Sustained Dechlorination of Vinyl Chloride to Ethene in *Dehalococcoides*-Enriched Cultures Grown without Addition of Exogenous Vitamins and at Low pH

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Supporting Information

ABSTRACT: Trichloroethene (TCE) bioremediation has been demonstrated at field sites using microbial cultures harboring TCE-respiring *Dehalococcoides* whose growth is cobalamin (vitamin B₁₂)-dependent. Bioaugmentation cultures grown ex situ with ample exogenous vitamins and at neutral pH may become vitamin-limited or inhibited by acidic pH once injected into field sites, resulting in incomplete TCE dechlorination and accumulation of vinyl chloride (VC). Here, we report growth of the *Dehalococcoides*-containing bioaugmentation culture KB-1 in a TCE-amended mineral medium devoid of vitamins and in a VC-amended mineral medium at low pH (6.0 and 5.5). In these cultures, *Acetobacterium*, which can synthesize 5,6-dimethylbenzimidazole (DMB), the lower ligand of cobalamin, and *Sporomusa* are dominant acetogens. At neutral pH, *Acetobacterium* supports complete TCE dechlorination by *Dehalococcoides* at millimolar levels with a substantial increase in cobalamin (~20-fold). Sustained dechlorination of VC to ethene was achieved at pH as low as 5.5. Below pH 5.0, dechlorination was not stimulated by DMB supplementation but was restored by raising pH to neutral. Cell-extract assays revealed that vinyl chloride reductase activity declines significantly below pH 6.0 and is undetectable below pH 5.0. This study highlights the importance of cobamide-producing populations and pH in microbial dechlorinating communities for successful bioremediation at field sites.

INTRODUCTION

Dehalococcoides and *Dehalogenimonas* spp., the only known bacteria capable of reductive dechlorination of vinyl chloride (VC) to nontoxic ethene,^{1–4} require H₂ or formate (*Dehalogenimonas*) as electron donor and conserve energy through organohalide respiration. Organohalide respiration depends on reductive dehalogenases (RDases) that harbor a cobamide as a prosthetic group. However, *Dehalococcoides* spp. cannot synthesize their own cobamide and thus import the vitamin from their surroundings. *Dehalococcoides*-containing cultures and isolates are typically grown in the presence of cobamides like cobalamin (vitamin B₁₂).^{5,6} Cobamides are composed of a corrin ring, a central chelated cobalt ion, two axial ligands (upper and lower ligands), an aliphatic side chain, and a phosphodiester bond, which links the tetrapyrrole ring and the α -ribazole (i.e., the ribonucleoside with the lower axial ligand).^{7,8} The best known cobamide is cobalamin, which contains 5,6-dimethylbenzimidazole (DMB) as the lower axial ligand. A recent study,⁹ using *Dehalococcoides mccartyi* strain GT, demonstrated that the vinyl chloride reductase (VcrA)-mediated dechlorination of VC to ethene is achieved only when the lower ligand of the cobamide is DMB or 5-methylbenzimidazole.⁹ The cobamide requirement of *Dehalococcoides* may be satisfied via direct cobalamin addition or via interspecies cobalamin transfer from microbial community

partners. Additionally, *Dehalococcoides* can synthesize cobalamin via a salvage pathway that replaces the lower ligands of other cobamides with DMB.^{8–12} Currently, only prokaryotes are known to produce cobamides with 17 different lower axial ligand structures.⁷ For example, members of the family Veillonellaceae, such as *Sporomusa*, produce cobamides with a phenolic lower base,^{13,14} while members of the family Eubacteriaceae, such as *Acetobacterium woodii* and *Eubacterium limosum*, exclusively produce cobamides with a DMB lower ligand.^{15,16}

The commercialized bioaugmentation enrichment culture KB-1 dechlorinates chlorinated ethenes, including the dry-cleaning solvent tetrachloroethene (PCE) and the industrial solvent trichloroethene (TCE). In the laboratory, the original KB-1 parent culture (TCE/M_1998_parent, Figure S1) is maintained with TCE (electron acceptor) and methanol (electron donor). This culture is dominated by several distinct strains of *Dehalococcoides*^{17,18} and also contains methanogens and acetogens. The historically dominant acetogens in the parent culture, which can grow using methanol as electron

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donor, belong to the genera *Sporomusa* and *Acetobacterium*.^{2,19,20} In KB-1, *Dehalococcoides* consumes acetate (carbon source) and H₂ (electron donor) produced by the acetogens, thus facilitating their growth. The most abundant RDase is VcrA²¹ whose activity is dependent on cobalamin availability. Hence, dechlorinating enrichment cultures such as KB-1 are typically grown in a medium amended with cobalamin and other vitamins to support steady dechlorination.

Another important factor influencing the performance of dechlorinating cultures is pH. Extensive dechlorination generates HCl, which can lead to a drop in pH as the buffering capacity of the medium is exhausted. A drop in pH to below pH 6.0 causes a significant reduction in dechlorination rates and extent.^{22–25} Lower pH may impact the dechlorinators directly or may impact associated fermenting and cobalamin-producing organisms in the culture. *Dehalococcoides* populations recently studied by Yang et al.²⁶ could not sustain reductive dechlorination at pH 5.5, yet the reason for the loss of dechlorination activity remains unclear. In addition, the enrichment cultures grown at pH 5.5, in which partial dechlorination of TCE to *cis*-dichloroethene (*cis*-DCE) was observed, were shown to harbor dechlorinating *Sulfurospirillum*, but not *Dehalococcoides* populations.²⁶

Addition of vitamins and cobamides to a field site can be very expensive; vitamin B₁₂ (cobalamin) for field site remediation applications is sold at around 1800 USD per 100 g.²⁷ Similarly, pH adjustment at field sites can also be difficult and costly. The first objective of this study was to determine if a TCE-dechlorinating enrichment culture like KB-1, that stoichiometrically dechlorinates TCE to ethene, could be successfully maintained without exogenous vitamins. We have established a subculture of KB-1 that has sustained dechlorination of TCE to ethene without addition of vitamins for over 6 years, indicating an adequate endogenous supply of vitamins in the mixed culture. The second objective of this study was to determine if *Dehalococcoides* populations can sustainably dechlorinate VC to ethene at progressively lower pH and to identify the reasons for decreased activity at low pH. Following progressive adaptation, sustained VC dechlorination to ethene was observed in KB-1 cultures maintained at pH ~ 6.0 and even at pH ~ 5.5 but not observed at lower pH. We determined that the rate of VC dechlorination to ethene by *Dehalococcoides* in KB-1 subcultures at pH below 5.0 was not limited by DMB or cobalamin availability but rather by a direct effect of pH on the VcrA-mediated dechlorination activity. The impact of these results is discussed in the context of bioremediation practice.

