Mutualistic interaction between dichloromethane- and chloromethane-degrading bacteria in an anaerobic mixed culture

Gao Chen,[1,2] Sara Kleindienst,[1,3,4] Daniel R. Griffiths,[5] E. Erin Mack,[6] Edward S. Seger,[7] Frank E. Löffler[1,2,3,8,9*]

[1]*Center for Environmental Biotechnology, University of Tennessee, Knoxville, TN 37996, USA.*
[2]*Department of Civil and Environmental Engineering, University of Tennessee, Knoxville, TN 37996, USA.*
[3]*Oak Ridge National Laboratory, University of Tennessee and Oak Ridge National Laboratory (UTORNL) Joint Institute for Biological Sciences (JIBS) and Biosciences Division, Oak Ridge, TN 37831, USA.*
[4]*Center for Applied Geosciences, Department of Geosciences, Eberhard Karls University Tübingen, Tübingen, 72074, Germany.*
[5]*Parsons, Denver, CO 80203, USA.*
[6]*Corporate Remediation Group, E. I. DuPont de Nemours and Company, Wilmington, DE 19805, USA.*
[7]*The Chemours Company, Wilmington, DE 19899, USA.*
[8]*Department of Microbiology, University of Tennessee, Knoxville, TN 37996, USA.*
[9]*Department of Biosystems Engineering and Soil Science, University of Tennessee, Knoxville, TN 37996, USA.*

***Abstract*—The microbial mixed culture RM grows with dichloromethane (DCM) as the sole energy source generating acetate, methane, chloride and biomass as products. Chloromethane (CM) was not an intermediate during DCM utilization consistent with the observation that CM could not replace DCM as a growth substrate. Interestingly, cultures that received DCM and CM together degraded both compounds concomitantly. Transient hydrogen (H_2) formation reaching a maximum concentration of 205 ± 13 ppmv was observed in cultures growing with DCM, and the addition of exogenous H_2 at concentrations exceeding 3000 ppmv impeded DCM degradation. In contrast, CM degradation in culture RM had a strict requirement for H_2. Following five consecutive transfers on CM and H_2, *Acetobacterium* 16S rRNA gene sequences dominated the culture and the DCM-degrader *Candidatus* Dichloromethanomonas elyunquensis was eliminated, consistent with the observation that the culture lost the ability to degrade DCM. These findings demonstrate that culture RM harbours different populations responsible for anaerobic DCM and CM metabolism, and further imply that the DCM and CM**

degradation pathways are mechanistically distinct. H_2 generated during DCM degradation is consumed by the hydrogenotrophic CM degrader, or may fuel other hydrogenotrophic processes, including organohalide respiration, methanogenesis and H_2/CO_2 reductive acetogenesis.

Introduction

Dichloromethane (DCM; CH_2Cl_2) is a naturally occurring compound and also intensively used as a solvent, paint-removal agent and synthetic intermediate in chemical synthesis. While the majority of DCM in the environment is attributed to anthropogenic releases, DCM emissions from a variety of sources including the oceans, wetlands and volcanoes contribute to the global DCM budget (for review, see Gribble, 2010). Chloromethane (CM; CH_3Cl) is the most abundant naturally occurring halocarbon in the atmosphere with an estimated annual global flux of about 2.8 Tg (Harper, 2000; Rhew *et al*., 2000). In addition, CM has served as a refrigerant and is used as a synthetic intermediate in the chemical industry (e.g., for the manufacture of silicone polymers), a foam-blowing agent and a pesticide (Seidel, 2007). DCM and CM cause stratospheric ozone destruction and are potent greenhouse gases (Butler, 2000; Hossaini *et al*., 2017). Both DCM and CM are toxic environmental pollutants (ATSDR, 1998, 2000) and have been detected in 252 (15%) and 120 (7%) of National Priority Listed (NPL) sites in the United States of America (USA) respectively (EPA, 2014). DCM was detected in groundwater samples taken throughout the contiguous United States in concentrations ranging from 0.02 to 100 µg L^{-1} (Moran *et al*., 2007).

Microorganisms and pathways for DCM and CM degradation under oxic conditions have been studied in detail and are well documented (Kohler-Staub and Leisinger, 1985; Leisinger and Braus-Stromeyer, 1995; Vannelli *et al*., 1999; McDonald *et al*., 2002; Nikolausz *et al*., 2006). In contrast, the anaerobic degradation of DCM and CM is far less clear. To date,*Dehalobacterium formicoaceticum* and *Acetobacterium dehalogenans* are the only known isolates capable of utilizing DCM and CM, respectively, as growth substrate in the absence of oxygen (Traunecker *et al*., 1991; Mägli *et al*., 1996). In contrast to chlorinated ethenes, chlorinated ethanes and chloroform, DCM and CM do not undergo direct hydrogenolysis (i.e., reductive dechlorination). Instead, based on physiological and biochemical evidence, DCM and CM are funnelled into the Wood-Ljungdahl pathway (reductive acetyl-CoA pathway) after chlorine removal and broken down to acetate, formate, carbon dioxide and inorganic chloride (Meßmer *et al*., 1993; Mägli *et al*., 1998).

Culture RM is a strictly anaerobic, DCM-degrading mixed culture that has been enriched with DCM as the sole energy source producing acetate, methane, chloride and biomass as products (Justicia-Leon *et al*., 2012). This culture has been maintained with DCM as the sole energy source for at least 50 consecutive transfers after establishment (Justicia-Leon *et al*., 2012). Phylogenetic and metagenomic analyses identified the DCM degrader and affiliated the organism with the family *Peptococcaceae*, most closely related to the genera of *Dehalobacter* and *Syntrophobotulus* but with 16S rRNA gene sequence identities not exceeding 94% and 93% respectively. Based on the taxonomic differences to its closest relatives, the DCM degrader in culture RM was characterized as *Candidatus* Dichloromethanomonas elyunquensis representing a new genus and species (Kleindienst *et al*., 2017). Although CM could not replace DCM as a growth substrate, CM was metabolized in cultures that received DCM and CM concomitantly. Detailed physiological studies revealed a H_2-mediated, mutualistic relationship between the DCM- and the CM-consuming populations, a finding with implications for bioremediation as well as for the ecology of organisms contributing to chlorinated C1 compound metabolism in pristine and contaminated environments.

Results

Degradation of DCM and CM in culture RM

RM cultures that received repeated feedings of DCM degraded DCM at a maximum observed rate of 160 ± 15 μmoles d^{-1} L^{-1}. Consistent with prior studies, stoichiometric amounts of chloride were released during DCM degradation, and acetate, methane and biomass were produced (Justicia-Leon *et al*., 2012; Kleindienst *et al*., 2017). CM was not detected as a possible degradation intermediate in DCM-fed cultures, suggesting that DCM does not undergo reductive dechlorination (hydrogenolysis), which would result in CM formation. In cultures where CM substituted DCM as the sole energy source, no growth occurred, no chloride was released, and CM remained in the vessels even after extended incubation periods of 3 months (data not shown). Interestingly, when DCM and CM were added together to culture RM, both DCM and CM were degraded (Fig. 1A). After a 2 week lag phase, DCM degradation occurred at a rate of 47.5 ± 5.7 μmoles d^{-1} L^{-1} and the degradation rate approximately doubled following the second and third DCM feedings (Fig. 1A). CM consumption by culture RM commenced shortly after the initiation of DCM degradation, and CM was degraded at a rate of 6.1 ± 1.3 μmoles d^{-1} L^{-1} (Fig. 1A). A significantly faster rate of 17.3 ± 0.4 μmoles d^{-1} L^{-1} was measured following the second CM feeding (Fig. 1A). Both CM and DCM were consumed to concentrations below

the detection limits of the analytical system, which were approximately 0.01 and 0.02 µmoles respectively. In cell-free control incubations, as well as in live cultures without DCM, some CM loss (approximately 10 µmoles) occurred over a 6 week long incubation period (Fig. 1B), presumably due to sorption to the rubber stoppers.

The observation that CM degradation only occurred in cultures that degraded DCM implied that CM degradation in culture RM was either dependent on a DCM metabolite or was a co-metabolic conversion possibly mediated by the DCM-degrading bacterium. To examine the effect of previously observed DCM degradation products, that is, acetate and formate, on CM degradation, each organic acid was amended individually to CM-fed cultures. No CM degradation occurred in cultures amended with 500 µmoles of acetate, but CM degradation was observed in cultures that received 500 µmoles of formate (Fig. 2A). The initial amount of CM (~20 µmoles) was also degraded in cultures that received 10 ml of H_2 (~410 µmoles), but additional CM was not utilized (Fig. 2B). Headspace measurements indicated that H_2 had been consumed to concentrations below 91 ± 11 ppmv (0.22 ± 0.03 µmoles), and CM degradation resumed after H_2 was replenished (Fig. 2B).

