BIOREMEDIATION OF CIS-DICHLOROETHENE BY *POLAROMONAS* SP. STRAIN JS666

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Because *Polaromonas* sp. JS666 is able to aerobically oxidize *cis*-dichloroethene (cDCE) as sole carbon and energy source, it is a promising candidate for use as a bioaugmentation agent at cDCE-contaminated sites. To test the feasibility of using JS666 for bioremediation, we conducted microcosm studies with subsurface material from six aerobic, cDCE-contaminated sites. Under favorable conditions, JS666 was able to degrade cDCE in every sediment or groundwater with which it was inoculated. Additionally, JS666 showed some success when challenged with an alternate carbon source and/or competitive microorganisms.

Further, a DNA-based probe was used in conjunction with quantitative PCR to track the abundance of JS666 in microcosms. We found the probe accurately and precisely tracks growth when suspected predation is not present. We were able to resolve the accuracy and precision of the probe and determine how measured JS666 cells correlated with variations in microcosm performance. Moreover, a positive result from this probe suggests that degradation can occur in suitable environmental, conditions, as the DNA-target does not persist long after cell death within environmental samples.

To design a more effective bioindicator – one that is based on a gene directly involved with cDCE degradation – fundamental knowledge about the metabolic pathways of the organism is necessary. While there are hypotheses of parallel pathways employed by JS666, currently the cDCE degradation pathway is not completely elucidated. In order to better understand the

genetic regulation of this organism, we performed a suite of studies in attempt to observe the pattern of expression of putative genes found to be upregulated during cDCE degradation under dynamic conditions. These experiments suggest that a putative haloacid dehalogenase gene is involved in degradation, and it may be possible to surmise which genes are involved with oxidative stress. However, we found that the window of response of JS666 was most likely too small to be able to make this approach useful.

Collectively, these studies suggest that it is possible to employ JS666 for bioaugmentation of aerobic, cDCE-contaminated sites, validate the utility of the DNA-based probe for site assessment, and further our understanding of the metabolic functioning of this organism.

BIOGRAPHICAL SKETCH

After graduating high school at the age of sixteen, Cloelle lived in Mendoza, Argentina to avoid applying to college. After returning home, and after working at an artisan pasta shop and a few bars, she finally decided to further her education. Cloelle started her undergraduate studies at the University of Vermont before transferring to Smith College. In 2004, she proudly joined the first class to receive a degree from an engineering program at an all-women's college in the United States. Cloelle got her B.S. in Engineering Science. She then went on to Cornell University to pursue advanced degrees in Environmental Engineering and received a M.S. in 2008 before continuing on to pursue a Ph.D. In addition to her studies, Cloelle enjoyed being a part of the outdoor education community at Cornell and taught various rock climbing courses.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
AFB	Air Force Base
ATP	adenosine triphosphate
bgs	below ground surface
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BTEX	benzene, toluene, ethylbenzene, xylenes
CAD	chloroacetaldehyde dehydrogenase
cDCE	*cis*-1,2-dichloroethene (-ylene)
CFU	colony forming units
CMO	cyclohexanone monooxygenase
CSIA	compound specific isotope analysis
CT	threshold cycle
CV	coefficent of variation
DART-PCR	data analysis for real-time polymerase chain reaction
DCA	1,2-dichloroethane
dH_2O	distilled water
Da	Daltons
1,1-DCE	1,1-dichloroethene
DNA	deoxyribonucleic acid
DNAPL	dense non-aqueous phase liquid
E. coli	*Escherichia coli*
EPA	Environmental Protection Agency
EtOH	Ethanol
Fe(III)	ferric iron
FID	flame-ionization detector
GC	gas chromatography
GSH	glutathione
GST	glutathione S-transferase
GW	Groundwater
H_3PO_4	phosphoric acid
HAD	haloacid dehalogenase
IAWTP	Ithaca Area Wastewater Treatment Plant
ISO	isocitrate lyase
JS666	*Polaromonas* sp. JS666
kb	thousand base pairs
kWh	kilowatt hours
LS/MS/MS	liquid chromatography / tandem mass spectrometry
LUC	exogenous internal reference luciferase
Mb	million base pairs
MBT	molecular biological tool
MCL	maximum contaminant level
MEHQ	hydroquinone monomethyl ether
Mn-(IV)	manganese dioxide

mRNA	messenger ribonucleic acid
MSM	minimal salts medium
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
NPL	National Priority List
OD600	optical density at 600 nm
P450	cytochrome P450
PCE	perchloroethene (-ylene), tetrachloroethene (-ylene)
PE	primary effluent
PNP	pyridoxamine 5'-phosphate oxidase
ppb	parts per bilion
PVC	polyvinyl chloride
qPCR	quantitative polymerase chain reaction
redox	oxidation-reduction
RPM	rotations per minute
RpoB	beta subunit of RNA polymerase
RT-PCR	real-time polymerase chain reaction
SJCA	St. Julien's Creek Annex
SO_4^{-2}	sulfate
SRS	Savannah River Site
SVE	soil vapor extraction
TCD	thermal conductivity detector
TCE	trichloroethene (-ylene)
tDCE	trans-1,2-dichloroethene
TMS	trace-metal solution
TSA	tripticase soy agar
VC	vinyl chloride

LIST OF SYMBOLS

k	specific substrate utilization rate
Ks	half-saturation constant
R_0	initial copy number in qPCR
Y	growth yield
σ	standard deviation of sample
μ	average value of samples

CHAPTER 1

1. INTRODUCTION

1.1. Context

Chlorinated ethenes such as perchloroethene (PCE) and trichloroethene (TCE) are commonly used in dry-cleaning and as industrial solvents, respectively. They have been introduced into the environment, largely though poor disposal techniques and accidental spills/leaks (1). PCE and TCE are dense, non-aqueous phase liquids (DNAPLs) that, if released into an aquifer, tend to sit below water. Chlorinated ethenes are volatile, slightly soluble and less viscous than water, all of which contribute to their mobility in the environment (3).

Incomplete anaerobic reductive dechlorination of chlorinated ethenes can lead to an accumulation of daughter products such as *cis*-1,2-dichloroethene (cDCE) that can migrate to aerobic zones where anaerobic degradation is no longer feasible without imposing changes to the system. In these circumstances, remediation by aerobic oxidation of cDCE would be more practicable. The bacterium *Polaromonas* sp. JS666 is the only isolate that can aerobically oxidize cDCE as its sole carbon and energy source. Because this organism does not require any additional amendments to degrade cDCE, JS666 is a potential bioaugmentation agent at sites where cDCE has migrated to aerobic environments (2).

1.2. Objectives

Collectively, the experimental studies constituting this dissertation were designed to address issues in the development of JS666 as a bioaugmentation agent for remediation of cDCE-contaminated aerobic sites. The focus of this work was three-fold. The first objective was to ascertain if JS666 could survive when introduced into subsurface materials containing indigenous microbiota and substrates. The second objective was to develop DNA-based

molecular tools for monitoring/tracking presence of JS666 in bioaugmented environments. Finally, experiments were conducted to gain a better understanding of this organism through monitoring dynamic expression of suspected pathway genes and housekeeping genes – the aim being to gain some insight into what genes' expressions coincide with onset of cDCE degradation. Molecular probes based on mRNA-biomarkers that are coincident with cDCE degradation could potentially be more useful targets to assess *in situ* activity than would be DNA-based probes.

1.2.1. Microcosm Assessment

Previous work has demonstrated that JS666 is able to degrade cDCE without the addition of any external carbon source. However, to be able to use an organism for bioaugmentation, it is imperative that it be robust enough to survive in the subsurface. Microcosms were constructed from subsurface materials from aerobic plumes at six different cDCE-contaminated sites. These microcosms were designed to address possible hurdles in the survival of JS666 in a variety of subsurface materials. These challenges included the presence of indigenous microorganisms, micronutrient and metals requirements, alternative co-substrates, buffering capacity of soil or groundwater, inoculation level, and concentration of cDCE. In all phases of study, activity was assessed principally through the monitoring of cDCE degradation.

1.2.2. DNA Probe

In addition to demonstrating that bioaugmentation is possible, it is also important to be able to monitor the organism once it is injected into a field site. To this end, additional microcosm studies were conducted that included the application of a DNA-based molecular probe (used in conjunction with real-time, quantitative polymerase chain reaction, qPCR) to track growth or

decay of JS666. A probe based on the species-specific isocitrate lyase gene was designed to assess JS666 quantities in microcosms.

1.2.3. Dynamic Expression Studies

DNA-based probes are convenient, but potential limitations are that DNA might persist long after cell death, and gene-presence (even in living cells) addresses metabolic *capability*, not *activity*. The intention of the final set of experiments described herein was to examine the pattern of expression of putative, cDCE-pathway degradative genes (and other genes found to be upregulated during cDCE degradation) under dynamic conditions. This would have two-fold utility: (i) to indicate mRNA markers potentially useful as indicators of cDCE-degradation activity; and (ii) to provide useful observations that might help elucidate the primary degradative pathway(s), including genes that are associated with the onset of cDCE degradation.

1.3. Organization

Chapter 2 presents a literature review supporting the exploration of JS666 for use in bioaugmentation and relevant research on the physiology and function of this organism. In Chapter 3, studies are presented in which the survival of JS666 and its ability to degrade cDCE in the subsurface were explored through microcosm experiments. In conjunction with the microcosm studies, the application of a DNA-based probe to track JS666 in the subsurface was examined, and these studies are presented in Chapter 4. In Chapter 5, a set of experiments to examine the pattern of expression of putative, cDCE-pathway degradation genes and other genes of potential interest are presented. Lastly, Chapter 6 presents a summary of this work and suggestions for future study.

1.4. REFERENCES

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CHAPTER 2

2. BACKGROUND

2.1. Use of Chlorinated Ethenes and Their Introduction Into the Environment

Chlorinated ethenes are among the most prevalent groundwater pollutants in the United States, and are on the US EPA's list of primary regulated drinking water contaminants (58). Tetrachloroethylene (PCE) has been found in 771 of the nearly 1500 National Priority List (NPL) sites and trichloroethylene (TCE) in 861, making TCE the most commonly occurring contaminant at such sites (40, 55, 56). Moreover, these numbers can only increase as more sites are evaluated (55, 56).

While chlorinated ethenes are not technically xenobiotic compounds, the vast majority of these chemicals encountered in the environment have anthropogenic origins (22, 40). Both PCE and TCE are used as metal degreasers and industrial solvents; additionally, they are used to create other chemicals and can be found in consumer products, such as refrigerants (55, 56). PCE is most widely used in dry cleaning. PCE and TCE are mainly found in the environment due to unintentionally direct release, after which they migrate through the subsurface (Figure 2.1).

Certain microorganisms can degrade PCE and TCE anaerobically *in situ* by the process of reductive dechlorination (6, 39). However, reductive dechlorination can stall at daughter compounds (lesser chlorinated ethenes) without proper conditions. This partial degradation will occur if the correct microorganisms are not present, if there is a lack of electron donor, or if the geochemistry changes such as with an oxygen recharge event (40). When this occurs, the daughter products cis-1,2-dichloroethene (cDCE) and vinyl chloride (VC) can accumulate (16, 40).

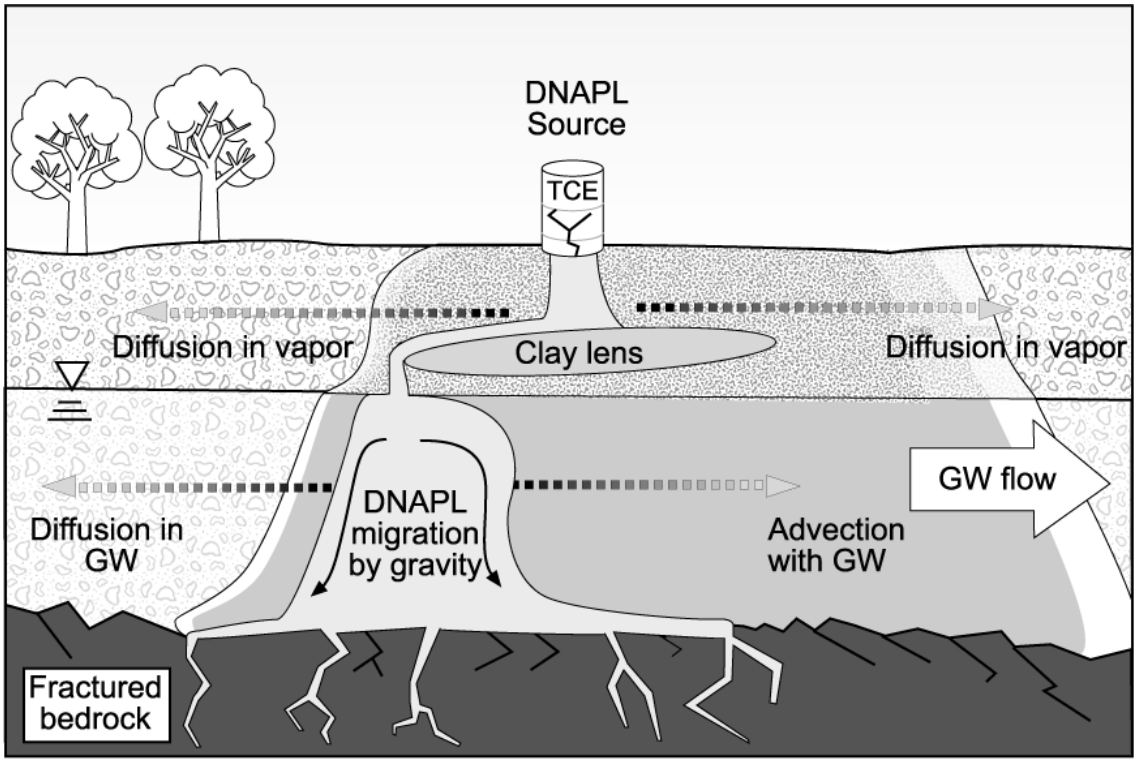

Figure 2.1: Example of DNAPL subsurface transport processes (58).

It is estimated that in 1993, over 11 million pounds of PCE were released into the air, 10,152 to water, and 8,027 to soil in the United States alone. In 1982, the amount of PCE released to the environment amounted to 80-90% of the annual use of PCE (55). The main mode of PCE release is evaporation, however it can also make its way into soil and ground water through leaking underground storage tanks or through poor industrial waste-disposal techniques (55).

Like PCE, the main path of TCE into the environment is through evaporation during use, but it also can leak from underground storage and poor disposal practices (56). Additionally, TCE is an intermediate in PCE degradation and may occur at sites where PCE was the original contaminant but reductive dehalogenation has occurred (6).

cDCE is mostly used as an intermediate in making other chlorinated chemicals, and can also be used as a solvent to extract oils, fats and other organic materials (53). It is commonly produced

as a by-product or impurity from other chemical manufacturing. Its release into the environment can be due evaporation from waste streams, leaching from waste disposal sites, or spills (53). However, the main source of cDCE at contaminated sites is likely not its direct discharge, but rather as a daughter product from the reductive dechlorination of PCE or TCE (40). Once in the atmosphere, cDCE is predicted to be removed photochemically undergo, however this process is unlikely to occur in water or soil systems (53).

Vinyl chloride (VC) is produced in the United States mainly to manufacture polyvinyl chloride (PVC) and other copolymers (57). Most VC found in the environment is either from spills, leaks from storage, improper disposal or from the break down of the higher chlorinated ethenes. VC is highly volatile, and once in the air, it can be degraded photochemically (57). The VC that doesn't volatilize will leach through the soil, and since VC is slightly soluble, some will dissolve into water and can migrate with groundwater.

While studies differ on the carcinogenicity of chlorinated ethenes – i.e., whether they are *known* or merely *suspected* human carcinogens – it is clear that they are toxic (40). The one chlorinated ethene about which there is no such debate is VC: it is a known human carcinogen, and as such has an MCL of 2 ppb in water, and an exposure limit of 1 ppm in the air per 8-hour work day (57). The toxicology of TCE has been widely studied due to its relative prevalence in groundwater. Even though it is unclear if TCE is carcinogenic in humans, it has been linked to increased risk of Parkinson's disease, breast cancer, and autoimmune syndromes (40). TCE, like PCE, has an MCL of 5 ppb in water and exposure limit of 100 ppm in the air per 8-hour workday (55, 56). While, cDCE has the highest MCL of the chlorinated ethenes discussed here (70 ppb in water), it is nonetheless toxic and in animal studies has been shown to cause central nervous system and respiratory depression in oral exposure. Studies of inhalation of cDCE suggest that

the heart, liver, and lung are potential targets for toxicity, and occupational exposure is limited to 200 ppm in the air (57).

2.2. Remediation of Chlorinated Ethenes

The cleanup of these and other volatile organic compounds is estimated to cost "more than $45 billion dollars (1996 dollars) over the next several decades" (58). There are a number of systems in place to treat sites contaminated with chlorinated ethenes, including pump-and-treat, whereby contaminated groundwater is pumped to the surface and treated *ex situ* by carbon adsorption, air-stripping, and biological reactors (59). Other systems include surfactant/co-flushing, *in situ* chemical oxidation, and various *in situ* thermal technologies that are appropriate for source-zone remediation (e.g., steam injection, electrical resistive heating, and thermal conductive heating) (59). Thermal treatments can help overcome some of the challenges of pump and treat – the low solubilities of chlorinated ethenes and their high octanol-water partition coefficients impede their removal via pumped-out groundwater. Of the technologies used to remediate contaminated sites (particularly of lower-concentration zones down-gradient of sources), *in situ* bioremediation, including monitored natural attenuation and bioaugmentation, is recognized as being a promising and cost-effective solution (36*,* 47).

Moreover, as part of a push to use more sustainable practices and "promote environmental stewardship," the EPA has been incorporating green remediation strategies into all phases of remediation. *In situ* bioremediation, as a relatively low-energy system, is an example of green remediation versus more "active-energy" systems that use mechanical equipment to treat contaminated soil and groundwater. Collectively, active-energy systems such as pump-and-treat, and thermal desorption, use an estimated 620 million kWh annually (60).

2.3. Bioremediation Lines of Evidence

Bioremediation depends on suitable conditions, including the presence of suitable organisms with the metabolic capacity to degrade the pollutants, and favorable geochemical conditions. Even if these conditions are met, a site may require amendments to stimulate degradation. In cases where microorganisms with the degradative capacity are absent, sites will require augmentation with microorganisms (20, 39, 46).

Site characterization is essential to successful bioremediation, which involves three lines of evidence: (i) demonstration of the reduction of contaminant mass; (ii) the potential for biodegradation, which can be demonstrated through geochemical data such as dissolved oxygen levels or redox potential; and (iii) demonstration that microbial activity is responsible for the contaminant reduction. The three requisite lines of evidence have been shown in successful bioaugmentation of sites impacted by chlorinated ethenes (19, 20, 39).

The final line of evidence is usually demonstrated through microcosms and/or column studies. Microcosm and column studies are time-consuming and expensive and do not necessarily reflect *in situ* conditions, as moving material from the subsurface to a lab is likely to change the conditions of the system (28). Molecular biological tools (MBTs) that can measure potential (or, better yet, current) microbial activity *in situ* can make a case for natural attenuation and enhanced natural attenuation as treatment methods, and they could replace the current use of microcosm and column studies (51). MBTs are usually molecular probes, which are detectable molecules that bind to a nucleic acid of interest. Compared to microcosms, MBTs require far smaller sample size; are capable of producing results in hours or days (instead of weeks or months); and are therefore less expensive to run, per sample. Therefore, for similar sampling/analysis costs, more locations at a site can be assayed with MBTs than with

microcosms, resulting in a far better characterization of the physical distribution of biodegradation potential (and possibly activity) at a site.

An MBT that relies on an organism-specific, DNA-based probe has benefits and drawbacks. DNA is relatively stable in the environment, which makes it easy to work with. However, it can persist for sometime after cell death, meaning that a positive result does not necessarily equate with *current* potential for degradation. Furthermore, presence of a gene does not guarantee that it is actively being expressed; therefore, DNA-based probes cannot be presumed to correlate with activity (13, 44). Nevertheless, this type of probe serves the important purpose of showing the capability for degradation and also how the target organism has become distributed in a bioaugmentation context. Currently, DNA-based probes, even those based on phylogeny and not function, are accepted as indication of potential degradation at bioaugmentation sites, and are both qualitative (indicating presence), and also quantitative (demonstrating abundance) (47).

Messenger RNA (mRNA) is potentially a useful bioindicator because changes in transcript levels reveal regulatory response of an organism to environmental changes more quickly than other cellular indicators. Moreover, the instability of mRNA, while making it difficult to work with, means that it is a time-sensitive indicator. The possibility of detecting a false positive, meaning detecting transcripts while the cell is inactive, is decreased. There have been a number of studies designed to examine expression of functional genes by extracting RNA from mixed culture and subsurface materials with the hope of tying those genes to active processes to monitor *in situ* metabolic activity (13, 26, 27, 34, 48).

Studies on a mixed enrichment culture containing *Dehalococcoides ethenogenes* strain 195 were completed in order to better understand potential differences in gene expression between the planktonic or biofloc cell attachment forms (48). This work tested whether or not

heterogeneity in the community attachment phases sampled – predominantly planktonic cells are expected from groundwater samples – may bias a sample's representation of the community expression as a whole. Rowe et al. discovered that upregulated genes of interest for *Dehalococcoides*, with the exception of *tceA*, which encodes the TCE reductive dehalogenase, were expressed similarly in both attachment forms; however 16S rRNA gene copies of other organisms had higher representation within the bioflocs (48). The potential for differentially expressed genes across attachment forms highlights that preliminary work is required for respective genes and cultures across different growth phases of interest. Understanding how various microbes are distributed between attachment phases ensures sampling adequately represents and quantifies the organisms of interest.

Studies on mixed microbial systems completed by Holmes et al. on the organism *Geobacter* in four subsurface materials showed the feasibility of measuring transcript levels of key genes (reported relative to constitutively expressed housekeeping genes) and relating them to metabolic activity correlated to ferric-iron reduction (27). Lee et al. used qPCR to measure transcript levels of the three reductive dehalogenase genes, *tceA*, *vcrA*, and *bvcA*, which likely corresponded to different *Dehalococcoides* species, in groundwater at a TCE-contaminated site at Fort Lewis, WA (34). These gene copy numbers were followed over a one-year period during biostimulation and bioaugmentation of the site. Over time, these biomarkers increased concurrent with biostimulation, changes in injection strategy, and degradation of TCE and production of VC and ethene (34).

However, changes in transcripts and their concentrations do not necessarily reflect the production of functional enzymes. Moreover, genes associated with degradation or transformation of compounds of interest may be constitutively expressed, meaning assays based

on their differential expression would not reveal their presence. Production of enzymes, which are the workhorses in degradation, have a number of complicating factors between mRNA synthesis and protein production. These include ribosome-binding efficiencies; competition for catalytic components, such as ribosomes and tRNA that can affect efficiency of translation; post-translational modifications; and stability of the protein product (42). Rowe et al. demonstrated that transcript numbers or population numbers of individual organisms may not necessarily reveal their metabolic contributions, as some organisms can carry vastly more ribosomes and convert disproportionate amounts of electron equivalents to end products (48). Conversely, proteins are a direct measure of what enzymes have been actually produced. There have been a number of studies showing the successful use of peptide biomarkers in environmental samples to track presence (14) and activity of organisms of interest (5, 63).

Chuang et al. developed a method to detect enzymes expressed by aerobic etheneotrophs and VC-assimilating bacteria with shotgun, mass-spectrometry-based, proteomic methods in ethene-enriched groundwater microcosms from a VC-contaminated site (14). They were able to confirm presence or absence of ethenotrophs via enzyme-detection in half of the samples analyzed, which were confirmed to have etheneotrophs by culture-based techniques. They attribute false-negatives to inefficient protein extraction. This demonstrates the feasibility – and some of the difficulty – of finding proteins of interest in environmental samples (14).

Moreover, it is possible to use biomarkers not only to provide evidence for remediation, but also evidence for presence of the contaminant of concern. Bansal et al. used peptides derived from two proteins, chlorite dismutase and perchlorate reductase, to show perchlorate presence (versus an alternative substrate) and biodegradation in both pure and mixed-cultures (5).

Wilkins et al. used peptide biomarkers to monitor biostimulation and population shifts of *Geobacter in situ* (63). This group used shotgun-proteomic methods to track the citrate synthase enzyme of *Geobacter* during two years of biostimulation at the Department of Energy (DOE) Integrated Field Research Challenge (IFRC) site in Rifle, CO. The level of this enzyme correlated to metabolic rates (and therefore activity) of *Geobacter* to reduce soluble U(VI) to insoluble U(IV). Further, the divergent regions of the citrate synthase can be used to track strain-level changes within the *Geobacter* community. Both activity of *Geobacter* and community composition are two important factors that influence the efficiency of U(VI) removal (63). Moreover, Wilkins points out that extraction of mRNA from large numbers of samples needed to characterize a contaminated site is a difficult and time-intensive process. Proteomic techniques are potentially easier and faster to apply to such large numbers of samples (63).

However, peptide detection suffers from some of the same drawbacks as DNA bioindicators. Like DNA, protein is long-lived in a system and can persist after function has ceased, so does not necessarily reflect instantaneous activity of an organism (28). And unlike DNA- or RNA-based probes, proteins cannot be amplified from samples, presenting challenges with respect to detection limit (14).

2.4. Microbial Transformation of Chlorinated Ethenes

While chlorinated ethenes are usually introduced into the ecosystem as either PCE or TCE, once introduced into the environment, they can undergo a variety of biological transformations, depending upon the biogeochemical environment.

2.4.1. Reductive Dehalogenation

Under anaerobic conditions, the chlorinated ethenes can be reductively dechlorinated by microorganisms in the genera *Dehalobacter*, *Dehalospirillum*, and *Dehalococcoides* that respire

chloroethenes as electron-acceptors (6). Reductive dechlorination sequentially replaces chlorine atoms with hydrogen, which serves as the electron donor, and proceeds most effectively at very low redox conditions – i.e., in methanogenic or sulfate-reducing environments (58). . Though communities of these organisms have been shown to completely reduce PCE to ethene, this process is often stalled at the daughter products cDCE or VC, potentially due to insufficient supply of electron donor, inadequate microbial-community composition, and/or unsuitable geochemical conditions (6, 19, 20, 39). This is particularly problematic as VC is a known human carcinogen.

2.4.2. Anaerobic Oxidation

Because anaerobic reductive dehalogenation produces daughter products, these lesser-chlorinated compounds are themselves an indicator of the process of reductive dechlorination. In theory, within anaerobic environments, practitioners should be able to perform a mass balance and account for all of the chlorinated compounds as either parent or daughter products. In reality, this is often easier said than done. While mass-balance at subsurface sites is difficult, there has been evidence to suggest that at some ostensibly anaerobic sites a lack of daughter compounds could be due to some other types of transformations other than reductive dechlorination (21). In an attempt to explain lack of mass-balance of these contaminants, researchers have proposed processes of anaerobic oxidation to explain the absence of daughter compounds (21). It has been suggested that cDCE and VC are oxidized under Fe(III)-reducing, humic-acid-reducing, Mn-(IV)-reducing, SO_4^{-2}–reducing, methanogenic, and/or mixed electron-acceptor conditions (Figure 2.2) (8, 10, 11, 12).

