Longitudinal study of vinyl chloride degrading *Dehalococcoides mccartyi*-containing cultures to promote horizontal gene transfer

Nadia Morson[1], Olivia Molenda[2], Katherine J. Picott[2], Ruth E. Richardson[3] and Elizabeth A. Edwards[1,2]#

[1]Department of Cell & Systems Biology, University of Toronto, Toronto, Ontario, Canada

[2]Department of Chemical Engineering & Applied Chemistry, University of Toronto, Toronto, Ontario, Canada

[3]School of Civil and Environmental Engineering, Cornell University, Ithaca, New York, United States of America

Corresponding Author: Elizabeth A. Edwards, elizabeth.edwards@utoronto.ca

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ABSTRACT

Vinyl chloride (VC) is a human carcinogen that accumulates in soil and groundwater due to incomplete dechlorination of chlorinated ethenes. Some strains of obligate organohalide respiring *Dehalococcoides mccartyi* can synthesize the VC reductase that catalyzes the dechlorination of VC to ethene. The gene encoding the VC reductase, *vcrA*, is found on a mobile genetic element called the *vcrA*-Genomic Island (GI) that may participate in horizontal gene transfer. We designed an experiment to try to induce horizontal gene transfer of the *vcrA*-GI by mixing two enrichment cultures: one containing the donor *D. mccartyi* strain with the *vcrA*-GI that could not fix nitrogen and the second containing the recipient strain devoid of the *vcrA*-GI that could fix nitrogen. Therefore, mixing the two cultures in medium without ammonium while providing VC as the sole electron acceptor was hypothesized to select for a mutant strain of *D. mccartyi* that could both fix nitrogen and respire VC. However, after over 4 years of incubation, no evidence for horizontal gene transfer of the *vcrA*-GI was found. Rather, we observed VC-dechlorinating activity attributed to the TCE reductase, TceA, in the recipient strain. We also observed that *D. mccartyi* can grow by scavenging low concentrations of fixed nitrogen sources. During this experiment we identified two additional *D. mccartyi* strains in the KB-1 TCE-enriched culture that could fix nitrogen. The presence of multiple strains of *D. mccartyi* with distinct phenotypes may enhance bioaugmentation success, but here it may have undermined attempts to force horizontal gene transfer of the *vcrA*-GI.

IMPORTANCE

Dehalococcoides mccartyi are a powerful bioremediation tool for the degradation of chlorinated solvent contamination in soil and groundwater. Only a few *D. mccartyi* strains have the ability to

dechlorinate toxic chlorinated compounds like vinyl chloride. Interestingly, the genetic ability to dechlorinate vinyl chloride is theorized to be shared among *D. mccartyi* strains. In this study we attempted to promote the genetic transfer of vinyl chloride degrading ability from one *D. mccartyi* strain to another. Although we did not observe this exchange, our findings suggest there may be restrictions of genetic transfer between specific clades or sub-groups of *D. mccartyi* strains. Developing our understanding of genetic transfer among *D. mccartyi* strains could allow for enhanced degradation of chlorinated solvent contamination *in situ*.

INTRODUCTION

Chlorinated solvents are among the most prevalent and persistent soil and groundwater contaminants in industrialized countries (1). Tetra- or perchloroethene (PCE) and trichloroethene (TCE) contamination originates from the on-going use of dry cleaning solvents and metal degreasing agents, respectively (2). These compounds, and their degradation intermediates such as vinyl chloride (VC), are known to have toxic or carcinogenic effects so their widespread soil and groundwater contamination poses a great concern to human health (3). In the late 1980s, following the discovery that microorganisms could completely dechlorinate these solvents under anaerobic conditions, bioremediation and bioaugmentation emerged as highly successful treatment options for these problematic pollutants (4). A microbial culture enriched from soil contaminated with chlorinated solvents in Southern Ontario, Canada, resulted in the KB-1™ mixed microbial consortium (5). KB-1™ has been used commercially for bioaugmentation for more than 20 years to remediate chlorinated compounds (6, 7). In the KB-1™ culture, chlorinated ethenes, such as PCE and TCE, are sequentially dechlorinated via *cis*-dichloroethene (cDCE) and VC to ethene (5, 8, 9). Chloroethene reductive dechlorination in KB-1™ is primarily

performed by multiple *Dehalococcoides mccartyi* strains in a growth-linked process (5, 8, 9). On the basis of 16S rRNA sequence similarity, *D. mccartyi* strains are categorized into three phylogenetic clades or sub-groups: Pinellas, Victoria and Cornell. The Pinellas clade is represented by strain CBDB1, the Victoria clade is represented by strain VS, and the Cornell clade is represented by the type strain, strain 195 (10, 11). Lab-grown KB-1 cultures enriched on different chlorinated substrates select for different strains of *D. mccartyi* that express different reductive dehalogenases (8). Reductive dehalogenases belong to a broad protein family (PF13486) and have been classified into Ortholog Groups (OGs) on the basis of >90% amino acid pairwise identity to facilitate comparisons (9, 12). In the TCE-enriched KB-1 culture, strains belonging to the Pinellas clade are dominant, and these strains express the VC reductases VcrA (OG 8) and BvcA (OG 28) (9). Recently, the TCE-enriched KB-1 culture was also found to contain low abundance Cornell clade strains, which express the TCE reductase TceA (OG 5) (9). VC reductases are the most critical since VC is the most toxic dechlorination intermediate and following VC dechlorination, complete dechlorination is achieved.

