

ORIGINAL ARTICLE

The corrinoid cofactor of reductive dehalogenases affects dechlorination rates and extents in organohalide-respiring *Dehalococcoides mccartyi*

Jun Yan^{1,2,3,4}, Burcu Şimşir^{1,2,5}, Abigail T Farmer⁶, Meng Bi^{1,2,5}, Yi Yang^{1,2,5}, Shawn R Campagna⁶ and Frank E Löffler^{1,2,3,4,5}

¹Department of Microbiology, University of Tennessee, Knoxville, TN, USA; ²Center for Environmental Biotechnology, University of Tennessee, Knoxville, TN, USA; ³Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN, USA; ⁴Joint Institute for Biological Sciences (JIBS), Oak Ridge National Laboratory, Oak Ridge, TN, USA; ⁵Department of Civil and Environmental Engineering, University of Tennessee, Knoxville, TN, USA and ⁶Department of Chemistry, University of Tennessee, Knoxville, TN, USA

Corrinoid auxotrophic organohalide-respiring *Dehalococcoides mccartyi* (*Dhc*) strains are keystone bacteria for reductive dechlorination of toxic and carcinogenic chloroorganic contaminants. We demonstrate that the lower base attached to the essential corrinoid cofactor of reductive dehalogenase (RDase) enzyme systems modulates dechlorination activity and affects the vinyl chloride (VC) RDases *BvcA* and *VcrA* differently. Amendment of 5,6-dimethylbenzimidazolylcobamide (DMB-Cba) to *Dhc* strain BAV1 and strain GT cultures supported *cis*-1,2-dichloroethene-to-ethene reductive dechlorination at rates of 107.0 (\pm 12.0) μ M and 67.4 (\pm 1.4) μ M Cl⁻ released per day, respectively. Strain BAV1, expressing the *BvcA* RDase, reductively dechlorinated VC to ethene, although at up to fivefold lower rates in cultures amended with cobamides carrying 5-methylbenzimidazole (5-MeBza), 5-methoxybenzimidazole (5-OMeBza) or benzimidazole (Bza) as the lower base. In contrast, strain GT harboring the *VcrA* RDase failed to grow and dechlorinate VC to ethene in medium amended with 5-OMeBza-Cba or Bza-Cba. The amendment with DMB to inactive strain GT cultures restored the VC-to-ethene-dechlorinating phenotype and intracellular DMB-Cba was produced, demonstrating cobamide uptake and remodeling. The distinct responses of *Dhc* strains with *BvcA* versus *VcrA* RDases to different cobamides implicate that the lower base exerts control over *Dhc* reductive dechlorination rates and extents (that is, detoxification), and therefore the dynamics of *Dhc* strains with discrete reductive dechlorination capabilities. These findings emphasize that the role of the corrinoid/lower base synthesizing community must be understood to predict strain-specific *Dhc* activity and achieve efficacious contaminated site cleanup.

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Introduction

The worldwide usage of chlorinated solvents for degreasing, chemical manufacturing and household applications made chlorinated solvents widespread groundwater contaminants (Abelson, 1990). Chlorinated solvents such as tetrachloroethene (PCE), trichloroethene (TCE) and dichloroethenes (DCEs) are toxic and probable human carcinogens, and recently TCE has been implicated in the development of Parkinson disease (ATSDR, 2003, 2004; Goldman *et al.*, 2012). The breakdown of polychlorinated ethenes to the human carcinogen vinyl

chloride (VC) is particularly troublesome, and exposure may occur through vapor intrusion into dwellings or consumption of tainted drinking water (Kielhorn *et al.*, 2000; EPA, 2012; NRC, 2013).

In bacterial organohalide respiration, chlorinated compounds serve as the terminal electron acceptors, a cornerstone process for chlorinated solvent bioremediation (Leys *et al.*, 2013). Although diverse bacterial groups contribute to reductive dechlorination of PCE and TCE to *cis*-1,2-dichloroethene (cDCE), only some *Dhc* strains dechlorinate DCEs and VC to benign ethene (Maymó-Gatell *et al.*, 1997; He *et al.*, 2003; Löffler *et al.*, 2013a, b). *Dhc* strain BAV1 and strain GT possess the reductive dehalogenase (RDase) enzyme systems *BvcA* and *VcrA*, respectively, that share 54% amino acid similarity (40% identity) and are responsible for reductive dechlorination of DCEs and VC to ethene in the respective *Dhc* strains (Krajmalnik-Brown *et al.*,

Correspondence: FE Löffler, Department of Microbiology, University of Tennessee, M409 Walters Life Sciences, Knoxville, TN 37996-0845, USA.

E-mail: frank.loeffler@utk.edu

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2004; Müller *et al.*, 2004; Sung *et al.*, 2006; McMurdie *et al.*, 2009; Tang *et al.*, 2013; Parthasarathy *et al.*, 2015). *Dhc* are obligate organohalide respirers and the RDases require a corrinoid cofactor (that is, coenzyme B₁₂) for activity (Yi *et al.*, 2012; Hug *et al.*, 2013). Remarkably, *Dhc* strains lack the entire set of ~20 genes involved in corrin ring biosynthesis, and growth of *Dhc* pure cultures strictly depends on exogenous cyanocobalamin (that is, vitamin B₁₂) (Löffler *et al.*, 2013b). Instead of *de novo* corrinoid biosynthesis, *Dhc* strains harbor the requisite genes for both the bacterial and the archaeal scavenging pathways that include *btuF*, *btuC* and *btuD* corrinoid transport, *cbiZ*, *cbiB*, *cobD* and *cobU* cobinamide salvage/activation and *cobT*, *cobC* and *cobS* lower base activation to access growth-supporting cobamides (Yi *et al.*, 2012; Men *et al.*, 2014a, b). In addition, the sequenced *Dhc* genomes harbor at least one ortholog of *cobA* that encodes the co(I)rrinoid adenosyltransferase responsible for attaching the upper adenosyl group ligand (Yi *et al.*, 2012).

