Identification of purinyl-cobamide as a novel corrinoid cofactor of tetrachloroethene reductive dehalogenases in *Desulfitobacterium* spp.

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Identification of purinyl-cobamide as a novel corrinoid cofactor of tetrachloroethene reductive dehalogenases in *Desulfitobacterium* spp.

A Thesis Presented for the Master of Science Degree

The University of Tennessee, Knoxville

Meng Bi

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ABSTRACT

Corrinoids (e.g. vitamin B₁₂ [cyanocobalamin]) are a group of structurally similar, cobalt-containing tetrapyrrole compounds involved in a number of important biochemical reactions. In organohalide respiration, vitamin B₁₂ analogues carrying different lower bases are obligate cofactors for the reductive dehalogenases (RDases) that catalyze reductive dechlorination reactions. The focus of this research was on the isolation and characterization of a novel natural corrinoid cofactor that enables the dechlorination-coupled energy conservation in organohalide-respiring Desulfitobacterium strains. Analysis of the purified corrinoid in the cyano form using a combination of high-performance liquid chromatography (HPLC), UV-Vis [Ultraviolet–visible spectroscopy] and ultra-performance liquid chromatography-high-resolution mass spectrometry (UPLC-HRMS) demonstrated that this novel corrinoid is different than the currently known naturally occurring corrinoids in the lower base structure. Analysis by $^{15}$N [nitrogen isotope 15] isotope labeling methods suggested a lower base with the molecular formula C₅H₃N₄ [purine] (ligand form). Proton ($^1$H) and correlation spectroscopy (COSY) NMR [Nuclear magnetic resonance] experiments corroborated that purine is the lower base, and Coα-purinyl-Coβ-cyanocobamide [cobalt alpha-purinyl-cobalt beta-cyanocobamide] (purinylcobamide) is a novel corrinoid. The dechlorination-supporting function of purinylcobamide was validated using corrinoid-auxotrophic Dehalobacter restrictus (Dhb) and Dehalococcoides mccartyi (Dhc) pure cultures expressing distinct RDases. Indistinguishable bacterial growth and dechlorination rates compared to vitamin B₁₂-amended cultures were observed in Dhb.
but not *Dhc* cultures, demonstrating distinct cofactor requirements. Overall, the discovery of purinylcobamide emphasizes that the diversity and functions of corrinoids, especially the lower base structures to organo-halide-respiring bacteria, need to be fully understood to implement successful bioremediation for chlorinated solvents contamination.
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CHAPTER I
INTRODUCTION AND GENERAL INFORMATION

Bioremediation of chlorinated solvents

Chlorinated solvents contamination

One of the biggest challenges in soil and groundwater remediation is the problem of cleaning up sites with chlorinated solvents. Chlorinated solvents are heavier than water and form dense non-aqueous phase liquids (DNAPLs), which are difficult to locate and recover once they are released into a subsurface environment [1]. Tetrachloroethene (PCE) and trichloroethene (TCE) are common chlorinated solvents and are widely used for dry-cleaning and metal degreasing applications [2]. The spillage and improper storage of PCE and TCE made them common groundwater contaminants [3]. During downward movement in saturated aquifers, they encounter the low permeability strata, causing lateral spreading of chlorinated solvents and formation of a DNAPL pools [4]. Due to their low solubility (PCE: 0.15 g/100 mL @ 20 °C; TCE: 1.280 g/L), PCE and TCE DNAPLs serve as long-term sources for continuous dissolution of contaminants into the passing groundwater, form a plume and eventually lead to the contamination of drinking water [5]. Chlorinated solvents such as PCE and TCE are probable human carcinogens. Additionally, exposure to TCE may increase the risk of developing Parkinson’s disease [6, 7].
Reductive dechlorination

Biodegradation and biotransformation play important roles for the detoxification of chlorinated solvents. Under anoxic conditions, PCE can be reduced to TCE, cDCEs, vinyl chloride (VC), and eventually the nontoxic end product ethene, each step is associated with the release of chloride [8]. In this process, chlorinated solvents are used as the terminal electron acceptors to generate energy for microbial growth. Organic acids such as pyruvate, lactate and acetate, as well as hydrogen, are used as either carbon sources or electron donors [9, 10]. The process of breaking down harmful chlorinated solvents under anoxic conditions by microbial respiration is called organohalide respiration [11]. The key enzymes involved in organohalide respiration are the reductive dehalogenases (RDases), which are membrane-associated iron-sulfur proteins that catalyze the cleavage of carbon-chlorine bonds [8]. The PCE RDases (PceA) that catalyze the reductive dechlorination of PCE to TCE or cDCE have been characterized in a diversity of organohalide-respiring bacterial genera include *Dehalococcoides mccartyi (Dhc)*, *Geobacter lovleyi*, *Desulfitobacterium spp.(Ds)*, *Sulfurospirillum multivorans (Smul)*, and *Dehalobacter restrictus (Dhb)* [12-18]. PceA are usually diverse in gene sequences among different species, and even different between different genera (Figure 1). For example, the PceA of Ds strain Y51 and strain JH1 is able to dechlorinate PCE to cDCE, while PceA of Ds strains Viet1, PCE1, KBC1 and JW can only degrade PCE to TCE.
**Figure 1.** Phylogenetic tree of PceA.

Maximum likelihood phylogeny of 19 reductive dehalogenases based on tetrachloroethene reductive dehalogenase protein sequence alignments.

Sequences were aligned by Geneious R8 CLUSTALW program. Bar: 0.2 nucleotide substitutions per site.
Additionally, the PceA of *Dhb* PER-K23 is highly similar to the PceA sequence of *Desulfitobacterium hafniense* and can dechlorinate PCE to cDCE as well.

**Biochemistry and molecular biology of RDases**

The *pceA* gene coding for PceA is the catalytic unit in the *pceABCT* operon. *pceB* encodes a PceA anchoring protein. *pceC* is believed to encode a putative transcriptional regulator which controls *pceA* gene expression, and it was demonstrated to be co-transcribed in a polycistronic manner with *pceAB* genes in *Ds* strain Y51 [19]. *pceT* encodes a trigger factor-like protein and acts as a chaperone for proper folding of the PceA preprotein and the export of PceA to the periplasm via the TAT [twin arginine] secretion pathway [20].

Most chlorinated compound reductive dehalogenases characterized so far are associated with the cytoplasmic membrane [11]. The N-terminal region of most reductive dehalogenases contains the TAT (twin arginine translocation) consensus sequence “RRXFXK,” which is involved in the protein translocation into or across the cytoplasmic membrane [21]. Most RDases contain a corrinoid cofactor in the catalytic center. They also contain consensus sequences for two 4Fe-4S [four iron-four sulfur] clusters which are in the C-terminal region of the reductive dehalogenases. For example, *Ds* strain Y51, the 4Fe-4S cluster-binding motif is comprised of “CXXCXXXCP” and “GXXCXXXCS” [22].
Corrinoids as cofactor for reductive dechlorination

Structure of corrinoids

The mechanism of reductive dechlorination remained in theory for years until recent research done by Bommer et al. and Payne et al. proved that the electron transfer in RDases mainly relies on corrinoid cofactors [23, 24]. Before taking a deeper look at the mechanism, it is important to firstly understand the composition of corrinoids. Corrinoids have large and complex chemical structures. One example is 5-deoxyadenosylcobalamin (Ado-B\textsubscript{12}), a derivative of B\textsubscript{12}. The ado-B\textsubscript{12} molecule has three parts: a central corrin ring system, a side chain, and a lower base (Figure 2) [25]. It is a typical corrinoid cofactor with a 5-deoxyadenosyl moiety serving as its upper (Co\textbeta) axial ligand and the 5' carbon covalently bound to the cobalt within the corrin ring [11, 26]. The lower (Co\textalpha) axial base of cobalt in Ado-B\textsubscript{12} is the N-7 of 5, 6-dimethylbenzimidazole (DMB). The DMB moiety is attached covalently to the corrin ring as part of a nucleotide loop to form phosphoribosyl-DMB, and is linked through a phosphate bridge to an aminopropanol moiety that is attached to a propionyl group extending from the D porphyrin of the corrin ring (Figure 3A and C) [25]. The deoxyadenosyl moiety of the upper ligand base can be replaced by a methyl group within the cell [27]. It is often prepared commercially as a cyano group, when corrinoid is extracted outside the cells. Cyanocobalamin (CN-B\textsubscript{12}) is known as vitamin B\textsubscript{12}, a form not found in nature but frequently used as a nutrient supplement for human and bacteria [28].
Figure 2. Structure of corrinoid.
Figure 3. Structure of vitamin B$_{12}$ and nor-pseudo B$_{12}$ (Keller, S, 2014).

A: the structure of cobalamin in the base-on form with DMB as lower base.

