirito 1996; Nguyen et al. 1998). This enzyme contains copper and is inactivated by light and acetylene, inhibited by copper chelators, and is sensitive to dioxygen. Whole cells of *M. trichosporium* OB3b expressing pMMO oxidized TCE at a rate of 4.1 nmol min⁻¹ mg of total cell protein⁻¹ with a *K₅* of 7.9 μM (Lontoh and Semrau 1998). The inhibitor and inactivator profile of pMMO is similar to that of another copper-containing bacterial monooxygenase, ammonia monooxygenase (AMO) from the nitrifying bacteria. In cells containing either pMMO or AMO a polypeptide with an apparent molecular mass of 27 kDa is preferentially labeled upon treatment with ¹⁴C₂H₂, suggesting that acetylene acts as a mechanism-based inactivator of the these enzymes and that the active site of each resides on the 27 kDa subunit (Hyman and Wood 1985; Prior and Dalton 1985). The similarity between the enzymes also extends to the genetic level with the genes encoding pMMO (pmoCAB) sharing extensive homology with the genes encoding AMO (amoCAB). AMO has also been shown to oxidize TCE in whole cell studies with a *Vₘₐₓ* of 10.9 nmol min⁻¹ mg of total cell protein⁻¹ and a *K₅* of 9.8-30 μM (Arp et al. 2001).

1.3.3. Soluble methane monooxygenase

sMMO has been purified to homogeneity from *M. capsulatus* (Bath) and from *M. trichosporium* OB3b and in each case the enzyme was found to consist of a
hydroxylase (component A), a regulatory protein (component B), and a NADH reductase (component B) (Lipscomb 1994). The hydroxylase component exists as a dimer with an \((\alpha\beta\gamma)_2\) configuration containing a \(\mu\)-hydroxo-bridged diiron cluster, where dioxygen is activated and substrate oxidation occurs. The reductase contains a 2[Fe-S] cluster and an FAD center which transfer electrons from NADH to the hydroxylase active site. It is thought that the regulatory protein induces a conformational change in the enzyme complex that alters the substrate specificity of the hydroxylase and/or optimizes the interaction between the reductase and the hydroxylase components, thus facilitating the transfer of electrons between these two components (Gallagher et al. 1999). A generally accepted catalytic cycle for sMMO has been proposed based on a number of kinetic and spectroscopic studies (Jin and Lipscomb 1999). In this scenario, the diiron cluster is first reduced to the diferrous state via electrons transferred from the reductase. Next, the diiron cluster interacts with \(\text{O}_2\) to form an Fe bound peroxo intermediate, followed by O-O bond cleavage to form a reactive O species (either Fe(V)=O\(^2^-\) or Fe(IV)=O) and \(\text{H}_2\text{O}\). The O-activated enzyme then abstracts an electron in the form of a hydrogen atom from the substrate, forming a radical intermediate and a diiron-bound hydroxy group. Recombination of the hydroxyl group with the substrate radical and release of the oxygenated product would complete the reaction cycle. In such a manner sMMO is proposed to catalyze the oxidation of methane to methanol.

Purified sMMO from \textit{M. trichosporium} OB3b catalyzes the oxidation of TCE at a rate of 680 nmol min\(^{-1}\) mg of hydroxylase\(^{-1}\) with a \(K_m\) of 35 \(\mu\text{M}\) (Fox et al. 1990). In comparison, ethylene was oxidized at a rate of 858 nmol min\(^{-1}\) mg of hydroxylase\(^{-1}\) with a \(K_m\) of 32 \(\mu\text{M}\). The stable observed products of TCE oxidation were: glyoxylate (5%), dichloroacetate (5%), formate (35%), chloral (6%), and CO (53%). With the exception of chloral, the identity and distribution of the stable products from sMMO catalyzed TCE oxidation nearly matched that observed upon the spontaneous breakdown of TCE epoxide in the experimental buffer. The authors proposed that the majority of TCE is oxidized to TCE epoxide, which undergoes spontaneous rearrangement and hydrolysis to yield glyoxylate, dichloroacetate, formate, and CO.
About 20% of the transformed TCE could be trapped as TCE epoxide with 4-(p-nitrobenzyl)pyridine in the vapor phase of the reaction vessels. Since TCE epoxide exhibits a half-life of 10-20 seconds in the experimental buffer, a significant amount could have been hydrolyzed prior to trapping. Kinetic analysis of the experimental data led the investigators to suggest that chloral is generated via chloride migration on an enzyme-bound intermediate (not TCE epoxide) within the active site of the hydroxylase.

### 1.3.4. Toluene 2-monooxygenase

Toluene 2-monooxygenase is composed of three protein components including: 1) a diiron hydroxylase component exhibiting an $\alpha_2\beta_2\gamma_2$ conformation, 2) a flavo-iron-sulfur protein that transfers electrons from NADH to the hydroxylase, and 3) a small regulatory component with no identifiable metal or organic cofactors (Newman and Wackett 1995). Purified toluene 2-monooxygenase from *B. cepacia* G4 catalyzes the oxidation of TCE at a rate of 37 nmol min$^{-1}$ mg of hydroxylase$^{-1}$ with a $K_m$ of 12 $\mu$M (Newman and Wackett 1997). In an experiment where 90% of the TCE was oxidized the product distribution was as follows: 41% CO, 21% formate, 10% glyoxylate and <1% dichloroacetic acid, trichloroacetic acid, and chloral. Approximately 10% of the TCE could be trapped as TCE epoxide using 4-(p-nitrobenzyl)pyridine. Again, it was suggested that the relatively short half-life of TCE epoxide prevented a higher trapping efficiency. In contrast to other TCE-degrading enzymes, chloral was not produced in significant amounts during TCE oxidation by toluene 2-monooxygenase; therefore, the authors suggested that TCE epoxide was the predominant reaction product released by this enzyme.

### 1.3.5. A potential mechanism for monooxygenase-catalyzed oxidation of TCE

Drawing on information gained from studies evaluating TCE oxidation by mammalian cytochrome P-450 enzymes (Miller and Guengerich 1982; Miller and
Guengerich 1983; Guengerich 1984; Bruckner et al. 1989), sMMO (Fox et al. 1990), and toluene 2-monooxygenase (Newman and Wackett 1997) a general mechanism for monooxygenase-catalyzed oxidation of TCE is shown in Figure 1.1. Abstraction of an electron from the π bond of TCE by an activated oxygen species, stabilized by the diiron center of the hydroxylase, would generate a transient radical, cationic intermediate. Rebound of the radical would form a [Fe-O]-bound transition intermediate. From the transition intermediate, 1,2-migration of a chloride or hydride group could occur resulting in haloaldehyde (chloral) formation. Alternatively, rearrangement of the [Fe-O]-bound transition intermediate could produce TCE epoxide. In this manner chloral and TCE epoxide formation could occur in parallel at the active site of the monooxygenase. Interestingly, chloral represents the majority of the stable products of TCE oxidation catalyzed by mammalian cytochrome P-450s (Miller and Guengerich 1982; Miller and Guengerich 1983), yet it accounts for only 6% of the stable products produced by sMMO (Fox et al. 1990) and was <1% of the TCE oxidation products produced by toluene 2-monooxygenase (Newman and Wackett 1997). It is presumed that localized environmental conditions within and around the active site of the various isozymes of cytochrome P-450 and other monooxygenases could influence the partitioning between epoxide or chloral formation.

1.4. Mechanism-based inactivators

1.4.1. General characteristics of mechanism-based inactivators

Mechanism-based inactivators are molecules that covalently modify an essential amino acid or prosthetic group within the active site of a particular enzyme, resulting in loss of enzymatic activity. Central to the definition of a mechanism-based inactivator is that it must be catalytically converted to a reactive species by its target enzyme and bind to the transforming enzyme before leaving the active site. Molecules that are enzymatically converted to a reactive species and released from the active site before inactivating that enzyme or other enzymes have been termed
Figure 1.1 Proposed mechanism for TCE oxidation by bacterial diiron monooxygenases

\[
\begin{align*}
\text{HOCl} & \\
\text{NADH} & \text{O}_2 \\
\text{Diiron monooxygenase} & \\
\text{Fe} & \text{OH} \\
\text{Fe} & \text{O} \\
\text{Fe} & \text{OH} \\
\text{Fe} & \text{O} \\
\text{chloride shift} & \\
\text{epoxidation} &
\end{align*}
\]

HCl + H$_2$ NAD$^+$

\[
\begin{align*}
\text{HCHO} & \\
\text{glyoxylate} & \\
\text{HOCl} & \\
\text{dichloroacetate} & \\
\text{HCOO} & \\
\text{formate} &
\end{align*}
\]
metabolically-activated inactivators (Ahern and Downing 1970). Mechanism-based inactivators have received much attention because of the specific and irreversible nature of their interaction with enzymes, and they have been utilized to investigate enzyme mechanisms, as therapeutic agents, and as inhibitors of select microbial processes (Walsh 1982; Silverman 1988; Ator and Montellano 1990; Ortiz de Montellano and Almira Correia 1991).

A set of seven criteria has been established in order to characterize a molecule as a mechanism-based inactivator (Walsh 1982; Silverman 1988). First, loss of enzymatic activity should be time-dependent, usually following pseudo first-order kinetics. Second, the rate of inactivation should exhibit saturation kinetics. Third, a physiological substrate for the enzyme should protect against inactivation. Fourth, inactivation should be irreversible. Fifth, a 1:1 stoichiometry of inactivator bound to the target enzyme should be realized following inactivation. Sixth, a catalytic activation step should be demonstrated. Seventh, there should be no lag time for inactivation. In practice it is difficult to unequivocally demonstrate each of these criteria, but it is possible to evaluate potential mechanism-based inactivators using these criteria as guidelines.

The potency of a mechanism-based inactivator can be described by its partition ratio. The partition ratio is the number of times that the inactivator is released from the active site of the target enzyme as a product per inactivation event (Waley 1985; Silverman 1988). A partition ratio of zero describes a perfect mechanism-based inactivator, one that covalently binds to the enzyme active site with each turnover event, while a large partition ratio describes an inefficient mechanism-based inactivator.

1.4.2. Alkynes and alkenes as mechanism-based inactivators of oxygenases

Given the reactive nature of the activated oxygen species and substrate intermediates involved in oxygenase catalyzed reactions and the broad substrate
ranges of these enzymes, it is hardly surprising that the oxidation of certain molecules can result in oxygenase inactivation. Indeed, a number of mechanism-based inactivators have been identified for monooxygenases and dioxygenases (Walsh 1984; Silverman 1988). Terminal alkynes and olefins are particularly well-studied and effective inactivators of cytochrome P-450 enzymes. Oxidation of alkenes by cytochrome P-450s results in formation of a radical intermediate that can alkylate the heme or rearrange into an epoxide product (Ator and Montellano 1990). Meanwhile, it is thought that the result of alkyne oxidation depends on the placement of the activated O species, with addition of O to the sub-terminal carbon resulting in heme alkylation, while addition of O to the terminal carbon generates a ketene, which can undergo hydrolysis to form a carboxylic acid or inactivate the enzyme by covalently binding to protein residues (CaJacob et al. 1988). It is important to note that the epoxide products of alkene oxidation do not damage cytochrome P-450s (Kunze et al. 1983). With alkenes the resulting heme adduct is a N-(2-hydroxyalkyl) moiety and with alkynes an N-(2-oxoalkyl) adduct is formed (Ator and Montellano 1990). Additionally, regiospecificity is observed during inactivation, in that olefins bind almost exclusively to the nitrogen of pyrrole ring D, whereas terminal alkynes (with the exception of acetylene) react with the nitrogen of pyrrole ring A (Kunze et al. 1983). Ortiz de Montellano et al. (1983) have provided evidence that the major determinant of the partition ratio between heme alkylation and epoxide formation upon terminal alkene oxidation by cytochrome P-450s is the orientation of the π bond relative to the heme. Generally, alkynes exhibit lower partition coefficients than alkenes with cytochrome P-450s.

Acetylene is also a well-known inactivator of several bacterial monooxygenases (Hyman and Arp 1988). Acetylene is a robust inactivator of ammonia monooxygenase in nitrifying bacteria. Loss of AMO activity in *N. europaea* cells exposed to acetylene was shown to be a time-dependent process requiring a catalytically favorable environment (i.e. O₂ was required) (Hyman and Wood 1985). Incubations of *N. europaea* cells with [¹⁴C]C₂H₂ resulted in covalent attachment of radiolabel to a single membrane polypeptide (27 kDa) (Hyman and Wood 1985).
experiment provided the initial evidence that the 27 kDa polypeptide contains the active site of AMO. Acetylene has also been demonstrated to act as a mechanism-based inactivator of sMMO and pMMO (Dalton and Whittenbury 1976; Stirling and Dalton 1977; Prior and Dalton 1985). $[^{14}C]C_2H_2$ specifically binds to a single polypeptide of 27 kDa when incubated with methanotrophic cells grown under conditions of copper sufficiency (expressing pMMO), while $[^{14}C]C_2H_2$ is covalently bound to a single polypeptide of 54 kDa in cells grown in copper-deficient environments (expressing sMMO) (Prior and Dalton 1985). Very low concentrations of acetylene (0.01 to 0.03%) are required to inactivate AMO and both forms of MMO (Dalton and Whittenbury 1976; Hynes and Knowles 1978).

The effects of longer chain terminal alkynes and alkynes with internally located triple bonds on oxygenase activity in *N. europaea* and methanotrophs has also been examined (Dalton and Whittenbury 1976; Stirling and Dalton 1977; Arp et al. 1996). 1-Alkynes up to C$_{10}$ are effective inactivators of AMO, sMMO, and pMMO. With AMO, 1-hexyne was found to be the most effective inactivator, whereas with sMMO and pMMO the potency of inactivation tends to decrease with increasing chain length $> C_2$. 2-Alkynes were not found to be inactivators of AMO, while 3-hexyne was as efficient an inactivator as acetylene. 3-Heptyne and 4-octyne inactivated AMO, but with less efficiency than acetylene. With sMMO and pMMO, internal alkynes were generally less effective than their terminal counterparts.

1.4.3. **Mechanism-based inactivators as experimental tools for TCE biodegradation**

Alkynes, in their capacity as mechanism-based inactivators, have found applications within the field of bioremediation as a tool to investigate the role that specific enzymes or microorganisms play in the transformation of xenobiotic compounds. For example, TCE degradation by both methanotrophs and *N. europaea* can be inhibited in the presence of acetylene (Arciero et al. 1989; Alvarez-Cohen and McCarty 1991). Indeed, Istok et al. (Istok et al. 1997) have utilized acetylene to examine the capacity of indigenous populations of methanogens and nitrifiers to
degrade various CAHs \textit{in situ} via a “push-pull” test. In another study it was suggested that phenylacetylene might be used to differentiate between AMO, sMMO, and pMMO activity \textit{in situ} (Lontoh et al. 2000).

Alkynes have been used to investigate the role of oxygenases in the degradation of CAHs in other groups of bacteria, as well. Propyne treatment has been shown to inhibit degradation of chlorinated C$_2$ and C$_3$ alkenes by propene monooxygenase in \textit{Xanthobacter} strain Py2 (Ensign et al. 1992). Interestingly, acetylene was shown to strongly inhibit TCE degradation by cells of the toluene-oxidizing bacterium, \textit{P. mendocina} KR1, yet acetylene treatment only resulted in partial inhibition of TCE degradation by two other toluene-oxidizing strains, \textit{B. cepacia} G4 and \textit{Pseudomonas} sp. strain ENVBF1 (McClay et al. 1995).

Acetylene has also been used to examine the toxicity associated with TCE oxidation by microorganisms. For example, radiolabeling of cell polypeptides of \textit{N. europaea} during [$^{14}$C]TCE incubations is prevented by the addition of acetylene to inactivate AMO (Rasche et al. 1991). Additionally, indices of the general cell health of methanotrophs, such as formate utilization and cell culturability, are not affected upon TCE exposure in cells pre-treated with acetylene (Alvarez-Cohen and McCarty 1991; van Hylckama Vlieg et al. 1997). Through such experiments investigators have firmly established that many of the toxic effects associated with TCE degradation are not caused by TCE itself, but rather a reactive metabolite of TCE oxidation.

1.5. Oxygenase inactivation upon TCE transformation

1.5.1. Toluene dioxygenase

A common feature of \textit{in vitro} TCE oxidation, catalyzed by bacterial and mammalian oxygenases, is turnover-dependent inactivation. Loss of toluene dioxygenase activity has been observed \textit{in vivo} during TCE transformation in several strains of \textit{P. putida} (Wackett and Gibson 1988; Heald and Jenkins 1994). With purified toluene dioxygenase from \textit{P. putida} F1, radiolabel was incorporated into each of the enzyme components upon incubations with [$^{14}$C]TCE (Li and Wackett 1992). Additionally, it was determined that 6% of the non-volatile products formed during
TCE oxidation resembled NADH-adducts. The authors suggest that the adducts arise from a covalent interaction between acyl chloride intermediates formed during TCE transformation with the NADH added to the reaction mixture. Interestingly, oxidation of one mol of TCE required 7.8 mol of NADPH, indicating that significant uncoupling occurs during the reaction.

1.5.2. **Soluble methane monooxygenase**

With purified sMMO, inactivation of each of the protein components accompanied TCE oxidation, and it was determined that complete inactivation occurred upon transformation of 200 molecules of TCE per molecule of hydroxylase (Fox et al. 1990). Several observations from this study provide suggestive evidence that the destructive species is a diffusible intermediate originating from TCE epoxide breakdown. First, the addition of cysteine to the reaction mixture protected each enzyme component from inactivation. Second, incubations performed with sMMO and [14C]TCE resulted in covalent attachment of radiolabel to each of the sMMO protein components, and the amount of radiolabel associated with each component corresponded roughly with the surface area of each. It was calculated that 14C-label from 1 out of every 30 molecules of [14C]TCE that had been transformed was covalently attached to a protein component within the reaction mixture.

Although the identity of the reactive species was not determined in the sMMO - TCE incubations, several lines of evidence led the authors to speculate that short-lived hydrolysis product(s) of either chloral and/or TCE epoxide could be the damaging agent. Incubations with TCE and sMMO but without NADH (to prevent turnover) did not result in enzyme inactivation. Additionally, the addition of cysteine to the reaction mixture resulted in a decrease in enzyme inactivation, yet was accompanied by an increase in concentration of TCE epoxide within the reaction mixture, suggesting that TCE epoxide itself is not the destructive species. Finally, it was determined that the presence of chloral did not cause sMMO inactivation. Since incubations containing sMMO and chloral did not result in enzyme inactivation, it is unlikely that potential intermediates of chloral degradation, such as dichloroacetyl chloride or
dichlorocarbene, can account for the damage. However, glyoxylic chloride and formyl chloride, two intermediates of TCE epoxide hydrolysis, are extremely electrophilic and could react rapidly with certain protein side groups.

### 1.5.3. Toluene 2-monooxygenase

Turnover-dependent inactivation of purified toluene 2-monooxygenase is also observed during TCE oxidation (Newman and Wackett 1997). Incubations with toluene 2-monooxygenase and \[^{14}C\]-TCE resulted in covalent modification and inactivation of each of the enzyme components, although the small regulatory component contained a higher density of radiolabel per surface area than the other components. Addition of cysteine had a protective effect on toluene 2-monooxygenase activity during TCE turnover, but the effect was limited mainly to the reductase component. The hydroxylase component lost the same percentage of activity during TCE oxidation with or without cysteine addition. This observation contrasts with the situation for sMMO in that the active site-containing component was not protected by cysteine addition, and it can be interpreted to suggest that inactivation is mediated by both a diffusive, reactive intermediate and a reactive species that interacts with the hydroxylase before it can escape the active site of the enzyme.

Inactivation of purified toluene 2-monooxygenase was observed in incubations containing only the enzyme components, O\(_2\), and NADH (Newman and Wackett 1997). Auto-inactivation of oxygenases has been documented and linked to reactive oxygen species formed upon the uncoupling of electron transfer from NAD(P)H to oxidizable substrates (Bernhardt and Kuthan 1981). Indeed, toluene 2-monooxygenase utilized 2.5 \(\mu M\) NADH per \(\mu M\) of TCE transformed, indicating that uncoupling could occur with this enzyme system. Accordingly, it was found that catalase and superoxide dismutase could protect toluene 2-monooxygenase from inactivation occurring in the absence of substrate. A similar phenomenon is thought to cause loss of methane monooxygenase activity in methanotrophs that have been aerated in the absence of substrate (Fox et al. 1989; Alvarez-Cohen and McCarty...
1991; Roslev and King 1995; Chu and Alvarez-Cohen 1999). However rates of oxygenase auto-inactivation are much slower than inactivation rates associated with TCE turnover and the two types of inactivation are usually distinguishable from one another (Newman and Wackett 1997; Chu and Alvarez-Cohen 1999).

1.5.4. Cytochrome P-450 monooxygenases

The most detailed studies concerning oxygenase inactivation during TCE oxidation have been conducted with mammalian cytochrome P-450 enzymes. The exact nature of the destructive species remains unknown, but the investigations have yielded several important mechanistic insights. It is recognized that transformation of TCE by cytochrome P-450 can result in four distinct outcomes: 1) formation of CO, formate, and glyoxylate via TCE epoxide 2) formation of chloral 3) suicidal heme destruction and 4) intermediates that covalently attach to various cell components (Miller and Guengerich 1983). As mentioned earlier, chloral is a major stable product of TCE oxidation by many cytochrome P-450 oxygenases (Miller and Guengerich 1983; Guengerich 1984); however, there has been considerable debate concerning the role of TCE epoxide in the formation of chloral (Bruckner et al. 1989). Although it has been argued that TCE epoxide is an obligate precursor of chloral formation by cytochrome P-450s, kinetic data and studies with bacterial monooxygenases support the mechanism proposed by Miller and Guengerich in which an enzyme bound TCE intermediate can either release from the active site as TCE epoxide or undergo a chloride shift to yield chloral as presented in Scheme 1 (Miller and Guengerich 1982; Fox et al. 1990; Newman and Wackett 1997).

Miller and Guengerich (1982) have also postulated that the heme-bound TCE intermediate could rearrange to form a covalent attachment with the prosthetic heme. They suggest that a radical intermediate attacks a porphyrin pyrrolic nitrogen resulting in a destructive heme adduct. Incubations with TCE epoxide and cytochrome P-450 do not result in heme alkylation (Miller and Guengerich 1982). A similar mechanism has been proposed to account for the inactivation of cytochrome P-450 by ethylene, alkynes, and vinyl fluoride, where the structure of the modified hemes has been
elucidated (Ortiz de Montellano et al. 1982). As such, trichloroethylene would act as a mechanism-based inactivator of cytochrome P-450 oxidases. Again the conformation of the P-450 active site and the environmental conditions surrounding the prosthetic heme would be expected to influence the rearrangement pathway for the bound TCE intermediate: chloral, TCE epoxide, or heme alkylation. Therefore, the extent to which TCE oxidation results in suicidal inactivation of individual cytochrome P-450 isozymes (or perhaps other classes of oxygenases capable of TCE transformation) will likely vary from enzyme to enzyme and will depend upon the localized conditions surrounding the enzyme during the transformation.

