# Identification of functional genes in a chloroform and dichloromethane-degrading microbial culture

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**Running Head:** Functional genes in a CF and DCM-degrading culture.

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**Abstract**

Chloroform (CF) and dichloromethane (DCM) are groundwater contaminants of concern due to their high toxicity and inhibition of important biogeochemical processes. Biotransformation of CF and DCM have been well documented but always independent of one another. CF is dechlorinated to DCM by organohalide-respiring bacteria using reductive dehalogenases (RDases), while known DCM-degraders either ferment or mineralize DCM, both of which use the *mec* cassette to facilitate the entry of DCM to the Wood-Ljungdahl pathway. The SC05 culture, used commercially for bioaugmentation, is the first and only known stable enrichment culture to transform CF and DCM simultaneously. Here we use metagenomic and metaproteomic analysis to identify the functional genes involved in each of these transformations. A single *Dehalobacter* metagenome assembled genome contains the genes for an RDase, named *acdA*, and a complete *mec* cassette encoded on a single contig. AcdA and several of the Mec proteins were also highly expressed. Furthermore, AcdA displayed high dechlorination activity on CF and other chloroalkanes but did not show significant dechlorination of DCM. Overall, the high expression of Mec proteins and the activity of AcdA suggest a *Dehalobacter* capable of dechlorination of CF to DCM, and subsequent mineralization of DCM using the *mec* cassette.

**Importance**

Chloroform (CF) and dichloromethane (DCM) are harmful groundwater contaminants. SC05 is the first anaerobic microbial culture that can effectively and simultaneously remove both CF and DCM. Here we identify the key CF and DCM-degrading pathways in the culture, which are both found in a *Dehalobacter* strain. This *Dehalobacter* contains and expresses all the genes necessary for both CF dechlorination and DCM mineralization, which has never been observed. Identifying these functional genes will improve our knowledge of how CF and DCM bioremediation progress and provide means to monitor the process when SC05 is deployed in the field.

**Keywords:** chloroform, dichloromethane, bioremediation, *Dehalobacter*, reductive dehalogenase, *mec* cassette

## Introduction

Chlorinated alkanes like chloroform (CF) and dichloromethane (DCM) are prevalent and particularly persistent groundwater contaminants. Although CF and DCM are naturally occurring compounds, high concentrations in the sub-surface occur due to their use in industrial processes (1, 2). In addition, they are also generated as by-products in wastewater treatment (1, 2). These chlorinated compounds are toxic and carcinogenic to humans, and they inhibit biogeochemical cycling as well as bioremediation efforts targeting other compounds at mixed-contaminant sites (1, 3–5). Under anoxic conditions, CF and DCM tend to accumulate due to slow co-metabolic and abiotic transformation rates, but mixed microbial cultures can remove these compounds from anaerobic contaminant sites, particularly through bioaugmentation (1, 2, 5).

Numerous enrichment cultures and isolated organisms targeting CF and DCM have been described, but typically these cultures are specific to either CF or DCM. All known CF-dechlorinating cultures include strains of *Dehalobacter restrictus* (6–9), with the exception of *Desulfitobacterium* sp. strain PR (10). *Dehalobacter* and *Desulfitobacterium* are both organohalide-respiring bacteria (OHRB)—organisms that use organohalides as terminal electron acceptors in their respiratory processes. These strains efficiently respire CF through reductive dechlorination, yielding DCM as a product (6–10). These reactions are catalyzed by a family of enzymes called reductive dehalogenases (RDases). Though RDases are ubiquitous among OHRB, each of the known CF-respiring organism expresses its own unique enzyme.

To classify similar RDases, the enzymes are organized into ortholog groups (OGs) (11, 12). Each OG comprises the RDases that share more than 90% amino acid sequence identity (12). All known CF-dechlorinating organisms possess RDases that cluster in a single group: OG 97 (similarity matrix in Figure S1). It is worth noting that enzymes within OG 97 also demonstrate the capacity to dechlorinate various structurally related halomethanes and chloroethanes, although each enzyme has distinct substrate preferences. Members of OG 97 that are capable of CF dechlorination include CfrA from *Dehalobacter* sp. CF (13), TmrA from *Dehalobacter* sp. UNSWDHB (14), ThmA from *Dehalobacter* sp. THM1 (8), CtrA from *Desulfitobacterium* sp. PR (10), and most recently RdhA D8M_v2_40029 from *Dehalobacter* sp. 8M (15). While some of these enzymes have demonstrated the ability to dechlorinate DCM to chloromethane (CM), it is typically a minor transformation product and has not been shown to sustain growth of these organisms (7, 10).

