

ORIGINAL ARTICLE

Refined experimental annotation reveals conserved corrinoid autotrophy in chloroform-respiring *Dehalobacter* isolates

Po-Hsiang Wang¹, Shuiquan Tang^{1,8}, Kayla Nemr¹, Robert Flick¹, Jun Yan^{2,3,4,5,6}, Radhakrishnan Mahadevan¹, Alexander F Yakunin¹, Frank E Löffler^{3,4,5,6,7} and Elizabeth A Edwards¹

¹Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Ontario, Canada; ²Key Laboratory of Pollution Ecology and Environmental Engineering, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, Liaoning, China; ³Department of Microbiology, University of Tennessee, Knoxville, TN, USA; ⁴Center for Environmental Biotechnology, University of Tennessee, Knoxville, TN, USA; ⁵Joint Institute for Biological Sciences (JIBS), Oak Ridge National Laboratory, Oak Ridge, TN, USA; ⁶Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN, USA and ⁷Department of Civil and Environmental Engineering, University of Tennessee, Knoxville, TN, USA

Two novel chlorinated alkane-respiring *Dehalobacter restrictus* strains CF and DCA were isolated from the same enrichment culture, ACT-3, and characterized. The closed genomes of these highly similar sister strains were previously assembled from metagenomic sequence data and annotated. The isolation of the strains enabled experimental verification of predicted annotations, particularly focusing on irregularities or predicted gaps in central metabolic pathways and cofactor biosynthesis. Similar to *D. restrictus* strain PER-K23, strains CF and DCA require arginine, histidine and threonine for growth, although the corresponding biosynthesis pathways are predicted to be functional. Using strain CF to experimentally verify annotations, we determined that the predicted defective serine biosynthesis pathway can be rescued with a promiscuous serine hydroxymethyltransferase. Strain CF grew without added thiamine although the thiamine biosynthesis pathway is predicted to be absent; intracellular thiamine diphosphate, the cofactor of carboxylases in central metabolism, was not detected in cell extracts. Thus, strain CF may use amino acids to replenish central metabolites, portending entangled metabolite exchanges in ACT-3. Consistent with annotation, strain CF possesses a functional corrinoid biosynthesis pathway, demonstrated by increasing corrinoid content during growth and guided cobalamin biosynthesis in corrinoid-free medium. Chloroform toxicity to corrinoid-producing methanogens and acetogens may drive the conservation of corrinoid autotrophy in *Dehalobacter* strains. Heme detection in strain CF cell extracts suggests the ‘archaeal’ heme biosynthesis pathway also functions in anaerobic *Firmicutes*. This study reinforces the importance of incorporating enzyme promiscuity and cofactor availability in genome-scale functional predictions and identifies essential nutrient interdependencies in anaerobic dechlorinating microbial communities.

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Introduction

Halogenated organic compounds include a wide array of naturally occurring and man-made chemicals with moderate to high toxicity. Many make excellent hydrophobic solvents. For example, chloroform (CF) and 1,1,1-trichloroethane (1,1,1-

TCA) are frequent groundwater contaminants because of their widespread industrial use as solvents and historically poor disposal practices (Furukawa *et al.*, 2005). CF is also one of the most abundant organohalides in the atmosphere (estimated annual global flux is from 0.7 to 4 million t) (Harper, 2000; Cappelletti *et al.*, 2012). In fact, CF in the environment is primarily of natural origin (~90%; McCulloch, 2003), produced by algae in marine systems (Nightingale *et al.*, 1995; Scarratt and Moore, 1999) and by termites in terrestrial systems (Khalil *et al.*, 1990; Laternus *et al.*, 2002). In addition to neurotoxicity and carcinogenic activity, CF and 1,1,1-TCA are also strong inhibitors of many

Correspondence: EA Edwards, Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, ON M5S 3E5, Canada.
E-mail: elizabeth.edwards@utoronto.ca

⁸Current address: Zymo Research Corporation, Irvine, CA, USA.
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microbial processes essential for biogeochemical cycling, such as methanogenesis, acetogenesis and reductive dechlorination of chlorinated ethenes (Bagley *et al.*, 2000; Duhamel *et al.*, 2002), and are ozone-depleting substances (Slaper *et al.*, 1996).

Members of the *Dehalobacter* genus are anaerobes found in sediments of river and marine ecosystems (Zanaroli *et al.*, 2010) as well as groundwater aquifers (Grostern and Edwards, 2006). *Dehalobacter* are specialized in organohalide respiration (Holliger *et al.*, 1998) or organohalide fermentation (Justicia-Leon *et al.*, 2012; Lee *et al.*, 2012), and are active participants in the global halogen cycle. Based on 16S ribosomal RNA (rRNA) gene sequences (>99% average nucleotide identity), all characterized strains described to date belong to the type species *Dehalobacter restrictus* and use H₂ or formate as an electron donor to reductively dechlorinate a variety of chlorinated and brominated hydrocarbons (Holliger *et al.*, 1998; Sun *et al.*, 2002; Grostern and Edwards, 2009; Yoshida *et al.*, 2009; Grostern *et al.*, 2010; Deshpande *et al.*, 2013; Tang and Edwards, 2013; Nelson *et al.*, 2014; Wang *et al.*, 2014). *D. restrictus* strains CF and DCA were enriched from a mixed culture referred to as ACT-3 that dechlorinates 1,1,1-TCA via 1,1-dichloroethane (1,1-DCA) to chloroethane (CA) and CF to dichloromethane (DCM). ACT-3 is also used commercially for bioaugmentation (<http://sirem-lab.com/products/kb-1>). Reductive dehalogenases (RDases) are corrinoid-dependent enzymes that catalyze organohalide dehalogenation (Jugder *et al.*, 2016). In ACT-3, the dechlorination of CF and 1,1,1-TCA is catalyzed by CfrA (CF reductase subunit A) from strain CF; the dechlorination of 1,1-DCA is catalyzed by DcrA from strain DCA. CfrA and DcrA were the only two RDases found expressed of the 19 RDase genes in ACT-3 (Tang and Edwards, 2013).

As organohalide-respiring bacteria like *Dehalobacter* are key factors in sustaining the global halogen cycle (Krzmarzick *et al.*, 2012) and in bioremediation (Jackson, 2004; Justicia-Leon *et al.*, 2012, 2014; Jugder *et al.*, 2016), realizing their metabolic and physiological characteristics is of great importance. This characterization enables more accurate activity predictions, as illustrated by a recent study that found that *Dehalococcoides*, another genus of organohalide-respiring bacteria, requires a specific form of corrinoid cofactor to degrade the carcinogen vinyl chloride (Yan *et al.*, 2015). The closed genomes of strains CF and DCA were previously assembled from the metagenome of the ACT-3 mixed culture (Tang *et al.*, 2012) and available under NCBI accession numbers CP003869 and CP003870. These two highly similar genomes (>90% whole-genome average nucleotide identity) were then compared with three other available *Dehalobacter* genomes (strains PER-K23, E1 and UNSWDHB) to categorize common and unique genome-inferred physiological and metabolic traits (Tang *et al.*, 2016). The genomes differed primarily

in the complement of RDases and the completeness of the corrinoid biosynthesis pathway. However, all five genomes harbor the nearly identical complement of genes ascribed to central metabolism (the TCA cycle) and amino-acid and cofactor biosynthesis. All strains appear to have the same defects in serine, biotin and thiamine biosynthesis, whereas they all harbor a complete archaeal heme biosynthesis pathway (Tang *et al.*, 2016).

Recent advances in bioinformatics and sequencing technology have generated a large number of genomes and metagenomes from a wide variety of environments that are being mined for functional and ecological traits. However, bioinformatic analyses, while powerful, are often insufficient to reliably predict many metabolic features of an organism because of the considerable number of mis-annotations and hypothetical genes in every genome (Sañudo-Wilhelmy *et al.*, 2014). A relevant example is the recent discovery of the anaerobic biosynthesis genes of 5,6-dimethylbenzimidazole (DMB; Hazra *et al.*, 2015), the lower ligand of the best known corrinoid, cobalamin (vitamin B₁₂). The genes encoding 5-hydroxybenzimidazole synthase (*bzaABF*) were previously wrongly annotated as thiamine biosynthesis genes because of the homology-based annotation (Hazra *et al.*, 2015).

