Interspecies metabolite transfer and aggregate formation in a co-culture of \textit{Dehalococcoides} and \textit{Sulfurospirillum} dehalogenating tetrachloroethene to ethene

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Received: 26 June 2020 / Revised: 4 December 2020 / Accepted: 16 December 2020 / Published online: 21 January 2021
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Abstract
Microbial communities involving dehalogenating bacteria assist in bioremediation of areas contaminated with halocarbons. To understand molecular interactions between dehalogenating bacteria, we co-cultured \textit{Sulfurospirillum multivorans}, dechlorinating tetrachloroethene (PCE) to \textit{cis}-1,2-dichloroethene (cDCE), and \textit{Dehalococcoides mccartyi} strains BTF08 or 195, dehalogenating PCE to ethene. The co-cultures were cultivated with lactate as electron donor. In co-cultures, the bacterial cells formed aggregates and \textit{D. mccartyi} established an unusual, barrel-like morphology. An extracellular matrix surrounding bacterial cells in the aggregates enhanced cell-to-cell contact. PCE was dehalogenated to ethene at least three times faster in the co-culture. The dehalogenation was carried out via PceA of \textit{S. multivorans}, and PteA (a recently described PCE dehalogenase) and VcrA of \textit{D. mccartyi} BTF08, as supported by protein abundance. The co-culture was not dependent on exogenous hydrogen and acetate, suggesting a syntrophic relationship in which the obligate hydrogen consumer \textit{D. mccartyi} consumes hydrogen and acetate produced by \textit{S. multivorans}. The cobamide cofactor of the reductive dehalogenase—mandatory for \textit{D. mccartyi}—was also produced by \textit{S. multivorans}. \textit{D. mccartyi} strain 195 dechlorinated cDCE in the presence of norpseudo-B$_{12}$ produced by \textit{S. multivorans}, but \textit{D. mccartyi} strain BTF08 depended on an exogenous lower cobamide ligand. This observation is important for bioremediation, since cofactor supply in the environment might be a limiting factor for PCE dehalogenation to ethene, described for \textit{D. mccartyi} exclusively. The findings from this co-culture give new insights into aggregate formation and the physiology of \textit{D. mccartyi} within a bacterial community.

Introduction
Microbial communities interact in numerous ways, involving the transfer and consumption of metabolic products. Molecular hydrogen, for example, is an important electron carrier in syntrophic communities, in which hydrogen is...
produced by e.g., fermentation and taken up by hydrogen-consuming prokaryotes. This hydrogen consumption leads to a lower hydrogen partial pressure, which allows otherwise thermodynamically unfavorable reactions to proceed. Therefore, the involved bacteria are physiologically dependent on each other [1–3]. The probably carcinogenic groundwater pollutant tetrachloroethene (PCE), a remnant of e.g., bleaching, dry-cleaning and metal degreasing, is completely, but often slowly, dechlorinated to ethene in communities involving hydrogen transfer from fermenting bacteria to the obligately hydrogen-consuming Dehalococcoides mccartyi [4–7]. PCE and other organohalides such as hexachlorobenzene or polychlorinated biphenyls are used as terminal electron acceptors by D. mccartyi and other bacteria in an anaerobic respiratory chain coupling the dehalogenation to energy conservation via electron transport phosphorylation [6]. This process is termed organohalide respiration (OHR) and involves the corrinoid-containing reductive dehalogenases (RDases) as terminal reductases [8–10]. However, D. mccartyi is characterized by low growth rates and dehalogenates PCE slower [11] compared to other dehalogenating species such as Sulfitrospirillum multivorans. D. mccartyi is strictly dependent on several specific vitamins, most importantly cobamides [12]. Besides being restricted to hydrogen as electron donor, D. mccartyi uses only acetate plus bicarbonate as carbon source and organohalides as electron acceptors. In addition, these bacteria are not able to de novo synthesize corrinoids, the obligate cofactor of RDases [11, 13]. While proteins for complete corrinoid biosynthesis are usually not encoded in the genomes of D. mccartyi [14], different studies revealed its ability to salvage and remodel corrinoids, enabling D. mccartyi to restore dechlorination [15–17]. The functionality of the corrinoid and thus the RDase is directly dependent on the type of the lower base in D. mccartyi. Only three types of corrinoids have so far been described to be functional in D. mccartyi strain 195: 5,6-dimethylbenzimidazolyl-cobamide ([DMB]-Cba), 5-methylbenzimidazolyl-cobamide and 5-methoxybenzimidazolyl-cobamide ([5-OMeBza]Cba). Nonfunctional corrinoids e.g., 5-hydroxybenzimidazolyl-cobamide or 7-adeninyl-cobamide ([AdelCba) can be converted to functional ones by replacement of the lower ligand in D. mccartyi when 5,6-dimethylbenzimidazole (DMB) is provided [16, 17].