Recent studies revealed that *Dhc* strains have specific corrinoid requirements that corrinoid producers such as the methanogen *Methanosarcina barkeri* strain Fusaro, the acetogen *Sporomusa ovata* (DSMZ 2662) and the ferric iron reducer *Geobacter sulfurreducens* cannot fulfill, unless 5,6-dimethylbenzimidazole (DMB) is amended to the medium (Yan *et al.*, 2012, 2013). Studies with *Dhc* strain 195 revealed that TCE reductive dechlorination was only sustained by DMB-cobamide (Cba), 5-MeBza-Cba and 5-OMeBza-Cba, but not by Bza-Cba, 5-hydroxybenzimidazolyl-cobamide (5-OHBza-Cba or factor III) or phenolic cobamides (Phe-Cba and *p*-Cresol-Cba) (Yi *et al.*, 2012; Men *et al.*, 2014b). Apparently, the cobamide lower base (Figure 1) determines whether *Dhc* can or cannot use chlorinated hydrocarbons as electron acceptors. To explore the effects of the lower base on *Dhc* reductive dechlorination activity in more detail, we prepared naturally occurring cobamides through guided biosynthesis (Allen and Stabler, 2008; Mok and Taga, 2013), and investigated their impact on *Dhc* growth and reductive dechlorination activity. Pure cultures of *Dhc* strain BAV1 and strain GT harboring the *bvcA* and *vcrA* genes, respectively, and capable of growth with *c*DCE and VC were amended with the different cobamides and reductive dechlorination rates and extents were analyzed.

Materials and methods

Chemicals

DMB (≥99%), 5-methylbenzimidazole (5-MeBza; 98%), 5-methoxybenzimidazole (5-OMeBza; 97%), benzimidazole (Bza; 98%), *c*DCE (≥99.5%), VC (≥99.5%), ethene (≥99.9%) and betaine (≥99%) were purchased from Sigma-Aldrich (St Louis, MO, USA). Yeast extract and casitone were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA).

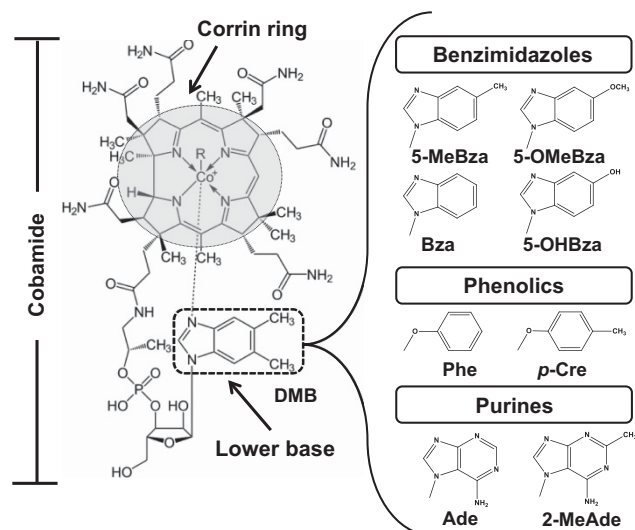


Figure 1 General structure of the corrin ring system and several naturally occurring lower bases. Ade, adenine; 2-MeAde, 2-methyladenine; *p*-Cre, 4-methylphenol; Phe, phenol.

Dehalococcoides mccartyi (*Dhc*) cultures

Dhc strain BAV1 (ATCC BAA-2100) and strain GT (ATCC BAA-2099) were grown in 160 ml serum bottles containing 100 ml defined, completely synthetic mineral salts medium amended with 5 mM acetate as carbon source, 10 ml hydrogen as electron donor, 5 µl neat *c*DCE (0.60 mM aqueous phase concentration) or 2 ml VC (0.52 mM aqueous phase concentration) as electron acceptor as previously described (Yan *et al.*, 2013). Following equilibration, triplicate bottles were amended with the Wolin vitamin mix (Wolin *et al.*, 1963) not containing cyanocobalamin (vitamin B₁₂). The vessels received 36.9 nM of a purified cobamide. For inoculation, *Dhc* strain BAV1 or strain GT cell suspensions were centrifuged and the pellets were suspended in cyanocobalamin-free mineral salts medium to avoid cyanocobalamin carryover (Yan *et al.*, 2012). Cultures that failed to dechlorinate *c*DCE or VC received 10 µM DMB from a filter-sterilized 3 mM aqueous stock solution to restore dechlorination activity. All culture vessels were incubated statically in dark at 30 °C. The *c*DCE-to-ethene dechlorination rates were calculated based on VC and ethene quantification, each step associated with the release of one chloride ion, and reported as µmol of Cl⁻ released per liter per day.

Cobamide biosynthesis, extraction and purification

DMB-, 5-MeBza-, 5-OMeBza- and Bza-Cba were obtained via guided cobamide biosynthesis using *Sporomusa* sp. strain KB-1 (GenBank accession no. AY780559.1) cultures supplied with a single lower base compound (200 µM). To achieve higher yields, 800 ml cultures of strain KB-1 were grown in 1.2 l glass vessels using a modified medium (Yan *et al.*, 2013) amended with yeast extract (2 g l⁻¹), casitone (2 g l⁻¹) betaine (50 mM) and a cyanocobalamin-free

