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Characterization of Vinyl Chloride Degrading Bacteria From a Serial Anaerobic-Aerobic Chloroethene Bioreactor

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Chloroethenes are among the most common groundwater contaminants in the United States. In anoxic groundwater, chloroethenes can be reduced to vinyl chloride (VC), a known carcinogen. The goal of this research was to characterize bacteria that could mineralize VC from a microbial community within a serial aerobic-anaerobic chloroethene bioreactor. Isolations for organisms capable of VC degradation were performed. The ability to degrade VC was first indicated by accumulation of chloride. Gas chromatography assay showed that VC was degraded in cultures capable of chloride accumulation. The epoxyalkane:coenzyme M transferase (EaCoMT) gene which codes for an enzyme previously shown to function in VC mineralization, was observed in several mixed VC degrading cultures. Pure cultures isolated on nutrient rich media from EaCoMT positive cultures did not retain ability to mineralize VC. A confirmed VC degrading pure culture without the EaCoMT gene was isolated but the species identity could not be ascertained.

Acknowledgments

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1. Literature Review and Introduction

Chloroethenes are widespread contaminants in groundwater. Chlorinated ethenes are two carbon alkenes with one or more chlorine substitutions. Perchloroethylene (PCE) is the completely chlorinated ethene, and has been used extensively as a dry cleaning solvent (4, Figure 1.1). Trichlorethene (TCE) is the tri-substituted chloroethene. There are three dichloroethene isomers: *cis*-1,2-dichloroethene (*cis*-DCE), *trans*-1,2-dichloroethene (*trans*-DCE), and 1,1-dichloroethene (1,1-DCE). Vinyl Chloride (VC) is the mono-substituted chloroethene and is listed by the Merck index as a known carcinogen (25). Chloroethenes have been used as solvents for fats, waxes, resins, oils, rubber, paints, and varnishes (4). Since their introduction in 1925 (18), their common use and improper disposal have led to the accumulation of chloroethenes in groundwater. The U.S. produced 4.791 billion tons of PCE from 1983 to 1993 (7). The estimated use of PCE is as follows: 55% for chemical intermediates, 25% for metal cleaning and vapor degreasing, 15% for dry cleaning and textile processing, and 5% for unspecified uses (Figure 5.16) (6). A survey conducted in 1989 indicated that 88.9% of dry cleaners in the U.S. used PCE (2). Although discharge of PCE into U.S. waters requires a permit (37), it has been estimated that 80-90% of PCE used in the U.S. is released into the environment (31).

1.1 Reductive Dechlorination

Chloroethenes have high densities and low water solubility, which allow them to form dense non-aqueous phase layers (DNAPLs) that accumulate on the bottom of the water table and to slowly partition into groundwater. Under anaerobic conditions PCE and TCE have been shown to be reductively dechlorinated by both mixed cultures (35) and the pure culture *Dehalococcoides ethenogenes* strain 195 (24). PCE is considered non-biodegradable under aerobic conditions, but TCE has been described to be degraded by non-specific oxygenases (24).

Reductive dechlorination is the process of removal of chlorine atoms from a chlorinated molecule usually under reducing conditions. Many organisms reduce chloroethenes fortuitously through a process of co-metabolism where an organism growing on appropriate carbon source will transform other molecules because of relaxed enzyme specificity when reductive dechlorination is coupled to energy production it is referred to as dehalorespiration (17). Dehalorespiration is the process by which chlorinated compounds, such as chloroethenes, serve as terminal electron acceptors. Dehalorespiration can sustain bacterial growth because it is coupled to energy production. Reductive dechlorination can dechlorinate highly substituted chloroethenes such as PCE to ethene (ETH), but complete dechlorination is rare. Intermediates such as TCE, DCE isomers, and VC are the major end products of reductive dechlorination. VC is the most problematic because it is a known carcinogen (25).

Dehalococcoides ethenogenes has been observed under anaerobic conditions to reductively dechlorinate PCE to ETH but VC can accumulate as an end product as well (24). When *D. ethenogenes* PCE- grown cultures were transferred into media containing VC or trans-DCE, products accumulated slowly which indicated that those compounds did not support

growth (24). Two enzymes have been characterized that facilitate the PCE anaerobic dechlorination pathway. PCE-reductive dehlogenase (PCE-RDase) in the presence of specific reductants can dechlorinate PCE to TCE (21). The other enzyme TCE-dehalogenase was found to dechlorinate TCE to ethane (21). Although under some conditions *D. ethenogenes* can dechlorinate PCE to ETH, accumulation of VC is still a problem in terms of a bioremediation scheme.

The natural distribution of PCE degrading bacteria has recently studied. Surveys based on 16S rRNA genes presence have shown that PCE degrading bacteria can be found not only at contaminated sites, but are also present in pristine locations (20). Samples were taken from pristine streams, contaminated streams, and contaminated groundwater systems such as Cape Canaveral, FL. A two step polymerase chain reaction (PCR) protocol was performed to initially screen samples for possible PCE degraders. The first round of PCR amplified only the bacterial 16S rRNA sequences and the first round PCR amplicons were used as the template for the second round. The second round of PCR amplified only a conserved region of the 16S rRNA gene specific for known PCE degrading bacteria. PCE containing serum vials were inoculated with samples that passed the PCR screen. The 16S rRNA gene of PCE degrading cultures was fully sequenced to determine their identity. The fact that PCE-degrading cultures were found in pristine water systems is interesting because it implies that PCE-degrading bacteria have an enzymatic system(s) with multiple functions (20). The enzymes necessary for degradation of PCE may also be used for metabolism of other molecules found naturally in the environment.

1.2 Vinyl Chloride Aerobic Degradation

VC is the most problematic product of the PCE mineralization pathway and is very common in subsurface ecosystems (5). VC has been found in 496 (of 1430) of the sites listed on the EPA National Priorities List (16). As early as 1984, it was realized that VC was accumulating in groundwater systems due to reduction of chloroethenes by anaerobic bacteria (30, 36).

Dehalogenation of VC has been observed in anaerobic mixed cultures (27). Aerobic VC mineralization has been observed in many organisms whose genome contains genes which code for monoxygenase activity such as methane, propane, isoprene, and ammonia utilizing bacteria. Hartmans and DE Bont (14) studied *Mycobacterium aurum* L1 which is the first VC degrading bacterium described that utilized VC aerobically as a sole source of carbon and energy. In 2000, Verce et al. (34) characterized an isolate of *Pseudomonas aeruginosa* that was able to grown in high concentrations of VC and release stoichiometric amounts of chloride (34). Coleman et al. (9) surveyed the natural distributions of aerobic VC degrading organisms at contaminated sites and found 11 *Mycobacterium* strains and one *Nocardiodes* strain that were capable of VC degradation.

The molecular identification of chlorinated ethene degrading bacterium involves two important steps. The first step is the identification of the bacterium capable of degrading the chlorinated ethene. Once the identity of the bacterium is known then the next step is characterization of the metabolic pathway that is used for degradation. With currently 10,000 full or partial sequences stored on Genbank, which is the largest depository of DNA sequences,

the 16S rRNA gene sequence is an accepted standard by which species of bacterium can be identified. Classical bacteriological characterization which entails biochemical metabolic tests is also used to identify bacteria. The metabolic pathway of aerobic VC degradation can be examined with analytical, biochemical, and modern molecular methods.

1.3 Molecular Identification and Characterization

The 16S rRNA is a structural component of the small subunit of the ribosome. The sequence of the gene which codes for the 16S rRNA is highly conserved within the bacterial domain. Because the 16S rRNA gene has been extensively studied there is a wealth of sequence data presently stored in databases. Verce et al. (34) sequenced the 16S rRNA gene to confirm the identity of their isolate (strain MF1) that could aerobically grow on VC. Strain MF1 was identified as *Pseudomonas aeruginosa* (34). Genomic DNA was extracted from the isolate. The 16S rRNA gene was then PCR amplified with primers based on the *Escherichia coli* 16S rRNA gene. The PCR amplicon was then purified and sequenced by dye terminator sequencing. The entire 1540 bp sequence was determined. Strain MF1 was the first *Pseudomonas* species to be isolated that can use VC as a sole source of carbon and energy (34).

Although aerobic bacteria such as *Mycobacterium* species and *Pseudomonas* species have been isolated can be cultivated on VC as the sole carbon and energy source, the natural diversity and distribution of VC degraders has not been investigated prior to the work of Coleman et al. (9). A microcosm consists of minimal salts broth medium without a labile carbon source in a sealed serum vial with the chloroethene amended to the vial. Twenty-three of thirty-seven microcosms that were inoculated with samples taken from known contaminated sites were found

to include bacteria that were capable of growth on VC. After isolation of a bacterium capable of VC degradations, the 16S rRNA gene was PCR amplifed and partial sequences were used to identify the species of each bacterium. All isolates were found to be *Mycobacterium* species except one which was found to be the first *Nocardioides* species capable of growth on VC. Even though the medium and temperatures used for isolation were similar to Verce et al. (34), no Pseudomonads were isolated from environmental samples. The possible explanation for this is the differences in the selection process. Coleman et al. (9) used VC as their initial enrichment; where as Verce et al. (34) used ethane and ethene to isolate organisms that could degrade VC. Also, Verce's samples originated from sewage sludge and Coleman's samples came from contaminated groundwater. Coleman et al. (9) inferred that Mycobacteria are better candidates for the natural attenuation of VC in the environment than pseudomonads because pseudomonads can survive on more energy sources than mycobacteria.

Mycobacterium, *Pseudomonas*, and *Nocardioides* are the only three known taxa with the ability to metabolize VC. With the age of molecular biology, the metabolic pathways that allow for VC degradation are finally becoming understood.

1.4 Metabolic Pathway Analysis

Hartmans *et al.* (15) first examined the metabolic pathway of VC degradation in *Mycobacterim aurum* L1. Loss of VC along with accumulation of chloride ions within a closed enrichment culture is determinative for VC degradation. Hartmans *et al.* (15) found that the initial step of VC metabolism is catalyzed by alkene monoxygenase which transforms VC into the highly reactive epoxide chlorooxirane (Figure 1.2). Alkene monooxygenase has a binuclear

iron binding site that is similar to methane and propene monooxygneases. (28) Hartmans *et al*. (15) also postulated that epoxyethane dehydrogenase catalyzed the next step in VC metabolism but the evidence was not conclusive.

The characterization of epoxide metabolism in the VC catabolic pathway was slowed mainly by the instability of the epoxide. Early research on the ethene-assimilating *Mycobacterium* strain E20 (13) suggested that coenzyme A (CoA) and nicotinamide adenine dinucleotide (NAD^+) dependent enzyme converted epoxyethane into acetyl-CoA, but activities were very low. There have been other enzymes that have been found to transform epoxides (11). In propene-assimilating bacteria *Xanthobacter* strain Py2 and *Rhodococcus* strain B-276, there is an unusual system of epoxyalkane:CoM transferase (EaCoMT) that catalyzes propene assimilation (14).

Coleman and Spain (11) first revisited early work that proposed that a NAD^+ dependent enzyme was involved in the metabolism of the epoxide. Cell extracts were tested for epoxyethane metabolism with various cofactors to determine the type of enzyme involved. No significant loss of epoxyethane was observed even in the presence of glutathione, NAD^+, or CoA. Epoxyethane metabolism was rapid when CoM (2-mercaptoethanesulfonate) was present. This suggested the presence of an epoxyalkane:coenzyme M transferase enzyme (EaCoMT) (1). Based on the conserved regions of the propene assimilating genes of both *Xanthobacter* strain Py2 and *Rhodococcus* strain B-276, PCR primers were designed to assay for the presence or absence of an EaCoMT enzyme in VC/ETH assimilating *Mycobacterium* strain JS60. Using the primers CoM-F1 and CoM-R1 yielded an amplicon of 981 base pairs from JS60. After the EaCoMT gene fragment from JS60 was sequenced, new primers CoM-F1L and CoM-R2 which

anneal to the *etnE* gene, were designed which produced an amplicon of 893 bp which would later be used for Southern blotting experiments. Analysis of the flanking DNA near the EaCoMT gene shows a total of seven open reading frames, five of which code for products related to ETH and VC degradation. The EaCoMT gene is found in the *etnE* locus which is 1109 bp long. The entire *etn* operon is 5447 base pairs in length (11). Downstream of the *etnE* locus, the *etnA* codes for the monoxygenase β-subunit. Next in line the *etnB* locus codes for the monoxygenase coupling- effector protein. The *etnC* locus codes for the monoxygnease α-subunit, and the *etnD* codes for the monoxygenase reductase.

The *etnE* (EaCoMT) gene was first cloned into an *E. coli* T7 based expression system vector (pET-21a) and subsequently transformed into *E. coli*, but the gene could not be expressed (11). The same gene was then cloned into a *Mycobacterium smegmatis* (non-VC degrading strain) with the constitutive *hsp* 60 promoter on the pMV261 vector. The strain was named mc2155 (pMV-CoM). Epoxyethane and chloroxirane were metabolized by the bacteria expressing the *etnE* gene but no metabolites of the epoxides were produced by *Mycobacterium smegmatis* without the *etnE* gene.

The natural distribution of the EaCoMT gene was observed by Coleman and Spain (10). Isolates taken from contaminated sites where analyzed for the presence of the EaCoMT pathway. A phylogenetic tree was made based upon EaCoMT and 16S rRNA gene alignments. The phylogenetic tree suggests that the lateral transfer of many genes among *Mycobacterium* strains has occurred (23). It was also hypothesized that the EaCoMT gene was found on a plasmid because the propene assimilating bacteria (*Rhodococcus* strain B-276 and *Xanthobacter* strain Py2) carry their propene assimilating genes on plasmids. Plasmids were found in

Mycobacterium JS60 by Coleman and Spain (10). The plasmids were probed with EaCoMT gene probes made from PCR amplifications of VC/ETH assimlating JS60 strains. The plasmids identified to contain the EaCoMT genes ranged in size from approximately 200-300 kb. The plasmid carrying the *etn* operon has now been found in VC assimilating *Nocardioides* sp. strain JS614 (22). To date all published VC assimilating strains code for the EaCoMT enzyme except the *Pseudomonas* strains.

1.5 Introduction

All published VC assimilating strains have been isolated from chloroethene contaminated environmental sites. Chloroethene degradation can be performed in serial anaerobic-aerobic bioreactors (32). The theory behind these systems is the coupling of reductive dechlorination with aerobic mineralization. Anaerobic bacteria perform reductive dechlorination of PCE and TCE. DCE isomers and VC are the major end products of anaerobic dechlorination in this system. VC can be further mineralized to chloride and ethene. ETH is probably mineralized completely but the concentration of ETH has not been assayed in the system. The data shows that VC concentration is lower in the aerobic chamber than the outflow from the anaerobic chamber. This data suggests aerobic bacteria are degrading VC. No aerobic VC degrading bacteria have been isolated from a serial anaerobic-aerobic chloroethene bioreactor in the published literature. Since the initial inoculums of the bioreactor came from a chloroethene contaiminated site, *Mycobaterium*, *Nocardioides*, and or *Pseudomonas* species may be present as well as uncharacterized species. The goal of this research was to isolate and characterize bacteria capable of growth on VC. Isolation and characterization of the aerobic VC degrading

bacteria from the aerobic camber of the serial anaerobic-aerobic chloroethene bioreactor may provide valuable information essential to the development of an effective chloroethene biodegradation scheme.