B: The nor-pseudo B$_{12}$ cofactor of the tetrachloroethene (PCE) reductive dehalogenase (PceA) from *Smul* in the base-on form with an adeninyl-moiety as lower base, the methyl group at position 176 in cobalamin is replaced by a hydrogen atom, which is marked by a black dashed circle in nor-pseudo B$_{12}$ (B), compared to cobalamin (A, red dashed circle). X: upper ligand; C: base 5,6-dimethylbenzimidazole (DMB) [29].
Most known corrinoids have conserved structure in the central ring and nucleotide loop, except for nor-pseudo B\textsubscript{12} which lacks of the methyl group attached to carbon 176 on the nucleotide loop (Figure 3B).

The variety of corrinoids cofactors are distinct from their lower bases (Figure 2). In the past 50 years, 16 different types of lower bases were discovered indicating 16 different corrinoids: 5-hydroxybenzimidazolylcobamide was discovered by Lezius and Barker from \textit{Methanobacillus omelianskii} in 1965 [30]; 5-methoxybenzimidazolylcobamide (Factor III\textsubscript{m}) was discovered in 1973 by Ljungdahl \textit{et al.} [31]; 2-methylsulfinyladenine, 2-methylsulfonyladenine and phenolyl cobamide were all discovered in the late 80s [32]. According to the literature, the various types of lower bases can be classified into three major categories: benzimidazole-, purine-, and phenol-type corrinoids. Table 1 (Appendix A) shows all the naturally occurring corrinoids with different moieties as lower bases. Even though many different types of corrinoids have been discovered, new corrinoids with distinguished lower bases have not been reported since the discovery of guanylcobamide and hypoxanthylcobamide produced by \textit{Desulfovibrio vulgaris} 20 years ago[33].

\textbf{Corrinoid anaerobic biosynthetic pathway}

The synthesis of the corrinoid cofactor is restricted to certain \textit{Bacteria} and \textit{Archaea} [28]. There is no evidence that any eukaryote is able to synthesize corrinoids. Early study elucidated the two distinct biosynthesis pathways of cobalamin (B\textsubscript{12}), which are divided into aerobic and anaerobic pathways. The
aerobic pathway requires molecular oxygen and has been studied in 
*Propionibacterium freundii* [28]. In 1996, Raux first revealed the anaerobic
adenosylcobamic acid synthetic operon in *Salmonella enterica* [34]. *S. enterica*
produces cobalamin for four cobalamin dependent reactions: the function of
methyltransferase, the cleavage of ethanolamine, formation of the nonessential
hyper-modified Q base, and a cofactor for propanediol degradation [35].
According to Raux et al., *S. enterica* contains a complete *cbi* operon and *cob*
operon, which are responsible for the synthesis of ado-cobamic acid [27]. Figure
28 (Appendix B) shows the function of each gene in *cbi/cob* operons, with
comparison to the aerobic corrinoid biosynthesis pathway.

**The cobT gene determines the structural variability of corrinoids**

The range of cobamides that can be synthesized in several bacteria are
limited by molecular factors encoded by the *cobT* gene. The CobT is 5,6-
dimethylbenzimidazole phosphoribosyltransferase that catalyzes the activation of
the lower ligand base by phosphoribosylation to form a ribosylated product [36,
37]. The a-ribosylated product is incorporated as the lower ligand of the
cobamide [38]. X-ray crystallographic studies of *Salmonella enterica* CobT
showed that benzimidazoles, purines, and phenolic compounds can be bound in
the active site, and all but the phenolic compounds can be used as substrates for
phosphoribosylation [39]. A recent study by Amrita B. Hazra demonstrated that
CobT enzymes can activate a range of lower ligand substrates, and the majority
of the enzymes tested preferentially attach 5,6-dimethylbenzimidazole (DMB),
the lower ligand of cobalamin[40]. This suggests that many B\textsubscript{12}-homologue producing bacteria in pure culture may produce cobalamin when DMB is available in the environment.

One exception occurs with \textit{Smul}. According to the research reported by Schubert et al. (2014), \textit{Smul} produced nor-B\textsubscript{12} when DMB replaced adenine as its lower base [29]. This nor-B\textsubscript{12} has very low catalytic activity, and hinders the maturation of pre-PceA and causes decreased RDase activity [29]. As mentioned above, characteristic of the nor-structure is a demethylation in the nucleotide loop of the cobamide. We can therefore conclude that the structural inconsistency leads to deactivation of catalytic capability of corrinoid cofactor. A demethylated B\textsubscript{12} will not support reductive dechlorination.

\textit{Mechanism of corrinoid dependent reductive dechlorination}

The PceA harbors a deeply buried corrinoid, which supports reductive chlorine elimination, and the RDases achieve organohalide substrate reduction via chlorine–corrinoid interaction [23, 24]. The work of Payne et al. suggested that RDases cleave carbon-chlorine bonds through a direct contact between the corrinoid cobalt and chlorine. Cobalt passes an electron to chlorinated solvents and cause cobalt-chlorine fission by either homolytic or heterolytic mechanism [23]. During the dechlorination catalytic reaction, the lower base has been implicated in stabilizing dechlorination. Bommer et al. observed that corrinoids are usually converted from “base-on” form to “base-off” conformation in order to
allow the accessibility of the substrate to the catalytic center [24]. The base-off conformation of the DMB lower base in reductive dehalogenase NprdhA is “fastened” by four direct hydrogen bonds [23]. The lower base anchors the corrinoid to the reductive dehalogenase protein and stabilizes the interaction between cobalt and the chlorinated substrate [23]. If high concentrations of DMB (25 µM) are supplied exogenously to Smul, the lower base synthesis pathway is shut down and DMB replaces the lower base of nor-pseudo B_{12} (an adenine) [29, 41]. This treatment severely affects PCE-dependent growth and PceA activity in Smul [29]. This research indicated the important function of the lower base in reductive dechlorination.

*Desulfitobacterium* spp.

**General features**

The genus of *Desulfitobacterium* (*Dsf*) encompasses strict anaerobes belonging to the *Firmicutes* phylum, *Clostridia* class, *Clostridiales* order and *Peptococcaceae* family. *Dsf* isolates were obtained from sites contaminated with halogenated compounds, such as waste water sludge and fresh water sediments [17]. Many of the *Dsf* strains can dechlorinate chlorinated organic compounds such as PCE and TCE through the reductive dechlorination pathway. Strains of *Dsf* are phylogenetically distinct from other reductively dehalogenating bacteria, such as *Sulfurospirillum*, *Geobacter* and *Dhc*, but related to *Dhb* [14, 42]. *Dsf* strains are Gram-positive, slightly curved rods, motile and with a cell size varying
from 2 to 7 mm. The majority of $Dsf$ species can form spores, while some of the strains such as PCE1 and $D.\ \text{metallireducens}$ do not sporulate. The optimal temperature for growth of $Dsf$ range from 25° to 38°C and pH from 6.5 to 7.8. Their G+C content varies from 45 to 49 mol% [17].

Electron donors and acceptors

Most $Dsf$ strains utilize pyruvate and lactate as electron donors and some of the strains can use $H_2$ and acetate [9]. When supplied with an electron donor, most $Dsf$ isolates have the capability to use a variety of electron acceptors including chlorophenolic compounds and chloroalkenes [43, 44]. They can also utilize substrates other than chlorinated solvents such as fumarate, thiosulfate, sulfite, nitrate, nitrite, dimethylsulfoxide (DMSO), trimethylamine N-oxide, As(V), and soluble forms of Fe(III) as growth-supporting electron acceptors [45]. The major chlorinated solvents $Dsf$ can respire are chlorinated ethenes and chlorophenols. Three $D.\ \text{hafniense}$ strains Y51, TCE1, and PCE-S, dechlorinate PCE to cDCE via TCE but do not dechlorinate chlorophenols [9]. In contrast, $Dsf$ strain DCB-2 utilizes chlorophenols in organohalide respiration but not chloroethenes[9]. $Dsf$ strains PCE1, Viet1 and KBC1 dechlorinate PCE to yield TCE and also dechlorinate chlorophenols [46, 47].

Bacteria producing uncharacterized corrinoids

$D.\ \text{hafniense}$ strain Y51 ($Dsf$ strain Y51) is one of the $Dsf$ strains that has been well studied. The Y51 genome has been sequenced and genome analysis
showed that strain Y51 encodes the complete cobyric acid synthesis pathway [42]. Previous research also mentioned that strain Y51 is able of producing its own RDase cofactor [17, 42]. Dsf strain Viet1 also does not rely on exogenous B$_{12}$ for growth in medium amended with PCE as electron acceptor. PCE dechlorination is not affected (i.e., enhanced or inhibited) by the presence of B$_{12}$ (Figure 4). This evidence suggests that Dsf are corrinoid-producing organisms. Even though the earliest Dsf strain was discovered in the 1980s, the type of corrinoid produced by this species remains unknown [48]. The goal of this study was to identify the corrinoid cofactor produced by Dsf. We studied its structure and biological features through cultivation methods and chemical analysis. We are also interested in studying the whether the unknown corrinoid produced by Dsf support reductive dechlorination of corrinoid-auxotrophic bacteria such as Dhb and Dhc.
Figure 4. PCE utilization in Viet1 cultures.