RM cultures provided with 10 ml of H_2 (~410 µmoles) as the sole electron donor and with CO_2 as electron acceptor produced 44.1± 3.6 µmoles of methane and 58.6 ± 3.4 µmoles of acetate (Supporting Information Fig. S1). Stoichiometric calculations indicated that the electrons generated from H_2 oxidation were fully recovered in methane and acetate (Supporting Information Table S1), indicating hydrogenotrophic methanogenesis and H_2/CO_2-reductive acetogenesis were the predominant electron-consuming processes in culture RM. In cultures amended with 470.0 ± 23.2 µmoles of formate as electron donor and with CO_2 as electron acceptor, a similar pattern of methane (70.8 ± 5.9 µmoles) and acetate (48.9 ± 5.7 µmoles) formation was observed (Supporting Information Fig. S2A and Table S1). H_2 was transiently produced in formate-grown cultures (Supporting Information Fig. S2B) indicating formate conversion to CO_2 and H_2. Acetate added either as a sole substrate or together with DCM could not be utilized by culture RM (data not shown).

Abiotic reductive dechlorination of chlorinated C1 hydrocarbons, including DCM and CM, by corrinoids with the cobalt atom (Co) in the reduced Co^{+1} state has been reported (Krone *et al.*, 1989). Homoacetogens are known corrinoid producers (Stupperich *et al.*, 1988); however, the abiotic reductive dechlorination of DCM and CM was slow, incomplete and required corrinoid concentrations orders of magnitude higher than the nM concentrations expected in microbial cultures (Krone *et al.*, 1989). To examine if fortuitous reductive dechlorination was responsible for the observed CM degradation activity, we tested CM degradation in axenic

cultures of *Acetobacterium woodii* and *Sporomusa ovata*, both known corrinoid producers (Stupperich *et al*., 1988). Both cultures did not degrade CM during active growth with H_2 and CO_2 (data not shown), and the fortuitous reductive dechlorination of CM to methane mediated by corrinoids produced by acetogens in culture RM could be ruled out.

Distinct microorganisms are responsible for DCM and CM degradation

qPCR assays specifically targeting the 16S rRNA gene of the DCM degrader *Candidatus*Dichloromethanomonas elyunquensis demonstrated that this organism did not grow in cultures amended with either CM and H_2 or H_2 alone (Supporting Information Fig. S3), indicating that a distinct member of culture RM is responsible for CM degradation. After five consecutive transfers in medium amended with CM and H_2, the culture lost its ability to degrade DCM, and the 16S rRNA gene of *Candidatus* Dichloromethanomonas elyunquensis was no longer detectable with qPCR (data not shown). These observations demonstrate that co-metabolic degradation of CM by the DCM degrader cannot explain CM disappearance and, thus, another distinct microorganism (or microorganisms) in culture RM must be responsible for CM degradation in the presence of H_2.

Repeated transfers of culture RM to medium amended with H_2 as the sole electron donor and with CO_2 as the electron acceptor (i.e., without DCM) resulted in complete loss of DCM degradation activity, which is consistent with the observation that *Candidatus*Dichloromethanomonas elyunquensis cannot grow via H_2/CO_2 reductive acetogenesis (Kleindienst *et al*., 2017). In contrast, the ability of the culture to degrade CM was maintained suggesting homoacetogens (i.e., bacteria capable of H_2/CO_2 reductive acetogenesis) or methanogens were involved in CM degradation. To explore the role of methanogens in CM degradation, the methanogenesis inhibitor 2-bromoethanesulfonate (BES) was added over seven consecutive transfers in medium amended with H_2 as the electron donor and with CO_2 as electron acceptor (i.e., without CM additions). The resulting culture produced no methane but maintained its ability to degrade CM in the presence of H_2 without lag phase (Supporting Information Fig. S4). Non-methanogenic, CM/H_2-grown cultures released stoichiometric amounts of chloride and acetate was the only detectable product (Supporting Information Fig. S5), indicating that methanogens were not involved in CM degradation.

To elucidate structural community changes in response to enrichment with DCM or with CM plus H_2 in the presence/absence of BES, 16S rRNA gene amplicon sequencing was performed. With DCM as the sole energy source, the DCM-degrading bacterium *Candidatus*Dichloromethanomonas elyunquensis was the dominant population and

contributed about half of the total sequences. In contrast, no sequences representing this organism were detected in the amplicon dataset generated with DNA extracted from cultures grown with CM and H_2 (Table 1), a finding supported by qPCR targeting the *Candidatus*Dichloromethanomonas elyunquensis 16S rRNA gene. When grown with CM and H_2, sequences of the genus *Acetobacterium* dominated, representing 36.9% and 27.7% of all sequences in cultures with and without the methanogenesis inhibitor BES, respectively (Table 1). In cultures without BES, the genus *Methanospirillum* contributed up to 10.7% of the total amplicons and no other methanogen sequences were detected (Table 1). *Methanospirillum* spp. are known to utilize H_2 as electron donor and CO_2 as electron acceptor to synthesize methane. BES effectively inhibited the growth of *Methanospirillum* as evidenced by the lack of methane production and the absence of *Methanospirillum* 16S rRNA gene sequences (Table 1).

Table 1. Community composition of RM cultures grown with DCM, or CM plus H_2 in the presence/absence of BES, as determined by 16S rRNA gene amplicon sequencing.

	Relative sequence abundance (%)[a]		
Taxon	DCM	CM/H_2 + BES	CM/H_2
Methanospirillum	7.1	0.0	10.7
Bacteroidales	7.3	12.5	23.7
Acetobacterium	1.4	36.9	27.7
Candidatus Dichloromethanomonas elyunquensis	46.0	0.0	0.0
Desulfovibrio	4.0	7.5	1.3
Geobacter	1.5	6.8	0.1
Sulfuricurvum	6.7	16.3	0.8
Pseudomonadaceae	1.3	0.0	0.1
Treponema	2.2	5.2	4.8
Synergistales	7.5	2.1	6.8
Mollicutes	0.3	1.4	0.2
WPS-2	10.5	7.3	20.2
Others	4.2	3.9	3.6

a. The total sequence reads obtained from cultures grown with DCM, CM/H_2+BES and CM/H_2 in bicarbonate-buffered medium were 65 055, 154 455 and 128 211 respectively. Only taxa with greater than 1% relative abundance are shown and reported to the lowest taxonomic rank possible.

H_2 formation during DCM degradation and effect of H_2 on DMC degradation

To elucidate why a hydrogenotrophic CM-degrading organism was maintained in culture RM during the extended enrichment phase with DCM as the sole energy source, H_2concentration measurements were carefully performed in cultures growing with DCM. $H_2$was produced during DCM consumption and the H_2 concentration increased from 4 ± 1 ppmv to a maximum concentration of 205 ± 13 ppmv (Fig. 3). Concomitant with H_2formation, methane and acetate were produced (data not shown) suggesting that methanogens and homoacetogens were actively consuming H_2. These findings indicate that the H_2 partial pressures measured in the cultures

represent compensation concentrations due to simultaneous H_2 production and consumption. Following DCM depletion, H_2concentrations decreased to a concentration of 38 ± 3 ppmv (Fig. 3), which falls within the reported range of H_2 consumption threshold concentrations (i.e., 6 – 120 ppmv) for hydrogenotrophic CO_2 reduction to methane (Löffler *et al*., 1999) (Fig. 6). In DCM-free controls, some H_2 was formed over a 6 week incubation period, presumably generated from the biomass introduced with the inoculum; however, the H_2 concentrations did not exceed 45 ± 4 ppmv (data not shown).

To explore the effect of H_2 on DCM degradation, replicate cultures were incubated with and without H_2. Whereas DCM was degraded in cultures without exogenously added H_2 (Fig. 4A), H_2 amendments to DCM-fed cultures prevented DCM degradation (Fig. 4B). Since H_2 was consumed by organisms capable of H_2/CO_2 reductive acetogenesis, repeated H_2 additions were required to maintain inhibitory H_2 partial pressures in the culture vessels (Fig. 4B). When the H_2 addition was halted and the H_2 concentrations dropped below approximately 3000 ppmv, DCM degradation commenced (Fig. 4C). Following DCM consumption, H_2 was consumed to a threshold concentration of $\sim 780 \pm 140$ ppmv (Fig. 4C). Because BES abolished methanogenesis, H_2/CO_2 reductive acetogenesis was the only H_2-consuming process. Thus, the measured H_2 threshold concentration of $\sim 780 \pm 140$ ppmv represents the consumption threshold concentration of the hydrogenotrophic CO_2-to-acetate-reducing population with the highest H_2 affinity present in the culture.