In addition to heme alkylation, reactive intermediates formed during TCE oxidation by cytochrome P-450s can migrate from the active site and covalently modify proteins, DNA, RNA, lipids, and other cellular components (Banerjee and Van Duuren 1978; Miller and Guengerich 1983; Bruckner et al. 1989). Although the identity of the reactive metabolite(s) has not been firmly established, acyl chlorides (breakdown products of TCE epoxide) or an unknown, unstable reaction product of the enzyme-bound TCE intermediate are leading candidates (Miller and Guengerich 1983; Halmes et al. 1996). TCE epoxide itself was largely discounted as the reactive species by Miller and Guengerich (1983) because: 1) TCE epoxide was produced by only 1 of eight P-450 isozymes at detectable levels, yet metabolites from each enzyme formed protein adducts 2) levels of TCE epoxide formation did not strictly correlate with the amount of adduct formation 3) an epoxide hydroxylase inhibitor, 3,3,3-trichloropropylene, increased TCE epoxide levels in liver microsomal protein incubations containing TCE, without a concomitant increase in adduct formation. Similarly, Miller and Guengerich (1983) did not observe a correlation between adduct formation and chloral production during the oxidation of TCE by cytochrome P-450s.

Several recent studies have provided evidence that acyl chlorides, formed upon TCE oxide hydrolysis, can covalently bind to cytochrome P450s. First, polyclonal antibodies that recognize dichloroacetylated lysine residues bound to a 50-kDa polypeptide, thought to be cytochrome P450 2E1, following incubations of TCE with mice microsomal liver fractions (Halmes et al. 1996). Subsequently, this antibody
preparation has been used to detect dichloacetyl-protein adducts in circulating proteins of TCE-exposed rodents (Halmes et al. 1996; Halmes et al. 1997). Cai and Guengerich (1999; 2000) confirmed the role of acyl chlorides in TCE-mediated adduct formation when they identified stable N^6-formyllysine, N^6-(dichloroacetyl)lysine, and N^6-glyoxyllysine adducts on proteins following incubations with TCE oxide and following TCE oxidation by several cytochrome P450 enzymes. Concentrations of TCE oxide in the low-mM range were shown to irreversibly inactivate aldolase and glucose-6-phosphate dehydrogenase, two enzymes that contain catalytically essential lysine groups. In a separate study, Cai and Guengerich (2001) provided evidence that the majority (80%) of protein adducts formed upon incubation with TCE oxide were unstable with a t_{1/2} of approximately 1 hour. Furthermore, they found that enzymes containing serine (α-chymotrypsin), cysteine (papain), or tyrosine and cysteine (D-amino acid oxidase) as essential amino acids exhibited time-dependent loss of activity upon TCE oxide incubations, but enzymatic activity could be recovered within 4-5 hours of the treatment, unlike proteins containing essential lysine groups. Additionally, these researchers were able to demonstrate that TCE oxide interacts with human P450 2E1, resulting in formyl- or dichloroacetyl-adduction to lysines at positions 87, 251, and 487 (Cai and Guengerich 2001). From these studies, the authors suggest that a dominant means of adduct formation upon TCE oxidation is through rearrangement of TCE oxide to acyl chlorides, which can form stable adducts with lysine residues or relatively unstable, ester type adducts with cysteine, serine, tyrosine, or threonine residues.

1.6. Cytotoxicity and carcinogenicity associated with TCE metabolism in mammalian cells

In a number of experiments with intact hepatocytes, reactive metabolites of TCE oxidation have been observed to migrate across the plasma membrane and covalently bind to extracellular proteins or DNA (Bruckner et al. 1989). A reactive intermediate(s) that possess sufficient stability to pass through the plasma membrane would certainly have time to diffuse throughout a cell and target a variety of cellular
constituents. Consequently, it is understandable that the relationship between cell toxicity and TCE oxidation seems rather complex.

Studies with rodents have indicated that TCE metabolism can produce toxic effects, most notably liver, lung, and kidney tumors [Goeptar et al. 1995]. The underlying cause of TCE-mediated carcinogenicity has not yet been determined, and in fact, there is still considerable debate whether or not the mechanism is genetic or epigenetic. Currently, three hypothesis are generally recognized which may explain the carcinogenic effects associated with TCE metabolism: 1) peroxisome proliferation induced by TCE metabolites (Ogino et al. 1991; Bull et al. 1993), 2) genotoxicity of GSH conjugates (Dekant et al. 1988; Goeptar et al. 1995), and 3) genotoxicity of reactive intermediates of TCE oxidation (Fahrig et al. 1995).

1.6.1. Peroxisome proliferation and genotoxicity of GSH-TCE conjugates

Peroxisome proliferation describes the extensive induction of peroxisomal and microsomal enzymes involved in β-oxidation of fatty acids in response to a diverse group of chemicals. Rats and mice appear to be particularly sensitive to this response, and hepatic, testicular, and pancreatic carcinomas have been linked to peroxisome proliferation in these animals (Reddy et al. 1980). It has been suggested that peroxisome proliferation leads to increased production of reactive oxygen species that can damage cellular components, including DNA. Alternatively, peroxisome proliferation could exert carcinogenic effects by inducing hepatic cell proliferation or via the action of the peroxisome proliferator-activated receptor, which can alter gene expression.

Metabolism of TCE has been demonstrated to elicit peroxisome proliferation in rodents, and it is largely believed that two metabolites of TCE oxidation, trichloroacetate and dichloroacetate, are the chemicals that actually induce the response (Bruschi and Bull 1993; Bull et al. 1993). Additionally, both trichloroacetate and dichloroacetate are capable of inducing liver tumors in mice (Herren-Freund et al. 1987; Bull et al. 1990). However, peroxisome proliferation cannot be initiated in cultured human hepatocytes (Elcombe 1985). In fact, the bulk of the research suggests
that peroxisome proliferation, if it is present at all, occurs to a much lesser extent in humans (Kluwe 1994). The contribution of peroxisome proliferation to TCE-mediated genotoxicity in humans remains to be determined.

In mammals, it has been demonstrated that a minor portion of TCE metabolism proceeds via a reductive pathway in which glutathione-S-transferase catalyzes the formation of a GSH-TCE conjugate (chlorovinyl glutathione), which is further transformed into a dichlorovinyl cysteiny1 adduct (Dekant et al. 1988; Henschler 1995; Moore and Harrington-Brock 2000). Metabolic products of the dichlorovinyl cysteiny1 adduct include dichlorovinyl mercapturic acid (which is excreted) or an unstable, reactive thiol (1,2- or 2,2-dichlorovinyl thiol) that can covalently modify cellular constituents or breakdown to form another reactive electrophile, chloroethenethione. The dichlorovinyl thiols have been shown to covalently bind to DNA, cause single strand breaks, and are mutagenic in bacterial test strains (Bruckner et al. 1989; Lash et al. 2000). However, the GSH-dependent pathway is involved in only a small fraction of TCE metabolism (approximately 1% or less), leading some investigators to postulate that products or intermediates of oxidative TCE metabolism may exert a greater toxicological effect.

### 1.6.2. Genotoxicity of reactive intermediates of TCE oxidation catalyzed by cytochrome P-450 monooxygenases

Cytotoxic effects associated with TCE oxidation by cytochrome P-450 monooxygenases include: adduction of reactive metabolites to DNA and proteins, loss of hepatic enzyme activity, single strand breaks in DNA, membrane damage, and cell necrosis (Miller and Guengerich 1983; Bruckner et al. 1989). Because of the link between TCE metabolism and tumor formation in rodents, the genetic toxicology of TCE oxidation has been studied most extensively (Fahrig et al. 1995). The capacity of $[^{14}\text{C}]$TCE to bind to DNA in vitro following bioactivation by cytochrome P-450s has been well documented (Banerjee and Van Duuren 1978; Di Renzo et al. 1982; Bergman 1983; Miller and Guengerich 1983). However, evidence for covalent modification of DNA in vivo upon TCE oxidation is somewhat limited. In two of the
pioneering studies, low levels of radiolabel were found associated with hepatic DNA extracted from rats and mice that had been exposed to $[^{14}\text{C}]$TCE, but the authors of each study suggested that the signal may have been caused by protein contamination (Parchman and Magee 1982; Stott et al. 1982). Bergman (Bergman 1983) also observed radiolabel in DNA extracted from a variety of tissues in mice that had been repeatedly injected with $[^{14}\text{C}]$TCE. However, radiolabel associated with DNA from all of the tissues examined, with the exception of liver, was attributed to metabolic incorporation (a process whereby radiolabel is incorporated into various macromolecules via C1 and C2 anabolic pathways). This study provided the first weak evidence for formation of adducts between TCE metabolites and hepatic DNA in vivo. Since then, several studies have provided data that suggests that TCE metabolites bind to DNA at low levels (Mazullo et al. 1992; Kautianinen et al. 1997). Kautianinen et al. (1997) found much higher levels of protein-TCE adducts (1.4 ng/g of protein) than DNA-TCE adducts (1.5 pg/g of DNA) in mice exposed to TCE, a general trend that has also been observed by other investigators (Miller and Guengerich 1983).

Recently, Cai and Guengerich (2001) used HPLC/MS to examine DNA adducts formed in incubations containing TCE epoxide and either one of each of the four nucleosides or an 8-mer oligonucleotide. With the nucleoside incubations, TCE oxide-derived adducts were detected only with deoxyguanosine, and the identity of the adduct was not determined due to the instability of the complex. With the oligonucleotide incubations, two formyl groups were found attached to the 8-mer (most likely to the two dGuo residues in the oligonucleotide). The oligonucleotide adducts were quite unstable though, with a $t_{1/2}$ of 30 min under the conditions utilized. The authors suggest that DNA adducts formed in vivo during TCE oxidation may also be labile, perhaps explaining the previously described imbalance between TCE-derived adducts found in DNA vs. proteins.

In addition to DNA adduct formation, there is evidence that TCE oxidation can lead to DNA strand breaks in vivo, induction of recombination in yeast, chromosome loss in fungi, and aneuploidies (Fahrig et al. 1995). However, TCE oxidation does not seem to cause gene mutations or structural chromosomal aberrations (Fahrig et al.
Overall, the genotoxic characteristics of TCE are rather atypical in that carcinogens usually test positive for a variety of indicator tests (especially gene mutation tests), whereas TCE has only been linked to a few, specific genotoxic effects (i.e. recombination induction and aneuploidy induction).

Of particular interest, TCE oxidation by mammalian liver microsomal preparations elicits virtually no genotoxic effects as determined by a suite of bacterial indicator tests. For example, TCE yielded negative results (with or without the S-9 liver microsomal protein mix) with the Umu test, in which a chemical is tested for its ability to induce expression of the *umuC-lacZ* reporter fusion in *Salmonella typhimurium* strain TA1535/pSK1002 via the SOS response system (Nakamura et al. 1987). TCE also yielded negative results (with or without the S-9 mix) with the SOS chromotest, Rec assay, and Ara test (Fahrig et al. 1995). The rec assay measures the survival rate of *recE*<sup>+</sup> vs. *recE* (recombination-defective) strains of *Bacillus subtilis* in the presence of the test chemical. The Ara test examines the mutation rate caused by the test compound by monitoring the number of L-arabinose resistant revertants in *Salmonella typhimurium*. The results of these tests have been interpreted to signify that TCE and the reaction products of TCE oxidation by mammalian cytochrome P-450 monooxygenases have little or no genotoxic effect on bacteria. It should be noted that any DNA-damaging species formed during TCE oxidation would have to cross the bacterial membrane to elicit an effect in these tests.

### 1.7. Cytotoxicity associated with TCE oxidation in bacteria

A link between cellular toxicity and TCE oxidation in bacteria was initially proposed by Wackett and Gibson (Wackett and Gibson 1988) to explain the decrease in TCE consumption over a relatively short time span (20 min) by *P. putida* F1. Since then, turnover-dependent loss of activity has become a hallmark of aerobic TCE degradation by microorganisms (Oldenhuis et al. 1991; Rasche et al. 1991; Ensign et al. 1992; van Hylckama Vlieg et al. 1997). As discussed previously, TCE turnover results in enzyme inactivation with each of the TCE-degrading, bacterial oxygenases that have been purified to date (Fox et al. 1990; Li and Wackett 1992; Newman and
Wackett 1997); therefore, it has been generally accepted that TCE acts as a mechanism-based or metabolically activated inactivator of oxygenase activity in vivo. In fact, the ability of certain bacterial strains to sustain constant rates of TCE oxidation over relatively short time periods has been interpreted to signify a relative lack of toxicity associated with the reaction (Ewers et al. 1990; Folsom et al. 1990; Sun and Wood 1996).

1.7.1. Effect of TCE oxidation on general indices of cell health

Toxicity associated with TCE oxidation in bacterial cells is not limited to the transforming oxygenase, as the reaction has also been shown to elicit general cellular damage. For example, growth rates of *P. putida* strains (Wackett and Householder 1989; Heald and Jenkins 1994), *M. trichosporium* OB3b (Sipkema et al. 2000), *B. cepacia* G4 (Mars et al. 1996), and various other bacteria (Leahy et al. 1996) have been reported to decline in the presence of TCE. Two independent mechanisms of toxicity seem to be at play when cells are exposed to TCE. First, cells exposed to high concentrations of TCE can suffer immediate toxic effects, most likely a consequence of the solvent properties of TCE. Second, toxic effects associated with TCE have been shown to be turnover dependent in many cases. For example, TCE vapors caused the doubling time of toluene-induced cells of wild type *P. putida* F1 growing on arginine amended medium to increase from 1.5 h to 5.0 h, but did not affect the growth rate of toluene-induced cells of a *P. putida* F1 mutant that does not express toluene dioxygenase (Wackett and Householder 1989). Similarly, the doubling time of uninduced, wild type *B. cepacia* G4 grown on glucose was unaltered by the presence of TCE (up to 530 μM), whereas the doubling time of a mutant of this strain, *B. cepacia* G4 5223 PR1, that constitutively expresses toluene 2-monoxygenase increased approximately two fold in the presence of TCE (Shields et al. 1994).

Further evaluation of the effects of TCE oxidation on general cell health in methanotrophic bacteria has revealed that cell viability and respiratory activity can be severely compromised. Chu and Alvarez-Cohen (1999) utilized microscopic analysis of 5-cyano-2,3-ditolyl tetrazolium chloride-stained cells to examine the effect of TCE
oxidation on the respiratory activity of two methanotrophs, *M. trichosporium* OB3b and strain CAC-2. They observed a linear decrease in respiratory activity as a function of the amount of TCE degraded until less than 5% of the original respiratory activity remained. The authors suggested that once cells had lost $\geq 95\%$ of their respiratory activity they were essentially incapable of cell division or recovery of TCE-degrading activity. In a separate study, the viability of *M. trichosporium* OB3b was examined following degradation of several chlorinated ethenes, including TCE (van Hylckama Vlieg et al. 1997). Colony counts on agar plates were used to assess the viability of cells, and it was found that the viability of cultures decreased exponentially with the amount of TCE degraded per unit of biomass. In contrast, loss of sMMO activity proceeded in a linear relationship with the amount of TCE degraded in the assays. These observations led the authors to conclude two important points. First, the predominant toxic effect associated with TCE oxidation in *M. trichosporium* OB3b was loss of cell viability rather than inactivation of sMMO. Second, TCE-mediated inactivation of sMMO in *vivo* must proceed via a process in which most of the oxidized intermediates are released from the active site of the enzyme.

1.7.2. $^{14}$C TCE radiolabeling studies

To identify potential targets of reactive TCE intermediates, $^{14}$C TCE labeling studies have been performed with various microorganisms. SDS-PAGE analysis of $^{14}$C TCE-exposed cell extracts of *M. trichosporium* OB3b revealed nonspecific binding of radiolabel to various polypeptides (Oldenhuis et al. 1991). Although the $\alpha$-subunit of the hydroxylase component of sMMO accounted for 10% of the bound radiolabel, the $\beta$- and $\gamma$-subunits of the hydroxylase were not labeled. With a similar SDS-PAGE-$^{14}$C TCE experiment performed with *Neuropaea* cells, up to 15 individual bands were radiolabeled and most of the radioactivity detected on the gel ran with the dye front, suggesting that small molecules were also labeled (Rasche et al. 1991). A 27-kDa polypeptide thought to contain the active site of ammonia monooxygenase was one of the heaviest labeled bands observed on the gel. When ammonia monooxygenase was inhibited with allylthiourea or inactivated with
acetylene during the $[^{14}\text{C}]\text{TCE}$ incubations, cellular proteins were not radiolabeled, indicating that TCE turnover was required to produce the modifying agent.

Wackett and Householder examined the distribution of $[^{14}\text{C}]\text{TCE}$ modified cellular constituents in $P$. putida F1 (Wackett and Householder 1989). Following $[^{14}\text{C}]\text{TCE}$ exposure, cells were fractionated and the $[^{14}\text{C}]\text{TCE}$ equivalents incorporated (nmol) into various macromolecules were determined as follows: protein (113), RNA (15), DNA (3), lipid (2), and small molecules (33). Cell fractions from $[^{14}\text{C}]\text{TCE}$-treated $P$. putida F4 (a F1 derivative that lacks toluene dioxygenase activity) yielded insignificant amounts of radiolabel. An insight into the identity of the reactive TCE intermediates was also obtained in this study. HPLC analysis of acid hydrolysate of TCE-modified proteins from $P$. putida F1 revealed two major peaks with elution profiles matching those of glyoxylic acid and formic acid, leading the authors to suggest that the glyoxylyl chloride and formyl chloride (breakdown products of TCE epoxide) could react with nucleophilic amino acids side chains of proteins.

1.7.3. Transformation capacity and TCE oxidation

In order to design effective, aerobic bioremediation systems for TCE, a number of biochemical and kinetic models have been developed to describe the process (Alvarez-Cohen and McCarty 1991; Criddle 1993; Ely et al. 1995; Sipkema et al. 2000). These models often incorporate parameters or equations that describe the kinetics of TCE conversion, reductant flow, inhibition between TCE and the growth substrate for the oxygenase active site, and more recently, the toxicity associated with TCE turnover. As a measure of toxicity many of the models utilize a parameter termed transformation capacity ($T_c$), which is defined as the maximum mass of a compound that can be degraded per mass of cells prior to inactivation (Alvarez-Cohen and McCarty 1991). The $T_c$ for TCE transformation has been especially well-used in studies involving methanotrophs with values ranging from 0.3 to 4.1 umol of TCE transformed per mg of cells (dry weight) (Chang and Alvarez-Cohen 1996; van Hylckama Vlieg et al. 1997; Sun et al. 1998). With methanotrophs, the addition of formate to the TCE consumption assays usually increases the $T_c$ of the cells by
roughly two-fold. Apparently, formate provides the cells with extra reductant (NADH) enabling further oxidation of TCE by sMMO (alternatively the extra reductant could be utilized by protective or repair mechanisms to assuage TCE-related damage). Reported Tc values (umol TCE mg cells⁻¹) for TCE transformation by other bacteria include: 0.24 for a phenol-oxidizing consortia, 0.06 for a toluene-oxidizing culture, 0.05 for a propane-utilizing mixed culture, 0.06 for *N. europaea*, and approximately 0.44 for *B. cepacia* PR123 (a derivative of *B. cepacia* G4 that constitutively expresses toluene 2-monooxygenase) (Chang and Alvarez-Cohen 1995; Sun et al. 1998). Two characteristics of Tc values are worth mentioning. First, they are not static values and can vary significantly depending on the experimental conditions with differences of up to 20 fold. Second, the Tc provides a measure of specific enzymatic activity over time; it does not assess the overall physiological status of the cell. Therefore, it may not quantify toxicity at the cellular lever, but rather at the level of oxygenase activity.

1.8. **Research objectives**

Aerobic cometabolism is an attractive technology for remediation of halogenated aliphatic hydrocarbons. The approach is potentially cost-effective and results in complete mineralization of many target compounds, including the common and persistent groundwater pollutant TCE. From a biological standpoint, degradation of CAHs by aerobic cometabolism is often constrained by the toxicity that is associated with the initial oxidation reaction (Wackett and Householder 1989; Oldenhuis et al. 1991; Rasche et al. 1991; Hyman et al. 1995; van Hylckama Vlieg et al. 1997; Chu and Alvarez-Cohen 1999). Yet the mechanisms, targets, and physiological implications of these toxic effects are for the most part poorly understood. The primary goal of the research described in this dissertation was to investigate the physiological effects of TCE oxidation on the well-studied bacterium *B. cepacia* G4.

With both cytochrome P-450s and bacterial oxygenases it has been suggested that TCE may act as a mechanism-based inactivator. As such, it is advantageous to compare and contrast the effects of TCE oxidation on a particular oxygenase to the
action of known mechanisms-based inactivators. Acetylene and other alkynes are potent mechanism-based inactivators of both cytochrome P-450 monooxygenases and many bacterial monooxygenases involved in TCE degradation including AMO, sMMO, pMMO, and propene monooxygenase (Dalton and Whittenbury 1976; Colby et al. 1977; Stirling and Dalton 1977; Hyman and Wood 1985; Silverman 1988; Ensign et al. 1992). Yet, it has been reported that acetylene is a weak inhibitor of TCE-degrading activity in B. cepacia G4 (Dalton and Whittenbury 1976; Colby et al. 1977; Stirling and Dalton 1977; Hyman and Wood 1985; Silverman 1988; Ensign et al. 1992; McClay et al. 1996). I was intrigued by this observation in light of the fact that B. cepacia G4 was also reportedly relatively impervious to TCE-related toxicity (Nelson et al. 1987; Folsom et al. 1990; Folsom and Chapman 1991). Therefore, I systematically examine the effects of acetylene and other alkynes on the toluene-oxidizing activity of this microorganism in Chapter 2.