Several studies on dechlorinating communities containing D. mccartyi in association with fermenting, acetogenic and/or methanogenic bacteria revealed higher dechlorination and growth rates than those of pure cultures [16, 18–26]. It is hypothesized that cross-feeding and a constant supply of growth factors such as corrinoids and biotin enhance growth [27]. Within these communities, non-dechlorinating fermenting bacteria provide hydrogen, acetate, and CO₂ from e.g., lactate or butyrate fermentation. The fermenting bacteria are dependent on hydrogen consumers which keep the hydrogen partial pressure low [18, 21, 22, 28]. For example, co-culture experiments revealed an interspecies hydrogen transfer between Desulfovibrio desulfuricans fermenting lactate and D. mccartyi dechlorinating TCE [15]. Acetogens like Sporomusa ovata and sulfidogens (Desulfovibrio), produce different types of corrinoids which can be used by D. mccartyi [29, 30]. An interspecies cobamide transfer was also shown between Methanosarcina barkeri strain Fusaro and D. mccartyi strain BAV1, GT and FL2, when DMB was present [25, 26]. In the only co-culture of D. mccartyi (strains BAV1 and FL2) with a PCE-dechlorinating bacterium, Geobacter lovleyi, a corrinoid transfer was also observed [31]. In this co-culture, however, hydrogen had to be supplemented. All these studies showed more robust growth of D. mccartyi in co-cultures, resulting in higher dechlorination rates and cell yields than in pure cultures and sometimes, depending on the cobamide metabolism of the co-culture, a different degree of the dechlorination extent [32]. However, a PCE-dechlorinating bacterium providing hydrogen, acetate and corrinoids to D. mccartyi, to our knowledge, has not been described in the literature. Thus, further information on D. mccartyi co-cultures is needed to gain insights into complex microbial communities involving D. mccartyi. Relatively simple co-cultures provide a model community in which metabolic transfers are easier to follow. Recently, the PCE to cDCE-respiring S. multivorans, capable of de novo corrinoid production [33–35], was shown to produce hydrogen and acetate under fermentative growth conditions, e.g., with pyruvate or lactate and without electron acceptor present [36]. In the same study, a co-culture of S. multivorans with a methanogen dependent on hydrogen as electron donor was established. Therefore, S. multivorans is a promising partner for a co-cultivation with D. mccartyi. In addition, it is of interest, whether D. mccartyi is able to take up and utilize norpseudo-B12 ([AdelNCba), so far known to be produced exclusively by S. multivorans and S. halorespirans [35, 37]. In this study, we investigated the physiological interaction between D. mccartyi strain BTF08 and 195 in co-culture with S. multivorans dechlorinating PCE to ethene by using dechlorination analytics, microscopy, and proteomics.

Materials and methods

Cultivation of pure cultures

D. mccartyi cultures BTF08 and 195 (maintained at the UFZ Leipzig) were cultivated in 200 mL serum bottles containing 100 mL bicarbonate-buffered mineral salt medium with 5 mM acetate and 148 mM vitamin B₁₂ (cyanocobalamin, ca. 200 µg/L), reduced with Na₂S·7 H₂O (final concentration, 0.25 g/L) [38]. Anoxic atmosphere was established by 30
cycles of gassing and degassing with nitrogen, and CO₂ was added to a final atmosphere of N₂:CO₂ (75:25 v/v 150 kPa). After autoclaving the medium and adjusting the pressure to ~100 kPa with nitrogen and CO₂, we applied in addition, hydrogen [50 kPa] to avoid possible electron donor limitation. PCE (>99% purity, Sigma Aldrich, Steinheim, Germany) and cDCE (97% purity, Sigma Aldrich, Steinheim, Germany) served as electron acceptors and were added with a microliter syringe (Hamilton, Bonaduz, Switzerland) to a final concentration of 0.35 mM (aqueous-phase concentration). Re-feeding of the cultures was done with the same amount of PCE or cDCE after complete conversion to ethene. After maximally three re-feeding steps, cultures were transferred [10% (v/v)] into fresh medium. To evaluate the effect of different types and concentrations of B₁₂ on dechlorination activities, D. maccartyi cultures received 54 nM norpseúdo-B₁₂ ([Adε]Cba) or 5-OMeBza-B₁₂ ([5-OMeBza]Cba), each. Norpseúdo-B₁₂ was extracted as described previously [39] from 6 L of S. multivorans grown anaerobically with 40 mM pyruvate and 10 mM PCE as described elsewhere [40]. In brief, crude cell extract was acidified to a pH of ≤5 with acetic acid before adding 0.1 M potassium cyanide and boiling the sample for 15 min. Afterwards, the sample was centrifuged and the supernatant kept on ice. The cell pellet was resuspended in 10 mL ultrapure water (UPW) and the same extraction procedure was applied twice. The combined supernatant was mixed with Amberlite XAD4 (0.25 g XAD4 per mL extract; Sigma-Aldrich) and incubated on a shaker overnight. The supernatant was removed and the cobamide-loaded XAD4 material was washed with 10 volumes of UPW. Elution of cobamide was done for 1 h on a shaker with 1 volume of methanol which was repeated twice. A vacuum concentrator was used to completely dry the eluates which were then resuspended in 2 mL UPW. The sample was applied on a column containing 3 g aluminum oxide, recovered with 50 mL UPW, and dried. Resuspension of purified norpseúdo-B₁₂ was done with UPW. 5-OMeBza-B₁₂ was obtained in the same way from 6 L of Desulfotobacterium hafniense DCB2 grown anaerobically with 40 mM pyruvate and 10 mM ClOHPA (3-chloro-4-hydroxy-phenylacetate) on a medium amended with 25 µM 5-OMeBza [41]. S. multivorans (DSMZ 12446) was cultured in the same mineral salt medium as D. maccartyi [38] with 40 mM lactate and 10 mM nominal PCE in hexadecane (2 mL PCE/hexadecane per 100 mL medium) but without the addition of acetate and hydrogen [38]. All cultivation experiments were performed statically at 28 °C in the dark and in biological triplicates.

Co-cultivation of S. multivorans and D. maccartyi

Co-cultures of S. multivorans and D. maccartyi strain BTF08 or 195 were maintained in the same mineral salt medium as the pure cultures, without acetate and hydrogen. Initially, the inoculum of the pure cultures into the co-culture was 10% of each pure culture. The medium contained 25 mM lactate (D/L, >85% purity, Sigma-Aldrich, Steinheim, Germany) as the electron donor for S. multivorans and 0.35 mM PCE (aqueous-phase concentration) as the electron acceptor. Lactate (25 mM) and PCE (0.35 mM) was re-fed after depletion of substrates was measured. The B₁₂-dependence of the co-cultures was tested with 148 nM vitamin B₁₂ (>98% purity, Sigma-Aldrich, Steinheim, Germany) serving as the positive control and without vitamin B₁₂ amendments. Cultures without vitamin B₁₂ received 1 µM DMB (>99% purity, Sigma-Aldrich, Steinheim, Germany), where indicated. The DMB dose used had only minimal negative effect on S. multivorans growth [39]. For isotope fractionation experiments, S. multivorans and Sm/BTF08 co-cultures were cultivated in 50 mL serum bottles with 25 mL medium. Replicate bottles (triplicates for each time point) were inoculated at the same time and the dehalogenation process was stopped at different time points by addition of 3 mL 2 M Na₂SO₄ (pH 1.0).