Discussion

The experiments with culture RM demonstrated that CM is not an intermediate of DCM degradation and CM could not replace DCM as a growth substrate. Apparently, DCM does not undergo reductive dechlorination (i.e., the replacement of one chlorine substituent with a hydrogen atom), a common bacterial strategy to take advantage of chlorinated compounds under anoxic conditions (Smidt and De Vos, 2004). The absence of CM as a degradation intermediate is consistent with a previous investigation of a DCM-degrading methanogenic mixed culture, where ^{14}C-DCM tracer studies identified radiolabelled CO_2, methane and acetate, but not CM (Freedman and Gossett, 1991). Similarly, CM was not detected in a mixed culture containing a chloroform-to-DCM-respiring *Dehalobacter* sp. and a population degrading DCM to acetate (Lee *et al*., 2012; Wong *et al*., 2016). Based on these observations, it was surprising that CM degradation occurred in culture RM in the presence of DCM. The experimental efforts ruled out

the direct involvement of *Candidatus*Dichloromethanomonas elyunquensis in CM degradation, indicating that CM was not co-metabolized by the DCM degrader.

H_2 requirement and the involvement of an *Acetobacterium* sp. in CM degradation

To date, *Acetobacterium dehalogenans* strain MC is the only pure culture capable of utilizing CM under anoxic conditions (Traunecker *et al*., 1991). *Acetobacterium dehalogenans* grows autotrophically with H_2 and CO_2 generating acetate and has the unique ability to also utilize CM as the sole electron donor and energy source (Traunecker *et al*., 1991). The enzyme system catalysing CM dehalogenation has yet to be characterized, but it was proposed that the CM dehalogenase functions analogously to *O*-demethylases, which are corrinoid-dependent methyltransferases (Meßmer *et al*., 1996; Kaufmann *et al*., 1998; Kreher *et al*., 2010). Experiments with cell extracts of strain MC demonstrated the presence of all Wood-Ljungdahl pathway enzymes and the ability to synthesize acetate from either CM alone or CO_2 and H_2 (Meßmer *et al*., 1993, 1996). Therefore, it was proposed that CM is initially converted to methyl-tetrahydrofolate (CH_3-THF), a Wood-Ljungdahl pathway intermediate, and then fermented to acetate and CO_2 (Meßmer *et al*., 1993, 1996). CM metabolism by *Acetobacterium dehalogenans* strain MC does not require H_2, and the oxidation of CH_3-THF to CO_2 via the reversal of the Wood-Ljungdahl pathway generates the reducing equivalents required for the reduction of CO_2 to acetate (Diekert and Wohlfarth, 1994). In contrast, the CM-degrading population in culture RM has a strict requirement for H_2 and no CM degradation occurred without H_2 or formate additions. The findings that (i) CM degradation required H_2 or formate (Fig. 2), (ii) CM degradation in cultures grown with H_2/CO_2commenced without apparent lag phase (Supporting Information Fig. S4) and (iii) acetate was the only product generated in non-methanogenic cultures grown with CM and H_2(Supporting Information Fig. S5) suggest that the CM-degrading bacterium in culture RM is most likely a bacterium capable of H_2/CO_2-reductive acetogenesis (i.e., is a homoacetogen).

The dependency of CM degradation on an electron donor (i.e., H_2 or formate) is puzzling because the CM-degrading bacterium in culture RM should be able to produce reducing equivalents via the oxidative direction of the Wood-Ljungdahl pathway, as was observed in *Acetobacterium dehalogenans* strain MC (Meßmer *et al*., 1993, 1996). Possibly, *Acetobacterium dehalogenans* and the CM-degrading bacterium in culture RM use mechanistically different strategies to degrade CM. Mechanistic understanding of carbon-chlorine bond cleavage is lacking, but a plausible scenario is the conversion of CM to CH_3-THF, which is funnelled to the Wood-Ljungdahl pathway (Meßmer *et al*., 1993, 1996). In contrast to *Acetobacterium dehalogenans*, the CM-degrading bacterium in culture RM might not be able

to oxidize CH_3-THF via the reversal of the pathway. Therefore, H_2 is required to reduce CO_2 to carbon monoxide (CO), and CO and CH_3-THF are converted via the reductive direction of the Wood-Ljungdahl pathway to generate acetate. In cultures amended with CM and CO, but lacking H_2, CM was degraded (Supporting Information Fig. S6), consistent with the hypothesis that H_2 is needed for the reduction of CO_2 to CO. The CM-degrading bacterium is not available in pure culture, and it remains to be determined if CM degradation is linked to energy conservation or CM is co-metabolized during growth with H_2/CO_2 or CO/CO_2. *Acetobacterium woodii* and *Sporomusa ovata* are model organisms to study H_2/CO_2 reductive acetogenesis, and both bacteria cannot degrade CM during active growth on H_2/CO_2, indicating that CM catabolism is not a shared feature of homoacetogens. Should future isolation efforts be successful, detailed physiological and biochemical experiments can reveal interesting new insights into anaerobic CM metabolism in homoacetogens.

Based on the strict requirement for H_2 and the formation of acetate, chloride and biomass as products, the CM-degrading population is likely to metabolize CM according to Eq. 1 (Table 2). This reaction is energetically more favourable than the fermentation of CM to acetate (Eq. 2, Table 2) by *Acetobacterium dehalogenans* under standard conditions (Traunecker *et al*., 1991). H_2 concentrations influence the thermodynamics associated with H_2-dependent CM conversion to acetate (Eq. 1, Table 2); however, the reaction is still favourable even under very low H_2 partial pressures (<1 ppmv) (Supporting Information Fig. S7), indicating that this reaction might occur in natural anoxic environments.

Table 2. Reactions involved in anaerobic DCM and CM metabolism and associated free energy changes.[a]

		kJ mol^{-1} DCM or CM	
Equation	Reaction	$\Delta G^{\circ\prime}$	ΔG^{b}
(1)	$CH_3Cl + CO_2 + H_2 \rightarrow CH_3COO^- + 2\ H^+ + Cl^-$	−141.7	−112.1
(2)	$4\ CH_3Cl + 2\ CO_2 + 2\ H_2O \rightarrow 3\ CH_3COO^- + 7\ H^+ + 4\ Cl^-$	−113.8	−115.7
(3)	$3\ CH_2Cl_2 + 4\ H_2O + CO_2 \rightarrow 2\ HCOO^- + CH_3COO^- + 9\ H^+ + 6\ Cl^-$	−225.6	−243.0
(4)	$3\ CH_2Cl_2 + 4\ H_2O \rightarrow 2\ H_2 + CO_2 + CH_3COO^- + 7\ H^+ + 6\ Cl^-$	−227.9	−259.3

a. The calculations used published DG8f values (Thauer et al., 1977; Dolfing and Janssen, 1994; Widdel and Musat, 2010).
b. Gibbs energy changes (ΔG) were calculated under the following conditions: [DCM]=1.5 mM; [CM]=0.2 mM; [Cl^-]=33 mM; [CO_2]=0.2 atm=10^{-6} atm ~ 1 ppmv; T=258°C and pH=7.

The community analysis of cultures grown with CM plus H_2 revealed a strong enrichment of *Acetobacterium* (Table 1), suggesting an *Acetobacterium* population as a potential candidate responsible for CM degradation in the mixed culture. Among the 16S rRNA gene sequence-based operational taxonomic units (OTUs) assigned to the genus *Acetobacterium*, one OTU

(OTU-970) dominated, accounting for 98.6% (929 out of 942), 98.9% (56 125 out of 56 745) and 99.2% (35 238 out of 35 511) of total *Acetobacterium* sequence reads when grown with DCM, CM/H_2 + BES and CM/H_2, respectively (Table 1). OTU-970 represented a 250-bp long 16S rRNA gene fragment that shared 100% identity with the 16S rRNA gene of many *Acetobacterium* species including *Acetobacterium dehalogenans* strain MC. Although these data suggest a possible affiliation with the genus *Acetobacterium*, the strict requirement for H_2 indicates physiological differences between the CM-degrader in culture RM and the characterized anaerobic CM-degrading bacterium *Acetobacterium dehalogenans* strain MC.

H2 evolution during DCM degradation

Careful gas phase measurements in RM cultures growing with DCM revealed H_2 formation during DCM degradation, even though H_2 scavengers (i.e., methanogens and homoacetogens) constantly consumed H_2. Transient H_2 formation during DCM degradation is not unprecedented and has been observed in a DCM-degrading methanogenic enrichment culture, where DCM-derived H_2 was shown to support methanogenesis (Freedman and Gossett, 1991). The mechanism of H_2 formation is unclear but H_2 could be evolved directly from DCM metabolism via a so far unknown mechanism, or indirectly from a DCM degradation product, presumably formate. In pure culture studies with the DCM-degrading anaerobe *Dehalobacterium formicoaceticum*, acetate and formate were identified as DCM degradation products (Mägli *et al*., 1996, 1998), suggesting that formate conversion is a plausible mechanism for H_2 generation. Based on the physiological and biochemical evidence obtained from studies of *Dehalobacterium formicoaceticum*, DCM was proposed to lose both chlorine substituents by a yet unknown mechanism prior to being funnelled into the Wood-Ljungdahl pathway to generate acetate and formate in a fermentative manner according to Eq. 3 (Table 2) (Mägli *et al*., 1996, 1998).