Likewise, no DCM-degrading culture is known to use reductive dechlorination or organohalide respiration for DCM degradation; the four strains with established DCM metabolism each use one of two anaerobic DCM-degradation pathways. DCM fermentation was first discovered in *Dehalobacterium formicoaceticum* strain DMC and uses the Wood-Ljungdahl pathway (WLP) to produce formate and acetate from DCM (16, 17). *Candidatus* Formimonas warabiya and *D. formicoaceticum* strain EZ94 are also

thought to use this pathway (18–20). The second pathway, DCM mineralization, also harnesses the WLP to assimilate DCM, but instead produces carbon dioxide and hydrogen as end products (21). This mechanism has only been shown in *Candidatus* Dichloromethanomonas elyunquensis (21–23). Both pathways involve the *mec* cassette in the initial DCM assimilation step, which encodes methyltransferases, corrinoid proteins, and accessory proteins that can transform DCM to methylenetetrahydrofolate in both pathways before they diverge later in the WLP (24).

SC05 is the only known microbial culture that dechlorinates CF to DCM and concurrently mineralizes DCM to carbon dioxide and hydrogen (25). An electron-donor free subculture known as SC05-UT was previously established, which produces sufficient hydrogen through DCM mineralization to power its CF dechlorination (26). From this culture, an additional subtransfer was made and maintained on DCM alone (known as DCME) (26). In each of these cultures, the same *Dehalobacter* amplicon sequence variant (ASV) increases in abundance while the culture consumes CF and DCM, as well as DCM alone. These findings suggested a multifunctional *Dehalobacter* that can produce its own electron donor from mineralization of DCM (its dechlorination product), but the mechanisms for both CF dechlorination and DCM mineralization have remained uncharacterized in this culture. Here we search for functional genes in SC05-UT and DCME to 1) identify and characterize the RDase performing CF dechlorination and 2) provide insight into the mechanism of DCM degradation in the SC05-UT *Dehalobacter*.

## Results & Discussion

### 1. AcdA is the expressed RDase in SC05-UT

To identify the CF dechlorinating RDase in SC05-UT, its metagenome was first assembled and then searched for RDases using an in-house database (https://rdasedb.biozone.utoronto.ca) (12). Of the 51 identified *rdhA* genes, 27 were from contigs in the *Dehalobacter* MAG (Table S1). Closed *Dehalobacter* genomes typically encode ~20-25 RDases, but each strain often only expresses one for respiration (12, 27, 28). Through proteomics, only one RDase was identified (ID: JAWDGN_38927, average protein abundance rank: 3) (Dataset S3A, deposited in the following repository: https://borealisdata.ca/dataverse/SC05-metaproteogenomics).

Like other characterized *rdhA* sequences, this gene contains a twin-arginine transport (TAT) sequence, two iron-sulfur cluster binding motifs, and a downstream *rdhB* gene (Figure S3). The *rdhB* gene is followed by two additional genes that encode a superfamily endonuclease domain and a 4Fe-4S binding protein before a terminator sequence to end the operon (Figure S3).

This uncharacterized RDase was discussed in the context of divergent isotope fractionation in CF-dechlorinating cultures, wherein it was referred to as KB-1® Plus CF RdhA (29), but no biochemical analysis was performed at that time. The RDase clustered with OG 97, the ortholog group containing all five known CF-dechlorinating RDases,

with its highest amino acid similarity (96.7%) to TmrA (WP_034377773) (29). This classification, in addition to its lone expression from the metagenome's roster of putative RDases, suggests the identification of yet **a**nother **c**hloroform **d**ehalogenase, AcdA.

## 2. AcdA dechlorinates many chloroalkanes, but not DCM

To explore AcdA's dechlorinating activity, we expressed the native gene heterologously in *Escherichia coli*. Its expression was much lower than what has been previously seen for TmrA using the same method (30). This could be because the native *Dehalobacter* gene was expressed rather than codon-optimizing for the expression host, or that the enzyme itself might be less soluble. Regardless, the enzyme was enriched by nickel-affinity chromatography, and the protein concentration used in assays was adjusted using the estimated purity. The expressed AcdA was assayed against known OG 97 substrates including CF; 1,1,1-trichloroethane (TCA); 1,1,2-TCA; and 1,1-dichloroethane (DCA) (Figure 1). DCM was also tested as a substrate to investigate if there was sufficient activity to contribute to DCM mineralization (Figure 1A). Enzyme-free negative controls were used for each substrate, no product was detected in any reaction except for two CF and 1,1-DCA replicates in which less than 0.5 nmol of DCM and vinyl chloride (VC) were detected, respectively (Dataset S4, deposited in the following repository: https://borealisdata.ca/dataverse/SC05-metaproteogenomics). TmrA was used as a positive control and for comparison against a known CF-reductase. The enzyme activity is reported here as the nmol of product measured per second of the reaction per mg of RDase.

Generally, AcdA displayed comparable activity levels on all tested substrates to TmrA (Figure 1). Similar to TmrA and other OG 97 enzymes, AcdA sequentially dechlorinates 1,1,1-TCA to 1,1-DCA, and then to chloroethane (CA), though it dechlorinates 1,1-DCA at a much slower rate. Interestingly, the enzyme assay was able to predict 1,1,1-TCA and 1,1-DCA as growth substrates for SC05-UT. We confirmed that the culture could be sustained on 1,1,1-TCA as the sole electron acceptor and CA accumulated as a final dechlorination product (Figure S4). AcdA also dechlorinated 1,1,2-TCA via two different reaction pathways yielding a mixture of 1,2-DCA and VC at a ratio of 1:1.5. This is a much higher ratio of VC than most OG 97 RDases produce, and it may be of interest to investigate the cause of this mechanistic switch.

