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Research Article

The PceA-orthologous reductive dehalogenase of *Dehalococcoides mccartyi* CBDB1 is involved in 1,2,4-trichlorobenzene respiration

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Abstract

The reductive dehalogenation of halogenated benzenes by anaerobic bacteria is of great environmental and biotechnological importance; however, the role of specific reductive dehalogenases in the dehalogenation of different isomers has not been studied in detail. Here, we cultivated the obligate organohalide-respiring *Dehalococcoides mccartyi* strain CBDB1 with either 1,2,3- or 1,2,4-trichlorobenzene (TCB) as electron acceptor and investigated the transcription of its 32 reductive dehalogenase (*rdhA*) genes using RNA-sequencing. The chlorobenzene reductive dehalogenase gene *cbrA,* and *rdhA* cbdbA80 were the two most highly expressed *rdhA* genes with 1,2,3-TCB. In the presence of 1,2,4-TCB, *cbrA* was the most highly expressed *rdhA* followed by *rdhA* cbdbA1588, encoding an orthologue of the tetrachloroethene reductive dehalogenase PceA of *D. mccartyi* strain 195. RNA-sequencing also allowed for the detection of small RNAs and an unannotated protein. Proteomics confirmed the synthesis of RdhA CbdbA1588 during respiration with 1,2,4-TCB and also with hexachlorobenzene, which is dehalogenated via 1,2,4-TCB. Dehalogenase activity assays with cell extracts from 1,2,4-TCB-grown cultures indicated a higher activity towards 1,2,4-TCB and a ten-fold higher activity towards 2,3-dichlorophenol compared to that in extracts from 1,2,3-TCB-grown cultures. These findings demonstrate the functionality of RdhA CbdbA1588 and further support a role in 1,2,4-TCB dechlorination by strain CBDB1.

Keywords *Dehalococcoides mccartyi*, reductive dehalogenase, transcriptome, proteome, trichlorobenzene, PceA, sRNA

Introduction

Dehalococcoides mccartyi strain CBDB1 is an obligate organohalide-respiring anaerobic bacterium. It oxidizes hydrogen as electron donor and uses halogenated compounds as electron acceptors. The strain is particularly versatile towards the dehalogenation of haloaromatic compounds such as halogenated benzenes and phenols (Adrian et al. 2000, Adrian et al. 2007a, Jayachandran et al. 2003, Wagner et al. 2012, Yang et al. 2015), chlorinated dibenzo-*p*-dioxins (Bunge et al. 2003), polychlorinated biphenyls (PCBs) (Adrian et al. 2009), and others (Cooper et al. 2015, Reino et al. 2023). In addition, the bacterium dechlorinates tetrachloroethene (PCE) to *cis*- and *trans*-dichloroethene (Marco-Urrea et al. 2011). Organohalide respiration (OHR) in *D. mccartyi* is facilitated by a quinone- and cytochrome-independent protein complex (Kublik et al. 2016, Seidel et al. 2018, Türkowsky et al. 2018). This OHR complex consists of the reductive dehalogenase catalytic subunit, RdhA, and the membrane anchor, RdhB, the hydrogenase subunits, HupL and HupS, the iron-sulfur cluster protein, HupX, the OHR molybdoenzyme, OmeA and the membrane anchor protein OmeB (Seidel et al. 2018). The electron flow from the hydrogenase via HupX to the reductive dehalogenase is coupled to the translocation of protons from the cytoplasm to the periplasmic side of the membrane, thus generating a proton motive force that is essential for energy conservation through the ATPase (Hellmold et al. 2023, Adrian et al. 2025).

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The genomes of *D. mccartyi* strains have a large number of *rdhA* and associated *rdhB* genes (Türkowsky et al. 2018). This might be the basis for the observed broad dehalogenation capacity of the bacterium and is in accordance with its dominant occurrence in anoxic compartments of sites heavily polluted with a variety of aliphatic and aromatic organohalogens (e.g. Nijenhuis et al. 2018). Of the 32 RdhAs of *D. mccartyi* strain CBDB1 only one has been characterized so far, namely the chlorobenzene reductive dehalogenase CbrA, which has been shown to dechlorinate 1,2,3-trichlorobenzene (TCB) to 1,3-dichlorobenzene (DCB), and 1,2,3,4-tetrachlorobenzene to 1,2,4-TCB (Adrian et al. 2007b). Since these are the only products of these reactions, CbrA most likely removes exclusively the middle of three adjacent chlorines from the benzene ring (so-called doubly-flanked dechlorination). High expression of *cbrA* was detected by RT-qPCR during growth with 1,2,3- and 1,2,4-TCB and with 2,3-dichlorodibenzo-*p*-dioxin (Wagner et al. 2009, Wagner et al. 2013) and by proteomics during dehalogenation of different bromobenzenes (Wagner et al. 2012), but not during dehalogenation of bromophenol blue (Yang et al. 2015) or 2,3-dichlorophenol (DCP) (Morris et al. 2007). So far, an orthologue of *cbrA* was observed only in *D. mccartyi* strain DCMB5 (dcmb_86, Pöritz et al. 2013). This strain is also able to use a range of haloaromatic compounds, such as 1,2,3-TCB and chlorinated dibenzo-*p*-dioxins, as electron acceptors (Bunge et al. 2008, Pöritz et al. 2015). However, it cannot respire 1,2,4-TCB, 2,3-dichlorodibenzo-*p*-dioxin or 2,3-DCP, which would require the removal of a chlorine from a position on the aromatic ring flanked by only one adjacent chlorine (Pöritz et al. 2015). To our knowledge, strain CBDB1 is the only reported *D. mccartyi* strain that can use 1,2,4-TCB as electron acceptor for growth, forming a mixture of 1,3- and 1,4-dichlorobenzene as dechlorination products. Therefore, this strain is a suitable candidate to identify the reductive dehalogenase responsible for removing the singly-flanked chlorine.

Almost all *rdhAB* genes in the genome of strain CBDB1 are located proximally to genes encoding predicted transcriptional regulatory proteins, strongly suggesting these gene products have a role in regulating transcription of the cognate *rdhA* gene (Kube et al. 2005). Typically, these genes encode either MarR-type (RdhR) (Krasper et al. 2016) or two-component systems (TCS) comprising a histidine kinase, RdhS, and its cognate response regulator, RdhP (Kruse et al. 2016).

Previous gene expression studies using qPCR (e.g. Fung et al. 2007, Low et al. 2015), microarrays (e.g. Johnson et al. 2008, Hug et al. 2011, Lee et al. 2012) or RNA sequencing (RNA-seq) (Wang et al. 2014) helped to associate distinct *rdhA* genes of *D. mccartyi* to specific dechlorinating reactions. For example, the contribution of different RdhA proteins to the complete dechlorination of PCE to ethene is well known in *D. mccartyi*: PceA (Magnuson et al. 1998) or PteA (Zhao et al. 2017) and TceA (Magnuson et al. 2000) are involved in the sequential dechlorination to vinyl chloride, which is finally dechlorinated by VcrA (Parthasarathy et al. 2015). In contrast, the role of individual reductive dehalogenases in the sequential dechlorination of hydrophobic aromatic compounds such as hexachlorobenzene (HCB) is not understood yet.