Wolin vitamin mix (Wolin *et al.*, 1963). After 72–96 h of static incubation at 30 °C in the dark, cells were harvested from 1.6 to 2.4 l culture suspensions by centrifugation at 17 600 *g* for 15 min at room temperature. Cell pellets were suspended in 15 ml deionized water, briefly incubated in a sonication water bath to achieve homogeneous suspensions and 5 ml aliquots were transferred to sterile 50 ml plastic tubes. Total corrinoids were extracted in the cyano form (that is, a cyanide group as the upper β -ligand) and purified using the potassium cyanide extraction method (Yan *et al.*, 2013). Cobamide-containing fractions were separated by high-performance liquid chromatography (HPLC) and manually collected from the detector outlet according to retention time and diode array detector (DAD) response to remove any remaining traces of native phenolic cobamides (that is, Phe-Cba and *p*-Cre-Cba). Cobamides were purified as described above. Cobamide absorbance was measured with a Lambda 35 UV/VIS spectrometer (PerkinElmer, Waltham, MA, USA) at 361 nm, and cobamide concentrations were estimated using a molar extinction coefficient of 28 060 mol⁻¹ cm⁻¹ (Pratt, 1972). The purified cyano form of DMB-Cba was indistinguishable from commercial cyanocobalamin. All experiments used purified DMB-Cba in control incubations to verify the absence of any inhibitory compounds introduced during the extraction process.

HPLC and liquid chromatography–mass spectrometry analysis

Cobamide purity and authenticity (for example, distinct singular peaks and *m/z* values) were analyzed in a combined approach employing an Agilent (Santa Clara, CA, USA) 1200 HPLC system and a Thermo Fisher Scientific (Waltham, MA, USA) Orbitrap Exactive Plus LC/MS system (Supplementary Figures S1 and S2). For HPLC analysis, 20 μ l samples were injected onto an Eclipse XDB-C18 column (5 μ m, 4.6 \times 150 mm) (Agilent) and separated with a flow rate of 1 ml per min at 30 °C using 0.1% (v/v) formic acid (\geq 88%, w/v) in water (eluent A) and 0.1% (v/v) formic acid in methanol (eluent B) as mobile phases. The column was equilibrated with 82% eluent A/18% eluent B, and a linear change to 75% A/25% B was applied following sample injection over a 12-min time period. Then, the eluent composition decreased immediately to 25% A/75% B over 3 min followed by a 5-min hold before the column was equilibrated to initial conditions. Cobamides were detected at 361 nm with an Agilent 1260 Infinity DAD and quantified by comparing integrated peak areas with 4-point calibration curves generated with purified cobamides. Liquid chromatography–mass spectrometry analysis was performed using a Dionex Ultimate 3000 system (Thermo Fisher Scientific) with an inline DAD fitted to an Exactive Plus Orbitrap Mass Spectrometer with an electrospray ionization source (Thermo Fisher Scientific). For liquid chromatography–DAD–mass spectrometry analysis, 10 μ l aliquots of each sample

were injected onto a Kinetex XB-C₁₈ column (2.6 μ m, 2.1 \times 100 mm) (Phenomenex, Torrance, CA, USA) and separated at a flow rate of 0.2 ml per min at 30 °C using 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B) as mobile phases. The gradient started with 100% A, changed linearly to 85% A after 2.8 min, 75% A after 5.2 min, 90% A after 5.44 min and 100% A after 6.8 min with a 4.2-min hold to achieve column equilibration to starting conditions. The mass spectrometer was operated in full scan mode with a mass range of 750–1800 *m/z* and a resolution of 140 000. All ion fragmentation was performed on each ion packet in a subsequent event after each full scan using a normalized collision energy of 20 eV with a stepped normalized collision energy of 50%. Electrospray ionization was performed in positive mode with the sheath gas set at 25 arbitrary units, the auxiliary gas at 10 arbitrary units, the spray voltage at 4000 V and a capillary temperature of 350 °C. The DAD was set to detect at 361 nm as well as over a 3D field from 190 to 800 nm.

Corrinoid extraction from *Dhc* cultures

Dhc cells and culture supernatant were separated by filtration using a 47 mm diameter 0.22 μ m pore size membrane (Pall Life Sciences, Port Washington, NY, USA). Intracellular corrinoids were extracted and purified following the KCN extraction protocol (Yan *et al.*, 2013). Supernatant-associated corrinoids were reduced into the upper ligand-free, cobalt(II) form by reductants (for example, HS⁻, L-cysteine, dithiothreitol) present in the medium (Assaf-Anid *et al.*, 1994; Chiu and Reinhard, 1996; Lesage *et al.*, 1998). To convert cob(II)amides back to the oxidized cyanocob(III)amide forms, which can be separated and quantified with the established HPLC method, culture supernatants were reacted with ambient air in the presence of KCN for 24 h (Schneider and Stroinski, 1987). This method was validated by extracting a mixture of cobamides with different lower bases from 100 ml of medium, and the average recovery efficiencies from triplicate vessels were 93.4%, 90.6% and 91.1% for DMB-Cba, 5-OMeBza-Cba and 5-Bza-Cba, respectively. Following the 24-h incubation, culture supernatants were loaded onto a C₁₈ Sep-Pak cartridge and washed with 40 ml distilled water. Absorbed corrinoids were eluted with 3 ml methanol, vacuum dried and suspended in 0.5 ml distilled water.

Cobamide uptake in *Dhc*

Triplicate 100 ml cultures of *Dhc* strain BAV1 and strain GT were grown with 5 μ l *c*DCE and a cobamide mixture containing equimolar concentrations of DMB-Cba, 5-OMeBza-Cba and 5-Bza-Cba (36.9 nM each). As soon as *c*DCE was completely dechlorinated to ethene, cobamides remaining in the culture supernatants were recovered, purified and quantified as described above.

