Understanding the metabolism of tetrachloroethene-respiring *Dehalobacter restrictus*: from genome analysis, corrinoid cofactor biosynthesis to regulation of reductive dehalogenases

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B. Abstract

Tetra- and trichloroethene (PCE, TCE) are organohalides polluting the environment as a result of inappropriate disposal by various industries. Although such compounds are also produced naturally in the environment in low quantity, their anthropogenic pollution is a major source of concern because they pose many undesirable effects on human health. *Dehalobacter restrictus* represents the paradigmatic member of the genus *Dehalobacter*, which in recent years has proved to be a major player in the biodegradation of a growing number of organohalides, both *in situ* and in the laboratory. *D. restrictus* grows only through anaerobic respiration of PCE and TCE as the only electron acceptors and hydrogen as the electron donor by a process known as organohalide respiration (OHR). To this day, only a single reductive dehalogenase (PceA/RdhA), the key enzyme in the OHR process, has been characterized on genetic and biochemical levels previously (Maillard et al., 2003). However, recent genome analysis of *D. restrictus* has revealed the presence of 25 *rdhA* genes. ***Chapter*** 2 of this thesis describes a functional genomics approach on *D. restrictus* where a global proteomic analysis allowed to consider general metabolic pathways relevant to OHR, while the dedicated genomic and transcriptomic analysis focused on the diversity, composition and expression of *rdh* gene clusters. Genome analysis revealed a complete corrinoid biosynthetic pathway, WL pathway for CO_2 fixation and hydrogenases. Some of these were also identified in proteomic analysis along with main PceABCT, RdhA14 and a few RdhK. OHR bacteria (OHRB) have developed different strategies to satisfy their need of corrinoid (Cobalamin/Vitamin B12 derivatives), as it is an essential cofactor of RdhAs forming the basis for ***Chapter 3***. *De novo* corrinoid biosynthesis represents one of the most sophisticated metabolic pathways in nature requiring more than 25 enzymes. Obligate OHRB as *Dehalococcoides spp.* and *D. restrictus* which absolutely need the corrinoid, cannot produce it and need external supplementation. Genome analysis revealed that in contrast to *Dehalococcoides mccartyi*, the genome of *D. restrictu*s surprisingly has the complete series of genes for biosynthesis of corrinoid, however a single non-functional gene could account for the corrinoid auxotrophy. Comparative genomics within *Dehalobacter* spp. revealed that one of the five operons associated with the biosynthesis of corrinoid is unique to *D. restrictus*. This operon-2 encodes several

enzymes involved in the transport and salvaging of corrinoids. The expression of operon-2 was highly up-regulated upon corrinoid starvation both at the transcriptional and proteomic level, in correlation with the presence of an upstream cobalamin riboswitch. These results highlight the importance of operon-2 in corrinoid homeostasis in *D. restrictus* and the augmented salvaging strategy this bacterium adopted to retrieve this essential cofactor. **Chapter 4** finally analyses the diversity of RdhK proteins in *D. restrictus* belonging to the CRP-FNR family of transcriptional regulators. Earlier studies in *Desulfitobacterium* spp. have allowed the identification and characterization of a transcriptional regulator, CprK known to be involved in the regulation of *cpr* gene cluster involved in OHR. Moreover recent genome analysis in *D. restrictus*, revealed the presence of 25 *cprK*-like *rdhK* genes found to be located in the direct neighbourhood of the *rdh* gene clusters strongly suggesting they could be implicated in regulating OHR in *D. restrictus*. A combination of *in silico*, *in vivo* and *in vitro* analyses have been attempted to characterize the role of a few RdhK proteins and understand the tri-partite interaction of the RdhK with the putative organohalide and the putative-DNA binding regions (dehaloboxes). However, further efforts are still needed to elucidate the network regulating OHR metabolism in *D. restrictus.*

C. Résumé

Le tétra- et le trichloroéthène (PCE, TCE) sont des composés organo-halogénés polluant l'environnement à la suite de décharges inappropriées par diverses industries. Bien que de tels composés sont également produits naturellement dans l'environnement en faible quantité, leurs émissions anthropiques est une source majeure de préoccupation car ils posent de nombreux effets indésirables sur la santé de l'homme. *Dehalobacter restrictus* représente une espèce bactérienne modèle du genre *Dehalobacter*, qui au cours des dernières années s'est avérée être un acteur majeur dans la biodégradation d'un nombre croissant de composés organo-halogénés, tant *in situ* qu'en laboratoire. *D. restrictus* croit uniquement grâce à la respiration anaérobie du PCE et du TCE comme seul accepteur d'électrons avec l'hydrogène comme seul donneur d'électrons, un processus appelé en anglais 'organohalide respiration' (OHR). A ce jour, une seule déhalogénase réductrice (RdhA), l'enzyme-clé du processus OHR, a été caractérisée aux niveaux

génétique et biochimique. Toutefois, l'analyse génomique récente de *D. restrictus* a révélé la présence de 25 gènes de type *rdhA*. Le **chapitre 2** de cette thèse décrit une approche de génomique fonctionnelle à partir de *D. restrictus*. Une analyse protéomique globale a permis d'examiner les voies métaboliques générales en relation au processus OHR, tandis que une analyse génétique et transcriptionnelle a révélé la diversité, la composition et l'expression de gènes présents dans les clusters de gènes *rdh*. L'analyse du génome a révélé une voie de biosynthèse corrinoïde complète, WL voie pour la fixation de CO2 et hydrogénases. Certains d'entre eux ont également été identifiés dans l'analyse protéomique avec PceABCT principale, RdhA14 et quelques RdhK. Les bactéries de type OHR (appelées OHRB) ont développé différentes stratégies pour satisfaire leur besoin du cofacteur corrinoïde (dérivé de la vitamine B_{12}), étan donné qu'il s'agit d'un cofacteur essentiel des enzymes RdhA (**chapitre 3**). La biosynthèse *de novo* des corrinoïdes représente l'une des voies métaboliques les plus compliqués nécessitant plus de 25 enzymes. Les bactéries OHR obligatoires telles que *D. restrictus* et *Dehalococcoides* spp. ont un besoin absolu de corrinoïde et ne peuvent le produire elles-mêmes. Le génome de *D. restrictus* a révélé que contrairement à *Dehalococcoides* spp., *D. restrictus* étonnamment possède la série complète des gènes de biosynthèse du corrinoïde, cependant un gène non-fonctionnel pourrait expliquer son auxotrophie. D'autres analyses de génomique comparative des espères du genre *Dehalobacter* a révélé qu'un des cinq opérons associés à la biosynthèse du corrinoïde est unique chez *D. restrictus*. Cet opéron encode plusieurs enzymes impliquées dans le transport et le recyclage de corrinoïde, et l'expression de ces gènes s'est révélée hautement induite en conditions de manque de corrinoïde, tant au niveau transcriptionnel que protéomique, en corrélation avec la présence de riborégulateurs en amont. Ces résultats soulignent l'importance de cet opéron dans l'homéostasie du corrinoïde chez *D. restrictus* et la stratégie adoptée par cette bactérie pour récupérer ce cofacteur essentiel. Le **chapitre 4** présente enfin des analyses sur la diversité des protéines RdhK de *D. restrictus* appartenant à la famille de régulateurs de transcription de type FNR-CRP. Des études antérieures sur *Desulfitobacterium* spp. ont permis l'identification et la caractérisation du régulateur de transcription CprK en tant qu'acteur de la régulation des gènes *cpr* impliqués dans le processus OHR. Dans le génome de *D. restrictus*, 25 gènes de type *rdhK* ont été identifiés dans le voisinage direct des groupes de gènes *rdh*, suggérant qu'ils peuvent être impliqués

dans la régulation du processus OHR chez *D. restrictus*. Une combinaison d'analyses *in silico, in vivo* et *in vitro* ont été tentées pour caractériser le rôle de quelques protéines RdhK et comprendre l'interaction tripartite de la protéin RdhK avec les composés organo-halogénés ainsi que les régions de l'ADN potentiellement ciblées. Toutefois, de nouvelles tentatives sont encore nécessaires pour élucider le réseau de régulation du processus OHR chez *D. restrictus*.

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I. List of keywords

Dehalobacter restrictus, organohalide respiration metabolism, biodegradation, genome, reductive dehalogenase, corrinoid biosynthesis, cobalamin riboswitches, corrinoid auxotrophy, functional genomics, CRP-FNR transcriptional regulator proteins.

J. List of abbreviations

Generic abbreviations

% per cent

Amp ampere

Amp^R ampicillin resistance gene

APS ammonium persulphate

AU Arbitrary units

cm centimetre

Da Dalton

DNA deoxyribonucleic acid

DTT dithiothreitol

g gram

x *g* earth's gravitational acceleration

IPTG isopropyl-β-D-thiogalactopyranoside

K_A association constant

K_D dissociation constant

kb kilobase

kDa kilo Dalton

L litre

LB Luria-Bertani broth

log10 logarithm base 10

M molar

mg milligram

mL millilitre

mM millimolar

ng nanogram

nM nanomolar

°C degrees centigrade

PAGE poly acrylamide gel electrophoresis

PBS phosphate buffered saline

PCB polychlorinated biphenyls

PCR polymerase chain reaction

pH -log10[H+]

pKa -log10 Ka

psi pounds per square inch

RNA ribonucleic acid

RT-PCR reverse transcriptase-polymerase chain reaction

SDS sodium dodecyl sulphate

SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TAE Tris-Acetate-EDTA buffer

TBE Tris-Borate-EDTA buffer

TEMED Tetramethylethylenediamine

Tris Tris(hydroxymethyl)aminomethane

UV ultra-violet

V volt

ΔG Gibb's free energy

ΔH Change in enthalpy

ΔS Change in entropy

µg microgram

µL microlitre

µM micromolar

Specific abbreviations

OHR organohalide respiration

OHRB organohalide respiring bacteria

rdhA reductive dehalogenase gene

pceA tetrachloroethene reductive dehalogenase gene

cprA chlorophenol reductive dehalogenase gene

RdhA reductive dehalogenase protein/enzyme

HGT horizontal gene transfer

IR inverted repeats

ORF open reading frame

Cbl-RS cobalamin riboswitch

CS corrinoid salvaging

CT corrinoid transporter

LFQ label free quantitation

NLA nucleotide loop assembly

UCB upper corrinoid biosynthesis

DB dehalobox

ITC isothermal titration calorimetry

ITF intrinsic tryptophan fluorescence

EMSA electrophoretic mobility shift assay

DMB 5, 6-dimethylbenzimidazole

Organohalides

PCE tetrachloroethene (perchloroethylene)

TCE trichloroethene

DCE dichloroethene

ClOHPA 3-chloro-4-hydroxyphenylacetic acid

CB chlorobenzene

CE chloroethene

CP chlorophenol

DCA dichloroethane

DCB dichlorobenzene

DCP dichlorophenol

PCP pentachlorophenol

TCA trichloroethane

TCB trichlorobenzene

TCP trichlorophenol

TeCA tetrachloroethane

VC vinyl chloride

Chapter 1
General Introduction

1 General Introduction

1.1 Organohalides

1.1.1 Natural presence in environment

When a halogen (chlorine or bromine) in its inorganic and ionic form is incorporated into an organic compound it is known as an organohalide. Organohalides are required naturally by many microbes as they are involved in various biological functions such as in chemical defense against predators and as pheromones (Oberg, 2002; Barre et al., 2010). Naturally present organohalides could be produced abiotically from volcanic activites, forest fires or could also be synthesized biologically by fungi, plants, termites, marine algae, mushrooms and bacteria (Gribble, 2003).

1.1.2 Uses of organohalides and their contamination into the environment

Not only microbes and algae but even humans over the years have realized the remarkable ways in which organohalides can be used. Thereby from the early 20th century, the exploitation of organohalides has been on the rise due to their application in agriculture and industry as pesticides, solvents, preservatives, and many other functions. Trichloroethene (TCE) and tetrachloroethene (PCE) are widely used solvents in the dry cleaning industry. Along with the benefits the introduction of organohalides has brought to humans and microbes, there are many disadvantages too. Accidental spills, careless handling, and leaking storage tanks led to the release of these compounds into the environment which is a serious issue because of the negative impact they have on all life forms. Chlorinated ethenes (in this case PCE and TCE) are characterized by their low solubility and a density much greater than water, thereby forming Dense Non-Aqueous Phase Liquids (DNAPL) due to their seepage into subsurface in contaminated sites.

1.1.3 Health risks

One of the primary groundwater and soil contaminants constitute chlorinated aliphatic organohalides such as PCE and TCE (Moran et al., 2007) and are identified as “dangerous” by the EU Water Framework Directive (Lepom et al., 2009). PCE not only affects the groundwater, aquatic life and consumers of such polluted water but also workers in industries with high PCE exposure. PCE and TCE are both toxic and suspected carcinogens, known to cause immune system and liver dysfunctions, thus their presence in groundwater is of significant risk to human health (Christensen et al., 2013; Guha et al., 2012; Mattei et al., 2014; Vlaanderen et al., 2013). Exposure to PCE and TCE also happen to workers in professional dry cleaning, surface cleaning or paint industries where PCE is used for its many well-touted properties and applications. The most likely route of human exposure is through inhalation or to a less extent direct skin contact. A recent report by ECSA (The European Chlorinated Solvent Association, Belgium, 2012) indicates that PCE is suspected to cause cancer in humans and is also

toxic to aquatic life with long-lasting effects. Thereby the proper disposal of such organohalides and eventual breakdown is very important to study.

1.1.4 Evolution of organohalide detoxification mechanisms

Organohalides are not only anthropogenic pollutants (recent rise) but have been present naturally from a long time. There is growing evidence that organohalides are important in global elemental cycles as well. For example, in order to complete the chlorine cycle the covalent bond between the carbon and the halogen atom must be cleaved and the chlorine ion must be released into the environment. The main contribution to this process is achieved by microbial dehalogenation (stripping off a halogen atom per reaction from the multi-halogenated compounds). During such a process, organohalides such as PCE can either be partially or completely dehalogenated into eventually harmless ethene. Also given that organohalides may have been present on earth since the origin of life (Vetter and Gribble, 2007), it is not surprising that diverse microbial populations with ability to degrade a wide array of organohalides exist in the environment. Over the years, presence of such natural organohalides could have helped them evolve enzymatic mechanisms for organohalide detoxification or as well as specialize towards various substrates (Mohn and Tiedje, 1992; Fetzner and Lingens, 1994; Holliger and Schraa, 1994; Holliger et al., 1998a; Villemur et al., 2006; Taş et al., 2010; Maphosa et al., 2012a). This specialized group of bacteria is able to reductively dehalogenate organohalides under anaerobic conditions and use the energy conserved via a proton gradient pump for bacterial growth, a process named organohalide respiration (OHR) and the bacteria which use this mode of respiration are therefore termed as organohalide-respiring bacteria (OHRB).

1.2 Organohalide respiration

1.2.1 Terminology and thermodynamics

As an emerging field, a clear and generally accepted nomenclature is required for efficient communication within the field. At a recently held Royal Society meeting in July 2011 in Kavli centre, UK, it was discussed and they reached the consensus that terms ‘dehalorespiration’ and ‘halorespiration’ previously used to describe this respiratory process is now commonly discouraged, and instead should be replaced by the term ‘organohalide respiration’ (OHR). Furthermore, a clear classification system is needed for the key reductive dehalogenases, and a proposal for this is described in a paper by Hug and co-workers (Hug et al., 2013).

Several species of microaerophilic, facultative or obligate anaerobic bacteria are able to conserve energy via the reductive dehalogenation of organohalides by OHR metabolism (Mohn and Tiedje, 1992; Schumacher and Holliger, 1996; van de Pas et al., 2001a; Smidt and de Vos, 2004). Reductive dechlorination is a process in which the removal of a halogen substituent (mostly chlorine) from an organic molecule occurs with the addition of a proton (H^+) and concomitant transfer of two electrons. The first dechlorination step in bioremediation of the recalcitrant tetrachloroethene

(PCE) is obligatory anaerobic in nature whereas the latter steps involving the less chlorinated ethenes could be also catalysed aerobically following an oxidative mechanism (Pant and Pant, 2010; Mattes et al., 2010; Nelson et al., 1987; Vogel et al., 1987). Therefore understanding the different steps of PCE dechlorination is of high significance for bioremediation purposes. Organohalides are excellent electron acceptors as the standard redox potential range lies between +250 and +600mV (El Fantroussi et al., 1998), thereby ensuring that it is thermodynamically favourable. Oxidation of H_2 is coupled to reductive dehalogenation, which in overall is an exergonic reaction yielding between -130 to -180 kJ/mol of Gibbs free energy per mole of chlorine removed and thereby a thermodynamically favourable reaction (Smidt and de Vos, 2004),

1.2.2 Phylogenetic diversity of OHRB and their capabilities

OHRB couple the reductive dehalogenation of a large variety of halogenated aromatic and aliphatic hydrocarbons to energy conservation and hence to microbial growth. OHR can be described as a process in which the oxidation of an electron donor and subsequent electron transport occurs across the cytoplasmic membrane leads to the terminal reduction of organohalides (Holliger et al., 1993; Louie and Mohn, 1999; Smidt and de Vos, 2004). Such a transport of electrons across the membrane along with the generation of a proton gradient allows for energy conservation for anabolic reactions. The enzyme class involved for the terminal reduction of the halogenated compound is known as reductive dehalogenases (RdhA or RDase) (Fetzner and Lingens, 1994; Holliger and Schumacher, 1994; Holliger et al., 1998a; Neumann et al., 1994a). For the reduction of PCE the enzyme is named PceA as the one characterized from *Dehalobacter restrictus* (Maillard et al., 2003) or in *D. hafniense* strain Y51 (Suyama et al., 2002).

OHRB are affiliated to five distinct phylogenetic branches namely the Gram-positives with low GC content (belonging to the *Firmicutes*), the *δ*-, *γ*- and *ε*-*Proteobacteria*, and the *Chloroflexi*. Anaerobic bacteria that can grow with chloroethenes as final electron acceptors include members of the genera *Dehalobacter* (Holliger et al., 1998b), *Dehalococcoides* (Maymó-Gatell et al., 1999), *Desulfitobacterium*, *Desulfuromonas* (Sung et al., 2003), *Geobacter* (Wagner et al., 2012), and *Sulfurospirillum* (Neumann et al., 1994b). The well-studied organisms, *Sulfurospirillum multivorans* and *Dehalobacter restrictus* dechlorinate PCE to 1, 2-*cis*-dichloroethene (DCE). *S. multivorans* is a Gram-negative anaerobic spirillum which belongs to the ε-subdivision of *Proteobacteria*. A few bacterial species belonging to the *δ-proteobacteria* also perform OHR such as *Anaeromyxobacter, Geobacter, Desulfuromonas, Desulfovibrio* spp. (Richardson, 2013). For example, both *Geobacter lovleyi* strain SZ (Sung et al., 2006) and *Desulfuromonas michiganensis* respire PCE to DCE (Sung et al., 2003). The *Dehalobacter* genus belongs to the phylum *Firmicutes,* along with the genus *Desulfitobacterium*; however, OHR is the sole system of energy conservation in the genus *Dehalobacter*, unlike in *Desulfitobacterium* which is metabolically versatile in

nature (Schumacher and Holliger, 1996; Holliger et al., 1998b; Villemur et al., 2006; Maphosa et al., 2010; Prat et al., 2011).

Although the above-mentioned strains can utilize tetrachloroethene (PCE) or trichloroethene (TCE) as the electron acceptor, they cannot dechlorinate DCE or VC to ethene. *Dehalococcoides mccartyi* 195, a member of the phylum *Chloroflexi* is the only strain so far isolated in pure culture which can perform the complete dechlorination from PCE to ethene (Maymó-Gatell et al., 1999). Strains isolated from consortia containing *Dehalococcoides* spp. such as strain ANAS1 and strain ANAS2 (Lee et al., 2011) cannot dechlorinate PCE but can dechlorinate TCE to ethene. Strains BAV1 (Krajmalnik-Brown et al., 2004), VS (Müller et al., 2004) and FL2 (He et al.) are known to dechlorinate DCE isomers to VC or ethene. Strain BAV1 degrades PCE or TCE only co-metabolically, whereas strain VS and strain GT (Sung et al., 2006) are reported to couple TCE dechlorination to energy conservation. A new genus within the Chloroflexi branch emerged with the isolation and characterization of bacteria such as *Dehalogenimonas* spp. *D. alkenigignens* IP3-3T (Maness et al., 2012) and *D. lykanthroporepellens* BL-DC-9T (Moe et al., 2009) can both reductively dehalogenate chlorinated alkanes such as 1,2-dichloroethane, 1,2-dichloropropane, and 1,1,2-trichloroethane. The *Dehalogenimonas* genus seems to share several phenotypic features with the *Dehalococcoide*s and *Dehalobacter* genus such as strict anaerobic respiration, requirement for H_2 as electron donor, exclusive use of halogenated compounds as electron acceptors. However, based on a few other features as Gram-negative staining, small irregular cell morphology and differentiated resistance to antibiotics as well as other genotypic, chemotaxonomic and phenotypic properties, they can be differentiated from or compared with the related *Dehalococcoides* genus (Adrian et al., 2000; He et al., 2003; Sung et al., 2006; Yan et al., 2009). Therefore three genera known so far such as the *Dehalobacter*, *Dehalococcoides* and *Dehalogenimonas* possess a restricted metabolism and are categorised under "obligate OHRB", i.e. derive their energy from OHR only. Whereas, the remaining OHRB such as *Desulfitobacterium*, *Geobacter*, *Sulfurospirillum* and *Anaeromyxobacter* spp. can be categorised as "facultative OHRB".

1.3 Reductive dehalogenases as key enzymes in OHR metabolism

Most of the biochemically characterized reductive dehalogenases (RdhAs) have been isolated from OHRB as a 48-65 kDa monomer containing one corrinoid cofactor and two iron-sulfur (FeS) clusters. Examples of purified chloroethene RdhAs are PceA of *D. restrictus* (Maillard et al., 2003), PceA of *S. multivorans* (Neumann et al., 1996, 2002), PceA of *Desulfitobacterium* sp. strain PCE-S (Miller et al., 1998) and of *D. hafniense* strain PCE1 (van de Pas et al., 2001b), TceA of *Dehalococcoides mccartyi* strain195 (Magnuson et al., 2000) and the vinyl chloride RdhA (VcrA) of *Dehalococcoides* sp. strain VS (Müller et al., 2004). The purified chlorophenol RdhAs are the CprA enzymes of *Desulfitobacterium dehalogenans* (van de Pas et al., 1999), of *D. hafniense* strain DCB-2 (Christiansen et al., 1998) and of *D. chlororespirans* (Krasotkina et al., 2001).

1.3.1 Biochemical features of reductive dehalogenases

The RdhA enzymes harbour several conserved features, including a corrinoid cofactor, two iron-sulfur clusters, and a signal peptide targeting the protein to the Twin-arginine translocation (Tat) system, indicating that the protein is transported across the cell membrane. While the biochemical activity of several of these enzymes has been determined, the structure of two RdhA enzymes has only recently been elucidated (Bommer et al., 2014; Payne et al., 2015).

1.3.2 Structural insights of RdhA enzymes

Recently the crystal structure of PceA from *Sulfurospirillum multivorans* was elucidated (Bommer et al., 2014). The structure opens new avenues in understanding this important OHR metabolism as well as answer mechanistic questions. So far, studies were restricted to the use of only crude extracts or purified RdhA enzymes to study substrate specificities, however the recent paper by Bommer and co-workers (Bommer et al., 2014) brings forward the active site information. They also found out that the corrinoid cofactor is deeply buried and thus stays protected in the PceA enzyme. Along similar lines, Payne and co-workers recently described the crystal structures of a new class of corrinoid-dependent reductive dehalogenases (NpRdhA) found in aerobes (Payne et al., 2015). Both these papers contribute towards understanding the relationship of the RdhA enzyme active site, corrinoid cofactor and correlating with substrate preferences. Payne and co-workers suggest that the NpRdhA mechanism is fundamentally different from other B12-containing enzymes and from the hydrolytic dehalogenases (Banerjee and Ragsdale, 2003; Brown, 2005; de Jong and Dijkstra, 2003). They propose that the NpRdhA uses the cobalamin cofactor to attack the substrate halogen atom itself, leading to Cobalt–halogen bond breakage concomitant with protonation of the leaving group. It is believed that distinct variations on this theme could occur within the RdhA family to account for the varied substrate specificity (aliphatic versus aromatic organohalides) as well as the dihalo-elimination catalysed by some enzymes (Grostern and Edwards, 2009).

1.3.3 Iron-sulfur clusters in reductive dehalogenases

FeS clusters have been characterized by EPR analysis only for two RdhAs, namely for PceA of *D. restrictus* and CprA of *Desulfitobacterium dehalogenans*. FeS clusters present in other RdhAs have been postulated based on stoichiometry obtained from iron and sulfur extractions as well as indications of FeS cluster binding motifs from sequence information. However, sequence information is not sufficient to predict the type of FeS clusters. The CprA protein purified from *D. dehalogenans* has one [4Fe-4S] cluster and one [3Fe-4S] cluster as elucidated from EPR analysis, of which one had a low and the other a high redox potential (van de Pas et al., 1999). Upon redox titration CprA showed a signal ascribed to a [3Fe-4S] cluster which appeared with a midpoint redox potential of $E_{m,7.8}$ of +70 mV, whereas the signal from the [4Fe-4S] cluster appeared with $E_{m,7.8}$ at −440 mV. The latter redox potential is similar to that of the two [4Fe-4S] clusters found in *D. restrictus* PceA (-480 mV) (Schumacher et al.,

1997). The two FeS clusters of the *D. restrictus* PceA can be assumed to function as electron transfer devices rather than as storage of electron equivalents because of their low redox potential.

1.3.4 Corrinoid as a cofactor of the reductive dehalogenases

The active site of typical RdhAs contains a corrinoid cofactor. It has been previously shown that the $Co^{(I)}$ form of corrinoids even in its free form can reductively dechlorinate PCE and other chlorinated ethenes (Glod et al., 1997). The corrinoid cofactor is thereby responsible for the catalytic activity of the RdhA and this has been shown by light-reversible inactivation in the presence of propyl iodide and the corresponding loss of dechlorination activity (Neumann et al., 1996; Schumacher et al., 1997). Studies on *S. multivorans* have shown that the corrinoid in PceA is absolutely essential for its dechlorination activity and its loss resulted in a subsequent lack of dechlorination (Siebert et al., 2002). Upon purification and structural characterization of this particular corrinoid, it was identified as a novel one, namely norpseudovitamin B_{12} (Kräutler et al., 2003). It has been proven that norpseudovitamin B_{12} is essential for the reductive dechlorination activity of PceA in *S. multivorans* (Neumann et al., 2002; Siebert et al., 2002). A recent study has shown that when *S. multivorans* is supplied with corrinoids other than norpseudovitamin B_{12}, it significantly reduces growth and PceA dechlorination activity of S. multivorans, even though it is eventually incorporated into the PceA (Keller et al., 2013). The midpoint redox potential of PceA in *S. multivorans*, pH 7 ($E_{m,\ 7.0}$) of the $Co^{(II/I)}$ and $Co^{(III/II)}$ couples were measured by UV/vis spectrometry at -490 mV and -140 mV, respectively. The CprA enzyme of *D. dehalogenans* demonstrated a $Co^{(II)}$ EPR signal which disappears with a midpoint reduction potential of $E_{m,7.8}$ of −370 mV upon reductive titration (van de Pas et al., 1999). The cobalt content in *D. restrictus* PceA was analysed upon extraction of the corrinoid using cyanolysis. By comparing the absorbance maxima at 279, 361, 520 and 550 nm and the retention time in reversed-phase HPLC, this corrinoid was similar to the commercially available cyano-form of cobalamin (5, 6-dimethylbenzimidazole cobinamide) which was treated analogously. EPR anaylsis on PceA of *D. restrictus* exhibited a $Co^{(II)}$ signal characteristic for cobalamin in the base-off/His-off form, suggesting that the cobalt is not coordinated with a lower ligand. The midpoint redox potential of $Co^{(II/I)}$ was estimated at -350 mV (Schumacher et al., 1997). EPR spectra of VcrA-MBP reconstituted with either aquahydroxocobinamide or dicyanocobinamide were identical to each another, but significantly different from the spectrum of the cobalamin-reconstituted enzyme. This indicated that the 5,6-dimethylbenzimidazole (DMB) base of the cobalamin is likely serving as a ligand to the VcrA-bound cofactor. Recent studies also showed that DMB was essential for *D. mccartyi* reductive dehalogenation (Men et al., 2014a) unlike in *Sulfurospirillum*-type microorganisms where the lower ligand is an adenine derivative and for which it has been shown that addition of DMB in the medium led to a substantial loss of PceA activity (Keller et al., 2013).

1.4 Functional diversity of *rdhA*/RdhA in OHR bacterial genera

OHRB genome sequences are now available for either the isolates or in some cases metagenomes of the stable communities in which they live and respire. Analysis of these genomes suggests that some of the classes (the δ-proteobacteria and the *Desulfitobacterium* spp.) are metabolic generalists i.e., facultative OHRB with broad electron acceptor ranges (e.g. sulfate, iron, nitrate, dimethylsulfoxide (DMSO)), while the *Dehalobacter* and *Dehalococcoides* (including the *Dehalogenimonas*) genera are obligate OHRB that are also restricted to H_2 as their electron donor. What the latter they lack in overall metabolic diversity they make up for in *rdhA*/RdhA diversity (Hölscher et al., 2004; Kruse et al., 2013; Mukherjee et al., 2014; Rupakula et al., 2013; Seshadri et al., 2005).

A reductive dehalogenase gene (*rdhA*) is annotated based on some particular properties. A *rdhA* gene upon translation into amino acid sequence is characterized by the presence of a Twin-arginine translocation (Tat) signal peptide which harbors a conserved twin-arginine motif (RRxFLK) that is usually found in complex redox proteins that are exported to or across the cytoplasmic membrane (Berks, 1996; Berks et al., 2003; Palmer et al., 2005; Sargent et al., 2006). It is also characterized by the presence of FeS cluster binding motifs (two conserved cysteine motifs: $CX_{2-12}CX_2CX_3CP$). No conserved motif for binding the corrinoid cofactor ($DXHX_2G$) (as defined by Drennan et al., 1994) is present, in contrast to other classical corrinoid-dependent enzymes such as methyltransferases and isomerases (Ludwig and Matthews, 1997).

The first report on the purification of a RdhA enzyme mediating the reductive dehalogenation of a chlorinated aliphatic compound was presented by Neuman and co-workers in 1996 (Neumann et al., 1996). However cloning and sequencing was performed later. Maillard and co-workers characterized the PceA from *D. restrictus* in 2003 (Maillard et al., 2003). Originally starting from the N-terminal sequence of the PceA of *D. restrictus* and from a conserved amino acid stretch encoded in two already sequenced *rdhA* genes, a degenerate PCR approach allowed the isolation of the gene encoding PceA (Maillard et al., 2003). However, N-terminal sequencing of the purified protein did not correspond to the 5'-end of encoded *rdhA* gene sequences which led into the identification of the Tat signal peptide that is cleaved upon translocation of the RdhA enzymes (Maillard et al., 2003).

With rapid advances in genome sequencing and its ease to access, several OHR bacterial strains and metagenomic profiles of OHR communities have been sequenced. This effort provides with a direct insight into the *rdhA* gene pool diversity among the various genomes and environments. The advent of genome sequencing has allowed for the identification of novel *rdhA* genes which were otherwise not known in OHRB strains. Increasing numbers of *rdhA* gene sequences are being identified and deposited in databases, however very few of them have their functional role verified or substrate range identified. Recently Hug and co-workers have analysed the diversity among the many RdhA sequences available. They compared these sequences with the few

enzymes that have been biochemically characterized (Hug et al., 2013). Emerging studies clearly show that sequence homology alone cannot be used to determine their respective substrate range as two almost identical enzymes could have differing substrates as seen by Buttet and workers (Buttet et al., 2013). Thereby identification of a new *rdhA* gene needs to be associated with its corresponding substrates and hereon presented below are a few partially or fully characterized *rdhA*/RdhA examples from selected OHRB.

1.4.1 *Sulfurospirillum* spp.

Apart from the known pceA genes of S. multivorans (Neumann et al., 1996) and S. halorespirans (Luijten et al., 2003), putative rdhA genes have been identified using the degenerate primer approach such as the rdhA-Sm isolated in S. multivorans (Regeard et al., 2004) and the Dm2-rdmA isolated in S. halorespirans strain PCE-M2 (Smidt et al., 2000b). In Sulfurospirillum spp., a clear illustration of the difficulty to assign the substrate to the amino acid sequence of a given RdhA has been presented by Maillard and co-workers. A PCE-degrading enrichment culture, SL2 (Maillard et al., 2011a) has been identified which mainly contained Sulfurospirillum spp. and further subcultures thereof produced lineages with varied capacities of degrading either PCE to TCE only or PCE to DCE. Functional genotyping allowed distinguishing two new PceA encoding genes which were similar to the pceA in S. multivorans (Buttet et al., 2013). Buttet and co-workers show that relatively few changes in amino acid sequence between $PceA_{TCE}$ and $PceA_{DCE}$ (i.e. 93% identity) affected their specific substrate range i.e., $PceA_{TCE}$ dechlorinated PCE to TCE while $PceA_{DCE}$ dechlorinated PCE further till DCE. Also the PCE-dechlorination activity of $PceA_{TCE}$ was found to be five times higher than that of $PceA_{DCE}$ (Buttet et al., 2013). A third rdhA gene known as rdhA2 was identified to be present always at DNA level however never expressed in the enriched cultures. Also the substrate of rdhA2 is not known.

The first purified PceA enzme was from *S. multivorans* which catalyzed the reductive dechlorination of PCE to TCE and cis-1,2-DCE. Biochemical characterization of the main PceA enzyme from *S. multivorans* was thoroughly done Neumann and co-workers. The PceA enzyme was purified when cultivated with pyruvate and fumarate in the presence of yeast extract (Neumann et al., 1996). It had a native molecular mass of 58 kDa from gel filtration and SDS-PAGE revealed a single protein band of 57 kDa. It mediates the reductive dechlorination of PCE to *cis*-DCE. Atomic absorption spectroscopy along with difference in the UV-vis absorbance maxima (A_{580}-A_{650}) of the enzyme have allowed to estimate the cobalt and corrinoid content to be respectively 1.0 and 1.1 mol/mol of protein. *S. multivorans* PceA is localized in the periplasm (John et al., 2006) anchored most probably to the cytoplasmic membrane (Neumann et al., 1998) via the small hydrophobic protein PceB. The PceA enzyme of *S. multivorans* harbours a unique type of corrinoid cofactor, a norpseudo-B12 (Kräutler et al., 2003) which is not found in the RdhA of other OHRB identified so far. Much later, the *S. multivornas* genome paper was published (Goris et al., 2014).

1.4.2 *Desulfitobacterium* spp.

The metabolically versatile *Desulfitobacterium* genus in contrast to *Dehalococcoides* spp. and *Dehalobacter* spp. has only a limited number of *rdhA* genes per bacterial isolate. However within the *Desulfitobacterium* spp. depending on the type of chlorinated compounds they degrade, the numbers of *rdhA* genes they possess differ. I.e., for example aromatic-degrading *Desulfitobacterium* spp. such as *D. hafniense* PCP-1, strain DCB-2, *D. dehalogenans*, *D. chlororespirans* seem to possess relatively higher numbers of *rdhA*s than aliphatic-degrading *Desulfitobacterium* spp. such as strains PCE-S, TCE-1 and Y51. Thereby the pattern in the types of chlorinated compounds they can degrade is reflected in their gene diversity and numbers as well.

Also, the *Desulfitobacterium* spp. seem to have distinct preference not only for the type of chlorinated compounds they can degrade (i.e. either aromatic or aliphatic as described above or both) but specificity as well towards the chlorine substituent they can remove; for example, *D. dehalogenans* (Utkin et al., 1995), *D. hafniense*, and *D. chlororespirans* (Sanford et al., 1996; Christiansen et al., 1998; Krasotkina et al., 2001) dehalogenate at the *ortho*-position to a hydroxy group. *D. dehalogenans* can also dehalogenate hydroxy-PCBs (Wiegel et al., 1999). On the other hand, *D. hafniense* PCP-1 catalyzes dehalogenation of chlorophenols (Dennie et al., 1998) or anilines with chlorine groups at the *ortho*, *meta*, and *para* positions. The purified *D. chlororespirans* RdhA enzyme can catalyze the dechlorination of a hydroxy-PCB (3,3′,5,5′-tetrachloro-4,4′-biphenyldiol) (Krasotkina et al., 2001).

Several RdhAs have been purified from *Desulfitobacterium* spp., including the 3-chloro-4-hydroxy-phenylacetate *rdhA* from *D. hafniense* (Christiansen et al., 1998) and *D. dehalogenans* (van de Pas et al., 1999), the 3-chloro-4-hydroxybenzoate dehalogenase from *D. chlororespirans* (Krasotkina et al., 2001), the haloalkane (PCE and TCE) dehalogenases from *Desulfitobacterium* strain PCE-S (Miller et al., 1998) are just a few examples.

D. hafniense strain DCB-2 is capable of dehalogenating different chloroaromatic compounds and its genome harbors seven chlorophenol reductive dehalogenase (*cprA)*-like genes, five of which (*cprA1-cprA5*) are predicted to encode functional proteins (Nonaka et al., 2006; Kim et al., 2012). CprA3 and CprA5 of *D. hafniense* strain PCP-1 have been associated with stereospecific dechlorinating activites towards highly chlorinated phenols (Bisaillon et al., 2010; Thibodeau et al., 2004).

Miller and co-workers characterized PceA from *Desulfitobacterium* sp. strain PCE-S (Miller et al., 1998). It was purified up to 165-fold homogeneity and shown to catalyse the redutive dechlorination of PCE to *cis*-DCE showing a specific activity of 650 nkat/mg protein. The apparent K_M values of the enzyme for PCE, TCE and methyl viologen were10 µM, 4 µM and 0.3 mM respectively.

Desulfitobacterium sp. strain PCE1 has been shown to possess two RdhAs, of which one is specific for PCE as substrate and the other for chlorophenols (van de Pas et al., 2001b). The RdhA substrate specificity in cell extracts of strain PCE1 cultivated with

either PCE or 3-chloro-4-hydroxy-phenyl acetate (ClOHPA) as electron acceptor showed differences. Correspondingly, the two RdhAs differed from each other in terms of their N-terminal amino acid sequence, substrate spectrum and behaviour on anion exchange columns. Purification of the ClOHPA RdhA from strain PCE1 has revealed a 48 kDa protein resembling the CprA enzyme isolated earlier from *D. dehalogenans* and *D. hafniense* strain DCB-2 (Christiansen et al., 1998; van de Pas et al., 1999).

D. hafniense strain PCP-1 harbors several RdhAs: two enzymes have been originally isolated, one inducible RdhA enzyme for the *ortho*-dechlorination of 2,4,6-trichlorophenol (Boyer et al., 2003), and another responsible for the *meta*-dechlorination of various chlorophenols (Thibodeau et al., 2004). This indicated that the two RdhAs have a similar substrate range i.e. both dechlorinate chlorophenols but are stereospecific. Recently, yet another RdhA (CprA3) has been purified and characterized from strain PCP-1 which resembles the former RdhA from Boyer and co-workers (Boyer et al., 2003) because of its high *ortho*-dechlorinating activity, however the CprA3 had affinity toward higher chlorinated phenols such as pentachlorophenol (Bisaillon et al., 2010).

PceA from *D. hafniense* strain Y51 was purified and characterized (Suyama et al., 2002; Furukawa et al., 2005). The purified enzyme reductively dechlorintaed PCE to *cis*-DCE at a specific activity of 113.6 nmol/min/mg protein. The apparent K_m values for PCE and TCE were 105.7 and 535.3 µM, respectively. In addition to PCE and TCE, the enzyme exhibited dechlorination activity for various chlorinated ethanes such as hexachloroethane, pentachloroethane, 1,1,1,2-tetrachloroethane and 1,1,2,2-tetrachloroethane.

1.4.3 *Dehalococcoides* spp.

The apparent redundancy in *rdhA* genes can be rather considered as a genuine property of OHR bacteria. For example, the OHR obligate *Dehalococcoides* genus for which different genomes are already available displays between 17 and 36 *rdhA* genes (McMurdie et al., 2009) among which most of them have unknown substrate range. The obligate OHRB *Dehalococcoides mccartyi* strain 195 is known to have 18 *rdhA* homologs regeard (Regeard et al., 2005; Seshadri et al., 2005), *D. mccartyi* strain CBDB1 has 32, strain BAV1 has 11, and strain VS 36 different *rdhA* sequences - the highest number of *rdhAs* in any genome till date, and strain VS has the most unique (15 *rdhA*) among the three *D. mccartyi* strains (Löffler et al., 2013; McMurdie et al., 2009). While 32 of the 96 *rdhA* are unique to an individual strain, the remaining genes have at least one predicted *Dehalococcoides* ortholog.

Recently Hug and co-workers conducted phylogenetic analysis of all the available OHRB's RdhA sequences and observed that most RdhA cluster into two major clades in trees constructed by Hug *et al.*, (Hug and Edwards, 2013), the largest of which (Cluster 1, 85 sequences) contains only *Dehalococcoides*-derived RdhA such as VcrA (Müller et al., 2004) and BvcA (Krajmalnik-Brown et al., 2004), TceA and PceA (Magnuson et al., 1998) and CbrA (chlorobenzene RdhA) (Adrian et al., 2007). The

second cluster contains two sequences from *Dehalococcoides*, in addition to all presently available non-*Dehalococcoides* RdhA such as those belonging to the genera *Desulfitobacterium*, *Dehalobacter*, *Geobacter* and *Shewanella* (Hug et al., 2013).

Magnuson and co-workers partially purified two membrane-bound RdhAs from *D. mccartyi* 195, a bacterium which dechlorinates PCE to ethene (Magnuson et al., 1998). The 51 kDa PceA exclusively reduced PCE to TCE, whereas the 61 kDa TceA dechlorinated TCE and *cis*-DCE (Magnuson et al., 2000) finally leading to VC accumulation which is eventually and slowly dechlorinated to ethene in a co-metabolic process.

In *Dehaloccoides* spp. RdhAs could be preliminarily characterized for their activity and substrate range, but diffculties in obtaining sufficient biomass has hampered their further biochemical studies. Substrates for the BvcA (Krajmalnik-Brown et al., 2004) and MbrA (Chow et al., 2010) enzymes were inferred from transcriptional analysis, although biochemical confirmation is still missing. Adrian and co-workers identified the first chlorobenzene RdhA, CbrA (Adrian et al., 2007) using a combination of clear native polyacrylamide gel electrophoresis (CN-PAGE), enzyme assays, and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) peptide identification. However a few years later many new techniques were developed enabling functional attribution without requiring large amounts of biomass. On similar lines, Tang and co-workers further developed these approaches and used blue native PAGE (BN-PAGE) for *rdhA* substrate identification from *Dehalococcoides* spp. cultures. Use of BN-PAGE substantially improved recovery of RdhAs dechlorinating activity after electrophoresis, resulting in higher sensitivity and enabling analysis of a wider range of substrates (Tang et al., 2013). In cultures of *Dehaloccoides* sp. strain BAV1, BvcA was the only RdhA detected, revealing that this enzyme has a broader substrate range and catalyzes the dechlorination not only of vinyl chloride, but also of all DCE isomers and 1,2-dichloroethane (Tang et al., 2013).

The VC reductive dehalogenase, VcrA, of *Dehaloccoides* sp. strain VS was partially purified. It reduces TCE to *cis*-DCE as well, but very slowly and occurred at only 5% of the reduction rate for DCE to VC. PCE was not transformed during the course of the enzyme assay. Thus, the enzyme has preferred substrate specificity to VC and DCE over TCE and PCE. VcrA has a calculated molecular mass of 53 Da (Müller et al., 2004).

Parthasarathy and co-workers report the heterologous production of vinyl chloride reductase VcrA from *D. mccartyi* strain VS, fused with maltose binding protein and expressed in *E.coli*. Cofactors reconstitution was successful and the reconstituted enzyme had kinetic properties that were similar to those obtained from the native VcrA enzyme purified from *D. mccartyi* strain VS. VcrA was also found to catalyze a novel reaction, the environmentally important dihaloelimination of 1,2-dichloroethane to ethene along with its already known abilities of reductively dechlorinating VC, 1,1-DCE, *cis*- and *trans*-1,2-DCE.

1.4.4 *Dehalobacter* spp.

Following the characterization of *pceA* gene of *D. restrictus* (Maillard et al., 2003), Regeard and co-workers used a degenerate primer approach and isolated two new putative *rdhA* genes from *D. restrictus*: *rdhA1*-Dr and *rdhA2*-Dr (Regeard et al., 2004). Interestingly the authors found that the *rdhA2-Dr* isolated from *D. restrictus* was almost 100% identical to *rdhA-DP* gene isolated from *Desulfitobacterium* sp. strain PCE1.

Another *Dehalobacter* sp. isolate, strain E1, is known for dechlorinating hexachlorocyclohexane (HCH) in a co-culture with *Sedimentibacter* (van Doesburg et al., 2005). When the genome was studied extensively, 10 putative RdhA encoding genes were identified suggesting that strain E1 has a greater dechlorination potential than previously observed (Maphosa et al., 2012b).

The diversity of *Dehalobacter*-derived *rdhA* sequences has been studied in a co-culture containing *Dehalobacter* spp. and *Acetobacterium* spp. (Grostern et al., 2009). Three new *rdhA* genes have been identified encoding WL-RdhA1 which shows 95% to PceA of *D. restrictus* and two partial sequences, WL-RdhA2 and WL-RdhA3. The two latter sequences were identified using degenerate PCR and had respectively 74% and 96% identity on amino acid level with WL-RdhA1 (Grostern and Edwards, 2009).

The PCE reductive dehalogenase (PceA) from *D. restrictus* was among the early RdhAs to be purified and biochemically characterized (Schumacher and Holliger, 1996; Schumacher et al., 1997; Maillard et al., 2003). It was purified anaerobically from *D. restrictus* membranes using detergents such as OGP and Triton X-100, KCl, and subsequent chromatography techniques of HPLC followed by size exclusion. It had a molecular mass of 60 kDa. PceA reductively dechlorinates PCE and TCE to *cis*-DCE with a specific activity of 250 ± 12 nkat/mg of protein. The K_m values for PCE, TCE, and methyl viologen were 20.4 ± 3.2, 23.7 ± 5.2, and 47 ± 10 μM, respectively. The PceA was oxygen sensitive with a half-life of 280 ± 10 min upon exposure to air. PceA reductive dehalogenase activity of *D. restrictus* seems to be 100-200 times higher than PceA of *D. hafniense* strain Y51 although they are 99% identical thus aruguing for a better quality preparation of the former. However, PceA of *D. restirctus* has 3-10 times lower activity than that of the PceA proteins isolated from *D. hafniense* strain PCE-S (Miller et al., 1998) and *S. multivorans* (Neumann et al., 1998).

1.4.4.1 Dechlorination potential of the *Dehalobacter* genus

The *Dehalobacter* genus as a whole seems to have a wide range of dechlorination potential though the isolated strains individually seem to have restricted ranges (Holliger et al., 1998b; Wild et al., 1996; Sun et al., 2002). Various studies have been conducted with *Dehalobacter* pure culture isolates, some with mixed cultures and others with novel and uncharacterized *Dehalobacter* spp. found in environmental samples. A preliminary argument for their involvement in the dechlorination of various compounds is the increase in the copy number of *Dehalobacter* 16S rRNA gene which is an indirect measure of growth, thereby predicting respiration of these organohalides by the corresponding *Dehalobacter* spp.. From the following studies, it is possible to

speculate about the involvement of *Dehalobacter* spp. in dechlorinating the following compounds: dichloromethane (Grostern et al., 2010), chloroform (Grostern et al., 2010; Lee et al., 2012), dichloroethane, 1,1,1-trichloroethane (Grostern and Edwards, 2006), and other trichloroethanes (Sun et al., 2002), trichlorobenzenes (von Wintzingerode et al., 1999), dichlorobenzenes (Nelson et al., 2011), tetrachlorophthalides (Yoshida et al., 2009), β-hexachlorocyclohexane (van Doesburg et al., 2005), and 1,2-dichloroethene (Imfeld et al., 2010).

Dehalobacter spp. are either isolated in pure culture such as *D. restrictus* strain PER-K23 (Holliger et al., 1998b) or are present as co-cultures such as *Dehalobacter* spp. strain CF (Grostern et al., 2010; Tang and Edwards, 2013), strain DCA (Grostern and Edwards, 2009; Grostern et al., 2009), strain E1 (Maphosa et al., 2012b), strain UNSWDHB (Deshpande et al., 2013; Lee et al., 2012), and strain FTH1 (Yoshida et al., 2009). Recently a *Dehalobacter* sp. strain TCP1 was isolated from a digester sludge sample and is able to dechlorinate 2,4,6-trichlorophenol (2,4,6-TCP) to 4-monochlorophenol (4-MCP) (Wang et al., 2013). Two other *Dehalobacter* isolates have been reported: *Dehalobacter* sp. TEA able to dechlorinate PCE and TCE (Wild et al., 1996) and *Dehalobacter* sp. TCA1 dechlorinating 1,1,1-trichloroethane to chloroethane (Sun et al., 2002), both strains being however only poorly characterized on biochemical and genetic level. Many studies have described cocultures or enrichment cultures where *Dehalobacter* spp. have been considered as the key player in the dechlorination of several other organohalides. A coculture containing *Dehalobacter* sp. E1 and *Sedimentibacter* sp. has been obtained for the dechlorination of β -hexachlorocyclohexane (β-HCH) to benzene and chlorobenzene (van Doesburg et al., 2005) and the metagenome was analysed (Maphosa et al., 2012b).

1.4.5 Heterologous production of reductive dehalogenases

For a long time, study of RdhA had been exclusively done on natively purified proteins from the OHRB itself. However, direct protein purification from OHRB is impractical and unlikely to routinely yield the high levels required for an array of detailed biophysical studies as it is usualy accompanied by low cellular yields in the *Dehalococcoides* and *Dehalobacter* spp., oxygen sensitivity of RdhAs to date are key hurdles to purification and characterization of an RdhA's substrate range. In addition, obligate OHRB contain several homologues of *rdhA* genes that might be coexpressed and therefore making it difficult to analyze for the specific substrate range of the respective *rdhA* gene products. For example, even when fed a single substrate *Dehalococcoides* spp. strains have been shown to express multiple RdhAs simultaneously, further confounding purification of individual RdhAs from OHRB cultures (Johnson et al., 2008; Waller et al., 2005, 2012). Such limitations could be overcome by the functional heterologous expression system for *rdhA* genes. In addition, availability of heterologous production platforms could further allow functional analysis of RdhA enzymes for example by facilitating approaches such as site-directed mutagenesis.

Thereby earlier studies such as heterologous expression of the PceA of *D. hafniense* Y51 and *S. multivorans* into *Escherichia coli* were attempted yet unsuccessful (Suyama et al., 2002; Neumann et al., 1998). Dechlorinating activity was not observed and it was suggested that the enzyme was not folded properly and/or that the proper cofactor was absent when expressed in *E. coli*.

Recently Sjuts and co-workers studied a robust heterologous system to enable production of the cofactor- reconstituted PceA from *D. restrictus* in milligram quantities from *E. coli*. They used Strep-tagged PceA fused to a cold-shock induced trigger factor and performed purification under anaerobic conditions by StrepTactin affinity chromatography. Thereafter the trigger factor (TF) could be removed through limited proteolysis followed by characterization of the purified PceA by UV-Vis and EPR spectroscopy. There were however discrepancies between the natively purified PceA characterised by Schumacher and co-workers (Schumacher and Holliger, 1996; Schumacher et al., 1997; Holliger et al., 1998b) as well as the current study by Sjuts and co-workers (Sjuts et al., 2012). The recombinant PceA analysis revealed that the protein binds methylcobalamin in the base-on form after proteolytic cleavage of the trigger factor, unlike the base-off conformation reported for the PceA originally purified from *D. restrictus*. Moreover, EPR spectroscopy revealed the purified TF-PceA fusion protein to be only partially reconstituted with 4Fe-4S clusters. Thereby though this study demonstrates an efficient platform for heterologous over-production of PceA however the enzyme was eventually inactive (Sjuts et al., 2012) and could not be used for further biophysical analysis.

Following the previous study, two years later, the PceA of *D. hafniense* strain Y51 was heterologously produced in a catalytically active form in *Shimwellia blattae* (ATCC 33430), a Gram-negative γ-proteobacterium (which does not dechlorinate but can produce cobamide *de novo*) (Mac Nelly et al., 2014). Mac Nelly and co-workers observed enhanced catalytic activity of the PceA when the dedicated PceT chaperone was coproduced as well as when the lower ligand (5,6-dimethylbenzimidazole) and cobalamin were externally supplemented to the *S. blattae* cultures. Using this technique, the authors studied RdhA3 from *D. hafniense* DCB2. It was heterolously expressed in *S. blattae* and helped in identifying that RdhA3 used substrates such as PCE, 3,5-dichlorophenol (DCP), 2,3-DCP, and 2,4-DCP (Mac Nelly et al., 2014) i.e., allowed to characterize the substrates of an enzyme whose substrate spectrum has not been reported before.

Yet another successful production of RdhA, however homologously was performed by Lohner and co-workers (Lohner and Spormann, 2013). They transformed plasmid inserts containing respective *rdhA* genes from *Shewanella* itself into the wild-type (WT) *Shewanella sediminis* strain HAW-EB3 (Zhao et al., 2005) (AS1028) through bi-parental mating using *E. coli* WM3064 as conjugal donor. Analysis of mutants carrying in-frame deletions of all five *rdhA* genes showed that only deletion of Ssed_3769 *rdhA* resulted in the loss of PCE dechlorination activity suggesting that Ssed_3769 is a functional RdhA in *Shewanella* spp. However it was found that the *S.*

sedimins performs reductive dechlorination as a co-metabolic process and cannot depend on it solely for its growth.