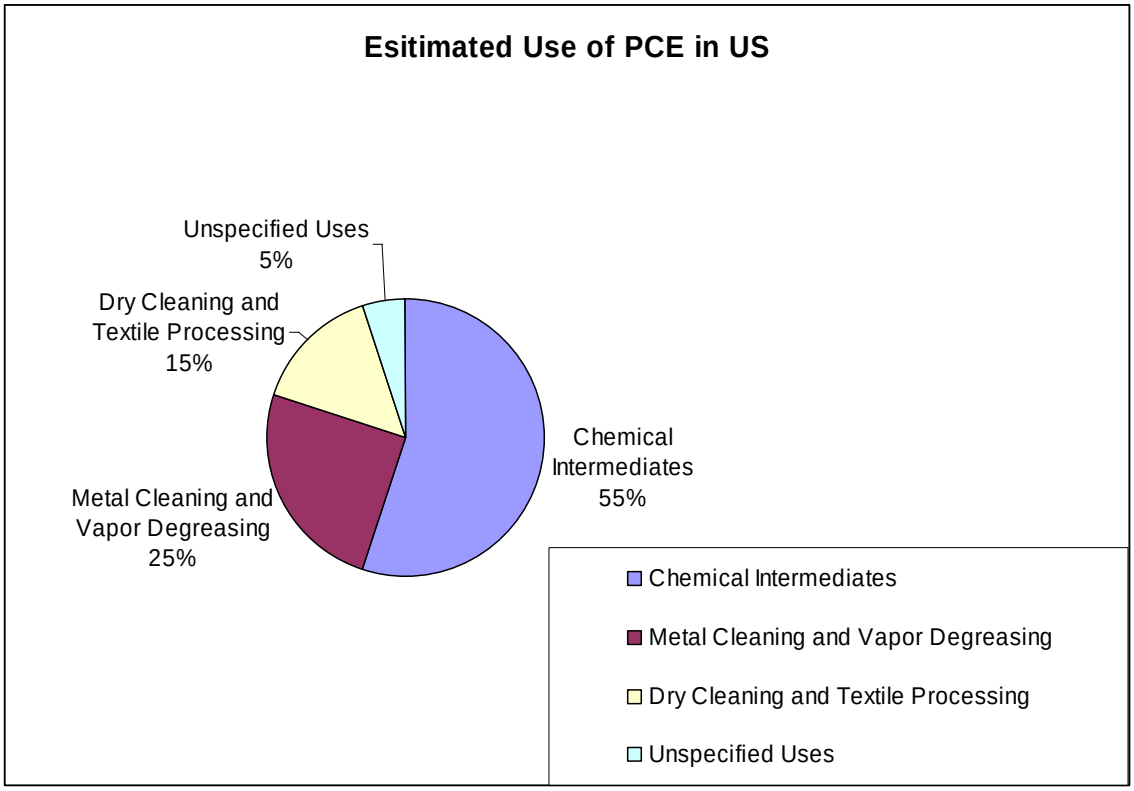

Figure 1.1 Estimated Use of PCE in US Adapted from (6)

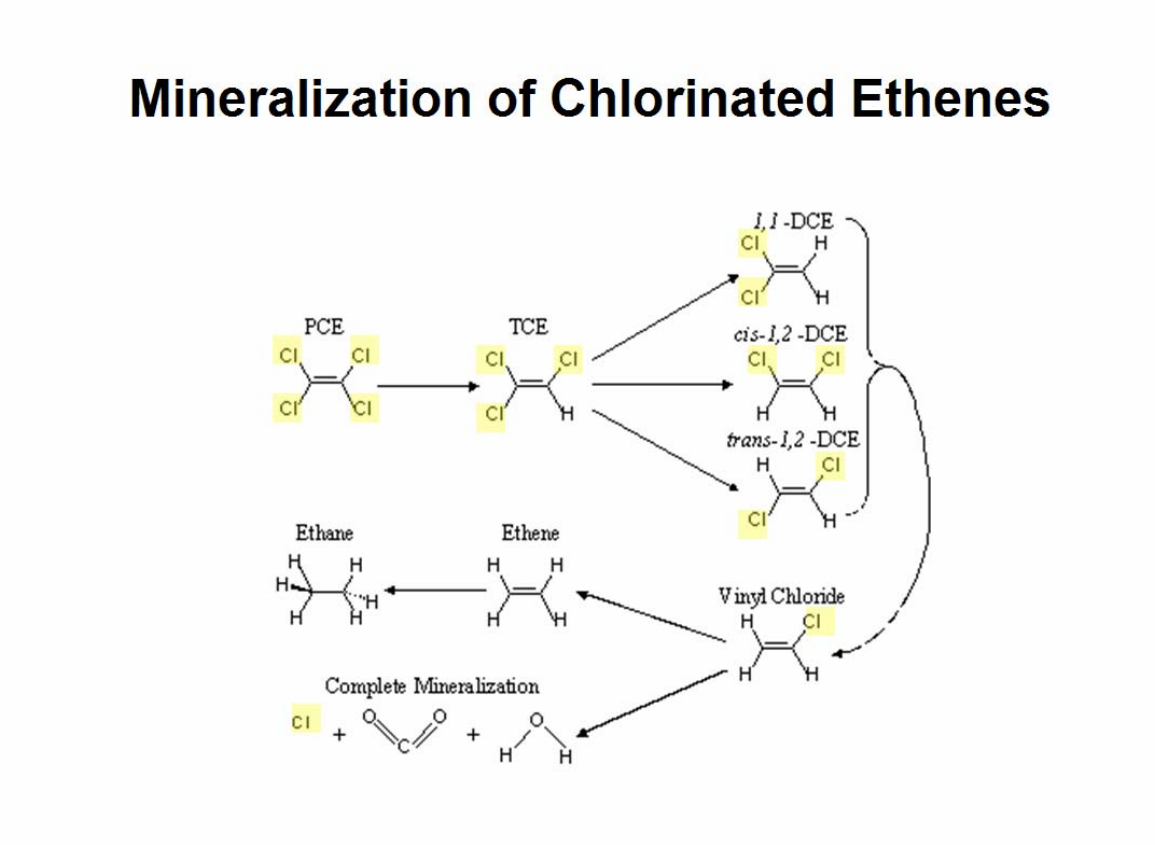

Figure 1.2 Mineralization of Chlorinated Ethenes. PCE, TCE, and DCE dechlorination by bacteria occur most favorably under anaerobic reducing conditions. VC complete mineralization performed by bacteria occurs most favorably under aerobic conditions. (http://gis.esri.com/library/userconf/proc00/professional/papers/PAP553/p553.htm)

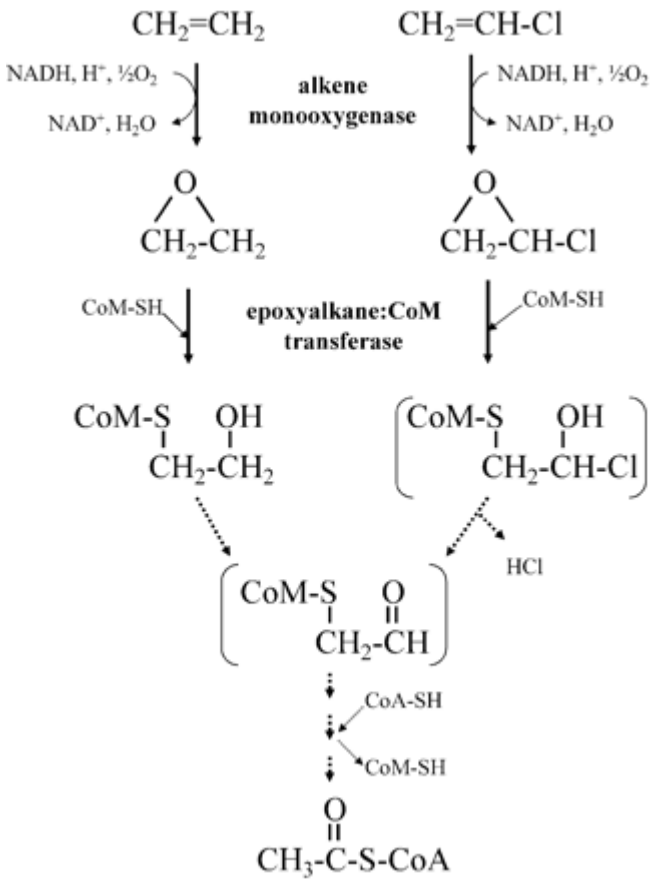

Figure 1.3 Proposed pathways of vinyl chloride and ethene assimilation in Mycobacterium strains. Intermediates that have not been identified are in brackets, and hypothetical reactions are indicated by dotted lines (Taken from Coleman and Spain (11))

2. Materials and Methods

2.1 VC Microcosm Culture Methods

Minimal Salts Broth (15) was prepared to a volume of 10 liters and stored at 4°C until use. Agarose was added (15 g/l) to make minimal salts agar (MSA) plate. Inoculating fluid was taken from the aerobic reactor of a chlorinated ethenes serial anaerobic-aerobic bioreactor that had been established (32). An aliquot (100 µl) from the aerobic reactor was plated onto MSA plates. The plates (16 total) were placed in a sealed 9 L vessel. VC was injected into the vessel to make a 1% vol/vol atmosphere. A control vessel containing 6 plates (3 from each aerobic reactor) were placed in a 3 L sealed vessel without added VC. The sealed vessels were incubated at room temperature for one month. Colonies growing on the MSA plates within the VC atmosphere vessel were inoculated into liquid microcosms. Microcosms were setup in 70 ml serum vials sealed with butyl rubber stoppers and aluminum crimp tops. MSB (5 ml) was poured onto each MSB plate. A sterile swab was used to suspend the colonies. The swab was dipped into a 70 ml serum vial that contained 30 ml of MSB. The 5 ml of MSB on the MSA plates was transferred to the 70 ml microcosm making the final aqueous volume of the microcosm 35 ml (Figure 2.1). Cultures were numbered and fed VC (see section 2.2.2, Figure 2.2). Microcosms were placed on a rotary shaker at 150 rpm at room temperature for three

months. In subsequent microcosm generations, controls were added (killed inoculum and no inoculum controls).

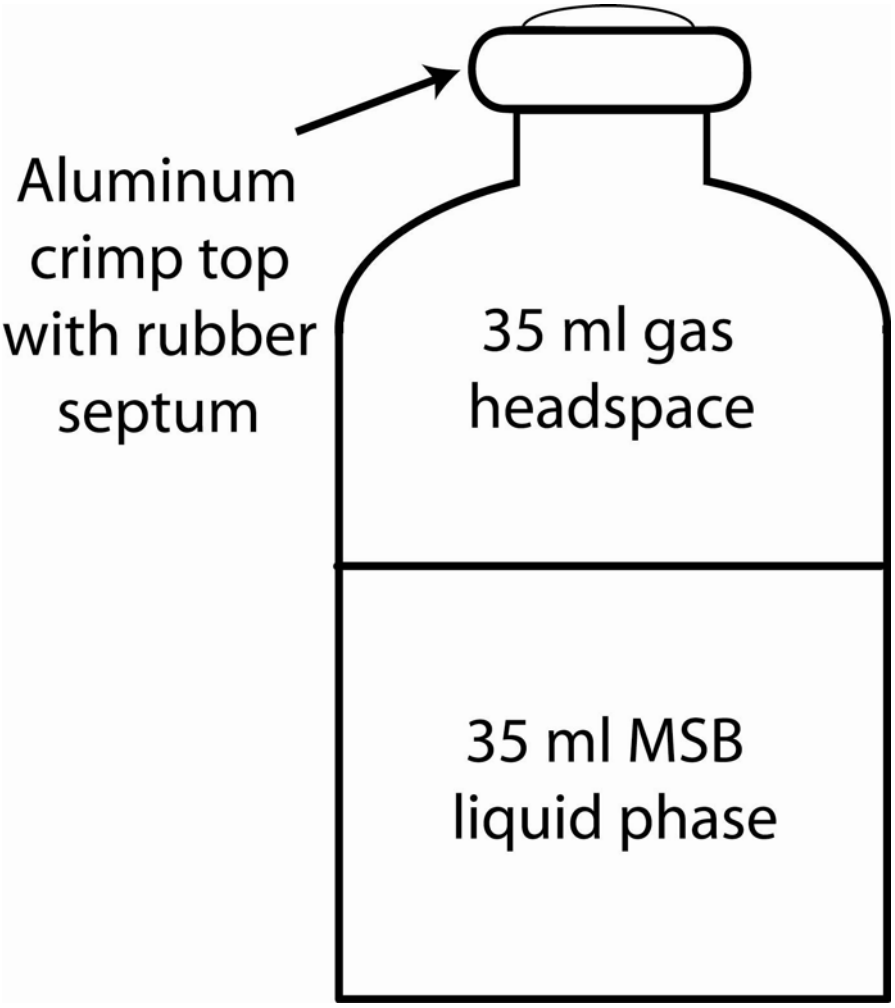

Figure 2.1 Microcosm Setup. The microcosms were established with a 70 ml serum vial. The serum vial was autoclaved containing 35 ml of MSB liquid medium with the rubber septum seal taped on. Once serum vial with sterile MSB cooled to room temperature, the culture was inoculated. The microcosm was sealed by crimping an aluminum top over the septum. The center of the aluminum crimp top could be removed to add VC or sample the liquid phase with a needle and syringe.

2.2 VC Microcosm Maintenance

2.2.1 VC Microcosm Generation 1 (VCG1)

Initially microcosms received 0.35 ml (1% vol/vol) of VC as the sole carbon source at atmospheric pressure weekly but since no growth was observed dosage of VC was increased to 10 ml VC volumes sampled at bottle pressure (200-500ppm final concentration in headspace) each week. The VC was transferred to the gas phase of the microcosms via a gas tight Hamilton syringe with shut off valve (Figure 2.2). New sterile needles were used to inject through the rubber seals into the microcosm serum vials. Optical densities were monitored weekly by extracting 1 ml of shaken/vortexed aqueous phase of microcosm and recording the absorbance at 600 nm on an analytical spectrophotometer after blanking with MSB.

The nomenclature of microcosm cultures was set as first the generation number and then the book number and finally the culture number. For example cultures from generation 1 were named VCG1-ANJ2-#.