AcdA is most active on CF as a substrate which is unsurprising given that the SC05-UT culture originated from a CF contamination site (25). While there is a small amount of DCM dechlorination observed (0.79 ± 0.08 nmol/s/mg), this activity is 100-fold lower than that of CF (68 ± 14 nmol/s/mg). CtrA and TmrA also display nominal dechlorination activity on DCM (10; Figure 1A), but this is predicted to be co-metabolic. Neither of their native organisms have demonstrated the ability to grow on DCM alone (7, 10). We do not believe that AcdA is dechlorinating DCM in the SC05-UT culture, but there is evidence of other DCM degrading machinery in the metagenome.

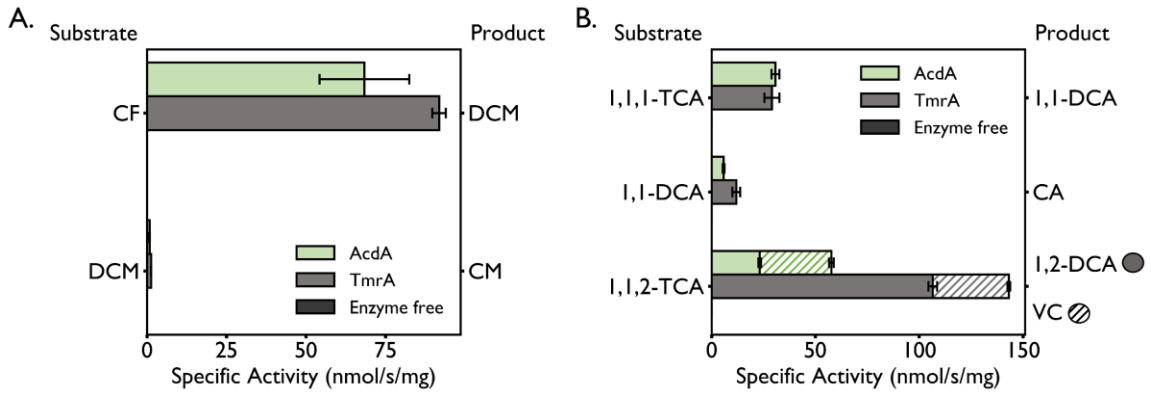


**FIGURE 1.** AcdA and TmrA specific activity on chlorinated methanes (A) and ethanes (B). Specific activity is the amount of product (nmol) per second per mg of RDase in the reaction, enzyme free negative control is in nmol/s. Reactions were incubated at RT for 1 h with no headspace. Activity on 1,1,2-TCA is stacked depending on the product of the reaction – 1,2-DCA (solid) or VC (striped). Error bars are the standard deviation between triplicate reactions. CF = chloroform; DCM = dichloromethane; CM = chloromethane; TCA = trichloroethane; DCA = dichloroethane; CA = chloroethane; VC = vinyl chloride.

### 3. Only the *Dehalobacter* MAG contains the *mec* cassette

To identify the mechanism for DCM degradation in SC05-UT, we searched both the SC05-UT and DCME metagenomes for the *mec* cassette: a ten-gene cassette identified specifically in the genomes of all known DCM-degrading microorganisms. The DCME metagenome was sequenced from a DNA sample taken after the subculture had been enriched with DCM only for more than one year. Only one instance of the *mec* cassette was found in each metagenome—on a contig assigned to the *Dehalobacter* MAG (Table S2). This contig, interestingly, also encodes *acdA* less than 8 kbp upstream of *mec*A (Figure S3), which further supports the cassette's assigned taxonomy and suggests a region of high expression in the genome. There are a number of strong promoters in this region, including immediately upstream of *acdA*, but notably there is no promoter predicted near *mecA* (Figure S3). It is possible the terminator between the nearest promotor and *mecA* is not very strong, allowing *mec* cassette expression, or perhaps a promoter exists that has remained undetected by our methods.

Though a bin in the DCME metagenome was assigned Dehalobacteriia—the class containing Dehalobacteriales, which includes the DCM degrading *Dehalobacterium* spp. and *Ca.* F. warabiya—this bin did not contain a *mec* cassette (Table S3). The Dehalobacteriia class contains two orders other than Dehalobacteriales: UBA7702 and UBA4068, neither of which degrade DCM nor contain the machinery to do so (31). The DCME Dehalobacteriia metagenome-assembled genome (MAG) is of the order UBA7702, one of the two non-DCM degrading orders, so is likely a fermentative scavenger in the culture. Moreover, this bin was detected in the DCME metagenome (fed

DCM only), but it was not seen in the SC05-UT metagenome (fed CF) (Table S3). This microbe may be inhibited by CF, like many fermenters are, and thus proliferated during DCM enrichment due to removal of the CF pressure. Additionally, at the time of metagenome sequencing, the relative abundance of the Dehalobacteriia strain was between 2-22% abundance in DCME but was at < 0.4% abundance in SC05-UT as detected by amplicon sequencing (Figure S2). At such low abundance in the parent culture, and lacking the *mec* cassette, we do not expect this organism to be the DCM degrader in either culture.

