Trichloroethylene Remediation by Engineered Soil Bacteria

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Trichloroethylene Remediation by Engineered Soil Bacteria

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Trichloroethylene (TCE) is a toxic pollutant that has become a widespread problem by seeping into groundwater across the developed world. Clean-up of sites contaminated with TCE is extremely difficult due to the absence of an efficient and cost-effective method for clean-up. Bioremediation efforts include a variety of potential microbial candidates with various metabolic capabilities as clean up options of contaminated sites. *Cupriavidus necator*, a soil bacterium was found to possess the ability to degrade TCE via a phenol-dependent pathway. Previous research by Ayoubi and Harker (1998) created a strain (MM02) capable of constitutive TCE degradation but the underlying genetic alteration causing constitutive production of the phenol hydroxylase pathway (PHL) and TCE breakdown was poorly characterized. We attempted to gain further understanding of the alterations that occurred in the PHL pathway to cause TCE to break down and replicate constitutive TCE degradation in a new strain with reduced foreign elements that may be introduced into the environment. Strain MM02 possessing this constitutive degradation activity and strain MM01 were sequenced and compared to discover the source of this variation. A 210 base-pair deletion in the beginning of the PHL operon was identified and is likely the cause of this altered activity. The new strain of *C. necator* (MM14) was created using traditional bacterial mating methods and included a cleanly introduced kanamycin resistance gene and its associated promoter which could drive constitutive expression of the PHL pathway. The TCE degradation abilities of strains MM01, MM02, and MM14 were evaluated through the TCE degradation assay and gas chromatography. We had difficulty accurately measuring the concentration of TCE due to its volatile nature and dramatically altered the method ultimately reducing variation and capturing TCE concentrations in assays. When accurate readings were obtained, none of the strains measured exhibited quantifiable TCE degradation activity when compared to controls. Our results showed .08% of the degradation by strain MM02 measured previously (P. J. Ayoubi, 1997). Based on our findings, we were unable to replicate the TCE degradation caused by MM02 and our genetically modified strain also failed to breakdown TCE.

Keywords: Trichloroethylene, *Cupriavidus necator*, phenol hydroxylase, bioremediation, suicide plasmid, tri-parental mating
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INTRODUCTION

Trichloroethylene (TCE) is one of the most commonly observed organic contaminants in groundwater in the developed world (Fischer et al., 1987; Ohlen et al., 2005). In the United States, TCE is present in more than 700 (out of a total of approximately 1,300) Environmental Protection Agency (EPA) Superfund sites (Chiu Weihsueh A. et al., 2013). TCE is extremely volatile, nearly insoluble in water, and highly miscible with a variety of organic solvents (Waters et al., 1977). Additionally, it is neither flammable nor explosive at room temperature. These properties make TCE beneficial in a wide variety of industries as a universal degreasing agent, extractant, spot cleaner, ingredient in paints and solvents, intermediate to refrigerants, and even anesthetic (Chiu Weihsueh A. et al., 2013; Ohlen et al., 2005; Waters et al., 1977; Wu C & Schaum J, 2000).

Production and use of TCE is now mostly restricted in the United States, but this wasn’t always the case. Worldwide production of TCE in 1973 was estimated to be 2.260 billion pounds (McConnell et al., 1975; Waters et al., 1977). As a result, TCE contamination is significant in air, soils, and subsequently groundwater in the developed world. Figure 1 shows a map of EPA Superfund sites across the country with known TCE contamination in groundwater. Most of these sites have been placed on the national priority list for superfund site cleanup. One study completed between 1988 and 1997 detected TCE (>0.01µg/L) in 10% of randomly sampled individuals in urban environments within the United States (Chiu Weihsueh A. et al., 2013; Wu C & Schaum J, 2000). In 1982 the same researchers detected TCE in 8/8 breast milk samples from urban areas within the United States (Wu C & Schaum J, 2000). These exposure levels are the result of widespread environmental TCE release. In 2001 TCE was only detectable in the blood of 1% of people tested in urban areas, indicating a decrease in exposure over time as use has decreased (Centers for Disease Control and Prevention, 2009). The downward trend of TCE release into the
environment is a positive sign. However, without methods in place to remove existing contamination, there is a lasting risk to health and limitation of important natural resources.

**TCE contamination in the environment**

Once released into the environment, TCE and its biproducts can be absorbed by and cause harm to anyone who is exposed to them (Wu C & Schaum J, 2000). In anoxic groundwater TCE is broken down into vinyl chloride, a known carcinogen, as well as other harmful compounds (Figure 2). This leaves the aquifer unfit for human use (Ordaz et al., 2017). In addition to harmful biproducts, TCE alone is harmful to the nervous system, liver, kidney, immune system, and reproductive organs. It is known to cause liver cancer, kidney cancer, and non-Hodgkin lymphoma (Chiu Weihsueh A. et al., 2013). Because of pressure put on regulators by producers of TCE, regulations weren’t even proposed until 2011 and the proposed total restriction of TCE manufacture and distribution is still being debated because of the chemical’s economic value (Shrader-Frechette & Biondo, 2020). Much of the environmental TCE released in the United States is a result of TCE being volatilized in vapor degreasing, incinerated with hazardous waste, leached out of landfills, or is a part of industrial discharge of wastewater (Wu C & Schaum J, 2000). In a toxicological review of TCE, the EPA reported that total TCE release into the environment in 1988 was greater than 57 million pounds; by 2010 the annual release rate was lowered to 2.4 million pounds (Chiu Weihsueh A. et al., 2013). The newly amended Toxic Substances Control Act puts into place regulations to further eliminate production, distribution, and use of TCE but contaminated sites still require treatment (Bergeson, 2000). Because of its widespread use, large quantities of TCE ended up in soils and groundwater where, because of its density and volatility, TCE saturates the soil as it migrates downward until settles at the bottom of aquifers as a dense non aqueous phase liquid (DNAPL) (P. J. Ayoubi, 1997; Freeberg et al., 1987). As a result of
TCE’s volatility, contamination often spreads in the form of plumes that require intervention to stop further spreading.

Environmental breakdown of TCE

In anaerobic conditions TCE undergoes reductive dichlorination (Figure 2) (P. Pant & Pant, 2010). In some environments TCE and its biproducts would continue to break down into ethene. However, without sufficient electron donors for energy metabolism or organic material to reduce other electron receptors TCE and vinyl chloride build up in groundwater (P. Pant & Pant, 2010). Without an effective method to further degrade these chemicals, the groundwater becomes unusable. Investigators have attempted to develop methods for removing these contaminants from the environment, but most are not suitable for wide-scale use (P. Ayoubi & Harker, 1998; Sims et al., 1992). The EPA has identified and sequestered sites of contamination as Superfund sites (Figure 1) to prevent human exposure, but cleanup is difficult and these sites often end up abandoned. These sites, along with many smaller sites, are inaccessible until work is presented that suggests a feasible method for toxin removal. The degree of the problem makes chemical or catalytic oxidation (common methods of chemical remediation) costly and ineffective as a means of treating contamination (Kim et al., 1996). As a result, bioremediation was deemed the most suitable method of TCE removal. This involves utilizing bacterial metabolic pathways to selectively remove toxins from the environment (Viebahn et al., 2009). Methods exist that could possibly tackle a problem of this scale if a clean degrader of these types of chemicals could be created (Sims et al., 1992). Modern genetic methods make the creation and application of these microbes as a biocontrol a viable option for TCE removal (Kern et al., 2007).
**Cupriavidus necator as a tool to degrade TCE**

