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Regulation of reductive dehalogenase gene transcription in *Dehalococcoides mccartyi*

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The remarkable capacity of the genus *Dehalococcoides* to dechlorinate a multitude of different chlorinated organic compounds reflects the number and diversity of genes in the genomes of *Dehalococcoides* species encoding reductive dehalogenase homologues (*rdh*). Most of these genes are located in the vicinity of genes encoding multiple antibiotic resistance regulator (MarR)-type or two-component system regulators. Here, the transcriptional response of *rdhA* genes (coding for the catalytic subunit) to 2,3- and 1,3-dichlorodibenzo-*p*-dioxin (DCDD) was studied in *Dehalococcoides mccartyi* strain CBDB1. Almost all *rdhA* genes were transcribed in the presence of 2,3-DCDD, albeit at different levels as shown for the transcripts of *cbrA*, cbdbA1453, cbdbA1624 and cbdbA1588. By contrast, 1,3-DCDD did not induce *rdhA* transcription. The putative MarR CbdbA1625 was heterologously produced and its ability to bind *in vitro* to the overlapping promoter regions of the genes cbdbA1624 and cbdbA1625 was demonstrated. To analyse regulation *in vivo*, single-copy transcriptional promoter–*lacZ* fusions of different *rdhA* genes and of cbdbA1625 were constructed and introduced into the heterologous host *Escherichia coli*, and expression levels of the fusions were measured. The cbdbA1625 gene was cloned into a vector allowing a regulation of expression by arabinose and it was transformed into the strains containing the *rdh*-promoter–*lacZ* fusion derivatives. CbdbA1625 was shown to downregulate transcription from its own promoter resulting in a 40–50% reduction in the β-galactosidase activity, giving the first hint that it acts as a repressor.

1. Introduction

The obligately organohalide-respiring *Dehalococcoides mccartyi* strains isolated so far are able to dehalogenate a variety of organohalides ranging from chlorinated aliphatic to chlorinated aromatic compounds. These include notoriously recalcitrant compounds such as polychlorinated biphenyls or dibenzo-*p*-dioxins. From an evolutionary standpoint, this broad substrate specificity is logical and because nature harbours a multitude of halogenated compounds [1]; however, each of these is present at relatively low concentrations compared with the abundance of electron acceptors such as sulfate or carbonate that provide the basis for other anaerobic respiratory processes. Recent results [2] demonstrated a positive correlation between the number of *Dehalococcoides*-like Chloroflexi and the natural organochlorine content in forest soils. It is thought that the high number of up to 36 non-identical genes encoding putative reductive dehalogenases (*rdh*) in the *D. mccartyi* genomes [3–5] forms the genetic basis for the capability to attack chlorinated compounds with a broad range of electronic and steric properties. Reductive dehalogenases consist of a catalytic cobalamin- and [Fe–S] cluster-containing subunit facing the outer side of the cytoplasmic membrane and a putative membrane anchor, encoded by the *rdhA* and *rdhB*

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Table 1. Number of *rdhAB* genes and of genes encoding putative MarR-type and two-component system regulators in the genomes of *D. mccartyi* strains.[a]

strain	*rdhAB* genes	genes encoding TCS regulators		genes encoding MarR-regulators	
		total	adjacent to *rdhA* genes	total	adjacent to *rdhA* genes
CBDB1	32	24	14	16	10
195	17	19	10	8	3
BAV1	11	13	5	4	1
GT	20	3	1	12	7
VS	36	21	11	14	10

[a]According to references [3,4] and genome searches using BioCyc tools [11]. The regulator-encoding genes were usually located upstream and in opposite orientation to the *rdhA* genes.

genes, respectively. Owing to the large number of *rdhAB* operons, it can be assumed that their synthesis is controlled by regulatory events.

This assumption is supported by data obtained with another group of organohalide-respiring bacteria, the chlorophenol-dechlorinating members of the Gram-positive genus *Desulfitobacterium* [6]. In contrast to *Dehalococcoides*, these bacteria are facultative organohalide respirers, and regulation must confer an adequate response to the presence of alternative electron acceptors such as nitrate or chlorinated compounds in the environment or even to fermentative growth conditions. The *o*-chlorophenol reductive dehalogenase-encoding genes *cprBA* were specifically induced in the presence of chlorinated phenols by high affinity binding of the CRP/FNR-type transcriptional activator CprK to target sequences (dehaloboxes) in the promoter regions within the *cpr* gene cluster [7]. CprK is activated by an allosteric mechanism. Binding of *o*-chlorophenols leads to conformational changes, which are required for specific DNA binding [8]. An examination of the genome sequences of *D. mccartyi* and relatives for the presence of putative regulators of the CRP/FNR-type revealed that they are present in the genomes of *D. mccartyi* strains 195 and CBDB1 and of *Dehalogenimonas lykanthroporepellens* BL-DC-9, but no orthologues were annotated in the genomes of the *D. mccartyi* strains GT, VS and BAV-1, ruling out a general role in *D. mccartyi* organohalide respiration.

In the *vcrAB* operon, encoding a vinyl chloride (VC) reductive dehalogenase, in *D. mccartyi* strain VS a gene is present coding for a putative regulator of the NosR/NirI-type [9]. It shows some similarity to CprC, which is also encoded in the *cpr* gene cluster of the *o*-chlorophenol-respiring *Desulfitobacterium dehalogenans* [10]. Although closely related orthologues (94–95% identity) are found in the genomes of the two *D. mccartyi* strains 195 and CBDB1, these are not associated with *rdh* genes. Interestingly, two other types of regulators are frequently encoded in the vicinity of *rdhAB* genes in all described *D. mccartyi* genomes: two-component system (TCS) regulators and MarR-type regulators (table 1).

TCS regulators typically consist of a histidine kinase (HK) and a response regulator (RR). HKs possess a modular architecture with diverse input domains linked to a conserved catalytic core, which allows the coupling of a variety of input signals to output responses through a conserved autophosphorylation and phosphotransfer pathway [12]. Localization of the sensory domains on extracytoplasmic or membrane-spanning regions allows direct signal perception at the cell periphery; however, the 14 putative HKs encoded in the vicinity of *rdh* genes in strain CBDB1 have no transmembrane helices and are predicted to be cytoplasmic proteins [3]. For 13 of these HKs, at least one Per–Arndt–Sim (PAS) fold is predicted as part of the input domain. PAS domains can sense a variety of signals such as oxygen, light, redox potential or the presence of small molecules [13] and can bind cofactors such as haem or flavin. In general, upon signal recognition, a histidine residue in the HK is autophosphorylated, and the phosphoryl group is transferred to an aspartate of the RR. The RR proteins in *D. mccartyi* predicted to be involved in organohalide respiration possess a signal receiver domain with a conserved aspartate and a C-terminal effector domain forming a DNA-binding winged helix, which could mediate activation of transcription of target genes [14]. The range of signals sensed by the cognate sensor kinase can be extended by the so-called TCS connector proteins, transmitting signals from other regulatory circuits to TCS regulators by protein–protein interaction [15,16]. Therefore, the TCS regulators encoded in the genome of *D. mccartyi* might represent important target molecules for the integration of different signals such as the redox state of the cell or the presence of organochlorines.