When aligned to all known *mec* cassettes, the SC05-UT *mec* cassette is syntenic to the other DCM-degraders, except for an endonuclease inserted between *mecH* and *mecI* that does not appear to interrupt either gene (Figure 2). The SC05-UT cassette is most similar to that of *Dehalobacter* sp. UNSWDHB (Figure 2). Though the *Dehalobacter* sp. UNSWDHB genome encodes a "full" cassette of all 10 *mec* genes, the organism lacks the ability to degrade DCM (7). This may be due to its truncated *mecE*, which encodes a critical methyltransferase for assimilation of DCM into the WLP (24). Two other strains of *Dehalobacter* (sp. CF and sp. DCA) also contain three genes homologous to *mecABC*, but cannot degrade DCM for lack of the remaining *mec* genes (6, 27). Despite this precedent for partial, nonfunctional, *mec* cassettes in CF-dechlorinating *Dehalobacter* strains, the SC05-UT *mec* cassette is the first reported to be found in full—and seemingly functioning—in a *Dehalobacter* genome.

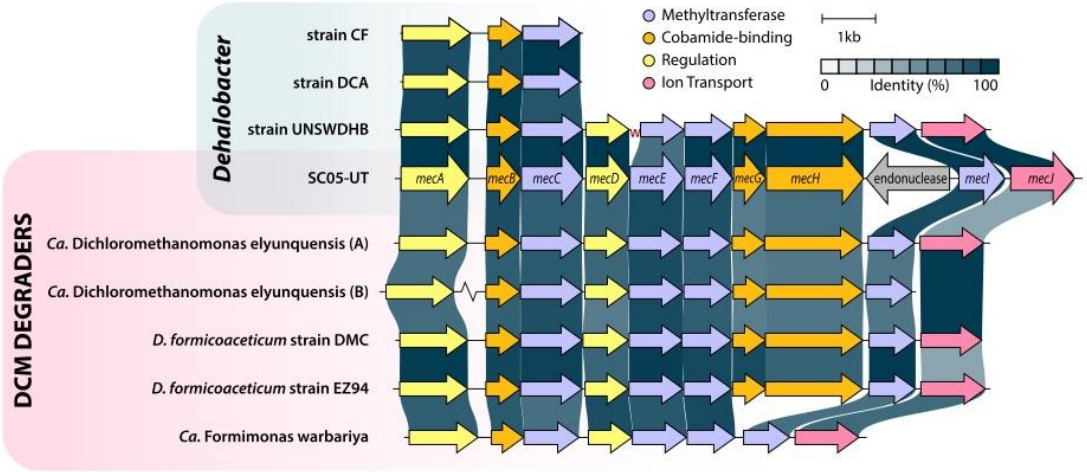


**FIGURE 2**. Alignment of all known *mec* cassettes in DCM degrading organisms and strains of *Dehalobacter*. Genes are colour-coded by functional category, and the *mecE* truncation in strain UNSWDHB is represented by a red squiggle. Connections between genes are colored based on percent amino acid identity. Figure was made using clinker (32).

## 4. Expression of DCM degradation genes

To determine if the encoded *mec* genes were expressed in SC05-UT (while CF and DCM were degraded) and DCME (while DCM alone was degraded), a proteomic workflow was performed on each culture. All detected peptides and proteins are shown in Dataset S3 (Deposited in the following repository: https://borealisdata.ca/dataverse/SC05-metaproteogenomics). All protein hits mapping to *Dehalobacter* are filtered in Table S4.

Most of the proteins encoded by the *mec* cassette are expressed under both culture conditions (Figure 3). The most highly expressed are MecE, MecF, MecB and MecC, which each rank in the top ten proteins expressed in at least one culture. The only Mec proteins not detected in either condition are MecA, MecI, and MecJ. Notably, MecI and MecJ are located after the inserted endonuclease found in the cassette which may interrupt their expression (Figure 2). A terminator sequence is predicted to exist within the endonuclease sequence, which could account for this effect (Figure S3). Additionally, MecA was not detected in *D. formicoaceticum* strain EZ94, and MecJ has never been detected in studies of either *D. formicoaceticum* strain or *Ca.* Dichloromethanomonas elyunquensis (20, 24). Overall, high expression of the key methyltransferases and corrinoid proteins show the possibility of DCM assimilation to methylenetetrahydrofolate in SC05-UT, which could then enter the WLP for further metabolism.