MATERIALS AND METHODS

Microorganisms and Chemicals. Chemical reagents were purchased through Sigma-Aldrich Canada (Oakville, ON, Canada), Fischer Scientific Canada (Ottawa, ON, Canada), and BioShop (Burlington, ON, Canada) at the highest purity available. Gases were purchased from Praxair (Mississauga, ON, Canada). *p*-Cresolyl cobamide¹⁴ and the pure culture of *Sporomusa* sp. KB-1 were provided by Prof. Frank E. Löffler (University of Tennessee Knoxville).

Analytical Procedures. Sampling, DNA extraction, quantitative polymerase chain reaction (qPCR), 16S rRNA gene amplicon sequencing, cobamide extraction, and measurement protocols are detailed in the [Supporting Information](#).

Medium Preparation. A bicarbonate-buffered FeS-reduced mineral medium^{28,29} was used to maintain enrichment

cultures and set up new experiments. The medium was prepared either without any vitamins or with addition of one of two vitamin stock solutions: (1) a complete stock containing 11 vitamins (as described in Edwards and Grbić-Galić³⁰) or (2) the same stock prepared without any vitamin B₁₂. The medium was autoclaved and purged while cooling with a gas mix containing 80% N₂ and 20% CO₂. The pH of the medium was 6.9 ± 0.1. If required, the pH was adjusted using a 6N HCl solution. Except for the *Sporomusa* growth inhibition assay, methanol was always added as electron donor and carbon source. TCE was fed in solution with methanol, and VC (pure gas) was fed using a gastight syringe. The TCE/methanol solution was prepared at a ratio of 5:1 electron equivalents (eeq) of donor to acceptor (methanol 6 eeq/mol; TCE to ethene: 6 eeq/mol).

Vitamin-Free Enrichment Cultures. Preparation of the initial TCE-dechlorinating enrichment cultures grown and maintained without the addition of exogenous vitamins is described in the doctoral thesis of Islam (2014, available at <http://hdl.handle.net/1807/90156>).³¹ Briefly, three parallel sets of triplicate cultures (100 mL) were started with inoculum from a 4 L TCE-to-ethene-dechlorinating culture derived from the KB-1 parent culture TCE/M_1998_parent (T3MP1), i.e., an enrichment culture maintained since 1998 with TCE as acceptor and methanol (M) as donor. Cells were triple-washed in medium devoid of vitamins, i.e., centrifuged (8000 rpm, 4°C), resuspended, and centrifuged again after discarding the supernatant before inoculation. The first set received medium containing the regular set of vitamins (controls), the second set received medium with all vitamins except cobalamin, and the third set received medium that was completely vitamin-free. The cultures were routinely reamended with donor and acceptor and periodically transferred into the same three medium formulations. Following the experiments described in Islam (2014),³¹ the cultures (i.e., triplicate 100 mL cultures grown at the three different medium formulations described above) were combined and scaled up to 700 mL and are referred to as TCE/M_Vit(+), TCE/M_Vit(–), and TCE/M_B12(–) ([Figure S1](#)).

Low pH Enrichment Cultures. A long-term pH adaptation experiment is described in the Master's thesis of Li (2012, available at <http://hdl.handle.net/1807/33289>),³² in which transfers from TCE/M_1998_parent (T3MP1) ([Figure S2](#)) were grown over multiple feedings in media at pH 7 (control), pH 6.0, and pH 5.5. In these cultures, the electron acceptor added was VC rather than TCE to maintain specific selection pressure for *Dehalococcoides*. Triplicate enrichment cultures (50 mL) kept at the three pH conditions were maintained with regular VC/methanol feedings for about two and a half years, prior to combining triplicates and scaling up to three 500 mL cultures. These scaled-up cultures are referred to as VC/M_pH7, VC/M_pH6, and VC/M_pH5.5 ([Figure S2](#)).

Growth Experiments. Two major growth experiments were performed to test the impacts of pH and B₁₂: one using 10% dilution transfers set up at pH 7.0 and 5.5 (amended with either TCE or VC) and another using 1% dilution transfers (amended with TCE) (see [Figure S1](#) for experimental flow). All experiments were set up in an anaerobic chamber (Coy) with a 10% CO₂, 10% H₂, and 80% N₂ atmosphere. For the 10% dilution transfers at pH 7.0 and 5.5 ([Figure S1](#)), inoculum from the TCE/M_B12(–) enrichment culture (10 mL for TCE dechlorination assays and 5 mL for VC dechlorination

assays) was added to autoclaved 160 mL narrow-necked glass serum bottles containing defined mineral medium without cobalamin (90 mL for TCE dechlorination assays and 45 mL for VC dechlorination assays). Subsequently, the serum bottles were sealed with rubber stoppers and crimped. The electron donor (methanol) and electron acceptor (TCE or VC) were added in the same way as for the enrichment cultures, at a ratio of 5 to 1 electron equivalents of donor to acceptor, unless otherwise specified. If necessary, additional electron donor was added to ensure complete dechlorination. Once the TCE transfers set up at pH 5.5 had partially dechlorinated the added TCE to ethene, they were subsequently aliquoted to six 10 mL glass vials to a final volume of 5 mL each, sealed with rubber stoppers and crimped. DMB (10 μ M) was added to two bottles, and the pH was adjusted to 7.0 in another two bottles using a saturated bicarbonate solution ($\sim$ 1.3 M). The remaining two bottles were kept as controls at pH 5.5. The growth of *Sporomusa* and *Acetobacterium* in the absence of TCE was investigated in 10% dilution transfers from the TCE/M_B12(–) culture that were fed methanol only (Figure S1).