Here we describe the isolation, growth, morphological characteristics, and electron donor and electron acceptor substrate ranges of strains CF and DCA. Guided by previous genomic and transcriptomic analyses (Rupakula *et al.*, 2013; Tang *et al.*, 2016), we experimentally tested specific genomic predictions, particularly where annotations were uncertain or inconsistent with the observed phenotypes. Tang *et al.* (2016) classified and curated genome annotations of five *Dehalobacter* strains including strains CF and DCA. As the orthologous proteins from the five strains are highly similar, typically sharing >98% protein sequence identity, strain CF was chosen as an appropriate model organism for pathways common to all sequenced strains. We explored the predicted gaps in the TCA cycle, potential defects in amino-acid biosynthesis and were able to refine annotations of genes involved in serine biosynthesis. We also evaluated functionality of biosynthetic pathways for biotin, corrinoid, heme and thiamine. These experimental efforts verify genome annotations and bring previous proteomic data of ACT-3 and strain PER-K23 cultures (Rupakula *et al.*, 2013; Tang and Edwards, 2013) into context.

Materials and methods

Isolation and growth conditions

For strain isolation, two highly enriched subcultures, *Dehalobacter*-1,1,1-TCA/H₂ and *Dehalobacter*-1,1-DCA/H₂, were constructed by feeding transfers from the parent mixed culture ACT-3 (Tang and Edwards,

2013) with either 1,1,1-TCA or 1,1-DCA (2 mM nominal) as the electron acceptor in minimal mineral medium (MM medium; Edwards and Grbić-Galić, 1994). H₂ was provided as the electron donor (25% in the headspace; 20 mM nominal). Sodium acetate (5 mM) was provided as the carbon source. Specifically, for dilution-to-extinction transfers, inside an anoxic chamber, 7 ml of MM medium, 3 ml of ACT-3 mixed culture supernatant (described in Supplementary Information), sodium acetate (5 mM), 2 µl of neat chlorinated substrate (1,1,1-TCA or 1,1-DCA), and 6.4 ml of H₂/CO₂ (80% v/v) were added to a 25 ml butyl rubber stopper-sealed and crimped Bellco glass tube. After autoclaving, 10 µl of 1000x filter-sterilized vitamin solution (Supplementary Information) and 20 µl of autoclave-sterilized iron sulfide (FeS) slurry (~8 g l⁻¹; Supplementary Information) were injected into each tube. The enriched subcultures were serially diluted from 10⁻¹ to 10⁻⁹. To minimize carryover, inocula were pelleted down to remove supernatant, and were resuspended in an equal volume of sterile MM medium before inoculation. The diluted transfers were incubated at room temperature and dechlorination was monitored by gas chromatography as described in Supplementary Information. Growth conditions for experiments evaluating electron donors, electron acceptors and vitamin requirement were similar to the conditions for these dilution-to-extinction transfers with some modifications (Supplementary Information). The average volumetric dechlorination rates of cultures were determined from the total amount of dechlorinated products generated per day in each bottle divided by the culture volume when >90% of the chlorinated substrates were depleted in the three replicates.

Isolate purity tests

Contamination of the isolate cultures with other microorganisms was investigated by making transfers (10⁻³ dilution) into MM medium described above but excluding the chlorinated substrates (to detect homoacetogens), or replacing chlorinated electron acceptors with 5 mM NaNO₃ (for nitrate reducers), 5 mM NaSO₄ (for sulfate reducers), a mixture of methanol, ethanol and lactate (for fermenting microbes), or 0.1 g l⁻¹ yeast extract. These new transfers were incubated at room temperature for 1 month and then examined by restriction fragment length polymorphism (Supplementary Information) and epifluorescence microscopy with 4',6-diamidino-2-phenylindole dihydrochloride staining (to look for any growth) using standard protocols as described in Supplementary Information. Two pairs of specific primers targeting *cfrA* and *dcrA* (Supplementary Information) developed previously (Tang and Edwards, 2013) were used to examine the potential cross-contamination of strains CF and DCA in their isolate cultures.

Enzyme activity assays

Established assays for malate dehydrogenase, succinate dehydrogenase, NADP-dependent malic enzyme (NDME), and RDase activity were conducted using cell extracts prepared from *Dehalobacter* strain CF and from *Escherichia coli* strain K-12 (Supplementary Information). Cell extracts from both cultures were adjusted to a final total protein concentration of 50 µg ml⁻¹ in each assay. Serine hydroxymethyltransferase (SHMT) and threonine aldolase activity assays were conducted using purified recombinant SHMT from strain CF (accession number AFV06891). Cloning, expression and purification of strain CF SHMT is described in Supplementary Information. Protein concentrations of cell extracts were determined using the Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Mississauga, ON, Canada) with a linear range spanning from 20 to 2000 µg ml⁻¹ of protein. Specific activity is reported as µmol of substrate consumed or product generated per min per mg of protein, as well as in ncat mg⁻¹.

Extraction and derivatization of intracellular thiamine species

Thiamine can exist as free and phosphorylated forms within a cell. To measure the concentration of intracellular thiamine species, a protocol involving derivatization into thiochromes was used (Supplementary Information; Leonardi and Roach, 2004).

Corrinoid purification, measurement and structure characterization

Corrinoid content in strain CF culture was quantified using a *Lactobacillus* growth-based B₁₂ assay following established protocols with some modifications (Yan *et al.*, 2012, 2013). For the B₁₂ assay, strain CF cells grown in corrinoid-free medium were transferred twice (10⁻² dilution) to exclude any carryover. The third transfer was sampled over time during growth and the samples were assayed using the growth-based *Lactobacillus* auxotroph assay. In this assay, *Lactobacillus* growth increased linearly over a corrinoid concentration range from 2 to 20 ng l⁻¹ (Supplementary Figure S1). To determine total corrinoid content in cultures, pH of the samples was adjusted to 5–6 with 3% acetic acid, and the samples were incubated in a boiling water bath for 30 min, followed by centrifugation at 16 000 g for 15 min at 4 °C. The supernatants were diluted 10–50 times and stored at -20 °C until use. The corrinoids produced by strain CF were extracted and derivatized following previous established protocols (Yan *et al.*, 2012, 2013), and characterized using ultra performance liquid chromatography-mass spectroscopy (Supplementary Information).

Analytical procedures

Gas chromatography to measure concentrations of chlorinated substrate analyses, high pressure liquid chromatography for measuring thiamine derivatives and acetaldehyde, and ultra performance liquid chromatography-mass spectroscopy for biotin, corrinoid, heme and amino-acid detection are described in the Supplementary Information.

Results and Discussion

Isolation and characterization of *Dehalobacter* strains

Isolation and verification of purity. Isolation of the strains CF and DCA was achieved via repeated dilution-to-extinction transfers from the original mixed parent culture, ACT-3. The addition of autoclaved sterile mixed culture supernatant prepared from the ACT-3 parent culture was required to sustain the growth of the 10^{-9} dilution transfers (that is, detection of dechlorination activity) (Supplementary Figure S2). Nineteen successive 10^{-9} dilution transfers were performed. To replace mixed culture supernatant in the growth medium for the isolate cultures, a chemically defined medium (Supplementary Information) similar to that used for the isolation of *D. restrictus* strain PER-K23 (Holliger *et al.*, 1998) was tested. Cultures of the two isolates were able to deplete 1 mM chlorinated substrate (1,1,1-TCA or 1,1-DCA) after three successive 10% transfers (v/v) without mixed culture supernatant in MM medium when 0.1 mM of each arginine, histidine and threonine were provided (Figure 1a). These three amino acids may not represent the only viable combination required for growth but other combinations were not explored. The transfer cultures of the two isolate strains stopped dechlorinating when any of the three amino acids was excluded from the medium (data not shown). Based on the highly similar 16S rRNA gene sequences of strains CF and DCA to strain PER-K23 (99.3% average nucleotide identity), and their shared carbon source requirement, strains CF and DCA belong to the type species *D. restrictus*.