The multiple antibiotic resistance regulator (MarR) protein family is distributed throughout the bacterial and archaeal domains and mediates cellular responses to changing environmental conditions, such as antibiotic or peroxide stress and adaptation to the catabolism of aromatic compounds [17]. The latter fact is interesting in the light of the capability of strains of *D. mccartyi* and its close relatives to use chlorinated aromatics for organohalide respiration. In general, the MarRs of aromatic compound metabolism act as repressors. Binding of the aromatic ligand releases the repressor from the promoter and leads to the induction of the regulated gene(s). Structurally, the reported aromatic ligands range from salicylate (MarR) [18], 3-chlorobenzoate [19], 2-methylhydroquinone [20] to flavonoids [21]. The genes encoding MarR proteins are generally part of the gene cluster that they regulate and are divergently oriented to them. This is also the case for most *rdhA* gene-associated *marR* genes in *D. mccartyi* genomes.

The typical structural element of MarR proteins is the winged helix–turn–helix DNA recognition fold. Some structures of MarRs have been resolved with and without bound ligand, providing evidence for the displacement of the

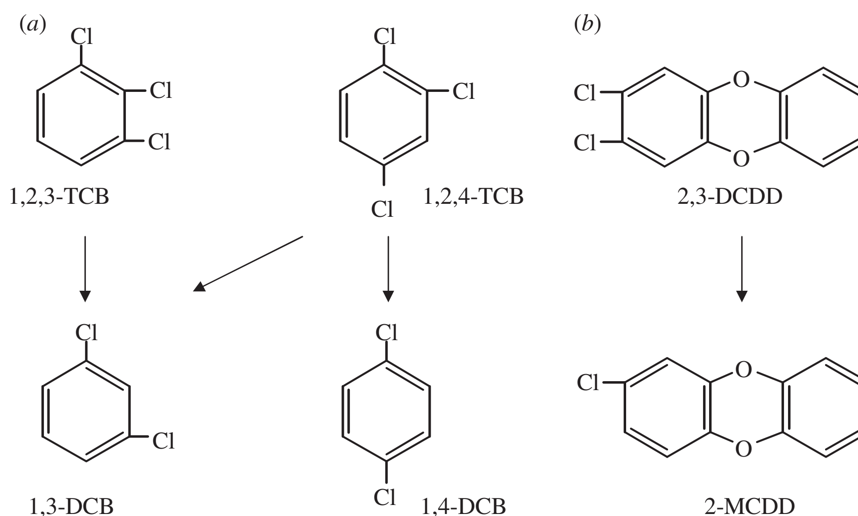

Figure 1. Scheme of (*a*) reductive dechlorination of 1,2,3- and 1,2,4-trichlorobenzene (TCB) to 1,3- and 1,4-dichlorobenzene (DCB) [32] and (*b*) 2,3-dichlorodibenzo-*p*-dioxin (DCDD) to 2-monochlorodibenzo-*p*-dioxin (MCDD) [33].

DNA-binding helix upon ligand binding [22]. Interestingly, the *rdh*-associated regulators form a separate branch in the phylogenetic tree of MarRs (see the electronic supplementary material, figure S1). It is also interesting that putative sensory and catalytic proteins in organohalide respiration seem to have co-evolved, as indicated by the conservation of gene clusters comprising a specific *rdh* gene and its associated regulatory gene in different *D. mccartyi* genomes.

First indications of a differential expression of *rdhA* genes in response to specific compounds came from transcription analyses of *D. mccartyi* in pure and mixed cultures that dechlorinate chlorinated ethenes. During VC respiration by strain BAV1, the gene *bvcA* was highly expressed, suggesting its gene product functions as a VC reductase, and indeed, it was detected in several VC-dechlorinating mixed cultures [23,24]. Another gene encoding a functional VC reductase was also induced by VC [9]. In addition, albeit at lower levels, transcripts of further *rdhA* genes were formed, suggesting that multiple *rdhA* genes are induced by a single chlorinated ethene [24]. Studies with trichloroethene (TCE)- and tetrachloroethene (PCE)-grown cells of strain 195 indicated that the genes encoding the PCE and TCE reductive dehalogenases (PceA, TceA) were transcribed to the highest levels among all 17 *rdhA* genes [25], and the corresponding proteins were identified with a high coverage in the proteome of the respective cells [26]. Furthermore, *rdhA* gene expression levels depended on the growth phase and the PCE respiration rate [27–29]. Consequently, they were used as biomarkers for active PCE or TCE dechlorination in technical and groundwater systems [30,31].

The *D. mccartyi* strain CBDB1 is adapted to organohalide respiration with chlorinated aromatic compounds such as different chlorinated benzenes, phenols, biphenyls and even chlorinated dibenzo-*p*-dioxins [32–35]. Figure 1 shows three selected chloroaromatic compounds and the dechlorination route observed with strain CBDB1. With regard to the dechlorination of polychlorinated dibenzo-*p*-dioxins, strain CBDB1 can even dechlorinate the most toxic congeners 1,2,3,7,8-penta- and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to 2,7- or 2,8-dichlorodibenzo-*p*-dioxin (DCDD), resulting in a strong reduction in the toxicity [33]. The transcriptional response of all 32 *rdhA* genes of strain CBDB1 during dechlorination of 1,2,3- and 1,2,4-trichlorobenzenes (TCBs) was investigated using a combined reverse transcription (RT)-PCR/terminal restriction fragment length polymorphism analysis (t-RFLP) approach [36]. This approach enabled the simultaneous detection of 29 of 32 *rdhA* transcripts. The transcription of the remaining three *rdhA* genes was also detected by reverse transcription-quantitative PCR (RT-qPCR). In summary, the upregulation of all 32 *rdhA* genes could be observed following the addition of 1,2,3- or 1,2,4-TCB. However, a differential upregulation of at least two *rdhA* genes, cbdbA1453 and cbdbA1624, in response to 1,2,3- and 1,2,4-TCB, respectively, was suggested by the results. RT-qPCR revealed that the transcript levels of ten analysed *rdhA* genes differed by several orders of magnitude. As expected, the *rdhA* gene *cbrA*, encoding a chlorobenzene reductase [37], was transcribed at the highest level in the presence of both TCBs [36]. Although *cbrA* is co-localized with genes encoding a TCS system, cbdbA1624 and cbdbA1453 are preceded by *marR* genes suggesting that different regulatory principles might be involved in controlling the dechlorination of 1,2,3-TCB and 1,2,4-TCB.