The development of alkynes, other than acetylene, as specific inactivators of toluene 2-monooxygenase activity in B. cepacia G4, and the observation that short chain alkenes are also oxidized by this enzyme, without any signs of inactivation, provided a means to dissect the effects of TCE oxidation on toluene 2-monooxygenase activity, general respiratory activity, cell energetics, and cell culturability in B. cepacia G4. The effects of TCE cometabolism on these indexes of cell health are characterized in Chapter 3. Insights gained from the research presented in Chapters 2 and 3 led us to propose that B. cepacia G4 possess cellular defense mechanisms and/or repair systems that act to limit the toxicity associated with TCE oxidation. Therefore, I initiated investigations to examine the physiological and genetic determinants of B. cepacia G4 that are involved in mediating the toxic effects associated with TCE oxidation in Chapter 4.
Chapter 2

Inactivation of Toluene 2-Monooxygenase in *Burkholderia cepacia* G4 by Alkynes

Chris M. Yeager, Peter J. Bottomley, Daniel J. Arp, and Michael R. Hyman

Published in *Applied and Environmental Microbiology*, American Society for Microbiology, February 1999, Vol. 65, p. 632-639
2.1. Abstract

High concentrations of acetylene (10-50% [vol/vol] gas phase) were required to inhibit growth of *Burkholderia cepacia* G4 on toluene, while 1% (vol/vol) (gas phase) propyne or 1-butyne completely inhibited growth. Low concentrations of longer chain alkynes (C₅ to C₁₀) were also effective inhibitors of toluene-dependent growth, and 2- and 3-alkynes were more potent inhibitors than their 1-alkyne counterparts. Exposure of toluene-grown *B. cepacia* G4 to alkynes resulted in the irreversible loss of toluene- and o-cresol-dependent O₂ uptake activities, while acetate- and 3-methylcatechol-dependent O₂ uptake activities were unaffected. Toluene-dependent O₂ uptake decreased upon addition of 1-butyne in a concentration- and time-dependent manner. The loss of activity followed first-order kinetics, with apparent rate constants ranging from 0.25 min⁻¹ to 2.45 min⁻¹. Increasing concentrations of toluene afforded protection from the inhibitory effects of 1-butyne. Furthermore, oxygen, supplied as H₂O₂, was required for inhibition by 1-butyne. These results suggest that alkynes are specific, mechanism-based inactivators of toluene 2-monooxygenase in *B. cepacia* G4, although the simplest alkyne, acetylene, was relatively ineffective compared to longer alkynes. Alkene analogs of acetylene and propyne—ethylene and propylene—were not inactivators of toluene 2-monooxygenase activity in *B. cepacia* G4 but were oxidized to their respective epoxides, with apparent Kᵣ and V_max values of 39.7 μM and 112.3 nmol min⁻¹mg of protein⁻¹ for ethylene and 32.3 μM and 89.2 nmol min⁻¹mg of protein⁻¹ for propylene.

2.2. Introduction

Molecules which inactivate an enzyme after undergoing a catalytic transition in the active site are known as mechanism-based inactivators or suicide substrates (Walsh 1982; Silverman 1988; Ator and Montellano 1990). Posing as a substrate, the inactivator binds to the target enzyme and is catalytically converted into a reactive species that can covalently bind to an active site amino acid or prosthetic group, causing a concurrent loss of enzyme activity. Due to the specificity and irreversible
nature of this interaction, mechanism-based inactivators are versatile tools that have been used as probes for enzyme mechanisms, as therapeutic agents, and as inhibitors of microbial processes (Walsh 1982; Silverman 1988; Ator and Montellano 1990; Ortiz de Montellano and Almira Correia 1991).

Alkynes are a well known class of mechanism-based inactivators of a number of oxygenase enzymes, including several bacterial monooxygenases (Hyman and Arp 1988; Silverman 1988). For example the simplest alkyne, acetylene, is a potent inactivator of ammonia monooxygenase (AMO) from the nitrifying bacterium *Nitrosomonas europaea*. Inhibition of AMO activity in *N. europaea* by acetylene has been shown to be a saturable, time- and O₂-dependent reaction and incubation of cells with ¹⁴C₂H₂ results in the specific, covalent radiolabeling of a membrane polypeptide which is thought to contain the active site of the enzyme (Hyman and Wood 1985). Similar kinetic and radiolabeling results obtained from studies with methanotrophic bacteria indicate that acetylene is a mechanism-based inactivator of both the particulate (pMMO) and soluble (sMMO) forms of methane monooxygenase (Dalton and Whittenbury 1976; Stirling and Dalton 1977; Prior and Dalton 1985). In spite of the differences among these three enzymes, they are all inactivated by low concentrations of acetylene (0.01-0.03%) (Dalton and Whittenbury 1976; Hynes and Knowles 1978), and are all capable of oxidizing the chlorinated solvent trichloroethylene (TCE) (Arciero et al. 1989; Oldenhuis et al. 1989; Tsein et al. 1989; DiSpirito et al. 1992).

A variety of other microorganisms are also known to oxidize TCE through the activity of nonspecific oxygenase enzymes. Among these, most attention has been given to the toluene-oxidizing organism *Burkholderia cepacia* G4. This organism initiates metabolism of toluene via successive hydroxylations at the ortho and then the adjacent meta position of the aromatic ring, immediately followed by meta cleavage of the catechol intermediate (Arciero et al. 1989; Oldenhuis et al. 1989; Shields et al. 1989; Tsein et al. 1989; DiSpirito et al. 1992; Newman and Wackett 1995). Genetic and biochemical studies strongly suggest that the enzyme toluene 2-monooxygenase is singularly responsible for both of the hydroxylation reactions required to initiate
toluene catabolism and for the cometabolic oxidation of TCE by *B. cepacia* G4 (Shields et al. 1991; Newman and Wackett 1995; Newman and Wackett 1997). Furthermore, biochemical analysis of the purified enzyme and sequence comparisons indicate that toluene 2-monoxygenase is part of a family of binuclear-iron enzymes that contains several other hydrocarbon- and TCE-oxidizing oxygenases, including the well characterized sMMO (Wilkins 1992; Fox et al. 1994; Newman and Wackett 1995).

Despite the strong catalytic and structural similarities between toluene 2-monoxygenase and sMMO, these two enzymes appear to differ considerably in their sensitivity to acetylene. While sMMO-catalyzed reactions such as TCE oxidation are known to be readily inactivated by acetylene (Prior and Dalton 1985; Alvarez-Cohen and McCarty 1991), a recent study suggested that this compound is a weak inhibitor of the TCE-degrading activity of *B. cepacia* G4 (Prior and Dalton 1985; Alvarez-Cohen and McCarty 1991; McClay et al. 1996). These observations suggested two possibilities to us. First, it may be that acetylene exerts its inhibitory effects on toluene oxidation through a different mechanism than the inactivation-based mechanisms observed with several other bacterial oxygenases. Second, it is possible that acetylene acts as a conventional, albeit unusually weak, mechanism-based inactivator of toluene-oxidizing activity. The aim of the present study was to resolve these questions by examining the effects of acetylene and other alkynes on the toluene-oxidizing activity of *B. cepacia* G4.

2.3. Materials and Methods

2.3.1. Chemicals and reagents

Acetylene was generated from calcium carbide (technical grade; Aldrich, Milwaukee, WI). Propyne (97%), 1-hexyne, phenylacetylene, 3-phenyl-propyne, 1-ethynylcyclohexylamine, toluene, o-cresol, 3-methylcatechol, propylene, and propylene oxide were also obtained from Aldrich. 1- and 2-Butyne, 1- and 2-pentyne, 2- and 3-hexyne, and 1-decyne were obtained from Farchan Laboratories, Inc
(Gainesville, FL). Other reagents and their sources include N,N-dimethylformamide (Sigma, St. Louis, MO), ethylene (Airco, Murray Hill, NJ), and ethylene oxide (MG Industries, Malvern, PA). All other chemicals were of reagent grade or better.

2.3.2. **Bacterial strain and culture conditions**

*B. cepacia* G4 was kindly provided by Dr. Malcolm Shields (University of West Florida, Pensacola) and was maintained on minimal medium agar plates containing 20 mM lactate. The minimal medium contained (per liter) 0.5 g NH₄NO₃, 0.2 g MgSO₄·7H₂O, 0.05 g CaCl₂·2H₂O, 0.01 g Na₂EDTA, 0.005 g FeCl₃, 50 ml of 1 M KH₂PO₄/K₂HPO₄ (pH 7.0), and 10 ml trace elements solution (0.143 g H₃BO₃, 0.102 g MgSO₄·7H₂O, 0.032 g ZnSO₄·7H₂O, 0.01 g CoCl₂·6H₂O, 0.008 g CuSO₄·5H₂O, 0.005 g Na₂MoO₄·2H₂O per liter). Liquid cultures were grown overnight with shaking (200 rpm) at 30°C in glass serum vials (160 ml) containing minimal media (60 ml) and either lactate (20 mM) or toluene (94 μmol, 1 mM aqueous phase; added neat). The vials were sealed with butyl rubber stoppers. At 4 h before harvest, additional toluene (94 μmol) was added to toluene-grown bacteria. Lactate-grown cells were not amended before harvest. Cells were pelleted by centrifugation (6000 x g, 10 min) and resuspended in 30 ml of phosphate buffer (50 mM KH₂PO₄-K₂HPO₄, [pH 7.0]). Cells were then recentrifuged, resuspended in 30 ml phosphate buffer, and pelleted again. Finally, the washed cells were resuspended in phosphate buffer (1 ml), yielding a final concentration of 2 to 4 mg protein per ml. The concentrated cell suspension was stored at room temperature for ≤ 3 h before use. Toluene-oxidizing activities remained constant over this storage period.

2.3.3. **Analytical and other methods**

Hydrocarbons were analyzed with a Shimadzu (Kyoto, Japan) GC-8A chromatograph equipped with a flame ionization detector and a stainless steel column (0.3 x 122 cm) packed with Porapak Q 80-100 mesh (Alltech, Deerfield, IL). The column temperature was 135°C for the experiment described in the legend to Fig. 6.
Propylene and propylene oxide were determined using a column temperature of 125°C, whereas ethylene and ethylene oxide were determined at a column temperature of 100°C. The injector and detector temperatures were set at 200°C for all analyses. Substrate consumption was determined by peak area quantification using a HP3395 integrator (Hewlett Packard, Palo Alto, CA). Data for determination of stoichiometry and kinetic constants were obtained by comparison of propylene and epoxide peak heights to standard curves constructed from known amounts of the authentic compounds. The concentration ranges for primary substrates used to construct standard curves were as follows: 0.1 to 5.0 µmol for propylene oxide, 0.2 to 10.0 µmol for ethylene oxide, and 0.45 to 8.9 µmol for propylene. All standard curves were linear over these substrate ranges, with an $r^2$ value of ≥ 0.98.

The aqueous concentration of toluene in two-phase systems was calculated with a dimensionless Henry’s constant that was determined empirically to be 0.343 at 30°C (Gossett 1987). The concentrations of ethylene and propylene in the aqueous phase were calculated with Henry’s constants derived from the data of Wilhelm et al. (Wilhelm et al. 1977). The presence of B. cepacia G4 cells at the concentrations used in these experiments did not affect the partitioning of toluene, ethylene, or propylene between the liquid (1.5 ml phosphate buffer) and gas (8.5 ml) phases compared to cell-free controls. Protein concentrations were determined with the biuret assay (Gornall et al. 1949) following cell solubilization in 3 N NaOH for 30 min at 65°C. Bovine serum albumin was used as the standard.

## 2.3.4. Growth inhibition by alkynes

Toluene- or lactate-grown cells were cultivated in glass serum vials (160 ml) sealed with Teflon-lined butyl stoppers (Supelco, Bellefonte, PA). Prior to inoculation, the required concentrations of toluene, lactate, and alkynes were added to the vials through the stoppers by use of sterile syringes. The vials were then incubated under experimental conditions (30°C with shaking [200 rpm]) for at least 2 h to allow equilibration of the hydrocarbons between the gas and liquid phases. The incubations
were initiated by the addition of concentrated, toluene-grown *B. cepacia* G4. The cells were injected into the sealed vials through the stoppers to give an initial optical density at 600 nm of 0.05. Samples (1 ml) were aseptically withdrawn from sealed vials throughout the experiment to monitor cell growth, as determined by measurements of optical density at 600 nm. In all experiments, acetylene, propyne, and 1-butyne were added as gases. Other alkynes and aromatics were added from stock solutions prepared in N,N-dimethylformamide. Abiotic control incubations were also established for each experimental condition to determine the effect of repeated puncturing of the stoppers on the concentrations of each substrate and each inhibitor. Headspace analysis by gas chromatography revealed that the abiotic losses of all substrates were less than 5% of the initial values.

### 2.3.5. Inhibition of O₂ uptake by alkynes

O₂ uptake measurements were determined with a Clark (Yellow Springs, Ohio)-style O₂ electrode mounted in a glass water-jacketed reaction vessel (1.6 ml) maintained at 30°C. In all assays, the reaction chamber was filled with phosphate buffer before the addition of cells or other reactants. A basal rate of cellular respiration was established by measuring O₂ uptake in the absence of substrate and this value was subtracted from all substrate-induced rates to yield the substrate-dependent O₂ uptake rate. To determine rates of alkyne-dependent inactivation of toluene-dependent O₂ uptake, tangents were drawn to the nonlinear O₂ electrode traces at selected times following addition of each inactivator. These rates were plotted against time and were fitted to a first order exponential decay curve to determine the apparent rate constant for activity loss (k_{obs}) at each inactivator concentration.

The specificity of alkyne inactivation in *B. cepacia* G4 was determined by measuring the effects of alkyne exposure on various substrate-specific O₂ uptake rates. Concentrated toluene-grown cells (130 µg of protein) were added to phosphate buffer (final reaction volume, 1 ml) in glass serum vials (10 ml) sealed with Teflon-lined butyl rubber stoppers. Specific alkynes (0.45 μmoles) were added separately to individual vials with a gas-tight syringe, and the vials were incubated for 30 min in a
shaking water bath (30°C, 150 rpm). A control vial to which no alkyne was added was included. After the reaction period, the cells were sedimented in a microcentrifuge (30 sec at 14000 x g), washed twice with phosphate buffer, and resuspended in phosphate buffer (150 μl). Samples of the washed cell suspension were then examined for residual O₂ uptake activities in the presence of toluene (200 μM), o-cresol (200 μM), 3-methylcatechol (400 μM), or acetate (1 mM). Residual activity was determined by comparing the substrate-dependent O₂ uptake rate obtained from alkyne-treated cells with the corresponding rate determined from cells recovered from the control vial.

2.3.6. Requirement of O₂ for inactivation

A culture of toluene-grown B. cepacia G4 (500 ml) was harvested and resuspended in phosphate buffer (8 ml). A portion of this concentrated cell suspension was used to completely fill an O₂ electrode chamber, which was quickly (< 1 min) driven anaerobic by endogenous respiration. A 5.5 cm capillary inlet filled with the same concentrated cell suspension prevented atmospheric O₂ from entering the electrode chamber during the experiment. At various times after the chamber became anaerobic, cell samples (20 μl) were removed and added to another O₂ electrode chamber containing phosphate buffer and toluene (400 μM) to measure toluene-dependent O₂ uptake rates. At 30 min after the initiation of the experiment, 1-butyne (120 μM, final concentration) was added to the anaerobic cell suspension, and at 54 and 77 minutes, H₂O₂ (400 nmol) was introduced into the chamber as a source of O₂. In a control experiment, the procedure was repeated without addition of 1-butyne. In all cases, the additions of substrates and inhibitors to the electrode chambers involved dilution of the reaction mixture by 2% or less. Both electrode chambers were maintained at 30°C with a circulating water bath.
2.3.7. Alkyne and alkene oxidation

Consumption of short chain alkenes and alkynes by *B. cepacia* G4 in resting-cell assays was examined by gas chromatography. Concentrated toluene-grown *B. cepacia* G4 cells (300 μg of protein) were added to phosphate buffer (final reaction volume, 1ml) in glass serum vials (10 ml) sealed with Teflon-lined butyl rubber stoppers. Heat-inactivated cells (95° C, 15 min) were added to one vial as a control. In two other vials, cells were preincubated (30 min, 30° C) with 1-butyne (4.5 μmol) to inactivate toluene 2-monooxygenase activity prior to the addition of alkenes. Reactions were initiated by addition of 10 μl (0.45 μmol) of either ethylene, propylene, acetylene, or propyne with a gas-tight microsyringe. Reaction mixtures were incubated at 30° C with shaking (150 rpm); periodically, headspace samples (20 μl) were analyzed by gas chromatography as described above. Liquid samples (4 μl) were removed at select time points for comparative analysis of soluble products against epoxide standards (see above).

The epoxide nature of the products obtained from the transformation of ethylene and propylene by *B. cepacia* G4 was confirmed by acid hydrolysis. Epoxides undergo C-O bond cleavage under acidic conditions to yield 1,2-diols, which are not detectable by gas chromatographic analysis of headspace samples (100 μl) under the conditions described above (epoxides are detectable). Therefore, headspace samples from reaction vials in which ethylene or propylene had been consumed were analyzed by gas chromatography before and after the addition of 9 N H₂SO₄ (50 μl).

For examination of the stoichiometry of propylene oxidation to propylene oxide, cells (300 μg of protein) and propylene (5.5 μmol) were incubated at 30° C with shaking (150 rpm) for 4 h. Headspace (20 μl) and liquid (4 μl) samples were analyzed by gas chromatography (see above) at selected times.

2.3.8. Kinetic analysis of propylene and ethylene oxidation

Various amounts of ethylene or propylene (0.45 to 25 μmol) were added to sealed glass serum vials (10 ml) containing phosphate buffer (final reaction volume,
1 ml). The alkenes were allowed to equilibrate between the gas and liquid phases by shaking (150 rpm) for 2 h at 30°C. Concentrated toluene-grown *B. cepacia* G4 cells (400-600 µg of protein) were added to initiate the reaction. After 20 min, liquid samples (4 µl) were removed for analysis of epoxide formation by gas chromatography. The rates of epoxide formation (v) were plotted against the corresponding initial propylene or ethylene concentration (S) and fit by least-squares regression analysis to the Michaelis-Menten equation \[v = \frac{V_{max} \times S}{K_m + S}\] to determine the apparent K<sub>s</sub> and V<sub>max</sub>.

2.4. Results

2.4.1. Inhibition of growth by alkynes

In the presence of 1% (vol/vol) acetylene (gas phase), the growth curve of *B. cepacia* G4 was similar to a control containing no acetylene. However, the presence of 10, 25, and 50% acetylene progressively inhibited growth (Fig 2.1A). Acetylene concentrations remained constant throughout the experiments, as determined by gas chromatography (data not shown). Growth of *B. cepacia* G4 on lactate was largely unaffected by the presence of acetylene at all concentrations tested (Fig 2.1B). In contrast to acetylene, considerably lower concentrations of 1-butyne were required to inhibit the growth of *B. cepacia* G4 on toluene (Fig 2.1C). 1-Butyne exhibited inhibitory effects at levels as low as 0.05%, while 1.0% completely halted growth over the full time course of the experiment. Similar results were obtained with equivalent concentrations of propyne (data not shown). The growth of *B. cepacia* G4 on lactate was unaffected by the addition of 1% propyne or 1% 1-butyne (Fig 2.1D). Additionally, *B. cepacia* G4 grew in the presence of toluene and 1% 1-butyne upon the addition of 20 mM lactate (data not shown).
Fig. 2.1. Growth of *B. cepacia* G4 in the presence of various alkynes. Growth substrates were 1 mM toluene (A and C) and 20 mM lactate (B and D). Symbols in panels A and B represent initial amounts (volume/volume) (gas phase) of acetylene added to growth vials: □, 0%; ●, 1%; ▲, 10%; ◆, 25%; and ▼, 50%. Symbols in panel C represent initial amounts (volume/volume) (gas phase) of 1-butyne added to growth vials: □, 0%; ●, 0.01%; ▲, 0.05%; ◆, 0.1%; and ▼, 1.0%. Symbols in panel D: □, no alkyne; ●, 1% propyne; ▲, 1% 1-butyne.
Fig. 2.1

A

B

C

D

Absorbance at 600 nm

Time (h)
Table 2.1. Inhibition of toluene-dependent growth of *B. cepacia* G4 by alkynes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>45 µmolb</th>
<th>4.5 µmol</th>
<th>0.45 µmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetylene</td>
<td>0.35±.03 (−)</td>
<td>0.35±.02 (−)</td>
<td>0.35±.02 (−)</td>
</tr>
<tr>
<td>propyne</td>
<td>0.04±.00 (++)</td>
<td>0.10±.01 (+)</td>
<td>0.30±.09 (+)</td>
</tr>
<tr>
<td>1-butynẽ</td>
<td>0.04±.00 (++)</td>
<td>0.06±.02 (++)</td>
<td>0.27±.05 (+)</td>
</tr>
<tr>
<td>2-butynẽ</td>
<td>0.03±.00 (++)</td>
<td>0.03±.00 (++)</td>
<td>0.04±.00 (++)</td>
</tr>
<tr>
<td>1-pentynẽ</td>
<td>0.04±.01 (++)</td>
<td>0.04±.01 (++)</td>
<td>0.19±.02 (+)</td>
</tr>
<tr>
<td>2-pentynẽ</td>
<td>0.04±.00 (++)</td>
<td>0.04±.00 (++)</td>
<td>0.04±.01 (++)</td>
</tr>
<tr>
<td>1-hexynẽ</td>
<td>0.05±.01 (++)</td>
<td>0.05±.01 (++)</td>
<td>0.18±.04 (+)</td>
</tr>
<tr>
<td>2-hexynẽ</td>
<td>0.04±.00 (++)</td>
<td>0.04±.00 (++)</td>
<td>0.05±.01 (++)</td>
</tr>
<tr>
<td>3-hexynẽ</td>
<td>0.04±.00 (++)</td>
<td>0.04±.01 (++)</td>
<td>0.08±.01 (+)</td>
</tr>
<tr>
<td>1-decyne</td>
<td>0.05±.01 (++)</td>
<td>0.07±.03 (++)</td>
<td>0.23±.10 (+)</td>
</tr>
<tr>
<td>phenylacetylene</td>
<td>0.03±.00 (++)</td>
<td>0.18±.02 (+)</td>
<td>0.29±.01 (+)</td>
</tr>
<tr>
<td>3-phenylpropyne</td>
<td>0.05±.01 (++)</td>
<td>0.23±.03 (+)</td>
<td>0.37±.06 (−)</td>
</tr>
<tr>
<td>1-ethynylcyclo-hexylamine</td>
<td>0.29±.06 (+)</td>
<td>0.38±.02 (−)</td>
<td>0.39±.04 (−)</td>
</tr>
</tbody>
</table>

*B. cepacia* G4 was grown as a batch culture with 94 µmol of toluene in the presence of the indicated amount of inhibitor for 9.5 hrs. The *A*₆₀₀ at time zero was ca. 0.05. Values are mean ± standard deviation for triplicate experiments. Symbols in parentheses indicate levels of inhibition. Complete inhibition (++) is defined as a final *A*₆₀₀ of ≤0.075, partial inhibition (+) is defined as a final *A*₆₀₀ of between 0.075 and 0.30, and no inhibition (−) is defined as a final *A*₆₀₀ of ≥0.30. The final *A*₆₀₀ of cells grown in the absence of an alkyne was 0.341 ± 0.014.