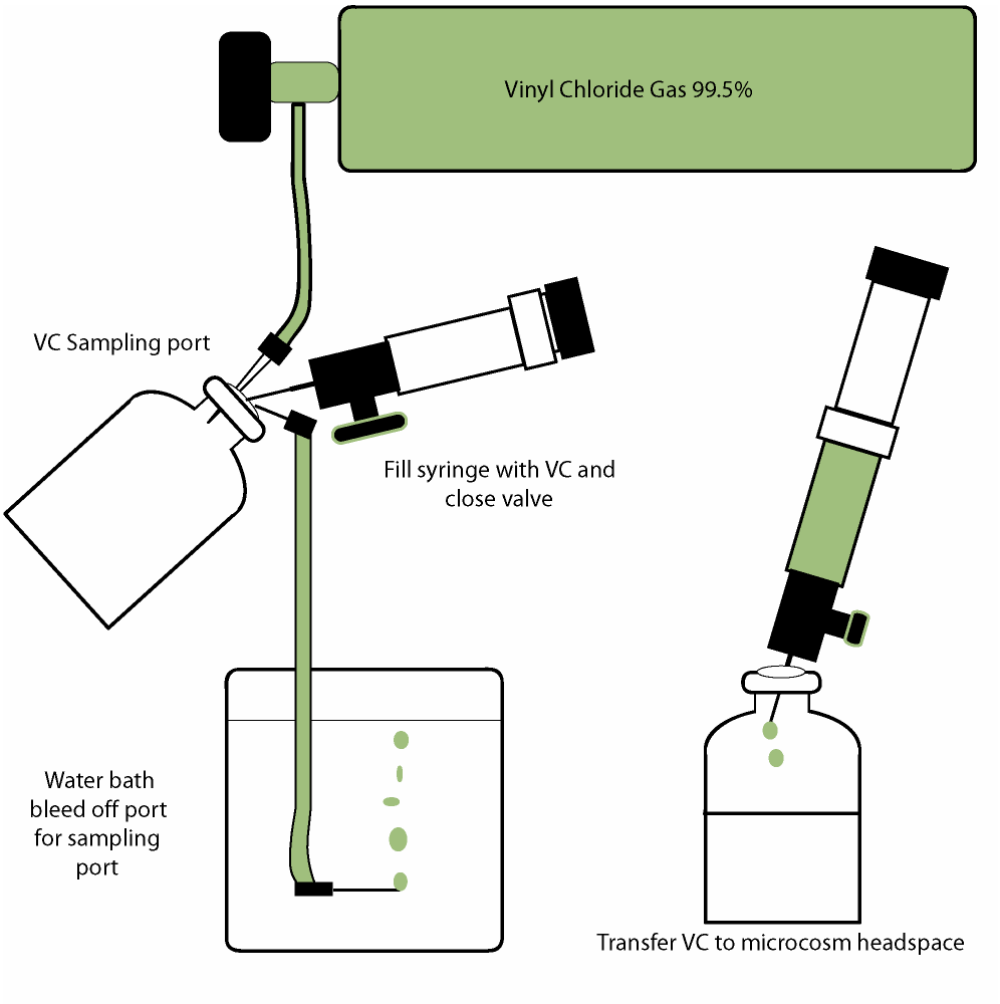

Figure 2.2 Transfer of VC to Microcosms. Transfer of VC (99.5% pure) gas to microcosms headspace was performed using a Hamliton (Reno, NV) gas tight syringe with shut off valve. The VC gas was extracted from a sampling point which was purged with VC prior. VC gas was extracted from the sampling port with the syringe. If the gas was sampled at atmospheric pressure the valve was opened once out of the sampling port to release excess gas under pressure. If a more concentrated sample of VC was needed the valve on the VC gas tank was opened up to release bottle pressure gas into the sampling port. The syringe was then used to extract the gas but the gas in the syringe was not equilibrated to atmospheric but rather added directly to the headspace of the microcosm.

2.2.2 VC Microcosm Generation 2 (VCG2)

VCG1 microcosms were subcultured into VCG2. Three ml of inoculum was taken from the VC Microcosm Generation 1 (VCG1). Controls of no inoculum and killed inoculum were setup as controls. Microcosms were fed 10 ml (sampled at bottle pressure) of VC gas. Chloride concentration was monitored (see section 2.3.1). Microcosm cultures from generation 2 were VCG2-ANJ2-#.

2.2.3 VC Microcosm Generation 3 (VCG3)

All microcosm vials were autoclaved with desired amount of MSB added. A control vial (VCG3-FHD2-007-1) with no inoculum was setup to monitor loss of VC by other than degradation such as leakage through the rubber crimped seal of the serum vial. Another control vial (VCG3-FHD2-007-2) with autoclaved inoculum was also setup to compare biomass. The autoclaved inoculum vial was fed 10 ml of VC sampled at atmospheric pressure. Vial ANJ2-8-3, which had been observed to accumulate biomass on the surface of the MSB, increase chloride concentration, as well as had yielded a PCR amplification of the EaCoMT gene fragment, was used to isolate pure strains. An aliquot (100 µl) of the vial was plated with sterile glass beads onto a Tryptic Soy Agar plate (TSA) (Bacto Liverpool, NSW, Australia) (see section 6.2.2 Medium). Four colony morphologies were noted and an isolate of each type was streaked for pure isolations. Each of the four isolates was allowed to grow on TSA (Appendix A) for 5 days at 25° C.

Each of the four morphologies was inoculated into 35 ml of MSB via a sterile inoculation loop by touching the loop to the isolated colonies on TSA plates and transferring it to the

microcosm vial (FHD2-007-3 through FHD2-007-6). The same four pure isolated colonies were inoculated into 10 ml of Tryptic Soy Broth (TSB) and placed on a rotary shaker (150 rpm) for 3 days at room temperature. The TSB cultures were then centrifuged at 16,110 x *g* for 5 min at room temperature. The supernatant was poured off and the cells were suspended in 1 ml of sterile MSB. The inoculum was vortexed and transferred to 2 ml microcentrifuge tubes. The inoculum aliquot (1 ml) of each isolate suspended in MSB was then added to 34 ml of sterile 1× MSB (FHD2-007-7 through FHD2-007-10). The microcosm's initial chloride concentrations were recorded for all vials. All vials were supplemented with 10 ml of VC sampled at bottle pressure on day 1 (~200 ppm). Microcosm cultures from generation 3 were named VCG3-FHD2-#.

2.2.4 VC Microcosm Generation 4 Setup and Maintenance (VCG4)

Mixed culture ANJ2-46-11 was inoculated onto TSA medium. Colonies were isolated based on color, size, and morphology. The dominant morphology was a white colony. Yellow and white colonies were seen in lower numbers. After streaking for isolation of each of the colonies, a confluent lawn of cells was grown overnight at room temperature on TSA media. A Gram stain was also performed on the isolated colonies. Sterile water (2 ml) was spread onto each lawn plate and cells were suspended with a sterile loop. For the high inoculum microcosms, one ml of the homogenate was inoculated into each sterile MSB microcosm. The MSB microcosm was a sterile glass 70 ml vial with 35 ml of MSB sealed with a rubber butyl lid and aluminum crimp top. For the low inoculum microcosm, 100 µl of the homogenate was inoculated into sterile MSB microcosm vials. Initial chloride measurements were recorded

before VC was amended to the headspace of the microcosm. VC (10 ml) sampled at bottle pressure was amended to the headspace of the microcosms (200-500ppm). Four chloride measurements were taken over a one month period. Microcosm cultures from generation 4 were named VCG4-FHD1-#.

2.3 Chloride and VC Assays

2.3.1 Microcosm Chloride Assay

Chloride ion concentrations were measured with an ion-selective electrode (Thermo Orion Beverly, MA). The chloride selective electrode only measures free chloride ions. A millivolt meter (Corning Corning, NY) was used to observe the mV output of the electrode. A 10,000 ppm stock solution was prepared from analytical grade NaCl. Dilutions (10^{-1}) of the stock to make 1000, 100, 10, and 1 ppm standards were used to generate a standard curve. One ml sample size was used. Millivolt readings were transformed into part per million concentrations using the slope equation of the standard curve. Standards were read from lowest concentration to highest concentration. All measurements were made in clean glass test tubes. The tubes were cleaned with reagent alcohol (EtOH and Isopropanol) then stored at 100° C to drive off residual containments and solvents. Room temperature, clean test tubes were used at all times. The electrode was washed with water and dabbed dry with Kim-wipes between each sample or standard.

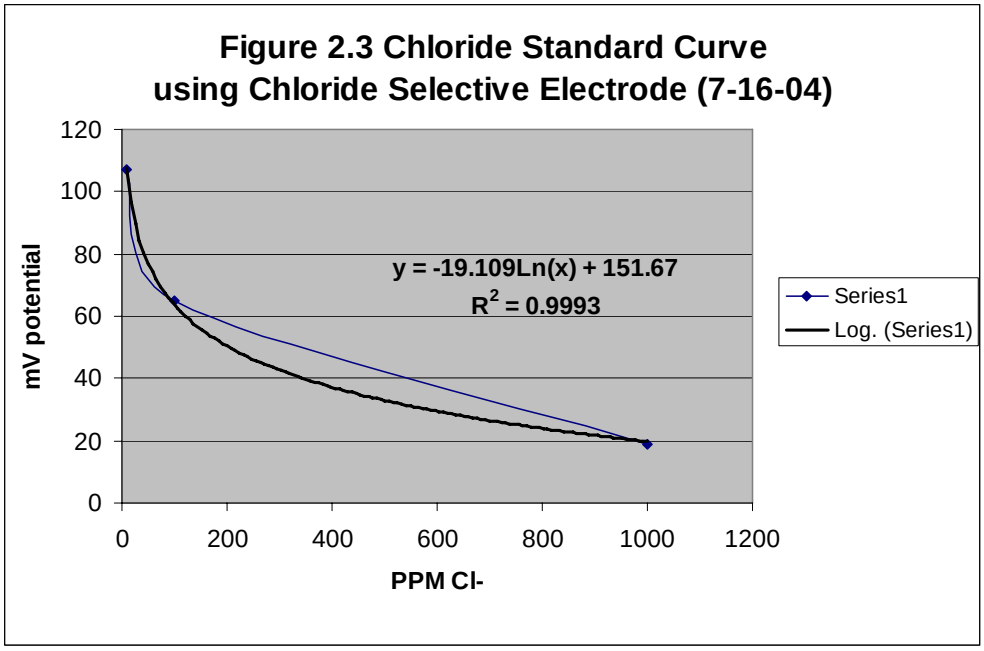

Figure 2.3 Chloride Standard Curve using Chloride Selective Electrode. Chloride standards were prepared in water (10, 100, and 1000 ppm). Standard curves were performed for each sampling. The slope equation was used to calculate the concentration of chloride in samples from the mV response of the chloride selective electrode.

2.3.2 VC Gas Chromatography Assay

All separations were made on a Buck Scientific Gas Chromatograph Model 910 (East Norwalk, CT) equipped with an electron capture detector (ECD). Nitrogen was used as both the carrier gas and the makeup gas. A 6 ft. by 1/8 in. packed Haysep D 60/80 mesh was used for VC assays. PeakSimple NT software (SRI instruments Torrence, CA) was used for data collection and calculation. Chromatograms were stored by sample name. The protocol for analysis used a linear temperature of 50° and 15 psi of nitrogen carrier and makeup gas. The retention time for VC was found to be 0.50 min. VC standards in methanol were used. Commercial VC standards (1 ml of 200 ppm in methanol) (Sigma-Aldrich, St. Louis, MO) were used. The unopened VC

standards were stored in -70° freezer. Upon cracking the vial the contents were quickly transferred to a sealed crimp top vial via a Hamlton gas tight syringe (Reno, NV). Various volumes of the 200 ppm VC standard were injected and peak areas were recorded from 1ppm standard (1 µl of 200 ppm) to the 500 ppm standard (500 µl of 200 ppm). Using the assumption that the same weight of analyte will produce the same signal on the electron capture detector, headspace responses were compared to standards using the line equation of the standards. To assay the VC concentration in the headspace of the microcosm, 200 µl was extracted from the headspace with a gas tight Hamlton 1 ml syringe (Reno, NV) with a valve. To determine the analyte concentration within the vial the response was factored to the injection volume. An injection volume of 200 µl produced the most repeatable results. The peak response from a 200 µl injection (response is proportional to µg of analyte) was then multiplied by 5 to determine the concentration of analyte found in one ml of headspace (PPM = µg/ml).

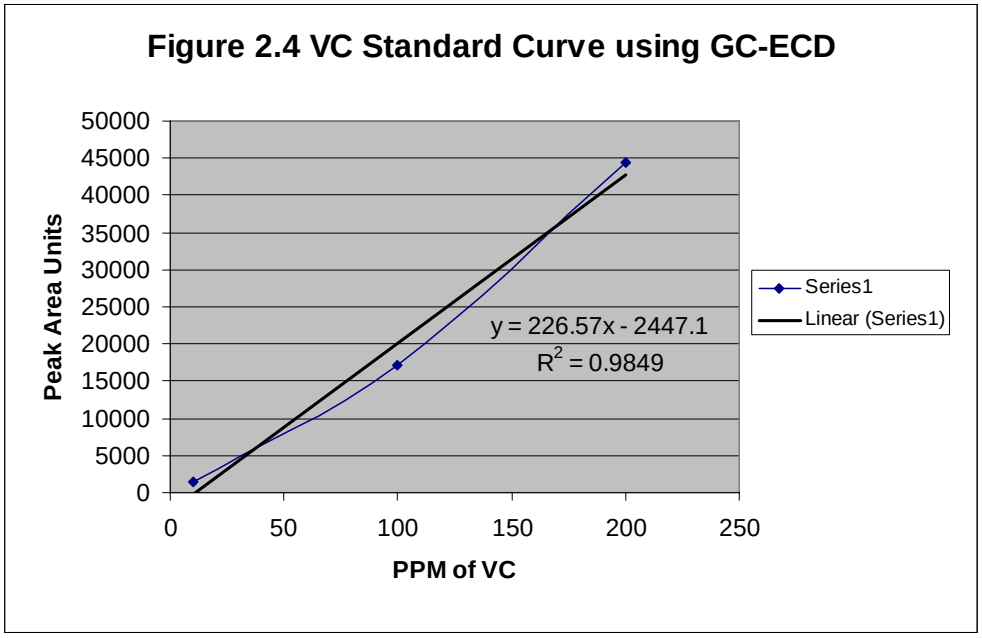

Figure 2.4 VC Standard Curve using GC-ECD. Known commercial standards (10, 100, 1000 ppm) of VC in methanol were separated by gas chromatography and detected with an electron capture detector (ECD). The slope equation obtained from standards was used to calculate the concentration of VC in headspaces of microcosms.

2.3.3 VC Degradation Kinetics Methods

To determine the rate at which VC was degraded, kinetics studies were used. A control microcosm was setup for comparison. The control contained 35 ml MSB with no inoculum and was sealed with rubber stopper and aluminum crimp. VC (10 ml sampled at atmospheric pressure) was amended to the control microcosm. The control contained the same aqueous phase and same gas phase volume as the experimental microcosm. The microcosm to be examined was first assayed for residual VC. The VCG2-ANJ2-46-11 was then opened to release headspace gas and allow oxygen in. The concentration of VC in VCG2-ANJ2-46-11 was then taken to assay for residuals. VC (10 ml sampled at bottle pressure) was administered to the headspace via a Hamlton gas tight syringe with a valve (Reno, NV). Between assays of the VC in the headspace

the GC was allowed to run through a column conditioner temperature program which ramped up to 200° for 40 min to drive off all residuals. Solvent (50µl of pentane) was also run through to clean off residuals. The Hamilton syringe used to sample the headspaces was also cleaned in between runs with pentane (99% HPLC grade Sigma, St. Louis, MO). The microcosm (VCG2-ANJ2-46-11) was all ways run first in order to achieve a run with the least carry over. In between the microcosms and the standard run the column was ramped to 150° and back down to 50°.

2.4 EaCoMT Fragment PCR Amplification

2.4.1 PCR Materials

Sterile vials for both genomic DNA extraction and PCR amplification were obtained. Pure water (18 MΩ) was autoclaved prior to use. PCR reactions materials included 10× *Taq* Buffer with Magnesium from New England Biolabs (Beverly, MA), dNTPs (20mM each dNTP) from New England Biolabs (Beverly, MA), and *Taq* polymerase was obtained from New England Biolabs (Beverly, MA) or Eppendorff (Hamburg, Germany). Primers were synthesized at the MUSOM DNA Core Facility based on those reported in Coleman and Spain (11). The forward primer or CoM-F1L (see section 6.1.1) and the reverse primer or CoM-R2E (see section 6.1.1) were designed by Coleman and Spain from conserved regions of EaCoMT genes of *Mycobacterium* strain JS60, *Rhodococcus* strain B-276, and *Xanthobacter* strain Py2 (GenBank accession numbers AY243034, AF426826, and X79863).