Microscopy suggested no growth in chlorinated substrate-free transfer cultures amended with yeast extract, a mixture of methanol, ethanol and sodium lactate, or with electron acceptors nitrate and sulfate. To corroborate the purity of isolates, DNA was extracted from isolate cultures and the 16S rRNA gene was amplified by general bacterial primers 27f and 1541r. Restriction fragment length polymorphism patterns reflected the presence of the only two *Dehalobacter* 16S rRNA genes in the isolate cultures (Supplementary Figures S3A-C). Strain CF cultures did not dechlorinate 1,1-DCA, and strain DCA cultures did not dechlorinate CF and 1,1,1-TCA. Moreover, *cfrA* was only amplified from strain CF DNA, and *dcrA* was only amplified from strain DCA

DNA (Supplementary Figure S3D), confirming that the two *D. restrictus* strains were indeed distinct.

Morphology. Microscopic images of samples of both isolate cultures stained with 4',6-diamidino-2-phenylindole dihydrochloride revealed a uniform cellular morphology, further supporting the culture purity (Figures 2a and b). Consistent with the three previously described *Dehalobacter* isolates (strains PER-K23, TCA1 and UNSWDHB; Holliger *et al.*, 1998; Sun *et al.*, 2002; Wong *et al.*, 2016), the cells of strains CF and DCA are either straight or curved rods (Figures 2a and b) approximately 2–6 μm long with a diameter of 0.4–0.6 μm (Figure 2c). Similar to strain PER-K23 (Holliger *et al.*, 1998), strain CF stains Gram-negative (Supplementary Figure S4), whereas it is phylogenetically Gram-positive. This negative staining may result from the presence of a proteinaceous S-layer on the exterior surface of *Dehalobacter* cells (Figure 2d; Holliger *et al.*, 1998). In some strain CF cells, an electron-dense granule was also observed (Figure 2d).

Electron donors and acceptors. According to annotations, *Dehalobacter* genomes possess various oxidoreductase genes involved in fermentation (Tang *et al.*, 2016), suggesting that they may be able to use electron donors other than H_2 or formate. The only genes for electron-accepting reactions in anaerobic respiration identified in the genomes were RDases. A broad range of electron donors and acceptors were tested to evaluate the substrate spectra of the both strains CF and DCA. Of the many electron donors tested, the two strains were only able to oxidize H_2 and formate (Supplementary Table S1A). The ability of both strains to use formate as the electron donor is consistent with the expression of *Dehalobacter* formate dehydrogenase (EC 1.2.1.2) in both 1,1,1-TCA- and 1,1,-DCA-fed ACT-3 mixed cultures (Tang and Edwards, 2013). However, the ability to use formate appeared to be conditional on the addition of ACT-3 mixed culture supernatant, yeast extract or casamino acids to the MM medium. Dechlorination with formate as electron donor was not sustained in medium that was not supplemented (data not shown). This conditional formate utilization may result from the inability to synthesize molybdopterin, the cofactor for formate dehydrogenase. Genome annotation previously revealed that none of the strains possess complete molybdopterin biosynthesis genes (Tang *et al.*, 2016). Therefore, strains CF and DCA may rely on molybdopterin supplementation from other community members in ACT-3 to respire formate. Unfortunately, the absence of commercially available molybdopterin standards has hampered our attempt to study this further.

We determined that strain CF could use 1,1,2-trichloroethane (1,1,2-TCA) as electron acceptor in addition to 1,1,1-TCA and CF. Strain DCA could also use 1,1,2-TCA in addition to 1,1-DCA (Supplementary Table S1B). However, the tolerance of strain

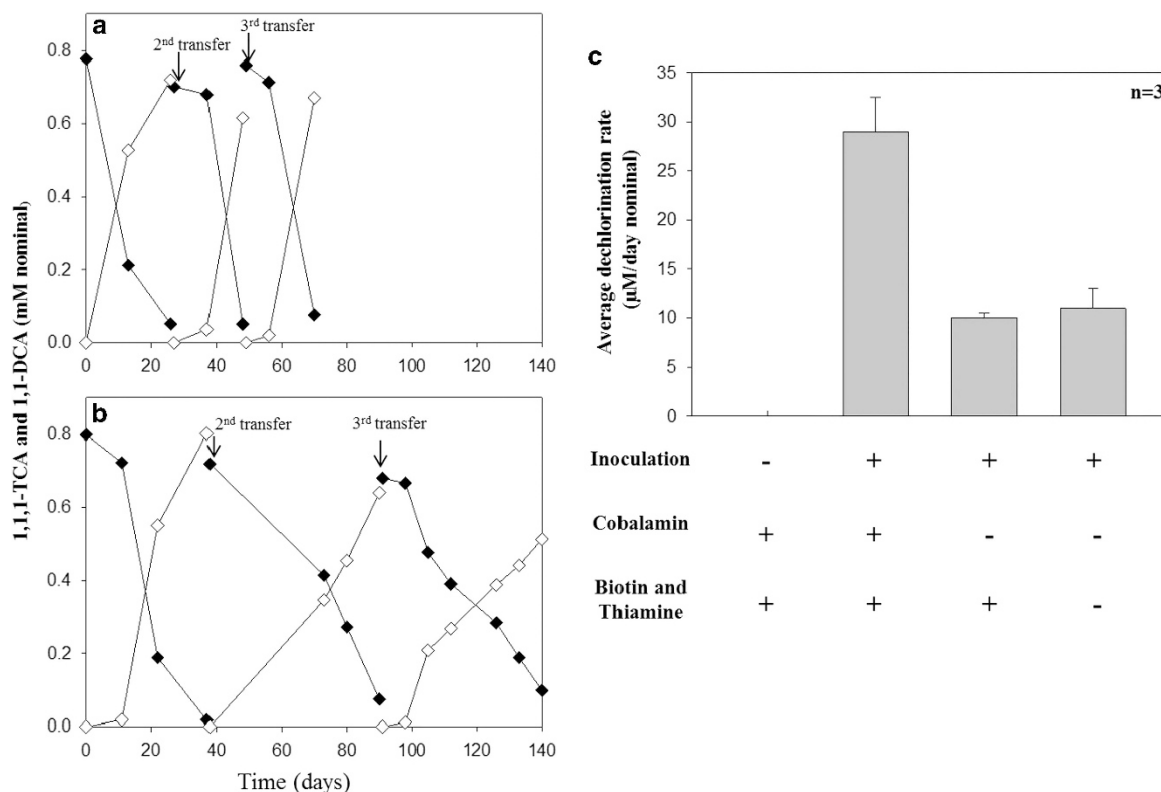


Figure 1 Dechlorination over three serial 10% transfers of strain CF. (a) cultures grown on chemically defined medium amended with the three vitamins (biotin, cobalamin and thiamine see Supplementary Information for exact composition). (b) cultures grown in medium lacking biotin, cobalamin and thiamine. (c) average dechlorination rates for strain CF cultures grown on medium with different vitamin combinations noted below. Symbols: \blacklozenge , 1,1,1-TCA; \diamond , 1,1-DCA. Data are mean \pm s.e. of three replicates in each experiment.

CF to aqueous CF concentrations ($[CF]_{aq}$) is much lower (below 0.42 mM) than to $[1,1,1-TCA]_{aq}$ and $[1,1,2-TCA]_{aq}$ (above 1 mM). The mixed culture was much less sensitive to CF, tolerating above 1 mM. No other chlorinated ethene or ethane tested was dechlorinated by the isolates (Supplementary Table S1B). As we could amend with higher concentrations, 1,1,1-TCA was used as the electron acceptor for strain CF in experiments to verify genome annotations. The genomes of strains CF, DCA and UNSWDHB contain a complete operon (*pceABCT*) in their genomes encoding an ortholog of the characterized tetrachloroethene (PCE) RDase PceA from strain PER-K23 (Tang *et al.*, 2016; Wong *et al.*, 2016) (>89% amino-acid identity). However, none of the strains was found to dechlorinate PCE. This lack of activity may result from the loss of a functional transcription factor to induce PceA expression or from a mutation at key catalytic residues.