Here we have used RNA-seq analysis to determine the *rdh* gene expression profile in 1,2,4-TCB- compared to 1,2,3-TCB-respiring cells of *D. mccartyi* CBDB1. Transcription of one *rdhA* gene (cbdbA1588) was shown to be induced specifically by 1,2,4-TCB, which was confirmed by RT-qPCR. The synthesis of the respective gene product was studied by proteomic analyses. To support its potential role in sequential dechlorination reactions, HCB-respiring cultures were also included. The functionality of the synthesized RdhA was demonstrated in reductive dehalogenase activity assays.

Material and methods

Cultivation

The cultivation of *D. mccartyi* strain CBDB1 was carried out at 30 °C under an atmosphere of nitrogen-carbon dioxide (80 : 20) in 60 ml or 150 ml volumes in 100 ml or 200 ml serum bottles, respectively. Reducing conditions were achieved by adding Ti(III)-citrate (1.5 mM) to the carbonate-buffered synthetic medium (Adrian et al. 2000). Typically, 1×10^7 cells ml^{-1} were used for inoculation of culture. Hydrogen (added to the medium with 0.5 bar overpressure for 10 s) served as electron donor and sodium acetate (5 mM) as carbon source (Adrian et al. 2000). Due to the low solubility of 1,2,3- and 1,2,4-TCB in aqueous solution (100 and 270 μM in water, respectively, https://pubchem.ncbi.nlm.nih.gov/source/hsdb/1105) and to their toxicity, strain CBDB1 was cultivated in two-liquid phase cultures overlaid with 200 or 400 mM 1,2,3- or 1,2,4-TCB dissolved in hexadecane (the added volume resulted in a nominal initial concentration of 10 mM). Based on the partition coefficient between water and hexadecane (Holliger et al. 1992), approximately 20–40 μM TCB was dissolved in the medium. In one of two proteome experiment, HCB was applied as an alternative electron acceptor, in which 1,2,4-TCB was transiently formed as a dechlorination intermediate (Jayachandran et al. 2003). Due to the lower solubility of HCB in hexadecane, 170 mg HCB crystals were added to empty bottles before 60 ml of medium was added (nominal HCB concentration 10 mM). The medium was overlaid with hexadecane to allow HCB to associate with, and partly dissolve in, the hexadecane phase. Addition of hexadecane led to a three-fold higher cell yield (6×10^8 ml^{-1}) compared to cultures grown in the presence of crystalline HCB without hexadecane addition. Growth of strain CBDB1 was monitored by microscopic counting of cells stained with 4′, 6-diaminido-2-phenyl-indole (DAPI) (2.5 μg ml^{-1}) and fixed on agarose-coated slides (Adrian et al. 2007a), using an Axioskop equipped with an AxioCam Mrc digital camera and AxioVision software (Zeiss, Oberkochen, Germany).

To allow a complete adaptation of the cells to the electron acceptor being used, separate cultivation lines were established for each chlorobenzene isomer. Cells were grown in a preculture with the selected chlorobenzene until stationary phase was reached. These precultures were then used to inoculate (10% vol/vol) freshly prepared medium containing the same electron acceptor. Triplicate cultures were harvested at a cell density of 1.0×10^8–1.0×10^9 cells ml^{-1} and subjected to transcriptome or proteome analysis.

Cell harvest

For the quantification of transcripts by reverse transcription quantitative PCR (RT-qPCR), cells from 2 ml of culture were used for RNA extraction. The samples were centrifuged (8415 × *g* for 20 min at 4°C); the cell pellets were suspended in 100 μl of residual supernatant and stored at −80°C until further processing. For RNA-seq replicate 150-ml cultures were grown to the transition from the

exponential to the stationary growth phase. Although this state is prone to metabolic changes as reflected by the up- and down-regulation of genes involved in different anabolic processes, membrane transport or stress response (Johnson et al. 2008), the functionally relevant reductive dehalogenase gene transcripts reach a high abundance in *D. mccartyi* (e.g. Johnson et al. 2008, Geiner-Haas et al. 2021). In addition, the cell number was high enough (1.6×10^8 cells ml^{-1} with 1,2,4-TCB and 4×10^8 cells ml^{-1} with 1,2,3-TCB) to ensure sufficient RNA to be extracted for the transcriptome analysis and the RNA integrity number (RIN) control. The cultures were divided in 100 and 50 ml portions (for RNA-seq and RIN analyses, respectively) and harvested. In the two separately performed proteome experiments, cells from 30 ml volumes of triplicate 60-ml cultures were harvested in the stationary phase (5.3×10^8 cells ml^{-1} on average). Cultures were centrifuged, first at $3660 \times g$ for 45 min and 4°C to collect cell material, after which the cells were suspended in 2 ml of residual supernatant and again the sample was centrifuged at $9520 \times g$ for 30 min and 4°C. The cell pellets were stored in 100 μl of residual supernatant and kept at −80°C until further processing.

RNA extraction and RT-qPCR

The Total RNA Mini Kit (A&A BIOTECHNOLOGY, Gdansk, Poland) was used for RNA extraction, which was carried out exactly according to the manufacturer's instructions. The RNA was finally eluted with 100 μl of distilled, diethylpyrocarbonate-treated H_20, and was then treated with 2 units of DNase I (New England Biolabs, Frankfurt [Main], Germany) to remove residual DNA before being stored at −80°C. To perform RT-qPCR, cDNA was synthesized using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) using random hexamer primers and according to the manufacturer's instructions. The synthesized cDNA served as template for quantitative PCR amplifications with primers (Table S1 in the Supplementary material) targeting the genes of interest. The amplification of cDNA was performed in QuantiTect® SYBR® *Green* PCR master mixes (Qiagen, Hilden Germany) with a primer concentration of 0.5 μM and using a Rotor Gene RG-3000 qPCR cycler (Corbett Research, Sydney, Australia). Plasmids carrying fragments of the respective genes (Table S1) were serially diluted and included as external standards in the qPCR runs, as described previously (Wagner et al. 2009). The data were normalized by dividing the amount of *rdhA* transcript by the amount of transcript of the housekeeping gene *recA*.

RNA concentration and quality control

For transcriptome analyses the extracted RNA was concentrated by precipitation. Ammonium acetate (2.5 mM) and 3.5 volumes of ethanol (99%, −20°C) were added to 100 μl of eluted RNA and the mix was stored at −80°C for at least 30 min. The RNA was collected by centrifugation at 21 400 × g for 20 min and at 4°C. The RNA pellet was washed with ice-cold ethanol (70%) and dried. The dried pellet was stored at −80°C until shipping or was dissolved in 15 μl of distilled, diethylpyrocarbonate-treated H_20 for the determination of the RIN. RIN analysis was performed using the RNA 6000 Nano Kit and 2100 Bioanalyzer G2938A (Agilent Technologies, Waldbronn, Germany) according to the manufacturer´s instructions. The evaluation of the RNA quality was based on the ratio of the ribosomal bands, the image of the entire electrophoretic trace and the presence or absence of degradation products (RIN number 1 referred to the most degraded, 10 to the best preserved RNA). For the transcriptome analyses samples with RIN numbers > 6.1 were used. The extraction, purification and concentration procedure yielded 7–33 μg of purified RNA per culture replicate (in average 20 μg).