*C. necator* has become a model organisms for biodegradation of chloro-aromatic compounds and is well known for its resistance to heavy metal contaminated environments (Pérez-Pantoja et al., 2008; Vicentin et al., 2018). It’s well characterized metabolic pathways have led to the discovery of its ability to degrade many common pollutants and have led to a great amount of research into a variety of uses in the field of bioremediation. *Cupriavidus necator* JMP134 was identified as a soil microbe capable of TCE degradation via two pathways, a chromosomal phenol-dependent pathway and a plasmid-encoded 2,4-dichlorophenoxyacetic acid (2,4-D) pathway. The phenol-dependent pathway is so designated because phenol is required as an inducer of the TCE degradation genes. These genes encode a multi-subunit phenol hydroxylase (PHL) enzyme. The genes encoding PHL activity are thought to be linked to a set of genes encoding catechol 2,3-dioxygenase activity (CD) (Harker & Kim, 1990; Kim et al., 1996). It is not understood whether these genes function as an operon or if they share a promoter, causing them to be expressed together. The PHL pathway contains all 6 genes essential to degradation of TCE and would be suitable for TCE degradation unlinked from the CD genes. A similar PHL enzyme in *Ralstonia sp.* KN1 was observed to degrade TCE by forming a TCE epoxide that would then be spontaneously degraded into glyoxylate, formate, or carbon monoxide. These biproducts are then broke down to water, carbon dioxide and other molecules (Ishida & Nakamura, 2000). This breakdown pathway is illustrated in Figure 3. The PHL pathway in *C. necator* is expected to break down TCE using this same pathway. The nontoxic biproducts of this process would be suitable for bioremediation of TCE in groundwater. Dr. Alan Harker and his group, seeking to utilize *C. necator*’s phenol dependence, sought to create a constitutive degrader of TCE by developing a version of the PHL gene cluster that does not require phenol induction and could safely be introduced to contaminated groundwater (Kim et al., 1996). To do this, strain JMP134 was cured of plasmid pJP4, containing
the 2,4-D pathway to produce strain AEO106. This ensured any PHL activity observed was due to the PHL pathway (Harker et al., 1989). A rifampin resistant derivative, AEO101 was also developed (Kim et al., 1996). This strain possesses both PHL and CD activity. To identify genes involved in TCE degradation, the cells were subjected to Tn5 transposon mutagenesis. The Tn5 transposon introduced kanamycin resistance. Transposon-containing strains that were observed to possess no detectable PHL or CD enzyme activity were confirmed to have transposons inserted at the same genetic locus. Strain AEK301 (MM01) was selected for further study. The genes responsible for phenol metabolism in JMP134 were then cloned by complementation, uncoupled from a regulatory gene, and sub-cloned into the pMMB67EH vector to create plasmid pYK3011 (P. J. Ayoubi, 1997). Plasmid pYK3011 conferred both PHL and CD activities. When pYK3011 was further digested using an XhoI restriction site, the sub-cloned plasmid pYK3021 conferred constitutive PHL activity as well as TCE degradation, but no observable CD activity. The separation of these genes indicated that genes involved in PHL activity alone are those necessary for TCE degradation. The reason for constitutive PHL expression is unknown.

We aimed to confirm the constitutive PHL activity exhibited by strain AEK301/pYK3021 (MM02) and examine the sequence to determine its cause. A better understanding of what caused PHL activity would allow it to be replicated in a strain that lacks foreign elements, making it suitable for in situ treatment of groundwater contaminated with TCE. We sought to expand on this previously completed work by implementing more recently developed methods. We expected that a functioning promoter inserted just prior to the PHL operon will produce constitutive PHL activity. Insertion of this identified promoter into strain MM03, MM01 that has been selected for streptomycin resistance, will create a clean strain to use in bioremediation efforts without introducing unnecessary foreign elements to the environment. Success in this research would
create methods to facilitate clean-up of complicated contamination sites. Methods of bioremediation are very limited in their abilities without further research into specific contaminants (Sims et al., 1992). Bacterial metabolisms can potentially mitigate other toxic contaminants if bacterial strains containing functional genes that remove these toxins from our environment were examined further (Kobayashi, 1995; Seo et al., 2009).

**METHODS**

*Bacterial Strain growth and analysis*

This study required growth and manipulation of a variety of strains of *C. necator* and *E. coli*. The bacterial strains included in this study are listed in Table 1 with relevant characteristics and antibiotic resistances. We obtained *C. necator* parent strains from Alan Harker and *E. coli* DH5α strains from Joel Griffitts. Antibiotics were used in the following quantities; kanamycin: 30 µg/ml; streptomycin: 200 µg/mL; chloramphenicol: 30 µg/mL; ampicillin: 100 µg/mL; rifampicin: 50 µg/mL. We grew MM01 and MM02 on MMO (minimal salts media) supplemented with 20 mM sodium citrate or phenol or on tryptone nutrient broth (TNB) selectively. We grew all strains in LB Lennox media (broth or 1.5% agar plates) if not otherwise specified. We incubated *C. necator* strains at 30°C, and *E. coli* strains at 37°C. When both strains were grown together, conditions for *C. necator* were favored as it was the strain that was to be used to create the final product of this experiment. We grew broth cultures on a shaker table at the appropriate temperature while shake at 180 rotations per minute. We prepared freezer stocks of all strains in 2 mL vials with 15% glycerol LB and stored them in replicates of 3 at -80°C. These stocks were used to produce fresh cultures whenever one was needed. We inoculated fresh cultures in autoclaved media and allowed them to grow overnight into the exponential growth phase with relevant antibiotics before experiments were conducted. We allowed freezer stocks of
*C. necator* two nights to grow to saturation in broth before fresh cultures were grown to use in experiments.

We sought to understand *C. necator*’s growth patterns in detail to ensure measurements were being taken when cellular activity levels were at their highest. This allowed us to measure the greatest rates of TCE degradation by each strain. We prepared growth curves for *C. necator* strains in LB broth with appropriate antibiotics to determine the length of time of bacterial growth phases. We prepared 50 mL cultures of each strain and 3 mL of each strain was removed to measure optical density every 2 hours over a 28-hour period. We took OD$_{600}$ measurements in clear 5 ml test tubes before the sample was returned to the culture vessel. We used the Eppendorf Biophotometer Plus spectrophotometer to take OD$_{600}$ measurements in. These were plotted against time to produce visual curves of the three stages of bacterial growth.