Analytical methods

Dhc cells were harvested onto 0.22 μm membrane filters (Merck Millipore Ltd, Darmstadt, Germany) and the genomic DNA was extracted from the filters using the MO BIO Soil DNA Isolation kit (MO BIO, Carlsbad, CA, USA) as previously described (Yan *et al.*, 2012). *Dhc* 16S rRNA gene copies were enumerated by quantitative PCR with a ViiA 7 real-time PCR system (Life Technologies, Grand Island, NY, USA) using primer pair *Dhc*1200F/*Dhc*1271R and probe *Dhc*1240probe following established protocols (Ritalahti *et al.*, 2006). Chlorinated ethenes and ethene were analyzed with an Agilent 7890 gas chromatograph equipped with a flame ionization detector and a DB-624 capillary column (60 m \times 0.32 mm \times 1.8 μm) (Sung *et al.*, 2006).

Results

The lower base affects dechlorination rates

The cobamides DMB-Cba, 5-MeBza-Cba, 5-OMeBza-Cba and Bza-Cba supported complete dechlorination of *c*DCE to ethene in *Dhc* strain BAV1 cultures. The highest *c*DCE-to-ethene dechlorination rates of 107.0 ± 12.0 and 74.3 ± 1.0 $\mu\text{M Cl}^-$ released per day were measured in the presence of DMB-Cba and 5-MeBza-Cba, respectively (Figure 2a), and the initial amount of $72.2 (\pm 1.4)$ $\mu\text{mol cDCE}$ was completely dechlorinated to ethene in 12–18 days. Longer time periods of 38 and 86 days were required to achieve complete *c*DCE and VC reductive dechlorination to ethene in strain BAV1 cultures amended with 5-OMeBza-Cba or Bza-Cba because of lower *c*DCE dechlorination rates of $33.2 (\pm 2.6)$ and $16.8 (\pm 1.1)$ $\mu\text{M Cl}^-$ released per day, respectively (Figure 2a). Strain GT cultures amended with DMB-Cba and 5-MeBza-Cba dechlorinated *c*DCE to ethene at rates of $67.4 (\pm 1.4)$ and $26.7 (\pm 1.9)$ $\mu\text{M Cl}^-$ released per day, respectively (Figure 2b). Similar to strain BAV1

cultures, *c*DCE dechlorination rates decreased in strain GT cultures amended with 5-OMeBza-Cba or Bza-Cba; however, the VC-to-ethene dechlorination step occurred at such low rates that VC, rather than ethene, was formed as dechlorination end product (Figure 2b). In strain GT cultures amended with 5-OMeBza-Cba or Bza-Cba, no more than 23% (17.5 μmol) of the total VC produced from *c*DCE dechlorination was further dechlorinated to ethene over a 77-day incubation period (Figure 2b).

The lower base affects *Dhc* growth yields

Enumeration of *Dhc* cell numbers after *c*DCE-to-ethene dechlorination was complete (that is, in all BAV1 cultures and DMB-Cba- or 5-MeBza-Cba-amended GT cultures) or ceased (that is, in 5-OMeBza- or Bza-Cba-amended GT cultures) revealed that strain BAV1 cultures reached similar growth yields of $1.38 (\pm 0.09)$ to $1.57 (\pm 0.03) \times 10^8$ cells per $\mu\text{mol Cl}^-$ released with all cobamides tested (Table 1). For *Dhc* strain GT, the highest final cell densities of $1.29 (\pm 0.22) \times 10^8$ and $1.30 (\pm 0.41) \times 10^8$ cells per ml were measured in cultures amended with DMB-Cba and 5-MeBza-Cba, respectively, and these results were consistent with the growth yield expected from complete *c*DCE-to-ethene dechlorination (Table 1). With 5-OMeBza-Cba or Bza-Cba, strain GT cultures reached cell yields of $6.95 (\pm 1.04)$ and $7.72 (\pm 0.87) \times 10^7$ cells per $\mu\text{mol Cl}^-$ released, respectively, that were on average 31% and 23% lower compared with growth yields of $0.92 (\pm 0.15)$ and $1.10 (\pm 0.24) \times 10^8$ cells per $\mu\text{mol Cl}^-$ released measured in DMB-Cba or 5-MeBza-Cba amended cultures, respectively. The lower growth yields suggested that the VC-to-ethene reductive dechlorination step was uncoupled from growth in strain GT cultures amended with 5-OMeBza-Cba or Bza-Cba.

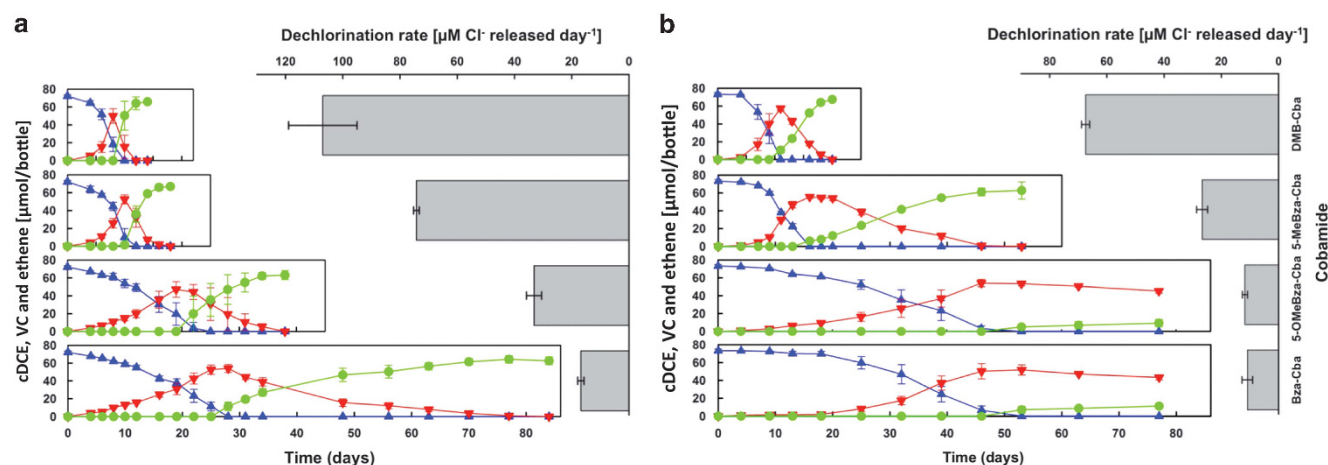


Figure 2 Reductive dechlorination of *c*DCE in (a) *Dhc* strain BAV1 harboring the *BvcA* RDase and (b) strain GT harboring the *VcrA* RDase. Triplicate cultures were amended with cobamides carrying different benzimidazole derivatives as lower bases at initial concentrations of 36.9 nM. Blue triangles, *c*DCE; red inverted triangles, VC; green circles, ethene. Error bars represent the s.d. of triplicate cultures.