Quantitative (q)PCR analysis of cell growth

Quantitative PCR (qPCR) was applied to count Sulfurospirillum and Dehalococcoides 16S rRNA gene copies. DNA was extracted from 1 mL co-culture taken from different time points during the cultivation experiment using the NucleoSpin Tissue DNA extraction kit according to the manufacturer’s instructions (Macherey-Nagel, Düren, Germany). Cell numbers were quantified by direct epifluorescence microscopy of Sybr-green stained cells immobilized on agarose-coated slides to generate standard curves for qPCR as described previously [42]. For the corresponding standard curves, D. maccartyi and S. multivorans cell suspensions with known cell numbers (1 × 10⁴ to 1 × 10⁸ and 1 × 10⁷ to 1 × 10¹⁰, respectively) were subjected to qPCR. The qPCR reaction mixture contained 1 µL of gDNA or DNA standard, 6.25 µL 1× KAPA SYBR Fast master mix (Sigma Aldrich, Steinheim, Germany) and 0.208 µM forward and reverse primer. Primers used were Dhc_sp_16S_fw (5′-GTATCGACCCTCTCTGTCGCC-3′) and Dhc_sp_16S_rev (5′-GCAAGTTCTCTGACTTAAACGG TCCT-3′) for D. maccartyi and Smul_16S_fw (5′-AGGCT AGTTTACTAGAACCTTTGAG-3′) and Smul_16S_rev (5′-C AGTCTGATTAGAGTGCTCAG-3′) for S. multivorans. The conditions of the PCR program were as followed: 95 °C for 2 min (initial denaturation) followed by 40 cycles of 55 °C (S. multivorans primer) or 60 °C (D. maccartyi primer) for 20 s (annealing), 72 °C for 30 s (elongation) and 95 °C for 10 s (denaturation). Each qPCR program included a melting curve for verification of specific target DNA amplification and primer efficiency was tested for used primers (R² Sim: 99.7%; R² Dhc: 98.8%, see Supplementary Fig. S1). The cell numbers of samples were calculated by comparing the sample CT
values with the C_T values of the standard curve obtained from microscopical cell counting (Supplementary Fig. S1). All samples were conducted in three biological replicates with two corresponding technical replicates and three technical replicates for the calibration curve.

**Analytical methods**

Ethene and chlorinated ethenes were quantified gas-chromatographically with a flame ionization detector (Clarus 500, Perkin Elmer, Rodgau, Germany) and a CP-PoraBOND Q FUSED SILICA 25 m × 0.32 mm column (Agilent Technologies, Böblingen, Germany). Samples from the cultures (1 mL of the gas phase and 1 mL of the liquid phase) were transferred to GC vials with fast tight syringes, the sampling proceeded via a headspace sampler (HS 40, Perkin Elmer; needle temperature 100 °C, vial pressurized with 0.1 kPa nitrogen for 2 min). The GC vial with the liquid phase was heated in the HS 40–80 °C for 5 min. Samples were transferred through the transfer line (100 °C) into the GC. Ethene and chlorinated ethenes were separated with 4 min at 60 °C rising to 280 °C in 10 °C min⁻¹ steps. The injector temperature was fixed at 250 °C and the detector temperature at 300 °C. Standard curves of ethene and each chlorinated ethene were recorded for peak area quantification and retention times were compared to known standards. Nonane served as an internal standard during measurements. The retention times were: PCE (detection limit [dl] 2.1 µM), 15.1 min; TCE (dl 2.5 µM) 13.2 min; cDCE (dl 3.1 µM), 11.3 min; VC (dl 3 µM), 6.0 min; ethene (dl 2.5 µM), 1.3 min. Due to the low solubility of PCE, it was added to the medium followed by stirring for at least 8 h to allow it to completely dissolve. This lead to a reliable quantification of only the initial PCE concentration, but not of the re-fed PCE, especially since a portion of the re-fed PCE is rapidly degraded by the now higher abundant *S. multivorans* cells. The concentrations of chlorinated ethenes (in µM or mM) were calculated by summing up the concentrations in the gas phase and liquid phase. Dechlorination rates were calculated according to the average amount of stoichiometrically converted PCE to the corresponding end products VC (in the case of *D. mccartyi* strain 195, where the formation of co-metabolically produced ethene was negligible) or ethene (for *D. mccartyi* strain BTF08) divided by time in days. For example the conversion of 400 µM PCE into 400 µM ethene over 10 days resulted in a dechlorination rate of 40 µM PCE/day. Hydrogen (dl 0.8 µM) was measured gas-chromatographically using a thermal conductivity detector (AutoSystem, Perkin Elmer, Rodgau, Germany) equipped with a Carboxen-1010 PLOT capillary column 30 m × 0.53 mm (Supelco, München, Germany) column. Separation of analytes was done at a temperature of 35 °C (detector at 150 °C). For this, the culture headspace (1 mL) was sampled with gas-tight syringes (Hamilton, Bonaduz, Switzerland) and injected directly into the injection valve of the GC. Concentrations were calculated using calibration curves ranging from 4 to 50 µmol hydrogen. Organic acids (lactate, acetate, pyruvate, and succinate) were analyzed using a reversed-phased HPLC (Merck-Hitachi, Darmstadt, Germany) equipped with a diode array detector (L-7450). Samples (1 mL) from the liquid phase of the cultures were filtered (MiniSart RC4, 0.2 µm, Sartorius, Göttingen, Germany) and acified with 2.5 µL H_2SO_4. Of this sample, 20 µL was injected into the HPLC. Monitoring of organic acids was based on their absorption at 210 nm. The column was operated at a temperature of 50 °C and 5 mM (v/v) H_2SO_4 served as mobile phase with a flow rate of 0.7 mL min⁻¹. The retention times were: lactate (dl 0.2 mM), 10.61 min; pyruvate (dl 0.01 mM) 7.92 min; acetate (dl 0.4 mM), 12.88 min; succinate (dl 0.15 mM), 9.81 min. Peak areas and retention times were related to standards ranging from 1 to 10 mM or 0.5 to 2 mM (for pyruvate).