Before beginning our molecular work, we sought to better understand the work completed previously. To do this, we submitted strains MM01 and MM02 to the BYU Sequencing Center for full genomic sequencing. We extracted genomic DNA using the Qiagen genomic tip kit and associated protocol. The kit’s filter tips became clogged and we aided movement of liquid through the filter by adding a small amount of pressure through a syringe inserted at the top of the filter tube, being careful not to tear the filters. We then submitted the samples to the DNA sequencing center where prior to sequencing the DNA was sheared to appropriate length on a Megaruptor 2, underwent AMPure cleanup and library prep, was size selected on the Elf system from Sage science. Bacterial genomes were run as circular consensus sequence reads on a PacBio Sequel II system and were loaded onto the Sequel II according to SMRt Link software. Obtained genomes were analyzed on Geneious Prime 2021.0.3. We trimmed sequence files with BBDuk before completing a De novo assembly of the fragments.
Molecular work to create a clean constitutive degrader of TCE

We first sought to confirm that MM01 would accept selected DNA introduced by a simple mating. We used a Griffitts lab tri-parental mating method to mate strain MM10, MM05, and MM01. Each strain’s specific characteristics are described in Table 1. Strain MM10 contained a mobile mScarlet gene that if accepted into the recipient bacteria would cause colonies to fluoresce bright red. We spun down 2 mL dense culture of each strain at max speed in a centrifuge for 30 seconds, and resuspended each in .5 mL LB mixed with a pipette tip. To confirm the antibiotics had been totally removed from solution we then spun down the strains once more for 30 seconds at max speed and resuspended each in 200 µL of clean LB. We plated 70 µL of each strain on plain LB and allowed them to grow for about 5 hours before we moved them to a fresh plate containing kanamycin and streptomycin to select for cells that experienced a successful mating. We let these cultures grown for two days, waiting for individual colonies to appear. We checked the color of individual colonies to determine whether the mScarlet was successfully introduced. The gradient of color visible in the colonies was indicative of the plasmid copy number in that specific colony. Successful uptake of the mobile mScarlet plasmid, indicated by bright red fluorescence visible in the bacteria, would confirm that we were able to mate our plasmid into MM01 to drive TCE degradation.

After we confirmed that we would be able to successfully introduce genetic elements into C. necator, we selected a promoter to ligate and transform into MM01. We selected plasmid pJG194, a 2.2 KB easy to mobilize suicide vector containing a Kanamycin resistance gene to donate a promoter to drive TCE production (Griffitts & Long, 2008). We ligated a C. necator homology region including the start codon and about 20 base pairs upstream to the region immediately following the kanamycin resistance gene. In order to do this, we created primers
oMM01 and oMM02 (Table 2) to amplify the homology region from MM01 with the addition of EcoRI and HindIII restriction sites surrounding the homology region that we planned to integrate into pJG194. We ordered all oligos from Thermo Fisher Scientific and dissolved them to a 100 µM concentration in TE buffer. To amplify this region, we ran 40 µL PCR reactions containing 24 µL H20, 8 µL 5X Q5 buffer, 1.2 µL 10mM dNTP mix, .5 µL Q5 polymerase, 2.5 µL primer 1, 2.5 µL primer 2, and 1.3 µL template DNA. We then ran the reactions with the following conditions: 96ºC for 1 minute, 30 cycles of 96ºC, 65ºC, and 72ºC for 15 seconds each, 72ºC for one minute, and a 4ºC hold until the products were retrieved shortly after the cycle was completed. We loaded 3 µL of the PCR products with 3 µL loading dye to be checked on a 1% agarose gel made with TAE buffer and stained with Sybr Safe DNA gel stain. We stored any products needed for downstream application at -20ºC. We ran all gels at 150 volts for 30-60 minutes with appropriate ladders in TAE buffer before visualizing them on a UV light. We ordered all ladders and loading dye from New England Biolabs and SYBR Safe DNA gel stain from VWR.

We cleaned up all PCR products using an abbreviated column clean-up method using the Zymo Research plasmid mini-prep kit developed by Joel Griffitts. The modified protocol is as follows: Add 200 µL endo-wash buffer using a p-200 pipette. Pipet up and down 5-6 times before transferring contents to a spin column. Centrifuge at 13,000 for 30 seconds. Dump the flow through into a waste beaker and tap the tube upside down on a clean paper towel several times. Replace column and add 400 µL Plasmid Wash Buffer. Centrifuge for 30 seconds at 13,000. Move the column to a clean, labeled microcentrifuge tube. Add 50 µL of elution buffer and allow to stand for 1 minute. Centrifuge at 13,000 for one minute. Save flow through and test 3 µL on a gel to confirm desired product.
We mini-prepped the plasmid to prepare it for a restriction digest and ligation with the cleaned PCR product. We used the Zymo Research plasmid mini-prep kit according to the manufacturer’s instructions. We then performed a restriction digest on both the MM01 cleaned PCR product and the mini-prepped plasmid pJG194. We used restriction enzymes EcoRI and HindIII to cut the plasmid just after the kanamycin genes and strain MM01 around the desired homology region. These sites were introduced on primers oMM01 and oMM02 (Table 2) around the homology region in MM01. For each restriction digest, we created a 30 µL reaction containing 6 µL H2O, 3 µL 10x Cutsmart buffer, 17 µL DNA solution, 2 µL EcoRI enzyme, and 2 µL HindIII enzyme. The reactions were then incubated at 37ºC for 4 hours in a heat block. We verified all digests on a 1% agarose gel as described above. We cleaned the restriction digest products using the abbreviated method for the Zymo Research plasmid mini-prep kit.

We then used the prepared vector and insert to ligate the kanamycin resistance gene and homology region from MM01 together on plasmid pMM11. We ligated them by combining them in a 15 µL reaction containing 6.5 µL H2O, 1.5 µL 10x T4 ligase buffer, 3 µL cleaned vector, and 3 µL cleaned insert. We incubated the ligation at room temperature for 120 minutes. We also prepared a negative control containing the vector and TE buffer rather than the insert to confirm a successful ligation. After 120 minutes, we heat inactivated the ligation by placing it in an incubator at 65ºC for 5 minutes before proceeding directly into a transformation after which the ligation products were stored at -20ºC. We transformed the product of the ligation, pMM11, into chemically competent *E. coli* DH5α to create strain MM11 to use in a mating into *C. necator*. Chemically competent *E. coli* DH5α cells were provided by Joel Griffitts. We allowed the heat inactivated ligation to return to room temperature and added 7 µL to the chemically competent *E. coli* cells that had been thawing on ice for less than one hour. We immediately mixed the tube and
then placed it on ice for 7 minutes. We then immediately moved the cells to a float rack that was placed in a 42°C water bath for 42 seconds. We quickly returned the tube to the ice where it remained for 2 minutes. We added 500 µL of room temperature LB broth to the tube which was taped sideways on a shaker at 37°C for one hour. We plated 150 µL each of a 1:10 dilution in LB and undiluted culture on LB kanamycin plates. We dried the plates before being incubated at 37°C for 24 hours.

After 24 hours, we collected a clump of cells from the emerged colonies to check for a successful transformation using colony PCR. We collected each colony on a toothpick and spun it in 50 µL ddH2O to suspend it. We used a Dreamtaq master mix to run the colony PCR according to manufacturer protocols. We used 2.5 µL of primers oMM03 and oMM04 (Table 2) and 1.5 µL of the bacteria suspended in water in this colony PCR. We then ran the reactions with the following conditions: 95°C for 1 minute, 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, followed by 72°C for 5 minutes, and a 4°C hold until the products were retrieved shortly after the cycle was completed. Dreamtaq Master Mix was ordered from ThermoFisher Scientific. We checked PCR products on a 1% agarose gel with beside a 1 KB ladder from New England Biolabs.