Interestingly, the operon which encodes VcrA was found on a mobile genetic element, called the *vcrA*-Genomic Island (GI) (13). In the KB-1 TCE-enrichment culture, the *vcrA*-GI was identified in a circularized and extrachromosomal state within the cell (14), and these findings suggest that the *vcrA*-GI can be transferred between *D. mccartyi* through horizontal gene transfer (HGT) (13, 15). However, there is no direct evidence of HGT of the *vcrA*-GI, and the mechanism of transfer between *D. mccartyi* remains unknown. In 2012, we had an idea to try to induce HGT of the *vcrA*-GI by mixing the KB-1 TCE-enrichment culture with another mixed culture, called Donna II (16). We called the mixture of the two enrichment cultures DKB (Donna + KB-1). The KB-1 TCE-enriched consortium was considered to be the ideal *vcrA*-GI donor given the

high abundance of the *vcrA*-containing *D. mccartyi* strain in this culture. The Donna II culture is a mixed microbial consortium that contains only one *D. mccartyi* strain, strain 195, that dechlorinates PCE to VC via organohalide respiration utilizing PceA (OG 30), a PCE dehalogenase, and TceA (16). Subsequently, VC is dechlorinated to ethene slowly via co-metabolism (15), since *D. mccartyi* strain 195 does not contain the *vcrA*-GI or any other VC reductases and cannot grow on VC. Of particular interest for experimental design, *D. mccartyi* strain 195 was identified to have a complete nitrogen fixation operon (17). Whereas the *vcrA*-GI-containing *D. mccartyi* strains from TCE-enriched KB-1 do not have a nitrogen fixation operon.

Armed with this understanding, we designed an experiment to try to induce HGT of the *vcrA*-GI, where the KB-1 TCE-enrichment culture would contain the donor strain of the *vcrA*-GI and *D. mccartyi* strain 195 in Donna II would be the recipient strain. Since *D. mccartyi* strain 195 is able to fix atmospheric nitrogen, it does not require fixed nitrogen sources, such as ammonium, for growth. By applying the selective pressure of providing only VC as the sole energy source (electron acceptor) in medium without ammonium, we anticipated a hybrid *D. mccartyi* strain which could fix nitrogen and contain the *vcrA*-GI would emerge.

After 4 years of co-incubation, continuous monitoring and re-feeding as needed, we did not observe the predicted hybrid strain of *D. mccartyi* and found no evidence for horizontal gene transfer of the *vcrA*-GI into *D. mccartyi* strain 195. Rather, under the highest selective pressure of VC dechlorination without ammonium provided in the DKB culture, we observed: (i) TceA evolved substrate range, (ii) scavenging or recycling of nitrogen sources rather than fixing nitrogen, and (iii) little to no growth or dechlorination. Additionally, we discovered that the KB-1 TCE-enrichment culture contains two *D. mccartyi* strains from the Cornell clade that can fix

nitrogen, and we demonstrate that nitrogen fixation occurs in the KB-1 TCE-enriched culture when grown without ammonium.

MATERIALS AND METHODS

Enrichment cultures

The KB-1 culture originated from microcosms prepared with aquifer materials from a TCE-contaminated site in southern Ontario in 1996 (5). The KB-1 TCE-enrichment culture is maintained with 100 mg/L TCE as the electron acceptor and methanol (MeOH) as the electron donor, referred to as KB-1/TCE-MeOH, as previously described (18–20). The Donna II culture was created from an enrichment culture seeded with digester sludge from a wastewater treatment plant in Ithaca, USA. The Donna II culture was maintained, at Cornell University, with 110 µM of PCE as the electron acceptor and butyrate as the electron donor, as previously described (16, 21). The "DKB" culture was created at Cornell University, in 2012, when 1000 mL of KB-1/TCE-MeOH and 700 mL of Donna II were combined. The DKB culture was maintained with PCE and butyrate, similar to the Donna II culture. In 2015, the DKB culture was shipped to the University of Toronto, and was subsequently used as inoculum to create DKB sub-cultures grown under different conditions of varying selective pressures, as described below.

Experimental Set-up and Monitoring

DKB sub-cultures were fed either PCE or VC as electron acceptor, and butyrate as electron donor. Additionally, DKB sub-cultures were either cultured in medium with ammonium (▲), or without ammonium (△) requiring nitrogen fixation. Therefore, four experimental conditions were created: (i) DKB PCE ▲, (ii) DKB PCE △, (iii) DKB VC ▲, and (iv) DKB VC △ (Figure

S1). Additionally, a KB-1 control culture, KB-1/TCE-MeOH, was used as inoculum for sub-culture triplicates into medium without ammonium and amended with VC, referred to as KB-1 VC △; this condition was expected to be a negative control for KB-1 growth under VC-degrading, ammonium-limiting conditions (Figure S1). To create each sub-culture, each 2 mL sample of culture (KB-1/TCE-MeOH or DKB) was centrifuged at 13,000 × g for 15 minutes at room temperature, the supernatant was discarded, and the pellet was resuspended by flicking and pipetting in 2 mL of anaerobic, autoclaved distilled H_2O, reduced with FeS. This process was repeated three times, to wash ammonium from the pellet. Following, the 2 mL volume of washed inoculum was transferred into 198 mL of anaerobic mineral medium (20) with (10 mM) or without NH_4Cl in 250 mL serum bottles sealed with butyl rubber stoppers (Geo-Microbial Technologies Inc.). An additional 500 µL of vitamin stock (22) was added to each bottle during set-up, and 50 µL was added for each 20 mL of medium added during medium amendments. The culture was purged with N_2:CO_2 (80:20) gas, and the headspace was over-pressurized with N_2:CO_2 for 3 seconds. The DKB sub-cultures were amended periodically with 310 µelectron equivalents (µeeq) of gaseous VC or neat PCE as electron acceptor, and 4 × eeq sodium butyrate stock as electron donor. The KB-1/TCE-MeOH sub-cultures, KB-1 VC △, were maintained with 310 µeeq VC as electron acceptor, and 5 × eeq of methanol and ethanol mixture (50:50 on an eeq basis) as electron donor. Cumulative electron acceptor consumed was monitored over time. Acceptor and donor were re-amended as needed when depleted in each sub-culture. Triplicate bottles were created for each of the four test conditions. In 2016, to reduce number of bottles monitored, only one of the triplicate cultures from the DKB sub-cultures with ammonium was maintained; prior to this time, these triplicates were behaving similarly. Therefore, from 2016 onward, 8 DKB sub-cultures and three KB-1 VC △ cultures were maintained and analyzed.