We also searched the metagenomes and metaproteomes for genes representing each step of the WLP, all of which were found encoded in the *Dehalobacter* MAG, and expressed in the proteomic data (Figure 3, Table S2). The WLP is a critical pathway for carbon assimilation and energy conservation in many organohalide-respiring bacteria (27, 33, 34). This pathway becomes doubly crucial when the *mec* cassette is in use, as it must also accept the methylenetetrahydrofolate produced from DCM assimilation and convert it to carbon dioxide or acetate. In SC05-UT, while degrading CF and DCM, as well as in DCME while degrading DCM alone, this *Dehalobacter* expresses all WLP enzymes (Figure 3). The expression of the WLP, in addition to the Mec proteins, positions the *mec* cassette as the likely mechanism of DCM assimilation in SC05-UT.

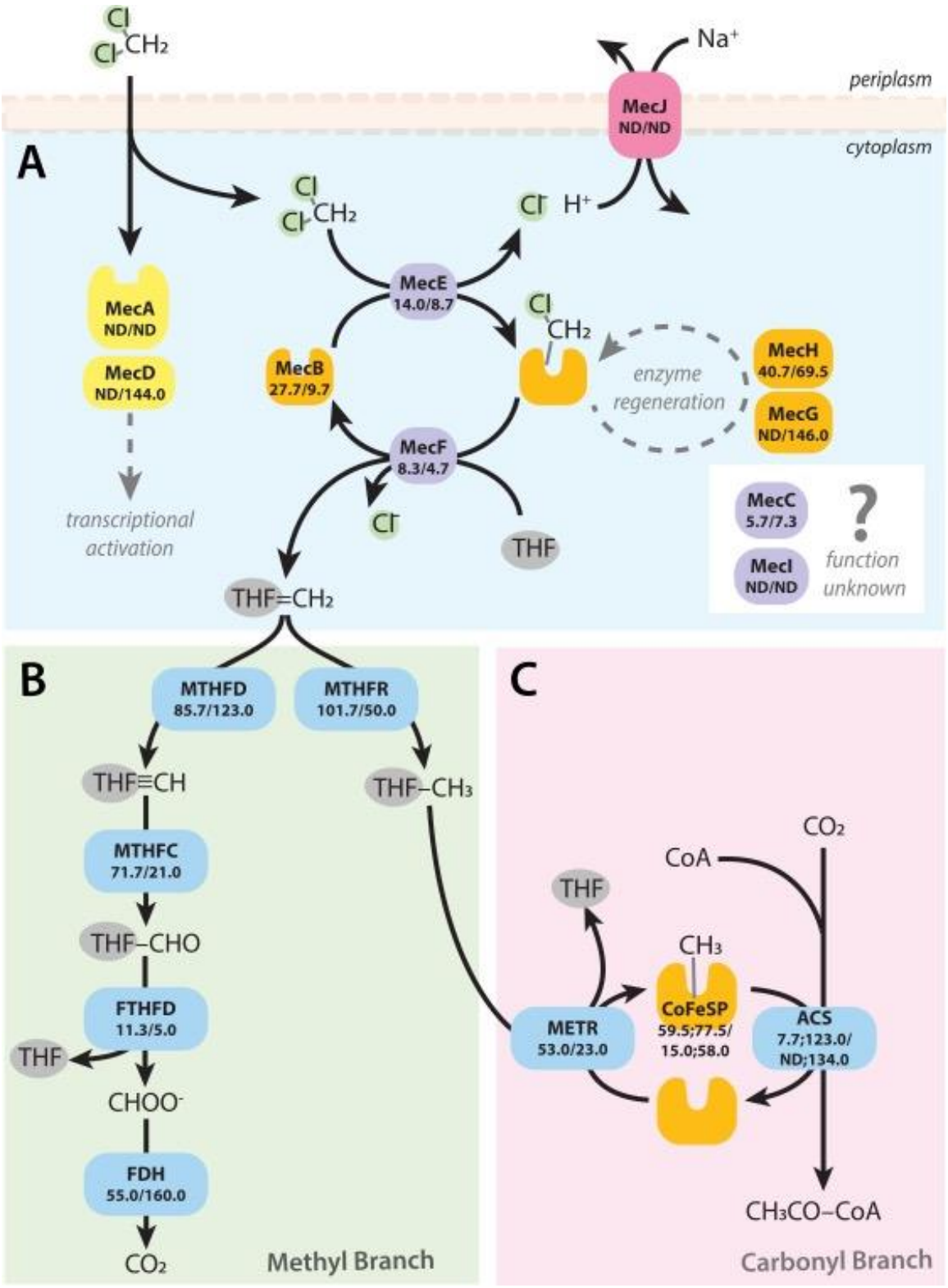


**FIGURE 3**. Mec cassette and Wood-Ljungdahl pathway expression in SC05-UT and DCME. Average rank shown beneath protein code (SC05-UT/DCME). Expression of multi-copy genes are separated with ";". THF = tetrahydrofolate; MTHFD = methylene-

THF dehydrogenase; MTHFC = methenyl-THF cyclase; FTHFD = formyl-THF deformylase; FDH = formate dehydrogenase; MTHFR = methylene-THF reductase; METR = 5-methyl-THF:corrinoid/iron-sulfur protein methyltransferase; CoFeSP = corrinoid/iron-sulfur protein; ACS = acetyl-coA synthase. Locus tags and enzyme descriptions shown in Table S2.