A remakarkable study by Bommer and co-workers led to the crystallization of PceA of Sulfurospirillum multivorans (Bommer et al., 2014). In this study, the PceA enzyme was homologously produced and purified from a S. multivorans mutant strain. The mutant strain produced a PceA variant, which contained a C-terminal Strep-tag (PceA-Strep). This shows a very good example of homologous recombination for the first time of a RdhA enzyme within a OHRB, and using such a strategy hereon could help produce RdhAs in strains which have their own complete corrinoid biosynthesis pathway as well as the de novo capacity to synthesise RdhAs.

Around the same time, the crystral structure of an oxygen-tolerant RdhA was reported by Payne and co-workers (Payne et al., 2015) as a result of heterologous recombination using *Bacillus subtilis* to express the RdhA, NprdhA from *Nitratireductor pacificus* pht-3B.The authors combined structure determination with EPR spectroscopy and simulation, to eventually highlight that a direct interaction between the cobalamin cobalt and the substrate halogen underpins catalysis.

1.5 Genetic environment and function of *rdhA* genes/proteins

In most of the OHRB genomes studied till now, one universal finding about *rdhA* genes is the presence in the direct vicinity of a second open reading frame named *rdhB* which codes for a predicted small hydrophobic membrane protein. The *rdhB* genes are located either upstream of *rdhA* like in *D. dehalogenans cprBA* cluster (Smidt et al., 2000a) or downstream of it such as the *pceAB* genes of *S. multivorans* or *D. restrictus* (Maillard et al., 2003; Neumann et al., 1998). Co-transcription of *rdhA* and *rdhB* genes has been shown in both types of clusters indicating a functional link of the two gene products. RdhB, despite a very low level of sequence identity, displays consensually 2 or 3 transmembrane helices strongly indicating a role in anchoring RdhA at the membrane as it was predicted for various OHRB (Smidt et al., 2000a; Maillard et al., 2003). Several attempts have been done to tackle the physiological function of PceB without success (John et al., 2006; Prat et al., 2011).

1.5.1 The *cprTKZEBACD* gene cluster

The genetic environment around the *cprA* gene has been studied in *D. dehalogenans* and they found an eight gene cluster, *cprTKZEBACD*, with all genes with the same orientation except for *cprT* (Smidt et al., 2000a). CprC has been postulated to belong to the NirI/NosR transcriptional regulator family (Wunsch and Zumft, 2005; Zumft, 1997) whereas CprK has been proposed to belong to the CRP-FNR family of transcriptional regulators (Körner et al., 2003). CprD and CprE have been predicted to be molecular chaperones of the GroEL type, whereas CprT has shown some similarity with the Trigger Factor, another general molecular chaperone.

1.5.2 The *pceABCT* gene cluster

The *pceABCT* gene cluster has been originally isolated and characterized from *D. restrictus* and *D. hafniense* strain TCE1 (Maillard et al., 2005), and a very similar genetic structure has also been identified in *D. hafniense* strain Y51 (Futagami et al., 2006a), the *dcaABCT* in *D. dichloroeliminans* (Maillard et al., 2005), and in sequences deposited in databases originating from other OHR bacteria (Duret et al., 2012). Next to PceA that was described above, this gene cluster encodes PceB, the predicted membrane anchor, PceC and PceT, both homologs of CprC and CprT described in the *cpr* gene cluster. The PceT protein has been recently shown to play the role of a dedicated Tat chaperone involved in folding of the PceA protein (Maillard et al., 2011b; Morita et al., 2009).

A common feature of reductive dehalogenases is the presence of an N-terminal Tat (twin-arginine-translocation) signal peptide which contains the conserved S/TRRXFLK sequence (Berks, 1996) conferring them the capacity to be translocated through the cell membrane. The fundamental difference of the Tat protein translocation system from the general secretory (Sec) transport system is that proteins are transported across the cytoplasmic membrane in their folded form. Proteins with the twin-arginine signal sequence often bind complex cofactors, such as iron-sulphur clusters, molybdopterin guanine dinucleotide or molybdopterin (Palmer et al., 2005; Sargent et al., 2006). It is assumed that the maturation of these complex cofactor-containing enzymes proceeds with the help of several assisting proteins. Indeed, reductive dehalogenase-encoding genes are often linked with genes whose products show high similarity to molecular chaperons and trigger factors (TF) (Maillard et al., 2011a). In the *Desulfitobacterium* genus, there were two complete TF chaperones and a third TF-like protein that lacks the N-terminal domain required for Ribosome binding (Morita et al., 2009) i.e. the PceT which is present as a part of the *pceABCT* gene cluster. The PceT lacks the N-terminal domain and is the “dedicated” chaperone for the PceA. It has been shown to bind specifically to the signal peptide of PceA. PceT has 99% identity to other putative TF proteins found in the dechlorinating *pceABCT* gene clusters in *D. restrictus* PER-K23, *D. dichloroeliminans* DCA1, and *D. hafniense* DCB2 (Duret et al., 2012).

1.5.3 Horizontal gene transfer of *rdh* gene clusters

Genomic analyses and subsequent expression profiling studies in various labs have provided key data for piecing together not only the genetics and enzymology of OHR, but also the regulation, origins, and horizontal movement of the key class of enzymes, the reductive dehalogenases (RdhAs).

Maillard and co-workers identified and isolated a new catabolic and active transposon containing the *pceABCT* gene cluster in *D. hafniense* TCE1 (Maillard et al., 2005). They found two insertion sequences (ISDha1) surrounding the *pceABCT* gene cluster which are completely identical to each other and are consequentially new members belonging to the IS256 family. Promoter mapping of the *pceA* gene in strain TCE1,

revealed the presence of a strong promoter partially encoded in the right inverted repeat of ISDha1. It was observed from sequence comparison that *Desulfitobacterium* sp. strains TCE-1, PCE-S and Y51 possess the same transposon structure including 100% identity of the *pceABCT* gene cluster,whereas only the *pceABCT* gene cluster is conserved in *D. restrictus.*

Similar looking transposon structures have been also identified in other OHRB such as *D. hafniense* strain Y51 (Futagami et al., 2006b), and the *dcaABCT* of *D. dichloroeliminans* strain DCA1 (Marzorati et al., 2007). Interestingly when comparing PceA and DcaA amino acid sequence only 88% identity was observed, while an overall 97% identity was calculated from DNA sequence of the complete gene clusters. This could suggest that both strains might have acquired the transposon structure by horizontal gene transfer and in the following each strain might have adapted its catalytic subunit for PCE or 1,2-dichloroethane (1,2-DCA) as substrate, respectively (Duret et al., 2012).

In the *Dehalococcoides* spp., horizontal gene transfer evidence was brought about by the identification of genomic islands (GEIs) (Kube et al., 2005; Seshadri et al., 2005; Regeard et al., 2005; McMurdie et al., 2009). Comparative genome analysis withitn the *Dehalococcoides* spp. genus, showed that of the 96 *rdhA*s, 91 were located in hyper plasticity regions (HPRs). Only three core *rdhA* orthologous groups were identified within which only one of them was syntenic among the *Dehalococcoides* genus. They identified low numbers of the core *rdhA*s in contrast to the very high numbers of *rdhAB* in the rest of the genome for each *Dehalococcoides* spp. (owing to even a max. of 36 per strain at times as found in strain VS). Followed with the observation that such *rdhA*s mostly colocalised within GEIs strongly suggested towards active horizontal gene transfer within the genus for adaptaion to the niches they are present in, i.e. to enable their only feasible form of metabolism, OHR. Such an adaptation allowed these organisms to allow multiple mechanisms of recombination however confined soley within the HPRs and maintain an otherwise stable and syntenic genome for a small, free-living organism.

Around the same time, Regeard and co-workers, studied the genome of *D. mccartyi* strain 195, and elucidated that it contains 18 copies of putative *rdhA* genes including the well-characterized *tceA* gene however within regions containing atypical signatures from the rest of the genome. This was concluded from genome-analysis using a bioinformatics tool basing on the frequency of oligonucleotides which then resulted in the form of genomic signatures realving regions of local disruptions (indicating foreign DNA) from the rest of the host genome's signature along the genome of of *D. mccartyi* strain 195. And surprisingly 15 of the 18 *rdhA*s including the *tceA* was found within such disrupted local signatures/atypical regions indicating horizontal gene transfer to the *D. mccartyi* (Regeard et al., 2005).

Comparative and metagenome analyses of *Dehalococcoides* genomes highlight two high plasticity regions (HPR) around the origin of replication but otherwise the strains show high synteny and identity (1029 genes conserved in all strains) (McMurdie et al.,

2007, 2009, 2011; Taş et al., 2010). McMurdie and co-workers found that within the *Dehalococcoides* spp. strains VS and BAV1 which encoded the *vcrAB* and *bvcAB* respectively, these were found embedded in distinct GEIs with different predicted integration sites, suggesting that these genes were acquired horizontally and independently by distinct mechanisms. More generally genomic plasticity seems to have played a role in the redundancy of *rdhA* genes present across the *Dehalococcoides* genus (McMurdie et al., 2009). While 32 of the 96 known *Dehalococcoides rdhA* are unique to an individual strain, the remaining genes have at least one predicted ortholog in another *Dehalococcoides*. Most ortholog pairs are present in the same hyper plastic region (HPR) and are supported by local synteny. Moreover the *vcrABC* gene cluster in *Dehalococcoides* sp. strain VS has been postulated to be part of a genomic island (McMurdie et al., 2011).

However, in *S. multivorans,* the two putative phage sequences were found close to the OHR region (Goris et al., 2014), and phages have been reported to eventually play a role in horizontal gene transfer of *rdhAs* within *D. mccartyi* (McMurdie et al., 2011; Pöritz et al., 2013; Waller et al., 2012) however no evidence of their role in Sulfurospirillum has yet been documented.

1.5.4 Transcription regulators and regulation of *rdhA* gene transcription in OHRB

Various types of transcriptional regulators seem to exist in OHR bacteria such as the putative two component regulatory systems of the MarR type (multiple antibiotic resistance) identified next to *rdhA* genes in *Dehalococcoides* genomes (Kube et al., 2005; Seshadri et al., 2005; Wagner et al., 2013) or the CprK type discovered in *Desulfitobacterium* spp (Smidt et al., 2000a; Gábor et al., 2006).

Within the *cpr* gene cluster of *D. dehalogenans* described earlier in this chapter, an open reading frame, *cprK*, has been proposed to encode a member of the CRP/FNR family of transcriptional regulatory proteins (Smidt et al., 2000a). In recent years, CprK has been the subject of intensive research down to the molecular and mechanistic levels (Smidt et al., 2000a; Gábor et al., 2006, 2008; Gupta and Ragsdale, 2008; Joyce et al., 2006; Kemp et al., 2013; Levy et al., 2008; Mazon et al., 2007; Pop et al., 2004, 2006). Rich in cysteine residues but apparently lacking motifs for FeS clusters CprK acts like a redox switch. In the reduced state, the effector domain binds to its ligand, 3-chloro-4-hydroxyphenylacetate (ClOHPA) with high affinity, promoting specific helix-turn-helix DNA-binding interactions with promoter regions of several *cpr* genes thereby activating transcription of these genes. However, in the presence of oxygen or absence of the substrate, the redox switch CprK is inactivated thereby preventing transcription of *cprTKZEBACD* gene cluster downstream.

Waller and co-workers performed transcriptional analysis of the multiple *rdhA*s identified in a *Dehalococcoides*-containing mixed culture KB1 in the presence of different electron acceptors: TCE, *cis*-DCE, VC, and 1,2-DCA. Their results have shown that only two (*bvcA* and *vcrA*) of a total of fourteen amplified *rdhA* genes were

transcribed consistently in response to the above four compounds, whereas in the presence of each individual compound, multiple *rdhA* genes were simultaneously transcribed (Waller et al., 2005). These findings suggest that multiple *rdhA* genes are induced by a single chlorinated substrate and that multiple RdhAs contribute to degradation of aliphatic chlorinated compounds in KB1. Many other studies have indicated differential *rdhA* expression patterns too (Villemur et al., 2002; Gauthier et al., 2006; Rahm et al., 2006; Wagner et al., 2009; Chow et al., 2010; Bisaillon et al., 2011). No data however has been produced to elucidate the possible regulation mechanisms.

To answer such similar questions, Johnson and co-workers in their studies of a TCE-fed enrichment culture that contained *Dehalococcoides* spp., have reported increased levels of *tceA* mRNA in starved cells after amendment of TCE, *cis*-DCE, *trans*-DCE, or 1,1-DCE but not PCE or VC (Johnson et al., 2005). These findings suggest that a molecular mechanism of *tceA* transcription regulation is occurring by which only specific chlorinated substrates are recognized. Curiously, unlike most other *rdhA* genes, there were no genes with similarity to transcription regulators adjacent to *tceA*, so it is also possible that regulatory circuits encoded elsewhere in the chromosome play a role in regulation.

The genus *Dehalococcoides* spp. has the ability to dechlorinate a multitude of organohalides which is also reflected in the number and diversity of *rdhA* genes in their genomes. However most of these *rdhAB* genes are located in the vicinity of multiple antibiotic resistance regulator (MarR)-type or two-component system regulators. In 2005, Kube and co-workers, analysed the genome of *D. mccartyi* strain CBDB1's and identified 28 histidine kinase, 34 response-regulator genes as well as 16 regulators of the MarR-family, among which 15 of the two-component regulatory systems and 13 of the MarR-type regulators were found in association with *rdhAB* genes (Kube et al., 2005).

Recently Wagner and co-workers studied the transcriptional response of *rdhA* genes in *D. mccartyi* strain CBDB1 to organohalides as 2,3- and 1,3-dichlorodibenzo-p-dioxin (DCDD). They found that almost all *rdhA* genes were transcribed in the presence of the former organohalide, however at different levels. In contrast the latter organohalide did not induce *rdhA* transcription. Wagner and co-workers heterologously produced the putative MarR and demonstrated its *in vitro* binding ability to the promoter regions of the genes *cbdbA1624* (*rdhA*) and *cbdbA1623 (rdhB*). To analyse further the regulation *in vivo*, they used transcriptional promoter-lacZ fusions of different *rdhA* genes and of MarR gene (*cbdbA1625*) and studied in *E. coli* for their expression levels. The MarR 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 (Wagner et al., 2013).

1.6 Corrinoid biosynthesis, metabolism and regulation in OHR bacteria

Corrinoids are complex organometallic cofactors associated with three families of enzymes: the adenosylcobalamin (AdoCbl) dependent isomerases, the cobalamin-dependent methyltransferases, and the reductive dehalogenases (RdhAs). While the reaction mechanism of the two former families is relatively well understood, it was not known till recently on how corrinoids catalyze the reduction of organohalides in RdhAs (Banerjee and Ragsdale, 2003). The role of corrinoid cofactor in the RdhA from chlorophenol and PCE-degrading organisms is of special interest, since it does not mediate the usual rearrangement or alkyl transfer but a reduction reaction by transferring electrons from the super-reduced form of cobalt (Co^{I}) of the corrinoid to the substrate i.e., which involves formation of radical intermediates (Kräutler et al., 2003; Ludwig and Matthews, 1997; Schumacher et al., 1997). This reaction represents a completely new type of biochemical reaction, in which the cobalt of the corrinoid appears to be subjected to a change in its redox state in the course of the dehalogenation.

1.6.1 Corrinoid biosynthesis by OHRB

Corrinoids function as enzyme cofactors in a wide variety of organisms but are produced solely by a subset of prokaryotes. A recent bioinformatic analysis revealed that while 76% of 540 sequenced bacterial genomes contain corrinoid-dependent enzymes, only 39% of these genomes contain the complete corrinoid biosynthesis pathway (Zhang et al., 2009). One major biologically active form of corrinoid is cobalamin, which is composed of a highly modified cobalt-containing tetrapyrrole linked to a nucleotide loop that houses an unusual base called 5,6-dimethylbenzimidazole. This base usually acts as a lower ligand to the cobalt atom, whereas the upper cobalt ligand is provided by either a water (aquocobalamin), a hydroxyl group (hydroxocobalamin), a methyl group (methlycobalamin), or a 5′-deoxyadenosyl group (adenosylcobalamin, AdoCbl). The cobalt-containing modified tetrapyrrole moiety is referred to as the corrin ring and differs in structure from other tetrapyrroles, because it has undergone contraction as part of the biosynthetic process. The biosynthesis pathway for corrinoid is complex consisting of about 30 reactions till the synthesis of the end product AdoCbl. It has also been studied that *btuFCD* genes encoding a high-affinity corrinoid transporter are present in 76% of sequenced bacterial genomes, possibly indicating that most bacteria are capable of taking up corrinoids from the environment (Zhang et al., 2009) especially, if they cannot synthesize it. Based on these experimental and bioinformatic results, corrinoid salvaging is likely to be widespread in microbial communities (Degnan et al., 2014; Seth and Taga, 2014).

1.6.1.1 Obligate OHRB

From genome analyses it has been known that obligate OHRB such as *Dehalococcoides* spp. which absolutely need the corrinoid (Löffler et al., 2013; Schipp et al., 2013; West et al., 2008), lack the complete corrinoid biosynthesis pathway and

thereby cannot *de novo* synthesize it. In *D. mccartyi* strain 195, studies have shown that it cannot synthesize corrinoids *de novo* and needs corrinoid supplementation in the growth medium (Johnson et al., 2009).

To cope with corrinoid auxotrophy, *Dehalococcoides* spp. have been known to salvage corrinoids either from the environment or in co-cultures (Gray and Escalante-Semerena, 2009; Yan et al., 2012, 2013; Yi et al., 2012; Löffler et al., 2013) using specialized gene products involved in re-modelling the nucleotide loop and lower ligand assembly such as adenoslycobinamide amidohydrolase, CbiZ (Gray and Escalante-Semerena, 2009).

1.6.1.2 Facultative OHRB

Unlike the obligate OHRB, the facultative OHRB like *Sulfurospirillum* spp. (Neumann et al., 2002; Siebert et al., 2002; Keller et al., 2013), *Geobacter lovleyi* (Wagner et al., 2012; Yan et al., 2012) and *Desulfitobacterium* spp. do encode a complete corrinoid biosynthesis pathway and can produce the corrinoid *de novo* (Suyama et al., 2001; Nonaka et al., 2006)

Upon elucidating the genome of *D. hafniense* strain Y51 (Nonaka et al., 2006), the complete anaerobic corrinoid biosynthetic pathway was confirmed. Later it was shown for other *Desulfiitobacteruim* spp. as well such as strain TCE-1 by Choudhary and co-workers (Choudhary et al., 2013) who further studied the regulation of these corrinoid biosunthesis genes by riboswitches. Kim and co-workers showed the presence of the corrinoid biosynthesis pathway as well in *D. hafniense* strain DCB-2 (Kim et al., 2012)

The PceA enzyme of *S. multivorans* harbours a unique type of corrinoid cofactor not found in other organohalide-respiring bacterial classes to date, a norpseudo-B12 (Kräutler et al., 2003), which is essential for reductive dechlorination in *S. multivorans* (Siebert et al., 2002) and it has been shown that this cofactor is synthesized *de novo* by *S. multivorans* (Keller et al., 2013). Neumann and co-workers found that the rates of abiotic dechlorination of halogenated compounds other than chloroethenes, when tested with the heat-inactivated PceA from *S. multivorans* were higher than those for the usual cyanocobalamin, thereby leading to the prediction that the cofactor in the PceA enzyme was certainly not a cyanocobalamin (Neumann et al., 2002). It has been reported that the *S. multivorans* strain K was able to synthesize the corrinoid unlike another variant strain N for which the absence of dechlorinating activity was due to the lack of a few corrinoid biosynthesis or uptake genes (Siebert et al., 2002). Eventually, Goris and co-workers identified the entire *de novo* corrinoid biosynthetic pathway as well as observed a very close clustering of both these genes and genes for reductive dehalogenation which has not been found in any other OHRB so far, for example neither in the *D. hafniense* strains (Nonaka et al., 2006; Kim et al., 2012; Choudhary et al., 2013) nor in *Geobacter lovleyi* SZ (Wagner et al., 2012).

1.6.1.3 Regulation of corrinoid metabolism by riboswitches

Recent advances have been made toward understanding how corrinoids can regulate gene expression in bacteria by interacting with cobalamin riboswitches (Cbl-RS) (Winkler and Breaker, 2005). Riboswitches are RNA elements located in the 5'-

untranslated region of transcripts that act to control the expression of downstream genes after transcription initiation. Cbl-RS are typically composed of two parts, an aptamer domain that binds cobalamin and an expression platform that usually translates cobalamin binding into the shutdown of the transcription or translation process. The genes controlled by Cbl-RS are often involved in corrinoid metabolism and transport, but can also regulate genes involved in cobalt transport, cobalamin-independent ribonucleotide reductases, and glutamate and succinate fermentation (Rodionov et al., 2006; Vitreschak et al., 2004). Johnson and co-workers studied the transcriptional analysis of *D. mccartyi* strain 195 and found that it adjusts its metabolism according to the corrinoid forms available for uptake. Genes *cobT*, *cobS*, *cobC* and *cobU* are predicted to encode proteins which build the lower ligand base that is attached to cobyric acid (Escalante-Semerena, 2007; Warren et al., 2002) and are present downstream of a putative Cbl-RS. These genes were found to be down-regulated upon exposure to the spent medium from a dechlorinating consortium but not upon exposure to excess cyanocobalamin, which meant that the corrinoids taken up in the former case was different from the usual cobalamin (Johnson et al., 2009).

The Rfam database (http://rfam.sanger.ac.uk/) (Griffiths-Jones et al., 2003) has been used to identify putative Cbl-RS in the genome of OHR bacteria. From the comparative genome analysis, sixteen Cbl-RS have been predicted in *D. hafniense* strain TCE1 and strain Y51, whereas eleven in strain DCB-2 (Choudhary et al., 2013). It is quite unusual to find such a high number of Cbl-RS in a single strain. For example in *Dehalococcoides* spp. (strains 195, BAV1, and VS), only two Cbl-RS have been found (Johnson et al., 2009). Studies on *D. hafniense* strain TCE1 showed that the transcription of several Cbl-RS dependent genes were indeed repressed after addition of cobalamin in cells cultivated in a medium depleted of corrinoids (Choudhary et al., 2013).

Chapter 2

The restricted metabolism of the obligate organohalide respiring bacterium *Dehalobacter restrictus* – lessons from tiered functional genomics

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2 The restricted metabolism of the obligate organohalide respiring bacterium *Dehalobacter restrictus* – lessons from tiered functional genomics

2.1 Introduction

Dehalobacter restrictus strain PER-K23 has been isolated from a tetrachloroethene (PCE) dechlorinating enrichment culture originally obtained from sediment of the Rhine River mixed with anaerobic granular sludge (Holliger et al., 1998b). *D. restrictus* is a Gram-positive member of the *Firmicutes* growing exclusively via organohalide respiration (OHR) with H_2 as electron donor, PCE or trichloroethene (TCE) as sole electron acceptors, and acetate as carbon source. The key catalytic enzyme in OHR with PCE, the reductive dehalogenase PceA, has been purified and shown to harbour a corrinoid and two 4Fe/4S clusters (Maillard et al., 2003). In *D. restrictus*, the PceA enzyme is encoded by a gene that is part of the *pceABCT* gene cluster which has been shown to be highly conserved in several other OHR strains belonging to the genus *Desulfitobacterium* (Duret et al., 2012; Maillard et al., 2005). The newly available genome sequence of *D. restrictus* was obtained and revealed a high number of 25 predicted reductive dehalogenase homologue (*rdhA*) encoding genes (Kruse et al., 2013), though only PCE and TCE have been recognized as physiological substrates. This observation clearly raises the question of the true bioremediation potential of *D. restrictus*.

Two other *Dehalobacter* isolates have been reported: *Dehalobacter* sp. TEA able to dechlorinate PCE and TCE (Wild et al., 1996), and *Dehalobacter* sp. TCA1 dechlorinating *1,1,1*-trichloroethane to chloroethane (Sun et al., 2002), both strains being however not yet characterized in detail on biochemical and genetic level. Many studies have described cocultures or enrichment cultures where *Dehalobacter* spp. have been considered as the key player in the dechlorination of several other organohalides. A coculture containing *Dehalobacter* sp. E1 and *Sedimentibacter* sp. B4 has been obtained for the dechlorination of □-hexachlorocyclohexane (□-HCH) to benzene and chlorobenzene (van Doesburg et al., 2005). The draft genome of *Dehalobacter* sp. E1 has been recently reconstituted and was shown to harbour 10 putative *rdhA* genes, including a gene cluster with high similarity to *pceABCT* present in *D. restrictus*, although strain E1 has not been shown to grow on PCE .

Further organohalides such as dichloroethane, chloroform, dichlorobenzenes or *4,5,6,7*-tetrachlorophthalide were shown to be dechlorinated by enrichment cultures dominated by *Dehalobacter* spp (Grostern et al., 2009, 2010; Nelson et al., 2011; Yoshida et al., 2009), suggesting that the degradation potential of the genus *Dehalobacter* is largely beyond PCE and TCE. Finally, fermentation of

dichloromethane by members of *Dehalobacter* has been shown (Justicia-Leon et al., 2012; Lee et al., 2012), suggesting that not necessarily all members of this genus are obligate OHR bacteria (OHRB).

The apparent redundancy in *rdhA* genes can be rather considered as a genuine property of OHRB that are otherwise restricted in their metabolism. For example genomes of members of the OHR-obligate *Dehalococcoides* genus for which 5 different genomes are already available (and 3 more pending) display between 10 and 36 *rdhA* genes (McMurdie et al., 2009), most of which have unknown substrate range. In contrast, completed genomes of members of the metabolically versatile *Desulfitobacterium* genus revealed the presence of only a limited number of *rdhA* genes with *D. hafniense* DCB-2 harbouring a maximum of 7 copies (Kim et al., 2012; Nonaka et al., 2006). While the composition of the genes associated with *rdhA* genes is strongly varying in the genomes of OHRB, *rdhA* subunits are almost invariably accompanied by a short open reading frame, *rdhB*, with the exception of the recently sequenced genome of *Dehalogenimonas lykanthroporepellens* (Siddaramappa et al., 2012). Despite a very low level of sequence similarity, RdhB proteins display consensually 2 or 3 transmembrane helices strongly indicating a role in anchoring the catalytic subunit in the membrane.

Recently proteomics and transcriptomics studies were used to study the metabolism of the two OHRB, *Desulfitobacterium hafniense* strain TCE1 (Prat et al., 2011) and Y51 (Peng et al., 2012), respectively, under different growth conditions, both confirming the apparent lack of regulation of the *pceA* gene that was postulated earlier (Maillard et al., 2005). Most omics studies involving OHRB have however focused on members of the *Dehalococcoides* genus. This genus, although phylogenetically distant to *Dehalobacter*, inhabits similar ecological niches and is exclusively dependent on OHR metabolism with H_2 as electron donor. These studies have employed both transcriptomics using full genome microarrays and proteomics to identify key components of the metabolism of OHRB under different growth conditions or growth phases (Fung et al., 2007; Johnson et al., 2008, 2009; Morris et al., 2006, 2007; Rahm et al., 2006).

In addition to genes directly linked to reductive dehalogenation, the genome of *D. restrictus* furthermore encodes one formate dehydrogenase, eight hydrogenase complexes, among which three uptake hydrogenases (Hup-type) and one energy-conservation hydrogenase (Ech-type) and one hydrogenase-3 (Hyc-type) (Kruse *et al*., submitted paper), similar to what has been described for *Dehalococcoides*. No data is yet available, however, concerning the role of these enzymes in the metabolism of *D. restrictus*.

Detailed studies of the metabolism of members of the *Dehalobacter* genus have so far been hampered by the lack of full genome information. Hence, the recently elucidated genome sequence of *D. restrictus* now provides the necessary basis for detailed studies of the metabolism of this obligate OHR bacterium using a tiered functional genomics approach.

2.2 Material & Methods

2.2.1 Bacteria and growth conditions

Dehalobacter restrictus strain PER-K23 (DSM 9455) was cultivated as described earlier (Holliger et al., 1998b; Maillard et al., 2003). Anaerobic serum flasks were supplemented with hydrogen as electron donor, inoculated with 2% (v/v) inoculum, and finally 1% (v/v) of 2 M PCE solution in hexadecane was added as electron acceptor. Nine batch cultures of *D. restrictus* were cultivated in 300 mL medium at 30°C under agitation (100 rpm) and their growth was monitored by chloride production and not optical density as it is biased by precipitation of medium component. The true nature of organohalide respiration (i.e. the link between dechlorination and growth) was already demonstrated for *D. restrictus* (Holliger et al., 1998b). Triplicate cultures were each harvested at three different growth stages of chloride release (20, 30, and 40 mM) that we have defined as the exponential (E), late-exponential (LE) and stationary (S) phases (Table S1). Aliquots of 50 mL culture were collected for transcriptomic analysis, while the rest of each culture was harvested for proteomic analysis. For RNA extraction, 50 mL was collected by 2 min centrifugation at 4600 *g* at 15°C, the pellet was readily resuspended in 1 mL of LifeGuard^{TM} (MoBio, Carlsbad, CA, USA), incubated for 1 min and flash-frozen in liquid nitrogen. The remaining 250 mL of culture were centrifuged for 10 min as above for proteomic analysis. The pellet was washed in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, and then flash-frozen in liquid nitrogen. All biomass samples were stored at -80°C until use. *E. coli* DH5α was cultivated on standard liquid or solid LB medium containing 100 $\mu g \cdot l^{-1}$ ampicillin when transformed with derivatives of the pGEM-T Easy vector (Promega, Duebendorf, Switzerland).

2.2.2 Sequence analysis

All sequences mentioned in this study are taken from the recently published genome of *D. restrictus* strain PER-K23 (Genbank XXX, Kruse *et al*., submitted paper). The annotation of specific genes was verified using manual search with BlastP (Altschul et al., 1990). Rho-independent transcription terminators were identified with TransTerm from the Nano+Bio-Center of Kaiserslautern Technical University (http://nbc11.biologie.uni-kl.de) using default parameters. Protein sequences were aligned using ClustalX 2.0 (Larkin et al., 2007). The RdhA tree was built with MEGA5 (Tamura et al., 2007).

2.2.3 RNA Extraction

RNA was extracted using the TRIzol method according to (Prat et al., 2012) with the following modification. The DNaseI treatment was stopped by adding 1× DNase stop solution and incubating for 10 min at 65°C. RNA concentration was estimated using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Ecublens, Switzerland).

2.2.4 Reverse transcription

Two µg of RNA were added to 4.5 µg of random hexamer (Microsynth GmbH, Balgach, Switzerland) in a volume of 85 µl. This mixture was incubated at 70°C for 5 min and then placed on ice. A 75 µl of reverse transcription (RT) mix contained, 32 µl of 5× buffer, 8 µl of 10 mM dNTPs, 19.2 µl of 25 mM $MgCl_2$, 4 µl of RNasin (40 $U \cdot \mu l^{-1}$) and 8 µl of ImProm-II reverse transcriptase (Promega). The RT was performed as follows: 25°C for 5 min, 42°C for 60 min, and 70°C for 15 min in a T3 Thermocycler (Biometra, Goettingen, Germany).

2.2.5 Primer design

Specific primers were designed for each *rdhA* gene present in the *D. restrictus* genome by targeting unique regions. The primers were chosen such that the amplified products would fall in a size range suitable for quantitative PCR (qPCR, see below). Primer sequences and expected amplicon sizes are given in Table S1.

2.2.6 End-point PCR approaches

Different PCR strategies were applied in this study: standard endpoint PCR (sPCR), multiplex endpoint PCR (mPCR) and quantitative PCR (qPCR, see below). Standard PCR reactions were carried out in 10 µl containing 4.25 µl ddH_2O, 1 µl 10× buffer, 0.3 µl dNTPs at 10 mM each, 0.4 µl 25 mM $MgCl_2$, 1 µl each primer at 10 µM, and 0.05 µl Taq polymerase at 5 $U \cdot \mu l^{-1}$ (Peqlab, Erlangen, Germany). Two µl of genomic DNA or cDNA were added as template. For mPCR, a solution with 8 different primers (4 targets) was prepared containing 10 µM of each primer. Two µl of that solution was added in the standard reaction mix. Standard PCR and mPCR were performed in a Thermocycler (Biometra) using the following conditions: 5 min of initial denaturation at 95°C, followed by 30 cycles of 1 min denaturation at 95°C, 1 primer annealing at 52°C, and 1 min elongation at 72°C. A final extension step of 10 min at 72°C was added at the end. The PCR products were routinely analyzed in 1.5% (w/v) agarose gels stained with GelRed (Biotium, Hayward, CA, USA). DNA was visualized using the Syngene gel imaging system (Syngene, Cambridge, UK).

2.2.7 Cloning and sequencing of PCR products

PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hombrechtikon, Switzerland) according to manufacturer's instructions. The products were then A-tailed following instructions from the pGEM T-Easy vector manual (Promega), and finally ligated into pGEM T-Easy overnight at 16°C. The ligated products were cloned by heat shock transformation of $CaCl_2$-competent *E. coli* DH5α. Transformants were screened using colony PCR with primers T7 and SP6, and positive clones were cultivated overnight at 37°C followed by plasmid preparation with the QIAprep Spin Miniprep kit (Qiagen). Plasmid inserts were verified by sequencing using the BigDye Terminator 3.1 kit on the ABI Prism 3130 Genetic Analyzer according to manufacturer's instructions (Applied Biosystems).

2.2.8 Quantitative PCR

Standards for qPCR were prepared from plasmids containing the gene targets as follows. One µg of plasmid DNA was digested with 5 units of ScaI restriction enzyme (Promega) for 2 h at 37°C. The linearized plasmid was dephosphorylated during 1 h at 37°C by adding 1 µl shrimp alkaline phosphatase (Takara, Clontech Laboratories, Mountain View, CA, USA), followed by purification with the QIAquick PCR purification kit (Qiagen). The DNA concentration was measured with the Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Serial dilutions (10^{-1} to 10^{-8} copies·µl^{-1}) of the purified sample were finally prepared and used as standards. A typical 10 µl qPCR reaction contained 5 µl of KAPA SYBR FAST Universal 2× qPCR master mix (KAPA Biosystems, Woburn, MA, USA), 0.2 µl of each primer at 10 µM, 2.1 µl of ddH_2O, and 2.5 µl of template DNA (standards or samples). The reactions were performed in the Rotor Gene qPCR machine (RG-3000, Corbett Research, Qiagen) using the following program: 2 min of initial denaturation at 95°C, then 40 cycles of 30 s denaturation at 95°C, 30 s primer annealing at 58°C, and 20 s elongation at 72°C. Fluorescence was measured at the end of each elongation step. Each run consisted of triplicate reactions for both the standards and the samples. Run performances are given in Table S2 for each considered gene target. The obtained data were expressed as transcript copy number per µl (cn·µl^{-1}) of initial cDNA samples.

2.2.9 Protein extraction and SDS-PAGE

Cell pellet was transferred to two mL low binding micro centrifuge tubes (Eppendorf, Nijmegen, The Netherlands) prior to protein extraction. Protein extraction was done in 500 µL SDT-lysis buffer (100 mM Tris-HCl pH 7.6, 4% SDS, 0.1 M dithiotreitol). Cells were lysed by sonication, using a Branson sonifier equipped with a 3 mm tip (six pulses of 30 s with 30 s rest on ice in-between each pulse, strength of the pulse was increased stepwise from setting 2 to 4). Proteins were denatured by boiling for 5 min, followed by 10 min centrifugation at 15700 *g*. Protein concentrations were determined using the Bradford method (Bradford, 1976). Finally SDS-PAGE was performed with gels containing 10% acrylamide using a MiniProtean III system (Bio-Rad, Veenendaal, The Netherlands). Samples containing 10 µg protein were mixed with 2× loading buffer (100 mM Tris-HCl pH 6.8, 200 mM dithiotreitol, 4% SDS, 0.2% bromophenol blue and 20% glycerol) and briefly heated to 95°C before loading on gels. Gels were stained with Coomassie Brilliant Blue.

2.2.10 In-gel trypsin digestion

For the growth phase experiment each lane was cut in five slices of approximately equal size. Each slice was cut into approximately 1 mm^3 pieces and transferred to independent 500 µL low binding micro centrifuge tubes (Eppendorf). All solutions were prepared using 50 mM NH_4HCO_3 unless otherwise stated. Tubes were briefly centrifuged and the liquid phase removed between each step. Proteins were reduced by incubating in 50 mM dithiotreitol for 1 h at 60°C while slowly shaking, and

alkylated by incubation in 100 mM iodoacetamide for 1 h in the dark at room temperature, washed once and incubated with 20 ng trypsin (sequencing grade, Roche Diagnostics, Almere, The Netherlands) over night at room temperature. Samples were sonicated in a water bath for 30 min before the supernatant was transferred to fresh 500 µL low binding micro centrifuge tubes. To increase the yield the gel pieces were covered with 10% trifluoroacetic acid in H_2O and sonicated for another 30 min. Then an equal volume of a solution containing 15% acetonitrile and 1% trifluoroacetic acid in H_2O were added. The samples were sonicated for 1 min, before supernatants were combined in the low binding micro centrifuge tubes mentioned above. Peptides were concentrated using StageTip C18 columns essentially as described in (Rappsilber et al., 2007). Finally the volume was reduced to 10 µL using a SpeedVac vacuum centrifuge, and increased to 25 ul with 0.1% (v/v) formic acid. Samples were measured by nLC-MS/MS with a Proxeon nLC and a LTQ-Orbitrap mass spectrometer as described in (Lu et al., 2011).

2.2.11 LC-MS data analysis

LC-MS runs with all MS/MS spectra obtained were analyzed with MaxQuant 1.2.2.5 (Cox and Mann, 2008) using default settings for the Andromeda search engine (Cox et al., 2011)}, except that extra variable modifications were set for de-amidation of N and Q. A protein database was generated based on the genomes of *D. restrictus* and *Dehalobacter* sp. E1 (Maphosa et al., 2012b), using the Artemis genome browser, and combined with a database that contains sequences of common contaminants as for instance BSA (P02769, bovine serum albumin precursor), trypsin (P00760, bovine), trypsin (P00761, porcine), keratins K22E (P35908, human), K1C9 (P35527, human), K2C1 (P04264, human) and K1CI (P35527, human) (Rutherford et al., 2000). The label-free quantification (LFQ) as well as the match between runs options (with ± 2 min retention time deviation) were enabled. De-amidated peptides were allowed to be used for protein quantification and all other quantification settings were kept default.

Filtering and further bioinformatic analysis of the MaxQuant/Andromeda workflow output and the analysis of the abundances of the identified proteins were performed with the Perseus 1.2.0.16 module (available at the MaxQuant suite). Accepted were peptides and proteins with a false discovery rate (FDR) of less than 1% and proteins with at least 2 identified peptides of which one should be unique.

Reversed hits were deleted from the MaxQuant result table as well as all results showing a LFQ value of 0 for both sample and control. Zero values for one of the two LFQ columns were replaced by a value of 5 to make sensible ratio calculations possible. Relative protein quantification of sample to control was done with Perseus 1.2.0.16 by applying a two sample T-test using the “LFQ intensity” columns obtained with threshold 0.10 and S0=1.

2.3 Results

2.3.1 Proteomic analysis of *D. restrictus* along growth phases

The genome of *D. restrictus* strain PER-K23 was predicted to encode 2826 proteins (Kruse *et al*., submitted paper). Using a combined protein database generated from the genomes of *D. restrictus* and *Dehalobacter* sp. E1, we identified 1055 proteins by proteome analysis (Table S3 and Fig S2), of which 15 have been previously annotated as pseudogenes in *D. restrictus*, and one was newly discovered (Table S4). Data obtained from biological triplicates taken at the designated exponential (E), late-exponential (LE), and stationary (S) phases (see Material and Methods) were used to calculate the relative abundance ratio of proteins at stationary versus exponential phase (S/E); late-exponential versus exponential phase (LE/E), and stationary versus late-exponential phase (S/LE). The S/E protein abundance ratios of only 38 proteins were considered as statistically different (with False Discovery Rate < 0.1), and corresponded to ratios between 25- to 3000-fold). However, in a mere qualitative approach, we considered a three-fold in-/decrease in relative protein abundance as cut-off to define the proteins which differed between growth phases (Table 1). This selection allowed investigating general trends in protein changes across the different growth phases. The largest differences were seen between stationary and exponential phases, where the production of 29% of all identified proteins seemed to be regulated. Comparing late-exponential and exponential phase, or stationary and late-exponential phase, only 16 and 18% of all identified proteins were produced at different levels, respectively. In the following, we focused on selected proteins and metabolic pathways most directly linked to the organohalide respiratory lifestyle of *D. restrictus* (Table 2), but the complete data set is given in (Table S8). The housekeeping enzyme RNA polymerase (RpoB, Dehre_0495) was detected at stable levels throughout all growth phases.

Figure 1. Metabolic map showing selected pathways of *D. restrictus*. (Next page)

Metabolic pathways, both predicted from *D. restrictus* genome and analyzed by functional genomics are presented in a simplified bacterial cell. The cytoplasmic membrane is depicted in grey, the cytoplasm in blue shading. *D. restrictus* genomic loci (Dehre_#) are indicated in parentheses, with black label showing proteins detected in the proteomic analysis and those not detected indicated in red. Three important pathways are given in details: the Wood-Ljundahl pathway (WL), the menaquinone biosynthesis pathway (MK) and the corrinoid biosynthesis pathway (Cbl).

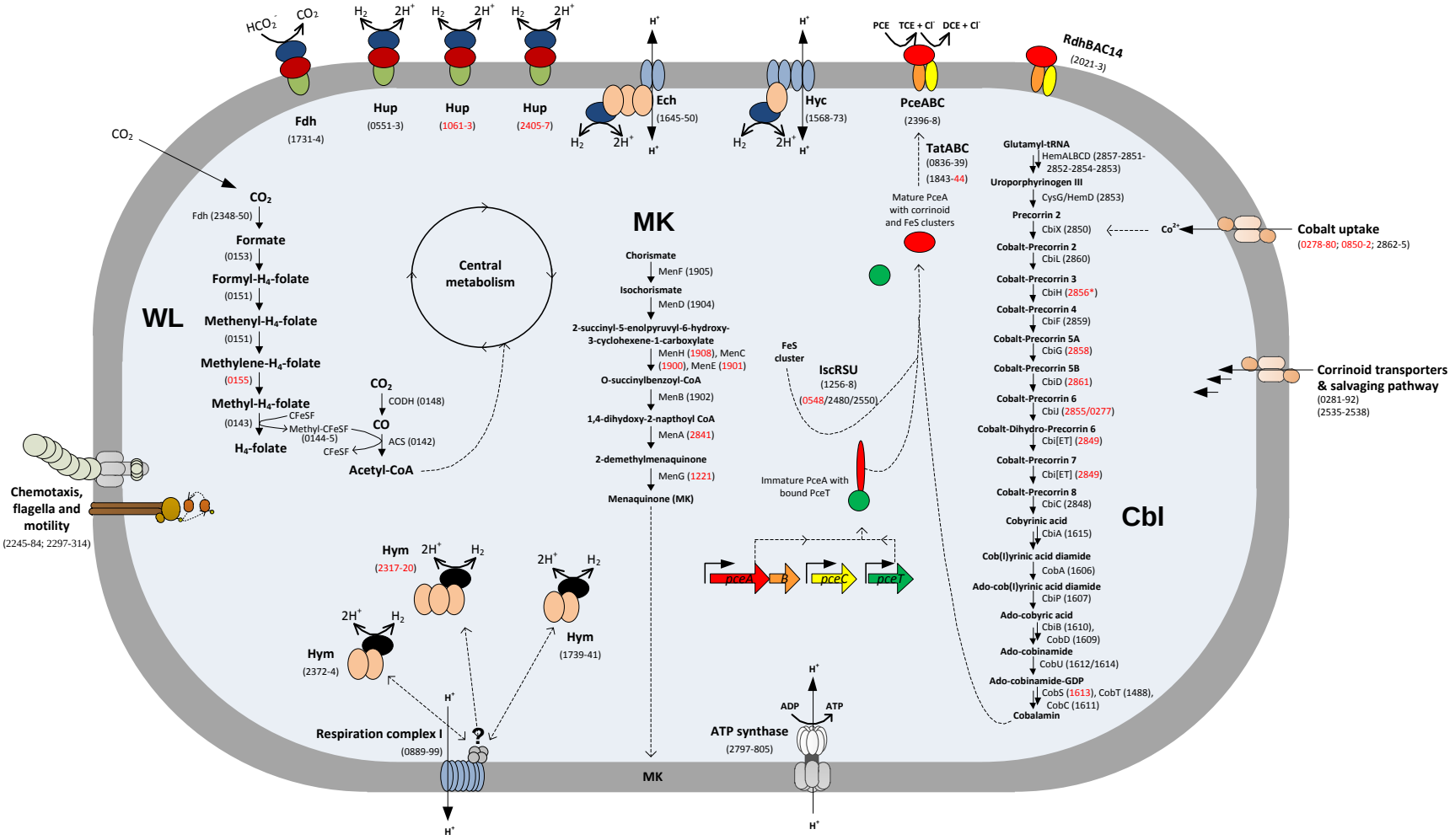
Fdh
(1731-4)
Hup
(0551-3)
Hup
(1061-3)
Hup
(2405-7)
Ech
(1645-50)
Hyc
(1568-73)
PceABC
(2396-8)
RdhBAC14
(2021-3)
TatABC
(0836-39)
(1843-44)
Mature PceA with corrinoid and FeS clusters
WL
Central metabolism
MK
Chorismate
MenF (1905)
Isochorismate
MenD (1904)
2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate
MenH (1908), MenC (1900), MenE (1901)
O-succinylbenzoyl-CoA
MenB (1902)
1,4-dihydroxy-2-napthoyl CoA
MenA (2841)
2-demethylmenaquinone
MenG (1221)
Menaquinone (MK)
Fdh (2348-50)
Formate
(0153)
Formyl-H4-folate
(0151)
Methenyl-H4-folate
(0151)
Methylene-H4-folate
(0155)
Methyl-H4-folate
(0143)
H4-folate
CFeSF
Methyl-CFeSF
(0144-5)
CFeSF
CODH (0148)
CO
ACS (0142)
Acetyl-CoA
FeS cluster
IscRSU
(1256-8)
(0548/2480/2550)
Immature PceA with bound PceT
Glutamyl-tRNA
HemALBCD (2857-2851-2852-2854-2853)
Uroporphyrinogen III
CysG/HemD (2853)
Precorrin 2
CbiX (2850)
Cobalt-Precorrin 2
CbiL (2860)
Cobalt-Precorrin 3
CbiH (2856*)
Cobalt-Precorrin 4
CbiF (2859)
Cobalt-Precorrin 5A
CbiG (2858)
Cobalt-Precorrin 5B
CbiD (2861)
Cobalt-Precorrin 6
CbiJ (2855/0277)
Cobalt-Dihydro-Precorrin 6
Cbi[ET] (2849)
Cobalt-Precorrin 7
Cbi[ET] (2849)
Cobalt-Precorrin 8
CbiC (2848)
Cobyrinic acid
CbiA (1615)
Cob(I)yrinic acid diamide
CobA (1606)
Ado-cob(I)yrinic acid diamide
CbiP (1607)
Ado-cobyric acid
CbiB (1610), CobD (1609)
Ado-cobinamide
CobU (1612/1614)
Ado-cobinamide-GDP
CobS (1613), CobT (1488), CobC (1611)
Cobalamin
Cbl
Cobalt uptake
(0278-80; 0850-2; 2862-5)
Corrinoid transporters & salvaging pathway
(0281-92)
(2535-2538)
Chemotaxis, flagella and motility
(2245-84; 2297-314)
Hym
(2317-20)
Hym
(2372-4)
Hym
(1739-41)
Respiration complex I
(0889-99)
ATP synthase
(2797-805)
ADP
ATP
pceA
B
pceC
pceT

Figure 1

2.3.1.1 Reductive dehalogenases

The genome of *D. restrictus* contains 25 genes predicted to encode reductive dehalogenase homologues (*rdhA*) (see below). Overall, a total of 86 genes are potentially associated with reductive dehalogenase expression and maturation, including genes that are predicted to encode putative membrane anchors, transcriptional regulators, chaperones, and other *rdh* associated genes. Two of the reductive dehalogenase catalytic subunits (RdhA) were detected in the proteome: RdhA14 (Dehre_2022) and RdhA24 (PceA, Dehre_2398). The former shows a very high amino acid sequence identity (89%) with RdhA2 from *Desulfitobacterium hafniense* DCB-2 (Dhaf_0693) (Kim et al., 2012), while the latter is the biochemically characterized PceA (Maillard et al., 2003) (Table S5). All four proteins encoded by the *pceABCT* gene cluster (Dehre_2398 to Dehre_2395) were also identified in the proteome (Table 2). PceA was among the most abundant proteins at all growth stages (data not shown). The protein abundance ratio of PceA, PceB and PceC remained within the three fold cut-off value when comparing any of the three growth phases considered. The absence of regulatory component in the direct vicinity of the *pce* gene cluster (see below) suggests that PceA is constitutively expressed, although it needs to be further investigated. PceT, however, was the only member of the gene cluster that seemed to be regulated as the relative protein abundance ratios were 0.12, 0.43 and 0.28 for S/E, LE/E and S/LE, respectively. Although the value for LE/E did not exceed the cut-off value, the data suggests that PceT was most abundant at exponential phase and then became slightly less abundant at later growth stages (Figure 1 and Table 1).

2.3.1.2 Hydrogenases

Hydrogen is the only electron donor that *D. restrictus* has been shown to utilize. The key role of hydrogenases is underscored by the fact that the genome of *D. restrictus* is predicted to encode eight multi-subunit hydrogenase complexes. Three of these (Dehre_0551-0553; 1061-1063 and 2405-2407) belong to the group of periplasmic membrane-bound Ni/Fe uptake hydrogenases (Hup) consisting of three subunits, a membrane-bound *b*-type cytochrome, a Fe/S cluster protein and the catalytic subunit (Table S8). Two membrane-bound energy-conserving Ni/Fe hydrogenases (Dehre_1568-1573 and 1645-1650) resemble the Hyc and Ech clusters found in *Dehalococcoides mccartyi* 195 (Seshadri et al., 2005). These two hydrogenase complexes each consist of six subunits, a large and small subunit, and four subunits resembling elements of the proton-translocating respiration complex I (Table S8). The three Fe-only hydrogenases (Hym) consist of the catalytic unit and two or three subunits predicted to be involved in electron transfer. Unlike what was observed in *D. mccartyi* 195 (Seshadri et al., 2005), none of the Fe-only complexes contains any predicted transmembrane region, which suggests that they are either located in the cytoplasm or form a complex with other membrane-bound proteins

A constant amount of the large and small subunits from one of the Hup-type hydrogenases (Dehre_0552-0553) was detected throughout all growth phases. We did

not detect the *b*-type cytochrome subunit (Dehre_0551), possibly as a consequence of its strong association with the membrane. Both putative energy-conserving hydrogenase complexes were detected in the cells. We detected the large and small subunit of the Hyc-type hydrogenase (Dehre_1568-1569), but none of the four subunits (Dehre_1570-1573) predicted to be involved in electron transfer and proton transport across the cell membrane. Interestingly, the small subunit (Dehre_1568) was most abundant at late exponential phase and least abundant in stationary phase with S/E, S/LE and LE/E ratios of 0.24, 0.03, and 9.74, respectively, whereas the abundance of the large subunit did not differ between growth phases (Table S8). All but one (Dehre_1649) component of the Ech complex (Dehre_1645-1650) were detected. The only protein that differed in abundance between growth phases was Dehre_1647, predicted to encode an NADH-ubiquinone oxidoreductase. This protein became gradually more abundant at later growth stages with S/E, S/LE and LE/E ratios of 8.4, 1.8 and 4.8, respectively. We detected both 3-subunit Fe-only hydrogenases (Dehre_1739-1741 and Dehre_2372-2374) in the proteome, but none of the components of the 4-subunit complex (Dehre_2317-2320). The abundance of Dehre_1739-1741 did not change with the growth phases, whereas Dehre_2372-2374 showed a weak trend of decreasing abundance at later growth phases, most pronounced for Dehre_2373 with S/E, S/LE, and LE/E ratios of 0.30, 0.32 and 0.96, respectively (Table S8).

2.3.1.3 Corrinoid synthesis and uptake

The genome of *D. restrictus* encodes a seemingly complete de novo corrinoid biosynthesis pathway starting from glutamyl-tRNA (Figure 1)

This pathway is encoded by two distinct gene clusters in *D. restrictus*: cluster I (Dehre_2848-2865), the upper pathway, and cluster II (Dehre_1606-1615), corresponding to the lower pathway. One additional gene (Dehre_1488) belonging to the lower pathway is located elsewhere in the genome (Table 2).

Cluster I contains all genes necessary for the synthesis of cobyrinic acid starting from glutamyl-tRNA. This pathway, however, appears to be incomplete since cbiH (Dehre_2856) encoding precorrin-3B C17-methyltransferase displays a frame-shift mutation, and consequently is annotated as a pseudogene. We identified several proteins of the corrinoid synthesis pathway until cobyrinic acid, except CbiH and all enzymes responsible for the conversion of cobalt-precorrin-5A to cobalt-precorrin-8. From the upper pathway, only HemA (Dehre_2857) and HemL (Dehre_2851) showed a decreasing relative abundance from exponential to stationary phases with S/E ratios of 0.07 and 0.32, respectively. Most enzymes of the lower corrinoid synthesis pathway encoded by cluster II were found in stable amounts throughout the growth phases with exception of CbiB and CobS. CbiB (Dehre_1610) is responsible for the conversion of adenosylcobyric acid to adenosylcobinamide and was found in slightly increasing amounts at stationary phase (S/E: 3.26), while CobS (Dehre_1613) which is responsible for the conversion of adenosylcobinamide-GDP to adenosylcobalamin was not detected at all.