In RM cultures grown with DCM in the presence of BES, acetate was the only measurable product and formate was not observed. As discussed above, RM cultures readily utilized formate with the transient formation of H_2 (Supporting Information Fig. S2). Hence, it is possible that formate generated from DCM was quickly oxidized and evaded detection. Therefore, H_2 detected in DCM-grown RM cultures might be generated from formate oxidation catalysed by enzyme systems such as the formate-H_2-lyase complex, which consists of a formate dehydrogenase and a hydrogenase, and links formate oxidation to proton reduction (McDowall *et al*., 2014; Trchounian and Sawers, 2014). The genes encoding a cytoplasmic formate dehydrogenase and a putative group 4 membrane-bound, H_2-evolving [NiFe]-hydrogenase complex have been identified in the genome of *Candidatus*Dichloromethanomonas elyunquensis (Kleindienst *et al*., 2016), implying that the specific DCM-degrader has the potential to convert formate to

CO_2 and H_2. However, formate alone could not support growth of *Candidatus* Dichloromethanomonas elyunquensis, although it was rapidly consumed in the mixed culture generating methane and acetate with transient formation of H_2 (Supporting Information Fig. S2). An alternate scenario would be that another microorganism (or microorganisms) in culture RM transform(s) formate and generates H_2. Although the direct source of H_2 in DCM-degrading RM cultures remains to be determined, the overall DCM degradation process leading to acetate and H_2 formation is summarized in Eq. 4 (Table 2).

H2 inhibition of DCM degradation and mutualistic interactions between DCM- and CM-degrading populations in culture RM

Growth experiments using RM cultures amended with DCM and H_2 revealed a strong inhibitory effect of H_2 on DCM degradation (Fig. 4). Because of the continuous consumption of H_2 by methanogens and/or homoacetogens, the H_2 concentration leading to an inhibitory effect on DCM degradation could not be precisely determined in the mixed culture. Based on the measured DCM and H_2 consumption profiles, we estimated that inhibition occurs at H_2 partial pressure exceeding 3000 ppmv (Fig. 4C). Such a high H_2 concentration seems unlikely in natural environments as well as contaminated sites, where H_2 concentrations in the low ppbv range are expected (Lovley and Goodwin, 1988; Mazur and Jones, 2001). H_2inhibition of DCM degradation is not unique to culture RM and has been observed in another mixed culture that required H_2 to support chloroform reductive dechlorination to DCM, but H_2 impacted subsequent consumption of DCM (Lee *et al*., 2012). The reasons why H_2 exerts an inhibitory effect on DCM degradation are unclear and cannot be explained by thermodynamics. Even under standard conditions with a H_2 concentration of 1 atm ($\sim 10^6$ppmv), the Gibbs free energy change ($\Delta G^{\circ\prime}$) of DCM fermentation associated with H_2production (Eq. 4) is still substantially negative (–227.9 kJ mol^{-1}), indicating favourable thermodynamics. In contrast, the conversion of formate to CO_2 and H_2 ($HCOO^- + H^+ \rightarrow CO_2 + H_2$, $\Delta G^{\circ\prime} = -3.5$ kJ mol^{-1}) is impeded by H_2 and the Gibbs free energy change becomes positive when H_2 concentrations exceed 0.02 atm ($\sim 2 \times 10^4$ ppmv) under actual reaction conditions (i.e., $[CO_2] = 0.2$ atm, $[HCOO^-] = 1$ mM, $T = 25°C$ and pH = 7). While *Candidatus*Dichloromethanomonas elyunquensis has the capability to oxidize formate, the genes encoding formate transporters could not be identified in the draft genome (Kleindienst *et al*., 2016). Thus, elevated H_2 concentrations could prevent formate oxidation, leading to formate accumulation inside the cells and impact DCM degradation. RM cultures amended with formate showed visual turbidity indicative of growth (Supporting Information Fig. S2); however, *Candidatus* Dichloromethanomonas elyunquensis 16S rRNA genes did not increase, indicating this organism cannot utilize formate as a growth substrate.

The CM degrader was maintained over numerous (at least 50) consecutive transfers with DCM as the sole energy source and H_2 was evolved in DCM-grown cultures indicating that the CM degrader can grow via H_2/CO_2 reductive acetogenesis. The H_2 generated in DCM metabolism supported CM degradation by a hydrogenotrophic organism in culture RM, most likely a homoacetogenic *Acetobacterium* sp. The finding that DCM metabolism generates H_2 also explains why hydrogenotrophic methanogens and homoacetogens were stably maintained in the mixed culture RM amended with DCM as the sole energy source. The inhibitory effect of H_2 and the absence of any known formate transporters suggest that the DCM-degrader, *Candidatus* Dichloromethanomonas elyunquensis, oxidizes formate and generates H_2 inside the cell during DCM degradation. These observations suggest interspecies H_2 transfer from the DCM degrader to a hydrogenotrophic partner population, for example, the CM degrader, occurs in culture RM (Fig. 5). In turn, the hydrogenotrophic partner(s) prevent(s) H_2 from reaching inhibitory levels enabling DCM degradation to proceed. The mutualistic interactions based on interspecies H_2-transfer between the DCM- and the CM-degrading populations are depicted in Fig. 5. In addition to methanogens and homoacetogens, the CM degrader serves as a H_2 scavenger in the presence of CM. In such a mutualistic relationship, *Candidatus* Dichloromethanomonas elyunquensis generates H_2during DCM metabolism and the CM degrader, presumably an *Acetobacterium* sp., consumes the H_2 and enables continued DCM utilization.

Environmental implications

The feasibility of bioaugmentation for chloroform and DCM detoxification has been demonstrated (Justicia-Leon *et al*., 2014), and the findings reported here highlight that chloroform remediation can be sustained without biostimulation (i.e., electron donor addition) because the degradation of the daughter product DCM generates the H_2 required for chloroform reductive dechlorination. A logical extension of this concept is that DCM serves as an indirect electron donor (i.e., a source of H_2 and acetate) to support other reductive processes such as nitrate reduction, organohalide respiration, iron reduction, sulfate reduction, methanogenesis and reductive acetogenesis (Fig. 6). Depending on the thermodynamics of the redox reaction, H_2 is consumed to different threshold concentrations (Fig. 6) (Cord-Ruwisch *et al*., 1988; Lovley and Goodwin, 1988; Löffler *et al*., 1999; Luijten *et al*., 2004). In the absence of more favourable electron acceptors such as nitrate, H_2 generated during DCM degradation can effectively fuel organohalide respiration. For example, DCM has been demonstrated to serve as an electron donor to support tetrachloroethene (PCE) reductive dechlorination to ethene in a mixed culture containing a PCE-to-ethene organohalide-respiring bacteria and at least one microbe capable of

fermenting DCM (Kanitkar, 2012). Such mutualistic interactions could explain natural attenuation scenarios where productive contaminant removal is observed without intervention (e.g., biostimulation) (Simsir *et al*., 2017). The observation that anaerobic DCM metabolism can directly impact hydrogenotrophic processes in both pristine and contaminated environments emphasizes the need for in-depth understanding of chlorinated C1 compound metabolism and its ecological impacts on the microbiology.

Experimental procedures

Chemicals

CM (purity > 99.95%) and DCM (purity > 99.95%) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Acros Organics (Thermo Fisher Scientific, Fair Lawn, NJ, USA) respectively. H_2 gas mixtures at the concentrations of 10, 50 and 100 ppmv were purchased from Airgas (Radnor, PA, USA) and were used as standards. All other chemicals used were analytical reagent grade or higher, unless otherwise specified.