**Conclusions & Implications**

SC05-UT is the only known culture to simultaneously dechlorinate CF to DCM and to mineralize DCM to carbon dioxide. The SC05-UT *Dehalobacter* population is the first reported to encode both an RDase and the complete *mec* cassette. Here we introduce AcdA—another unique chloroform dechlorinating RDase in OG 97 that expands the arsenal of biochemical tools against chloroalkane contaminants. In addition to AcdA, the Mec cassette and WLP proteins were among the most highly expressed in the metaproteome, indicating likely mechanisms for CF and DCM metabolism by a *Dehalobacter* population. A single *Dehalobacter* strain may be capable of both metabolisms simultaneously, or subtle strain differences may account for these observations. A closed *Dehalobacter* genome—or closed genomes of multiple strains—is required to solidify these findings in the future. Fundamentally, this metagenome contributes to a growing understanding of chloroalkane metabolism, particularly regarding DCM mineralization, but development of a functional assay for DCM assimilation by the Mec cassette is necessary to unequivocally demonstrate this activity. Understanding this culture's unique metabolism will enable it to be applied in the field more effectively and the functional genes presented here could act as potential biomarkers to survey for successful bioaugmentation.

## Materials and Methods

All chemicals and primers were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. All gasses were supplied from Linde Canada Inc. (Mississauga, ON, Canada).

### Microbial cultures and growth conditions

SC05-UT is a derivative of SC05, an established enrichment culture used commercially for bioaugmentation (also known as KB-1® Plus CF**)** originally sampled from a contaminated groundwater site. It dechlorinates CF to DCM and concurrently degrades DCM to carbon dioxide. This culture was maintained in a defined mineral medium without addition of external electron donor for over two years before sampling, as previously described (26). DCME is a more recent subculture originally transferred from SC05-UT, this culture was enriched by feeding DCM alone for over 400 days before subsequent analysis, as previously described (26).

### Metagenome sequencing and assembly

Each enrichment culture was sampled twice in November 2020 by pelleting 300 mL of SC05-UT and 400 mL of DCME, and DNA was extracted using the Kingfisher Duo Prime MagMax microbiome kit (Thermo Scientific, Waltham, MA). DNA was sequenced by Genome Quebec using both Illumina MiSeq and PacBio Sequel II technologies. Aliquots of each DNA sample were also used for amplicon sequencing of the V6-V8 region by Genome Quebec as previously described (26). Illumina reads were trimmed using Trimmomatic and FastQC (35, 36). Long and short reads were assembled together using hybridSPAdes (37). Additional quality control, mapping and binning were performed using Anvi'o Snakemake (38–40). Assembly statistics can be seen in Table S5.

### Metagenome binning and annotation

Each metagenome was binned using metabat2, maxbin2, and CONCOCT, and ultimately DAS Tool for dereplication and selection of quality bins within each bin set (41–44). All DAS Tool–defined bins were > 60% complete and < 25% redundant before curation. Genes were annotated used metaerg, which harnesses prodigal to identify protein-encoding genes and HMMER to assign gene function (45). Gene annotations and assigned locus tags for the SC05-UT and DCME metagenomes are shown in Dataset S1 and Dataset S2, respectively (Deposited in the following repository: https://borealisdata.ca/dataverse/SC05-metaproteogenomics). RDase genes were identified and classified using the RDase database (https://rdasedb.biozone.utoronto.ca), using a 90% sequence similarity cutoff to assign each RDase an OG (12). Each gene in the *Dehalobacterium formicoaceticum mec* cassette was searched for in the metagenome contigs using BLAST to identify homologous proteins (46). Promoters and terminators were predicted using ProPr v2.0 (47).

**Protein extraction**

Protein was extracted using an adapted protocol by Murdoch *et al* (24). Briefly, 200 mL of each culture was passed through 0.22 µm Sterivex membrane filter (MilliporeSigma, Burlington, MA) units to capture cells. The filter outlets were then capped with parafilm and 0.5 mL of boiling sodium dodecyl sulfate (SDS) lysis solution (4% SDS in 100 mM Tris/HCl buffer, pH 8.0) was added to each filter unit and incubated at room temperature for one hour on a laboratory shaker. Lysate was extracted from the filter by back pressure using a 3 mL syringe, and filters were rinsed with 0.5 mL of fresh lysis buffer. Lysate mixtures were centrifuged at 21000 x g for 15 min, and supernatant was collected for subsequent trichloroacetic acid precipitation and two acetone washes of the pellet. Protein pellets were air-dried and resuspended in a denaturation buffer (8 M urea, 100 mM Tris–HCl pH 8.0), reduced with 5 mM dithiothreitol (DTT) for 10 min at RT, then alkylated with 10 mM iodoacetamide for 10 min at RT. Samples were diluted to 4 M urea with water for Bradford Assay quantification (Bio-Rad Laboratories, Inc, Hercules, CA) and trypsin digestion. Protein extracts were stored at -80 °C for subsequent high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