A. PCE dechlorination curve with exogenous B$_{12}$. PCE (white square) was completely dechlorinated to TCE (black diamond) in 7 days. B. PCE dechlorination curve without exogenous B$_{12}$. PCE (white square) was completely dechlorinated to TCE (black diamond) in 7 days.
CHAPTER II

CHARACTERIZATION OF NATIVE CORRINOID PRODUCED IN

DESFITOBACTERIUM

Materials and Methods

Chemicals

PCE (≥99.5%), TCE (≥99.5%), DMB (≥99%), betaine (≥99%) and Vitamin B$_{12}$ (≥98%), were purchased from Sigma-Aldrich (St Louis, MO, USA). Casitone was purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Benzimidazolylcobamide (Bza-Cba), 5-methylbenzimidazolylcobamide (5-MeBza-Cba) and 5-methoxybenzimidazolylcobamide (5-OMeBza-Cba) were obtained via guided cobamide biosynthesis [41].

Cultures

Pure cultures of Dsf strain Y51, JH1, PCE1, Viet1 were grown statically in 2.2 L culture bottles (Chemglass Life Sciences, Vineland, NJ, USA) containing 1.8 L of reduced, phosphate buffered, defined anoxic mineral medium amended with a cyanocobalamin-free Wolin vitamin mix at 30°C in the dark [49]. The defined medium at pH 7.2 contains the following components per liter (unless stated otherwise): 7.8 g K$_2$HPO$_4$; 1.2 g KH$_2$PO$_4$; 0.5 g of MgSO$_4$·6H$_2$O, 0.3 g of NH$_4$SO$_4$, 0.3 g of K$_2$SO$_4$, 0.015 g of CaSO$_4$·H$_2$O, 0.05 mg of resazurin. Trace solution (per liter): HCl (25%, solution w/w) 10 mL, 1.5g FeCl$_2$·4H$_2$O, 0.19 g
CoCl$_2$$ \cdot $ 6 H$_2$O; 0.1 g MnCl$_2$$ \cdot $ 4 H$_2$O; 70 mg ZnCl$_2$; 6 mg H$_3$BO$_3$; 36 mg Na$_2$MoO$_4$$ \cdot $ 2 H$_2$O; 24 mg NiCl$_2$$ \cdot $ 6 H$_2$O; 2 mg CuCl$_2$$ \cdot $ 2H$_2$O; 0.048 g of Na$_2$S$ \cdot $ 9H$_2$O, 0.035 g of L-cysteine, 6 mg Na$_2$SeO$_3$$ \cdot $ 5 H$_2$O, 8 mg Na$_2$WO$_4$$ \cdot $ 2 H$_2$O and 0.5 g NaOH [13, 50].

The vitamin solution contains the following (in mg/L): biotin, 0.05; folic acid, 0.02; pyridoxine, 0.1; riboflavin, 0.05; thiamine, 0.1; cyanocobalamin, 0.1; nicotinamide, 0.55; P-aminobenzoic acid, 0.25; lipoic acid, 0.05; and pantothenic acid, 0.05); CH$_3$COONa$ \cdot $ 3 H$_2$O, 0.68; and peptone, 0.05. The gas phase consisted of H$_2$ : CO$_2$ (90 : 10, v/v) at a pressure of 1.5 $\times$ 10$^5$ Pa. Sodium pyruvate (10 mM) was amended as electron donor for strain Y51. PCE (0.5mM) was provided as electron acceptor.

Sporomusa sp. strain KB-1 (GenBank accession no. AY780559.1) cultures were supplied with a single lower base (200 µM). To achieve higher biomass yields, 800 mL cultures of strain KB-1 were grown in 1.2 liter glass vessels using a modified medium [51] amended with yeast extract (2 g L$^{-1}$), casitone (2 g L$^{-1}$), betaine (50 mM) and a cyanocobalamin-free Wolin vitamin mix. After 72-96 hours of static incubation at 30$^\circ$C in the dark, cells were harvested from 1.6-2.4 L of culture suspension by centrifugation at 17,600 x 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 following an established protocol [52].
Smul cultures were grown in the same medium as Dsf except for a replacement of phosphate buffer with bicarbonate buffer (2.52 g/L of NaHCO₃). Smul cultures were also supplied with 10 mM sodium pyruvate and 0.5 mM PCE. Geobacter sulfurreducens were grown in the same medium with Smul, except for a replacement of sodium pyruvate with 5 mM acetate and 10 mL H₂. The pH was adjusted to 7.2-7.3 with CO₂ at a pressure of 1.5 ×10⁵ Pa in both medium.

**Preparation of corrinoid standards**

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 liter glass vessels using a modified mineral salts medium [51] amended with yeast extract (2 g L⁻¹), casitone (2 g L⁻¹), betaine (50 mM) and a cyanocobalamin-free Wolin vitamin mix [53]. After 72-96 hours of static incubation at 30°C in the dark, cells were harvested from 1.6-2.4 L culture suspensions by centrifugation at 17,600 x 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 following the KCN extraction and purification protocol [41]. The cobamide-containing fractions were separated using high performance liquid chromatography (HPLC) and manually
collected from the diode array detector outlet according to retention time and
detector response.

5-hydroxybenzimidazolylcobamide [5-OHBza-Cba (factor III)], nor-pseudo
vitamin B\textsubscript{12} and phenolic cobamides (Phe-Cba and p-Cre-Cba) were extracted
and purified from \textit{Methanosarcina barkeri} strain Fusaro (DSM 804), \textit{Smul} (DSM
12446) and \textit{Sporomusa} sp. strain KB-1 cells, respectively, following the KCN
extraction and purification [41, 54].

Concentrations of purified corrinoid standards were estimated with a
Lambda 35 UV-Vis spectrometer (PerkinElmer, Waltham, MA, USA) using a
molar extinction coefficient of 28,060 mole\textsuperscript{-1} cm\textsuperscript{-1} as described [55].

\textit{Intracellular corrinoids extraction and purification from Dsf cultures}

Cells were harvested from 1.8 liter pure cultures of \textit{Dsf} strains Y51, JH1,
PCE1 and Viet1 grown with sodium pyruvate and PCE by centrifugation at
15,000 x g for 20 min at 4 °C. Supernatants were discarded and cell pellets were
suspended in 5 ml of deionized water in 50 mL sterile falcon tubes. The pH was
adjusted to 5-6 with 3\%(v/v) glacial acetic acid, and KCN stock solution (200 mM,
0.651 g KCN dissolved in 50 mL milli-Q water) was added to reach a final
concentration of 10 mM [52]. Each tube was incubated for 20 min in a boiling
water bath. Following centrifugation at 15,000 x g for 20 min, the supernatants
were collected and previous steps were repeated on the pellets one more time to
increase the corrinoid yields. The combined supernatants were loaded onto a
Sep-Pak C18 cartridge (Waters Corp, Milford, MA, USA), which had been
previously equilibrated with 2 ml 100% methanol and 40 mL deionized water. Following sample loading, the cartridge was washed with 10 ml of deionized water (20 interstitial volumes) and 7.5 ml of 10% methanol (15 interstitial volumes). The cartridge was eluted with 4 ml of 100% methanol in the final step. The pink-colored solution obtained from the final elution step was vacuum dried and the residues were suspended in 1 ml sterile, deionized water.

**HPLC analysis**

Corrinoids were analyzed using an Agilent 1200 series HPLC system equipped with an Eclipse XDB-C18 column (5 mm, 4.6 × 250 mm) and a diode array detector set at a detection wavelength of 361 nm. Samples were injected and separated at a flow rate of 1 mL per min at 30°C using 0.1% (v/v) formic acid (≥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. Corrinoid fractions were quantified by comparing integrated peak areas to 4-point calibration curves generated with purified corrinoid standards.
**LC/MS analysis**

LC-MS analysis was performed using a Dionex Ultimate 3000 system equipped with an Exactive Plus Orbitrap Mass Spectrometer and an electrospray source (Thermo Scientific). Samples were injected into a Kinetex XB-C18 column (2.6 µm, 2.1 x 100 mm) (Phenomenex, Torrance, CA) 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% after 5.2 min, and 90% after 5.44 min, before the column was equilibrated at 100% eluent A. The mass selective detector full scan range was 50-1,800 m/z with a resolution of 140,000, a normalized collision energy of 20%, and stepped normalized collision energy of 50%. Electrospray ionization settings were sheath gas 25 V, auxiliary gas 10 V, spray voltage 4,000 V, and capillary temperature of 350°C.

*m* stands for mass and *z* stands for charge number of ions. In this mass analysis, an electron is taken from molecules to create single charged ions. Thus, *m/z* represents mass divided by charge number and the horizontal axis in a mass spectrum is expressed in units of *m/z*. Since *z* is almost always 1 in LC-MS analysis, the *m/z* value is considered to be the molecular mass in this study [56].
**Guided vitamin B$_{12}$ biosynthesis**

Pure cultures of *Ds* strain Y51 were grown in 160-mL serum bottles containing 100 mL reduced, bicarbonate buffered, defined mineral medium with 0.5 mM PCE as electron acceptor, 20 mM sodium pyruvate as carbon source and electron donor, and an addition of 25 μM DMB. Cultures that did not receive DMB were established as the control group. Strain Y51 cells were harvested following complete dechlorination of PCE to cDCE. Intracellular corrinoids produced in DMB-amended and control groups were extracted and purified from cell pellets using the described method [41].