Transcriptome analysis

cDNA libraries suitable for sequencing were prepared from 400 ng of total RNA. RNAs were fragmented using Mg^{2+} ions for 2 min 45 sec (NEBNext Magnesium RNA fragmentation module) and were treated with T4 PNK for phosphorylation/dephosphorylation and RppH for decapping followed by NEBNext® Multiplex Small RNA Library Prep (New England Biolabs). In total 15 PCR cycles were performed with 15 sec elongation time. Libraries were quantified by Qubit™ dsDNA HS Assay Kit 3.0 Fluorometer (Thermo Fisher) and quality was checked using a 2100 Bioanalyzer with High Sensitivity DNA kit (Agilent Technologies) before pooling. Sequencing of pooled libraries, spiked with a PhiX control library, was performed at a minimum of 20 million reads per sample in single-end mode with 150 cycles on the NextSeq 500 platform (Illumina). Demultiplexed FASTQ files were generated with bcl2fastq v2.20.0.422 (Illumina)

Raw sequencing reads were subjected to quality and adapter trimming via Cutadapt (Martin 2011) v1.15 using a cutoff Phred score of 20 and discarding reads without any remaining bases (parameters: –nextseq-trim=20 -m 1 -a AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC). Afterwards, all reads longer than 11 nt were aligned to the *D. mccartyi* CBDB1 reference genome (RefSeq assembly accession: GCF_000009025.1) using the pipeline READemption (Förstner et al. 2014) v2.0.3 with segemehl version 0.3.4 (Hoffmann et al. 2009) and an accuracy cut-off of 95% (parameters: -l 12 -a 95). Afterwards, READemption coverage was applied to generate positional genomic coverage files based on full regions spanned by alignments. READemption gene_quanti was applied to quantify aligned reads overlapping genomic features by at least 10 nt (-o 10) on the sense strand. Features consisted of RefSeq annotations (CDS, riboswitch, RNase_P_RNA, rRNA, tmRNA, tRNA; annotation version RS_2025_03_18 for assembly GCF_000009025.1) supplemented with manual small RNA (sRNA)/small open reading frame (sORF) annotations based on inspection of genomic coverages (see above) in the Integrated Genome Browser (IGB) (Freese et al. 2016). Based on these counts, differential expression analysis for the two conditions was conducted via DESeq2 (Love et al. 2014) version 1.24.0. Read counts were normalized by DESeq2 and fold-change shrinkage was conducted by setting the parameter betaPrior to TRUE. Differential expression of transcripts was assumed at adjusted p-value after Benjamini-Hochberg correction (p_{adjust}) < 0.05 and log2fold-change ≥ 2. The similar size of all *rdhA* genes (∼ 1480 nt) allowed the comparison of their expression levels not only between the two, but also within one condition. For figure generation using IGB, genomic coverage files were normalized by DESeq2 size factors.

Proteome analysis

Protein extraction, tryptic digestion and peptide purification were performed as described before (Greiner-Haas et al. 2021). For

proteome analysis, 5% (v/v) of the eluate was used. Samples were lyophilized and stored at −80°C. For liquid chromatography-tandem mass spectrometry, the samples were desalted by using SOLAμ SPE plates (Thermo Fisher Scientific, Waltham, MA, USA). Peptides solubilized in 0.1% (v/v) formic acid were separated using a 100 min nonlinear gradient from 3.2 to 40% (v/v) acetonitrile and 0.1% (v/v) formic acid on a C18 analytical column (Acclaim PepMap100, 75 μm inner diameter, 25 cm, Thermo Fisher Scientific, Waltham, MA, USA) in a UHPLC system (Ultimate 3000 RSLCnano, Dionex/Thermo Fisher Scientific, Idstein, Germany) at a flow-rate of 300 nL/min and 35°C. Mass spectrometry was performed on a Q Exactive HF MS (Thermo Fisher Scientific, Waltham, MA, USA) with a TriVersa NanoMate (Advion, Ltd., Harlow, UK) source in LC chip coupling mode. Mass spectrometer full scans were measured in the Orbitrap mass analyzer within the mass range of 350-1550 m/z, at 60 000 resolution using an automatic gain control of 3×10^6 and maximum fill time of 50 ms. An MS/MS isolation window for ions in the quadrupole was set to 1.4 m/z. MS/MS scans were acquired from the top ten ions of charge state 2–6 using the HCD mode at normalized collision-induced energy of 28%, with a resolution of 15 000, an automatic gain control target of 1×10^5, and maximum injection time of 100 ms. The exclusion time to reject masses from repetitive MS/MS fragmentation was set to 30 s.

Proteome Discoverer (v2.1, Thermo Fisher Scientific) was used for protein identification and quantification. The acquired spectra were searched by Sequest HT against a *D. mccartyi* strain CBDB1 database containing 1454 nonredundant protein-coding sequences. Enzyme specificity was selected as trypsin with up to four missed cleavages allowed. Peptide ion tolerance was set to 10 ppm and MS/MS tolerance to 0.2 Da. Only peptides with a false discovery rate (FDR) < 0.01 and a SeQuest HT XCorr > 2.0 were considered as identified. Quantification of proteins was performed using the average of the top three peptide MS1 areas. Protein quantification was considered successful for proteins quantified in at least two of three replicates; otherwise, they were classified as identified.

After log transformation, protein abundances were median-normalized and scaled, so that the global minimum is zero.

Preparation of crude extracts and dehalogenase activity assay

A total volume of 200–300 ml of culture containing $\sim 2 \times 10^8$ cells ml^{-1} was harvested under anoxic conditions and 4 °C in an anaerobic chamber (Coy™, Grass Lake Michigan, USA; 95% N_2: 5% H_2 atmosphere). Cells were pelleted by centrifugation at 3660 × g for 45 min, suspended in 2 ml of residual supernatant and again centrifuged at 9520 × g for 30 min. The cell pellets were maintained in 100 μl of residual supernatant and kept at −80 °C until use. For cell lysis, the pellets were suspended in 500 μl 50 mM MOPS, pH 7, including 5 μg ml^{-1} DNase, 0.2 mM phenylmethylsulfonyl fluoride and 2 mM sodium dithionite and the suspension was incubated with 1.5% (w/v) digitonin (Sigma–Aldrich, Darmstadt Germany) for 45 min. Lysis was completed by gentle agitation followed by sonication on ice (30 W power for 2×30 s with 0.5 s pulses). Residual cells and cell debris were removed by centrifugation at 15 000 g for 30 min at 4 °C. The resulting supernatant (cell-free extract) was used immediately for the dehalogenase activity assay. Determination of the protein concentration was done using the Lowry method. The dehalogenase activity assay was performed under anoxic conditions in 1 ml of 50 mM Tris-HCl. The assay contained 1 mM methyl viologen, 2 mM titanium (III) citrate and either 50 μM TCBs or 200 μM 2,3-DCP. The reaction was started by the addition of cell-free extract (10–15 μg protein). After 2.5 h, hexane extraction was employed to stop the reaction and to extract and quantify the chlorobenzene or chlorophenol isomers by gas chromatography and flame-ionization detection (GC-2010 FID, Shimadzu Scientific Instruments). The chlorophenols were acetylated before extraction by hexane. The assay (300 μl) was mixed with 600 μl 0.05 M $NaHCO_3$, pH 9.9 and 60 μl of acetic acid anhydride and incubated for one hour at RT before hexane extraction. The identification of the compounds was based on matching their retention time to authentic standards and quantified as reported previously (Pöritz et al. 2015). Enzyme assays were performed in triplicate.