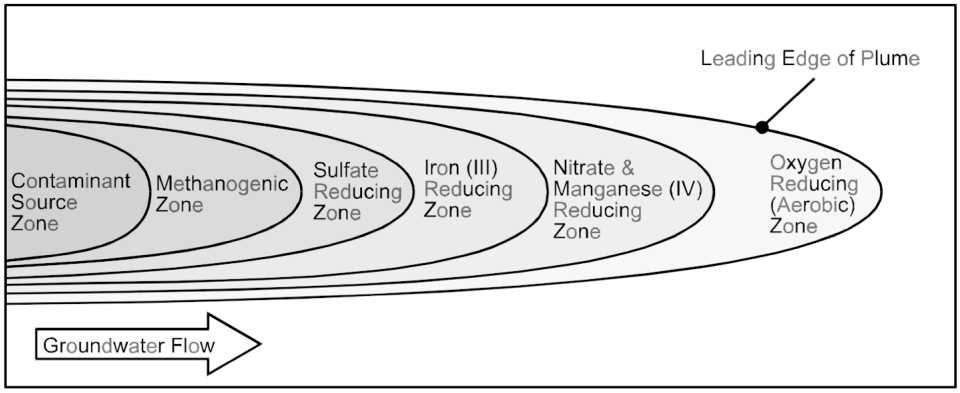

Figure 2.2: Redox zones of contaminant plume in aerobic aquifer (58).

No isolates have been identified from any of these studies. More recently, Gossett suggested that seemingly anaerobic environments could actually contain almost immeasurably low levels of oxygen capable of supporting aerobic oxidation of chlorinated ethenes. He demonstrated sustained aerobic oxidation of VC in systems subject to steady, slow flux of oxygen that maintained O_2 concentrations as low as 0.02 mg/L (21). This suggests that in the study of Hata et al., where cDCE and VC oxidation occurred both in the presence and absence of Fe(III) or in the absence of added electron acceptors, the acceptor might actually have been oxygen (24). Moreover, the many studies by Bradley et al. used relatively undefined systems, which would have provided a number of potential substrates for cometabolic, aerobic oxidation of cDCE, such as methane or even VC (8, 10, 11). While the study conducted by Gossett does not disprove anaerobic oxidation, it does indicate that more robust studies may be required to demonstrate its occurrence.

It has been suggested that ideal conditions for chloroethene biodegradation would occur in stratified redox conditions, whereby the higher chlorinated ethenes would be reduced in anaerobic zones to VC, which would then be oxidized in adjacent aerobic zones (58). VC oxidation readily occurs in aerobic environments; the organisms that perform this transformation

are nearly ubiquitous (15). However, there is the possibility that other daughter products might migrate to and/or accumulate in these aerobic zones where oxidation may not occur as readily. While aerobic VC-oxidizers seem relatively abundant, cDCE-oxidizers (at least those that do not require co-substrates) appear to be uncommon (16).

2.4.3. Cometabolic Aerobic Oxidation

cDCE and VC can be completely mineralized by many aerobic bacteria possessing non-specific or promiscuous oxygenases, but these processes are cometabolic and require presence of a cosubstrate such as methane, propane, toluene, phenol, or ammonia as a carbon and/or energy source (4, 6). This process is generally net energy consuming and potentially damaging to the mediating microorganisms through production of toxic products or intermediates, which can cause reduced activity and viability proportional to degradation (4, 6). The toxic effects of cometabolic oxidation of chlorinated solvents are well documented, despite the specific product responsible being unknown (4). Additionally, cometabolic degradation may be inefficient or fail because of enzyme inhibition or competition (4). Competitive enzyme inhibition occurs with the presence of multiple substrates, primary and cometabolic, and creates an apparent reduced affinity for each, resulting in a reduced transformation of all compounds (4). Non-competitive inhibition has been observed with higher chlorinated solvents, such as PCE (4). Finally, a lack of energy-producing substrates that generate reductants such as NAD(P)H, which are generally required for oxygenases, can inhibit cometabolic degradation of compounds (4).

As a consequence, such removal mechanisms are unreliable and difficult to sustain as bases for bioremediation (62). In cases where aerobic cometabolism has been observed, it was considered a fortuitous occurrence at the edges of plumes where the systems had become aerobic and there was still the presence of other co-contaminants as primary substrates (6, 7). Otherwise,

cometabolic oxidation occurs primarily in engineered systems where organisms are given oxygen and primary substrates (6).

2.4.4. Growth-Coupled Aerobic Oxidation

Bradley and Chapelle (9) have demonstrated that there are indigenous microorganisms in black-water stream sediments that are capable of aerobic oxidation of cDCE. However, degradation was not maintained through transfers, nor were any organisms isolated. More recently, Schmidt et al. began characterizing an enrichment culture created from groundwater at a cDCE-contaminated site (concentrations greater than 1 mg/L) in Germany (49). Initially, this site had been predominately contaminated by PCE and TCE. These had been anaerobically transformed to cDCE, which was then aerobically degraded within the plume (49). Aerobic cDCE degradation as a sole carbon and electron source had been maintained in the cultures through subsequent transfers into carbon-free minimal media, however to date, no isolate has been identified. Zhao et al. conducted further studies to explore the ability of this enrichment culture (referred to as the FT-culture) to degrade cDCE in the presence of other chloroethenes (65). They looked at the effects of PCE, TCE, trans-1,2-dichloroethene (tDCE), 1,1-dichloroethene (1,1-DCE), and VC on the degradation of cDCE. The FT-culture was inhibited by each of the chloroethenes, to varying degrees. It was able to slowly degrade cDCE in the presence of PCE, tDCE, and VC but was almost completely inhibited by TCE and 1,1-DCE. Additionally, the FT-culture was shown to degrade VC but none of the other chloroethenes tested (65).

Coleman et al. sought and found aerobic bacteria that use VC and cDCE as sole carbon and energy sources (15, 16). This work produced 12 isolates (11 *Mycobacterium* and 1 *Nocardioides* species) that can grow on VC, suggesting that such microbes are common in the aerobic zones of

VC-contaminated plumes. Also from this work, one organism, *Polaromonas* sp. JS666, was isolated that is able to aerobically oxidize cDCE as carbon and energy source. This enrichment culture was created from granular activated carbon from a pump-and-treat plant in Dortmund, Germany that was processing groundwater contaminated with cDCE, along with PCE and TCE (16). Thus far, JS666 remains the only isolate capable of growth-coupled, aerobic mineralization of cDCE. The implication is that such growth-coupled cDCE-oxidizers are far less common than are VC-oxidizers – or perhaps it is that VC-oxidizers are easier to culture than cDCE-oxidizers. Since JS666 apparently requires no exotic growth factors, it is considered a promising bioaugmentation agent for aerobic sites where cDCE has accumulated.

The early work performed by Coleman et al. characterizing JS666 revealed that this organism is non-motile, yellow-pigmented, catalase-negative and oxidase-positive, with an optimum temperature between 20-25°C (16). JS666 shares a 98% sequence identity to the 16S rRNA gene of *Polaromonas* sp. GM1, a psychrotolerant arsenite-oxidizing bacterium (45), and a 97% 16S rRNA nucleic acid identity with *Polaromonas naphthalenivorans* CJ2, which is able to degrade naphthalene (32). Neither organism possesses the ability to degrade cDCE. Moreover, polaromonads tend to be slow growing, psychrophilic or tolerant, and there has been increased evidence this genus is important in xenobiotic contaminant degradation (41, 64).

Coleman et al. determined the kinetics of cDCE metabolism by JS666 have a specific substrate utilization rate, k, of 12.6 ± 0.3 nmol/min/mg of protein and a half-saturation constant, K_S, of 1.6 ± 0.2 μM when grown at 20°C with continuous agitation. Additionally, the calculated growth yield, Y, on cDCE was found to be 6.1 ± 0.4 g of protein per mol cDCE. By using k and Y, the authors estimated the doubling time of JS666 to be 150 h. However when this was measured directly, they found it to be 74 ± 8 h, again grown at 20°C. The authors attribute this discrepancy

to an underestimation of k because of differences in the growth phases of the two experiments which lead to an overestimation of active protein during the substrate depletion assay versus exponential-growth assays (16).

JS666 is able to transform tDCE, TCE, VC, 1,2-dichloroethane (DCA) and ethene after being grown on cDCE (16). When JS666 was grown on succinate, the activity associated with these transformations was much lower, suggesting this ability is induced by cDCE (Table 2.1). None of these other compounds was used for growth by JS666 in this study (16). However, later experiments have demonstrated that JS666 can grow on DCA as a sole carbon source, though this pathway seems to be poorly regulated (30, 41, 50).

Table 2.1: Activity of cDCE-grown and succinate-grown JS666 cells with chloroethenes, ethene, and DCA as substrates (16).

Test substrate	Specific activity (nmol/min/mg of protein)	
	cDCE-grown cells	Succinate-grown cells
cDCE	16.8 ± 4.8	0.9 ± 0.6
tDCE	4.0 ± 0.7	0
TCE	5.2 ± 0.9	0.4 ± 0.4
VC	6.6 ± 0.6	1.5 ±0.7
DCA	12.5 ± 1.9	1.1 ± 0.8
Ethene	2.9 ± 1.7	0.6 ± 0.7

Data are averages ± standard deviations based on three experiments.

2.5. Bioinformatics and Omics-Based Approaches to Understanding JS666

Bioinformatics uses databases of biological data and nucleic acid sequences, statistics, and various computer algorithms to broaden understanding in molecular biology. It is an interdisciplinary and ever-expanding field. These techniques allow for DNA, protein, and genome analyses and gene-expression analysis, among others, to investigate biological data (37).

The first step to understanding protein function is often with comparative genomics, whereby previously studied and characterized genomes can be compared to newly sequenced genes.

Comparative genomics uses databases of sequenced genomes to search for homologous sequences. Newly discovered genes or genes of unknown function can be compared to previously studied genes, and this relationship can be used to ascribe putative gene function. Additional evidence of gene function can be found by examining the genomic context (i.e. neighboring genes) of the organism for clues. Synteny, or conserved gene order, can provide indication of the relationship between the genes of unknown function, since genes encoding for enzymatic steps of a pathway are often found in close proximity. However, to confirm the functions of enzymes requires rigorous experimental analysis, which could include gene knockouts, enzyme assays, or heterologous expression in another host. Moreover, inaccurate annotation in bioinformatic databases is quite prevalent (30). Errors in annotation are propagated by an increase in genomic sequencing without confirmation of gene or protein function.

There are three levels to understanding of protein function: phenotypic, cellular, and molecular function (35). The overall effect that a protein has on the organism is its phenotypic function. This can be determined through *in vivo* studies such as gene knockout or heterologous gene expression. How a protein interacts at the cellular level is its cellular function. Cellular patterns of expression, as determined by assays such as 2D-gel electrophoresis, can offer insight to how various proteins are expressed under various conditions (35). These proteins can then be identified using tandem mass spectrometry (MS/MS). Finally, the biochemical activity of a protein defines its molecular function. To understand reactions that an enzyme can catalyze, it's necessary to isolate and purify the protein of interest (35). Crude extracts of proteins can be purified in a number of ways, including on a variety of columns, by centrifugation, or salt-precipitation. Once a protein is purified, it can be studied in isolation or with other proteins, but in a controlled environment.

Using a combination of bioinformatic and phenotypic analyses, Mattes et al. explored the metabolic capabilities of JS666. The JS666 genome has two plasmids (pPol360 and pPol338), which are 360 kb and 338 kb, respectively (Figure 2.3). The entire genome is 5.9 Mb with 5,569 predicted protein-encoding genes. They identified a number of potential putative genes involved in xenobiotic and/or hydrocarbon metabolism, including 16 monooxygenases, 4 cytochrome P450s, 21 glutathione *S*-transferases, 9 dehalogenases and a number of dioxygenases, hydrolases, and hydratases. Additionally, Mattes et al. found a complete set of dichloroethane (DCA) catabolic genes, which are also expected to be important in metabolism of chlorinated alkenes, even though the organism's ability to grow on DCA seems poorly regulated (30, 41, 50).

A number of other catabolic genes in the JS666 genome are located near mobile genetic elements (40). This suggests that these genes were acquired recently or the organism rearranged the genes needed to degrade xenobiotic compounds. While JS666 has maintained its ability to use cDCE as a sole carbon and energy source through many transfers, this phenotype can be suppressed when exposed to alternate substrates. Mattes et al. postulate that this could be due to either limited or ineffective regulation of the newly acquired or evolved pathway or the inherent instability of plasmid- or transposon-carried genes (40).

Jennings et al. used an integrated omics approach to study gene expression in JS666 when degrading cDCE, versus non-chlorinated substrates such as glycolate (31). This work was accomplished with proteomics (2-D gel electrophoresis, followed by LS/MS/MS) in conjunction with transcriptomics (full genome microarrays) and metabolomics. Putative gene functions were suggested using bioinformatics tools.

Several putative degradative genes were upregulated, as well as several genes that encode for proteins typically involved in stress-responses (Table 2.2). In addition to up-regulation and

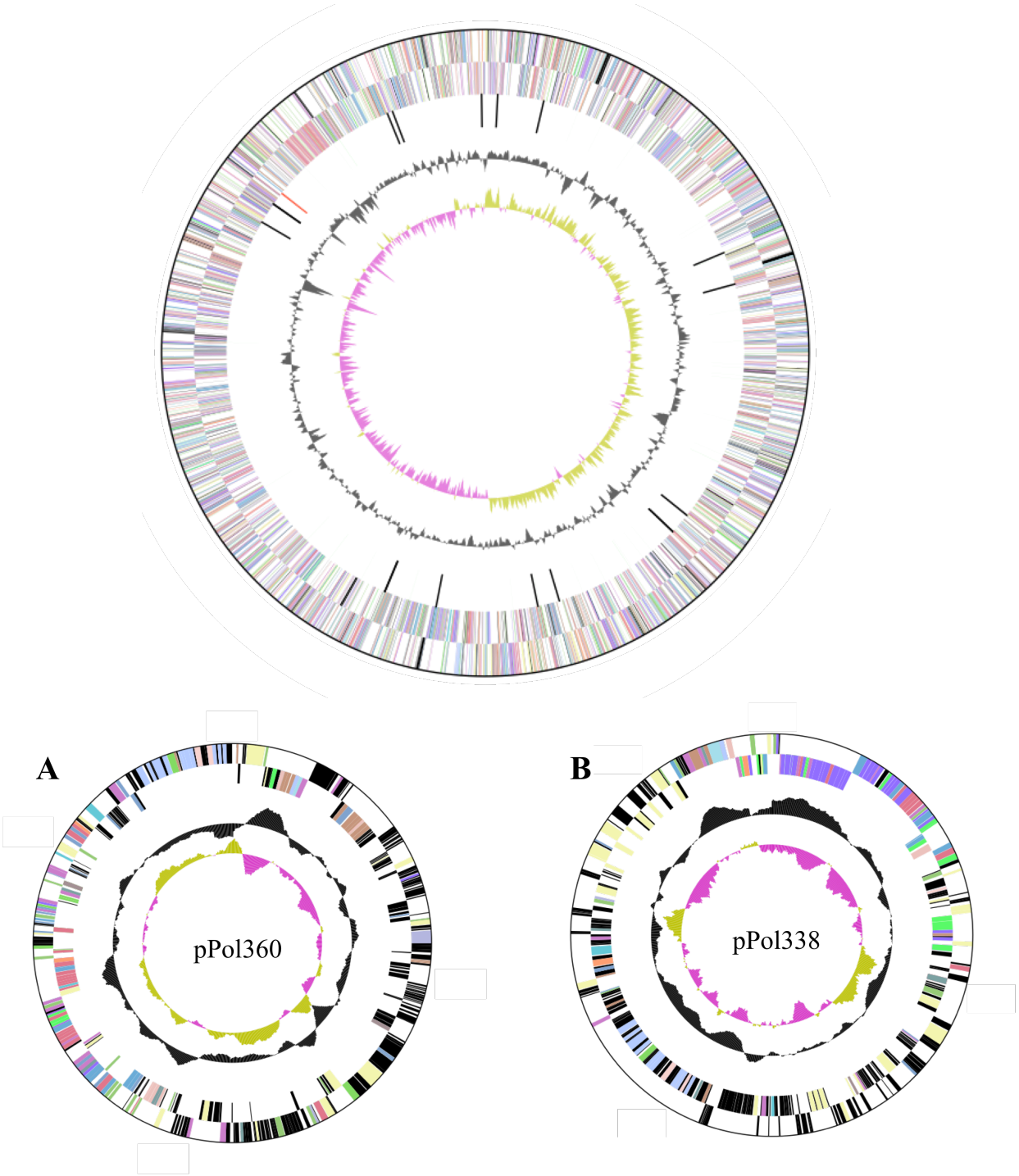

Figure 2.3: The chromosome of *Polaromonas* sp. strain JS666, which consists of a 5.2 Mb chromosome and two plasmids, (A) a 360-kb plasmid (pPol360) and (B) The 338-kb plasmid (pPol338). The rings from outside to center represent genes on forward strand (color by COG categories), genes on reverse strand (color by COG categories). Images from the Joint Genome Institute.

homology to known or putative enzymes, the physical location of genes in relation to one another and hierarchical clustering of transcripts suggests genes important, both directly and indirectly, to cDCE degradation. Further, a number of other interesting putative genes were identified, including universal stress proteins and membrane-associated proteins that can be difficult to detect in 2D-gel electrophoresis. Indeed, genes that are thought to be involved in the cell wall, membrane, and envelope syntheses were "statistically over-represented" in an enrichment analysis of the transcriptomic data (30). cDCE, being an organic solvent, most likely has some physical toxicity associated with it that would compromise the membrane of JS666.

Proteins extracted from JS666 grown on either cDCE or glycolate were compared by overlaying the individual 2-D gel separation and differing proteins were excised and analyzed using LC/MS/MS and the NCBI (nr) database for putative identification (31). Microarray data comparing RNA extracted from cDCE- and glycolate-grown cultures confirmed five of the proteins found by the previous proteomics study (Table 2.2) (31).

The omics approaches raised many hypotheses and suggested alternative pathways, but did not clearly elucidate the cDCE degradative pathway(s). These upregulated gene transcripts could bear further scrutiny in the hope of better understanding their actual function, and how they are related to metabolic and stress gene regulation.

Table 2.2: Summary of upregulated transcripts in JS666 when grown on cDCE versus the reference substrate glycolate. Upregulated proteins from a separate proteomic study are shown here in bold.

Locus Tag	P-Value	Fold Change	Gene Description
Bpro3336	0.005	111	ABC transporter, extracellular ligand-binding receptor
Bpro0645	**0.012**	**99.8**	**glutathione S-transferase-like**
Bpro0646	**0.012**	**87.5**	**pyridoxamine 5'-phosphate oxidase-related**
Bpro3335	0.009	70	ABC transporter, inner-membrane translocator
Bpro0530	**0.011**	**53.3**	**haloacid dehalogenase, type II**
Bpro5186	**0.013**	**51.5**	**haloacid dehalogenase, type II**
Bpro0531	0.015	40.9	sodium/solute symporter
Bpro5185	0.017	39.2	sodium/solute symporter
Bpro3334	0.01	30.8	ABC transporter, inner-membrane translocator
Bpro3333	0.013	27.1	ABC transporter, ATPase component
Bpro3332	0.005	18.3	ABC transporter, ATPase component
Bpro2396	0.017	14.8	heme peroxidase
Bpro5565	**0.024**	**10.1**	**cyclohexanone monooxygenase**
Bpro3866	0.027	3.6	universal stress protein (UspA)
Bpro5301	0.01	3.5	cytochrome P450
Bpro2732	0.018	3.4	Transposase
Bpro4792	0.03	3.2	Transposase
Bpro4575	0.024	3.1	Transposase
Bpro3227	0.04	3	universal stress protein (UspA)

2.6. Predicted Protein Function of Upregulated Transcripts in JS666 by cDCE

2.6.1. Cyclohexanone Monooxygenase (CMO), Bpro5565

Monooxygenases catalyze the reaction between molecular oxygen and an organic substrate, resulting in the addition of one oxygen atom to the substrate and reduction of the other oxygen atom to water (38). These enzymes require a reductant cofactor, such as NAD(P)H. Bpro5565, found on plasmid pPol338, shares a 66% amino-acid sequence identity with a CMO in *Brachymonas petrolevorans*, and its function was confirmed by expression in *Escherichia coli* (*E. coli*) and purified enzyme assays (2). This type of monooxygenase oxidizes cyclohexanone

to ε-caprolactone, as part of a cyclohexanol degradation gene cluster (2, 41). CMO is known more broadly as a Baeyer-Villiger monooxygenase.

Jennings postulates that the function of CMO in JS666 is to catalyze DCE epoxidation. In whole cell assays, epoxyethane was produced from ethene, suggesting the presence of a monooxygenase (16), and on-going knockout studies with CMO suggests that this enzyme is important for cDCE degradation. However, there was no CMO activity in crude cell extracts (30), and further studies with recombinant *E. coli* expressing CMO conducted by Alexander demonstrated no CMO activity with cDCE or ethene, but the recombinant was able to transform cyclohexanone to ε-caprolactone (2). Alexander hypothesized that the upregulation of CMO during cDCE degradation is due to regulatory linkage with a putative epoxide hydrolase, and that CMO is not responsible for the initial attack on cDCE (2). Rather, this hydrolase, which was also upregulated, has been proposed to act on a cDCE epoxide (2, 30, 50).

2.6.2. Haloacid Dehalogenase (HAD), Bpro0530, Bpro5186

Dehalogenases catalyze the removal of the halogen from a molecule, cleaving the carbon-halogen bond. HADs replace the halogen with a hydroxyl through the hydrolysis of the α-halogenated carboxylic acid (30). There are two paralogous HADs (99% amino acid identity) in the JS666 genome, one of which is on a plasmid (Bpro5186), and they share a 58% and 54% amino-acid identity with HADs of *Agrobacterium tumefacians* RS5 and *Pseudomonas* sp., respectively. Both have confirmed activity by expression in *E. coli* (30). Moreover, such duplication of metabolically significant genes would aid JS666 in growth on cDCE giving it an advantage over other microorganisms due to elevated expression of these genes (40). In purified cell extracts from JS666, putative HAD enzyme (Bpro0530) transformed chloroacetic acid to glycolate (30).

2.6.3. Cytochrome P450 (P450), Bpro_5301

P450 is a widely occurring monooxygenase that is also able to catalyze hydroxylation of saturated carbon-hydrogen bonds and the epoxidation of double bonds, among other functions (43). As such, P450s are aerobic catalysts for many types of organic molecules (23). The reaction that is catalyzed by P450 acting upon an alkene (such as cDCE) produces an epoxidation, an aldehyde rearrangement and/or a suicide complex that deactivates the protein (17). In JS666, Bpro5301 has an amino-acid identity of 76% to a *Mycobacterium* hydroxylase that is responsible for the attack on C_5 to C_{10} alkanes (41). Mattes et al. theorize that the n-alkane degradation pathway in JS666 is initiated by this putative cytochrome P450 (41).

More recently, Shin completed a number of studies that strongly suggest that P450 is responsible for the initial steps of cDCE degradation in JS666 (50). These studies included oxygen-uptake and oxygen-limited experiments, use of P450-specific inhibitors, heterologous gene expression, and cell-free extracts. JS666 only degraded cDCE in the presence of oxygen – a requirement of monooxygenases – and did not degrade cDCE while P450 was inhibited with metyraprone or phenylhydrazine. Further, resting-cell experiments with cDCE, DCA and their intermediate substrates showed high oxygen uptake with cells grown on cDCE. This is significant because the enzymes involved in cDCE degradation would only use oxygen if they were already induced. Additionally, recombinant *E. coli* expressing P450 along with adjacent genes were able to transform both DCA and cDCE.

Shin demonstrated that dichloroacetaldehyde is an intermediate of cDCE degradation and a product of P450 transformation of cDCE (50). The products of cDCE transformation by P450 were accounted for by 60% as dichloroacetaldehyde, 5% as a cDCE epoxide, and the final 35% most likely an irreversible suicide complex.

2.6.4. Glutathione S-Transferase (GST), Bpro0645

Glutathione S-Transferases (GST) have a multitude of functions, and are involved in detoxification and dehalogenation processes (3). There is evidence of bacteria producing GST to relieve epoxide stress, and glutathione is involved in protein synthesis, degradation, and folding, protection against oxidative stress, as well as a number metabolic processes (3, 61). The gene Bpro0645 in JS666 shares a 66% amino-acid identity with a putative GST in *Pseudomonas mendocina*. In JS666, GST could be involved in epoxide transformation, direct dehalogenation of cDCE, or simply to relieve oxidative stress (30). However, Shin reports a recombinant *E. coli* expressing GST that is able to transform the GST substrate 1-chloro-2,4-dinitrobenzene but not cDCE (50). This suggests that GST is not involved in direct dehalogenation in JS666.

Interestingly, van Hylckama Vleig proposed constructing an organism that is able to oxidize 1,2-dichloroethenes with a non-specific monooxygenase coupled with a GST to transform the resulting epoxide (61). This organism would then use glyoxl (which they reported as being a poor growth substrate) as a carbon and energy source.

2.6.5. Pyridoxamine 5'-Phosphate Oxidase (PNP), Bpro0646

PNP is an oxidase that catalyzes the final step in the vitamin B6 metabolism pathway. Vitamin B6 is thought to be involved in a number of important metabolic processes including sustaining glutathione (GSH) levels, and alleviating oxidative stress (61). Bpro0646 has an amino-acid identity of 52% with the putative PNP of *Acaryochloris marina.*

2.6.6. ABC Transporters, Bpro3332-3336

The superfamily of ATP-binding cassette (ABC) transporters are found in all three kingdoms of life, which suggests they are essential to cellular regulation (33). These proteins catalyze solute transport against concentration gradients across membranes, for import and export –

though never both at once – using the energy from ATP (25). While the transporter is specific to a substrate, there are countless different substances that are moved across membranes (25). They have a conserved structure of two nucleotide-binding domains (the "molecular motors" that harness ATP energy) and two transmembrane domains, which span the membrane forming a channel (33). ABC transporters are involved in translocation of amino acids and other essential nutrients, ions, and xenobiotics, which means they function in osmotic homeostasis, nutrient uptake and resistance to toxins and antibiotics (33).

Jennings et al. found that JS666 grown on cDCE showed upregulation of putative ABC transporter components (Bpro3332-3336), which had high sequence similarity (72-87%) to ABC-type transporters in *Janthinobacterium* and *Ralstonia* sp. (30). However, because ABC transporters have such diverse function, their specific roles in JS666 are unknown. The presence of a binding receptor suggests that this particular transporter is an importer. Further, there is evidence that the ABC transporter in JS666 functions as an amino acid importer, which would mean involvement in nutrient uptake (30). It is also possible that these enzymes are involved with moving cDCE or other metabolites across membranes, though gram-negative bacteria, like JS666, have an outer membrane comprised of lipopolysaccharide that allows slow penetration of hydrophobic molecules, such as cDCE (18).

Jennings also postulates that the ABC transporters could be involved in transporting cDCE-degradation metabolites from the periplasm to the cytoplasm, or possibly even exporting periplasmic proteins that are synthesized in the cytoplasm and then transported to the periplasm (30). Such a phenomena has been proposed in other systems where the transport mechanism of proteins to the periplasm is unknown, as with glutathione S-transferase and peptide methionine

sulphoxide reductase in *Ochrobactrum anthropi* (52). It is also possible that these enzymes function to export chloride ions.

2.7. Proposed cDCE Degradation Pathways by JS666

Taken together, the work completed by Jennings et al. identified at least two possible scenarios: (i) dechlorination of cDCE by glutathione *S*-transferase (GST) that feeds into a pathway involving chloroacetaldehyde dehydrogenase (CAD) and haloacid dehalogenase (HAD) that oxidizes the glutathione conjugate to glycolate, or (ii) cDCE oxidation through epoxidation catalyzed by the cyclohexanone monooxygenase (CMO), where the epoxide is then converted into a glycol compound through a hydrolase enzyme before being dehydrogenated to form glyoxylate (Figure 2.4) (30).