The 1% dilution transfer experiments (Figure S1) were initiated to minimize cobalamin and DMB carry-over. In these experiments, the conditions were similar to those of the 10% dilution transfers, except that the inoculum (1 mL) from the TCE/M_Vit(–) was first pelleted down, washed twice with sterilized defined (cobalamin-free) medium, resuspended in 1 mL of the same cobalamin-free medium, and then added to serum bottles containing 99 mL of cobalamin-free medium.

Cell-Extract Dehalogenase Activity Assay. The cell-extract dehalogenase activity assay was performed following previously established protocols under anaerobic conditions.³³ All reagents were pre-reduced or kept anaerobically before the assays. Briefly, 100 mL of the TCE/M_B12(–) culture was harvested and pelleted down by centrifugation at 9000g for 20 min at 4 °C under anaerobic conditions. The supernatant was removed and the pellets were resuspended in 0.3 mL of 100 mM Tris–HCl buffer (pH 7.5) containing 12.5 mM NaCl, 2.5% glycerol, and 1% digitonin (detergent). The suspensions were loaded into a 2 mL O-ring-capped plastic microcentrifuge tube containing 50 mg of glass beads (particle size $\leq$ 106 μ m). To lyse the cells, the tube was vortexed at maximum intensity for 2 min and incubated on ice for 1 min. The step was repeated four times. The cell lysate was then centrifuged at 16 000g for 15 min at 4 °C to remove cell debris, beads, and unbroken cells, and the supernatant was used in the assays. The dehalogenase activity assays (1 mL) were performed in capped 2 mL glass vials. Tris–HCl buffer (50 mM) was used for the assays at pH 6.0 and 7.0, and sodium acetate-acetic acid buffer (50 mM) was used for the assays at pH 4.75, 5.0, and 5.5. Ti(III) citrate-reduced methyl viologen (1 mM) was used as the artificial electron donor, and *cis*-DCE (0.5 mM) was used as the electron acceptor; these were added to the buffers before the addition of cell extracts. The cell extracts were added into the vials to a final protein concentration of 10 μ g/mL to start the reaction. The vials were immediately capped, incubated for 2 h, and sampled for analysis of dechlorination products via gas chromatography (see Supporting Information). Protein concentration was determined using a microplate Bradford (Sigma-Aldrich Bradford Reagent) protein assay with bovine serum albumin standards.

***Sporomusa* Growth Inhibition Assays.** For this experiment, Na₂S (0.2 mM), instead of FeS, was used as the reductant in the medium to avoid turbidity and facilitate cell

density measurements; resazurin was excluded from the medium to avoid interference from the pink color that develops during cell density measurements after exposure to air. In an anaerobic chamber, the active *Sporomusa* sp., strain KB-1 pure culture (2 mL; OD_{600nm} $\sim$ 1), was transferred into 2 mL sterile O-ring-capped plastic microcentrifuge tubes and pelleted down by centrifugation at 10 000g for 10 min at room temperature. The supernatants were removed, and the pellets were washed with a sterile cobalamin-free defined medium (1 mL) twice. Afterward, the pellets were resuspended in sterile defined media containing varying cobalamin concentrations (0, 5, 10, 25, and 50 μ g/L) to a final cell density (OD_{600 nm}) of 0.04 (as determined by the no-substrate control included in the experiment); 5 mL of the resuspended cells at each cobalamin concentration was transferred into the sterile 10 mL rubber-stopper-sealed glass tubes by sterile plastic syringes and needles. The tubes were then purged with 20% CO₂/80%N₂ to remove H₂, followed by the addition of methanol to a final concentration of 20 mM. In some assays, H₂ (20% in head space), formate (10 mM), or lactate (10 mM) was added. The glass tubes were incubated at 30 °C for 1 day, and 0.2 mL of each culture was sampled and loaded into a 96-well optical plate (Falcon). The cell density in each well was determined using a TECAN Infinite M200 plate reader at 600 nm.

Accession Numbers. 16S rRNA amplicon sequences have been deposited into NCBI under bioproject PRJNA376155 SRA study SRP144609 accession numbers SRR9839478–86. The draft genomes of *Sporomusa* sp. KB-1 and *Acetobacterium* sp. KB-1 are available in IMG under genome ID 2512047088 and NCBI under accession number CP030040, respectively.

■ RESULTS AND DISCUSSION

We first describe the results of the long-term cultivation experiment with cultures grown with TCE in mineral minimal medium without exogenous vitamins and without cobalamin. Then, we describe the results of the long-term cultivation experiment with cultures grown with VC at pH 7.0, 6.0, and 5.5. Next, we describe the microbial community of these cultures as determined by 16S rRNA gene amplicon sequencing and describe the hypotheses that led to subsequent experiments to investigate the dynamic relationship between the acetogens in this culture in light of *Dehalococcoides* cobamide dependence and the sensitivity of reductive dechlorination to pH below 6.0.

Reductive Dechlorination of TCE to Ethene in Enrichment Cultures Grown without Exogenous Vitamins. Dechlorination of TCE to ethene was sustained over 800 days (Figure 1) in all of the cultures, TCE/M_Vit(+), TCE/M_B12(–), and TCE/M_Vit(–), regardless of the type of vitamin amendment. In general, the cultures with or without exogenous vitamins performed similarly (Figure 1A,C). The average long-term dechlorination rates for these three cultures, taken as the slope of the linear regression of the data over 800 days as shown in Figure 1, were 69 $\pm$ 2.4, 66 $\pm$ 2.2, and 57 $\pm$ 1.3 μ mol Cl[–] released/L/d, respectively. The average dechlorination rate for TCE/M_Vit(–) is slightly lower than that for TCE/M_Vit(+). Although this could be related to the high metabolic cost associated with the synthesis of the required cobamides, in practice, these differences are within the expected variability in dechlorination rates that may be observed during this type of experiments. Altogether, these long-term data demonstrate that the entire vitamin require-

Figure 1. Long-term and sustained dechlorination of TCE to ethene (left) and corresponding microbial community composition shifts (right) for the scaled-up TCE- and methanol-fed enrichment cultures TCE/M_Vit(+) (A), TCE/M_Vit(−) (B), and TCE/M_B12(−) (C). TCE was fed every 4–6 weeks, at a nominal concentration of 1.4–1.5 mM. Dechlorination rates obtained from linear regressions are shown with 95% confidence intervals; the R^2 values of the regressions are 0.98, 0.99, and 0.98 for (A), (B), and (C), respectively. On the right, two amplicon sequencing events are shown for DNA extracted on day 279 and day 777.

ments of the culture could be met by organisms present in the KB-1 culture.