In this study, we report the results of transcription analyses of *rdhA* genes in response to two chlorinated dibenzo-*p*-dioxins. The two congeners 1,3- and 2,3-DCDD were chosen because they are dechlorinated by strain CBDB1 in one step to 2-monochlorodibenzo-*p*-dioxin (MCDD) (shown for 2,3-DCDD in figure 1). We also addressed the possible role of MarR-type regulators in organohalide respiration and focused on cbdbA1625, a *marR* gene preceding the *rdhA* gene cbdbA1624, which shows a specific transcriptional response to 1,2,4-TCB [36]. The putative MarR CbdbA1625 was heterologously produced in *Escherichia coli* and analysed *in vitro* for its ability to interact with the intergenic region between cbdbA1624 and cbdbA1625. Furthermore, promoter–reporter gene fusions were developed to analyse the functionality of certain *marR* and *rdhA* gene promoters in the *E. coli* host and to investigate the regulation of the system *in vivo* by the MarR CbdbA1625.

2. Material and methods

(a) Bacterial strains, plasmids and culture conditions

Dehalococcoides mccartyi strain CBDB1 was grown in Ti(III) citrate-reduced, carbonate-buffered synthetic medium with

hydrogen as electron donor and 5 mM acetate as carbon source as described previously [36]. Two-liquid phase cultures (50 ml) supplemented with 1,2,3-TCB (200 mM, with a nominal concentration of 10 mM) dissolved in hexadecane served as inoculum. 1,3- and 2,3-DCDD (AccuStandard, New Haven, CT, USA) were added from 300 μM stock solutions in acetone to several replicates of empty 100 ml serum bottles and in parallel to 20 ml gas chromatography (GC) vials at final concentrations of 15–40 μM. The acetone was evaporated in a stream of filter-sterilized N_2 gas before the bottles and vials were closed with Teflon-coated butyl rubber stoppers, filled with 50 ml and 3 ml, respectively, of sterile, anaerobic medium and inoculated to 10% (v/v) with the pre-culture resulting in 5×10^7 cells ml^{-1} as determined by qPCR targeting the 16S rRNA gene [36]. Cultures were statically incubated at 30°C.

The *E. coli* strains, plasmids and phages used in this study are given in table 2. The *E. coli* strains were grown at 37°C in nutrient or lysogeny broth (LB) supplemented with the appropriate antibiotics, diagnostic substrates or inducers according to standard procedures [42]. BL21(DE3)-CodonPlus-RIL served as host for the cbdbA1625 expression vectors derived from pASK-IBA5 and pASK-IBA3 (IBA, Göttingen, Germany). *Escherichia coli* XL1-Blue MRF′ (Stratagene, Amsterdam, The Netherlands) was used as host for the cloning vectors pGEM T-Easy and pRS551. The λ-phage-sensitive *E. coli* strain MC1061 was used to produce a heterogeneous phage lysate for the subsequent transfer of the promoter–reporter gene constructs into the chromosome of *E. coli* MC4100.

(b) Measurement of chlorinated dibenzo-*p*-dioxins

The dibenzo-*p*-dioxins were analysed by GC with a Shimadzu 14A gas chromatograph and a DB608-megabore-capillary column (30 m, 0.331 mm i.d., 0.5 μm film thickness, J&W Scientific, Folsom, CA, USA). 2-MCDD was quantitatively determined by solid-phase microextraction from the headspace of 3-ml cultures as described before [43]. Subsequently, the dichlorodibenzo-*p*-dioxins were extracted from the 3-ml cultures by the addition of 3 ml of hexane and shaking (250 r.p.m.) at 30°C for 12 h. The extraction was repeated twice. The combined hexane extracts were supplemented with 50 μl of the internal standard 5,6-dibromoacenaphthene (0.25 mM) dissolved in 2,2,4,4,6,8,8-heptamethylnonane (HMN), concentrated to the HMN phase in a stream of nitrogen and diluted with 200 μl of hexane. The samples were analysed by GC–flame ionization detector, at a split ratio of 1 : 10 and injector and detector temperatures of 250°C and 280°C, respectively. The oven temperature programme was as follows: 3 min at 170°C, 1°C per min to 175°C, 5°C per min to 290°C, hold for 5 min.

(c) Transcription experiments, nucleic acids extraction and reverse transcription

For each transcription experiment, several replicate 50 ml cultures and 3 ml cultures were inoculated by 10% (v/v) with cells from one selected pre-culture. Duplicate 50 ml cultures were removed immediately after inoculation and after 24, 48, 72 and 168 h and completely harvested for nucleic acid extraction. The concentration of chlorinated dibenzo-*p*-dioxins was followed in the 3 ml cultures, which were incubated in parallel, extracted and analysed in duplicate at each time point. Cultures (50 ml) without the addition of the chlorinated electron acceptors served as controls. Chromosomal DNA of *Dehalococcoides* sp. strain CBDB1 was extracted from 1 ml of the 50 ml cultures using the NucleoSpin tissue kit (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer. Forty-five millilitres of the liquid culture was harvested by low-speed centrifugation and subjected to RNA isolation according to the procedure described previously [36], including the addition of *Coleoptera* luciferase mRNA (Promega, Mannheim, Germany), which served as an internal standard for normalization owing to losses during mRNA preparation and reverse transcription inefficiencies. Contaminating DNA was removed using a DNaseI kit (Fermentas, St Leon-Rot, Germany) with a treatment period of 3 h. RNA was stored at −80°C until cDNA synthesis was performed. Within each experiment, equal amounts of sample RNA in the range of 0.1–1 μg were subjected to reverse transcription using random hexamer primers and the RevertAid H minus first strand cDNA synthesis kit (Fermentas), according to the manufacturer's recommendations.