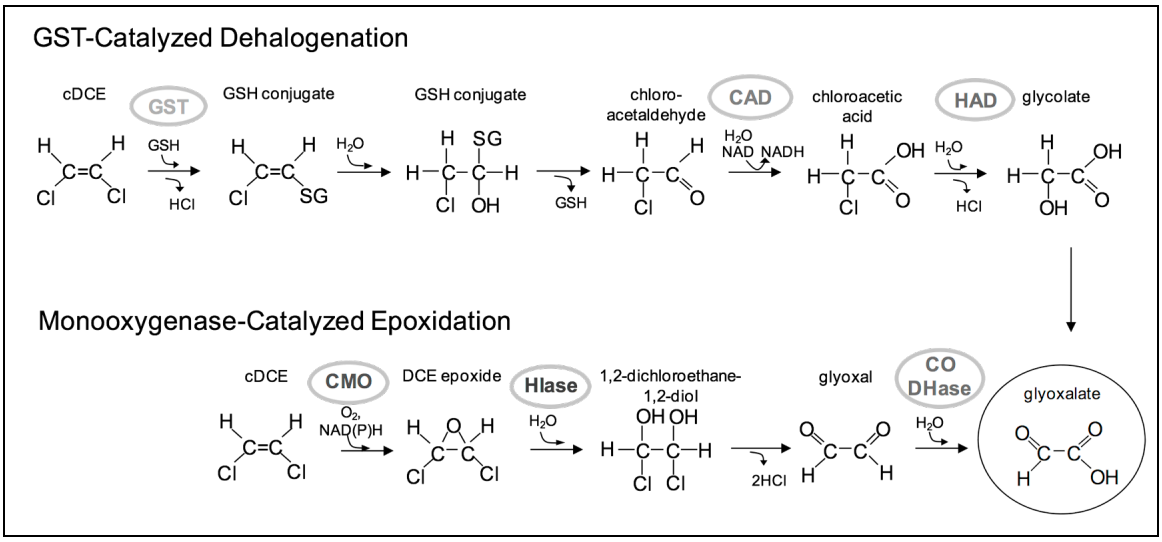

Figure 2.4: Proposed cDCE-oxidation pathways by Jennings (31)

These proposed pathways support the observed dual-phenotypical behavior of JS666, where one pathway enables productive, growth-coupled oxidation and the second pathway is cometabolic-like, destructive to the cell and difficult to maintain. Here, the cometabolic-like pathway likely produces an epoxide that is potentially damaging to JS666, which would explain

the reduced activity that is observed on occasion. Further, observation of dual phenotypes supports the hypothesis put forth by Mattes et al. that JS666 has either limited regulation of a newly acquired pathway or has a pathway with the instability of plasmid or transposon carried genes (40). Poorly regulated or unstable pathways exhibit varied phenotypes depending on which pathway is being expressed. In JS666, this is highly dependant on culturing conditions. Also, the hierarchical clustering of the induced transcripts shows the GST and CMO are not grouped together, further reinforcing the notion of two pathways (30).

Shin also proposed dual pathways for cDCE degradation by JS6666 (Figure 2.5) (50). Here P450 initiates both the productive pathway and destructive pathway, transforming approximately 60% into dichloroacetaldehyde, 5% into DCE epoxide, and the final 35% most likely into an irreversible complex. Based on bioinformatic evidence, Shin proposes that these pathways evolved recently in a progenitor capable of degrading dichloroacetaldehyde by recruiting the P450 gene from alkane-assimilating bacteria (50).

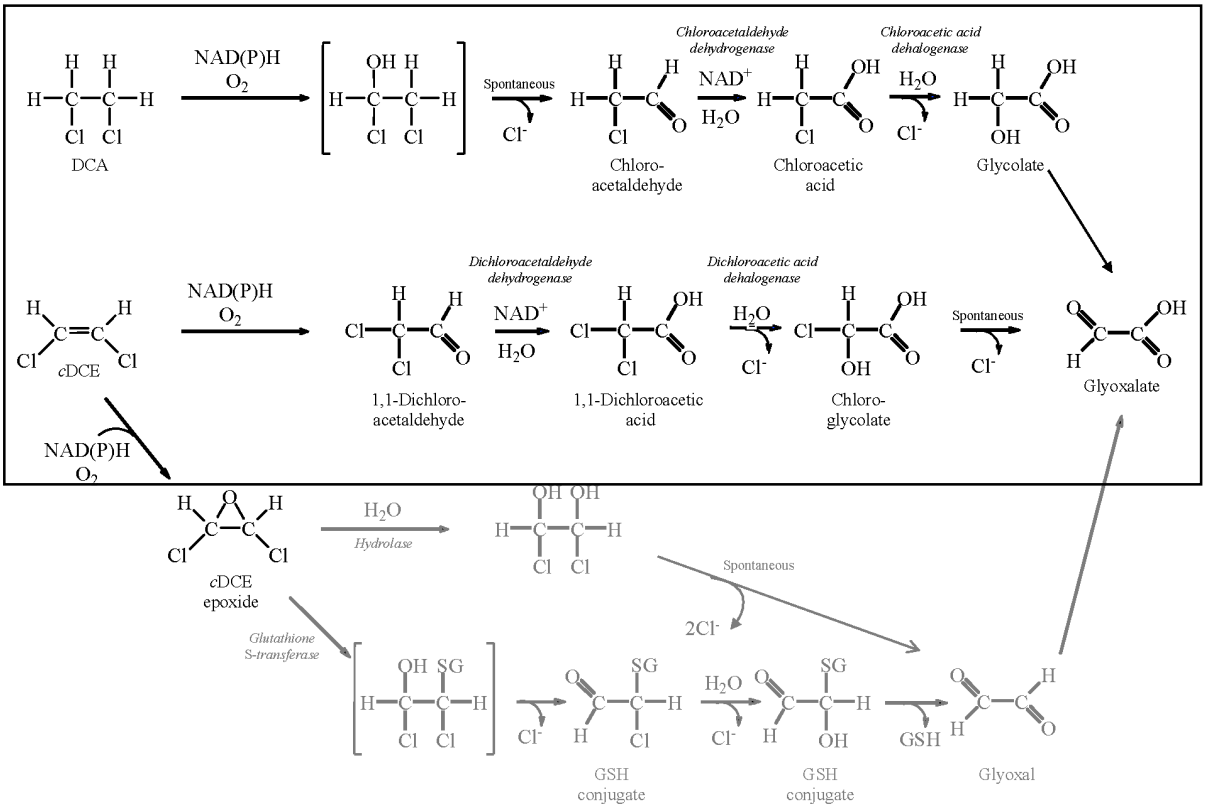

Figure 2.5: Proposed *c*DCE and DCA degradation pathways initiated by P450. Pathways boxed and in bold were supported by the studies conducted by Shin (50).

Carbon-isotopic fractionation studies can lend further insight into possible pathways (31, 53). Compound specific isotope analysis (CSIA) measures the change in ratio of heavy isotopes to light isotopes (here, $^{13}C/^{12}C$) that occurs during biological degradation. The type of bond that is initially broken (more specifically, the type of bond broken in the first irreversible step) in degradation of chlorinated ethenes and ethenes dictates the degree of fractionation and can be described with the Rayleigh equation (31). For example, fractionation values associated with VC epoxidation (aerobic cleavage of C=C bond) are between -7.0 ± 0.3‰ to -8.2 ± 0.1‰ (31), whereas reductive dehalogenation of VC (anaerobic cleavage of the C-Cl bond) is much lower, between -21.5‰ and -31.1‰ (1, 53). Tiehm et al. reported a fractionation value from aerobic enrichment cultures that degraded cDCE cometabolically (with VC as a cosubstrate) between

-9.8 ± 1.7‰ and -7.1 ± 0.9‰ depending on the temperature of the experiment (53). Like VC, values for anaerobic reductive dehalogenation of cDCE (cleavage of C-Cl bond) are lower, with ranges between -14.1‰ to -29.7‰ (1, 53).

CSIA results from studies conducted with JS666 aerobically degrading cDCE are varied (1, 31). Abe et al. measured the aerobic fractionation of cDCE by JS666 and reported a value of -8.5 ± 0.10‰, suggesting that epoxidation is the initial step (1). However, the Jennings et al. study produced much lower fractionation values, between -17.4‰ to -22.4‰, which are much closer to those seen in anaerobic dechlorination, suggesting a C-Cl breakage as the initial step (31). Because both types of fractionation were observed with JS666, it supports the hypothesis that there are two pathways used by the organism to degrade cDCE, which are highly dependent on culturing technique (29): one by growth-coupled oxidation where the C-Cl bond is cleaved initially; and another that cleaves the C=C bond, which produces an epoxide that reduces the activity of JS666. Interestingly, the CSIA enrichment factor found by Schmidt et al. for their cDCE-oxidizing culture was -15.2 ± 0.5‰, also suggesting that their culture does not utilize an epoxidation pathway alone (47).

2.8. Summary

Accumulation of cDCE at aerobic sites remains an environmental concern; however only a handful of cultures have been identified that are able to oxidize cDCE as a sole carbon and energy source (6, 16, 49). Of those cultures, only two have maintained the ability to degrade cDCE through subsequent transfers and only one isolate has been identified. As such, *Polaromonas* sp. JS666 is a promising candidate for bioaugmentation in oxic, cDCE-contaminated sites. However, no studies yet exist to show its efficacy in environmental

materials. To successfully employ this organism in bioaugmentation, a fundamental understanding of the requirements and capabilities of JS666 is needed.

Moreover, no studies exist testing biomarkers to detect the spread and persistence of JS666 in the subsurface. Tracking bioaugmented JS666 on site could help inform on the ability of this organisms to survive, migrate and compete in soil and groundwater. Additionally, a molecular biological tool can potentially be used for site assessment to screen for the presence of indigenous, JS666-like microbes, if they exist.

Finally, the cDCE degradation pathway(s) of JS666 remains inconclusive. This means that biomarkers based on degradative genes, which could potentially indicate the *activity* of JS666 *in situ*, are yet to be developed. This is important in evaluating sites where cDCE is being oxidized. There are no convenient degradation products to monitor as indicators of metabolic activity, as there are at anaerobic sites where reductive dechlorination is occurring. Once developed, MBTs that can measure the biological response of an organism *in situ* could replace the current use of microcosms or column studies.

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CHAPTER 3[1]

3. MICROCOSM ASSESSMENT OF *POLAROMONAS* SP. JS666 AS A BIOAUGMENTATION AGENT FOR DEGRADATION OF CIS-1,2-DICHLOROETHENE IN AEROBIC, SUBSURFACE ENVIRONMENTS

3.1. Abstract

Chlorinated ethenes such as tetrachloroethene and trichloroethene have been widely used as dry-cleaning and degreasing solvents. Under anaerobic conditions, microorganisms reduce these parent compounds to less-chlorinated daughter products such as *cis*-1,2-dichloroethene (cDCE), and often further to ethene. This process can be stalled at cDCE, due to insufficient supply of reductants and/or inadequate microbial-community composition. Recently, a novel bacterium, *Polaromonas* sp. JS666, was isolated that is able to aerobically oxidize cDCE as sole carbon and energy source. As such, it is a promising candidate for use as a subsurface, bioaugmentation agent at sites where anaerobic bioremediation is inappropriate or has stalled and cDCE has migrated to, and accumulated within, aerobic zones, or where it is practical to impose aerobic conditions.

Subsurface sediments or groundwaters from six such cDCE-contaminated sites were used to construct microcosms. In every sediment or groundwater inoculated with JS666, the organism was able to degrade cDCE, provided that the pH remained circum-neutral. Even when JS666 was challenged with an alternate carbon source, or in the presence of competitive/predatory microorganisms, there was a measure of success. Collectively, these microcosm studies suggest

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that JS666 is a viable candidate for the bioaugmentation of aerobic, cDCE-contaminated sites. A minimum inoculation level in excess of 10^5 cells per ml is recommended for field applications. At this level of inoculation, 100 liters of inoculum culture grown to an OD600 of 1.0 should be able to treat a 10-m x 30-m x 80-m (24,000-m^3) plot.

3.2. Background

Chlorinated ethenes have been widely used as dry-cleaning and degreasing solvents. Therefore, it is not surprising to find them among the most prevalent groundwater pollutants in the United States and on the US EPA's list of primary regulated drinking water contaminants (5). Generally introduced into the ecosystem as tetrachloroethene (PCE) or trichloroethene (TCE), they are reduced to less-chlorinated daughter products under anaerobic conditions by microorganisms in the genera *Dehalobacter, Dehalospirillum,* and *Dehalococcoides* that respire chloroethenes as electron-acceptors with H_2 as donor (2, 9). Though communities of these organisms have been shown to completely reduce PCE to ethene, this process is often stalled at the daughter products *cis*-1,2-dichloroethene (cDCE) or vinyl chloride (VC), due to insufficient supply of electron donor and/or inadequate microbial-community composition. When this occurs, the daughter products can migrate to aerobic zones **(**10**)**.

cDCE and VC can be completely mineralized by many aerobic bacteria possessing non-specific monooxygenases (2). However, these processes are cometabolic, and thus require presence of co-substrates (e.g., methane or toluene). In the many cases where aerobic cometabolism has been observed in the field, it was generally a fortuitous occurrence at the edges of plumes where the systems had become aerobic and there was still the presence of other co-contaminants as primary substrates (2, 4, 10, 23). When cometabolic oxidation of cDCE is exploited in engineered bioremediation, oxygen must usually be administered along with a co-

substrate because the additional oxygen demand resulting from the latter can otherwise cause a site to become anaerobic (13). The cometabolic oxidation process is generally damaging to the mediating microorganisms through production of highly reactive epoxide intermediates (1, 2), adding to the difficulty of its application. Nonetheless, in recent years numerous researchers have explored bioremediation strategies involving cometabolic processes, including the use of bioaugmentation (13, 19, 20, 21). In principle, bioaugmentation with a growth-coupled, aerobic cDCE oxidizer could be employed with the same recirculation well technology as has been used in cometabolism-based aerobic remediation.

Early work by Bradley and Chapelle showed that indigenous microorganisms in black-water stream sediments were capable of aerobic oxidation of cDCE without any additional co-substrates, though no causative organism was isolated (3). More recently, Coleman et al. sought and found aerobic bacteria that use VC and cDCE as sole carbon and energy sources (6, 7). This work produced 12 isolates (11 *Mycobacterium* and 1 *Nocardioides* species) that can grow on VC, suggesting that such microbes are common in the aerobic zones of VC-contaminated plumes. However, only one organism, *Polaromonas* sp. JS666, was isolated that is able aerobically to oxidize cDCE as its carbon and energy source. The implication is that such growth-coupled cDCE-oxidizers are far less common than are VC-oxidizers, or alternatively, VC-oxidizers are easier to culture than cDCE-oxidizers.

Since JS666 apparently requires no exotic growth factors, it is considered a promising bioaugmentation agent for aerobic sites where cDCE has accumulated. The six sites from which subsurface sediment or groundwater were obtained for the studies reported herein, are representative of such problematic sites where cDCE has stalled in aerobic zones. Despite the numerous studies (cited earlier) in which aerobic oxidation of cDCE has been observed, its

occurrence is neither ubiquitous nor assured. In that sense, aerobic cDCE oxidation is analogous to reductive dechlorination in that some sites would benefit from bioaugmentation. Aerobic remediation might be preferred over anaerobic reductive dechlorination in situations where the cDCE concentration is low (but above some maximum allowable concentration), requiring little oxygen for its depletion; where the aquifer is aerobic; and/or where the byproducts of anaerobic biological activity (methane, sulfides, reduced iron, etc.) are undesirable.

Here we present a first step in the evaluation of JS666 as bioaugmentation agent; namely, the assessment of JS666's survival in microcosms constructed from subsurface sediments or groundwaters from aerobic plumes at six cDCE-contaminated sites. The microcosm studies were designed to assess the survival of JS666 in a variety of environmental conditions. These included presence of indigenous microorganisms and alternative co-substrates, micronutrient and metals requirements, buffering capacity of sediment or groundwater, inoculation level, and concentration of cDCE. Groundwater or sediment that was not amended with buffered medium tested the organism's tolerance to the material's native ability to buffer against HCl production as cDCE was degraded, and also the material's ability to provide necessary trace metals and micronutrients (at least in the short-term). Biotic factors, such as predation, phages and competition from indigenous microbiota, were addressed by adding municipal primary effluent to some microcosms, representing a condition of possible competition, predation, and/or viral infection. Primary effluent also contributed a diverse array of alternative substrates. In all phases of study, activity was assessed principally through the monitoring of cDCE degradation. Subsequently, a pilot-scale field study using JS666 as a bioaugmentation agent was begun in late October of 2008, in St. Julien's Creek Annex, Chesapeake, VA. Site water downgradient from a

bioaugmentation well was obtained two months after inoculation, and microcosm results from it are presented as evidence of JS666's dispersal and survival in the field.

3.3. Materials & Methods

3.3.1. Culturing Techniques

JS666 cultures were grown on cDCE in carbon-free minimal salts medium (MSM) modified from Hartmans et al. to contain 20 mM phosphate, 10 mM ammonium, and 0.02 mM chloride (12). Resulting pH was approximately 7.1 to 7.2. Pure cultures were maintained through 5% v/v transfers into 100 ml MSM in 160-ml serum bottles. Bottles were sealed with Teflon®-lined butyl-rubber stoppers and aluminum crimp-caps. Four microliters of neat cDCE (99% [TCI America]) was added as the sole carbon and energy source at an initial nominal concentration (i.e., ignoring partitioning to headspace) of 51 mg/L. After approximately two and one-half spikes of cDCE were degraded, 5 ml of culture was transferred into approximately 95 ml of fresh MSM and cDCE. Trypticase soy agar (Becton Dickinson) at one-quarter strength in 15 g/L agar (Fisher Scientific) was used as a non-selective medium in purity-checks. After any handling that could have potentially contaminated the culture and before inoculation, a small amount of culture was streak-plated to check for abnormal colony morphologies indicative of contamination. JS666 forms tight, yellowish-white colonies, and colony morphology was confirmed once by microscopy and restriction-fragment-length polymorphism (31). Cultures were stored inverted at 22ºC in the dark on an orbital shaker at 160 RPM. All cultures were grown with ambient levels of oxygen.

3.3.2. Sediment and Groundwater Types

3.3.2.1. Sediments

Subsurface sediment samples were obtained from four, aerobic, cDCE-contaminated sites: Savannah River Site (SRS), SC; Robins AFB, GA; Hill AFB, UT; and an Aerojet facility, CA. Available characteristics and constituents are summarized in Table 3.1.

Table 3.1: Subsurface groundwater characteristics within sample areas.

Parameter	SRS	Robins	Hill	Aerojet	SJCA
Sample Well	CRP 44A	BIA8 Area	U1-175	3651	MW 04S
Dominant site lithology	Silty-sand	Fine to coarse-grained sands, interlayered silts and clays	Sand, gravelly sand with silts	Fine to coarse-grained sands, interlayered gravel, silts and clays	Brown and tan, fine to coarse silty sand
max PCE, mg/L	<0.005	<0.02	--	<0.002	<0.01
max TCE, mg/L	<0.005	0.46	--	0.41	<0.01
max cis-1,2-DCE, mg/L	0.53	0.3	0.086	0.0035	0.78
max VC, mg/L	<0.005	0.05	--	ND	<0.002
max Sulfate, mg/L	4.3	<5	42.6	3.4	26.9
max Nitrite, mg/L	<0.1	0.4	0.2	ND	--
max Nitrate, mg/L	<0.1	0.4	103.9	11	--
max Fe, mg/L	--	0.04	1.94	0.2	--
pH	4.4-5.1	6.4*#	8.5*#	7.4*#	6.0*
Conductivity, µS/cm	26	24-63	1132	325	1460
ORP, mV	122	165-335	64.5-335	63	28.5
DO, mg/L	0.9-3.7	6-11	3.6-10.5	2.01	3.05
Depth to water, ft bgs	3-5	6-9	81	92	1.7

*Measured in lab, rather than *in situ*.
Sediment-sample pH was measured by creating a slurry of sediment with distilled water.

The sediment samples were dried (103˚C for approximately 24 h) and analyzed gravimetrically to determine the moisture content of each sediment-type. Since microcosms should not be constructed from heat-dried sediment, the measured moisture content was used to compute the weight of native material needed to achieve the desired dry weight of sediment in microcosms, which was 50 g dry wt per bottle. All sediment and groundwater samples were shipped on ice to the laboratory and stored at 4ºC in the dark for later use. The native pH was determined for each of the sediments used in preparing the microcosms by mixing 50% (by dry weight) sediment with distilled water (dH_2O) as no site-specific groundwater was available. pH measurements were taken after the slurry had equilibrated and were made with an Accumet micro-electrode with a calomel reference. Additionally, pH measurements were taken from individual microcosms at the conclusion of each experiment to assure that pH never became prohibitively low (below 6.5) (14).

3.3.2.2. Groundwaters

Groundwaters were obtained from two aerobic, cDCE-contaminated sites: Ft. Lewis, WA and St. Julien's Creek Annex (SJCA), Chesapeake, VA. Fort Lewis groundwater had a native pH of 6.90, whereas SJCA groundwater native pH was approximately 6.0. Both waters exhibited little to no buffering capacity. Note that SJCA groundwater samples were obtained on two different occasions. The first SJCA samples were from MW04S, a well in the location that would six months later become the test plot for a bioaugmentation study with JS666. The second SJCA samples were obtained two months after bioaugmentation with JS666, and were taken from three wells – two (MW04 and MW05) immediately down-gradient from the bioaugmentation location, and one (MW15) outside the influence of bioaugmentation.

3.3.2.3. IAWTP Primary Effluent

Primary effluent was collected from the Ithaca Area Wastewater Treatment Plant, and was stored at 4ºC in the dark for later use.

3.3.3. Microcosms

All microcosms were prepared aseptically under a laminar-flow hood, with sterilized spatulas in autoclaved 160-ml serum bottles and contained either 50 g (dry wt) sediment or 50 ml groundwater. For each sediment treatment, MSM or dH_2O was aseptically added to yield a total of 50 ml of liquid (including the moisture contributed from sediment). The microcosms constructed with groundwater and amended with MSM were created with 45 ml of groundwater and 5 ml of 10X concentrated MSM. Non-amended microcosms were pH-neutralized when necessary, however microcosms amended with MSM required no other neutralization beyond that provided by the phosphate-buffer component of the MSM. Each microcosm had 2.3 µl of cDCE (59 mg/L nominal concentration) delivered via syringe through ethanol-swabbed, flamed septa. The initial oxygen in the air-headspace volumes of the microcosms was more than ten times the requirement for complete oxidation of the added cDCE. All experiments were conducted at 22ºC in the dark, agitated at 60 RPM. Every microcosm study with site materials included uninoculated controls. In the various microcosm treatments described below, "inoculation level" refers to the initial JS666 concentration achieved in microcosms. The experimental treatments depicted in Table 3.2 were performed in duplicate with the various subsurface sediments or groundwaters for a total of 29 treatments (48 total bottles) to achieve the following objectives:

- To test the efficacy of bioaugmentation of subsurface sediments or groundwaters from six sites;
- To study the effects of inoculum level and cDCE concentration;
- To explore the effects of primary effluent (as a source of competing and predatory microorganisms and alternative substrates) on the success of JS666 as a bioaugmentation agent.

Table 3.2: Experimental set-up.

Objective	Material	Inoculation Level			cDCE Level		MSM buffer	pH Adjusted	Primary Effl (v/v)	
Bioaugmentation of Subsurface Material	SRS Sediment	1X			1C		√			
	Robins Sediment	1X			1C		√			
	Robins Sediment	1X			1C			√		
	Hill Sediment	1X			1C		√			
	Hill Sediment	1X			1C			√		
	Aerojet Sediment	1X			1C		√			
	Aerojet Sediment	1X			1C					
	Ft. Lewis GW	1X			1C		√			
	Ft. Lewis GW	1X			1C					
Effects of Inoculum Level & cDCE Level	MSM-Only	1X	0.1X	0.01X	1C	0.1C	√			
	SRS Sediment	1X	0.1X	0.01X	1C	0.1C	√			
	SJCA GW	1X	0.1X		0.17C		√			
	SJCA GW	1X	0.1X		0.17C			√		
Effects of Primary Effluent	SRS Sediment	1X	0.1X		1C		√		10%	1%

Note: For reference, "1X" = 7 x 10^6 cells/ml (3.5×10^8 copies/bottle) and refers to the added JS666 concentrations in microcosms; "1C" = 59 mg/L cDCE (nominal concentration).

3.3.4. Analytical Methods

Total quantities of cDCE in bottles were measured from 100-µl headspace samples by gas chromatography (Perkin-Elmer, Autosystem GC) with a flame-ionization detector and a packed column (1% SP-1000 on 60/80 Carbopack B [Supelco]). The cDCE detection limit was approximately 0.03 mg/L. cDCE levels were quantified through comparison to a standard curve created from known additions (measured gravimetrically) to replicate serum bottles, containing,

as appropriate, either dH_2O or sediment and MSM. Standards created in sediment and allowed to equilibrate for 24 to 48 hours showed a 5% difference in headspace concentration over those created in dH_2O, demonstrating neither significant cDCE sorption to the sediment matrix nor salting out, relative to the analytical precision of the procedure (coefficient of variation of 4 to 7%). Additionally, oxygen was monitored using a thermal conductivity detector (TCD) to assure that it was never limiting to oxidation of cDCE.

Optical density of cultures at 600 nm (OD600) was measured with an Eppendorf Biophotometer. The biomass concentration of more dense (mature) cultures could be estimated from this technique. A 1-ml aliquot of culture was aseptically sampled and put into a standard, 1-cm-pathlength plastic cuvette and placed in the Biophotometer. Optical density was used to estimate the initial target inoculations for each microcosm. This was done by establishing a correlation between OD600 and cell counts from real-time qPCR using primers targeting the isocitrate lyase gene of JS666 as developed by Jennings (14). This method has a detection limit of approximately 20 copies/reaction, which correlates to 700 copies/ml of pure, undiluted culture.

3.4. Results & Discussion

3.4.1. Bioaugmentation of Subsurface Sediment or Groundwater, With or Without Buffered Medium

SRS, Robins, Hill, and Aerojet sediments and Ft. Lewis and SJCA groundwaters were prepared as described in Table 3.2. Results are shown in Figure 3.1 and Figure 3.2. Within 12 days, cDCE was depleted in all inoculated, MSM-amended microcosms except those prepared from SRS sediment, which degraded cDCE more slowly. SRS microcosms are estimated to have required about 20 days for complete degradation, but were terminated after 16 days. No

degradation was seen in any of the uninoculated microcosms. This indicates that degradation was due to JS666 and not native organisms in the site materials.