**Compound-specific stable isotope analysis**

Determination of the carbon isotope composition of the chlorinated ethenes in pure culture of *S. multivorans* and in co-culture of *S. multivorans* and *D. mccartyi* strain BTF08 was done using gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS; Thermo GC Trace 1320 combined with Thermo Finnigan MAT 253 IRMS, Bremen, Germany) [43]. All samples were analyzed in technical triplicates. 2 mL liquid phase were taken from the respective sample and transferred to a He-flushed 10 mL crimped vial. Of these, 1 mL was taken from the headspace via an autosampler (Thermo TriPlus RSH Autosampler, Bremen, Germany) and injected in a gas chromatograph with a split ratio of 1:5. Chlorinated ethenes were separated on a DB-MTBE column (60 m × 0.32 mm × 1.8 µm, J&W Scientific, Waldbronn, Germany) and the following temperature program: 40 °C for 5 min, increase to 250 °C by 20 °C min⁻¹ and hold for 5 min using helium as carrier gas at a flow rate of 2.0 mL min⁻¹ (Injector at 250 °C). The carbon isotope composition is given in the δ-notation (‰) relative to the Vienna Pee Dee Belemnite standard [44]. Carbon isotope fractionation was calculated using the Rayleigh equation (Eq. 1) where R_R and R_T represent the isotope values and C_0 and C_T the concentrations at time 0 and t [45, 46].

\[
\ln \frac{R_T}{R_0} = \epsilon C \times \ln \frac{C_T}{C_0}
\]  

(1)

The carbon isotope enrichment factor (ε) relates changes in the concentration of the isotopes to changes in their
isotope composition. A two-tailed T-Test was used to calculate the 95% confidence interval based on the slope. Standard deviations were obtained from at least triplicate measurements (<0.5‰).

**B₁₂ extraction and MS analysis**

The B₁₂ content of three independently cultivated 100 mL cultures was analyzed. For this, the culture volume was reduced to 20 mL using a vacuum concentrator and 0.1 M potassium cyanide was added. After boiling the samples for 20 min, cell debris was removed by centrifugation (10 min, 6700 × g, 8 °C). The supernatant was applied onto a C-18 column (CHROMABOND C-18 ec, Macherey-Nagel, Düren, Germany) equilibrated with 5 mL 100% (v/v) methanol and 5 mL UPW. Washing of the column was done twice with 5 mL UPW and B₁₂-types were eluted with 5 mL 100% (v/v) methanol. The eluate was completely dried in a vacuum dryer and dissolved in UPW prior to analysis. The extract was injected to ultrahigh performance nano-flow liquid chromatography (UHPLC) (Ultimate 3000, Thermo Fisher, Waltham, USA) coupled to mass spectrometer (LC–MS, Orbitrap Fusion, Thermo Fisher, Waltham, USA) via heated electrospray ionization (HESI-II, Thermo Fisher, Waltham, USA). The UHPLC was equipped with a Hypersil Gold C18 column (150 × 2.1 mm, 3 µm film thickness; Thermo Fisher, Waltham, USA) and a C18 guard column (10 × 2.1 mm, Waters, Milford, USA). Chromatographic separation using a gradient method with 0.1% formic acid (A) and methanol (B) as mobile phase was applied as following: 5% B for 1 min, 60 min gradient to 90% B, 4 min at 90% B, 1 min gradient to 5% B and 4 min at 5% B with a constant flow of 0.2 mL min⁻¹ and 25°C column oven temperature. Injection volume was 5 µL. Ionization was set to positive ion mode at 3.5 kV, 35 arbitrary units (Arb) sheath gas, 10 Arb auxiliary gas, 325 °C ion transfer tube temperature and 275 °C vaporizer temperature. Orbitrap resolution for precursor scan (MS1) was set to 120.000 with a scan range of 300–1600 m/z. Data evaluation was done on the original mass spectra comparing with predicted masses of corrinoids (Supplementary Tables S1 and S2, for cobalamin standards see Supplementary Fig. S2).

**Scanning electron microscopy and fluorescence in situ hybridization**

Field emission-scanning electron microscopy (FE-SEM) was performed with co-cultures of *S. multivorans* and *D. mccartyi* strains BTF08 and 195 and pure *S. multivorans* and *D. mccartyi* strain BTF08 cultures. All of the cultures were treated the same way for FE-SEM as follows. Cells of 5 mL culture were incubated for 15 min with 2.5% glutaraldehyde and pre-fixed for 2 h on poly-L-lysine coated cover slides (12 mm, Fisher Scientific, Schwerte, Germany). Cover slides were washed three times with 0.1 M sodium cacodylate (pH 7.2) (>98% purity, Sigma Aldrich, Steinheim, Germany) and post-fixed for 1 h with 1% osmium tetroxide in the same cacodylate buffer. After fixation, samples were dehydrated using different ethanol concentrations (35%/50%/70%/80%/95%/100% v/v) and incubated for 10 min at each step. Critical point drying was done in a Leica EM CPD200 Automated Critical Point Dryer (Leica, Wetzlar, Germany), followed by coating with 6 nm platinum in a BAL-TEC MED 020 Sputter Coating System (BAL-TEC, Balzers, Liechtenstein). Imaging of the samples was done with a Zeiss-LEO 1530 Gemini field emission-scanning electron microscope (Carl Zeiss, Oberkochen, Germany) at different magnifications and at 10 kV acceleration voltage. Fluorescence in situ hybridization (FISH) was performed as described previously [47, 48]. In brief, samples were fixed with formaldehyde (2% v/v final concentration) for 2 h at 4°C and filtered on polycarbonate membrane filters (47 µm diameter, 0.2 µm pore size, Nucleopore). Filters were stored at −20°C until further processing. FISH detection of *D. mccartyi* strain BTF08 and 195 was done with Cy3-labeled DHC1259t and DHC1259c probes and *S. multivorans* detection with FITC-labeled probes SULF F220ab [49]. Imaging of un-aggregated cells was done with an epifluorescence microscope (Olympus, BX51) combined with an Olympus XM10 camera. Images were analyzed via Cell-F software. Aggregates were visualized using a confocal laser scanning microscopy (CSLM, Olympus FV1000).