**Results**

**HPLC and UV-Vis analysis of Dsf corrinoid extracts**

HPLC-DAD analysis of the cyano form (e.g., a cyanide group as the upper β-ligand) of the corrinoids extracted from cells of all four *Ds* strains revealed the presence of a predominant corrinoid fraction with a retention time of 11.79 min (Figure 5). The retention time of this corrinoid fraction (11.79 min) is different from that of vitamin B$_{12}$ (15.02 min) and vitamin B$_{12}$ analogues (11.23 min [nor-pseudo-B$_{12}$], 13.34 min [Bza-Cba], 13.88 min [5-OMeBza-Cba], 13.13 min [Factor III], 13.89 min [5-MeBza-Cba], 17.23 min [Phe-Cba], and 17.64 min [p-Cre-Cba]), suggesting that the corrinoid produced in *Ds* strains is not identical to any of these corrinoids standards (Figure 6). UV-Vis spectra were acquired simultaneously during HPLC separation over a wavelength range from 250 to
Figure 5. HPLC chromatograms of corrinoids

HPLC chromatogram of corrinoids produced by Desulfitobacterium spp. strain Y51 (A), strain JH1(B), strain PCE1(C) and strain Viet1(D). The only corrinoid peak shown in all chromatograms is at 11.79 min. The other peaks are non-corrinoid metabolites.
Figure 6. HPLC retention time of the unknown corrinoid and corrinoid standards. HPLC chromatograms of corrinoid standards compared with the unknown corrinoid. A. I is nor-Pseudo B₁₂; II is Ben-Cba; III is OMeBza-Cba; IV is phenolic corrinoids; B. V is Factor III; VI is MeBza-Cba; VII is cobalamin (B₁₂). All standards are at a concentration 5 mg/L except for IV, which had a concentration of 2 mg/L. C. unknown corrinoid extracted from strain Y51 cultures with a retention time of 11.79 min does not match the retention time of any of the standards in Figures 1A and 1B.
The Dsf corrinoid fraction exhibited a maximum adsorption wavelength at 361 nm indicative of Co(III), which completely matched with that of authentic vitamin B\(_{12}\) but different than the maximum adsorption wavelength of cyanocobinamide (355 nm) (Figure 7) [57]. The initial characterization conducted with HPLC and UV-Vis demonstrated that these Dsf strains produced a non-vitamin B\(_{12}\) cobamide.

**LC-MS analysis of Dsf corrinoid extracts**

Mass spectra acquired by high resolution liquid chromatography mass spectrometry revealed strong base peaks at \(m/z = 1329.54\) (Figure 8) in all Dsf corrinoids extracts, while vitamin B\(_{12}\) (tested as positive control) displayed a base peak at \(m/z = 1355.59\) (Figure 9) characteristic of the molecular weight of B\(_{12}\). All Cba standards used in this study were also analyzed and base peaks with \(m/z\) values corresponding to their calculated molecular weight were detected (Figure 9). The MS analysis combined with HPLC and UV-Vis results confirmed that these four organohalide-respiring Dsf strains produced a similar type of corrinoid as RDase cofactor to catalyze the PCE-to-cDCE reductive dechlorination reaction. Further, the measured molecular weight (1329.54) of Dsf corrinoid does not equal to that of vitamin B\(_{12}\) or any known naturally occurring corrinoid, indicating Dsf strains synthesize a novel type of corrinoid. We deduced the molecular formulae of the unknown corrinoid as (i) C\(_{59}\)H\(_{82}\)CoN\(_{16}\)O\(_{14}\)P or (ii) C\(_{60}\)H\(_{82}\)CoN\(_{14}\)O\(_{15}\)P, mainly based on the possibility of demethylation on the nucleotide loop (Figure 10).
Figure 7. UV-Vis absorbance curve.

UV-Vis of corrinoid produced by *Dsf* cultures compared standard corrinoids. The unknown corrinoid (red dashed line) and vitamin to B$_{12}$ (blue solid line) share the same maximum UV absorbance at a wavelength of 361 nm, but a distinctly different absorption maximum at a wavelength of 355 nm was recorded for cyanocobinamide (green dash-dotted line).
Figure 8. LC/MS spectra of unknown corrinoid.

Confirmation of corrinoid authenticity by measuring $m/z$ values using LC/MS analysis. The molecular weights for the cyano form of unknown corrinoid in all Dsf (A: strain JH1, B: strain PCE1 C: strain Viet1 D: strain Y51) is 1329.54
Figure 9. Chromatograms of cobalamin LC-MS standards.

A. DMB-Cba; B. 5-OMeBza; C. 5-Me-Bza; D. Bza-Cba.
Figure 10. Proposed structures of unknown corrinoid purified from Dsf cultures.

Based on LC-MS analysis, two possible structure for the unknown corrinoid were deduced. A. A formula of $\text{C}_{59}\text{H}_{82}\text{CoN}_{16}\text{O}_{14}\text{P}$, with 9H-purine as lower base.

B. A formula of $\text{C}_{60}\text{H}_{82}\text{CoN}_{14}\text{O}_{15}\text{P}$, a nor type of corrinoid that lacks a methyl group at position 176 of the nucleotide loop (purple) with 5-OHBza as the lower base.
**Guided vitamin B_{12} biosynthesis**

We first investigated the structure of the non-lower base portion of the *Dsf* corrinoid(s). Demethylation at the carbon 176 position in the side chain that connects the corrin ring to the α-glycosidic bound lower base moiety represents an unusual “Nor”-type corrinoid recently discovered in *Smul* (Figure 3) [29]. Distinct shift in molecular weight (a decreased \( m/z \) value of 12) caused by the replacement of methyl group with a proton can be captured by mass-spectrometry to provide initial assessment of possible side chain modifications, but knowledge of the lower base structure is required. Guided cobamide biosynthesis, in which biosynthesis of non-native cobamides is induced by the addition of the corresponding lower base compounds, was performed in *Dsf* strain Y51 cultures. *Dsf* native corrinoid completely vanished in the DMB amended culture and a single corrinoid fraction with identical retention time to authentic vitamin B_{12} emerged (Figure 13). A strong base peak at \( m/z = 1355.59 \) identical to vitamin B_{12} as revealed by mass spectrum corroborated that the addition of DMB enabled vitamin B_{12} biosynthesis in *Dsf* strain Y51 (Figure 12).

Additional test was performed with Bza as the lower base compound and Bza-Cba was the only corrinoid synthesized in *Dsf* strain Y51 (Figure 13). These results clearly demonstrated that *Dsf* strains synthesize the “normal” type corrinoid without demethylation in the side chain. With both DMB and Bza, strain Y51 was able to dechlorinate PCE as efficiently as with its native corrinoid (Figure 11). Based on this information, the lower base structure of the *Dsf*
Figure 11. PCE reductive dechlorination in strain Y51

PCE (white square) was dechlorinated to TCE (black diamond) and cDCE (black triangle) with 25 µM DMB amended to the medium in the Y51 culture.
Figure 12. LC-MS of B12 and the unknown corrinoid with DMB as the lower base.

Confirmation of corrinoid authenticity by measuring m/z values using LC/MS analysis. The molecular weights for the cyano form of standard B12 (A) is 1355.59; the unknown corrinoid with DMB as lower base (B) is also 1355.59
Figure 13. HPLC chromatograms of corrinoid obtained from strain Y51 cultures.

HPLC chromatogram of B\textsubscript{12} standards (A), the unknown corrinoid (B), the corrinoid obtained from benzimidazole-amended strain Y51 pure cultures (C), the corrinoid from DMB-amended strain Y51 pure cultures (D).
corrinoid can be faithfully deduced as C₅H₃N₄ (in ligand form) or C₅H₄N₄ (in free form), presumably representing purine (Figure 10A).

Interestingly, the addition of DMB into PCE-dechlorinating Dsf strain Y51 cultures switched strain Y51’s native corrinoid completely into vitamin B₁₂ or Bza-Cba, respectively, without apparent effects on culture growth and dechlorination activity. These results demonstrated that the PceA RDase of strain Y51 has a flexible corrinoid requirement, which can be fulfilled by multiple types of corrinoids. However, the type of corrinoid cofactor, more specifically the lower base structure, does not appear to influence the catalytic capacity of PceA since cDCE is always the dechlorination end product.
CHAPTER III

DETERMINATION OF LOWER BASE STRUCTURE BY $^{15}$N-LABELLING AND NMR SPECTROSCOPY

Material and Methods

Chemicals

$^{15}$N labeled NH$_4$Cl (99%) was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA. USA).