Phylogenetic tree construction

RdhA protein sequences were retrieved from GenBank.Tree building was based on the fast maximum likelihood tree estimation program provided by NGPhylogeny.fr (Lemoine et al. 2019) using the integrated multiple alignment (MAFFT) and curation (BMGE) tools and the maximum likelihood-based inference of phylogenetic trees (PhyML) with Smart Model Selection (SMS) including subtree pruning and regraphing (SPR) for the topology search and SH-like alRT (Shimodaira–Hasegawa-like approximate Likelihood Ratio Test) for the evaluation of branch support. Newick Display was used for tree rendering and iTOL (Letunic and Bork 2021) for the final tree presentation.

Results and discussion

RdhA gene transcription in the presence of 1,2,3-TCB or 1,2,4-TCB as electron acceptor

Of the 1488 genes encoding proteins and structural RNAs as well as 9 regulatory elements (riboswitches and T-box leaders) annotated in the genome of strain CBDB1 only 21 were not transcribed (mean value of read numbers ≤ 1, see Table S2 in the Supplementary material). A differential abundance analysis (Table S2 and Fig. S1 in the Supplementary material) revealed similar transcription levels for ∼80% of all genes between the two conditions. Among the genes not differentially transcribed were those encoding the main components of the OHR complex, as expected. This complex is essential for the respiration with both TCBs, including the Hup hydrogenase (CbdbA129), OmeA, OmeB (CbdbA193, CbdbA195) and the main RdhA, CbrA. They showed similar transcript levels that were not significantly different ($p_{adjust} \geq 0.05$, Table S2). All 32 *rdhA* genes were transcribed (Table S2). Their expression varied over several orders of magnitude. Fig. 1 summarizes only those ten most abundant *rdhA* transcripts that reached ≥ 100 read counts in at least one cultivation condition. The transcripts of *cbrA* and *rdhA* cbdbA80 were the two most abundant *rdhA* transcripts in 1,2,3-TCB-grown cultures. In 1,2,4-TCB-grown cultures, the *cbrA* transcript was in the same range, but the *rdhA* cbdbA80 transcript was less abundant. Instead, a third *rdhA*, cbdbA1588, an orthologue of *pceA* (Det0318 in the genome of *D. mccartyi* 195, 94%

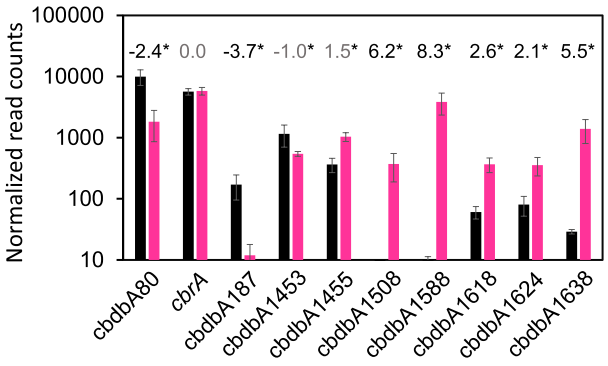

Figure 1 Differential *rdhA* transcription in cells grown with either 1,2,3-TCB (black bars) or 1,2,4-TCB (purple bars) as electron acceptor. *RdhA* genes with normalized read numbers of > 100 in at least one condition are shown (mean values and SD from triplicate cultures). Note the log-scale of the y-axis. The log2-fold changes (1,2,4-TCB vs. 1,2,3-TCB) (>2/<-2 in black, otherwise in gray) are given above the bars. ∗Significance of differences (Benjamin-Hochberg adjusted p-values < 0.05). For the significance of differences of all *rdhA* genes see also Fig. S1 in the Supplementary material).

amino acid sequence identity over the whole sequence length), was found to be the second most highly expressed *rdhA* and one of the most highly up-regulated genes in the genome during cultivation with 1,2,4-TCB (Fig. S1), as opposed to its negligible transcription with 1,2,3-TCB. This was validated by RT-qPCR (Fig. S2 in the Supplementary material). The strong transcription of *cbrA* confirms earlier RT-qPCR results (Wagner et al. 2009, Wagner et al. 2013) and supports a general function of CbrA in the dehalogenation of halogenated benzenes. The high transcription level for *rdhA* cbdbA80 with both TCBs is in accordance with the reported abundance of this RdhA in cells respiring with a variety of halogenated benzenes (Wagner et al. 2012) or phenols (Morris et al. 2007, Yang et al. 2015). However, *rdhA* cbdbA1588 seems to play a specific role for the dechlorination of 1,2,4-TCB. Other differentially expressed *rdhA* genes revealed much lower transcript levels and a specific function in TCB respiration cannot be concluded (Fig. 1).

Transcription of *rdh operons*

An IGB-based visual inspection of the transcriptomes revealed the expression of *rdhAB* genes as transcriptional units. The transcription profiles of the genomic regions containing the three most abundant *rdhAB* transcripts, cbdbA80/cbdbB3 and *cbrA*/cbdbA85 (Fig. 2) and cbdbA1588/cbdbA1587 (Fig. 3) are shown. TCS genes encoding a sensor histidine kinase RdhS and a DNA-binding response regulator RdhP are located upstream of each of these *rdhAB* genes (*rdhSP* cbdbA79/cbdbA78, cbdbA82/cbdbA83, Fig. 2, and cbdbA1590/cbdbA1589, Fig. 3) and are also strongly transcribed as separate transcriptional units, which is characteristic for regulatory genes. However, it is still unclear whether they act similarly in *rdhA* gene regulation as has been recently described for the TCS regulator in the PCE regulon of *Sulfurospirillum multivorans* (Esken et al. 2020).

The *rdhSP* cbdbA82/cbdbA83 mRNA is part of a polycistronic transcript (Fig. 2) and additionally comprises cbdbA81, encoding the CbiZ-like protein RdhF, with a putative function in remodeling the lower ligand of corrinoids (Kruse et al. 2016).

The *rdhAB* transcripts of cbdbA80/cbdbB3 and *cbrA*/cbdbA85 both possessed extended 3´-UTRs (Fig. 2). The cbdbA80/cbdbB3 transcript continued into a 626 nt-long 3´-UTR (Fig. 2). The abundant transcript *cbrA*/cbdbA85 extended into a shorter 3´-UTR (46 nt), ending with a rho-independent terminator sequence, immediately followed by a transcript, which might be a sRNA candidate (Fig. 2). Since 3´-UTRs are a recognized source of regulatory sRNAs (Chao and Vogel 2016, Ponath et al. 2022), future studies should address their possible role in the remarkable abundance and stability of these two *rdhAB* transcripts.

The remaining *rdhA* transcripts with normalized read numbers > 100 (Fig. 1) are mostly encoded upstream of inversely oriented regulatory genes: cbdbA187 lies upstream of TCS-encoding genes, while five others (cbdbA1453, cbdbA1455, cbdbA1508, cbdbA1618 and cbdbA1624) are located upstream of *rdhR* genes encoding a putative MarR-type regulator. All of these regulatory genes were transcribed (Table S2), further supporting the notion of their role in *rdhAB* gene regulation (Kube et al. 2005).