Reductive Dechlorination of VC to Ethene in Enrichment Cultures Grown at pH 6.0 and pH 5.5. The reductive dechlorination of VC to ethene in the cultures VC/M_pH7, VC/M_pH6, and VC/M_pH5.5 is shown in Figure 2A. The observed dechlorination rates for these cultures, taken as the slope of the linear regression of the data set shown for each culture (~ 400 days), are 7.9 ± 0.29 , 8.8 ± 0.25 , and $2.0 \pm 0.08 \mu\text{mol Cl}^- \text{ released/L/d}$, respectively. The enrichment culture originally set at pH 6.0, described in the Master's thesis of Li (2012),³² initially dechlorinated VC more slowly than the culture at pH 7.0 but eventually maintained a rate comparable to that of the enrichment culture maintained at pH 7.0 (Figure 2). The average dechlorination rate for VC/M_pH6 is slightly higher than that for VC/M_pH7, yet in practice these

differences may be due to the nature of the experimental set up, i.e., occasional small ethene leaks during sampling in the pH 7.0 bottle or differences in feeding amounts (gaseous VC is fed directly), or may be related to changes in the culture composition through the period of adaptation to pH 6.0. In the culture maintained at pH 5.5 (see Figure S3 for pH measurements), the observed dechlorination and ethene production rates were about 4 times lower than those at pH 7.0 and 6.0. The maintenance of low pH batch cultures required careful monitoring as acid buildup from dechlorination progressively lowered the pH; at low pH, the carbonate buffering system is not as robust as at neutral pH. Overall, we found that the enrichment culture KB-1 was able to dechlorinate VC to ethene at $5.5 \leq \text{pH} \leq 7.0$ (Figure 2A) and that this activity was mediated by *Dehalococcoides* (Figure 2B), which is different from the observations reported by Yang et al.²⁶ Perhaps, certain *Dehalococcoides* populations are more resistant to low pH than others.

Microbial Community Composition Revealed by Amplicon Sequencing. Two snapshots of the microbial community composition in the enrichment cultures used in this study are shown in the right-hand panels in Figures 1 and 2. As expected, *Dehalococcoides* was the dominant genus in these cultures, accounting for about 25–50% of the total microbial population based on 16S rRNA gene copies. As previously reported,^{2,19,20} the two major acetogens were *Acetobacterium* and *Sporomusa*. On day 279 (first DNA sampling), *Sporomusa* was more abundant in the enrichments amended with vitamins (TCE/M_Vit(+)) than in the nonamended ones. *Sporomusa* was also more abundant than *Acetobacterium* in the sample from VC/M_pH5.5. The relative abundance of *Sporomusa* increased in the TCE/M_Vit(−) enrichment cultures between day 279 and day 777. We also noted that the pH in this culture, TCE/M_Vit(−), and in the TCE/M_Vit(+) had fallen to 4.7–4.8 (as a result of progressive HCl accumulation from dechlorination). The pH was adjusted back to 7.0, but perhaps this pH drop, which likely occurred over a period of 2–4 weeks after feeding, had already affected the microbial community. Nevertheless, these observed differences in the relative abundance of *Acetobacterium* and *Sporomusa* led us to propose two hypotheses: (1) *Sporomusa* outcompetes *Acetobacterium* at pH below pH 6.0 and (2) *Sporomusa* outcompetes *Acetobacterium* when exogenous vitamins (particularly vitamin B₁₂) are present. To test the pH hypothesis further, we carried out a point biserial (pb) correlation analysis between the microbial abundance data and pH above or below 6.0. We used the data shown in Figures 1 and 2, along with two samples from the parent KB-1 culture and a VC/methanol-fed KB-1 subculture (see Table S2). The relative abundance of *Acetobacterium* was negatively correlated ($r_{\text{pb}} = -0.75$) with pH < 6.0 (anytime within 3 weeks prior to DNA extraction). In contrast, the relative abundance of *Sporomusa* was positively correlated ($r_{\text{pb}} = 0.77$) with pH > 6.0. *Acetobacterium* also seemed to be more abundant in cultures without added vitamin B₁₂ (Figure 1B,C). Additional growth experiments (described below) were initiated to further investigate these trends.

Dechlorination of TCE at pH 7.0 and pH 5.5 in Cultures Grown without Exogenous Cobalamin (Vitamin B₁₂). Using a 10% inoculum from culture TCE/M_B12(−), we examined the growth of *Dehalococcoides*, *Acetobacterium*, and *Sporomusa* (measured by qPCR) during the dechlorination of TCE to ethene in parallel experiments at

Figure 2. Long-term and sustained dechlorination of VC to ethene in enrichment cultures maintained at pH 7.0, 6.0, and 5.5 $\pm$ 0.2 (A) and their corresponding microbial community composition on day 94 (B). Dechlorination rates obtained from linear regressions with 95% confidence intervals are shown; the R^2 values of the linear regressions are 0.96, 0.97, and 0.96, for the pH 7.0, pH 6.0, and pH 5.5 enrichment cultures, respectively.