Table 1 Growth of *Dhc* pure cultures amended with different cobamides.

<i>Dhc</i> strain (Electron acceptor)	Cobamide ^a	DMB ^b	<i>Dhc</i> cell density (16S rRNA gene copies per ml)		Growth yield (cells (μmol Cl ⁻¹) ⁻¹) ^c
			Initial	Final	
BAV1 (cDCE)	DMB-Cba	–	4.26 ± 0.87 × 10 ⁶	1.82 ± 0.25 × 10 ⁸	1.38 ± 0.09 × 10 ⁸
	5-MeBza-Cba	–	4.26 ± 0.87 × 10 ⁶	2.09 ± 0.51 × 10 ⁸	1.54 ± 0.39 × 10 ⁸
	5-OMeBza-Cba	–	4.26 ± 0.87 × 10 ⁶	1.85 ± 0.39 × 10 ⁸	1.42 ± 0.20 × 10 ⁸
	Bza-Cba	–	4.26 ± 0.87 × 10 ⁶	2.07 ± 0.10 × 10 ⁸	1.57 ± 0.03 × 10 ⁸
GT (cDCE)	DMB-Cba	–	5.22 ± 0.54 × 10 ⁶	1.29 ± 0.22 × 10 ⁸	0.92 ± 0.15 × 10 ⁸
	5-MeBza-Cba	–	5.22 ± 0.54 × 10 ⁶	1.30 ± 0.41 × 10 ⁸	1.10 ± 0.24 × 10 ⁸
	5-OMeBza-Cba	–	5.22 ± 0.54 × 10 ⁶	4.97 ± 0.99 × 10 ⁷	6.95 ± 1.04 × 10 ⁷
	Bza-Cba	–	5.22 ± 0.54 × 10 ⁶	5.72 ± 0.38 × 10 ⁷	7.72 ± 0.87 × 10 ⁷
GT (VC)	DMB-Cba	–	2.61 ± 0.27 × 10 ⁶	0.96 ± 0.06 × 10 ⁸	1.17 ± 0.15 × 10 ⁸
	5-MeBza-Cba	–	2.61 ± 0.27 × 10 ⁶	1.10 ± 0.10 × 10 ⁸	1.39 ± 0.20 × 10 ⁸
	5-OMeBza-Cba	–	2.61 ± 0.27 × 10 ⁶	2.08 ± 0.01 × 10 ⁶	No growth
	5-OMeBza-Cba	+	2.61 ± 0.27 × 10 ⁶	1.21 ± 0.34 × 10 ⁸	1.45 ± 0.33 × 10 ⁸
	Bza-Cba	–	2.61 ± 0.27 × 10 ⁶	2.37 ± 0.00 × 10 ⁶	No growth
	Bza-Cba	+	2.61 ± 0.27 × 10 ⁶	1.22 ± 0.08 × 10 ⁸	1.48 ± 0.11 × 10 ⁸

Abbreviations: Bza, benzimidazole; Cba, cobamide; cDCE, *cis*-1,2-dichloroethene; *Dhc*, *Dehalococcoides mccartyi*; DMB, 5,6-dimethylbenzimidazole; 5-MeBza, 5-methylbenzimidazole; 5-OHBza, 5-hydroxybenzimidazole; 5-OMeBza, 5-methoxybenzimidazole; VC, vinyl chloride.

^aCobamide concentration was 36.9 nM.

^bDMB was supplied at 10 μM.

^cGrowth yield was estimated from the final cell density after complete or stalled cDCE or VC dechlorination.

Impact of the lower base on dechlorination extent

VC stall and lower growth yields suggested that 5-OMeBza-Cba and Bza-Cba were not fully functional in strain GT, and VC was not used as a growth substrate. The key feature distinguishing *Dhc* strain GT from strain BAV1 is the VC RDase: strain GT uses *VcrA* whereas strain BAV1 uses *BvcA* for cDCE and VC reductive dechlorination (Krajmalnik-Brown *et al.*, 2004; Müller *et al.*, 2004; Sung *et al.*, 2006; Tang *et al.*, 2013; Parthasarathy *et al.*, 2015). To further investigate the impact of the lower base on the catalytic activity of the *VcrA* RDase, growth experiments in medium amended with VC as electron acceptor were performed. Consistent with the observation in cDCE-dechlorinating cultures, only DMB-Cba and 5-MeBza-Cba sustained VC dechlorination in strain GT cultures, and rates of 40.0 (±5.1) and 16.5 (±2.8) μM VC per day, respectively, were observed. Ethene production was negligible (<0.8% of the total VC added) in strain GT cultures amended with 5-OMeBza-Cba or Bza-Cba (Figure 3). In contrast, strain BAV1 cultures that received 5-OMeBza- or Bza-Cba completely dechlorinated VC to ethene. Enumeration of *Dhc* cells with quantitative PCR confirmed the inability of 5-OMeBza-Cba and Bza-Cba to support growth of strain GT with VC as an electron acceptor. In vessels with initial cell densities of 2.61 (±0.27) × 10⁶ cells per ml (that is, cells introduced with the inoculum), strain GT cell numbers decreased to 2.08 (±0.01) × 10⁶ and 2.37 (±0.09) × 10⁶ cells per ml with 5-OMeBza-Cba or Bza-Cba provided as corrinoid cofactor (Table 1). In contrast, strain GT cell numbers increased to 0.96 (±0.06) × 10⁸ and 1.10

(±0.10) × 10⁸ cells per ml in cultures amended with DMB-Cba and 5-MeBza-Cba, respectively, corresponding to growth yields of 1.17 (±0.15) × 10⁸ and 1.39 (±0.20) × 10⁸ cells per μmol VC dechlorinated (Table 1).