2.4.2 PCR Methods for MSB Microcosm Cultures

DNA was extracted from sealed serum vials with visible biomass growth in MSB and VC as the sole carbon source. Serum vials were shaken or vortexed. One ml of the culture was transferred into a sterile 1.5 ml centrifuge tube. The tubes were centrifuged at micro centrifuge max speed (16,110 x *g*) for 5 min at room temperature. Supernatant (900 µl) was extracted out of the 1 ml of liquid to be boiled. The tubes with cell pellet were then placed into a wet bath and boiled at 100°C for 8 min. Tubes were then centrifuged at max micro centrifuge speed (16,110 x *g*) for 3 min to pellet solid mass. Tubes were then stored on ice until PCR reaction setup. Template DNA was taken from supernatant.

Boiling prep DNA extraction was found to be an unnecessary step to amplify the EaCoMT fragment. Using a flamed sterile loop (1 µl), visible biomass from the aqueous surface of the microcosm was transferred into the PCR reaction tube. A 5 min hot start at 94°C was successful in lysing the cells to expose genomic DNA for PCR amplification.

PCR reaction setup was performed on ice. Reactions (50 µl) were prepared was materials described in section 2.4.1. Primers were diluted to form a stock mix (100 pmol/ µl). Each primer was then diluted 1/10 for working concentration. For the 50 µl reactions the setup was as follows: 5µl of 10× buffer with magnesium, 1µl of forward primer, 1µl of reverse primer, 1µl of 20 mM dNTP mix, 0.5 µl of *Taq* polymerase (2.5 U), variable amount of genomic DNA template from boiling (derived empirically by performing initial template gradient amplification), and water to a final volume of 50 µl. Reactions (25 µl) were also used by cutting the recipe in half to conserve reagents. Negative controls were PCR reactions with *E. coli* or no template DNA, and

positive controls were reactions with VCG1-ANJ2-8-3 biomass that yield amplicon if the PCR reaction was setup properly. The thermocycler protocol contained 3 cycles. Cycle one was the hot start of 94°C for 5 min. Cycle two was 94°C for 30 seconds to denature the strands of DNA, 30 seconds at 60°C (annealing temperature empirically derived) to allow the primers to anneal to the template, and 30 seconds at 72°C allow for primer extension by *Taq* polymerase. Cycle two was repeated 30 times. Cycle three was at 72°C for 10 min to allow complete extension of products. Thermal cycling was done in a Bio-Rad Gene Cycler (Hercules, CA), and completed amplifications were stored at -20°C until analyzed by agarose gel electrophoresis.

2.4.3 PCR Methods for Pure Cultures Isolated on Nutrient Rich Media

R2A medium (Bacto Liverpool, NSW, Australia) was prepared according to manufacturers specifications. Isolation plates were poured and allowed to stand for 24 hrs. Aliquots (100 µl) were removed from a microcosm (VCG1-ANJ2-8-3) that had previously yielded an amplicon of the EaCoMT gene fragment. The 100 µl of microcosm fluid was diluted with 900 µl of sterile H_2O. A sub-sample (100 µl) of the dilution was plated onto R2A agar plates. Sterile glass beads were used to spread the dilution evenly over the agar surface. Isolation plates were incubated for 24 hrs at room temperature. Eight colony morphologies were selected for subculturing. The colonies were streaked for isolation on R2A agar plates and incubated at room temperature for at least 24 hrs. Separate and distinct colonies were lifted with a sterile wooden toothpick and transferred into one ml of sterile water and then centrifuged at 16,110 x *g* for 5 min to pellet the cells. The supernatant was poured off and the pellet was

suspended to be boiled at 100° C for 8 min to lyse the cells. PCR was performed with the same conditions that were used for the amplifications from the microcosms. A template gradient was performed from a volume of 10 µl of template to 0.01 µl of template. The genomic DNA isolation step was also disregarded for some experiments and biomass was used as the template with no extra preparation. The thermocyler protocol was the same that was used with amplifications from mixed cultures. Negative controls were PCR reactions with *E .coli* or no template DNA, and positive controls were reactions with VCG1-ANJ2-8-3 biomass that yield amplicon if the PCR reaction was setup properly.

2.5 Agarose Gel Electrophoresis Materials and Methods

PCR amplification reactions were separated and visualized on 1% agarose gels. One percent agarose gels were prepared by adding 0.50 grams of molecular biology grade agarose to 50 ml of 1× TAE (Appendix A) Buffer in a flask. The mixture was heated and stirred until all the agarose was melted. The gel was slowly cooled on lab benchtop with a swirling motion. Once the agarose solution was cool enough to be poured, 2 µl of Ethidum Bromide (20 mg/ml) was added to stain the DNA for visualization. The molten agarose was then poured into a mini-gel mold and allowed to solidify with a comb in place to form wells. When solid, the gels were loaded into a gel electrophoresis box and submerged in 1× TAE buffer. PCR reactions were mixed with a 6× loading dye (Promega Madison, WI) (to insure both proper loading and adequate run time on the gel). One kilobase DNA ladder (Promega Madison, WI) was mixed with 6× loading dye and used as a linear DNA size standard. Ladder was loaded into the outer wells for comparison. Amplicons with loading dye were loaded into the wells. Gel

electrophoresis was performed at 100V until the orange tracking dye reached the end of the gel. Gels were first visualized on a small UV light box at 302 nm. Images of the gels were taken using Alpha Innotech (San Leandro, CA) FluorChem™ IS-990 gel imaging system.

2.6 Cloning of EaCoMT Gene for Sequencing

2.6.1 Amplicon Purification

QIAquick® PCR product spin kits were used to clean up amplicons generated from PCR with the EaCoMT primers (see 6.1.1). The manufactures protocol (Buffers table) was used to clean up the amplicons for cloning. PCR reactions were combined, and 100 µl of amplicon products were added to each QIAquick® spin column. Buffer PB (500 µl) was added to each of the spin columns. The DNA was bound to the spin column by centrifugation for 60 seconds the binding of the DNA to the silica resin is due to attraction of charge between the silica and DNA. The column wash was then discarded, and 0.75 ml of buffer PE was added to the columns and centrifuged for 1 min. The eluate was discarded and the column was centrifuged for an additional min. The column was put into a clean 1.5 ml tube. The DNA was then eluted with 50 µl of Buffer EB and centrifuged for 1 min and then repeated to elute as much DNA from the column as possible.

2.6.2 Purification of EaCoMT Amplicon

The purified amplicon then was run on a low melting point 1.5% agarose gel. A double well was used in pouring the gel (two wells of the comb taped to double the size of the well).

Purified amplicon (40 µl) was mixed with 10 µl of load dye and run on the gel along with 1 kb DNA marker. The band at 900 bp band was then cut from the gel with a sterilized razor while viewing over a UV light box. Gel containing the band of interest was placed into a 2 ml microcentrifuge tube for extraction of the DNA from the gel. The MinElute™ (Qiagen Valencia, CA) extraction protocol was followed. Buffer QC (1.2 ml) was added to the tube. The tube was then incubated at 50°C for 10 min with frequent vortexing to dissolve the gel. The mixture color was yellow which signified that the mixture was at the proper pH to perform the extraction (no pH correction was needed). The mixture was then split into two tubes at 1 ml per tube. Isopropanol (200 µl) was added to each tube and the contents were gently mixed by pippetting. Each mixture was added to a MinElute™ column. The DNA was subjected to the silica column by centrifuge for 1 min at 16,110 x g. The column wash was discarded and 500 µl of buffer QG was added to each column and then centrifugation for 1 min at 16,110 x g. The eluate was discarded and the column the columns were centrifuged at 16,110 x g for one additional min to insure that potential residual ethanol was removed. The MinElute™ columns were placed into clean 1.5 ml tubes, and the DNA was eluted by adding 100 µl of Buffer EB to the center of the membrane. The columns were centrifuged one final time to collect the DNA. After extraction from the gel the amplicon was ethanol precipitated.

The ethanol precipitation protocol used was as follows for 100µl sample (DNA to be precipitated): add 10 µl of 5M ammonium acetate, add 275 µl of 100% EtOH, add 1 µl of glycogen, vortex 10 seconds, flash freeze at -80°C, spin 15 min at 16,110 x g, pull liquid out

with vacuum and leave pellet, add 1ml of 70% EtOH, turn upside down once, spin 15 min at 16,110 x *g*, pull liquid out with vaccum, air dry 15 min, and finally reconstitute in water.

The concentration of amplicon extracted from the gel for ligation into the vector was assayed by a Nanodrop ND-1000 Spectrophotometer (Montchanin, DE) and by visual estimation on an agarose gel against DNA samples of known concentration. The amplicon was observed to be at a concentration of approximately10 ng/µl.

2.6.3 Ligation into Plasmid Vector

A Qiagen® PCR Cloning kit (Qiagen Valencia, CA) was used for cloning of the amplicon into the pDrive cloning vector. Initially 2× ligation master mix, pDrive cloning vector, and the distilled water provided by the manufacturer were thawed on ice. A sample (4 µl of the 10 ng/µl purified amplicon) was added to the ligation mix which was a 3 molar excess of amplicon to vector. pDrive cloning vector (1 µl of 50 ng/ µl) was added and 5 µl of 2× ligation master mix was added last to have a total of 10 µl ligation volume. The mixture was then mixed briefly and incubated for 2 hrs in a 4°C refrigerator.

2.6.4 Transformation of pDrive plasmid

One tube of Qiagen EZ competent cells were thawed on ice and a tube of SOC medium were thawed to room temperature. Ligation mix (2 µl) was added to the competent cells (50µl) which were then mixed gently and placed on ice for 5 min. The competent cells with the ligation

mix were then heated in a 42°C water bath for 30 seconds without shaking. Next, the mix was incubated on ice for 2 min. SOC medium (250 µl) was added to the mixture. The transformants (100 µl) were then plated on Luria Bertani plates containing ampicillin (100 µg/ml), X-gal (40 mg/ml), and IPTG(100mM). The plates were allowed to incubate inverted at 37°C overnight.

2.6.5 Screening of Clones

The preliminary screening of the transformants was blue/white clone screening. A *lacZ*' gene is located at the cloning site of the pDrive cloning vector. The intact *lacZ*' gene codes for a subunit of β-galactosidase that allows the cell to metabolize X-gal. A colony that contains an uninterrupted *lacZ*' gene will turn blue and a colony that contains a *lacZ*' gene that is interrupted by the insertion of an amplicon is white. The plates were incubated at 4°C for 30 min to enhance the contrast between blue and white colonies.

The conformation screening was performed by PCR amplification using the M13 sequencing primers that flank the amplicon insert site on the pDrive vector. Ten white colonies and 2 blue colonies were selected. A sterile 10 µl pipette was used to touch a colony and transfer to a PCR reaction tube. Reaction volumes of 25 µl were used for M13 screening. The M13 PCR reaction was setup with the following volumes: 2.5 µl 10× buffer, 1 µl of 10 pmol/ul M13 Forward primer, 1µl of 10 pmol/µl M13 Reverse primer, 0.5 µl of 10 mM dNTP mix, 19 µl H_20, and 1 µl of 1.25 U/µl taq polymerase. *Taq* polymerase was added after the PCR block reached 94°. The thermocyler protocol was three cycles. Cycle one was a 2 min hot start at 94°C. Cycle

two was 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 min. Cycle two was repeated for 30 cycles. Cycle 3 was a 10 min extension at 72°C. The amplicons of the M13 colony screen were then ran on a 1.5% agarose gel to determine the size of the insert into the plasmid that the colony is carrying.

2.6.6 Plasmid Prep

A Qiagen® Plasmid Mini gravity fed prep (Qiagen, Valencia, CA) was used to isolate plasmid DNA for sequencing. Colonies that contained the insert of the size of interest were then cultured in 10 ml of LB broth overnight. An aliquot (3 ml) was pelleted by centrifugation at 16,110 x *g* in a microcentrifuge. The LB broth was poured off and the pellet was suspended in 0.3 ml of buffer P1 with RNase A. The pellet was mixed with the buffer. Buffer P2 (0.3 ml) was added and mixed with incubation at room temperature for 5 min. Buffer P3 was chilled before addition of 0.3 ml to the mixture. The tube was incubated on ice for 5 min. The tube was then centrifuged at max speed (16,110 x *g*) for 10 min and the supernatant was removed. The Qiagen-tip 20 gravity fed column was equilibrated with 1 ml of buffer QBT and allowed to empty by gravity flow. The supernatant collected earlier was added to the equilibrated gravity column and was allowed to flow through the silica resin column by gravity flow. The Qiagen-tip 20 was then washed four times with wash buffer QC to ensure purity of the plasmid for sequencing. The DNA was then eluted from the column by addition of 0.8 ml of buffer QF. The eluted DNA was collected in a sterile microcentrifuge tube. The DNA was then precipitated

with 0.7 ml of room temperature isopropanol and centrifuged immediately at 16,110 x *g* for 30 min in a microcentrifuge at room temperature. The DNA pellet was then washed with 1 ml of 70% ethanol and allowed to air dry to remove residual ethanol. The pellet was suspended in 20 µl of water and stored at -20°C prior to sequencing.

2.7 Sequencing of EaCoMT Gene

Purified plasmid containing the amplicon of interest was sent to Marshall University DNA Core Facility (Huntington, WV). M13 primer sequencing was performed with a LI-COR (Lincoln, NE) automated dye primer, simultaneous bi-directional sequencer. The sequence data were manually edited to remove possible automated mistakes.

2.8 Sequence comparison

Sequences generated were aligned using BLAST alignment software and were analyzed using T-COFFEE, Version_1.41. (http://www.ch.embnet.org/software/TCoffee.html)

2.9 16SrRNA Gene PCR Amplification from VCG4-FHD1-103-6

Primers 27F and 1513R (see section 6.1.2) were used to amplify the 16S rRNA from pure culture FHD1-103-6 which was isolated from VCG2-ANJ2-46-11. PCR amplification was setup

as follows:1 µl DNA template, 1 µl 20 mM dNTP mix, 5 µl 10× buffer, 1 µl of 10 µM 27F primer, 1 µl of 10 µM 1513R primer, 3 µl DMSO, 0.5 µl *Taq* polymerase, 37.5 µl sterile H_2O. One ml of VCG4-FHD1-103-6 microcosm was stored in 50% glycerol at 80 ºC and the remaining MSB was pelleted at 4000 rpm (2,880 x *g*) for 20 min. QiaAMP DNA purification kit (Qiagen Valencia, CA) was used according to manufacturer's protocol to purify genomic DNA. The annealing temperature used for 16S rRNA gene amplification was 58°.

2.10 Method of Sequencing the 16S rRNA gene amplicon

PCR products were labeled with a Big Dye Termintor kit (Applied Biosystems, Foster City, CA) using a standard labeling temperature program. The labeling reaction was then purified to remove unincorporated nucleotides. The purified labeled template was sequenced with both the 27F and 1513R primers (see section 6.1.2) on an ABI 310 automated capillary column sequencing machine (Applied Biosystems, Foster City, CA).