DNA Extraction

Samples were taken from KB-1/TCE-MeOH, Donna II, DKB, and DKB sub-cultures at various times throughout the experiment for DNA extraction. For each DNA sample, a volume of 20 mL of liquid culture was removed anaerobically. The 20 mL aliquot was centrifuged in a sealed Falcon conical centrifuge tube at 6870 × g for 20 minutes at room temperature, using a swinging-bucket rotor. Aerobically, the supernatant was decanted, and the pellet was resuspended immediately using 60 µL of Solution C1 and 500 µL of liquid from the PowerBead tube, from the DNeasy PowerSoil Kit (Qiagen). All subsequent kit instructions were followed. Final DNA elution volume was 50 µL of Solution C6 (elution buffer). DNA was quantified by NanoDrop or using the High-Sensitivity DNA kit with the Qubit® 3.0 Fluorometer (ThermoFisher Scientific). All DNA extracts were stored at -80°C.

RNA Extraction and cDNA synthesis

RNA was extracted from 10 mL of the KB-1 VC △ cultures and the KB-1/TCE-MeOH culture. Cells were pelleted by centrifuging at 5000 × g at 4°C for 15 minutes. Pellets were then resuspended in 500 µL of supernatant and stabilized with 1 mL of RNAprotect Bacteria Reagent (Qiagen). The RNeasy® Protect Bacteria Mini Kit (Qiagen) was used to extract RNA from the cells by physical disruption via bead-beating. The kit procedure was followed, and RNA was eluted in 30 µL of RNase-free water. Following, RNA was DNase-treated using 10 µL of DNase A and 70 µL of DNase buffer for 30 seconds. To purify, the RNA was cleaned up using the RNeasy® MinElute® Cleanup kit (Qiagen) and eluted in 15 µL of RNase-free water. Following, cDNA was synthesized from the RNA using the Invitrogen Superscript® VILO cDNA synthesis

kit (Invitrogen), and procedure was followed according to kit instructions. cDNA was quantified using the High-Sensitivity DNA kit with the Qubit® 3.0 Fluorometer. All cDNA extracts were stored at -80°C.

PCR for 16S rRNA and functional genes

To track the growth of the different *D. mccartyi* strains in the DKB sub-cultures, we designed strain-specific biomarkers. The abundance of all *D. mccartyi* strains was quantified by the 16S rRNA gene, using primers Dhc1f and Dhc264r (10), since there is a single 16S rRNA gene copy per genome. To quantify the *vcrA*-containing strain from KB-1/TCE-MeOH and all extrachromosomal *vcrA*-GIs, we used primers vcrA670f and vcrA440r targeting the *vcrA* gene (23). The tceA500f and tceA795r (24) primers were used for tracking *tceA*-containing *D. mccartyi* strains in KB-1/TCE-MeOH and *D. mccartyi* strain 195. To quantify strain 195, we designed primers for a unique *rdhA* (DET_RS00960), that is truncated (truncated *rdhA*, "t*rdhA"*) and predicted to encode a non-functional reductive dehalogenase. It is only 1200 bp long compared to functional *rdhA* with lengths of approximately 1500 bp. As well, this gene contains two iron-sulfur cluster binding domains $(CX_2CX_2CX_3CP)_2$ but does not contain commonly conserved motifs: a twin-arginine TAT membrane export sequence (RRXFXK) nor a cobalamin binding domain. Primers for t*rdhA* were designed for the *D. mccartyi* strain 195 genome, using Primer2 (25) in Geneious 8.1.9 (26). To track nitrogen fixation (*nif)* genes, we quantified the *nifD* gene which encodes the nitrogenase molybdenum-iron protein α-chain. The *nifD* primers used in this study were used to characterize nitrogen fixation in *D. mccartyi* strain 195 (17). These *nifD* primers were also used for transcription analysis of nitrogen fixation genes in KB-1 VC △ and KB-1/TCE-MeOH, by reverse transcription (RT)-quantitative (q)PCR. Lastly,

we went back to archived DNA samples to quantify the vinyl chloride reductase, *bvcA* (Figure S2), using primers bvcA318f and bvcA555r (27). More information on primers can be found in Supplementary Information (Table S2).

As a qPCR standard for absolute quantification, a plasmid with concatenated sequences corresponding to the *D. mccartyi* 16S rRNA gene, *vcrA*, *tceA*, and *bvcA* was used, as previously described (14). This concatenated plasmid allowed us to calculate accurate ratios of these *rdhA* genes to 16S rRNA gene copies. For *nifD* and t*rdhA* qPCR standards, regions were PCR amplified, purified and cloned into *Escherichia coli* (TOPO™ TA Cloning™ Kit for Sequencing, Invitrogen) (Additional methods in Supplemental Information).

All qPCR reactions were prepared in a UV PCR cabinet and each qPCR reaction was run in duplicate or triplicate, using a CFX96 real-time PCR detection system. Each 20 µL qPCR reaction was prepared in UV-treated UltraPure nuclease-free water containing 10 µL of EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA), 0.5 µL of each primer (forward and reverse, from 10 µM stock solutions), and 2 µL of DNA template or standard plasmid dilution series, from 10^1 to 10^7 copies of plasmid per µL. Thermocycler program: initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 98°C for 5 s, varied annealing temperatures (Table S2), followed by extension for 10 s at 72°C. All qPCR standards and quality metrics (efficiency, standard curve details, etc.) can be found in Supplementary Information (Table S3), with calculations of absolute gene abundances per mL of culture (Table S4). To compare *nifD* expression among different experimental conditions, the absolute *nifD* gene abundances of genomic DNA and cDNA were determined. Using this data, we calculated *nifD* transcript per gene (TPG) ratios, as the ratio of transcript copies per mL of culture to gene copies per mL of culture.