We next tested whether the addition of DMB could rescue the VC-to-ethene dechlorination phenotype in strain GT cultures that had received 5-OMeBza-Cba or Bza-Cba. At 3 weeks following DMB addition to inactive cultures, all VC was dechlorinated to ethene, whereas no ethene formation occurred in cultures without DMB amendment (Figure 4a). To verify whether the restoration of VC dechlorination activity following DMB addition was because of corrinoid remodeling, intracellular corrinoids from strain GT cells were extracted and purified. DMB-Cba was only detected in corrinoid extracts of cells collected from vessels amended with DMB, and DMB-Cba accounted for nearly half of the total intracellular cobamides in strain GT cells grown in medium with 5-OMeBza-Cba or Bza-Cba (Figure 4b). Cell enumeration with quantitative PCR demonstrated that additional growth occurred following restoration of *VcrA* activity. Cultures that initially received 5-OMeBza-Cba or Bza-Cba and were then amended with DMB to restore the VC dechlorinating phenotype produced 1.21 (±0.34) × 10⁸ and 1.22 (±0.08) × 10⁸ cells per ml culture, respectively (Table 1). The growth yields in 5-OMeBza-Cba and Bza-Cba cultures following DMB addition were 1.45 (±0.33) × 10⁸ and 1.48 (±0.11) × 10⁸ cells per μmol VC dechlorinated, respectively, and are comparable to those observed in strain GT cultures amended with DMB-Cba (1.17 (±0.15) × 10⁸ cells per μmol VC dechlorinated; Figure 4c).

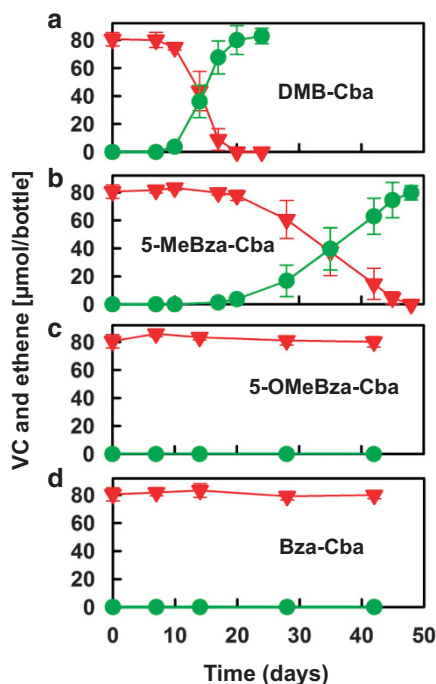


Figure 3 Reductive dechlorination of VC in *Dhc* strain GT cultures amended with an initial concentration of 36.9 nM (a) DMB-Cba, (b) 5-MeBza-Cba, (c) 5-OMeBza-Cba or (d) Bza-Cba. Red inverted triangles, VC; green circles, ethene. Error bars represent the s.d. of triplicate cultures.

Cobamide transport in *Dhc*

To explore whether differential transport of cobamides with distinct lower bases across the *Dhc* surface layer and/or cytoplasmic membrane could explain the observations, cDCE-dechlorinating *Dhc* cultures were supplied with an equimolar mixture of DMB-Cba, 5-OMeBza-Cba and Bza-Cba. Following cDCE conversion to ethene in strain BAV1 and strain GT cultures, the molar concentration ratios of DMB-Cba/5-OMeBza-Cba/Bza-Cba were 1.06:1.00:1.00 and 1.00:1.00:1.02 in strain BAV1 and strain GT culture supernatants, respectively, and therefore not significantly different than the initial conditions. Furthermore, statistically identical concentrations of DMB-Cba (24.4 (±1.2)/24.1 (±1.9) nM), 5-OMeOBza-Cba (23.1 (±1.1)/24.0 (±1.0) nM) and Bza-Cba (23.2 (±1.3)/24.6 (±1.5) nM) were recovered from strain BAV1/strain GT culture supernatants (Figure 5). These findings indicate that surface layer and membrane transport did not discriminate between the three benzimidazole-type cobamides. Intracellular cobamides were also analyzed, and DMB-Cba, 5-OMeBza-Cba and Bza-Cba were present in similar amounts ranging from 2×10^3 to 5×10^3 molecules per strain BAV1 and strain GT cell.

Discussion

Corrinoids in complete form (that is, cobamides) are essential enzyme cofactors exclusively synthesized