3.0 Results

3.1 Chloride Analysis Results VC Microcosm Generation 1 and 2

Chloride analysis revealed large accumulations of chloride in VC microcosm Generation 1 (VCG1) cultures after large feedings of VC (Figure 3.1). As high as 3-fold increases in chloride concentration were observed in VCG1-ANJ2-8-1 over a period of 42 days. VC microcosm Generation 2 was setup with inoculum from VCG1 with the addition of sterile and killed controls (Figure 3.2). Six of 8 of the VC G2 experimental cultures showed significant increases in chloride accumulation over the 47 day period. Culture VCG2-ANJ2-46-5 exhibited an accumulation rate of 4.4 ppm of Cl^- per day (Figure 3.3)

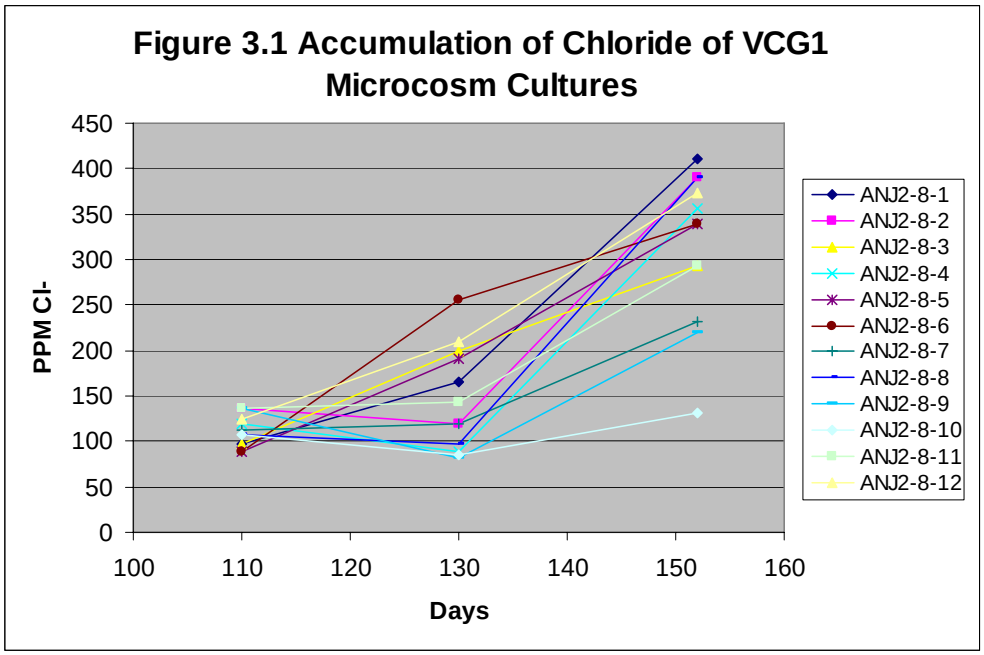

Figure 3.1 Accumulation of Chloride of VCG1 Microcosm Cultures. Microcosm cultures were setup with inoculum from aerobic chlorinated ethene bioreactor. VC was transferred to the headspace of the microcosm. No controls were setup in this generation. VCG1-ANJ2-8-1 through VCG1-ANJ2-12 were each setup with different 1 ml aliquots from aerobic bioreactor. Chloride concentrations were obtained by comparison of mV response of sample to mV response of known standards.

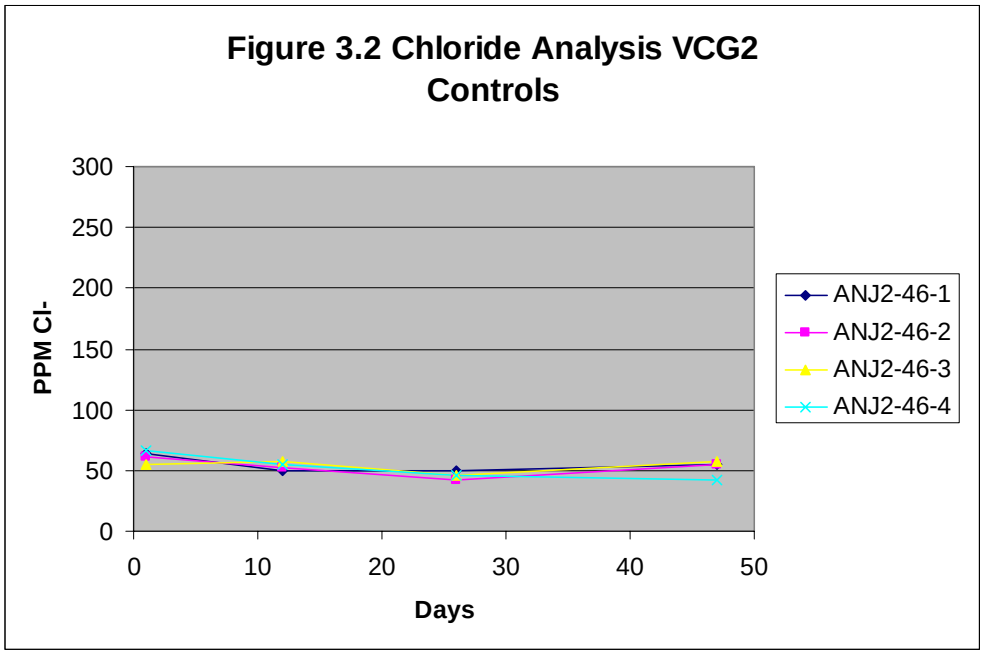

Figure 3.2 Chloride Analysis VCG2 Controls. VCG2-ANJ2-46-1 and VCG2-ANJ2-46-2 contained no inoculum but VC was transferred to the headspace. VCG2- ANJ2-46-3 and VCG2-ANJ2-46-4 contained autoclaved-killed inoculum with VC in the headspace. Chloride concentrations were obtained by comparison of mV response of sample to mV response of known standards.

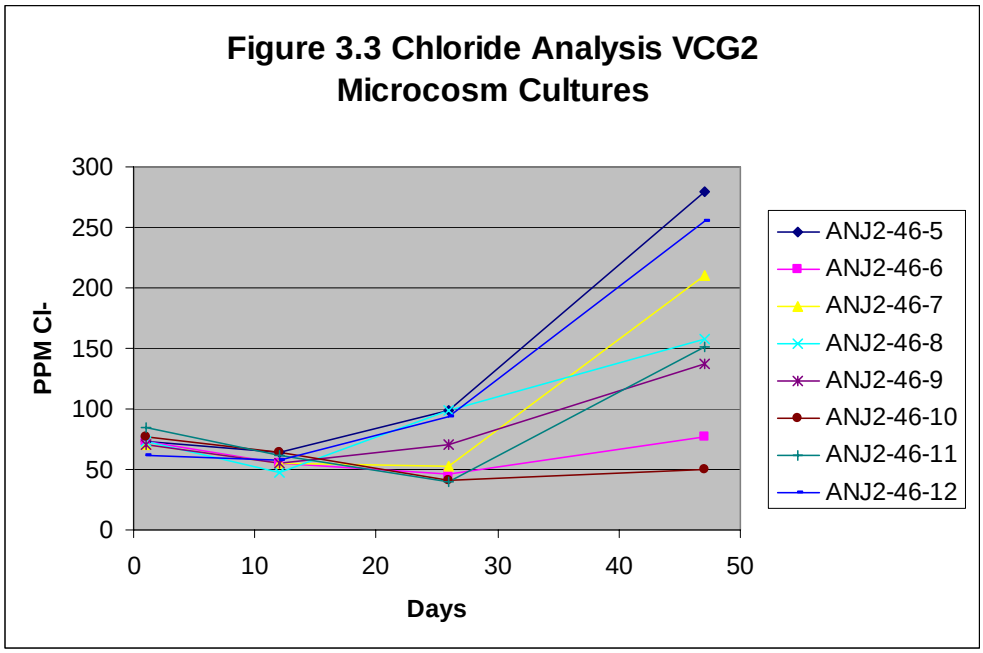

Figure 3.3 Chloride Analysis VCG2 Microcosm Cultures. VCG2-ANJ2-46-5 through VCG2-ANJ2-46-12 are subcultures of VCG1-ANJ2-8-5 through VCG1-ANJ2-12 respectively. Chloride concentrations were obtained by comparison of mV response of sample to mV response of known standards.

3.2 Chloride Analysis Results VC Microcosm Generation 3

Generation 3 cultures were attempts at growing pure isolates bearing the capability of degrading VC. The cultures were not mixed cultures as were generations one and two but they were isolated colonies that were grown on a nutrient rich medium (TSA). Colonies were selected based on colony morphology and grown to a higher biomass on TSB before inoculation into a MSB VC enrichment culture. No VC Generation 3 pure isolates from VCG1-ANJ2-8-3, which is a VC degrading mixed culture, accumulated any significant amount of chloride compared to the control cultures (Figure 3.4 and 3.5). Using chloride accumulation as a sign of metabolism of VC, no pure cultures were isolated with the ability to degrade VC.

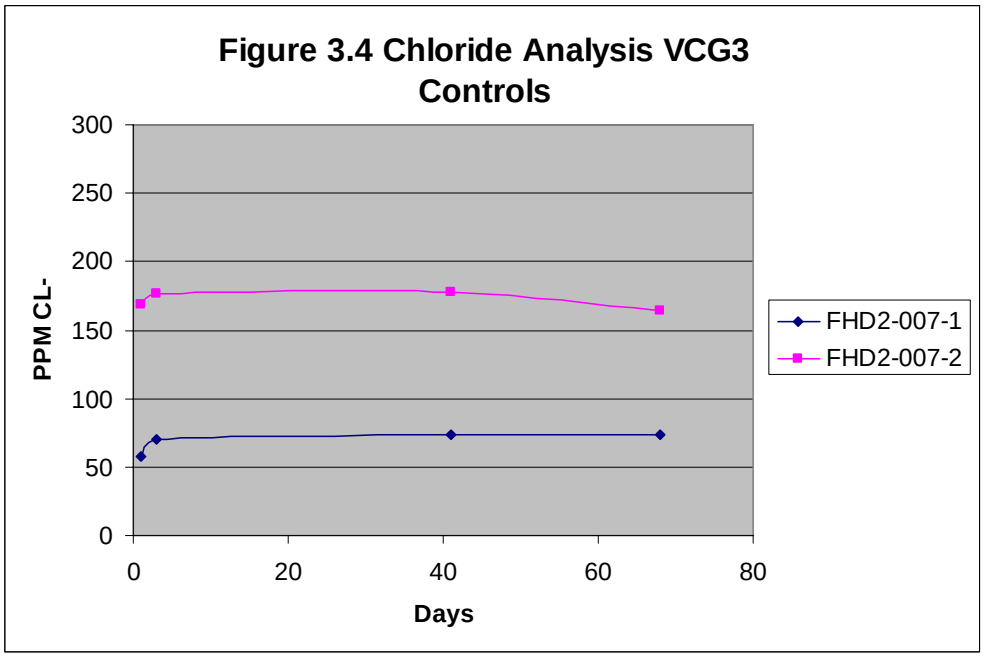

Figure 3.4 Chloride Analysis VCG3 Controls. VCG3-FHD2-007-1 is a control with no inoculum but VC was transferred to the headspace. VCG3-FHD2-007-2 contained autoclaved-killed inoculum with VC in the headspace. Chloride concentrations were obtained by comparison of mV response of sample to mV response of known standards.

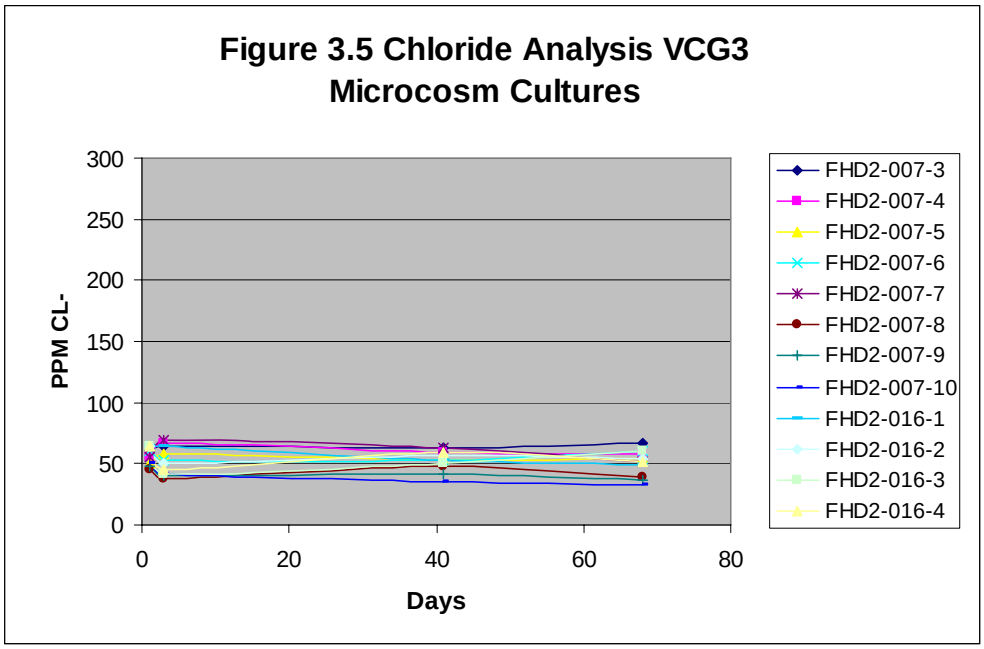

Figure 3.5 Chloride Analysis VCG3 Microcosm Cultures. VCG3-FHD2-007-3 through VCG3-FHD2-016-4 are pure cultures isolated on nutrient rich medium from VCG1-ANJ2-8-3 (VCG1 microcosm culture with EaCoMT gene present). Chloride concentrations were obtained by comparison of mV response of sample to mV response of known standards.

3.3 Chloride Analysis Results VC Microcosm Generation 4

Over the 27 day sampling period, increases as high as 50 ppm were observed in VCG4-FHD1-103-6 and VCG4-FHD1-103-12 (Figure 3.8). Optical densities were not recorded, but biomass accumulation was visually observed. VCG4-FHD1-103-6 and VCG4-FHD1-103-12 are pure cultures isolated from VCG2-ANJ2-46-11 which both stained Gram negative. VC Generation 4 culture FHD1-103-6 showed an average chloride accumulation rate of 1.9 ppm per day.

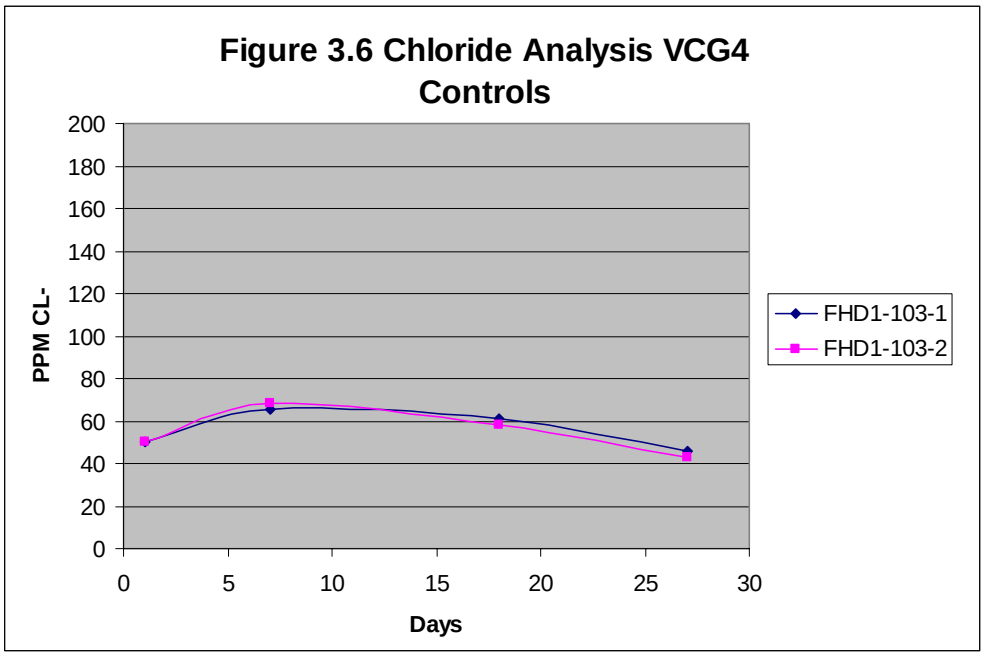

Figure 3.6 Chloride Analysis VCG4 Controls. VCG4-FHD1-103-1 is a control with no inoculum but VC was transferred to the headspace. VCG4-FHD1-103-2 contained autoclaved-killed inoculum with VC in the headspace. Chloride concentrations were obtained by comparison of mV response of sample to mV response of known standards.