Culture for NMR spectroscopy

Dsf strain Y51 cultures were grown in multiple 2.2 L serum bottles containing 1.8 L MMYPF medium (per liter: K$_2$PO$_4$, 7.8 g; KH$_2$PO$_4$, 1.2 g; sodium citrate, 0.5 g; MgSO$_4$·7H$_2$O, 0.1 g; yeast extract, 2.0 g, resazurin 1.0 mg; pH 7.2) [15]. The total Y51 MMYPF culture reached 20 L. Sodium pyruvate (40 mM) served as carbon source and electron donor. Dicyanocobinamide (0.5 μM) was added to stimulate corrinoid production. Each bottle was spiked with neat TCE (400 μL, 2.3 mM aqueous phase concentration) three times during incubation to achieve high biomass yields.

$^{15}$N labeling experiment

Pure cultures of Dsf strain Y51 and Geobacter sulfurreducens strain PCA were grown under described conditions (Chapter II) with 0.3 g/L $^{15}$NH$_4$Cl as the
only nitrogen source. Each culture was transferred (1% inoculum, v/v) twice in 160 mL bottles containing 100 mL medium before scaling up into 1.8 liter vessels for LC-MS analysis.

**Corrinoid extraction and purification**

Total intracellular corrinoids were extracted and purified from the cell pellets harvested from 1.8 liter of *Dsfl* strain Y51 and *G. sulfurreducens* strain PCA cultures as described in Chapter II.

**NMR spectroscopy**

For NMR analysis, an aliquot of methanol-d4 (250 μL) was added to the purified *Dsfl* strain Y51 corrinoid (~300 μg). The mixture was used to collect $^1$H and COSY NMR spectra using an INOVA 600 MHz NMR (Varian NMR Systems, Palo Alto, CA) spectrometer. The $^1$H NMR experiments consisted of 1024 scans, using PRESAT solvent suppression multiple peak selection, and a total measuring time of 1 hour. The gradient-selected COSY NMR experiments consisted of 256 scans per increment, at 96 scans per increments. The delay time between scans was 1.5 seconds, and the total measuring time was 1 hour.
Results

**H-NMR and COSY NMR spectroscopies**

Starting with authentic vitamin B\textsubscript{12}, a $^1$H NMR spectrum was generated using the previously stated parameters. Unknown corrinoid (XB\textsubscript{12}, 700 µg) was obtained from 20 L of Y51 samples. The structure of cyanocobalamin (vitamin B\textsubscript{12}) was previously characterized by Kurumaya et al. \cite{58}, and this information was used to assign the peaks to the standard. By superimposing the spectrum of the XB\textsubscript{12} and vitamin B\textsubscript{12} (Figure 29. Appendix B), changes amongst peaks could be easily observed. The superimposed spectra revealed that the only significant changes occurred in the aromatic region, which was primarily associated with the DMB portion of vitamin B\textsubscript{12}.

Next, a homonuclear correlation spectroscopy (COSY) experiment was performed on both the XB\textsubscript{12} (Figure 15) and vitamin B\textsubscript{12} (Figure 14). This 2-dimensional (2D) NMR experiment reveals couplings of similar nuclei, within three bonds, in order to further understand proton connectivity. By observing the two spectra, separately, a significant difference can be seen in the aromatic region, between δ 9.0-6.0. The aromatic protons of the XB\textsubscript{12} lack correlation with other protons in the compound. This observation indicated that the aromatic protons of this compound cannot have any adjacent protons. In comparison, the vitamin B\textsubscript{12} COSY spectrum shows that the aromatic protons of DMB have a direct correlation between the benzyl protons and adjacent methyl groups.
Figure 14. \( \text{B}_{12} \) two-dimensional nuclear magnetic resonance correlation spectroscopy (2D COSY NMR).
Figure 15. $\text{XB}_{12}$ two-dimensional nuclear magnetic resonance correlation spectroscopy (2D COSY NMR).
Figure 16. XB\textsubscript{12} lower base spectrum of $^1$H-NMR.
Due to the limited amount of pure XB\textsubscript{12} sample, a \textsuperscript{13}C signal to locate the position of C atoms in the lower base could not be obtained. However, the COSY and \textsuperscript{1}H-NMR results strongly suggest that the lower base of XB\textsubscript{12} is purine, since purine is the only naturally occurring compound that matches the analytical data (Figure 16). The chemical shifts of three hydrogen atoms A, B, and C in Figure 16 correlate with the three hydrogen atoms in the purine structure.

\textit{\textsuperscript{15}N-labeling isotope labelling}

The initial characterization of unknown corrinoid (Chapter II) revealed that the novel corrinoid produced by \textit{Dsf} has a lower base with a deduced molecular formula of C\textsubscript{5}H\textsubscript{4}N\textsubscript{4} (ligand free form), which presumably is purine. Corrinoid with purine as the lower base contains 15 nitrogen atoms that are different than previously reported naturally occurring corrinoids that carry an adenine (16 N atoms), benzimidazole derivative (13 N atoms) or phenol derivative (11 N atoms) as the lower base. Based on the differences in nitrogen atom numbers, a \textsuperscript{15}N-labeling experiment with \textit{Dsf} strain Y51 was conducted to further validate that the lower base is indeed purine. \textit{Geobacter sulfurreducens} produces factor III (5-OHBza-Cba), a corrinoid with 13 nitrogen atoms, was included in the analysis as a control.

The mass spectra obtained with \textsuperscript{15}N-labeled and -unlabeled factor III validated the labeling approach. As expected, the molecular weight of \textsuperscript{15}N-labeled factor III increased by 13.18 (expected value = 18), compared to its unlabeled molecular weight 1343.32 (Figure 17). For \textsuperscript{15}N-labeled \textit{Dsf} Y51
Figure 17. LC/MS spectrum of factor III
corrinoid, the mass spectrum exhibited a base peak with an \( m/z \) value (i.e., molecular weight) of 133.49, which corresponds to a 14.95 (expected value = 15, Figure 19, Appendix B) increase in molecular weight (Table 2). The \(^{15}\text{N}\)labeling results confirmed that the lower base is not adenine, a benzimidazole or phenol derivative, but purine.

Discussion

\(^{15}\text{N}\)-labelling and NMR spectroscopy analysis determined that purine is the lower base of the novel corrinoid produced by \( Dsf \), which is named purinyl-cobamide (Co\( \alpha \)-purinyl-Co\( \beta \)-cyanocobamide, or P-Cba). Even though purinyl-cobamides have been isolated from resting cells or cell-free extracts of several \( Propionibacterium \) spp. by guided cobamide biosynthesis\cite{41}, it is the first time to discover naturally produced purinyl-cobamides. To date, 16 naturally occurring lower bases have been identified (Table 1). Here, the discovery of purine as of a novel lower base and P-Cba as a novel corrinoid cofactor of PceA RDases involved in organohalide-respiring in \( Dsf \) is reported.

Vitamin B\(_{12}\) is commonly added to medium formulations to grow corrinoid-auxotrophic OHRB such as \( Dhc \) and \( Dhb \) \cite{10, 59}. At the external supply of a suitable lower base, \( Dsf \) strain Y51 is able to effectively produce and utilize guided biosynthesized cobamides (e.g., vitamin B\(_{12}\)) as RDase cofactor (Chapter II). However, natively produced vitamin B\(_{12}\) was not observed in the absence of DMB suggesting DMB biosynthesis pathway is absent in the organohalide-respiring \( Dsf \) strains. The recently resolved anaerobic DMB biosynthesis pathway
**Figure 18.** LC/MS spectrum of factor III $^{15}$N labeling

**Figure 19.** LC/MS spectrum of XB$_{12}$ following $^{15}$N isotope labeling.

XB$_{12}$ $^{15}$N isotope labeling reveals $m/z$ value of 1344.49 mass units.
studied with *Eubacterium limosum* consists of four enzymatic steps that are limited to a very narrow subset of anaerobic microorganisms [60]. Not surprisingly, genes homologous to anaerobic DMB biosynthesis genes identified in the B\textsubscript{12}-producing *Eubacterium limosum* and *Acetobacterium woodii* are completely missing in all sequenced *Dsf* genomes, which implies that cobalamin cannot be produced. Instead, *Dsf* strains use purine as the lower base to functionalize the corrinoid enabling these bacteria to use chlorinated compounds such as PCE and TCE as the growth substrates (i.e., energy sources).
CHAPTER IV
DOES P-CBA SUPPORT *DEHALOBACTER* AND *DEHALOCOCCOIDES* REDUCTIVE DECHLORINATION ACTIVITY?

Materials and Methods

*Chemicals and chemical analysis*

CDE (≥99.5%), VC (≥99.5%), ethene (≥99.9%) and were purchased from Sigma-Aldrich (St Louis, MO, USA). Peptone was purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). P-Cba was prepared with *Dsf* strain Y51 and quantified as described in Chapters II and III.