As an exception, the *rdhA* cbdbA1638 gene is not directly associated with a regulatory gene. The orthologue DET1545 in *D. mccartyi* strain 195 was reported to be expressed during the entry into the stationary phase or under growth-limiting conditions (Johnson et al. 2008, Rahm and Richardson 2008, Lee et al. 2012, Mansfeldt et al. 2014). The apparent up-regulation of cbdbA1638 in the 1,2,4-TCB-incubated cultures possibly reflects the typical fluctuations that occur in the transition phase, suggesting that these cells were slightly closer to the entry point of the stationary phase.

sRNAs and a small open reading frame encoded in intergenic regions

In order to identify non-coding RNAs that might be involved in other levels of expression control, such as in post-transcriptional regulation, the RNA-seq data were screened for putative sRNAs. An IGB search for transcription in intergenic regions revealed three abundant monocistronic transcripts (Tables S2 and S3 in the Supplementary material). Based on their high abundance, predicted size, secondary structure and σ^{70}-binding sites, as well as their sequence conservation (87.5–100% identity) in *D. mccartyi* genomes, we annotated these transcripts as putative *D. mccartyi* sRNAs, designated sRNA1 to sRNA3. They do not match any Rfam family (Ontiveros-Palacios *et al*. 2025). However, sRNA1 has characteristics of 6S RNA, a global regulator of σ^{70}-dependent transcription (Sharma et al. 2010, Wassarman 2018). It shows a very high transcript level (the third highest abundance after 16S and 23S rRNA under both conditions, Table S2) and a size of 188 nt. In addition, the predicted hairpin structure with a central asymmetric loop and small antisense transcripts (putative product RNAs) (Fig. S3 in the Supplementary material), all suggest this represents the 6S RNA. sRNA1 and sRNA2 are encoded in the core region of the genome, encoding the essential metabolic functions, whereas sRNA3 is part of a 12 kb syntenic region conserved in all *D. mccartyi* genomes and localized at the end of high plasticity region 2 (McMurdie et al. 2009), relatively close to *rdhA* cbdbA1638, which showed a concordant transcriptional response. In addition, one so far unannotated ORF was transcribed, encoding a 23 amino acid long protein, pep-

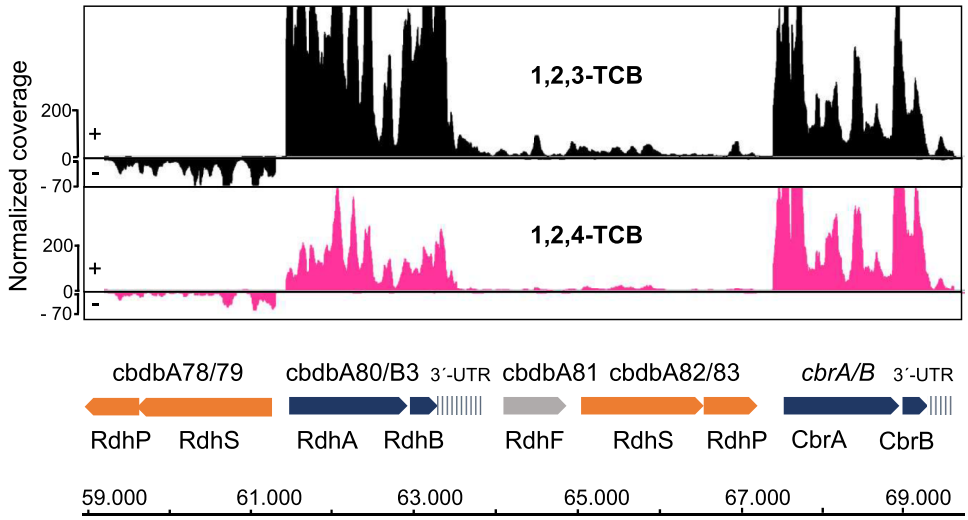

Figure 2 Transcription profiles of the genomic region (positions 59000–69 600) harbouring the *rdhA*/*rdhB* genes cbdbA80/cbdbB3 and *cbrA*/*cbrB* during cultivation with either 1,2,3- or 1,2,4-TCB. The coverages of transcripts are shown for the forward (+) and reverse (-) strands as normalized read numbers per nucleotide of the reference genome. The "Integrated Genome Browser" (v. 9.0.1) was used for data visualization (Freese et al. 2016). The genes in this region are colored according to their function: *rdhA*/*B* in blue, *rdhS*/*P* in orange, and *rdhF* in gray. RdhP and RdhS are the response regulator and sensor histidine kinase of two component-system and RdhF is a CbiZ-like protein, which all are postulated to be involved in OHR (Kruse et al. 2016). Both *rdhA* transcripts belong to the most highly expressed protein-coding sequences in the genome of strain CBDB1 (Table S2 in the Supplementary material). Note the extended 3´-untranslated regions (3´-UTR) of the bicistronic *rdhA*/*B* transcripts.

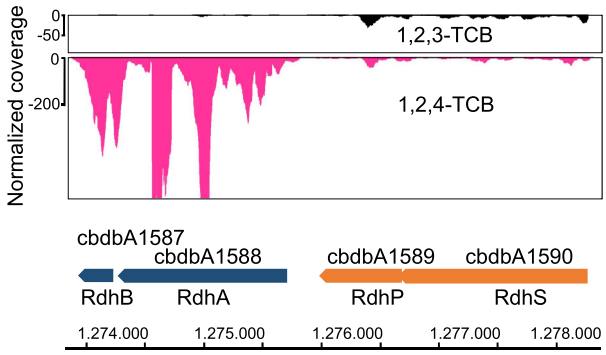

Figure 3 Transcription profiles of the genomic region (positions 1 273 800–1 278 500) harbouring the *rdhA*/*rdhB* genes cbdbA1588/cbdbA1587 (blue) during cultivation with 1,2,3- and 1,2,4-TCB, indicating their distinct transcription only in the presence of 1,2,4-TCB. In contrast, the associated genes encoding the response regulator RdhP and the sensor histidine kinase RdhS (orange) of a two component-system are transcribed under both conditions. The coverages of transcripts are shown for the reverse strand as normalized read numbers per nucleotide of the reference genome. The "Integrated Genome Browser" (v. 9.0.1) was used for data visualization (Freese et al. 2016).

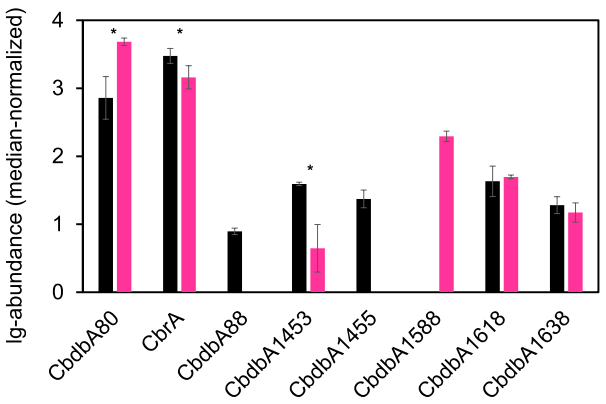

Figure 4 Abundance of RdhAs in the proteome of cells grown with 1,2,3-TCB or 1,2,4-TCB (black and purple bars, respectively) as electron acceptors. Cells were harvested in the early stationary phase. Mean values and SD from three biological replicates are shown. Only RdhAs present in at least two of three replicates were quantified and displayed. ∗Significantly different median-normalized abundances (Benjamin-Hochberg adjusted *P*-values < 0.05).

tides of which were detected in the LC/MS library of the proteome (Tables S3 and S4 in the Supplementary material).