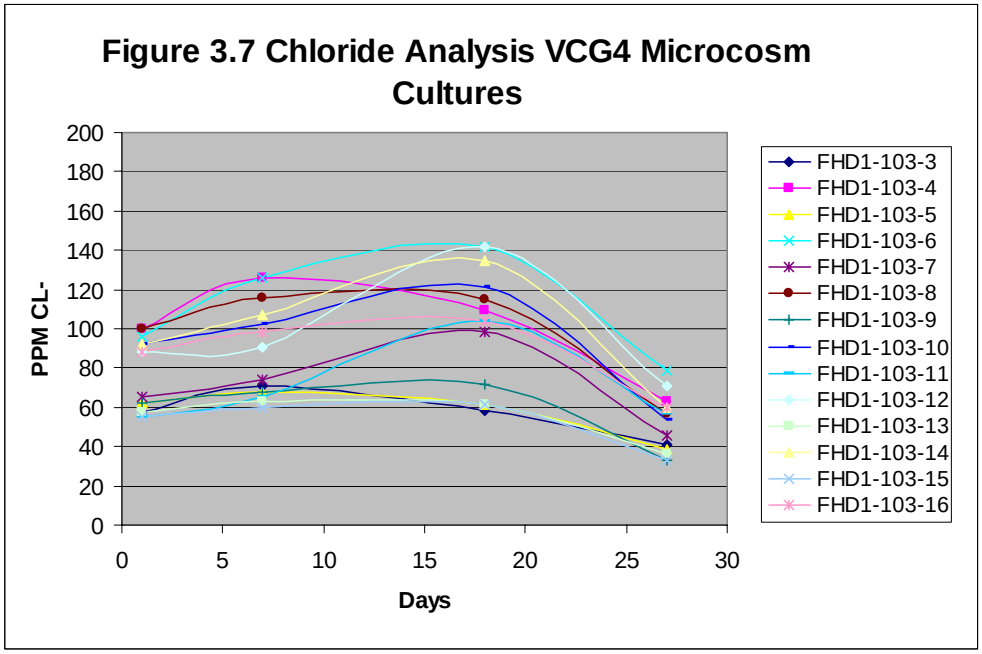

Figure 3.7 Chloride Analysis VCG4 Microcosm Cultures. VCG4-FHD1-103-3 through VCG-FHD1-103-16 are pure cultures isolated from VCG2-ANJ2-46-11 (VC degrading and Cl- accumulating mixed culture without the EaCoMT gene present). Chloride concentrations were obtained by comparison of mV response of sample to mV response of known standards.

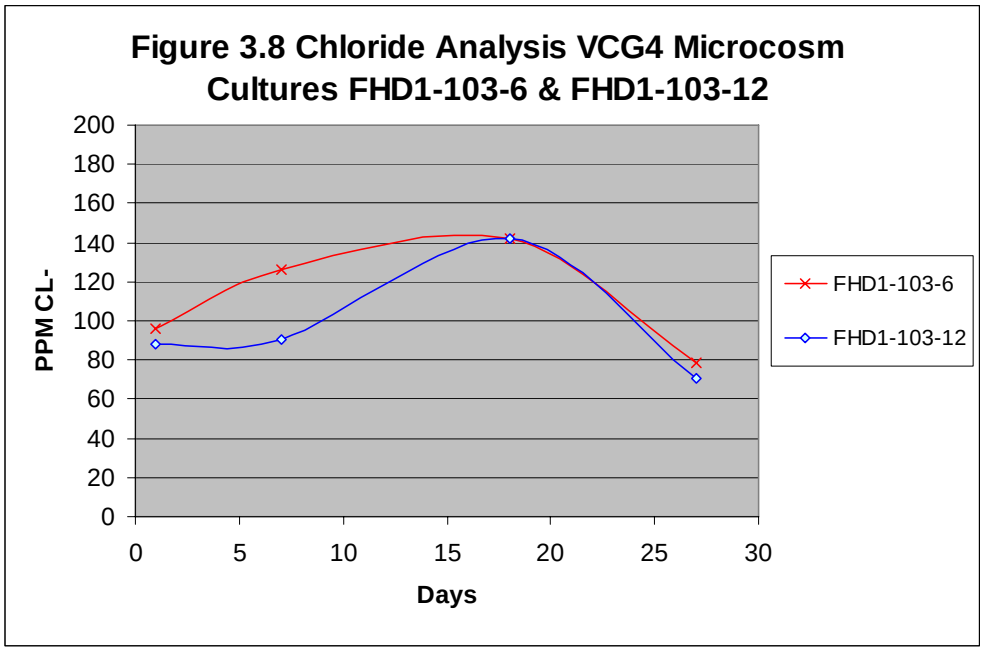

Figure 3.8 Chloride Analysis VCG4 Microcosm Cultures FHD1-103-6 and FHD1-103-12. VCG4-FHD1-103-6 and VCG4-FHD1-103-12 are pure cultures isolated from VCG2-46-11. Chloride concentrations were obtained by comparison of mV response of sample to mV response of known standards.

3.4 VC Degradation Kinetics of VCG2-ANJ2-46-11

Culture VCG2-ANJ2-46-11 was selected for a VC degradation kinetics study due to the high amount of biomass floating on the surface of the growth media. An initial estimate of VC degradation curves showed VCG2-ANJ2-46-11 had the ability to degrade VC, but the resolution of the measurements were poor (no figure). The problem was resolved by flushing the gas tight syringe between injections with solvent and ramping the GC oven to 150° to push off any VC residuals left in the system.

A clean serum vial was inoculated with 10 ml of VC sampled at atmospheric pressure at time zero and used as a control (Figure 3.9). Culture VCG2-ANJ2-46-11 was assayed at time zero to have 19 ppm of residual VC left in the headspace. VCG2-ANJ2-46-11 was then fed 10 ml at bottle pressure and assayed to contain 193 ppm of VC in the headspace (Figure 3.9). After 4.75 hrs 87% of the VC was lost from the headspace. The VC was degraded at a rate of 42 ppm per hr. The concentration of VC in VCG2-ANJ2-46-11 after 24 hrs was observed to be 26 ppm of residual VC. The VC concentration in the control vial did not significantly change. The average concentration of the control vial was 59 ppm of VC (Figure 3.9).

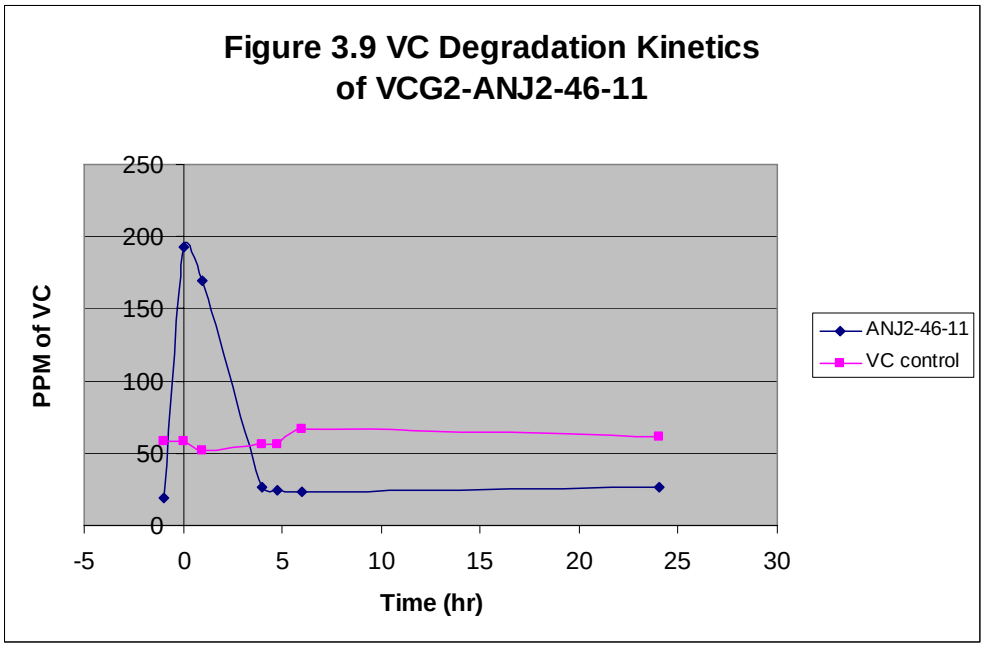

Figure 3.9 VC Degradation Kinetics of VCG2-ANJ2-46-11. VCG2-ANJ2-46-11 was fed 10 ml of VC at bottle pressure at time 0 hr. VC control was fed 10 ml of VC sampled at atmospheric pressure at time -1 hr. VC headspace concentration was monitored once per hr for first 5 hrs and then again at 24 hrs by sampling the headspace of the microcosm with a gas tight syringe and observing the electron capture detector response upon separation of analytes with gas chromatography. Headspace VC concentration (ppm) was calculated with slope equation from VC standard curve (Figure 2.4)

3.5 PCR Amplification of EaCoMT Gene from Mixed Culture ANJ2-8-3

A PCR amplicon of approximately 900 bases was amplified using EaCoMT specific primers (11) (see Appendix A section 6.1.1). A total of 47 reactions produced the 900 base pair fragment from culture VCG1-ANJ2-8-3. No PCR amplification using EaCoMT primers were observed using template from ANJ2-46-11 in a culture that has been shown to both accumulate

chloride and degrade VC. Inoculum from VCG1-ANJ2-8-11 was grown in TSB and inoculated back into MSB with VC added to the headspace. The resulting culture ANJ2-75-1 was able to accumulate chloride but no amplicon using EaCoMT specific primers was observed. Amplification from mixed culture was found to be possible either by boiling biomass before thermocycling or by lysing cells directly in the thermocycler.

3.6 Cloning of EaCoMT

Initially the unpurified amplicon produced with the EaCoMT-specific primers was transformed into the pDrive vector without the gel purification step. Screening of the first cloning attempt resulted in many different sizes of inserts. A single amplicon was viewed on the gel but a smear was located near the 900 bp amplicon. The second cloning attempt included recovery of the 900 bp amplicon from the gel to insure that the amplicon of interest was the only fragment ligated into the vector to be transformed into the competent cells. M13 PCR screening showed a 900 bp insert between the M13 primer sites in 8 of 10 white colonies selected and none of the blue colonies. Colony #7 was grown to the appropriate cell mass and the plasmid was recovered for sequencing.

3.7 Sequencing EaCoMT from Mixed Culture VCG1-ANJ2-8-3

A 496 bp read of the pDrive-EaCoMT #7 plasmid was first obtained. A second plasmid prep was performed to obtain the full length of the EaCoMT sequence (Figure 3.10). The full

insert (1113 bp) was sequenced from the pDrive-EaComT #7 colony plasmid on the second attempt. The sequence was 98% match to the *Mycobacterium mageritense* strain JS625 epoxyalkane:coenzyme M transferase gene which is the essential gene for the epoxide metabolism formed during VC degradation.

```
T-COFFEE, Version_1.41(Fri Jun 28 14:24:48 MDT 2002)
Notredame, Higgins, Heringa, JMB(302)pp205-217,2000
                    CPU TIME:0 sec.
                       SCORE=99

                    AY243043    :  99
                    Heath2      :  99

AY243043    --------------------------GGACACGGATTCGCTCAATTCCCCAAGGGGGAA
Heath2      AACTACCCCAACCCGCGCTGGTACGACGGACACGGATTCGCTCAATTYCCCA--RGGGAA

AY243043    TTTGTCTATGACGCGATCAGTAGGGAAGCGTTCGAAGATGCGGTTCTGGCCATCGTCCAC
Heath2      TTTGTCTATGACGCGATCAGTAGGGAAGCGTTCGAAGACGCGGTCCTGGCCATCGTCCAC

AY243043    GACCAAGAAGCGGCTGGACTCGACATCATTTCCGACGGCAAGGTCTATGGCGGGGACTCA
Heath2      GACCAAGAAGCGGCTGGACTCGACATCATTTCCGACGGC-AGGTCTATGGCGGGGACTCA

AY243043    CCGTATGCATCGATCATTTATCACTACTACGAGCGGATGACCGGATTCAGACCGTCGGGA
Heath2      CCGTATGCATCGATCATTTATCACTACTACGAGCGGATGACCGGATTCAGACCGTCGGGA

AY243043    ACGAATATCGGACTGCCGATCTATTCCACCCTGTACTCGCCGATCGTGGAATCCGAAGTG
Heath2      ACGAATATCGGACTGCCGATCTATTCCACCCTGTGCTCGCCGATCGTGGAATCCGAAGTG

AY243043    CGTCGCGAACATCCGTTCCATCTCGCGACGCTGCGAGCCACTCGGAAGGCAACGAAGAAG
Heath2      CGTCGCGAACATCCGTTCCATCTCGCGACGCTGCGAGCCACTCGGAAGGCAACGAAGAAG

AY243043    CCGGTCAAGGTGTCCTACGTGGGTATTCAGGTGCTGGCCGCTGCGGCGACGAACAATTTT
Heath2      CCGGTCAAGGTGT-CTACGTGGGTATTCAGGTGCTGG-CGCTGCGGCGACGAACAATTTT

AY243043    TACTCCGAAGAACGTGAACTGGGCATGGCGATCGCCAAGGCGTTCAAAGAGGACTTCAAG
Heath2      TACTCCGAAGAACGTGAACTGGGCATGGCGATCGCCAAGGCGTTCAAAGAGGACTTCAAG

AY243043    GAGATCGAGCAGAACGGCTGCGACATCATCCAACTCGACGAGTTCGTCTGGCCCTATGGC
Heath2      GAGATCGAGCAGAACGGCTGCGACATCATCCAACTCGACGAGTTCGTCTGGCCCTATGGC

AY243043    ATCGGCGACTGGGAGGTCGAGGCGATCAATTTCGCCATGGAGGGCATCAGCTGCGACTTC
Heath2      ATCGGCGACTGGGAGGTCGAGGCGATCAATTTCGCCATGGAGGGCATCAGCTGCGACTTC

AY243043    TGGGTACACACCTGCTGGGGAAACTACTCCGGCACGCCGGGTTATTTGCCGGAGGAGAAC
Heath2      TGGGTACACACCTGCTGGGGAAACTACT-CGGCACGCCGGGTTATTTGCCGGAGGAGAAC

AY243043    GAAAAGGAATTCGGTGCCTGGGTCCTGGATAAGAGGACCAGCCACTCGGCCGGAACTGAG
Heath2      GAAAAGGAATTCGGTGCCTGGGTCCTGGATAAGAGGACCAGCCACTCGGCCGGAACTGAG

AY243043    CGTGCGGCTGCAATCTTCCCGAAGGTGCTGCAAACCAATATCACCGCGTTGAACTACGAG
Heath2      CGTGCGGCTGCAATCTTCCCGAAGGTGCTGCAAACCAATATCACCGCGTTGAACTACGAG

AY243043    GTGGGTCGCACTGGGCCTGATGATCTCAAACCTCTCGTCGACAACAACTGGGACCGCCCG
Heath2      GTGGGTCGCACTGGGCCTGATGATCTCAAACCTCTCGTCGACAACAACTGGGACCGCCCG

AY243043    TTTGTCGCCGGTGTCATCGAC------------------------
Heath2      TTTGTCGCCGGTGTCATCGACGTCAAGAGCACGATCACCGAAACTG
```

Figure 3.10 Alignment of Heath2 to EaCoMT sequence of AY243043.
Heath2 sequence was obtained by amplifying an EaCoMT gene fragment from VCG1-ANJ2-8-3. The resulting amplicon was then cloned and sequenced. The sequence matches the EaCoMT gene sequence from known VC degrading strains. Highlighted bases are ambiguous bases.