Clone Library Preparation and Sequencing of *tceA*

A clone library of *tceA* was generated from one of the DKB VC △ cultures. As control, a *tceA* clone library was generated in parallel using the KB-1/TCE-MeOH culture. The two *tceA* clone libraries were generated using the TOPO™ XL-2 Complete PCR Cloning Kit (Invitrogen). PCR primers were designed to amplify the 2200 bp *tceAB* region (Table S2). Blunt end *tceA* PCR products were produced using the Platinum SuperFi polymerase, bands were extracted from an agarose gel, and cloned into One Shot™ OmniMAX™ 2 T1R Chemically Competent *E. coli* cells. 50 µL or 100 µL of transformed *E. coli* were spread on 50 µg/mL Kanamycin LB plates, with 40 µL of 40 mg/mL X-gal in DMF solution and 40 µL of filter-sterilized 0.1 M IPTG solution for blue-white selection. Following, 10 colonies were selected each from the DKB sub-culture and from the KB-1/TCE-MeOH culture and transferred cultures onto a patch plate for further analysis. From the patch plate, colonies were incubated in to 10 mL of 50 µg/mL Kanamycin LB broth, and plasmids were extracted using a QIAprep Spin Miniprep Kit (Qiagen). Each plasmid was PCR amplified using the T3 and T7 cloning primers to confirm successful transformation. For the first round of Sanger sequencing, plasmids were sequenced using the T3 and T7 cloning primers, at the SickKids Center for Applied Genomics (TCAG) sequencing/synthesis facility (Toronto, Canada). Ten sequences each from the forward and reverse were then aligned, and two consensus sequences were generated at a 99% nucleotide identity threshold. Using the consensus sequences, primers were designed (Table S2) for the remaining 845 bp. For the second round of sequencing, the final 99% nucleotide identical consensus sequences were generated by aligning these 20 sequences. The cloned *tceA* sequences from this study, the *tceA* published on NCBI (AAW39060), and the *tceA* from the Donna II

metagenome (IMG-M taxon ID: 2032320001) were aligned using the MUSCLE aligner (28) in Geneious 8.1.9.

TceA protein modelling

Protein models were independently produced for TceA from the *Dehalococcoides mccartyi* 195 isolate and the TceA from one of the DKB VC △ cultures. Each sequence was first trimmed to remove the TAT signal peptide sequence predicted by SignalP-5.0 (29). The models were produced from three different predictive modelling servers, Robetta, I-TASSER, and Phyre2, and assessed based on QMEAN and MolProbity quality scores determined by Swiss-Model assessment tool (30–35). The Robetta models had the highest quality scores and were used for further analysis; however, all models were used to validate the predicted spatial locations of the mutated residues. The cobalamin cofactor was modelled into the active site using AutoDock Vina, the docking position consistent with known crystal structure positions was taken (36). The substrate access channels were predicted using the CAVER 3.0 plugin in PyMol v2.3.4, only the channels predicted on the catalytic face of the cobalamin cofactor were kept (37).

Nitrogenase operon gene and protein alignments

The *D. mccartyi* strain 195 nitrogen fixation operon (DET_RS05950-5990), encoding the nitrogenase enzyme, was aligned with the nitrogenase operons of the two KB-1/TCE-MeOH strains (KBTCE2: B1773_05150-05185, KBTCE3: B1774_04800-04835). An alignment of these operons was performed using the built-in MUSCLE aligner (28) in Geneious 8.1.9.

RESULTS & DISCUSSION

Growth of DKB sub-cultures under varying degrees of selective pressure

To analyze the effects of experimental conditions on growth and dechlorination we analyzed the cumulative electron acceptor consumed over the course of the experiment (Table S1). Cultures grown without ammonium (△) did not consume as much electron acceptor as cultures with ammonium (▲) (Figure 1A). The DKB PCE ▲ culture consumed twice as much PCE as the DKB PCE △ cultures, while the DKB VC ▲ culture consumed nearly six times more VC than the DKB VC △ cultures over the same extended time period. The DKB VC △ cultures, which were grown with the highest selective pressure, also dechlorinated least of all DKB sub-cultures (Figure 1A). These findings confirmed that the selective pressures on the DKB sub-cultures to induce HGT of the *vcrA*-GI were effective. However, early in the experiment, we noticed that KB-1 VC △ cultures were able to dechlorinate VC even without ammonium provided in the growth medium (Figure 1A, Figure S3). This condition was intended to be a control, and dechlorination and growth were not anticipated. As dechlorination continued, we suspected that the KB-1 VC △ culture may contain nitrogen-fixing microbes, later justified by the high abundance of *D. mccartyi nifD* copies/mL of culture (Figure 1F). Further analysis of nitrogen fixation activity in the KB-1/TCE-MeOH and KB-1 VC △ cultures are addressed in this paper.

We also tracked the abundance of key genes of *D. mccartyi* strains predicted to be involved in HGT of the *vcrA*-GI (Table 1). We tracked *vcrA* and t*rdhA* (Figure 1C and 1D), representing the donor, *D. mccartyi* strain KBTCE1, and recipient, *D. mccartyi* strain 195, respectively. Additionally, we tracked genes such as the *D. mccartyi* 16S rRNA gene, which indicated growth of all *D. mccartyi* (Figure 1B), and *tceA* and *nifD* for the other strains in the DKB cultures that were not directly involved in the HGT experiment (Figure 1E-F).

The abundance of key biomarkers of HGT strains, *vcrA* and t*rdhA* (Figure 1C and 1D), reveal different trends in the DKB cultures. Interestingly, *vcrA* was highly abundant (10^7 to 10^9 copies/mL of culture) in all cultures except the DKB VC △ replicate cultures. In DKB VC △ 1 and 2 cultures, *vcrA* copies/mL decreased over time, whereas in the DKB VC △ 3 culture, *vcrA* abundance remained around 10^6 copies/mL. Conversely, an increase in t*rdhA* copies/mL was observed in DKB VC △ 1 and 2 cultures, whereas it was below or near the detection limit of 10^3 copies/mL in the DKB VC △ 3 culture throughout the experiment. In the DKB VC △ cultures, the trends in *tceA* and *nifD* biomarkers were similar to that of t*rdhA*. The *D. mccartyi* 16S rRNA trends also support the differences between the three DKB VC △ cultures. These findings suggest that the dominant strains in DKB VC △ 1 and 2 cultures are more similar to *D. mccartyi* strain 195, and the dominant strains in the DKB VC △ 3 culture are similar to *D. mccartyi* strain KBTCE1 (Figure S4). It was predicted that the HGT product, the "hybrid" *D. mccartyi* strain, would contain both *vcrA* and t*rdhA* in high abundance, which was not observed. Therefore, these results do not support the existence of the predicted hybrid strain that acquired the *vcrA*-GI.