Proteomics of cells grown with 1,2,3-TCB, 1,2,4-TCB or HCB as electron acceptor

Proteomics was used to examine the differential synthesis of RdhA proteins. In the first experiment, strain CBDB1 was cultivated with either 1,2,3-TCB or 1,2,4-TCB and harvested in the stationary phase. A total number of 855 and 881 proteins (averages of triplicates) were detected in cells grown with 1,2,3- and 1,2,4-TCB, respectively. Eight RdhAs were detected in at least one condition (Fig. 4, Table S4 in the Supplementary material). Five of these were detected under both conditions, two and one with only 1,2,3-TCB or 1,2,4-TCB, respectively. The three most abundant RdhAs detected corroborated the transcriptome data: RdhA CbdbA80 and CbrA were the most abundant during growth in the presence of either TCB. Interestingly, the abundances of CbrA and

RdhA CbdbA80 were significantly different between the two conditions, with RdhA CbdbA80 exhibiting the highest abundance with 1,2,4-TCB and CbrA with 1,2,3-TCB. RdhA CbdbA1588 was abundant in samples cultivated with 1,2,4-TCB, whereas it was not detectable in 1,2,3-TCB cultures. The next most abundant RdhAs were CbdbA1618, CbdbA1638, CbdbA1453 and CbdbA1455, which also corresponds well with the transcriptional data for their cognate genes (Fig. 1). Functional RdhA(s) induced by a specific compound often persist for a comparatively long time in cultures, even after the depletion of this compound, or the transfer to a different organohalogen, requiring induction of another functional RdhA (Franke et al. 2020). To reduce the abundance of possible "background", stable RdhAs and to verify the specific induction of RdhA CbdbA1588 by 1,2,4-TCB, a second, long-term experiment was set up. Starting from one preculture incubated with 1,2,3-TCB, three culture lines were each established in triplicate. One was incubated with 1,2,3-TCB, a second with 1,2,4-TCB and the third with HCB as a control. Strain CBDB1 dechlorinates HCB via pentachloro- and 1,2,3,5 and 1,2,4,5-tetrachlorobenzene intermediates to 1,3,5- and 1,2,4-TCB; the latter compound is finally dechlorinated to 1,3- and 1,4-DCB (Jayachandran et al. 2003) and the expression of RdhA CbdbA1588 has been reported (Schiffmann et al. 2014, Seidel et al. 2018). All established cultures were grown to the stationary phase and transferred a further three times into fresh medium. From the second transfer on, all replicate cultures were sampled in the stationary phase and the proteomes were analyzed (Table S5 in the Supplementary material). The total number of proteins in each transfer was similar regardless of the cultivation with either 1,2,3-TCB, 1,2,4-TCB or HCB. The mean values varied from 323 in the second, to 605 in the third and 331 in the fourth transfer (for details see Table S5). Twelve RdhAs were detectable (Table 1). CbrA and RdhA CbdbA80 were the two most abundant RdhAs in the presence of all three electron acceptors. This is in agreement with published data for a range of halogenated benzenes (Wagner et al. 2012, Schiffmann et al. 2014, Kublik et al. 2016, Seidel et al. 2018). Our data further support a role for both RdhAs in the reductive dehalogenation of halogenated benzenes, although experimental evidence for RdhA CbdbA80 carrying out such a reaction is still lacking.

As anticipated, RdhA CbdbA1588 was not detected in any replicate incubated with 1,2,3-TCB, whereas it was formed without exception in all replicates containing 1,2,4-TCB and HCB (Table 1). In the samples from the second transfer of each of the three replicates of the HCB cultures, 1,2,4-TCB was quantified by gas chromatography as having a concentration of 2.8, 4.7, and 6.7 μM in the aqueous phase for replicates 1 through 3, respectively. In light of the comparative transcriptome and proteome data we conclude that induction and synthesis of RdhA1588 in HCB-incubated cultures is triggered by the transiently formed 1,2,4-TCB and has a crucial role in the efficient dechlorination of HCB. Among the somewhat less abundant RdhAs were again CbdbA1453, CbdbA1455, CbdbA1638, and CbdbA1618 and additionally CbdbA1624; however, they exhibited some variation in their respective abundance and substrate affiliation. In addition, some RdhAs were only occasionally detectable, such as CbdbA88 with 1,2,3-TCB in the first (Fig. 4) and only in one replicate with HCB in the second experiment (Table 1), CbdbA1508 and CbdbA1491 only in selected transfers of the second experiment (Table 1). This variability might be due to the limiting amount of protein extractable from such a slow-growing and small bacterium like *Dehalococcoides*, which might lead to the poor reproducibility in detecting less abundant RdhA proteins.

Functionality of RdhA CbdbA1588

The observed strong up-regulation in synthesis of RdhA CbdbA1588 does not *per se* indicate that the RdhA is active and involved in 1,2,4-TCB dechlorination. Since the enrichment and purification of RdhA CbdbA1588 from strain CBDB1 is hard to achieve due to the abundant synthesis of two other RdhAs and the heterologous expression is still challenging (Parthasarathy et al. 2015), we have used an indirect approach to study its functionality using 2,3-DCP as a substrate. The orthologous *pceA* gene (DET0318) in *D. mccartyi* strain 195 of cbdbA1588 is strongly induced by PCE and also by 2,3-DCP (Fung et al. 2007). Strain 195 and strain CBDB1 both can dechlorinate 2,3-DCP (Adrian et al. 2007a). RdhA CbdbA1588 was the most abundant RdhA in the proteome during growth of CBDB1 with 2,3-DCP as electron acceptor (Morris et al. 2007), suggesting it is responsible for 2,3-DCP dechlorination. Based on this hypothesis, we tested 2,3-DCP dechlorination by crude extracts from 1,2,4-TCB- and 1,2,3-TCB-grown cells. Each extract was analyzed with 1,2,3-TCB, 1,2,4-TCB and 2,3-DCP as substrate (Table 2). Whereas the specific dehalogenase activity towards 1,2,3-TCB did not significantly differ between extracts originating from 1,2,3-TCB- and 1,2,4-TCB-cultures, the specific activity towards 1,2,4-TCB increased two-fold in extracts from 1,2,4-TCB-grown cultures. In addition, the extract derived from 1,2,4-TCB-grown cultures showed a ten-fold higher activity towards 2,3-DCP, clearly correlating with the presence of RdhA CbdbA1588 and suggesting its functionality. Notably, extracts from 1,2,4-TCB-grown cells showed an increased ratio of the dechlorination products 1,4-DCB versus 1,3-DCB from 1,2,4-TCB (Table 2), further supporting a contribution of RdhA CbdbA1588 to the dechlorination of 1,2,4-TCB. However, even in the absence of RdhA CbdbA1588 (cell-free extract derived from 1,2,3-TCB-grown cells), a relatively low, but distinct, activity towards both 1,2,4-TCB and 2,3-DCP was observed, perhaps suggesting a broader substrate spectrum of CbrA and/or RdhA CbdbA80. This argues in favour of a potential concerted action of different RdhAs on 1,2,4-TCB as electron acceptor, with the PceA orthologue being the prerequisite for the productive dechlorination of singly-flanked chlorines. This is also suggested by the observation that *D. mccartyi* strain DCMB5, a close relative of strain CBDB1, which harbours *cbrA* and *rdhA* cbdbA80 orthologues, but has no *pceA* orthologous gene in its genome (Pöritz et al. 2013), is unable to grow with 1,2,4-TCB as electron acceptor and produced only negligible amounts of 1,4-DCB from 1,2,4-TCB (Pöritz et al. 2015). Interestingly, *D. mccartyi* strain 195, which harbours a *pceA* gene in its genome, but lacks *cbrA* or *rdhA* cbdbA80 orthologues, is also unable to use 1,2,4-TCB as electron acceptor (Fennell et al. 2004), which might be due to the 6.3% sequence difference between PceA and RdhA CbdbA1588. The effect on the substrate spectrum of even a lower amino acid sequence variation of a RdhA has been recently observed (Yan et al. 2021). In addition, the regulatory capacity of a strain might influence its adaptive response to specific halogenated substrates, as suggested by the observation that strain 195 was able to dechlorinate 1,2,4-TCB, when PCE had been added as an inducer (Fennell et al. 2004). Another *D. mccartyi* strain, BTF08,