We were then mated our plasmid, pMM11, from strain MM11 into MM12, a streptomycin resistant strain of C. necator. By doing this, we could select for pMM11 on LB plates containing appropriate levels of streptomycin and kanamycin. We grew MM05, MM11, and MM12 overnight into dense cultures in LB with the appropriate antibiotics. We moved 2ml of these cultures into microcentrifuge tubes that were centrifuged to pellet the cells at 13,000 RCF for 1 minute. We dumped the supernatant and placed the tubes upside down on a clean paper towel for 1 minute. After 1 minute, we tapped the tubes on the paper towels to remove any excess media and we
resuspended the pellet in 500 µL LB broth. In order to ensure we had removed any antibiotics present, we spun the resuspended cells at 13,000 for one minute before one more dumping the supernatant and tapping on a clean paper towel. We resuspended the cells in 200 µL fresh LB. We performed the mating by combining 70 µL of each cell type into a fresh microcentrifuge tube and plating 150 µL of the resulting solution was plated on LB agar plates without any antibiotics. We placed these plates in a 30°C incubator for 6 hours. After 6 hours, we pipetted 3 mL of LB with 15% glycerol onto the plates and used a sterile spreader to resuspend the cells into the broth. We plated 150 µL of this solution, a 1:10 dilution, and a 1:100 dilution onto LB agar plates containing appropriate concentrations of streptomycin and kanamycin. We incubated these plates at 30°C until individual colonies emerged. We placed the remaining solution in a freezer at -80°C for use if the dilutions did not yield individual colonies.

We sought to confirm that the product of this mating was a new strain of *C. necator* with a kanamycin resistance gene and its associated promoter driving the PHL operon and consequentially, constitutive TCE degradation. After 48 hours, we recovered colonies of our new strains and performed colony PCR using the procedure listed above with primers oMM04 and oMM05 (Table 2) to verify our desired product. PCR products were visualized using agarose gels electrophoresis and a 1 KB ladder. Each mating that was identified as successful was streaked to single colonies on LB plates containing Kanamycin and Streptomycin. From these we selected individual colonies to create permanent freezer stocks of the new strain MM14 by suspending a colony in 1 mL LB with 15% glycerol and storing them at -80°C in triplicate. We also submitted the amplified insert from strain MM14 for sequencing after cleaning it. We cleaned up the final colony PCR product abbreviated Zymo Research plasmid mini-prep protocol. After clean up, we submitted the DNA to Eton Biosciences with primer oMM04 for Sanger sequencing. The DNA
was pre-mixed with the primer and water before being same day shipped to Eton Biosciences who conducted Sanger sequencing on the insert. The received sequence was compared to the expected insert sequence using Geneious Prime 2021.0.3.

*Measuring TCE degradation by C. necator*

We first attempted to replicate the TCE degradation assay previously used with these strains of *C. necator*, but had great difficulty obtaining accurate measurements of any type. We prepared TCE stocks by gently pipetting 5 mL of TCE into a 160 mL serum vial filled with 150 mL ddH20. We immediately sealed the vial and avoided disturbing the solution in order to allow the TCE to remain in the bottom of the vial as a dense non-aqueous phase liquid (DNAPL). We allowed the stock to sit at room temperature for a minimum of 5 days to allow the TCE to dissolve into the water and create a saturated solution. Every version of the TCE degradation assay that was attempted required saturated solution inserted into vials containing cultured *C. necator* before degradation was measured.

Our first attempt with this assay was identical to the assay recorded in the methods of previously published work by Kim et al. (1996). We prepared cultures in MMO and inoculated strains with phenol and sodium citrate accordingly to induce TCE degradation by each strain. We then injected TCE into each vial through a septum. The vial was then mixed and the TCE was allowed to degrade over time. At a specified timepoint, N-pentane was injected into the reaction vessels to stop the reaction and allow TCE to be measured at that point. When this method was unable to produce consistent measurements, we began making changes in an attempt to reduce error. Because we were not interested in phenol dependent degradation and this requirement had been removed from strain MM02, we decided to eliminate this requirement in the degradation assay. By doing this, we anticipated eliminating any degradation by strain MM02 because this
strain requires phenol induction. This strain was expected to be a negative control in further testing. With this requirement eliminated, we switched over to using LB broth for simplicity. At this point we decided it would be prudent to only use the “no-headspace assay” described in previous work in an attempt to eliminate any volatile release of TCE and only measure concentrations observed in the liquid phase. Because TCE is an extremely volatile chemical, the no-headspace assay was originally developed to attempt to understand the types of loss that would be observed in a normal system. This assay is very similar to the TCE degradation assay described above, except in this case the bacteria and TCE are combined in a vial that does not contain any air. N-pentane is still required to stop the reaction and TCE samples are withdrawn to be measured by gas chromatography. This assay also failed to produce consistent results with any strain or in controls injected with only ddH2O and consistent levels of TCE.

When this assay also failed to provide consistent results from any strains or control vials, we reached out to labs currently using assays that measure co-metabolism of various chemicals by *C. necator*. We worked with Dr. David Freedman at Clemson University to develop a functioning TCE degradation assay based on current work in his lab. We adapted his protocols to work with our lab setup and resources. Figure 4 includes a graphical representation of our final methods for the newly developed TCE degradation assay. There were several elements of the assay that had to be changed because of variation of gas chromatography equipment between Dr. Freedman’s lab and our own. Most significantly, we were unable to take measurements in a time-series from a single vessel containing *C. necator* and TCE. In our method a new sample containing 25 µL TCE was prepared for each time point, and the measurements were taken in real time as the TCE was degrading. This introduced some variation between samples within replicates for a single strain of bacteria. In this protocol, we eliminated the requirement to stop the reaction and move TCE to a
separate vial to be measured. Elimination of this step should eliminate a significant source of variation observed in our measurements.

Our new protocol allowed TCE measurements to be taken in real time from a headspace vial containing cultured bacteria and TCE. In this method, we grew a 30 mL culture of *C. necator* in LB broth overnight before separating this dense culture into 4 headspace vials in 5 ml increments. 25 µL of TCE stock solution was then injected into the surface of the media before the vial was immediately sealed crimp sealed with a Teflon-butyl septum. Each vial’s starting concentration was expected to be around 250 µg/L TCE. The Km and Vmax for TCE degradation by MM02 whole cells were determined to be 630 µM and 22.6 nmol/min/mg of total protein, respectively (P. Ayoubi & Harker, 1998). Our concentration was selected because it was the starting point for the functioning degradation assay in Dr. Freedman’s lab that we modeled our assay after. After each replicate was prepped, we briefly shook the vials to ensure the TCE was mixed evenly throughout the vial. Blanks were also included containing consistent levels of TCE in ddH2O to represent TCE levels in this setting without any degradation. Total TCE concentrations were then measured in 1 hour increments using a Gas Chromatography flame ionization detector. Samples were heated beyond the boiling point of TCE before a headspace sample was extracted from the vial to quantify the concentration of TCE at that time point. TCE had an expected retention time of 3.357 minutes under these conditions. 4 replicates of each strain and the blanks were run over 4 days and the mean values at each time point were used for further analysis. 20 ml headspace vials and lids containing Teflon lined septa were purchased from Thermo Fisher Scientific.

Our new TCE degradation assay aimed to be able to confirm the TCE degradation activity previously observed in strain MM02 and compare it to the degradation activity of MM14
containing our newly inserted promoter. We compared the observed degradation activity of strains MM01, MM02, and MM14 to determine if the product of our experiment was eliminating similar levels of TCE as strain MM02 which had been previously measured. TCE degradation means were plotted in GGplot2 to visually demonstrate the levels of degradation that were being observed compared to the blanks. We analyzed the results in R using a linear regression to compare the slope of the different strains degradation. We also plotted the mean value of each strain at each time point with standard deviation.