Complete nitrogenase operons identified in two KB-1 strains

In 2012 when this experiment was conceived, the only *D. mccartyi* strain that had been identified to fix nitrogen was the *D. mccartyi* strain 195 isolate (17). Shortly after the DKB experiment had begun, metagenomic sequencing of the KB-1/TCE-MeOH culture revealed that it contained more than three strains of *D. mccartyi* (14). The *vcrA*-GI-containing strain, KBTCE1, was the most abundant strain belonging to the Pinellas clade, as previously described. The other two strains, KBTCE2 and KBTCE3, were both *tceA*-containing Cornell strains, which were not previously detected in the culture. Upon genome analysis, strains KBTCE2 and KTBCE3 were

found to contain complete nitrogenase operons, encoded by *nif* genes, and were therefore predicted to fix nitrogen. The nitrogenase operons in strains KBTCE2 and KBTCE3 share 99.0% nucleotide pairwise identity (PID) with the Cornell strain 195 isolate nitrogenase operon (DET_RS05950-RS05990) (Figure 2A, Table S5). The KBTCE2 and KBTCE3 nitrogenase operons are almost identical to each other, with one single nucleotide polymorphism (SNP) in the *nifK* gene (Figure 2A), which is the α-subunit of the iron-molybdenum protein in the nitrogenase complex (38).

To determine if KBTCE2 and KBTCE3 strains could fix nitrogen, we quantified the *nifD* gene copies and transcripts in the KB-1 VC △ cultures and in KB-1/TCE-MeOH, also referred to here as KB-1 TCE ▲. In the KB-1 VC △ cultures, the absolute abundance of *nifD* transcripts was 2.3 (± 1.7) times more than the KB-1 TCE ▲ culture, even though the absolute *nifD* gene abundance was 24 (± 9.6) times less (Figure 2B). *D. mccartyi* strains KBTCE2 and KBTCE3 in the KB-1 VC △ cultures are *tceA*-containing strains and do not contain *vcrA*. As a result, these nitrogen fixing strains are not be able to obtain energy from VC in these experimental conditions. This is different from the KB-1 TCE ▲ culture in which strains KBTCE2 and KBTCE3 can obtain energy through the dechlorination of TCE. It is a conundrum as to how these strains obtain energy when grown in the presence of VC as the sole electron acceptor in the KB-1 VC △ cultures, especially since nitrogen fixation is an energetically expensive process (39). This activity may point towards the function of the constitutively expressed reductive dehalogenase (OG 15) observed under “starvation” conditions in *D. mccartyi* strain 195 (DET_RS07915)(40) and in KB-1 (DQ177510)(27, 41). It may be that RdhA from OG 15 can sustain the population with an unknown electron acceptor, and what is perceived as “starvation”. Further work is

required to determine if a reductive dehalogenase from OG 15 is present in the KB-1 VC △ cultures and its role in providing energy to *D. mccartyi* strains.

The *nifD* transcripts-per-gene (TPG) ratio was calculated for the KB-1 VC △ cultures and for KB-1 TCE ▲ (Figure 2C). These TPG ratios were compared to approximate TPG ratios of the *D. mccartyi* strain 195 isolate (17) and the PW4 groundwater aquifer-derived enrichment culture dominated by Cornell strains (42) (Figure 2C, Table S6). There was an increase in *nifD* transcription when ammonium was limiting, inferring that nitrogen fixation occurred. Therefore, we identified two additional strains of *D. mccartyi* that can fix nitrogen, from the KB-1/TCE-MeOH culture. *D. mccartyi* strains KBTCE2 and KBTCE3, along with *D. mccartyi* strain 195, are all the only strains that are currently known to fix nitrogen, and all belong to the Cornell clade (9). The nitrogen fixation characteristic of *D. mccartyi* may be specific to the Cornell clade.

D. mccartyi strain 195 TceA predicted VC-dechlorinating activity

D. mccartyi strain 195 in the Donna II culture contains *tceA.* The KB-1/TCE-MeOH culture was found to harbour at least two *tceA*-containing strains, KBTCE2 and KBTCE3 (9). The *tceA* gene encodes the TCE reductive dehalogenase, TceA, which catalyzes the reductive dechlorination of TCE to cDCE and cDCE to VC (43). TceA can also dechlorinate 1,2-dichloroethane (1,2-DCA) to ethene and trace amounts of VC (20). However, TceA has not been shown to dechlorinate VC to ethene coupled to organohalide respiration. Our data suggest that we observed organohalide respiration of VC by TceA in the DKB experiment. In the DKB VC 1 △ and VC 2 △ cultures, we observed an increase in *tceA* and 16S rRNA gene copies, with a decrease in *vcrA* gene copies (Figure 1), accompanied by VC dechlorination (Figure S5). Perhaps this observed activity arose

from a mutation in *tceA* which would result in VC-dechlorinating activity. To further test this hypothesis, we generated a clone library of *tceA* from the DKB VC △ 1 culture (Table S7), and from the KB-1/TCE-MeOH culture as a control. The cloned *tceA* from the KB-1/TCE-MeOH culture was identical (100% nucleotide PID) to the *tceA* in published KBTCE2 and KBTC3 genomes (Figure S6). The cloned *tceA* sequence from the DKB VC △ 1 culture was identical to the *tceA* in *D. mccartyi* strain 195 from the Donna II metagenome (Figure S7). Therefore, the observed VC-dechlorinating activity was not the result of a mutation during this experiment. The Donna II strain 195 *tceA* sequence was next aligned to the sequence from the isolated *D. mccartyi* strain 195. In this alignment, two single nucleotide polymorphisms (SNPs) were identified, resulting in two non-synonymous amino acid substitutions in TceA: V261A and I481T (Figure 3, Figure S8). To explore whether these two mutations could have an impact on the substrate specificity and activity of the protein, we created TceA structure models of the *D. mccartyi* strain 195 isolate and Donna II *D. mccartyi* strain 195. Models were created independently through three different prediction servers: Robetta, I-TASSER, and Phyre2, resulting in six models to compare (30, 31, 33). Since there are only two known crystal structures (44, 45) and neither hold high sequence identity to TceA, the accuracy of each model is restricted. Therefore, several models were created to increase confidence in the predicted locations of each mutation (Table S8). Although the models generated differed, the location of the I481T mutation was consistently near the active site in both the *D. mccartyi* strain 195 isolate and the Donna II strain 195 TceA models (Figure 3B). Whereas the location of the V261A mutation had some variability but was always predicted to be removed from the active site. It is unlikely that the V261A mutation plays a role in altering the enzymatic activity (Figure 3A).