**Protein extraction and proteome analysis**

Samples for proteome analyses were taken ~2 weeks after one re-feeding with PCE or cDCE (pure *D. maccarthyi* strain BTF08 cultures) or 12 h after the third re-feeding with PCE (co-cultures *Sm*/BTF08, Supplementary Fig. S3). When harvested, PCE, TCE, and cDCE were the dominant chlorinated ethenes in the pure cultures, while cDCE and VC were the dominant chlorinated ethenes in the co-cultures (Supplementary Fig. S3). Samples were processed as described previously [50]. Briefly, protein extraction was performed in lysis buffer (20 mM HEPES, 1 mM sodium vanadate, 1 mM β-glycerol phosphate, 2.5 mM sodium pyrophosphate, 8 M urea [51]) by three freeze/thaw-cycles and ultrasonic bath treatments. Ten micrograms of protein dissolved in 100 µL UPW was precipitated with the fivefold amount (v/v) of ice-cold (−20°C) acetone. Protein pellets were dissolved in 50 µL SDS sample buffer (2% SDS, 2 mM beta-mercaptoethanol, 4% glycerol, 40 mM Tris-HCl pH 6.8, 0.01% bromophenolblue), heated to 90°C for 5 min and separated on an SDS gel (12.5%). The gel was run until the samples entered the separating gel. Afterwards, a
Interspecies metabolite transfer and aggregate formation in a co-culture of *Dehalococcoides* and *Sulfurospirillum*.

Growth and dechlorination in *Sulfurospirillum* (Sm)/ *Dehalococcoides* (Dhc) co-cultures

The growth characteristics and the PCE to cDCE dechlorination pattern of *S. multivorans* cultivated in the co-culture medium (Supplementary Fig. S4) were comparable to those in the routinely used medium [55]. With lactate as electron donor and PCE as electron acceptor, a co-culture of *S. multivorans* and *D. mccartyi* BTF08 maintained over 10 transfers under the same conditions dechlorinated PCE to stoichiometric amounts of ethene within 8 days after inoculation (Fig. 1A). The dechlorination rate (PCE to ethene) of the Sm/BTF08 co-culture was 4.5-fold higher compared to the *D. mccartyi* strain BTF08 pure culture (Table 1). In the latter, PCE was completely reduced to ethene within 35 days at a rate of 9 ± 0.3 μM/day (Supplementary Fig. S5A). The culture was re-fed with PCE at day 8 and day 12 after inoculation. After re-feeding, the dechlorination increased (Fig. 1A) and complete dechlorination of PCE occurred within 3 days. PCE was dechlorinated to cDCE within 2 days. To check whether *S. multivorans*, known for its high dehalogenation rate [55],
was responsible for this fast dechlorination, the stable carbon isotope fractionation patterns (which largely differ between *D. mccartyi* and *S. multivorans*) of pure and co-cultures were compared. The isotope fractionation pattern of PCE in the co-culture did not change significantly, and ranged from $-30.2 \pm 0.09$ to $-29.5 \pm 0.12\%e$ at 70% of transformed PCE (Supplementary Fig. S6A). A low but significant fractionation was measured in the pure culture of *S. multivorans* (from $-29.2 \pm 0.05$ to $-27.6 \pm 0.11\%e$ at 56% of transformed PCE, Supplementary Fig. S6A). The isotope enrichment factors for the *S. multivorans* pure and the co-culture were in the same range while differing largely from that of a *D. mccartyi* strain BTF08 pure culture ($Sm/BTF08$ co-culture: $\varepsilon_C = -0.4 \pm 0.3\%e$; *S. multivorans* pure

**Fig. 1** Dechlorination of chlorinated ethenes, growth and metabolite analysis of *S. multivorans/ D. mccartyi* co-cultures with vitamin B12 amendment. A PCE dechlorination of *Sm/BTF08* and B *Sm/195*. C Growth curve of *Sm/BTF08* and D *Sm/195*. E Lactate consumption and acetate production of *Sm/BTF08* and F *Sm/195*. Arrows indicate the time points of re-feeding the culture with PCE (A–D) or lactate (E, F). Broken red lines with open symbols represent theoretical, not analytical, values of the PCE concentration as added to the culture. Please note the secondary y-axes for *D. mccartyi* cell numbers in C, D. Negative controls were run with autoclaved cells (abiotic controls, C). Standard deviation of three independent biological replicates ($N = 3$) are represented by error bars (not visible when smaller than the used symbol). $\sum$ - mass balance; sum of PCE, TCE, cis-DCE, VC and ethene.
Table 1 Dechlorination rates of different co-culture set-ups of *S. multivorans* and *D. mccartyi* BTF08 or 195 from PCE to the products VC (*D. mccartyi* strain 195) or ethene (*D. mccartyi* strain BTF08).

| Organism(s)          | Electron donor | Dechlorination rate (µM day⁻¹) |
|----------------------|----------------|-------------------------------|
| **With vitamin B₁₂** |                |                               |
| *D. mccartyi* BTF08  | H₂             | 9 ± 0.3                       |
| *D. mccartyi* 195    | H₂             | 15 ± 0.4                      |
| Sm/BTF08             | Lactate        | 41 ± 2                        |
| Sm/195               | Lactate        | 48 ± 1                        |
| **Without vitamin B₁₂** |                |                               |
| Sm/BTF08             | Lactate        | –                             |
| Sm/195               | Lactate        | 9 ± 0.04                      |
| **Without vitamin B₁₂ with DMB** |        |                               |
| Sm/BTF08             | Lactate        | 36 ± 2                        |
| Sm/195               | Lactate        | 8 ± 0.5                       |

– No dechlorination to VC or ethene observed.