Microorganisms and cultivation

Culture RM capable of utilizing DCM as the sole energy source originated from pristine freshwater sediment collected from Rio Mameyes in Luquillo, Puerto Rico (Justicia-Leon *et al*., 2012). Routine cultivation of culture RM occurred in 160 ml glass serum bottles containing 100 ml of bicarbonate-buffered (30 mM, pH 7.3) basal mineral salt medium (Löffler *et al*., 1996, 2005). The vessels were sealed with black butyl rubber stoppers (Bellco Glass, Vineland, NJ, USA) under a headspace of N_2/CO_2 (80/20, vol/vol) and provided with 5–10 µl neat DCM (78–156 µmoles) as the sole growth substrate prior to inoculation from a DCM-grown culture (5%, vol/vol). Upon DCM depletion, the cultures received 1 or 2 additional doses of DCM. To study CM degradation, a volume of 0.5 ml neat CM gas (~20.4 µmoles) was injected into 160 ml culture vessels. Cultures received 10 ml (~410 µmoles) of H_2 to study its effect on CM and DCM degradation. BES (2 mM) was added to inhibit methanogenesis. All culture vessels were incubated at 30°C in the dark without agitation. To quantitatively monitor inorganic chloride release during DCM and CM degradation, the reduction of the chloride background in the medium was required, and chloride salts were substituted by equimolar concentrations of bromide salts.

DNA extraction and quantitative real-time polymerase chain reaction (qPCR)

16S rRNA gene-targeted qPCR was used to monitor growth of the DCM degrader, that is,*Candidatus* Dichloromethanomonas elyunquensis, in cultures grown on DCM, CM/H_2, H_2 and formate respectively. For DNA extraction, 5 ml of culture suspension samples were collected over a typical growth cycle and filtered onto 0.22 µm Durapore membranes (Millipore, Cork, Ireland). DNA was extracted using the PowerSoil DNA isolation kit (MO BIO, Carlsbad, CA, USA) following the manufacturer's protocol. qPCR primers and a probe specifically targeting the 16S rRNA gene of *Candidatus* Dichloromethanomonas elyunquensis have been reported (Kleindienst *et al*., 2017). qPCR followed published protocols (Ritalahti *et al*., 2006; Kleindienst *et al*., 2017) and was conducted using an ABI ViiA7 real-time PCR system (Life Technologies) equipped with ViiA7 Software (Life Technologies). Briefly, every 20 µl-reaction mixture contained 10 µl of 2X Taqman Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), 2 µl of DNA template (DNA concentrations between 20 and 100 ng μl^{-1}), and forward and reverse primers and probe at final concentrations of 300 nM each. The PCR thermal cycling protocol was as follows: 50°C for 2 min, then at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Calibration curves used serial 10-fold dilutions of plasmid DNA carrying a cloned 16S rRNA gene of the DCM degrader (pCR 2.1-TOPO vector; Invitrogen, Carlsbad, CA, USA), and spanned a concentration range from 3.44×10^8 to 34.4 target gene copies.

16S rRNA gene amplicon sequencing

Cultures (30 ml) were transferred at least 3 times on the specific substrate(s), that is, DCM, CM/H_2 ± BES. DNA was extracted using the PowerSoil DNA isolation kit as described above. 16S rRNA genes were amplified using general PCR primers 515F/806R (515F: 5′-GTG CCAGCMGCCGCGGTAA-3′, and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′ with barcodes) targeting the V4 region of both bacterial and archaeal 16S rRNA genes and the DNA library was prepared according to procedures described by (Caporaso *et al*., 2011, 2012). 16S rRNA gene amplicon sequencing was performed on the Illumina Miseq platform (San Diego, CA, USA). Sequencing data were analysed by QIIME v.1.9.1 software package (Caporaso *et al*., 2010). Raw sequencing reads were jointly paired, demultiplexed and trimmed at a length of 250 bp. Chimeric reads were identified and removed. After quality checking, a total number of 65 055, 154 455, and 128 211 sequences were obtained from DNA amplicon samples of cultures grown on DCM, CM/H_2+BES and CM/H_2 respectively. Open reference OTUs were picked via the default UCLUST pipeline (Edgar, 2010) and were filtered at 0.005% threshold. Taxonomy assignment was performed using RDP classifier trained against the Greengenes 16S rRNA gene database (version May, 2013) (DeSantis *et al*., 2006).

Analytical methods

DCM, CM and methane (CH_4) were measured by manual headspace injections (0.1 ml) into an Agilent 7890 gas chromatograph (GC) (Santa Clara, CA, USA) equipped with a DB-624 column (60 m length, 0.32 mm i.d., 1.8 µm film thickness) and a flame ionization detector (FID). The GC inlet was maintained at 200°C, the GC oven temperature was kept at 60°C for 2 min followed by an increase to 200°C at a ramping rate of 25°C min^{-1}, and the FID detector was operated at 280°C as described (Amos *et al*., 2007).

Organic acids were quantified by high-performance liquid chromatography (HPLC). Aqueous samples were acidified with 1 M H_2SO_4 in a ratio of 40:1 (vol/vol) prior to HPLC analysis. Acetate and formate were analysed using an Agilent 1200 series HPLC system equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and a UV detector set to 210 nm. The separation occurred at a column temperature of 30°C, and the eluent (4 mM H_2SO_4) was delivered isocratically at a rate of 0.6 ml min^{-1} for 25 min.

Chloride ion (Cl^-) was measured with an Ion chromatograph (IC) using a Dionex ICS-2100 system equipped with a 4 mm × 250 mm IonPac AS18 hydroxide-selective anion-exchange column (Sunnyvale, CA, USA). The column temperature was held at 30°C, the 10 mM KOH eluent was delivered at the rate of 1 ml min^{-1} and an ERS 500 suppressor (4 mm) was set at a current of 57 mA.

To follow the consumption of H_2, 1 ml of headspace sample was injected into an Agilent 3000A MicroGC (Santa Clara, CA, USA), which has a H_2 detection limit of ~50 ppmv For monitoring H_2 formation during DCM degradation, 0.5 ml of headspace gas was injected into a Peak Performer 1 Reducing Compound Photometer (Peak Laboratories, Mountain View, CA, USA) with a H_2 detection limit of ~8 ppbv. H_2 partial pressures were expressed in parts per million by volume (ppmv; 1 ppmv ≈ 0.1 Pa ≈ 10^{-6} atm). A H_2 partial pressure of 100 ppmv in the headspace corresponded to approximately 0.25 µmoles of H_2 per bottle at 25°C (Löffler *et al*., 1999).

Acknowledgements

This work was supported by The Chemours Company and the Strategic Environmental Research and Development Program (SERDP). We thank Dr. Sandra Studenik, Friedrich-Schiller-Universität Jena, Germany, for sharing the full-length 16S rRNA gene sequence of *Acetobacterium dehalogenans* strain MC and valuable discussions.

Conflict of interest

The authors declare no conflict of interest.

Supporting Information

Supporting Information may be found in the online version of this article at the publisher's website: https://doi.org/10.1111/1462-2920.13945

References

Amos, B.K., Christ, J.A., Abriola, L.M., Pennell, K.D., and Löffler, F.E. (2007) Experimental evaluation and mathematical modeling of microbially enhanced tetrachloroethene (PCE) dissolution. *Environ Sci Technol* **41**: 963–970.

ATSDR (1998) *Toxicological profile for chloromethane*. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service. [WWW document]. https://www.atsdr.cdc.gov/toxprofiles/tp106.pdf

ATSDR (2000) *Toxicological profile for methylene chloride*. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service. [WWW document]. https://www.atsdr.cdc.gov/toxprofiles/tp14.pdf

Butler, J.H. (2000) Better budgets for methyl halides? *Nature* **403**: 260–261.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., *et al*. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**: 335–336.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., *et al*. (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA* **108**: 4516–4522.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., *et al*. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J***6**: 1621–1624.

Cord-Ruwisch, R., Seitz, H.J., and Conrad, R. (1988) The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. *Arch Microbiol* **149**: 350–357.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., *et al*. (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* **72**: 5069–5072.

Diekert, G., and Wohlfarth, G. (1994) Energetics of acetogenesis from C1 units. In *Acetogenesis*. Drake, H.L. (ed.). Boston, MA, USA: Springer, pp. 157–179.

Dolfing, J., and Janssen, D.B. (1994) Estimates of Gibbs free energies of formation of chlorinated aliphatic compounds. *Biodegradation* **5**: 21–28.

Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**: 2460–2461.

EPA (2014) TOXMAP®: Environmental Health Maps [WWW document]. https://toxmap-classic.nlm.nih.gov/toxmap/main/index.jsp

Freedman, D.L., and Gossett, J.M. (1991) Biodegradation of dichloromethane and its utilization as a growth substrate under methanogenic conditions. *Appl Environ Microbiol* **57**: 2847–2857.

Gribble, G.W. (2010) Naturally occurring organohalogen compounds - A comprehensive update. In *Progress in the Chemistry of Organic Natural Products*. Gribble, G.W. (ed.). Vienna: Springer-Verlag Wien, pp. 12–13.

Harper, D.B. (2000) The global chloromethane cycle: biosynthesis, biodegradation and metabolic role. *Nat Prod Rep* **17**: 337–348.