*Cultures*

The corrinoid-auxothrophic *Dehalococoides mccartyi* (Dhc) strain BAV1 (ATCC BAA-2100) and strain GT (ATCC BAA-2099), and *Dehalobacter restrictus* (Dhb) strain PER-K23 (DSM 9455) were grown in reduced, bicarbonate buffered, defined mineral medium. Serum bottles (160 mL) containing 100 mL of medium received 10 mL H₂ as electron donor and 5 mM sodium acetate as carbon source as described [13, 49]. *Dhc* and *Dhb* cultures were spiked with 5 µL neat cDCE (aqueous phase concentration 0.5 mM) and 8 µL neat PCE (aqueous phase concentration 0.78 mM) respectively, as electron acceptor. In addition, *Dhb* cultures were amended with 0.5 g/L peptone to supply essential amino acids.
Each vessel received a *Dhc* or *Dhb* inoculum and 36.9 nM of vitamin B$_{12}$ or purified P-Cba. Prior to inoculation, *Dhc* or *Dhb* cell suspensions were centrifuged and the pellets were suspended in vitamin B$_{12}$-free mineral salts medium inside an anoxic chamber to avoid vitamin B$_{12}$ carryover [61]. All culture vessels were prepared in triplicate and incubated statically in the dark at 30°C.

**Corrinoid extraction and purification**

Cells were harvested by filtration using a 47 mm diameter 0.22 μm pore size membrane (Pall Life Sciences, Port Washington, NY, USA) from 100 mL *Dhc* and *Dhb* culture suspensions following complete dechlorination of cDCE to ethene or PCE to cDCE. Total intracellular corrinoids were extracted following the KCN extraction protocol and purified using a C18 Sep-Pak cartridge (Waters Corp, Milford, MA) [41].

**HPLC and LC-MS analysis**

The corrinoids extracted from *Dhc* and *Dhb* cells were characterized using an Agilent 1200 HPLC system and a Thermo Fisher Orbitrap Exactive Plus UPLC-HRMS system based on retention times and ion transitions as described in chapters II and III.

**DNA extraction**

Cells from *Dhc* or *Dhb* pure cultures were collected from 1 mL culture suspensions by filtration using 13 mm diameter 0.22 μm pore size membrane
filters (Merck Millipore Ltd., Darmstadt, Germany) as described in chapter II. Genomic DNA was extracted from the filter using the Mo BIO PowerSoil DNA Isolation kit (Mo Bio, Carlsbad, CA) following manufacturer’s guidelines [61].

**Quantitative PCR (qPCR)**

*Dhc* 16S rRNA gene targeted quantitative PCR (qPCR) was performed with established protocols using the primer set Dhc1200F/Dhc1271R and probe Dhc1240probe [62].

*Dsf* strain Y51 shares 98.7% identical *pceA* nucleotide sequence with *Dhb* strain PER-K23. Therefore qPCR primers and TaqMan probe can be designed based on the identical region of *pceA* genes of Y51 (DSY2839) and PER-K23 (AJ439607.2). Primers and probes were designed using Primer Express software (Applied Biosystems, Foster City, CA). DNA regions of the RDase genes were used to create a sensitive and specific qPCR assay that fulfilled the criteria (amplicon length 50 -150 bp and a primer and probe T_m of 58 - 60°C and 68 - 70°C, respectively [62]). The G+C content of the primers and probe were between 30 to 80 mol %, with no more than three consecutive G or C bases in either the primer or the probe sequences. Specificity of the primers and probe was verified using BLAST analysis against the database and by comparison with the *pceA* gene sequence of *Dsf* strain Y51 and *Dhb* PER-K23 [42]. The primers and probe sequences are: forward primer 5’-CGGACAAGCCGAGAAAATTC-3’ (Df_pceA_1223F), reverse primer 5’- GCATCCGACATTTTTTTCG-3’ (Df_pceA_1286R) and probe 6FAM-TACGCGAGTTCTGCCG-MGB
A qPCR standard was obtained using plasmid DNA from *Escherichia coli* K12 host cells with plasmid pMK-RQ kanR (Life technologies, Carlsbad, CA) carrying partial sequence of the *pceA* gene (1656 bp) from *Dsf* strain Y51.

The qPCR assays contained: 10 μL of Taqman 2X master mix (Applied Biosystems, Foster City, CA), 300 nM probe, 300 nM of each primer, 2 μL DNA sample or standard and nuclease-free water (Invitrogen) to achieve a total volume of 20 μL. Prior to adding the DNA, aliquots (18 μL) of the reaction mix were dispensed into a MicroAmp® fast optical 0.1mL 96-well reaction plate (Applied Biosystems, Foster City, CA). All solutions were prepared in a dimly lit room to prevent light-activated degradation of the fluorescently labeled oligonucleotide probes. Template DNA (2 μL) was added to each tube, and the tubes were sealed with optical adhesive film (Applied Biosystems, Foster City, CA). Gene copy values in the standard curve ranged from 1×10^1-1×10^8 gene copies per μL. Samples to be assayed contained 20 to 100 ng μL^−1 DNA. All tubes were maintained in the dark prior to loading on the ViiA 7 real-time PCR system (Life Technologies, Grand Island, NY). qPCR cycles were: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15s at 95°C and 1 min at 58°C. qPCR calibration curves were obtained following established methods [62]. pMK-RQ vector with 1656 bp targeted gene was extracted for the standard DNA. The copies of *pceA* genes in each sample were determined by comparing the Ct value with a standard curve following established procedures [63]. The standard
curve generated using the Df_pceA_1223F, Df_pceA_1286R primer sets and Df_pceA_1247 probe targeting pceA RDase genes in Dsf and Dhb is illustrated in Figure 20. The standard curve had the following parameters: slope -3.297, Y-inter -31.448, $R^2$ 0.989, eff% 101.068.

![Standard curve](image)

**Figure 20.** qPCR standard curve for *Dehalobacter pceA* genes.

**Results**

**Effect of P-Cba on Dhb cultures**

Both vitamin B$_{12}$ (positive control) and P-Cba supported dechlorination of PCE to cDCE in *Dhb* strain PER-K23 cultures. PCE-to-cDCE dechlorination rates of $486.16 \pm 37.91$ and $455.27 \pm 35.25$ µM Cl$^-$ released day$^{-1}$ were measured in the presence of B$_{12}$ and P-Cba, respectively (Figure 21). In both treatments, the initial amount of $64.44 \pm 2.32$ µmoles PCE was completely dechlorinated to cDCE within 4-6 days. A significantly lower dechlorination rate of $2.88\pm0.99$ µM...
Cl⁻ released day⁻¹ was observed in negative control incubations without corrinoid, indicating that corrinoid additions were essential to support reductive dechlorination activity in Dhb and Dhc cultures. Dhb cultures that received corrinoid additions reached comparable final cell density after the completion of PCE-to-cDCE dechlorination, and 3.9 ± 2.6×10⁸ cells/mL were measured in cultures amended with P-Cba, and 3.2 ± 1.1×10⁸ cells/mL were measured in cultures amended with vitamin B₁₂.

In negative control experiments, 9.2 ± 1.6×10⁶ cells/mL were measured, indicating a low growth in a corrinoid depleted condition. Enumeration of Dhb cell numbers revealed that Dhb cultures amended with P-Cba reached similar growth yields with cultures amended with B₁₂. The former reached growth yield of 4.2±2.4×10⁸ cells per µmole Cl⁻ released, while the latter reached 3.3±0.9×10⁸ cells per µmole Cl⁻ (Figure 22). In the negative control, Dhb growth yields 5.9±5.4×10⁶ cells per µmole Cl⁻. In P-Cba-amended Dhb cultures, prolonged incubation after the complete dechlorination of PCE-to-cDCE did not lead to further reductive dechlorination activity and VC or ethene formation was not observed. Total intracellular corrinoids were extracted from P-Cba amended Dhb cultures to verify the uptake and utilization of P-Cba by Dhb. HPLC analysis
Figure 21. PCE-dechlorinating Dhb cultures amended with P-Cba.

Reductive dechlorination of PCE in Dhb strain PER-K23 cultures amended with an initial concentration of 36.9 nM of vitamin B\textsubscript{12} (A), P-Cba (B), no corrinoid amendment (C). Open square, PCE; black diamond, TCE; black triangle, cDCE. Error bars represent the standard deviations of triplicate samples.
Figure 22. Growth PCE-dechlorinating *Dhb* cultures amended with P-Cba or B_{12}.

*Dhb* cell numbers were determined with *pceA* targeted qPCR.
revealed the presence of a corrinoid fraction with identical retention time and UV-Vis spectrum as the P-Cba standard, demonstrating that P-Cba without structural modification served as the PceA RDase cofactor in the *Dhb* cultures (Figure 23).