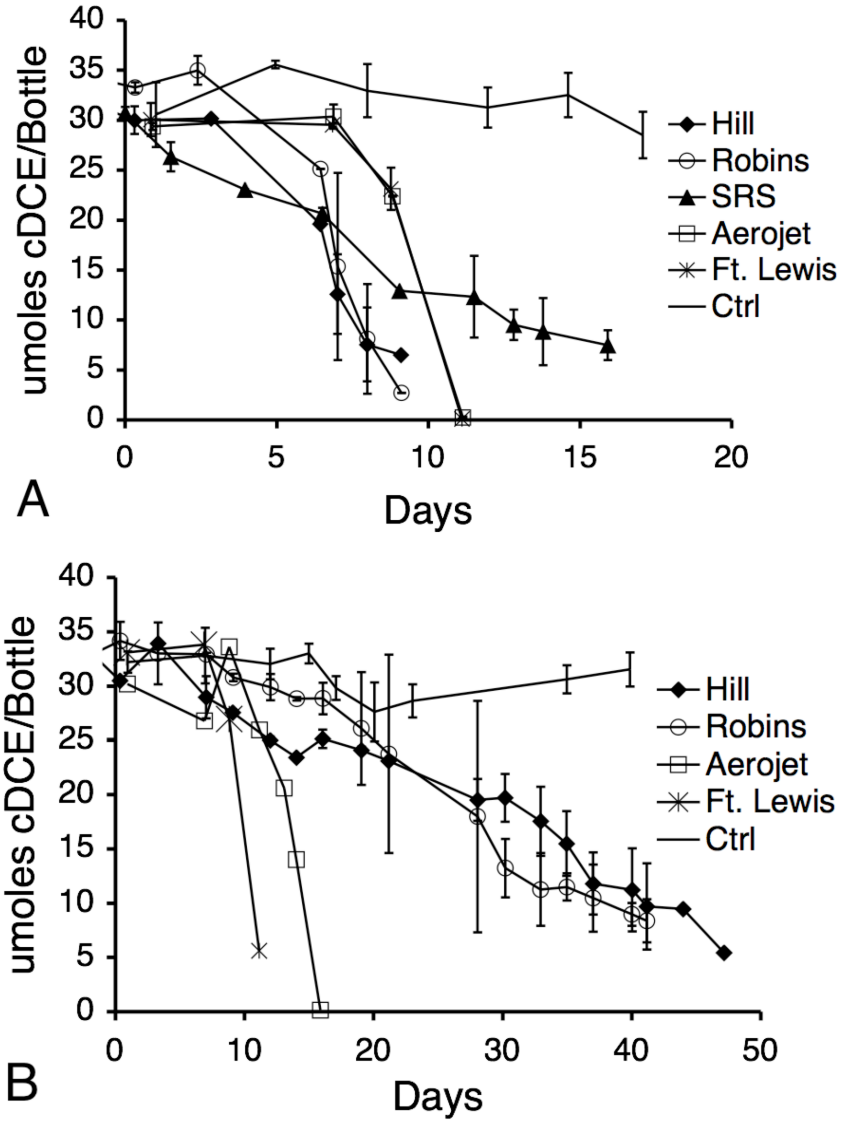

Figure 3.1: cDCE degradation in microcosms constructed from (A) five subsurface sediments or groundwaters with buffered MSM; or (B) four subsurface sediments or groundwaters without buffered MSM (i.e., dH_2O only). With the following exceptions, error bars represent the standard deviations of duplicate microcosms: unbuffered Aerojet and Ft. Lewis microcosms each had a duplicate that did not experience cDCE degradation; only the successful microcosms of these two are shown. Error bars in the controls represent the standard deviations in ten or six uninoculated controls, respectively. Uninoculated controls for Hill soil with dH_2O were not created, as there was insufficient sediment available. However the absence of cDCE degradation in Hill controls with MSM (included with controls of Figure 3.1A) assures us that this material does not support degradation without inoculation.

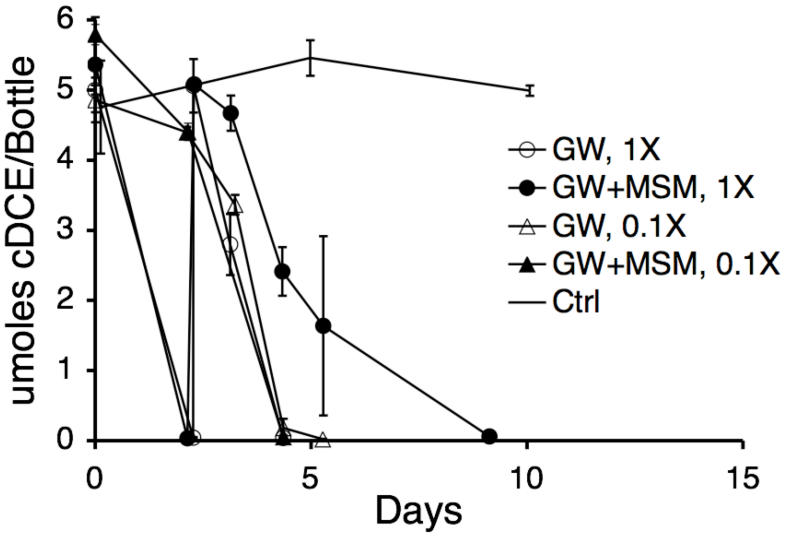

Figure 3.2: cDCE degradation in SJCA groundwater-only microcosms (GW) and SJCA groundwater amended with MSM (GW + MSM) at two inoculation levels (1X = 7×10^6 cells/ml and 0.1X = 7×10^5 cells/ml). The SJCA groundwater used here was obtained from MW04S six months prior to commencing a bioaugmentation study at the site. Microcosms were prepared with 9.7 mg/L cDCE. No degradation was seen in any of the uninoculated controls. Error bars represent the standard deviations of duplicate microcosms. Note that the 1X-inoculated microcosms were re-spiked with cDCE on Day 2, when analyses showed that the initially added cDCE had been depleted.

Microcosms prepared with Robins and Hill sediments in dH_2O and pH-neutralized with NaOH and H_3PO_4, respectively, prior to inoculation slowly and steadily degraded cDCE over 40 to 42 days (Figure 3.1B), when the experiment was terminated. Aerojet and Ft. Lewis microcosms without MSM were not pH-neutralized prior to inoculation. One of these Ft. Lewis groundwater microcosms was able to fully degrade the added cDCE, however this result was not replicated in its duplicate bottle (not shown). Similar lack of replication was also observed in the non-MSM-amended Aerojet microcosms (not shown). However, out of the 33 total treatments, these were the only two whose duplicates failed to agree. The SJCA groundwater-only microcosms, which were pH-neutralized with NaOH, showed similar degradation as that seen in the SJCA groundwater amended with MSM (Figure 3.2). No degradation was seen in uninoculated

microcosms. Collectively, these data suggest that subsurface materials exhibiting conditions favorable to JS666, most notably aerobic conditions with circum-neutral pH, support degradation by JS666 inocula without further need for amendment.

3.4.2. Effects of Inoculum Level and cDCE Concentration

The success of bioaugmentation could logically be dependent on inoculum size. Ramadan et al. suggest an inoculation level for bioremediation on the order of 4×10^4 to 4×10^5 cells per ml (17). Most of our microcosms had been inoculated with a culture density close to 7×10^6 cells per ml, with SJCA-groundwater microcosms also showing success at one-tenth that inoculum level. The SJCA-groundwater microcosms at the usual inoculation level degraded the added cDCE within 2 days, and those at the lower inoculation level, within 4 to 5 days (Figure 3.2).

To further explore the issue of inoculum level – recognizing that determining minimum effective inoculum level would be an important consideration for field application – we inoculated SRS sediment microcosms (amended with MSM) at $1/10^{th}$ (7×10^5 cells/ml) and $1/100^{th}$ (7×10^4 cells/ml) the usual level, as well as at the higher level (7×10^6 cells/ml). SRS sediment was considered a good choice for this study, because it was the material that had performed the worst in our earlier suite of microcosm studies (Figure 3.1), and thus would be expected to provide a challenging test of inoculum level. All inoculations were made with pure transfer culture.

It was already known from operation of the transfer cultures and from the microcosm studies of the previous section that JS666 is able to degrade high concentrations of cDCE (ca. 59 mg/L). However, we felt it important to investigate performance at cDCE concentrations more realistically encountered at contaminated sites as these lower levels might also contribute to inoculation failure (11). SRS microcosms with MSM were therefore prepared at $1/100^{th}$ the

usual level of cDCE (590 μg/L) as well as at the higher level (59 mg/L). Degradation was observed in all no-soil (i.e., MSM-only) controls, at the higher level of cDCE, and all levels of inoculation (Figure 3.3A). As inoculum level decreased, it took progressively longer for cDCE degradation to become noticeable, but the maximum rates ultimately achieved with each inoculum level were about the same. In SRS-sediment microcosms at the usual level of cDCE (Figure 3.4A), the effect of decreasing inoculum was also to slow cDCE degradation. However, maximum rates at the two lower inoculum levels never approached the rate observed at the highest level. Because of poor replication at the mid-inoculation level, it is difficult to determine whether or not there was a significant difference between the middle and lowest inoculum levels in Figure 3.4A, but both – while evidencing sustained degradation of cDCE – were clearly inferior to the highest inoculum level, which is probably to be expected.

In the no-soil (i.e., MSM-only) microcosms with $1/100^{th}$ the usual cDCE concentration, there was degradation at all inoculation levels (Figure 3.3B), though degradation at the lowest inoculation level appears to have stalled after about 50% of the substrate was degraded. For the sediment microcosms at this cDCE concentration, degradation was observed at all inoculation levels, while none was seen in the uninoculated-sediment controls (Figure 3.4B). Low levels of cDCE are more difficult to measure precisely. Because of this, degradation trends were noisy and thus could not be concluded to be significantly different. However, it is clear that JS666 is able to degrade these lower levels to below our detection limit, which is less than 0.3 μM (30 ppb) nominal concentration, and well below the MCL (70 ppb).

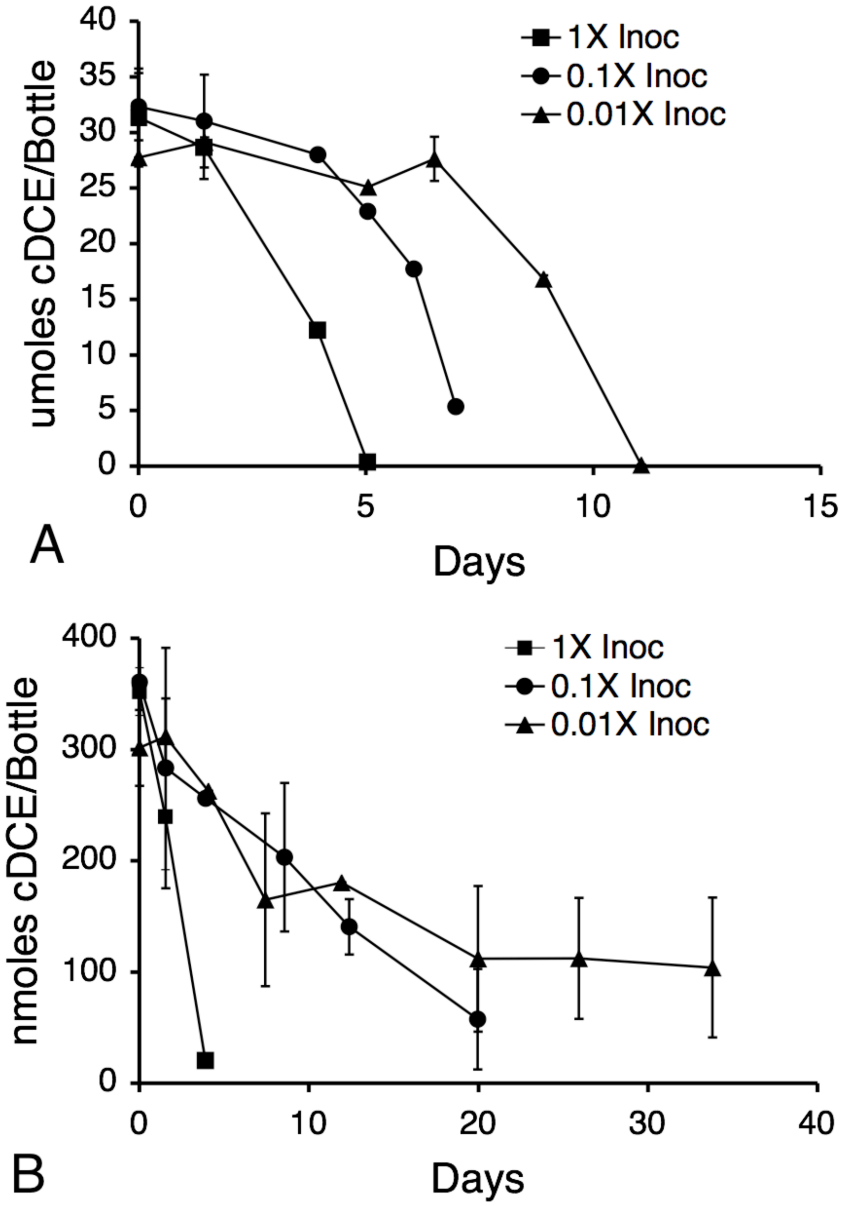

Figure 3.3: cDCE degradation in no-soil, MSM-only microcosms at three inoculation levels of serial dilution (1X = 7×10^6 cells/ml, 0.1X = 7×10^5 cells/ml, and 0.01X = 7×10^4 cells/ml). Microcosms were prepared with either: (A) 59 mg/L cDCE, or (B) 590 μg/L cDCE. No uninoculated controls were created because the medium was sterile. Error bars represent the standard deviations of duplicate microcosms.

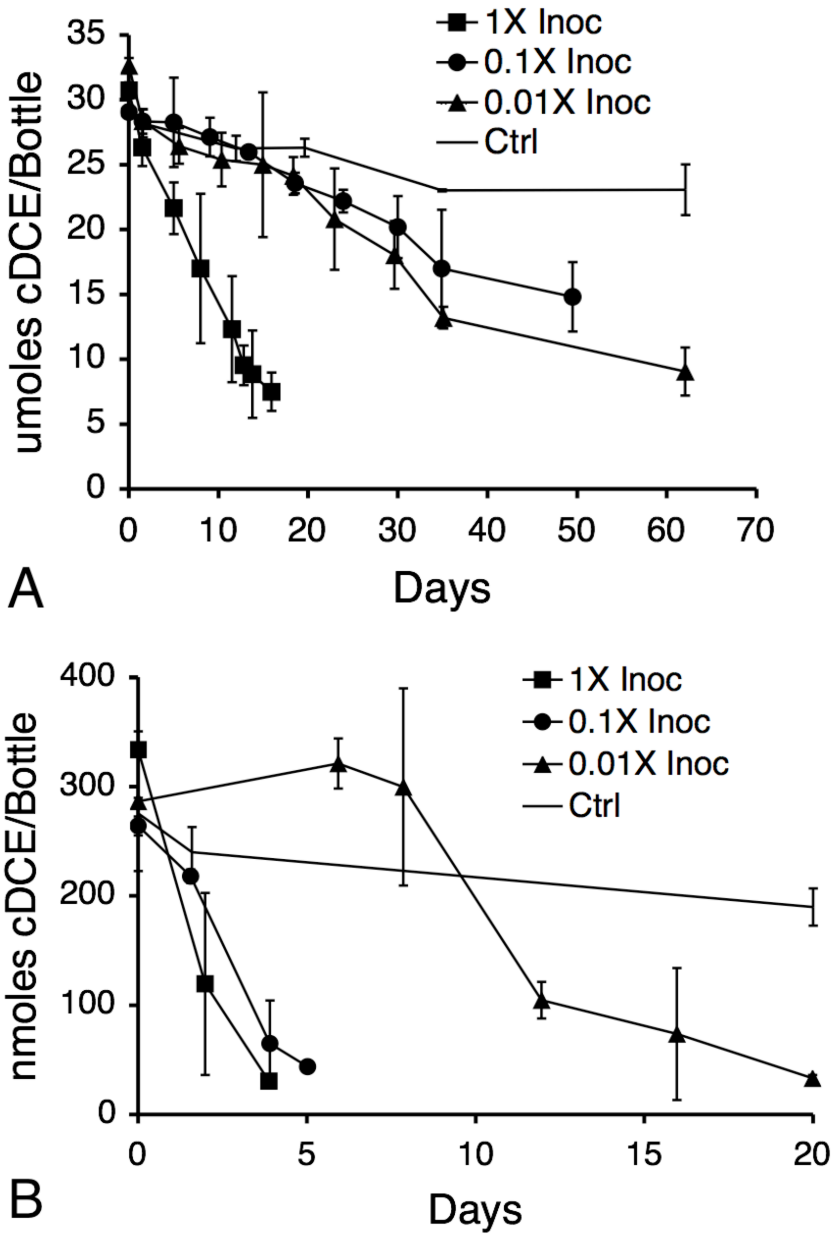

Figure 3.4: cDCE degradation in SRS-sediment microcosms (with MSM) at three inoculation levels of serial dilution (1X = 7×10^6 cells/ml, 0.1X = 7×10^5 cells/ml, and 0.01X = 7×10^4 cells/ml). Microcosms were prepared with either: (A) 59 mg/L cDCE, or (B) 590 μg/L cDCE. Error bars represent the standard deviations of duplicate microcosms.

As a perhaps more rigorous test of JS666 survival, we obtained groundwater from three wells within the SJCA pilot-scale field site, two months after bioaugmentation with JS666. Two samples were from wells (MW04 and MW05) ca. 4 weeks travel time downgradient of the bioaugmentation injection well; the other was from a well (MW15) in a control area outside of the bioaugmentation plot. MW04 and MW05 groundwaters had undetectable ($<1\times10^3$ copies/ml, by real-time qPCR) JS666 levels and 2×10^3 per ml, respectively, two months after bioaugmentation; JS666 was not detected in MW15, as to be expected since it was outside the augmentation area. Duplicate microcosms were prepared from each sample, as received from the site, and results are shown in Figure 3.5. The microcosms prepared from MW04 and MW05 groundwaters showed complete degradation of cDCE within 25 days, whereas the microcosms prepared from MW15 groundwater showed no cDCE degradation. Measurements at the end of the study showed JS666 levels in MW04 microcosms to have risen to 5×10^3 per ml, and JS666 levels in MW05 microcosms to have increased five-fold (to 10^4/ml), demonstrating growth of JS666 in both.

We conservatively recommend a minimum inoculation level in excess of 10^5 per ml for field applications – somewhat higher than the lowest level attempted here, and considerably higher than what was demonstrated to be active *in situ* in the SJCA field study, though lower than what has been implemented at other field demonstrations (18). Moreover, our estimations of biomass levels *in situ* at the SJCA are likely to be much lower than actual levels as only groundwater was sampled for JS666 quantification, which ignores any biomass sorbed to sediment. At the recommended level of inoculation (assuming soil porosity of 0.25), we estimate that 100 liters of inoculum culture grown to an OD600 of 1.0 would be able to treat a 10-m x 30-m x 80-m (24,000-m^3) plot.

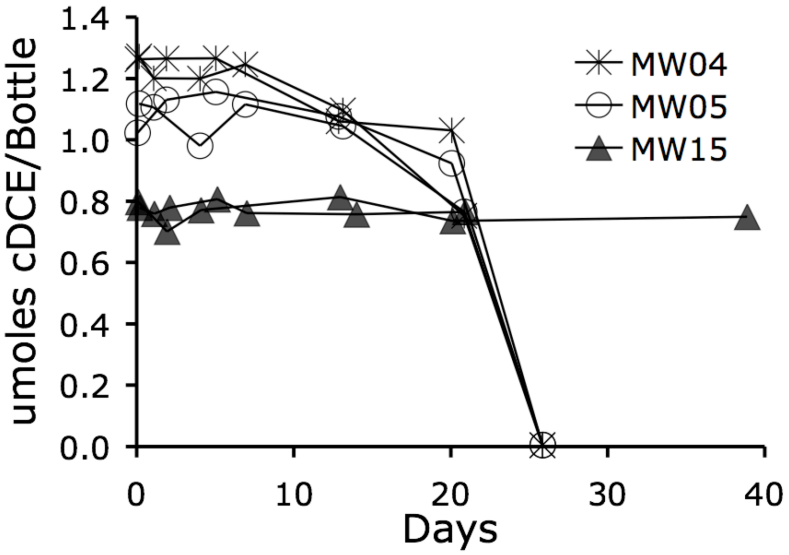

Figure 3.5: Activity assays from monitoring wells within the SJCA pilot-scale field study. Microcosms were created from three as-is groundwaters sampled from monitoring wells two months after bioaugmentation. In each case, duplicate microcosms are shown. MW04 and MW05 were about four weeks' travel downgradient of the bioaugmentation location, and MW15 was a control well outside the test plot.

3.4.3. Microcosms Amended with Municipal Primary Effluent

In the microcosm studies presented above, JS666 was able to degrade cDCE in all subsurface sediments or groundwaters investigated, so long as pH was controlled. We further challenged JS666 by adding municipal primary effluent (PE) to some microcosms. PE represents a source of diverse, alternative substrates as well as a likely source of potential microbial competitors, predators, and phages.

In SRS-sediment microcosms amended with MSM and PE (Figure 3.6), cDCE degradation was clearly observed in all cases (two different inoculum levels and two different levels of PE), but there was no consistent trend with inoculum level or PE concentration. While cDCE degradation rate with 1% PE occurred more rapidly at the standard inoculum level than at the $1/10^{th}$ level. The opposite was true in microcosms with 10% PE. Despite the lack of a clear trend, the results do suggest that JS666 can function in mixed-culture, mixed-substrate

environments, albeit more slowly. We consider the application of as much as 10% PE to be a rather severe test, in comparison to the likely microbial environment at prospective subsurface application sites as the number of protozoa in primary effluent far outweighs that of typical sediment samples (16).

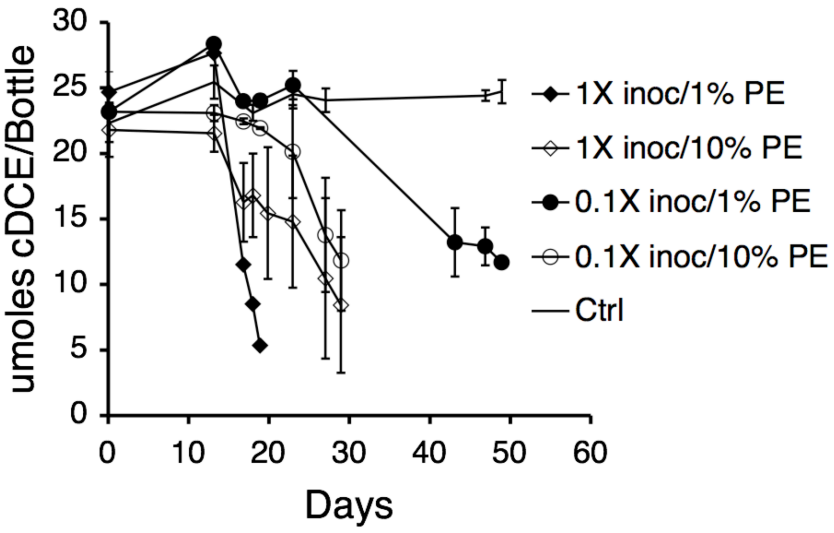

Figure 3.6: SRS-sediment microcosms amended with MSM and municipal primary effluent (at 1% and 10% PE v/v), inoculated with JS666 at two levels (1X = 7×10^6 cells/ml and 0.1X = 7×10^5 cells/ml). Error bars represent the standard deviations of duplicate microcosms.

3.5. Conclusions

Subsurface sediments and groundwater from six different cDCE-contaminated sites were used to construct microcosms. In every subsurface sediment or groundwater inoculated with JS666, the organism was able to degrade cDCE, provided that the pH in the system remained circum-neutral. Even when JS666 was challenged with an alternate carbon source, or in the presence of competitive/predatory microorganisms, there was a measure of success. Collectively, these microcosms studies suggest that JS666 is a viable candidate for the bioaugmentation of aerobic, cDCE-contaminated sites.

3.6. Acknowledgements

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CHAPTER 4[2]

4. MICROCOSM ASSESSMENT OF A DNA-PROBE APPLIED TO AEROBIC DEGRADATION OF CIS-1,2-DICHLOROETHENE BY *POLAROMONAS* SP. STRAIN JS666

4.1. Abstract

A molecular biological tool based upon an organism-specific DNA sequence does not necessarily indicate *in situ* activity, but serves important functions of evaluating the potential for biodegradation and mapping the distribution of an organism. Currently, DNA-based probes are accepted as evaluative tools for site assessment. However, these techniques are far from standardized, and information on precision is usually lacking.

Here we present the development and evaluation of a DNA-probe for *Polaromonas sp*. strain JS666, a bacterium that couples growth to aerobic oxidation of *cis*-1,2-dichloroethene (cDCE), and is therefore a promising candidate for bioaugmentation at sites where cDCE has accumulated in aerobic zones. The DNA probe was used in conjunction with quantitative PCR to track the abundance of JS666 in microcosms. This series of studies has allowed explicit resolution of the accuracy and precision of the probe and its correlation with variations in microcosm performance. We determined that the method is sufficient to monitor distribution of JS666 at bioaugmented sites. We found that within environmental, mixed cultures, the DNA target does not persist long after cell death, demonstrating that positive result from the probe is a strong indicator that degradation can occur in suitable environmental conditions. Finally, absent suspected predation, the probe accurately and precisely tracks growth. Collectively, the studies

[2] This chapter was originally published as: **Giddings, C. G. S., L. K. Jennings, and J. M. Gossett.** 2010. Microcosm Assessment of a DNA Probe Applied to Aerobic Degradation of cis-1,2- Dichloroethene by *Polaromonas* sp. Strain JS666. Ground Water Monitoring & Remediation. **30:**97-105, and has been updated to reflect changes suggested by my Special Committee since publication.

appear to validate the utility of the molecular probe for site assessment in a bioaugmentation context.

4.2. Background

Chlorinated ethenes are among the most prevalent groundwater pollutants in the United States, and are on the US EPA's list of primary regulated drinking water contaminants (37). The clean up of these and other volatile organic compounds is estimated to cost "more than $45 billion dollars (1996 dollars) over the next several decades" (37). Of the technologies used to remediate contaminated sites, *in situ* bioremediation is recognized as being a promising and cost-effective solution (19, 32). However, bioremediation depends on suitable conditions, including the presence of proper organisms with the metabolic capacity to degrade the pollutants, and favorable geochemical parameters. Even if those conditions are met, a site may require amendments to stimulate degradation. In cases where microorganisms with the degradative capacity are absent, sites will require augmentation with microorganisms (10, 21, 27).

Site characterization is essential to successful bioremediation, which typically involves three lines of evidence: (i) demonstration of the reduction of contaminant mass, (ii) the potential for biodegradation, which can be demonstrated through geochemical data such as dissolved oxygen levels or redox potential, and (iii) demonstration that microbial activity is responsible for observed reduction in contaminant levels (37). This final line of evidence is often demonstrated through microcosms and/or column studies and is especially important in aerobic systems where, unlike reductive dehalogenation, there are no easily distinguishable daughter products to demonstrate biodegradation (11). However, microcosm and column studies are time-consuming and expensive and do not necessarily reflect *in situ* conditions, as the act of sampling a site may change the condition of the system (16). It has been recently shown that compound-specific

isotopic analysis can be useful in demonstrating aerobic biooxidation of cDCE, though few labs currently have the capability to perform these analyses (1). Further, contaminant concentrations can be affected by multiple organisms, which presumably degrade by various pathways and mechanisms. Because an isotope enrichment factor depends on the initial irreversible transformation step, the mixed-effect of a microbial consortium may be observed (1). For this reason, Abe et al. have suggested a duel isotope approach be employed to characterize environmental samples (1).

Molecular biological tools (MBTs) that can quantify the presence of desirable microorganisms (or, better yet, their activities) *in situ* are useful at both the decision-making stage when evaluating alternative technologies, as well as in the monitoring of an implemented bioremediation technology (36). MBTs can conceivably be based on DNA (targeting either phylogeny or genes of a degradative pathway), mRNA (targeting up-regulation of degradative genes), or proteins (targeting the degradative enzymes themselves). At present, there have been a limited number of studies conducted exploring the potential of mRNA-based probes (14, 28, 29, 30), and even fewer that have explored their use in soil and groundwater (3, 15). Though mRNA-based probes would appear conceptually to have greater potential to correlate to activity than would DNA-based probes, mRNA is very short lived and difficult to recover (15). Because of this, the technology for their use has not yet reached practical utility and will likely be most effective in tandem with other molecular techniques and/or other more traditional methods of detection.