Hossaini, R., Chipperfield, M.P., Montzka, S.A., Leeson, A.A., Dhomse, S.S., and Pyle, J.A. (2017) The increasing threat to stratospheric ozone from dichloromethane. *Nat Commun* **8**: 15962.

Justicia-Leon, S.D., Ritalahti, K.M., Mack, E.E., and Löffler, F.E. (2012) Dichloromethane fermentation by a *Dehalobacter* sp. in an enrichment culture derived from pristine river sediment. *Appl Environ Microbiol* **78**: 1288–1291.

Justicia-Leon, S.D., Higgins, S., Mack, E.E., Griffiths, D.R., Tang, S., Edwards, E.A., and Löffler, F.E. (2014) Bioaugmentation with distinct *Dehalobacter* strains achieves chloroform detoxification in microcosms. *Environ Sci Technol* **48**: 1851–1858.

Kanitkar, Y. (2012) Evaluation of dichloromethane as an electron donor for reductive dechlorination of tetrachloroethene to ethene. Master thesis. Clemson University. Paper 1456. Clemson, SC, USA. [WWW document]. http://tigerprints.clemson.edu/all_theses/1456

Kaufmann, F., Wohlfarth, G., and Diekert, G. (1998) *O*-demethylase from *Acetobacterium dehalogenans*-substrate specificity and function of the participating proteins. *Eur J Biochem* **253**: 706–711.

Kleindienst, S., Higgins, S.A., Tsementzi, D., Konstantinidis, K.T., Mack, E.E., and Löffler, F.E. (2016) Draft genome sequence of a strictly anaerobic dichloromethane-degrading bacterium. *Genome Announc* **4**: e00037–e00016.

Kleindienst, S., Higgins, S.A., Tsementzi, D., Chen, G., Konstantinidis, K.T., Mack, E.E., and Löffler, F.E. (2017) '*Candidatus* Dichloromethanomonas elyunquensis' gen. nov., sp. nov., a dichloromethane-degrading anaerobe of the *Peptococcaceae* family. *Syst Appl Microbiol* **40**: 150–159.

Kohler-Staub, D., and Leisinger, T. (1985) Dichloromethane dehalogenase of *Hyphomicrobium* sp. strain DM2. *J Bacteriol* **162**: 676–681.

Kreher, S., Studenik, S., and Diekert, G. (2010) Ether cleaving methyltransferases of the strict anaerobe *Acetobacterium dehalogenans*: controlling the substrate spectrum by genetic engineering of the N-terminus. *Mol Microbiol* **78**: 230–237.

Krone, U.E., Thauer, R.K., and Hogenkamp, H.P.C. (1989) Reductive dehalogenation of chlorinated C1-hydrocarbons mediated by corrinoids. *Biochemistry* **28**: 4908–4914.

Lee, M., Low, A., Zemb, O., Koenig, J., Michaelsen, A., and Manefield, M. (2012) Complete chloroform dechlorination by organochlorine respiration and fermentation. *Environ Microbiol* **14**: 883–894.

Leisinger, T., and Braus-Stromeyer, S.A. (1995) Bacterial growth with chlorinated methanes. *Environ Health Perspect* **103**: 33–36.

Löffler, F.E., Sanford, R.A., and Tiedje, J.M. (1996) Initial characterization of a reductive dehalogenase from *Desulfitobacterium chlororespirans* Co23. *Appl Environ Microbiol* **62**: 3809–3813.

Löffler, F.E., Tiedje, J.M., and Sanford, R.A. (1999) Fraction of electrons consumed in electron acceptor reduction and hydrogen thresholds as indicators of halorespiratory physiology. *Appl Environ Microbiol* **65**: 4049–4056.

Löffler, F.E., Sanford, R.A., and Ritalahti, K.M. (2005) Enrichment, cultivation, and detection of reductively dechlorinating bacteria. *Methods Enzymol* **397**: 77–111.

Lovley, D.R., and Goodwin, S. (1988) Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. *Geochim Cosmochim Acta* **52**: 2993–3003.

Luijten, M.L.G.C., Roelofsen, W., Langenhoff, A.A.M., Schraa, G., and Stams, A.J.M. (2004) Hydrogen threshold concentrations in pure cultures of halorespiring bacteria and at a site polluted with chlorinated ethenes. *Environ Microbiol* **6**: 646–650.

Mägli, A., Wendt, M., and Leisinger, T. (1996) Isolation and characterization of *Dehalobacterium formicoaceticum* gen. nov. sp. nov., a strictly anaerobic bacterium utilizing dichloromethane as source of carbon and energy. *Arch Microbiol* **166**: 101–108.

Mägli, A., Messmer, M., and Leisinger, T. (1998) Metabolism of dichloromethane by the strict anaerobe *Dehalobacterium formicoaceticum*. *Appl Environ Microbiol* **64**: 646–650.

Mazur, C.S., and Jones, W.J. (2001) Hydrogen concentrations in sulfate-reducing estuarine sediments during PCE dehalogenation. *Environ Sci Technol* **35**: 4783–4788.

McDonald, I.R., Warner, K.L., McAnulla, C., Woodall, C.A., Oremland, R.S., and Murrell, J.C. (2002) A review of bacterial methyl halide degradation: biochemistry, genetics and molecular ecology. *Environ Microbiol* **4**: 193–203.

McDowall, J.S., Murphy, B.J., Haumann, M., Palmer, T., Armstrong, F.A., and Sargent, F. (2014) Bacterial formate hydrogenlyase complex. *Proc Natl Acad Sci USA* **111**: E3948–E3956.

Meßmer, M., Wohlfarth, G., and Diekert, G. (1993) Methyl chloride metabolism of the strictly anaerobic, methyl chloride-utilizing homoacetogen Strain MC. *Arch Microbiol* **160**: 383–387.

Meßmer, M., Reinhardt, S., Wohlfarth, G., and Diekert, G. (1996) Studies on methyl chloride dehalogenase and *O*-demethylase in cell extracts of the homoacetogen strain MC based on a newly developed coupled enzyme assay. *Arch Microbiol* **165**: 18–25.

Moran, M.J., Zogorski, J.S., and Squillace, P.J. (2007) Chlorinated solvents in groundwater of the United States. *Environ Sci Technol* **41**: 74–81.

Nikolausz, M., Nijenhuis, I., Ziller, K., Richnow, H.H., and Kästner, M. (2006) Stable carbon isotope fractionation during degradation of dichloromethane by methylotrophic bacteria. *Environ Microbiol* **8**: 156–164.

Rhew, R.C., Miller, B.R., and Weiss, R.F. (2000) Natural methyl bromide and methyl chloride emissions from coastal salt marshes. *Nature* **403**: 292–295.

Ritalahti, K.M., Amos, B.K., Sung, Y., Wu, Q., Koenigsberg, S.S., and Löffler, F.E. (2006) Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple *Dehalococcoides* strains. *Appl Environ Microbiol* **72**: 2765–2774.

Seidel, A. (2007) Chlorocarbons and chlorohydrocarbons. In *Kirk-Othmer Encyclopedia of Chemical Technology*, 5th ed. Seidel, A. (ed.). Hoboken, NJ, USA: Wiley, p. 226.

Simsir, B., Yan, J., Im, J., Graves, D., and Löffler, F.E. (2017) Natural attenuation in streambed sediment receiving chlorinated solvents from underlying fracture networks. *Environ Sci Technol* **51**: 4821–4830.

Smidt, H., and De Vos, W.M. (2004) Anaerobic microbial dehalogenation. *Annu Rev Microbiol* **58**: 43–73.

Stupperich, E., Eisinger, H.J., and Kräutler, B. (1988) Diversity of corrinoids in acetogenic bacteria. *P*-Cresolylcobamide from *Sporomusa ovata*, 5-methoxy-6-methylbenzimidazolylcobamide from *Clostridium formicoaceticum* and vitamin B12 from *Acetobacterium woodii*. *Eur J Biochem* **172**: 459–464.

Thauer, R.K., Jungermann, K., and Decker, K. (1977) Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol Rev* **41**: 100–180.

Traunecker, J., Preuß, A., and Diekert, G. (1991) Isolation and characterization of a methyl-chloride utilizing, strictly anaerobic bacterium. *Arch Microbiol* **156**: 416–421.

Trchounian, A., and Sawers, R.G. (2014) Novel insights into the bioenergetics of mixed-acid fermentation: can hydrogen and proton cycles combine to help maintain a proton motive force?*IUBMB Life* **66**: 1–7.

Vannelli, T., Messmer, M., Studer, A., Vuilleumier, S., and Leisinger, T. (1999) A corrinoid-dependent catabolic pathway for growth of a *Methylobacterium* strain with chloromethane. *Proc Natl Acad Sci USA* **96**: 4615–4620.