Figure 3. TCE dechlorination profiles in 10% dilution transfers of the TCE/M_B12(-) at pH 7.0 (A) and pH 5.5 (B) and associated cell and corrinoid abundances. Corresponding cell abundances per mL of culture as determined by qPCR are shown for pH 7.0 in panel (C) and pH 5.5 in panel (D). Cobalamin and *p*-cresolyl cobamide concentrations are shown in panel (E). Attempts to reactivate pH 5.0 culture samples from day 16 in panel B with DMB addition or pH adjustment are shown in panel (F). In panels (A) and (B), initial and final pH measurements are shown. Error bars correspond to the range of duplicate experimental bottles except for the qPCR samples at $t = 0$ day in which a single sample was measured and error bars correspond to the range for technical duplicates. The * symbol indicates growth greater than 5-fold as compared to the measurements obtained at $t = 0$ day. In (E), cobalamin and *p*-cresolyl cobamide were measured at $t = 16$ days (final time point for the transfers set up at pH 5.5). In (F), the pH indicated below the bars corresponds to the pH measured in the bottles after DMB addition or pH adjustment; in the “no treatment” and “10 μM DMB” bottles, the pH fell from 5.5 to 5.0 as a result of dechlorination.

pH 7.0 and 5.5. Gene copies per mL were converted to cells per mL using the number of 16S rRNA gene copies found in the genomes of *Acetobacterium* sp. KB-1 (5 copies, draft

genome in NCBI under accession number CP030040) and *Sporomusa* sp. KB-1 (13 copies, draft genome in IMG under genome ID: 2512047088). *Dehalococcoides* only possesses a

Table 1. Cell growth, Yield, and Cobalamin Production Measured in a 1% Dilution Transfer of the TCE/M_Vit(−) Enrichment Culture Dechlorinating TCE to Ethene in the Absence of Any Added Vitamins^a

measurement	inoculum (<i>t</i> = 0 day)	final (<i>t</i> = 60 days)	yield (cells per $\mu\text{mol Cl}^-$ released)	fold change
<i>Dehalococcoides</i> (cells/mL)	$2.4 \pm 0.4 \times 10^7$	$1.7 \pm 0.05 \times 10^8$	$1.1 \pm 0.04 \times 10^8$	7.3
<i>Acetobacterium</i> (cells/mL)	$5.7 \pm 0.2 \times 10^3$	$3.6 \pm 0.04 \times 10^5$		63
total cobalamin (ng/L)	40 ± 5	750 ± 140		19

^aDechlorination data is shown in Figure S5. Cell growth and cobalamin concentrations are reported as the mean $\pm$ range of duplicate measurements.

single copy of the 16S rRNA gene per genome. As shown in Figure 3A,B, complete dechlorination of TCE to ethene was only observed at pH 7.0. At pH 5.5, TCE was dechlorinated to VC, but VC was not further dechlorinated to ethene. In these bottles, the dechlorination of TCE (0.75 mM per bottle) to VC at pH 5.5 caused the pH to drop to 5.0. This drop in pH likely impacted the ability of the *Dehalococcoides* population to dechlorinate VC to ethene. Nonetheless, an increase in the cell numbers of *Dehalococcoides* per mL of culture (as compared to the measurements obtained at *t* = 0 day) was observed at both pH 7 and 5.5 (Figure 3C,D; Table S3); at pH 5.5, the growth of *Dehalococcoides* was proportional to observed dechlorination (two-thirds of the electron acceptor equivalents were consumed at pH 5.5 compared to pH 7). In the transfers set up at pH 7.0, there was a 5-fold increase of *Acetobacterium*, but no increase of *Sporomusa*. In the transfers set up at pH 5.5, *Acetobacterium* and *Sporomusa* both showed a small 2-fold increase (Figure 3C,D; Table S3). Slow to no growth of *Acetobacterium* at pH 5.5 was expected as the pH growth range for other *Acetobacterium* spp. is between 6.0 and 8.0, with a pH optimum around 7.0.^{34–37} In contrast, the pH growth optimum for *Sporomusa* spp. is between 6.2 and 7.4,³⁸ with a pH growth range as low as 5.5 and as high as 8.7.^{38–40} For *Sporomusa silvacetica*, the closest relative of *Sporomusa* sp. KB-1, relatively rapid growth was reported for pH between 5.5 and 7.5.³⁹

Cobamide Profile in Cultures Grown at pH 7.0 and 5.5. Since *Acetobacterium* spp. are reported to be cobalamin producers,^{15,16} we hypothesized that the amount of cobalamin in the cultures would be proportional to the relative abundance of the *Acetobacterium* population. Thus, we extracted the cobamides from the aforementioned KB-1 transfers grown at pH 7.0 and at pH 5.5, respectively (Figure 3E; mass spectroscopy (MS) spectra in Figure S4). Consistently, in the cultures grown at pH 5.5 in which VC accumulated, the measured concentration of *p*-cresolyl cobamide was $\sim 88 \mu\text{g/L}$, which is ~ 20 times higher than the measured concentration of cobalamin ($\sim 4 \mu\text{g/L}$). In contrast, in the cultures grown at pH 7.0, in which VC was completely dechlorinated and the growth of *Acetobacterium* was measureable (5-fold increase), the average concentrations of *p*-cresolyl cobamide and cobalamin are comparable ($\sim 25 \mu\text{g/L}$). The higher cell density of *Acetobacterium* and higher cobalamin concentration in the cultures grown at pH 7.0, as compared to the cultures grown at pH 5.5, support our hypothesis that *Acetobacterium* is a DMB/cobalamin producer in the KB-1 enrichment cultures. To further confirm that *Acetobacterium* sp. strain KB-1 can supply DMB/cobalamin to *Dehalococcoides*, we inoculated medium without cobalamin with a 1% transfer from the culture TCE/M_Vit(−). In the previous experiment (10% dilution transfers), some DMB or cobalamin could have carried over with the inoculum. For this experiment, the inoculum was centrifuged and washed twice with anaerobic mineral medium

devoid of vitamins to remove cobalamin and DMB carry-over. After the duplicate KB-1 cultures had dechlorinated $\sim 0.5 \text{ mM}$ TCE to ethene (1.34 mM Cl^- released), we harvested one of the cultures (dechlorination data shown in Figure S5) and measured cobalamin concentrations. The measured concentration ($\sim 750 \text{ ng/L}$, 0.55 nM) is almost 20-fold higher than the observed concentration before TCE dechlorination occurred (Table 1). Nevertheless, it is surprising that nanomolar levels of cobalamin could support the dechlorination of TCE to ethene. For this experiment, the yield of *Dehalococcoides*, 1.1×10^8 cells per $\mu\text{mol Cl}^-$ released, is in the same order of magnitude as previously published yields of *Dehalococcoides* pure cultures, which range between 1.5×10^7 and 2.9×10^8 cells per $\mu\text{mol Cl}^-$ released.⁴¹ Moreover, the cell density for *Acetobacterium* increased by 63-fold (Table 1).