The genome of *D. restrictus* contains several gene clusters predicted to be involved in cobalt and corrinoid uptake. One predicted ABC-type cobalt transporter (Dehre_0850-0852) and two ECF-type cobalt transporters (Dehre_0278-0280 and Dehre_2862-2865) are present in *D. restrictus*. While none of Dehre_0850-0852 or of Dehre_0278-0280 were detected in the proteome, we identified both CbiQ (Dehre_2862) and CbiN (Dehre_2864) proteins from the transport system encoded in corrinoid synthesis gene cluster I. Both showed a decreasing trend when going from exponential to stationary phases with S/E ratio of 0.04 and 0.03, respectively (Table 2). Two gene clusters (Dehre_0281-0292 and Dehre_2535-2538) are predicted to encode proteins possibly involved in uptake of various corrinoid precursors as part of salvaging pathways. From the first cluster three proteins (Dehre_0286, 0289 and 0291) were detected. Their protein abundance ratio did not change over time, except for Dehre_0289 which was only detected during exponential phase (Table 2), while from the second cluster, all proteins except the membrane-associated Dehre_2536, were detected. Only Dehre_2538 showed an LE/E ratio exceeding the 3-fold cut-off (5.63). Interestingly this protein is predicted to encode a CbiZ homologue which salvages cobinamides and converts it back to cobyric acid (Woodson and Escalante-Semerena, 2004).

2.3.1.4 Additional elements of the general energy metabolism

Constant amounts of six proteins (Dehre_2797-2802) out of the ten subunits of the ATP synthase (Dehre_2797-2806) were detected in the proteome. The proton-translocating respiration complex I encoded in the genome of *D. restrictus* consists of 11 subunits (Dehre_0889-899) instead of the canonical 14 (Kruse *et al*., submitted paper), lacking the components NuoEFG that usually receive electrons from NADH. Three subunits (NuoBCD, Dehre_890-892) were clearly detected in the proteome, whereas none of the membrane components could be seen.

The genome encodes enzymes of a putative Wood-Ljungdahl pathway for CO_2 fixation (Dehre_0130-0155 and Dehre_2348-2351). Most proteins belonging to this pathway were detected in the proteome (Table S8). They were observed at constant level throughout the growth phases with the exception of proteins representing the carbonyl branch of the Wood-Ljungdahl pathway and the acetyl-CoA synthase/CO dehydrogenase (ACS/CODH) complex. Generally these proteins showed a gradual and significant increase in relative abundance towards later growth stages with S/E ratios between 25 and 175 (Table 1).

We also identified a putative three component formate dehydrogenase (Fdh), consisting of a membrane-bound *b*-type cytochrome, a Fe/S cluster protein and the catalytic subunit (Dehre_1730-1734), which were detected at all growth phases (Table S8)

The catalytic unit contains probably a selenocysteine as it is encoded by two in-frame genes (Dehre_1733-1734) separated by a UGA stop codon. The genomic loci Dehre_2245-2284 and 2297-2314 contain large numbers of genes involved in the synthesis of flagella, motor proteins and chemotaxis (Figure 1 , Table S8).

In the proteome, we identified 32 out of 62 proteins encoded in these genomic regions. Sixteen of them were less abundant in stationary than in exponential phase, only three increased in abundance, and the remaining 13 were equally abundant during stationary and exponential phase Table S8, indicating that the cells are reducing their motility when entering the stationary phase. Proteins showing significant changes in abundance between stationary and exponential phase are displayed in Table 1.

Generally, many proteins associated with regulation of transcription, chemotaxis, and sensing, were among the proteins displaying significant changes in their abundance. The protein showing the greatest change in abundance, with an S/E value of 2954, is Dehre_1215, annotated as $ComF_B$, an uncharacterized protein possibly involved in development of late competence (Kovacs et al., 2009; Londono-Vallejo and Dubnau, 1993). The gene cluster containing the *$comF_B$* gene (Dehre_1214-1220) in *D. restrictus* contains genes predicted to encode an RNA helicase, an ABC transporter, and two genes encoding proteins of unknown function. We detected the periplasmic component of the ABC transporter and one of the hypothetical proteins in the proteome, the latter increasing in abundance at later growth phases (Table S8). The genome of *D. restrictus* encodes other competence factors such as $ComE_A$ and $ComE_C$ (Dehre_0586-0587), and $ComF_A$ (Dehre_2784), suggesting that it is capable of natural competence. None of these additional proteins, however, were detected in the proteomic analysis. Two gene clusters encoding pili (Dehre_1166-1175 and Dehre_1272-1289) possibly involved in DNA uptake are also present.

Another protein (Dehre_0668) that was among those with the strongest increase in abundance in stationary phase (Table 1) has high similarity with RelE toxin and builds with Dehre_0667 a toxin/antitoxin addiction module system which could be involved in modulating the persistence of cell growth in unfavourable growth conditions (Engelberg-Kulka and Glaser, 1999). The antitoxin component (Dehre_0667) was however never detected in the proteome. The direct vicinity of Dehre_0668 displays several phage- or plasmid-related genes, suggesting that Dehre_0667-0668 could have been acquired by horizontal gene transfer and represent a phage-like defence mechanism (Lioy et al., 2010).

Table 1. Detected proteins showing a significant in/decrease in abundance (expressed as S/E ratio) during the transition from exponential (E) to stationary (S) phases. The S/LE and LE/E ratios are also indicated.

Locus tag (Dehre_#)	Annotated function	Protein abundance ratio [1]		
		S/E	S/LE	LE/E
Proteins displaying significant increase in S/E ratio				
1215	Late competence development protein ($ComF_B$)	2953.811	17.777	166.162
0983	Cupin-domain protein	1979.457	15.449	128.126
0318	Uncharacterized protein conserved in bacteria	844.603	4.175	202.285
2151	Aspartyl/glutamyl-tRNA (Asn/Gln) amidotransferase	664.710	7.106	93.547
0568	Similar to acyl-coenzyme A synthetase/AMP-fatty acid ligase	441.340	8.693	50.772
0109	Predicted transcriptional regulator	388.111	13.908	27.906
1963	Uncharacterized protein conserved in bacteria	368.862	11.267	32.737
0668	RelE-type toxin (TA system)	264.301	36.410	7.259
0856	Response regulator with CheY-like and AraC-type domains	262.331	23.632	11.100
2645	Uncharacterized domain 1 protein	234.981	15.909	14.770
2325	Hypothetical protein	226.171	90.056	2.511
1400	Nitrogen regulatory protein PII	225.530	16.512	13.659
0147	CODH/ACS, maturation factor	175.038	3.096	56.531
1786	Hypothetical protein	121.475	15.275	7.952
0146	CODH/ACS, maturation factor	120.810	4.518	26.741
1237	Acetate-CoA ligase	72.992	2.649	27.551
0264	Hypothetical protein	70.763	5.316	13.312
2205	YGGT protein family	51.320	10.788	4.757
2544	Hypothetical protein	50.437	7.759	6.501
0651	Predicted transcriptional regulator	48.623	2.241	21.694
1310	Transcription antitermination factor NusB	36.586	10.353	3.534
0143	Pterin-binding enzyme	33.101	2.499	13.248
2265	Response regulator containing CheY-like receiver	29.886	6.504	4.595
2560	Transcriptional regulator	26.901	8.803	3.056
0144	CODH/ACS, γ-subunit (CFeSP)	25.194	1.640	15.367
0198	Bacterial nucleoid DNA-binding protein	24.830	7.296	3.403

Proteins displaying significant decrease in S/E ratio				
2864	Cobalt transport protein	0.034	0.150	0.227
1795	Predicted transcriptional regulator containing CBS domains	0.015	0.029	0.500
2895	Uncharacterized protein conserved in bacteria	0.002	0.158	0.013
0194	Transcription-repair coupling factor	0.002	0.005	0.515
*	LSU ribosomal protein L34p	0.003	0.004	0.663
0258	Excisionase-like DNA-binding domain	0.003	0.009	0.355
2307	Uncharacterized protein conserved in bacteria	0.005	1.000	0.005
2254	Chemotaxis protein stimulating methylation of MCP proteins	0.006	0.008	0.757
2873	Trypsin-like serine protease	0.006	0.009	0.715
1962	Predicted Fe-S oxidoreductase	0.009	0.038	0.228
2146	rRNA (uracil-5-)-methyltransferase (RumA)	0.009	1.000	0.009
2280	Hypothetical protein	0.010	6.675	0.001

[1] Ratios above 1 mean increase in protein abundance, ratios below 1 mean decrease.

Table 2. Proteomic analysis of selected metabolic pathways of *D. restrictus*

Locus tag (Dehre_#)	Protein	Annotated function	Protein abundance ratio S/E	S/LE	LE/E
Proteins associated with organohalide respiration					
2022	RdhA14	Reductive dehalogenase	0.336	0.535	0.628
2025	RdhK15	CPR/Fnr-type regulator	1.199	3.130	0.383
2048	RdhK20	CPR/Fnr-type regulator	0.469	0.396	1.184
2395	PceT	Chaperone (Trigger factor)	0.122	0.284	0.429
2396	PceC	FMN-binding domain	0.686	0.799	0.859
2397	PceB	Membrane anchor	0.530	0.679	0.781
2398	PceA	PCE reductive dehalogenase	0.803	1.166	0.689
Proteins associated with corrinoid synthesis and uptake					
0286		ABC-type iron transporter, substrate-binding component	0.661	1.260	0.833
0289		Mg/Co protoporphyrin IX chelatase	0.066	1.000	0.066
0291	NodI	ABC-type Nod export system, ATP-binding	0.523	2.899	1.516
1488	CobT	Nicotinate-nt-DMB phosphoribosyltransferase	0.202	3.177	0.642
1606	CobA	Cob(I)yrinic acid a,c-diamide adenosyltransferase	1.193	1.009	1.203
1607	CbiP	Cobyric acid synthase	0.675	1.104	0.745
1608		Phosphoglycerate mutase	0.832	1.115	0.927
1609	CobD	L-Thr-O-3-phosphate decarboxylase	0.989	0.841	0.832
1610	CbiB	Adenosylcobinamide-phosphate synthase	3.259	0.378	1.232
1611	CobC	Alpha-ribazole-5'-phosphate phosphatase	1.252	0.813	1.017

Proteins associated with corrinoid synthesis and uptake (continues)					
1612	CobU/CobP	Cobinamide kinase/phosphate guanylyltransferase	0.735	1.711	1.258
1614	CobU/CobP	Cobinamide kinase/phosphate guanylyltransferase	1.137	1.101	1.252
1615	CbiA	Cobyrinic acid a,c-diamide synthase	0.691	1.006	0.695
2535	BtuF	ABC-type Cbl/$Fe3^+$ transporter, substrate-binding component	1.455	0.851	1.238
2537	BtuD	ABC-type Cbl/$Fe3^+$ transporter, ATPase component	0.580	1.242	0.720
2538	CbiZ	Adenosylcobinamide amidohydrolase	2.451	2.297	5.629
2848	CbiC	Precorrin-8x methylmutase	0.830	0.937	0.886
2850	CbiX	Sirohydrochlorin cobalt chelatase	1.681	2.083	0.807
2851	HemL	Glutamate-1-semialdehyde 2,1-aminomutase	0.321	0.499	0.642
2852	HemB	D-aminolevulinic acid dehydratase	0.593	0.760	0.780
2853	CysG/HemD	Uroporphyrinogen-III synthase/C-methyltransferase	0.860	0.870	0.989
2854	HemC	Porphobilinogen deaminase	0.649	0.718	0.903
2857	HemA	Glutamyl-tRNA reductase	0.069	8.234	0.568
2859	CbiF	Precorrin-4 C11-methyltransferase	1.061	0.901	0.956
2860	CbiL	Precorrin-2 C20-methyltransferase	1.079	1.000	1.078
2862	CbiO	ECF-type cobalt transporter, ATPase component	0.040	0.959	0.038
2864	CbiN	ECF-type cobalt transporter, bipartite component	0.034	6.677	0.227
Proteins belonging to the Wood-Ljundahl pathway					
0140		Predicted RNA-binding protein	38.372	1.108	34.630
0142		CODH/ACS, α-subunit	10.343	1.982	5.217
0143		Pterin-binding enzyme	33.101	2.499	13.248
0144		CODH/ACS, γ-subunit	25.194	1.640	15.367
0145		CODH/ACS, δ-subunit	10.735	1.337	8.032
0146		CODH/ACS, maturation factor	120.810	4.518	26.741
0147		CODH/ACS, maturation factor	175.038	3.096	56.531
0148		CODH/ACS, β-subunit	5.366	1.532	3.502
0150		Pterin-binding enzyme	1.276	1.027	1.243
0151		Methylene-H_4F-DH/methenyl-H_4F cyclohydrolase	2.258	1.091	2.070
0152		Methenyl-H_4F cyclohydrolase	0.626	0.529	1.183
0153		Formyl-H_4F synthetase	0.929	0.827	1.123
2348		Formate dehydrogenase, α-subunit	0.650	1.057	0.615
2349		NADH:ubiquinone oxidoreductase	0.184	0.207	0.889

2.3.2 Diversity and composition of *rdh* gene clusters in *D. restrictus*

Multiple rdh *gene clusters in D. restrictus*. A thorough analysis of the *D. restrictus* genome (Kruse *et al*., submitted paper) has revealed the presence of 25 reductive dehalogenase homologue encoding genes (*rdhA*), among which 20 are in full length, 4 harbour one or several frame-shifts (*rdhA04*, *05*, *13*, and *21*), and one is a partial gene (*rdhA25*) (see Table S5). The biochemically characterized reductive dehalogenase PceA (Maillard et al., 2003) is encoded by *rdhA24*. While most *rdhA* genes are grouped in two genomic regions (*rdhA01-10* and *rdhA13-23*), a detailed analysis of the genetic structure around them allowed defining 13 clusters consisting of one to six *rdhA* surrounded by genes encoded on the same strand. It is however rather unlikely that these clusters represent actual operons as several rho-independent transcription terminators were predicted within the clusters (Figure 2). Three general *rdh* genetic organizations can be considered here. Together with the well-characterized *pceABCT* cluster (*rdhA24*), two other *rdhA* are embedded in a similar configuration (*rdhA20* and -*22*), albeit harbouring an additional *rdhK* subunit at the 3'-end. Seven *rdhA* genes are accompanied by *rdhB* and *rdhC* subunits, five of them in the orientation *rdhABC* (*rdhA02*, *-05*, *-06*, *-13*, and *-17*) and two as *rdhBAC* (*rdhA14* and *-21*). Finally the remaining *rdhA* subunits are only accompanied by their respective *B* subunit exclusively in the orientation *rdhBA*. Most of *rdh* gene clusters are also associated with one *rdhK* subunit in various orientations. The *rdhK* encoded proteins clearly belong to the large family CRP/Fnr regulatory proteins from which CprK members of *D. dehalogenans* and *D. hafniense* DCB-2 were extensively studied and represents the paradigmatic DNA-binding regulatory protein for the respective chlorophenol reductive dehalogenase (*cpr*) operons (Kemp et al., 2013; Gábor et al., 2006, 2008; Gupta and Ragsdale, 2008; Levy et al., 2008; Mazon et al., 2007; Joyce et al., 2006; Pop et al., 2004, 2006; Smidt et al., 2000a). Screening of the genome of *D. restrictus* for RdhK proteins encoding genes revealed 25 paralogues from which 22 are located within the 13 *rdh* gene clusters, and the remaining 3 in their direct vicinity. This strongly suggests that RdhK are regulatory proteins dedicated to OHR metabolism.

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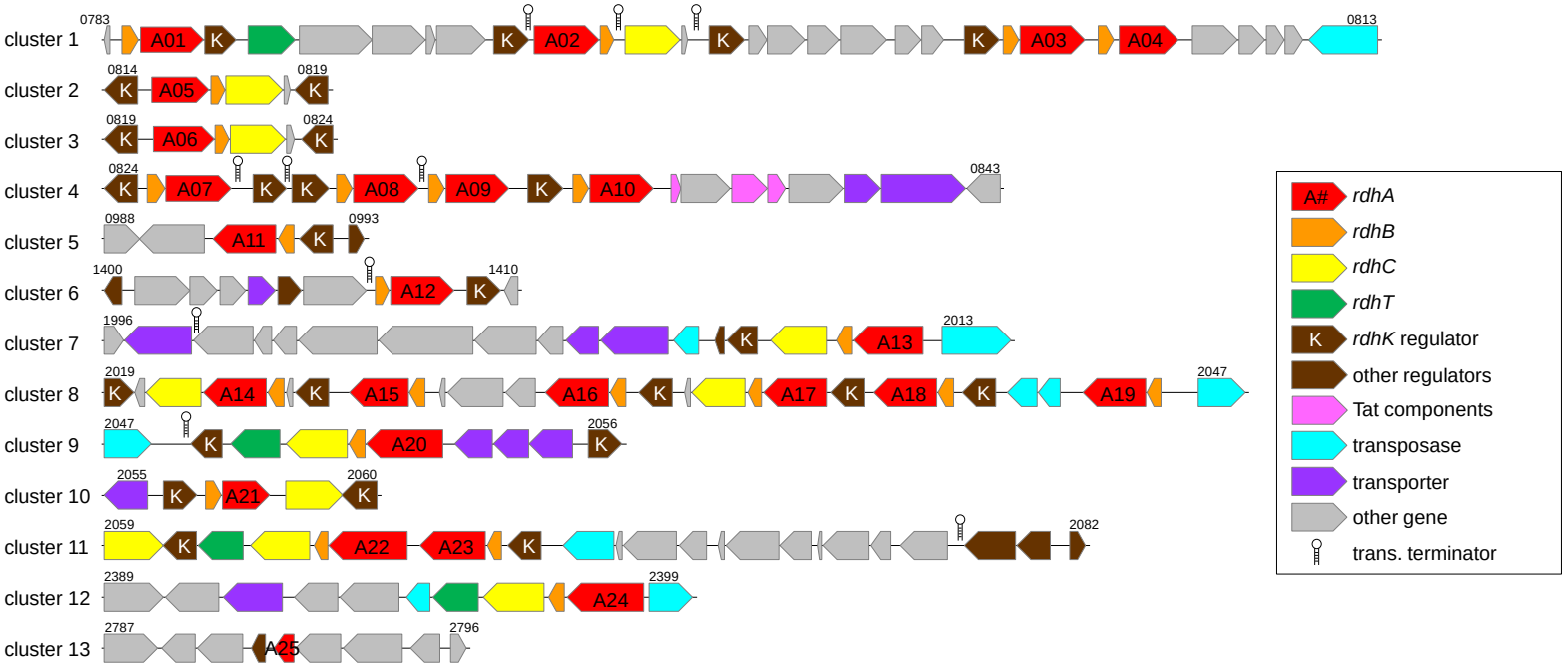

Figure 2. Genetic map of *D. restrictus* gene clusters containing reductive dehalogenase genes (*rdhA*, red numbered arrows).

For each *rdh* cluster, all the genes present on the same DNA strand were considered together with the direct flanking genes in opposite orientation.
The numbers indicated above each cluster are the corresponding loci in *D. restrictus* genome (Dehre_#)

2.3.3 Diversity of *D. restrictus* RdhA proteins

Protein sequence alignment of RdhA subunits of *D. restrictus* with selected sequences from other OHRB revealed several interesting features (Figure 3). Firstly, a strong correlation could be established between the level of sequence identity (see also Table S6) and the genetic organization of the predicted *rdh* operons. Indeed the dominating group of 14 RdhA proteins encoded by minimal *rdhBA* operons forms a separate branch, which also contains the well-characterized chlorophenol reductive dehalogenase (CprA) of *D. dehalogenans*. All three *rdhABCT* predicted operons in *D. restrictus* also cluster together, however, with homology to enzymes with different substrate specificities. PceA (RdhA24) is highly similar to other PceA enzymes from members of the closely related genus *Desulfitobacterium*, but also highly similar (88% sequence identity) to DcaA of *D. dichloroeliminans,* as already reported (Duret et al., 2012; Marzorati et al., 2007). In contrast, both RdhA20 and -22 of *D. restrictus* have a rather strong sequence identity with CprA5 and RdhA3 of *D. hafniense* strain PCP-1 and strain DCB-2, respectively, which have been shown to use 3,5-dichlorophenol (Bisaillon et al., 2011; Kim et al., 2012), these two latter enzymes being encoded in a similar genetic structure (*rdhABCT*). Interestingly two pairs of RdhA proteins (RdhA03 with -04; RdhA16 with -19) show a very high level of sequence identity (Table S6). Another striking feature is the high conservation degree of RdhA proteins between *D. restrictus* and the newly available RdhA sequences identified in the metagenome of the β-HCH dechlorinating co-culture containing *Dehalobacter* sp. E1 (*Dhb*E1 in Figure 3) (Maphosa et al., 2012b). Indeed 5 out of 9 *Dhb*E1 proteins have identical counterparts in *D. restrictus* (99-100% sequence identity), while 3 RdhA have highly similar homologues (70-92% identity) in *D. restrictus*. One last sequence (*Dhb*E1_1222) is partial.

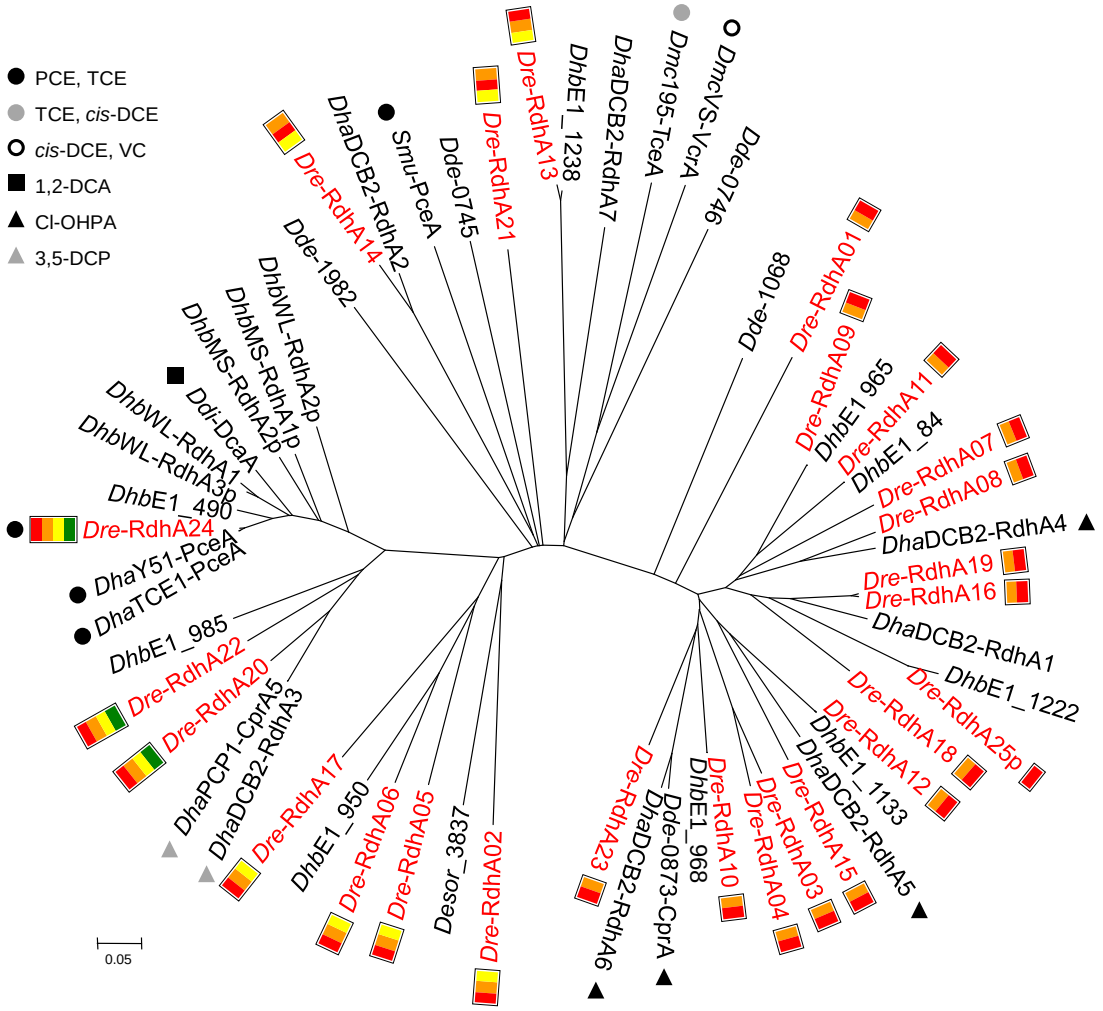

Figure 3. Diversity analysis of *D. restrictus* RdhA proteins.

All *D. restrictus* RdhA proteins are indicated in red together with their genetic structure: *rdhA* (red box), *rdhB* (orange), *rdhC* (yellow), and *rdhT* (green). Protein sequences were aligned with selected RdhA proteins from other OHRB. When known, the corresponding substrates are also indicated. Legend: *Dre*: *Dehalobacter restrictus*; *Dhb*: *Dehalobacter* spp. (strains E1, MS and WL); *Dha*: *Desulfitobacterium hafniense* (strains DCB-2, PCP-1, TCE1 and Y51); *Dde*: *Desulfitobacterium dehalogenans*; *Ddi*: *Desulfitobacterium dichloroeliminans*; *Desor*: *Desulfosporosinus orientis*; *Dmc*: *Dehalococcoides mccartyi* (strains 195 and VS); *Smu*: *Sulfurospirillum multivorans*.

2.3.4 Transcriptomic analysis of *D. restrictus* reductive dehalogenase genes

2.3.4.1 Screening of *rdhA* gene transcription by RT-multiplex PCR

From the global proteomic analysis, only two RdhA proteins were clearly detected: the main PCE reductive dehalogenase (PceA) and RdhA14, albeit at a much lower abundance. A specific approach was then conducted in order to evaluate the transcriptional level of the 24 full-length *rdhA* genes in *D. restrictus* along the growth phases. First a RT-multiplex PCR method was developed allowing screening groups of *rdhA* genes at mRNA level in the triplicate cultures collected at the exponential (E), late-exponential (LE) and stationary (S) growth phases. Figure 4 illustrates the qualitative data obtained for a combination of four *rdhA* genes using that method (the

complete set of data is presented in Fig S3). Five *rdhA* gene transcripts (*rdhA08*, *-14*, *-16*, *-19*, and *-24*) were strongly amplified, however, showing various transcription levels. The *pceA* gene (*rdhA24*) was clearly dominant and was still detected in the RNA samples collected in stationary phase (Figure 4 and Fig S3). Those five *rdhA* genes were further analyzed by RT quantitative PCR.

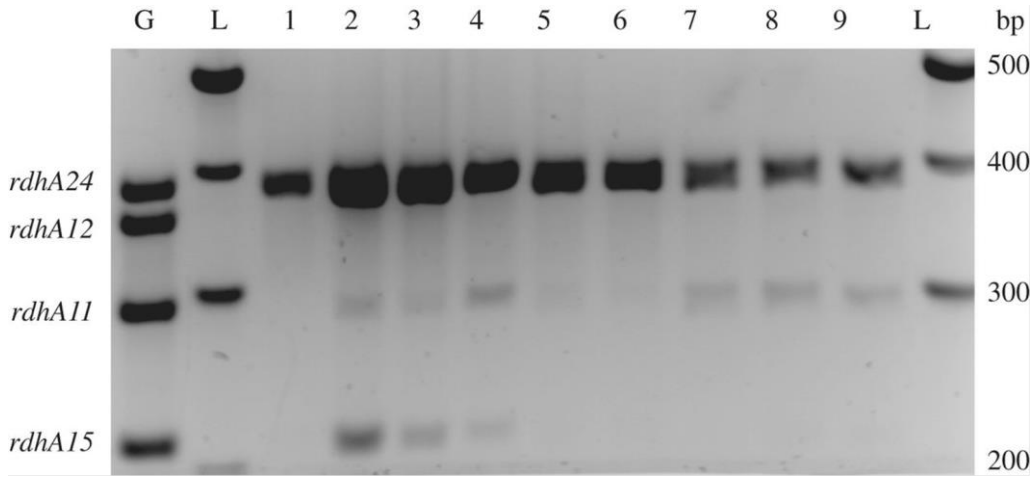

Figure 4. Growth phase dependent transcription level of rdhA genes in *D. restrictus* analyzed by RT-multiplex PCR.

One multiplex PCR result is depicted as illustration. The targeted genes are indicated on the left of the gel. G: positive control on genomic DNA; L: 100 bp ladder from which the corresponding fragment sizes are indicated on the right. Samples 1-3, 4-6, and 7-9 were taken from triplicate cultures harvested in exponential, late exponential and stationary phases, respectively.

2.3.4.2 Quantitative assessment of selected *rdhA* gene transcription by RT-qPCR

Based on individual standards for each target gene, transcript copy numbers per µl of cDNA samples were measured for *rdhA08*, *-14*, *-16*, *-19* and *-24* (*pceA*) along with *rpoB* (Dehre_0495), which was chosen as a constitutively expressed housekeeping gene (Figure 5, see Table S2 for qPCR parameters). A decrease in transcription level was generally observed for all genes along the growth phases, some of them dropping below the detection limit of the method applied. These data confirmed the trend observed by the qualitative multiplex PCR approach. In the exponential phase, the *pceA* gene (*rdhA24*) was highly transcribed in comparison with all other genes considered (between 51- and 3688-fold, depending on the gene, see Table S7 for details). Although decreasing, *pceA* remained strongly transcribed even at stationary phase. The level of transcription of the remaining *rdhA* genes decreased with the following order: *rdhA19* > *rdhA14* >> *rdhA16* > *rdhA08*.

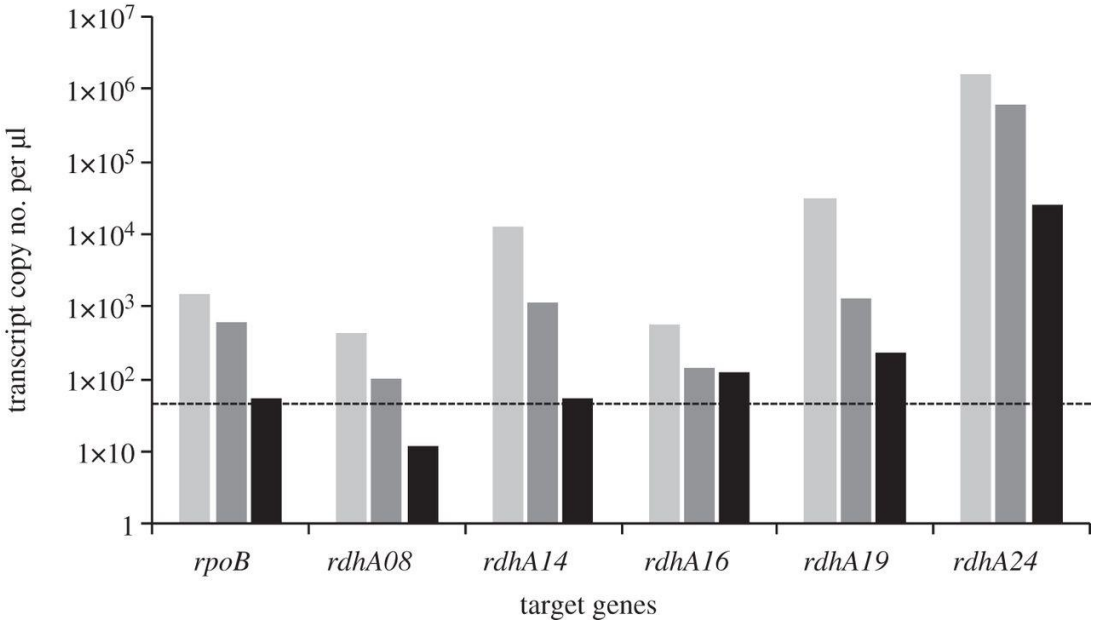

Figure 5. Growth phase dependent transcription level of selected *rdhA* genes in *D. restrictus* analyzed by RT-qPCR.

The graph depicts the gene copy number of selected *rdhA* genes along with *rpoB* as control obtained from one culture replicate harvested in exponential (light grey), late exponential (dark grey), and stationary (black) phases, respectively. The same trend was observed for all replicates. Standard deviation of qPCR replicates was below 15% of the measured data. The dotted line (50 copies / µl) displays the lower detection limit that was generally considered for the data obtained.

2.4 Discussion

Although *D. restrictus* was among the first OHRB to be isolated, a significant part of its metabolism remained largely unresolved, mainly due to the lack of the genome sequence, but also due to the restricted conditions in which this bacterium has been found to grow, namely exclusively by anaerobic respiration with hydrogen as electron acceptor and PCE or TCE as unique terminal electron acceptors. We recently obtained the genome sequence of *D. restrictus* strain PER-K23 (Kruse *et al*., submitted paper) which allowed us in the present study to consider general questions about its metabolism and a more specific investigation line focusing on the key players in OHR, the reductive dehalogenases.

The 2.9 Mb genome of *D. restrictus* can be considered to occupy an intermediate position among OHRB between the reduced genome size of the OHR obligate *Dehalococcoides* genus (~1.4 Mb) and the largely redundant genomes of the versatile *Desulfitobacterium* genus (> 5 Mb). Metabolically, however, *D. restrictus* is closer to *Dehalococcoides*, suggesting that, besides additional genetic information responsible for peptidoglycan synthesis and motility, some parts of *D. restrictus* genome may be not functional or encode for yet unsuspected metabolic pathways. A remarkable example is the presence of a complete cobalamin biosynthetic pathway, an essential cofactor for OHR metabolism. Indeed based on the anaerobic pathway described by Roessner (Roessner and Scott, 2006) and the cobinamide salvaging pathway studied by Escalante-Semerena and co-workers (Gray and Escalante-Semerena, 2009; Woodson

and Escalante-Semerena, 2004; Woodson et al., 2003; Zayas et al., 2007), all genes were clearly identified in *D. restrictus*, although it cannot grow without a supply of vitamin B_{12} in the medium (Holliger et al., 1998b; Rupakula et al., 2013). The proteomic data obtained here showed that about half of the proteins of the biosynthetic pathway mostly from the upper pathway were not detected, indicating that under the growth conditions applied, *D. restrictus* used the corrinoid amended and possibly modified it according to its needs. On genetic level, the frame-shift mutation observed in *cbiH* (Dehre_2856) needs to be confirmed, but could also be a reason why *D. restrictus* is not able to synthesize cobalamin *de novo*. Preliminary proteomic data obtained from cells which were partially depleted of vitamin B_{12} revealed that the production of corrinoid transporters and proteins of the salvaging pathway increased significantly rather than the biosynthetic proteins (J. Maillard & T. Kruse, unpublished data), suggesting that the biosynthetic pathway is not functional in *D. restrictus*.

Enzymes belonging to the Wood-Ljungdahl (WL) pathway for CO_2 fixation were clearly detected on the proteomic level in *D. restrictus*. A significant increase in the carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) complex (Dehre_0143-8, corresponding roughly to the carbonyl branch of the pathway) was observed in the late-exponential and stationary phases when compared to the exponential phase. This suggested that acetate might be depleted in the medium already during the late-exponential phase, and that *D. restrictus* could partially assimilate CO_2 via the acetyl-CoA synthase. This is however contrasting with a previous data set where heterotrophic CO_2 assimilation (probably via the pyruvate:ferredoxin oxidoreductase, PFOR) has been postulated for an enrichment of *D. restrictus* (Holliger et al., 1993). Several homologues of the latter enzyme were also detected in the proteome (Table S8). The role of the WL pathway and more generally the assimilation of CO_2 have been questioned for other OHRB such as various isolates of *Desulfitobacterium* (Kim et al., 2012; Kreher et al., 2008; Nonaka et al., 2006; Prat et al., 2011) or of *Dehalococcoides* (Ahsanul Islam et al., 2010; Marco-Urrea et al., 2012; Tang et al., 2009). In *D. hafniense* strains, components of the WL pathway have been shown to participate in the use of phenyl methyl ethers as electron donors (Kreher et al., 2008). There, however, the methyl branch was mainly used together with *O*-demethylases. For strain TCE1 it has been reported that components of this pathway increased in abundance when H_2 and PCE were used as a combination of electron donor and acceptor (Prat et al., 2011). The work by Tang and co-workers suggested that the WL pathway was not involved in CO_2 fixation in *Dehalococcoides* (Tang et al., 2009). As illustrated by these examples, the role of the WL pathway in OHRB might be diverse, and further dedicated experiments are required to fully understand why *D. restrictus* recruits it at late growth phases.

The presence of eight different hydrogenases underscores the central role of hydrogen in the metabolism of *D. restrictus*. The genomes of *Desulfitobacterium hafniense* DCB-2 (Kim et al., 2012) and Y51 (Nonaka et al., 2006) encode like *D. restrictus* three Hup-type hydrogenases. The fact that we only detected one of them (Dehre_0551-0553) at relatively constant abundance across growth phases indicates that (i) the three

Hup complexes have different roles in the metabolism, and (ii) the detected Hup plays a role in the core metabolism. Concerning the three Fe-only hydrogenases (Hym), two complexes were present in stable abundance at all growth phases, whereas one was not observed at all. Unlike what is predicted for Hym in *Dehalococcoides mccartyi* 195 (Morris et al., 2006; Seshadri et al., 2005), we did not find any membrane-associated components in the Hym-type hydrogenases in *D. restrictus*. We therefore suggest that these enzymes are located in the cytoplasm, where they might be involved in generating reducing equivalents (e.g. NADH, FADH) for biosynthetic reactions or maybe directly in generating a proton motive force with respiration complex I, as speculatively indicated in Figure 1, Table S8.

The respiration complex I in *D. restrictus* lacks the NuoEFG subunits that usually receive electrons from NADH. The electron donor for this type of respiration complex I is not yet known, but it has been speculated that they act as a docking station able to receive electrons from various electron donors (Moparthi and Hagerhall, 2011). It is interesting that we find two large membrane-bound putatively proton-translocating hydrogenase complexes, Hyc and Ech in *D. restrictus* like in *D. mccartyi* 195, whereas the more closely related *Desulfitobacterium hafniense* Y51 and DCB-2 only contain a Hyc homolog (Kim et al., 2012; Nonaka et al., 2006; Seshadri et al., 2005). The role of these remains unclear, however, disrupting *hyc* in *Desulfitobacterium dehalogenans* resulted in loss of ability to utilize *3*-chloro-*4*-hydroxyphenylacetic acid and nitrate as electron acceptor when formate was used as electron donor, suggesting a role in the electron transport chain (van de Pas et al., 2001a). It has been suggested that Ech and Hyc may play a role in generating low potential electrons for OHR by reverse electron flow. It was however observed that the expression of both Hyc and Ech decreased when *D. mccartyi* 195 was cultivated under lower partial pressure of hydrogen. Since hydrogen is a stronger reductant at higher partial pressure, the opposite would have been expected if they played a role in reverse electron flow (Morris et al., 2006). Our findings suggest that different hydrogenases play specific and central roles in the metabolism of *D. restrictus*, but elucidating the exact role of the individual hydrogenases requires further studies.

Significant changes in the protein content between exponential and stationary phases were observed for various unrelated proteins for which the predicted function was often not clear. For example, $ComF_B$ (Dehre_1215) and a cupin-domain containing protein (Dehre_0983) were identified with more than 1000-fold increase in the stationary *vs.* exponential phase (Table 1). The former protein is predicted to play a role in the late development of competence, although no other competence protein was detected. Competence represents a general strategy for bacteria to survive in unfavourable conditions such as during stationary phase (Claverys et al., 2006). The latter protein has no clear predicted function, but might be part of an operon involved in the shikimate pathway responsible for the biosynthesis of aromatic amino acids. The list of proteins that in/decreased significantly between exponential and stationary phase clearly indicates that the cells are adjusting their metabolism when shifting from one growth phase to another.

The discovery of 25 reductive dehalogenase (*rdhA*) genes in the genome of *D. restrictus* was surprising given its currently known substrate range for reductive dehalogenation (Kruse *et al*., submitted paper), but is in line with what has been observed in all available genomes of *Dehalococcoides mccartyi*. The detailed analysis of the *rdh* gene clusters we present here, together with the transcriptional and proteomic data on the components of these clusters, helped us to consider their diversity, evolution and function in *D. restrictus* during growth on PCE. Analysis of the sequence similarity of *D. restrictus* RdhA proteins along with the best characterized RdhA proteins revealed at least three groups of enzymes. The largest and relatively deep-branching first group contains 16 RdhA proteins which are affiliated to the characterized *ortho*-chlorophenol dechlorinating enzymes (CprA) of *Desulfitobacterium* isolates (Pas et al., 1999; Krasotkina et al., 2001). Within this group, two gene duplication events must have occurred recently, as the couples RdhA16/19, and RdhA03/04 show 98 and 81% sequence identity, respectively (Table S6). Further synteny analysis revealed that the sequence conservation was extended to the corresponding *rdhB* genes (data not shown). A second deep-branching group of RdhA sequences contains *D. restrictus* PceA (RdhA24) and two slightly more distant members (RdhA20 and -22). These proteins form a closely related family together with some of the best characterized enzymes, namely PceA of several *D. hafniense* isolates (Maillard et al., 2005; Miller et al., 1997; Suyama et al., 2002), DcaA of *D. dichloroeliminans* (Marzorati et al., 2007) and CprA5 (dechlorinating 3,5-dichlorophenol) of *D. hafniense* PCP-1 (Thibodeau et al., 2004). The last 7 RdhA proteins build up a group of highly heterogeneous enzymes for which no characterized counterpart is yet available. Among them however, four *D. restrictus* RdhA proteins (RdhA02, -05, -06 and -17) show 45% sequence identity with a putative RdhA identified in the genome of *Desulfosporosinus orientis* (Desor_3837, (Stackebrandt et al., 1997)). The genetic organization around *rdhA* genes is tightly correlated with the sequence diversity of their encoded proteins. Indeed both deep-branching groups of *D. restrictus* RdhA show uniform genetic structures, *rdhBA* and *rdhABCT*, respectively. The rather heterogeneous third group is made of either *rdhABC* or *rdhBAC* operons. This strongly indicates a possible evolutionary line in which a few individual *rdh* operons might have been acquired by horizontal gene transfer, followed by several rounds of gene duplication.

Functional investigation of the *rdh* gene clusters along the growth curve of *D. restrictus* on PCE clearly revealed that the PCE reductive dehalogenase (PceA, RdhA24) was dominating both at transcriptional and proteomic levels, with only little changes along the growth phases. This can explain why only PceA could be purified from *D. restrictus* in earlier studies. On proteomic level RdhA14 was the only other reductive dehalogenase detected but at an estimated PceA/RdhA14 ratio of 212 during exponential phase. While all subunits encoded by the *pceABCT* operon were identified, neither RdhB nor RdhC belonging to the *rdhBAC14* operon were detected, possibly as a result of their lower expression and high hydrophobicity. On transcriptional level, the results are somehow contrasting. While *rdhA14* was also detected at a copy number

ratio similar to the proteomic data (Table S7, B), other *rdhA* genes were also significantly transcribed, and among them *rdhA19* at a slightly higher level than *rdhA14*, although not detected in the proteome. Whether this is due to the sensitivity of the proteomic analysis or to a possible post-transcriptional regulation remains to be investigated. Similar to several omics studies on *Dehalococcoides* (Johnson et al., 2008; Morris et al., 2007; Rahm et al., 2006; Wagner et al., 2009; West et al., 2008), a relatively tight regulation seems to operate in *D. restrictus* for *rdhA* candidates, among which only a few of them are steadily expressed. In contrast to *Dehalococcoides* however, where mostly two-component systems and MarR-type regulators are likely to regulate the expression of *rdhA* genes (Kube et al., 2005), in *D. restrictus*, as well as in the closely related *Desulfitobacterium* isolates, numerous CprK activating regulators (so-called RdhK) are present in or in the direct vicinity of *rdh* gene clusters. Only two of them however were detected in the proteomic analysis (Dehre_2025 and 2048), suggesting that their expression level remains low in the cell or that they are themselves regulated.

Additional proteins encoded in *rdh* gene clusters were also detected in the proteome. The TatA and TatB components of two Tat systems (Dehre_0836, 0839 and 1843) were detected. Interestingly the former system is encoded directly downstream of *rdhA10* and surprisingly contains an ApbE homologue (Dehre_0837) involved in thiamine biosynthesis. Also possibly linked to the translocation of RdhA proteins across the cytoplasmic membrane, a SppA homologue (Dehre_0809) was detected. The corresponding gene is located directly downstream of *rdhA04* and its product is possibly involved in the degradation of signal peptides (such as the Tat signal peptides of RdhA proteins) after they have been cleaved from the mature proteins (Bolhuis et al., 1999; Wang et al., 2008).

Our multi-level study of *D. restrictus* metabolism revealed rather elaborate genomic and proteomic features despite its restricted physiology recognized so far, suggesting that there is much more to discover especially in the energy metabolism of this bacterium. In addition, the high number of reductive dehalogenase genes raises the question of a wider bioremediation spectrum via organohalide respiration for *D. restrictus*.

Chapter 3
Functional genomics of corrinoid starvation in the organohalide-respiring bacterium *Dehalobacter restrictus* strain PER-K23

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3 Functional genomics of corrinoid starvation in the organohalide-respiring bacterium *Dehalobacter restrictus* strain PER-K23

3.1 Introduction

Corrinoids are essential cofactors for a wide variety of enzymes that facilitate reactions ranging from rearrangements, methyl group transfers, and reductive dehalogenation. A recent bioinformatic study has revealed that while 76% of 540 sequenced bacterial genomes contain corrinoid-dependent enzymes, only 39% of these genomes encode the complete corrinoid biosynthesis pathway, suggesting that the salvage of corrinoids from the environment is an important process for many bacteria (Zhang et al., 2009). Both aerobic and anaerobic corrinoid biosynthesis pathways have been described showing few but significant differences, notably in tetrapyrrole ring contraction and the step at which cobalt is inserted into the ring (Moore and Warren, 2012; Scott, 2003) This pathway is complex and consists of approximately 30 reactions (see (Moore and Warren, 2012) for a recent review).

Organohalide respiration (OHR) is an anaerobic bacterial respiration process of environmental interest as many anthropogenic halogenated organic compounds can be used as terminal electron acceptors by organohalide-respiring bacteria (OHRB), which remove the halogens and therefore contribute to bioremediation of environments polluted with these compounds (Leys et al., 2013) . The key enzyme in OHR is the reductive dehalogenase (RdhA) (Hug et al., 2013), which strictly depends on corrinoid cofactors for the dehalogenation reaction. While the reaction mechanism has not yet been solved, RdhAs represent an unusual family of corrinoid enzymes as no typical corrinoid binding motif is present reflecting the base-off/his-off conformation of the corrinoid in the enzyme (van de Pas et al., 1999; Schumacher et al., 1997). In recent years, corrinoid biosynthesis regained substantial interest in the scientific community as exemplified by the following studies: an unusual corrinoid cofactor (norpseudo-B_{12}) has been identified in the tetrachloroethene (PCE) RdhA of *Sulfurospirillum multivorans* (Kräutler et al., 2003) ; the lack of exogenous corrinoid had an effect on the RdhA activity of *Desulfitobacterium hafniense* when cultivated with alternative electron acceptor (Reinhold et al., 2012) ; many essential corrinoid biosynthetic genes have been found on a plasmid in *Geobacter lovleyi* (Wagner et al., 2012) ; the involvement of the bacterial community accompanying members of *Dehalococcoides mccartyi* for corrinoid supply has been highlighted (Hug et al., 2012; Men et al., 2014b; Yan et al., 2012, 2013) modifying the lower ligand on the corrinoid had a severe effect on the activity of the PCE RdhA of *S. multivorans*(Keller et al., 2013).

Contrasting situations have been observed regarding the ability of OHRB to produce corrinoid cofactors *de novo*. Both genome analysis and physiological studies have shown that the obligate OHR *D. mccartyi* is strictly dependent on exogenous corrinoid supply and that only 5,6-dimethylbenzimidazole can serve as nucleotide loop in corrinoid cofactors (Hug et al., 2012; Men et al., 2014b; Yan et al., 2012, 2013). On the contrary, the facultative OHRB *S. multivorans* strain K and *D. hafniense* strains encode the full corrinoid biosynthetic pathway in their genome and have been shown to grow without any supply of corrinoid in the medium (Neumann et al., 2002; Siebert et al., 2002; Nonaka et al., 2006; Kim et al., 2012; Choudhary et al., 2013).

Dehalobacter restrictus strain PER-K23 is an obligate OHRB only able to grow by dechlorinating tetra- and trichloroethene (PCE and TCE, respectively). It was first isolated from Rhine river sediment and since then always cultivated in the presence of exogenous vitamin B_{12} (cyanocobalamin) (Holliger et al., 1998b) . The PCE RdhA (PceA) of *D. restrictus* has been extensively studied and revealed a 60-kDa enzyme containing a corrinoid cofactor and two 4Fe-4S clusters with estimated redox potential of -350 mV ($Co^{1+/2+}$) and -480 mV ($4Fe\text{-}4S^{2+/1+}$), respectively, and a specific dechlorination activity of 250 nkat/mg (Schumacher et al., 1997; Maillard et al., 2003). Spectrophotometric analysis has suggested that cyanocobalamin was present in the RdhA (Holliger et al., 1998b). Analysis of the newly published genome of *D. restrictus* revealed the presence of a complete set of corrinoid biosynthetic genes where one gene, *cbiH*, is truncated due to a 101-bp deletion, likely responsible for the corrinoid auxotrophy of *D. restrictus* (Kruse et al., 2013; Rupakula et al., 2013) (Figure 1).

This present study aims to explore in detail the effect of corrinoid starvation on *D. restrictus* with a combination of comparative genomics, as well as transcription and proteome analysis.

3.2 Materials and methods

3.2.1 Bacteria, plasmids and growth conditions

Dehalobacter restrictus strain PER-K23 (DSM 9455) was cultivated as described earlier (Holliger et al., 1998b; Maillard et al., 2003; Rupakula et al., 2013). Anaerobic serum flasks of 500 mL were supplemented with hydrogen as electron donor, inoculated with 2 % (v/v) inoculum, and finally 1 % (v/v) of 2 M PCE dissolved in hexadecane was added as electron acceptor. Batch cultures of *D. restrictus* were cultivated in 300 mL medium at 30°C under gentle agitation (100 rpm), and chloride release was used as an indicator of growth. Cultures for proteomic analysis were prepared in triplicate with high, mid and low concentration of cyanocobalamin corresponding to 250, 50 and 10 µg/L, respectively.

Escherichia coli DH5α was cultivated on liquid or solid LB medium containing 100 µg/L ampicillin after transformation with derivatives of the pGEM-T Easy vector (Promega, Duebendorf, Switzerland).

3.2.2 Sequence retrieval and genome analysis

All sequences mentioned in this study were taken from the recently published genome of *D. restrictus* strain PER-K23 (Kruse et al., 2013) (the annotation of which was derived from JGI project #402027) and from other *Dehalobacter* spp. genomes including *Dehalobacter* sp. E1 (Maphosa et al., 2012b), *Dehalobacter* sp. DCA and sp. CF (Tang et al., 2012) , *Dehalobacter* sp. FTH1 (RefSeq PRJNA199134, JGI genome project), *Dehalobacter* sp. UNSWDHB (Deshpande et al., 2013). The annotation of selected genes was verified using a manual search with BLAST (Altschul et al., 1990). Protein sequences were aligned using ClustalX v.2.0 (Larkin et al., 2007). Sequence maximum likelihood tree analysis was done with MEGA5 (Tamura et al., 2011). Cobalamin riboswitches (Cbl-RS) were identified using Rfam (Griffiths-Jones et al., 2003) and initially aligned using ClustalX and then corrected manually as described earlier (Choudhary et al., 2013). Comparative genome analysis was performed using the Artemis Comparison Tool (Carver et al., 2005).

3.2.3 Transcription analysis

RNA was extracted using the TRIzol method according to (Prat et al., 2012) with the following modification. The DNaseI treatment was stopped by adding the DNase stop solution and incubating for 10 min at 65°C. RNA concentration was estimated using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Ecublens, Switzerland). Reverse transcription was performed as described in (Rupakula et al., 2013).

Primers targeting each gene present immediately downstream of the five Cbl-RS of *D. restrictus* were designed. PCRs and cloning using the pGEM-T Easy vector, cloning, clone selection, sequencing and quantitative PCR were performed as described earlier (Rupakula et al., 2013a). Primer sequences, amplicon sizes, plasmids and quantitative PCR specifications are given in Table S1.

3.2.4 Protein extraction and SDS-PAGE

Cells were harvested by 10 min centrifugation at 12000 × *g*, washed twice with 25 mL 20 mM Tris–HCl (pH 7.5), and then flash-frozen in liquid nitrogen. All biomass samples were stored at -80°C until use. Cell pellets were resuspended in 0.5 mL lysis buffer (100 mM Tris/HCl, pH 7.5, 4% sodium dodecyl sulfate, and 0.1 M dithiothreitol) and then transferred to 2-mL protein LoBind tubes (Eppendorf, Hamburg, Germany). Protein extraction was done as described earlier (Rupakula et al., 2013). Protein concentration was determined with the Qubit® protein assay kit (Invitrogen, Eugene, OR, USA) following the manufacturer's instructions. Protein samples were stored at -20°C until use. SDS-PAGE was done following standard procedures (S. Eschbach, 1990). In brief, 15 µg of proteins from each sample were loaded in separate lanes in gels containing 10% SDS. Gels were stained with Coomassie brilliant blue R250 (Merck, Darmstadt, Germany) and scanned using a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA, USA). The Quantity One basic software package was used to quantify the intensity of lanes. Series of gels were

prepared and analyzed until less than 5% differences in the intensity between any lanes were achieved.