Widdel, F., and Musat, F. (2010) Energetic and other quantitative aspects of microbial hydrocarbon utilization. In *Handbook of Hydrocarbon and Lipid Microbiology*. Timmis, K.N. (ed.). Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 729–763.

Wong, Y.K., Holland, S.I., Ertan, H., Manefield, M., and Lee, M. (2016) Isolation and characterization of *Dehalobacter sp*. strain UNSWDHB capable of chloroform and chlorinated ethane respiration. *Environ Microbiol* **18**: 3092–3105.

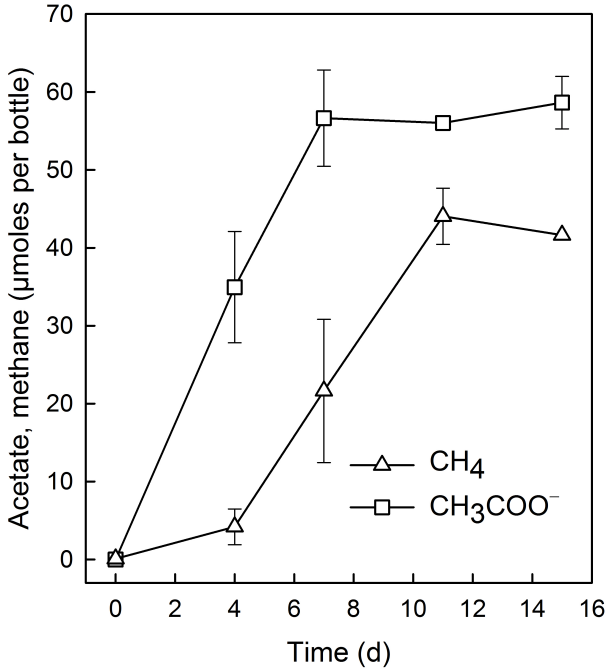

Figure S1. Methane and acetate formation in H_2/CO_2-grown RM cultures. Cultures were grown in anoxic, bicarbonate-buffered (30 mM, pH 7.3) basal salts medium under a N_2/CO_2 (80/20, vol/vol) atmosphere amended with 10 mL of H_2 (~410 µmoles) as the electron donor and CO_2 as the electron acceptor. The data represent the averages of triplicate incubations and the error bars represent the standard deviations.

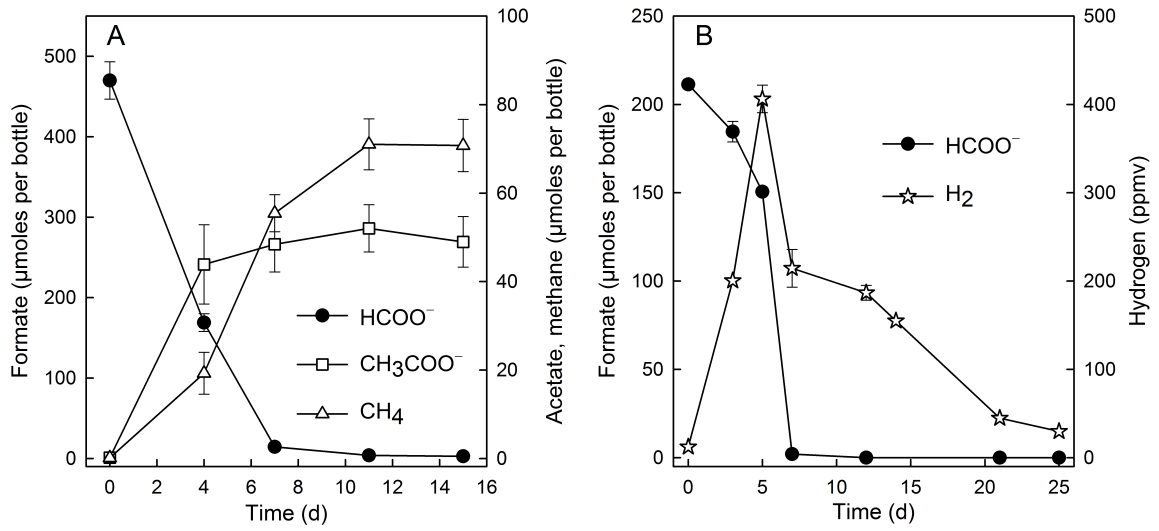

Figure S2. Conversion of formate to methane and acetate (A) and transient H_2 formation during formate utilization (B) in RM cultures.

(A) Formate (solid circles) was rapidly consumed in RM cultures, producing methane (open triangles) and acetate (open squares).

(B) The transient formation of H_2 (open stars) was observed during formate utilization. A H_2 partial pressure of 100 ppmv corresponds to approximately 0.25 µmoles of H_2 in the cultivation vessel.

Cultures that received 5 mM formate generated visible turbidity indicative of growth. The data represent the averages of triplicate incubations and the error bars represent the standard deviations.

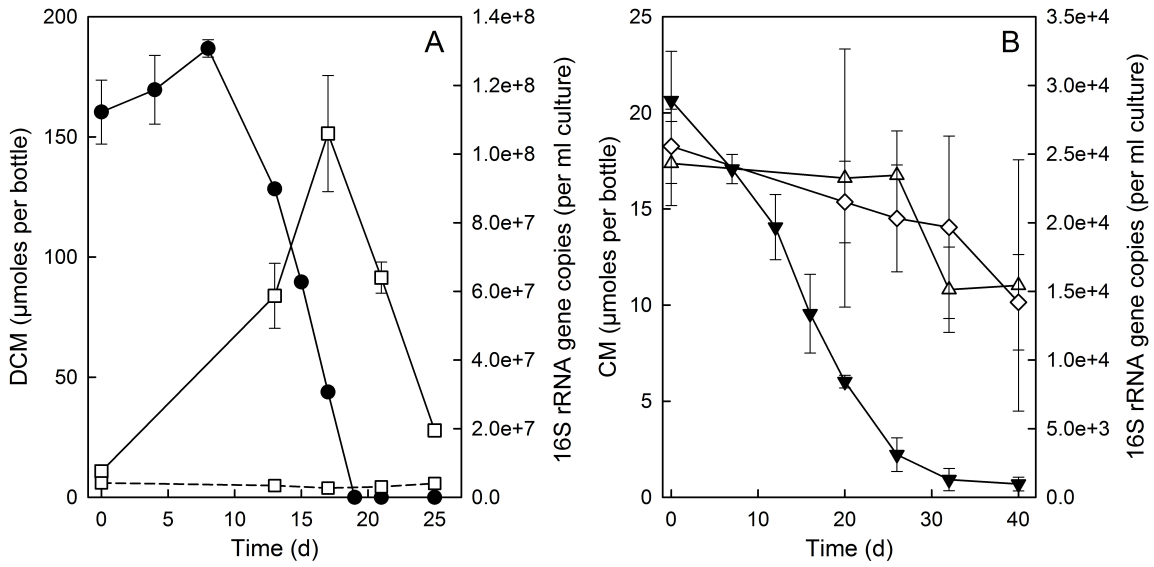

Figure S3. Growth of *Candidatus* Dichloromethanomonas elyunquensis in culture RM in bicarbonate-buffered basal salts medium with DCM as the sole electron donor (A), with CM and H_2 (B), or with H_2/CO_2 (B) as determined by qPCR enumeration.

(A) The copy number of *Candidatus* Dichloromethanomonas elyunquensis 16S rRNA genes (open squares, solid line) increased along with DCM consumption (solid circles), but decreased immediately after DCM was depleted. In DCM-free controls, 16S rRNA gene copy numbers did not increase (open squares, dashed line).

(B) No increase of *Candidatus* Dichloromethanomonas elyunquensis 16S rRNA genes (open triangles) occurred during CM degradation (solid inverted triangles) in the presence of H_2. With H_2 as the electron donor and CO_2 as electron acceptor, no increase of *Candidatus* Dichloromethanomonas elyunquensis 16S rRNA genes was observed (open diamonds). These qPCR results demonstrated that the DCM-degrader *Candidatus* Dichloromethanomonas elyunquensis was not responsible for CM degradation. The data represent the averages of triplicate incubations and the error bars represent the standard deviations.