An MBT that relies on an organism-specific DNA sequence also has benefits and drawbacks. DNA is relatively stable in the environment, which makes it easier to work with. However, it is not directly a measure of activity and might remain after cell death (24), though one might

expect DNA from dead cells to be degraded relatively rapidly (i.e., over days rather than weeks) within environmental, mixed cultures (6, 34). DNA probes can serve the important purposes of showing the capability for biodegradation and also the spatial/temporal distribution of an organism following enhancement and/or bioaugmentation (34).

Currently, DNA probes, even those based on phylogeny and not function, are accepted as indication of potential for degradation at bioaugmentation sites, and are both qualitative (indicating presence), and also quantitative (demonstrating abundance) (32). The three requisite lines of evidence, including evidence from DNA-based probes, have been presented in successful bioaugmentation of sites impacted by chlorinated ethenes through the process of reductive dechlorination (21, 27, 34). In aerobic remediation where the end products are difficult to measure, the ability to monitor organisms could contribute to evidence that the process is microbially mediated (9). However, these techniques are far from standardized, and each probe requires an amount of examination to determine its utility. Work conducted with DNA-based probes in the context of aerobic remediation of contaminants such as chlorobenzenes and BTEX include both probes based upon specific catabolic genes (5) and also upon entire suites of oxygenases employed by a number of different organisms (4). Studies involving MBT application for the aerobic remediation of chloroethenes have been limited to cometabolic systems probed for the genes of enzymes with broad substrate specificity such as nonspecific monooxygenases and are not quantitative (13).

Coleman et al. isolated a beta-proteobacterium, *Polaromonas* sp. JS666, which is able aerobically to oxidize cDCE as carbon and energy source (7). The organism is the first of its kind to be isolated. Since JS666 apparently requires no exotic growth factors, it is considered a promising bioaugmentation agent for aerobic sites where cDCE has accumulated. The genome

of JS666 has been sequenced, and the results are discussed by Mattes et al. (22). Initial work exploring the potential of JS666 as a bioaugmentation agent has been completed, namely successful cDCE oxidation in microcosm studies constructed with various subsurface materials and inoculated with the organism (12). Additionally, a pilot study using JS666 as a bioaugmentation agent began in October of 2008 at St. Julien's Creek Annex (SJCA), Chesapeake, VA.

Here we present the development and application of a DNA-based probe based on the isocitrate lyase gene of JS666, known from the genome to be present at only one copy per cell. This probe was used in conjunction with real-time, quantitative PCR (qPCR) to track the abundance of JS666 in microcosms (and is currently being applied in the ongoing SJCA field study). The microcosm studies allowed explicit resolution of the accuracy and precision of the probe, and determination of the extent to which probe results correlate with variations in microcosm performance.

4.3. Materials & Methods

4.3.1. Culturing Technique

JS666 cultures were grown on neat cDCE (99%, TCI America) in carbon-free minimal salts medium (MSM) at a pH of approximately 7.1 to 7.2, as described elsewhere (12). Pure cultures were maintained through a series of 5% v/v culture transfers into 100 ml MSM in 160-ml serum bottles and fed a nominal concentration of cDCE of 51 mg/L. Additionally, purity checks by streak-plating were routinely carried out.

4.3.2. Sediment and Groundwater Types

Subsurface sediment or groundwater samples were obtained from aerobic, cDCE-contaminated sites at Savannah River (SRS), SC and SJCA, VA, respectively. Moisture content of the SRS

sediment was determined gravimetrically from heat-dried sediment samples, and pH of both the sediment and groundwater were measured using an Accumet micro-electrode with a calomel reference. Additionally, pH measurements were taken from individual microcosms at the conclusion of each experiment to assure that pH never became prohibitively low. All sediment and groundwater samples were shipped on ice to the laboratory and stored at 4ºC in the dark for later use.

4.3.3. Microcosms

All microcosms were prepared aseptically under a laminar-flow hood, with PCR-clean spatulas in autoclaved 160-ml serum bottles and contained either 50 g (dry wt) sediment or 50 ml groundwater. For each sediment treatment, MSM was aseptically added to yield a total of 50 ml of liquid (including the moisture contributed from soil). Microcosms amended with MSM required no other neutralization beyond that provided by the phosphate-buffer component of the MSM. cDCE-fed microcosms were administered 2.3 µl of cDCE (59 mg/L nominal concentration) via syringe through ethanol-swabbed, flamed septa. All experiments were conducted at 22ºC in the dark, agitated at 60 RPM. The experimental treatments summarized in Table 4.1 were performed to achieve the following objectives:

- To determine if the probe could usefully detect growth;
- To gain a measure of the precision of the probe in soil, as well as to provide variation in cDCE-degradation performance to attempt correlation with probe results; and
- To investigate JS666 die-off (including endogenous decay and cell death due to predation or toxicity) and microbiostasis.

Table 4.1 Set-up of experimental studies

Objective	Material	Inoculation Level	cDCE Level	MSM Buffer
Growth and Probe Correlation	MSM-Only (in duplicate)	1X 0.1X 0.01X	1C	√
Probe Accuracy and Precision in Soil	SRS Sediment (in quadruplicate)	1X	1C	√
Starvation & Die-off	SRS Sediment (in triplicate)	10X	0	√
	SJCA GW (in duplicate)	1X 0.05X	0 0	

Note: For reference, "1X" = 7×106 cells/ml (3.5×108 copies/bottle); "1C" = 59 mg/L cDCE (nominal concentration).

4.3.4. Analytical Methods

Total quantities of cDCE in bottles were measured from 100-µl headspace samples by gas chromatography with a flame-ionization detector and a packed column, as described in detail elsewhere (12). Standards to quantify cDCE were created in either dH_2O or sediment and MSM.

Optical density at 600 nm (OD600) in a Biophotometer was used to estimate the initial target inoculations for each experimental subculture culture. This was done with an established correlation between OD600 and cell counts from real-time qPCR using primers targeting the isocitrate lyase gene of JS666.

4.3.4.1. Sampling Procedure and Nucleic Acid Extraction.

DNA was extracted from pure cultures or from microcosms using the UltraClean Soil DNA Kit (MoBio, Carlsbad, CA). Microcosms were sampled in a manner so that the analyzed sample had the same proportions of supernatant and sediment as in the microcosms. Moist sediment was sampled with a PCR-clean spatula and weighed. Based upon the previously measured moisture content of the sediment, the weights of dry solids and water in this sediment sample were calculated. Next, with a disposable, sterile syringe, an appropriate mass of supernatant was withdrawn from the microcosm and added to this sediment sample to achieve a

supernatant/solids ratio identical to that of the microcosm as a whole. Total sample size was approximately 0.5 g (but precise mass was noted for later calculations). Liquid cultures (without soil) were sampled more simply: 0.5 ml of liquid was withdrawn by sterile disposable syringe. This small amount was used to avoid having to pelletize the cells to discard the supernatant, as the culture does not cohere easily, and instead extract DNA from the entire sample to prevent inadvertently disposing of cells. Pure culture DNA was used to create standards for each of the primer probes. All DNA extractions were stored at -20ºC until later use.

The total concentration of DNA in each sample was quantified with fluorometry using the intercalating reagent PicoGreen (Invitrogen). A Fluoroskan Ascent spectrophotometer (Thermo Labsystems) measured fluorescence of PicoGreen bonded to double-stranded DNA at an excitation wavelength of 485 nm and emission wavelength of 538 nm. Lambda DNA (Invitrogen) was employed as a primary standard. Since DNA in extracts from pure JS666 cultures was assumed to be entirely comprised of JS666 DNA, the DNA concentrations of JS666-DNA stocks were fluorometrically determined by applying the lambda DNA standard curve. Standard curves for JS666 were generated using serial dilutions of DNA of known concentration extracted from pure cultures and applied in the standardization of qPCR analyses.

4.3.4.2. Probe Development and Real-Time qPCR.

Polaromonas sp. JS666's genome was sequenced by the Joint Genome Institute Microbial Sequencing Program (Genbank accession numbers CP000316-CP000318), which facilitated the selection of target genes for the molecular probe. The chromosomal gene, isocitrate lyase, was selected as the target for our molecular probe. Although a cDCE-specific degradative gene would perhaps have been a preferred target, the cDCE-degradative pathway has not been elucidated in this organism. Jennings et al and Mattes et al. have suggested the involvement of

glutathione-S-transferases and monooxygenases, respectively, but JS666 has several genes of each type and the role of any specific one in cDCE degradation remains unproven (18, 22). Its isocitrate-lyase gene is a functional gene in the glyoxylate cycle, and has ample variability and sequence stability for the design of strain-specific primers. Additionally, it has more sequence variability than the 16S rRNA gene. The Beacon Designer 4 software program aided in the design of JS666 primers and optimized efficiency for real-time PCR assays. The isocitrate lyase primer set AceA 276F (TGCCGCTGACAACAACAC) and AceA 414R (ATCAATGCCTTTGGAGTGC) has an amplicon length of 139 bp.

The specificity of the primers was confirmed with database searches in Genbank. It was also tested with conventional PCR with both the isocitrate lyase and a 16S rRNA primer set (8f/1492r) as a positive control on DNA extracted from a number of sources. The DNA came from pure JS666 culture, SRS sediment and groundwater, two topsoil samples with high organic carbon content from Ithaca, New York collected 4 inches bgs, *Escherichia coli*, primary effluent collected from the Ithaca Area Wastewater Treatment Plant, and a mixed dehalogenating culture that contained *Dehalococcoides ethenogenes.* Additionally, a negative control containing DNA-clean water was carried through the experiment. Gel electrophoresis with ethidium bromide staining was used to determine the presence of any non-specific amplification from the primer sets. Amplification was only observed in the reactions carried out with JS666 and the isocitrate lyase primer sets and all DNA with the 16S rRNA primer sets, and no amplification was observed in the negative controls, demonstrating that the primer set was specific to JS666 for our purposes.

The DNA, and therefore the number of target genes in each sample, were quantified by qPCR with a thermocycler (iCycler Detection System, BIO RAD) with the intercalation agent iQ

SYBR Green (BIO RAD). The reactions were carried out under the following conditions: 2 min at 50ºC followed by 3 min at 95ºC; next 40 cycles (denaturation at 95ºC for 15 sec, annealing and extension at 63ºC for 1 min), where fluorescence was measured after every cycle. Each reaction was performed in triplicate, and a melt curve was completed following the amplification reactions to confirm the specificity of the primers and the reactions.

To provide a normalization parameter for eventual field studies, we designed a technique for quantitatively measuring both JS666 and total eubacterial 16S rRNA targets. The 16S rRNA primers employed, 799F (GGTAGTCYAYGCMSTAAACG) and 1044R (GACARCCATGCASCACCTG) have a similar annealing temperature to that of the isocitrate lyase primers, and were therefore run with the same protocol (2).

4.3.4.3. qPCR Applied to Sediment Systems.

To overcome soil-matrix inhibition to the PCR reaction, DNA extracts from sediment or groundwater were diluted. To determine the minimum level of dilution required, the following procedure was performed for sediment or groundwater. A known amount of JS666 was used to inoculate a sediment sample, as measured by viable plate counts and LIVE/DEAD microscopy (SRS soil) or qPCR on pure culture (SJCA groundwater). Next, a DNA extraction was performed. This DNA was diluted 1-, 5-, 10-, 20-, 50-, 100-, and 200-fold and the copy-number was measured using qPCR. These were compared against the expected amount of DNA as determined by a liquid extraction performed on the same inoculum. We determined that DNA extracted from SRS and SJCA materials required a 50-fold and a 5-fold dilution, respectively (Figure 4.1, Figure 4.2). All microcosms were sampled for DNA at the completion of each experiment.

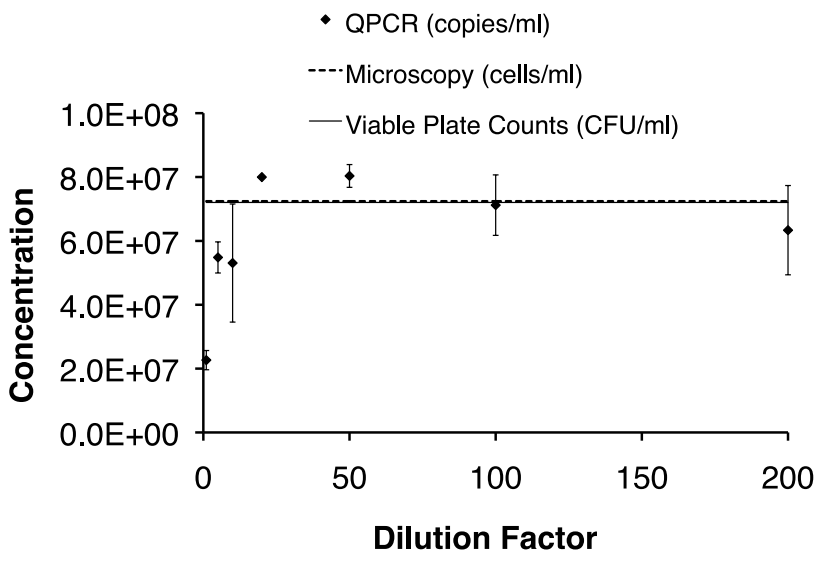

Figure 4.1: Copy numbers of diluted JS666-DNA in soil measured by qPCR as compared to expected values from heterotrophic plate counts and LIVE/DEAD microscopy. Error bars represent the standard deviation of duplicate measurements (17).

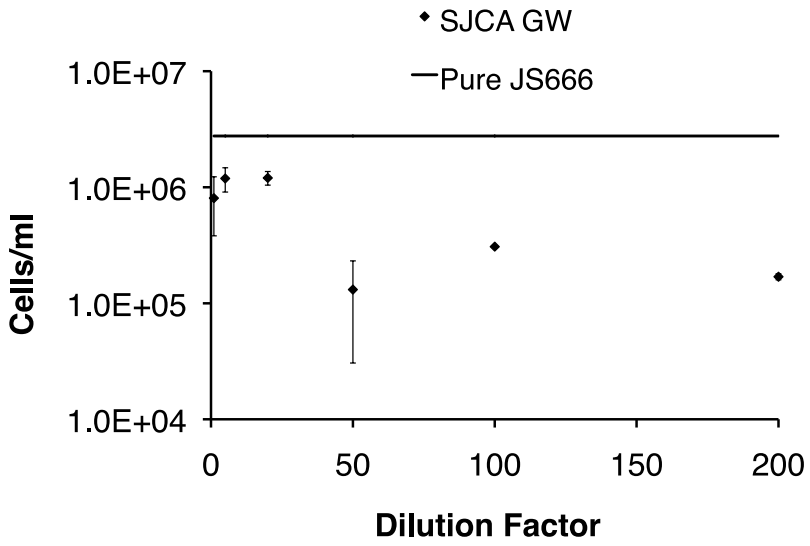

Figure 4.2: JS666 concentrations (cells/ml) of diluted JS666-DNA in SJCA groundwater measured by qPCR. These are compared to expected values from DNA extracted from pure culture. Error bars represent the standard deviation of triplicate measurements.

Preliminary work on the recovery of JS666 from sediment by qPCR was done by comparing the qPCR copy-numbers to the numbers of cells in the inoculating cultures as determined by heterotrophic plate counts and viable microscopic counts, as previously described (17). Serial dilutions of JS666 pure cultures were prepared by making 10-fold dilutions of a source culture. This resulted in four JS666 cultures labeled A-D (most concentrated to least concentrated) and one blank. The concentration of cells in the diluted cultures was determined by heterotrophic plate counts and LIVE/DEAD microscopic counts. Heterotrophic plate counts were carried out on 1/4-strength Trypticase soy agar (TSA) plates. LIVE/DEAD staining was used to obtain direct microscopic counts of the live cells and apparently dead cells with damaged membranes.

To test for differences in extraction efficiencies, a MoBio DNA Soil Isolation kit was used to extract DNA from 0.5-ml aliquots of the JS666 cultures and approximately 0.2 ml of the same JS666 cultures applied to approximately 0.3 g of SRS sediment (0.41 g dry weight, exact measurements noted for analysis).

4.3.4.4. Expression Data Analysis (DART).

To damp-out errors associated with plate-to-plate variation in standard curves, fluorescence data generated by the iCycler was analyzed using the DART-PCR technique as outlined and developed by Pierson et al. (25). The DART-PCR tool uses linear regression to extrapolate an initial fluorescence level, R_0, in each well (33). The JS666 standard conversion factor between initial fluorescence (R_0) and ng of DNA per reaction was created for qPCR. This number, in units of R_0/ng DNA, was found by averaging pure JS666-DNA samples of known concentrations. Measured concentration of DNA was converted to copies of target gene per microliter of sample (Equation 4.1 and Equation 4.2), where the size of the JS666 genome is 5.9

Mb as reported by the Joint Genome Institute Microbial Sequencing Program. The total mass of DNA (grams) per mole of JS666 cells is thus given by

$$\left(\frac{g\ DNA}{mol}\right) = \left(5.9\times10^{6}\ \ bp\right)\times 660\left(\frac{daltons}{bp}\right) \qquad \text{Equation 4.1}$$

and therefore the number of copies per µl is found by

$$\left(\frac{copies}{\mu l}\right) = \frac{6.02\times10^{23}\left(\frac{copies}{mol}\right)\times C_{DNA}\left(\frac{g}{\mu l}\right)}{\left(\frac{g\ DNA}{mol}\right)} \qquad \text{Equation 4.2}$$

where C_{DNA} is the concentration of DNA as measured by fluoroskan.

This number was found for the isocitrate lyase gene and the 16S rRNA by averaging 13 pure JS666-DNA samples of known concentrations (as determined with the fluoroskan) and was $1.97\times10^{-9} \pm 4.43\times10^{-10}$ and $4.65\times10^{-10} \pm 8.79\times10^{-11}$ R_0/gene copy, respectively.

4.4. Results & Discussion

4.4.1. Accuracy and Precision

The accuracy of the DNA probe and recovery of JS666 DNA was first assessed by inoculating sediment samples with aliquots of serially diluted, pure-culture JS666, and then comparing the copy number of isocitrate lyase genes (copies/ml) determined from qPCR to the cell counts obtained from heterotrophic plate counts (CFU/ml) and direct microscopic counts (cells/ml). Given that there is a single copy of the isocitrate lyase gene per cell, the cell counts should directly correlate with the copy numbers calculated from qPCR. Results are presented in Figure 4.3. Although there is some discrepancy in the most-concentrated of the serial dilutions, there is generally satisfactory agreement among enumeration methods.

The test for differences in extraction efficiencies (between sediment and pure liquid cultures) using a MoBio DNA Soil Isolation kit showed that the efficiency in sediment was actually elevated nearly ten-fold relative to that in pure liquid culture ($1.6\times10^7 \pm 7.5\times10^6$ vs. $3.3\times10^5 \pm 1.2\times10^4$ copies/ml). This might be due to soil's protection of the DNA, preventing excessive DNA damage during the bead-beating portion of the extraction procedure; or alternately the sediment aided lysis of cells. Given adequate dilution of the DNA sample, though counter to what might be expected, the presence of sediment did not appear to impede DNA extraction.

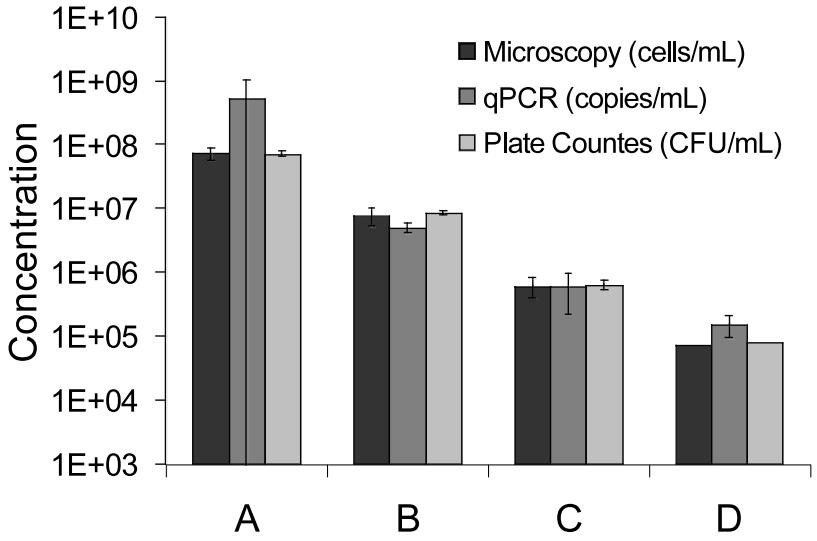

Figure 4.3: Comparison of copy numbers calculated from qPCR of JS666/sediment samples to cell counts from microscopy and heterotrophic plating (17). JS666 cultures in sediment were extracted in duplicate from duplicate sediment samples. Samples A, B, C, and D are serial dilutions of JS666. Error bars represent standard deviations of duplicate measurements.

A microcosm study prepared with SRS sediment and amended with MSM was constructed in quadruplicate to assess the over-all precision of the qPCR method (including the soil-extraction step). Initially, each of the four bottles was extracted in triplicate immediately following inoculation, yielding a sample size of 12 extractions. The calculated copy numbers per bottle were not found to differ statistically for any of the extractions or bottles ($\alpha = 0.01$). The average

copy number in each bottle was 3.0×10^8 ($\pm 1.3 \times 10^8$ std dev) per bottle, which corresponds to a coefficient of variation (CV) of 43%. This also confirmed that our calculated inoculation levels (target level of 3.5×10^8 copies/bottle, as per Ramadan et al. [31]) were achieved. When using a fluoroskan following Picogreen staining of double-stranded DNA to calculate the total DNA (ng) in each bottle, the CV was much lower, only 20%. The total DNA in the bottle was $1.1 \times 10^6 \pm 2.2 \times 10^5$ ng. The large difference between these CVs is most likely due to error-propagation in the qPCR process. Due to the mathematics involved (i.e., extrapolation backwards many cycles from the log-linear phase), small variations in efficiencies will translate into large variations in the estimate of initial copy number. Additionally, the CV of 43% includes not only the imprecision of the qPCR procedure itself, but also of the DNA extraction and comparison across plates. The R_0/rxn of replicate samples of pure JS666 DNA had intra-run (within plate) CVs of 0.6 - 1.5%, whereas the inter-run (across plates) CV was 21%. This suggests that simply comparing samples across qPCR plates accounts for approximately half of the variance seen in the overall process.

Currently, there is no standard procedure for nucleic-acid extraction from sediment systems, and comparing across studies is difficult. qPCR has been demonstrated to be sensitive and accurate, but inter-run variability (i.e. across plates) tends to be high (8, 26, 35). Moreover, most researchers report only variations in their inter-run CT values which, when converted to absolute numbers, would have significantly higher variation (35). For example, Powell et al. found in their intra-run assays that the CVs calculated on the CT values was between 1.2% and 1.4% for the differing gene assay, but much higher when calculated on the number of copies per μl (26). These CVs were 16% and 18%, respectively, and even higher when calculated for inter-run values: 25% to 38% (26).

4.4.2. Tracking Growth and Degradation Performance

To determine whether the probe could usefully track cell growth within microcosms (in the absence of complicating factors such as predation), MSM-only microcosms were constructed in duplicate and inoculated at three different levels – 1X (7×10^6 cells/ml or 3.5×10^7 per bottle), 0.1X, and 0.01X – with an initial, nominal cDCE concentration of 59 mg/L.

Coleman et al. report a yield coefficient for JS666 of 6.1 g protein/mol cDCE (7). If we assume a protein mass per cell of 1.55×10^{-13} grams, as for *E.* coli, then a rough estimate of expected cellular yield is 4×10^7 cells formed per μmol cDCE degraded (23). In Figure 4.4A, the 1X, 0.1X and 0.01X microcosms each completely degraded 30 μmol, for an expected synthesis of 1.2×10^9 cells per bottle. Measured qPCR-based JS666 levels in the various microcosm-types at conclusion of the study agree reasonably well with quantities of cDCE degraded. The two 1X replicates showed an average increase of 4.8×10^8 cells above inoculum level, whereas the 0.1X and 0.01X microcosms showed increases of 1.6×10^9 and 1.0×10^9 cells per bottle, respectively (Figure 4.4B). The average of all six bottles provides a yield of $4.9\times10^7 \pm 2\times10^7$ cells/μmol cDCE degraded, which is in accord with protein-based yield estimates.

Precision is, of course, only one issue of importance in the utility of a MBT. Another question is whether or not the MBT can adequately track the performance of the targeted organism. The SRS sediment with MSM microcosms prepared in quadruplicate that had been used to assess MBT precision at time zero, were subsequently monitored over time, with expected variations in cDCE-degradation performance observed among them that could be tested against results from DNA probing.

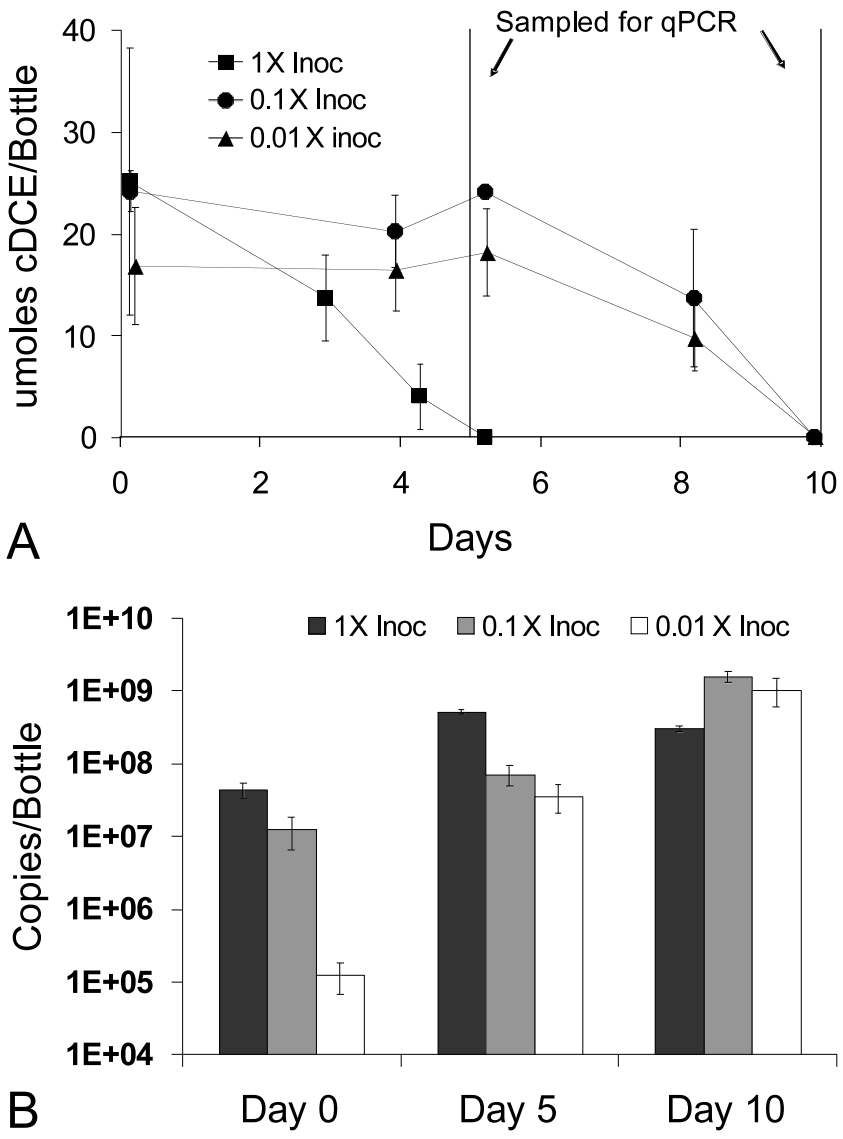

Figure 4.4: (A) cDCE degradation in no-soil, MSM-only microcosms at various inoculation levels. Microcosms were prepared with 59 mg/L cDCE and inoculated at target values of 1X (3.5×10^8 cells/bottle), 0.1X or 0.01X; (B) Corresponding qPCR data. DNA was extracted in duplicate. Error bars represent the standard deviations of duplicate microcosms and duplicate DNA extractions.