(d) PCR amplification of *rdhA* targets from DNA or cDNA and t-RFLP analysis

Thirteen degenerate primer pairs were used to amplify fragments of all 32 *rdhA* sequences encoded in the genome of strain CBDB1 using exactly the same primers and procedure as described previously [36]. Each primer pair was designed to recognize clusters of up to five specific *rdhA* genes or transcripts. The forward primers were labelled with 6-carboxyfluorescein to allow a subsequent t-RFLP analysis as described previously [36]. Briefly, the amplicons obtained with each degenerate primer pair were divided into aliquots, which were subjected to separate digestions with at least two appropriate restriction enzymes (*Msp*I, *Rsa*I, *Alu*I, *Mbo*I, *Fnu*DII, *Pst*I or *Bsu*RI; Fermentas). The labelled terminal restriction fragments were separated on a genetic analyser 3100 (PE Applied Biosystems, Langen, Germany), and sizes were determined by comparison with an internal size standard (ROX Genescan 500). The fragments were assigned to specific *rdhA* transcripts on the basis of t-RFLP profiles obtained with the same set of PCR primers and restriction enzymes from genomic DNA of strain CBDB1 and compared with computational digests of the respective *rdhA* gene sequences.

(e) Quantification of genes and transcripts

The copy number of the *rdhA* genes *cbrA*, cbdbA1624, cbdbA1453, cbdbA1588 and of the respective transcripts, of the 16S rRNA gene and of luciferase mRNA were measured by qPCR using total DNA or cDNA as template and the QuantiTect SYBR green kit (Qiagen, Hildesheim, Germany), the primers, reaction mixtures, cycling conditions and external standards for quantification as described previously [36]. Samples and external standards were analysed in duplicate. The transcription rate of the *rdhA* genes was calculated as the ratio of the copy number of the *rdhA* transcript, which was normalized for the recovered luciferase transcript number, and the copy number of the corresponding *rdhA* gene.

(f) Mapping the transcriptional start sites

Total RNA was extracted from cells of strain CBDB1 grown for 94 h in the presence of 50 μM 1,2,4-TCB and from *E. coli* strains LS5 and LS13 grown to an OD_{600} of 0.25. Primer extension analyses were carried out using the 5′-rapid amplification of cDNA ends according to the manufacturer's recommendations (Roche, Mannheim, Germany). Total RNA (50 ng from strain CBDB1 and 1 μg from *E. coli* strains) was subjected to cDNA synthesis using the cbdbA1624- and pRS551-specific SP1 primers, respectively. The 3′-end of cDNA was tailed with poly-dATP and subjected to nested PCR with the corresponding SP2 and SP3 primers (see the electronic supplementary material, table S1) and sequencing.

Table 2. *Escherichia coli* strains, plasmids and phages used in this study. The plasmids were derived by standard cloning procedures from vectors.

E. coli strain, plasmid, or phage	description/genotype (insert size)	reference or source
strains		
MC1061	F^- Δ(*ara-leu*)*7697* [*araD139*]$_{B/r}$ Δ(*codB-lacI*)*3 galK16 galE15* λ^- *e14*$^-$ *mcrA0 relA1 rpsL150*(*strR*) *spoT1 mcrB1 hsdR2*($r^- m^+$)	[38]
MC4100	F^- *araD 139* Δ (*argF-lac*) U 169 *ptsF25 deoCl selA1 fibB530 rpsL* 150λ^-	[39]
XL1-Blue MRF′	Δ(*mcrA*) *183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*[F′*proAB lacI*^{q}*Z*Δ*M15* Tn*10*(Tet^r)]	Stratagene, Amsterdam, The Netherlands
BL21(DE3)-CodonPlus-RIL	B F^- *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ (DE3) *endA* Hte [*argU ileYleuW* Cm^r]	Statagene, Amsterdam, The Netherlands
LS4	MC4100 λ RS_pRS551, carrying the promoter-less *lacZ* of pRS551	this study
LS5	MC4100 λ RS_P_{1625}, carrying a chromosomal P_{1625}–*lacZ* promoter fusion	this study
LS11	MC4100 λ RS_P_{1455}, carrying a chromosomal P_{1455}–*lacZ* promoter fusion	this study
LS13	MC4100 λ RS_P_{1624}, carrying a chromosomal P_{1624}–*lacZ* promoter fusion	this study
plasmids		
pP1–P2[a]	Amp^R, comprising complete intergenic region of cbdbA1624/1625 PCR-amplified with primer pair P1/P2 (269 bp)	this study
pP1–P3[a]	Amp^R fragment of intergenic region of cbdbA1624/1625 PCR-amplified with primer pair P1/P3 (97 bp)	this study
pP4–P5[a]	Amp^R fragment of intergenic region of cbdbA1624/1625 PCR-amplified with primer pair P4/P5 (106 bp)	this study
pIR-CBDBA84[a]	Amp^R fragment of intergenic region of cbdbA83/84 PCR-amplified with primer pair CbdbA84-for/rev (350 bp)	this study
pIR-CBDBA1453[a]	Amp^R fragment of intergenic region of cbdbA1454/1453 PCR-amplified with primer pair CbdbA1453-for/rev (475 bp)	this study
pCBDBA1625-IBA5[b]	Amp^R, Cm^R, PCR-amplified cbdbA1625 cloned into BsaI site of pASK-IBA5 (482 bp)	this study
pCBDBA1625-IBA3[b]	Amp^R, Cm^R, PCR-amplified cbdbA1625 cloned into BsaI site of pASK-IBA3 (485 bp)	this study
pRS551_P_{1624}::*lacZ*[c]	Kan^R, complete cbdbA1625–cbdbA1624 intergenic region (247 bp), cbdbA1624–*lacZ* promoter fusion	this study
pRS551_P_{1625}::*lacZ*[c]	Kan^R cbdbA1624–cbdbA1625 intergenic region, truncated 5′ by 14 bp (233 bp), cbdbA1625–*lacZ* promoter fusion	this study
pRS551_P_{1455}::*lacZ*[c]	Kan^R complete cbdbA1456–cbdbA1455 intergenic region (251 bp), cbdbA1455–*lacZ* promoter fusion	this study
pBAD1625[d]	Amp^R, *marR* gene cbdbA1625 cloned into *Eco*RI/ HindIII restriction sites in front of arabinose inducible pBAD promoter	this study
phages		
λ RS_*lacZ*[e]	λ RS45 (*lacZ*)	this study
λ RS_P^e_{1624}	λ RS45 (P_{1624}::*lacZ*), cbdbA1624–*lacZ* promoter fusion	this study
λ RS_P^e_{1625}	λ RS45 (P_{1625}::*lacZ*), cbdbA1625–*lacZ* promoter fusion	this study
λ RS_P^e_{1455}	λ RS45 (P_{1455}::*lacZ*), cbdbA1455–*lacZ* promoter fusion	this study

The plasmids were derived by standard cloning procedures from vectors [a]pGEM T-Easy (Promega), [b]661 pASK-IBA 3 and 5, respectively (IBA), [c]pRS551 [40] and [d]pBAD30 [41].
[e]The phage variants were obtained by homologous recombination using λ RS45 [40].