3.8 PCR Amplification of EaCoMT Gene from Isolated Colonies

No amplicons were observed using EaCoMT specific primers from either isolated colonies on solid media or isolated colonies in MSB amended with VC even if the PCR conditions were empirically optimized.

3.9 PCR Amplification and Sequencing of 16Sr RNA gene from VCG4-FHD1-103-6

A single amplicon was obtained of ~1500 bp. Only ~100 bp of sequence using the 1513R primer was obtained. BLAST nucleotide sequence analysis did not yield a possible microbial match to a 16S rRNA gene. The ABI 310 automated sequencer was later serviced and the capillary column was replaced. No further data for this amplicon are available.

3.10 Summary of Results

Bacteria capable of mineralization of VC were isolated by metabolic ability (degradation of chloride) and by presence of metabolic end products (chloride). Organisms isolated were assayed for presence of genes which code for known VC degrading enzymes. Figure 3.11 summarizes the basic results of this research. Although many cultures were observed to accumulate chloride only five cultures were assayed positive for the EaCoMT genes. Also interestingly, all VCG3 cultures which are from isolated colonies on nutrient rich media from

EaCoMT positive culture VCG1-ANJ2-8-3 not did not have the ability to accumulate chloride but were negative for the presence of the EaCoMT gene.

Culture #	Parent Culture #	Approximate amount of VC fed*	Increase in Cl- (ppm per day)	EaCoMT gene presence
VCG1-ANJ2-8-3	----------	1000-2500	6.3	**Yes**
VCG1-ANJ2-8-4	----------	1000-2500	5.4	**Yes**
VCG1-ANJ2-8-5	----------	1000-2500	4.4	**Yes**
VCG2-ANJ2-46-5	VCG1-ANJ2-8-5	1300-3300	4.3	No
VCG2-ANJ2-46-6	VCG1-ANJ2-8-6	1300-3300	>1	**Yes**
VCG2-ANJ2-46-7	VCG1-ANJ2-8-7	1300-3300	2.7	No
VCG2-ANJ2-46-8	VCG1-ANJ2-8-8	1300-3300	1.6	No
VCG2-ANJ2-46-9	VCG1-ANJ2-8-9	1300-3300	1	No
VCG2-ANJ2-46-10	VCG1-ANJ2-8-10	1300-3300	>1	**Yes**
VCG2-ANJ2-46-11	VCG1-ANJ2-8-11	1300-3300	1.6	No
VCG2-75-2	VCG1-ANJ2-46-11	200-500		No
VCG2-75-3	VCG1-ANJ2-46-11	200-500	3.6	No
VCG3 (all cultures)	VCG1-ANJ2-8-3	200-500	0	No
VCG4-FHD1-103-12	VCG2-46-11	200-500	2.7	No

Table 3.11 Summary of choride results versus presence of EaCoMT gene.
* Approximate amount of VC fed is based on one 10 ml feeding of VC per week at bottle pressure which has been empirically shown to be between 200-500 ppm concentration when added to a 70 ml microcosm.

4. Discussion

4.1 Chloride Accumulation and Optical Density

Monitoring chloride accumulation proved to be an effective method for identifying VC degrading mixed cultures. Coleman and Spain (11) observed a *Mycobacterium* strain JS60 with a chloride accumulation rate of 40.25 ppm of chloride per day. Similar accumulation rates were observed by Verce et al. (34) in *Pseudomonas aeruginosa*. The average chloride accumulation rate of VCG2 degrading cultures was 2.7 ppm of chloride per day and the highest rate of accumulation was from culture VCG2-ANJ2-46-5 of 4.4 ppm of chloride per day. There is no standardization such as weight or number of cells in culture to compare the JS60 culture to the VCG2-ANJ2-46-5 culture. Standardization would be needed to compare these cultures because Coleman and Spain grew JS60 on ethene to build up a large biomass. Once a large biomass of ethene grown JS60 was achieved JS60 was cultured in the presence of VC. The optical density (OD at 600 nm) or biomass of culture JS60 was 0.05 before introduction of VC. The OD rose to 0.5 after ten days of degradation of VC. Growth on VC has been found to be much slower than on ethene. JS60 can grow to an OD of 1.0 in 70 hrs with ethane but growth on VC for 70 hrs only results in an OD of 0.06. Culture ANJ2-75-3 had a chloride accumulation rate of 3.6 ppm per day (Figure 4.1) and during a 94 day period only changed in OD by 0.06 (Figure 4.2). Hydrophobic cultures tend to grown on either the surface of the media or on a surface unlike

typical liquid cultures that replicated and fill the entire volume of the culture. Due to the hydrophobic nature of these organisms large increases in OD at 600 nm are not common. ANJ2-75-3 was fed 10 ml of VC (sampled at atmospheric pressure) three times which is approximately 300 ppm of VC fed. Stoichiometric VC to chloride observations were difficult to achieve because of the difficulty of administering known amounts of VC gas.

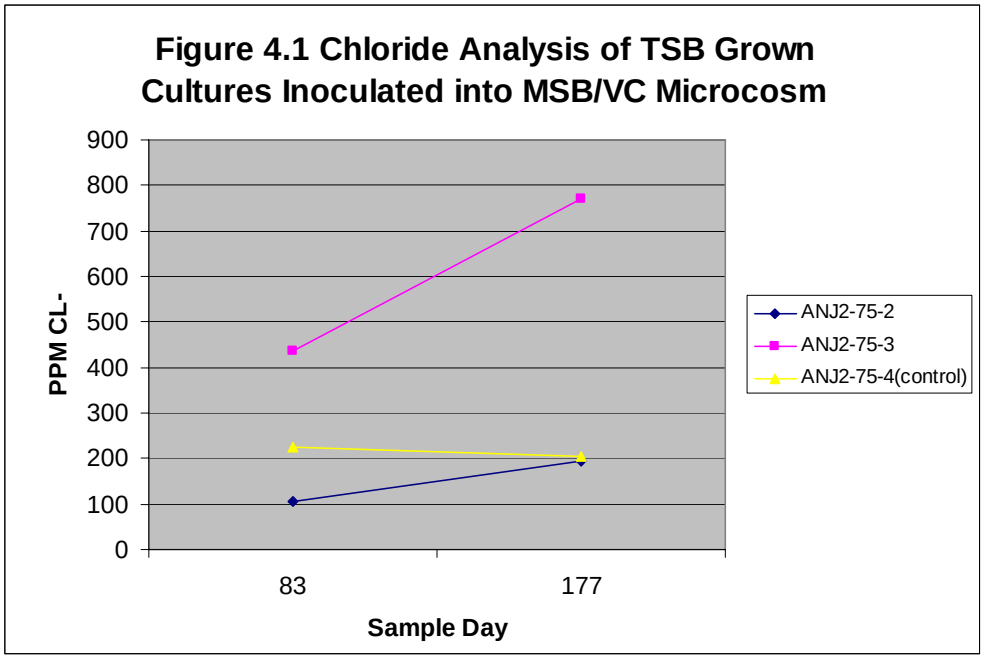

Figure 4.1 Chloride Analysis of TSB Grown Cultures Inoculated into MSB/VC Microcosm. ANJ2-75-3 is a microcosm culture that was established by growing inoculum from VCG2-46-11 on TSA and then inoculation back into a MSB/VC microcosm. Small OD changes (Figure 4.2) were observed chloride concentration increased 300 ppm over this sampling time.

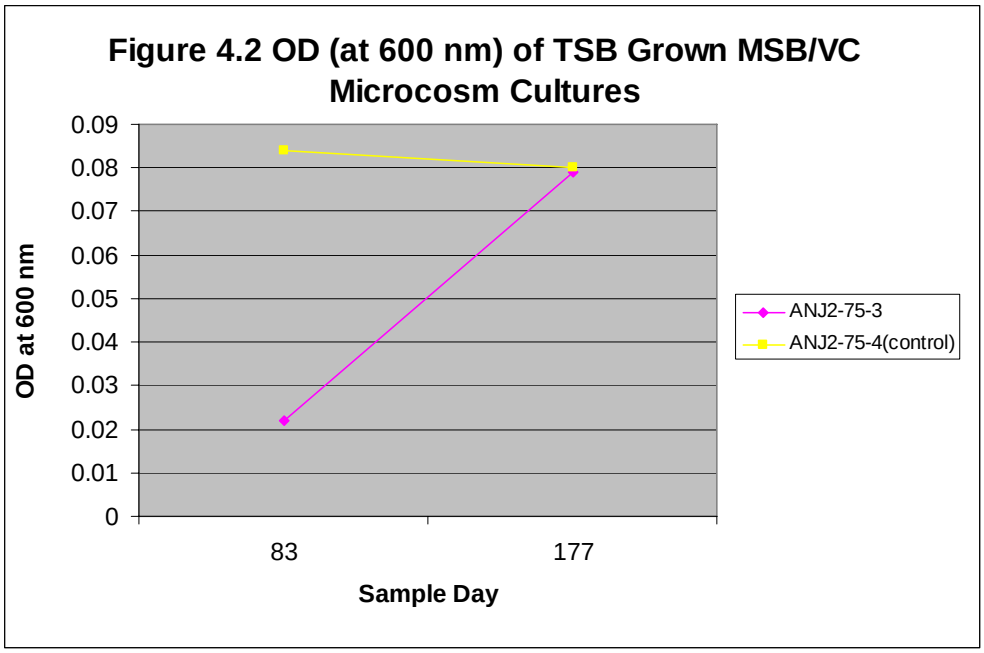

Figure 4.2 Optical Density (OD) at 600 nm of mixed TSB grown cultures. ANJ2-75-3 is a microcosm culture that was established by growing inoculum from VCG2-46-11 on TSA and then inoculation back into a MSB/VC microcosm.

VC Kinetic observations have been published numerous times, but the focus of this study was to identify VC degrading organisms from the aerobic bioreactor of a serial PCE degrading system. Coleman and Spain (11) described JS60's growth on VC as consumption and did not show the amount of VC fed to the culture. The difference in the amount of VC fed and the difference in starting optical densities should be able the account for the differences in chloride accumulation rates between *Mycobacterium* cultures such as JS60 and the VC degrading cultures observed in this study. Coleman and Spain (11) also used a precise lamp-combustion procedure (3) for chloride analysis which yielded a standard deviation of 0.1 ppm which is far more sensitive that the chloride ion selective electrode. Deviations aside chloride accumulation observations taken using a chloride ion selective electrode were useful in identifying VC degrading mixed cultures.

VCG3 of pure isolates from mixed culture VCG1-ANJ2-8-3, which is the mixed culture yielded amplification of the EaCoMT gene, were not observed to accumulate chloride. No pure isolates of VCG3 contain the EaCoMT gene that is found in the mixed culture that they were isolated from (VCG1-ANJ2-8-3). This may also suggest that the organisms responsible for VC degradation in the mixed culture (VCG1-ANJ2-8-3) may be noncultivable.

4.2 VC Degradation

Culture VCG2-ANJ2-46-11 was selected for VC degradation kinetics because of its high amount of floating biomass. Headspace VC concentration monitoring by GC-ECD was performed to assess the amount of VC consumed by the culture. VCG2-ANJ2-46-11 was able to decrease the headspace concentration of VC by 42 ppm per hr, but chloride accumulation was not measured during that same time course. The consumption rates of published strains JS60 and *Pseudomonas aeruginosa* strain MF1 were based on aqueous concentrations of VC. Gas chromatograph response to a headspace sample was calibrated to give the total mass of the compound in the bottle. The headspace and aqueous phases were assumed to be in equilibrium. VCG2-ANJ2-46-11 was a mixed culture that had been growing in the presence of VC for 163 days. *Pseudomonas aeruginosa* strain MF1 (34) was able to degrade more VC each time it was fed. With MF1 initial feedings were 166 ppm and it was able to handle feedings up to 456 ppm without any observed inhibition. MF1 showed an initial lag of 80 days before it began to degrade VC. MF1 was observed to degrade VC at a rate of 1 ppm per hr, but it is unclear if the kinetics experiment was conducted early in the culturing process or later (34). VCG2-ANJ2-46-11 showed a VC consumption rate of 42 ppm from the headspace, but comparing the VC consumption rate of VCG2-ANJ2-46-11 is subject to the caveat that only the headspace concentration was monitored as a sign of VC degradation in the culture. Oxygen may be the rate limiting factor for VC degradation. Strain MF1 could be starved of VC for 24 days and still resume consumption of VC unlike other mycobacteria. But if MF1 is deprived of oxygen for 2.5 days then it completely looses it ability to consume VC even if the culture remains viable. The

reason for this loss of metabolic ability is not understood (34). Kinetics on MF1 and JS60 where performed on cultures that had been growing for extended periods of time. Kinetics on VCG2-ANJ2-46-11 were performed after equilibrating the headspace of the vial to atmospheric levels of oxygen. Immediately after sealing the vial with the rubber butyl top and aluminum crimp, the VC concentration was assayed to contain a residual amount of VC (20 ppm) due to the partitioning of the gas between aqueous and gas phases of the microcosm. VC was then administered to the headspace of the vial and assayed for concentration. Oxygen availability could influence VC consumption rates which could explain the observed differences in VC consumption rate between VCG2-ANJ2-46-11 and other published VC degrading cultures. VC consumption was measured in VCG2-ANJ2-46-11, but is difficult to compare to previously published experiments due to the differences in the experimental design.

4.3 EaCoMT Sequence from Mixed Culture VCG1-ANJ2-8-3

The sequence of the EaCoMT gene amplified from mixed culture VCG1-ANJ2-8-3 was 98% identical to the *Mycobacterium mageritense* strain JS625 epoxyalkane:coenzyme M transferase gene which has been shown to be the essential gene for the epoxide metabolism during the aerobic degradation of VC. The sequence obtained (Figure 3.10 or see section 7.1) was a read through from the M13 primer sites on the p-Drive-EaCoMT plasmid. VCG1-ANJ2-8-3 contains an organism coding for the EaCoMT gene and the mixed culture also showed accumulation of chloride indicative of aerobic VC degradation.