**Corrinoid transfer in co-cultures and the effect of the lower corrinoid ligand**

*D. mccartyi* strains rely on externally provided corrinoids for dehalogenation and growth. This was also tested for *D. mccartyi* strains BTF08 and 195, which only showed negligible cDCE dechlorination after 100 days without addition of corrinoid (Fig. 2A, D). Therefore, it was of interest whether *S. multivorans* is able to provide functional corrinoids for *D. mccartyi* strains BTF08 and 195. In Sm/BTF08 co-cultures without the amendment of vitamin B₁₂, stoichiometric dechlorination of PCE to cDCE was obtained (~30 µmol/bottle/day) (Fig. 2B). No further dechlorination of cDCE to VC or ethene was detected, indicating that *S. multivorans* alone was responsible for the dechlorination. When cDCE dechlorination stalled in the Sm/BTF08 co-culture, 1 µM 5,6-dimethylbenzimidazole (DMB) was added, which resulted in subsequent cDCE dechlorination (Fig. 2C). Ethene production rates from cDCE (36 ± 2 µM/day) were similar to the co-culture amended with vitamin B₁₂ (41 ± 2 µM/day). The dechlorination rate increased after re-feeding with PCE to 8 µmol/bottle/day. In the Sm/BTF08 co-culture with DMB but without vitamin B₁₂ amendment, three different types of corrinoids were found by mass spectrometric analysis: [Ade]NCba, [DMB]NCba and [DMB]Cba (Supplementary Fig. S7).

Interestingly and in contrast to the Sm/BTF08 co-culture, the Sm/195 co-culture without vitamin B₁₂ amendment (-B₁₂) dechlorinated PCE to VC, although at low rates (Fig. 2E). After 35 days, PCE was re-fed, and the cDCE to VC dechlorination rate increased fourfold (3.6 µmol/bottle/day). The only corrinoid detected in the Sm/195 co-culture -B₁₂ was [Ade]NCba (Supplementary Fig. S8). No significant increase in cDCE dechlorination was observed when 1 µM DMB was added to the Sm/195 co-culture (Fig. 2F). Similar to the Sm/BTF08 co-culture, [Ade]NCba, [DMB]NCba and [DMB]Cba were found by mass spectrometric analysis when DMB was added to the Sm/195 co-culture (Supplementary Fig. S9). To confirm that *D. mccartyi* strain 195 can use [Ade]NCba for dechlorination of cDCE to VC, we amended a pure culture with [Ade]NCba isolated from *S. multivorans* (Supplementary Fig. S10A).
Dechlorination of 50 µmol cDCE to VC was completed within 22 days (Supplementary Fig. S10A). *D. mccartyi* strain 195 pure cultures amended with [5-OMeBza]Cba showed slightly faster dechlorination and the cDCE was converted within 16 days into VC (Supplementary Fig. S10B). Only the amended corrinoid types were detected in the cultures via MS, impurities and rearrangement of corrinoids could be therefore excluded (Supplementary Fig. S11 and S12).

**Electron microscopy and FISH analysis of formed cell aggregates**

After about 25 transfers on lactate and PCE, all co-cultures formed spherical aggregates up to 2 mm in diameter (Fig. 3A). FE-SEM was applied to uncover the cell morphology and cell distribution in these aggregates. After preparation for FE-SEM, the sizes of the aggregates were lower (30–200 µm, Fig. 3B, C). Electron micrographs of both co-cultures revealed a compact network of *S. multivorans* and *D. mccartyi* cells coiled around net-forming filament-like structures. The cells were embedded in an extracellular matrix (ECM) which might aid cell-to-cell contact (Fig. 3D, Supplementary Fig. S13). *S. multivorans* could be distinguished from *D. mccartyi* by FISH with specific oligonucleotide probes targeting 16S rRNA and 3-dimensional imaging showed a spatial organization and an almost equal distribution of both species within the aggregates (Fig. 3E, Supplementary Fig. S14). The high resolution of the confocal laser scanning microscopy enabled visualization of single cells and revealed the same morphologies as in the electron micrographs. In the FISH pictures, the sizes of the aggregates ranged from 50 to 100 µm in diameter.

*S. multivorans* revealed a typical helical rod-shaped cell structure and a size of 2–5 µm by 0.5 µm at magnifications of around ×10,000 and ×20,000 in FE-SEM as described previously [55] (Fig. 4, Supplementary Fig. S13). However, the typical polar flagellum was only observed in a few cells and several flagella seemed to be detached from cells, possibly a part of the ECM (Fig. 4, Supplementary Fig. S13). *D. mccartyi* showed an atypical cell morphology in the co-cultures. Microscopic analysis of the pure culture revealed a disc-shaped irregular coccus of 0.5 µm diameter (Fig. 4a, Supplementary Fig. S15), as previously described [11], whereas the *D. mccartyi* strains in the co-culture
showed 0.5 µm large barrel-like cells with a flattened cell pole at one side and a ring-shaped septum (Fig. 4, Supplementary Fig. S13). Visual analysis of cells separated from aggregates in the co-culture identified unequivocally both organisms and confirmed the presence and unusual morphology of *D. mccartyi* (Supplementary Fig. S15 and S16).

**Proteomics of pure and co-cultures**

We applied a label-free shotgun proteomics approach to identify changes in protein abundances during PCE dechlorination in pure and co-cultures (with and without vitamin B12) with *D. mccartyi* strain BTF08 and *S. multivorans*. The clustering approach nMDS based on quantified proteins revealed a significant separation between pure and co-cultures (*Dhc* BTF08 proteins $p = 0.001$, *Sm* proteins $p = 0.004$, Supplementary Fig. S17). A multi-level pattern analysis was applied to determine indicator proteins for a given condition (i.e., co-culture vs. pure culture) to better understand the functional changes occurring during the dehalogenation in the co-culture (Supplementary Excel File S2).

Of the 20 RDases encoded in the genome of *D. mccartyi* strain BTF08 [57], only two RDase proteins were identified, the gene products of btf_1393 (a hitherto not annotated RdhA) and btf_1407 (the vinyl chloride reductase VcrA). The two reductive dehalogenases PceA (PCE reductive dehalogenase) and TceA (TCE reductive dehalogenase), putatively involved in dechlorination of PCE to cDCE, were not identified under any condition. While VcrA was one of the most abundant proteins under all tested conditions (Fig. 5, Supplementary Fig. S18, Supplementary Excel File S1), the gene product of btf_1393 was more abundant in the two co-cultures (Fig. 5, Supplementary Excel File S2). A BLASTp search against the NCBI nr database revealed that the btf_1393 amino acid sequence was almost identical (99% or 497/498 amino acid sequence identity over the whole length) to an RdhA from *D. mccartyi* 11a5, encoded by 11a5_1355 and characterized as a novel PCE RDase PteA [56, 58]. In *S. multivorans*, the proteins encoded in the organohalide respiratory gene were present.
among all conditions as described previously [59, 60] (Supplementary Excel File S1).