To determine if CfrA can also dechlorinate 1,1,2-TCA, we conducted RDase enzyme assays using cell extracts of pure strain CF culture grown on 1,1,1-TCA that is known to express only CfrA. These cell extracts not only dechlorinated 1,1,1-TCA, but also CF and 1,1,2-TCA, confirming substrate promiscuity of CfrA (Table 1). Heat-treated cell extracts did not catalyze 1,1,2-TCA

dechlorination. Moreover, the fact that strain DCA is able to respire 1,1,2-TCA, and that DcrA is the only RDase detected in 1,1-DCA grown cells (Tang and Edwards, 2013) suggest DcrA also dechlorinates 1,1,2-TCA. 1,1,2-TCA is therefore a common substrate for four similar RDases, including CfrA and DcrA described above, TmrA from strain UNSWDHB (Wong *et al.*, 2016), and CtrA from *Desulfitobacterium* sp. strain PR (Zhao *et al.*, 2015). These chlorinated alkane-dechlorinating RDases define a protein cluster (ortholog group) based on >90% amino-acid identity (Hug and Edwards, 2013; Tang *et al.*, 2016). Interestingly, each strain and corresponding active RDase have slightly different substrate preferences supporting a previous contention that differentiation of organohalide-respiring strains is driven by chlorinated substrates (Islam *et al.*, 2010; Tang *et al.*, 2016).

Experimental verification of genome annotations of strain CF

Missing TCA cycle genes. The TCA cycle in all five sequenced *Dehalobacter* genomes is predicted to be incomplete because the genes encoding malate

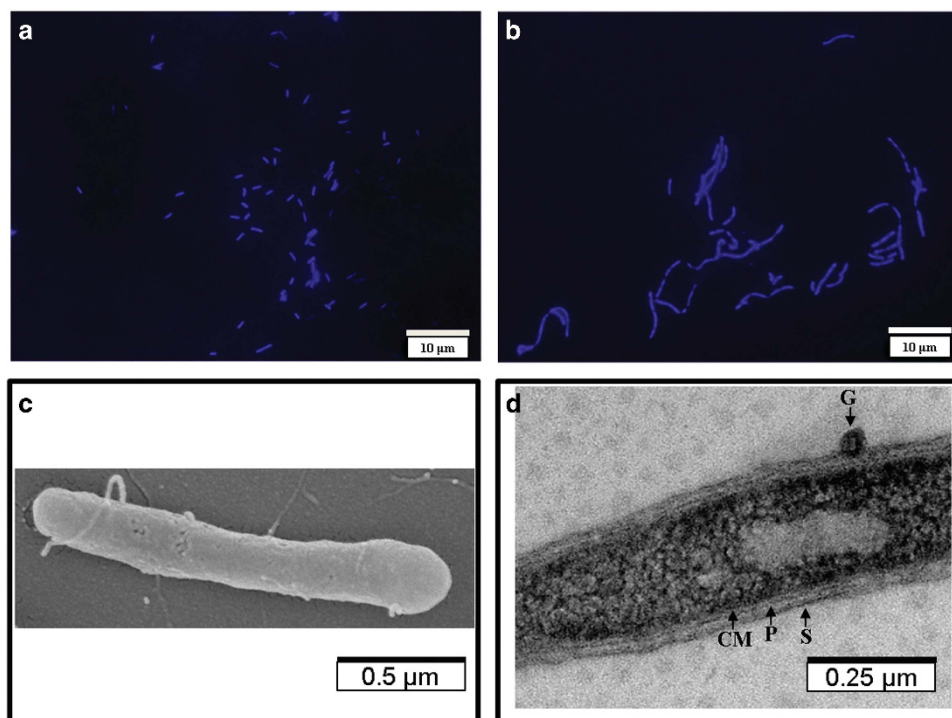


Figure 2 Microscopic images of *Dehalobacter* strains. Epifluorescence microscopic images of strains CF (a) and DCA (b) at x1000 magnification. (c) Scanning electron microscopic image and (d) transmission electron microscopic image of the strain CF cell. Scale bars are shown in each panel. Note the long rods in Figure 2b are formed by multiple strain DCA cells lining up end-to-end. CM, the cytoplasmic membrane; G, the electron-dense granule; P, the peptidoglycan layer; S, the proteinous S-layer.

Table 1 Dechlorinating activity observed in strain CF cell-free extracts

Substrate	Product	Dechlorination activity (nmol day ⁻¹ per mg protein) ^a
CF	DCM	868 ± 60
1,1,1-TCA	1,1-DCA	302 ± 26
1,1,2-TCA	1,2-DCA (major)	322 ± 28
	VC (minor)	7 ± 0.6

Abbreviations: CF, chloroform; DCM, dichloromethane; 1,1-DCA, 1,1-dichloroethane; 1,1,1-TCA, 1,1,1-trichloroethane; 1,1,2-TCA, 1,1,2-trichloroethane; VC, vinyl chloride.

^aData are mean ± s.e. of three replicates in each experiment.

dehydrogenase and succinate dehydrogenase were not found (Figure 3, pathways marked with red x). If these genes are truly absent, neither fumarate nor malate could be synthesized. *Dehalobacter* strains need the TCA cycle to synthesize oxaloacetate and 2-oxoglutarate for amino-acid biosynthesis (Mifflin and Lea, 1977). The absence of the two enzymes may be associated with the amino-acid requirement of *Dehalobacter*. Alternatively, a gene annotated as NDME (EC 1.1.1.40) is present in all *Dehalobacter* genomes and may have a role in compensating for missing TCA cycle genes. If active, this predicted NDME gene product could catalyze pyruvate–malate conversion (Figure 3). Moreover, the annotated malic

enzyme is also expressed in the ACT-3 mixed culture and proteome of strain PER-K23 (Rupakula *et al.*, 2013, Tang and Edwards, 2013). We assayed cell extracts of strain CF and *E. coli* (positive control) for malate and succinate dehydrogenase as well as NDME activities (Table 2). The latter activity was detected in both strain CF (0.06 μmol min⁻¹ mg⁻¹ or 1 nkat mg⁻¹) and *E. coli* (0.01 μmol min⁻¹ mg⁻¹ or 0.17 nkat mg⁻¹), whereas malate dehydrogenase and succinate dehydrogenase activities were only detected in *E. coli* cell extracts. Consistent with annotation, enzyme assays revealed that the activities of malate and succinate dehydrogenase are absent in *Dehalobacter* genomes. However, the NDME can salvage the synthesis of malate and fumarate from pyruvate (Figure 3).

Serine biosynthesis. Strains CF, DCA and PER-K23 were found to require arginine, histidine and threonine for growth, yet the biosynthesis pathways for these three amino acids appear complete in all the five *Dehalobacter* genomes. Rather, their genomes are missing the gene encoding phosphoserine phosphatase (PSP; EC 3.1.3.3), the enzymes that catalyzes the last step of the conventional serine biosynthesis pathway (Greenberg and Ichihara, 1957), whereas other serine salvage pathways are not present (Supplementary Figure S5A). A defect in serine biosynthesis should disable biosynthesis of glycine and cysteine (Supple-