In KB-1, dominant methanogens from the *Methanomicrobiales*, such as *Methanoregula*, may produce factor III⁴² (with 5-hydroxybenzimidazole as lower ligand) while *Desulfosporosinus* and *Dehalobacter* may produce purinyl-cobamide as predicted by Yan et al.⁷ Sulfate reducers like *Desulfovibrio*, although not in high abundance, may produce cobamides with guanine or hypoxanthine as lower ligand, as demonstrated previously for *Desulfovibrio vulgaris*.⁴³ These cobamides, in any case, are not functionally equivalent to cobalamin for VcrA, which catalyzes the dechlorination of VC to ethene. In fact, supplementing other cobamides like factor III, phenyl cobamide or purinyl-cobamides, to *Dehalococcoides* isolate cultures that synthesize the VcrA and BvcA enzymes (which can dechlorinate VC to ethene) resulted in incomplete dechlorination and VC accumulation.⁹ Nevertheless, *Dehalococcoides* is able to synthesize cobalamin via a salvage pathway that replaces the lower ligands of other cobamides with DMB.^{9,13}

Relationship between *Acetobacterium* and *Dehalococcoides* during TCE Reductive Dechlorination. As demonstrated earlier, the growth of *Acetobacterium* in KB-1 is correlated to an increase in cobalamin content, which supports efficient dechlorination of VC to ethene (Table 1). Analyses of the KB-1 metagenome (NCBI accession PRJNA376155) are consistent with this observation, revealing that only *Acetobacterium* sp. strain KB-1 contains a complete anaerobic DMB biosynthesis operon (*bza*ABCDE; Figure S6). The operon arrangement and gene annotation are identical to those of *E. limosum* strain ATCC 10825 and *A. woodii*, two well-known cobalamin producers^{16,44,45} (Figure S6); the encoded proteins are most similar to the ones found in *Acetobacterium dehalogenans* (Table S4).

We were next interested in studying the growth of *Acetobacterium* in the absence of dechlorination to further understand the relationship between *Acetobacterium* and *Dehalococcoides*. We performed a 10% dilution transfer of the culture TCE/M_B12(−) into medium free of vitamins amended with methanol but not TCE, at both pH 7.0 and 5.5. Contrary to our expectation, we did not observe growth of

Acetobacterium (Figure S7). Measurable growth (an order of magnitude) was observed only for *Sporomusa*, regardless of pH. *Dehalococcoides* was not expected to grow at either pH tested, yet we expected to observe measurable growth for *Acetobacterium* analogous to the growth that we observed at pH 7.0 with added TCE (Figure 3C). While it is clear that DMB/cobalamin produced by *Acetobacterium* is beneficial to *Dehalococcoides*, any potential mechanism that may facilitate the growth of *Acetobacterium* during active dechlorination by *Dehalococcoides* remains to be further explored.

When cobalamin is absent at field sites, VC dechlorination by *Dehalococcoides* can be expected to be highly dependent on the biosynthesis of DMB/cobalamin by cobalamin-producing organisms such as *Acetobacterium*. The role that native *Acetobacterium* and other cobamide-producing populations have at chlorinated ethene-contaminated sites should be investigated. Environmental practitioners working on bio-stimulation via cobalamin addition may find it beneficial to first assess if native cobalamin-producing populations may support in situ VC dechlorination. *Acetobacterium* and other cobamide-producing organisms can be quantified, via qPCR of the 16S rRNA gene for example, in addition to *Dehalococcoides* 16S rRNA genes, *Dehalococcoides* RDase genes, and 16S rRNA genes for other dechlorinating organisms. Cobamide-producing organisms such as *Acetobacterium* may indeed serve as additional biomarkers for successful field site reductive dechlorination of VC.

Cobalamin in Methanol-Fed Enrichment Cultures May Inhibit *Sporomusa*. According to our findings, when enrichment cultures are starved of TCE (Figure S7) or experience low pH (Figures 2B and 3D), *Sporomusa* growth is favored. *Sporomusa* can also efficiently grow at neutral pH as shown in Figure S7. An earlier study⁴⁶ reported that the addition of 10 μM DMB ($\sim 1400 \mu\text{g/L}$) to growth medium inhibited the growth of *Sporomusa* on methanol since the methanol methyltransferase requires either phenyl cobamide or *p*-cresolyl cobamide as a cofactor.^{46–48} We hypothesized that cobalamin, the cobamide with DMB as the lower ligand, may also inhibit the growth of *Sporomusa* on methanol. When 50 $\mu\text{g/L}$ cobalamin was added to the medium, the growth of *Sporomusa* was completely inhibited (Figure 4). When 25 $\mu\text{g/L}$ cobalamin was added to the medium, the growth of *Sporomusa*

Figure 4. Inhibition of *Sporomusa* growth on methanol by cobalamin. Assays tested 0, 5, 10, 25, and 50 $\mu\text{g/L}$ cobalamin + 20 mM methanol; 50 $\mu\text{g/L}$ cobalamin + 10 mM sodium formate; 50 $\mu\text{g/L}$ cobalamin + 33 mM (nominal) hydrogen gas; and 50 $\mu\text{g/L}$ cobalamin + 10 mM sodium lactate (Lac). Growth was measured using OD_{600nm} after 24 h. NC stands for the no-substrate control. Above the dotted line, no inhibition is observed. Error bars represent the standard deviation of triplicate experiments.

was lower relative to the control. This is about the same concentration as measured in the pH 7.0 experiment shown in Figure 3 in which the growth of *Sporomusa* was not apparent (Figure 3C). The growth of *Sporomusa* was not inhibited by cobalamin when grown with H₂, formate, or lactate instead of methanol (Figure 4), suggesting that the inhibition by cobalamin is specific to growth on methanol, which requires the *p*-cresolyl cobamide- or phenyl cobamide-specific methanol methyltransferase. It is possible that the use of methanol as the sole electron donor in the KB-1 enrichment cultures may play a role in the enrichment of the DMB/cobalamin-producing *Acetobacterium* population, whose production of cobalamin represses the growth of the *p*-cresolyl cobamide-producing *Sporomusa* population.

Stalled Dechlorination of VC to Ethene Was Restored after pH Adjustment.