3.2.5 Gel digestion and peptides purification

In-gel digestion of proteins and purification of peptides were done following a modified version of the protocol described earlier (Rupakula et al., 2013). Disulphide bridges in proteins were reduced by covering whole gels with reducing solution (10 mM dithiothreitol, pH 7.6, in 50 mM NH_4HCO_3), and the gels were incubated at 60°C for 1 h. Alkylation was performed for 1 h by adding 25 mL of iodoacetamide solution (10 mM iodoacetamide in 100 mM Tris-HCl, pH 8.0). Gels were thoroughly rinsed with dd H_2O water in between steps. Each lane of SDS-PAGE gels was cut into three equally sized slices, and each slice was cut into approximately 1 mm^3 cubes and transferred to separate 0.5 mL protein LoBind tubes (Eppendorf, Hamburg, Germany). Enzymatic digestion was done by adding 50 µL of trypsin solution (5 ng/µL trypsin in 50 mM NH_4HCO_3) to each tube, and incubating at room temperature overnight with gentle shaking. Extraction of peptides was performed with manual sonication in an ultrasonic water bath for 1 s before the supernatant was transferred to a clean protein LoBind tube. Additional peptides were recovered by adding 25 µL of 2.5% (v/v) trifluoroacetic acid to the gel pieces, which were sonicated for 2 s before the supernatant was combined with the first supernatant obtained. Peptides were purified with a C18 Empore disk as previously described (Rappsilber et al., 2007). Acetonitrile in the samples was removed by using a concentrator vacuum centrifuge. Finally, sample volume was adjusted to 50 µL with 0.1% (v/v) formic acid.

3.2.6 nLC-MS/MS and data analysis

Peptides derived from extracted and digested proteins were analyzed by nLC-MS/MS (Biqualys, Wageningen, The Netherlands) as described earlier (Lu et al., 2011). MaxQuant v.1.3.0.5 (Cox and Mann, 2008) with default settings for the Andromeda search engine (Cox et al., 2011) in the label free quantitation mode was used to analyze MS and MS/MS spectra, except that extra variable modifications were set as described before (Rupakula et al., 2013). A protein database of *D. restrictus* was generated from the whole genome sequence (Kruse et al., 2013) using the Artemis genome browser (release 15.0.0). Also, a contaminant database including sequences of common contaminants like trypsin, BSA and human keratins (Rutherford et al., 2000) was used. Further filtering and bioinformatics analysis was performed with Perseus software v. 1.3.0.4 as described before (Smaczniak et al., 2012). Also, protein groups with a logarithmic label-free quantitation (LFQ) intensity of zero for all treatments were deleted from the MaxQuant result table. Subsequently, remaining Log LFQ zero values were replaced by 5 (slightly below the lowest value measured) in order to make sensible ratio calculations possible. Students T-test was used to identify significant differences in the proteome when comparing logarithmic LFQ values obtained from two culture conditions.

3.3 Results

Corrinoids are essential as a growth factor for *D. restrictus* (Holliger et al., 1998b). The corrinoid present in the PCE reductive dehalogenase (PceA) of *D. restrictus* is similar to the type added to the medium, i.e. cyanocobalamin (Maillard et al., 2003). Detailed analysis of the genome of *D. restrictus* revealed a seemingly complete corrinoid biosynthesis pathway. Compared to other *Dehalobacter* genomes, however, a 101-bp fragment was found to be missing in the *cbiH* gene of *D. restrictus* (Kruse et al., 2013; Rupakula et al., 2013) (Fig S4). The present study aimed specifically at obtaining a better understanding of the corrinoid metabolism in *D. restrictus*.

3.3.1 Growth of *D. restrictus* under corrinoid-limiting conditions

The full corrinoid biosynthetic pathway was described earlier (Rupakula et al., 2013). A modified and extended version of it is depicted in Figure 7. Briefly, the pathway can be divided in two branches, namely the upper corrinoid biosynthesis (UCB) and the nucleotide loop assembly (NLA), which are connected at the level of ado-cobyric acid. In the present study, batch cultures were cultivated with addition of 250 µg/L cyanocobalamin to the growth media. An experiment was performed to assess the effects of lowering the initial corrinoid concentration in the medium (250, 50, 10, 1 µg/L and no corrinoid) on dechlorination, which for this obligate OHRB is also a direct measure for growth (Figure 6). The extent of PCE dechlorination was the same in cultures provided with 50 or 250 µg/L corrinoid demonstrating that the former was enough to reach the maximum dechlorination capacity. In contrast, the chloride release was only half of the maximum in cultures supplemented with 10 µg/L corrinoid, implying that availability of corrinoids was a limiting factor. Further lowering the corrinoid concentration to 1 or 0 µg/L resulted in negligible levels of dechlorination, and therefore growth was assumed to be abolished in these cultures.

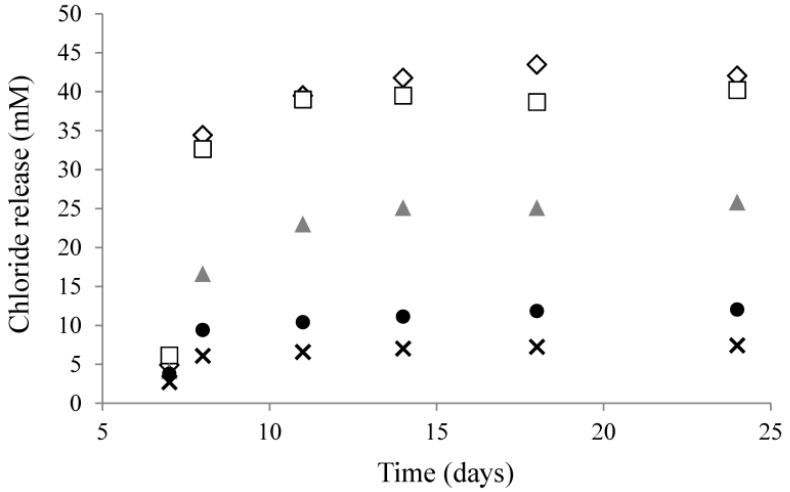


Figure 6. Corrinoid starvation effect on PCE dechlorination by *D. restrictus* by lowering the initial concentration of corrinoid supplemented into the medium.

Corrinoid concentration Legend: 250 µg/L (white diamonds), 50 µg/L (white squares), 10 µg/L (grey triangles), 5 µg/L (black circles) and 1 µg/L (black crosses).

3.3.2 Corrinoid metabolic gene arrangement in *D. restrictus*

The complete corrinoid biosynthetic and uptake pathway is genetically encoded in *D. restrictus*. These genes can be divided into four functional groups depending on the part of the pathway they encode for (Figure 7). The first group denoted as the upper corrinoid biosynthesis (UCB) pathway genes contains genes required to synthesize ado-cobyric acid. The second group consists of genes required for synthesis and the nucleotide loop assembly (NLA) of corrinoids, and the third functional group comprises the corrinoid salvaging (CS) pathway, i.e. genes involved in remodeling corrinoid intermediates salvaged from the environment into ado-cobyric acid (*cbiZ* gene family). The fourth group harbors both cobalt and corrinoid transporter encoding genes (CT) (Figure 8). Most genes associated with corrinoid metabolism are arranged in the genome of *D. restrictus* in five gene clusters (referred to as operon-1 to -5), which are roughly organized according to the function they play in corrinoid biosynthesis (Figure 3). Most proteins involved in the UCB pathway are encoded in operon-5 with the exception of the three last steps that are catalyzed by the product of genes present in operon-3. This latter operon also codes for the enzymes involved in the NLA pathway. Within operon-3, the locus Dehre_1608 was initially annotated as a phosphoglycerate mutase, but shows also sequence homology with archaeal-type homoserine kinase (with conserved domain TIGR02535) involved in the synthesis of threonine. Here, it was proposed to act as an L-threonine kinase (in analogy to PduX in *Salmonella* (Fan and Bobik, 2008)), which might therefore be involved in the production of aminopropanol-phosphate. No *pduX* homolog could be identified in *D. restrictus*, suggesting that this function is fulfilled by the gene product of Dehre_1608. Hence, we propose to name it *cbiU*. Operon-1 contains a homolog of *cbiJ* (besides the *cbiJ*/*cysG* gene, Dehre_2855, present in the conserved biosynthesis operon-5), and a set of genes coding for the energy-coupling factor-type CbiMNQ cobalt transporter. An additional, albeit different *cbiMNQO* gene cluster is also present at the 5'-end of operon-5 together with the genes for the UCB pathway. Operon-2 harbors a combination of genes coding for transporters (with sequence homology to FepBCD/BtuCDE ABC-type transporters) likely involved in corrinoid transport, the genes for two different salvaging enzyme (CbiZ) paralogues (Dehre_0282 and _0285), a gene cluster encoding the cobaltochelatase CobN (Dehre_0287), and several subunits of a magnesium chelatase complex. All proteins encoded in operon-2 share between 50 and 77% sequence identity with homologous proteins present in *Acetobacterium woodii*. Finally, operon-4 contains a gene cluster coding for an ABC-type corrinoid transporter (BtuFCD) and another copy of *cbiZ* (Dehre_2538). Two additional genes potentially involved in corrinoid biosynthesis (*cobT*/Dehre_1488 and *cobB*/*cobQ*, Dehre_2360) are located elsewhere in the genome and not in one of the five operons.

3.3.3 Comparative genomics of corrinoid operons in *Dehalobacter* spp.

The genome of *D. restrictus* was compared with newly available genomes of *Dehalobacter* spp. strains DCA, CF, E1, FTH1, and UNSWDHB with regard to the

organization of corrinoid operons (Table 1 and Table S2). Synteny maps for operon-1 and -2 (Fig S5), and for operon-3, -4 and -5 (Fig S6, Fig S7, Fig S8 respectively) are given as supplementary material. Operon-1 is conserved in *D. restrictus* and *Dehalobacter* sp. E1 but absent in all other genomes. Operon-2, which is directly following operon-1 in *D. restrictus*, is lacking in all other *Dehalobacter* spp. for which genome sequences are available to date. However, a detailed analysis of *Dehalobacter* sp. E1 revealed that the sequence conservation with *D. restrictus* is extended slightly beyond operon-1 but is interrupted within the homolog of Dehre_0282, which corresponds to the first gene in *D. restrictus* operon-2. The deletion in strain E1 goes beyond the last gene of operon-2, as a 5'-truncated version of Dehre_0297 is again found in strain E1 (Fig S5, panel C). *D. restrictus* operon-3 to -5 are fully conserved in all *Dehalobacter* spp. with the exception of another deletion in the proximal region of operon-5 in strain E1 (Fig S8).

Table 3. Comparative genomics of corrinoid operons in *Dehalobacter* spp.

		Dehalobacter spp.				
	Dehalobacter restrictus	sp. E1	sp. DCA	sp. CF	sp. FTH1	sp.UNSWDHB
Operon-1	+	+	-	-	-	-
Operon-2	+	-	-	-	-	-
Operon-3	+	+	+	+	+	+
Operon-4	+	+	+	+	+	+
Operon-5	+	partial	+	+	+	+
cbiH	deletion	intact	intact	intact	intact	Intact

Figure 7. Predicted corrinoid biosynthesis pathway of *D. restrictus*. (Page 63)

Based on the genome annotation, a map of the corrinoid biosynthesis was predicted including four pathways: the upper corrinoid biosynthesis (UCB, in yellow), the nucleotide loop assembly (NLA, in red), cobalt and corrinoid transporters (in blue) and corrinoid salvage (in green). The PCE reductive dehalogenase (PceA and associated proteins PceB and PceC) is also depicted. The enzymes catalyzing each reaction are given colour boxes indicating the protein name (when available) and corresponding gene loci (with the Dehre_# prefix removed). CbiH in *D. restrictus* is likely to be non-functional as the corresponding gene is truncated (yellow box with white background

Figure 8. Arrangement of genes associated with corrinoid biosynthesis and uptake in the genome of *D. restrictus*. (Page 64)

Genes involved in corrinoid metabolism are grouped in five operons (operon-1 to -5) located in different places of the genome. All five operons are predicted to be regulated by cobalamin riboswitches (Cbl-RS1 to 5) located directly upstream of the first gene of each operon. Genes are depicted as arrows in colours corresponding to four distinct pathways (see also Figure 1): upper corrinoid biosynthesis (UCB, in yellow), nucleotide loop assembly (NLA, in red), cobalt and corrinoid transporters (in blue) and corrinoid salvage (in green). Grey arrows depict genes that have no clear function in corrinoid biosynthesis. Gene names and corresponding gene loci (with the Dehre_# prefix removed) are given when available. Notes: (1) cbiU was newly annotated as a possible L-threonine kinase encoding gene. (2) A 101-bp deletion makes *cbiH* non-functional in *D. restrictus*.

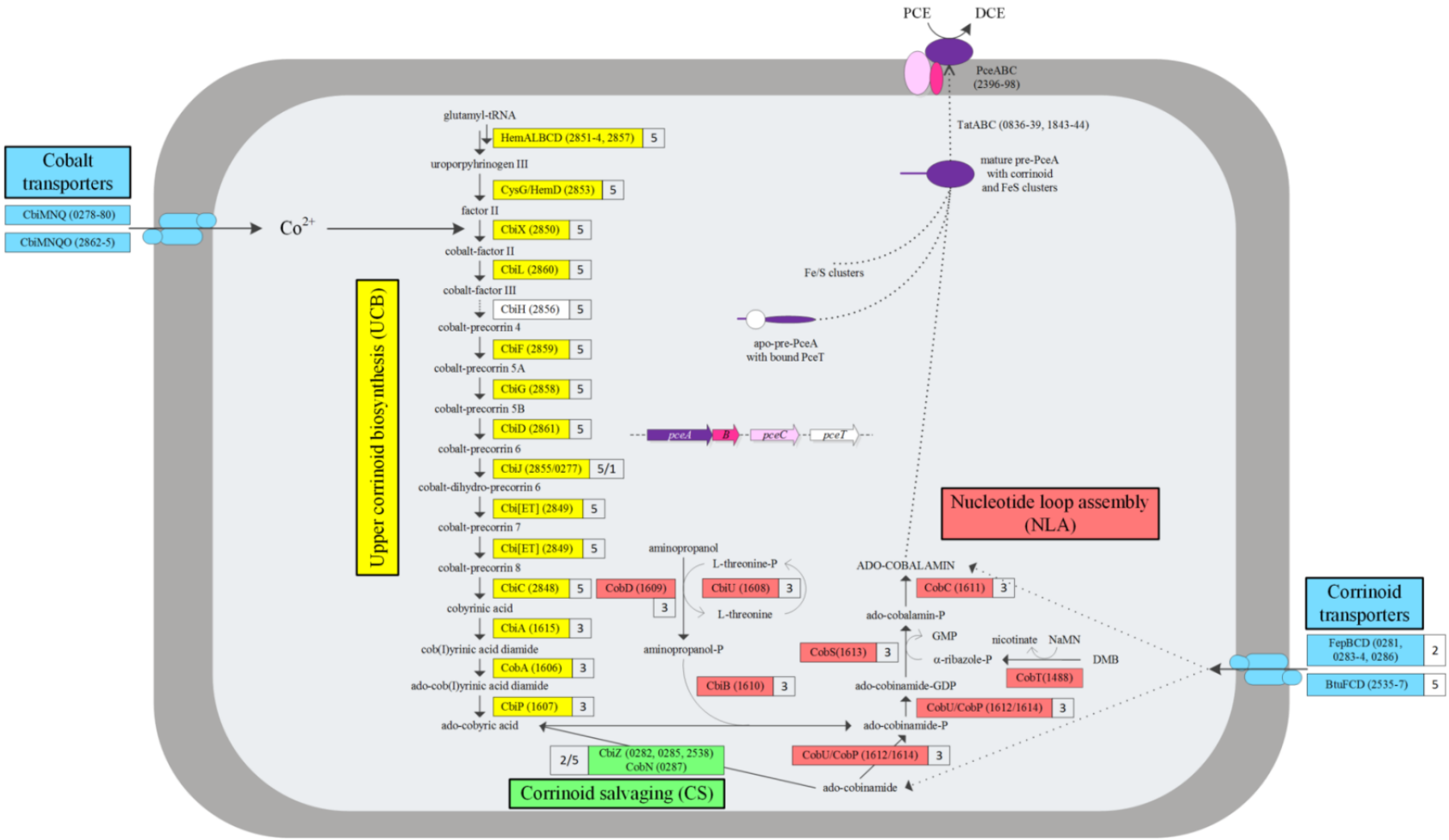
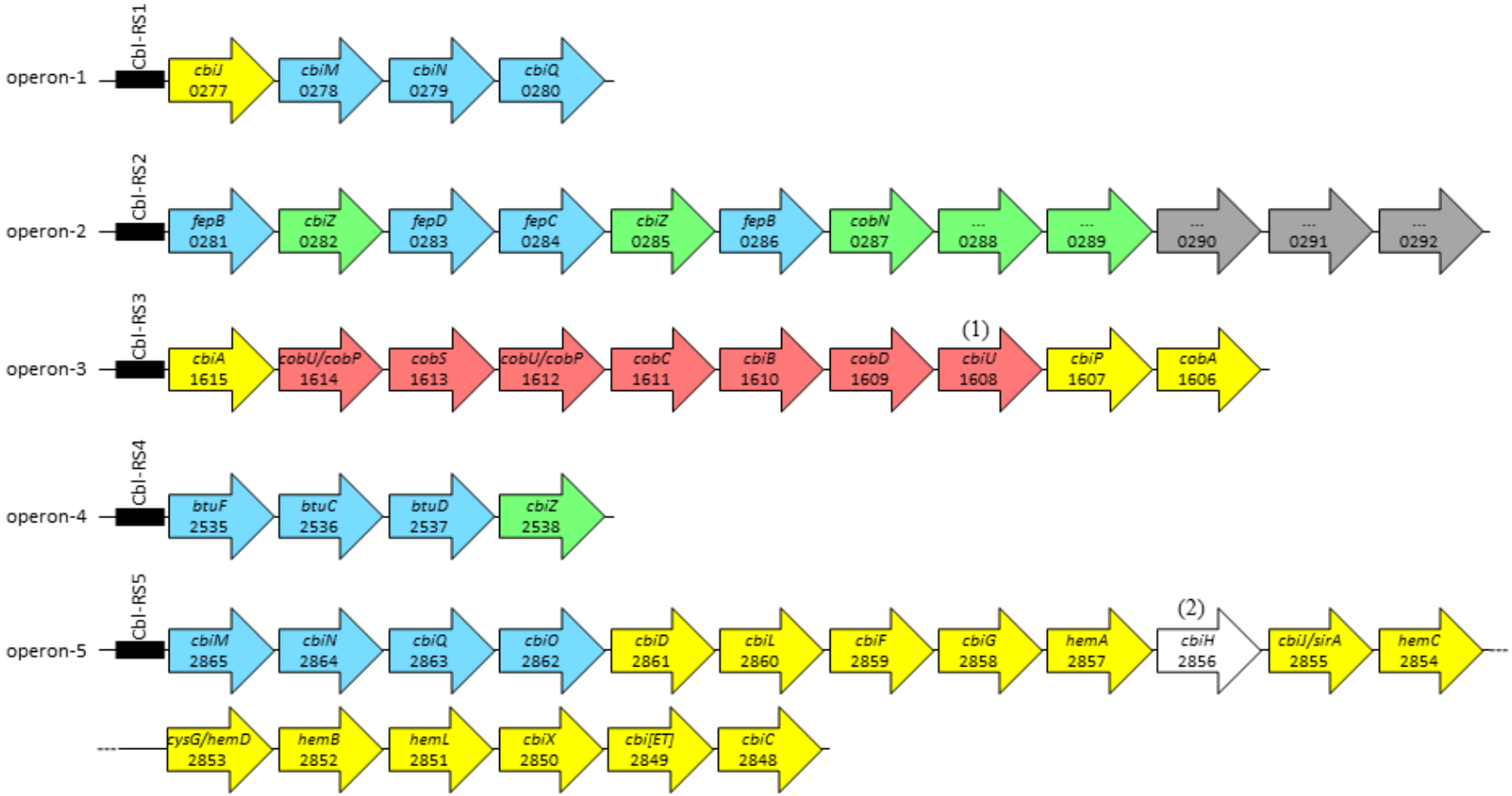

PCE
DCE
PceABC
(2396-98)
TatABC (0836-39, 1843-44)
mature pre-PceA
with corrinoid
and FeS clusters
Fe/S clusters
apo-pre-PceA
with bound PceT
Cobalt
transporters
CbiMNQ (0278-80)
CbiMNQO (2862-5)
Co2+
Upper corrinoid biosynthesis (UCB)
glutamyl-tRNA
HemALBCD (2851-4, 2857) 5
uroporpyhrinogen III
CysG/HemD (2853) 5
factor II
CbiX (2850) 5
cobalt-factor II
CbiL (2860) 5
cobalt-factor III
CbiH (2856) 5
cobalt-precorrin 4
CbiF (2859) 5
cobalt-precorrin 5A
CbiG (2858) 5
cobalt-precorrin 5B
CbiD (2861) 5
cobalt-precorrin 6
CbiJ (2855/0277) 5/1
cobalt-dihydro-precorrin 6
Cbi[ET] (2849) 5
cobalt-precorrin 7
Cbi[ET] (2849) 5
cobalt-precorrin 8
CbiC (2848) 5
CobD (1609) 3
cobyrinic acid
CbiA (1615) 3
cob(I)yrinic acid diamide
CobA (1606) 3
ado-cob(I)yrinic acid diamide
CbiP (1607) 3
ado-cobyric acid
pceA
B
pceC
pceT
aminopropanol
L-threonine-P
CbiU (1608) 3
L-threonine
aminopropanol-P
CbiB (1610) 3
Nucleotide loop assembly
(NLA)
ADO-COBALAMIN
CobC (1611) 3
ado-cobalamin-P
GMP
CobS(1613) 3
α-ribazole-P
nicotinate
NaMN
DMB
CobT(1488)
ado-cobinamide-GDP
CobU/CobP (1612/1614) 3
ado-cobinamide-P
CobU/CobP (1612/1614) 3
ado-cobinamide
2/5
CbiZ (0282, 0285, 2538)
CobN (0287)
Corrinoid salvaging (CS)
Corrinoid
transporters
FepBCD (0281,
0283-4, 0286) 2
BtuFCD (2535-7) 5

Figure 7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cbiJ 0277
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cbiN 0279
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cbiZ 0282
fepD 0283
fepC 0284
cbiZ 0285
fepB 0286
cobN 0287
... 0288
... 0289
... 0290
... 0291
... 0292
operon-3
CbI-RS3
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cobU/cobP 1614
cobS 1613
cobU/cobP 1612
cobC 1611
cbiB 1610
cobD 1609
(1)
cbiU 1608
cbiP 1607
cobA 1606
operon-4
CbI-RS4
btuF 2535
btuC 2536
btuD 2537
cbiZ 2538
operon-5
CbI-RS5
cbiM 2865
cbiN 2864
cbiQ 2863
cbiO 2862
cbiD 2861
cbiL 2860
cbiF 2859
cbiG 2858
hemA 2857
(2)
cbiH 2856
cbiJ/sirA 2855
hemC 2854
cysG/hemD 2853
hemB 2852
hemL 2851
cbiX 2850
cbi[ET] 2849
cbiC 2848

Figure 8

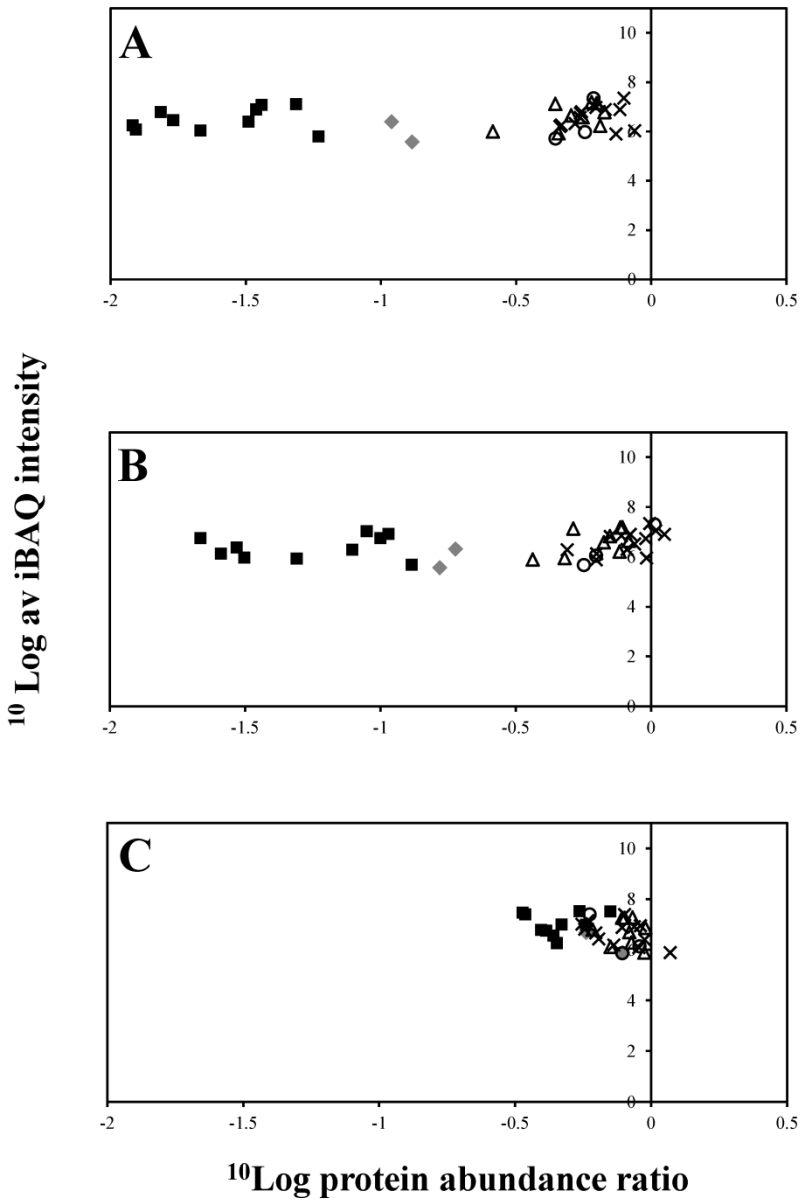

Figure 9. Proteins associated with corrinoid biosynthesis and uptake, extracted from *D. restrictus* cells cultivated in the presence of different cyanocobalamin concentrations.

(A) Proteomics analysis of cells cultivated in the presence of high (250 µg/L) *vs.* low (10 µg/L) corrinoid concentrations; (B) high (250 µg/L) *vs.* mid (50 µg/L) corrinoid concentrations; (C) mid (50 µg/L) *vs.* low (10 µg/L) corrinoid concentrations. Proteins encoded by corrinoid operon-1 (grey diamonds), operon-2 (black squares), operon-3 (white triangles), operon-4 (white circles) and operon-5 (black crosses) are shown. Logarithmic average of iBAQ (Intensity based absolute quantitation) value is plotted against the log value of protein abundance ratio based on LFQ value. CbiM (Dehre_2878) was left out because it could not be identified in two replicates of cultures under low corrinoid concentration.

3.3.4 Identification of cobalamin riboswitches in *D. restrictus*

Upstream of each of the five corrinoid biosynthesis-related operons in *D. restrictus* a distinct cobalamin riboswitch (Cbl-RS) was identified using Rfam. These five sequences were manually refined in a similar way as done previously for the Cbl-RS sequences of *Desulfitobacterium hafniense* (Choudhary et al., 2013). The alignment of structurally conserved regions of *D. restrictus* riboswitches (Cbl-RS01 to -RS05) was compared to *E. coli btuB* Cbl-RS (Fig S11). In contrast to *E. coli* Cbl-RS, which is regulated at the level of translation (Nahvi et al., 2004), all five *D. restrictus* Cbl-RS sequences end with a predicted transcriptional terminator, suggesting that the regulation operates at the level of transcription.

3.3.5 Transcriptional analysis of corrinoid biosynthesis operons in *D. restrictus*

The transcription of genes located directly downstream of the Cbl-RS in *D. restrictus* was analyzed for cells cultivated in the presence of high (250 µg/L) and low (10 µg/L) corrinoid concentration, and after corrinoid replenishment from low to high concentrations (Figure 6). Analysis of corrinoid-starved *D. restrictus* RNA revealed a higher transcription level of these genes, confirming an active regulation of the respective riboswitches at transcriptional level. Two hours after corrinoid replenishment, transcription of all selected genes was again repressed to the same level as observed under high corrinoid concentration. However, individual responses were significantly different. Indeed, the most pronounced effect was observed for two genes, namely Dehre_0277 (73-fold repression) and _0281 (346-fold), corresponding to the first genes in operon-1 and -2 in *D. restrictus*, respectively.

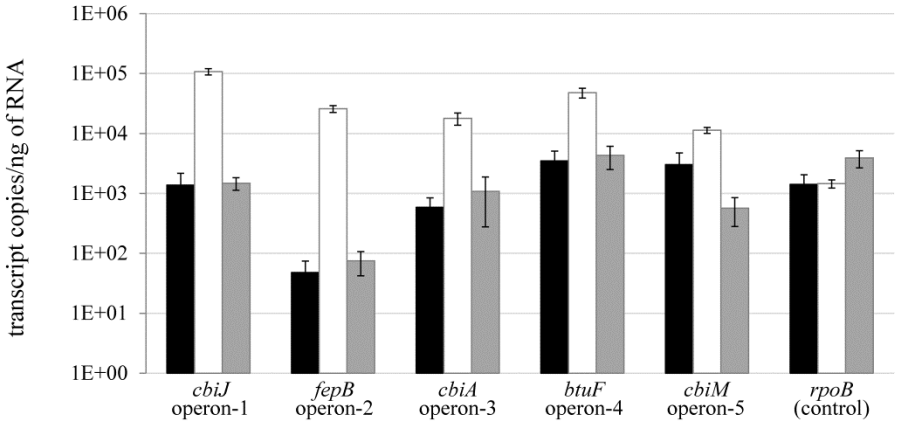

Figure 10. Transcriptional analysis of cobalamin riboswitch-dependent genes in *D. restrictus*.

The transcription of the genes located directly downstream of the cobalamin riboswitches in operon-1 to -5 was analyzed. The housekeeping gene *rpoB* was used as unregulated control. The black bars indicate the transcriptional level under corrinoid standard conditions (250 µg/L), the white bars under corrinoid starvation conditions (10 µg/L), and the grey bars show the transcriptional level 2 h after replenishment of the latter cultures with 250 µg/L cyanocobalamin. The data show the mean of triplicate cultures with standard deviation.

3.3.6 Proteome analysis of corrinoid starvation in *D. restrictus*

Comparative whole-proteome analysis was done on *D. restrictus* PER-K23 cells cultivated in the presence of 250 (high), 50 (mid) or 10 (low) µg/L cyanocobalamin. A total of 1195 proteins were detected, corresponding to 42% of the predicted 2826 proteins encoded on the genome (Kruse et al., 2013). The majority of the detected proteins (1175) was identified in cells from all the tested cyanocobalamin concentrations (Table S4). Normalized LFQ protein intensities were used to compare relative abundances of proteins between different cyanocobalamin treatments. The abundance of 44 proteins differed significantly ($P<0.01$) between high (250 µg/L) and low (10 µg/L) corrinoid concentration, and another 29 proteins showed more than 10-fold changes, albeit not significant due to high variation between triplicates (Fig S12). Similar results were found in the comparison of high *vs*. mid and mid *vs*. low cyanocobalamin concentrations (Table S12).

Proteins associated with cobalamin biosynthesis were further analyzed. A complete *de novo* corrinoid biosynthesis pathway was predicted to be encoded on the genome of *D. restrictus* starting from glutamyl-tRNA to cobalamin (Figure 7) (Kruse et al., 2013; Rupakula et al., 2013). CbiD (cobalamin biosynthesis protein, Dehre_2861), CbiJ (precorrin-6x reductase, Dehre_0277) and Cbi[ET] (precorrin-6Y methyltransferase, Dehre_2849) were identified in the proteome, revealing a complete biosynthetic pathway from cobalt-precorrin 5B to ado-cobinamide (Table S4). None of these proteins were detected in a previously analyzed proteome from *D. restrictus* (Rupakula et al., 2013). However, several proteins belonging to different parts of the corrinoid biosynthesis pathway including CobS (cobalamin 5'-phosphate synthase, Dehre_1613), CbiJ/CysG (precorrin-6x reductase, Dehre_2855), CbiH (precorrin-3B C17-methyltransferase, Dehre_2856) and CbiG (cobalamin biosynthesis protein, Dehre_2858) were also not found in the current proteome analysis. The lack of CbiH in the proteome is in line with the observation of a 101-bp deletion in *cbiH* likely leading to a non-functional gene (Fig S4). An incomplete cobalamin biosynthesis pathway might explain why *D. restrictus* requires exogenous corrinoids supplied to the growth medium.

Previously, the presence of one ABC-type cobalt transporter (Dehre_0850-0852) and two energy-coupling factor-type cobalt transporters (Dehre_0278-0280 and Dehre_2862-2865) was predicted in the genomic study of *D. restrictus* (Rupakula et al., 2013). Here, CbiM (Dehre_0278), CbiQ (Dehre_0280), CbiO (Dehre_2862) and CbiN (Dehre_2864) were identified in the proteome dataset obtained in this study (Table S4).

Relative abundance of proteins associated with corrinoid biosynthesis and salvaging pathways was further analyzed. Interestingly, nearly all proteins associated with corrinoid biosynthesis and salvaging pathways were up-regulated under corrinoid limiting growth conditions (Figure 10). As expected the overall largest change in the abundance of proteins related to corrinoid biosynthesis and salvaging pathways was observed when comparing the proteome of cells cultivated at high *vs*. low

concentration of cyanocobalamin (Figure 10A). The corrinoid metabolism differed more strongly when comparing cells cultivated in the presence of high *vs*. mid than mid *vs*. low concentrations (Figure 10B and Figure 10C).

Proteins encoded by operon-2 showed the largest change in protein abundance ratios with on average 46-fold up-regulation when comparing cells cultivated at low *vs*. high corrinoid concentrations (Figure 10A). Operon-2 encodes proteins predicted to be involved in corrinoid salvaging or corrinoid transport. Among these are two predicted CbiZ proteins, amidohydrolases required for salvaging the corrinoid precursor cobinamide, which were up-regulated 80-fold (Dehre_0285) and 58-fold (Dehre_0282) under corrinoid starvation, respectively (Table S4). Furthermore, proteins encoded in operon-1 including an energy-coupling factor-type cobalt transporter (Dehre_0278-0280) which is likely to be involved in the cobalt uptake process, and a precorrin-6x reductase (Dehre_0277), were on average 8-fold up-regulated when comparing cells cultivated in the presence of low *vs*. high corrinoid concentrations. Fewer changes were found for proteins encoded by the three remaining corrinoid-related operons under the different corrinoid conditions.

3.3.7 Functionality of the lower ligand pathway in *D. restrictus*

To investigate the functionality of the lower ligand pathway in *D. restrictus* it was supplemented either with dicyanocobinamide (Cbi, corrinoid with no lower ligand) only or Cbi together with 5,6-dimethylbenzimidazole (DMB). *D. restrictus* was cultivated in the presence of Cbi over a few transfers still demonstrated dechlorination and growth. Corrinoid extraction and analysis were performed by S. Keller and T. Schubert (University of Jena, Germany) as described previously (Keller et al., 2013) and revealed that *D. restrictus* produces different types of corrinoids, when it is cultivated without DMB as shown in Figure 11. However, when DMB is present most of the corrinoid forms disappear and exclusively cyanocobalamin is formed.

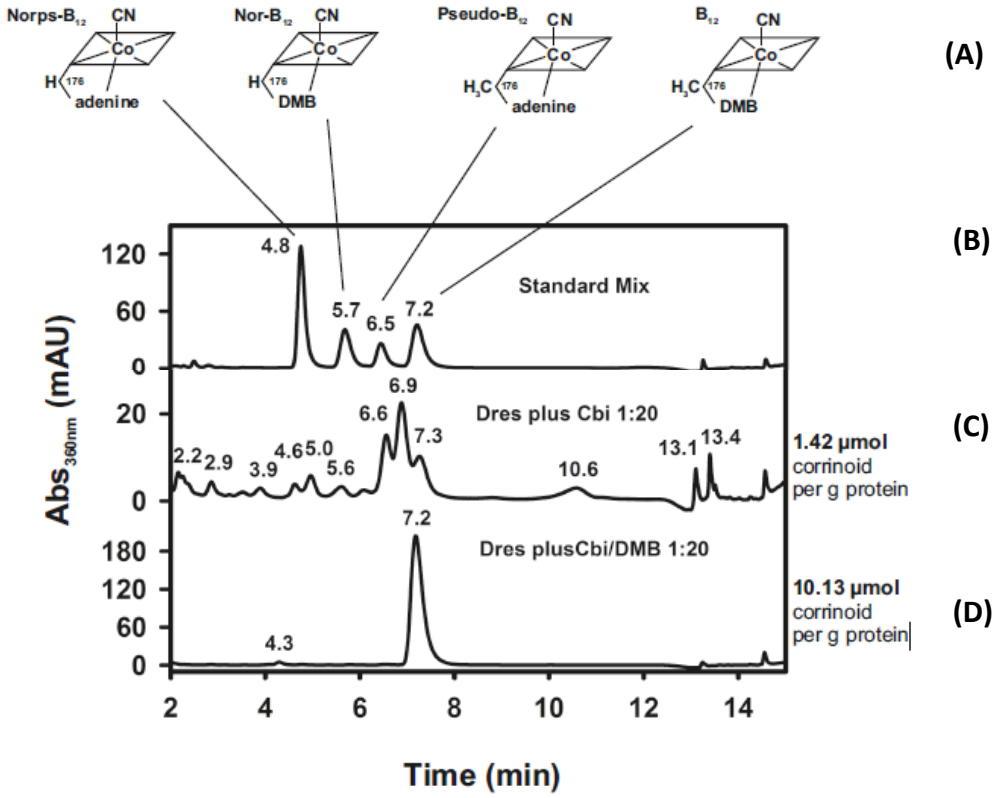

Figure 11. HPLC analysis of corrinoid extracts from *D. restrictus* cells cultivated with cobinamide (Cbi) with or without DMB.

(A). Standard mix of various corrinoids used for HPLC analyses.
(B). HPLC analyses of corrinoids in standard mix. All peaks marked with a retention time (x-axis) and with an absorbance A_{360nm} (y-axis).
(C). HPLC showing *D. restrictus* cultivated with dicyanocobinamide (Cbi) (corrinoid with no lower ligand)
(C). HPLC showing *D. restrictus* cultivated with Cbi and dimethylbenzimidazole (DMB).

3.4 Discussion

3.4.1 Corrinoid biosynthesis of *D. restrictus* and other OHRB

In the present study, ≥ 50 µg/L of cyanocobalamin was required for *D. restrictus* to reach its maximum PCE dechlorination, confirming that this organism depends on externally supplemented corrinoids (Holliger et al., 1998a). The genome of *D. restrictus* encodes a complete set of corrinoid biosynthesis genes, with the exception of a non-functional *cbiH* gene, suggesting that tetrapyrrole ring contraction does not occur here and represents a dead-end in the biosynthesis pathway. Comparative genomic analysis among other *Dehalobacter* spp. revealed that an intact *cbiH* gene is present in all other genomes. However, little is known about the capacity of other members of this genus to *de novo* synthesize corrinoids since they only have been studied under growth conditions with external addition of cyanocobalamin or in co-cultures (Grostern and Edwards, 2006; Yoshida et al., 2009; Grostern et al., 2010; Maphosa et al., 2012a; Deshpande et al., 2013). Similarly, strains of *Dehalococcoides*

mccartyi, which are also obligate OHRB, are corrinoid-auxotroph (Löffler et al., 2013). Unlike *D. restrictus*, the corrinoid auxotrophy in *D. mccartyi* strains is due to the lack of the complete biosynthetic pathway. Instead they rely on uptake of extracellular corrinoids via the salvaging pathway and on remodelling of incomplete or non-functional corrinoids in the presence of appropriate free lower ligands, among which 5,6-dimethlybenzimidazole plays a key role (Yi et al., 2012; Yan et al., 2012; Schipp et al., 2013; Yan et al., 2013; Men et al., 2014a). Interestingly, facultative OHRB such as *Sulfurospirillum multivorans* (Kräutler et al., 2003), *Desulfitobacterium hafniense* (Nonaka et al., 2006; Choudhary et al., 2013) or *Geobacter lovleyi* (Wagner et al., 2012) are all capable of *de novo* biosynthesis of corrinoids.

The genome of *D. restrictus* encodes five well-organized operons containing most of corrinoid biosynthesis-associated genes. Comparing the genomes of the sequenced *Dehalobacter* spp. revealed that *D. restrictus* harbours an extra set of genes (operon-2) coding for putative corrinoid transporters and salvaging enzymes (CbiZ and cobaltochelatases), suggesting an augmented capacity for corrinoid uptake and remodelling compared to other *Dehalobacter* spp. The importance of *cbiZ* genes in remodelling corrinoids has been already demonstrated for *D. mccartyi* (Kube et al., 2005; Seshadri et al., 2005; Men et al., 2014b). The role of operon-2 in *D. restrictus* was evidenced by the significant up-regulation of the corresponding enzymes when corrinoid concentration in the medium was lowered. This result clearly showed that *D. restrictus* has developed a unique strategy to cope, at least partially, with its lack of corrinoid biosynthesis under unfavourable corrinoid conditions. The presence of additional *cbiZ* genes in operon-2 raises the questions of the functional redundancy *vs*. specificity of multiple CbiZ proteins within a single strain, and of the origin of the additional *cbiZ* genes present in *D. restrictus*. While *in vitro* biochemical investigations would be required to answer the first question, a detailed analysis of CbiZ sequence homology (Fig S9) revealed that the two additional CbiZ proteins in *D. restrictus* show a high level of sequence identity with CbiZ homologues present in *Acetobacterium woodii*, a corrinoid-producing bacterium (Stupperich et al., 1988), which has been well-characterized for the Wood-Ljungdahl pathway that also requires corrinoids as an essential cofactor (Ragsdale and Pierce, 2008). A high degree of genomic synteny was further identified between the corrinoid operon-2 of *D. restrictus* and the corresponding region of the genome of *A. woodii* (GenBank NC_016894.1, (Poehlein et al., 2012)). The genetic structure is almost fully conserved, and the sequence identity of the encoded proteins ranges between 59 and 77% (Fig S10). This suggests that *D. restrictus* most probably acquired operon-2 by horizontal gene transfer and successfully exploited this operon to compensate for the partial deletion of *cbiH*.

3.4.2 Effect of corrinoid starvation on *D. restrictus* metabolism

Reduction of corrinoid provided in the growth medium strongly inhibited PCE dechlorination by *D. restrictus*. It also had a profound effect on *D. restrictus* corrinoid metabolism both at the level of transcription and at the proteome level. While changing from high, (250 µg/L) corrinoid concentration to an intermediate concentration (50 µg/L), *D. restrictus* responded by up-regulating proteins associated with corrinoid transport and salvaging pathways encoded in operon-1 and -2, allowing the strain to reach the same PCE dechlorination level as observed during high corrinoid concentration. Decreasing the corrinoid concentration even further to 10 µg/L showed, however, that, while the extent of PCE dechlorination was strongly affected, the amount of corrinoid-associated proteins did not notably change when compared to cells cultivated in the presence of 50 µg/L. This indicates that at corrinoid concentrations as low as 10 µg/L, *D. restrictus* was not able to compensate the lack of externally provided corrinoids by increased corrinoid transport and salvaging.

The presence of cobalamin riboswitches directly upstream of the five corrinoid operons in *D. restrictus* already suggested an active repression at the level of transcription by cyanocobalamin. Similar to transcriptional studies on *D. mccartyi* (Johnson et al., 2009)and *D. hafniense* (Choudhary et al., 2013), the cobalamin riboswitches of *D. restrictus* responded to addition of excess cyanocobalamin, and the level of repression correlated well with the proteomic data, showing the strongest effect for operon-1 and -2.

D. mccartyi strain 195, another corrinoid-auxotroph, requires a concentration of 25 µg/L cyanocobalamin to support optimal TCE dechlorination rates and growth yield(He et al., 2007), a value that is similar to what was observed for *D. restrictus*. Therefore, and in addition to the ecogenomic biomarkers defined by Maphosa *et al*. (Maphosa et al., 2012a), one could consider the physiological threshold of corrinoid concentration as a possible diagnostic tool to delineate the reductive dechlorination potential by corrinoid-auxotrophic OHRB in anaerobic environments. Meanwhile, the production of the PCE reductive dehalogenase (PceA, Dehre_2398) in *D. restrictus* showed no significant change under different corrinoid concentrations, which strongly suggests that the amount of available corrinoid and not of the apo-enzyme represents the main limiting factor for PCE dechlorination.

Preliminary experiments done to investigate the functionality of the lower ligand biosynthesis pathway in *D. restrictus* gave unexpected results. It was observed that the addition of 5,6-dimethylbenzimidazole (DMB) had a significant effect on the level of cyanocobalamin synthesized in *D. restrictus.* However *D. restrictus* was still able to dechlorinate in the absence of DMB, i.e., when cultures were supplemented with cobinamide only, producing various unidentified corrinoid derivaties. This suggests the possibility of *D. restrictus* to synthesize its own lower ligand, which is contrasting to what was observed earlier in *Dehalococcoides* spp. pure cultures or co-cultures, where the addition of DMB was required, irrespective of the presence of a few genes from the lower ligand and salvaging pathways.

Taken altogether, our results support the hypothesis that, besides the partial deletion of *cbiH* in *D. restrictus* (Rupakula et al., 2013; Kruse et al., 2013; Rupakula et al., 2014), which already represents a crucial checkpoint in the corrinoid biosynthesis pathway, the energetic cost of *de novo* corrinoid biosynthesis might explain why *D. restrictus* has developed enhanced corrinoid transport and salvaging strategies. *D. restrictus* corrinoid metabolism represents an intermediate situation between the true corrinoid-auxotrophic and obligate organohalide-respiring *D. mccartyi*, which lacks the corrinoid biosynthesis pathway completely (He et al., 2007; Men et al., 2012; Yan et al., 2013; Men et al., 2014b), and the facultative OHRB able to produce corrinoids *de novo*.

Chapter 4

RdhK regulatory proteins in *D. restrictus* and their possible role towards regulating the reductive dehalogenases

4 RdhK regulatory proteins in *D. restrictus* and their possible role towards regulating the reductive dehalogenases

4.1 Introduction to regulatory proteins in OHRB

4.1.1 CRP-FNR family of transcriptional regulators

Bacteria live in extremely diverse environments. These environments are not stable and expose bacteria to rapid changes and often a paucity of resources. Bacteria must have the capacity to respond quickly to transitory conditions and imbalanced resources by activating alternative gene programs in order to make use of short-lived opportunities or to shut off unneeded metabolic routes. Herein comes the importance of transcriptional regulators in controlling the expression of genes, for example reductive dehalogenases.

In contrast to the obligate OHRB *Dehalococcoides* spp. and *Dehalobacter* spp., *Desulfitobacterium* spp., are facultative organohalide-respiring bacteria, and here the regulation is needed to confer an adequate response to the presence of alternative electron acceptors such as nitrate, sulfite, or organohalides.

Transcriptional analysis has shown that a number of genes were transcribed alongside the chlorophenol reductive dehalogenase (CprA) when induced with 3-chlorophenylacetic acid as electron acceptor, which led to the identification of the *cpr* gene cluster (Smidt et al., 2000a). Within this gene cluster, it became apparent that the proteins encoded were supportive of CprA. Based on sequence comparison, the genes in the *cpr* gene cluster have been named *cprTKZEBACD*, and most have been proposed to play a role in regulation or to function as molecular chaperones. However, many *rdhA* genes are only associated with *rdhB*, suggesting that regulation of *rdhA* genes and maturation of the gene products are highly diverse (Futagami et al., 2008). Sequence similarity searches revealed CprK to belong to the CRP-FNR (cAMP receptor protein and fumarate and nitrate reduction regulatory protein) family of transcriptional regulators. Many of the transcripts identified were found to be polycistronic, containing a number of the *cpr* genes transcribed at once.

Thereby due to the large number of *rdh* operons in *D. restrictus*, it can be assumed that their synthesis is also controlled by regulatory events.

The CRP-FNR proteins usually consist of 230 to 250 amino acids. The proteins are characterized by a C-terminally-located helix-turn-helix (HTH) structural motif consisting of two helices joined by a turn, which fits into the major groove of DNA. Regulatory proteins bind via this motif in the promoter region of target genes and thus exert function of activators or repressors. In addition to the HTH motif, CRP-FNR regulatory proteins have a large nucleotide-binding domain (Kolb et al., 1993;

Körner et al., 2003; Antelmann and Helmann, 2011) that extends from the N-terminus over roughly 170 residues. Thus, by definition, members of the CRP-FNR family are characterized by the presence of both domains.

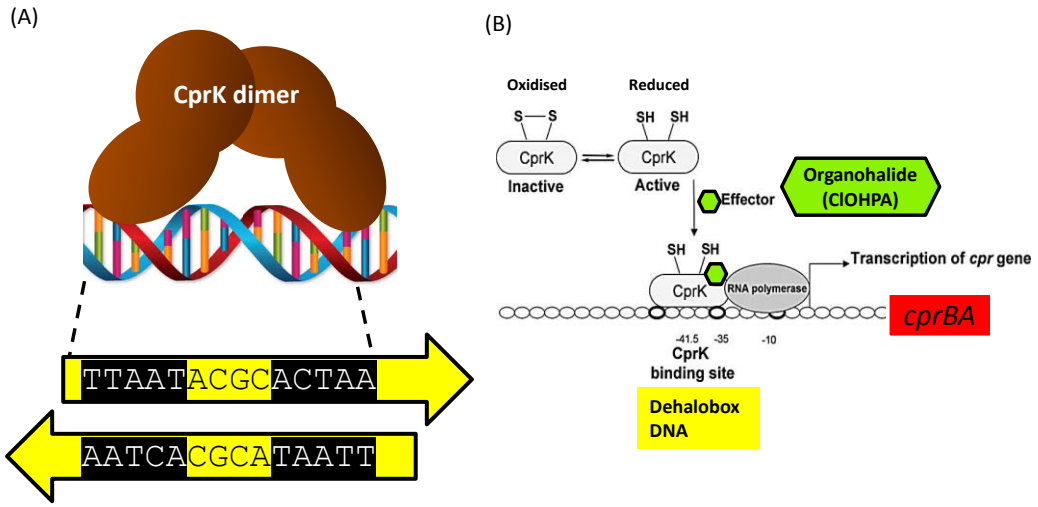

Figure 12. Model for transcriptional regulation of OHR by CprK in *D. dehalogenans* and *D. hafniense* strain DCB-2.

Panel (A) shows general mechanism of CprK binding to the DNA. The two monomers are connected via intermolecular disulphide bonds between C_{11} of one monomer with C_{200} of another monomer, such that eventually each of the monomer's C-terminal DNA binding domains, recognise the inverted repeat TAATT-N4-ACTAA (indicated in yellow arrows) within the palindrome, allowing binding along the major groove of DNA.
Panel (B) shows detailed model of CprK. It is a tripartite interaction between the CprK protein-Organohalide-dehalobox DNA, which regulates the expression of the reductive dehalogenase gene cluster downstream. Here, CprK is required to be reduced (active form) and can then bind to its specific organohalide (ClOHPA). This causes changes in the DNA-binding region of CprK, allowing CprK to bind to the dehalobox (DNA-motiff containing the specific inverted repeats) positioned around -41.5 bp upstream from the +1 transcription start site of the gene downstream. Panel (B) has been adapted from (Gábor et al., 2008).

4.1.2 Transcriptional regulation by CprK/K1 proteins

The transcriptional activator CprK1 from *Desulfitobacterium dehalogenans*, activates transcription of genes encoding proteins involved in reductive dehalogenation of chlorinated aromatic compounds. 3-chloro-4-hydroxyphenylacetate (ClOHPA) is a known ligand that binds tightly to CprK1 and induces binding to a specific DNA sequence motif (so-called dehalobox (DB), TTAAT-N_4-ATTAA) located in the promoter region of *cpr* genes (Smidt et al., 2000a).

The first characterization of CprK was performed by Pop and co-workers in 2004 (Pop et al., 2004). Gene *cprK* within the *D. dehalogenans cpr* gene cluster was cloned, actively overexpressed in *E. coli*, and purified to homogeneity. EMSA, DNA footprinting studies, and promoter-lac fusion experiments were performed to further characterize CprK with its dehalobox and ligand. It was also shown through

gel filtration that CprK was present as a dimer (Gábor et al., 2006). *D. hafniense* DCB-2 also contains a gene cluster essentially similar to the well-characterized *cpr* gene cluster of *D. dehalogenans*.

For *D. hafniense* CprK, many other substrate molecules, such as 3-chlorophenylacetate, 4-hydroxyphenylacetate, 3-chloro-4-hydroxybenzoate, 3-chlorobenzoate, 3,5-dichloro-4-hydroxy benzoate and 2,4-dichlorobenzoate, were also tested by EMSA for binding to CprK. However, no significant binding or mobility shift was observed (Pop et al., 2004). This suggests that the chlorine group and the hydroxyl group are important for specific interactions between ClOHPA and CprK.