We attempted to eliminate addition of multiple antibiotic resistance genes into the final product of this experiment by determining if selection could be conducted using C. necator’s higher copper tolerance. We expected that a certain level of copper introduced into media would be toxic to most strains of bacteria, but C. necator would be resistant, as suggested by the genus Cupriavidus. We compared growth of strains MM01 and MM05 in the presence of a gradient of concentrations of CuSO₄ to determine whether this was a feasible method for selection. We prepared a 1 M solution of CuSO₄ and grew overnight cultures of MM01 and MM05. Typical copper tolerance assays result in cell death of intolerant strains at a 200 µM-500 µM concentration (Shi et al., 2020; Vicentin et al., 2018). 4 replicates of each strain were inoculated with identical amounts in a gradient varying between 100 µM and 800 µM. We grew cells overnight and culture density was measured with a spectrophotometer. Cells that were unable to develop into dense cultures in the presence of the CuSO₄ were observed to be experiencing copper toxicity.

RESULTS

Confirmation of culture characteristics

Step one of the experiment was to confirm antibiotic resistance properties and the ability to grow each strain in appropriate media. Previous attempts by other labs to work with strains
MM01 and MM02 generated some concern about stock contamination and actual antibiotic resistance as opposed to that detailed in literature. In our testing, all strains grew as expected with antibiotics as specified in Table 1 in their designated media. Initial testing was conducted using MMO media and TSB broth as indicated in previous work (P. Ayoubi & Harker, 1998). However, cultures grown in LB broth with appropriate antibiotics grew to equal densities in the same period of time. LB Lennox broth was deemed sufficient to continue this experiment and maintain clean, viable stocks. Cells were checked under a microscope to confirm that the stocks were not contaminated by other visibly different bacteria and to verify that growing cells were rod shaped bacillus as *C. necator* should be. Microscopy and media growth techniques preceded later sequencing efforts to confirm that strains MM01 and MM02 were of clean stocks. Growth curves were produced for strains MM01 and MM02 to determine the appropriate length of time before these cultures were measured for TCE degradation these are shown in Figure 5. The exponential growth phase of *C. necator* lasts from 2 until 18 hours. The 16-hour growth phase was suitable to induce TCE degradation due to high rates of cellular activity. Therefore, the cultures were grown overnight before being manipulated or analyzed in TCE degradation efforts.

*Determination of further methods*

A variety of molecular methods were used to move in an insert into the desired strain of *C. necator*. The methods section describes the tri-parental mating that allowed a fragment of interest to be moved from a donor to a recipient strain by utilizing the mechanisms of a specific helper strain, in this case MM05. A tri-parental mating introduced a red fluorescent mScarlet gene on a plasmid with a high rate of replication into strain MM01. The colonies that were produced by this mating fluoresced bright red, representative of a successful mating. Thus, MM01 was a suitable recipient for manipulation via this type of crossover mating. Plasmid
pJG194 was selected to donate a kanamycin gene including its promoter into the region immediately preceding the PHL operon to drive constitutive expression. The movement required a ligation, and transformation of a PHL homology region into the plasmid to produce a donor containing both required mating elements.

The genus *Cupriavidus* implies a relatively high tolerance of heavy metals including copper. If copper tolerance could be used as a selective pressure, it would eliminate one of the antibiotic resistance genes required to select for the end product of a tri-parental mating. The copper tolerance assay described above was used to determine if this selective pressure was adequate to identify a clean product strain. When growth of MM01 and MM05 were compared in media containing concentrations of CuSO₄ in a gradient, there was no concentration tested at which *Cupriavidus necator* outperformed the control bacteria, *E. coli*. No difference in *C. necator*’s growth was visible in the presence of copper. Therefore, the experiment was continued using antibiotic resistance as a selective mechanism rather than copper tolerance.

Strains MM01 and MM02 were sequenced in an attempt to further confirm the identity of the bacterial stocks of MM01 and MM02, as well as understand in greater detail the work completed by previous researchers. The sequencing results demonstrated that the stocks did contain cultures of *C. necator* with the documented molecular changes from parent strain *Cupriavidus necator* JMP134 with plasmid pJP4 removed. MM01 and MM02 were confirmed to be very similar strains with a few expected minor differences. The Tn5 transposon inserted into MM09, the immediate predecessor to MM02, was located in the PHL operon region (Figure 7). This transposon was also confirmed to be absent in MM01, as expected. The deletion resulted in one of the more significant regions of variance between the sequences of these two strains. The homology regions between MM01 and MM02 are present in Figure 7. The other large
region of variation observed in these strains was the insertion to reintroduce PHL operon activity in MM02 via plasmid pYK3021. There was no identifiable mutation that explained the constitutive TCE degradation activity in MM02. There were small alterations throughout the PHL operon that may have conferred degradation activity, but no region was identified definitively to be the cause. Identification of the mutation that drove degradation was not necessary to accomplish the goals of this project. Instead, expression of the PHL operon to drive constitutive TCE degradation could be created by insertion of an active promoter into this region and measured by an assay that compares the degradation abilities of different strains.

Creation of strain MM14

A clean constitutive TCE degrading strain of *C. necator* was created through mating. The homology region at the beginning of the PHL operon in MM01 was successfully amplified using PCR, cleaned, and checked on a gel to confirm fragment length (Figure 6). MM07 was mini prepped and both the vector and insert were cleaned before being digested by EcoRI and HindIII. This digest was confirmed on a gel (Figure 6) before fragments were ligated together to create plasmid MM11. This plasmid was then transformed into *E. coli* DH5α and the product was confirmed on a gel. The expected product was around 600 base pairs and was visible on gel 3 in Figure 6. Once MM11 containing the kanamycin resistance gene and the PHL homology region was properly made, it needed to be mated into a strain of *C. necator*. Strain MM12, a streptomycin resistant version of strain MM01 was mated with MM11 to produce a kanamycin and streptomycin resistant strain with a kanamycin resistance gene and its promoter placed just before the PHL operon. The successful mating was verified using gel 4 in Figure 6. PCR was used to confirm the desired product. Primer oMM04 was a reverse primer located at the end of the integrated vector. Primer oMM05 was sequence native to MM01 located just before the
region where the insert was meant to be inserted. The resulting bright band confirmed that the insert was in the proper region of the genome and the entire insert was successfully introduced in the correct orientation. After confirming the mating was successful, the insert was sequenced to ensure that the correct genes were inserted. Sequencing efforts produced the expected sequence for this strain, deemed MM14. The kanamycin promoter was successfully inserted to drive expression of the PHL operon and phenol induction was eliminated to create a strain that, with further testing, could be introduced into the environment.

**TCE degradation assay**

To quantify the degradation of MM14 compared to the previously observed degradation in MM01 and MM02, all three strains were subjected to degradation assays. All assays were run with a negative control to ensure the bacteria was the source of observed diminishing TCE concentrations. This test took place in MMO media with either phenol or sodium citrate present to selectively induce TCE degradation. Replication as observed in previous work could not be replicated and it was difficult to contain consistent levels of TCE in the controls over time. TCE levels were highly variable due to difficulty maintaining consistent levels of TCE when switching liquids between containers and repeatedly pulling from stocks with a needle through the septum. Modifications were made as described above to limit error but this too produced results with enough error that no accurate comparison of degradation could be made as TCE was not being contained. It was assumed that the error originated in the loss of volatile TCE through seams in vials, septa, and syringes. The no-headspace assay was substituted for the regular TCE degradation assay in an attempt to eliminate loss of TCE through volatile gas release. The no-headspace assay eliminated the presence of air in the vials containing TCE but still required the addition of n-pentane to stop the reaction before an aliquot to be measured was moved into
sealed vials. The no-headspace assay was unable to produce consistent TCE measurements and it was decided that the test required further troubleshooting to produce meaningful data.