Gaseous alkynes (acetylene, propyne, and 1-butynẽ) were assumed to behave as ideal gases, i.e., 45 µmol = 1 ml of gaseous alkyne = 1% (vol/vol) gas phase in experimental vials.

Interestingly, linear alkynes with interior triple bonds were more potent inhibitors of growth than their terminal counterparts. The aromatic alkynes phenyl-acetylene and 3-phenyl-propyne, suppressed growth at the higher concentrations tested but were less effective at lower concentrations.

A yellow product was visible in vials containing ≤ 4.5 µmol of phenylacetylene or 3-phenyl-propyne following the incubation period, suggesting that a meta-ring
cleavage product had accumulated. It is possible that ring cleavage of the aromatic alkynes resulted in a dead end yellow product or that these aromatic alkynes inhibited metabolism of 2-hydroxy-6-oxohepta-2,4-dienoic acid (the product of ring cleavage in toluene metabolism by \textit{B. cepacia} G4). The cyclic alkyne ethynylcyclohexylamine was an extremely weak inhibitor of growth.

2.4.2. \textbf{Inactivation of toluene-dependent O}_2 \textbf{uptake by alkynes}

To further investigate the effect of alkynes on toluene-grown \textit{B. cepacia} G4, toluene-dependent O$_2$ uptake by cells (from concentrated stock) was examined in the presence of specific concentrations of 1-butyne. After addition of toluene (200 \(\mu\)M), a constant rate of O$_2$ uptake was established. The addition of 1-butyne resulted in a time-dependent loss of O$_2$ uptake activity, and the rate of inactivation depended on the concentration of 1-butyne (Fig 2.2). After the addition of 1-butyne, the rates of O$_2$ uptake eventually stabilized (in reactions containing sufficient O$_2$) at levels comparable to the basal rate of O$_2$ uptake obtained prior to the addition of toluene.

The addition of \textit{o}-cresol to cells inactivated with 1-butyne did not stimulate O$_2$ uptake; however, the addition of 3-methylcatechol resulted in an O$_2$ uptake rate that was within 10\% of that determined when 3-methylcatechol was added to untreated cells (Fig 2.2). Acetylene also inhibited toluene-dependent O$_2$ uptake by \textit{B. cepacia} G4 in a time- and concentration-dependent manner, although much higher concentrations were required to produce an observable effect (data not shown).

The time-dependent loss of toluene-dependent O$_2$ uptake activity in \textit{B. cepacia} G4 during exposure to 1-butyne was slowed by increasing concentrations of toluene. Cells incubated in the presence of 70 \(\mu\)M 1-butyne with 150, 250, 400, and 750 \(\mu\)M toluene lost 71, 56, 39, and 24\% of their respective toluene-dependent O$_2$ uptake activities over a 4-min period (data not shown). In the absence of 1-butyne, O$_2$ uptake rates were constant (until O$_2$ became limiting, at \(\geq 5\)min) and similar (within 3\% of each other) over the range of toluene concentrations examined in this experiment (150-750 \(\mu\)M). The time- and concentration-dependent inactivation of toluene-dependent
Figure 2.2. Effects of 1-butyn on substrate-dependent O$_2$ uptake by *B. cepacia* G4. The time at which a given substrate or inactivator was added to the O$_2$ electrode chamber containing cells (130 µg of protein) is depicted next to each trace by arrowheads. Abbreviations: 3-Meth, 3-methylcatechol; 1-Buty, 1-butyn.
O₂ uptake by 1-butyne could also be described as a pseudo-first-order reaction (Fig 2.3A). The observed rate constant for inactivation, kₐₙₖ, was determined at each concentration of 1-butyne tested as described in Materials and Methods. The kₐₙₖ values ranged from 0.25 min⁻¹ to 2.45 min⁻¹, and although inactivation rates were dependent upon inactivator concentration, saturation was not observed (Fig 2.3B). Even at the highest concentration (1.1 mM 1-butyne) at which reliable rates of inactivation could still be determined, the kₐₙₖ was still proportional to the concentration of inactivator.

The effects of 1-, 2-, and 3-hexyne on toluene-dependent O₂ uptake were also investigated. Addition of 0.45 μmol (281 μM) of each of these alkynes to a reaction mixture of cells and toluene (200 μM) resulted in a rapid, time-dependent loss of activity (data not shown). Following complete inactivation of toluene-dependent O₂ uptake activity by each hexyne isomer, 3-methylcatechol (400 μM) stimulated O₂ uptake in treated cells to within 10% of the 3-methylcatechol-dependent rate determined in the absence of alkynes (as was demonstrated for cells inactivated with 1-butyne [Fig 2.2]). The kₐₙₖ values for 2- and 3-hexyne were approximately twice that of 1-hexyne, consistent with the increased effectiveness of 2- and 3-hexyne relative to 1-hexyne as inhibitors of growth (Table 2.1). These results suggest that subterminal alkynes are more effective inactivators of toluene 2-monooxygenase activity in B. cepacia G4 than their N-terminal counterparts.

The specificity of alkyne-based inactivation was further examined by comparing toluene-, o-cresol-, 3-methylcatechol-, and acetate-dependent O₂ uptake rates after exposure of toluene-grown cells of B. cepacia G4 to acetylene, 1-butyne, or 1-, 2-, or 3-hexyne. While toluene- and o-cresol-dependent O₂ uptake activities were irreversibly inactivated after exposure of the cells to each alkyne, 3-methylcatechol- and acetate-dependent O₂ uptake activities were uninhibited (Fig. 2.4). Surprisingly, cells incubated in the presence of alkynes (with the exception of acetylene) exhibited higher levels of 3-methylcatechol-dependent O₂ uptake activity than the control cells. Organic solvents, such as ethanol or acetone, are known to protect catechol 2,3
Figure 2.3. Kinetics of inactivation of toluene-dependent O$_2$ uptake activity by 1-butyne. (A) The time course of inactivation of toluene-dependent O$_2$ uptake in the presence of various micromolar concentrations of 1-butyne: ■ 46, ● 93, ▲ 231, ◇ 555, and ▼ 1,156. Cells (130 mg protein) were mixed with phosphate buffer and toluene (200 μM) in an O$_2$ electrode chamber (1.6 ml) prior to 1-butyne addition. O$_2$ uptake rates were determined at the indicated time points following 1-butyne addition as described in Materials and Methods and plotted as the log of the percentage of remaining activity. (B) Rate of inactivation ($k_{obs}$) versus 1-butyne concentration. Values for $k_{obs}$ were determined as described in Materials and Methods.
Figure 2.4. Irreversible effects of alkyne exposure on substrate-dependent O₂ uptake. Cells (130 µg protein) were incubated in the presence of an alkyne (indicated on the x axis) for 30 min and washed twice in phosphate buffer, and the remaining O₂ uptake activities stimulated by 200 µM toluene (black bars), 200 µM o-cresol (white bars), 400 µM 3-methylcatechol (hatched bars), or 1mM acetate (stippled bars) were calculated relative to that of a control (see Materials and Methods). Cells were exposed to 45 µmol of acetylene and 0.45 µmol of the other alkynes. Values are the mean ± standard deviation for three trials (acetylene treated cells, n=2).
dioxygenases from O₂-dependent autoinactivation (Buswell 1975). Perhaps linear alkynes other than acetylene acted in a similar manner in these experiments.

### 2.4.3. Requirement of O₂ for inactivation

Since O₂ is a required cosubstrate in monooxygenase catalyzed reactions, the inactivation potential of 1-butyne was examined under anaerobic conditions. Toluene-grown cells of *B. cepacia* G4 retained full toluene-dependent O₂ uptake activity when incubated anaerobically for 24 min in the presence of 1-butyne (120 μM) (Fig. 2.5).

---

**Fig. 2.5.** Requirement of O₂ for inactivation of toluene-dependent O₂ uptake activity by 1-butyne. Toluene-dependent O₂ uptake by anaerobically incubated *B. cepacia* G4 exposed (●) or not exposed (■) to 1-butyne. A dense suspension of cells was kept anaerobic by mixing in an O₂ electrode chamber throughout the course of the experiment. 1-Butyne (120 μM) was added at 30 min. H₂O₂ (400 nmol) was added at 54 and 77 min. Toluene-dependent O₂ uptake rates were measured after transfer of 20-μl samples of the dense cell suspension into another O₂ electrode chamber as described in Materials and Methods. The trace at the bottom represents the amount of O₂ (nanomoles) in the dense cell suspension over the course of the 1-butyne amended experiment.
When H₂O₂ (400 nmol) was added to the anaerobic cell suspension, a measurable amount of O₂ was produced and rapidly consumed. Immediately following the addition of H₂O₂, cellular toluene-dependent O₂ uptake activity rapidly decreased to a lower constant level, which was approximately 50% of the activity before the addition of H₂O₂. A further addition of H₂O₂ (400 nmol) resulted in the loss of an additional 25% of the original toluene-dependent O₂ uptake activity. In contrast, cells incubated under anaerobic conditions and exposed to 800 nmol of H₂O₂ in the absence of 1-butyne retained 85% of their toluene-dependent O₂ uptake activity, demonstrating that the addition of H₂O₂ to B. cepacia G4 cells, which could result in the production of potentially damaging oxygen species, does not cause a substantial loss of toluene-dependent O₂ uptake activity. These results indicate that the cosubstrate O₂ is required for the loss of toluene 2-monoxygenase activity in the presence of 1-butyne.

2.4.4. Alkene oxidation by B. cepacia G4

Since alkenes are effective mechanism-based inactivators of certain monoxygenases, such as cytochrome P-450 (Stirling and Dalton 1977; Prior and Dalton 1985), I examined the possibility that the alkene analogs of acetylene and propyne—ethylene and propylene—could inactivate monoxygenase activity in B. cepacia G4. Initial experiments indicated that ethylene and propylene were not inactivators of toluene 2-monoxygenase activity in B. cepacia G4 but were rapidly consumed by toluene-grown cells (data not shown). To further examine the substrate efficacy of alkenes versus alkynes, the consumption of acetylene, propyne, ethylene, and propylene by toluene-grown B. cepacia G4 was monitored with resting cell assays. Both ethylene and propylene were consumed by B. cepacia G4, whereas acetylene and propyne remained at constant levels (Fig 2.6). Additionally, the presence of 1-butyne completely inhibited the consumption of ethylene and propylene (Fig 2.6). The consumption of both ethylene and propylene was accompanied by the accumulation of a single reaction product for each, as determined by gas-phase sampling. The retention time of these products matched those of ethylene oxide and propylene oxide, respectively, and the epoxide nature of these products was confirmed.
by their disappearance from the gas phase after the addition of H$_2$SO$_4$ (see Materials and Methods). A ratio of 0.96 for propylene consumed to propylene oxide produced was observed over a 4-h time course in a separate resting-cell assay (data not shown).

Figure 2.6. Alkene and alkyne consumption by B. cepacia G4. Cells were incubated with 0.45 μmol of each compound as described in Materials and Methods. 1-Butyne (4.5 μmol) was added to inactivate toluene 2-monoxygenase activity in selected samples. Symbols: □, ethylene; ○, propylene; ■, ethylene plus 1-butyne; ●, propylene plus 1-butyne; ▼, ethylene (heat killed cells); ◆, acetylene; ▲, propyne.
2.4.5. Kinetics of ethylene and propylene oxidation

The kinetics of ethylene and propylene oxidation by toluene-grown *B. cepacia* G4 were further investigated with short-term, whole cell assays. The amounts of propylene oxide and ethylene oxide produced from a range of initial propylene and ethylene concentrations were determined following 20-min incubations. The rates of propylene oxide and ethylene oxide production were constant over this time course for all concentrations tested (data not shown). $K_s$ and $V_{max}$ values were 39.7 μM and 112.3 nmol min$^{-1}$ mg of protein$^{-1}$ for ethylene and 32.3 μM and 89.2 nmol min$^{-1}$ mg of protein$^{-1}$ for propylene (values are averages from duplicate experiments [≤20% variation]).

2.5. Discussion

In this study it was demonstrated that a variety of alkynes act as potent inhibitors of the toluene-dependent growth of *B. cepacia* G4. Using representative alkynes it was also demonstrated that toluene 2-monooxygenase activity is specifically inactivated by these compounds. Based on both the kinetic and growth studies, it was concluded that alkynes represent a general class of mechanism-based inactivators for toluene 2-monooxygenase activity in *B. cepacia* G4.

The simplest kinetic model for a mechanism-based inactivator is presented in Fig. 2.7 (Silverman 1988).

$$
E + I \xrightarrow{k_1} E \cdot I \xrightarrow{k_2} E \cdot X \xrightarrow{k_4} E - X' \\
\xrightarrow{k_3} E + P
$$

**Fig 2.7.** Kinetic model for a mechanism-based inactivator. I, inactivator; E, enzyme; P, product (any transformed species of I that diffuses away from the active site); E \cdot I, reversible enzyme-inactivator complex; E \cdot X, reactive intermediate; E - X', inactivated enzyme. k represents the rate constant for each reaction step.
According to this model a number of kinetic criteria should be met to describe a molecule as a mechanism-based inactivator (Walsh 1982; Silverman 1988; Ator and Montellano 1990), and in this study many of these requirements were satisfied. First, inactivation of toluene 2-monoxygenase activity in B. cepacia G4 was time-dependent, and the loss of enzyme activity followed pseudo-first-order kinetics (Fig 2.3A). Second, a substrate for the target enzyme, toluene, protected against inactivation by 1-butyne, suggesting that there is a competitive interaction between these two compounds for the active site in toluene 2-monoxygenase. Third, the inactivation of toluene 2-monoxygenase activity in B. cepacia G4 by alkynes was irreversible (Fig 2.4). Fourth, a catalytically favorable environment for toluene 2-monoxygenase was required for alkyne-dependent inactivation. This result addresses the most characteristic feature of mechanism-based inactivators, which is that the target enzyme is required to catalytically activate the inactivator. Like all other monoxygenases, toluene 2-monoxygenase has a requirement for O₂ and a source of reductant to effect catalysis. Since it is not possible to limit the availability of reductant to the enzyme in whole-cell experiments, I demonstrated that O₂ is a necessary cosubstrate for 1-butyne-dependent inactivation of toluene 2-monoxygenase activity in B. cepacia G4 (Fig 2.5). Although the H₂O₂ used in this experiment may directly provide both O₂ and reductant to the monooxygenase via a “peroxide shunt” (Newman and Wackett 1995), this possibility supports rather than detracts from my conclusion that the alkyne-dependent inactivation of toluene 2-monoxygenase activity requires a catalytically functional form of the enzyme.

Although many of the important criteria of mechanism-based inactivation were satisfied in regards to the effects of alkynes on toluene 2-monoxygenase activity in B. cepacia G4, other aspects of this type of inhibition were not demonstrated in this study. For instance, an additional kinetic criterion is that the inactivation reaction should exhibit saturation kinetics. This criterion requires that the rate of enzyme inactivation is proportional to inactivator concentration at low inactivator concentrations and that it approaches a constant, maximal value at higher
concentrations. Although I demonstrated a strong concentration dependence for inactivation of toluene-dependent \( \text{O}_2 \) uptake in \( B. \text{cepacia} \) G4 by 1-butyne, the reaction did not appear to be saturable under the conditions tested (Fig 2.3B). This apparent lack of saturation could be due to intrinsic features of toluene 2-monoxygenase. For example, the maximal rate of inactivation could be fast relative to the formation of the enzyme-inactivator complex; alternatively, inactivator transformation by the target enzyme might not be preceded by a conventional enzyme-inactivator binding step. It is also possible that the rate of inactivation of toluene 2-monoxygenase activity in \( B. \text{cepacia} \) G4 with alkynes can be saturated at higher concentrations. However, at the highest alkyne concentrations tested in these experiments, the observed rates of inactivation were the highest rates that I could determine given the finite response time of the \( \text{O}_2 \) electrode.

Another feature of mechanism-based inactivation that was not demonstrated in this study was a 1:1 ratio of covalent attachment of inactivator to target enzyme. This criterion is infrequently demonstrated for mechanism-based inactivators because the necessary experimental approaches usually require sources of enzyme with defined specific activities and an identifiable (usually radiolabeled) inactivator. Attempts with \( ^{14}\text{C}_2\text{H}_2 \) labeling in \( B. \text{cepacia} \) G4 resulted in an inefficient, nonspecific labeling of proteins and other cellular constituents (data not shown). Unfortunately, a \( ^{14}\text{C} \)-labeled alkyne other than acetylene is not commercially available, so the covalent modification of toluene 2-monoxygenase was not investigated with longer alkynes. The unresolved kinetic considerations discussed above would benefit from additional studies with purified toluene 2-monoxygenase.

It is of interest that acetylene appears to be an ineffective mechanism-based inactivator of toluene 2-monoxygenase activity in \( B. \text{cepacia} \) G4 relative to other alkynes. Two possible explanations for the poor inactivation capacity of acetylene are that the partition ratio for acetylene is large and that the dissociation constant for acetylene is very high. The partition ratio represents the number of times that the activated inactivator is released as product per inactivation event (Waley 1985; Silverman 1988), or \( k_3/k_4 \) as depicted in Fig. 2.7. Therefore, a large partition ratio
describes an inefficient inactivator. In this study, there was no evidence of acetylene transformation by \textit{B. cepacia} G4 during growth experiments or resting cell assays (Fig 2.6), suggesting that acetylene is not acting as a mechanism-based inactivator with a large partition ratio. If the dissociation constant for acetylene is high relative to that of propyne, it seemed reasonable that a similar pattern could be demonstrated for the alkene analogs of these compounds - ethylene and propylene. The results indicated that these alkenes were not inactivators of toluene 2-monooxygenase activity in \textit{B. cepacia} G4, but they were oxidized by this enzyme to their respective epoxides, with no evidence of further breakdown. Surprisingly, the apparent $K_s$ values for ethylene and propylene (39.7 $\mu$M and 32.3 $\mu$M, respectively) were quite similar. In comparison, two previous studies reported apparent $K_s$ values for TCE of 3 and 6 $\mu$M in \textit{B. cepacia} G4 (Folsom et al. 1990; Landa et al. 1994), while the $K_m$ for TCE with purified toluene 2-monooxygenase from this organism was reported to be 12 $\mu$M (Newman and Wackett 1997). Further analysis indicated that acetylene was a poor inhibitor of ethylene and propylene oxidation (data not shown). These results suggest that acetylene binds poorly to toluene 2-monooxygenase, whereas $C_2$ alkenes, such as ethylene and TCE (Newman and Wackett 1997) exhibit a much higher affinity for the enzyme.

Much of the current interest in \textit{B. cepacia} G4 results from the rapid rate of TCE degradation that is supported by toluene-grown cells of this microorganism. While the results from the current study are consistent with earlier observations that acetylene is a relatively weak inhibitor of TCE degradation by \textit{B. cepacia} G4 (McClay et al. 1996), they also extend these earlier studies and provide two important observations that could be of use in future studies of TCE biodegradation. First, as noted earlier, several TCE-oxidizing monooxygenase systems are inactivated by low concentrations of acetylene (Dalton and Whittenbury 1976; Hynes and Knowles 1978). Since toluene 2-monooxygenase activity is relatively immune to inactivation by acetylene, this effect could potentially be exploited to determine which type of enzyme activity is responsible for TCE oxidation in undefined microbial mixtures. A similar approach could also be used with other compounds containing internal triple bonds. For
example, it was demonstrated that 2-alkynes are potent inactivators of toluene-oxidizing activity in *B. cepacia* G4, whereas these compounds are known to be largely ineffective inactivators of both sMMO and AMO (Dalton and Whittenbury 1976; Stirling and Dalton 1977; Arp et al. 1996). Second, the observation that *B. cepacia* G4 can readily oxidize both ethylene and propylene supports the general observation that this activity is common to TCE-degrading strains. Methane- (Colby et al. 1977), propane- (Hou et al. 1983), and ammonia- (Hyman et al. 1988) oxidizing bacteria are all capable of cometabolic alkene epoxidation, while other TCE-degrading bacteria, such as *Xanthobacter Py2* (Ensign et al. 1992), can grow on simple alkenes. Although these coincident catalytic activities are perhaps unsurprising given the structural similarities between TCE and simple n-alkenes, my confirmation that this theme extends to toluene-oxidizing bacteria suggests that alkene-oxidizing activity might represent the basis for a non-toxic assay for determining the TCE-degrading “potential” of an undefined microbial population.

2.6. Acknowledgments

I thank Evan DeSzoike for performing preliminary growth experiments and Malcolm Shields for providing *B. cepacia* G4.

Funding of this study was provided by the Office of Research and Development, U.S. Environmental Protection Agency, under agreement R-815738 through the Western Region Hazardous Substance Research Center.
Chapter 3

Cytotoxicity Associated with Trichloroethylene Oxidation in *Burkholderia cepacia* G4

Chris M. Yeager, Peter J. Bottomley, and Daniel J. Arp

Published in *Applied and Environmental Microbiology*,
American Society for Microbiology
3.1. Abstract

The effects of trichloroethylene (TCE) oxidation on toluene 2-monooxygenase activity, general respiratory activity, and cell culturability were examined in the toluene-oxidizing bacterium *Burkholderia cepacia* G4. Nonspecific damage outpaced inactivation of toluene 2-monooxygenase in *B. cepacia* G4 cells. Cells that had degraded approximately 0.5 μmol TCE (mg cells⁻¹) lost 95% of their acetate-dependent O₂ uptake activity (a measure of general respiratory activity), yet toluene-dependent O₂ uptake activity had decreased only 35%. Cell culturability also decreased upon TCE oxidation; however, the extent of loss varied greatly (up to 3 orders of magnitude) with the method of assessment. Addition of catalase or sodium pyruvate to the surface of agar plates increased enumeration of TCE-injured cells by as much as 100 fold indicating that the TCE-injured cells were ultra-sensitive to oxidative stress. Cell suspensions that had oxidized TCE recovered the ability to grow in liquid minimal media containing lactate or phenol, but recovery was delayed substantially when TCE degradation approached 0.5 μmol (mg cells⁻¹) or 66% of the cells’ transformation capacity for TCE at the cell density utilized. Furthermore, among *B. cepacia* G4 cells isolated on LB agar plates from cultures that had degraded approximately 0.5 μmol TCE (mg cells⁻¹) up to 90% were ToF variants, no longer capable of TCE degradation. These results indicate that a toxicity threshold for TCE oxidation exists in *B. cepacia* G4, and that once a cell suspension has exceeded this toxicity threshold, the likelihood of reestablishing an active, TCE-degrading biomass from these cells will decrease significantly.