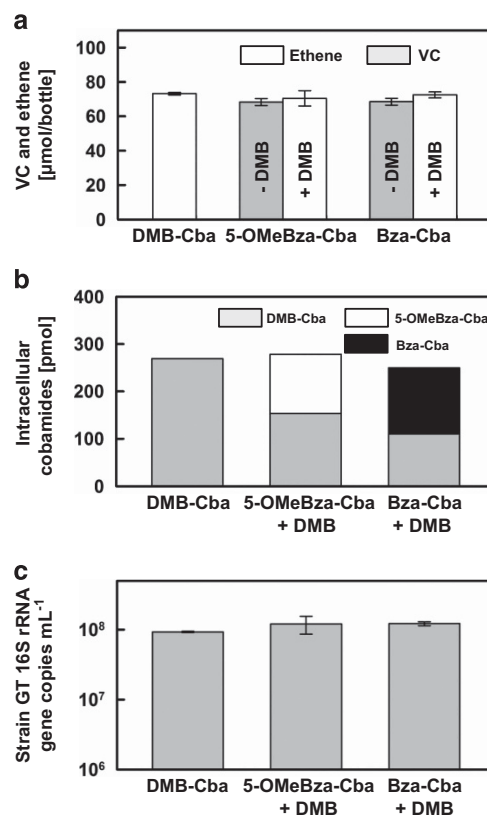


Figure 4 Restoration of the VC dechlorination phenotype by DMB addition to inactive *Dhc* strain GT cultures. (a) Comparison of VC-to-ethene dechlorination activity in 5-OMeBza-Cba- or Bza-Cba- (36.9 nM each) amended strain GT cultures in the presence or absence of 10 µM DMB. (b) Quantification of intracellular cobamides extracted from strain GT biomass grown with 5-OMeBza-Cba or Bza-Cba in the presence of DMB and collected from 100 ml cell suspensions. (c) Comparison of the final cell densities in 5-OMeBza-Cba- or Bza-Cba-amended strain GT cultures with or without DMB (see Table 1).

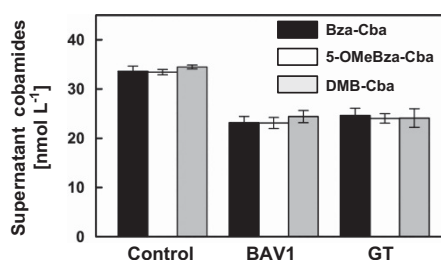


Figure 5 Quantification of supernatant-associated cobamides recovered from cDCE-dechlorinating *Dhc* strain BAV1 and strain GT cultures supplied with an equimolar mixture of DMB-Cba, 5-OMeBza-Cba and Bza-Cba (36.9 nM each).

by a subset of the *Archaea* and *Bacteria*, even though these molecules play critical roles for the majority of organisms in all domains of life (Schneider and Stroński, 1987; Banerjee, 1999; Martens *et al.*, 2002; Gruber *et al.*, 2011; Miles *et al.*, 2011; Parks *et al.*, 2013). Naturally occurring corrinoids carry a variety of lower bases, and as many as 16 benzimidazole, nucleobase and phenol derivatives have been identified (Stupperich *et al.*, 1990; Allen and

Stabler, 2008). Microorganisms such as *Escherichia coli* (ATCC 11105) and *Lactobacillus leichmannii* (ATCC 4797) are capable of utilizing natural corrinoids with at least 11 different lower bases to fulfill their nutritional requirements (Thompson *et al.*, 1950; Burkholder, 1951; Schneider and Stroiński, 1987). The human gut anaerobe *Bacteroides thetaiotaomicron* grows with indistinguishable doubling times with DMB-Cba, 5-MeBza-Cba, 5-OMeBza-Cba, Bza-Cba, pseudo-B₁₂ (that is, Cba with adenine as the lower base) or 2-methyladeninyl-Cba as corrinoid cofactor (Degnan *et al.*, 2014). *S. ovata* and *Sporomusa* sp. strain KB-1, which natively synthesize phenolic-type cobamides (that is, Phe-Cba and *p*-Cre-Cba), were also capable of utilizing cobamides with benzimidazole-type lower bases (that is, DMB-Cba, 5-MeBza-Cba, 5-OMeBza-Cba, Bza-Cba) during growth with H₂/CO₂, betaine or fructose as substrate (Mok and Taga, 2013; Yan *et al.*, 2013). Some inhibitory effects were observed in *S. ovata* cultures amended with 5-MeBza when methanol or 3,4-dimethoxybenzoate served as growth substrates, suggesting that the structure of the lower base affected cobamide function and essential metabolic pathways (Mok and Taga, 2013; Yan *et al.*, 2013). Reduced versatility in terms of the lower base utilization was observed with the corrinoid-auxotrophic *Dhc* strains that exhibited a clear preference for cobamides with DMB or 5-MeBza lower bases to perform organohalide respiration with *c*DCE and VC as electron acceptors.

The PCE-to-*c*DCE RDase PceA of *Sulfurospirillum multivorans* and the *ortho*-dibromophenol RDase NpRdhA of *Nitratireductor pacificus* strain pht-B shed first light on the structure of RDase enzyme systems and provided details on interactive corrinoid-RDase binding (Bommer *et al.*, 2014; Payne *et al.*, 2014). In both RDases, the corrinoid cofactor (norpseudo-B₁₂ for PceA and DMB-Cba for NpRdhA) is involved in the catalytic cleavage of carbon-chlorine bonds. The lower bases adenine in norpseudo-B₁₂ or DMB in DMB-Cba are uncoordinated and in the base-off conformation to anchor the cofactors deeply inside the RDase scaffold, suggesting that the lower base is not directly involved in catalysis (PDB ID=4UR0, 4RAS). Interestingly, kinetic studies on non-RDase cobamide-dependent enzymes suggested varying affinities of the apoprotein to cobamides with different lower bases (Barker *et al.*, 1960; Lengyel *et al.*, 1960; Tanioka *et al.*, 2010). For example, both the mammalian (sheep) kidney and bacterial (*Propionibacterium shermanii*) methylmalonyl-CoA mutase have high affinity to DMB-Cba (K_m values of 0.021 and 0.024 μM , respectively), whereas the K_m values for Bza-Cba were about an order in magnitude greater (Lengyel *et al.*, 1960). In contrast, the glutamate mutase of *Clostridium tetanomorphum* preferred Bza-Cba over DMB-Cba with K_m values of 0.24 and 18 μM , respectively (Barker *et al.*, 1960). Preference for a cobamide with a specific lower base was also observed with the methionine synthase of

Spirulina platensis strain NIES-39, which bound pseudo-B₁₂ with a K_m of 0.07 μM , and a much higher K_m of 16.0 μM was determined for DMB-Cba (Tanioka *et al.*, 2010). These observations suggest that the affinity of the enzyme to cobamides with different lower bases affects the assembly of the functional holoenzyme, which can explain the observed lower base effects on reductive dechlorination rates and extents.