The crystal structure of PceA, a PCE reductive dehalogenase, from the organohalide-respiring bacterium *Sulfurospirillum multivorans* depicts a 'letterbox' opening with restricted access to buried hydrophobic binding pocket (Bommer et al., 2014). To visualize where the substrate may enter the active site in our models, we predicted all of the tunnels with access to the catalytic face of the cobalamin cofactor using Caver 3.0 (37). The I481T mutation is situated at the mouth of the major substrate access channel in each model (Figure 3B). Access channels can have a drastic impact on the substrate scope of an enzyme as they act as a filter for the active site. It has been established in directed evolution research that mutations in the substrate channel lead to a change in enzyme activity (46), thus it is speculated that this mutation in TceA has altered substrate preferences. Predictive modelling is a powerful tool in gaining insight as to the potential impact of these mutations; however, biochemical characterization and mutagenesis experiments need to be done to unequivocally determine the substrate range of the Donna II *D. mccartyi* strain 195 TceA. Further, to determine if the TceA variants in strains KBTCE2 and KBTCE3 can also contribute to energy conservation from VC to fuel observed nitrogen fixation.

Horizontal gene transfer of the *vcrA*-GI was not observed

The overall objective of this experiment was to promote HGT of the *vcrA*-GI from *D. mccartyi* strain KBTCE1 into strain 195. As previously mentioned, criteria that would suggest HGT occurred would have been the presence of the *vcrA* and t*rdhA* genes in high abundance, while VC dechlorination was observed (Table 1). These criteria were not met in any DKB sub-cultures. To further confirm that the *vcrA*-GI had not integrated into the *D. mccartyi* strain 195 genome, we PCR-amplified the integration site at the *ssrA* gene locus (13). We did not observe *vcrA*-GI integration in any DKB sub-cultures at the *ssrA* locus (Figure S9). As a result, we definitively

concluded that HGT of the *vcrA*-GI did not occur in under the experimental conditions of the DKB experiment.

An evolved substrate range for TceA rather than HGT of the *vcrA*-GI is the most likely explanation for our observations. Although the *tceA* mutations examined here occurred prior to this experiment, such mutations are a clear signal of positive selection in *rdhA*, as observed in *vcrA* which have been under positive selective pressures for an estimated 4000 years (13). It is also possible that the experimental conditions in the DKB experiment drove evolution of the TceA substrate range through mutations to other components of the electron transport chain. Recently, VC respiration with TceA has been reported in strain 195 and FL-2 when sufficient vitamin B12 is provided (Löffler, F.E., personal communication). There are more possible interactions that could explain our observations.

We thought that selective pressures that maintain the *vcrA*-GI would be sufficient to promote HGT. Natural selective pressures driving the uptake and maintenance of genomic islands or plasmids carrying genes to enhance bacterial fitness are common in soil environments (47, 48). However, it has become clear in this longitudinal study of the DKB sub-cultures that these specific selective pressures were not conducive to the desired gene transfer outcome. First and foremost, a consistent trigger of *vcrA*-GI circularization from the genome remains unknown. In fact, the *vcrA*-GI may not be under control of the cell, but rather acting as a selfish DNA element. Selfish DNA elements are found to be under the control of their own trigger stimulus, rather than as a result of a stimulus originating from the host cell or the environment (49). Therefore, it is possible that the selective pressure in this experiment presumed to promote the transfer of the *vcrA*-GI had little to no effect on the circularization and HGT of the *vcrA*-GI. Furthermore, we have additional evidence that *D. mccartyi* clade speciation plays a role in strain

compatibility for HGT donors and recipients. To date, there are no Cornell strains that contain the *vcrA*-GI, but rather it is unique to the Pinellas and Victoria clades. There are many clade-specific characteristics, including nitrogen fixation which is distinct to the Cornell clade, as previously described. These clade-specific characteristics underscore a lack of HGT between clades. HGT between clades may also be prevented by cellular defense mechanisms such as CRISPR-Cas systems (9, 14). CRISPR-Cas systems are involved in adaptive defense to protect the host bacterium from invading mobile genetic elements, and were found in *D. mccartyi* Pinellas strains KBVC1 (14), KBDCA3 (14), DCMB5 (50), CBDB1 (51) and GT (52). Some of these CRISPR-Cas systems have been shown to target the *vcrA*-GI, which would prevent HGT of the *vcrA*-GI from occurring (14). Although there are no complete CRISPR-Cas systems in the KBTCE1, KBTCE2 or KBTCE3 genomes, CRISPR-Cas genes were identified in the KB-1/TCE-MeOH culture that were not assembled into a genome (9). Therefore, it is possible that an unassembled *D. mccartyi* strain with a CRISPR-Cas system could influence HGT in the KB-1/TCE-MeOH culture. However, since there were no known CRISPR-Cas systems in the *D. mccartyi* strains involved in this experiment, there may be more reasons as to why HGT of the *vcrA*-GI did not occur. In fact, these reasons may be at play in preventing HGT within the KB-1/TCE-MeOH culture itself: there is no evidence of *vcrA*-GI HGT between the Pinellas and Cornell strains now known to exist within this culture. In the family of KB-1 cultures enriched on different substrates, more than 8 *D. mccartyi* genomes were closed (9). These genomes revealed that clade-specific, and even strain-specific, characteristics provide *D. mccartyi* populations with ecological advantages to persist within mixed communities and to prevent gene transfer of mobile genetic elements between dissimilar strains.

While this experiment started off as a simple attempt to promote HGT, the breadth and depth of knowledge of *D. mccartyi* has developed immensely since the experiment began in 2012. In the time since the DKB culture was created at Cornell University, *D. mccartyi* was renamed (11), the number of closed genomes of *D. mccartyi* in NCBI quintupled from 5 to 25 (9, 23, 50–53), prophages (27), mobile genetic elements (14), and CRISPR-Cas systems (14) were identified in KB-1, and most importantly, the genomes in the KB-1/TCE-MeOH (9) and Donna II cultures (IMG-M taxon ID: 2032320001) were sequenced. Considering all of these advancements, the findings of the DKB experiment contribute to this knowledge and will influence attempts to induce HGT in the future.