Table 1 Abundance of RdhAs in the proteomes of cells from the 2nd, 3rd and 4th transfer of cultures grown with either 1,2,3-TCB, 1,2,4-TCB or HCB as electron acceptor.

RdhA (name/ locus tag)	Electron acceptor								
	1,2,3-TCB			1,2,4-TCB			HCB		
	2nd	3rd	4th	2nd	3rd	4th	2nd	3rd	4th
cbdbA80	27.9	25.5	29.1	30.3	28.0	30.0	29.7	28.1	29.8
CbrA	30.0	28.8	29.7	29.2	26.7	28.7	30.4	29.6	29.0
cbdbA1453	27.6	28.1	27.8	25.8	id.	26.2	27.8	26.7	25.7
cbdbA1588	–	–	–	27.2	25.9	25.5	24.2	25.3	26.9
cbdbA1624	26.6	28.4	26.6	25.2	id.	26.7	27.3	26.9	25.6
cbdbA1638	–	id.	24.7	24.0	id.	24.7	24.9	id.	24.2
cbdbA1455	id.	id.	24.8	–	–	–	23.4	id.	–
cbdbA1618	–	–	id.	23.9	–	id.	–	–	25.7
cbdbA1092	id.	id.	–	–	–	–	–	–	–
cbdbA1491	–	–	25.5	–	–	27.6	id.	id.	id.
cbdbA1508	–	–	–	25.6	–	–	–	–	id.
cbdbA88	–	–	–	–	–	–	–	id.	–

Shown are the $\log_2$transformed, median-normalized MS1 areas. Only RdhAs present in at least two of three replicates were quantified and mean values are given. id., peptides identified in one replicate; -, not detected.

Table 2 Specific dechlorination activities of crude extracts from cells grown with either 1,2,3-TCB or 1,2,4-TCB as electron acceptor

Cells grown with[a]	Specific dehalogenase activity (pkat mg^{-1})[b] determined with			Ratio of 1,4-DCB vs. 1,3-DCB formed from 1,2,4-TCB
	1,2,3-TCB	1,2,4-TCB	2,3-Dichlorophenol	
1,2,3-TCB	171.0 ± 28.3	38.0 ± 2.7	103.6 ± 6.2	1.4 ± 0.1
1,2,4-TCB	142.8 ± 53.7	77.1 ± 4.0	1070.0 ± 433.0	2.3 ± 0.3

[a] Cultures were incubated with the indicated electron acceptor (nominal 10 mM concentration) and harvested for cell extract preparation at a cell density of 1.6×10^8–2.4×10^8 ml^{-1}.
[b] Activity was calculated based on the concentration of the dechlorination product(s) formed after 2.5 h incubation (protein concentration: 0.71–0.77 mg ml^{-1}). Mean value and SD of triplicate assays are shown, each measured in duplicate. The ratio of 1,4-DCB versus 1,3-DCB formation from 1,2,4-TCB as assay substrate is given as the mean value and SD of two (1,2,3-TCB) and three (1,2,4-TCB) independent experiments.

also encodes a PceA orthologue (Bft_1454) in its genome (Pöritz et al. 2013), but the strain does not respire with 1,2,4-TCB (Kaufhold et al 2013). During PCE respiration, the strain does not synthesize PceA, but rather the RdhA Btf_1393 (Franke et al. 2020), a close homologue of PteA (Zhao et al. 2017) (Fig. 5). This suggests the suppression of *pceA* expression, at least under the tested conditions. Nevertheless, *pceA* might prove to be a useful biomarker if targeted on the transcript or protein level for the indication of an ongoing remediation in anoxic habitats contaminated with multiple compounds such as PCE and halogenated aromatics, particularly those with singly-flanked chlorines.

PceA is one representative of the (at least) five orthologue groups (OG) of RdhAs (Molenda et al. 2020) dehalogenating both aromatic halogenated compounds and tetrachloroethene (Fig. 5). They vary in their specific aromatic substrates and site-specific removal of halogens. PcbA1 (OG 67), PcbA4 and PcbA5 (OG 35) dechlorinate PCBs (Wang et al. 2014), while PteA (OG 16) debrominates polybrominated diphenyl ethers (PBDE) (Zhao et al. 2021). The PCE-dehalogenase MbrA (OG 52) of strain MB additionally dehalogenates PBDEs (metabolically) and PCBs (co-metabolically) (Xu et al. 2022). Interestingly, RdhA CbdbA80 in strain CBDB1 is a homologue of MbrA (Fig. 5). Its functional similarity to MbrA is suggested by the ability of strain CBDB1 to dehalogenate PCE at least partly to *trans*-DCE (Marco-Urrea et al 2011), a property that has been first assigned to MbrA (Chow et al. 2010). The abundant synthesis of RdhA CbdbA80, and particularly its up-regulation in the presence of 1,2,4-TCB and HCB (Table 1), suggests a role in the dechlorination of chlorinated benzenes and possibly indicates an even broader substrate spectrum of MbrA orthologues. Based on the presence of *pceA* orthologues in all three *D. mccartyi* clades and on the number of mutations it was calculated that *pceA* was present before clade separation (Molenda et al. 2020), demonstrating the importance of this OG during *D. mccartyi* evolution. The members of most other PCE and aromatics dehalogenating RdhAs such as PteA, MbrA, and PcbA show a high pairwise identity in different strains regardless of clades and are associated with mobile genetic elements (Molenda et al. 2020), suggesting their facile horizontal transfer. This, together with their broad substrate spectrum, may be important factors for the rapid establishment of *D. mccar-*