**Effect of P-Cba on Dhc dechlorination activity and growth**

In BAV1 cultures, the initial amount of 83.00±1.7 µmoles cDCE was completely dechlorinated to ethene within 17 days in vessels amended with vitamin B$_{12}$, with the highest cDCE-to-ethene dechlorination rates of 245.77±28.36 µM Cl$^{-}$ released day$^{-1}$. However, in the P-Cba treatment, VC-to-ethene dechlorination step occurred at such low rates that VC, rather than ethene, was formed as end product after 32 days of incubation (Figure 24, 26). The cDCE-to-VC dechlorination rates were 35.30±7.82 µM Cl$^{-}$ released day$^{-1}$ in the presence of P-Cba. Similar to strain BAV1 cultures, cDCE dechlorination rates decreased in strain GT cultures amended with P-Cba amended as corrinoid cofactor (Figure 25, 27). cDCE-to-VC dechlorination rates of 16.24±5.07 µM Cl$^{-}$ released day$^{-1}$ in the presence of P-Cba were measured while cDCE-to-ethene dechlorination rates of 237.91±36.49 µM Cl$^{-}$ released day$^{-1}$ were observed in the presence of vitamin B$_{12}$.

Enumeration of *Dhc* cell numbers after cDCE-to-ethene dechlorination was complete or ceased revealed that *Dhc* cultures BAV1 and GT reached growth yields of 9.66±0.48×10$^{5}$ cells per µmole Cl$^{-}$ released and 6.70±1.62×10$^{5}$ cells per µmole Cl$^{-}$ released in vitamin B$_{12}$ amended cultures, respectively (Figures 26,27). While the growth yields are 9.62±1.21×10$^{5}$ cells per µmole Cl$^{-}$
Figure 23. HPLC chromatogram of P-Cba and B\textsubscript{12} as corrinoid cofactor of \textit{Dhb} reductive dechlorination.
Figure 24. Reductive dechlorination of cDCE in Dhc strain BAV1 cultures.

Reductive dechlorination of cDCE in Dhc strain BAV1 cultures amended with an initial concentration of 36.9 nM of vitamin B$_{12}$ (A), P-Cba (B), no corrinoid (C). black triangle, cDCE; open inverted triangle, VC; black circle, ethene. Error bars represent the standard deviations of triplicate samples.
Figure 25. Reductive dechlorination of cDCE in *Dhc* strain GT cultures.

Reductive dechlorination of cDCE in *Dhc* strain GT cultures amended with an initial concentration of 36.9 nM of vitamin B$_{12}$ (A), P-Cba (B), no corrinoid (C). black triangle, cDCE; open inverted triangle, VC; black circle, ethene. Error bars represent the standard deviations of triplicate samples.
Figure 26. Growth of *Dhc* strain BAV1 with cDCE.

Quantification of intracellular corrinoids extracted from strain BAV1 biomass grown with B$_{12}$, P-Cba and no corrinoid (Negative control, NC).
Figure 27. Growth of *Dhc* strain GT with cDCE.

Quantification of intracellular corrinoids extracted from strain GT biomass grown with B$_{12}$, P-Cba and no corrinoid (Negative control, NC).
released and 5.68±2.24×10^5 cells per µmole Cl− in BAV1 and GT P-Cba culture, respectively.

P-Cba does not support complete dechlorination of cDCE in Dhc strains tested. Instead of ethene, VC is the major end product observed at the end of the 32-day incubation period in both Dhc strain GT and strain BAV1 cultures. The cDCE to VC dechlorination rate in Dhc strain BAV1 cultures amended with P-Cba (16.24±5.07 µM Cl− released day⁻¹) was significantly lower than the cDCE to ethene dechlorination rate (237.91±36.49 µM Cl− released day⁻¹) in the vitamin B_{12}-amended culture. Nevertheless, growth yields of Dhc strains GT and BAV1 in P-Cba and vitamin B_{12} amended microcosms were similar. Due to incomplete reductive dechlorination, the Dhc cultures amended with P-Cba could not take advantage of VC as an electron acceptor, and consequently lower cell yields were obtained. Therefore, P-Cba does not appear to be an ideal cofactor for Dhc reductive dechlorination.

**Effect of different electron acceptors on P-Cba production in Dsf strain Y51**

As mentioned in the materials and methods section, sodium fumarate and PCE were supplied to study P-Cba production Dsf strain Y51 using different electron acceptors. Cell density and corrinoid production were measured after 10 days of incubation, when PCE was completely reduced to cDCE, while fumarate was reduced to succinate. In the fumarate treated cultures, cell density reached 9.12×10^8 cells mL⁻¹, while cell density was 2.88 ×10^8 cells mL⁻¹ in the PCE amended cultures. Corrinoid yields were 5.46 and 4.91 mg/L in fumarate- and in
PCE-amended cultures, respectively, and the corrinoid production rates were $8.13 \times 10^9$ and $2.35 \times 10^{10}$ molecules·cell$^{-1}$ in fumarate and PCE-amended cultures, respectively. Corrinoid production under dechlorination conditions was about ten-fold higher compared to fumarate reduction, reflecting a higher demand for P-Cba for organohalide respiration.

**Effect of P-Cba on Dhc pure cultures**

In *Dhc* strain BAV1 cultures, the initial amount of $83.00 \pm 1.7$ µmoles cDCE was completely dechlorinated to ethene within 17 days at the presence of vitamin B$_{12}$ (36.9 nM), with the highest cDCE-to-ethene dechlorination rates at $245.77 \pm 28.36$ µM Cl$^{-}$ released day$^{-1}$. In contrast, cDCE dechlorination occurred at a much lower rate of $35.30 \pm 7.82$ µM Cl$^{-}$ released day$^{-1}$ in the P-Cba amended cultures, and VC was the end product after 32 days of incubation without ethene formation (Figure 24, 25). Similar to strain BAV1, cDCE dechlorination rate decreased significantly in strain GT cultures amended with P-Cba as the corrinoid cofactor. The cDCE-to-VC dechlorination rates were $16.24 \pm 5.07$ µM Cl$^{-}$ released day$^{-1}$ in the presence of P-Cba, while cDCE-to-ethene dechlorination rates of $237.91 \pm 36.49$ µM Cl$^{-}$ released day$^{-1}$ were measured in cultures amended with vitamin B$_{12}$. In negative control incubations, cDCE to VC dechlorination rates of $8.20 \pm 5.08$ µM Cl$^{-}$ released day$^{-1}$ were observed.

Enumeration of *Dhc* cell numbers after 32 days of incubation revealed that *Dhc* strain BAV1 and strain GT cultures amended with B$_{12}$ reached growth yields of $9.66 \pm 0.48 \times 10^8$ cells per µmole Cl$^{-}$ released and $6.70 \pm 1.62 \times 10^8$ cells per
μmole Cl⁻ released, respectively. Consistent with the observed reductive dechlorination extents, lower growth yields of $9.62\pm1.21 \times 10^7$ cells per μmole Cl⁻ released and $5.68\pm2.24 \times 10^7$ cells per μmole Cl⁻, respectively, were determined in strain BAV1 and strain GT cultures that received P-Cba.

**Discussion**

The functionality of P-Cba as a cofactor for RDases was validated in *Dhc* and *Dhb* cultures. Both species are obligate organohalide-respiring and corrinoid auxotrophic bacteria that strictly depend on external corrinoid supply to enable growth with suitable chloroorganic electron acceptors. Interestingly, P-Cba could replace vitamin $\text{B}_12$ in *Dhb* cultures and similar PCE-to-cDCE reductive dechlorination activities were observed; however, P-Cba did not support VC reductive dechlorination in *Dhc* cultures.

A previous study revealed that *Dhc* strains exhibit a stringent corrinoid/lower base requirement and prefer benzimidazole type corrinoids [52]. The study suggested that the methyl substitutions of the benzimidazole backbone at the 5 and 6 positions are critical to support dechlorination activities in *Dhc* cultures. With vitamin $\text{B}_12$ (i.e., with DMB as the lower base) as the most favorable corrinoid cofactor, decreased reductive dechlorination rates in the order 5-MeBza-Cba > 5-OMeBza-Cba > Bza-Cba were observed in pure cultures of *Dhc* strains 195, BAV1 and GT[52]. Purine lacks methyl substitutions in the pyrimidine ring that may hinder the binding of P-Cba to the BvcA and VcrA RDases. BvcA and VcrA RDases share lower similarities (19.9% identity) to the
PceA in Dsf strain Y51, and subsequently inhibited RDase maturation and export and affected cDCE dechlorination rates in Dhc strains BAV1 and GT.

The corrinoid requirement in Dhb has not been investigated in B$_{12}$. Dhb and Dsf strain Y51 harbor almost identical (98.7% amino acid identity) PceA RDases to couple growth with PCE-to-cDCE dechlorination. Thus, it is not surprising that these enzyme systems share structural features and use similar corrinoid cofactors. Statistically indifferent reductive dechlorination rates and final cell densities demonstrated that P-Cba is a fully functional equivalent to vitamin B$_{12}$ for corrinoid-auxotroph Dhb.