3.2. Introduction

Trichloroethylene (TCE), a suspected human carcinogen, is one of the most widespread groundwater contaminants. Although TCE can be reductively dehalogenated under anaerobic conditions, a common end product of this reaction is vinyl chloride, which is more water soluble than TCE and a known human carcinogen
(Bouwer and McCarty 1983; Vogel and McCarty 1985; Infante and Tsongas 1987). Removal of TCE from polluted sites or industrial discharge is therefore of great concern. Although TCE has not been shown to support microbial growth under aerobic conditions, a number of bacteria are capable of degrading this compound via aerobic cometabolism. In each case examined to date, a non-specific oxygenase catalyzes TCE transformation. Bacterial strains that use oxygenases to grow on methane, butane, propene, ethylene, ammonia, toluene, phenol, isoprene, and dichlorophenoxyacetic acid have been characterized as TCE-degraders (Arciero et al. 1989; Tsein et al. 1989; Zylstra et al. 1989; Ewers et al. 1990; Harker and Kim 1990; Shields et al. 1991; DiSpirito et al. 1992; Ensign et al. 1992; Hamamura et al. 1997). Although some of these bacteria exhibit rapid initial rates of TCE conversion, the process is invariably non-sustainable in the absence of a growth supporting substrate (Wackett and Gibson 1988; Alvarez-Cohen and McCarty 1991; Oldenhuis et al. 1991; Heald and Jenkins 1994).

From the biological standpoint, aerobic cometabolism of TCE by microorganisms is largely dependent on two factors: 1) cellular energy requirements and 2) the toxicity often associated with TCE oxidation. Energetic problems can be overcome by carefully controlling the concentrations of cometabolite and growth substrate (Chang and Alvarez-Cohen 1995; Ely et al. 1995), or by selecting or designing microorganisms that are capable of expressing the TCE-degrading oxygenase while growing on a non-competitive substrate (Shields et al. 1994; McClay et al. 1995; Munakata-Marr et al. 1996). Perhaps more problematic is the toxicity that is often associated with TCE cometabolism.

Aerobic cometabolism of TCE is typically plagued by turnover-dependent loss of activity. Whole cell studies with the ammonia-oxidizing bacterium Nitrosomonas europaea, the methanotroph Methylosinus trichosporium OB3b, and the toluene-oxidizing bacterium Pseudomonas putida F1 have shown that the rate of TCE degradation decreases rapidly with time (Wackett and Gibson 1988; Oldenhuis et al. 1991; Rasche et al. 1991; van Hylckama Vlieg et al. 1997). The effects of TCE oxidation have also been examined with purified oxygenases from M. trichosporium
OB3b (soluble methane monooxygenase), *P. putida* F1 (toluene dioxygenase), and *Burkholderia cepacia* G4 (toluene 2-monooxygenase). In each case, TCE turnover results in enzyme inactivation, and is accompanied by covalent modification of each of the components of the oxygenase complex (Fox et al. 1990; Li and Wackett 1992; Newman and Wackett 1997).

[^14C]TCE labeling studies with *N. europaea* and *P. putida* F1 indicate that reactive intermediates of TCE oxidation can alkylate not only components of the transforming oxygenase, but other cellular constituents as well, including DNA, RNA, lipids, proteins, and various small molecules (Wackett and Householder 1989; Rasche et al. 1991). Furthermore, it has been noted that general cellular damage can accompany TCE oxidation in a variety of bacteria (Wackett and Householder 1989; Oldenhuis et al. 1991; Heald and Jenkins 1994; Hyman et al. 1995); however, the effect of TCE transformation on oxygenase activity has often been the focus of these studies and characterization of the cytotoxic damage incurred during the reaction was not vigorously pursued. Two recent studies examining the effects of TCE oxidation in methanotrophs have been an exception. Van Hylckama Vlieg et al. (1997) reported that the predominant toxic effect of TCE degradation by *M. trichosporium* OB3b was not methane monooxygenase inactivation, but rather general cellular damage resulting in an apparent decrease in cell viability. In that study, cell viability was determined by colony formation on LB plates, which decreased exponentially with the amount of TCE degraded. In another study, the respiratory activity (as measured by microscopic analysis of 5-cyano-2,3-ditolyl tetrazolium chloride-stained cells) of two methanotrophs, *M. trichosporium* OB3b and CAC-2, was analyzed following TCE degradation (Chu and Alvarez-Cohen 1999). The respiratory activity was found to decrease in a linear relationship with the amount of TCE degraded.

Because cellular toxicity can ultimately limit TCE oxidation, considerable effort has been directed towards identifying bacterial strains that can sustain high rates of TCE degradation. From, these efforts, the toluene oxidizing bacteria *B. cepacia* G4 has emerged as a promising agent for TCE bioremediation (Munakata-Marr et al. 1996; Radway et al. 1998; Sun et al. 1998). Earlier studies had led to the assumption
that this microorganism was relatively impervious to TCE-related toxicity. For example, stable rates of TCE consumption by *B. cepacia* G4 were observed during short-term resting cell assays (Nelson et al. 1987; Folsom et al. 1990). Furthermore, steady rates of TCE degradation were obtained in bioreactors containing phenol- and toluene-fed cultures of *B. cepacia* G4 (Folsom and Chapman 1991; Landa et al. 1994). However, other observations were made which suggest that *B. cepacia* G4 may indeed incur damage during TCE degradation (Shields et al. 1994; Mars et al. 1996; Mars et al. 1998). For example, when *B. cepacia* G4 cells were cultivated in a toluene-fed batch reactor and exposed to TCE under non-growth conditions (toluene feed was suspended), a four-fold increase in the maintenance energy requirements of the cells was observed (Mars et al. 1996). In addition, TCE oxidation by purified toluene 2-monooxygenase from this organism leads to inactivation of the enzyme (Newman and Wackett 1997). Because of the conflicting data surrounding TCE-related toxicity in *B. cepacia* G4 and the potential value of this organism in TCE bioremediation efforts, I decided to systematically examine the physiological consequences of TCE cometabolism in whole cells of this microorganism.

3.3. Materials and Methods

3.3.1. Chemicals and reagents

Toluene and TCE were obtained from Aldrich. Other reagents and their sources include: bovine liver catalase (38,080 U/ml, cat.# 100429; ICN Biomedicals Inc., Aurora, OH), N,N-dimethylformamide (Sigma, St. Louis, MO), ethylene (Airco, Murray Hill, NJ), and 2-hexyne (Farchan Laboratories, Inc., Gainesville, FL).

3.3.2. Culture conditions

*B. cepacia* G4 was maintained and grown essentially as described previously (Yeager et al. 1999). Batch cultures were grown overnight at 30°C with shaking in sealed serum vials (160 ml) containing minimal media (60 ml) with toluene (94 μmol; 1.0 mM aqueous phase concentration) or lactate (20 mM). Cultures grown on toluene
in this manner attained an optical density at 600 nm (OD<sub>600</sub>) of approximately 0.25. Additional toluene (94 µmol) was added to toluene-grown cultures 4-5 h prior to harvesting for experimental assays, enabling the cultures to obtain a final OD<sub>600</sub> of 0.5-0.6. Lactate-grown cells were harvested from overnight cultures that had reached a final OD<sub>600</sub> of 1.0-1.3. A dimensionless Henry’s constant of 0.343 was used to calculate the aqueous phase concentration of toluene in two-phase systems at 30°C (Yeager et al. 1999). To collect cells for experimental assays, cultures were centrifuged, rinsed twice with 50 mM KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub> buffer, pH 7.0 (phosphate buffer) and resuspended in fresh phosphate buffer. The cell suspension was stored at room temperature for ≤2 h before use.

### 3.3.3. Determining transformation capacities

Toluene-grown cells of *B. cepacia* G4 were added to serum vials (10 ml) that were sealed with Teflon-lined butyl rubber stoppers and contained either ethylene (3.4, 6.7, 8.9, or 13.4 µmol [40-157 µM initial aqueous concentration]) or TCE (1.3, 1.7, or 1.9 µmol [239-349 µM initial aqueous concentration]) in phosphate buffer. Additions were made to yield a final reaction volume of 1 ml with either 0.42, 1.05, 2.1, or 4.2 mg cells (dry weight) ml<sup>-1</sup>. The reaction vials were inverted and incubated at 30°C with shaking (150 rpm) for either 4 (TCE vials) or 8 h (ethylene vials), at which time consumption of the compounds had ceased. To monitor substrate consumption, headspace samples were analyzed by gas chromatography immediately following addition of cells and at the end of the incubation period. The T<sub>c</sub> for each compound at each cell density was determined by dividing the total amount of substrate consumed (µmol) by the amount of cells (mg dry weight) in the reaction vial. Control vials with heat-killed cells exhibited virtually no loss of ethylene or TCE when incubated under the conditions described above.

Because TCE concentrations above 400-500 µM were inhibitory to *B. cepacia* G4 (data not shown), the amount of TCE that could be added to a given reaction vial was limited (≤ 1.9 µmol [350 µM initial aqueous concentration]). Therefore,
incubations with TCE at a cell density of either 2.1 or 4.2 mg cells ml\(^{-1}\) required a second addition of TCE (1.7 \(\mu\)mol) after 2 h. The amount of TCE in the vials immediately prior to and after the second addition of TCE was determined by GC analysis of headspace samples.

### 3.3.4. TCE exposure for toxicity assays

Aliphatic hydrocarbons (1.36 \(\mu\)mol of TCE [250 \(\mu\)M initial aqueous concentration] or 1.36 \(\mu\)mol ethylene [16 \(\mu\)M initial aqueous concentration]) were added to phosphate buffer (=700 \(\mu\)l) in glass serum vials (10 ml) that were sealed with Teflon-lined butyl rubber stoppers. The reaction vials were incubated at 30°C with shaking (150 rpm) for at least 15 min to allow equilibration of the volatile hydrocarbons between the gas and liquid phases. Reactions were initiated by the addition of toluene-grown \textit{B. cepacia} G4 cells (final reaction volume, 1 ml) and incubated at 30°C with shaking. To monitor substrate consumption, headspace samples were analyzed by gas chromatography immediately following addition of cells and at the end of the incubation period. After the desired incubation period, the reaction mix was transferred to a 1.5 ml microfuge tube and centrifuged at 12000 rpm for 30 sec. The supernatant was removed, and the pellet was washed with phosphate buffer (700 \(\mu\)l), recentrifuged, and finally resuspended in fresh phosphate buffer (100 \(\mu\)l). Samples of this concentrated cell suspension were then used to assay cell viability, acetate-dependent \(O_2\) uptake, and toluene-dependent \(O_2\) uptake rates.

### 3.3.5. Assays for cell culturability and \(O_2\) uptake rates

To measure cell viability following hydrocarbon exposures, a 10-fold dilution series of the concentrated cell suspension was prepared in sterile phosphate buffer, and aliquots (50 \(\mu\)l) from select dilutions were plated in duplicate onto LB agar plates. Where indicated, catalase (210 units/plate; from a 38,080 U/ml sterile stock solution) or sodium pyruvate (60 mg/plate; from a 375 mg/ml filter sterilized stock solution prepared in \(dH_2O\)) was spread over the surface of LB agar plates and allowed to dry.
Colony counts were performed after the plates had been incubated at 30°C for 3 days. Alternatively, the number of culturable cells in each concentrated cell suspension was determined by a most probable number (MPN) technique. Four identical 10-fold dilution series were prepared from each cell sample. The dilutions were carried out beyond extinction ($10^{12}$) in tubes containing sterile minimal media with 20 mM lactate. Tubes were incubated for 6 days at 30°C and scored for growth. A MPN computer program was used to calculate the number of culturable cells ml$^{-1}$ in each sample (Woomer et al. 1990).

Cell viability was also assayed with the BacLight Live/Dead stain (Molecular Probes, Eugene, OR). TCE-treated cells were diluted in phosphate buffer, and a sample (1 ml) was stained according to the manufacturer’s directions. A portion of the stained cells (7 μl) was place on a slide under a coverslip and observed via epifluorescent microscopy. One hundred cells were examined from randomly chosen areas under the coverslip and scored as live or dead. Heat (70°C for 15 min) and alcohol (75% isopropanol) killed >95% of B. cepacia G4 cells as determined by this method (data not shown).

To examine toluene 2-monooxygenase activity following hydrocarbon exposure, the toluene-dependent O$_2$ uptake rates of treated cells were determined with a Clark (Yellow Springs, Ohio)-style O$_2$ electrode mounted in a glass water-jacketed reaction vessel (1.6 ml) maintained at 30°C. The reaction vessel was filled with phosphate buffer, and cells were added to determine a basal rate of cellular respiration. The basal respiratory rate was subtracted from the toluene-stimulated (250 μM) O$_2$ uptake rate to obtain the toluene-dependent O$_2$ uptake rate. Acetate-dependent O$_2$ uptake rates were determined similarly with addition of 1 mM acetate.

### 3.3.6. Effect of TCE oxidation on surrounding cells

*B. cepacia* TCS-100 is a Tn5 mutant of *B. cepacia* G4 that is resistant to tetracycline. This strain is phenotypically indistinguishable from wild type *B. cepacia* G4 in terms of toluene oxidation activity, TCE degradation activity, and growth rates.
on lactate, toluene and LB (Yeager, unpublished results). Toluene-grown cells of *B. cepacia* TCS-100 were mixed with toluene-grown wild type *B. cepacia* G4 cells at a ratio of 1:9 in a sealed serum vial (10 ml) containing phosphate buffer and TCE (1.36 μmol [250 μM initial aqueous concentration]). The cell density in the TCE reaction vial (1 ml final volume) was 2.1 mg cells (dry weight) ml⁻¹.

Prior to cell mixing toluene 2-monooxygenase was inactivated in select cell suspensions of *B. cepacia* TCS-100 and wild type *B. cepacia* G4 by incubating with 2-hexyne (5 μl from a 1:100 dilution of 2-hexyne in N,N-dimethylformamide) in sealed serum vials (10 ml) for 40 min at 30°C (Yeager et al. 1999). After the 2-hexyne incubation, cells were washed twice and resuspended in phosphate buffer. 2-Hexyne-treated cells did not degrade TCE (data not shown). Three combinations of TCS-100 and G4 cells were mixed and examined: (a) both strains treated with 2-hexyne; (b) TCS-100 treated and G4 untreated; (c) neither strain treated.

The TCE reaction vials containing both strains were incubated for 30 min at 30°C with shaking. Following the incubation, the reaction mixes were transferred to a 1.5 ml microfuge tube and centrifuged at 12000 rpm for 30 sec. Supernatants were removed, cells washed with phosphate buffer (700 μl), recentrifuged, and finally resuspended in fresh phosphate buffer (100 μl). The culturability of *B. cepacia* TCS-100 cells from the cell mixes was then determined by plating appropriate dilutions onto LB plates containing tetracycline (15 μg/ml), and counting colonies after incubating at 30°C for 3 days. Wild type *B. cepacia* G4 did not grow on plates containing tetracycline at 15 μg/ml.

### 3.3.7. Recovery of growth by *B. cepacia* G4 cells exposed to TCE

Toluene-grown *B. cepacia* G4 cells were incubated with TCE, as described in the "TCE Exposure for Toxicity Assays" section. At select time points, samples (50 μl) were removed from the TCE reaction mixture and added to a sterile glass serum vial (160 ml) containing minimal medium (60 ml) with 20 mM lactate or 2.5 mM
phenol. The inoculated vials were then incubated at 30°C with shaking and 1 ml portions were removed periodically to monitor the OD$_{600}$ of the culture.

### 3.3.8. Occurrence of Tol$^+$ variants upon TCE oxidation

Toluene-grown *B. cepacia* G4 cells that had been exposed to TCE for 0, 15, 30 or 60 min were diluted and spread onto LB plates. The percentage of TCE-treated cells able to grow on toluene was determined by streaking 100 colonies from the LB plates onto minimal media plates, which were then incubated in sealed, one-gallon polyethylene jars containing toluene vapors. Toluene vapors were supplied by adding neat toluene (150 µl) to a Durham tube, plugging the tube with cotton, and placing the tube in an empty Petri dish at the bottom of the polyethylene jar. The plates were scored for growth after 3 days incubation at 30°C. Ten *B. cepacia* G4 variants that were incapable of growth on toluene vapors were randomly chosen and analyzed for the presence of the TOM plasmid. Plasmid DNA was isolated as previously described (Kado and Liu 1981) and visualized following separation on a 0.6% agarose gel.

### 3.3.9. Analytical and other methods

Hydrocarbons were analyzed with a Shimadzu (Kyoto, Japan) GC-8A chromatograph equipped with a flame ionization detector and a stainless steel column (0.3 by 61 cm) packed with Porapak Q 80-100 mesh (Alltech, Deerfield, Ill.). To detect ethylene a column temperature of 100°C was utilized, and for TCE the column temperature was 155°C. The injector and detector temperatures were set at 200°C for all analyses. Hydrocarbons were quantified by comparison of peak heights to standard curves constructed from known amounts of authentic compounds.

The aqueous concentration of TCE in two-phase systems at 30°C was calculated with a dimensionless Henry’s constant of 0.494 (Gossett 1987). The concentration of ethylene in the aqueous phase at 30°C was calculated with a Henry’s constant of 9.35 (derived from the data of Wilhelm et al. (1977)).
The protein concentration of cell suspensions was determined by measuring the \( \text{OD}_{600} \) of appropriate dilutions of the suspensions and converting to protein (suspensions of \textit{B. cepacia} G4 cells with an \( \text{OD}_{600} \) of 1.0 contained 0.2 mg ml\(^{-1}\) of total cell protein). Protein concentrations were determined with the Biuret assay (Gornall et al. 1949) following cell solubilization in 3 M NaOH for 30 min at 65°C. Bovine serum albumin was used as the standard. The dry weights of culture samples were determined by resuspending cells in \( \text{dH}_2\text{O} \) in pre-weighed Eppendorf tubes, drying for 2 days at 55°C, and weighing the cell pellets. It was determined that 2.1 mg of \textit{B. cepacia} G4 cells (dry weight) contains approximately 1.0 mg protein.

3.4. Results

3.4.1. Effects of TCE transformation on toluene 2-monooxygenase activity, general respiratory activity and cell viability

TCE degradation by toluene-grown \textit{B. cepacia} G4 was examined in resting cell assays (Fig 3.1).

\textbf{Figure 3.1.} TCE and ethylene consumption by \textit{B. cepacia} G4. Toluene-grown cells (2.1 mg of cells) were harvested and incubated with 1.36 \( \mu \text{mol} \) of TCE (■) or ethylene (●). Additional TCE or ethylene was added where indicated (arrows). □, heat-killed cell control with TCE.
The initial rate of TCE consumption at 30°C was 15 nmol min\(^{-1}\) mg total cell protein\(^{-1}\). Folsom et al. previously reported a maximal TCE degradation rate of 8 nmol min\(^{-1}\) mg protein\(^{-1}\) with phenol-grown *B. cepacia* G4 at 26°C and Sun and Ward reported a rate of 9 nmol min\(^{-1}\) mg protein\(^{-1}\) at 25°C (Folsom et al. 1990; Sun and Wood 1996). After 4 h the rate of TCE depletion had slowed to essentially that of the control vial containing heat-killed cells. In contrast, *B. cepacia* G4 cells consumed ethylene at high rates during the entire assay (up to 6 h). The initial aqueous phase concentrations of TCE and ethylene in the reaction mixtures were 250 μM and 16 μM, respectively. I previously determined a K\(_r\) for ethylene of 39.7 μM for *B. cepacia* G4 (Chapter 2). Therefore, the nonlinear nature of ethylene consumption (Fig 3.1) is likely due to concentration-dependent kinetics. Indeed, at higher ethylene concentrations (>200 μM) a constant rate of ethylene consumption was observed (data not shown).

The transformation capacities (T\(_c\)) of resting cell suspensions of *B. cepacia* G4 for TCE and ethylene were determined. The T\(_c\) is defined as the maximum mass of compound that can be degraded per mass of cells prior to inactivation (Alvarez-Cohen and McCarty 1991). The T\(_c\) of *B. cepacia* G4 for ethylene was 10-18 times greater than that for TCE over a range of cell densities (Table 3.1). It was previously observed that *B. cepacia* G4 oxidizes ethylene to epoxyethane with no evidence of further breakdown of the product (Fig 2.6) and no evidence of toxicity (Fig 3.1). Therefore, ethylene transformation should provide a measure of the effects of reductant drain on cells that should also be applicable during TCE transformation (unless extensive uncoupling of NADH utilization occurs during TCE oxidation relative to ethylene oxidation). Since the T\(_c\) of resting cell suspensions of *B. cepacia* G4 for TCE was much lower than that of ethylene, it seemed likely that the time-dependent decrease in TCE degradation observed in Figure 3.1 was largely due to toxicity rather than reductant depletion. The decrease in the T\(_c\) of *B. cepacia* G4 for TCE with increasing cell density is addressed later.

To determine if an irreversible loss of toluene 2-monooxygenase activity occurred in *B. cepacia* G4 cells during TCE oxidation, rates of toluene-dependent O\(_2\)
Table 3.1. $T_c$ values of resting cell suspensions of *B. cepacia* G4 for TCE and ethylene at different cell densities

<table>
<thead>
<tr>
<th>Cell Density (mg of cells/ml)</th>
<th>Ethylene</th>
<th>TCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.42</td>
<td>9.0±2.7</td>
<td>0.95±0.14</td>
</tr>
<tr>
<td>1.05</td>
<td>10.4±1.0</td>
<td>0.92±0.10</td>
</tr>
<tr>
<td>2.1</td>
<td>11.0±0.2</td>
<td>0.75±0.07</td>
</tr>
<tr>
<td>4.2</td>
<td>10.6±0.2</td>
<td>0.61±0.07</td>
</tr>
</tbody>
</table>

$a$ $T_c$ is expressed as micromoles of substrate transformed per milligram of cells (dry weight). All values are the means from three trials ± standard deviations.

uptake were determined in cells exposed to TCE. Cells retained full toluene-dependent $O_2$ uptake activity during the first 20-30 min of TCE degradation, after which a slow, linear decrease in activity was observed (Fig 3.2A). The decrease in toluene-dependent $O_2$ uptake activity of cells during TCE oxidation could be attributed specifically to the loss of toluene 2-monooxygenase activity, since levels of 3-methylcatechol-dependent $O_2$ uptake (3-methylcatechol is the ultimate product of toluene oxidation by toluene 2-monooxygenase in *B. cepacia* G4) remained constant in the TCE-treated cells (data not shown). However, the data were not sufficient to distinguish between the loss of toluene 2-monooxygenase activity being a consequence of inactivation of a particular component of the oxygenase, decreased electron flow to the terminal oxygenase component, damage to the diiron center of the $\alpha$ subunit of the hydroxylase, or some other toxic effect. Acetate-dependent $O_2$ uptake rates were used to examine the effects of TCE transformation on the general respiratory status of *B. cepacia* G4 cells for several reasons. First, toluene-grown cells of *B. cepacia* G4 exhibited robust, stable levels of acetate-dependent $O_2$ consumption (acetate is an intermediate of toluene metabolism in this microorganism). Second, the majority of $O_2$ consumed during acetate metabolism is associated with central metabolism, and therefore acetate-dependent $O_2$ consumption is a good measure of the
general respiratory status of the cell. Upon TCE exposure the acetate-dependent O$_2$ uptake activity of toluene-grown *B. cepacia* G4 cells decreased steadily until <5% of the original activity remained (Fig 3.2B). TCE turnover was required to bring about the loss of acetate-dependent O$_2$ uptake activity, because cells incubated with TCE and 2-hexyne (a mechanism based inactivator of toluene 2-monoxygenase (Yeager et al. 1999)) retained >94% of their original activity during the incubation period.