Mazon and co-workers (Mazon et al., 2007) studied *D. hafniense* DCB-2 CprK1 interaction using different methods. They initially assembled a library of potential effector molecules which have been known to be dehalogenated by *D. dehalogenans* and *D. hafniense* so far. Then they used a combination of techniques to study the interaction. Native mass spectrometry was used to study oligomeric states of CprK1 as well as its interaction with the effectors-library and dehaloboxes. They also used intrinsic fluorescence, DNA binding assays and limited proteolysis where the latter was used to characterize both redox- and effector-induced structural changes of CprK1.

Experiments by Gabor and co-workers (Gábor et al., 2008) revealed that CprK4 is indeed capable of DNA binding in its effector-free form, and that cAMP or ClOHPA did not promote DNA binding to any extent. However, in the presence of compounds such as 3,5-dichlorophenol, 2,3-dichlorophenol and 2,4,6-trichlorophenol, over 80% of the DNA formed a complex with CprK4. These were the same set of compounds which were detected to not act as effectors for CprK1. Eventually CprK4 was suggested to have complementary function to CprK1. Effectors for CprK1 were detected as 2,4-dichlorophenol, 2-bromo-4-chlorophenol, 2,4,5-trichlorophenol and ClOHPA. In summary, their results indicated that a halogen substitution at the *meta* position in phenol derivatives resulted in the loss of effector activity on CprK1; in contrast, this appeared to be beneficial for the activity of CprK4.

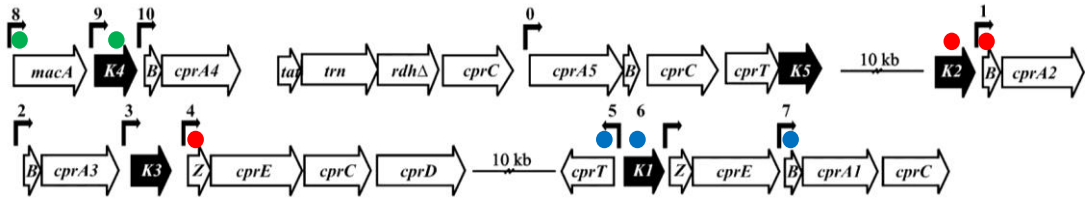

Figure 13. The *cpr* gene clusters in *D. hafniense* DCB-2

Figure adapted from (Gábor et al., 2008). Figure shows the reductive dehalogenase gene clusters in *D. hafniense* DCB-2. Numbered arrows indicate putative CprK-binding sites (dehaloboxes, DB), of which the interacting pairs are indicated with respective colour-filled circles. Red: CprK2, whose interaction was shown best with DB1 and DB4; Blue: CprK3 interacting with DB5, DB7; Green: CprK4 interacting with DB8.

Table 4. Biochemically characterised CprK proteins and their partners

Strain [1]	Protein name [3]	Dehaloboxes they interact with:	Corresponding genes:
DCB-2[1]	CprK1	DB5, DB6, DB7	*cprBAC1*, *cprT*, *cprZE*
	CprK2	DB1, DB3 > DB2, DB4	*cprBA2*, *cprK3* > *cprBA3*, *cprZECD*
	CprK4	DB8 > DB9, DB10	*macA* > *cprK4*, *cprBA4*
Dde[2]	CprK	IGR-1	*cprBA*
		IGR-7	*cprT*
		IGR-9	*cprZE*
		IGR-4	*cprCD*

[1] *D. hafniense* DCB-2
[2] *D. dehalogenans*
[3] The protein annotation defined by (Gábor et al., 2008) was used here. These proteins were annotated differently in (Kim et al., 2012): CprK1 = RdhK6; CprK2 = RdhK4; CprK4 = RdhK1.

Another technique used for observing CprK interaction is by coupling DB with β-galactosidase expression and activity (Kemp et al., 2013). This reporter system, allows direct quantification of gene products *in vivo*. Therefore the GFP_{UV} gene was placed under the control of the DB7 promoter. This reporter system proved highly efficient in sensing cellular levels of ClOHPA with an apparent K_D to be only slightly smaller than that obtained during *in vitro* experiments, however transport of ClOHPA across the cell membrane could have affected the cellular ClOHPA levels. Kemp and co-workers expressed CprK variants fused to GFP to enable identification of effector-binding site by *in vivo* assay and investigate the mechanism by which CprK distinguishes between the substrate ClOHPA and the dechlorination product OHPA. The halogen substituent was determined to contribute to affinity via van der Waals interactions, although the main detection mechanism was by sensing the effects of the electronegative halogen substituent on the phenol, (pK_a). Kemp and co-workers found that CprK dependent transcriptional activation was entirely dependent on an ionic interaction between the lysine K_{133} of the protein and a phenolic effector molecule, rather than the presence of a halogen atom, and this was supported by pK_a calculations of the CprK effector binding site. Also, the strict conservation of K_{133} within the CprK family suggests that its function is limited to sensing the presence of halogenated phenols.

4.1.3 Dehalobox specificity

CprK from *D. dehalogenans* was characterized in 2004 (Pop et al., 2004) as the first transcriptional regulator in OHR. Some of the experimentally demonstrated DNA-binding properties of CprK are as follows. CprK has been shown to bind DNA in the presence of ClOHPA. DNA footprinting analysis revealed that CprK protected a 14-bp pseudo palindromic sequence as shown in Figure 12 and Figure 13. This sequence is similar to the consensus sequences recognized by CRP and FNR. However, a electrophoretic mobility shift assay (EMSA) showed that a minimum of 32 bp are required for a gel shift in the *in vitro* assay, thereby

extending the DNA length by few more residues on both sides of the 14-bp protected region (AGATAAAAGTTAATACGCACTAATACTTGTGT). The apparent K_D for CprK for DNA is 190 ± 30 nM as determined in EMSA studies (Pop et al., 2004).

Each dehalobox contains a 5-bp palindromic sequence, separated by 4 spacing nucleotides; the deduced consensus sequence of the dehalobox is TTAAT-N_4-ATTAA (Smidt et al., 2000a). In 2006 Gabor *et al.,* identified three putative CprK1 binding sites (Gábor et al., 2006). This was confirmed by electrophoretic mobility shift assay (EMSA) using intergenic region of *cprT-cprK1* (DB5), *cprK1-cprZ* (DB6) and *cprE-cprB* (DB7) as putative binding targets, in the presence of 25 μM ClOHPA (Gábor et al., 2006, 2008). By keeping the DNA concentration constant during the EMSA, it was observed that protein concentrations as low as 1 nM CprK1 was sufficient to observe protein-DNA complexes on the gel, suggesting a high affinity of CprK1 for its target DNA sequence. The dissociation constant (K_D) was deduced to be 90 nM between CprK1 and DB7(Gábor et al., 2006). Each dehalobox differs slightly from the other, and this affects the affinity of CprK1 to the DNA. CprK1 was found to have the greatest affinity for DB7, which has a perfect palindromic sequence (Gábor et al., 2008). Eventually the *D. hafniense* DCB-2 genome sequencing led to the discovery of more dehaloboxes than the three known from Gabor and co-workers in 2006. The genome has been found to contain at least ten dehaloboxes (Gábor et al., 2008) and around twenty CRP-FNR type transcriptional regulators, five of which show high sequence similarity with CprK of *D. dehalogenans*, and were also clustered around potential reductive dehalogenase genes (Pop et al., 2004) implicating a role for these five CprK paralogs towards regulating these *rdh* genes.

4.1.4 Dehalobox specificity as inferred from CprK sequence alignments with CRP-FNR proteins

Dehalobox specificity can also be understood from analysing the CprK protein structure and the important amino acid residues involved in DNA recognition. Green and co-workers demonstrated that position of certain residues within the motif EXXSR in the related FNR protein from *E. coli* were responsible for the specificity towards the target DNA sequence TTGAT-N4-ATCAA (FNR-box) (Green et al., 2001). Later, Gabor and co-workers (Gábor et al., 2008) also found similar motifs containing amino acid residues that confer DNA-binding specificity by comparing the CRP protein of *E. coli*, the FNR regulator of *E. coli*, CprK of *D. dehalogenans* (Joyce et al., 2006) and the CprK1–K5 proteins identified in *D. hafniense* DCB-2. This along with secondary structure prediction, showed that in the α-helixes of the HTH domain, CprK1 and CprK2 possess a conserved motif VXXSR while in CprK4 the corresponding sequence is VXXSK, and in CprK3 and CprK5 it is VXXCK.

4.1.5 Redox regulation of CprK1/CprK

CprK1 can distinguish between the substrate i.e., 3-chloro-4-hydroxyphenylacetate (ClOHPA) and the product, 4-hydroxyphenylacetate (OHPA) of CprA, which is remarkable given the high degree of similarity between both compounds, i.e. they differ only by a single chlorine substituent. It was estimated from indirect *in vitro* measurements that CprK1 distinguishes them to a 10^4–fold degree (Pop et al., 2004; Joyce et al., 2006; Mazon et al., 2007; Leys et al., 2013). This ability to differentiate between substrate and product allows for the tight regulation of the *cpr* gene cluster, whereby *cpr* genes are not expressed when the RdhA specific substrate (ClOHPA) is absent. Purified CprK of *D. dehalogenans* was in-activated by oxidation however was reversibly activated upon addition of 2 mM dithiothreitol. This inactivation led to the suggestion of a redox-control mechanism by Pop *et al.*, (Pop et al., 2004), whereby the cell is able to prevent *cpr* gene cluster and eventually prevent the *cprA* gene expression under aerobic conditions.

Redox regulation of CprK occurs through a thiol/disulfide redox switch, which includes two classes of cysteine residues. Under oxidizing conditions, C_{11} and C_{200} form an intermolecular disulfide bond, while C_{105} and C_{111} form an intramolecular disulfide *in vitro*. The intermolecular disulfide bond between the C_{11} of one monomer to C_{200} of the other monomer, seemingly cause the DNA binding domains of the two monomers to be asymmetrically arranged. This position of the HTH motif in CprK1 is incompatible with tight binding to the target DNA. A rotation of the DNA-binding domains was performed *in silico* into a position similar to DNA-bound CRP, which was found to be only possible by breaking the disulfide bonds between C_{11} and C_{200}. Gupta and co-workers report that C_{11} is involved in redox inactivation *in vivo*. Upon replacement of C_{11} with serine, alanine, or aspartate, CprK loses its DNA binding activity (Gupta and Ragsdale, 2008). Thus it was believed that C_{11} plays a dual role in its involvement in a redox switching mechanism and in maintaining the correct tertiary structure that promotes DNA binding.

Further in 2006, Gabor and co-workers (Gábor et al., 2006) investigated the potential redox regulation suggested earlier by Pop and co-workers (Pop et al., 2004). They created single amino acid mutants of CprK1 from *D. hafniense* DCB-2 i.e., modifying the following residues; C_{11} and C_{200} to serine to make CprK1-C_{11}S and CprK1-C_{200}S variants, respectively. It was found that in aerobic conditions, both CprK1 variants were able to form dimers in the absence of disulfide bridges, and *in vivo* activity coupled to β-galactosidase expression assays showed that CprK1-C_{11}S performed similarly to CprK1 wild type in the absence and presence of ClOHPA. If redox regulation of CprK1 was physiologically relevant, it was postulated that both variants would show different β-galactosidase expression patterns and accordingly it was observed. The variant CprK1-C_{200}S was found to have a higher activity in both the absence and presence of ClOHPA than both CprK1 wild type and CprK1-C_{11}S, therefore it was suggested that a higher level of CprK1-C_{200}S must be present within the cells due to increased expression.

Alongside this evidence and the lack of conservation of C_{11} and C_{200} between CprK homologues, Gabor and co-workers (Gábor et al., 2006) eventually refuted the hypothesis of CprK redox regulation proposed by Pop and co-workers.

Mazon and colleagues found that upon reduction of the intra-molecular disulfide bridge in oxidized CprK1, it activates the protein, but this alone is not sufficient for DNA binding (Mazon et al., 2007). Activation of CprK1 is a typical example of allosteric regulation; the binding of a potent ligand molecule (ClOHPA) to reduced CprK1 induces local changes in the N-terminal substrate binding domain, which subsequently may lead to changes in the hinge region and as such to structural changes in the DNA binding domain that are required for specific DNA binding (Mazon et al., 2007).

4.1.6 Sequence and crystal structure analysis of CprK1

The *D. hafniense* DCB-2 CprK1 crystal structure was published in 2006 by Joyce and co-workers (Joyce et al., 2006). They identified the structures of oxidized CprK1 from *D. hafniense* DCB-2 in complex with ClOHPA and reduced CprK from *D. dehalogenans* in its unliganded form. It was observed that both the CprKs exhibit high structural similarity to two other proteins, i.e. the cAMP receptor protein (CRP) (Harman, 2001; Fic et al., 2006) and PrfA, virulence regulator in *Listeria monocytogenes* (Eiting et al., 2005). CprK is made up of two identical subunits forming an asymmetric dimer. Each monomer is folded in two distinct domains: the N-terminal effector binding domain and the C-terminal DNA binding domain containing a helix turn-helix motif. Further, an intermolecular disulfide bond between C_{11} and C_{200} connects the effector binding domain of one monomer to the DNA binding helix of the opposite monomer. In addition to this intermolecular disulfide bond, CprK from *D. dehalogenans*, but not CprK1 from *D. hafniense*, has also an intramolecular disulfide bond between C_{105}, which is conserved in FNR and most other CprK homologs and C_{11} (Pop et al., 2004, 2006). In the x-ray structure of ligand-bound oxidized CprK1 from *D. hafniense*, one ClOHPA molecule is bound per monomer in the β-barrel of the effector binding domain. The observed ClOHPA binding site was found to be at a position, similar to the position where the binding site of cAMP ligand was detected in CRP (Joyce et al., 2006). Upon further comparative analysis of the liganded oxidised CprK1 structure from *D. hafniense* DCB-2 and the ligand-free reduced CprK from *D. dehalogenans*, it was found that both the structures were not compatible with tight DB binding.

Mazon and co-workers compared the two crystal structures and postulated that the binding of ClOHPA to CprK1 caused reorientation of the N-terminal effector binding domain's β-barrels with respect to the central α-helix at the dimer interface. This consequentially caused further conformational changes in the C-terminal DNA binding domains allowing tight DB binding. They found crucial features that enable such structural changes and suggested the presence of the chlorine group and the ortho position to be important along with the *pKa* dependent mechanism (i.e.

formation of phenolate group) which allowed DNA binding. However, the crystal structure of effector-bound and reduced CprK1 protein was not available by then to prove this hypothesis. Later, Levy and co-workers (Levy et al., 2008) reported crystal structures of *D. hafniense* CprK in the ligand-free (both oxidation states), ligand-bound (reduced state) and DNA-bound states, enabling a complete understanding of CprK's redox-dependent activity and structural description of the allosteric molecular rearrangements.

The crystal structure by Joyce and co-workers (Joyce et al., 2006) showed the presence of two ligand-binding pockets per dimer of CprK1, similar to the structure of CRP. Within the ligand binding pocket, a total of six hydrogen bonds are indicated to form between the ligand-binding domain and ClOHPA. Three of these hydrogen bonds are formed between the hydroxyl group of ClOHPA and Tyr-76, Lys-133, and the backbone of Gly-85 of one monomer. This area was deduced to be important for ClOHPA recognition. The chlorine atom is positioned in a hydrophobic pocket with van der Waals contacts to the central α-helix residues Tyr-130, Leu-131, and Val-134, with no direct halogen-bonding interactions, between ligand and protein (Joyce et al., 2006).

It was therefore presumed unlikely that these interactions alone could account for the 10^4-fold preference of CprK1 for the *ortho*-halogen atom in ClOHPA (as compared to HPA). To explain the ability of CprK1 to sense ClOHPA and HPA to such different degrees, a *pKa*-dependent mechanism has been suggested, which was first put forward in 1999 by Palfey and co-workers (Palfey et al., 1999). The *pKa* of the phenolic moieties of ClOHPA and HPA are different (ClOHPA *pKa* = 8.5, HPA *pKa* = 10.2), and this difference in *pKa* could mean the difference between the ionisation/non-ionisation of the ligand, which in turn would affect the ability of the Lys-133 residue to form a salt bridge with the ligand (Joyce et al., 2006; Mazon et al., 2007).

4.1.7 Diversity and features of RdhK regulatory proteins in *D. restrictus*

The genome analysis of *D. restrictus* indicated the presence of 25 different CRP-FNR like regulatory proteins, denoted as RdhK. Among these 22 *rdhK* genes were found in the close vicinity of *rdh* gene clusters, thereby suggesting the possible involvement of RdhK proteins in regulating the *rdh* gene clusters, as described so far for CprK. The goal of the present study was to understand the diversity of the CRP-FNR like RdhK regulatory proteins from *D. restrictus* along with identifying their putative DNA targets (dehalobox located upstream of the *rdh* genes) and the organohalides they would respond to. Using bioinformatic analysis, putative dehaloboxes were predicted upstream some of the *rdh* gene clusters. A set of RdhK proteins were chosen among the 25 identified in the genome based on criteria such as the induction of the adjacent *rdhA* genes or detection of these RdhK proteins in previous proteomic studies. The chosen RdhK proteins were heterologously produced and purified either as His-tagged or tagless proteins. Biochemical characterisation of these RdhK proteins has been attempted to identify the tri-partite

interaction with particular dehaloboxes and particular organohalides. Various combinations were attempted using electrophoretic mobility shift assay (EMSA), as well as isothermal titration calorimetry (ITC) or intrinsic tryptophan fluorescence (ITF) quenching. The Chapter 4 presents an overview of the methods used and results obtained in characterisation of some RdhK proteins in *D. restrictus* in order to understand their possible role in regulating the transcription of *rdh* gene clusters and in turn regulating OHR metabolism.

4.2 Methods

4.2.1 Sequence retrieval and genome analysis

All sequences mentioned in this study were taken from the recently published genome of *D. restrictus* strain PER-K23 (Kruse et al., 2013), the annotation of which was derived from JGI project #402027. The annotation of selected genes was verified using a manual search with BLAST (Altschul et al., 1990). Protein sequences were aligned using ClustalX v.2.0 (Larkin et al., 2007). Sequence maximum likelihood tree analysis was done with MEGA5 (Tamura et al., 2011). RdhK sequences from the genome of *D. restrictus* were numbered according to the numbering system of the *rdhA* genes present in their direct vicinity (Rupakula et al., 2013).

4.2.2 Putative dehaloboxes search

Intergenic regions upstream *rdhAB* or *rdhBA* gene operons were used for detection of inverted repeats, which was performed with the Palindrome tool (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=palindrome) using different sets of criteria (minimum length of palindrome: 3-8; maximum length of palindrome: 14-18; maximum gap between repeated regions: 8; number of mismatch allowed: 0-1). Palindrome sequences were inspected manually and selected based on their similarity to the consensus dehalobox sequence established for CprK and the *cpr* operon (Smidt et al., 2000a; Pop et al., 2004; Gábor et al., 2006). A consensus dehalobox sequence for *D. restrictus* was obtained using the WebLogo 3 program (http://threeplusone.com/weblogo/). Alignment of the consensus sequence TTAAT-N_4-ATTAA was performed followed by box shading to highlight the conserved residues.

4.2.3 Bacterial strains, plasmids and growth conditions

Dehalobacter restrictus strain PER-K23 (DSM 9455) was cultivated as described earlier (Holliger et al., 1998b; Maillard et al., 2003; Rupakula et al., 2013). Anaerobic serum flasks of 50 mL were supplemented with hydrogen as electron donor (80:20% in mixture with CO_2), inoculated with 5% (v/v) inoculum, and finally 1% (v/v) of 2 M PCE dissolved in hexadecane was added as electron acceptor. *D. restrictus* was cultivated at 30°C under gentle agitation (100 rpm), and chloride release was used as an indicator of growth. *Escherichia coli* DH5α, *E. coli* Rosetta λDE3 and *E. coli* BL21 were cultivated on liquid or solid LB medium

containing the appropriate antibiotics required by the accompanying plasmids (at final 30 µg/mL chloramphenicol, 100 µg/mL ampilicillin, 100 µg/mL kanamycin into media). Plasmids pGEM-T Easy (Promega) was used for cloning the intergenic regions containing the dehaloboxes while plasmid pET24d (Novagen) was used for cloning rdhK genes chosen from *D. restrictus* for expression.

4.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to visualise DNA restriction fragments and PCR products. Agarose gels were prepared fresh in 0.5× TAE buffer (containing 20 mM Tris acetate, 0.5 mM EDTA, pH 8.2 - 8.4). GelRed (Biotium, Hayward, CA, USA) was added 1:10000 dilution) to the melted gel solution before casting the gels. The gels were then loaded with 2× Promega loading buffer along with the DNA sample and electrophoresed at 90 V for 45 min. DNA was visualized using the Syngene gel imaging system (Syngene, Cambridge, UK).

4.2.5 DNA quantification

Plasmid DNA and PCR products was quantified using the Nanodrop apparatus (NanoDrop ND-1000). DNA purity was verified using absorbance ratios of 260 nm / 280 nm and 260 nm / 230 nm.

4.2.6 The intergenic regions of *rdh* gene clusters: primer design and cloning

Specific primers were designed for each candidate intergenic region identified upstream of the *rdhAB or rdhBA* operons in *D. restrictus* for targeting the putative dehalobox sequences. Primer sequences and expected amplicon sizes are given in Table 5.

Primers were designed by using Primer 3 (http://frodo.wi.mit.edu/primer3) and parameters were verified using PrimerStats (Stothard, 2000), and obtained from Microsynth (Switzerland). Intergenic regions (IGR) containing putative dehalobox sequences were numbered according to the corresponding *rdhAB*/*rdhBA* operons. PCR protocol was followed as described previously (Rupakula et al., 2013). PCR products were purified with the QIAquick PCR purification kit (Qiagen, Switzerland) according to the manufacturer's instructions. The products were then A-tailed following instructions from the pGEM-T Easy vector manual (Promega), and finally ligated into pGEM-T Easy at insert:vector ratio of 3:1 overnight at 16ºC.

Table 5. Primers for the amplification of intergenic regions upstream of *rdh* gene in *D. restrictus*.

Dehalobox Target [1]	Primer	Primer sequence (5'-3')	Product size (bp)
cpr-DB07 [2]	ARP109F	TCTGGGCATTGTGTCCATTA	221
	ARP111R	CCCGCGGTTTAATAAAGAGA	
DB01	ARP050F	CATGTGCATACCTCCTTGTTAAATC	281
	ARP051R	CATAATTTCACCTCCCTACCTCA	
DB02	ARP052F	AGTTTTTATTGGGGAACTTACCTTC	162
	ARP053R	CATAACCAAACCCTCCTTTTTTTCT	
DB03	ARP054F	CATTTGTAAAATATTATCA	131
	ARP055R	CATCATTTTCACCTCCTCTTTTAG	
DB04	ARP056F	GCATATCCAACGCTTAATATGGA	326
	ARP057R	CATTTTCACCTCCTCTCTCAGC	
DB05	ARP058F	AAAAATCAAACTCCCTAATATTGATTAT	288
	ARP059R	CATTCCGACTGAGGTTTTTAAGAAA	
DB06	ARP060F	AAAAATCAAACTCCCTTTCGATAGATTT	326
	ARP061R	CATACCTTATCTCCTCCTTAAAATAT	
DB07	ARP062F	AAAAATCAAACTCCTATATCGTGGATT	256
	ARP063R	CATTTGTTTCCTCACCTCCTTAATTA	
DB08	ARP022F	TTATACTAGACTAAGTCTGGTTTTGTT	92
	APR023R	CATTGTTTTACACCTCCTATCTTC	
DB09	ARP064F	ATACTGTTTTCTCGAAAGAAGCTGCT	406
	ARP065R	CATTTTTTCACCTCCTTTAATTTCACAAA	
DB10	ARP066F	AATTAAGTTTGACAATTCATTTTGTACAA	254
	ARP067R	CATCATTTTCACCTCCCTTTTGATAATT	
DB11	ARP068F	ATACCGCCGAAAGGCGGTTT	312
	ARP069R	CATTCTCACCTCCTTAAATTTTG	
DB12	ARP070F	AACCTTATATTTATAACGAAACGATTGAT	245
	ARP071R	CACGTTAACACCCCCTTTCCATACTTT	
DB13	ARP072F	GAAAACACGCTCCCAAGTGCA	427
	ARP073R	AATTGGAATGGAGTGAACAGGATG	
DB14	ARP074F	CTTTGGATTGTTGATGGTAAAAAAAT	249
	ARP075R	CACGTCTCCCTTCTTCTCTACTTT	
DB15	ARP024F	TAGAAACTTCCATCTGTTATCTGAAA	515
	APR025R	CATATTTCTCCTCCTTTATTTTTTATTAGC	
DB16	ARP026F	AAGGTAAAATCAAAATCCCTGCTTC	328
	APR027R	TTATCTTTTCACCTCCCTTTTTATGC	
DB17	ARP076F	AATTTAAAAAAATAAGCACCTTGTAGTT	308
	ARP077R	CATAACCTAGCTCTCCTTTCCAAA	
DB18	ARP028F	TATAGTTATAAAAAACTGCTTGCTCAG	222
	APR029R	CATTATATTTCACCTCCTTTCTACTC	
DB19	ARP116F	CGGTGACAAGGTATATGATATT	280
	ARP117R	CATAGCTTTTTCACCTCCCTTATA	
DB20	ARP030F	CAATAAAAACCAAGCTTATGATTACC	295
	APR031R	CATGTAGTCTCTCCTCCCTTAA	
DB21	ARP080F	CTACGAAAAAATTTATCCACAAAGCTT	232
	ARP081R	CATGATTACGGACACCTAATC	
DB22	ARP082F	AATTATTTTTTCGTAATAGAACAATGCACT	699
	ARP083R	CATGAATGTATCTCCTCCTAAATGGTA	
DB23	ARP084F	TTGAAAAATAGAAAACAACAAAAATATAGTT	160
	ARP085R	CATATTTTTCACCTCCTTCCCAGTTAA	
DB24	ARP086F	CTGTTGGTGTGCTCATATATTGTAT	109
	ARP086R	CATTCGAATCTTCCTCCTTAATAAT	

[1] The complete intergenic region directly upstream of the *rdhAB* or *rdhBA* operons were targeted for cloning.
[2] *cpr*-DB07 was amplified from genomic DNA of *D. hafniense* DCB-2.

4.2.7 Plasmids for the expression of *rdhK* genes: primer design and cloning

Total DNA from *D. restrictus* was isolated as described previously (Maillard et al., 2003). Vector pWUR176 was obtained by cloning *cprK1* gene into pET24d (Gábor et al., 2006) in order to obtain tagless CprK1, and this vector was kindly provided by Proffessor Hauke Smidt anc co-workers, Wageningen University, Netherlands. *D. restrictus* DNA was used as a template for *rdhK*s. The expression plasmids were produced by PCR amplification of the *rdhK* genes using the corresponding primers and the PCR products were digested with respective restriction enzymes (Table 6), purified and ligated into pET24d vector that was digested in the same way. Ligated products were transformed into *E. coli* DH5α. Positive transformants were selected by colony PCR and verified by sequencing. Primers for sequencing the insert in pET24d plasmid were also chosen such that the forward and reverse primers bind approximately 100 bp upstream and downstream of the restriction sites, respectively (J. Maillard, unpublished data).

Table 6. Primers for the amplification and cloning of *rdhK* genes

Primer name	Target	Sequence (5'-3') [1]	Restriction site
pET24d-F	pET24d insert	AAGGGGTTATGCTAGTTATTGCTCAGCG	-
pET24d-R		CCGGTGATGCCGGCCACG	-
ARP036F	*rdhK08* Dehre_0827	GCGCCCATGGTTATGAAGGAAACTCTTAAAGAGCAACTTATCCC	*Nco*I
ARP037R		GCGCGAGCTCCCGTTTTTACTGTTCTCATTGATTATTTCCTTAAGCCG	*Sac*I
ARP038F	*rdhK09* Dehre_0828	GCGCCCATGGTAAAAAATGATATTAAGGAAGTTATTAGAATTTGCTTTGGAGAC	*Nco*I
ARP039R		GCGCGAGCTCCCAGTTTTCATCCACTCTTCAATCAAATCATGTAACC	*Sac*I
ARP050F	*rdhK14* Dehre_2019	GCGCCCATGGTTGACTATTCTATTTTCCCGTGGGAACCTTCA	*Nco*I
ARP051R		GCGCGAGCTCCCTAATTGGGATTCAACCCATGATTTGAAGTCTTTAG	*Sac*I
ARP112R [2]		CGCGCGGATCCTATAATTGGGATTCAACCCATGATTT	*EcoR*I
ARP040F	*rdhK15* Dehre_2025	GCGCCCATGGATGATAATAAGAAGCAAATTCCCCGACCTTATATG	*Nco*I
ARP041R		GCGCGAGCTCCCGTACTTTAAAACTTCATTAATCATAGAAGCTAATTTTGAAAG	*Sac*I
ARP113R [2]		CGCGCGGATCCTAGTACTTTAAAACTTCATTAATCATAGAAGC	*EcoR*I
ARP042F	*rdhK16* Dehre_2033	GCGCCCATGGGAGAAATTCTTAAAAGTTTTGTTCTGCCAGATAC	*Nco*I
ARP043R		GCGCGAGCTCCCGTAAGGTATGCCTTCCTCAAACAGTTC	*Sac*I
ARP114R [2]		CGCGCGGATCCTAGTAAGGTATGCCTTCCTCAAA	*EcoR*I
ARP044F	*rdhK18* Dehre_2041	GCGCCCATGGGAGAAGTTCTTAGAGGCTGTGTTCTACC	*Nco*I
ARP045R		GCGCGAGCTCCCGTACGTTATTCCCTCATCAATTAAACTTTTTAGCC	*Sac*I
ARP046F	*rdhK20* Dehre_2048	GCGCCCATGGAAAAAATAGCAAATAATTATGGGGCCTTGCCG	*Nco*I
ARP047R		GCGCGAGCTCCCTTCAATTAAGTTTTTAAGCCCTTGCAAGTCAAAGAC	*Sac*I
ARP048F	*rdhK24* Dehre_2041	GCGCCCATGGGGGAAGATATTTATACTTTGACTAAATTTTTAAATTATCCTTG	*Nco*I
ARP049R		GCGCGAGCTCCCTTCAGAATATAATTCAGGACGAATTAAGGTTTTG	*Sac*I
ARP115R [2]		CGCGCGGATCCTATTCAGAATATAATTCAGGACGAATTAA	*EcoR*I

[1] The respective restriction enzymes sites are underlined in the sequence of the primers. The corresponding ATG start codon is indicated in red.

[2] These primers were designed for cloning genes encoding tagless RdhK proteins.

Table 7. Plasmids used in this study

Plasmid	Description	Source or reference
pGEM-T Easy	Vector for direct (TA) cloning of PCR products, Amp^R	Promega
pGEM-T_DB#	Intergenic sequences (#1-24) were cloned into the pGEM-T vector	this study
pET24d	Expression vector (5.3 kb), pMB1 ori, Kan^R, IPTG-inducible T7 promoter	Novagen
pWUR176	*cprK1* gene cloned in pET24d (tagless)	(Gábor et al., 2006)
pRdhK08	*rdhK08* gene cloned in pET24d (His-tag at C-term.)	this study
pRdhK09	*rdhK09* gene cloned in pET24d (His-tag at C-term.)	this study
pRdhK14	*rdhK14* gene cloned in pET24d (His-tag at C-term.)	this study
pRdhK15	*rdhK15* gene cloned in pET24d (His-tag at C-term.)	this study
pRdhK16	*rdhK16* gene cloned in pET24d (His-tag at C-term.)	this study
pRdhK18	*rdhK18* gene cloned in pET24d (His-tag at C-term.)	this study
pRdhK20	*rdhK20* gene cloned in pET24d (His-tag at C-term.)	this study
pRdhK24	*rdhK24* gene cloned in pET24d (His-tag at C-term.)	this study
pRdhK16N	*rdhK16*gene cloned in pET24d (tagless)	this study

4.2.8 Competent cells preparation, transformation and sequencing

The resulting plasmids were transformed by heat-shock into $CaCl_2$-competent *E. coli* DH5α cells and both were performed using standard protocols (Sambrook, 1989). Transformants were screened using colony PCR with primers T7 and SP6 (pGEM-T derivatives) or pET24d-F and -R (pET24d derivatives), and positive clones were cultivated overnight at 37ºC followed by plasmid preparation with the QIAprep Spin Miniprep kit (Qiagen). Plasmid inserts were verified by sequencing using the BigDye Terminator v3.1 kit on the ABI Prism 3130 Genetic Analyzer according to the manufacturer's instructions (Applied Biosystems). DNA sequences were analysed using SeqMAN of the DNAStar package.

4.2.9 Heterologous production of proteins and collection of biomass

For the heterologous production of RdhK proteins, *E. coli* BL21 λDE3 containing the relevant plasmid expressing RdhK genes were cultivated in LB medium, supplemented with 30 μg/mL kanamycin (for pET24d-derived vectors), at 37 °C, with shaking at 180 rpm, until an absorbance (A_{600nm}) of 0.4-0.6 was reached. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 100 μM, and during induction of protein expression, cells were incubated at a temperature between 16-18 °C overnight with shaking at 180 rpm. Cells were harvested by centrifugation at 12'200 ×*g* (7000 rpm), for 20 min at 4 °C using an Avanti™ JLA 8.1 Centrifuge (Beckman Coulter). The wet weight of the biomass was measured. All biomass samples were stored at -80°C until further use. The biomass was thawed on ice. Biomass was resuspended in lysis buffer at a ratio of 10

mL per g biomass. Lysis buffer consisted of 50 mM Tris-HCl, pH 8.5, 1 mM DTT, a few DNAse crystals and one tablet of Complete™ proteinase inhibitor (Roche) per 50 mL. Cells were first homogenised then disrupted by three passages in a French® pressure cell press at 1000 psi. Cell debris was removed by centrifugation at 4°C and 16'000 ×*g* for 10 min. The soluble and insoluble fractions were separated by 1 h of centrifugation at 100'000 ×*g* (44'000 rpm), 4°C using TLA 120.2 rotor (Beckman Coulter). The pellet (insoluble fraction) was resuspended in 50 mM Tris-HCl pH 7.5 (10 mL/g) for later analysis by SDS-PAGE. Samples from soluble and insoluble fractions were stored at -20°C until further use for SDS-PAGE.

4.2.10 Purification of RdhK proteins by chromatography

4.2.10.1 His-tagged RdhK protein purification

After centrifugation, the RdhK proteins that were produced with a C-terminal His-tag were purified by one or two chromatographic steps by fast protein liquid chromatography (FPLC) using an AKTAPrime apparatus (GE Healthcare). The soluble crude extract was loaded onto a Ni-NTA affinity column (HisTrap™ HP; GE Healthcare) equilibrated with buffer NA (50 mM Tris/HCl buffer (pH 7.5), 10 mM imidazole, 150 mM NaCl, 1 mM DTT). Proteins were eluted with a 50-mL gradient of 0–1 M imidazole using buffer NB (buffer NA with addition 1 M imidazole). One mL fractions were collected. Eluted fractions were analysed by SDS-PAGE and fractions containing RdhK were pooled, 10-fold diluted in buffer QA (50 mM Tris/HCl buffer (pH 7.5), 1 mM EDTA, 1 mM DTT), and loaded onto an anion exchange column (HiTrap™ Q HP, GE Healthcare). RdhK proteins were eluted with a 50-mL gradient of 0–2 M NaCl using buffer QB (buffer QA with addition of 2 M NaCl). Eluted fractions were subjected to SDS-PAGE, and visualised with Coomassie R250 Brilliant Blue staining solution. Fractions containing RdhK were collected, pooled and concentrated using Microcon devices (10 kDa MW cut-off) to a final volume of 1-5 mL. Concentrated protein samples were dialysed overnight into dialysis buffer (50 mM Tris-HCl (pH 7.5), 20% glycerol, 100-500 mM NaCl, 1 mM DTT) in a 2-L tank. The RdhK protein samples were finally quantified using the NanoDrop apparatus. The purity of RdhK proteins was estimated by SDS-PAGE.

4.2.10.2 Tagless RdhK proteins purification by chromatography

The soluble fraction was applied to a HiPrep heparin FF column (GE healthcare) that was equilibrated with buffer HA (50 mM sodium phosphate buffer (pH 7.0), 10 mM DTT, 10 mM NaCl). Unbound proteins were washed with 8 column volumes of buffer HA, and RdhK proteins were subsequently eluted by a 50-mL gradient to 100% buffer HB (buffer HA with addition of 1 M NaCl). Eluted fractions were analysed by SDS-PAGE for the purity. Depending on their purity, the fractions containing the RdhK proteins were either pooled and directly dialysed overnight into dialysis buffer as above or applied through a second chromatography column for further purification such as the anion exchange column as described above.

4.2.11 Electrophoretic Mobility Shift Assays (EMSA)

Intergenic sequences of rdh gene clusters were amplified by PCR from the pGEM-T derived vectors using their respective primers (Table 5). These were further purified using Qiagen PCR Product purification kit following the manufacturer's instructions. Up to 200 µL of PCR product was subsequently loaded onto same filter and eluted with 30 µL water in order to concentrate the PCR products (100 nM DNA was needed for each EMSA experiment). 5× stock of TBE Buffer was prepared in 1 l of H2O with 54 g of Tris base, 27.5 g of boric acid, 20 mL of 0.5 M EDTA (pH 8.0) then corrected to pH 8.3. 5× stock was diluted to 0.5× working solution before use, which finally had 45 mM Tris-borate/1 mM EDTA. EMSA native gels were prepared according to Table 8, then casted for 2 h and pre-run for 30 min in 0.5× TBE electrophoresis buffer. The DNA/protein/organohalide reaction mixtures were loaded onto each lane and run at a constant voltage of 100 V.

Table 8. EMSA gel composition

Components (final concentration)	Stock concentration	Volume from stock (mL)
acrylamide -N,N'-methylene-bis-acrylamide (10%)	40%	5.00
TBE buffer (0.5×)	5×	2.00
glycerol (2.5%)	100%	0.50
APS (0.075%)	10%	0.15
TEMED (0.05%)	100%	0.01
EDTA, pH 8.3 (1 mM)	500 mM	0.04
water		12.30
	Total	20.00

Table 9. 2× EMSA reaction buffer

Components (final concentration)	Stock concentration	Volume from stock (mL)
glycerol (40%)	100%	8.0
Tris/HCl, pH 8.5 (100 mM)	1 M	2.0
$MgCl_2$ (10 mM)	1 M	0.2
EDTA (5 mM)	500 mM	0.2
NaCl (500 mM)	1 M	10.0
	Total	20.0

Table 10. EMSA reaction mixture

Components (final concentration)	Stock concentration	Volume from stock (μL)
EMSA reaction buffer (1×)	2×	7.5
DTT (10 mM)	100 Mm	1.5
DNA (100 ng)	1000 ng/μL	1.0
PCE (25 μM), or	125 μM in H_20	3.0
ClOHPA (25 μM)	125 μM in H_20	
RdhK protein (400 nM)	4 μM	1.5
Water		0.5
	Total	15.0

2× reaction buffer for EMSA was prepared according to Table 9, using 40% glycerol, 100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 500 mM NaCl, which was further used into a standard EMSA reaction buffer at final 1×, along with DNA (100 nM minimum), protein and DTT to a final reaction volume of 15 μL. Protein molarity was calculated and added according to the protein:DNA ratio that needs to be tested. The samples were incubated at 25ºC for 30 min to allow complex formation, and then loaded onto a native 10% PAGE gel with 16 μL ladder (ThermoScientific DNA ladder, 100 bp), and run at 100 V constant for 40-45 min. Then the gel was placed in a staining case to which 45 mL Tris-borate (TBE) electrophoresis buffer was added, along with 0.9 μl of ethidium bromide from a diluted stock to reach a final concentration of 0.2 μg/mL. After 30 min staining in the dark, the gel was placed in the UV-illuminator for visualisation.

4.2.12 Isothermal titration calorimetry (ITC) analysis

ITC experiments were performed at 25°C using the VP-ITC apparatus (MicroCal, Malvern) in the laboratory of Prof. Dirk Fasshauer (University of Lausanne). The reaction cell (1.4 mL) was filled with a 9.5 μM purified RdhK protein that was previously dialyzed against 50 mM Tris/HCl (pH 7.5), 300 mM NaCl, and degassed by vacuum aspiration for 5 min prior to loading. The titrated ligand (concentration 95 μM) was prepared in the same buffer to avoid any interference and also degassed. The stirring speed was 300 rpm, and the thermal power of 21 injections of 10 μL was recorded every 240 s. VP-ITC dedicated software was used for data analysis.

4.2.13 Intrinsic tryptophan fluorescence (ITF) quenching analysis

ITF quenching spectra were recorded with the Fluorolog-3 instrument (HORIBA) with constant temperature at 20°C maintained using a water bath. Samples were analysed in a precision quartz cuvette with a 10-mm light path (Hellma). Excitation was carried out at 280 nm with a 3-5 nm slit and emission fluorescence recorded between 300-500 nm with a 3-5 nm slit. Ligand was added to a 700 μL of 0.9 μM

RdhK protein in 50 mM Tris-HCl buffer (pH 7.5), 50 mM DTT, 300 mM NaCl. To determine the dissociation constant of the RdhK protein for the ligand, titrations of the ligand was carried out. Initial fluorescence was adjusted to a value between 50'000 to 200'000 count per second (cps). Fluoresence quenching was plotted relatively to the initial fluorescence (I_0, without ligand). Subsequent fluorescence readings was recorded after each ligand addition (denoted as I) and normalised with respect to the I0 by the formula I0/I. The increasing volume in the cuvette after each ligand addition was taken into account as the dilution factor to calculate the relative fluorescence (Rel. Fluor). The calculated fluorescence (Calc. fluo.) was further used to fit the data and estimate the dissociation constant (K_D) for the respective RdhK ligand pair. Formula to calculate the fluorescence is the following: Calc. fluo. = 1 - [dilution factor * (protein concentration) * (ligand concentration)] / [K_D + ligand conc)].

4.3 Results and Discussion

CRP-FNR type transcriptional regulators regulate various metabolic pathways in bacteria and typically function in response to environmental changes. The presence of 1-7 CprK regulatory protein-encoding genes have been found in *Desulfitobacterium* spp. genomes for a facultative OHRB which needs to limit the OHR metabolism in response to the presence of the organohalides that can be used as terminal electron acceptor. However, the presence of many *rdh* gene clusters (including genes encoding RdhK proteins) in genome *D. restrictus* came as a surprise as it is known to be an obligate OHRB currently known to grow only with PCE and TCE as electron acceptors. Initial comparative genomic analysis of the available *Dehalobacter* spp. genomes showed the presence in *rdh* gene clusters of *rdhK* genes also in strains other than *D. restrictus* (*Dehalobacter* spp. strains DCA, CF, E1, data not shown). While the function of CprK of a facultative OHRB has been well-studied, little is known about the function of the 25 RdhK homologs in *D. restrictus*. A combined study using bioinformatics (*in silico*), physiological (*in vivo*) and biochemical (*in vitro*) approaches were conducted to attempt characterizing the possible organohalide ligands and the DNA targets (so-called dehaloboxes, DB, present upstream of the reductive dehalogenase (*rdhA*) they may regulate).

From Chapter 2, it was observed that most of *rdh* gene clusters in *D. restrictus* are associated with one *rdhK* gene located in relatively close vicinity and in various orientations. The *rdhK* encoded proteins clearly belong to the large family of CRP-FNR regulatory proteins, the closest relative being CprK and CprK1 proteins of *D. dehalogenans* and *D. hafniense* DCB-2, respectively, were extensively studied and represent the paradigmatic DNA-binding regulatory protein for the chlorophenol reductive dehalogenase (*cpr*) operon (Kemp et al., 2013; Gábor et al., 2006, 2008; Gupta and Ragsdale, 2008; Joyce et al., 2006; Levy et al., 2008; Mazon et al., 2007; Pop et al., 2004, 2006; Smidt et al., 2000a). Screening of the genome of *D. restrictus* for RdhK protein-encoding genes revealed 25 paralogs from which 22 are located

within the 13 *rdh* gene clusters defined previously (Chapter 2), and the remaining three in their direct vicinity. This strongly suggests that RdhK regulatory proteins play a role in regulating the OHR metabolism in *D. restrictus* by acting on the transcription of the *rdh* genes.

In the present study, six RdhK homologs present in the genome of *D. restrictus* were chosen for purification and characterisation based on the finding that the *rdhA* genes located in their direct vicinity were found to be expressed in standard growth conditions. In addition and upon alignment with the well-characterised CprK protein, conserved amino acid residues gave some clues about the organohalide specificity these RdhK proteins could bind to.

4.3.1 *In silico* analysis of diversity in RdhK and identification of putative dehalobox motifs

4.3.1.1 Genetic arrangement of *rdhK* in *D. restrictus*

Genetic arrangement of *rdhK* within *D. restrictus* was studied to predict which *rdhK* could respectively act on their adjacent *rdhAB* gene loci. Figure 14 shows the genetic map of *rdh* gene clusters of *D. restrictus* with emphasis on the *rdhK* paralogs (depicted in brown) and their position relatively to *rdhAB* and *rdhBA* operons. Besides the well-characterized *pceABCT* cluster (*rdhABCT24*), two other *rdhA* genes are embedded in a similar configuration (*rdhA20* and -*22*), albeit harbouring the *rdhK* subunit at the 3'-end instead of the 5'-end. Most of *rdh* gene clusters are also associated with one *rdhK* subunit in various orientations and different genetic distance of the respective *rdhAB* or *rdhBA* operons. In some cases there are more than one *rdhK* genes present around some *rdhAB*/*BA* operons thereby it is not clear on which *rdhAB/BA* the *rdhK* may eventually act as a transcriptional regulator. For example, *rdhK08* and -*09* are neighbour genes and followed by *rdhBA08* and-*09* operons and such targets were considered for the study. The *rdhK24* gene also presented an interesting case, since it was located downstream of the well-studied *pceA* gene (*rdhA24*) in the *pceABCT* operon and it has been known from earlier studies that PceA is absolutely essential for OHR in the obligate OHRB *D. restrictus* and thereby might not need to be regulated. Moreover RdhK24 was detected in proteomic datasets, hence identifying its target *rdh* operon is an important issue for understanding the OHR metabolism in *D. restrictus*.

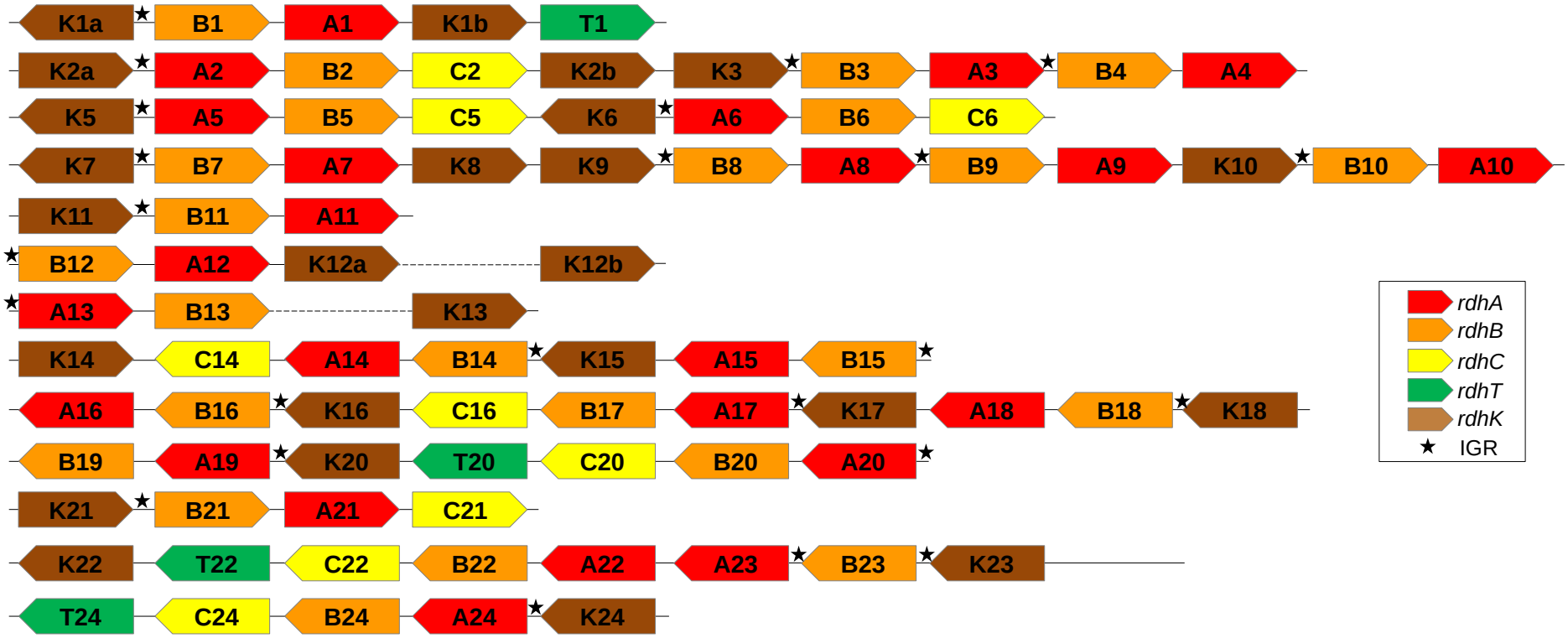

Figure 14. Genetic map of the *rdh* gene clusters in *D. restrictus* with emphasis on *rdhK* paralogs.

Modified from Figure 2 (Chapter 2). The *rdh* gene clusters are depicted with a color code: *rdhK* genes in brown, *rdhA* genes in red, *rdhB* genes in orange *rdhC* genes in yellow and *rdhT* genes in green. The *rdhK* genes accompany different types of *rdh* operons (*rdhAB/BA, ABC, BAC, ABCT*) and are present in various orientations with respect to *rdh* operon in their direct vicinity. The intergenic regions (IGR) selected for the present study are those located upstream of the *rdhAB* or *rdhBA* gene operons and are marked with a star.

4.3.1.2 Diversity of *rdhK*/RdhK in *D. restrictus*

To account for the RdhK diversity at amino acid level, sequence likelihood analysis was performed. Sequence similarity within all RdhK proteins of *D. restrictus* is illustrated in form of a tree in line with the well-characterised CprK proteins of *Desulfitobacterium* spp. (Figure 15). It can be seen that RdhK proteins of *D. restrictus* are very diverse and thereby suggesting that characterization of one or a few of their specific function (affinity for specific organohalide ligands and DNA targets) would not allow explaining the function for the rest of them, thereby they need to be tackled individually.

However, similarity between a few RdhK sequences was observed with shorter branch lengths in the tree analysis. For example, RdhK02b and RdhK03 of *D. restrictus* share 94% identity revealing a possible redundant function that is most probably due to a gene duplication event. RdhA03 and -04, which are located in the direct vicinity of RdhK03, also display a high identity level (81% identity on amino acid) which suggests that RdhK02b and -03 might act similarly on the transcription of *rdhA03* and -*04*. Sequence likelihood analysis also showed relatively short branch lengths between RdhK16 and -18 of *D. restrictus* (Figure 15) and RdhK1 of *D. hafniense* DCB-2 (as annotated by (Kim et al., 2012), see Table 4). Similarly, RdhK09, -11, -20 of *D. restrictus* also share sequence similarity with RdhK4 of *D. hafniense* DCB-2. Sequence similarity studies among the RdhK, along with sequence alignments for highlighting conserved amino acid residues identified in the well-characterised CprK could guide towards the prediction of their putative organohalide targets.

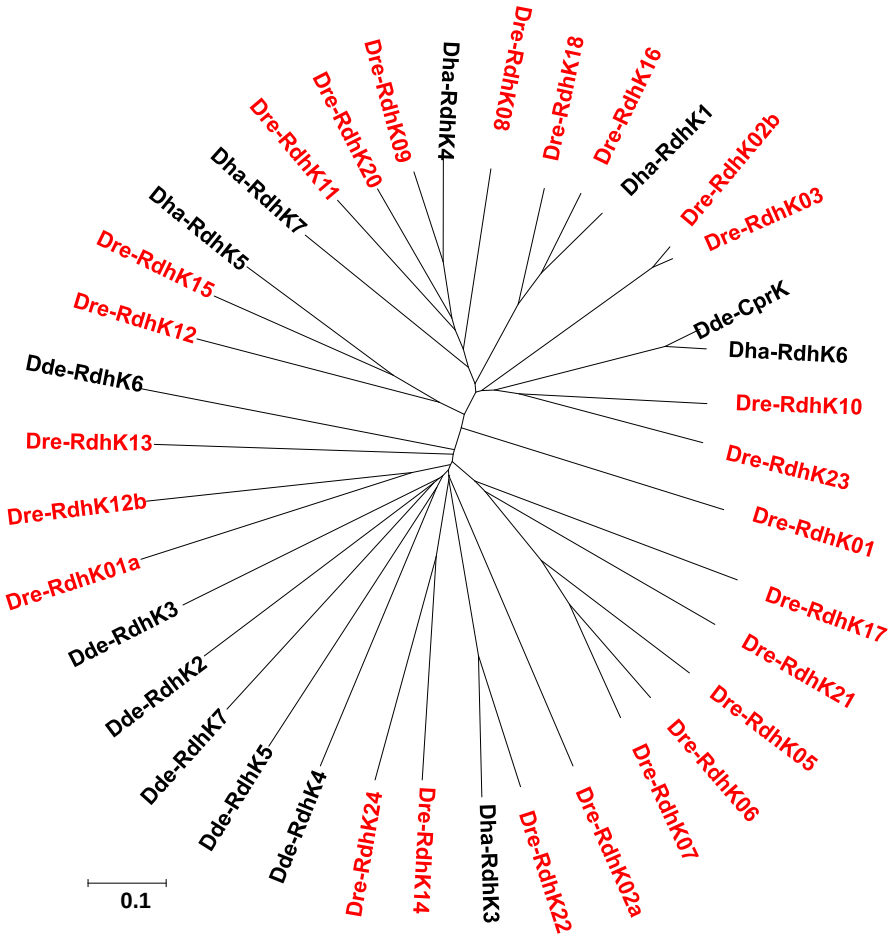

Figure 15. Sequence likelihood analysis of RdhK proteins in *D. restrictus* including other RdhK proteins present in *Desulfitobacterium* spp.