Our data revealed that the population of cobalamin/DMB-producing *Acetobacterium* is much less abundant in the dilution transfer cultures grown at pH 5.5 than in those grown at pH 7.0 (Figure 3), suggesting that *Dehalococcoides* may have faced cobalamin shortage to produce the functional form of VcrA for VC dechlorination. Given that *Dehalococcoides* can salvage cobalamin using *p*-cresolyl cobamide and DMB,¹² we wanted to test if DMB supplementation (10 μM ; $\sim 1400 \mu\text{g/L}$) alone could rescue the loss of VC dechlorination in these transfers. As shown in Figure 3F, DMB supplementation did not restore the dechlorination of VC to ethene at pH 5.5. However, increasing pH to 7.0 led to 80% conversion of VC to ethene in 5 days. This suggests that incomplete VC dechlorination by *Dehalococcoides* was not due to DMB shortage but due to acidic pH.

Effect of pH on the *Dehalococcoides*-Mediated Reductive Dechlorination of VC in 10% Dilution Transfers Amended with VC.

Analogous to the 10% dilution transfer experiments set up at pH 7.0 and 5.5 with TCE as electron acceptor, a 10% dilution transfer experiment with the culture TCE/M_B12(–), maintained at circumneutral pH, was performed to specifically test the dechlorination of VC at different pH values: 7.0, 6.3, 5.5, and 5.5 with 10 μM DMB. As shown in Figure 5A, complete VC dechlorination to ethene only occurred at pH 7.0 and pH 6.0. The dechlorination of VC to ethene was slower in the transfers set up at pH 5.5, but ethene was produced until the pH decreased below 5.5. *Dehalococcoides* growth was observed at each pH (Table 2). Even though the enrichment culture TCE/M_B12(–) is grown at circumneutral pH, *Dehalococcoides* was still able to grow and dechlorinate VC to ethene at a pH as low as 5.5. The addition of 10 μM DMB at pH 5.5 resulted in enhanced *Dehalococcoides* growth (Table 2), 10 times (pH 5.5 + DMB) vs four times (pH 5.5 without DMB addition), and a higher final pH, 4.8 (pH 5.5 + DMB) vs 4.0 (pH 5.5 without DMB addition). Interestingly, in the cultures incubated at pH 5.5 with 10 μM DMB, the pH decreased to 4.8 as opposed to 4.0 for the cultures incubated at pH 5.5 without DMB. This buffering effect is likely related to the documented inhibition of *Sporomusa* by DMB,⁴⁶ which resulted in reduced acetogenesis. In terms of yield, at pH 6.3 and 7.0, the yield of *Dehalococcoides* ($1.1 \pm 0.1 \times 10^7$ cells per $\mu\text{mol Cl}^-$ released) is in the same order of magnitude as previously published yields for *Dehalococcoides* pure cultures, which range between 1.5×10^7 and 2.9×10^8 cells per $\mu\text{mol Cl}^-$ released,⁴⁹ yet these published yields were measured in cultures generally grown with cyanocobalamin supplementation at 50 $\mu\text{g/L}$.^{1,41} The

Figure 5. Effect of pH on the reductive dechlorination of VC by *Dehalococcoides* in KB-1 enrichment cultures originally grown at circumneutral pH. Dechlorination of VC to ethene in 10% dilution transfers of the TCE/M_B12(–) enrichment culture amended with VC and methanol (A) and *Dehalococcoides* RDase activity in cell extracts, amended with *cis*-DCE, of the TCE/M_Vit(–) enrichment culture, measured at varying pH values (B). In (A), the initial pH of the transfers is shown in the figure legend and the final measured pH is shown next to the last measured time point; error bars represent the standard deviation of triplicate experiments. In (B), CE(–) stands for the control without cell extract; error bars represent the range of duplicate experiments.

Table 2. Changes in *Dehalococcoides* Cell Numbers per mL in 10% Dilution Transfers of the TCE/M_B12(–) Enrichment Culture after the Dechlorination of VC to Ethene at Varying pH Values^a

starting pH	final pH	<i>Dehalococcoides</i> cells/mL		Cl [–] released from VC (mmol)	yield (cells per μ mol Cl [–] released)	fold change
		<i>t</i> = 0 day mean	<i>t</i> = 22 days mean			
5.5	4.0	$7.1 \pm 0.3 \times 10^6$	$2.8 \pm 2.8 \times 10^7$	0.23	$4.5 \pm 6.1 \times 10^6$	4
5.5 + 10 μ M DMB	4.8	$7.1 \pm 0.3 \times 10^6$	$6.9 \pm 0.6 \times 10^7$	0.32	$9.7 \pm 0.9 \times 10^6$	10
6.3	6.2	$5.0 \pm 0.5 \times 10^6$	$1.2 \pm 0.1 \times 10^8$	0.54	$1.1 \pm 0.1 \times 10^7$	25
7.1	7.0	$2.6 \pm 0.6 \times 10^6$	$1.3 \pm 0.2 \times 10^8$	0.55	$1.1 \pm 0.1 \times 10^7$	50

^aMeasurements at *t* = 0 day correspond to the mean $\pm$ range of duplicate qPCR reactions for single DNA samples at each pH condition. Measurements at *t* = 22 days correspond to the mean and standard deviation of analyses performed on triplicate experimental bottles. Corresponding dechlorination data for each pH is shown in Figure 5A.

reported yields for the BAV1 and GT pure *Dehalococcoides* cultures grown with 1 μ g/L cyanocobalamin were $1.4 \pm 0.8 \times 10^7$ and $5.3 \pm 1.3 \times 10^7$ cells per μ mol Cl[–] release, respectively,¹⁴ which are comparable to the yield obtained in this study.

The negative impact of low pH on reductive dechlorination has been well documented.^{22–26,50} Although members of the genus *Sulfurospirillum* have been shown to reductively dechlorinate PCE and TCE to *cis*-DCE at low pH (pH 5.5),²⁶ this is the first time that growth of *Dehalococcoides* populations has been demonstrated at pH 5.5 after a rapid decrease from pH 7.0 to 5.5 (Table 2) and also after progressive decreases and long-term cultivation at pH 5.5 (Figure 2). Thus, *Dehalococcoides* is likely a key player in the complete chlorinated ethene detoxification at field sites even when pH is below 6.0.