Figure 4.5A and B present the time-course results for the quadruplicates. The microcosms were sampled in triplicate at inoculation, then at days 39, 45 and 59. These times correspond to when the microcosms had degraded all or most of the present cDCE, and then approximately 7 days and 15 days after degradation had completely ceased in all bottles. Given an analytical precision of ±43% (as determined earlier), the isocitrate-lyase gene copy number in bottles A and B at the day-39 sampling time (ca. 4×10^8 copies/bottle) were not significantly different, and they had performed similarly in cDCE degradation. However, bottle D had the lowest (1.4×10^8 copies/bottle) and bottle C had the highest (8.4×10^8 copies/bottle). This partially confirms what was observed in the cDCE-degradation patterns. At day 45, bottle D had significantly lower DNA levels than the others, though bottle D had performed the best in cDCE degradation, and bottle C had performed the worst.

It is possible that the levels of JS666 DNA that were observed are the result of sediment microbiostasis, which is a balance between growth, decay, and other pressures, such as predation or unfavorable abiotic effects (38). Some suppression mechanisms (e.g., predation and phage infection) would be expected to elevate in response to JS666 population levels, tending to equalize resulting observed populations so that any growth is not seen, especially in aerobic environments (20). However, protozoan grazing may become less of an issue in deeper aquifers as their presence decreases as oxygen becomes limiting (20). In our experience, SRS material was not a favorable environment for JS666 growth. Because of this, there was a prolonged incubation required to achieve cDCE degradation in SRS sediment that could lead to this stasis, which we were most likely observing.

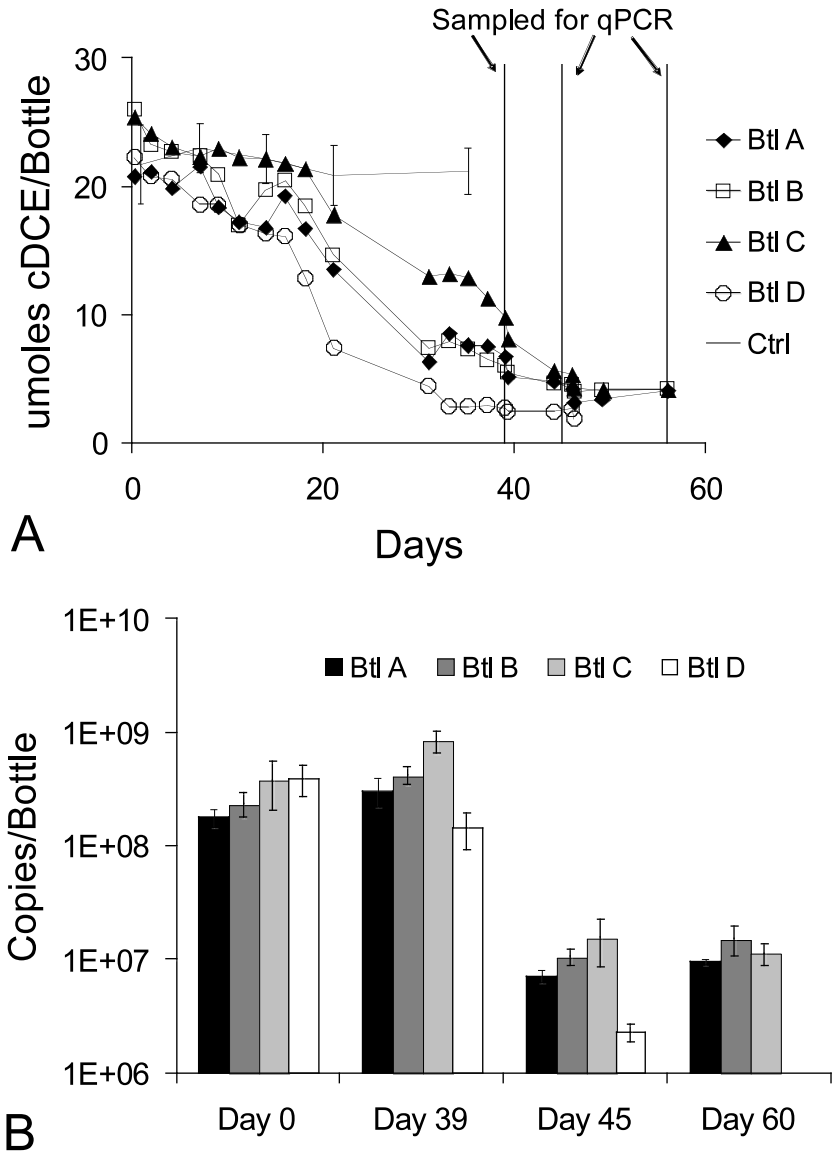

Figure 4.5: (A) cDCE degradation in SRS sediment inoculated with JS666, prepared in quadruplicate, and (B) corresponding average copies of JS666 per bottle from triplicate extraction samples, as enumerated via qPCR (data unavailable for bottle D for sample day 60). Error bars represent standard deviations of duplicate microcosms and duplicate DNA extractions.

Another microcosm study was constructed in SRS sediment without any cDCE or additional carbon source to monitor decline in probe-signal (both for the isocitrate lyase and the universal eubacterial 16SrRNA gene) over an extended period of starvation. Over four weeks, with no added carbon source, there was an obvious downward trend in JS666 DNA levels (Figure 4.6A), but not of the universal eubacterial 16S rRNA gene (Figure 4.6B), suggesting that there was no loss of total DNA or problems with extraction. Within one week, the total amount of JS666 present was reduced by almost three orders of magnitude from 1.6×10^9 to 5.0×10^6 cells/bottle, which supports the idea that any growth of JS666 would be difficult to detect due to rather robust mechanisms apparently operating in the SRS sediment system causing relatively rapid reduction of JS666 DNA. This suggests an explanation for the swift decline in DNA observed (Figure 4.5) between day 39 and day 45 in bottle D of the previous experiment following cessation of cDCE degradation, as the growth expected from the amount of cDCE degraded was never observed. Bottle D was the bottle that completed cDCE degradation first among the quadruplicates.

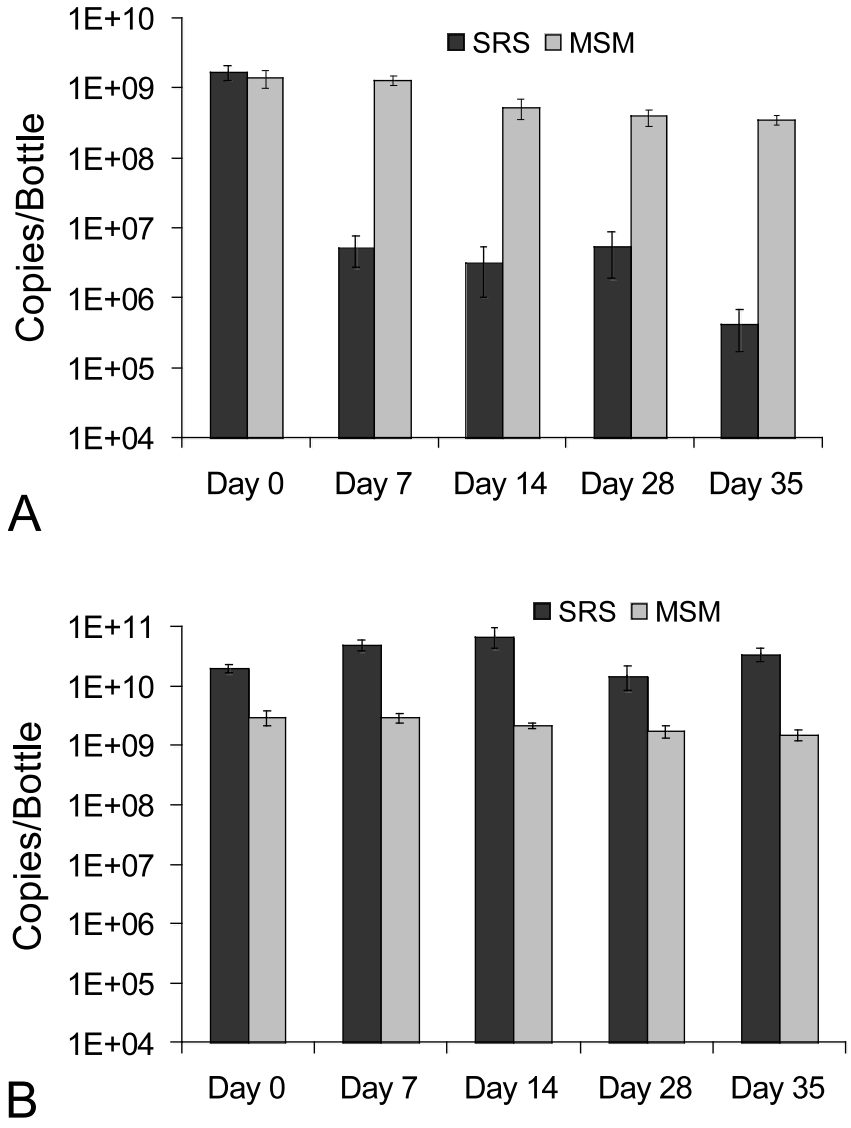

Figure 4.6: Average copies/bottle of (A) JS666-specific isocitrate lyase gene and (B) universal eubacterial 16S rRNA gene from microcosms constructed from Savannah River Site (SRS) soils + MSM, or MSM-only. Microcosms were inoculated with JS666 at 3.5×10^{9} copies/bottle and given no external carbon source. Bottles were sampled in triplicate; error bars represent standard deviations from average concentrations.

As a field study is currently underway, another starvation study was conducted with SJCA groundwater to determine the persistence of JS666 without cDCE as a substrate, and to provide another material for investigation of possible microbiostasis. In this study, groundwater purged with filtered air to remove and residual cDCE was inoculated at two levels. Again, probe-signal for both for the isocitrate lyase and the universal eubacterial 16S rRNA genes were monitored over an extended period of starvation. As with our earlier starvation study conducted with SRS soil, there was an obvious downward trend in JS666 DNA-target levels in SJCA groundwater following inoculation (Figure 4.7A), but not of the eubacterial 16S rRNA gene levels (Figure 4.7B). As with the previous study, JS666 declined to stasis levels on the order of magnitude of 10^4 copies/ml (or 10^5 copies/bottle), regardless of the inoculum level, which suggests that this could be the common level to which JS666 converges in environmental systems. This also lends support to the theory of microbial biostasis in natural environments. Moreover, these low levels did produce viable, active microcosms (and growth of JS666) when cDCE was subsequently administered (12), despite cell levels being at, or below detection limits.

We have determined that the method outlined here is likely to be sufficient to monitor distribution of JS666 at bioaugmented sites, and the precision is adequate to track a target whose concentration is expected to vary many orders of magnitude in application. Additionally, we found within environmental, mixed cultures, that the DNA target does not persist long in starved cells (Figure 4.6, Figure 4.7), especially within the time-scale of most remediation efforts, demonstrating that positive result from the probe is a strong indicator that degradation can occur if suitable environmental conditions are provided. Finally, absent suspected predation, the probe accurately and precisely tracks growth. Collectively, the studies appear to validate the utility of the molecular probe for site assessment in a bioaugmentation context.

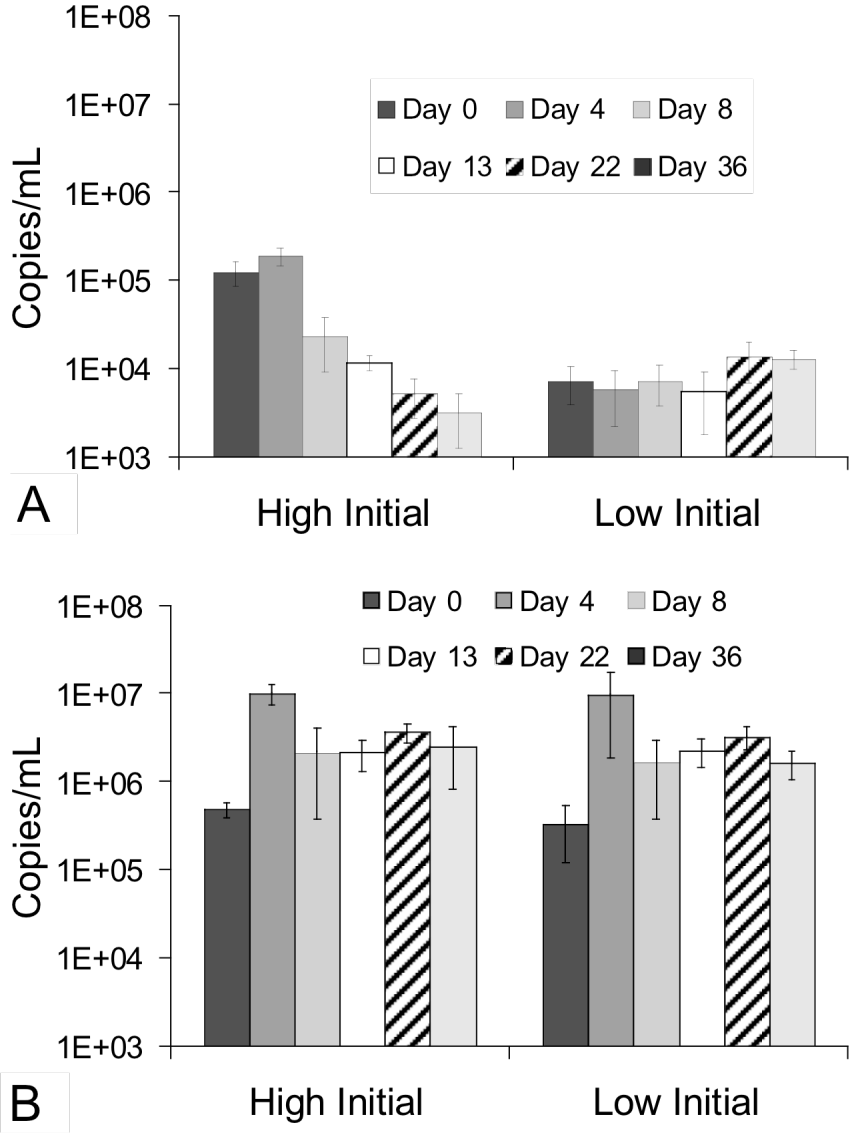

Figure 4.7: Average copies/bottle of (A) JS666-specific isocitrate lyase gene and (B) universal eubacterial 16S rRNA gene from microcosms constructed from SJCA groundwater, purged of residual cDCE. Microcosms were inoculated with JS666 at 3.5×10^{8} (High Initial) and 1.8×10^{7} (Low Initial) copies/bottle and given no external carbon source. Bottles were sampled in duplicate; error bars represent standard deviations of averaged concentrations.

4.5. Acknowledgements

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CHAPTER 5

5. DYNAMIC EXPRESSION OF PUTATIVE GENES IN THE AEROBIC, CIS-1,2-DICHLOROTHENE DEGRADATION PATHWAY OF *POLAROMONAS* SP. STRAIN JS666

5.1. Abstract

Molecular biological tools (MBTs) can measure the biological response of an organism *in situ.* When these tools are used to measure microbial activity in enhanced natural attenuation treatments, it may be possible to replace the current use of microcosms or column studies. However, in order to design effective bioindicators, fundamental knowledge about the metabolic pathways of the organism is necessary. Currently, the cDCE degradation pathway of *Polaromonas* sp. JS666 is not completely elucidated, although there are several hypotheses of parallel pathways functioning in the organism.

Here, we attempted to observe the pattern of expression of putative, cDCE-pathway degradative genes (and other genes previously found to be upregulated during cDCE degradation) under dynamic conditions. A series of batch experiments was conducted with JS666 exposed to changing conditions, and the response following starvation and change of substrate were observed. While these experiments suggest that the putative haloacid dehalogenase gene is most likely involved in degradation, and some inferences can be made about which genes are involved with oxidative stress, we found that the temporal window of response of JS666 was most likely too small to be able to make this type of approach useful.

5.2. Background

Chlorinated ethenes are frequently introduced into the environment as either tetrachloroethene [perchoroethylene (PCE)] or trichloroethene (TCE). Under anaerobic conditions, the higher-chlorinated ethenes can be biologically transformed through reductive dechlorination. This

process can be stalled at the daughter products *cis*-1,2-dichloroethene (cDCE) or vinyl chloride (VC) (3, 8, 9, 20) if there is insufficient supply of electron donor, lack of metabolic potential for complete transformation in the microbial community, and/or unsuitable geochemical conditions. This is particularly problematic as VC is a known human carcinogen with an MCL of only 2 ppb (29). The daughter products can accumulate in, or migrate to, aerobic zones where reductive dechlorination will no longer occur. cDCE and VC can be completely mineralized by many aerobic bacteria possessing non-specific monooxygenases, but these processes are often cometabolic, and so require presence of a co-substrate (e.g., methane or toluene) (3). This process is generally net energy consuming and potentially damaging to the microorganisms carrying out these reactions through production of highly reactive epoxide intermediates (1, 3). Thus, such removal mechanisms can be unreliable and difficult to sustain as bases for bioremediation (31). In cases where aerobic cometabolism has been observed, it was considered a fortuitous occurrence at the edges of plumes where the systems had become aerobic and there was still the presence of other co-contaminants serving as primary substrates (3).

Coleman et al. (5, 6) isolated the organism *Polaromonas* sp. JS666 that is able to aerobically oxidize cDCE as its sole carbon and energy source and remains the only isolate capable of this transformation. The implication is that such growth-coupled cDCE-oxidizers are not common, or at least are difficult to culture. Since JS666 apparently requires no exotic growth factors, it is considered a promising bioaugmentation agent for aerobic sites where cDCE has accumulated.

Initial work exploring the potential of JS666 as a bioaugmentation agent has been completed; namely, successful cDCE oxidation in microcosm studies constructed with various subsurface materials and inoculated with the organism (12). Additionally, a DNA-based probe used to track the presence of JS666 in the microcosms was shown to be successful within these microcosms

(11). The probe was based on a species-specific sequence of the isocitrate lyase gene, known from the genome (Genbank accession numbers CP000316-CP000318) to be present at only one copy per cell, and was designed to assess JS666 quantities in microcosms (17). While a cDCE-specific degradative gene would have been a preferred target, the cDCE-degradation pathway is yet to be elucidated. Moreover, the isocitrate-lyase gene probe cannot necessarily give any indication of activity or even the viability of JS666. DNA can remain stable and detectable for 2-3 weeks after the death of a cell (23), though in non-sterile, potentially hostile systems the persistence of DNA is lessened, and is most likely dependent upon the material into which the organism is inoculated.

Though inferences can be made from evidence presented by microcosm studies, they are time-consuming and expensive, limiting the number of samples assayed. It would be of obvious benefit to be able to use other methods to demonstrate *in situ* that JS666 is viable and active. Molecular biological tools (MBTs) that can measure potential cDCE oxidation activity *in situ* could make a case for natural attenuation and enhanced natural attenuation as treatment methods (28), replacing the current use of microcosms or column studies.

Measuring mRNA as a bioindicator is useful because changes in transcript levels can reveal regulatory response to environmental changes more quickly than other cellular indicators. Moreover, the instability of mRNA, while making it difficult to work with, means that it is a time-sensitive indicator. The possibility of detecting a false positive, meaning detecting transcripts while the cell is inactive, is decreased. There have been a number of studies designed to examine expression of functional genes by extracting RNA from subsurface materials with the hope of tying those genes to active processes, to monitor *in situ* metabolic activity (4, 13, 14). Work completed by Holmes et al. on the organism *Geobacter* in four subsurface materials

showed the feasibility of measuring transcript levels of key genes (reported relative to constitutively expressed housekeeping genes) and relating it to metabolic activity (ferric-iron reduction) (14).

However, fundamental questions remain unanswered that could inform the design of effective bioindicators for JS666 and for aerobic cDCE degradation. The objective of the work completed herein was to examine the pattern of expression of putative cDCE-pathway degradative genes (and other genes found to be upregulated during cDCE degradation) under dynamic conditions. Examining transcripts under conditions of both successful and unsuccessful degradation of cDCE – and during the process of adaptation to cDCE degradation – might provide evidence to help elucidate the primary cDCE-degradative pathway(s). This information would enhance our understanding of JS666 behavior, and could in turn lead to the development of improved MBTs for JS666. By extension, such tools might be useful in prospecting for in-situ cDCE-oxidation activity at sites not amended with JS666. These tools would be based on the degradative pathway and may better correlate with cDCE-degradation activity than does the current gene-probe.

Jennings et al. (16) used an integrated omics approach (proteomics, transcriptomics, and metabolomics) to study gene expression in JS666 (16). Microarray data comparing RNA extracted from cDCE-grown versus glycolate-grown cultures confirmed regulation of five of the proteins found through their proteomics study (Table 5.1).

Table 5.1 Upregulated transcripts determined from microarray data (17).

Locus Tag	P-Value	Fold Change	**Gene Description**
Bpro3336	0.005	111	ABC transporter, extracellular ligand-binding receptor
Bpro0645*	**0.012**	**99.8**	**glutathione S-transferase-like**
Bpro0646*	**0.012**	**87.5**	**pyridoxamine 5'-phosphate oxidase-related**
Bpro3335	0.009	70	ABC transporter, inner-membrane translocator
Bpro0530*	**0.011**	**53.3**	**haloacid dehalogenase, type II**
Bpro5186*	**0.013**	**51.5**	**haloacid dehalogenase, type II**
Bpro0531	0.015	40.9	sodium/solute symporter
Bpro5185	0.017	39.2	sodium/solute symporter
Bpro3334	0.01	30.8	ABC transporter, inner-membrane translocator
Bpro3333	0.013	27.1	ABC transporter, ATPase component
Bpro3332	0.005	18.3	ABC transporter, ATPase component
Bpro2396	0.017	14.8	heme peroxidase
Bpro5565*	**0.024**	**10.1**	**cyclohexanone monooxygenase**
Bpro5301	0.01	3.5	cytochrome P450

*identified as upregulated by cDCE in both microarray and proteomic experiments

Putative gene function was suggested using bioinformatics tools. A number of transcripts that were highly upregulated in the presence of cDCE share sequence homology with proteins that could be involved in degradation [e.g., cyclohexanone monooxygenase (CMO), haloacid dehalogenase (HAD), and glutathione S-transferase (GST)] or stress response [e.g., pyridoxamine 5'-phosphate oxidase (PNP) and GST]. Predicted protein function for Cyclohexanone Monooxygenase (CMO, Bpro5565), Haloacid Dehalogenase (HAD, Bpro0530, Bpro5186), Cytochrome P450 (P450, Bpro5301), Glutathione S-Transferase (GST, Bpro0645), and Pyridoxamine 5'-Phosphate Oxidase (PNP, Bpro0646) can be found in sections 2.6.1 through 2.6.5.

JS666 is known to produce an epoxide during oxidation of ethene (6); epoxidation is therefore one of the plausible first steps in cDCE oxidation. Epoxides are highly reactive and can be quite

damaging to cellular components. It is therefore understandable if there is an up-regulation of genes associated with oxidative stress caused by cDCE degradation. Further, cDCE, being an organic solvent, might be expected at high concentrations to compromise the membrane of JS666. This creates the possibility that some of these transcripts are upregulated as a response to solvent stress (15). These upregulated gene transcripts warrant further scrutiny to better understand how they are (or are not) correlated to up-regulation of metabolic genes.

In addition to degree of up-regulation of genes and their homology to known or putative enzymes, the physical location of genes in relation to one another and hierarchical clustering of transcripts hints at gene involvement in cDCE degradation. While bioinformatics tools are powerful, they can only generate hypotheses and the functionality of enzymes can only be confirmed by experimental methods. The omics approaches raised many hypotheses and suggested alternative pathways, but did not clearly elucidate the cDCE degradative pathway(s).

Because of the complexity involved in translation of mRNA into protein, changes in transcripts do not necessarily reflect the production of functional enzymes; meaning system perturbations that change mRNA levels do not always correspond to proportional shifts in protein product. Competition for catalytic components, such as tRNA or ribosomes, and the binding efficiency of the ribosomes can affect translation (22). Further, post-translational modifications and stability of the protein product also affect how mRNA concentrations map to protein production (22). Therefore, for the work conducted here, we chose to target the genes that were identified as upregulated by cDCE in both the microarray and proteomic experiments. However, a number of the interesting putative genes that were identified, including universal stress proteins and membrane-associated proteins, can be difficult to detect in 2D-gel electrophoresis. Genes that are thought to be involved in the cell wall, membrane, and envelope

synthesis were "statistically over-represented" in an enrichment analysis of the transcriptomic data (16).

Additionally, we targeted isocitrate lyase because of our previous work with this probe, and also cytochrome P450 (Bpro5301) because of its demonstrated importance in detoxification of compounds in various biosystems (7). Further, doctoral work completed by K. Shin suggests that P450 is responsible for the initial steps of cDCE degradation in JS666 (50). Studies with oxygen-uptake and oxygen-limitation demonstrated that JS666 only degraded cDCE in the presence of oxygen, which is a requirement of monooxygenases. Moreover, JS666 did not degrade cDCE while P450 was inhibited with metyraprone or phenylhydrazine. Additionally, recombinant *E. coli* expressing P450 along with adjacent genes were able to transform both DCA and cDCE.

While there is strong evidence that P450 is the monooxygenase responsible for the initial steps of cDCE transformation, on-going *in vivo* exploration of the cellular function of CMO suggests that this enzyme is important for cDCE degradation. Moreover, the genes that were identified as upregulated by cDCE in both the microarray and proteomic experiments may still be effective targets for biomarkers to indicate cDCE-degradation *in situ,* which warrants further exploration.

Work conducted with bioinformatics, integrated omics approaches, and enzyme assays (16, 21, 27), have lead to several hypothetical pathways to cDCE oxidation by JS666 with various initial steps (Figure 5.1).