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Table 1: Key features of *D. mccartyi* strains combined to try to promote horizontal gene transfer.

	D. mccartyi strain		
Feature	KBTCE1[a]	195	Predicted "hybrid"
Role in HGT	Donor strain	Recipient strain	HGT product
Source Enrichment Culture	KB-1	Donna II	DKB
Clade	Pinellas	Cornell	Cornell
vcrA-GI	*vcrA*-GI	--	*vcrA*-GI
Nitrogen Fixation Operon	0	1	1
Distinguishing Biomarkers[b]	*vcrA*	t*rdhA*	*vcrA* & t*rdhA*
Common Biomarkers[c]	16S	16S, *tceA*, *nifD*	16S, *tceA*, *nifD*

[a]When the study was initiated, a *vcrA*-containing Pinellas strain was known to dominate the KB-1 TCE-enrichment culture. Subsequently, genomes corresponding to three strains (KBTCE1, KBTCE2, and KBTCE3) were closed from the KB-1/TCE-MeOH culture (9). The KBTCE1 strain was most abundant when the culture was grown on TCE or VC.

[b] Key quantitative (q)PCR biomarkers used to distinguish strains.

[c] Biomarkers common to more than one strain, where 16S represents the *D. mccartyi* 16S rRNA gene.

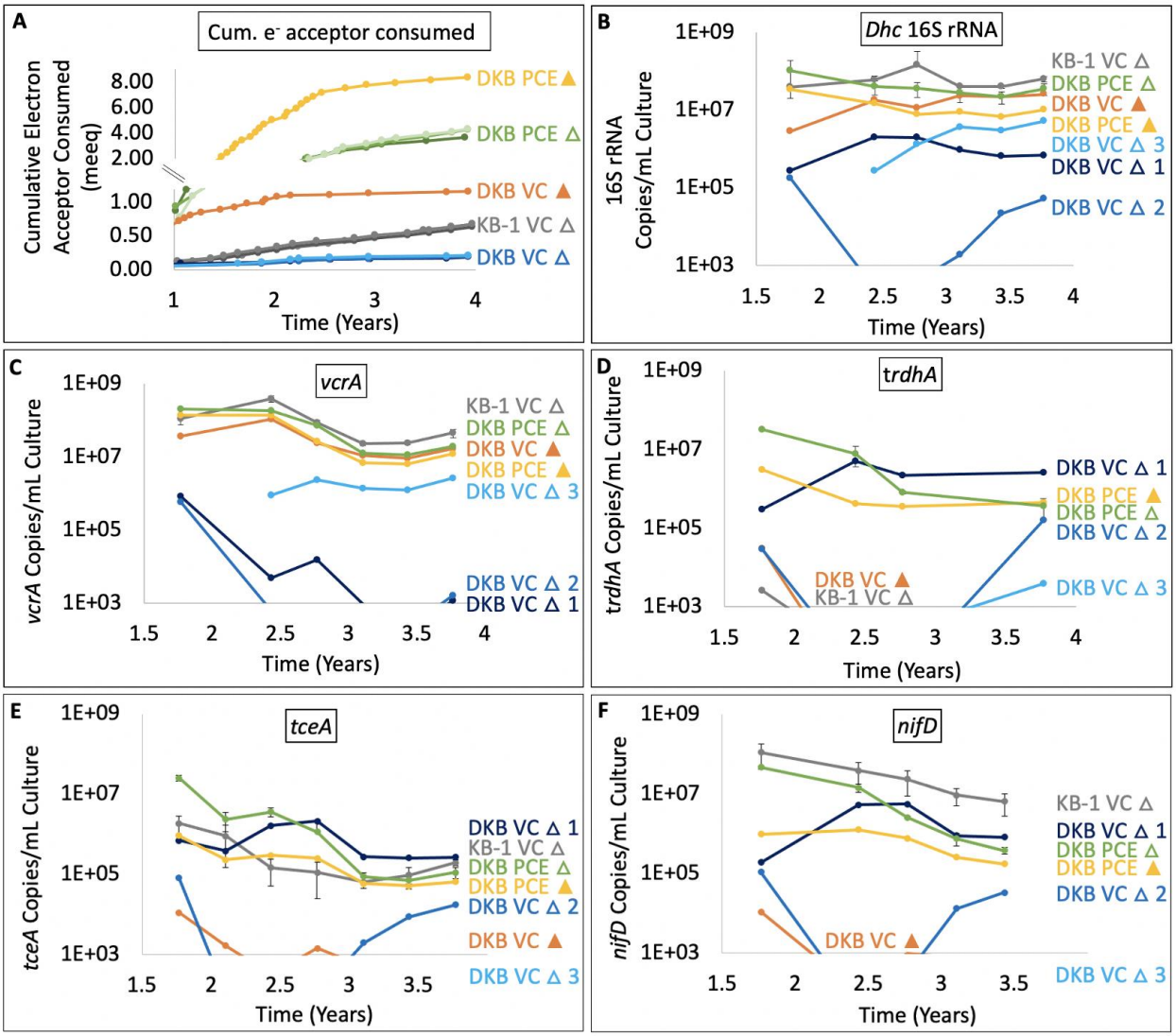

Figure 1: Cumulative electron acceptor consumed (meeq) and strain-specific biomarker tracking (gene copies/mL culture). (A) Cumulative electron acceptor consumed, given that 8 eeq are required per mol of PCE consumed, and 2 eeq per mol VC consumed. (B) *D. mccartyi* abundance, by 16S rRNA gene copies per mL of culture. (C) *vcrA* copies per mL of culture, quantifying both genomic and extrachromosomal *vcrA*-GI. (D) *D. mccartyi* strain 195-specific biomarker for a "truncated" *rdhA* (*trdhA*). (E) *tceA* and (F) *nifD* gene copies per mL of culture. Closed triangles represent ammonium provided in the medium (▲), and open triangles represent ammonium not provided (△) in growth medium. The detection limit was 1E+03. Error bars

represent the standard deviation (n=3), otherwise unaveraged triplicates are numbered (1,2,3) or a single bottle if not numbered (n=1).