Dechlorination in Cell Extracts at Different pH Values. To gain additional insight into the effect of pH on the *Dehalococcoides*-mediated reductive dechlorination, cell extracts, harvested from the TCE/M_Vit(–) enrichment culture while actively dechlorinating, were assayed at pH 4.75, 5.0, 5.5, 6.0, and 7.0. In this culture, the ratio of 16S rRNA gene copies to vinyl chloride reductase gene (*vcrA*) copies is about 1:1 with $\sim 1 \times 10^9$ copies per mL of culture (qPCR data shown in Figure S8). Titanium citrate-reduced methyl viologen and *cis*-DCE were used as the artificial electron donor and electron acceptor, respectively; *cis*-DCE was chosen as a suitable acceptor because *cis*-DCE can be more easily and reproducibly amended as opposed to VC, which is a gas at room temperature. The dechlorination activity of the cell

extracts declined sharply from pH 7.0 to 6.0 (Figure 5B). At pH < 5.0, the dechlorination activity was completely lost. These results are consistent with our previous findings in the 10% dilution transfers set up at pH 5.5: the pH dropped from 5.5 to 5.0 and VC dechlorination did not occur (Figure 3B). In accordance to the cell-extract assays, *VcrA* enzyme activity is not expected at such low pH. From earlier studies on the catalytic mechanism of RDases,^{51,52} it was possible to infer that RDases would be affected at low pH, yet the functional pH range of *Dehalococcoides* RDases had not been previously studied.

Implications for Environmental Science and Remediation. Vitamins are key molecules for various microbially mediated processes, and only a concentration of micrograms per liter is generally required. However, microbial vitamin biosynthesis has a high fitness cost, e.g., more than 30 genes are involved in the anaerobic biosynthesis pathway of cobalamin,⁵³ and not all microorganisms can synthesize the required vitamins. Thus, mixed microbial bioaugmentation cultures that do not require vitamin amendments are highly desirable at field sites to avoid the need for adding costly vitamins. Here, we have demonstrated that complete dechlorination of TCE to ethene occurs steadily in the KB-1 enrichment cultures grown without any exogenous vitamins (Figure 1). Vitamin autotrophy in mixed microbial communities is possible if the necessary vitamin-producing populations are present and maintained during cultivation. In KB-1, these vitamin-producing populations have been maintained owing to batch cultivation, infrequent medium changes, and the use of methanol as electron donor. At field sites, small amounts of

methanol may be available since methanol is a naturally occurring chemical released during decomposition of different plants and animal life and produced by methane oxidizing bacteria in anaerobic environments.⁵⁴ If electron donors, such as lactate, emulsified vegetable oil, or molasses, are added to a field site, cobamide-producing microorganisms are likely to be present along with other microbial populations. In fact, using metagenomics sequencing, a recent study detected the presence of *Acetobacterium* and *Eubacterium* populations, which are known to produce cobalamin, after field site biostimulation with a substrate composed of vegetable oil, cane molasses, and surfactants; at the site, TCE dechlorination to ethene was observed.⁵⁵ Lactate is frequently used at field sites and during cultivation of dechlorinating cultures. *Acetobacterium* spp. and other cobamide-producing organisms are able to use lactate as well as methanol.⁵⁶ Interestingly, dechlorinating cultures grown in the absence of cobalamin using lactate as electron donor were reported before, yet incomplete dechlorination and VC accumulation occurred.^{57,58} In the KB-1 cultures grown without exogenous vitamins, the presence of VcrA-containing *Dehalococcoides* populations along with DMB/cobalamin-producing *Acetobacterium* populations results in complete dechlorination to ethene. To our knowledge, this study is the first report of complete and sustained dechlorination to ethene in dechlorinating mixed cultures grown without any exogenous vitamins.

In a mixed microbial dechlorinating culture, and likely at field sites in which reductive dechlorination occurs, the pH impacts the rate and extent of dechlorination. In KB-1, the pH drops progressively as a result of dechlorination and acetogenesis. Our results indicate that slow dechlorination rates at low pH (<5.5) are mainly due to the loss of function of *Dehalococcoides* RDases. The activity of *Dehalococcoides* RDases in cell extracts is completely lost below pH 5.0 (Figure 5B). At pH < 6.0, an enzymatic limitation, and not a cobamide limitation, was found to affect the rates and extents of VC dechlorination to ethene. However, the activity is easily rescued by raising the pH slightly. Clearly, a pH above 5.0 is essential for the successful *Dehalococcoides*-mediated bioremediation of vinyl chloride (VC) at contaminated field sites regardless of the availability of DMB/cobalamin.

Our study on pH and cobamide dependencies in the TCE-dechlorinating mixed culture KB-1 also provides a mechanistic insight into the dynamic interactions between the populations of acetogens in a dechlorinating mixed culture. Interestingly, we found that the population of *Sporomusa* can grow at pH < 5.5, while the population of *Acetobacterium* can only grow at pH > 6.0. *Acetobacterium* is a beneficial syntrophic partner for *Dehalococcoides* since it can provide *Dehalococcoides* with H₂ and acetate, as well as DMB/cobalamin to support complete TCE dechlorination to ethene, at a physiological pH range (above 6.0) that closely matches the desirable pH range for efficient VC dechlorination to ethene; i.e., in adapted cultures, VC dechlorination rates at pH 6.0 and pH 7.0 were comparable. Our results demonstrate that cobalamin inhibits the growth of *Sporomusa* on methanol. Thus, in KB-1, *Acetobacterium* likely exports cobalamin as a mechanism to inhibit *Sporomusa* growth and compete for methanol. Interestingly, *Acetobacterium* and *Sporomusa*, organisms with an overlapping ecological role, seem to have developed different strategies for interspecies competition and have evolved to occupy different niches.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b02339.

Quantitative PCR (qPCR) primer sequences, list of samples from methanol-fed KB-1-derived enrichment cultures, calibration information for the qPCR assays, and comparison between the proteins encoded by the bza operon; figures and detailed analytical procedures including history and experiments performed, history of the VC and methanol-fed KB-1 enrichment cultures, cumulative ethene production, MS spectra of extracted cobamides, dechlorination of TCE to ethene, DMB biosynthesis operon, cell copies per mL of culture, and gene copy number per mL of culture (PDF)

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Notes

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