4.4 Isolation of Pure Culture Containing EaCoMT Gene

Screening for the presence of the EaCoMT gene in isolated colonies was the proposed method for identification of a pure culture capable of growth on VC. Numerous amplification attempts on isolated colonies (from VCG1-ANJ2-8-3) grown on media such as TSA and R2A, never yielded an amplicon. Coleman and Spain (11) isolated their VC assimilating bacteria on MSA plates in the presence of VC, and this was the only way to culture the *Mycobacterium* species and retain VC assimilating ability, but Verce *et al*. (34) were able to culture the *Pseudomonas aeruginosa* strain MF1 on nutrient rich media such as TSA and still after growth on TSA retain the ability to assimilate VC. To date the EaCoMT gene has not be found in *Pseudomonas aeruginosa.*

Mixed culture VCG1-ANJ2-8-3 contains organisms that carry the EaCoMT gene in the presence of VC growing in MSB liquid media, but no pure isolated colonies which contain the gene have been found on nutrient rich medium. Two possibilities arise from this observation. The first is that the organisms carrying the EaCoMT gene are non-cultivable on nutrient rich media and the second possibility is that the organisms that carry the plasmid containing the EaCoMT gene lose the gene because the ability to assimilate VC is not necessary for survival thus permanently switching to metabolizing the nutrients in the medium. One possibility is that the organism(s) are noncultivible under these conditions. This is a common phenomenon, for

example, *Vibrio cholera,* the causative agent of cholera, is not cultivible on nutrient rich media (12).

Even though there is a possibility that the organism(s) carrying the EaCoMT gene are noncultivible, other information suggests that the genes for VC assimilation may be located on a plasmid and they might not be passed on in each generation if not selected for. The EaCoMT genes have only been found in *Mycobacterium* species when grown on MSB with VC as the only carbon source (Jim Spain personal correspondence). The hypothesis behind this mechanism is that once the VC assimilating bacteria such as *Mycobacterium* species are introduced to a nutrient rich medium they no longer use the pathway, so the plasmid is selected out of the culture. The organisms utilizing the nutrient rich pathways grow much faster and make up the majority of the colony growing on the media. Smith and Bidochka (29) found three important implications with relation to bacterial plasmids. First they found that bacteria with larger plasmids grow slower and have a significantly longer lag phase. They also observed that in nonselective media the rate of plasmid loss was greater when the plasmid was larger. In addition to the first two observations they also found that the copy number was lower the larger the plasmid was in length. Their implications fit well with the hypothesis of plasmid loss on nonselective medium. The organisms do not completely loose the plasmid but just the majority of the population does not have it because their fitness in the given environment is decreased due to the negative selective pressure for use of the metabolic pathway. It is favorable to possess the ability to assimilate VC when VC is the only carbon or energy source available, but it is not favorable to retain that ability when it slows the growth when nutrients are plentiful. Under

these circumstances it is more favorable to grow quickly while the nutrients for energy are present as opposed to growing slowly.

4.5 Isolation of Pure Culture Capable of Degrading VC from VCG2-ANJ2-46-11

Pure cultures capable of accumulating chloride were isolated from culture VCG2-ANJ2-46-11. Mixed culture VCG2-ANJ2-46-11 did not yield an EaCoMT amplicon despite exhaustive attempts, but mixed cultures of VCG2-ANJ2-46-11 were able to be grown on nutrient rich media but still retained their ability to accumulate chloride in the presence of VC. VCG4 pure cultures which were subcultured from VCG2-ANJ2-46-11 were capable of accumulating chloride in the presence of VC. More time and multiple feedings of VC would have allowed the accumulation of chloride to be as high as observed by previous experiments such as VCG1 and VCG2.

4.6 Identification of a Pure Culture Capable of Degrading VC

VCG4-FHD1-103-6 was selected to be identified by the sequence of its 16S rRNA gene due to its chloride accumulation, visible biomass accumulation, and due to its morphology which was characterized as the major constituent of the cultivable microbial diversity of VCG2-ANJ2-46-11. The short sequence obtained from the 1513R and 27F primers was not enough to assign the organism to a taxon. The set of 16Sr RNA primers used (1513R and 27F) were designed to amplify most of the gene. Primers could have been designed to amplify shorter sections of the

gene and then the sequences used to assign the organism to a taxon instead of attempting to amplify and sequence the entire gene at once.

4.7 Summary

A mixed culture VCG1-ANJ2-8-3 yielded an amplicon whose sequence identity matched the EaCoMT gene which has been previously characterized (10). No pure culture was isolated from VCG1-ANJ2-8-3 on nutrient rich media that would yield the same amplicon. Chloride, optical density, and VC degradation kinetics observations were useful tools in detecting of VC degradation in mixed cultures.

Pure cultures were isolated from VCG2-ANJ2-46-11 that were able to accumulate chloride but did not show the presence of the EaCoMT gene when assayed by PCR. The lack of amplification of an EaCoMT fragment suggests that the microbial constituents of VCG2-ANJ2-46-11 that could degrade VC may be using a novel pathway that has yet to be characterized in the published literature. This observation is the most novel finding of this study. VCG2-ANJ2-46-11 subcultures were capable of growth on a nutrient rich medium and could still degrade VC. This information suggests that the genes of VC degradation are carried on the chromosome or a plasmid which does not need selection to be propagated to the next generation. The major constituent of VCG2-ANJ2-46-11 stains Gram negative. Based on the Gram negative characteristic and that previously isolated organisms capable of degrading VC aerobically, it could be speculated that these organisms are of the *Pseudomonas* genera. The 16Sr RNA

sequencing failed to generate a sequence capable of identifying the genera or species of the isolated pure culture VCG4-FHD1-103-6. The identity of VCG4-FHD1-103-6 is unknown.

5.0 Additional Figures

Figure 5.1 Alignment of EaCoMT gene sequence AY243043 from Mycobacterium mageritense strain JS625 to Heath2 sequence from mixed culture ANJ2-8-3

```
T-COFFEE, Version_1.41(Fri Jun 28 14:24:48 MDT 2002)
Notredame, Higgins, Heringa, JMB(302)pp205-217,2000
CPU TIME:0 sec.
SCORE=99
*
 BAD AVG GOOD
*
AY243043   :  99
Heath2     :  99

AY243043   ---------------------------GGACACGGATTCGCTCAATTCCCCAAGGGGGAA
Heath2     AACTACCCCAACCCGCGCTGGTACGACGGACACGGATTCGCTCAATTYCCCANNRGGGAA

Cons                                  ******************** ****   *****


AY243043   TTTGTCTATGACGCGATCAGTAGGGAAGCGTTCGAAGATGCGGTTCTGGCCATCGTCCAC
Heath2     TTTGTCTATGACGCGATCAGTAGGGAAGCGTTCGAAGACGCGGTCCTGGCCATCGTCCAC

Cons       ************************************ ***** ***************
```

```
AY243043    GACCAAGAAGCGGCTGGACTCGACATCATTTCCGACGGCAAGGTCTATGGCGGGGACTCA
Heath2      GACCAAGAAGCGGCTGGACTCGACATCATTTCCGACGGCNAGGTCTATGGCGGGGACTCA
Cons        ************************************** ********************

AY243043    CCGTATGCATCGATCATTTATCACTACTACGAGCGGATGACCGGATTCAGACCGTCGGGA
Heath2      CCGTATGCATCGATCATTTATCACTACTACGAGCGGATGACCGGATTCAGACCGTCGGGA

Cons        ************************************************************

AY243043    ACGAATATCGGACTGCCGATCTATTCCACCCTGTACTCGCCGATCGTGGAATCCGAAGTG
Heath2      ACGAATATCGGACTGCCGATCTATTCCACCCTGTGCTCGCCGATCGTGGAATCCGAAGTG

Cons        ********************************** ************************

AY243043    CGTCGCGAACATCCGTTCCATCTCGCGACGCTGCGAGCCACTCGGAAGGCAACGAAGAAG
Heath2      CGTCGCGAACATCCGTTCCATCTCGCGACGCTGCGAGCCACTCGGAAGGCAACGAAGAAG

Cons        ************************************************************
```

```
AY243043    CCGGTCAAGGTGTCCTACGTGGGTATTCAGGTGCTGGCCGCTGCGGCGACGAACAATTTT
Heath2      CCGGTCAAGGTGTNCTACGTGGGTATTCAGGTGCTGGNCGCTGCGGCGACGAACAATTTT

Cons        ************ ********************** **********************

AY243043    TACTCCGAAGAACGTGAACTGGGCATGGCGATCGCCAAGGCGTTCAAAGAGGACTTCAAG
Heath2      TACTCCGAAGAACGTGAACTGGGCATGGCGATCGCCAAGGCGTTCAAAGAGGACTTCAAG

Cons        ************************************************************

AY243043    GAGATCGAGCAGAACGGCTGCGACATCATCCAACTCGACGAGTTCGTCTGGCCCTATGGC
Heath2      GAGATCGAGCAGAACGGCTGCGACATCATCCAACTCGACGAGTTCGTCTGGCCCTATGGC

Cons        ************************************************************


AY243043    ATCGGCGACTGGGAGGTCGAGGCGATCAATTTCGCCATGGAGGGCATCAGCTGCGACTTC
Heath2      ATCGGCGACTGGGAGGTCGAGGCGATCAATTTCGCCATGGAGGGCATCAGCTGCGACTTC

Cons        ************************************************************
```

```
AY243043    TGGGTACACACCTGCTGGGGAAACTACTCCGGCACGCCGGGTTATTTGCCGGAGGAGAAC
Heath2      TGGGTACACACCTGCTGGGGAAACTACTNCGGCACGCCGGGTTATTTGCCGGAGGAGAAC

Cons        ***************************** ******************************


AY243043    GAAAAGGAATTCGGTGCCTGGGTCCTGGATAAGAGGACCAGCCACTCGGCCGGAACTGAG
Heath2      GAAAAGGAATTCGGTGCCTGGGTCCTGGATAAGAGGACCAGCCACTCGGCCGGAACTGAG
Cons        ************************************************************


AY243043    CGTGCGGCTGCAATCTTCCCGAAGGTGCTGCAAACCAATATCACCGCGTTGAACTACGAG
Heath2      CGTGCGGCTGCAATCTTCCCGAAGGTGCTGCAAACCAATATCACCGCGTTGAACTACGAG

Cons        ************************************************************

AY243043    GTGGGTCGCACTGGGCCTGATGATCTCAAACCTCTCGTCGACAACAACTGGGACCGCCCG
Heath2      GTGGGTCGCACTGGGCCTGATGATCTCAAACCTCTCGTCGACAACAACTGGGACCGCCCG

Cons        ************************************************************




AY243043    TTTGTCGCCGGTGTCATCGAC-----------------------
Heath2      TTTGTCGCCGGTGTCATCGACGTCAAGAGCACGATCACCGAAACTG
```

Cons **********

Figure 5.2 Amino Acid Alignment of Heath2 sequence to AY243043

```
T-COFFEE, Version_1.41(Fri Jun 28 14:24:48 MDT 2002)
Notredame, Higgins, Heringa, JMB(302)pp205-217,2000
CPU TIME:0 sec.SCORE=100
*
 BAD AVG GOOD
*
AY243043   : 100
Heath2N    : 100

AY243043   GHGFAQFPKGEFVYDAISREAFEDAVLAIVHDQEAAGLDIISDGKVYGGDSPYASIIYHY
Heath2N    GHGFAQXPXXEFVYDAISREAFEDAVLAIVHDQEAAGLDIISDGXVYGGDSPYASIIYHY

Cons       ****** *  ********************************** ***************


AY243043   YERMTGFRPSGTNIGLPIYSTLYSPIVESEVRREHPFHLATLRATRKATKKPVKVSYVGI
Heath2N    YERMTGFRPSGTNIGLPIYSTLCSPIVESEVRREHPFHLATLRATRKATKKPVKVXYVGI

Cons       ********************** ******************************** ****
```

```
AY243043    QVLAAAATNNFYSEERELGMAIAKAFKEDFKEIEQNGCDIIQLDEFVWPYGIGDWEVEAI
Heath2N     QVLXAAATNNFYSEERELGMAIAKAFKEDFKEIEQNGCDIIQLDEFVWPYGIGDWEVEAI

Cons        *** ************************************************************


AY243043    NFAMEGISCDFWVHTCWGNYSGTPGYLPEENEKEFGAWVLDKRTSHSAGTERAAAIFPKV
Heath2N     NFAMEGISCDFWVHTCWGNYXGTPGYLPEENEKEFGAWVLDKRTSHSAGTERAAAIFPKV
Cons        ******************** ***************************************


AY243043    LQTNITALNYEVGRTGPDDLKPLVDNNWDRPFVAGVID
Heath2N     LQTNITALNYEVGRTGPDDLKPLVDNNWDRPFVAGV--

Cons        ***********************************
```

6. Appendix A

6.1 PCR Primers

6.1.1 EaCoMT Primers

Primer Name: CoM-F1L (11)
Sequence: AAC TAC CCS AAY CCS CGC TGG TAC GAC
Td = 73° (nearest neighbor method)
Tm = 77.8° (%GC method)
Tm = 82.0° [2*(A+T)+(C+G)]
nmol/OD = 3.94 (nearest neighbor extinction coefficient)
µg/OD = 32.5
Composition A+T = 10 37.0%
G+C = 14 51.9%
OD = 8.9 $_{A260}$

Primer Name: CoM-R2E (Coleman and Spain 2003)

Sequence: GTC GGC AGT TTC GGT GAT CGT GCT CTT GAC
Td = 83.9° (nearest neighbor method)
Tm = 82.2° (%GC method)
Tm = 94.0° [2*(A+T)+(C+G)]
nmol/OD = 3.65 (nearest neighbor extinction coefficient)
µg/OD = 34
Composition A+T = 13 43.3%
G+C = 17 56.7%
OD = 9.6 $_{A260}$

6.1.2 16SrRNA Primers

Primer Name: 27F
Sequence: AGA GTT TGA TCM TGG CTC AG

Primer Name: 1513
Sequence: ACR CCN ACC TAG TGG AGG AA

6.2 Reagents and Medium

6.2.1 Analytical Reagents

Reagent	Composition
Agarose Gel	50 ml 1× TAE buffer 0.75 g Molecular Grade agarose 2 µl 20 mg/µl Ethidium Bromide
Methanol	99.8% (GC assay)
Pentane	99+%
TAE Buffer	0.04 M Tris-acetate 0.001M EDTA
VC 200ppm standard	200 µg/ ml (200 ppm) Supelco certified standard
VC pure gas	227g gas cylinder 99.5% purity

6.2.2 Medium

Bacto (Liverpool, NSW, Australia) Trypic Soy Agar (TSA)	15 g Pancreatic Digest of Casein 5 g Enzymatic Digest of Soybean Meal 5 g Sodium Chloride 15g Agar
Bacto (Liverpool, NSW, Australia) Trypic Soy Broth (TSB)	30g TSB dry media 1 Lof nanopure quality water Autoclave @120°C for 20 min
Minimal Salts Broth (MSB)	38.8g K_2HPO_4 18.9g Na_2H2PO_4 20.0g $(NH_4)SO_4$ 1.0g $MgCl_2$ 0.1g EDTA 0.02g $ZnSO_4$ 0.01g $CaCl_2$ 0.05g $FeSO_4$ 0.002g Na_2MoO_4

	0.004g $CoCl_2$ 0.01g $MnCl_2$ Dilute to 10 L with nanopure H_20
Minimal Salts Agar (MSA)	1L MSB 15g Agar

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