(g) Construction of plasmids and promoter–*lacZ* transcriptional fusions

Standard procedures were used for plasmid preparations, restriction enzyme digestions, ligations, transformations, phage transduction and gel electrophoresis [42]. The desired DNA fragments were amplified from genomic DNA of strain CBDB1 using the primers indicated in the electronic supplementary material, table S1, digested with restriction endonucleases as appropriate, ligated into linearized cloning vectors pGEM T-Easy or pRS551 (promoter fragments) or expression vectors pASK-IBA3/5 (cbdbA1625 gene). The plasmids and primers used in this study are listed in table 2 and the electronic supplementary material, table S1, respectively. The promoter–*lacZ* fusions were constructed by cloning the PCR-amplified intergenic regions into the *Eco*RI site of pRS551. Fusions with the correct orientation were then transformed into MC1061 cells, which were subsequently infected by λ-RS45. The resulting lysates of recombinant phages (table 2) were used to transduce MC4100 cells to a kanamycin (Kan)-resistant phenotype as described by Sawers & Böck [44]. The single-copy integration of the promoter–*lacZ* fusions and of the promoter-less *lacZ* gene (control) into the genomes of strains LS5, LS11, LS13 and LS4, respectively, was examined by PCR and sequencing.

(h) *In vivo* promoter probe assay

Strains harbouring single-copy promoter–*lacZ* transcriptional fusions (LS5, LS11, LS13) or the promoter-less *lacZ* gene (LS4) were inoculated into completely filled anaerobic Hungate tubes containing LB medium and were incubated statically at 37°C. To study the effect of the putative MarR-type regulator on gene expression, the appropriate strains were transformed with plasmids pBAD1625 (table 2) or pBAD30 [41] (vector control) and cultivated in LB supplemented with ampicillin (125 $\mu g\ ml^{-1}$). For the induction of CbdbA1625 synthesis, 0.02% (w/v) of filter-sterilized arabinose was added. The strains were grown in triplicate to an optical density (600 nm) of 0.4–0.6. To study the gene expression, 100 μl of cell suspensions were added to a 96-well plate, and β-galactosidase activity was measured as described [42] by recording the absorbance at 415 nm using a microplate reader (Bio-Rad Laboratories, München, Germany). The rate of *o*-nitrophenol formation was normalized to the optical density of the respective culture, and the specific activity was given in Miller units. In addition, strains were grown on MacConkey-lactose agar (Roth, Karlsruhe, Germany), and β-galactosidase activity of colonies was qualitatively indicated by the formation of a pink colour.

(i) Overproduction and purification of CbdbA1625–Strep tag fusion proteins

The constructed vectors pCBDBA1625-IBA3 and -IBA5 encoding a C- and N-terminal *Strep-tag* II fusion of CbdbA1625 (CbdbA1625-$Strep_C$ and -$Strep_N$, respectively) were transformed into *E. coli* BL21(DE3)-CodonPlus-RIL (table 2). The heterologous production of CbdbA1625 was induced by the addition of anhydrotetracycline (final concentration 0.2 $\mu g\ ml^{-1}$), when the cultures had reached an optical density of 0.5–0.8 at 600 nm. Cells were harvested after 3 h by centrifugation at 4000*g* at 4°C for 10 min, and the pellet was resuspended in 1 ml lysis buffer (100 mM Tris/HCl, pH 7.6, 1 mM EDTA, 2 mM dithiothreitol (DTT)). The cells were lysed using a French press (SLM Instruments) at 1260 psi after prior treatment with DNase I (5 $\mu g\ ml^{-1}$) and lysozyme (1 $mg\ ml^{-1}$). The protein was purified by chromatography on StrepTactin–Sepharose columns following the recommendations of the manufacturer (IBA). Protein concentration was measured photometrically at 280 nm using a NanoDrop (Thermo Scientific, Schwerte, Germany). Purified protein was either used immediately or stored on ice for maximally 2–3 days prior to use.

(j) Electrophoretic mobility shift assay

The templates for the electrophoretic mobility shift assay (EMSA) were produced by re-amplification from plasmids pP1–P2, pP1–P3, pP4–P5, pIR-CBDBA84 and pIR-CBDBA1453, which contained the complete intergenic regions, or portions thereof (table 2), using the indicated primers (see the electronic supplementary material, table S1). The PCR products were purified using the QIAquick PCR purification kit. Different concentrations (10, 3.3, 1.1, 0.4, 0.1 μM) of both N- and C-terminally tagged CbdbA1625-Strep protein were incubated with 13–18 nM of the target DNA in 1× reaction buffer (20% (w/v) glycerol, 50 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 2.5 mM EDTA, 250 mM NaCl, 3 mM DTT, 1 $\mu g\ \mu l^{-1}$ poly(dI -dC) for 30 min at 24°C in a reaction volume of 200 μl. The effect of TCBs was examined by the addition of 1,2,3-, 1,2,4- or 1,3,5-TCB each at a final concentration of 40 μM. Twenty microlitres of the reaction mixtures was separated by electrophoresis on a non-denaturating 8% (w/v) polyacrylamide gel in 0.5 × TBE (1.78 mM Tris, 1.78 mM boric acid, 0.04 mM EDTA; pH 8.0), and the DNA was visualized by GelRed nucleic acid gel stain (Biotrend, Köln, Germany).