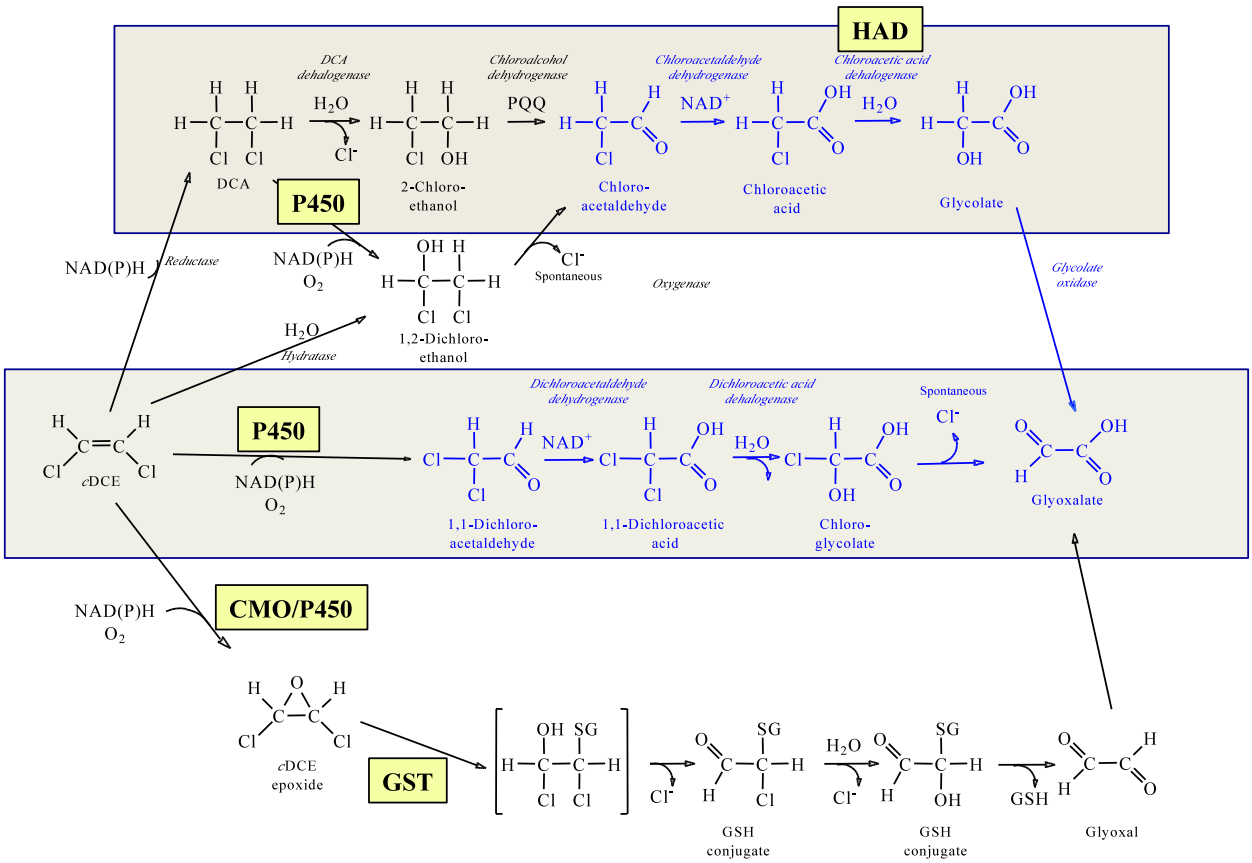

Figure 5.1: Predicted possible cDCE degradation pathways based upon bioinformatics, integrated omics approaches, and enzyme assays (27). Target genes are indicated. The reactions shown in blue have been established in cDCE-grown cells and cell extracts. Shaded pathways are supported by studies conducted by Shin (27).

5.3. Materials & Methods

5.3.1. Chemicals and Media

cDCE (>99%, stabilized with MEHQ) and 1,2-dichloroethane (DCA, 99.5%) were obtained from TCI. Ethanol (EtOH, anhydrous ethyl alcohol, 95.27%; methyl isobutyl ketone, 1.0%; ethyl acetate, 1.0%; hydrocarbon, 1.0%) and glycolic acid (gly, 70% aqueous solution) were obtained from Fisher Scientific. Minimal salts media (MSM) was used to grow JS666 as described elsewhere (12).

5.3.2. Culturing Technique

JS666 cultures were grown on neat cDCE in MSM at a pH of approximately 7.1 to 7.2, as described elsewhere (12). Pure cultures were maintained through a series of 5% v/v culture transfers into 100 ml MSM in 160-ml serum bottles and fed a nominal concentration of cDCE of 51 mg/L. [Note: concentrations of all volatiles are reported herein as "nominal" – i.e., ignoring partitioning to headspace.] Additionally, purity checks by streak-plating were routinely carried out.

5.3.3. Analytical Methods

5.3.3.1. GC.

Total quantities of cDCE, DCA, oxygen, and carbon dioxide in bottles were measured from 100-µl headspace samples by gas chromatography (Perkin-Elmer, Autosystem GC) with a flame-ionization detector (FID) and thermal conductivity detector (TCD). Samples were separated on a packed column (1% SP-1000 on 60/80 Carbopack B [Supelco]), and levels were quantified through comparison to standard curves created from known additions to replicate serum bottles containing distilled water (dH_2O). To measure oxygen, CO_2 and cDCE or DCA on one column, initially each sample was sent though the TCD, until oxygen and CO_2 had eluted, before being diverted to the FID to measure cDCE or DCA.

5.3.3.2. Nucleic Acid Extraction and Isolation.

RNA and DNA were isolated using the QIAGEN AllPrep DNA/RNA Mini Kit. Prior to cell lysis, each sample was amended with 2×10^{10} copies of luciferase control RNA (LUC, Promega) as exogenous internal reference mRNA, in order to ascertain recovery of the protocol (18). To remove DNA contamination, the RNA was treated with RNase-free DNase I (Fisher) digestion

protocol. The quality and quantity were assessed using an Agilent 2100 bioanalyzer RNA 6000 Nano assay (Agilent Technologies).

5.3.3.3. RT cDNA Synthesis.

cDNA was synthesized from 0.2 μg of RNA using an iScript cDNA synthesis kit (Bio-Rad) with random hexamer primers according to the manufacturer's instructions.

5.3.3.4. Quantitative Polymerase Chain Reaction (qPCR).

The cDNA, and therefore the number of transcripts from target genes in each sample, were quantified by real-time polymerase chain reaction (RT-PCR) with a thermocycler (iCycler Detection System, BIO RAD) with the intercalation agent iQ SYBR Green (BIO RAD), and primers specific for *Polaromonas* sp. JS666 targets and for the luciferase control). The reactions were carried out under the following conditions: 2 min at 50ºC followed by 3 min at 95ºC; next 40 cycles (denaturation at 95ºC for 15 sec, annealing and extension at 63ºC for 1 min), where fluorescence was measured after every cycle. Each reaction was performed in triplicate, and a melt curve was completed following the amplification reactions to confirm the specificity of the primers and the reactions. Primers for JS666 degradative, stress response, and house keeping genes (Table 5.2) were designed using the PrimerQuest software available at the IDT website (http://scitools.idtdna.com/Primerquest/). Primer specificity was checked by BLAST analysis. The sequence similarity of the two HAD genes was such that no primers could be designed to specifically target only one.

By enumerating luciferase as well as target transcripts, recovery efficiency and reverse transcription losses can be accounted for (18, 25). Additionally, by monitoring a housekeeping gene alongside upregulated genes, increases in the transcripts of interest can be attributed to cell activity during experimental conditions and not simply an increase in cell numbers. It is difficult

Table 5.2: Gene targets and primer sets

Locus Tag	Gene Description	Primers (F/R)
Bpro2101	isocitrate lyase (ISO)	5'TGCCGCTGACAACAACAC/ 5'ATCAATGCCTTTGGAGTGC
Bpro0645	glutathione S-transferase-like (GST)	5'CAAGCTTTACCGTGTCGCCATTTC/ 5'CAGGTCAATCTCCACCCGTTCAAA
Bpro5565	cyclohexanone monooxygenase (CMO)	5'ATTGTCAAAGACCCGGAAACTGCC/ 5'TAAATGGCGTAGTAGCCGCTGTCA
Bpro0646	pyridoxamine 5'-phosphate oxidase-related (PNP)	5'GTGCCGGATTTCATGGGCAACTTT/ 5'CAGATAGAGCAGGTCGCCATTGTCA
Bpro_0530/ Bpro_5186	Haloacid dehalogenase, type II (HAD)	5'GTTGACGAAGTGCGGCTGTTCAAA/ 5'TCTGATTCACCCAAGTGCAGGGTA
Bpro5301	cytochrome P450 (P450)	5'AGGACAGCTTGTTTGGTCCGTACT/ 5'ATCCATCGCAATGAACATCGGCAG
Bpro4442	beta subunit of RNA polymerase (RpoB)	5'TTGTGGAAGCCGATGCATTTGACC/ 5'ATCGCGTTCTTGATGCTTTCCAGC
	exogenous internal reference luciferase (LUC)	5'TACAACACCCCA ACATCTTCGA/ 5'GGAAGTTCACCGGCGTCAT

to know *a priori* which, if any, gene will be stably expressed (26), and so two possible housekeeping genes were chosen. These were isocitrate lyase (ISO) and the beta-subunit of RNA polymerase (RpoB). However, upon further investigation we found the RpoB expression levels to be difficult to quantify precisely and too variable for this purpose. For these reasons, ISO was chosen as the housekeeping gene.

5.3.3.5. Expression Data Analysis (DART).

To damp-out errors associated with plate-to-plate variation in standard curves, fluorescence data generated by the iCycler was analyzed using the DART-PCR technique as outlined and developed by Pierson et al. (24) and described previously (11).

5.3.4. Experimental Conditions.

All batch reactors were prepared aseptically in autoclaved 1-L serum bottles and contained 500 ml of MSM. Amendments were administered and samples taken via sterile syringe through ethanol-swabbed, flamed septa. All experiments were conducted at 22ºC in the dark, agitated at 60 RPM. Cultures were sampled for both RNA and DNA by withdrawing 10 ml of liquid by

sterile disposable syringe. The samples were centrifuged at 10,000*g* for 10 min at 4°C, and most of the supernatant discarded. They were then centrifuged again at 21,000*g* for an additional 5 min. The cell pellets were stored at -80°C until extraction unless otherwise noted. Because such large culture volumes were removed for RNA isolation, significant cDCE and DCA were also removed during the sampling. To account for such substrate losses that were not due to biodegradation, we present the GC-plots of degraded cDCE or DCA as the sum of incremental masses degraded, each divided by applicable liquid volume remaining during the incremental period (sum[-ΔMdeg/V]) vs. time. The values are negative so that the degradation line trends down. Note that without such a reporting method, remaining mass of cDCE or DCA would decline due to sampling losses alone, making it difficult to detect intervals during which no degradation occurred.

5.3.4.1. RNA Extraction Reproducibility and Precision.

As RNA extraction and reverse transcription can be a significant source of imprecision (10, 18), the reproducibility of RNA extraction and comparison of freeze-thaw at various points during the extraction and reverse transcription, were examined. RNA extractions were performed in quadruplicate on a pure JS666 culture that was actively degrading cDCE. Cell pellets were either frozen or further processed according to Table 5.3, for a total of four treatments (A-D). We found that RNA was degraded beyond quantification when it was not frozen before RNA isolation (treatments A & B).

This is most likely due to how unstable RNA is at ambient temperatures. It was found that freeze-thaw was less detrimental to the samples than letting them stay on ice between process steps, most likely due to the time required for the set-up of each step. There was no significant

Table 5.3: RNA processing methods. Check marks indicate when in the RNA isolation and reverse transcription process samples were frozen at -80°C (RNA) or -20°C (cDNA). Dash marks indicate processing without freezing.

Treatment	Cell Pellet Frozen	RNA Frozen	cDNA Frozen
A	-	-	✓
B	-	✓	✓
C	✓	-	✓
D	✓	✓	✓

difference between cDNA synthesized from RNA that had been frozen or not (treatments C & D). To be efficient in sample processing, it was desirable to accumulate samples for nucleic acid isolation and then reverse transcription. Therefore, treatment D was chosen for all subsequent samples. The total copies of ISO recovered (accounting for the exogenous internal reference LUC recovery) had a coefficient of variation of 28.5%. The relative expression of GST/ISO had a CV of 44.7%.

5.3.4.2. Dynamic Expression Studies.

To monitor highly upregulated transcripts such as those possibly associated with cDCE degradation and/or stress, batch experiments were conducted with JS666 exposed to changing conditions. The JS666 culture was either grown up on cDCE and subjected to starvation before re-exposure to cDCE or grown on the alternate substrate glycolate before being exposed to cDCE, DCA, or EtOH (Figure 5.2). Additionally, the second set of batch experiments included a glycolate-fed control culture. Both starvation and growth on glycolate have been demonstrated to create an extended lag prior to subsequent cDCE degradation.

5.3.4.2.1. Response Following Starvation

The hypothesis of this experiment was that the lag created by cDCE-starvation was correlated to the down-regulation of critical genes associated with cDCE-degradation. Monitoring transcript levels before starvation and throughout the lag period before resumption of cDCE

degradation would reveal genes whose up-regulation is coincident with the onset of cDCE degradation, and therefore associated with the determinative (presumably initial) steps of the metabolic pathway.

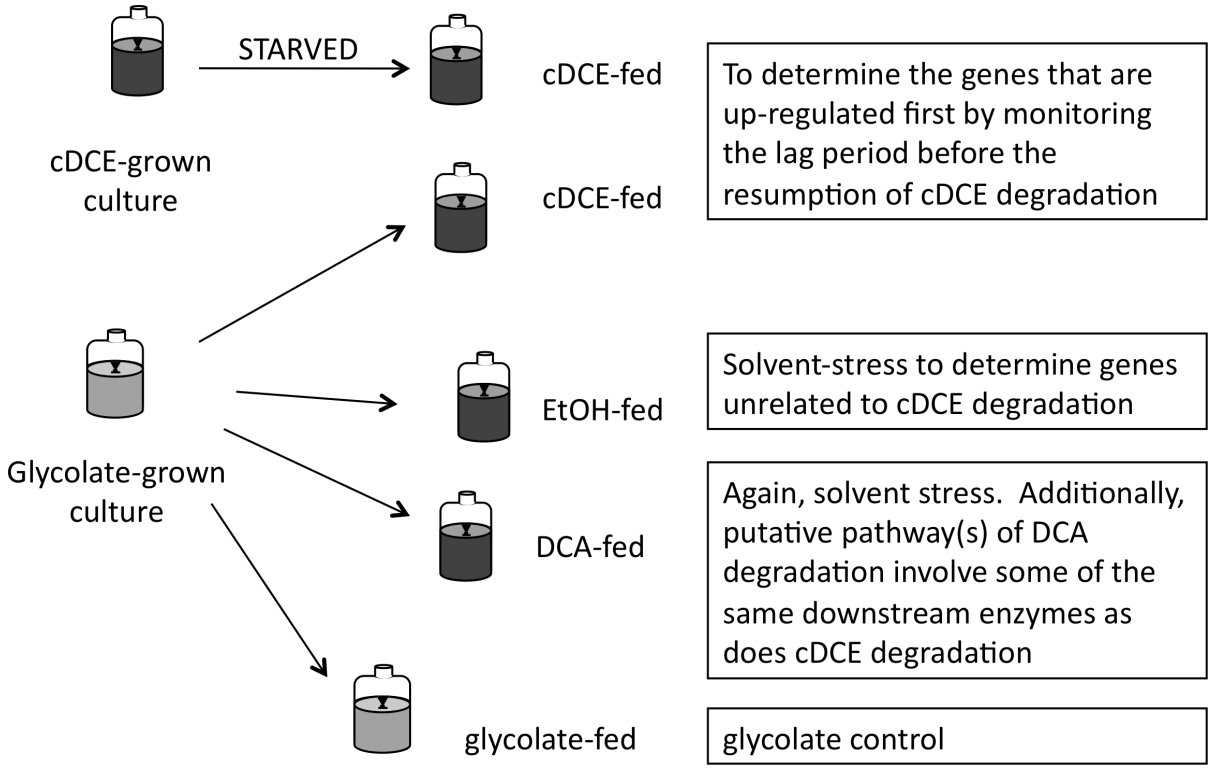

Figure 5.2: Experimental set-up for dynamic expression studies. The first study used JS666 grown-up on cDCE, which was starved before subsequent cDCE spikes. The second set of studies used JS666 that was grown on glycolate before being washed and resuspended in media containing DCA, EtOH, cDCE or glycolate.

The cDCE-starved cultures were prepared in duplicate and simply allowed to consume all the cDCE present (two spikes of 20 μl neat cDCE or approximately 100 mg/L total), and no additional cDCE was administered for approximately 72 hours, after which a third spike of cDCE was delivered. The cultures were sampled in triplicate for RNA before starvation, during starvation, during the lag prior to degradation, and as degradation commenced. Transcript

expression is reported as total copies/ml (accounting for luciferase internal reference recovery) and also normalized to the housekeeping gene ISO to account for cell activity.

5.3.4.2.2. Response Following Change of Substrate

When JS666 is grown on glycolate, it does not immediately degrade cDCE. It would seem that some of the genes required for cDCE degradation are down-regulated in the absence of cDCE and in the presence of glycolate. By switching these "down-regulated" cultures to various substrates, we hoped to observe which genes are associated with the critical steps of cDCE degradation and which are more general indicators of activity or are possibly stress-related. For this purpose, we selected cDCE, EtOH, and DCA as substrates provided to cultures immediately following growth on glycolate. All are "solvents," and might evidence up-regulation of genes unrelated to cDCE degradation. Though growth on EtOH has been shown to reduce lag to subsequent cDCE degradation, it does not eliminate lag. The putative pathway(s) of DCA degradation involve some of the same downstream enzymes as does cDCE degradation (Figure 5.1) (21).

A total of 1500 ml of JS666 culture was grown to high density on 10 mM of glycolate. The pH was adjusted to approximately 7.1 to 7.2 with 5N NaOH. After two days, another 1.35 ml of glycolic acid was administered and pH adjusted. The cultures were also aseptically amended with pure oxygen as needed. The culture was washed and resuspended in duplicate in MSM and given 260 μM DCA (25 mg/L), 3 mM EtOH, 520 μM cDCE (50 mg/L), or 10 mM glycolate. The cultures were streak-plated to confirm culture purity after any handling that could have introduced contamination. Each batch culture was sampled in triplicate for RNA approximately 12 hours post inoculation and as they degraded the substrates. The glycolate controls were sampled for RNA approximately 16 hours post-inoculation. The mean transcript level of each

target gene in the controls was normalized to the mean transcript level of the housekeeping gene ISO for that sample. Then for each experimental-treatment sample, its measured transcripts were normalized to ISO transcripts in that individual sample, and then reported relative to the ISO-normalized transcript values of the glycolate control. The standard deviation, σ, of a ratio of two averages of samples, $\left(\frac{\mu_Y}{\mu_X}\right)$, each with their own standard deviations, σ_Y and σ_X, is found by using the Taylor Expansion on a non-linear function of independent random variables (2). The general equation is:

$$Var[Y] = \sum_{i=1}^{n} \sigma_{X_i} \left(\frac{\partial g}{\partial X_i}\right)^2 \qquad \text{Equation 5.1}$$

where $Y = g(X_1, X_2 ... X_n)$ (2). Here $Y = g(\mu_X, \mu_Y) = \frac{\mu_Y}{\mu_X}$, which causes Equation 5.1 to reduce to the following:

$$\sigma = \left(\frac{\mu_Y}{\mu_X}\right) \times \left(\sqrt{\left(\frac{\sigma_Y}{\mu_Y}\right)^2 + \left(\frac{\sigma_X}{\mu_X}\right)^2}\right) \qquad \text{Equation 5.2}$$

Here, μ_x and μ_y represent the target genes normalized to the housekeeping gene ISO for each sample from the glycolate controls and the experimental treatments, respectively.

5.4. Results & Discussion

Experiments were conducted in an attempt to understand how the expression of selected genes in JS666 will vary with successful or unsuccessful degradation of cDCE (i.e., during adaptation following starvation or growth on non-cDCE substrate).

5.4.1. Response Following Starvation

JS666 cultures suffer a long cDCE degradation lag following a brief starvation period (Figure 5.3). It was hoped that in recovery from starvation, one or more of the gene transcripts that had been previously found to be upregulated when JS666 is growing on cDCE would coincide with the observed onset of cDCE degradation. This would be strong indication that this gene is most likely critical to degradation. However, this is not what was observed (Figure 5.4). During the starvation of the culture, there was a sharp drop in all transcript levels, no doubt reflecting generally low activity. As degradation resumed, all the targeted transcripts came up simultaneously, with the possible exception of GST and P450, which may have been upregulated later. Most likely the window of time for the up-regulation of critical gene(s) in this suite was too short relative to sampling intervals.

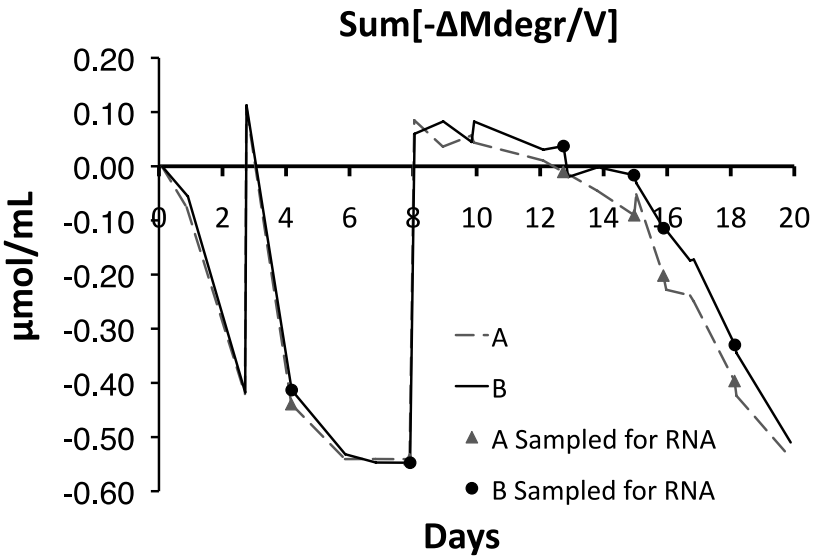

Figure 5.3: cDCE degradation in biological replicates subjected to starvation at day 6, reported as sum of mass degraded per remaining ml. Sample points indicate RNA extraction.

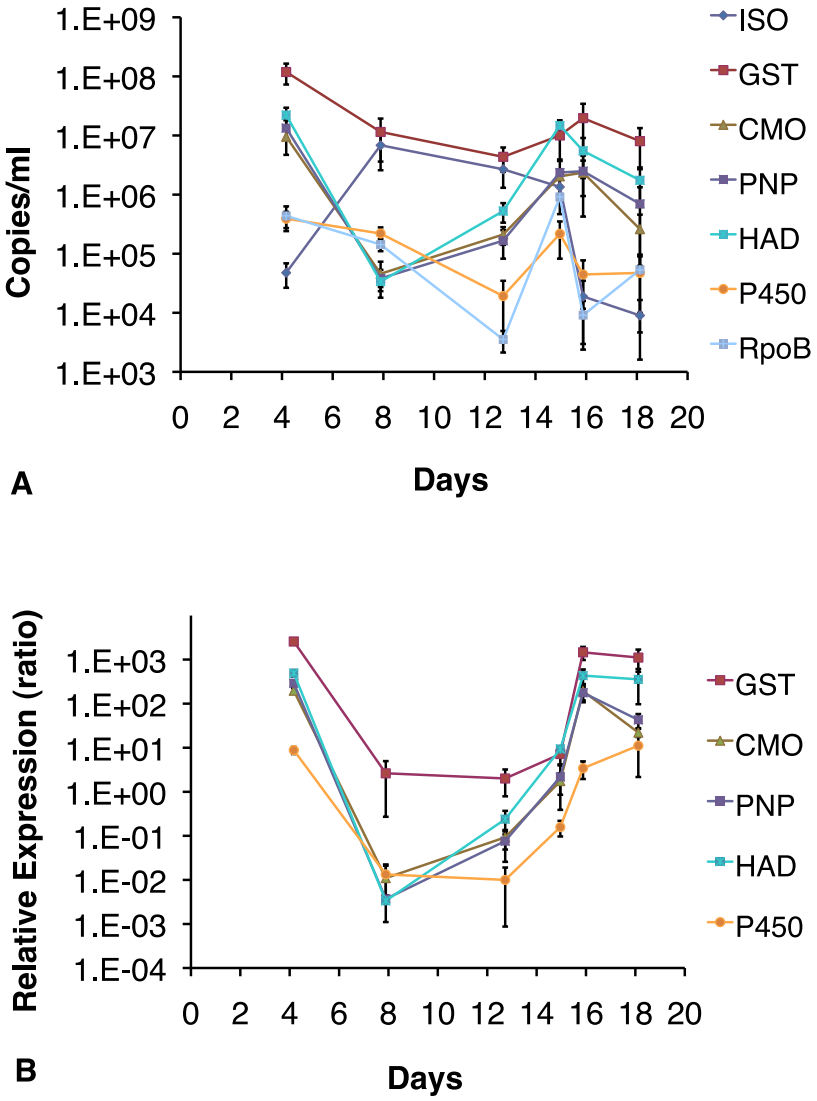

Figure 5.4: Gene expression profile for cDCE-starved cultures reported as A) total expression (copies/ml) accounting for internal reference RNA (luciferase RNA), B) relative gene expression normalized to ISO mRNA. Error bars indicate the standard deviation of triplicate extractions.

5.4.2. Response Following Change of Substrate

5.4.2.1. cDCE.

When the culture grown on glycolate was switched to cDCE, the expected lag before degradation was observed. The two biological replicate cultures did not closely track each other; thus results for each are plotted (Figure 5.5). We postulated that one or more of the gene transcripts that had been previously identified as being upregulated on cDCE would rise coincident with onset of cDCE degradation.

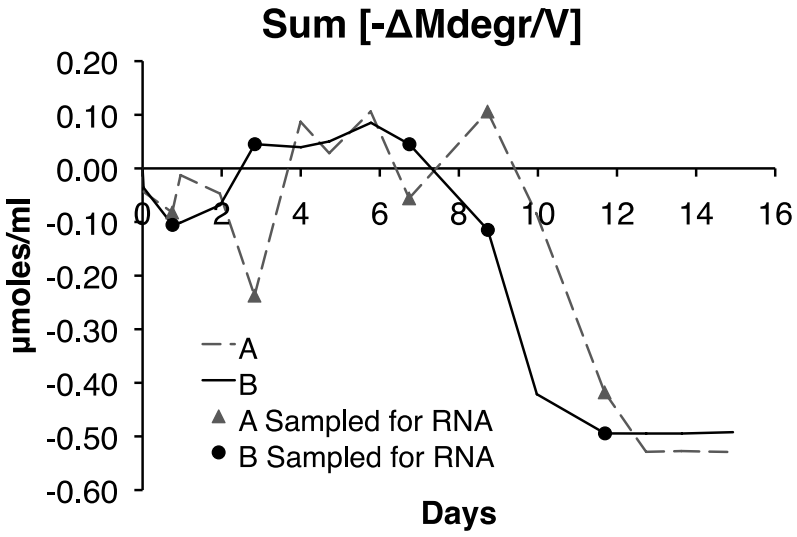

Figure 5.5: cDCE degradation in biological replicates inoculated with culture grown on glycolate, reported as mass degraded per remaining ml. Sample points indicate RNA extraction.

We observed that GST and HAD were upregulated first (Figure 5.6). Next, the CMO and PNP came up, which was right around the onset of cDCE degradation. Bottle B showed a sharp decrease in the relative expression of HAD at the final sampling point. A possible explanation is that bottle B depleted the cDCE present before bottle A, which would suggest that HAD transcripts are down-regulated quickly after cDCE depletion.