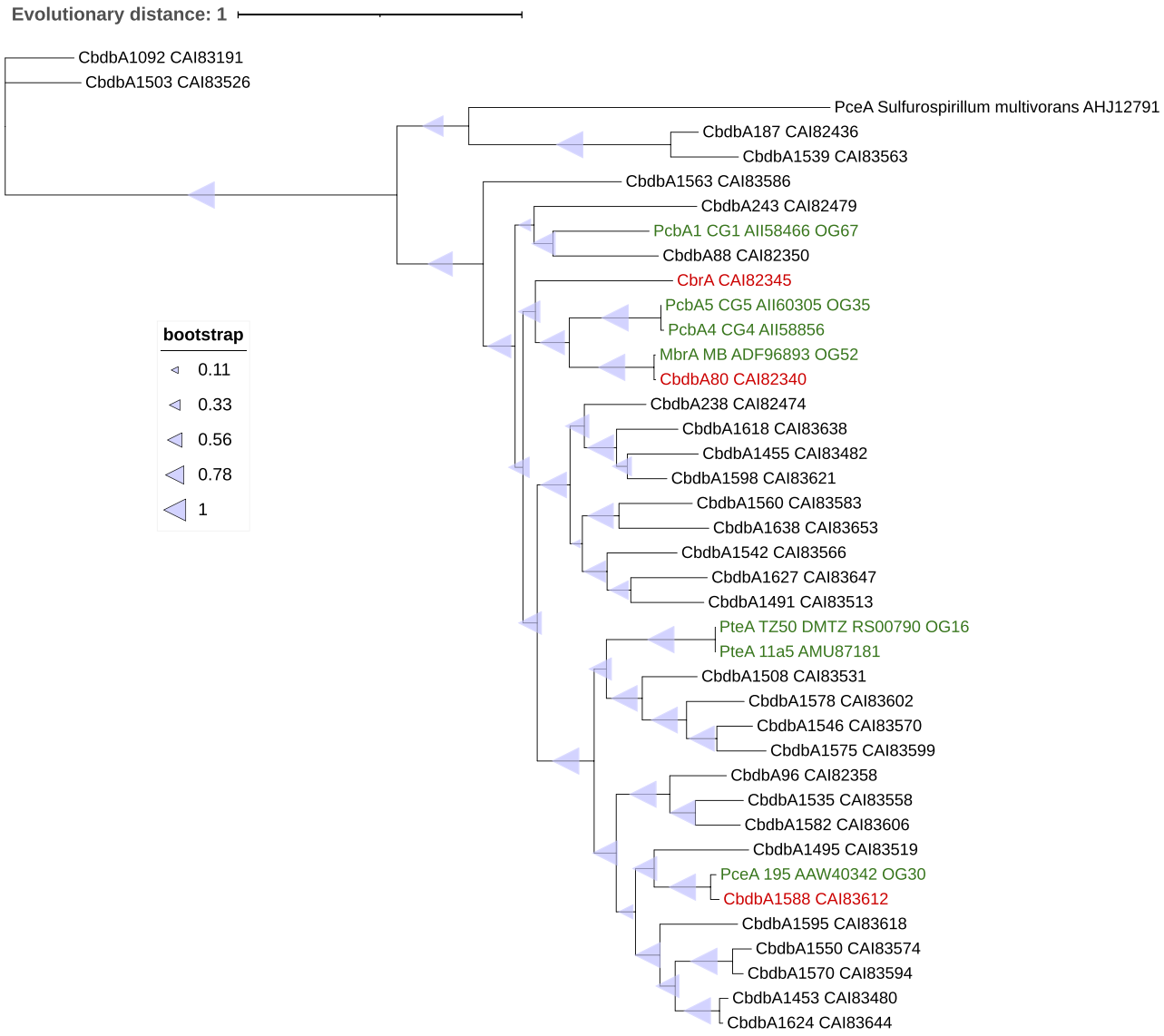

Figure 5 Phylogenetic tree of all 32 reductive dehalogenases (RdhAs) encoded in the genome of *D. mccartyi* strain CBDB1 and of functionally characterized RdhAs from other *D. mccartyi* strains, which are able to dechlorinate both tetrachloroethene (PCE) and aromatic halogenated compounds. PceA of *S. multivorans* was included for comparison. Tree construction was based on the fast maximum likelihood tree estimation program provided by NGPhylogeny (Lemoine et al. 2019). Accession numbers of amino acid sequences refer to the NCBI GenBank database. The RdhAs of strain CBDB1 and of PceA of *S. multivorans* are shown in black. The three most abundant RdhAs in strain CBDB1 during dehalogenation of 1,2,3- and 1,2,4-TCB and HCB are highlighted in red. The functionally characterized RdhAs, including their affiliation to specific *D. mccartyi* strains and to their RdhA OG (Molenda et al. 2020), are indicated in green. The evolutionary distance indicates substitutions per site. The bootstrap branch support of SH-like aLRT analysis is marked in blue.

tyi populations at anoxic sites heavily contaminated with multiple halogenated pollutants through human activity (e.g. Nijenhuis et al. 2018).

Conclusion

Our data demonstrate an isomer-specific regulation of *rdhA* gene expression during growth with 1,2,3- and 1,2,4-TCB as electron acceptors. The combination of transcriptomic and proteomic studies and dehalogenase activity measurements revealed the involvement of the PceA orthologue, CbdbA1588, in the dechlorination of 1,2,4-TCB and, consequently, sheds light on its role in the multistep dechlorination of HCB in strain CBDB1. The high 2,3-DCP reductive dehalogenase activity of cells expressing the *rdhA* cbdbA1588 gene and, additionally, its induction by 2,3-dichlorodibenzo-*p*-dioxin (Wagner et al. 2013), also corroborate that the gene product, CbdbA1588, might confer upon strain CBDB1 the capability to remove not only doubly-flanked, but also singly-flanked chlorines from the aromatic ring. This extends the spectrum of accessible electron acceptors in the field and possibly also increases the amount of energy that can be conserved by making use of the more efficient dehalogenation of a single polyhalogenated compound. Our study also reveals for the first time abundant putative sRNAs encoded in the *D. mccartyi* genome, one

of which is spatially proximal to an *rdh* operon encoded, providing an interesting target for future regulatory studies.

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Author contributions

F.G.H. (Investigation, Methodology, Writing – original draft), D.T. (Formal Analysis, Investigation, Writing – original draft), S.S. (Investigation), T.B. (Data curation, Formal Analysis, Writing – review & editing), N.J. (Data curation, Writing – review & editing), M.v.B. (Funding acquisition, Methodology), K.F. (Methodology), C.M.S. (Conceptualization, Methodology), G.S. (Conceptualization, Funding acquisition, Writing – review & editing), T.G. (Conceptualization, Data curation, Writing – review & editing), U.L. (Conceptualization, Funding acquisition, Methodology, Writing – review & editing).

Supplementary material

Supplementary material is available at *FEMSEC Journal* online.

Conflicts of interest

None declared.

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Data availability statement

The transcriptome data are available at NCBI Gene Expression Omnibus (GEO; Barrett et al. 2013) under accession number GSE306994.

(https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE306994).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al. 2025) partner repository with the dataset identifier PXD069682.

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