3. Results and discussion

(a) Transcription of *rdhA* genes in response to dichlorodibenzo-*p*-dioxins

The transcriptional response of the *rdhA* genes in strain CBDB1 to chlorinated dibenzo-*p*-dioxins was studied using the two congeners 2,3-DCDD and 1,3-DCDD. 2,3-DCDD was dechlorinated to 2-MCDD within 48 h (figure 2*a*). The transcription of clusters of *rdhA* genes was analysed by RT-PCR using 13 pairs of degenerate primers [36]. Almost no *rdhA* transcript was detectable at time-point 0 and in the control cultures without added dioxin. However, after 24 h of incubation with 2,3-DCDD, products were obtained, with 12 of the 13 primer pairs demonstrating a 2,3-DCDD-dependent induction of transcription (see the electronic supplementary material, figure S2). The abundance of products increased up to 48 h, when reductive dechlorination was complete, but remained relatively stable until the end of the experiment (168 h). t-RFLP was used to identify individual *rdhA* transcripts within the cluster-specific amplicons. As indicated in the electronic supplementary material, figure S2, altogether 29 individual *rdhA* transcripts were detected. The three *rdhA* transcripts of cbdbA80, cbdbA88 and cbdbA243 targeted by the cluster-3 primers were not detected previously in TCB-grown cells, presumably owing to their very low abundance [36]. In summary, the general transcription profile of *rdhA* genes strongly resembled the situation as reported for 1,2,3-TCB-grown cells [36]. Four *rdhA* genes belonging to different PCR-amplified clusters were selected for a quantitative transcription analysis: the three *rdhA* genes *cbrA*, cbdbA1453 and cbdbA1624, which previously had been shown to exhibit a strong response to TCBs [36], and cbdbA1588 encoding an orthologue of PceA, identified as a bifunctional PCE and 2,3-dichlorophenol reductive dehalogenase in strain 195 [25]. The 2,3-DCDD-dependent induction of all four genes could be demonstrated (figure 2*b*), whereas in the control without

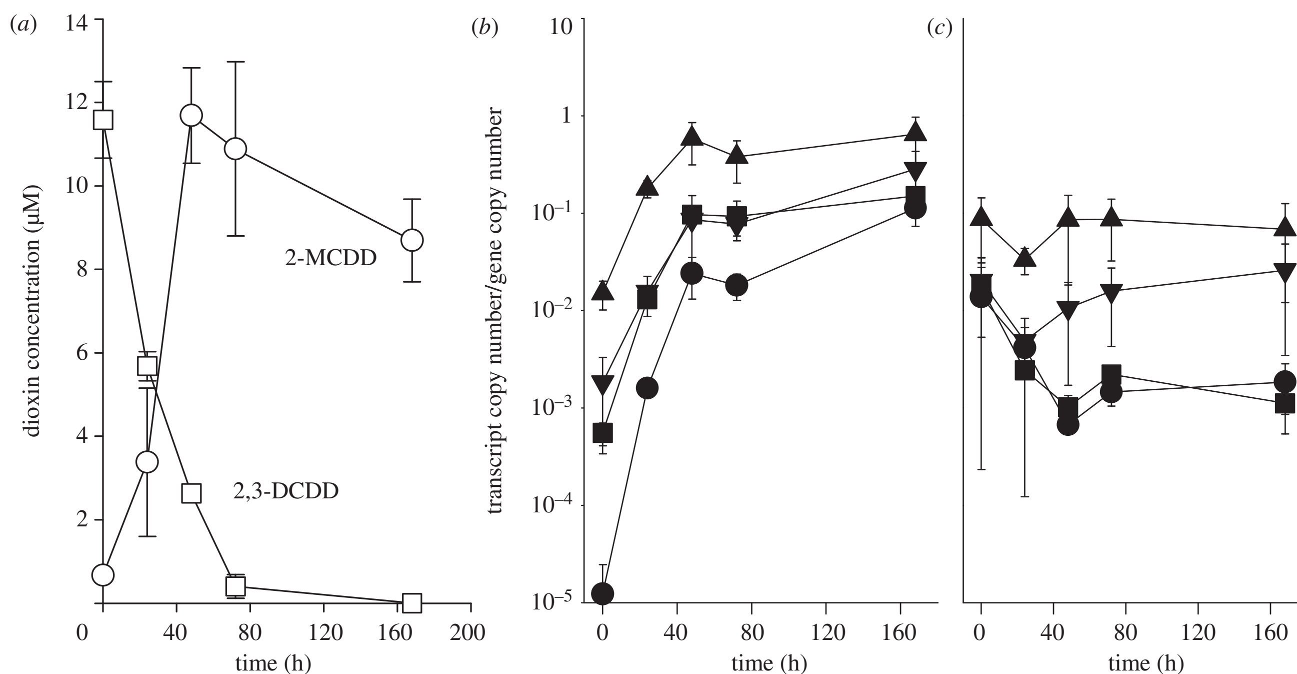

Figure 2. (*a*) Kinetics of reductive dechlorination of 2,3-dichloro (DCDD)- to 2-monochlorodibenzo-*p*-dioxin (MCDD) by strain CBDB1. Two 3 ml cultures were completely extracted for the analyses of chlorinated dibenzo-*p*-dioxins at each time-point shown. Quantitative transcription analysis of the four *rdhA* genes *cbrA* (filled triangles), cbdbA1624 (filled circles), cbdbA1453 (filled inverted triangles) and cbdbA1588 (filled squares) of strain CBDB1 in the presence of 2,3-DCDD (*b*), and (*c*) in the control not supplemented with an electron acceptor is shown. Mean values from duplicate cultures and s.d. are shown.

dioxin no induction of transcription was observed (figure 2*c*). It is noteworthy that after 48 h *cbrA* transcripts attained the highest level of the four genes followed by cbdbA1453, whereas the level of cbdbA1624 was one to two orders of magnitude lower, again agreeing with the transcription levels for 1,2,3-TCB-grown cells reported earlier [36]. Interestingly, cbdbA1588, which was expressed at a lower level in 1,2,3-TCB-grown cells [36], attained the same transcription level as cbdbA1453 in the presence of 2,3-DCDD. The transcript copy number of roughly one per cell determined for *cbrA* was very low. This might be due to the low concentration and the low water solubility (59 nM) [45] of 2,3-DCDD. However, the results emphasize a possible role of CbrA in reductive dechlorination of dioxins. It is likely, however, that other reductive dehalogenases are also involved, as suggested by the fact that so far only *D. mccartyi* strain DCMB5, which contains a close orthologue of *cbrA* (unpublished data, 2012), is not able to dechlorinate 2,3-DCDD [43].