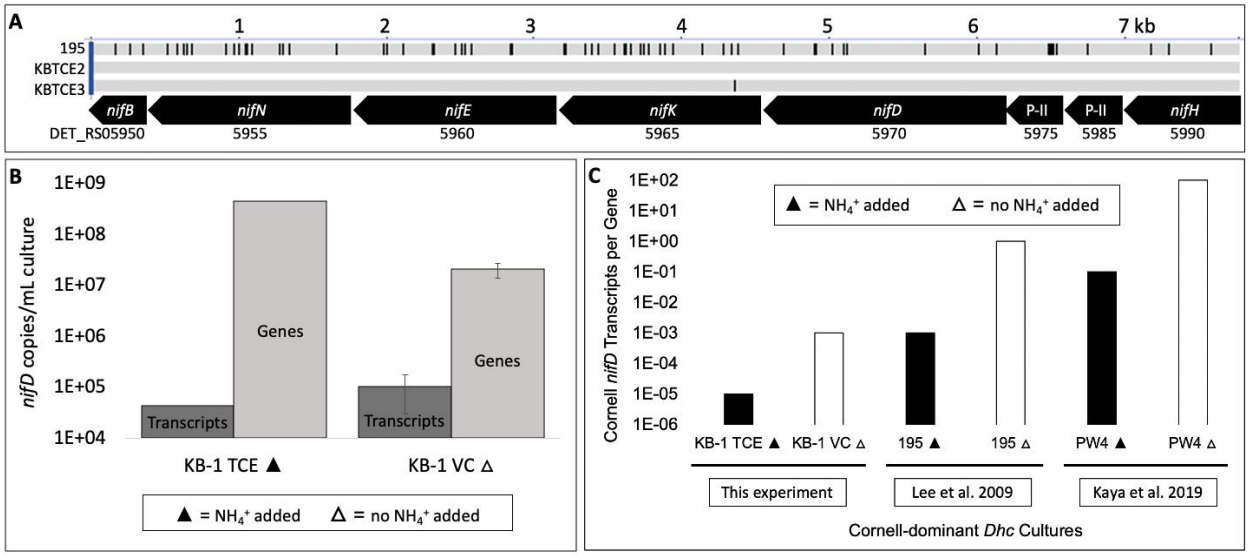

Figure 2: Nitrogen fixation in *D. mccartyi* (*Dhc*) in KB-1. (A) Alignment of nitrogenase genes (*nif*) in *D. mccartyi* strains 195 (DET_RS05950-5990), KBTCE2 and KBTCE3, where each black bar represents a misaligned nucleotide position. The top scale indicates nucleotide length in kilobase pairs (kb). (B) *nifD* transcripts and gene copies per mL of KB-1 grown with (▲) and without (△) ammonium provided. Error bars represent standard deviation (n=3). (C) *nifD* transcripts per gene (TPG) in KB-1, compared to approximate TPG ratios from Cornell-dominant *Dhc* cultures: pure strain 195 culture (17) and mixed PW4 culture (42), grown with (▲) and without (△) ammonium.

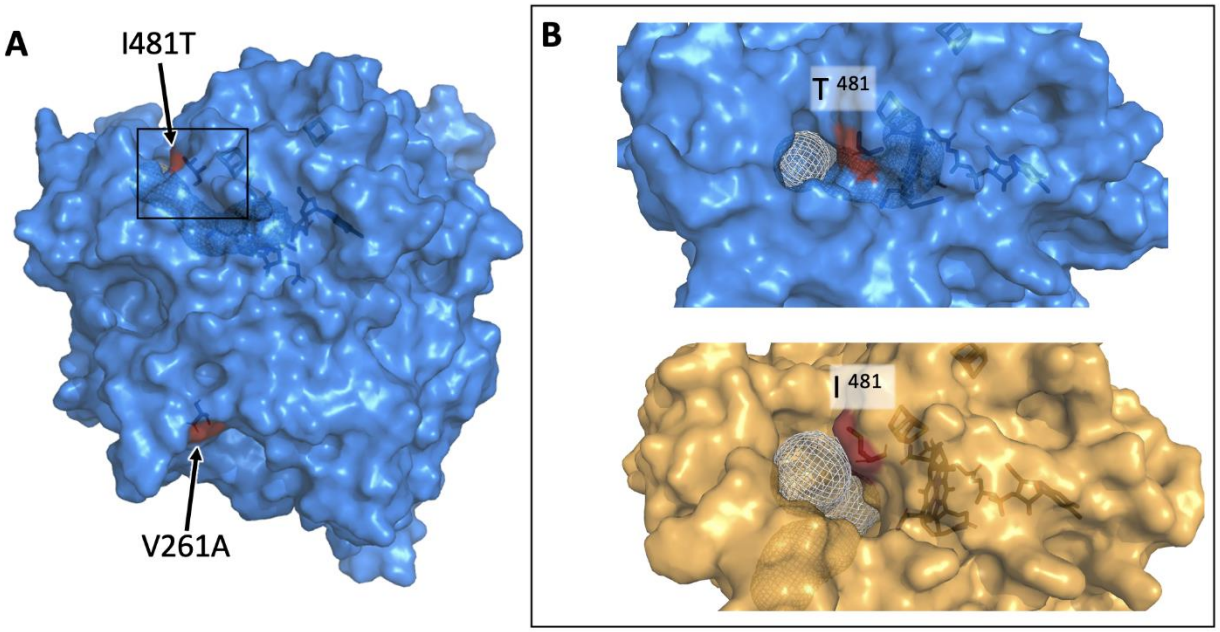

Figure 3: Protein models of the Donna II metagenome *D. mccartyi* strain 195 TceA (blue) and *D. mccartyi* strain 195 isolate TceA (yellow) with mutation sites highlighted in red. (A) The Donna II TceA mutant structure shows spatial locations of V261A and I481T mutations. (B) Close up of I481T mutation in the Donna II TceA and strain 195 isolate TceA, where the predicted substrate access channel is shown in white mesh. Models were produced using the Robetta online prediction server (30).