The development of an assay that allowed TCE measurements to be taken from the vial containing a culture of *C. necator* in its exponential growth phase, rather than attempting to stop the reaction or take multiple samples over time finally produced consistent results that could be compared between strains. This required a new sample to be prepared for each time point at which a measurement was to be taken. They were inoculated with identical amounts at the same time and measured at one-hour increments. Using the new degradation assay, consistent measurements were obtained from each strain as well as a control vial containing only ddH2O and TCE at a set quantity. Figure 8 shows the results of all strains in the degradation assay. The measured concentrations of all strains varied between .69 and 1.74 mM. The variation is largely due to differences error as observed in the controls and did not decrease over time as expected.

None of the three strains produced significant degradation especially when compared to the degradation measured in previous research efforts. To determine degradation, a linear regression was conducted on each strain and the slopes of the lines, indicating degradation, were compared to each other. Individually, there was no relationship between the concentration of TCE and time. Table 3 shows statistical values associated with each of these tests. The p-values for the relationship between each strain and time ranged from .11-.58 indicating that this relationship was not significant. Additionally, a linear regression was conducted with time and strain as explanatory variables for the difference from the original concentration. The interaction between time and strain had a marginally significant relationship (F = 12.98, P = .08657, R² = .3952). The equation found to best represent this relationship was Change in concentration = 1.017 + .0093(Strain) − .041(Hour) + .0021(Strain x Hour). In strains MM01 and MM02
there was a downward trend from hours 1-3 where concentrations for both strains decreased about .8 mM from the initial concentration. There was no observed degradation in strain MM14 over this time period. Comparison to rates of degradation previously observed in previous work showed any observed decrease was insignificant as it was .08% of degradation observed in strain MM02 before (P. J. Ayoubi, 1997).

DISCUSSION

Creation of strain MM14

Work attempting to apply the properties introduced by Ayoubi et al. struggled to confirm the presence of the claimed TCE degradation activity in MM01 and MM02 and introduced questions about the antibiotic resistances present and whether the stocks were pure cultures or if contamination had occurred (FJ Management, 2016). Our work confirmed that we could grow clean bacterial cultures and the strains possessed the recorded antibiotic resistances as displayed in Table 1. In addition, we confirmed that we would be able to maintain cultures in LB Lennox broth, which greatly simplified the remainder of the experiment. Previous work was completed using MMO and TNB media supplemented with minerals to promote different levels of cellular activity selectively. The change in media was an option because previous work had eliminated the need for aromatic induction by phenol of TCE degradation activity in strain MM02. We assumed that with this change of media strain MM02 would maintain constitutive degradation of TCE, while strain MM01 would lose (or exhibit reduced) activity in plain LB. The controls that were present in each experiment were attempts to verify these expected activity levels and provide a solid comparison for the newly created strain MM14.

We sought further clarity on the cellular components and PHL operon activity levels of strains MM01 and MM02 by sequencing their complete genomes. The results of these
sequencing efforts confirmed the identity and composition of these strains. We checked for expected sequence elements in each and compared them to one another to confirm that the work recorded by Ayoubi et al. properly represented the current strains (1997). There is not an explanation for how the MM02 is able to degrade TCE constitutively, this activity was fortuitously discovered and has not been well characterized. We attempted to better understand this activity by examining sequence obtained for strain MM02 compared to strain MM01 as well as reference database records of the PHL operon in *C. necator*. The differences between these strains in the region of the PHL operon are all listed in Table 4. The most significant difference observed in strain MM02 (which possesses the unexplained constitutive activity) was the deletion of bases 1-212 of the reference sequence of the PHL operon in *C. necator* (P. Ayoubi & Harker, 1998). The segment was present with high fidelity in strain MM01. This 212 base-pair region is known to contain a miscellaneous binding site and the promoter for the PHL operon. We were unable to determine why deletion of this promoter created constitutive expression of this operon. The other differences between the reference sequences and our observed strains were minor consisting of 1-2 base-pairs. Both strains had GC substitution at bp 5771. Any significant changes in gene expression are likely the result of the 212 base-pair deletion and not any of the other 1-2 base-pair minor differences that are noted.

After determining that the strains had not undergone alteration or contamination, we wanted to confirm that *C. necator* was a willing recipient of DNA fragments introduced in a simple mating. Previous work with *C. necator* JMP134 indicated that this was a possibility without too much difficulty (Saavedra et al., 2010; Tsutsui et al., 2009). The insertion of a replicating plasmid containing an mScarlet protein confirmed with brightly fluorescing colonies that mating a plasmid into MM01 could be done without any notable troubles. We then began
searching for a promoter that could be inserted prior to the PHL operon. A very simple suicide plasmid containing a kanamycin resistance gene was selected because it was likely that this gene would remain in its place after being moved into the *C. necator* genome which would limit transfer into other organisms in any environment it was introduced to. This plasmid, pJG194, could drive PHL expression with the promoter attached to the kanamycin resistance gene. This promoter would drive kanamycin resistance and as a result cause constitutive TCE degradation by activating the PHL operon constitutively.

A tri-parental mating identical to the one that introduced the mScarlet gene into *C. necator* was conducted to move the kanamycin resistance gene and homology region from the *E. coli* strain they were mated into strain MM01. The donor strain, *E. coli* DH5α containing pMM11, which includes the kanamycin resistance gene from pJG194 and PHL homology region was prepped with the end goal of completing this mating. It was designed as a suitable partner to move this plasmid into strain MM01 and create the desired activity. After the mating, the insert was sequenced to confirm its orientation and identity. Analysis of this insert showed the kanamycin gene was properly introduced in the region immediately before the PHL operon. The EcoRI and HindIII restriction sites were each present in the sequence that was obtained. This sequence was obtained with sanger sequencing and a single primer, as a result the quality of the sequence goes down over the length of the run and there are more possible errors in the later end of this sequence, but the genes and promoters that this project sought to identify were all confirmed to be present.

The end product of this molecular work was confirmed to be the insertion of a promoter just before the start codon of phlK, the first gene in the PHL operon, in strain MM01. The cross that should have resulted in this product is represented in Figure 9. This cross should have
upregulated operon expression and as a result TCE degradation should be constitutively expressed at a high rate as shown in the figure. Assuming the work done by Ayoubi et al. stands, this strain of bacteria, which we have labeled MM14, would be capable of degrading TCE contamination in the environment if the bacteria is provided with required nutrients for growth. This strain was the end goal of this project, however further work led to questions about its ability to actually degrade TCE.

Measuring TCE degradation

We attempted to recreate the TCE degradation assay using methods explained by Ayoubi et al to quantify degradation (1997). As explained in the methods section of this paper, the assay was modified multiple times to reliably measure the concentration of TCE in control vials. The modifications we made included changing the media required for this assay because we weren’t concerned about phenol induction of degradation activity, eliminating headspace, eliminating n-pentane to stop the reaction therefore requiring measurements to be taken in real time, and closing the cap immediately after inserting TCE in the vial through a gas-tight syringe rather than injecting through the septum and creating a hole. We eliminated the movement of TCE between vials in an attempt to prevent gaseous TCE from escaping. Each of these alterations were in an attempt to reduce error and be able to reliably measure TCE in vials over time, ultimately, we were able to take measurements of TCE. However, we were unable to replicate the degradation results that were observed previously. Dr. Harker’s work indicated that strain MM02 was able to degrade TCE from an initial concentration of 80 µM over a period of 180 minutes, at which point TCE concentration in a sample was measured to be 0 µM. Figure 8 shows the results of our degradation assay. After a period of 240 minutes, or 4 hours, there was no significant difference in degradation between any of the strains and the control. Some of the
problems we encountered were due to differences in equipment between what was used in previous work and what we currently have access to. We made as few modifications as possible in these areas. Others were the result of lack of detail in what protocols were available or inability to obtain precise measurements. This is where the greatest degree of change originated. We did all we could to maintain the tests as they were written, adjustments that were made were suggested by researchers who commonly work in these areas to put our work more in line with standard practice in the field.