The other congener, 1,3-DCDD, has been shown previously to be a transient intermediate in the dechlorination of 1,2,4-trichlorodibenzo-*p*-dioxin to 2-MCDD [33]. When we applied it directly to the culture, it was slowly dechlorinated only in the primary culture and surprisingly was not dechlorinated in a subculture which was inoculated to 10% (v/v) with the previous culture (data not shown). In addition, transcription of *rdhA* genes was not detectable in the primary culture by RT-PCR and RT-qPCR (data not shown), indicating that 1,3-DCDD was not an inducer of *rdhA* gene transcription. Thus, dechlorination of 1,3-DCDD might be a co-metabolic process similar to the co-metabolic conversion of VC to ethene by *D. mccartyi* strain 195 [46].

The transcription analyses of *rdhA* genes in *D. mccartyi* strain CBDB1 exposed to two dichlorinated dibenzo-*p*-dioxins revealed two findings. First, it confirmed for another class of chloroaromatic compounds that a single chlorinated compound induces a general transcriptional response. This possibly enables *Dehalococcoides* to recognize further chlorinated compounds present in its environment, also extending the spectrum of substrates to non-inducing organochlorines such as 1,3-DCDD. Second, the genes are expressed to different levels, strongly suggesting the occurrence of specific regulatory events. These might include a specific activation of target and/or repression of non-target genes.

(b) *In vitro* study of MarR interaction with the intergenic region of cbdbA1624 and cbdbA1625

The gene cbdbA1625 encoding a putative MarR-type regulator is oppositely oriented to the *rdhA* gene cbdbA1624 (figure 3), which is specifically regulated in the presence of 1,2,4-TCB [36]. To study whether CbdbA1625 exerts a regulatory function on the expression of both genes, the cbdbA1625 gene was cloned into the expression vectors pASK-IBA5 and pASK-IBA3 and Strep-CbdbA1625$_N$ and -$_C$ were purified as N- and C-terminally *Strep-tag* II-fusion proteins, respectively, from *E. coli* BL21(DE3)-CodonPlus-RIL. The complete intergenic region between cbdbA1624 and cbdbA1625 (P1–P2) and two sub-fragments (P1–P3 and P4–P5) were used in EMSA. After the incubation of the purified P1–P2 PCR product with different concentrations of either Strep-CbdbA1625$_c$ or -$_N$, a retardation of the DNA band was observed (shown for CbdbA1625$_C$ in figure 3*b*). Regardless of whether N- or C-terminally Strep-tagged CbdbA1625 was used, two protein–DNA complexes (C1, C2) appeared at protein concentrations between 0.4 and 3.3 µM, suggesting two specific binding sites for CbdbA1625 in the intergenic region. Additional discrete bands of lower electrophoretic mobility (C$_a$) appeared when the ratio of Strep-CbdbA1625 to the operator was further increased. Binding of Strep-CbdbA1625 to the cbdbA1624–cbdbA1625 intergenic region was shown to be specific, because no gel shift was observed when the amplified intergenic regions upstream of cbdbA1453 and *cbrA* were used in the

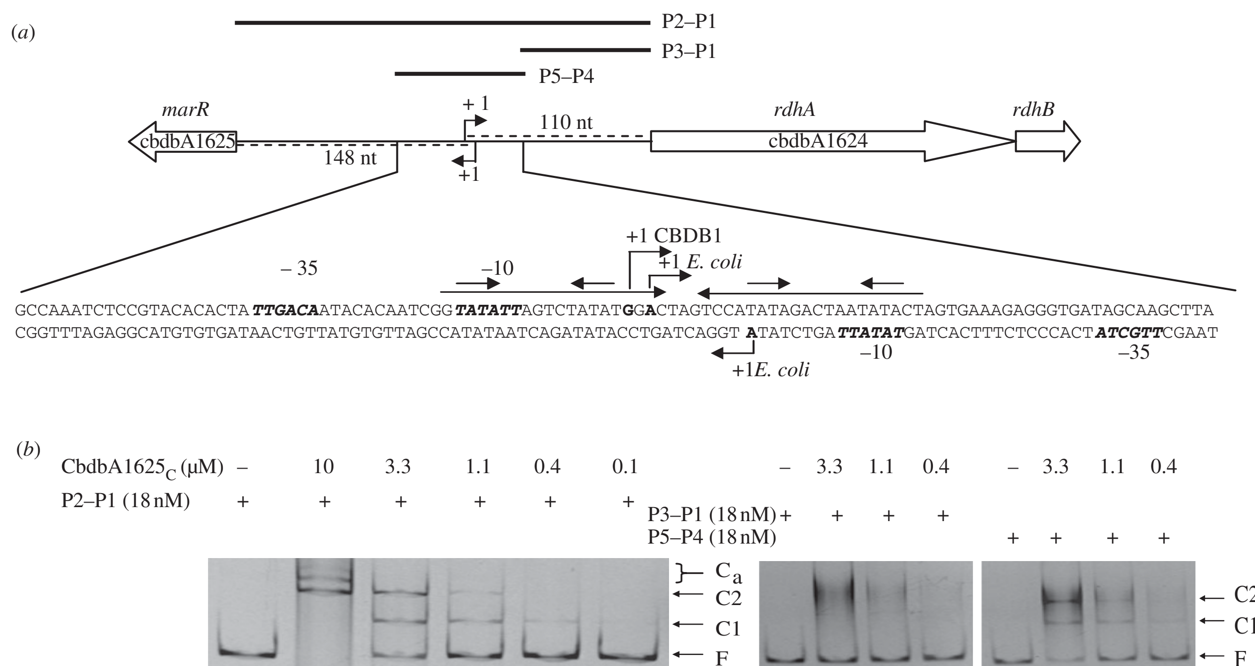

Figure 3. (*a*) Schematic of the intergenic region of cbdbA1624–cbdbA1625. The DNA sequence shown depicts the P5–P4 region. The long horizontal arrows above the sequence indicate inverted repeat sequences in the promoter region; the angled arrows indicate the experimentally determined transcriptional start sites; the short arrows above the inverted repeat sequences represent the putative recognition sequences of the regulator; bold and italic letters depict the consensus sequences of the −10 and −35 region predicted by the BPROM software (http://linux1.softberry.com/berry.phtml). The complete intergenic region (246 bp; P2–P1) and sub-fragments thereof (P3–P1/P5–P4) were amplified by PCR from total DNA of strain CBDB1 with primers binding in, or flanking, the intergenic region for EMSA. (*b*) Interaction of Strep-CbdbA1625$_C$ with the complete intergenic region (P2–P1) and the sub-fragments (P3–P1/P5–P4). EMSA was carried out in the presence (plus symbols) and absence (minus symbols) of Strep-CbdbA1625$_C$ protein. Free DNA (F) and retarded DNA–protein complexes (C1, C2, C$_a$) were visualized by staining with GelRed (Biotium, Hayward, CA, USA).

gel retardation assay (data not shown). To locate the binding site of CbdbA1625 within the intergenic region, two sub-fragments comprising the 3′-region (P1–P3) and the putative promoter region (P4–P5) (figure 3*a*) were used in an EMSA. Addition of both the C- and N-terminally Strep-tagged CbdbA1625 fusion proteins resulted in mobility shifts of the P4–P5 fragment (exemplarily shown for CbdbA1625$_c$ in figure 3*b*), which were comparable to the shift observed with the complete intergenic region. By contrast, no discrete shifted bands were identified when the P1–P3 fragment was used (figure 3*b*). Despite several attempts, disruption of the protein–DNA complexes could not be achieved by the addition of 1,2,4-, 1,2,3- or 1,3,5-TCB, suggesting that specific conditions or further regulatory components are required for signal perception (data not shown).