The effect of TCE oxidation on the culturability of *B. cepacia* G4 cells was examined by spreading TCE-treated cells on LB plates (Fig 3.2C). TCE degradation resulted in a precipitous loss of cell culturability (colony forming units). The culturability of the cells was found to decrease exponentially with the amount of TCE transformed (Fig 3.2C inset). *B. cepacia* G4 cells incubated with ethylene or without substrate did not exhibit a loss of culturability. Apparently, utilization of reductant for the oxidation of ethylene (and by inference also TCE) had little affect on cell culturability. Cells pre-treated with 2-hexyne and exposed to TCE remained culturable, again indicating that TCE transformation was required to elicit a toxic effect and that TCE, itself, was not the toxic agent. These results confirmed that TCE degradation in resting cell suspensions of *B. cepacia* G4 can cause extensive, non-specific cellular damage.

### 3.4.2. Further examination of the culturability of *B. cepacia* G4 cells following TCE transformation

Because measures of cell culturability are often influenced by the methodology utilized, several different techniques were used to examine the culturability of *B. cepacia* G4 cells that had been exposed to TCE. Colony counts from minimal media plates with 20 mM lactate that had been spread with TCE-treated cells yielded similar results to those obtained with LB plates – cell culturability decreased in an exponential fashion upon TCE transformation (data not shown). However, strikingly different results were observed when culturability was assayed on LB agar plates containing catalase (Table 3.2). The number of colony forming units obtained from cell suspensions exposed to TCE were up to two orders of magnitude higher when catalase...
Figure 3.2. Effects of TCE transformation on toluene 2-monooxygenase activity, general respiratory activity, and cell culturability. Toluene-dependent $O_2$ uptake activity (A), acetate-dependent $O_2$ uptake activity (B), and cell viability (C) in B. cepacia G4 cells following exposure to alkenes. Resting suspensions (2.1 mg of cells ml$^{-1}$) of toluene-grown cells were incubated with 1.36 µmol of TCE (■), ethylene (▲), or no substrate (○) for selected times, harvested, and assayed for toluene-dependent $O_2$ uptake activity, acetate-dependent $O_2$ uptake activity, and cell viability. Viability was measured by colony counts on LB agar plates. Toluene-grown cells were also pre-treated with 2-hexyne to inactivate toluene 2-monooxygenase and then incubated with TCE (♦). The inset in panel C depicts cell viability as a function of the amount of TCE degraded. For each graph (except the inset), values are the mean of two experiments.
Fig. 3.2

A

% Remaining Toluene-Dependent O$_2$ Uptake Activity

% Remaining Acetate-Dependent O$_2$ Uptake Activity

C

% Remaining viability

Inset: Log Cu5 vs TCE (nmol)
was added to the surface of the plates. Catalase addition did not affect the
culturability of non-treated cells. Furthermore, the addition of sodium pyruvate, a
compound that degrades H₂O₂ (Martin et al. 1976; Raymon et al. 1978; McDonald et
al. 1983), to the surface of LB plates also increased the number of colony forming
units obtained from TCE-treated cells (data not shown).

Data from a liquid media-MPN assay further showed that conditions on the
surface of agar plates are not optimal for the growth of TCE-treated cells (Table 3.2).
As measured by this technique, cell culturability was not impacted significantly until
larger quantities of TCE (>0.5 μmol) had been transformed, and even then, the loss of
culturability was far less than that observed on LB plates. The results imply that TCE-
treated cells were able to grow more readily in liquid broth than on the surface of agar
plates. Of particular note, the terminal MPN tubes containing cells that had been
exposed to TCE took longer to develop turbidity than those containing cells that had
not been incubated with TCE. Furthermore, there was a positive correlation between
the length of the delay before the appearance of turbidity and the amount of TCE
transformed.

Data obtained with the BacLight Live/Dead stain showed that TCE-treated B.
cepacia G4 cells remained impermeable to the nucleic acid stain, propidium iodide
(data not shown). Therefore, the toxicity associated with TCE oxidation in B. cepacia
G4 does not appear to be a consequence of extensive structural damage to the cell
membrane.

3.4.3. Recovery of B. cepacia G4 cells following TCE transformation

The aforementioned results suggested that cells of B. cepacia G4 could recover,
while bathing in liquid medium, from the damage accrued during TCE transformation.
To examine the recovery characteristics of B. cepacia G4 following TCE exposure, an
aliquot of TCE-treated cells was resuspended in minimal medium containing lactate
(20 mM) or phenol (2.5 mM initial aqueous concentration) and the ensuing growth
was monitored by OD₆₀₀ measurements. Toluene was not utilized in the recovery
Table 3.2. Cell culturability of *B. cepacia* G4 cells exposed to TCE for selected times

<table>
<thead>
<tr>
<th>TCE exposure time (min)</th>
<th>Average amt of TCE degraded (nmol)</th>
<th>Culturable cells ($10^9$) a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LB agar plates</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>1.69 ± 0.2</td>
</tr>
<tr>
<td>15</td>
<td>202 ± 64</td>
<td>0.48 ± 0.31</td>
</tr>
<tr>
<td>30</td>
<td>447 ± 58</td>
<td>0.0030 ± 0.0026</td>
</tr>
<tr>
<td>60</td>
<td>928 ± 232</td>
<td>0.00011 ± 0.00006</td>
</tr>
</tbody>
</table>

aToluene-grown cells (2.1 mg cells [dry weight]) were exposed to TCE for the indicated times and assayed for culturability either with a liquid medium-MPN technique or via colony formation on LB agar plates. All values are the means for three to five trials ± standard deviations.

bLB agar plates with catalase had 210 U of sterile bovine liver catalase spread on the agar surface.
experiments because it was toxic to *B. cepacia* G4 at aqueous concentrations >1.0 mM, therefore limiting the amount of growth that could be achieved in batch cultures with a single addition of substrate. Phenol was used instead, since it is less toxic than toluene yet still requires toluene 2-monooxygenase activity to be metabolized by *B. cepacia* G4.

Cells that had been exposed to TCE exhibited markedly longer lag periods before exponential growth was observed during the recovery experiment (Fig 3.3). Once exponential growth was observed, there were no obvious differences in the growth rates among the TCE-treated and non-treated cells. As a measure of recovery, I determined the time it took a culture to reach an OD$_{600}$ of 0.3 following TCE exposure in 3 independent trials. Cells exposed to TCE for 0, 15, 30, and 60 min required 11.2 ± 0.9, 13.5 ± 0.5, 17.1 ± 0.9, and 30.5 ± 3.0 h, respectively, to reach an OD$_{600}$ of 0.3 when lactate was used as the growth substrate for recovery. This method yielded more consistent results than was obtained with the liquid media-MPN assay. Data from both experiments indicate that the culturability of cells within a TCE-degrading cell suspension decreases rapidly once the cells have degraded larger quantities of TCE (>400-500 nmol TCE mg cells$^{-1}$). With phenol as the growth substrate, the recovery of TCE-treated cells was delayed even longer relative to the control cells. Cells exposed to TCE for 0, 15, 30, and 60 min required 14.8 ± 1.4, 18.4 ± 1.3, 24.0 ± 1.5, and 52.0 ± 3.4 h, respectively, to reach an OD$_{600}$ of 0.3 with phenol as the growth substrate.

### 3.4.4 Excessive TCE damage selects against toluene 2-monooxygenase activity in *B. cepacia* G4 populations

Since TCE oxidation by *B. cepacia* G4 can result in cellular injury and death, it is possible that TCE degradation by cultures of this organism could select against those cells in the population that exhibit toluene 2-monooxygenase activity. To examine this possibility, *B. cepacia* G4 cells were exposed to TCE for selected times, diluted and spread onto LB plates. Colonies that grew on the LB plates were then
Figure 3.3. Recovery of growth by *B. cepacia* G4 cells exposed to TCE. Cells harvested from cultures grown on toluene were incubated in phosphate buffer containing TCE (1.36 μmol) for 0 (■), 10 (●), 30 (▲), or 60 (●) min. TCE-treated cells were washed, and a set amount of the washed cells was added to vials containing minimal media with 20 mM lactate (A) or 2.5 mM phenol (B). The cultures were then incubated at 30°C with shaking, and growth was monitored by OD₆₀₀ readings.
streaked onto minimal media plates, which were incubated in the presence of toluene vapors. Among the colonies that grew on LB plates following TCE exposure, the percentage that were also capable of growth on toluene decreased with increasing TCE exposure times (Table 3.3).

Table 3.3. Tol+ phenotype among B. cepacia G4 cells that survive TCE exposure

<table>
<thead>
<tr>
<th>TCE exposure time (min)</th>
<th>% Colonies able to grow on toluenea</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>99.7 ± 0.6</td>
</tr>
<tr>
<td>15</td>
<td>99.3 ± 1.2</td>
</tr>
<tr>
<td>30</td>
<td>62.7 ± 35.4</td>
</tr>
<tr>
<td>60</td>
<td>9.5 ± 11.6</td>
</tr>
</tbody>
</table>

aDilutions of cells that had been exposed to TCE for various times were spread on LB agar plates. The percentage of colonies that formed on the LB agar plates that were also capable of growing on toluene was determined by streaking 100 of the colonies onto minimal-medium plates, which were then incubated in the presence of toluene vapors. All values are the means of three to five trials ± standard deviations.

Additionally, none of the Tol+ variants tested (n=12) exhibited toluene 2-monooxygenase activity (assayed by toluene-dependent O2 uptake and toluene consumption) when grown on lactate and induced with toluene (data not shown).

The genes required for toluene 2-monooxygenase activity have been localized to the TOM plasmid (approximately 108 kb) in B. cepacia G4 (Shields et al. 1995). To determine if the Tol+ phenotype observed among strains recovered from TCE-treated cell suspensions was due to instability of the TOM plasmid, the plasmid profile of 10 Tol+ strains was compared to that of wild type B. cepacia G4. The plasmid profile of each of the strains examined was identical to the pattern obtained from wild type cells (data not shown). Furthermore, the plasmid profile of each strain was similar to that previously published for wild type B. cepacia G4 (Shields et al. 1995; Mars et al.
1996), with two bands clearly visible. Therefore, the Tol' phenotype observed among cells surviving TCE exposure was not typically due to plasmid loss. The genetic basis of the Tol' phenotype was not determined.

3.4.5. Nature of the toxic TCE intermediates(s)

Data from previous studies suggest that the toxic species associated with TCE oxidation by bacterial monooxygenases is a short lived intermediate(s) of the reaction (Wackett and Householder 1989; Fox et al. 1990; Oldenhuis et al. 1991; van Hylckama Vlieg et al. 1996; Newman and Wackett 1997). With purified toluene 2-monoxygenase, the stable products of TCE oxidation are glyoxylate (10%), formate (21%), carbon monoxide (41%), and covalently modified oxygenase proteins (12%) (Newman and Wackett 1997). To test the possibility that the stable products of TCE oxidation are responsible for loss of cell viability and/or toluene 2-monoxygenase activity in B. cepacia G4, toluene-grown cell suspensions were incubated for 2 h with various amounts of glyoxylate, formate, and CO. None of the incubations altered the viability or toluene 2-monoxygenase activity of the B. cepacia G4 suspensions (data not shown). The incubations containing the highest levels of glyoxylate (1.5 µmol), formate (3.15 µmol), and CO (6.15 µmol) contained approximately 15 times the amount of products expected during one of the standard 60 min TCE degradation assays described in this manuscript. Additionally, fresh, toluene-grown B. cepacia G4 cells that were incubated with spent media from a 90 min TCE degradation assay with other B. cepacia G4 cells retained full viability and toluene 2-monoxygenase activity (data not shown). These results indicate that the toxic intermediate(s) formed during TCE oxidation by B. cepacia G4 is a short-lived species.

B. cepacia TCS-100 has a Tn5-OT182 cassette integrated into its chromosome (Yeager, unpublished results). This mini-transposon cassette confers tetracycline resistance on the host (wild type B. cepacia G4 is sensitive to tetracycline). Toluene-grown cells of this strain were mixed with toluene-grown wild type B. cepacia G4 at a ratio of 1:9 and incubated in the presence of TCE. Prior to cell mixing, B. cepacia TCS-100, both B. cepacia TCS-100 and wild type B. cepacia G4, or neither strain was
treated with 2-hexyne to inactivate toluene 2-monoxygenase. Following TCE exposure, the viability of \textit{B. cepacia} TCS-100 cells from the assay mixture was determined with LB plates containing tetracycline (15 \( \mu \text{g/ml} \)). The results are presented in Table 3.4. When both strains were pre-treated with 2-hexyne, TCE exposure resulted in a 11\% decrease in the viability of \textit{B. cepacia} TCS-100 cells as compared to the \textit{B. cepacia} TCS-100 control cells (no TCE added). However, the viability of 2-hexyne-treated \textit{B. cepacia} TCS-100 cells decreased 69\% when incubated with fully active wild type \textit{B. cepacia} G4 cells in the presence of TCE. Although this loss of viability was relatively minor when compared to that of \textit{B. cepacia} TCS-100 cells containing active toluene 2-monoxygenase (99.7\%), it indicates that TCE oxidation by a group of cells can have a negative impact on surrounding cells not transforming TCE. Similar results were obtained with a second mutant, \textit{B. cepacia} TCS-101, which has the TN5-OT182 cassette integrated into a different region of the

\begin{table}[h]
\centering
\begin{tabular}{lll}
\hline
2-Hexyne pretreatment\( ^a \) & \% TCS-100 cells able to grow on LB-Tet agar plates following TCE exposure\( ^b \) \\
\hline
Wild-type G4 & TCS-100 & \\
Yes & Yes & 89.0 \( \pm \) 8.5 \\
No & No & 0.3 \( \pm \) 0.25 \\
No & Yes & 31.5 \( \pm \) 3.2 \\
\hline
\end{tabular}
\caption{Diffusible nature of toxic TCE intermediate(s)}
\end{table}

\( ^a \)Cells pre-treated with 2-hexyne lacked toluene 2-monoxygenase activity and were incapable of TCE transformation.

\( ^b \)Toluene-grown cells of \textit{B. cepacia} G4 and \textit{B. cepacia} TCS-100 were or were not pretreated with 2-hexyne (as indicated). The cells were then mixed in a 9:1 ratio (G4-TCS-100) and exposed to TCE for 30 min. The culturability of the TCS-100 cells within the TCE-treated cell mixtures was determined by colony counts on LB agar plates containing tetracycline (15 \( \mu \text{g/ml} \)). All values are the means of three trials \( \pm \) standard deviations.
chromosome than *B. cepacia* TCS-100 (data not shown). It has been previously documented that reactive TCE intermediate(s) can diffuse outside of the transforming cell (van Hylckama Vlieg et al. 1996); however, to my knowledge, this study provides the first evidence that TCE oxidation by a group of cells can adversely effect the health of surrounding cells not participating in TCE oxidation.

The observation of decreasing $T_c$ with increasing cell density is consistent with production of a diffusible intermediate(s) that is more effectively toxic at high cell densities. Consistent with these observations, it was found that the $T_c$ of *B. cepacia* G4 for TCE decreases with increasing cell density (above 1 mg cells ml$^{-1}$), while the $T_c$ for ethylene by the same organism remains relatively constant over the range of cell densities tested (Table 3.1).

### 3.5. Discussion

Previous studies have reported conflicting data on the susceptibility of *B. cepacia* G4 to the toxicity that is often associated with aerobic cometabolism of TCE (Folsom et al. 1990; Folsom and Chapman 1991; Landa et al. 1994; Shields et al. 1994; Mars et al. 1996). The metabolic diversity and robust culturability of *B. cepacia* G4 allowed us to critically examine the toluene 2-monooxygenase activity, general respiratory activity, and culturability of this microorganism following TCE oxidation. TCE oxidation was shown to have a detrimental effect on each of these properties. Interestingly, the general respiratory activity and culturability of cells were more prone to damage during TCE transformation than was toluene 2-monooxygenase. In resting cell assays with *B. cepacia* G4, cells apparently retain the internal reductant pool and enzyme activities necessary to affect the oxidation of TCE, while general cell health as manifested by cell culturability or general respiratory activity can be severely compromised. These results underscore the idea that sustained rates of TCE oxidation by bacterial cells in short assays do not necessarily demonstrate a lack of toxicity associated with the reaction.

As reported with *M. trichosporium* OB3b (van Hylckama Vlieg et al. 1997), the culturability of *B. cepacia* G4 cells decreased exponentially upon TCE oxidation -
when determined by colony formation on LB plates. From studies performed primarily within the context of food microbiology and public health, it is known that catalase and pyruvate can increase enumeration of physically or chemically injured bacteria on agar plates (Martin et al. 1976; McDonald et al. 1983; Calabrese and Bissonnette 1990). It is thought that catalase and pyruvate act by preventing the accumulation of $\text{H}_2\text{O}_2$ in, and/or around, injured cells, which, in contrast to healthy cells, are apparently unable to tolerate even low levels of this reactive oxygen species (Martin et al. 1976; Raymon et al. 1978). In this study, it was found that the addition of catalase or sodium pyruvate to the surface of the LB agar plates increased the culturability of TCE-treated cells of *B. cepacia* G4 by as much as 100 fold.

Viable cell count estimates performed in this study with a liquid media-MPN assay also indicate that a dramatic decrease in cell culturability does not occur until a relatively large amount of TCE has been transformed (0.5 μmol mg cells$^{-1}$). From these results it appears that the culturability of *B. cepacia* G4 does not necessarily decrease exponentially during TCE oxidation; however, cellular injuries that render the bacteria ultra-susceptible to oxidative stress do accumulate in an exponential fashion. Thus, conditions that support the formation of $\text{H}_2\text{O}_2$ either intracellularly or in the external environment (i.e. the surface of agar plates) may hinder the recovery of TCE-injured bacteria.

The results from this study indicate that there is a critical level of damage, or a toxicity threshold, that a population of *B. cepacia* G4 cells can accumulate during TCE oxidation, beyond which cell culturability drops considerably. In their work with the methanotrophs *M. trichosporium* OB3b and CAC-2, Chu and Alvarez-Cohen (1999) suggest that general cellular damage proceeds in a linear relationship with the amount of TCE degraded, until a critical quantity of TCE is oxidized at which point cells can no longer recover. The recovery of both *M. trichosporium* Ob3b and CAC-2 was severely limited once the general respiratory activity of the cells decreased to <5% of its original level. Chu and Alvarez-Cohen (1999) also suggest that the $T_c$ for TCE provides a measure of the toxicity threshold exhibited by methanotrophs upon oxidation of this compound. When cells of *M. trichosporium* OB3b or CAC-2 had
degraded an amount of TCE that approached their respective Tc values, respiratory activity decreased approximately 95% and cell recovery was severely limited. With *B. cepacia* G4 I determined a Tc of 0.75 μmol mg cells⁻¹ for TCE at the cell density (2.1 mg cells ml⁻¹) used for most assays performed in this study. Yet, *B. cepacia* G4 cells that had degraded between 0.2 and 0.5 μmol TCE mg cells⁻¹ exceeded their toxicity threshold as determined by decreased acetate-dependent O₂ uptake rates and long recovery times. With *B. cepacia* G4, the toxicity threshold was clearly exceeded when the cells had degraded an amount of TCE that corresponded to approximately 66% of their Tc for this compound. While the Tc of *B. cepacia* G4 for TCE certainly describes the amount of TCE that can be degraded by non-growing cells of this microorganism, it does not correspond with the toxicity threshold of this organism. The general applicability of relating Tc values to the resuscitation potential of TCE-injured bacterial cultures will require further research.

The negative impact of TCE oxidation on cellular recovery was overtly pronounced in *B. cepacia* G4 cells when phenol was used as the growth substrate for recovery. Since phenol has biocidal properties (Sikkema et al. 1995) and can inhibit growth of *B. cepacia* G4 at high concentrations (Folsom et al. 1990) (my unpublished observations), it is possible that cells damaged extensively during TCE oxidation are unable to maintain the protective mechanisms or other physiological adaptations that are required for growth on phenol. Alternatively, TCE oxidation could selectively debilitate cells possessing toluene 2-monooxygenase activity, effectively enriching the number of Tol⁻ variants within a population. If so, the amount of time required for a TCE-treated population of cells to recover (grow) on phenol would increase relative to the time required for the cells to recover with lactate as the growth substrate. Indeed, it was found in this study that among *B. cepacia* cells that had oxidized approximately 0.5 μmol TCE (mg of cells⁻¹) a disproportionate number of the survivors subsequently isolated on LB agar plates (up to 90%) lacked toluene 2-monooxygenase activity (Tol⁻).

The emergence of Tol⁻ mutants from populations of *B. cepacia* cells originally containing toluene 2-monooxygenase activity has been documented previously. Mars
et al. (1996) found that mutants of *B. cepacia* G4 that had lost the TOM plasmid took over a pure culture that was exposed to TCE and starved for carbon and energy over a period of days. Tol' variants have also been detected within toluene-grown batch cultures of another bacteria, *Pseudomonas putida* 54G (Leddy et al. 1995; Mirpuri et al. 1997). In one study the percentage of Tol' variants within the toluene-grown population of *P. putida* 54G approached nearly 10% under certain conditions (Leddy et al. 1995). Furthermore, three types of Tol' variants were observed: one still harbored the 118 kb TOL-like plasmid (this 118 kb plasmid harbors the *meta* pathway for toluene catabolism in wild type *P. putida* 54G), a second type harbored a TOL-like plasmid of reduced molecular weight, and the third was cured of the plasmid altogether. In the current study the Tol' phenotype could not be attributed to the loss of the TOM plasmid. It seems plausible that the toluene-grown cell suspensions of *B. cepacia* G4 used in the TCE exposure assays contained a small percentage of Tol' members that nonetheless harbor the TOM plasmid, and that the toxicity associated with TCE oxidation acted to enrich these Tol' members of the population by selectively debilitating cells that did display toluene 2-monooxygenase activity. Regardless of the mechanism, this selective pressure could certainly compromise the likelihood of resuscitating a TCE-degrading population of *B. cepacia* G4 in instances where the toxicity threshold of the cells had been exceeded.