**Metaproteomics**

Chromatography was carried out using a PicoTip Emitter (New Objective, Littleton, MA) packed with Reprosil-Pur 120 C18-AQ, 3 µm (Dr. Maisch GmbH, Ammerbuch, Germany). The flow rate was set to 250 nL/min, and the eluents used were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient started at 0% B, followed by a linear gradient to 10% B over 5 min, a linear gradient to 40 % B over 48 min, a linear gradient to 95% B over 2 min, a hold at 95% B for 10 min, a linear gradient to 5% B over 2 min, and finally a re-equilibration under the initial conditions of 5% B for 14 min (total runtime 80 min). Liquid samples (5 µL) were injected on a Thermo Scientific Easy nLC 1000 (Thermo Fisher Scientific, Waltham, MA). MS detection was conducted using a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with a Nano Electrospray Ionization probe, operating in positive ionization mode, with spray voltage 2.5 kV, capillary temperature 275 °C, and s-lens RF level 55. Mass spectra were gathered using a data dependent method as follows; Full MS data was gathered over an m/z range from 400 to 2000 with the mass resolution set to 70k, AGC target 1e6, and a maximum injection time of 30 ms, while data dependant MS2 was gathered using a TOP10 approach, with resolution set to 17.5k, AGC target of 5e4, a maximum injection time of 50 ms, isolation window of 2.0 m/z and NCE 27. Data was processed through X!Tandem/ GPM for initial identification of peptides, and further analyzed through Scaffold. All detected peptides and proteins in SC05-UT and DCME are shown in Dataset S3 (Deposited at:

https://borealisdata.ca/dataverse/SC05-metaproteogenomics), and further filtered in Table S4.

**Reductive Dehalogenase Expression and Purification**

PCR reagents, restriction enzymes, Gibson Assembly master mix, the 1 kb DNA ladder, and *E. coli* DH5α were purchased from New England Biolabs (Ipswich, MA, USA). The *acdA* gene was PCR-amplified from gDNA extracted from the SC05-UT mixed culture as described above. The TAT sequence was not included in the amplified gene. The primers used for amplification included overhangs complimentary to the *p15TV-L* plasmid backbone as described previously (30, Table S8), such that gene could be inserted by Gibson Assembly into *p15TV-L* (Addgene #26093) linearized by BseRI digestion to produce the expression vector *p15TVL-AcdA*. The plasmid was verified with Sanger sequencing performed by The Centre for Applied Genomics at SickKids Hospital (Toronto, ON, Canada). AcdA was expressed following previously developed methods (30). Briefly, *p15TVL-AcdA* was transformed into *E. coli* BL21(DE3) *cat-araC*-$P_{BAD}$-*suf*, *ΔiscR::kan*, *ΔhimA*::$Tet^R$ ,with the *pBAD42-BtuCEDFB* plasmid to co-express the vitamin $B_{12}$ uptake pathway under arabinose control (48, 49). TmrA was expressed using the plasmid *p15TVL-tmrA* with *pBAD42-BtuCEDFB* co-expression in *E. coli* BL21(DE3) *cat-araC*-$P_{BAD}$-*suf*, *ΔiscR::kan*, *ΔhimA*::$Tet^R$, produced previously (30). The *pBAD42-BtuCEDFB* plasmid was generously provided by the Booker Lab (Pennsylvania State University, PA, USA). *E. coli* BL21(DE3) *cat-araC*-$P_{BAD}$-*suf*, *ΔiscR::kan*, *ΔhimA*::$Tet^R$, was generously provided by the Antony and Kiley Labs (St. Louis University School of Medicine, MO, USA; University of Wisconsin-Madison, WI, USA).

Large scale expression of AcdA was performed using 1 L of Luria Broth in a 2 L media bottle. To express AcdA, *E. coli* was first grown aerobically at 37°C, 170 rpm until the $OD_{600}$ reached 0.3-0.5. Expression of the Btu and Suf pathways were induced using a final concentration of 0.2% L-arabinose and the culture was supplemented with 3 µM of hydroxycobalamin hydrochloride. The culture was incubated again at 37°C, 170 rpm until the $OD_{600}$ reached 0.6-0.8. The bottle was then sealed with a silicon septum, put on ice, and purged with $N_2$ gas for 30 min. After purging, the culture was kept sealed and only handled under an anaerobic atmosphere of $N_2/H_2/CO_2$ (80:10:10 v/v). Supplements of 50 µM ammonium ferric citrate and 50 µM cysteine were provided and AcdA expression was induced using 0.5 mM Isopropyl β- d-1-thiogalactopyranoside (IPTG). The culture was incubated at 15°C, 170 rpm overnight (18-20 hr). Post-incubation, the culture was pelleted by centrifugation at 4500xg, 4°C for 20 min. The pellet was stored sealed and at -80°C until it was used for purification.