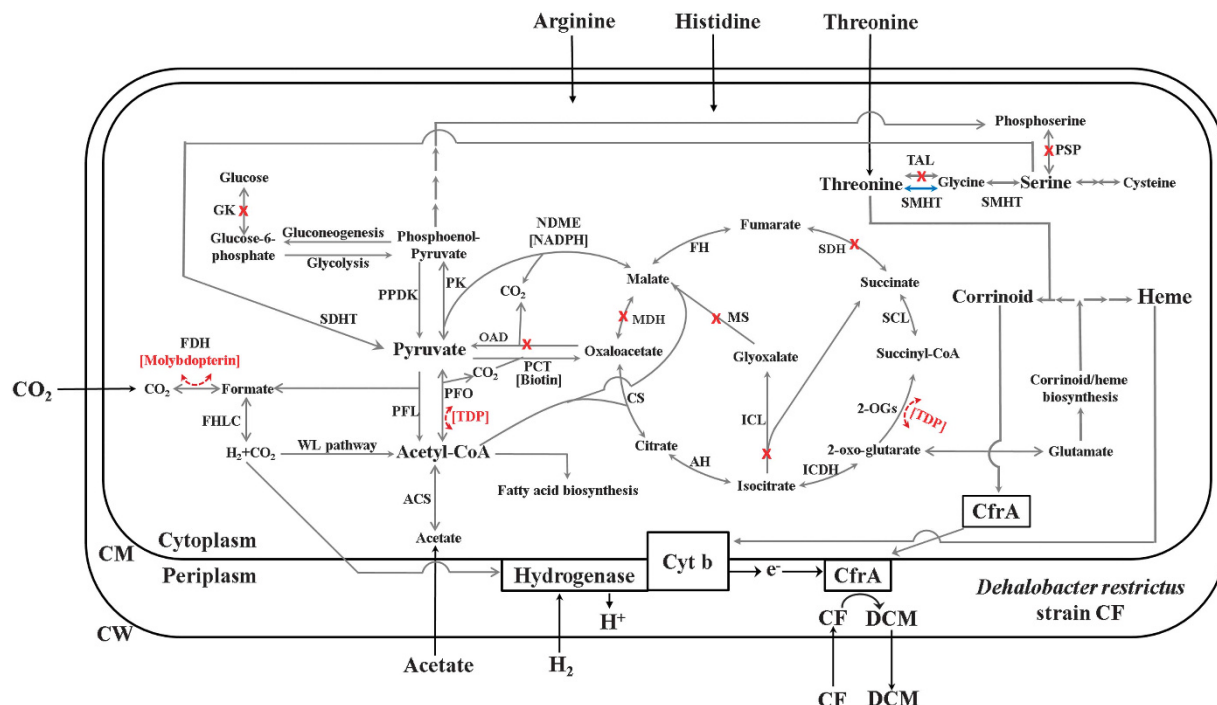


Figure 3 Schematic of the proposed metabolic map of strain CF. The red crosses (X) represent missing genes in the strain CF genome, whereas a red curved dashed line beside a reaction represents the absence of the essential cofactor. The black arrows represent nutrients imported from medium. Note that the threonine–glycine conversion (highlighted in blue) is catalyzed by promiscuous SHMT. Abbreviations for cofactors and enzymes involved in the reactions are listed below with full name: CfrA, chloroform reductive dehalogenase; PLP, pyridoxal phosphate; TDP, thiamine diphosphate; PK, pyruvate kinase; PPK, pyruvate phosphate dikinase; FDH, formate dehydrogenase (molybdopterin); FHL, formate hydrogen lyase complex (molybdopterin); ACS, acetate:CoA ligase (AMP-forming); PFL, pyruvate formate-lyase; PFO, pyruvate-flavodoxin oxidoreductase (TDP); PCT, pyruvate carboxyl transferase (biotin); NDME, NADP-dependent malic enzyme (NADP); CS, citrate synthase; AH, aconitate hydratase; ICDH, isocitrate dehydrogenase (NADP); 2-OGs, 2-oxoglutarate:ferredoxin oxidoreductase (TDP); SCL, succinyl-CoA ligase; FH, fumarate hydratase; GK, glucokinase; PSP, phosphoserine phosphatase; OAD, oxaloacetate decarboxylase (biotin); SDH, succinate dehydrogenase(heme); SDHT, serine dehydratase; MDH, malate dehydrogenase; ICL, isocitrate lyase; MS, malate synthase; TAL, threonine aldolase (PLP); SHMT, serine hydroxymethyltransferase (PLP). Other abbreviations: CM, cytoplasmic membrane; CW, cell wall; Cyt b, cytochrome b; WL pathway, Wood–Ljungdahl pathway.

Table 2 Enzymatic activities in cell extracts of *E. coli* and strain CF and promiscuous threonine aldolase activity of purified *Dehalobacter* SHMT

	Substrate	Detected product	Specific activity ($\mu\text{mol min}^{-1}$ per mg protein) ^a	
			<i>E. coli</i>	Strain CF
<i>Enzymatic activities in cell extracts</i>				
Malate dehydrogenase	Oxaloacetate	NAD	1.3 ± 0.2	ND
Succinate dehydrogenase	Succinate	DCPIP	0.03 ± 0.006	ND
NADP-dependent malic enzyme	Malate	NADPH	0.01 ± 0.001	0.06 ± 0.003
<i>Enzymatic activity of purified Dehalobacter SHMT</i>				
SHMT	L-serine	Glycine	NA	3.4 ± 0.2
Threonine aldolase	L-threonine	Acetaldehyde	NA	0.07 ± 0.003

Abbreviations: CF, chloroform; DCPIP, 2,6-dichlorophenolindophenol; *E. coli*, *Escherichia coli*; NA, not analyzed; NAD, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; ND, not detected; SHMT, serine hydroxymethyltransferase. ^aData are mean ± s.e. of three replicates in each experiment.

mentary Figure S5). However, strains CF and DCA are able to grow in a defined medium (supplemented with arginine, histidine and threonine; Figure 3) without cysteine, glycine and serine, suggesting the presence of an alternative

mechanism to produce serine, most likely from the three amino acids provided, as explained below.

Threonine can be converted to serine via glycine (Figure 3). However, threonine aldolase (EC 4.1.2.5), the enzyme catalyzing the threonine–glycine

conversion, is absent in the five *Dehalobacter* genomes. Interestingly, SHMT (EC 2.1.2.1), the enzyme catalyzing glycine-serine conversion, has been reported to possess promiscuous threonine aldolase activity in both *E. coli* and *Methanosarcina barkeri* strain Fusaro (Ogawa *et al.*, 2000; Buchenau and Thauer, 2004). SHMT is also expressed in the proteome of strain PER-K23 (Rupakula *et al.*, 2013). Furthermore, the SHMT of *M. barkeri* strain Fusaro shares >59% protein sequence identity to the sequences of the five *Dehalobacter* SHMTs, indicating *Dehalobacter* may utilize SHMT to synthesize serine from threonine. The lack of a PSP-coding gene can also be rescued by other promiscuous phosphatases in the haloacid dehalogenase (HAD) family. For example, it has been reported that histidinol phosphatase (HisB; EC 3.1.3.15) in histidine biosynthesis can rescue missing PSP in *E. coli* strain K-12 and *Thermococcus onnurineus* strain NA1 (Patrick *et al.*, 2007; Lee *et al.*, 2008; Yip and Matsumura, 2013). However, histidinol phosphatases (HisJ) in *Dehalobacter* genomes does not belong to the HAD family (Tang *et al.*, 2016).

To test if *Dehalobacter* can synthesize serine, the gene for SHMT (accession number AFV06891) from strain CF was cloned, heterologously expressed and purified (Supplementary Figure S5B). Strain CF SHMT revealed both threonine aldolase activity ($0.07 \mu\text{mol min}^{-1} \text{mg}^{-1}$ or 1.2 nkat mg^{-1}) and hydroxyl methyltransferase activity ($3.4 \mu\text{mol min}^{-1} \text{mg}^{-1}$ or 57 nkat mg^{-1} ; Table 2), suggesting that strain CF can synthesize serine from threonine via glycine. None of the enzyme-free or substrate-free controls showed any activity. Thus, the presence of a serine salvage mechanism enables serine production from threonine (also from glycine or cysteine) in strain CF. However, this does not explain why strain CF requires threonine as the biosynthesis pathway for threonine is complete. This discrepancy is further discussed in the section on thiamine section below.

In a recent comparative genomic analysis of >6000 sequenced bacteria from diverse environments, most *Firmicutes* were found to be serine auxotrophs based on annotated defects in the serine biosynthesis pathway (Mee *et al.*, 2014), consistent with previous annotation of *Dehalobacter* genomes (Tang *et al.*, 2016). However, we have now shown that these ostensible serine auxotrophs can actually synthesize serine using promiscuous enzymes, illustrating the shortcomings of typical bioinformatic analyses without experimental verification. Models constructed based on this comparative genomic analysis may assume that *Firmicutes* require serine from other community members when in fact they do not. For example, *Desulfitobacterium hafniense* strain Y51, another organohalide-respiring model organism and a member of *Firmicutes*, may also synthesize serine using promiscuous SHMT because (i) the organism lacks the PSP-coding gene in its genome (Nonaka *et al.*, 2006), (ii) its SHMT (accession number BAE86716) shares 75% protein

sequence identity with the homolog in strain CF and (iii) our data revealed that *D. hafniense* strain Y51 can grow in defined medium without amino-acid supplementation (Bi, 2015).