In summary, results from this study demonstrate that *B. cepacia* G4 does incur damage during TCE oxidation, with injuries that impair general cellular processes such as respiratory metabolism and cell culturability outpacing inactivation of toluene 2-monooxygenase. Additionally, there appears to be a critical amount of damage that *B. cepacia* G4 cells can accumulate during TCE oxidation, or a toxicity threshold, beyond which cellular recovery is severely limited. These findings have practical implications for the development of sustainable bioremediation systems for TCE degradation.
3.6. **Acknowledgements**

I thank Malcolm Shields for providing *B. cepacia* G4 and a special thanks to Miriam Sluis and Natsuko Hamamura for review and comments.

Funding for this study was provided by the office of Research and Development, U.S. Environmental Protection Agency, under Agreement PR-0345 through the Western Region Hazardous Substance Research Center.
Chapter 4

The Requirement of DNA Repair Mechanisms for the Survival of *Burkholderia cepacia* G4 upon Degradation of Trichloroethylene

Chris M. Yeager, Peter J. Bottomley, and Daniel J. Arp

Prepared for submission to *Applied and Environmental Microbiology*, American Society for Microbiology
4.1. Abstract

A Tn5-based mutagenesis strategy was used to generate a collection of trichloroethylene sensitive mutants (TCS) to identify repair systems or protective mechanisms that shield Burkholderia cepacia G4 from the toxic effects associated with trichloroethylene (TCE) oxidation. Single Tn5 insertion sites were mapped within open reading frames putatively encoding enzymes involved in DNA repair (UvrB, RuvB, RecA, and RecG) in seven of the 11 TCS strains obtained (four of the TCS strains had a single Tn5 insertion within a uvrB homolog). The data revealed that the uvrB disrupted strains were exceptionally susceptible to killing by TCE oxidation followed by the recA strain, whereas the ruvB and recG strains were just slightly more sensitive to TCE than wild type. The uvrB and recA strains were also extremely hypersensitive to UV light, and to a lesser extent mitomycin C and H2O2 exposures. The data from this study establish a link between DNA repair and the survivability of B. cepacia G4 cells following TCE transformation. A possible role for nucleotide excision repair and recombinational repair activities in TCE-damaged cells is discussed.

4.2. Introduction

Trichloroethylene (TCE), a suspected human carcinogen (National Cancer Institute 1976), has been used extensively as a metal degreaser, fumigant, and solvent for dry cleaning and other commercial applications. Because of its widespread use and persistence, TCE is one of the most commonly detected organic pollutants at hazardous waste sites and in municipal groundwater supplies in the United States (Love and Eilers 1982; Westrick et al. 1984). Although it has not been demonstrated that microorganisms can utilize TCE as a growth-supporting substrate under aerobic conditions, a number of bacteria are capable of degrading TCE cometabolically, whereby non-specific oxygenases catalyze the initial transformation (Wackett et al. 1989; Ensley 1991; Arp 1995).
The practicality of utilizing bacteria to degrade TCE via aerobic cometabolism has been questioned, however, due to the cytotoxicity that is almost universally associated with TCE degradation. Loss of TCE-degradative activity is often observed with whole cells during TCE transformation (Wackett and Gibson 1988; Oldenhuis et al. 1991; Rasche et al. 1991; van Hylckama Vlieg et al. 1997; Yeager et al. 2001), and each of the TCE-degrading enzymes that have been purified to homogeneity and examined to date (toluene dioxygenase, toluene 2-monoxygenase, and soluble methane monoxygenase) exhibit turnover-dependent inactivation upon TCE oxidation (Fox et al. 1990; Li and Wackett 1992; Newman and Wackett 1997). Additionally, TCE degradation can result in injuries that adversely affect more basic cellular functions, such as general respiratory activity and cell viability (Heald and Jenkins 1994; van Hylckama Vlieg et al. 1997; Chu and Alvarez-Cohen 1999; Yeager et al. 2001). Although the exact nature of the destructive species remains unknown, it has been proposed that acyl chlorides, generated from hydrolysis or rearrangement of TCE epoxide (monoxygenase catalyzed reactions) or TCE-dioxetane (dioxygenase catalyzed reactions), cause damage by alkylating various cellular constituents (Wackett and Householder 1989; Fox et al. 1990; Li and Wackett 1992; Newman and Wackett 1997; van Hylckama Vlieg et al. 1997).

Because cellular toxicity can potentially limit the sustainability of TCE biodegradation under aerobic conditions, a concerted effort has been directed towards identifying strains that resist inactivation during TCE transformation. Initial observations suggested that the toluene-oxidizing bacterium, *Burkholderia cepacia* G4, was such an organism (Folsom et al. 1990; Folsom and Chapman 1991; Landa et al. 1994). Subsequently, this strain (or derivatives of it) has become one of the best-known and well-studied microorganisms in terms of TCE bioremediation. Results from recent studies indicate that *B. cepacia* G4 is indeed susceptible to cellular damage as a result of TCE degradation, however. Newman and Wackett (1997) demonstrated that purified toluene 2-monoxygenase, which catalyzes TCE oxidation in *B. cepacia* G4, is inactivated during TCE oxidation in vitro. In another study, a four-fold increase in the maintenance energy requirements of *B. cepacia* G4 cells was
observed when they were cultivated in a toluene-fed batch reactor and exposed to TCE under non-growth conditions (Mars et al. 1996). The authors speculated that maintenance energy and growth conditions may play a role in influencing the extent to which *B. cepacia* G4 cells can repair damage incurred during TCE degradation, thus influencing the ultimate sustainability of TCE degradation. Finally, in Chapter 3 it was demonstrated that rapid rates of TCE degradation severely compromised the culturability and general respiratory activity of *B. cepacia* G4 cells, whereas inactivation of toluene 2-monooxygenase proceeds at a relatively slow pace *in vivo*.

The available data suggests that although *B. cepacia* G4 is indeed susceptible to toxic effects upon TCE oxidation, it is also likely to possess protective mechanisms and/or repair systems that influence the extent to which TCE degradation ultimately damages the cell. Without such repair or protective devices, or under physiological conditions that prevent them from functioning fully, cells would be particularly sensitive to TCE-mediated injury. In the current study transposon insertion mutagenesis was used to identify and characterize mutants of *B. cepacia* G4 that were ultra-susceptible to TCE-mediated cytotoxicity.

4.3. Materials and Methods

4.3.1. Bacterial strains and culture conditions

*B. cepacia* G4 was provided courtesy of Malcolm Shields (University of West Florida, Pensacola). All strains were maintained on minimal medium agar plates containing 20 mM sodium lactate (Materials and Methods, Chapter 3). To obtain cells for experimental assays, liquid cultures were grown overnight at 30°C with shaking in sealed serum vials (160 ml) containing minimal media (60 ml) with 20 mM sodium lactate. Alternatively, cells were grown on toluene (1.0 mM initial aqueous phase concentration) by providing two sequential additions of toluene (9 μl), as previously described (Materials and Methods, Chapter 3) To collect cells, cultures were centrifuged (8,000 x g for 10 min), rinsed twice with 50 mM KH₂PO₄-K₂HPO₄ buffer,
pH 7.0 (phosphate buffer) and resuspended in fresh phosphate buffer as a concentrated cell solution. The cell suspension was stored at room temperature for ≤1 h before use.

### 4.3.2. Transposon mutagenesis

Tn5-OT182 was introduced into *B. cepacia* G4 via conjugal transfer using an adaptation of a filter-mating technique (DeShazer et al. 1997). *E. coli* S17-1 (ATCC 47055) was grown overnight in 5 ml of LB containing tetracycline (15 μg/ml) and ampicillin (100 μg/ml). *B. cepacia* G4 was grown similarly without antibiotics. A portion of each culture (100 μl) was added to 3 ml of sterile 10 mM MgSO₄ solution, thoroughly mixed and filtered through a single 0.45 μm pore-size membrane filter disk (Millipore, type HA). Filter disks were subsequently placed upright on LB plates containing 10 mM MgSO₄ and incubated at 37°C for 12-14 h. The filters were then placed in sterile 15 ml culture tubes containing 0.85% NaCl and cells were washed from the filter by vortexing. Aliquots (100 μl) of the resulting cell suspension were spread onto minimal medium plates containing sodium lactate (20 mM) and tetracycline (15 μg/ml). Transconjugants of *B. cepacia* G4 were identified following incubation at 30°C for 72-96 h. Wild type *B. cepacia* G4 is sensitive to tetracycline and *E. coli* S17-1 cannot utilize lactate as a growth-supporting substrate. Control assays were performed using *B. cepacia* G4 or *E. coli* S17-1 alone and no colonies were observed on the selective plates.

### 4.3.3. Screening for TCE sensitive mutants

Tn5 insertion mutants of *B. cepacia* G4 were replica plated onto two sets of minimal medium plates, which were then incubated at 30°C for 4-5 days in sealed, one-gallon polyethylene jars containing toluene vapors. To select for TCE sensitive mutants, one of the replica plate sets was incubated in the presence of both toluene and TCE vapors. Toluene vapors were supplied by adding toluene (150 μl) to a Durham tube, plugging the tube with cotton, and placing the tube in an empty Petri dish at the bottom of the polyethylene jar. TCE vapors were supplied by including neat TCE (30
μl) in the Durham tube containing toluene. During the incubation period, the colony size of each mutant strain grown on toluene alone was monitored periodically by visual inspection and compared to that of the corresponding strain grown on toluene in the presence of TCE. Growth of all strains was impaired in the presence of TCE, but against this background, the inhibitory effect of TCE was exaggerated in certain strains, which were classified as "TCE sensitive". All strains identified as TCE sensitive were subjected to two more rounds of screening (as described above) to confirm the TCE sensitive (TCS) phenotype.

4.3.4. Cloning and sequencing DNA flanking the Tn5 inserts

Chromosomal DNA from TCS strains was isolated according to a standard protocol (Sambrook et al. 1989). Self-cloning of DNA flanking the Tn5 insert in the TCS mutants was accomplished by the method of Merriman and Lamont (Merriman and Lamont 1993). Approximately 4-5 μg of chromosomal DNA was digested with either EcoRI, XhoI, SalI, ClaI, HindIII, BamHI, or NheI, heated at 75°C for 20 min to inactivate the endonuclease, and precipitated with sodium acetate and absolute ethanol. The DNA pellets were washed in 70% ethanol, air dried, and resuspended in 30 μl dH2O. Twenty-five μl of the DNA suspension was self-ligated overnight at 12°C in a reaction volume of 50 μl containing 1 unit of ligase (Promega, Madison, WI). A portion of the ligated DNA (5 μl) was transformed into competent DH5α cells (Gibco BRL, Rockville, MD) as described by the supplier, and Ap'Tc' transformants were selected for isolation of plasmids. Plasmid preparations were performed as previously described (Lee and Rasheed 1990) and purified with the “Concert Rapid PCR Purification System” from Gibco BRL prior to sequencing.

Automated DNA sequencing was performed by the Central Services Laboratory, Center for Gene Research and Biotechnology at Oregon State University. Primers OT182-RT and OT182-LT were used to sequence the cloned DNA immediately flanking the Tn5 insertion site, as described by DeShazer et al. (DeShazer et al. 1997). OT182-RT and OT182-LT were synthesized by Gemini Biotech, LTD (Alachua, FL).
A primer walking strategy was used to determine the entire coding sequence of each gene that was disrupted by Tn5 insertion in the TCS mutants. Synthetic primers were ordered from Sigma Genosys (The Woodlands, TX) to prime these reactions.

4.3.5. **Recovery of growth by *B. cepacia* G4 cells exposed to TCE**

Toluene-grown cells (1 mg protein) were added to sealed serum vials (10 ml) containing phosphate buffer with 250 μM TCE (1 ml final reaction volume). The reaction vials were incubated at 30°C with shaking (150 rpm). At select time points, samples (50 μl) were removed from the TCE reaction mixture and added to a sterile glass serum vial (160 ml) containing minimal medium (60 ml) with 20 mM sodium lactate. The inoculated vials were then incubated at 30°C with shaking and 1 ml portions were removed periodically to monitor the OD₆₀₀ of the culture.

4.3.6. **Chemical and UV sensitivity assays**

Toluene-grown cells of wild type *B. cepacia* G4 and the TCS mutants were exposed to TCE, UV light, mitomycin C, or hydrogen peroxide. For chemical exposures cells (1 mg total cell protein) were added to sealed serum vials (10 ml) containing phosphate buffer and either TCE (250 μM), mitomycin C (0.25, 1.0, 5.0, or 25 mg/ml), or H₂O₂ (0.1, 0.5, or 2 mM). The final reaction volume was 1 ml for TCE and H₂O₂ treatments and 2 ml for the mitomycin C treatment. The reaction vials were incubated at 30°C with shaking for 30 min for mitomycin C and H₂O₂ treatments and 15, 30, or 60 min for the TCE treatment. The viability of the chemically treated cells was determined by plating appropriate dilutions onto R2A agar plates (Difco, Sparks, MD). For UV exposures cells were diluted, spread onto R2A plates, and the surface of each plate was exposed to UV light at 5, 10, 20, or 50 J/m².
4.3.7. **Analytical and other methods**

The aqueous concentrations of toluene and TCE in liquid-gas systems at 30°C were calculated with dimensionless Henry’s constants of 0.343 (Materials and Methods, Chapter 2) and 0.494 (Gossett 1987), respectively. Toluene (99.8%) and TCE (99+%) were obtained from Aldrich (Milwaukee, WI). The protein concentration of cell suspensions was determined by measuring the OD$_{600}$ of appropriate dilutions of the cells and applying an appropriate conversion factor (suspensions of *B. cepacia* G4 cells with an OD$_{600}$ of 1.0 contain 0.2 mg total cell protein ml$^{-1}$). Protein concentrations were determined with the Biuret assay (Gornall et al. 1949) following cell solubilization in 3 M NaOH for 30 min at 65°C. Bovine serum albumin was used as the standard. The dry weights of culture samples were determined by resuspending cells in dH$_2$O in pre-weighed Eppendorf tubes, drying for 2 days at 55°C, and weighing the cell pellets. It was determined that 2.1 mg of *B. cepacia* G4 cells (dry weight) contains approximately 1.0 mg protein.

Hydrocarbons were analyzed with a Shimadzu (Kyoto, Japan) GC-8A chromatograph equipped with a flame ionization detector and a stainless steel column (0.3 by 61 cm) packed with Porapak Q 80-100 mesh (Alltech, Deerfield, Ill.). To detect ethylene a column temperature of 100°C was utilized, and for TCE the column temperature was 155°C. The injector and detector temperatures were set at 200°C for all analyses. Hydrocarbons were quantified by comparison of peak heights to standard curves constructed from known amounts of authentic compounds.

4.3.8. **Nucleotide sequence accession numbers**

The nucleotide sequences determined in this study were deposited in GenBank under accession numbers: AY036066 (*recA*), AY036067 (*ruvB*), AY036068 (*uvrB*), and AY036069 (*recG*).
4.4. Results

4.4.1. Selection and genetic characterization of TCE sensitive mutants of
B. cepacia G4

To identify genetic loci that are involved in mediating the toxic effects
associated with TCE oxidation in B. cepacia G4, a Tn5 mutagenesis strategy was
employed. B. cepacia G4 cells were randomly mutagenized with Tn5-OT182, and
mutants were screened for TCE sensitivity by monitoring colony growth on minimal
media agar plates with toluene vapors supplied as a source of carbon and energy with
or without TCE (Fig 4.1).

Approximately 4500 mutants were initially screened for sensitivity to TCE. All
mutants whose growth was inhibited relative to their peers in the presence of TCE
were screened twice more to confirm the phenotype. Following the third round of
screening, 20 TCE sensitive mutants (TCS) were identified. To ensure that the Tn5-
OT182 cassette had inserted into the chromosome of each of the TCS mutants and that
it had not inserted into multiple sites, genomic DNA was isolated from each mutant
and digested with EcoRI. A southern blot was performed with the DNA fragments
from each mutant and a radiolabeled portion of pOT182 as the probe. Chromosomal
fragments from nine of the mutants did not hybridize with the probe. Further
characterization of these mutants was not pursued. A single radiolabeled band was
observed in lanes containing DNA from the other 11 mutants (data not shown),
indicating that each of these mutants contained a single copy of Tn5-OT182 inserted
in their respective chromosomes.

DNA flanking the Tn5-OT182 insertion site in each mutant was isolated by self-
cloning and subsequently sequenced (Merriman and Lamont 1993). The readable
sequences from each mutant were approximately 600 bp in length. A Blastx search
performed with these sequences revealed that the Tn5 insertion site in seven of the 11
TCS mutants had occurred within genetic loci predicted to code for proteins involved
in DNA repair (Table 4.1). These proteins, UvrB, RuvB, RecA, and RecG are
involved in several distinct DNA repair systems including nucleotide excision repair,
recombinational repair, and the SOS response system. Interestingly, four of the TCS mutants had a Tn5 insertion within a putative \textit{uvrB} gene. Sequence analysis revealed that each of the Tn5 insertions in the putative \textit{uvrB} gene had occurred at separate sites covering a 770 bp section of the gene with at least 65 bp between each insertion site (Fig 4.2). The sequencing data coupled with further Southern analysis (data not shown) indicated that the Tn5 insertions had occurred in the same \textit{uvrB} gene copy in

\begin{center}
\textbf{Figure 4.1.} Selection of TCE sensitive mutants of \textit{B. cepacia} G4. Colony growth of \textit{B. cepacia} G4 strains on minimal media agar plates with toluene vapors ± TCE vapors. The colonies were spotted in duplicate on each plate.
\end{center}
TCS-1, TCS-3, TCS-4, and TCS-13. The remaining four TCS mutants had insertion sites in genes associated with carbon metabolism. Because preliminary experiments indicated that the TCS mutants with Tn5 insertions in genes encoding putative DNA repair enzymes were the most sensitive to TCE damage and to limit the scope of this study, further analysis was limited to these strains.

Table 4.1. Blastx analysis of DNA sequences flanking the Tn5-OT182 insertion sites in TCE sensitive mutants of B. cepacia G4

<table>
<thead>
<tr>
<th>TCE Sensitive Mutant</th>
<th>Homologous sequence</th>
<th>Function of putative gene product</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCS1</td>
<td>uvrB</td>
<td>Subunit B of (A)BC excinuclease - nucleotide excision repair</td>
</tr>
<tr>
<td>TCS3</td>
<td>uvrB</td>
<td>See above</td>
</tr>
<tr>
<td>TCS4</td>
<td>uvrB</td>
<td>See above</td>
</tr>
<tr>
<td>TCS8</td>
<td>ruvB</td>
<td>Holliday junction DNA helicase - genetic recombination and recombinational repair</td>
</tr>
<tr>
<td>TCS12</td>
<td>recA</td>
<td>Genetic recombination and recombinational repair, regulation of the SOS response, stress response regulator</td>
</tr>
<tr>
<td>TCS13</td>
<td>uvrB</td>
<td>See above</td>
</tr>
<tr>
<td>TCS14</td>
<td>recG</td>
<td>Holliday junction DNA helicase - genetic recombination and recombinational repair</td>
</tr>
</tbody>
</table>
Figure 4.2. Schematic map of DNA fragments self-cloned from TCS mutants. The open triangles represent the location of the Tn5-OT182 insertions in each TCS mutant. The *recA*, *ruvB*, *uvrB*, and *recG* homologs are designated by black bars with the direction of transcription specified by the arrowhead. The nucleotide sequence of the *recA* upstream region is provided in the inset, with the putative transcriptional start site (+1) and –35 and –10 promoter sequences underlined. The putative LexA binding region (SOS box) is boxed.
Fig 4.2
4.4.2. Sequence analysis of the putative uvrB, ruvB, recA, and recG coding regions from *B. cepacia* G4

Further sequencing of the DNA fragments self-cloned from TCS-4, TCS-8, TCS-12, and TCS-14 was performed to characterize the coding regions of the Tn5-interrupted genes (Fig 4.2). In each case a single open reading frame (ORF) was identified. Analysis of the DNA fragment from TCS-8 revealed an ORF putatively encoding a 356 amino acid protein. The amino acid sequence was highly similar to RuvB proteins from a number of bacteria including: *Neisseria meningitidis* (76% identity), *Vibrio cholerae* (72% identity), *Pseudomonas aeruginosa* (70% identity), and *Escherichia coli* (70% identity). It is interesting that DNA sequence homologous to the ruvA gene was not found immediately upstream of the putative ruvB gene in *B. cepacia* G4, because bacterial ruvA and ruvB genes are almost always found together in an operon (Sharpl et al. 1999). A partial ORF was identified from the DNA fragments self-cloned from TCS-14. The complete sequence of the ORF was not determined (the 3' terminal region was not sequenced) due to the small size of the cloned fragments. The partial ORF was determined to encode 654 amino acids and amino acids 37-654 shared significant similarity with RecG proteins from *N. meningitidis* (56% identity), *P. aeruginosa* (57% identity), and *E. coli* (54% identity), which range in length from 680-693 amino acids. The ORF disrupted by Tn5 insertion in strain TCS-4 putatively encoded a 697 amino acid protein. The first 18 amino acids encoded by the ORF do not share significant similarity with any proteins in the Blastx database, but the remainder of the amino acid sequence was 68, 67, and 65% identical to UvrB proteins from *N. meningitidis*, *P. aeruginosa*, and *E. coli*, respectively.

The sequence obtained from the TCS-12 DNA fragment revealed a single ORF consisting of 1041 bp. The amino acid sequence encoded by this ORF was found to be 100% identical to the RecA protein from several strains of *Burkholderia vietnamiensis* and it shared 97-98% identity with RecA proteins from multiple strains of *B. cepacia*. The sequence upstream of the recA start codon was highly similar to that previously described in *Pseudomonas cepacia* (Nakazawa et al. 1990). A putative
SOS box (palindromic sequence that binds the LexA repressor protein of the SOS response system) was identified 100 bp upstream of the start codon, overlapping with a potential –10 promoter consensus sequence (Fig 4.2A). Analysis of the upstream regions (200-400 bp) of the putative ruvB, uvrB, and recG genes from B. cepacia G4 did not reveal the presence of sequences resembling the SOS box (CTG-N10-CAG).

Recently, Mahenthiralingam et al. (2000) found that DNA sequence analysis of recA genes from members of the B. cepacia complex provided a successful approach to taxonomically classify these organisms. The DNA sequence of the entire recA homolog from B. cepacia G4 exhibits 99% identity with recA sequences from known B. vietnamiensis strains and 93-94% identity with recA sequences from various B. cepacia strains. In addition, phylogenetic analysis of the 16S rRNA gene sequence from B. cepacia G4 obtained from the ribosomal database project (accession no. L28675) (Maidak et al. 1994) also suggests that this organism is more closely related to the B. vietnamiensis group (data not shown).