The tree shows sequence likelihood analysis of RdhK proteins from *D. restrictus* with respect to the well-characterized CprK but also to additional RdhK homologs found in *Desulfitobacterium* spp. The bacterial species are abbreviated as follows: Dde: *D. dehalogenans*; Dre: *Dehalobacter restrictus* (in red); Dha: *D. hafniense* strain DCB-2.

4.3.1.3 Conservation of key amino acid residues in *D. restrictus* RdhK proteins

Joyce and co-workers (Joyce et al., 2006) found that particular amino acid residues of CprK1 play important roles both in ligand binding and mechanistic aspects of DNA recognition. They found a few amino acids to be involved in binding the acetic acid moiety of ClOHPA, such as K/R_{86}, T_{90} and N_{92}. Sequence alignment of *D. dehalogenans* (*Dde*)-CprK with the RdhK proteins of *D. restrictus* is shown in Fig S14, and indicated in Table 11, highlighting where these residues were also conserved (RdhK02b, -03, -06, -08, -10 and -16), suggesting that they also possibly bind organohalides harboring an acetic acid moiety. Similarly, K_{133} was considered as important for binding to the phenolate moiety of ClOHPA. This residue is also conserved in most of the RdhK proteins of *D. restrictus* with exception of RdhK01a, -14, -22 and -24, suggesting that the latter proteins might bind to non-phenolate organohalides. More important residues and their conservation in RdhK proteins of *D. restrictus* are summarized in Table 11.

Table 11. Conservation of important amino acids in RdhK proteins

Amino acid residue [1]	**Functional importance in CprK**	***D. restrictus* RdhK proteins harbouring the conserved amino acids**
I_{14} I_{15}, P_{16}	contact with DNA-binding domain	- [5]
K_{86}/R_{86}	binding the acetic acid group	RdhK10
T_{90}	binding the acetic acid group	RdhK02b, -06, -10
N_{92}	binding the acetic acid group	RdhK02b, -03, -08, -10, -16
K_{133}	binding to phenolate moiety	All RdhK proteins except RdhK01a, -14, -22, -24
W_{106}	quenched by ClOHPA[2]	-
R_{155}	inter-domain salt bridge interaction [3]	RdhK08, to -11, -15, -18, -20, -23
H_{191}	binding to T_9 [4]	-
V_{192}	binding to a pyrimidine such as T_{20}	RdhK03, -4, -8 to -13, -15 to -18, -20, -23
T_{193}	binding to A_{18} and T_{19} [4]	All RdhK proteins except RdhK01a, -12b, -14, -15, -24
Y_{230}	binding to A_{18} and T_{19} [4]	-

[1] Amino acid residues of CprK1 of *D. hafniense* strain DCB-2.

[2] W_{106} was considered important and enabled performing ITF quenching experiments (Gupta and Ragsdale, 2008).

[3] Implicated in inter-domain salt bridge interaction of CprK - ClOHPA (Levy et al., 2008).

[4] Numbering corresponds to the dehalobox motif $T_9T_{10}A_{11}A_{12}T_{13}$-$N_4$-$A_{18}T_{19}T_{20}A_{21}A_{22}$ (Levy et al., 2008).

[5] All three are not conserved in RdhKs, but at least one of each is present in most RdhKs.

4.3.1.4 *In silico* dehalobox sequence search in *D. restrictus*

Intergenic regions (IGR) upstream of *rdhAB/BA* operons were selected for identification of short palindromes (inverted repeats of 14-bp) were conducted, targeting 5-bp palindromic sequences that are separated by 4-7 spacing nucleotides. The resulting palindromes were considered as candidate dehalobox sequences if the spacing between the palindrome and the predicted Pribnow box was around 20 nucleotides. Further, palindromic motifs were aligned to the consensual dehalobox sequence TTAAT-N_4-ATTAA of the *cpr* gene cluster of *Desulfitobacterium* spp. (Figure 16).

Strain	Downstream genes	Dehalobox	Pribnow	DB#	Ref.
		TTAAT ATTAA			
		***** *****			
Dde	*cprBA*	GTTAATACGCACTAAT_N21-TACAAT			(Smidt et al., 2000)
Dha	*cprT*	CTTATTGCACATTAAC-N21-TATAAT		DB1	(Gábor et al., 2006, 2008)
	cprZE	GTTAGTGCACTCTAAA-N21-TAGAAT		DB2	
	cprBAC	GTTAATACACATTAAT-N21-TACAAT		DB3/DB7	
Dre	*rdhBA01*	GTTAAATCATTTTAA-N23-TATAGT		DB01	Current study
	rdhAB02	GTTTTTTATGCAAAAG-N20-TATAAT		DB02	
	rdhBA03	AATATTATCATTTATA-N18-TAGACT		DB03	
	rdhBA04	TTCAATTTAGATTGAA-N22-TAGATT		DB04	
	rdhBA07	ATTATTTAAAAATAAA-N22-AATAAT		DB07	
	rdhBA10	TTGAATTCCATTTCAT-N23-TATTAT		DB10	
	rdhBA11	TTTTTCTTCATAAAAA-N22-TGTAAT		DB11	
	rdhBA18	GGATTAGTTGAAATCA-N18-TAAAAT		DB18	
	rdhBA19	GAAGACGTTTGTCTTG-N22-TAAAAT		DB19	
	rdhBA21	ACAAAATCCCTGTTGT-N23-TATTTT		DB21	
	rdhAB22	GTTTGGCATTATAAAT-N24-TATAAT		DB22	
	rdhBA23	TTTAAACCGATTTAAA-N22-TATAAT		DB23	

Figure 16. Prediction of putative dehalobox sequences upstream of *rdhAB* or *rdhBA* operons in *D. restrictus* and comparison with well-characterised dehaloboxes from *Desulfitobacterium* spp.

The well-characterized dehalobox (DB) of *D. dehalogenans* (Dde), and three DB sequences of *D. hafniense* DCB-2 (Dha) were aligned with the predicted dehalobox motifs identified in the promoter region of some rdh gene clusters of *D. restrictus* (Dre).

Figure 17. Consensus sequence of identified dehaloboxes from *D. restrictus*.

The consensus was created using the predicted dehaloboxes sequences of *D. restrictus* using the online software (http://weblogo.threeplusone.com/create.cgi)

From Figure 16 and Figure 17 it is clear that the dehalobox motifs of *D. restrictus* are very diverse and not as conserved as those in *Desulfitobacterium* spp., however in each of the DB, a few of the important residues of consensus sequence TTAAT-N_4-ATTAA are conserved, suggesting that the mechanism of DNA recognition for *D. restrictus* RdhK proteins might be similar to the one of CprK. Since, dehalobox prediction was only possible for a few *rdh* gene clusters of *D. restrictus,* the entire intergenic regions (IGR) upstream of the 24 *rdhAB/BA* operons were consequently cloned into pET24d vectors (Table 7) and used as template to amplify the DNA targets for interaction studies with RdhK proteins.

4.3.2 Attempt for *in vivo* analysis of *rdhA* substrate specificity

The diversity of RdhK regulatory proteins in *D. restrictus* raises questions about their possible function in the transcription of various *rdhA* when the cells are exposed to organohalides other than PCE and TCE. This was attempted by spiking the *D. restrictus* cultures with various mixes of organohalides to detect differential transcription patterns of *rdhA* genes if any. This would provide clues to the ability of *D. restrictus rdhA* genes to respond to a variety of organohalides despite its presently known incapacity to grow with other electron acceptors than PCE and TCE. Thereby, *in vivo* studies were performed by addition of various organohalides to *D. restrictus* cultures growing on PCE. Initially, a mixture of chloroethanes (CA) was chosen to be spiked into a culture of *D. restrictus*. Differential transcription of the *rdhA* genes was observed with respect to control samples (unspiked cultures) as well as at different time points after the spike. In Figure 18, it was observed that the following *rdhA* genes were transcribed: *rdhA01*, *-02*, *-07*, *-08 -09*, *-14*, *-15*, *-17*, *-20*, *-22*, *-23* and *-24*. However, they were equally transcribed in the non-spiked controls. Thereby, from transcriptional analysis of *rdhA* genes upon CA spike experiments, it was not conclusive of which particular *rdhA* was being transcribed in response to CA as there was not significant up-regulation of any particular *rdhA* gene over time. Moreover, the observed *rdhA* genes were also detected to be transcribed in previous experiments performed in standard conditions (see Chapter 2). As this approach was inconclusive, another approach was developed, wherein the focus was on characterising the RdhK regulatory proteins and identifying their binding partners (organohalides and dehaloboxes). Previous studies on CprK of *Desulfitobacterium* spp. have demonstrated that this type of proteins is relatively easy to produce, purify and characterise. The present study aimed to identify the ligands of a few selected RdhK proteins as a prerequisite to investigate the potential substrate range of RdhA enzymes in *D. restrictus*.

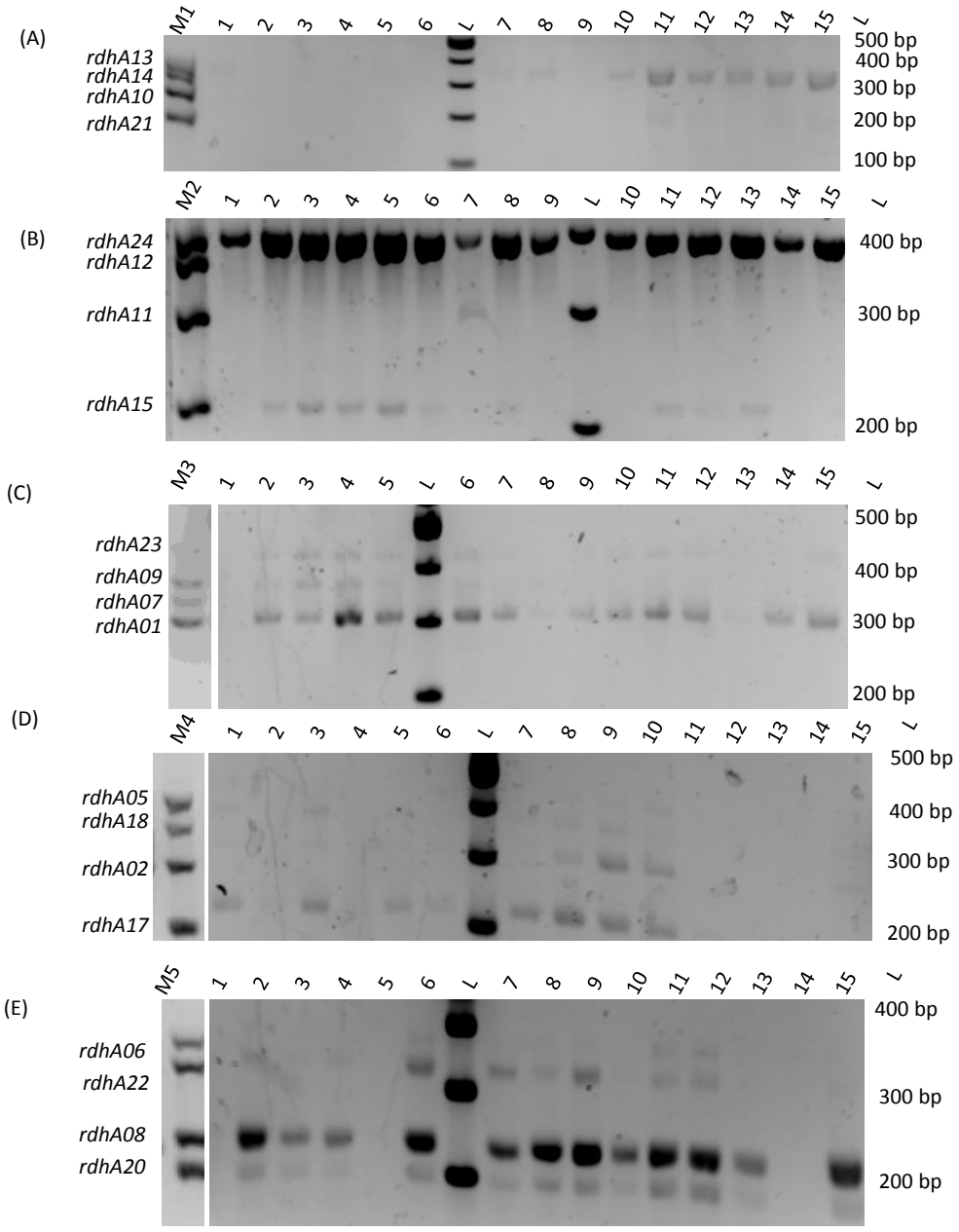

Figure 18. Analysis of differential transcription of *rdhA* genes in cells of *D. restrictus* spiked with a mixture of chloroethanes.

A mixture of chloroethanes (CA) containing 1,2-dichloroethane (DCA), 1,1,2-trichloroethane (TCA) and 1,1,2,2-tetrachloroethane (TeCA) was spiked into a culture of *D. restrictus* growing on PCE. Multiplex PCR (mPCR) results are shown for the targeted genes which are indicated on the left of each gel. Lanes M1- M5 represent the positive control performed on genomic DNA using the respective multiplex primers sets; L: 100 bp ladder from which the corresponding fragment sizes are indicated on the right. Samples 1-3, 4-6, 7-9 and 10-12 were taken from triplicate cultures, respectively. The cultures 1-3 were harvested at time 0 (T0, before CA spike), 4-6 after 30 min, 7-9 after 2 h, 10-12 after 24 h of the CA spike, respectively. Samples 13 and 14 were non-spiked control cultures and sample 15 was taken from a control culture spiked with hexadecane. Samples 13-15 were collected at the end of the experiment, (after 24 h), to synchronise with the last sample collection time point.

4.3.3 RdhK protein production and purification

4.3.3.1 Selection criteria for *in vitro* analysis of particular RdhK proteins

In Chapter 2, the transcriptional analysis of *rdhA* genes under standard PCE dechlorination conditions (Rupakula et al., 2013), revealed that the following *rdhA* genes were transcribed: *rdhA08, rdhA14, rdhA15, rdhA16, rdhA19, rdh20* and *rdhA24*. Thereby, for the present study, it was hypothesized that their adjacent *rdhK* genes, i.e. *rdhK08, rdhK09, rdhK14, rdhK15, rdhK16, rdhK18, rdhK20,* and *rdhK24* could be involved in transcriptional regulation of their cognate *rdhA* genes. So these *rdhK* genes were selected for expression and biochemical studies. The detection of a few RdhK proteins in the proteome of *D. restrictus* came as a surprise for a bacterium that was known to respire only on PCE. The following RdhK proteins were detected in proteomic studies conducted earlier from cells cultivated in PCE-dechlorinating conditions: RdhK12b, RdhK15, RdhK18, RdhK20, RdhK21 and RdhK24 (proteomic datasets; Chapters 2, 3). It also suggested that these proteins are involved in the regulation of PceA, and might therefore interact with PCE. The detection of RdhK24 in proteomic analysis could be correlated with *in silico* sequence analysis where RdhK24 lacks the K_{133} residue thereby could be predicted to bind to non-phenolic organohalides such as PCE (Table 11). Moreover, in PCE-dechlorinating conditions only two RdhA proteins were detected: RdhA14 and Rdh24 (Rupakula et al., 2013). This could again implicate back to the point that their respective putative regulator proteins within their vicinity; RdhK14 and RdhK24 could interact with PCE as well, using it as an effector. *In silico* amino acid analysis and alignments guided towards which organohalides to initially test the respective RdhKs with for further biophysical studies.

4.3.3.2 RdhK protein production and purification

The plasmids for the expression of the selected *rdhK* genes of *D. restrictus* were generated (Table 7). The plasmids were then transformed into competent cells of *E. coli* BL21 and the expression of the genes induced with IPTG. The induction was eventually optimised to an IPTG concentration of 0.1 mM overnight at 16-18ºC. Non-induced, induced, soluble and pellet fractions were collected to be analysed on SDS-PAGE (summarised in Figure 19). RdhK08, -15, -16, -18, -20, -24 were expressed mostly in soluble form rather than as inclusion bodies when induced overnight at 16ºC. RdhK09 however, was not soluble and RdhK14 was poorly induced, thereby they were not considered further.

Similar to RdhK08 purification displayed in Figure 20, all His-tagged RdhKs were purified first by Ni-NTA affinity chromatography (Figure 20, A-B), followed by anion exchange chromatography on a Q-HP column (Figure 20, C-D). All fractions of RdhK proteins purified from the Q column were collected and tested for their purity. Only RdhK16 was purified differently. During anion exchange chromatography, the His-tagged RdhK16 did not bind and was collected in the flow-through, while most of the contaminant proteins was bound. The flow-through containing RdhK16 was finally concentrated in a centrifugation device.

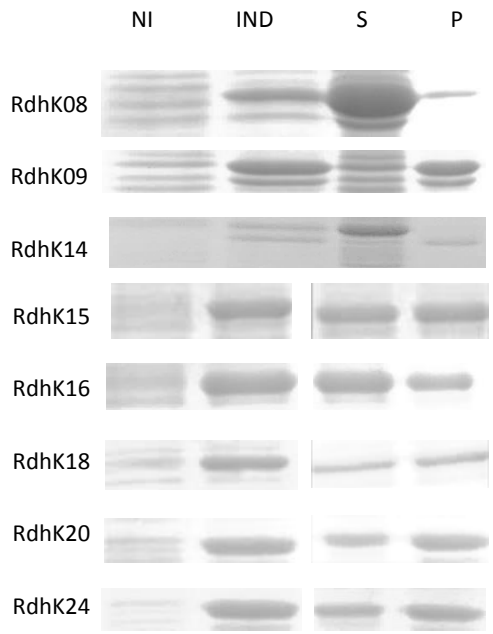

Figure 19. Production and solubility of RdhK proteins.

SDS-PAGE analysis of RdhK proteins induced at 0.1 mM final IPTG. RdhK proteins are indicated on the left. Legend: NI: non-induced sample; IND: induced sample; S: soluble fraction; P: pellet (insoluble fraction).

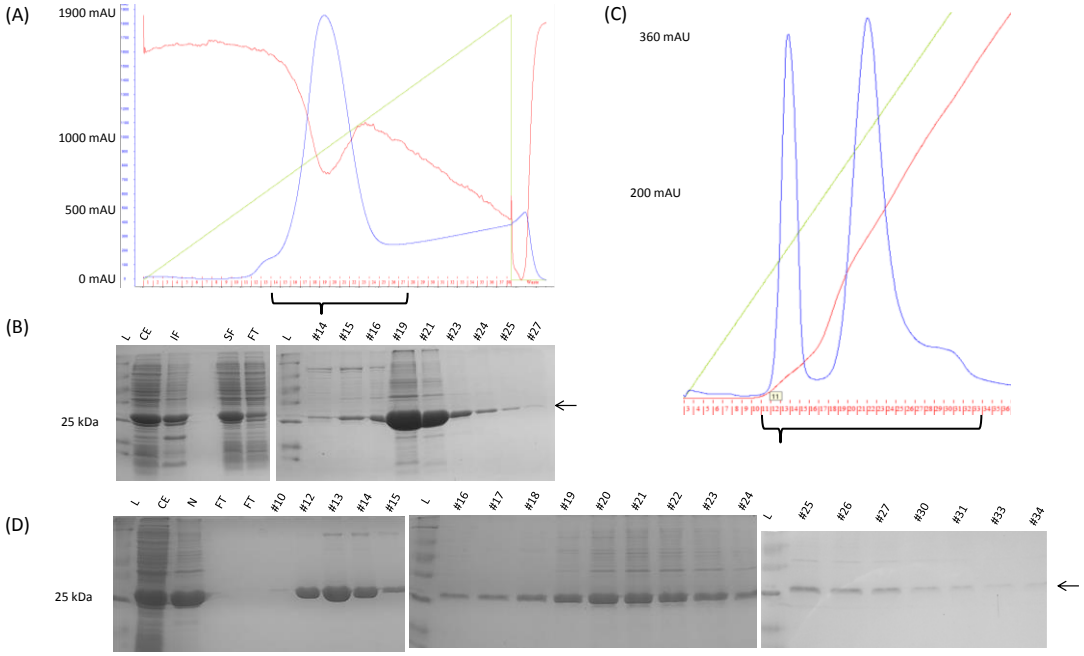

Figure 20. Purification of His-tagged RdhK08 protein.

(A). Ni-NTA affinity chromatography indicating the absorbance (A_{280nm}) in blue, the imidazole gradient in green and the eluted fractions in red. (B). SDS-PAGE analysis of the eluted fractions obtained from the purification by Ni-NTA affinity. The arrow indicates the purified RdhK08. (C). Anion exchange chromatography indicating the absorbance (A_{280nm}) in blue, the salt gradient in green and the eluted fractions in red. (D). SDS-PAGE analysis of the anion exchange chromatography. The arrow indicates the purified RdhK08. Additional legend: L: protein ladder; CE: crude extract; IF: insoluble fraction; SF: soluble fraction; FT: flow-through; #: elution fractions.

4.3.3.3 Purification of tagless RdhK proteins by heparin affinity chromatography

It was predicted that the His-tag could possibly interfere with the interaction of purified RdhK proteins when targeting more sensitive analyses like isothermal titration calorimetry (ITC), intrinsic tryptophan fluorescence quenching or mass spectrometry, thereby tagless protein purification of these targets was attempted by virtue of their innate DNA-binding property on heparin affinity chromatography.

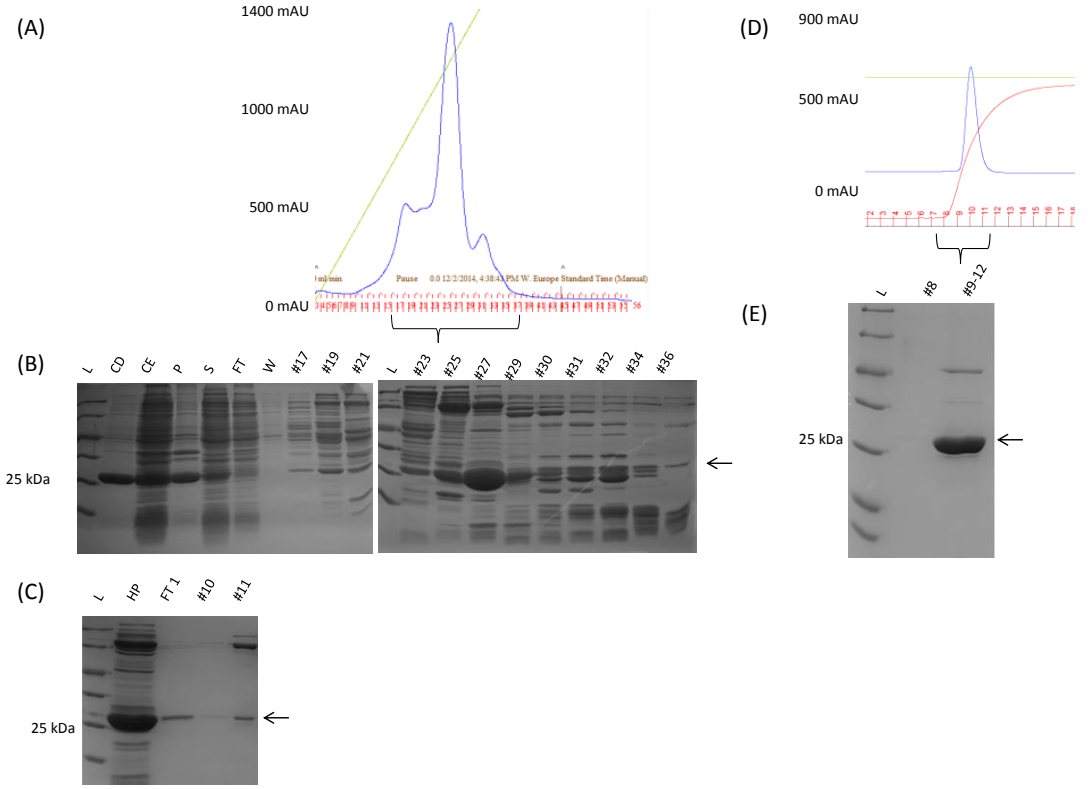

Figure 21. Tagless CprK1 purification by heparin affinity chromatography.

(A) Elution chromatogram from heparin affinity column, indicating the absorbance (A_{280nm}) in blue, the salt gradient in green and the eluted fractions in red on the x-axis. (B) SDS-PAGE analysis of the purification procedure. The arrow indicates the purified CprK1. (C. SDS-PAGE analysis of the CprK1 2nd step purification procedure (anion exchange chromatography). The arrow indicates the purified CprK1. (D) CprK1 was further purified and concentrated using a 3rd chromatography step (heparin affinity again) for further purification and concentration. Chromatogram is presented, indicating the absorbance (A_{280nm}) in blue, the salt gradient in green and the eluted fractions in red on the x-axis. (E) SDS-PAGE analysis of the heparin column. The arrow indicates the purified and concentrated CprK1. Legend: L: protein ladder; CD: cell debris; CE: crude extract ; P: pellet (insoluble fraction); S: soluble fraction; FT: flow-through; W : Wash ; #: elution fractions; HP: heparin collected CprK1 fractions; FT1: flow-through (10× diluted CprK1);

Soluble protein fractions containing tagless CprK1 was first loaded onto the heparin column, from which the eluted fractions were examined on SDS-PAGE and the most pure fractions containing CprK1 were pooled (Figure 21, A-B). This sample was further diluted 10× with QA buffer and then loaded onto a Q column. Again, the protein did not bind to it and was released in a pure form in the flow-through (FT)

(Figure 21, C). This sample was loaded again on the heparin column for further purification and concentration (Figure 21, D-E).The same heparin-based strategy was employed for purification of a tagless version of RdhK16. Purified RdhK16 was finally dialysed against 50 mM Tris-HCl (pH 7.5) containing 500 mM NaCl and 10 mM DTT. High salt concentration was required to maintain RdhK16 in a soluble form.

4.3.3.4 Summary of purified RdhK proteins

A few *rdhK* genes were cloned, heterologously expressed in *E. coli* and the corresponding proteins purified by chromatography. However despite several efforts, RdhK09 could not be produced in a soluble form and RdhK14 was poorly induced. Thereby these proteins were not targeted for further analysis. Also we observed that upon purification RdhK15 was precipitating and could not be recovered from the column. Similarly, Gabor and co-workers (Gábor et al., 2008) had troubles producing a few CprK homologs such as CprK3 and CprK5 due to solubility issues. However, purification of the remaining RdhK08, -16, -18, -20, -24 proteins in a pure and soluble form were successfully obtained for further biochemical characterisation studies. SDS-PAGE analysis of the purified RdhK proteins is shown in Figure 22 where it can be clearly seen that all RdhK proteins were relatively pure and had little or no contaminants. The concentration of each RdhK protein was estimated by spectrophotometry and their specific extinction coefficient, as given in Table 12.

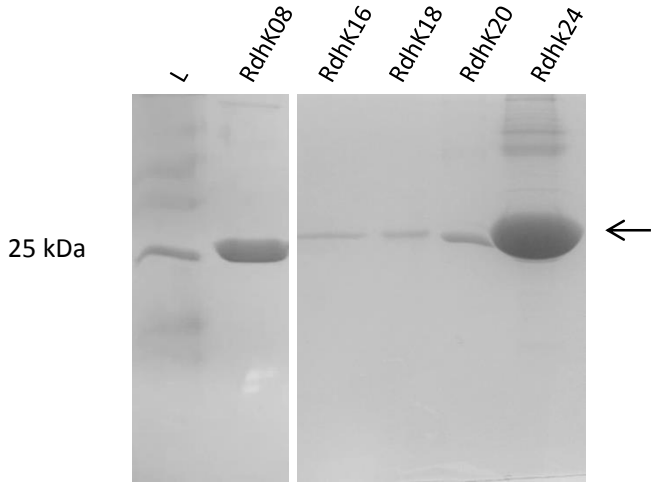

Figure 22. Purified RdhK proteins

SDS-PAGE analysis to check the purity of RdhK proteins after chromatography purification and dialysis. The arrow points to the purified proteins. Legend: L; Ladder.

Table 12. Concentration of purified *D. restrictus* RdhK proteins

Protein	Extinction coefficient at A_{280nm} (M^{-1} cm^{-1})	Concentration (mg/mL)	Concentration (μM)
CprK1	23610	0.70	33.21
RdhK08	14650	1.27	47.12
RdhK16	22330	9.15	343.93
RdhK18	24890	8.20	308.56
RdhK20	17920	7.08	273.44
RdhK24	30580	16.54	609.71

4.3.4 Electrophoretic mobility shift assays (EMSA) for RdhK

Tagless CprK1 from *D. hafniense* DCB-2 in combination with the dehalobox DB07 (Table 7) and ClOHPA as ligand were used to optimise the setup of EMSA in the laboratory and as positive control in all EMSA experiments. Figure 23 shows the results for EMSA for CprK1. Free DNA of DB07 is indicated with an arrow at a size of 221 bp and the DNA-protein complex (in the presence of ClOHPA) is visible above the 500-bp mark, at best with protein:DNA molar ratios of 8:1 to 15:1. These were thereon used as positive control for EMSA.

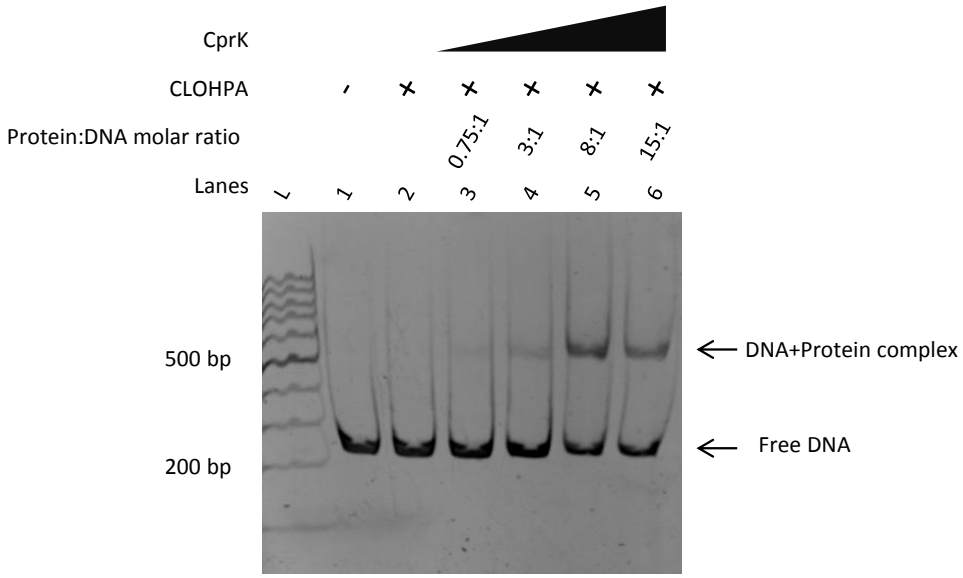

Figure 23. EMSA displaying interaction of purified CprK1 with DB07 of *D. hafniense* DCB-2.

EMSA was performed in the presence or absence of 100 µM ClOHPA using 100 nM DB07 free DNA (221 bp) and increasing amounts of CprK1 protein. Legend: L; 100bp DNA Ladder; Lane 1: DNA only control; Lane 2: Protein+DNA only control; Lanes 3-6: Protein+DNA+ClOHPA experiments varying the molar ratio of protein:DNA in each lane as indicated. Concentrations of CprK1 are as follows from lanes 1-6: 0, 300, 75, 300, 800, 1500 nM, respectively.

Table 13. Reaction mixtures for EMSA with CprK1 and *D. restrictus* RdhKs

Protein	Dehalobox DNA	Protein:DNA ratio	Organohalide	Final organohalide concentration
CprK1 [1]	DB07 [2]	0.75:1 to 50:1	ClOHPA	25 µM
RdhK08	DB07 to -10	0.75:1 to 50:1	PCE, ClOHPA	12.5 - 100 µM
RdhK16 [1]	Dre-DB14, -20, -24	3:1 to 200:1	PCE, ClOHPA, 2-chlorophenol, chloroform, chlorobenzene, OH mixture [3]	12.5 -100 µM
RdhK18	Dre-DB16, -18, -20	15:1 to 100:1	PCE, ClOHPA	12.5 - 100 µM
RdhK20	Dre-DB16, -18, -20	15:1 to 100:1	PCE, ClOHPA	12.5 - 100 µM
RdhK24	Dre-DB24	5:1 to 100:1	PCE, ClOHPA	12.5 - 100 µM

[1] Tagless purified RdhK proteins, while the remaining are his-tagged.

[2] DB07 of *D. hafniense* DCB-2.

[3] Organohalides (OH) mixture composed of 1,2-dichloroethane, 1,1,2-trichloroethane, 1,1,2,2-tetrachloroethane, 1,2-dichloropropane, chloroform, 3,5-dichlorophenol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol.

Similarly various dehaloboxes in *D. restrictus* selected for the study were cloned, purified, and sequenced. Then their purified PCR products along with purified RdhK and CprK1 proteins were used in EMSA. Following this, different combinations of dehaloboxes and organohalides were assayed for interaction using EMSA at various ligand concentrations and protein:DNA ratios. A summary of the various combinations tried is given in Table 13, where RdhK08, -16, -18, -20, -24 of *D. restrictus* were tested with putative dehaloboxes in their vicinity along with organohalides such as PCE or ClOHPA. No visible complex formation was observed among the RdhK-DB pairs under the conditions of the organohalide used, its concentration, and the Protein:DNA molar ratios tested (data not shown). When using higher protein:DNA ratios (50:1 to 200:1), it was observed that a lot of DNA did not enter the gels (data not shown). This could be explained the possible aggregation of proteins in the EMSA reaction. In conclusion, so far EMSA was not a successful approach for the identification of the binding partners of RdhK proteins

One such an example gel is shown in

Figure 24, where RdhK16 was tested with ClOHPA and various DB's as DB14 to -20, by maintaining a constant Protein:DNA molar ratio of 3:1 throughout the experiment.

After many EMSA trials, it was perceived that testing individual organohalides among a plethora of combinations with RdhK proteins in a tri-partite system where neither the DB was known nor the organohalide it binds to, posed a challenge in amplifying the permutations and combinations that need to be tested in order to reach a right triplet interaction of RdhK-DB-Organohalide. Henceforth, we tested the use of EMSA as a

screening approach using a mixture of organohalides within the same experiment to check a group of organohalides at a time. Here, RdhK16 in combination with an array of dehaloboxes was tested with such mixtures as shown in Table 13.

No clear complex formation was observed.

In order to minimise the combinations to test by two-fold, titration methods were eventually attempted, wherein a singular organohalide could be titrated along a range of concentrations with a given RdhK protein. As well, di-partite interaction could be studied in such methods where DNA was not required for visualisation as in EMSA. Thereby focussed RdhK-organohalide interaction studies were further performed using isothermal titration calorimetry and intrinsic tryptophan fluorescence quenching methods.

Figure 24. EMSA investigating interaction of purified RdhK16 with some dehaloboxes of *D. restrictus.* (Next page)

(A). EMSA was performed in the presence or absence of 100 µM of the organohalide using 100 nM of the DB, with various combinations of DB. The RdhK:DB molar ratios tested as well as the unbound-DB product sizes as indicated in each numbered lane. (B). EMSA result is shown. DNA only controls were placed next to DNA+protein+organhalide samples for each dehalobox tested. Legend: L: 100 bp DNA ladder; Lane 1: CprK+DB07+ClOHPA positive control; Lanes 4,5; DB14;, Lanes 6,7: DB15; Lanes 8,9: DB16; Lanes 10,11: DB17; Lanes 12,13: DB18; Lanes 14,15: DB19; Lanes 16,17: DB20. Protein:DNA molar ratio of 3:1 was respected throughout the latter samples as shown in panel

(A)

Lanes :	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Protein :	CprK	CprK	CprK	-	RdhK16	-	RdhK16	-	RdhK16	-	RdhK16	-	RdhK16	-	RdhK16	-	RdhK16
Dehalobox :	db07	db07	db16	db14	db14	db15	db15	db16	db16	db17	db17	db18	db18	db19	db19	db20	db20
Protein:DNA molar ratio :	3:1	3:1	3:1		3:1		3:1		3:1		3:1		3:1		3:1		3:1
Organohalide :	CLOHPA	PCE	CLOHPA	-	CLOHPA	-	CLOHPA	-	CLOHPA	-	CLOHPA	-	CLOHPA	-	CLOHPA	-	CLOHPA
Free DNA size :	212bp	212bp	330bp	250bp	250bp	515bp	515bp	330bp	330bp	308bp	308bp	222bp	222bp	280bp	280bp	295bp	295bp

(B)

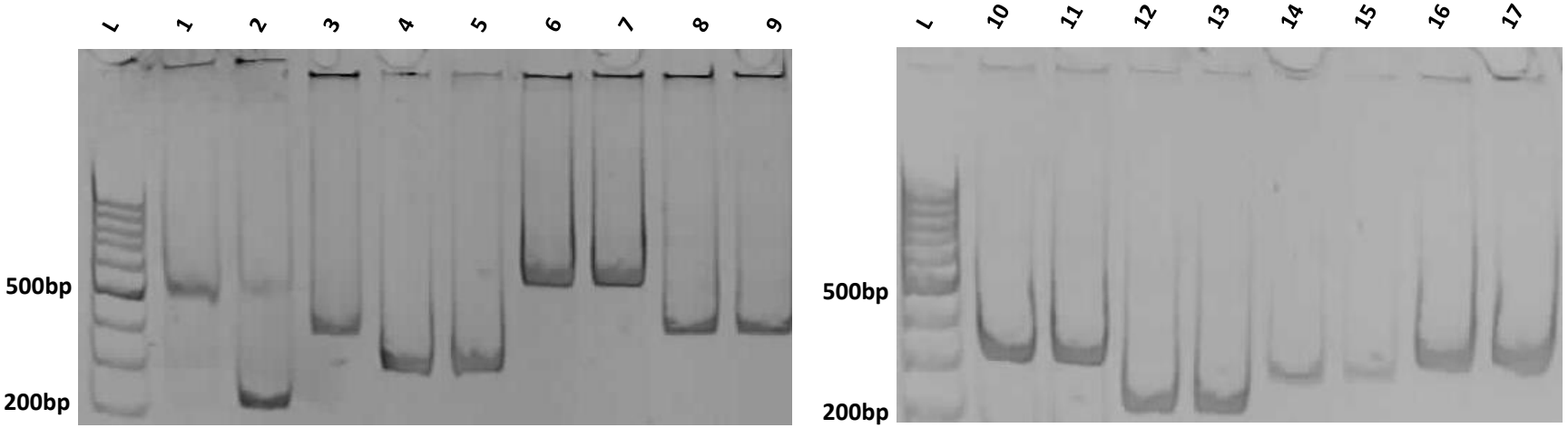

Figure 24

4.3.5 Isothermal titration calorimetry (ITC) for RdhK

Isothermal titration calorimetry (ITC) was established using the positive control CprK1 whose properties were verified to match those in previous studies (Gupta and Ragsdale, 2008; Levy et al., 2008; Pop et al., 2006). ITC was carried out at 25°C using a VP-ITC apparatus (MicroCal). Proteins were purified to a high degree and dialysed in a buffer in which the ligands were also dissolved. The heat of dilution from injecting the buffer into the protein solution was found to be negligible (data not shown).

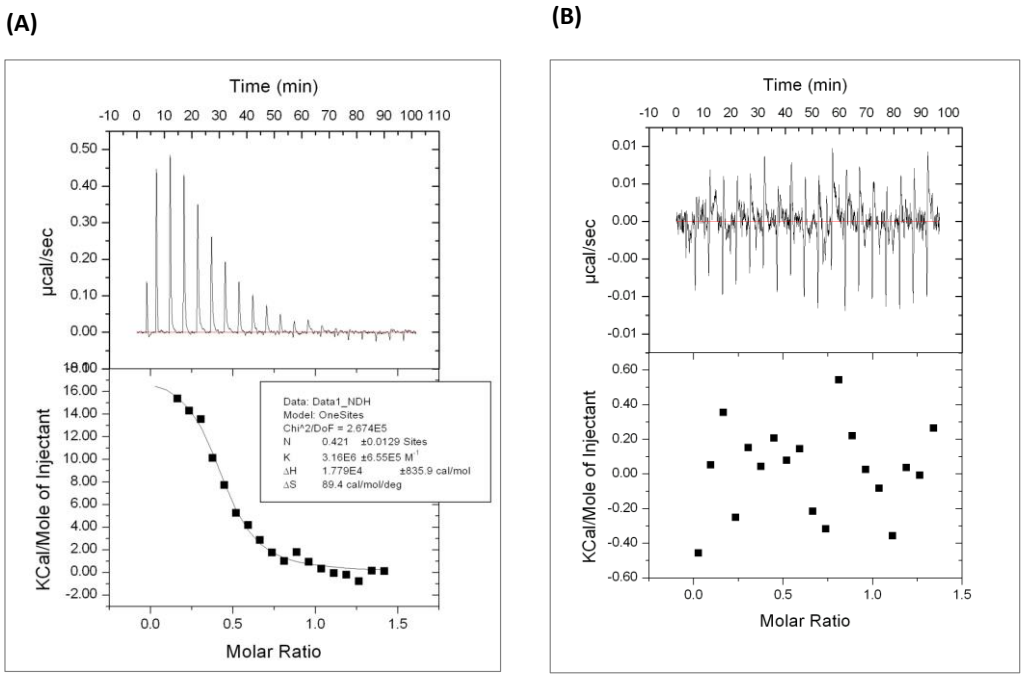

Figure 25. ITC to study the interaction of CprK1 and RdhK16 *vs*. ClOHPA

(A) Titration of CprK1 with ClOHPA.The experiment was best fitted with the one site model.
(B) Titration of RdhK16 with ClOHPA. No binding isotherms were observed

Upon injection of 100 µM ClOHPA into the chamber containing 10 µM CprK1, an exothermic reaction was monitored (Figure 25, A) confirming the results as obtained by (Levy et al., 2008). ITC gave an N = 0.403 ± 0.008 sites, K = 4.46E6 ± 7.43E5 M^{-1}, ΔH = 2.199E4 ±2622.7 cal/mole, ΔS = 104 cal/mole/deg, with a calculated K_D of 0.22 µM.

Similar values for CprK1 dissociation constants using ITC were obtained by Gupta and co-workers, i.e. in ranges of 0.29-0.49 and 0.34-0.68 µM for the oxidized and reduced proteins, respectively (Gupta and Ragsdale, 2008) Similarly, Pop and coworkers determined the affinity of CprK1 to ClOHPA under two conditions: in the presence of target DNA (K_D, 0.4 µM) and by ITC measurement in the absence of DNA (K_D, 3.4 µM) (Pop et al., 2004).

The tagless RdhK16 protein was eventually tested in a similar way with various organohalides individually such as ClOHPA (Figure 25 B), chloroform, 2-chlorophenol, PCE and chlorobenzene (data not shown). No interaction could be measured for any of the ligands tested. In order to test the use of ITC as a screening

method, CprK1 was tested with mixtures of organohalides. Two mixtures were made distinctly based on their chemical groups to which the selected organohalides belong, i.e. aromatic or aliphatic compounds. Both mixtures were tested with or without ClOHPA within the mixture to assess the possibility of using ITC as a screening platform and trace back the original binding isotherms of CprK1 towards ClOHPA. It was observed that the organohalides mixtures interferred with the interaction of CprK1 with ClOHPA (Fig S13). Thereby, since inconclusive binding isotherms were observed for both the mixtures of organohalides, it was decided that ITC could not be further used as a screening method to identify the ligand specificity of uncharacterised RdhK proteins.

4.3.6 Intrinsic tryptophan fluorescence (ITF) quenching of RdhK proteins

Intrinsic tryptophan fluorescence (ITF) quenching was applied next as it is more sensitive than ITC, therefore requiring less protein. However, this technique provides a rather undefined description of the protein-ligand interaction, as it is based solely on conformational changes around tryptophan residues of the target protein upon binding to the ligand. A single tryptophan (W_{106}) is situated at the bottom of the β-barrel in CprK, which is not in close contact with the ClOHPA-binding site (Joyce et al., 2006). It is therefore most likely that binding of ClOHPA triggers a conformational change that results in the fluorescence quenching, providing a way to measure ligand-binding activity, as published previously (Pop et al., 2006). Table 14 provides the list of selected RdhK proteins of *D. restrictus* including the number of tryptophan and tyrosine residues, making them likely to be analysed by ITF quenching.

CprK1 was again used as a positive control for setting up ITF quenching experiments. The apparent affinity constant of CprK1 for ClOHPA (K_D) obtained was found similar to those obtained for CprK previousy, thereby allowing to proceed ahead with RdhK proteins of *D. restrictus*. The results are presented in Figure 26. ITF quenching experiments demonstrated up to 60% of quenching upon titration of the reduced form of CprK1 with ClOHPA, revealing an apparent K_D of 10.9 μM as shown in Table 14. This was similar to results obtained by Joyce and co-workers using ITF quenching experiments, where CprK1 demonstrated up to 55% quenching upon titration of oxidized *D. hafniense* CprK1 with CHPA, leading to an apparent K_D of 4.1 ± 0.4 μM (Joyce et al., 2006).

ITF experiments with ClOHPA for RdhK16, -18, -20 from *D. restrictus* did result in ITF quenching, however in the higher micromolar range of ligand concentration as it can be seen in Table 14 and Figure 26. Bovine serum albumin protein (BSA) was used as a protein for which ClOHPA is not a specific ligand, revealing a K_D value in similar range as for RdhK proteins of *D. restrictus*, thereby reflecting the non-specificity of these proteins towards ClOHPA. This is also a possible reason behind why their binding to DNA in the presence of the ClOHPA could not be detected using EMSA. Therefore, ITF quenching of RdhK proteins could only be attributed to unfolding of the proteins here.

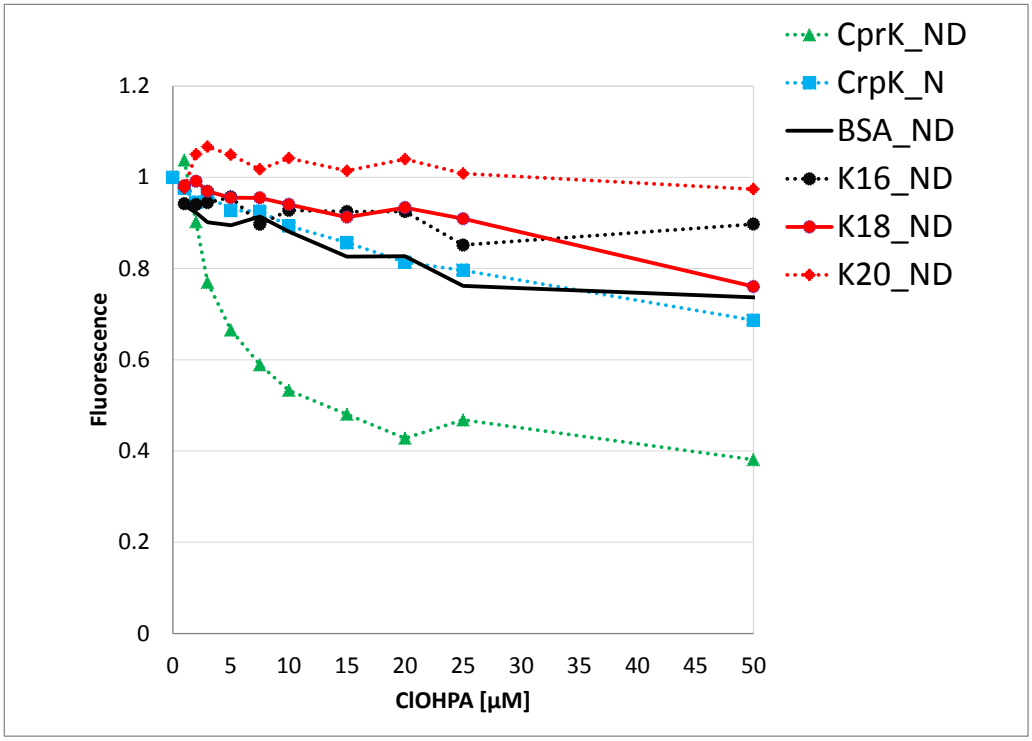

Figure 26. Intrinsic tryptophan fluorescence (ITF) quenching analysis.

CprK1 of *D. hafniense* strain DCB-2, and RdhK16, -18, -20 proteins of *D. restrictus* were tested with ClOHPA as ligand by intrinsic tryptophan fluorescence quenching experiments. The y-axis is the normalised fluorescence. The x-axis displays the concentration of ClOHPA. The proteins tested are shown in the legend on the right of the figure. The buffers used are indicated next to the proteins and are the following: Tris-HCl 50 mM (pH 7.5) with addition of 300 mM NaCl (N); of 10 mM DTT (D); of 300 mM NaCl and 10 mM DTT (ND). Bovine serum albumin (BSA) was used as negative control.

Table 14. Apparent dissociation constants (K_D) for RdhK-ClOHPA estimated by intrinsic tryptophan fluorescence quenching

Protein	CprK1	CprK1	CprK1	BSA	RdhK16	RdhK18	RdhK20
Buffer	ND	D	N	ND	ND	ND	ND
K_D /µM [1]	10.9	6.7	140.9	131.1	416.1	162.6	625.0
Protein /µM	0.9	0.9	0.9	1	15.23	17	7
#Trp	1	1	1	2	1	1	0
#Tyr	14	14	14	21	13	15	14

[1] Apparent dissociation constants (K_D) were calculated by fitting the fluorescence quenching data of the proteins for the ligand ClOHPA.

4.4 Perspectives

Although, ITC helped narrow down the permutations of experiments that need to be done, since di-partite-interaction between only the Protein (RdhK) and the organohalide (ClOHPA) could be tested with a titration allowing a gradient of concentrations to be tested in the same experiment. However, very soon the high amounts of protein needed for ITC became limiting, especially not knowing the organohalide it could bind with. In order to identify the ligand specifity of RdhK proteins in the near future, a more sensitive and accurate method is required. A technique such as Quadrupole Time Of Flight Electro-Spray Ionisation Mass Spectrometer (Q-TOF ESI MS) represent a promising alternative. The technique uses the difference in masses between ligand-bound and ligand-free RdhK proteins to assess ligand binding. This approach could be used to screen many ligands with very little amount of purified proteins and would represent a useful prerequisite for the biochemical approaches like ITC or EMSA used here without success.

Moreover, this chapter brings on challenges pushing towards a combination of more narrow and broad spectrum analysis, such as use of *in vivo* β-galactosidase assays, *in vitro* promoter probe experiments along with chromatin immune precipitation (CHIP-Seq) with antibodies to the respective RdhK protein targets in order to understand interaction *in vivo*. This would allow tackling the challenging regulation network of the OHR metabolism that is likely to be at play in *D. restrictus*.

Chapter 5
Concluding Remarks and Perspectives

5 Concluding Remarks and Perspectives

The present PhD thesis focuses on *Dehalobacter restrictus* strain PER-K23 (Holliger et al., 1993, 1998b), which is an obligate OHR bacterium (OHRB) whose tetrachloroethene reductive dehalogenase enzyme (PceA) (Maillard et al., 2003) consists of two 4Fe-4S clusters and a corrinoid cofactor (Schumacher et al., 1997). *D. restrictus* has been isolated from an enrichment culture dechlorinating tetrachloroethene (PCE) originally obtained from sediment of the Rhine River mixed with anaerobic granular sludge. *D. restrictus* is respiring exclusively with H_2 as electron donor, PCE or TCE as sole electron acceptors and can only use acetate as carbon source. In *D. restrictus*, the PceA enzyme is encoded by a gene that is part of the *pceABCT* gene cluster which has been shown to be highly conserved in several other OHR strains belonging to the genus *Desulfitobacterium* (Maillard et al., 2005; Duret et al., 2012). The genome sequence of *D. restrictus* was recently obtained (Kruse et al., 2013) and revealed a high number of 25 *rdhA* genes, though only PCE and TCE have been recognized as physiological substrates. This observation clearly raised the question of the true bioremediation potential of *D. restrictus* when comparing it with the wider dechlorination potential the entire *Dehalobacter* genus seems to harbour. Such an observation also raised questions behind the functional redundancy, diversity and expression of the 25 *rdhA* genes forming the basis of **Chapter 2**. The 2.9 Mb genome size of *D. restrictus* can be considered to occupy an intermediate position among OHRB between the reduced genome size of the OHR obligate *Dehalococcoides* genus (approx. 1.4 Mb) and the largely redundant genomes of the versatile *Desulfitobacterium* genus (greater than 5 Mb). Metabolically, however, *D. restrictus* is closer to *Dehalococcoides* spp., suggesting that, besides additional genetic information responsible for peptidoglycan synthesis and motility, some parts of the *D. restrictus* genome may be not functional or encode for yet unsuspected metabolic pathways. A remarkable example is the presence of a complete biosynthetic pathway for corrinoid, genes belonging to the WL pathway for CO_2 fixation and the presence of eight different hydrogenases. The discovery of 25 reductive dehalogenase (*rdhA*) genes in the genome of *D. restrictus* (Kruse et al., 2013) was surprising given its restricted substrate range, but is in line with what has been observed in all available genomes of *Dehalococcoides mccartyi* containing 17-36 *rdhA* gene copies per genome. The detailed analysis of the *rdh* gene clusters of *D. restrictus* is presented in **Chapter 2**, together with the transcriptional and proteomic data on the components of these clusters, which contributed towards understanding their diversity, evolution and function in *D. restrictus* during growth on PCE. Omics approaches were used to evaluate the expression of the 24 full-length *rdhA* genes in *D. restrictus* along the growth phases; first a RT-multiplex PCR method was developed allowing screening groups of *rdhA* genes at mRNA level in cultures collected at the exponential, late exponential and stationary phases. Five *rdhA* gene transcripts (*rdhA*08, -14, -16, -19, and -24) were strongly detected, however, showing various transcription levels. The *pceA* gene (*rdhA*24) was clearly dominant and was still detected in the RNA samples collected in stationary phase. A decrease in transcription

level was generally observed for all genes along the growth phases. RT quantitative PCR data confirmed the trend observed by the qualitative multiplex PCR approach. The level of transcription of the remaining *rdhA* genes followed an order: *rdhA*19 > *rdhA*14 >> *rdhA*16 > *rdhA*08. From proteomic analysis, two of the RdhA proteins were detected: RdhA14 and RdhA24 (PceA). All four proteins encoded by the *pceABCT* gene cluster were identified in the proteome with PceA being the most abundant protein at all growth stages. PceT (a dedicated chaperone for PceA) was most abundant at exponential phase and then became slightly less abundant at later stages. Also, a few RdhK regulatory proteins, belonging to the CRP-FNR family of transcriptional regulators were detected in the proteomic analysis. Perspectives for the work presented in **Chapter 2** would include a thorough analysis of the function and expression conditions of the remaining *rdhA* genes by cultivating *D. restrictus* in the presence of PCE together with another organohalide and by monitoring the dynamic transcription of individual *rdhA* genes over time. This could be easily monitored by collecting the RNA intermittently and applying the relatively quick semi-quantitative multiplex PCR on cDNA. This would allow for screening the differential *rdhA* gene transcription when compared with standard conditions.