Biosynthesis of organic cofactors. The growth reliance of *Dehalobacter* on the three amino acids can also result from defects in cofactor biosynthesis. It is possible that (i) *Dehalobacter* cannot synthesize specific cofactors or (ii) *Dehalobacter* is not able to transform certain cofactor pre-cursors into functional forms. For example, thiamine is converted to thiamine monophosphate or thiamine diphosphate (TDP) intracellularly to be functional (Leonardi and Roach, 2004). In genomes of strains CF and DCA, the biosynthetic pathway for biotin appears to be missing 4/5 genes and for thiamine the pathway is missing 5/9 genes (Tang *et al.*, 2016). To determine if these pathways are truly incomplete, growth of strain CF was tested in the absence of these cofactors. We also tested the growth of strain CF in the presence and absence of cobalamin as genome annotation predicted a complete corrinoid biosynthesis pathway. Growth of strain CF was sustained in the absence of these three cofactors (biotin, cobalamin and thiamine) in cultures that were spun down and transferred three successive times (10% v/v inoculum; Figure 1b). Removal of cobalamin decreased the dechlorination rate by about a factor of 3 (from 30 to 10 μM per day nominal), whereas exclusion of biotin and thiamine from the medium did not affect the growth rate (11 μM per day nominal; Figure 1c). When grown with the three vitamins, the cell yield for strain CF determined from microscopic cell counts, which is $5.73 (\pm 1.79) \times 10^{12}$ ($n=9$) cells/mol Cl^- released, is comparable to the qPCR-determined cell yield of strain CF in CF-fed mixed culture ACT-3, which is $6 (\pm 0.03) \times 10^{12}$ /mol Cl^- released (Grostern *et al.*, 2010).

Biotin biosynthesis and metabolism

Biotin is considered vital for organisms in all domains of life because of its essential role in fatty acid biosynthesis (cofactor for acetyl-CoA carboxylase; Lin and Cronan, 2011), yet no known biotin biosynthesis pathway was identified in the five *Dehalobacter* genomes even though both strain PER-K23 (Holliger *et al.*, 1998) and strain CF (Figure 1c) can grow without added biotin. It was reported that biotin-independent malonate decarboxylases (Hoenke *et al.*, 1997) can circumvent the need for biotin in fatty acid biosynthesis; however, a homolog of this gene was not found in the five *Dehalobacter* genomes. Biotin is also an essential cofactor for pyruvate carboxyl transferase (Figure 3) that can convert pyruvate to oxaloacetate for amino-acid biosynthesis, and the pyruvate carboxyl transferase coding gene is annotated in all the five *Dehalobacter* genomes (Tang *et al.*, 2016). Moreover, traces of biotin may be sufficient to support growth.

It was reported that as few as 1000 biotin carboxyl carrier proteins are present in each *E. coli* cell (Li and Cronan, 1993). Accordingly, medium with a biotin concentration of $0.4 \mu\text{g l}^{-1}$ should be enough to support the growth of an *E. coli* culture to a cell density of 10^9 cells ml^{-1} .

To determine if biotin is present at sufficient quantities in our medium as a contaminant, we analyzed samples of the concentrated stock solutions used to make the MM medium by liquid chromatography-mass spectroscopy. We detected biotin in the 1000x vitamin stock solution (excluding biotin; referred to as biotin⁻) at a concentration of $15 \mu\text{g l}^{-1}$ (Supplementary Figure S6), indicating that (after 1000x dilution of the stock) the medium may contain $\sim 15 \text{ ng l}^{-1}$ biotin. This amount is low but may be sufficient to support the growth of strain CF cultures because they only reach a cell density $\sim 10^8$ cells ml^{-1} and require biotin for anabolism but not for respiration. Nevertheless, we cannot exclude the possibility that an uncharacterized biotin biosynthesis pathway is functioning in strain CF.

Thiamine biosynthesis and metabolism

The absence of a thiamine biosynthesis pathway in *Dehalobacter* genomes is consistent with the observation that the growth of strain PER-K23 required thiamine supplementation (Holliger et al., 1998). In contrast, the growth of strain CF seems not to require thiamine addition (Figure 1c). TDP is the cofactor of pyruvate-flavodoxin oxidoreductase and 2-oxoglutarate decarboxylase, two important carboxylases in central metabolism (Figure 3; Pohl et al., 2002). To test whether strain CF can synthesize TDP from thiamine, cultures of strain CF and *E. coli* grown on thiamine-containing medium ($25 \mu\text{g l}^{-1}$) were harvested for intracellular thiamine derivatives analysis. TDP was only detected in *E. coli* cell extracts, whereas thiamine was detected in both strain CF and *E. coli* cell extracts (Figure 4). Interestingly, although a gene annotated as thiamine pyrophosphokinase (EC 2.7.6.2) is present in the strain PER-K23 genome (Tang et al., 2016), the enzyme was not found to be expressed in the strain PER-K23 proteomes (Rupakula et al., 2013), consistent with the lack of TDP detection.

The lack of TDP indicates that strain CF only uses pyruvate formate-lyase for acetyl-CoA-pyruvate conversion, and not the TDP-dependent pyruvate-flavodoxin oxidoreductase (Figure 3). However, it is unclear if an organism could use only pyruvate formate-lyase to assimilate acetate and formate into pyruvate in defined medium. The only relevant study we found in literature did not test the effect of deleting the pyruvate-flavodoxin oxidoreductase-like *ydbK* (Nakayama et al., 2013) in their *E. coli* strain to exclude its contribution to pyruvate production (Zelcbuch et al., 2016). Moreover, threonine can be converted into pyruvate via serine (Figure 3). Furthermore, strain CF cultures grown

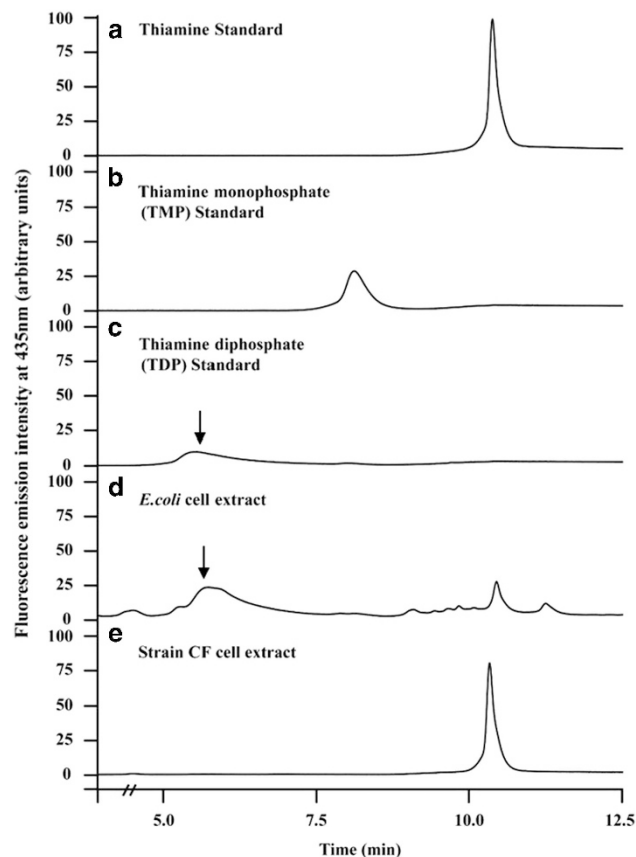


Figure 4 Determination of intracellular thiamine derivatives in strain CF cells. Chromatograms from high pressure liquid chromatography (HPLC) analysis with fluorescence detection (excitation at 365 nm and emission at 435 nm) of: (a) $1 \mu\text{M}$ thiamine standard solution; (b) $1 \mu\text{M}$ thiamine monophosphate; (c) $1 \mu\text{M}$ thiamine diphosphate; (d) cell extract of *E. coli*; and (e) cell extract of strain CF.

on pyruvate as the main carbon source dechlorinated at twice the rate ($53 \mu\text{M}$ per day nominal) of cultures grown on acetate ($29 \mu\text{M}$ per day nominal; amino acids supplemented in both conditions; Supplementary Figure S7). Therefore, strain CF likely requires amino-acid supplementation for growth partially because of difficulties in acetyl-CoA-pyruvate conversion, which in turn block synthesis of oxaloacetate and thus 2-oxo-glutarate (pre-cursors for amino acids; Figure 3; Miflin and Lea, 1977). Such nutrient requirements for strain CF portend a tightly entangled syntrophy in the ACT-3 microbial community.