Only a few proteins encoded in the genomes of *S. multivorans* or *D. mccartyi* are annotated to play a potential role in the formation of aggregates, e.g., via an ECM. The proteins which are part of a putative type II pili system in *D. mccartyi* (encoded by btf_1229 to btf_1240) were present in low to medium amounts among all conditions (about median [Med] to lower than Med minus one standard deviation [SD]) but not significantly more abundant in the co-cultures. Many proteins involved in flagellar motility of *S. multivorans* were lower abundant in the co-culture, with most of the Flg and Fli proteins not detected (Supplementary Excel File S1). Protein indicator analysis revealed further that several outer membrane porins of *S. multivorans* (encoded by SMUL_0494, SMUL0693, SMUL_0926, and SMUL_2351) were relevant proteins for the co-cultures. In addition, several molybdopterin oxidoreductases (e.g., a nitrate reductase) of *S. multivorans* were highly abundant only in the co-culture (Supplementary Table S3). Proteins related to cell division (tubulin/GTPase encoded by btf_0551, FtsZ, btf_0595, FtsH, btf_357, a cell division trigger factor, btf_0631) were more abundant or exclusively quantified in pure cultures (Fig. 5, Supplementary Table S4, Supplementary Excel File S2). A hypothetical protein encoded close to the tubulin (btf_0548) was also highly abundant (>Med+SD or >Med) only in cells of the pure culture, while it could not be quantified in any of the co-cultures.

The amidohydrolase CbiZ (btf_610), responsible for cleavage of the nucleotide loop of corrinoids, was quantified only in both co-culture conditions in average abundance (Fig. 5). The L-threonine 3-O-phosphate decarboxylase CobD, which synthesizes the linker of the lower base and the corrinoid ring of cobamides, could not be quantified in any culture.

**Discussion**

In this study, we investigated the dechlorination of PCE to ethene (or vinyl chloride) in co-cultures of *S. multivorans* with *D. mccartyi* BTF08 and 195. Dechlorination profiles, metabolite analysis and growth studies point to a biphasic physiology of the co-culture. In the first stage, when PCE or TCE serve as electron acceptors, *S. multivorans* grows by OHR with lactate as electron donor. In this phase, *D. mccartyi* does not contribute significantly to PCE dechlorination as indicated by the stable isotope fractionation pattern and the corresponding enrichment factors. This can be explained by the insufficient supply of *D. mccartyi* with hydrogen as electron donor due to the lack of (or very low) hydrogen production by *S. multivorans* during respiratory growth. In the second phase of the co-culture, when PCE has been completely converted to cDCE after less than 2 days, *S. multivorans* grows via fermentation of lactate mainly to acetate, CO₂ and hydrogen [36]. The fate of a part of the organic carbon and of the electrons generated by lactate oxidation could not be elucidated in this study. This part might have been used for biomass production and the generation of small amounts of organic acids, such as succinate, below the detection limit of the HPLC used here. It has been shown earlier that, for thermodynamic reasons, lactate can only be utilized fermentatively by *S. multivorans* in co-cultures, where hydrogen concentration is kept low by a syntrophic hydrogen consumer, while *S. multivorans* does not grow fermentatively on lactate in pure cultures [36]. In the co-culture described here, *D. mccartyi* utilizes H₂ produced by *S. multivorans* as electron donor for reductive dehalogenation of cDCE or VC (Fig. 6). As expected, PCE...
was completely dechlorinated to ethene in the Sm/BTF08 co-culture and mainly to VC and minor amounts of ethene in Sm/195. A similar slow and incomplete VC dechlorination was found for pure cultures of the latter strain [61] and co-cultures of D. mccartyi strain 195 with either Desulfovibrio vulgaris Hildenborough or Syntrophomonas wolfei [21, 22]. cDCE was presumably converted to ethene by VcrA of BTF08. This is supported by the proteomic analysis described here, in which VcrA of D. mccartyi strain BTF08 was highly abundant and the predominant RDase in all cultures. A highly similar VcrA ortholog of D. mccartyi strain VS (99% amino acid sequence identity) has already been biochemically characterized and described to dechlorinate cDCE and VC [62]. Interestingly, neither TceA, nor PceA, which had been suggested to be responsible for dechlorination of PCE to VC [57], were detected in any of the samples. An identical ortholog of the only other detected RDase (encoded by btf_1393) was recently characterized as PteA, a novel PCE RDase dechlorinating PCE to tCDE and VC [58]. D. mccartyi strain 195 was not subject to proteomic investigation in our study. However, previous studies suggested that TceA of this organism is involved in cDCE to VC dechlorination and that VC is further dechlorinated slowly by the same enzyme [61, 63].

Norpseudo-B₁₂, produced by S. multivorans, is used differently by the two D. mccartyi strains

Without amendment of vitamin B₁₂ the Sm/BTF08 co-culture was not capable of cDCE dechlorination to ethene, indicating that D. mccartyi strain BTF08 cannot use the norpseudo-B₁₂ ([Ade]NCba, ●) and [DMB]NCba (●) when DMB (maximally 1 µM) is amended. Both can be salvaged and remodeled by D. mccartyi into [DMB]Cba (● with a y-shaped linker). The interspecies cobamide transfer is indicated by yellow arrows. A discrimination between norcobamide or cobamide incorporation into the D. mccartyi RDases is not depicted here, since the data do not allow a conclusion on the cobalamin nucleotide loop type.
DMB. This indicates cDCE to VC conversion by strain 195 with [Ade]NCba produced by \textit{S. multivorans}. However, previous studies showed [Ade]Cba (or other cobalamins with adenine as lower ligand) to be nonfunctional in \textit{D. mccartyi} strain 195 with TCE as electron acceptor [17]. Trace amounts of DMB or cobalamin could be an explanation, but this explanation is unlikely given the same treatment but different dechlorination pattern of the co-cultures. Since RDase gene expression is often dependent on the electron acceptor present [14], cDCE in the \textit{Sm}/195 co-culture might have induced a yet unknown RDase dechlorinating cDCE to VC with [Ade]NCba as cofactor. Alternatively, TceA containing [Ade]NCba could catalyze cDCE to VC dechlorination, but not TCE dechlorination to cDCE. The mechanism of cobalamin transfer from \textit{S. multivorans} to \textit{D. mccartyi} is not yet known. Most likely, cobalamin is released upon cell lysis or degradation of periplasmic PceA and possibly excreted through porins of \textit{S. multivorans}, some of which were higher abundant in co-cultures.