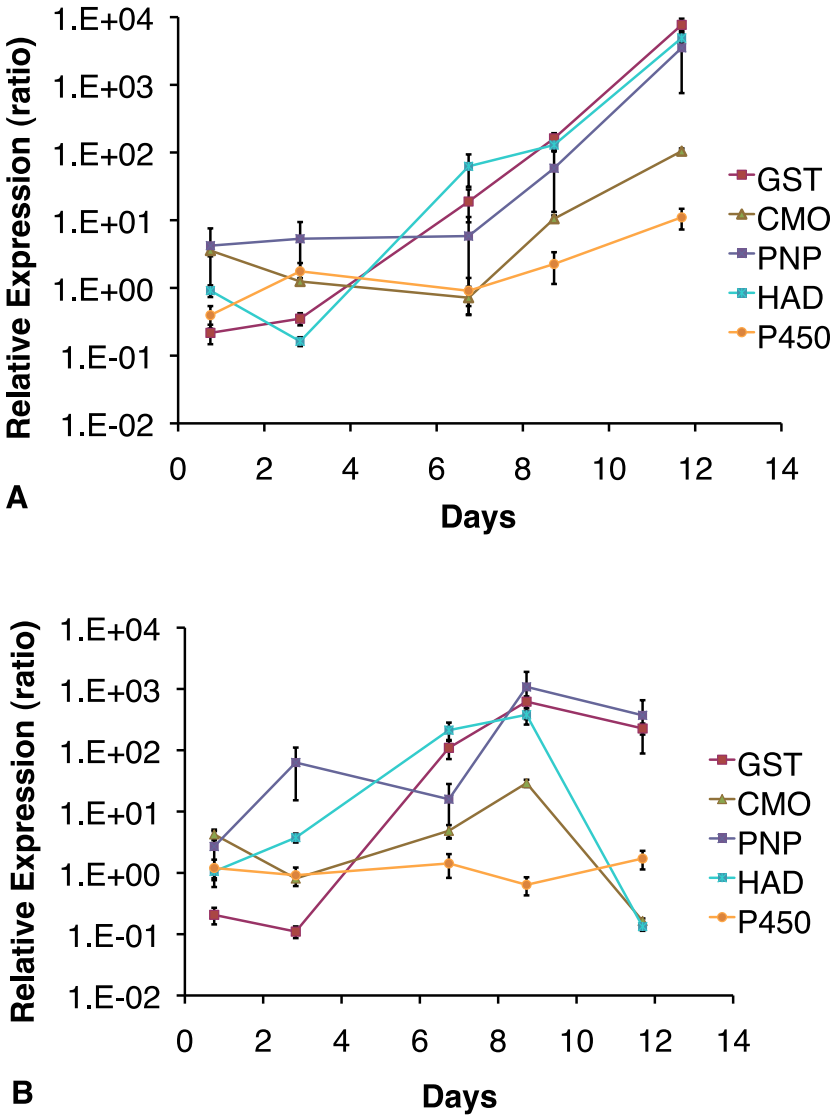

Figure 5.6: Relative gene expression of the target transcripts, normalized to the housekeeping gene, ISO, reported relative to the glycolate control, for replicates A and B. The final data point for CMO in bottle B is not available. The cultures were initially grown on glycolate before being washed and resuspended in media containing cDCE. Error bars indicate the standard deviation of triplicate extractions.

5.4.2.2. EtOH.

JS666 is known to be able to utilize ethanol as a substrate; moreover ethanol generally shortens the lag leading to cDCE degradation after re-exposure to cDCE (17). We did not measure ethanol directly, but measured both oxygen and carbon dioxide as surrogates for ethanol depletion. As ethanol was oxidized, there was an increase in carbon dioxide and a decrease in oxygen (Figure 5.7).

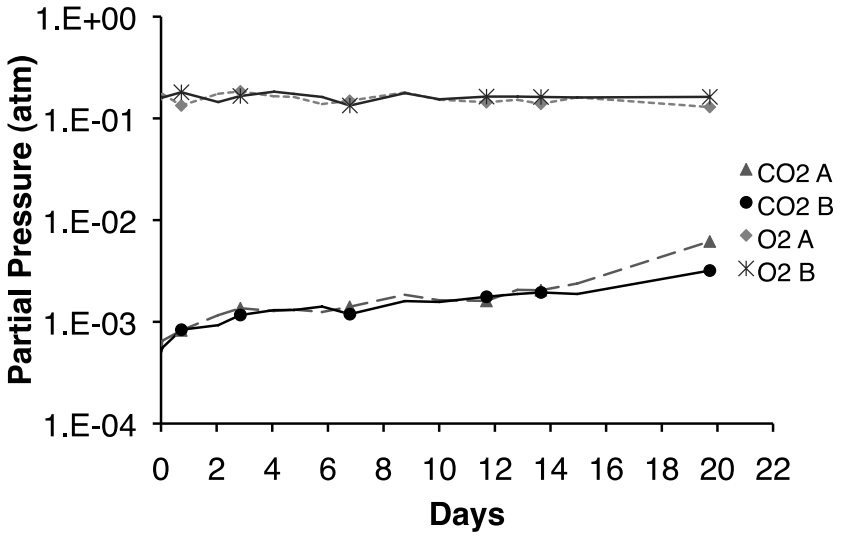

Figure 5.7: EtOH-degradation in biological replicates inoculated with culture grown on glycolate, using oxygen and carbon dioxide as surrogate measurements. Sample points indicate RNA extraction.

The corresponding transcript expression showed a relative increase of GST almost immediately (Figure 5.8), however degradation was delayed, suggesting that the GST may be involved in solvent or stress protection rather than degradation. The CMO and PNP transcripts both showed a relative increase that appears to correspond with onset of ethanol oxidation. This could indicate that the CMO is what was associated with degradation in the case of ethanol, and PNP is most likely involved with alleviating the stress associated with the oxidation of the added

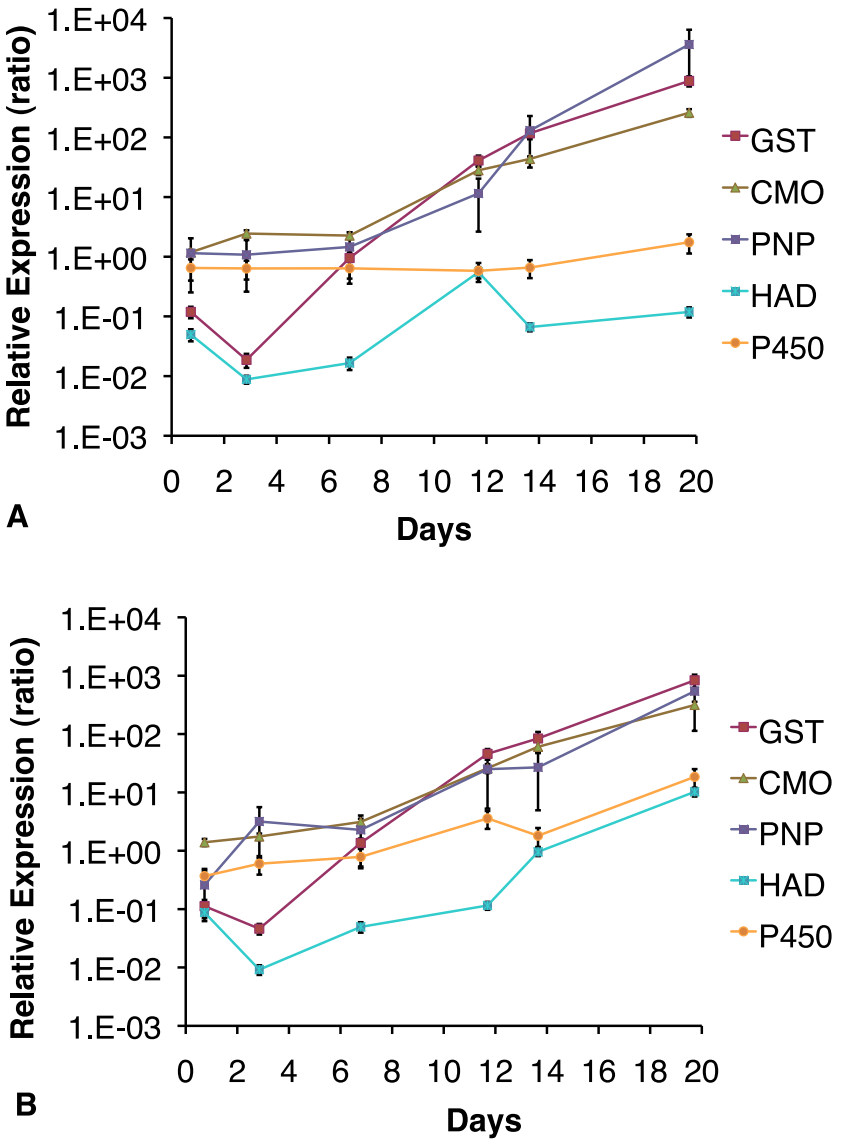

Figure 5.8: Relative gene expression of the target, normalized to the housekeeping gene, ISO, reported relative to the glycolate control, for replicate A and B. The cultures were initially grown on glycolate before being washed and resuspended in media containing EtOH. Error bars indicate the standard deviation of triplicate extractions.

ethanol mixture. It is also possible that CMO is being co-upregulated with a nearby putative alcohol dehydrogenase downstream from the CMO (27). This could help to explain how ethanol does not suppress JS666's ability to degrade cDCE. It's possible that CMO is involved with the initial steps of cDCE degradation, however these data are complicated by the impurity of the ethanol used in this study. While Beyer-Villiger monooxygenation reactions are defined for the oxygenation of ketones (32), and JS666 can grow on hydrocarbons (21), the additives to the ethanol used are toxic and most likely caused the lag in degradation observed here. The additives also most likely contributed to the stress response observed prior to ethanol degradation.

5.4.2.3. DCA.

According to the bioinformatic study conducted by Mattes et al. JS666 possesses a set of genes that are homologous to those known to encode DCA transformation in *Xanthobacter autotrophicus* (21). Presumably, the initial oxidation of cDCE would feed into this pathway. Additionally, JS666 has demonstrated sustained degradation of DCA (16). By giving JS666 DCA as a substrate, after having down-regulated the cDCE degradation pathway by growing the culture on glycolate, we hoped to understand which genes were associated with the later steps in cDCE degradation. However, our culture did not degrade DCA (Figure 5.9), therefore we decided to concentrate on the expression profiles of the first few days of DCA exposure in an attempt to capture the initial reaction of JS666 to this new substrate.

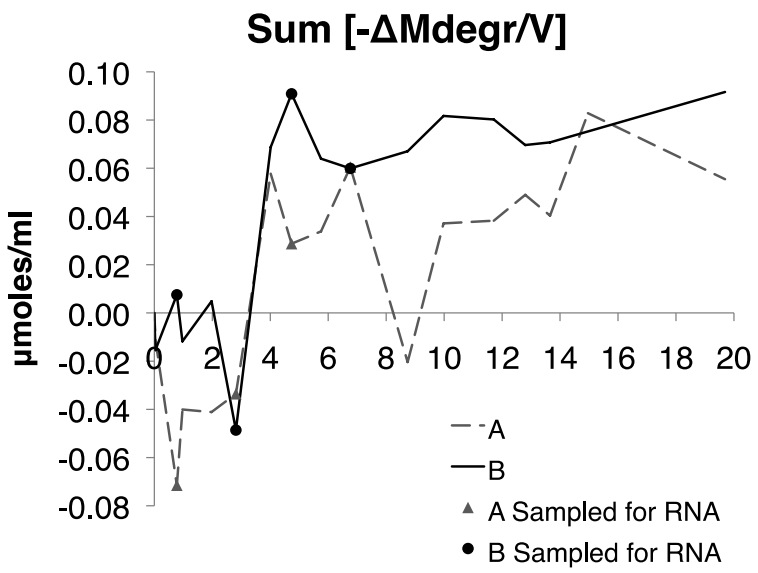

Figure 5.9: DCA-degradation in biological replicates inoculated with culture grown on glycolate. Sample points indicate RNA extraction.

Moreover, midway through GC-sampling of DCA, there was change in the instrument response, adding to the erratic DCA-degradation profile. However, it is obvious that there was little to no DCA degradation, which can be seen clearly in the later GC-sampling (Figure 5.9). In the days following the culture's exposure to DCA, the relative expression of PNP and GST rose, and the relative expression of CMO, HAD and P450 all fell (Figure 5.10).

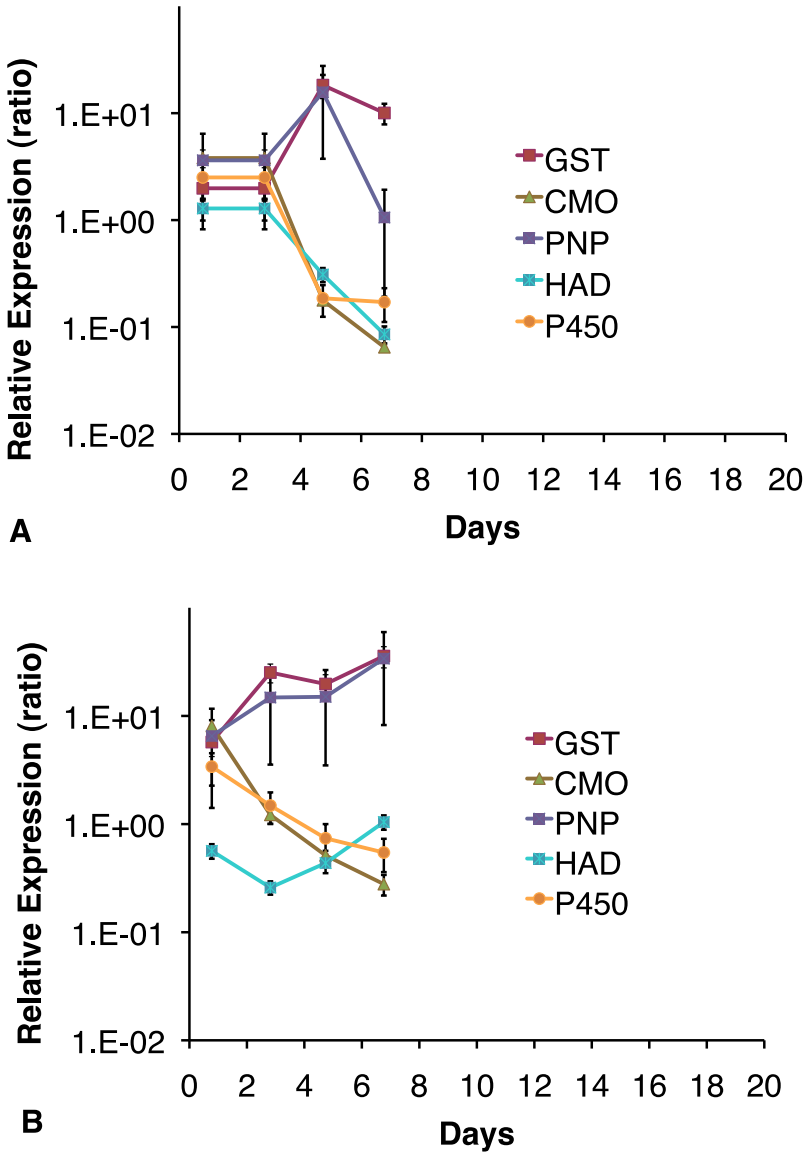

Figure 5.10: Relative gene expression of the target, normalized to the housekeeping gene, ISO, reported relative to the glycolate control, for replicate A and B. The cultures were initially grown on glycolate before being washed and resuspended in media containing DCA. Error bars indicate the standard deviation of triplicate extractions.

5.4.3. Summary Conclusions of Gly-fed Experiments

Taken together, these studies seem to suggest that HAD is involved in cDCE degradation because it was upregulated with cDCE degradation, and down-regulated with the lack of DCA degradation. This is consistent with what Shin concluded from cell-free extract studies, which is that cDCE degradation occurs through HAD catalyzed degradation of dichloroacetic acid (27), While this gene up-regulation is correlated with cDCE, it is not the first step in the process. However, since HAD seems to be down-regulated quickly after cDCE ceases, this may be a good gene for an MBT to look for JS666 degradation activity, if a species-specific HAD sequence is used as basis for the probe.

GST and PNP seem to be upregulated to alleviate solvent and/or oxidative stress, as rise in their transcription occurred under circumstances of exposure to – but not degradation of – substrates such as EtOH and DCA. GST was upregulated almost immediately in all treatments when there was no associated transformation. However, there is no way to rule out the role of GST in dechlorination, as GST could be involved in epoxide transformation or to relieve oxidative stress (16). PNP is an enzyme primarily associated with oxidative stress relief, and as it was upregulated with degradation, it is most likely associated with relieving stress due to oxidation. Finally, while not determinative, these results neither implicate nor rule out an important role for CMO. Moreover, because P450 has such a low degree of up-regulation under any circumstances, it is difficult to draw any conclusions about its possible role.

Other dynamic conditions to explore could include cultures that are initially grown on cDCE before being switched to DCA or dichloroacetaldehyde (an intermediate compound). Instead of looking for the up-regulation of genes known to be involved with cDCE degradation, what is down-regulated could inform which genes are involved with the primary steps of degradation.

Additionally, the glycolate-fed cultures switched to cDCE, DCA and EtOH studies could be repeated with a different suite of transcripts to look specifically at genes associated with the cell membrane. This would further differentiate genes that are associated with solvent effects over cDCE degradation identified previously with transcriptomics. However, this technique, which involves sampling over the time period prior to the onset of degradation and during degradation, presumably misses the temporal window of interest that is likely too small to make this approach useful. The amount of sampling required to accurately capture transcriptional response to dynamic conditions would be unfeasible due to long and unpredictable lags prior to degradation.

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CHAPTER 6

6. CONCLUSIONS

6.1. Summary

There are a significant number of plumes at chloroethene-contaminated sites where PCE and TCE have been dechlorinated to cDCE, but where the cDCE persists. Here, aerobic bioremediation of cDCE may be more cost-effective than trying to create anaerobic conditions for dehalogenation. Further, by employing an organism that can use cDCE as a sole carbon and energy source, the addition of an external cometabolite such as methane or toluene is not required. Collectively, the experimental studies contained herein were designed to aid in the development of JS666 as a bioaugmentation agent at sites where cDCE has accumulated in aerobic zones

In summary:

(i) We demonstrated that JS666 is able to survive in a variety of subsurface materials and maintain cDCE degradation.

(ii) We developed a DNA-based probe that is able to track JS666 in the subsurface and, when used in conjunction with quantitative PCR, can correlate the abundance of JS666 with variations in microcosm performance.

Both of these outcomes are imperative for advancing the application of JS666 as a bioaugmentation agent.

(iii) Finally, while some inferences can be made regarding the roles of various genes previously shown to be upregulated by cDCE, the time-window of JS666's dynamic response was too narrow, and its time-position too unpredictable, for our technique to be useful. Nevertheless, this work has helped further our understanding of degradation pathway(s) used by

JS666, which will ultimately aid in creating more robust MBTs and improve of JS666-bioaugmentation techniques.

6.1.1. Microcosm Assessment

The first study conducted, as outlined in Chapter 3, explored *Polaromonas* sp. JS666 as a bioaugmentation agent for cDCE degradation. Subsurface sediments or groundwaters from six aerobic, cDCE-contaminated sites were used to construct various microcosms, for a total of 29 types of treatments. These were designed to study the survivability of JS666 in the subsurface, as well as the effects of inoculum level, cDCE concentration, micronutrients, such as nitrogen and phosphate, and metals requirements, such as magnesium, zinc, and iron, the presence of primary effluent as a source of competing and predatory microorganisms and alternative substrates. This evaluation was an essential first step to determining the efficacy of this organism for bioaugmentation.

JS666 was able to degrade cDCE in every sediment or groundwater into which it was inoculated, provided that the pH remained circum-neutral. As inoculum level decreased, it took progressively longer for cDCE degradation to become noticeable, however the rates ultimately achieved with each inoculum level were similar. In the microcosms that included primary effluent, an alternate carbon source and/or competitive or predatory microorganisms, there also was a measure of success.

6.1.2. DNA Probe

In order to track JS666 once it is introduced into the environments, a DNA-probe specific to the organism was developed, as summarized in Chapter 4. First, a probe based on the isocitrate lyase gene, was developed for in soil and groundwater. Once the probe was determined to be specific to *Polaromonas* sp. JS666, DNA extraction efficiency and reproducibility were

determined. The probe was used in conjunction with quantitative PCR to track the abundance of JS666 in microcosms.

Though a DNA-based probe doesn't necessarily indicate *in situ* activity, we showed a strong correlation of increased DNA copy numbers (indicating cell growth in the absence of predation) to degradation of cDCE. Moreover, we demonstrated that, unlike in pure culture, the DNA-target is not persistent in subsurface material after cell death. This suggests that a positive result from the probe is a strong indicator that degradation can occur in suitable environmental conditions. Currently, DNA-based probes are accepted as evaluative tools for site assessment, and these studies seem to validate the use of this probe to monitor distribution of JS666 at bioaugmented sites.

We have determined that the method outlined in Chapter 4 is likely to be sufficient to monitor distribution of JS666 at bioaugmented sites. The precision of the probe is adequate to track a target whose concentration is expected to vary many orders of magnitude in application.

6.1.3. Dynamic Expression Studies

MBTs that can measure activity of an organism *in situ* may be able to replace the current use of microcosms or column studies. To be able to design an effective MBT, the metabolic pathway of an organism needs to be understood. However, the pathway by which JS666 degrades cDCE has not yet been elucidated.

Chapter 5 described the experiments that were conducted to better understand how the expression of selected genes known to be upregulated by cDCE in JS666 varied with successful or unsuccessful degradation of cDCE. Here, we attempted to observe the pattern of expression of these genes under dynamic conditions. Through this, we hoped to learn which genes were

associated with degradation and/or solvent toxicity, and to understand temporal variation of gene transcripts.

Collectively, the studies described in Chapter 5 suggest that the haloacid dehalogenase (HAD) gene is involved in cDCE degradation, which is consistent with cell-free extract studies conducted by Shin (9). However, HAD most likely does not catalyze the first step in cDCE degradation. While some suppositions can be made about genes involved with relieving oxidative stress, it is difficult to draw any definitive conclusions. The response of JS666 to changing conditions is unpredictable. Microcosms would need to be sampled too frequently to make this approach useful in teasing out temporal expression of transcripts.

6.2. Future Work

Because the materials employed in the microcosms constructed for the survivability studies were undefined and heterogeneous, it was difficult to understand why occasionally some microcosms would fail, as indicated by stalled cDCE degradation. Here, we only measured oxygen and pH as indicators of microcosm environment, both of which are important to JS666 function and viability. Further possible reasons for microcosm failure include lack of proper macronutrients or micronutrients; predation or competition from other organisms; or ineffective regulation of metabolic pathways in JS666 – none of which was monitored. While the objective of these studies was to determine if JS666 could survive and degrade cDCE in the environment, further studies are needed to better understand how to keep JS666 degrading cDCE indefinitely once it is introduced into the subsurface.

One of the biggest hurdles to employing an MBT is recovering enough material (target organism cells) from dilute environmental samples to apply the probe accurately. This can mean larger samples of soil or groundwater could be required. With groundwater, the sample can be

concentrated by filtering large amounts of water and extracting DNA off the filter. However, we do not know how JS666 partitions between soil and water, and this will likely vary for different types of soil. Additional experiments are required to account for partitioning and concentrations of the organisms on a site-to-site basis.

Because dynamic conditions are difficult to measure, future work examining transcript regulation could focus on comparing expression levels of the same suite of putative genes of JS666 actively growing in a variety of conditions. These would include growth on intermediate compounds, such as dichloroacetaldehyde, under limited macronutrient or micronutrient growth conditions, substrates that are known to promote the cometabolic-like phenotype, or with the addition of non-degradable solvents. Transcripts associated with growth on intermediate compounds could inform which genes are involved with the primary steps of degradation by comparing this profile to both cDCE-grown cells and cells grown on an a substrate such as glycolate that does not induce cDCE-degradation. Transcripts associated with various growth conditions could inform the role of various putative genes in the metabolic pathway of JS666. Finally, transcripts associated with solvents would inform which genes were upregulated solely to lessen solvent stress.

Additionally, transcript expression of genes that are membrane bound that were identified as being upregulated could be explored. In this study, we chose to target the genes that were identified as upregulated by cDCE in both the microarray and confirmed by proteomic experiments. However, membrane-associated proteins can be difficult to detect in 2D-gel electrophoresis. It is possible that one of these transcripts would make an effective bioindicator of cDCE degradation by JS666.

Microarrays are excellent at identifying differences in gene expression under various test conditions, but they are less accurate at quantifying the difference in relative expression. Microcosm studies using reverse transcript quantitative PCR would be more accurate for this purpose. Knowing this more accurately could give better understanding of how JS666 regulates the genes pertinent to cDCE degradation and may even help identify a better gene to use as a control for reporting relative gene expression under various test conditions.

Understanding the gene transcripts that are most directly associated with active degradation allows for the development of a molecular biological tool that can assay activity *in situ* – and possibly to prospect for other cDCE-oxidizers if they share similar genes and pathways. Additionally, this understanding might help elucidate the degradation pathway(s) used by JS666 and illuminate physiological characteristics that could suggest useful strategies for application of JS666-bioaugmentation technologies in the field. RNA-bioindicators based upon genes explored here and correlated to cDCE-oxidation, have the potential show metabolic activity occurring *in situ*, which offers compelling evidence of successful bioremediation. This could lead to better application of these organisms to environmental settings and to better stimulate, and manipulate environments to ensure successful remediation (1, 8).

While upregulated transcripts and expression patterns can point the way to a gene of interest, without further research into the proteins that are being expressed under these conditions, there is no way to know if these upregulated transcripts are even being translated, much less what their actual functions are. To understand what is occurring metabolically within this organism, further work examining proteins and their function would be required.

There have been a number of studies that have begun this work in JS666. Two protein expression analyses have been conducted in conjunction with transcription expression of genes

upregulated by cDCE versus non-inducing substrates (1, 5). Further, heterologous gene expression in *E. coli* has been completed for CMO, GST and P450 (1, 11). Additionally, inhibition studies and cell-free enzyme assays were conducted by Shin to confirm that P450 is responsible for the first steps of cDCE transformation in JS666 (11). Finally, on-going work involving *cmo*-negative strains of JS666 are being conducted to evaluate the role of CMO in the degradation of cDCE by the organism (12).

However, there remains a number of potential studies that could be conducted on other proteins that have been found to be expressed in response to cDCE, such as HAD, GST, PNP and the ABC transporters, as well as any proteins that are associated with the membrane of JS666 that have yet to be identified. Additionally, there are some interesting genes that have been co-regulated with CMO, including a putative epoxide hydrolase (Bpro_5566) (1) and putative alcohol dehydrogenase (Bpro_5304) that is potentially misannotated as it has not been shown to transform ethanol (12). The conflicting studies regarding the role of CMO in cDCE degradation by JS666 opens the way for studies on enzymes flanking CMO, as they may be the true genes of interest. Future studies could be conducted much in the same ways that previous studies have been performed. Heterologous gene expression, knockout studies and/or enzyme assays could help elucidate the function of the putative epoxide hydrolase or alcohol dehydrogenase in JS666.

Furthermore, purified proteins of interest can be used to test the affinity to cDCE, or other intermediate compounds in the transformation pathway, and through this, understand enzymatic function of the protein (7). Co-eluted proteins can give some indication of protein-protein interaction and are often functionally related (7). In fact, protein complexes often perform a novel function, rather than a combination of the original functions (3).

Additionally, X-ray diffraction and/or nuclear magnetic resonance are two techniques that can be used to determine the three-dimensional structure of the purified proteins. Structural motifs offer clues to the function of proteins and can be compared to a growing database of structures (7). To complement the growth in both proteomic and genomic databases, a number of analytical techniques have been developed to help process this data into usable and insightful information, where it is even possible to predict structure and dynamic function of proteins *in silico* and understand their interactions (6, 13). However, the outcome of such simulations is improved in combination with protein function assays so that the model can be modified to reflect empirical data (6).

The potential discovery of another organism that is able to aerobically use cDCE as a substrate would open the doors to comparative genomic and proteomic studies if an isolate were identified (9). Such a study could help predict metabolic pathway, identify potential horizontal gene transfer, or identify novel enzymes required for oxidizing cDCE, presuming that the organisms employ similar mechanisms. There are a number of similarities between the FT-culture and JS666, including inhibition by TCE, higher metabolic activity towards cDCE than VC (despite predictive thermodynamic calculations), optimal temperature range, and CSIA fractionation values (2, 10, 12, 14).

Finally, a number of studies could be constructed to explore how JS666 functions in a mixed culture, perhaps working from the FT-culture. Work conducted with *Dehalococcoides* shows the organism grows more robustly in a microbial consortium (9). This may help JS666 survive in other subsurface material and may even offset inhibition caused by other chlorinated solvents.

6.3. REFERENCES

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