Both the original assay and the assay with modifications failed to detect any significant degradation of TCE by any of the three strains. The margin of error we observed in controls containing only TCE and ddH20 was significantly reduced in the final assay. The results of this assay are represented in Figure 8. The differences in starting concentration were due to the nature of the TCE stock that was used to start each round of testing. Previous work indicates that the differences we observed between starting concentrations should not have significantly affected the degradation activity we observed. None of the strains showed significant degradation when compared to the degradation measured in MM01 and MM02 previously. Strains MM01 and MM02 had a marginally significant decrease in concentration from hours 1 to hour 3. However, this degradation is not significant when compared to expected levels of degradation. Strain MM14 demonstrated no change in level of degradation observed. The trends observed visually in Figure 8 were not supported by error margins or p values when the data was subjected to statistical analyses.

One concern of our project was that we were attempting to measure an activity we weren’t certain was present with a test that we weren’t sure could work. We attempted to coordinate work with other labs that possess functioning TCE degradation assays that measure
activity similar to what we were hoping to observe. We were unable to coordinate an opportunity
to send cultures of strains MM01, MM02, MM14, and a control to be tested. Before concluding
that TCE degradation activity is not present in any of the strains, we suggest testing for TCE
degradation activity in a lab that has a functioning assay and the chromatography equipment to
get reliable measurements. Additionally, our study would benefit from an assay that was able to
measure byproduct breakdown in addition to TCE degradation to conclude that toxins were
totally removed from the system and C. necator was suitable for environmental application. Our
work was based on observed pathways and expected protein behavior, but this work could be
validated more definitely. Research also indicated that in some cases enzymes are mass produced
in industry to make use of the activity extracellularly (Karigar & Rao, 2011). If work done on the
PHL operon indicated that this activity could be isolated, it seems possible that this enzyme may
be mass produced to obtain the desired activity rather than culturing large quantities of bacteria
to make use of this enzyme. This could potentially eliminate undesirable side effects and limit
transfer of bacterial DNA within the environment.

**Application in Bioremediation**

Over the last several decades, genetically engineered microorganisms have become more
prevalent in bioremediation efforts. The ability to select for specific resistances and metabolism
creates a much wider variety of organisms suitable for contaminated environments and common
pollutants. Genetic manipulation of bacteria for bioremediation is safer and much more cost
effective than many of the other commonly used techniques (G. Pant et al., 2021). The efficacy
of this type of work is limited by environmental condition in the area where the pollutant is
located. The pH, concentration, temperature, osmotic and hydrostatic pressure, salinity, and other
factors all affect the type of bioremediation that can occur within a contaminated site (Chen et
al., 2016; G. Pant et al., 2021; Sims et al., 1992). Bioremediation efforts of this type are greatly affected by the concentration of the chemical of interest, other toxins present in the environment, and other factors affecting growth and metabolism (Azubuike et al., 2016; Jan et al., 2014). In addition to limiting mechanical and chemical capabilities at bioremediation sites, these factors can influence the microbes that are able to grow in these environments. It is often preferred to promote growth of native bacteria over longer periods of time to allow pollutants to be broken down (L. Liu et al., 2019). Mechanisms exist that allow injection of nutrients and mixing of the contaminated area to facilitate treatment (Lee et al., 1988). Because of the complex chemical nature of these sites and limited microbial growth, this is not always a possibility. However, the variety and complexity of sites of contamination make even existing processes difficult to apply in many cases (Thomas & Ward, 1989).

Due to the complex nature of these sites, there is a significant amount of work that must be spent to determine if a method of bioremediation is suitable for a particular site (Wu et al., 2019). TCE contamination occurs in many similar sites at similar concentrations (citation). These similarities mean that large scale studies could be conducted to determine appropriate application techniques for a bacterial degrader like what we were attempting to create. Some of these studies have already been conducted as many techniques have been attempted. The TCE existing in soil and groundwater often volatilizes and spreads through soil as a plume around the source of the contamination. TCE spreads quickly in soil and water and persists for long periods of time, clinging to other molecules in the environment. The contamination is difficult to remove in its entirety. Some bioremediation efforts for organic volatile pollutants include ex-situ work, in which the contaminated area is treated in an artificial environment to ensure the pollutant is
totally removed from the environment before the natural resource can be used again (Sims et al., 1992).

Much of the current research into eliminating TCE contamination in ground water focuses on introduction of electron donors to facilitate reductive dechlorination. Chemicals like poly-\(\gamma\)-glutamic (\(\gamma\)-PGA) acid, soybean oil, and ferrous lactate are added to aquifers to facilitate reduction of TCE (Luo et al., 2021)(M.-H. Liu et al., 2021; Luo et al., 2021). While addition of these chemicals has been shown to reduce TCE in the environment, the aquifers often end up requiring further treatment to alter resulting state from the treatment. Some research has looked into inhibitions of methanogens to allow aerobic degraders to eliminate competition for introduced resources (Lin et al., 2021). Other work is being done to facilitate bioremediation by strains of bacteria by identifying functional genes in native bacteria (Gafni et al., 2020). Few groups are working with strains that co-metabolize TCE to create clean byproducts. Were this activity to be developed fully, it would greatly expand the field.