\textbf{Cell aggregates formed by \textit{D. mccartyi} and \textit{S. multivorans} could enhance interspecies hydrogen transfer}

The cell aggregates observed in all studied co-cultures but not in pure cultures are common for obligate syntrophic interactions often found in e.g., acetogenic and methanogenic communities [67–69]. The decrease of intermicrobial distances and establishment of cell-to-cell contacts, which were observed in co-cultures via FE-SEM, should lead to increased metabolite (e.g., hydrogen) fluxes between species according to Fick’s law [70], ultimately enhancing growth and dechlorination rates. Experimentally, this was shown, for example, during syntrophic propionate conversion of \textit{Pelotomaculum thermopropionicum} SI and \textit{Methanothermobacter thermoautotrophicus} ΔH where interspecies hydrogen transfer was calculated to be optimal in aggregates [71]. EPS-like substances and flagella most likely contribute to a stabilization of aggregates by adhesion and attachment of the cells [67, 72]. In addition, cellular extrusions such as pili and flagella might contribute to direct interspecies electron transfer (DIET). While \textit{S. multivorans} produces flagella in pure cultures [55], several structural flagellar proteins were downregulated in the co-culture, speaking against a use of flagellar for DIET in the co-culture of \textit{D. mccartyi} and \textit{S. multivorans}. Other proteins putatively involved in DIET are not encoded in the genomes of \textit{D. mccartyi} or \textit{S. multivorans}, arguing for an exclusive interspecies transfer of electrons via hydrogen. Aggregated \textit{D. mccartyi} cells embedded in an ECM were observed in a bioreactor community dechlorinating trichloroethene to ethene [73]. However in this study, \textit{D. mccartyi} formed typical disc-shaped cells, in contrast to the unusual, never before observed barrel-like morphology of \textit{D. mccartyi} in the co-cultures investigated in our study. This barrel-like morphology might be caused by the downregulation of proteins involved in cell division in the co-culture. One of these proteins, FtsZ, localized at the cell division site, was shown to play a key role in cytokinesis in \textit{E. coli}. It was shown to be responsible for septal invagination of the cell wall and cytoplasmic membrane by forming a ring-shaped septum followed by cell division [74–76]. Its down-regulation might hamper a complete membrane constriction resulting in slower cell division that might cause the observed barrel-like cell morphologies. However, the reasons for this downregulation and the different morphology remain obscure. Possibly the less frequent cell divisions and/or the barrel-like morphology give \textit{D. mccartyi} an advantage in cell-to-cell contact. In addition, this shape could represent a “sessile” state in which \textit{D. mccartyi} adjusts its metabolism to the given condition in the aggregates, including a downregulation of cell division. Since electron micrographs of co-cultures with \textit{D. mccartyi} are scarce, it is not possible to state whether the unusual cell morphology is specific for the partnership with \textit{S. multivorans} or whether it is found frequently for co-cultures containing \textit{D. mccartyi}. With \textit{S. wolfei} as syntrophic partner, disc-like cell structures typical for \textit{D. mccartyi} strain 195 were observed [21].

\textbf{Conclusion}

This study provides first insights into the interactions of \textit{S. multivorans} in association with \textit{D. mccartyi} and the formation of aggregates in which \textit{D. mccartyi} showed an unusual barrel-like morphology. Dechlorinating microbial communities sometimes reveal the presence of \textit{Sulfurospirillum} spp., but the functional role of these \textit{Campylobacterota} (formerly \textit{Epsilonproteobacteria}) is unexplored [77]. We observed that interspecies hydrogen and cobamide transfer in the co-culture resulted in fast and complete dechlorination of PCE to ethene. \textit{S. multivorans} could provide all growth factors required by \textit{D. mccartyi}, including hydrogen and cDCE as energy source, acetate as carbon source and cobamides as RDase cofactors (Fig. 6). It is the first study in which \textit{D. mccartyi} was provided with all nutrients required for growth by its syntrophic partner. Additionally, PCE to cDCE dechlorination was sped up by the fast dechlorination rate of \textit{S. multivorans}. This is of high interest for bioremediation attempts using \textit{Dehalococcoides}-containing mixed cultures, since electron donor and cobalamin limitations may impede \textit{Dehalococcoides} dechlorination activities.
Acknowledgements This work was supported by the Jena School for Microbial Communication (JSMC) and the DFG Research Unit FOR 1530 (sub-projects 1, 2, 5, and 6 were involved). The authors would like to thank Benjamin Scheer and Kathleen Eismann (Helmholtz Centre for Environmental Research, Leipzig) for skillful technical assistance in the lab, Susanne Linde (University Hospital Jena, Center for Electron Microscopy) for the field emission-scanning electron microscopic analysis and Dr. Elena Romano (University of Rome Tor Vergata, Department of Biology) for invaluable assistance in the confocal laser scanning microscope image acquisition and processing. Dr. Torsten Schubert (Friedrich Schiller University Jena) is gratefully acknowledged for insightful discussions on corninoid metabolism. Protein mass spectrometry was done at the Centre for Chemical Microscopy (ProVIS) at the Helmholtz Centre for Environmental Research. MvB and DT are grateful for support by the UFZ funded platform ProMetheus for proteome and metabolome analysis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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