Differential transport of cobamides into the cell can affect RDase maturation and is another possible explanation for the observed decrease in dechlorination rates. However, the experimental data indicated that DMB-, 5-OMeBza- and Bza-Cba were equally transported across the surface layer/cytoplasmic membrane in *Dhc* strains BAV1 and GT, suggesting that differential cobamide uptake cannot explain the observed decrease in dechlorination performance. *Dhc* strains lack a peptidoglycan cell wall, and a *btuB* homolog for controlling cobamide translocation across the outer membrane into the periplasmic space is absent in the sequenced *Dhc* genomes (Chimento *et al.*, 2003; Yi *et al.*, 2012; Löffler *et al.*, 2013b). Experimental evidence obtained with the human gut anaerobe *Bacteroides thetaiotaomicron* corroborated that BtuB, rather than the ATP-binding cassette corrinoid transporter system BtuFCD is the necessary component for selective cobamide transport (Degnan *et al.*, 2014).

An unexpected finding was that the activity of the VC RDases BvcA and VcrA was differentially influenced by cobamides with distinct lower bases. Apparently, lower bases can exert post-translational control over corrinoid-dependent enzyme systems, in this example RDases. An observation supporting this hypothesis was made in the PCE-to-*c*DCE dechlorinator *S. multivorans*. Following DMB addition, DMB replaced the native adenine lower base to generate nor-B₁₂ that inhibited PceA maturation and export and affected PCE dechlorination and growth of *S. multivorans* (Keller *et al.*, 2013). This finding further indicated that the lower base affects the binding between the cobamide and the apo-form of the RDase, and the incorporation of a cobamide with an unfavorable lower base affects RDase maturation. In *Dhc* strains BAV1 and GT, inefficient maturation and impaired export of BvcA and VcrA due to incorporation of a corrinoid cofactor carrying unfavorable 5-OMeBza or Bza lower bases may explain the decreased dechlorination rates, or even the complete loss of VC dechlorination ability in strain GT.

The pure culture studies with *c*DCE/VC-dechlorinating *Dhc* strains revealed that methyl substitutions of the benzimidazole backbone at the 5 and 6 positions affect *Dhc* strains harboring *vcrA* (strain GT) or *bvcA* (strain BAV1) differently. Apparently, the growth-supporting benzimidazole-type corrinoids are functionally not equivalent in *Dhc* strains with distinct RDases. In strain BAV1 (BvcA) and strain GT (VcrA), reductive dechlorination rates

decreased in the order DMB-Cba > 5-MeBza-Cba > Bza-Cba, and a similar trend was observed in *Dhc* strain 195 (Yi *et al.*, 2012). DMB-Cba supported the highest TCE dechlorination rates in strain 195 cultures, but no growth occurred with Bza-Cba. Furthermore, *Dhc* strains are unable to grow with 5-OHBza-Cba in axenic culture or during co-cultivation with a 5-OHBza-Cba-producing methanogen, indicating that substitution of the hydrophobic methyl group with a hydroxyl group in the 5 position and removal of the methyl group in the 6 position resulted in a nonfunctional lower base (Yi *et al.*, 2012; Yan *et al.*, 2013). Corrinoid cofactor-protein interactions are governed by hydrogen bonding, as revealed by the crystal structures of PceA and NpRdhA (Bommer *et al.*, 2014; Payne *et al.*, 2014). The findings presented here suggest that the methyl group substitutions in the 5 and 6 positions of the benzimidazole ring have relevant roles for VcrA and BvcA activity, and are possibly involved in stabilizing the mature RDase complex. Such methyl group-protein hydrophobic interactions provide a plausible explanation for the preference of the *Dhc* RDases BvcA (strain BAV1), VcrA (strain GT) and TceA (strain 195) for a corrinoid with DMB as the lower base.

The effects of the lower base on dechlorination rates and end points have implications for bioremediation practice. A common contaminated site management practice is to supply electron donor when dechlorination rates and extent following initial bioremediation treatment (that is, biostimulation alone or combined with bioaugmentation) decrease (Löffler and Edwards, 2006). The assumption is that hydrogen, the required electron donor for *Dhc*, is limiting and additions of fermentable carbon substrates increase hydrogen flux. Although repeated electron donor additions can sustain reductive dechlorination activity, evidence that hydrogen flux was actually limiting *Dhc* activity is difficult to ascertain. A carefully executed long-term study under conditions of sustained low hydrogen flux indicated that vitamin B₁₂ additions, not hydrogen, limited *Dhc* reductive dechlorination activity and VC to ethene conversion (Fennell *et al.*, 1997). In contaminated aquifers, organohalide-respiring *Chloroflexi* depend on corrinoid scavenging to acquire this essential cofactor from the environment. Depending on the site biogeochemical conditions and the type of substrate(s) used for biostimulation (that is, electron donors), microorganisms that produce the 'wrong' lower base(s) may dominate. In such scenarios, continued biostimulation and bioaugmentation will not sustain *Dhc* activity and lead to efficient contaminant detoxification. Knowledge of the exact lower base requirements of the keystone corrinoid-auxotrophic dechlorinators, as well as the biogeochemical conditions that favor the synthesis of required

growth factors by indigenous microbes, will enable strategies to overcome nutritional limitations of organohalide-respiring *Chloroflexi* and promote faster detoxification rates.

Conflict of Interest

The authors declare no conflict of interest.

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