These findings validate the function of P-Cba as an RDase cofactor for PceA catalytic activities in Dsf and Dhb, but not for Dhc RDases catalyzing the VC-to-ethene reductive dechlorination step. A large diversity of confirmed and putative RDases has been identified[64], and the results presented here emphasize that RDase-specific corrinoid cofactor requirements affect catalytic activity in terms of reductive dechlorination rates and extents.
CHAPTER V
CONCLUSION

Metabolically versatile *Desulfitobacterium* (*Ds*) strains play important roles in the bioremediation of toxic chlorinated contaminants. The corrinoid cofactor natively synthesized in *Ds* strains to fulfill the catalytic activity of tetrachloroethene reductive dehalogenases (PceA RDases) had not been previously identified and was investigated here to better understand corrinoid-dependent reductive dechlorination reactions. In this research, using a combined approach employing cultivation and sophisticated analytical techniques, the structure of the corrinoid cofactor produced in four PCE-dechlorinating *Ds* strains was resolved. Purinyl-cobamide (Coα-purinyl-Coβ-cyanocobamide) was identified as the corrinoid cofactor of PceA in *Ds* strains. Purine, a building block of nucleic acids, and thus a universal compound in biological systems, has not been implicated as a lower base in the assembly of naturally occurring corrinoids. We conclude that P-Cba represents a novel type of natural corrinoid to enable organohalide-respiration in *Ds*.

The role of P-Cba for reductive dechlorination was further validated with other organohalide-respiring genera. P-Cba was found to support dechlorination activities differently in the corrinoid auxotrophic *Dhc* and *Dhb* cultures. P-Cba acts as a functional equivalent of vitamin B$_{12}$ to sustain fast PCE dechlorination and microbial growth in *Dhb* cultures expressing a PceA RDase that shares 98.7% amino acid identity with the PceA of *D. hafniense*. In contrast, impaired
dechlorination activities compared to vitamin B\textsubscript{12}-fed cultures were observed in \textit{Dhc} strain BAV1 and strain GT cultures, which express the BvcA and VcrA RDases, respectively, demonstrating that P-Cba is not a favorable corrinoid cofactor for VC-dechlorinating \textit{Dhc} strains. Previous investigations with \textit{Dhc} pure or co-cultures demonstrated that \textit{Dhc} strains have a stringent corrinoid requirement with a preference for cobamides carrying a benzimidazole type of lower base with methyl substitution(s) in the 5 and/or 6 positions. This research demonstrated that a cobamide with purine as a lower base cannot sustain optimal \textit{Dhc} activity, presumably because of the lack of methyl group in the pyrimidine ring that affects the binding of corrinoid cofactor to the apo-form of RDases[52]. Interestingly, the PCE-dechlorinating \textit{Dhb} and \textit{Dsf} strains exhibit a more flexible corrinoid requirement than \textit{Dhc}. \textit{Dhb} was able to utilize vitamin B\textsubscript{12} and P-Cba and \textit{Dsf} strain Y51 was able to utilize vitamin B\textsubscript{12}, P-Cba and Bza-Cba in medium amended with PCE as electron acceptor. Apparently, the corrinoid/lower base requirements are not only organism but also RDase specific. Because of the involvement of distinct RDases at different PCE dechlorination steps, these results suggest that the final detoxification steps (i.e. cDCE and VC to ethene) are more corrinoid/lower base stringent than the initial steps in PCE/TCE dechlorinations. Unfavorable lower bases may hinder the complete PCE detoxification and explain the cDCE/VC stalls at many PCE/TCE contaminated sites.
Overall, the discovery, structural and functional determination of P-Cba suggest that the largely unexplored corrinoid metabolism in organohalide-respiring bacteria may expand our understanding of corrinoid diversity, function and biochemistry, and are relevant to the development of more efficient bioremediation strategies. The results presented here, together with recently published information[52], demonstrate the important role of the lower base for functionalizing the corrinoid and enable RDase activity. Prior to this study, 16 naturally occurring lower base structures were known, with the last novel structure reported in 1994 [33]. The experimental efforts with Dsf cultures revealed a new lower base that was identified as purine. Considering that the type of lower bases plays such an important roles for RDase function, knowledge about lower base and corrinoid diversity and biosynthesis is crucial for managing the reductive dechlorination process at sites impacted with chlorinated pollutants.
REFERENCES
15. Suyama, A., et al., Isolation and characterization of Desulfitobacterium sp strain Y51 capable of efficient dehalogenation of tetrachloroethene and


30. Lezius, A.G. and H.A. Barker, Corrinoid Compounds of Methanobacillus omelianskii. I. Fractionation of the Corrinoid Compounds and Identification


APPENDICES
## Appendix A

### Table 1. Characteristics of corrinoid cofactor

<table>
<thead>
<tr>
<th>Group</th>
<th>Lower base name</th>
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<th>Corrinoid name</th>
<th>Molecular weight</th>
<th>Host microorganisms</th>
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<td>benzimidazole</td>
<td>5,6-Dimethylbenzimidazole (DMB)</td>
<td>C₉H₁₀N₂</td>
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<td>Vitamin B₁₂ (Cobalamin)</td>
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<td>5-Methylbenzimidazole (5-MeBen)</td>
<td>C₈H₈N₂</td>
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<td>1341.34</td>
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<td>Benzimidazole (Ben)</td>
<td>C₇H₆N₂</td>
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</tr>
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<td>5-Hydroxybenzimidazole (5-OHBen)</td>
<td>C₇H₆N₂O</td>
<td>134.14</td>
<td>Factor III</td>
<td>1343.32</td>
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<td></td>
<td>5-Methoxybenzimidazole (5-MeOBen)</td>
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<td>5-Methoxy, 6-methylbenzimidazole</td>
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<td>1371.37</td>
<td><em>Clostridium</em> spp.</td>
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<td>purine</td>
<td>Adenine</td>
<td>C₅H₅N₅</td>
<td>135.13</td>
<td>Pseudo-vitamin B₁₂</td>
<td>1344.31</td>
<td><em>Methanococcales</em> sp.</td>
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<td>2-Methyladenine</td>
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<td>Corrinoid</td>
<td>Lower base</td>
<td>Molecular formula of lower base</td>
<td>Nitrogen atoms in lower base</td>
<td>Nitrogen atoms labeled in corrinoid</td>
<td>Expected MW increase</td>
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<td>Cyano-B$_{12}$</td>
<td>DMB</td>
<td>C$<em>9$H$</em>{10}$N$_2$</td>
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<td>Cyano-factor III</td>
<td>(5'-OHBen)</td>
<td>C$_7$H$_6$N$_2$O</td>
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Table 2. Expected mass shifts following $^{15}$N isotope labelling
**Figure 28.** Genes required for aerobic (late) and anaerobic (early cobalt insertion) corrin ring biosynthetic pathways (Warren, M.J., 2006).
Figure 29. $^1$H-NMR spectra of B$_{12}$ and the unknown corrinoid from Y51 cultures. B$_{12}$ standard (grey) and XB$_{12}$ (black) were performed by $^1$H-NMR.

$^1$H NMR (600 MHz, Methanol-d$_4$) $\delta$ 8.92 (s, 1H), 8.25 (s, 1H), 7.44 (s, 1H), 6.58 (d, $J$ = 3.5 Hz, 1H), 6.18 (s, 1H), 4.58 (s, 3H), 4.33 (dtt, $J$ = 13.4, 7.0, 3.4 Hz, 1H), 4.24 (s, 1H), 4.19 (d, $J$ = 11.4 Hz, 1H), 4.12 (s, 2H), 3.92 (dd, $J$ = 12.7, 3.2 Hz, 1H), 3.75 (td, $J$ = 11.4, 10.2, 4.8 Hz, 2H), 3.70 – 3.65 (m, 1H), 2.92 (dt, $J$ = 11.1, 6.2 Hz, 1H), 2.82 (dd, $J$ = 14.0, 9.6 Hz, 1H), 2.71 – 2.50 (m, 12H), 2.52 – 2.42 (m, 2H), 2.36 (s, 2H), 2.20 – 2.11 (m, 1H), 2.08 – 1.95 (m, 5H), 1.95 (s, 2H), 1.93 – 1.81 (m, 8H), 1.64 – 1.58 (m, 2H), 1.49 (s, 4H), 1.39 (d, $J$ = 14.6 Hz, 8H), 1.32 – 1.27 (m, 3H), 1.26 (d, $J$ = 6.3 Hz, 3H) 1.19 (s, 3H), 0.92 – 0.80 (m, 2H), 0.49 (s, 3H)
Meng Bi was born in the city of Qingdao, Shandong, China in 1988 to Rui Tang and Lu Bi. Her family lived in Qingdao for all her childhood and teenage life. She graduated from Qingdao No.9 High School. She entered Nanjing Agricultural University (NJAU) the following fall and majored in biotechnology, where she took courses in microbiology, biochemistry, genetics etc. After graduating from NJAU, she moved to Knoxville, Tennessee to join the University of Tennessee. She was accepted by the Department of Microbiology in 2011 and started her graduate study in the Löffler lab.

Meng has been working as graduate teaching assistant from 2011-2013, during which she assisted Micro 210. She has been a graduate research assistant from 2013-2015, during which she worked on identification of P-Cba as a novel corrinoid cofactor of tetrachloroethene reductive dehalogenases in Desulfitobacterium spp.