Corrinoid biosynthesis

Corrinoids, such as cobalamin, have a crucial role in methionine and ribonucleotide biosynthesis, as well as energy metabolism in organohalide-respiring bacteria (Miller et al., 1998; Maillard et al., 2003; Sañudo-Wilhelmy et al., 2014). Reductive dechlorination and growth of organohalide-respiring bacteria are usually enhanced with corrinoid

supplementation (Yan *et al.*, 2013). It is reported that strains TCA1 (Sun *et al.*, 2002) and UNSWDHB (Wong *et al.*, 2016) grow without corrinoid supplementation. Strain CF could also grow without corrinoid supplementation (Figure 1b). Consistent with these observations, genomes of strains CF, DCA (Tang *et al.*, 2016) and UNSWDHB (Deshpande *et al.*, 2013) all possess a complete cobinamide biosynthesis pathway, whereas strains PER-K23 (Rupakula *et al.*, 2013) and E1 (Maphosa *et al.*, 2012) do not. However, to date, no direct evidence for *de novo* corrinoid synthesis by *Dehalobacter* exists. Thus, the

corrinoid concentration in strain CF cultures grown in corrinoid-free medium was quantified using a microbial B₁₂ assay (Yan *et al.*, 2013). The significant increase of corrinoid concentration along with dechlorination confirms the ability of strain CF to synthesize corrinoid *de novo* (Figures 5a and b). We attempted to characterize the type of corrinoid using UPLC-ESI-MS, however, none of the known cobamides with a benzimidazolyl or phenyl type lower ligand was detected in the cell extract of strain CF culture grown on corrinoid-free medium based on *m/z* values and retention times of the known

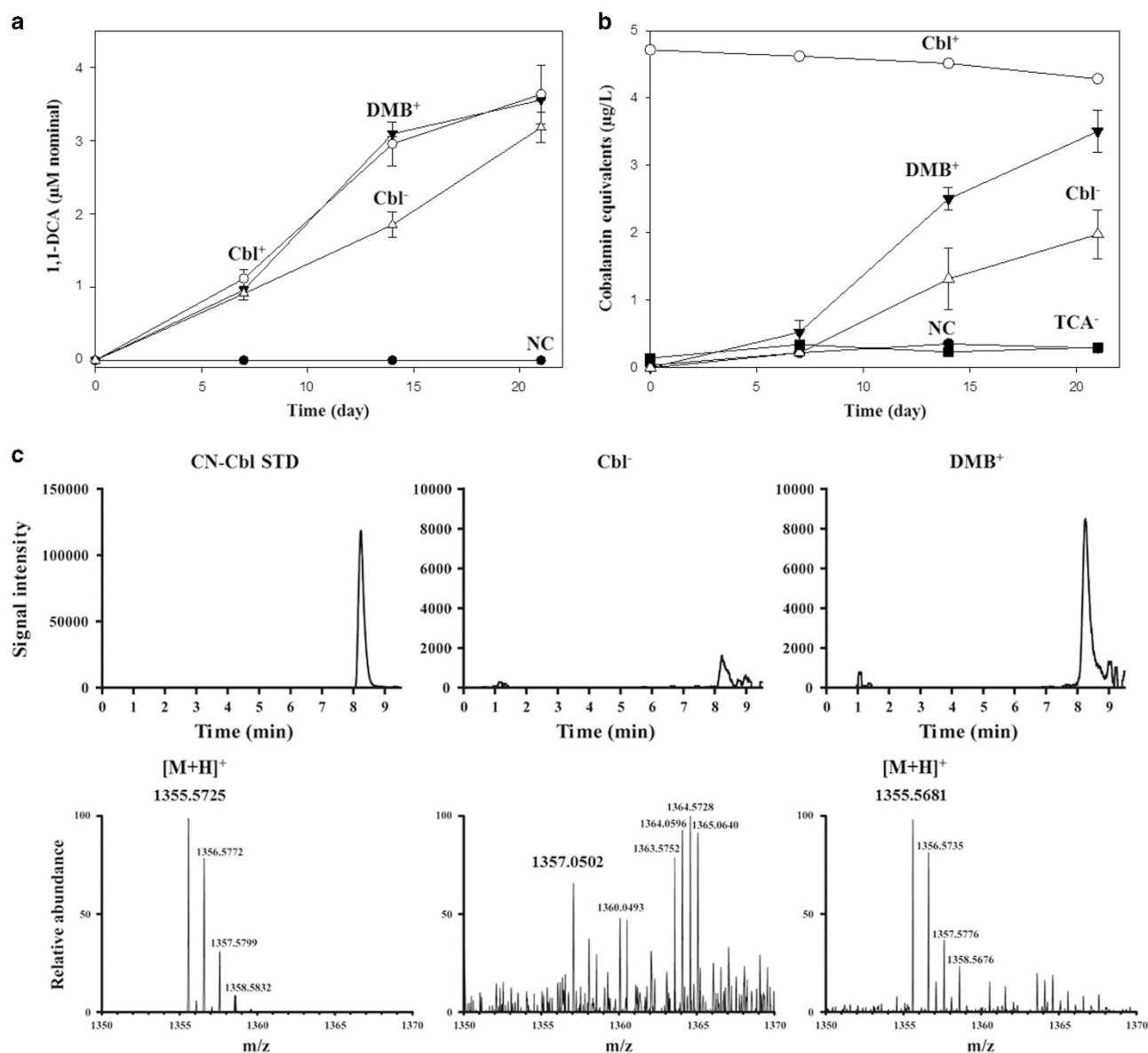


Figure 5 Corrinoid production and guided cobalamin synthesis by strain CF. (a) Dechlorination profiles (production of 1,1-DCA from 1,1,1-TCA) and (b) corresponding corrinoid production in cultures grown under different conditions as shown. ●, autoclaved negative control (NC); ■, 1,1,1-TCA-free control (TCA⁻); △, cobalamin- and DMB-free cultures (Cbl⁻); ▼, corrinoid-free and DMB-containing cultures (DMB⁺); ○, cobalamin-containing positive control (Cbl⁺). (c) UPLC-ESI-MS analysis of guided cobalamin synthesis in strain CF culture with DMB supplementation. The chromatograms on top panel shown are extracted ion chromatograms (*m/z* = 1355.57 ± 0.013) and the chemicals are ionized in positive mode. Bottom panel shows mass spectrum. CN-Cbl STD, cyanocobalamin standard. Data are mean ± s.e. of three replicates in each experiment.

cobamide standards (Supplementary Table S2). Nevertheless, cobalamin was identified in cell extracts of strain CF culture when 10 μM of the lower ligand compound DMB was supplemented to the corrinoid-free medium (Figure 5c). The guided cobalamin biosynthesis confirmed that strain CF possesses a functional cobinamide biosynthesis pathway to assemble cobalamin at the presence of DMB (Tang *et al.*, 2016). No cobinamide or cobalamin was identified in the commercial DMB and medium used for growth (Supplementary Figure S8). Thus, strain CF produces a cobamide that is yet to be identified, likely a purine species as the lower ligand as reported for strains of *Clostridium* (adenine) and *Desulfovibrio* species (guanine and hypoxanthine) (Hoffmann *et al.*, 2000; Guindon and Gascuel, 2003).

None of the *bza* genes involved in anaerobic DMB biosynthesis (Hazra *et al.*, 2015) was identified in the five *Dehalobacter* genomes (Tang *et al.*, 2016), consistent with the observation that no benzimidazole-type cobamide was identified from the strain CF culture. However, when DMB was provided, strain CF preferentially synthesized cobalamin rather than its native cobamide. This is in agreement with the discovery that DMB is a universally preferred substrate for the lower ligand-activating enzyme (CobT) in cobamide biosynthesis (Hazra *et al.*, 2013). Dechlorination rates in DMB-supplemented cultures were comparable to rates in cobalamin-supplemented cultures (Figure 5a), indicating that corrinoid synthesis in strain CF is limited by lower ligand availability but not by the availability of the porphyrin backbone. Furthermore, total corrinoid concentrations are higher in DMB-supplemented cultures than those without DMB (Figure 5b).