4.4.3. Growth characteristics of TCS strains following TCE exposure

It was previously found that monitoring the growth (OD600 measurements) of TCE-treated cells in liquid medium provides a consistent method of assessing general cellular damage incurred by cells that have oxidized TCE (Fig 3.3). Figure 4.3B exemplifies a typical growth curve of wild type B. cepacia G4 and select TCS cultures with sodium lactate supplied as the growth substrate. With the exception of TCS-14, the growth curves of the TCS strains were similar to that of wild type B. cepacia G4 in the absence of TCE treatment. TCS-14 cells tended to clump during growth on lactate until the cultures had reached an OD600 of approximately 0.4-0.5. Cell suspensions of each strain that had been exposed to TCE exhibited markedly longer lag periods prior to the onset of exponential growth (recovery time), but once exponential growth was observed there were no obvious differences in the growth rates among the TCE-treated and non-treated cell suspensions (Fig 4.3A). Relative to wild type B. cepacia G4, lag times were extended by approximately 8.0, 8.0, 2.3, 6.5, and 3.5 h in TCS1, TCS4, TCS8, TCS12, and TCS14, respectively. The growth curves of TCE-treated TCS-1
and TCS-4 cultures were almost identical. In fact, the phenotypes of each of the "uvrB mutants" (TCS-1, TCS-3, TCS-4, and TCS-13) were essentially indistinguishable from one another when tested throughout this study. Each of the TCS mutants degraded TCE at similar rates as wild type *B. cepacia* G4 (14 nmol min\(^{-1}\) mg total cell protein\(^{-1}\)) during the TCE exposure period.

![Graph A](image1.png)

**Figure 4.3.** Recovery of growth by TCE-treated cells. Wild type *B. cepacia* G4 (■), TCS1 (●), TCS4 (▲), TCS8 (◆), TCS12 (□), and TCS14 (○). Cells were grown overnight with toluene vapors and (A) incubated with (B) or without TCE for 30 min. A portion of each cell suspension was then added to vials containing minimal media with 20 mM sodium lactate, and culture growth (at 30°C with shaking) was monitored by OD\(_{600}\) readings.
4.4.4. Sensitivity to TCE, mitomycin C, hydrogen peroxide, and UV light

Since the TCS strains being examined were presumably deficient in DNA repair activity, I examined the survival of select TCS strains following exposure to several known DNA damaging agents or TCE (Fig 4.4). TCS-1 cells showed the most sensitivity to each of the agents tested, usually followed by TCS-12 then TCS-8 and TCS-14. When challenged with TCE or UV, TCS-1 and TCS-12 were exceedingly more sensitive than wild type *B. cepacia* G4, whereas, the response of TCS-1 and TCS-12 to H$_2$O$_2$ or mitomycin C was far less exaggerated relative to wild type cells. It is also interesting to note that most wild type cells (96%) remained culturable following 15 minutes of TCE exposure, whereas only 3 and 9% of TCS-1 and TCS-12 cells, respectively, remained culturable. With the exception of H$_2$O$_2$, TCS-8 and TCS-14 were slightly more susceptible to killing by the agents tested.

By selectively inactivating toluene 2-monooxygenase with alkynes, it was previously shown that the sensitivity of wild type *B. cepacia* G4 to TCE is turnover-dependent (51,52). Similarly, the sensitivity of TCS-1 and TCS-12 to TCE was insignificant when 2-hexyne was included in the incubations (data not shown, other TCS mutants were not tested).

4.5. Discussion

In mammals, TCE transformation is catalyzed by cytochrome P450-dependent monooxygenases and the genetic toxicity associated with the reaction has been studied extensively (9). From these studies it is known that $^{14}$C-TCE metabolites bind to DNA *in vitro* (Banerjee and Van Duuren 1978; Di Renzo et al. 1982; Bergman 1983; Miller and Guengerich 1983). There is also some evidence for covalent modification of DNA *in vivo* by $^{14}$C-TCE metabolites; however, the data are somewhat questionable, particularly because of the interference caused by the metabolic incorporation of C1 products formed during $^{14}$C-TCE oxidation.
Figure 4.4. Survival of cells upon exposure to TCE, UV light, H\textsubscript{2}O\textsubscript{2}, and mitomycin C. Wild type \textit{B. cepacia} G4 (■), TCS1 (●), TCS8 (♦), TCS12 (△), and TCS14 (□) with exposure to (A) TCE, (B) UV light, (C) mitomycin c, or (D) H\textsubscript{2}O\textsubscript{2}. Toluene-grown cells were exposed to each agent as described in the material and methods, diluted in phosphate buffer, and spread on R2A plates. After a 3 day incubation period at 30°C, the number of colonies per plate was determined relative to control plates, spread with untreated cells, to determine the surviving fraction.
Fig. 4.4

A

Surviving Fraction

TCE Exposure Time (min)

B

Surviving Fraction

UV Exposure Time (min)

C

Surviving Fraction

Mitomycin C (mg/L)

D

Surviving Fraction

H₂O₂ (mM)
(Bergman 1983; Fahrig et al. 1995). There is also limited evidence that TCE metabolites bind irreversibly to DNA in prokaryotes. Wackett and Householder (Wackett and Householder 1989) observed radiolabel incorporation into the DNA fraction of *P. putida* F1 cells upon $^{14}$C-TCE oxidation. In the current study, genetic and physiological characterization of TCE sensitive mutants of *B. cepacia* G4 revealed that functional DNA repair mechanisms play a role in the survival of TCE-damaged cells of this organism. Furthermore, the genetic identity of each TCS mutant, in conjunction with data obtained from the survival assays, provides insight into the DNA repair mechanisms involved and leads us to speculate that potentially lethal DNA adducts are formed during TCE degradation in *B. cepacia* G4.

In *E. coli*, cells require nucleotide excision repair (NER) working in combination with homologous recombination repair enzymes to fully recover from damage caused by many types of DNA lesions (Friedberg et al. 1995; Sancor 1996). When damage to DNA overwhelms excision repair capacity, DNA replication enzymes can stall at the blocking lesions. In this case, the replisome can either wait for the arresting lesion to be repaired via NER, or it can bypass the damage and restart downstream, leaving single-stranded gaps of approximately 1500 nucleotides. The gaps are subsequently filled by recombinational exchange with an undamaged sister duplex, a process termed daughter strand gap repair. Reassembly, maintenance, and re-initiation of the replication fork are thought to require the strand-exchange proteins, RecA, RecF, RecO, and RecR, and in daughter strand gap repair, RecG and/or RuvABC are required to promote branch migration and resolution of the Holliday junction recombination intermediates (Kowalczykowski et al. 1994; Friedberg et al. 1995; Pandya et al. 2000). RecG and RuvABC are thought to possess partially overlapping functions (Sharples et al. 1999). Indeed, *ruv* or *recG* single mutants are usually only modestly susceptible to UV and other DNA-damaging agents (as were TCS8 and TCS14 in the current study), while *ruv recG* double mutants are far more sensitive to such treatments (Lloyd 1991).
The preponderance of TCS mutants containing a Tn5 insertion in homologs of genes encoding enzymes involved in daughter strand gap repair (\textit{ruvB}, \textit{recG}, and \textit{recA}), re-initiation of the replication fork (\textit{recA}), and NER (\textit{uvrB}) provides suggestive evidence that \textit{B. cepacia} G4 cells utilize NER in conjunction with daughter strand gap repair to recover from damage accumulated during TCE oxidation. The extreme sensitivity of the "\textit{uvrB} mutants" (TCS1, TCS3, TCS4, and TCS13) to both TCE and UV exposure further underscores the importance of a functional nucleotide excision repair (NER) system for recovery of \textit{B. cepacia} G4 cells from these treatments. Without a functional NER system, bacterial cells cannot effectively remove a variety of DNA lesions, particularly bulky DNA adducts such as UV photoproducts, which ultimately results in the cessation of DNA synthesis and cell death (Friedberg et al. 1995; Sancor 1996). These observations certainly imply that DNA adducts accumulate \textit{in vivo} during TCE transformation by \textit{B. cepacia} G4.

The susceptibility of bacterial \textit{recA} mutants to UV and other DNA damaging agents can be linked to the inability of these strains to up-regulate the expression of SOS response genes upon sensing DNA damage. Indeed, RecA is known to stimulate the expression of at least 20 genes in \textit{E. coli}, including \textit{uvrB}, \textit{recA}, and \textit{ruvAB} and recent analysis of the \textit{E. coli} genome has revealed that there may be as many as 69 genes in the LexA-RecA regulon (Fernandez de Henestrosa et al. 2000). An SOS consensus region was identified upstream of the \textit{recA} coding region in \textit{B. cepacia} G4, yet found no evidence of the SOS consensus sequence upstream of either the \textit{uvrB}, \textit{recG}, or \textit{ruvB} homologs in this organism. These observations suggest that RecA does not directly regulate transcription of the \textit{uvrB}, \textit{recG}, and \textit{ruvB} homologs in \textit{B. cepacia} G4. Likewise, the \textit{uvrB} gene of \textit{P. aeruginosa} is not DNA damage-inducible nor is a SOS consensus region found upstream of its promoter (Rivera et al. 1996). Although other DNA repair enzymes may be part of the SOS regulon in \textit{B. cepacia} G4, it is likely that RecA plays a more pivotal role as a recombinase in protecting the cell from TCE-related damage.

Although TCS4 and TCS12 were each more susceptible to mitomycin C and H$_2$O$_2$ than wild type cells of \textit{B. cepacia} G4 (up to an order of magnitude difference in
survival), it was found that these strains were much more sensitive to TCE and UV exposures. NER mechanisms are not normally required to repair \( \text{H}_2\text{O}_2 \)-induced DNA damage in bacteria, thus it is not surprising that cells of TCS4 are less susceptible to damage from \( \text{H}_2\text{O}_2 \) than UV. Additionally, the effect of \( \text{H}_2\text{O}_2 \) on bacterial strains deficient in DNA repair activities can vary considerably from strain to strain. For example, \( \text{recA} \) mutants of \textit{E. coli} and \textit{Salmonella} are extremely sensitive to \( \text{H}_2\text{O}_2 \), yet \( \text{recA} \) mutants of \textit{Xanthomonas oryzae pv. oryzae} and \textit{Neisseria gonorrhoeae} exhibit the same level of resistance to \( \text{H}_2\text{O}_2 \) as their respective parental wild type strains (Mongkolsuk et al. 1998). Apparently, \( \text{recA} \) plays a limited role in protecting cells against oxidative stress in certain bacteria, including \textit{B. cepacia} G4. Because microbial resistance to \( \text{H}_2\text{O}_2 \) is a complex phenotype that depends on the action of numerous proteins including antioxidant enzymes, DNA binding proteins, DNA repair enzymes, and free-radical-scavenging agents (Demple and Harrison 1994; Martinez and Kolter 1997), the loss of RecA activity could certainly be masked by the actions of other defense systems. Indeed, under conditions of high cell density (as was utilized in the experiments described in this study) catalase often acts as the first line of defense for bacteria against \( \text{H}_2\text{O}_2 \) stress (Ma and Eaton 1992).

The weak response of TCS12 and TCS4 cells to mitomycin C in comparison to TCE and UV is more difficult to reconcile, because mitomycin C induces bulky DNA lesions, particularly DNA cross-links (Tomasz and Palom 1997). Damage caused by mitomycin C in \textit{B. cepacia} G4 could conceivably be constrained by some other factor such as alternative DNA repair mechanisms, uptake into the cell, efflux pumps, etc., any of which could mask the \( \text{uvrB} \) or \( \text{recA} \) phenotypes. Indeed, resistance to mitomycin C has been linked to drug export systems in both \textit{E. coli} and \textit{Streptomyces lavendulae} (Sheldon et al. 1999; Wei et al. 2001).

A connection between DNA repair mechanisms and the degradation of another chlorinated aliphatic hydrocarbon (CAH), dichloromethane, has been previously established. It was recently discovered that DNA polymerase I is essential for the growth of \textit{Methylobacterium dichloromethanicum} DM4 with dichloromethane (Kayser et al. 2000). DNA polymerase I exhibits polymerase, 5'-3' exonuclease, and 3'-5'
exonuclease activity, and as a DNA repair enzyme one of its primary functions is to fill the gap formed during excision repair processes. The authors suggested that DNA polymerase I could allow *M. dichloromethanicum* DM4 cells to grow on dichloromethane by aiding in the removal of DNA adducts formed between DNA and S-chloromethylglutathione, a proposed intermediate of dichloromethane conversion to formaldehyde (Kayser et al. 2000).

The results from the current study suggest that the recovery characteristics of TCE-damaged cells of *B. cepacia* G4 cells are dependent upon NER and additionally, recombinational repair enzymes. In Chapter 3 it was observed that *B. cepacia* G4 cells can accumulate a certain amount of damage during TCE oxidation, a toxicity threshold, before cell culturability is impacted significantly. A similar phenomenon has been reported for *Methylosinus trichosporium* Ob3B during TCE oxidation (Chu and Alvarez-Cohen 1999). In the current study, the culturability of wild type *B. cepacia* G4 on R2A plates did not decrease during a 15 min exposure to TCE, yet cell suspensions of the TCS1 and TCS12 lost 97 and 90% of their culturable members, respectively, during a similar treatment. It is possible that the toxicity threshold observed in *B. cepacia* G4 upon TCE transformation marks the point in time in which DNA damage overwhelms the functional capacity of the NER and recombinational repair systems of this organism. In light of these observations, it seems plausible that the ultimate TCE-or CAH-degrading potential of a given microorganism under aerobic conditions may be partially dependent on the efficiency of its DNA repair systems. The possibilities definitely warrant further exploration.

4.6. Acknowledgement

Funding for this study was provided by the office of Research and Development, U.S. Environmental Protection Agency, under Agreement PR-0345 through the Western Region Hazardous Substance Research Center.
Chapter 5. Summary

5.1. Inactivation of toluene 2-monooxygenase in *Burkholderia cepacia* G4 by alkynes

Degradation of TCE by microorganisms under aerobic conditions is catalyzed by non-specific oxygenases. In addition to this striking lack of substrate specificity, a feature that is seemingly ubiquitous among these enzymes is turnover-dependent loss of activity upon TCE oxidation (Fox et al. 1990; Li and Wackett 1992; Newman and Wackett 1997). This phenomenon also extends to mammalian cytochrome P-450 monooxygenases, where it has been studied extensively (Miller and Guengerich 1983; Bruckner et al. 1989).

I confirmed that acetylene was a weak inhibitor of toluene 2-monooxygenase activity in *B. cepacia* G4. Yet, longer-chain alkynes from C₅ to C₁₀ were effective inhibitors of toluene 2-monooxygenase. Through a series of whole-cell kinetic and physiological experiments evidence was provided that alkynes act as mechanism-based inactivators of toluene 2-monooxygenase in *B. cepacia* G4, with 2- and 3-alkynes yielding a more potent response than their 1-alkyne counterparts. Specifically, alkynes were shown to elicit an irreversible, time-dependent loss of toluene 2-monooxygenase activity that followed pseudo-first-order kinetics with apparent rate constants ranging from 0.25 min⁻¹ to 2.45 min⁻¹. It was also demonstrated that toluene protected toluene 2-monooxygenase against inactivation by alkynes. Finally, because O₂ was required for inactivation, it is likely that catalytic activation of alkynes is required to generate the destructive species. Several of the general criteria for mechanism-based inactivators were not addressed including: the inactivation reaction should be saturable, inactivation should proceed without an observable lag time, and demonstration of a 1:1 stoichiometry of inactivator covalently bound to the target enzyme.

It was suggested in Chapter 2 that acetylene is a poor mechanism-based inactivator of toluene 2-monooxygenase simply because it binds poorly to the enzyme.
Yet, it was demonstrated that the short chain alkenes, ethylene and propylene, were oxidized by \textit{B. cepacia} G4 to their respective epoxides with $K_s$ values of 32 and 40 $\mu$M, respectively. Neither ethylene nor propylene oxidation was accompanied by the loss of toluene 2-monoxygenase activity.

From the studies described in Chapter 2 I discovered that although acetylene was not an efficient inactivator of toluene 2-monoxygenase in \textit{B. cepacia} G4, longer chain, 2- and 3-alkynes, such as 2-hexyne, could be used as tools to specifically and effectively inactivate toluene 2-monoxygenase. Furthermore, the results provide tantalizing evidence that it may be possible to rapidly assess the role of particular monoxygenases or microorganisms in mixed cultures or in field studies using selective, alkyne-based enzyme inactivators.

5.2. Cytotoxicity associated with trichloroethylene oxidation in \textit{Burkholderia cepacia} G4

Data presented in Chapter 3 illustrate the effect of TCE oxidation on each of these physiological parameters. Interestingly, cellular damage manifested as a loss of general respiratory activity and decreased cell culturability outpaced loss of toluene 2-monoxygenase activity during TCE oxidation. TCE turnover was required to bring about cellular damage, because cells incubated in the presence of TCE and 2-hexyne (thus lacking toluene 2-monoxygenase activity) retained almost full levels of general respiratory activity and cell culturability. These results also confirm that the effect of alkynes on whole cells of \textit{B. cepacia} G4 is limited to toluene 2-monoxygenase. Cellular energetics were largely discounted as a major factor contributing to the general damage and loss of toluene 2-monoxygenase activity incurred during TCE oxidation, because \textit{B. cepacia} G4 cells that had transformed similar amounts of ethylene exhibited none of these detrimental effects. Furthermore, the transformation capacity, or maximum mass of a compound that can be degraded per mass of cells prior to inactivation, of \textit{B. cepacia} G4 for ethylene was shown to be 10-18 times greater than for TCE under the conditions tested. Barring the possibility that TCE oxidation results in high levels of NADH uncoupling relative to that of ethylene.
oxidation, I believe these experiments effectively provide a measure of the effects of reductant drain on cells that should be experienced during TCE transformation.

An intriguing observation was made concerning the effect of TCE oxidation on cell culturability during the investigations. It was found that measures of culturability of TCE-injured cells varied up to 3 orders of magnitude, depending on the method of assessment. Particularly, the results suggest that TCE-injured cells are ultra susceptible to damage from $\text{H}_2\text{O}_2$ on the surface of agar plates. These results indicate that TCE-damaged cells of \textit{B. cepacia} G4 are sensitive to other stresses that may not visibly effect healthy cells, and that the recovery of TCE-damaged cells can be greatly influenced by environmental factors.

A central theme that emerged from the study presented in Chapter 3 was that there appeared to be a toxicity threshold for \textit{B. cepacia} G4 cells during TCE oxidation. Cells that had degraded $\geq 0.5$ $\mu$mol of TCE (mg of cells$^{-1}$) exhibited significant loss of both of general respiratory activity and culturability, and recovery of growth by TCE-injured cells was severely compromised at and beyond this level of TCE oxidation. A similar phenomenon has been described for TCE degradation by methanotrophic bacteria (Chu and Alvarez-Cohen 1999). In the case of \textit{B. cepacia} G4, the transformation capacity for TCE exceeds the toxicity threshold by approximately two fold. In other words, cells will continue to degrade TCE long after they are effectively "dead". Thus, toxicity thresholds may be useful in designing sustainable, bioremediation systems by providing operational limits to the amount of TCE that can be degraded by an active biomass.

5.3. The requirement of DNA repair mechanisms for the survival of \textit{Burkholderia cepacia} G4 upon degradation of trichloroethylene

In Chapter 3 I maintained that the destructive species associated with TCE oxidation in \textit{B. cepacia} G4 was a short-lived intermediate of the reaction that was nonetheless capable of diffusing outside of the cell and injuring surrounding organisms. A reactive intermediate such as this would certainly have the opportunity to damage a variety of cellular constituents. Additionally, because the predominant
damage associated with TCE oxidation was directed towards general cellular processes, such as respiration and culturability, I hypothesized that cells harbor general mechanisms that could moderate these damaging effects. Therefore, I decided to take a genetic approach (as described in Chapter 4) in an effort to reveal the cellular targets of the reactive intermediate(s) formed during TCE oxidation and to concurrently identify protective mechanisms and/or repair also involved.

Of the 11 Tn5 insertion mutants that exhibited impaired levels of growth in the presence of TCE, seven of them had single Tn5 insertion sites in genes putatively encoding enzymes involved with DNA repair including: subunit B of the (A)BC excinuclease, RecA, and the DNA helicases, RecG and RuvB. The sensitivity of these strains to TCE was shown to be turnover-dependent, and was confirmed by several different assays. Furthermore, each of these strains was found to be ultra sensitive to several known DNA damaging agents (H2O2, UV light, and mitomycin C), as determined by cell culturability on R2A plates. The strains with Tn5 insertions in \textit{uvrB} and \textit{recA} homologs were especially susceptible to killing by TCE and UV light.

The data presented in Chapter 4 firmly establishes a connection between DNA repair and the survivability of TCE-injured cells of \textit{B. cepacia} G4. I suggest that nucleotide excision repair and recombinational repair mechanisms are directly involved in repairing DNA damage (presumably DNA adducts) incurred during TCE oxidation. Several observations led us to further propose that the toxicity threshold of \textit{B. cepacia} G4 for TCE may correspond to the point at which DNA damage accumulated during TCE oxidation overwhelms the cell’s capacity for repair. The extent to which TCE-induced DNA damage is responsible for cell death or loss of general respiratory activity in wild type cells of \textit{B. cepacia} G4 cannot be entirely ascertained from my data. Bacterial cells will shut down many basic cellular functions as a result of DNA damage (Friedberg et al. 1995); however, it is also possible that the general damage associated with TCE oxidation results from interactions between reactive TCE intermediate(s) and other cellular targets, such as lipids, proteins, and/or various small molecules. Yet it is a distinct possibility that the ability of a
microorganism to successfully sustain TCE degradation under aerobic conditions (in a non-suicidal fashion) may well depend upon the efficacy of its DNA repair systems.

5.4. Concluding remarks

The research presented in this thesis was undertaken to characterize the response of *B. cepacia* G4 to TCE oxidation. The data demonstrates that this organism is not a “wonderbug”, but is in fact susceptible to toxic effects upon TCE oxidation. As with methanotrophs (van Hylckama Vlieg et al. 1997; Chu and Alvarez-Cohen 1999), TCE-related damage is not constrained to the transforming oxygenase (as would be the case with a mechanism-based inactivator), but rather has a greater impact upon basic cellular processes, such as general respiratory activity and cell division. Although a smoking gun was not identified, DNA damage is almost certainly one of the debilitating consequences of TCE oxidation by *B. cepacia* G4. Hopefully, the insights gained from these basic cellular studies will enable scientists and engineers to better design and model bioremediation systems for the cleanup of TCE and other CAH pollutants.


Dekant, W., K. Berthold, S. Vamvakas, D. Henschler and M. W. Anders. 1988. Thioacetylating intermediates as metabolites of S-(1,2-dichlorovinyl)-L-cysteine and S-