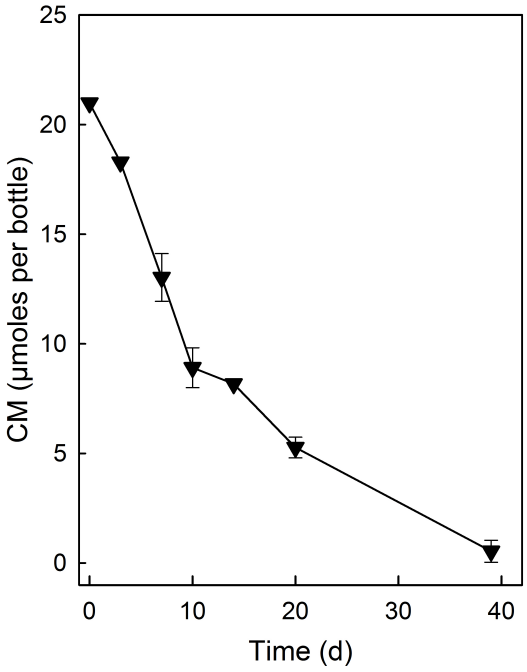

Figure S4. CM degradation in H_2/CO_2-grown, non-methanogenic RM cultures. Following seven consecutive transfers of the CM-degrading culture in medium amended with H_2 as the sole electron donor (i.e., without CM additions), CO_2 as electron acceptor, and 2-bromoethanesulfonate to inhibit methanogens, CM was degraded without lag phase as long as H_2 was provided to freshly inoculated cultures. The data represent the averages of duplicate incubations and the error bars represent the standard deviations.

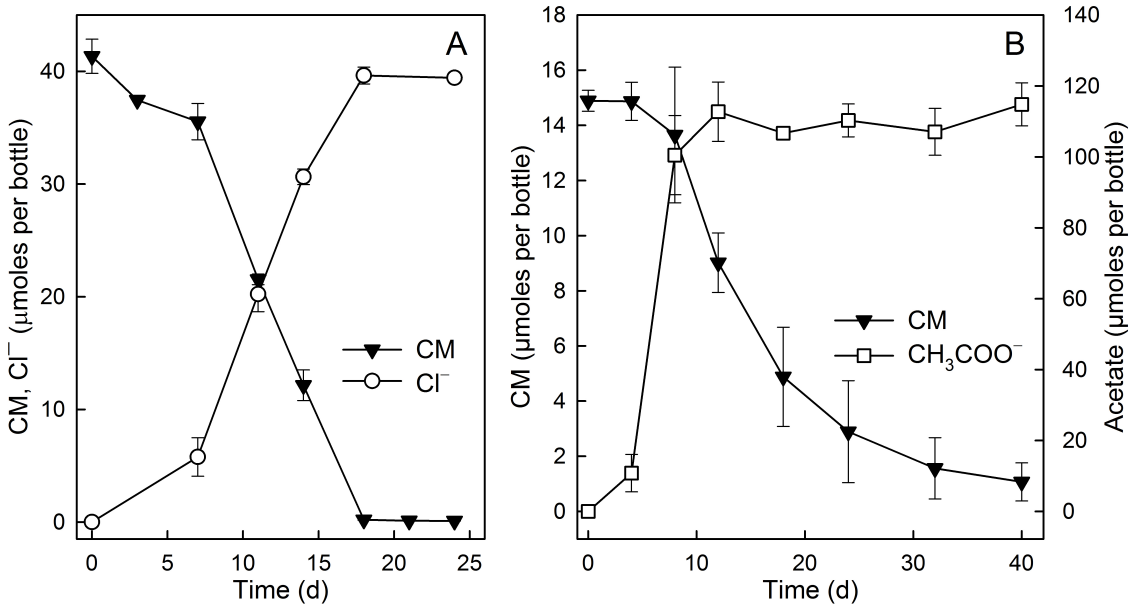

Figure S5. Chloride release and acetate production during CM degradation in RM cultures that received H_2 as electron donor.

(A) Stoichiometric amounts of chloride (open circles) were released during CM degradation (solid inverted triangles).

(B) Acetate (open squares) was the only detectable product in the CM/H_2-grown culture amended with 2-bromoethanesulfonate, an inhibitor of methanogenesis. The data represent the averages of triplicate incubations and the error bars represent the standard deviations.

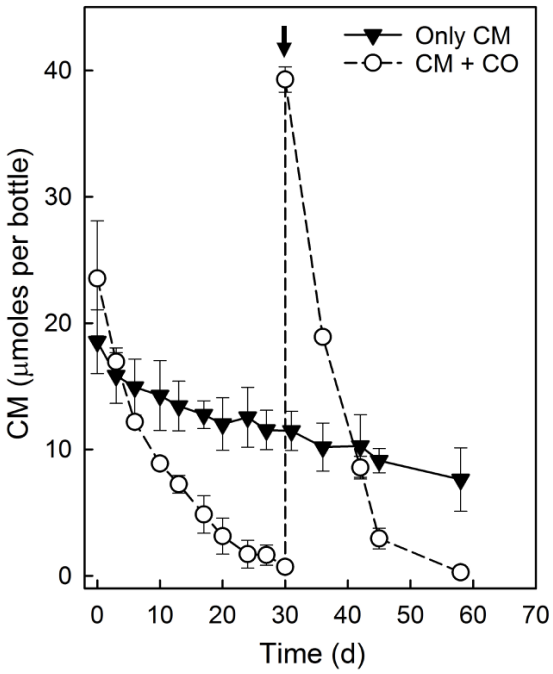

Figure S6. CM degradation in RM cultures amended with CO in the absence of H_2.

CM degradation in cultures amended with CO (5% [vol/vol] of headspace) (open circles, dashed line). In control vessels that received only CM but no CO, some CM loss (solid inverted triangles) was observed, presumably due to sorption to the rubber stopper. All cultures received 2 mM 2-bromoethanesulfonate to inhibit methanogenesis. The arrow indicates an additional CM spike. The data represent the averages of triplicate incubations and the error bars represent the standard deviations.

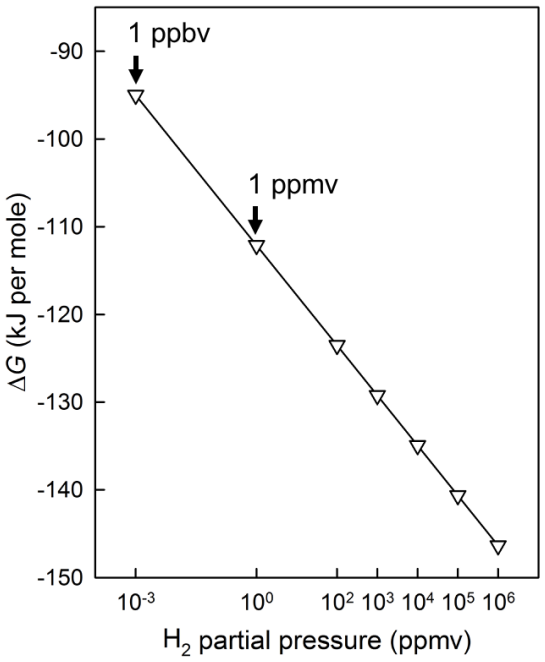

Figure S7. The Gibbs free energy change associated with H_2-dependent CM conversion to acetate ($CH_3Cl + CO_2 + H_2 \rightarrow CH_3COO^- + 2\ H^+ + Cl^-$, Equation 1 in Table 2) at different H_2 partial pressures. Calculations were performed assuming the following conditions: $[CH_3Cl]$ = 0.2 mM; $[CO_2]$ = 0.2 atm; $[CH_3COO^-]$ = 0.2 mM; $[Cl^-]$ = 33 mM; pH = 7.0.

Table S1. Stoichiometry of substrate consumption, product formation, and calculation of electron recovery in RM cultures grown with H_2 or formate as electron donors and CO_2 as electron acceptor as depicted in Figure S1 and Figure S2.

Substrate	Amount [µmoles]	Available e^- [µmoles]	Products	Amount [µmoles]	Required e^- [µmoles]	Electron recovery (%)
H_2	408.7	817.4	CH_3COO^-	58.6 ± 3.4	468.8 ± 27.2	100.5
			CH_4	44.1± 3.6	352.8 ± 28.8	
$HCOO^-$	470.0 ± 23.2	940.0 ± 46.4	CH_3COO^-	48.9 ± 5.7	391.2 ± 45.6	101.9
			CH_4	70.8 ± 5.9	566.4 ± 47.2	

The initial amounts of substrates were calculated from the volume of H_2 gas (10 mL) and the concentration of formate (5 mM) added to the culture vessels. Electrons available from substrate oxidation were calculated according to the following equations:

Hydrogen oxidation: $H_2 \rightarrow 2\ H^+ + 2\ e^-$

Formate oxidation: $HCOO^- \rightarrow H^+ + 2\ e^- + CO_2$

Acetate and methane were the only products detected in cultures that received H_2 or formate as electron donors. The electron requirements were calculated according to the following equations:

Acetate formation: $2\ CO_2 + 7\ H^+ + 8\ e^- \rightarrow CH_3COO^- + 2H_2O$

Methane formation: $CO_2 + 8\ H^+ + 8\ e^- \rightarrow CH_4 + 2\ H_2O$

All calculations used data generated from triplicate incubations.