The *Dehalobacter* strains enriched from CF- and 1,1,1-TCA-dechlorinating consortia have retained their ability to synthesize corrinoids, whereas strains PER-K23 and E1 enriched with other halogenated substrates (PCE and β -hexachlorocyclohexane) do not. Strains PER-K23 and E1 do have partial pathways, but specific functions or sections have been lost, perhaps during enrichment. CF and 1,1,1-TCA, or methylchloroform, are potent inhibitors of homoacetogens and methanogens, well-known corrinoid producers in anaerobic environments (Whitman and Wolfe, 1984; Stupperich *et al.*, 1988). In addition, most corrinoid biosynthesis genes are missing from the dominant fermenting microbe, a *Bacteroides* sp., in ACT-3 (Tang *et al.*, 2012, 2013). Strain CF may have conserved its corrinoid-synthesizing ability because of environmental corrinoid shortage, whereas other corrinoid-auxotrophic strains lost the ability as a result of an abundant supply from other community members or enrichment in laboratory medium containing B_{12} . For example, the β -hexachlorocyclohexane-dechlorinating strain E1 has only a partial cobinamide pathway and depends on the syntrophic relationship with a *Sedimentibacter* sp., which contains a complete cobinamide biosynthesis

pathway in its genome (Maphosa *et al.*, 2012). Moreover, PCE-dechlorinating strain PER-K23 possesses complete and functional cobinamide biosynthesis genes, except for a truncated CbiH gene, resulting in its corrinoid auxotrophy (Rupakula *et al.*, 2015). Thus 1,1,1-TCA and CF may indirectly contribute to the conservation of the corrinoid biosynthesis genes among *D. restrictus* genomes.

Heme biosynthesis

Heme (heme b) is a cofactor for cytochrome b, a subunit of hup-type Ni,Fe-hydrogenase complex found in all five *Dehalobacter* genomes (Tang *et al.*, 2016). The hup-type Ni,Fe-hydrogenase is expressed during growth of strain PER-K23 (Rupakula *et al.*, 2013) and in all ACT-3 subcultures (Tang and Edwards, 2013), although the membrane-bound cytochrome b subunit was not detected, perhaps because of the limit of tryptic digestion-based proteomics. All five *Dehalobacter* genomes were found to possess a complete archaeal heme biosynthesis pathway first characterized in the methanogenic archaeon *Methanosarcina barkeri* (Kuhner *et al.*, 2014; Tang *et al.*, 2016). Recently, another heme biosynthesis pathway in aerobic *Firmicutes* was characterized (Dailey *et al.*, 2015). However, one of the biosynthesis enzymes, HemY, which converts coproporphyrinogen III to coproporphyrin III is an oxygenase and thus cannot function in strict anaerobic *Firmicutes* including *Dehalobacter*. Accordingly, the so-called 'archaeal pathway' is still the only characterized pathway for anaerobic heme biosynthesis.

When heme (hematin form; 25 $\mu\text{g l}^{-1}$) was added to the medium, *Dehalobacter* strain CF dechlorinated faster than without heme addition (Figure 6a). To confirm that the heme biosynthesis pathway is functional in strain CF, cell extracts of culture grown in heme-free medium were analyzed by UPLC-ESI-MS. A peak with a retention time of 1.5 min and an m/z value of 633.0404 comparable to that of an authentic heme standard ($m/z = 633.0380$) was identified (Figures 6b and c), confirming that strain CF is able to synthesize heme, most likely via the identified archaeal pathway. Before this experiment, heme has never been included in the components used to make growth medium for *Dehalobacter*. Our experiment indicates that this 'archaeal pathway' is not only functioning in methanogenic archaea but also in some anaerobic *Firmicutes*.

Implications for microbial ecology

In this study, experiments were conducted to refine or confirm predicted annotations in a strict anaerobe. These improved annotations enhance the accuracy of protein annotations, not only for *Dehalobacter*, but also for many other microbes, especially anaerobes, with similar pathways and genes. The study also explained several metabolic interdependencies

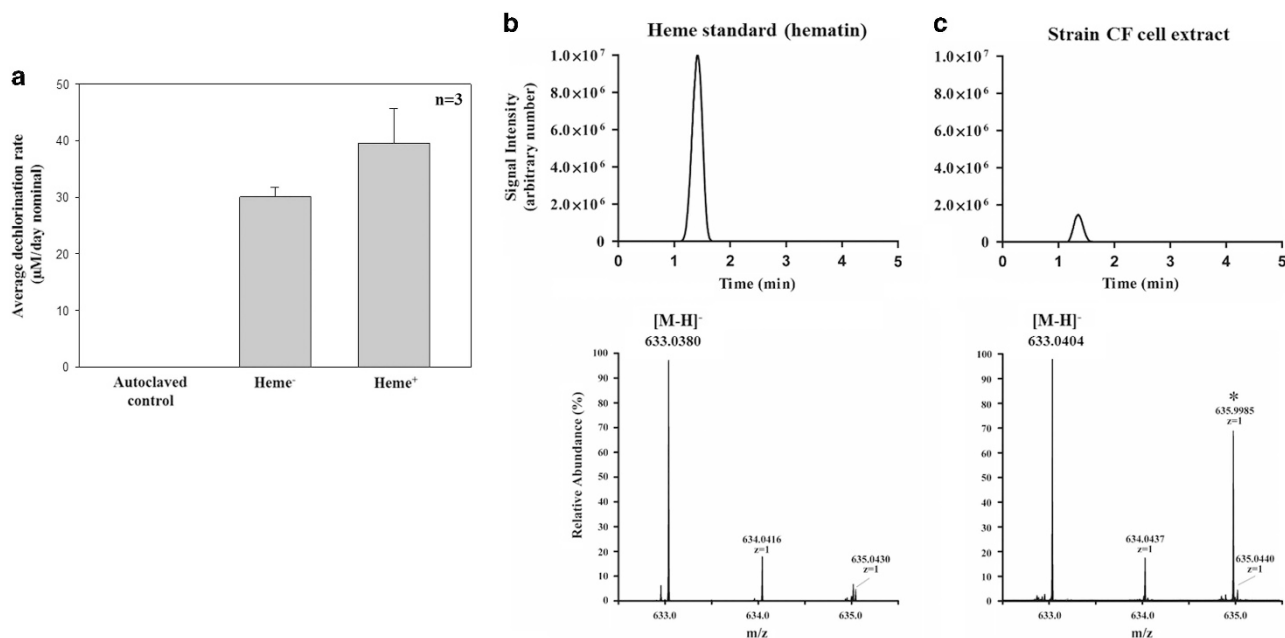


Figure 6 Heme production by strain CF and its influence on dechlorination. (a) Average dechlorination rates of strain CF cultures grown with/out heme supplementation. (b) Ultra performance liquid chromatography-mass spectroscopy (UPLC-MS) analysis of heme b standard (hematin form) and (c) heme in cell extract of strain CF. Chromatograms shown are extracted ion chromatograms ($m/z = 633.038 \pm 0.0063$) and the chemicals are ionized in negative mode. The * symbol in Figure 6c is an unknown organic present in strain CF cell extract. Data are mean \pm s.e. of three replicates in each experiment.

in *Dehalobacter*-containing mixed microbial communities. Consistent with curated genome annotations, CF-respiring *D. restrictus* strains are capable of *de novo* synthesis of the corrinoïd cofactor of RDases, which is in contrast to other *D. restrictus* strains where this ability has been lost. This study revealed that all sequenced *Dehalobacter* to date are capable of synthesizing heme (cofactor of Ni,Fe-hydrogenase) for organohalide respiration. 1,1,1-TCA and CF, as inhibitors of many fermentative or acetogenic corrinoïd producers, may indirectly contribute to the conservation of corrinoïd biosynthesis genes in *Dehalobacter* genomes. Experimental verification of genome annotation is a laborious effort, particularly for fastidious microorganisms, yet critical to resolving inconsistencies, discovering new functions, and enabling genome-scale metabolic modeling in microbial communities. This study illustrates two aspects of the interpretation of genome annotation that can improve the correspondence between bioinformatic predictions and the reality: (i) cofactor availability for corresponding metabolic reactions and (ii) the potential for enzyme promiscuity to rescue apparently missing pathways. These findings reinforce the importance of incorporating cofactor availability for corresponding enzymes in genome-wide function prediction as metabolic reactions require that both functional cofactors and corresponding enzymes be present. Moreover, biochemical characterization of poorly studied alternative metabolic pathways is urgently required to improve accuracy of genome annotations. Finally, the example of serine salvage by promiscuous SHMT

in strain CF suggests experimental verification is crucial to verify interpretations drawn from bioinformatic analyses.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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