CONCLUSION

The goal of our project was to create a clean, well-characterized strain of bacteria that could be introduced to sites contaminated with TCE as a method of bioremediation. The strain created by this study was well characterized by this work and lacked foreign elements other than the kanamycin resistance gene mated in just prior to the PHL operon. This was done intentionally in an attempt to limit introduction of foreign elements to the environment if this strain were to be put into practical use. Other work has been done using various types of bacteria to clean up chemical spill sites and make them suitable for human use. Methods for this work should be replicated to allow clean-up of TCE contaminated sites. Further studies need to be done to determine what nutrients, if any, need to be supplied to allow \(C.\) necator to grow in this
environment. All work done in this study was done in LB, a nutrient rich broth that provided more than the required nutrients to allow quick growth of large quantities of *C. necator*. In the environment, this would likely allow growth of many types of bacteria and thus, researchers would want to control which nutrients were introduced to the environment and limit those as much as possible.
FIG 1. Map of EPA superfund sites containing TCE contamination in groundwater. This does not include many other sites of TCE contamination or sites where TCE is present in other mediums. Map generated in GIS using EPA contamination data.
FIG 2. Pathway of reductive dechlorination that occurs when TCE is present in anaerobic conditions, like that of groundwater. Toxic biproducts remain because the last step, which would produce harmless ethene does not occur naturally. Aerobic co-metabolism is the fortuitous degradation of a molecule by non-specific enzymes produced by bacteria that is present on compounds they don’t usually grow on. Figure created with Biorender.com.
FIG 3. Expected pathway of TCE degradation by PHL genes in C. necator. This pathway was characterized in *Ralstonia sp.* KN1 (Harker et al., 1989). These byproducts would be favorable to those observed in Figure 2. Figure created with Biorender.com.
FIG 4. Step-by-step explanation of the new TCE Degradation Assay. Step 1 shows the preparation of a TCE stock as described in the methods section. Step 2 is to grow a dense overnight culture of the strain whose degradation activity is to be measured. In step 3 this culture (in mid exponential growth phase) is split into 5 mL aliquots in headspace vials before being inoculated with 25 µL TCE stock injected into the liquid. The cap is then immediately screwed in and the vials are labeled in 1 hour increments as shown in step 5. The vials are then mixed by shaking (step 6) briefly before gas chromatography measurements are taken in real time at the appropriate time point as shown in step 7. These steps are repeated over a 4-day period for each strain resulting in 4 samples at each time point for each strain measured.
FIG 5. Growth curves of C. necator over time. This curve represents the average of 4 samples taken over 26 hours with OD600 measurements taken every two hours. The greatest amounts of TCE degradation are observed in the exponential growth phase, which occurs between hours 2 and 18 (P. J. Ayoubi, 1997).
The PHL homology region was amplified, cleaned, ligated into pJG194, transformed into *E. coli* to create strain MM11, and then mated into *C. necator* to create strain MM14. All work has been validated on gels. We sequenced the insert from MM14 to confirm the desired product. Figure created with Biorender.com.
FIG 7. Alignment showing homology regions between MM01 and MM02 as well as the PHL genes’ layout in these strains. There are small regions of variance throughout the genomes and two large areas in the region containing the PHL operon. This is the region where the Tn5 transposon was inserted in strain MM09 as well as where plasmid pyk3021 was introduced to restore PHL activity.
FIG 8. TCE Degradation abilities of all degrading strains observed in this experiment. With the margin of error none of the strains measured produced degradation that was deemed significant. There is a downward trend in strains MM01 and MM02 between hours 1 and three. This decrease is .08% of the degradation observed in previous work and is irrelevant. Strain MM14 showed no degradation at any time point.
FIG 9. Map of crossover designed to produce constitutive TCE degradation in strain MM14. The final strain will contain the pJG194 kanamycin resistance gene inserted just prior to the homology region containing about 500 downstream of the phiK gene start codon.
**TABLES**

Table 1. Plasmids and strains relevant to this study and their pertinent characteristics.

<table>
<thead>
<tr>
<th>MM01</th>
<th><em>Cupriavidus necator</em> AEO 106 (Harker et al., 1989)</th>
<th>No antibiotic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM02</td>
<td><em>Cupriavidus necator</em> pYK301/3021 (Kim et al., 1996) Kan/Amp resistant, constitutive TCE degrader</td>
<td>Kanamycin Ampicillin</td>
</tr>
<tr>
<td>MM05</td>
<td>B001 from Joel Griffitts, <em>E. coli</em> DH5α containing plasmid pRK600</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>pRK600</td>
<td>Helper plasmid for tri-parental matings (Finan et al., 1986)</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>MM07</td>
<td><em>E. coli</em> DH5alpha containing pJG194, from Joel Griffitts</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>pJG194</td>
<td>Suicide plasmid containing kanamycin resistance gene (Griffitts &amp; Long, 2008)</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>MM09</td>
<td><em>Cupriavidus necator</em> AEK301 (Kim et al., 1996)</td>
<td>Rifampicin Kanamycin</td>
</tr>
<tr>
<td>MM10</td>
<td><em>E. coli</em> DH5alpha containing pKJ075, from Joel Griffitts</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>pKJ075</td>
<td>Contains mScarlet red fluorescent protein, self-replicating plasmid</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>MM11</td>
<td>Created in this study, <em>E. coli</em> DH5α with PHL homology and incorporated on plasmid pMM11.</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>pMM11</td>
<td>Plasmid containing kanamycin resistance gene and PHL homology region</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>MM12</td>
<td>Created in this study, MM01 selected on Sm</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>MM14</td>
<td>Final product of this study. MM01 with pMM11 incorporated.</td>
<td>Streptomycin Kanamycin</td>
</tr>
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Table 2. Primers used in this study and their recorded use.

<p>| | | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>oMM01</td>
<td>CGCGAATTCAACCAAGGAGATATCCCATG</td>
<td>F primer, amplifies homology region in MM01</td>
</tr>
<tr>
<td>oMM02</td>
<td>CGCAAGCTTGGTTGTTGATAGGCTT</td>
<td>R primer, amplifies homology region in MM01</td>
</tr>
<tr>
<td>oMM03</td>
<td>TATCGCCGCTCCCGATTCCG</td>
<td>F primer, confirms insert and vector combined</td>
</tr>
<tr>
<td>oMM04</td>
<td>GATTCTGTGGATAACCGTATTAC</td>
<td>R primer, confirms insert and vector combined</td>
</tr>
<tr>
<td>oMM05</td>
<td>GCCAGCAAGGAAGAAAGAC</td>
<td>F primer outside insert region in final product</td>
</tr>
</tbody>
</table>
Table 3. Statistical values describing degradation by each strain

<table>
<thead>
<tr>
<th>Strain</th>
<th>F Statistic</th>
<th>P value</th>
<th>Multiple R² Value</th>
<th>Equation of the line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>.9026</td>
<td>.44236</td>
<td>.3110</td>
<td>Concentration = 1.33 - .02(Hour)</td>
</tr>
<tr>
<td>MM01</td>
<td>5.722</td>
<td>.1392</td>
<td>.7410</td>
<td>Concentration = 1.51 - .04(Hour)</td>
</tr>
<tr>
<td>MM02</td>
<td>7.080</td>
<td>.1170</td>
<td>.7797</td>
<td>Concentration = 1.67 - .04(Hour)</td>
</tr>
<tr>
<td>MM14</td>
<td>.4247</td>
<td>.5815</td>
<td>.1751</td>
<td>Concentration = .98 - .01(Hour)</td>
</tr>
</tbody>
</table>
Table 4. Sequence differences between strains MM01 and MM02 compared to a reference sequence for the PHL operon (8620 bp).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation Type</th>
<th>New Sequence</th>
<th>Reference Sequence</th>
<th>Location in Reference Sequence (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM01</td>
<td>Substitution</td>
<td>G</td>
<td>C</td>
<td>5776</td>
</tr>
<tr>
<td>MM01</td>
<td>Substitution</td>
<td>GC</td>
<td>CG</td>
<td>7880-7881</td>
</tr>
<tr>
<td>MM01</td>
<td>Insertion</td>
<td>C</td>
<td>-</td>
<td>8511</td>
</tr>
<tr>
<td>MM02</td>
<td>Deletion</td>
<td>-</td>
<td>NA</td>
<td>1-212</td>
</tr>
<tr>
<td>MM02</td>
<td>Insertion</td>
<td>N (unspecified)</td>
<td>-</td>
<td>5091</td>
</tr>
<tr>
<td>MM02</td>
<td>Substitution</td>
<td>GC</td>
<td>CG</td>
<td>5777</td>
</tr>
<tr>
<td>MM02</td>
<td>Substitution</td>
<td>GC</td>
<td>CG</td>
<td>7880-7881</td>
</tr>
<tr>
<td>MM02</td>
<td>Insertion</td>
<td>C</td>
<td>-</td>
<td>8513</td>
</tr>
</tbody>
</table>
REFERENCES


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