All lysis and purification steps were done under an anaerobic atmosphere or in a sealed container. In a Coy anaerobic glovebox, the pellet was thawed and resuspended in 10 mL anaerobic lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton-X100, 5% glycerol, 1 mM tris(2-carboxyethyl)phosphine (TCEP), 50 µg/mL leupeptin, 2

µg/mL aprotinin, 10 mM $MgCl_2$], the cells were lysed using BugBuster® protein extraction reagent (MilliporeSigma), 300 µg/mL lysozyme, and 1 µg/mL DNase. The suspended mixture was sealed and incubated outside of the glovebox at RT, 70 rpm for 20 min to complete lysis. The crude lysate was clarified by centrifugation at 35 000xg, 4°C for 20 min. The clarified lysate was transferred into the glovebox and loaded onto a gravity column in with 1 mL nickel-nitrilotriacetic acid resin (Qiagen, Hilden, Germany) that was preequilibrated with wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 30 mM imidazole). The protein-bound column was washed with ~25 mL of wash buffer until protein was no longer detected in the flowthrough by Bradford reagent. AcdA was eluted with 10 mL of elution buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 300 mM imidazole). The elution was collected and concentrated using a 15 kDa cutoff filter tube (MilliporeSigma) by centrifugation at 3000xg, 4°C, 30 min. The protein was resuspended in 15 mL of storage buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM TCEP) and the centrifugation step was repeated. The concentrated protein was stored in liquid $N_2$ for long-term stability. The concentration was determined by a Bradford assay and the purity was estimated by SDS-polyacrylamide gel electrophoresis (PAGE) using Image Lab 6.1 software (2022, Bio-Rad Laboratories, Inc.). This expression and purification procedure was repeated for TmrA.

**Chloroalkane Reduction Assay**

AcdA dechlorination activity was assessed using an end-point assay. The substrates tested were chloroform; 1,1,1-TCA; 1,1,2-TCA; 1,1-DCA; and DCM. The reactions were performed in 2 mL glass vials with 500 µL inserts and sealed with Teflon lined caps such that there was no headspace. The reaction was carried out in 50 mM Tris-HCl pH 7.5 buffer with 5 mM Ti(III) citrate and 2 mM methyl viologen. A total of 0.31 µg of AcdA or 0.43 µg of TmrA (accounting for purity) was added to the reaction buffer and the respective substrates were suppled to a final concentration of 0.5 mM using saturated water stocks and glass syringes (Hamilton Company, Reno, NV). Once the substate was added, the vial was sealed and incubated upside down at RT for 1 hr. Each substrate was tested in triplicate with AcdA, TmrA, and in enzyme free negative controls. The assay was terminated by transferring 400 µL of the reaction into 5.6 mL of acidified (pH < 2) water in a 11 mL headspace autosampler vial immediately sealed with a Teflon coated septum. The samples were analyzed using gas chromatography with flame ionization detection (GC-FID) on an Agilent 7890A GC instrument equipped with an Agilent GS-Q plot column (30 m length, 0.53 mm diameter).

All samples were measured with the same instrument method. The vials were equilibrated to 70°C for 40 min in an Agilent G1888 headspace autosampler. The headspace was sampled, and 3 mL was injected via a heated transfer line and then onto the column by a packed inlet maintained at 200°C. Helium was used as the carrier gas at a flow rate of 11 mL/min. The oven was held at 35°C for 1.5 min, then the temperature

ramped up by 15°C/min to 100°C, then the rate was reduced to 5°C/min to 185°C where the temperature was held for 10 min. Finally, the temperature was increased by 20°C/min to 200°C held for 10 min to clear the column. The flame ionization detector was set to a temperature of 250°C. Data were collected and analyzed using Agilent ChemStation Rev. B.04.02 SP1 software. The chromatogram peak areas were translated to liquid concentration using external calibration for each substrate and product. The enzyme activity values were calculated by normalizing the nmol of product measured in the assay by the mass (mg) of purified RDase in the assay and the length of the assay incubation (s). The enzyme free control was reported in nmol product per length of incubation (s).

**Data Availability**

Illumina and PacBio metagenome sequencing data and the draft metagenomes are available under GenBank BioProject Accession PRJNA1013980. The assembled metagenomes of SC05-UT and DCME are available at NCBI under Genbank accession numbers JAWDGN000000000 and JAWDGO000000000, respectively. The versions used here are JAWDGN010000000 and JAWDGO010000000. Datasets S1-4 are deposited under the University of Toronto's Borealis data repository, at https://borealisdata.ca/dataverse/SC05-metaproteogenomics.

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**Contributor Roles**

E.A.E., R.M, O.B., and K.P. conceived of the project. O.B. established the microbial cultures, performed metagenomic sequencing and assembly and proteomic analyses. K.P. performed heterologous expression and enzyme assays. The manuscript was written by O.B., K.P. and E.A.E. All authors contributed to manuscript revision and have approved the submitted version.

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