Analysis of the DNA sequence of the P4–P5 region (figure 3*a*) indicated that it contained a perfect 40 bp palindrome, which comprised short inverted repeats in its half sites, the typical binding motif of MarRs [17]. A primer extension analysis using RNA extracted from 1,2,4-TCB-grown cells of strain CBDB1 mapped the transcriptional start site of cbdbA1624 to a position within this palindrome 110 bp upstream of the translational initiation codon (figure 3*a*). Primer extension analysis of the *marR* transcript cbdbA1625 failed to identify a signal, possibly indicating transcript levels below the detection limit of the assay.

(c) *In vivo* study of *rdhA* and *marR* transcription and interaction with MarR in a heterologous system

Transcriptional promoter–*lacZ* fusions were constructed for the two *rdhA* genes (cbdbA1624 and cbdbA1455) and the *marR* gene (cbdbA1625), and these were introduced in single copy into the genome of *E. coli* strain MC4100. Each DNA fragment resulted in detectable β-galactosidase enzyme activity, which varied in the range between 40 and 2800 Miller units (see the electronic supplementary material, figure S3) depending on the promoter region. This finding suggested that the putative promoters within these DNA sequences were recognized by the *E. coli* RNA polymerase. To provide further evidence for this proposal, we determined the transcriptional start sites of the transcriptional fusions P_{1624}-*lacZ* (strain LS13) and P_{1625}-*lacZ* (strain LS5) by primer extension. For P_{1624}, the transcription initiation site was mapped to a position almost identical (2 bp difference) to the transcriptional start of cbdbA1624 in the native host CBDB1, strongly suggesting that the *E. coli* RNA polymerase indeed recognized the *Dehalococcoides* promoter (figure 3*a*). The signal intensity was low perhaps owing to the unusually short distance between the putative −10 and −35 regions [47,48]. For P_{1625}-*lacZ*, the promoter was mapped 149 bp upstream of the putative translational start codon of cbdbA1625 (figure 3*a*). The transcriptional start and the predicted −10 region are also within the palindrome suggesting that the promoter regions of cbdbA1624 and cbdbA1625 overlap and are both under the control of the MarR CbdbA1625.

We selected the P1625–*lacZ* construct (strain LS5) because of its high β-galactosidase activity for a first analysis of *in vivo* regulation by CbdbA1625. The native MarR CbdbA1625 was cloned into the pBAD30 vector and transformed into strain LS5. After inducing expression of the cbdbA1625 gene on the plasmid in anaerobically grown cells of *E. coli* by adding 0.02% (w/v) arabinose, a 40–50% reduction in

Table 3. Influence of the MarR CbdbA1625 on the expression of cbdbA1625–*lacZ* and cbdbA1455–*lacZ* transcriptional fusions in the heterologous host *E. coli*. The strains LS5 and LS11 were transformed with pBAD1625, which carries the *marR* gene cbdbA1625 under the control of the P_{BAD} promoter, or pBAD30 (vector control) and grown to an OD_{600nm} of 0.4–0.6 in the presence or absence of 0.02% (w/v) of arabinose. The strains were analysed in biological triplicates. One out of three repeated experiments is shown with standard deviations.

		pBAD1625		pBAD30 (vector control)	
		promoter activity		promoter activity	
strains (transcriptional fusion)	addition of arabinose	(Miller units)	(%)	(Miller units)	(%)
LS5 (P_{1625}-*lacZ*)	—	1023.5 ± 145.1	100	1217.2 ± 164.4	100
	+	606.7 ± 103.5	59	1054.5 ± 185.7	87
LS11 (P_{1455}-*lacZ*)	—	937.5 ± 172.4	100	549.9 ± 108.9	100
	+	904.5 ± 156.3	97	535.5 ± 120.9	97

β-galactosidase enzyme activity was observed, whereas no significant reduction in the β-galactosidase activity occurred in a vector control (table 3). In addition, the interaction of CbdbA1625 was specific: no reduction in the β-galactosidase activity was observed for the cbdbA1455 promoter–*lacZ* fusion strain (table 3). These results demonstrate for the first time that a MarR of *D. mccartyi* is functional in a heterologous host and suggest that CbdbA1625 acts as repressor and auto-regulates its own transcription.

4. Concluding remarks

Our investigation of *rdh* gene expression in *Dehalococcoides mccartyi* strain CBDB1 demonstrated a general response to 2,3-dichlorodibenzo-*p*-dioxin. The levels of *rdhA* transcripts varied over several orders of magnitude, which was similar to the response to chlorinated benzenes. The fact that 1,3-DCDD did not induce *rdh* gene expression, despite being a substrate for reductive dehalogenases, highlights the importance of regulatory processes for the functionality of organohalide respiration and demonstrates specificity of regulation. As inferred from the genomic context of *rdh*-associated genes, MarR-type regulators might play specific roles in the regulation of a subset of the *rdhA* genes. This possibility was addressed by performing *in vitro* interaction studies involving the MarR CbdbA1625 and the respective *rdhA* and *marR* promoter regions. In addition, results of *in vivo* promoter probe assays suggest that CbdbA1625 functions as repressor. Given this is a typical regulatory mechanism of MarRs, we assume that *rdhA* genes regulated by MarR-type regulators undergo repression control and this may be the rule rather than the exception in the environmental life of *Dehalococcoides*. If halogenated compounds become available, a de-repression might enable specific induction of *rdhA* transcription. Negative auto-regulation would ensure a tightly regulated transcriptional response. However, to understand the regulation in more detail, future efforts must be directed towards elucidating the signal to which the regulator responds.

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