Two different strategies for obtaining corrinoids, are used by OHR bacteria, either *de novo* biosynthesis or salvaging it from the environment. One important observation developed from the genome of *D. restrictus* is the presence of the entire corrinoid biosynthesis pathway which came as a surprise (chapter 2), knowing that this bacterium cannot produce its corrinoid *de novo* and depends on external supplementation. This raised the question of the corrinoid metabolism of *D. restrictus*, which was the main question addressed in **Chapter 3**. The aim was to investigate the corrinoid metabolism of *D. restrictus* at the level of biosynthesis and regulation, and to compare it to contrasting situations in other *Dehalobacter* strains. Genome analysis identified the entire corrinoid biosynthesis pathway genes to be present with the exception of one non-functional gene (*cbiH*) involved in the contraction of the corrin ring, which could explain its corrinoid auxotrophy. However, comparative genome analyses showed that other *Dehalobacter* spp. have an intact *cbiH* gene. Proteomic analysis of *D. restrictus* revealed a strong up-regulation of two operons (named operon-1 and -2) containing genes involved in cobalt/corrinoid transport and salvaging as a response to corrinoid limitation. They contain multiple genes encoding transporters and CbiZ proteins. These two operons are found only in *D. restrictus* and generally not present in the genome of other *Dehalobacter* spp. Targeted transcriptional analysis further highlighted the importance of these two operons in *D. restrictus*. Taken together, these data suggest that *D. restrictus* has lost its ability of *de novo* corrinoid synthesis and instead evolved a strategy for augmented corrinoid uptake and modification to fulfill its corrinoid requirement. The types of corrinoids that can be used by *D. restrictus* were also tested in order to understand the functionality of the lower ligand and salvaging pathways identified in the genome. Feeding *D. restrictus* with cobinamide (corrinoid with no lower ligand) over repeated sub-cultivations still demonstrated dechlorination and growth. Thereby this suggested the possibility of *D.*

restrictus to synthesize its own lower ligand. This is contrasting to what was observed earlier in *Dehalococcoides* spp. pure cultures or co-cultures, where the addition of dimethylbenzimidazole (DMB) was important irrespective of the presence of a few genes from the lower ligand and salvaging pathways. Initial extraction and HPLC analysis of the corrinoids from *D. restrictus* cultivated with cobinamide showed peaks differing from cultures supplemented with cyanocobalamin, indicating differences in the types of corrinoid eventually synthesized by the bacterium. Also it would be interesting to explore the transcriptional regulation occurring in the presence of the cobinamide, if for example operon-3 which encodes a majority of the lower ligand pathway genes would be upregulated in such growth conditions. In a scenario where differential regulation of the corrinoid-operons is observed when supplemented with either cobalamin or cobinamide, it could suggest towards the different specificities between the cobalamin riboswitches controlling the regulation of these corrinoid-operons in *D. restrictus*. Following which, similar to the study by Choudhary *et al.*, 2013, it would be interesting to explore the substrate specificity of the five cobalamin-riboswitches located upstream each of the five corrinoid operons in *D. restrictus* by using In-line probing experiments. There are many unanswered questions in this context which could not be tackled within the timeline of the present PhD thesis, which however would be interesting to pursue in the next future. One specific topic would be to explore the apparently redundant salvaging pathway enzymes, CbiZ in *D. restrictus*. Indeed, besides the classical *cbiZ* homolog present in the genome of all *Dehalobacter* spp., there are two additional *cbiZ*-like genes in *D. restrictus*. It would be interesting to explore the exact function of all the corresponding CbiZ proteins in *D. restrictus* and identify the reason behind their redundancy.

From **Chapter 2**, it was also clear that most of *rdh* gene clusters in *D. restrictus* are associated with one *rdhK* subunit located in various orientation and vicinity. The *rdhK* encoded proteins clearly belong to the large family of CRP/FNR regulatory proteins from which CprK proteins of *Desulfitobacterium dehalogenans* and *Desulfitobacterium hafniense* DCB-2 were extensively studied and represent the paradigmatic DNA-binding regulatory protein for the respective chlorophenol reductive dehalogenase (*cpr*) operons. Screening of the genome of *D. restrictus* for RdhK protein encoding genes revealed 25 paralogs from which 22 are located within the 13 *rdh* gene clusters, and the remaining three in their direct vicinity. This strongly suggests that RdhK are regulatory proteins dedicated to OHR metabolism (Rupakula et al., 2013) forming the basis of **Chapter 4**. By spiking the *D. restrictus* culture with various organohalides, it was noticed that multiple *rdhA* genes are transcribed at the same time, thereby confounding our understanding of the specificity of single RdhA enzyme for an organohalide. Therefore, an alternative strategy was applied. The regulation of the *rdhA* genes was studied by targeting the RdhK regulatory proteins and identifying the ligand they could bind to. A selection of RdhK proteins was made using *in silico* analysis as well as according to the transcription of *rdhA* genes in their vicinity as observed from transcriptional analysis in standard conditions (PCE). The selected *rdhK* genes and their respective putative DNA targets so-called dehaloboxes

(DB) present in the intergenic region upstream of the *rdhAB/BA* operons were purified. Different combinations of DNA targets and organohalides for each RdhK were analysed by electrophoretic mobility shift assays (EMSA). However, after many EMSA trials, it was perceived that testing individual organohalides among a plethora of combinations with RdhK proteins and DNA targets in a tri-partite system posed a challenge in the number of permutations that need to be tested in order to reach the right triplet. Alternatively, the characterisation of merely the RdhK-organohalides duets was performed mainly using titration methods wherein the RdhK protein could be tested along with a concentration gradient of the organohalides using techniques as isothermal titration calorimetry (ITC) and intrinsic tryptophan fluorescence (ITF) quenching. From the few selected RdhK proteins, it was observed that these had higher dissociation constants (K_D) than CprK1 for ClOHPA (used as a positive control), and in the range like the one obtained for the negative control with bovine serum albumin, implying that the quenching observed was insignificant, and thereby the interaction between the RdhK-ClOHPA pairs could not be concluded. However this chapter brings on perspectives opening an array of other techniques that could be attempted to understand the ligand specificity of RdhK proteins and further their biochemical characterisation for understanding the regulation of OHR metabolism in *D. restrictus*. One such a technique would be the very sensitive Q-TOF mass spectrometry, where the differences in masses between ligand-bound and ligand-free RdhK proteins would indicate interactions between specific organohalides and RdhK proteins. Also, in order to screen for the binding regions of a particular RdhK, pull-down assays with specific antibodies targeting the RdhK proteins could be used in chromatin immuno-precipitation sequencing (CHIP-Seq) experiments (Mardis, 2007; Robertson et al., 2007; Blasco et al., 2012; Turkarslan et al., 2015). Here, heterologously produced RdhK proteins could be analysed with the entire *D. restrictus* genome in the presence of a single organohalide per experiment, where DNA targets could be identified upon sequencing the protein-bound DNA along with the antibody-precipitated RdhK proteins. It is indeed a challenging and interesting situation to understand the regulatory network in *D. restrictus*, where 25 *rdhA* genes are present with 25 *rdhK* genes in their vicinity and none of their substrates are known except for PceA. These studies would provide new insights into the regulatory mechanism of the OHR process in *D. restrictus*.

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Supplementary Figures and Tables

7 Supplementary Figures and Tables

7.1 Supplementary Figures and Tables to Chapter 2

Table S1. Oligonucleotides used in this study

Primer	Primer sequence (5'→3')	Target (Dehre_#)	Amplicon size (bp)
rpoB-f	GGAAAATCCGTTCTTTATGACG	rpoB (0495)	276
rpoB-r	TACCACATCATCGGACTTAACG		
A01-f	ACATGATTGCCTGTGTTTCG	*rdhA01* (0785)	306
A01-r	GGTATTACAATCGGCGCAAG		
A02-f	TCCAATACGGGGTTGTATGC	*rdhA02* (0793)	310
A02-r	GGAATGAGTTGTGCTCCGAC		
A03-f	TCATGACCCAAACTCTGAGATCC	*rdhA03* (0806)	248
A03-r	TTTCGCCAAATCAAACGC		
A04-f	CTTGGAGCTAACGGGGTTG	*rdhA04* (0808)	452
A04-r	ACAGATATAAAAGCTGTAGCGGC		
A05-f	TCCGACATCTCTTGAACTTGC	*rdhA05* (0815)	428
A05-r	GCTGAGAAATGTTTGGCACA		
A06-f	GCAAGGGCTCTTTTCTTGG	*rdhA06* (0820)	374
A06-r	GGTTACAAATGCCTGCCTTG		
A07-f	GAGAAAGACGCTGGATTTGC	*rdhA07* (0826)	337
A07-r	CCACTTCAGACGCCAATGAC		
A08-f	AACATTGTAAACGGCGTTCC	*rdhA08* (0830)	235
A08-r	CCTGTTACGGAGTCAATGCC		
A09-f	CCCGGATGAGATTTGTATCG	*rdhA09* (0832)	366
A09-r	CCGTAGCAAATATTGCGGTC		
A10-f	TTTTTCAGGCATCCTTTTGG	*rdhA10* (0835)	293
A10-r	GCTTGCCTTACGTCATCGTC		
A11-f	TTACGGCACGAAGTGTATGC	*rdhA11* (0990)	297
A11-r	GCAATTATGGTCTTGCCGTT		
A12-f	GGTAAACTAACCCCCGAAGC	*rdhA12* (1408)	363
A12-r	CGTTTATGGTTGACCAGCATT		
A13-f	ATTGCTGTACCCCATGAACC	*rdhA13* (2012)	389
A13-r	GCTCTATTCCAAGCGGACAA		
A14-f	ATATGGGTTACCGTGCTTGG	*rdhA14* (2022)	355
A14-r	GCAAAGCGTATTGGGATGAT		
A15-f	GAGCAGCCTCAGCTATTTCC	*rdhA15* (2026)	215
A15-r	CCATGAGGCAAATCAAGGAT		
A16-f	GGACAGTGGTCCAAGCTGAG	*rdhA16* (2031)	315
A16-r	GATACCTGATCCCGAGCAAA		
A17-f	GGAGAATTGAGCAGGATTGG	*rdhA17* (2037)	222
A17-r	GCTACGATTTCGACAAGCG		
A18-f	GGCTCTGATAAACGGAATGG	*rdhA18* (2039)	372
A18-r	GCTATTTACACTCGGGCCAA		
A19-f	AAGGACAATGGTCTAAATTGAA	*rdhA19* (2044)	315
A19-r	GATACCTGATCCCGAGCAAA		
A20-f	GGAGGTAGCATTGGATGTGG	*rdhA20* (2052)	199
A20-r	GGAGAGACGTGGTCAAGGTC		
A21-f	TAAGGATTATGGCCGTTTCG	*rdhA21* (2058)	217
A21-r	CCAATCACCTTTAATGCGGT		

A22-f	GAAAGAGGCACGTGTTCTGC	*rdhA22* (2064)	333
A22-r	GGAATAACCTGGAGCCGGTA		
A23-f	TTTGTTGGCACAGAAGATGC	*rdhA23* (2065)	418
A23-r	GCTCACCACGCAAGAAACTA		
A24-f	CCTCACCGTCAGTCATTTCC	*rdhA24* (2398)	392
A24-r	GGATTGTGAGGTAGCCGAAA		

Table S2. Parameters of the quantitative PCR runs

Target	*rpoB*	*rdhA08*	*rdhA14*	*rdhA16*	*rdhA19*	*rdhA24*
Std [1]	7.74	1.57	8.08	2.12	3.63	4.69
Efficiency	0.954	0.921	0.972	1.017	0.987	0.741
R^2	0.999	0.997	0.997	0.982	0.990	0.999

[1]Absolute concentration range of the plasmid standards (x 102 to 107 copy number/µl) used for the calibration of qPCR, according to the protocol described in Material and Methods.

Table S3. Number of proteins detected with 3-fold in/decreased abundance ratio across the growth phases (see also Table S1)

	Growth phase comparisons [1]		
	S/E	LE/E	S/LE
> 3-fold up	124	81	88
> 3-fold down	177	84	105
Total	302	165	193

[1] Growth phases: E, exponential phase; LE, late-exponential phase; S, stationary phase

Table S4. Correction of initially annotated pseudogenes in the genome of *Dehalobacter restrictus*

D. restrictus locus	Predicted function
Dehre_0219	Type III pantothenate kinase
Dehre_0312	Translation initiation factor 3
Dehre_0341	Tyrosyl-tRNA synthetase
Dehre_0460	Protein of unknown function
Dehre_0568	Hypothetical protein
Dehre_0631	DNA primase
Dehre_0734	Fic family protein
Dehre_0844	*2*-hydroxyhepta-*2,4*-diene-*1,7*-dioate isomerase
Dehre_1678	Cyclic β-*1,2*-glucan modification transmembrane protein
Dehre_1722	Preprotein translocase subunit (YajC)
Dehre_1888	*3*-deoxy-*D*-arabinoheptulosonate-*7*-phosphate synthase
Dehre_2482	Protein involved in intracellular sulphur reduction (DrsE)
Dehre_2674	ATPase-like histidine kinase
Dehre_2780	Peptide chain release factor 2
Dehre_2831	Asparaginyl-tRNA synthetase
Not assigned [1]	LSU ribosomal protein L34p

[1] This protein was detected although no gene was initially annotated as it is located at junction of the genome sequence.

Table S5. Properties of *rdhA*/RdhA present in the genome of *D. restrictus*

rdhA/ RdhA	Dehre_#	strand	*rdh* operon [1]	RdhA best match (identity) [2]
01	0785	+	**BA**KT	*Desulfitobacterium hafniense* DCB-2 RdhA4 (48%)
02	0793	+	(K)AB(C)	*Desulfosporosinus orientis* Desor_3837 (45%)
03	0806	+	KBA	*Desulfitobacterium chlororespirans* CprA (62%)
04	0808	+	BA	*Desulfitobacterium chlororespirans* CprA (59%)
05	0815	+	ABC	*Desulfosporosinus orientis* Desor_3837 (46%)
06	0820	+	ABC	*Desulfosporosinus orientis* Desor_3837 (45%)
07	0826	+	**BA**(K)	*Desulfitobacterium hafniense* DCB-2 RdhA4 (65%)
08	0830	+	KBA	*Desulfitobacterium hafniense* DCB-2 RdhA4 (83%)
09	0832	+	BAK	*Desulfitobacterium hafniense* DCB-2 RdhA1 (72%)
10	0835	+	KBA	*Desulfitobacterium chlororespirans* CprA (70%)
11	0990	-	KBA	*Desulfitobacterium hafniense* DCB-2 RdhA4 (69%)
12	1408	+	BAK	*Desulfitobacterium hafniense* DCB-2 RdhA5 (72%)
13	2012	-	ABCK	*Desulfitobacterium hafniense* DCB-2 RdhA7 (38%)
14	2022	-	BAC	*Desulfitobacterium hafniense* DCB-2 RdhA2 (89%)
15	2026	-	BAK	*Desulfitobacterium hafniense* DCB-2 RdhA5 (66%)
16	2031	-	KBA	*Desulfitobacterium hafniense* DCB-2 RdhA1 (83%)
17	2037	-	KABC	*Desulfosporosinus orientis* Desor_3837 (43%)
18	2039	-	KBA	*Desulfitobacterium hafniense* DCB-2 RdhA1 (73%)
19	2044	-	BA	*Desulfitobacterium hafniense* DCB-2 RdhA1 (83%)
20	2052	-	ABCTK	*Desulfitobacterium hafniense* DCB-2 RdhA3 (82%)
21	2058	+	KBAC	*Desulfosporosinus orientis* Desor_3837 (38%)
22	2064	-	ABCTK	*Desulfitobacterium hafniense* DCB-2 RdhA3 (69%)
23	2065	-	KBA	*Desulfitobacterium chlororespirans* CprA (71%)
24 (*pceA*)	2398	-	ABCT	*Desulfitobacterium hafniense* TCE1 PceA (99%)
25	2792	-	**A**’	*Desulfitobacterium chlororespirans* CprA (69%)

[1] Organization of putative *rdh* gene operons displaying the order and composition of *rdh* subunits. Letters in parentheses mean that the corresponding subunits might be disrupted from the *rdhA* transcriptional unit by a transcription terminator. RdhA25 is only a partial sequence (158 aa).
[2] Best matches for *D. restrictus* RdhA protein were obtained using BlastP and non-redundant protein sequences database (Altschul et al., 1990).

Table S6. Sequence identity level of RdhA proteins[1] in *D. restrictus*

	RdhA01	RdhA02	RdhA03	RdhA04	RdhA05	RdhA06	RdhA07	RdhA08	RdhA09	RdhA10	RdhA11	RdhA12	RdhA13	RdhA14	RdhA15	RdhA16	RdhA17	RdhA18	RdhA19	RdhA20	RdhA21	RdhA22	RdhA23	RdhA24	RdhA25
RdhA25	57	32	62	63	32	33	63	61	65	73	66	65	40	34	63	65	36	64	65	36	34	34	71	36	100
RdhA24	37	31	38	39	33	37	35	37	33	36	64	35	28	35	34	34	35	35	34	67	36	66	34	100	
RdhA23	49	28	65	61	33	31	61	60	63	72	28	65	35	30	63	65	33	64	65	37	28	32	100		
RdhA22	32	35	35	38	40	40	33	34	31	32	34	29	31	33	32	34	39	34	34	73	39	100			
RdhA21	27	34	31	32	33	37	31	32	32	34	25	32	38	30	32	32	41	32	32	35	100				
RdhA20	36	33	38	38	33	39	36	34	35	31	31	30	32	34	29	35	40	30	35	100					
RdhA19	50	31	59	55	30	33	65	68	71	63	69	59	31	25	59	98	34	77	100						
RdhA18	49	31	57	54	34	29	66	64	69	62	66	60	28	30	59	78	34	100							
RdhA17	28	35	30	32	52	51	33	33	30	34	31	33	28	30	31	34	100								
RdhA16	49	31	58	55	30	29	66	68	72	63	69	60	31	25	59	100									
RdhA15	47	32	57	55	33	32	57	56	58	60	56	66	34	33	100										
RdhA14	31	31	30	31	32	32	28	26	30	26	30	27	37	100											
RdhA13	35	37	34	32	26	31	30	36	35	38	30	41	100												
RdhA12	47	32	61	57	31	33	59	59	62	65	59	100													
RdhA11	48	30	59	55	28	30	66	65	75	62	100														
RdhA10	50	31	66	63	30	32	60	60	61	100															
RdhA09	49	30	59	56	33	34	69	66	100																
RdhA08	51	36	56	52	32	33	64	100																	
RdhA07	47	36	56	53	32	31	100																		
RdhA06	28	39	32	32	58	100																			
RdhA05	33	43	32	30	100																				
RdhA04	48	29	81	100																					
RdhA03	51	31	100																						
RdhA02	30	100																							
RdhA01	100																								

[1] Values given in the table represent the maximal sequence identity level between two individual sequences, as for some of them, only a partial coverage was considered by the online version of the software Blast2-sequences (http://blast.ncbi.nlm.nih.gov/).

Table S7. Transcript copy number ratio of selected *rdhA* genes of *D. restrictus*: A, across the growth phases; B, *rdhA24* (*pceA*) relative to other genes in the different growth phases.

A

Gene	Growth phase ratios		
	E/LE	LE/S	E/S
rpoB	2.5	11.2	27.5
rdhA08	4.5	8.3	37.0
rdhA14	11.0	20.9	229.1
rdhA16	4.1	1.1	4.7
rdhA19	25.1	5.5	137.1
rdhA24	2.8	22.7	63.0

B

Phase	*rdhA24/rpoB*	*rdhA24/rdhA08*	*rdhA24/rdhA14*	*rdhA24/rdhA16*	*rdhA24/rdhA19*
Exponential	1077	3688	130	2894	51
Late-exponential	955	5924	515	4265	463
Stationary	469	2167	473	215	111

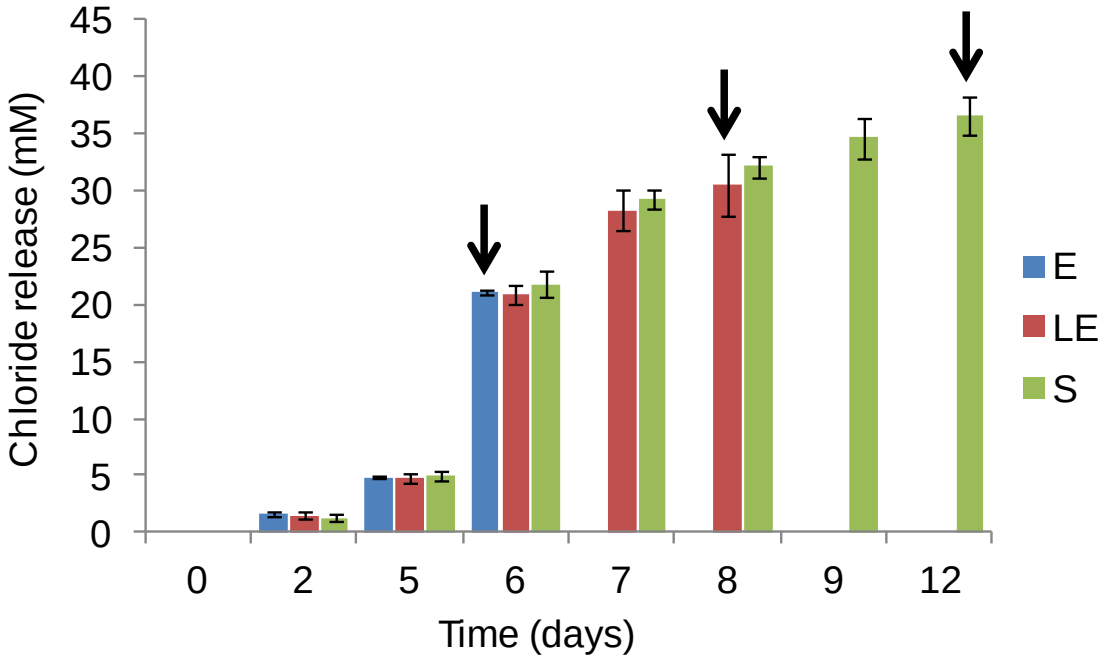


Fig S1. Follow-up of *Dehalobacter restrictus* batch cultures.

Nine bottles of 300 mL culture were cultivated and growth was monitored by following the chloride release during PCE dechlorination. Triplicate batches (the average including standard deviation being shown in different colours) were collected during the designated exponential phase (E, blue), the late-exponential phase (LE, red), and the stationary phase (S, green). Collection points are indicated by arrows.

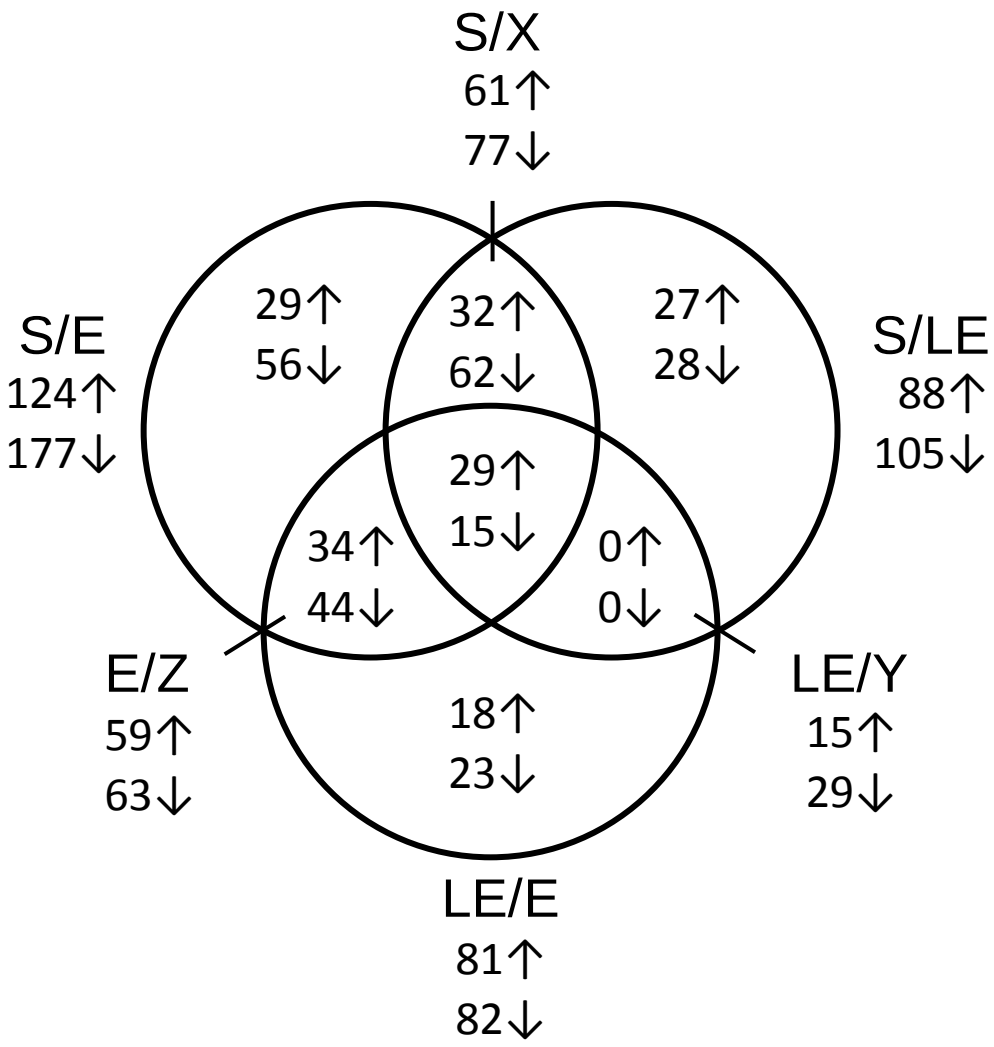


Fig S2. Venn diagram representing the number of common proteins detected with 3-fold in/decreased abundance ratio in the different growth phase comparisons.

E, exponential phase; LE, late-exponential phase; S, stationary phase; X, E+LE; Y, E+S; Z, LE+S.

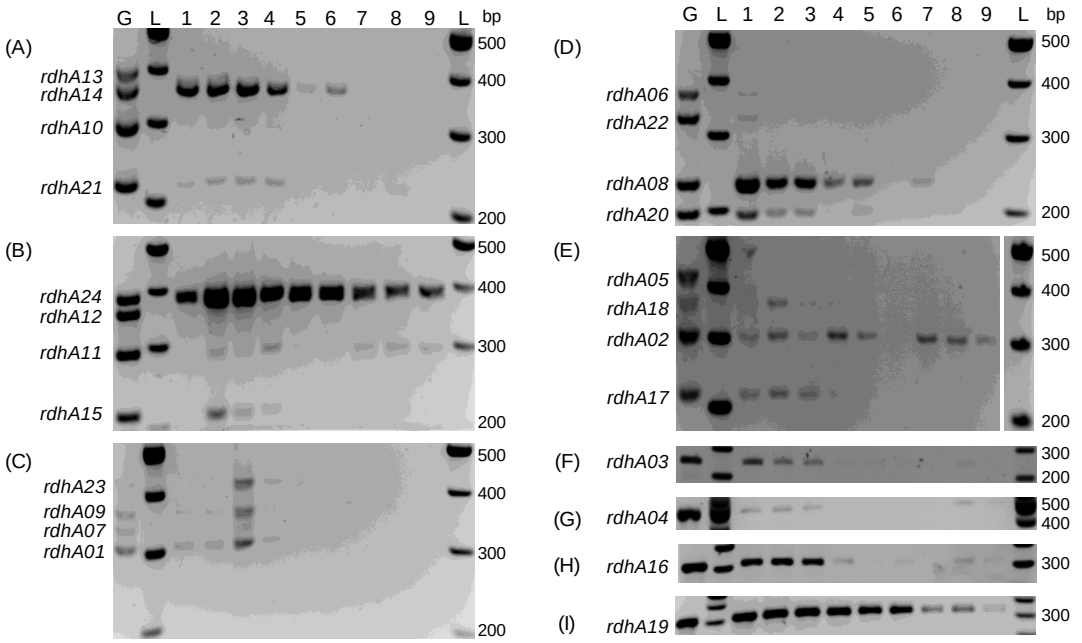


Fig S3. Complete data set of RT-multiplex PCR on *D. restrictus rdhA* genes.

Five combinations of rdhA genes (panels A-E) were screened using multiplex PCR, while four single rdhA genes were analyzed separately (panels F-I). The targeted rdhA genes are indicated on the left side of the gel pictures, the fragment size of the ladder on the right side. Legend: G, positive control with genomic DNA; L, 100-bp ladder; 1-3, RT-PCR samples from *D. restrictus* collected in exponential phase; 4-6, in late-exponential phase; 7-9, in stationary phase.

Table S8. Complete data set for growth phase dependent proteomic analysis of *D. restrictus*, between exponential (E), late-exponential (LE) and stationary (S) phases

Published in (Rupakula et al., 2013), as Table S5, Link to electronic version:
http://rstb.royalsocietypublishing.org/content/royptb/suppl/2013/02/27/rstb.2012.0325.DC1/rstb20120325supp2.pdf

7.2 Supplementary Figures and Tables to Chapter 3

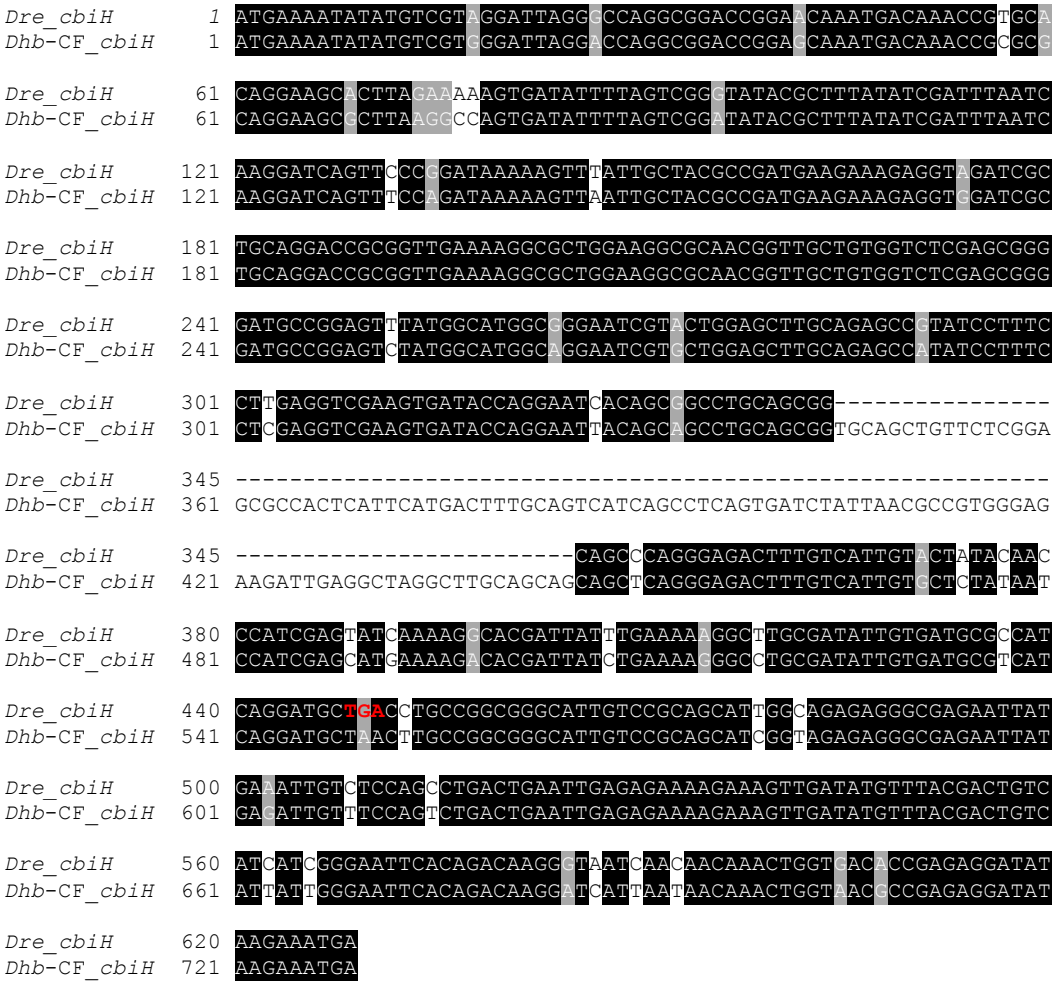


Fig S4. Sequence alignment of *cbiH* genes of *D. restrictus* (*Dre*) and *Dehalobacter* sp. CF (*Dhb*-CF).

A 101-bp deletion in *D. restrictus cbiH* gene created a frame-shift which terminates the corresponding polypeptide chain earlier (stop codon in red).

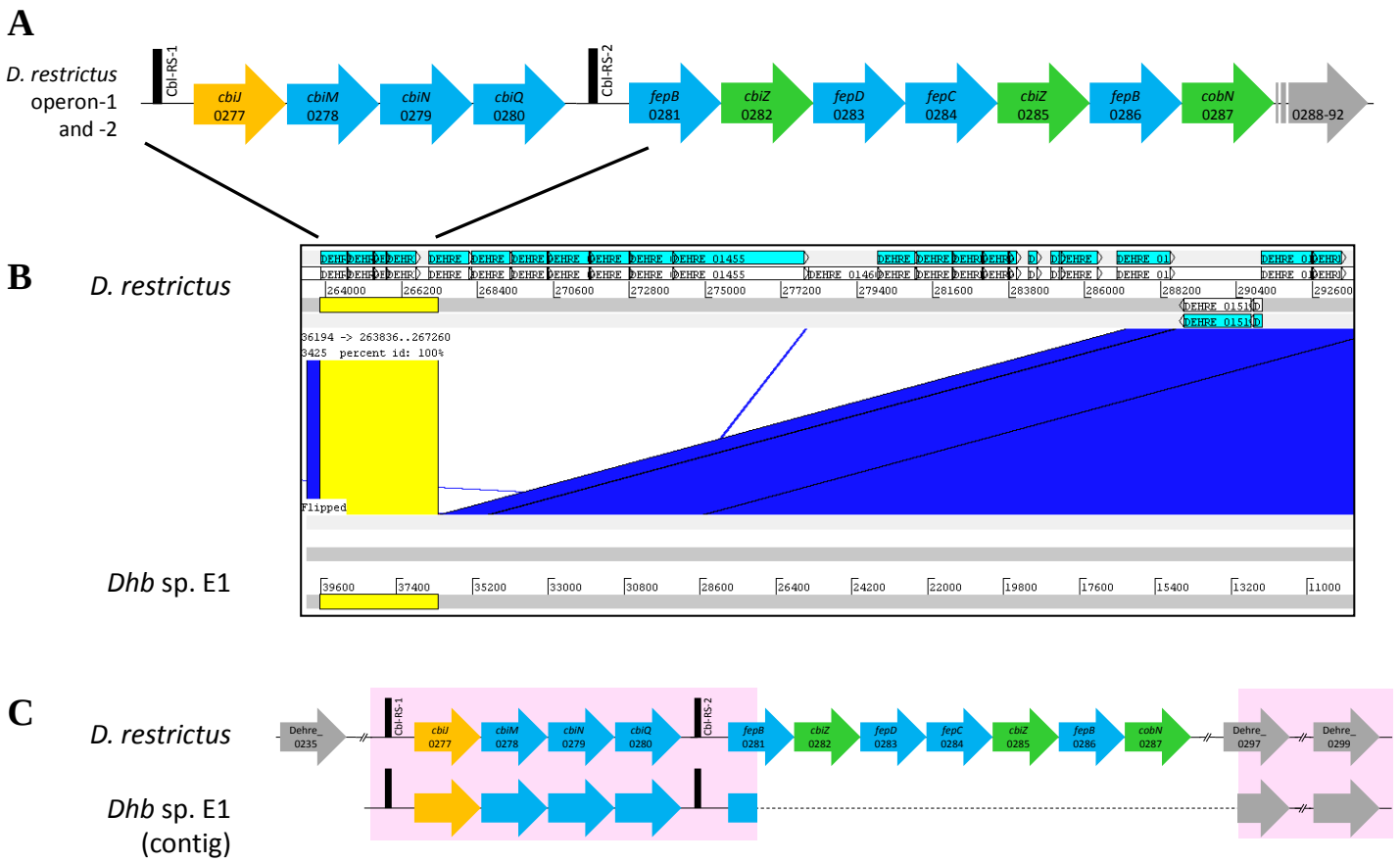


Fig S5. Synteny map of corrinoid operon-1 and -2 in *D. restrictus* and *Dehalobacter* sp. E1.

The gene arrays of corrinoid operon-1 and -2 of *D. restrictus* are preceded by their respective cobalamin riboswitches. (**B**). The yellow box in the synteny map indicates a nearly complete conservation of the genetic structure of operon-1 including the beginning of operon-2. The blue box indicates the conservation of genes beyond operon-2. Note the deletion in *Dehalobacter* sp. E1. (**C**) Alignment of the gene arrays of operon-1 and -2 in *D. restrictus* and *Dehalobacter* sp. E.

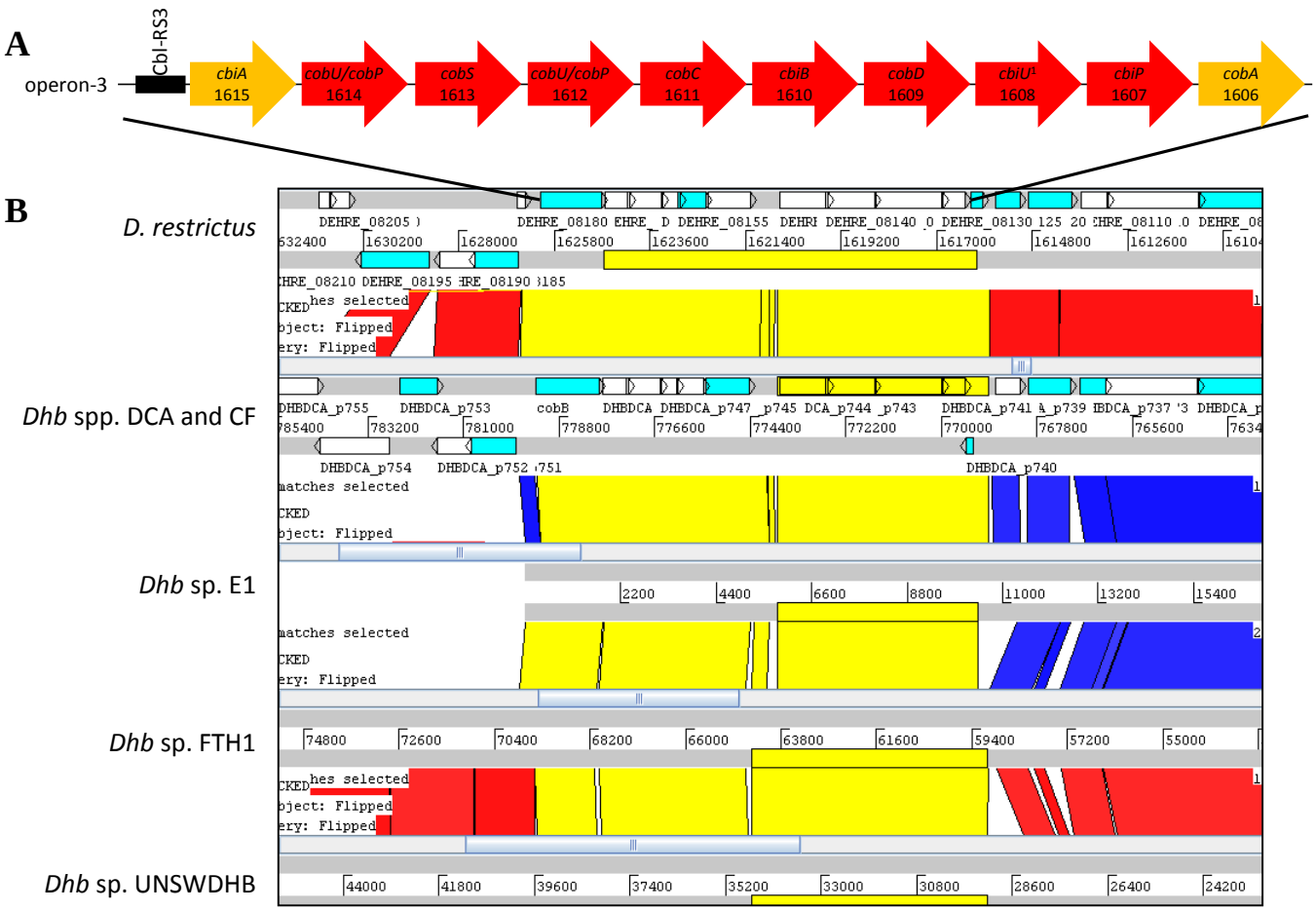


Fig S6. Synteny map of corrinoid operon-3 in *Dehalobacter* spp.

(A) The gene array of operon-3 of *D. restrictus* is preceded by the cobalamin riboswitch Cbl-RS3. (**B**) The yellow boxes in the map indicate a nearly identical genetic structure in other studied *Dehalobacter* genomes

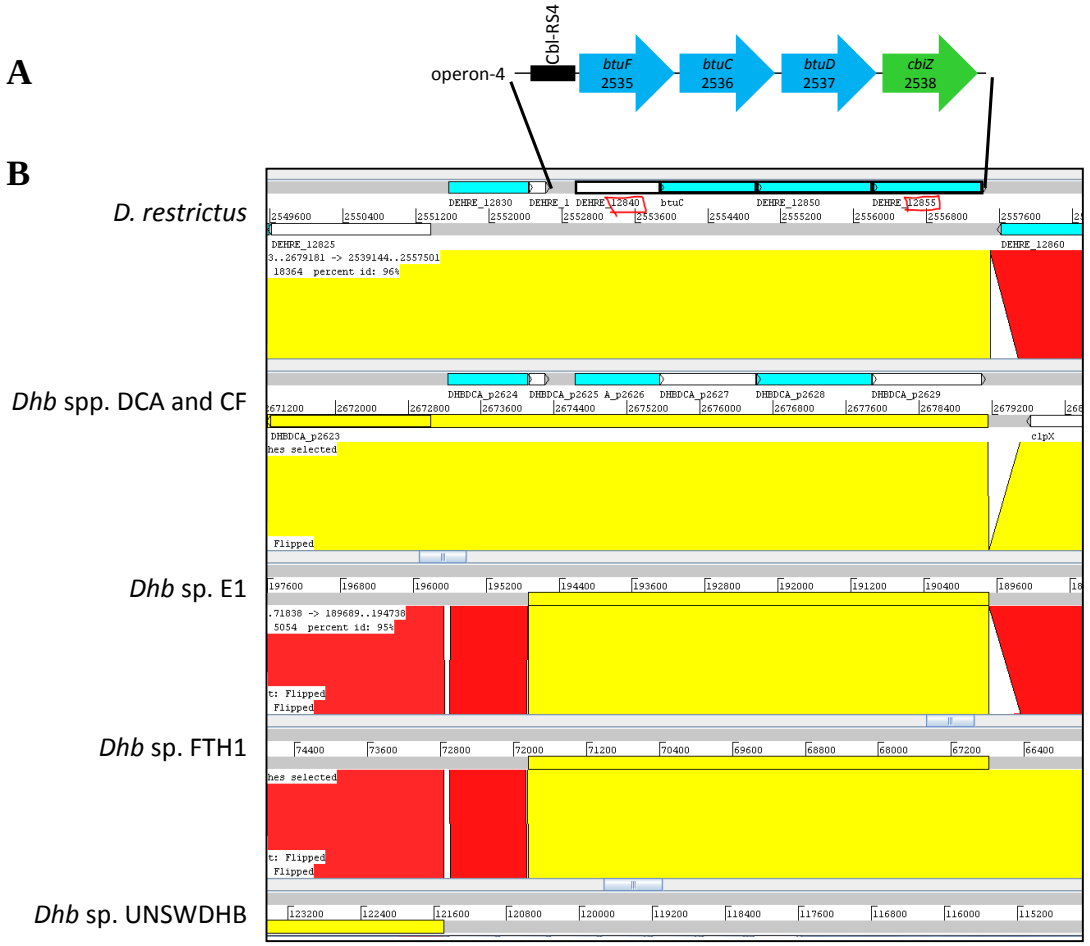


Fig S7. Synteny map of corrinoid operon-4 in *Dehalobacter* spp.

(A) The gene array of operon-4 of *D. restrictus* is preceded by the cobalamin riboswitch Cbl-RS4. (**B**) The yellow boxes in the map indicate a nearly identical genetic structure in other studied *Dehalobacter* genomes.

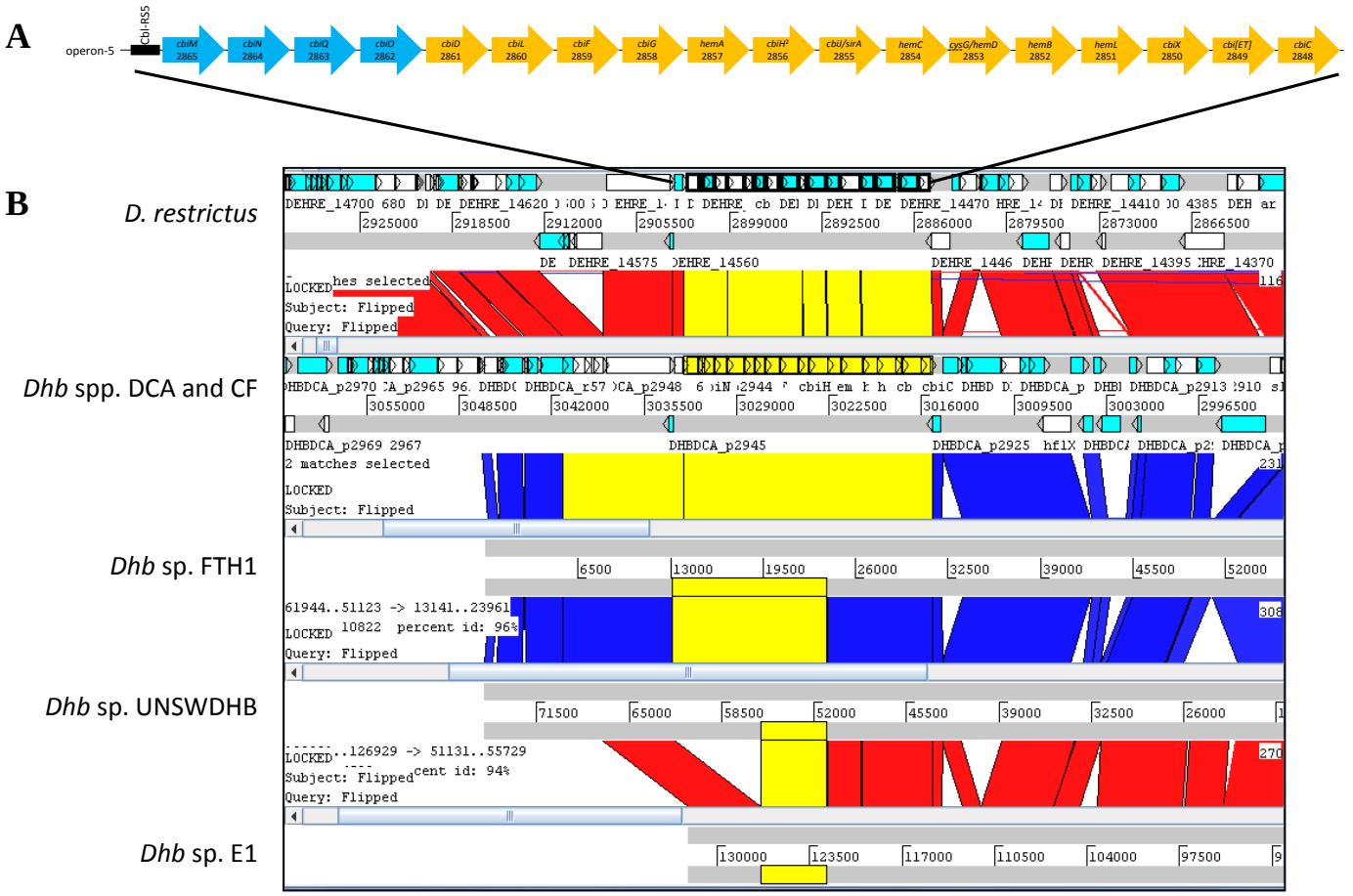


Fig S8. Synteny map of corrinoid operon-5 in *Dehalobacter* spp.

(A) The gene array of operon-4 of *D. restrictus* is preceded by the cobalamin riboswitch Cbl-RS4. (**B**) The colour boxes in the map indicate a nearly identical genetic structure in other studied *Dehalobacter* genomes

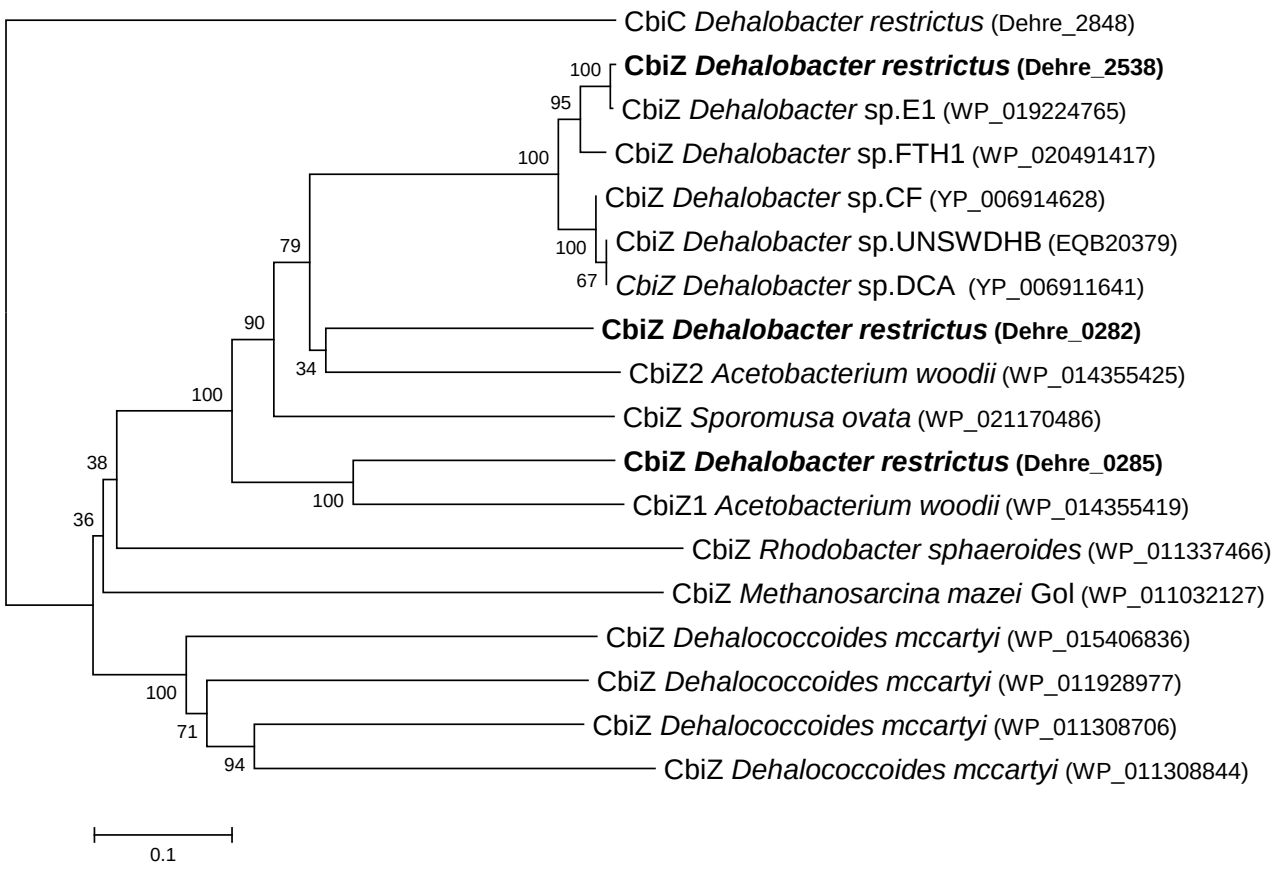


Fig S9. Maximal likelihood sequence analysis of CbiZ proteins.

The three CbiZ proteins of *D. restrictus* (in bold) were aligned with ClustalX and compared to CbiZ proteins of other *Dehalobacter* spp. and of well-characterized bacteria either producing corrinoid (from the genera *Acetobacterium*, *Sporomusa*, *Rhodobacter*, *Methanosarcina*) or known to salvage it (*Dehalococcoides mccartyi*). CbiC of *D. restrictus* was used to root the tree. Sequence reference numbers are given in brackets.

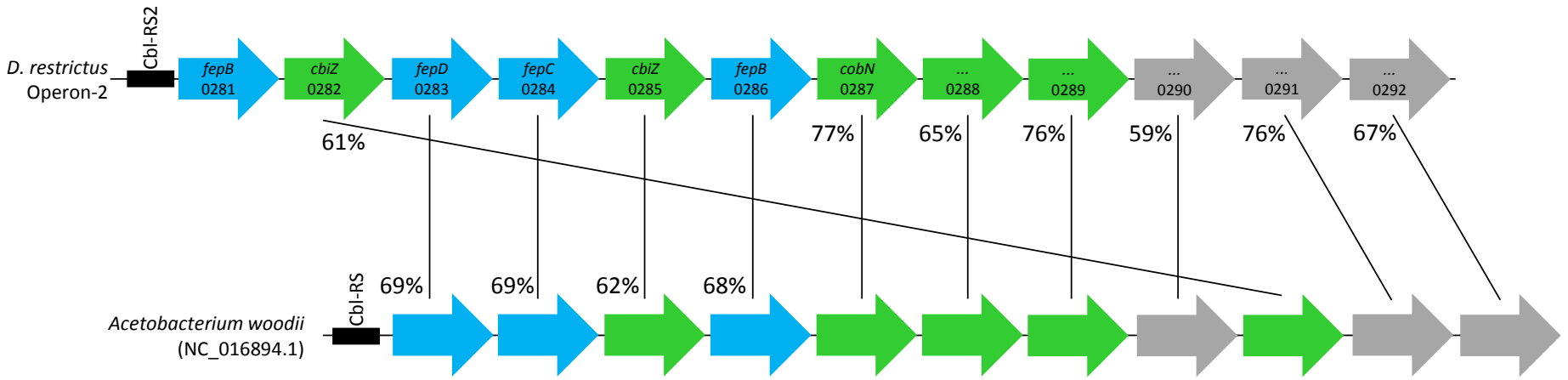


Fig S10. Synteny map of *D. restrictus* corrinoid operon-2 with a region of the genome of *Acetobacterium woodii* (DSM 1030).

A high level of sequence identity was calculated for the product of all depicted genes. With the exception of one single gene (*cbiZ*, corresponding to Dehre_0282), the genetic organization is fully conserved between *D. restrictus* and *A. woodii*. The first gene in operon-2 in *D. restrictus* (Dehre_0281) seems to be the result of a gene duplication as it is missing in *A. woodii*. Finally, a cobalamin riboswitch was also identified upstream of the gene cluster in *A. woodii*. Blue and green arrows depict genes whose product is involved in corrinoid transport and corrinoid salvaging, respectively. Grey arrows represent genes not associated with corrinoid metabolism.

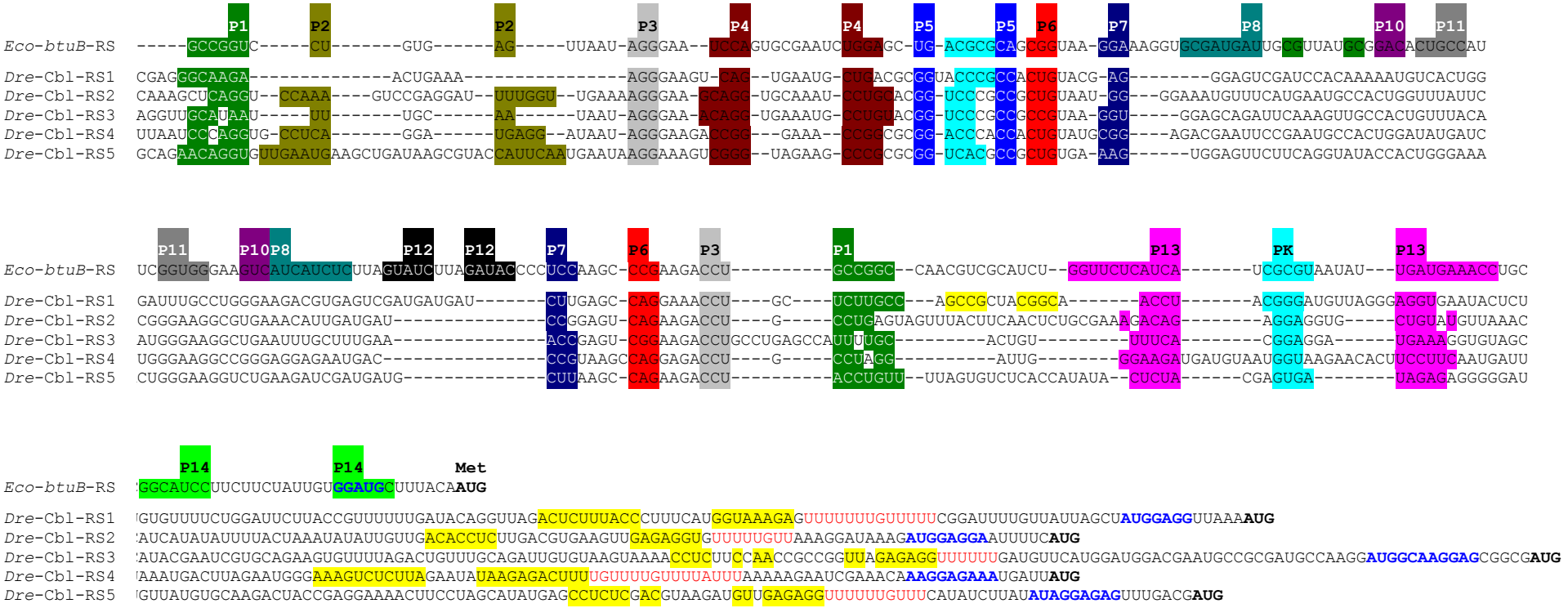


Fig S11. Sequence analysis of *D. restrictus* cobalamin riboswitches.

The five cobalamin riboswitches (Cbl-RS) were obtained using the Rfam toolbox, then automatically aligned using ClustalX together with *E. coli btuB* Cbl-RS, and finally manually refined to highlight the conserved secondary structures. The conserved hairpin structures are indicated by P1 to P14 colour shaded sequences. The terminal yellow shaded sequences in the lower panel indicate the putative transcriptional terminators located upstream of the ribosome-binding sequence (in blue) and the AUG start codon of the following gene (in bold).

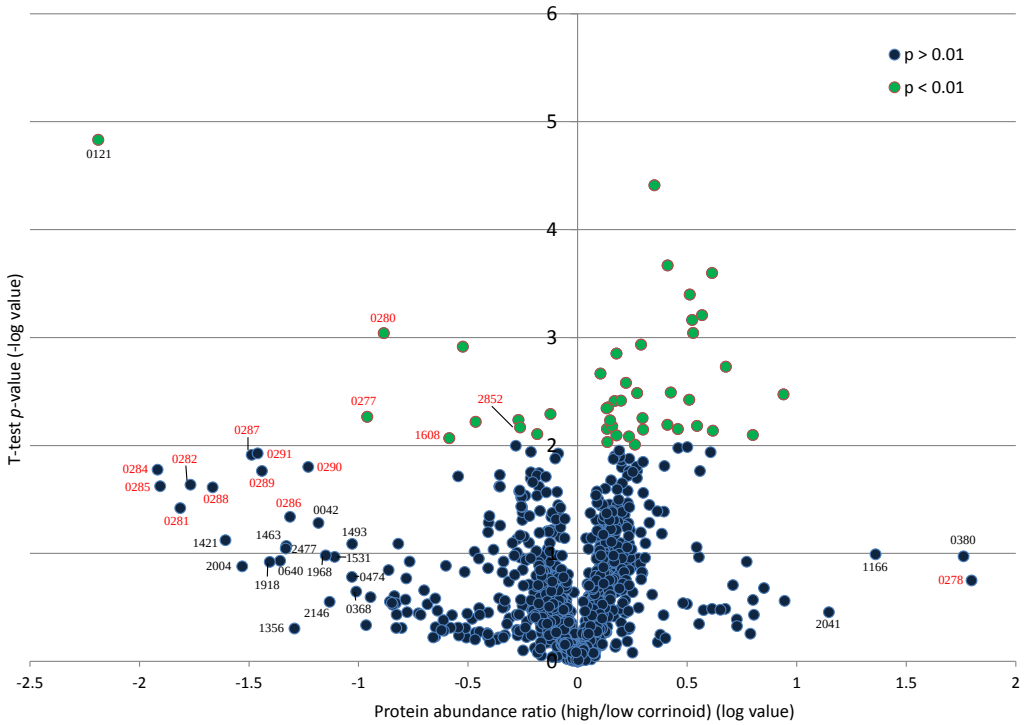


Fig S12. Volcano plot showing the whole-proteome data of *D. restrictus* cells cultivated with high (250 µg/L) *vs*. low (10 µg/L) cyanocobalamin in the medium.

Differences between two cobalamin conditions with *p* value >0.01 are considered as non-significant by T-test (dark dots). Proteins with *p* value <0.01 are shown with green dots. Proteins associated with corrinoid metabolism showing a *p* value <0.01, or a *p* value >0.01 but a fold change higher than 10 are indicated with a red locus tag (the number corresponds to the Dehre_# locus tag used throughout the study), other proteins unrelated to corrinoid metabolism but with fold change higher than 10 are indicated with a black locus tag

Table S9. Overview of corrinoid-related operons in the genomes of *Dehalobacter* spp

Dehalobacter restrictus

Operon	Original locus	NCBI locus tag	orientation	gene name	GI tag	Other tag	Protein length (aa)	Protein description
1	Dehre_0277	DEHRE_01400	+	*cbiJ*	570738077	AHF08944.1	240	precorrin-6x reductase
	Dehre_0278	DEHRE_01405	+	*cbiM*	570738078	AHF08945.1	250	ECF-type cobalt transport system, bipartite component
	Dehre_0279	DEHRE_01410	+	*cbiN*	570738079	AHF08946.1	109	ECF-type cobalt transport system, bipartite component
	Dehre_0280	DEHRE_01415	+	*cbiQ*	570738080	AHF08947.1	263	ECF-type cobalt transport system, permease component
2	Dehre_0281	DEHRE_01425	+	*fepB*	570738081	AHF08948.1	385	putative iron ABC-transporter, substrate-binding protein
	Dehre_0282	DEHRE_01430	+	*cbiZ*	570738082	AHF08949.1	367	adenosylcobinamide amidohydrolase
	Dehre_0283	DEHRE_01435	+	*fepD*	570738083	AHF08950.1	342	putative iron ABC transporter, permease component
	Dehre_0284	DEHRE_01440	+	*fepC*	570738084	AHF08951.1	404	putative iron ABC transporter, ATP-binding protein
	Dehre_0285	DEHRE_01445	+	*cbiZ*	570738085	AHF08952.1	373	adenosylcobinamide amidohydrolase
	Dehre_0286	DEHRE_01450	+	*fepB*	570740339	AHF11206.1	404	putative iron ABC transporter, substrate-binding protein
	Dehre_0287	DEHRE_01455	+	*cobN*	570738086	AHF08953.1	1262	cobaltochelatase
	Dehre_0288	DEHRE_01460	+	ND	643376646	WP_025205042.1	657	magnesium/cobalt protoporphyrin IX chelatase
	Dehre_0289	DEHRE_01465	+	ND	570738087	AHF08954.1	348	magnesium/cobalt protoporphyrin IX chelatase
	Dehre_0290	DEHRE_01470	+	ND	570738088	AHF08955.1	347	radical SAM domain-containing protein
	Dehre_0291	DEHRE_01480	+	ND	570738089	AHF08956.1	279	ABC-type Nod factor export system, ATPase component
	Dehre_0292	DEHRE_01485	+	ND	570738090	AHF08957.1	244	ABC-type Nod factor export system, permease component
3	Dehre_1606	DEHRE_08130	-	*cobA*	570739181	AHF10048.1	176	cob(I)yrinic acid a,c-diamide adenosyltransferase
	Dehre_1607	DEHRE_08135	-	*cbiP*	570739182	AHF10049.1	503	cobyric acid synthase
	Dehre_1608	DEHRE_08140	-	*cbiU*	570739183	AHF10050.1	373	L-threonine kinase
	Dehre_1609	DEHRE_08145	-	*cobD*	570739184	AHF10051.1	361	L-threonine-O-3-phosphate decarboxylase (product of which is the substrate of CbiB)
	Dehre_1610	DEHRE_08150	-	*cbiB*	570739185	AHF10052.1	321	adenosylcobinamide-phosphate synthase
	Dehre_1611	DEHRE_08155	-	*cobC*	570739186	AHF10053.1	200	alpha-ribazole-5'-phosphate phosphatase
	Dehre_1612	DEHRE_08160	-	*cobU/cobP*	570739187	AHF10054.1	131	cobinamide kinase/cobinamide phosphate guanylyltransferase
	Dehre_1613	DEHRE_08165	-	*cobS*	570739188	AHF10055.1	257	cobalamin 5'-phosphate synthase
	Dehre_1614	DEHRE_08170	-	*cobU/cobP*	570739189	AHF10056.1	184	cobinamide kinase/cobinamide phosphate guanylyltransferase
	Dehre_1615	DEHRE_08175	-	*cbiA*	570739190	AHF10057.1	474	cobyrinic acid a,c-diamide synthase
4	Dehre_2535	DEHRE_12840	+	*btuF*	570739982	AHF10849.1	305	ABC-type cobalamin/Fe3+ transport system, substrate-binding component
	Dehre_2536	DEHRE_12845	+	*btuC*	570739983	AHF10850.1	354	ABC-type cobalamin/Fe3+ transport system, permease component
	Dehre_2537	DEHRE_12850	+	*btuD*	570739984	AHF10851.1	423	ABC-type cobalamin/Fe3+ transport system, ATPase component
	Dehre_2538	DEHRE_12855	+	*cbiZ*	570739985	AHF10852.1	397	adenosylcobinamide amidohydrolase
5	Dehre_2848	DEHRE_14465	-	*cbiC*	570740257	AHF11124.1	208	precorrin-8x methylmutase
	Dehre_2849	DEHRE_14470	-	*cbi[ET]*	570740258	AHF11125.1	422	precorrin-6y C5,15-methyltransferase / precorrin-8w decarboxylase
	Dehre_2850	DEHRE_14475	-	*cbiX*	570740259	AHF11126.1	131	sirohydrochlorin cobalt chelatase
	Dehre_2851	DEHRE_14480	-	*hemL*	570740260	AHF11127.1	425	glutamate-1-semialdehyde 2,1-aminomutase (biosynthesis of d-aminolevulinic acid)
	Dehre_2852	DEHRE_14485	-	*hemB*	570740261	AHF11128.1	326	d-aminolevulinic acid dehydratase
	Dehre_2853	DEHRE_14490	-	*cysG/hemD*	570740262	AHF11129.1	500	uroporphyrinogen-III synthase / uroporphyrinogen-III C-methyltransferase
	Dehre_2854	DEHRE_14495	-	*hemC*	570740263	AHF11130.1	298	porphobilinogen deaminase
	Dehre_2855	DEHRE_14500	-	*cbiJ/sirA*	570740264	AHF11131.1	443	precorrin-6x reductase / sirohaem synthase
	Dehre_2856	DEHRE_14505	-	*cbiH*	-	-	227	precorrin-3B c17-methyltransferase
	Dehre_2857	DEHRE_14510	-	*hemA*	570740265	AHF11132.1	413	glutamyl-tRNA reductase
	Dehre_2858	DEHRE_14515	-	*cbiG*	570740266	AHF11133.1	352	opening of the ring A d-lactone and extrusion of the "C(2)" unit
	Dehre_2859	DEHRE_14520	-	*cbiF*	570740267	AHF11134.1	261	precorrin-4 c11-methyltransferase
	Dehre_2860	DEHRE_14525	-	*cbiL*	570740268	AHF11135.1	233	precorrin-2 c20-methyltransferase
	Dehre_2861	DEHRE_14530	-	*cbiD*	570740269	AHF11136.1	402	cobalt-precorrin-6a synthase
	Dehre_2862	DEHRE_14535	-	*cbiO*	570740270	AHF11137.1	292	ECF-type cobalt transport system, ATPase component
	Dehre_2863	DEHRE_14540	-	*cbiQ*	570740271	AHF11138.1	263	ECF-type cobalt transport system, permease component
	Dehre_2864	DEHRE_14545	-	*cbiN*	570740272	AHF11139.1	113	ECF-type cobalt transport system, bipartite component
	Dehre_2865	DEHRE_14550	-	*cbiM*	570740273	AHF11140.1	246	ECF-type cobalt transport system, bipartite component
	Dehre_1488	DEHRE_07530	+	*cobT*	570739088	AHF09955.1	372	nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase
	Dehre_2360	DEHRE_11955	-	*cobB/cobQ*	570739824	AHF10691.1	266	glutamine amidotransferase similar to cbiP/cobQ C-domain

	Dehalobacter sp.			*Dehalobacter* sp.			*Dehalobacter* sp. E1					*Dehalobacter* sp. FTH1							*Dehalobacter sp.*			
gene name	Locus	Max ID (%)	cover age (%)	Locus	Max ID (%)	cove rage (%)	Contig	start	end	Max ID (%)	cover age (%)	Contig	start	end	Max ID (%)	cover age (%)	GI tag	NCBI ref.seq	GI tag	NCBI ref.seq	Max ID (%)	cover age (%)
cbiJ	-	-	-	-	-	-	CANE01000026.1	39588	38869	100	100	-	-	-	-	-	-	-	-	-	-	-
cbiM	-	-	-	-	-	-	CANE01000026.1	38827	38078	100	100	-	-	-	-	-	-	-	-	-	-	-
cbiN	-	-	-	-	-	-	CANE01000026.1	38078	37752	100	100	-	-	-	-	-	-	-	-	-	-	-
cbiQ	-	-	-	-	-	-	CANE01000026.1	37676	36888	100	100	-	-	-	-	-	-	-	-	-	-	-
fepB	-	-	-	-	-	-	CANE01000026.1	36484	36182	98	26	-	-	-	-	-	-	-	-	-	-	-
cbiZ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
fepD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
fepC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cbiZ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
fepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cobN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ND	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ND	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ND	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ND	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ND	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cobA	DCF50_p799	99	100	DHBDCA_p741	99	100	CANE01000016.1	9592	10119	98	100	AQYY01000014.1	60102	59578	93	99	521981467	WP_020492738.1	530291266	EQB19828.1	98	100
cbiP	DCF50_p800	99	100	DHBDCA_p742	99	100	CANE01000016.1	8080	9588	99	100	AQYY01000014.1	61617	60109	96	100	521981468	WP_020492739.1	530291263	EQB19825.1	99	100
cbiU	DCF50_p801	91	100	DHBDCA_p743	91	100	CANE01000016.1	6921	8039	100	100	AQYY01000014.1	62776	61658	91	100	521981469	WP_020492740.1	530291264	EQB19826.1	91	100
cobD	DCF50_p802	98	100	DHBDCA_p744	98	100	CANE01000016.1	5861	6943	100	100	AQYY01000014.1	63836	62754	92	100	521981470	WP_020492741.1	530291298	EQB19860.1	98	100
cbiB	DCF50_p803	97	100	DHBDCA_p745	97	100	CANE01000016.1	4244	5206	98	100	AQYY01000014.1	65561	64605	94	100	521981471	WP_020492742.1	530291288	EQB19850.1	97	100
cobC	DCF50_p804	94	100	DHBDCA_p746	94	100	CANE01000016.1	3560	4156	93	100	AQYY01000014.1	66251	65670	87	100	521981472	WP_020492743.1	530291269	EQB19831.1	94	100
cobU/cobP	DCF50_p805	97	100	DHBDCA_p747	97	100	CANE01000016.1	3172	3564	97	100	AQYY01000014.1	66639	66247	89	100	521981473	WP_020492744.1	530291283	EQB19845.1	97	100
cobS	DCF50_p806	91	100	DHBDCA_p748	91	100	CANE01000016.1	2402	3172	93	100	AQYY01000014.1	67409	66639	86	100	521981474	WP_020492745.1	530291277	EQB19839.1	91	100
cobU/cobP	DCF50_p807	98	100	DHBDCA_p749	98	100	CANE01000016.1	1864	2415	97	100	AQYY01000014.1	67947	67396	92	100	521981475	WP_020492746.1	530291295	EQB19857.1	98	100
cbiA	DCF50_p808	98	100	DHBDCA_p750	98	100	CANE01000016.1	387	1754	98	96	AQYY01000014.1	69460	68066	93	98	521981476	WP_020492747.1	530291294	EQB19856.1	98	100
btuF	DCF50_p2637	99	100	DHBDCA_p2626	99	100	CANE01000001.1	194143	193304	100	92	AQYY01000005.1	71244	70405	97	92	521980149	WP_020491420.1	530291782	EQB20322.1	99	100
btuC	DCF50_p2638	99	98	DHBDCA_p2627	99	98	CANE01000001.1	193298	192240	100	100	AQYY01000005.1	70399	69341	97	100	521980148	WP_020491419.1	530291752	EQB20292.1	99	99
btuD	DCF50_p2639	96	100	DHBDCA_p2628	96	100	CANE01000001.1	192236	190968	98	100	AQYY01000005.1	69337	68069	97	100	521980147	WP_020491418.1	530291720	EQB20260.1	96	100
cbiZ	DCF50_p2640	93	99	DHBDCA_p2629	93	99	CANE01000001.1	190963	189776	100	100	AQYY01000005.1	68064	66874	96	100	521980146	WP_020491417.1	530291839	EQB20379.1	93	100
cbiC	DCF50_p2930	100	100	DHBDCA_p2926	100	100	CANE01000003.1	115747	115124	100	100	AQYY01000008.1	30567	31190	100	100	521980683	WP_020491954.1	570740257	AHF11124.1	100	100
cbi[ET]	DCF50_p2931	95	100	DHBDCA_p2927	95	100	CANE01000003.1	117081	115816	100	100	AQYY01000008.1	29234	30499	99	100	521980682	WP_020491953.1	530291351	EQB19909.1	96	100
cbiX	DCF50_p2932	97	100	DHBDCA_p2928	97	100	CANE01000003.1	117464	117072	100	100	AQYY01000008.1	28851	29243	99	100	521980681	WP_020491952.1	530291403	EQB19961.1	97	100
hemL	DCF50_p2933	97	100	DHBDCA_p2929	97	100	CANE01000003.1	118827	117553	100	100	AQYY01000008.1	27487	28761	97	100	521980680	WP_020491951.1	530291394	EQB19952.1	97	100
hemB	DCF50_p2934	98	100	DHBDCA_p2930	98	100	CANE01000003.1	119838	118861	100	100	AQYY01000008.1	26479	27453	98	100	521980679	WP_020491950.1	530291387	EQB19945.1	98	100
cysG/hemD	DCF50_p2935	96	99	DHBDCA_p2931	96	99	CANE01000003.1	121357	119858	100	100	AQYY01000008.1	24942	26438	96	100	521980678	WP_020491949.1	530291391	EQB19949.1	96	100
hemC	DCF50_p2936	97	100	DHBDCA_p2932	97	100	CANE01000003.1	122254	121361	100	100	AQYY01000008.1	24045	24938	98	100	521980677	WP_020491948.1	530291352	EQB19910.1	97	100
cbiJ/sirA	DCF50_p2937	90	99	DHBDCA_p2933	90	99	CANE01000003.1	123648	122320	100	100	AQYY01000008.1	22636	23961	97	100	521980676	WP_020491947.1	530291404	EQB19962.1	90	100
cbiH	DCF50_p2938	100	100	DHBDCA_p2934	100	100	CANE01000003.1	124328	123648	97	100	AQYY01000008.1	21956	22636	96	100	521980675	WP_020491946	530291343	EQB19901.1	100	100
hemA	DCF50_p2939	99	100	DHBDCA_p2935	99	100	CANE01000003.1	125623	124385	100	100	AQYY01000008.1	20661	21899	99	100	521980674	WP_020491945.1	530291348	EQB19906.1	100	100
cbiG	DCF50_p2940	97	100	DHBDCA_p2936	97	100	CANE01000003.1	126671	125616	100	100	AQYY01000008.1	19613	20668	100	100	521980673	WP_020491944.1	530291383	EQB19941.1	97	100
cbiF	DCF50_p2941	97	100	DHBDCA_p2937	97	100	CANE01000003.1	126928	126671	100	33	AQYY01000008.1	18831	19613	100	100	521980672	WP_020491943.1	530291363	EQB19921.1	97	100
cbiL	DCF50_p2942	97	100	DHBDCA_p2938	97	100	-	-	-	-	-	AQYY01000008.1	18133	18831	99	100	521980671	WP_020491942.1	530291398	EQB19956.1	97	100
cbiD	DCF50_p2943	96	100	DHBDCA_p2939	96	100	-	-	-	-	-	AQYY01000008.1	16940	18133	96	100	521980670	WP_020491941.1	530291349	EQB19907.1	97	100
cbiO	DCF50_p2944	99	100	DHBDCA_p2940	99	100	-	-	-	-	-	AQYY01000008.1	16042	16917	99	100	521980669	WP_020491940.1	530291369	EQB19927.1	99	100
cbiQ	DCF50_p2945	98	100	DHBDCA_p2941	98	100	-	-	-	-	-	AQYY01000008.1	15251	16039	97	100	521980668	WP_020491939.1	530291370	EQB19928.1	97	100
cbiN	DCF50_p2946	98	100	DHBDCA_p2942	98	100	-	-	-	-	-	AQYY01000008.1	14926	15264	98	100	521980667	WP_020491938.1	530291371	EQB19929.1	98	100
cbiM	DCF50_p2947	97	100	DHBDCA_p2943	97	100	-	-	-	-	-	AQYY01000008.1	14183	14920	98	100	521980666	WP_020491937.1	530291362	EQB19920.1	99	100
cobT	DCF50_p694	97	100	DHBDCA_p636	97	100	CANE01000007.1	70302	71417	100	100	AQYY01000012.1	58638	57523	97	100	521981239	WP_020492510.1	530293139	EQB21620.1	97	100
cobB/cobQ	DCF50_p1636	99	100	DHBDCA_p1627	99	100	CANE01000006.1	47756	48553	100	100	AQYY01000007.1	62219	63016	90	100	521980531	WP_020491802.1	530293473	EQB21936.1	99	100

Table S10. Oligonucleotides and plasmids used in this study

Primer	Primer sequence (5'-3')	Target gene	*D. restrictus* locus	Amplicon size (bp)
DRE1-f	TCATCCTTCTGGGAGAAACG	*cbiJ*	Dehre_0277	274
DRE1-r	GCTTCCTCCCAATTACATGC			
DRE2-f	TACCGGAGATTACGGACTGC	*fepB*	Dehre_0281	263
DRE2-r	ACACCTTGCCATCGTAGACC			
DRE3-f	CCATTTTAGACCGGACAACG	*cbiA*	Dehre_1615	235
DRE3-r	GGATTCTCTGGCCTGTATGG			
DRE4-f	TTGAAGAGCTGAAGGTGACG	*btuF*	Dehre_2535	173
DRE4-r	GGCGATGTCTTTGAGTTTGG			
DRE5-f	CTGCGGGTTGGTGTATTACC	*cbiM*	Dehre_2865	215
DRE5-r	GAACAAGATAGCGCCAAAGC			
RPOB-f	GGAAAATCCGTTCTTTATGACG	*rpoB*	Dehre_0463	276
RPOB-r	TACCACATCATCGGACTTAACG			

Table S11. Plasmids used in this study

Plasmid	Description	Reference
pGEM-T Easy	Vector for direct cloning of PCR products	Promega
pDRE1	Fragment of *cbiJ* (Dehre_0277) cloned into pGEM-T Easy	This study
pDRE2	Fragment of *fepB* (Dehre_0281) cloned into pGEM-T Easy	This study
pDRE3	Fragment of *cbiA* (Dehre_1615) cloned into pGEM-T Easy	This study
pDRE4	Fragment of *btuF* (Dehre_2535) cloned into pGEM-T Easy	This study
pDRE5	Fragment of *cbiM* (Dehre_2865) cloned into pGEM-T Easy	This study
pRPOB	Fragment of *rpoB* (Dehre_0463) cloned into pGEM-T Easy	This study

Table S12. Summary of proteome analysis

	Cobalamin concentration ratios		
	high/mid	mid/low	high/low
> 3-fold up	24	43	36
> 3-fold down	68	39	68
Total	92	82	104
up ($p<0.01$)	27	14	34
down ($p<0.01$)	11	33	10
Total	38	47	44

Table S13. Complete proteomic dataset for *D. restrictus*. High, mid and low stand for 250, 50 or 10 µg/L of cobalamin amendments to cultures, respectively.
Published in (Rupakula et al., 2014), as Table S4, Link to electronic version:
http://journal.frontiersin.org/file/downloadfile/148935/octet-stream/Table%204.XLSX/322/1/117786

7.3 Supplementary Figures and Tables to Chapter 4

Table S14. Relationships between *rdhA* and *rdhK* genes in *D. restrictus*

rdhA#	***rdhA* Dehre_#**	***rdhK* Dehre_#**	***rdhK#*** [1]	***rdh* cluster** [2]	**RdhK (*pI*)**	**RdhK MW (kDa)**
-		0779	K01a	*	6.74	26.99
1	0785	0786	K01b	BAKT	6.77	26.07
2	0793	0792	K02a	(K)AB(C)	7.94	26.70
-		0797	K02b	*	6.72	28.04
3	0806	0804	K03	KBA	6.53	28.20
4	0808	-	-	BA		
5	0815	0814	K05	KABC	5.71	26.95
6	0820	0819	K06	KABC	5.95	27.83
7	0826	0824	K07	BA(K)	6.41	26.92
8	0830	0827	K08	KBA	7.42	26.95
9	0832	0828	K09	BAK	7.94	27.46
10	0835	0833	K10	KBA	7.16	27.97
11	0990	0992	K11	KBA	7.87	27.16
12	1408	1409	K12a	BAK	8.39	26.83
-		1424	K12b	*	7.77	26.06
13	2012	2009	K13	ABCK	7.14	25.36
14	2022	2019	K14	BAC	8.22	26.27
15	2026	2025	K15	BAK	8.93	26.90
16	2031	2033	K16	KBA	7.42	26.60
17	2037	2038	K17	KABC	5.50	26.10
18	2039	2041	K18	KBA	8.34	26.58
19	2044	-	-	BA		
20	2052	2048	K20	ABCTK	8.46	25.89
21	2058	2056	K21	KBAC	8.79	25.46
22	2064	2060	K22	ABCTK	8.41	27.56
23	2065	2067	K23	KBA	8.88	28.06
24 (*pceA*)	2398	2401	K24	ABCT	7.38	27.13
25	2792	-	-	A (truncated)	-	-

[1] Numbering of *rdhK* genes was made with respect to the *rdhA* they are adjacent to (see **Figure 14**).
[2] A star indicates *rdhK* present outside the corresponding *rdh* gene cluster.

Table S15. Calculation of fluorescence quenching for CprK1 vs. ClOHPA

ClOHPA stock (mM)	Volume (mM)	ClOHPA final (mM)	Readings	Norm. readings	Relative fluor.	Calculated fluor.
0	0	0	150900	1	1	1
0.10	6.00	1	155040	1.027435	1.03771	0.927506
0.10	6.00	2	133530	0.884891	0.902588	0.866283
0.10	6.00	3	112740	0.747117	0.769531	0.813892
0.50	2.40	5	97100	0.643472	0.665351	0.728927
0.50	3.00	7.5	85540	0.566865	0.588973	0.648746
0.50	6.00	10	76690	0.508217	0.53312	0.587782
1.00	4.50	15	68630	0.454805	0.480501	0.50121
1.00	6.00	20	60550	0.401259	0.427943	0.442688
2.50	3.60	25	65860	0.436448	0.46809	0.400485
2.50	6.00	50	53130	0.352087	0.381135	0.293479
10.00	3.00	100	45490	0.301458	0.327835	0.224248
10.00	3.00	150	44470	0.294698	0.321958	0.198054
10.00	6.00	200	33750	0.223658	0.246583	0.184283
30.00	6.00	500	20750	0.137508	0.152978	0.158264
30.00	5.00	750	14860	0.098476	0.110375	0.152255
30.00	5.00	1000	12520	0.082969	0.093686	0.149218
30.00	5.00	1250	12200	0.080848	0.091965	0.147385
30.00	5.00	1500	9980	0.066137	0.075781	0.146159
30.00	10.00	2000	9100	0.060305	0.070104	0.144622

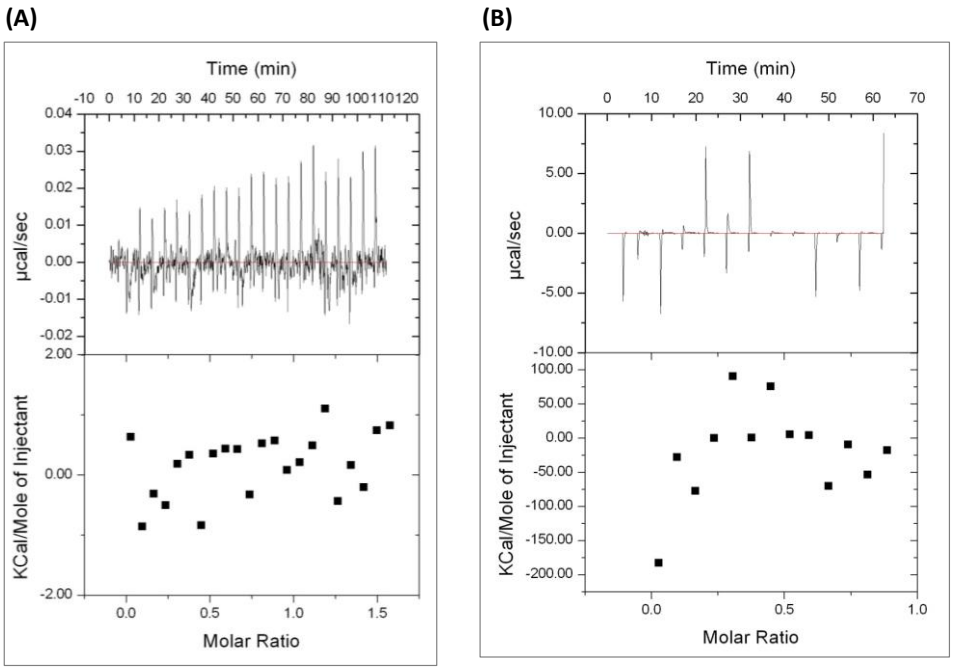
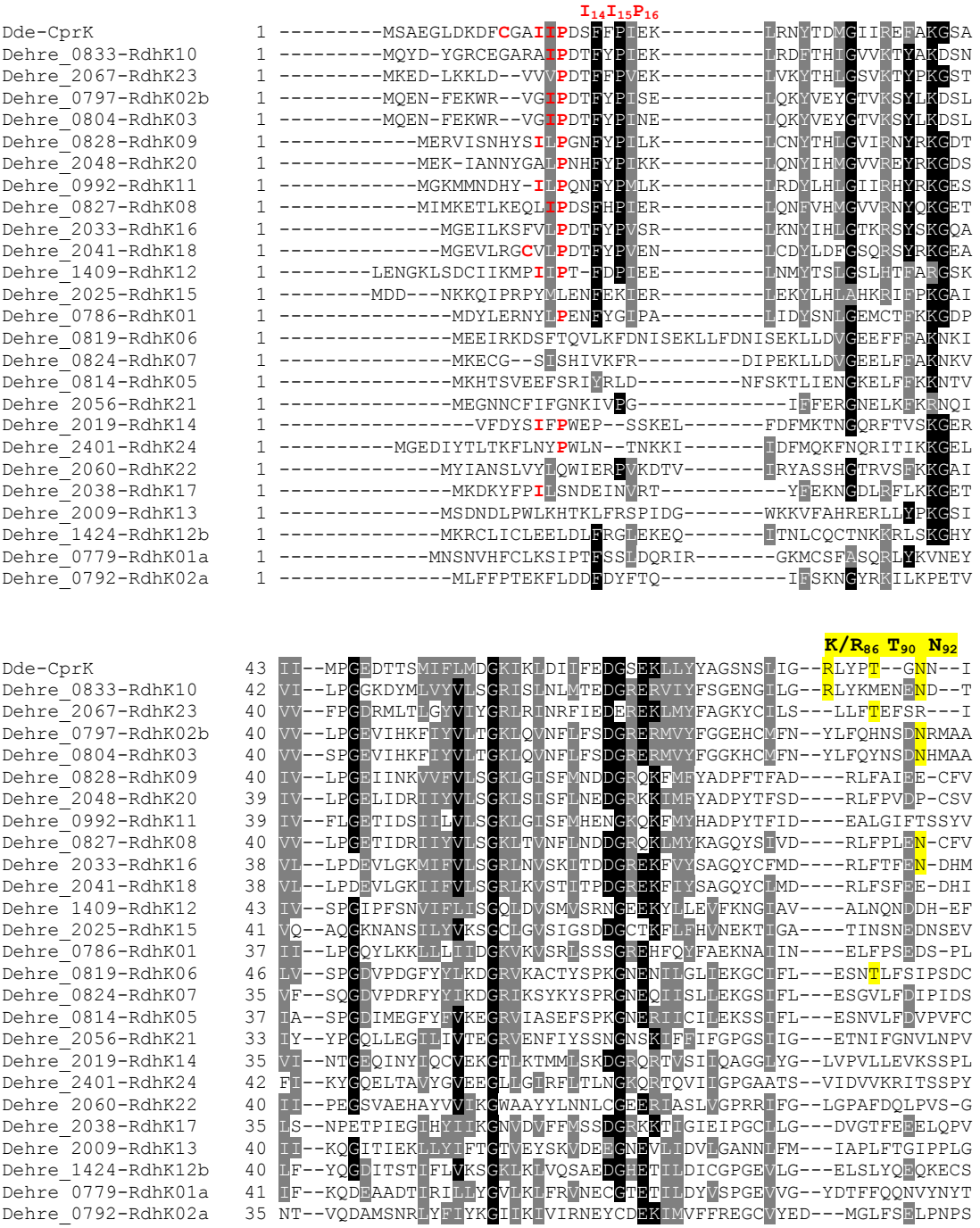


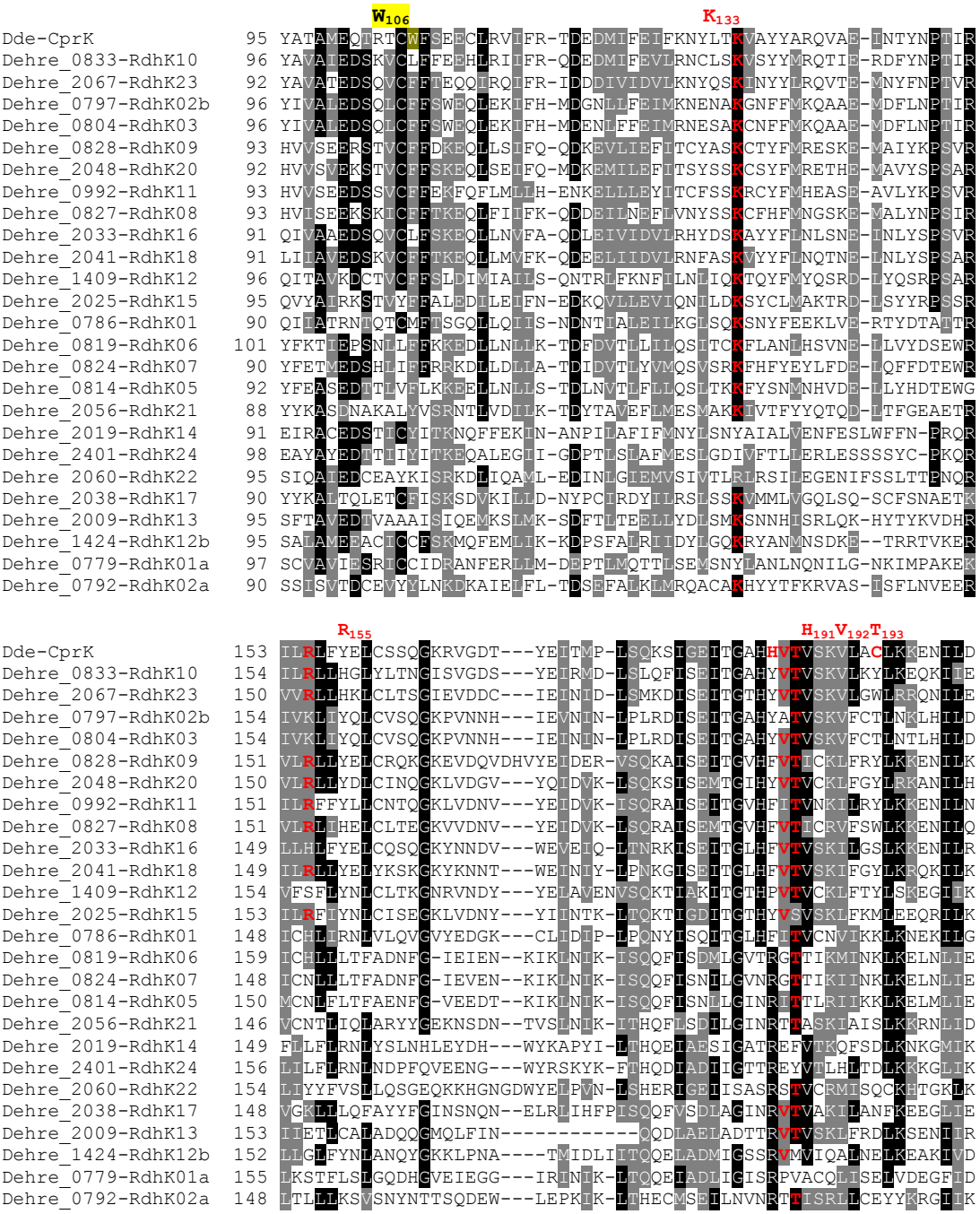
Fig S13. ITC to study interaction of CprK1 with ClOHPA in mixtures of organohalides.

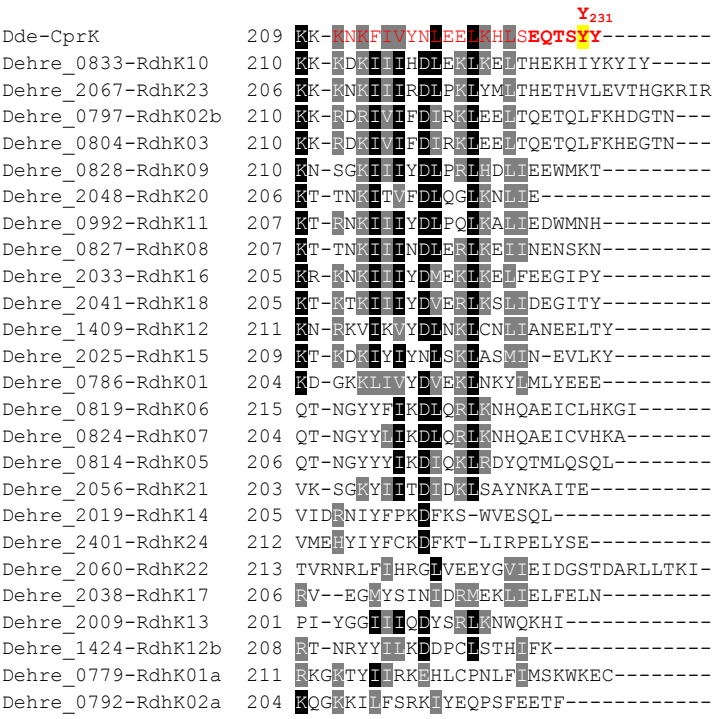
(A). Titration of CprK1 with ClOHPA and a mixture of Aromatic organohalides as; 2-, 3-, 4-chlorophenols, chlorobenzene and 1,2-dichlorobenzene (each at 100 µM final concentration). (B). Titration of CprK1 with with ClOHPA and a mixture of Aliphatic organohalides as: tetrachloroethene, 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane, 1,2-dichloroethane, dichloromethane and chloroform (each at 100 µM final concentration).

Fig S14. Alignment of RdhK in *D. restrictus* by alignment with crystal-studies derived important residues in CprK from *D. dehalogenans*.

Amino acid residues identified as important are mentioned in **Table 11**, from CprK studies and these have been highlighted with different colours in the alignment below.




W106
K133
R155
H191V192T193


Y231
Dde-CprK 209 KK-KNKFTVYNLEELKHLSEQTSYY---------
Dehre_0833-RdhK10 210 KK-KDKIILHDLEKLKELTHEKHIYKYIY-----
Dehre_2067-RdhK23 206 KK-KNKIIIRDLPKLYMLTHETHVLEVTHGKRIR
Dehre_0797-RdhK02b 210 KK-RDRIVIFDIRKLEELTQETQLFKHDGTN---
Dehre_0804-RdhK03 210 KK-RDKIVIFDIRKLEELTQETQLFKHEGTN---
Dehre_0828-RdhK09 210 KN-SGKIIIYDLPRLHDLIEEWMKT---------
Dehre_2048-RdhK20 206 KT-TNKITVFDLQGLKNLIE----------
Dehre_0992-RdhK11 207 KT-FNKIIIYDLPQLKALIEDWMNH---------
Dehre_0827-RdhK08 207 KT-TNKIIINDLERLKEIINENSKN---------
Dehre_2033-RdhK16 205 KR-KNKIIIYDVEKLKELFEEGIPY---------
Dehre_2041-RdhK18 205 KT-KTKIIIYDVERLKSLIDEGITY---------
Dehre_1409-RdhK12 211 KN-RKVIKVYDLNKLCNLIANEELTY--------
Dehre_2025-RdhK15 209 KT-KDKIYIYNLSKLASMIN-EVLKY--------
Dehre_0786-RdhK01 204 KD-GKKLIVYDVEKLNKYIMLYEEE---------
Dehre_0819-RdhK06 215 QT-NGYYFIKDLQRLKNHQAEICLHKGI------
Dehre_0824-RdhK07 204 QT-NGYYLIKDLQRLKNHQAEICVHKA-------
Dehre_0814-RdhK05 206 QT-NGYYYIKDIQKLRDYQTMLQSQL--------
Dehre_2056-RdhK21 203 VK-SGKYIITDIDKLSAYNKAITE----------
Dehre_2019-RdhK14 205 VIDSNIYFPKDFKS-WVESQL-------------
Dehre_2401-RdhK24 212 VMEHYIYFCKDFKT-LIRPELYSE----------
Dehre_2060-RdhK22 213 TVRNRLFIHRGLVEEYGVIEIDGSTDARLLTKI-
Dehre_2038-RdhK17 206 RV--EGMYSINIDRMEKLIELFELN---------
Dehre_2009-RdhK13 201 PI-YGGIIIQDYSRLKNWQKHI-------------
Dehre_1424-RdhK12b 208 RT-NRYYILKDDPCISTHIFK-------------
Dehre_0779-RdhK01a 211 RKGKTYIIRKEHLCPNLFIMSKWKEC--------
Dehre_0792-RdhK02a 204 KQGKKILFSRKIYEQPSFEETF------------

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PhD projects & Publications : (http://www.ncbi.nlm.nih.gov/pubmed/?term=rupakula+aamani)

- The restricted metabolism of the obligate organohalide respiring bacterium *Dehalobacter restrictus*: lessons from tiered functional genomics (Published, Rupakula et. al., 2013)
- Functional genomics of Corrinoid starvation in the organohalide-respiring bacterium *Dehalobacter restrictus* strain PER-K23. (Published, Rupakula et. al., 2015)
- Transcriptional regulation of Organohalide respiration in Dehalobacter restrictus strain PER-K23 (ongoing)

Areas of Experience (general & specific): Environmental Microbiology and Biotechnology, Molecular biology, Bioremediation, Biochemistry of Anaerobes, Organohalide Respiration, *Dehalobacter restrictus*, *Desulfitobacterium* spp., Corrinoid Biosynthesis and Salvage, Reductive Dehalogenases, Transcriptional regulation, Regulator proteins

Teaching Experience : Teaching assistant for Exercise and Laboratory for course "Microbiology for Engineers", EPFL and Training of apprentices.

SCIENTIFIC SKILLS

- Primer designing, Cloning, Transformation, Competent cells preparation, Sequencing
- RNA extraction, Reverse transcription, Quantitative PCR,
- DNA extraction and Optimization of method for cells with low biomass
- Heterologous Protein Expression in E.coli, protein purification, Troubleshooting,
- Gel electrophoresis (SDS, Native, gel shift mobility assays), Western blotting
- Chromatography techniques as : HPLC, affinity, gel exclusion, HIC
- Anaerobic bacterial cell cultivation
- Basic molecular biology & biochemistry techniques.
- Genomics: Functional annotation of genes and metabolic pathways
- Comparative genomics, Transcriptomics, Proteomics, Regulation of differential gene expression
- Identifying Protein-Ligand interactions using Electrophoretic mobility shift assays (EMSA), isothermal titration Calorimetry (ITC) intrinsic tryptophan fluorescence spectrometry (ITF), Mass Spectrometry.
- Organizational skills such Research project management, Methodology development & Writing scientific papers and reports

CONFERENCES & TALKS

A list of attended conferences and abstract details can be found here :http://infoscience.epfl.ch/search?ln=en&p=aamani+rupakula#

- INVITED TALK: Environmental Science Division, Rutgers, The state university oNew Jersey. (invited by Donna Fennel) "Understanding the metabolism of organohalide - respiring Dehalobacter restrictus : From genome analysis, corrinoid cofactor biosynthesis to regulation behind its prospected bioremediation substrate range." May 2014.
- TALK: DehaloCon - A Conference on Anaerobic Biological Dehalogenation, Jena, Germany, March 2014.
- POSTER: American Society of Microbiology (ASM) 2014, 114th General Meeting, Boston, Massachusetts, USA, May 2014
- TALK-POSTER: Swiss Society of Microbiology (SSM) - 72nd Annual Assembly, Fribourg, Switzerland, June 2014.
- POSTER: 71st Annual Assembly of the SSM, Interlaken, Switzerland, June 2013.
- TALK: Federation of European Microbiological Societies (FEMS) 2013, Leipzig, Germany, July 2013. VIDEO-RECORD (http://bit.ly/1AiDdDR)
- TALK-POSTER : SME 2013 : 5th Swiss Microbial Ecology Meeting, Murten, Switzerland, February 4-6, 2013.
- TALK: SSM, Joint Annual Meeting 2012, St.Gallen, Switzerland, June 2012.
- POSTER: SSM, 69th Annual Assembly, Zurich, June 2010.
- INVITED TALK: Semester Abroad Program (SAP) Scholarship Awardee, Vellore Institute of Technology, India, June 2010

LEADERSHIP EXPERIENCE & SOCIAL SKILLS

- Team-building and leadership experience in setting up and running student organisations and science associations throughout my career.
- Ability to grow and work in an interdisciplinary environment
- Problem-solving skills, Strong listening and expression skills
- Ability to initiate and foster collaborations, Maintain inter-personal skills
- Possess strong communication and Oral-presentation skills, Enjoy simplifying Science

Student Association President:

- Elected as President of the Indian Students Association, YUVA at EPFL, for the year 2013.
- Involved in Establishing and Pioneering the first-ever Indian Students Association, YUVA on EPFL University campus (http://yuva.epfl.ch/).
- Have been a volunteer and active core-team member from 2011 to March, 2014.
- Organized various Inter-Cultural events and helped facilitate incoming students into campus life.

Science Association Core Member:

- Active core-member of Science Association at N V Patel college of Pure & Applied sciences, Gujarat, India during my B.Sc Microbiology degree from June 2005-2008
- Organized competitions such as Scitoon making, Science Essays, Quiz, Crossword puzzles & updated the college notice board weekly on latest science developments.
- Got selected to represent the college in various national (India) and regional (Gujarat state) level conferences with talks and posters.

Collaborative initiatives & successes:

- Collaborated with Professor Hauke Smidt, Wageningen Univsersity, Netherlands(http://www.wageningenur.nl/en/Persons/Hauke-Smidt.htm) and his lab members (T. Kruse, Y. Lu, S. Boeren) for two projects as a part of my PhD Thesis at EPFL, Switzerland. These were successful and have been Published (http://www.ncbi.nlm.nih.gov/pubmed/?term=rupakula+smidt).

Languages:

- English (Native proficiency), French (Basic), Hindi, Telugu (Indian mother tongue, proficient),Setswana (Botswana national language, basic speaking ability)

Hobbies/Interests:

- Photography, Inter-cultural interests, Leading associations, organizing seminars and activities, Cooking, Travelling.

EDUCATION

Masters of science in Applied microbiology. — June 2008 — June 2010

Vellore Institute of Technology, India (School of Biotechnology, Chemical, Biomedical Engineering)
http://www.vit.ac.in/
Final cGPA: 8.9/10, (89%, First class)
Awards :

- Got "S" (highest) grade and 10/10 cGPA for my masters thesis research work performed in EPFL, Switzerland and its Defense-presentation VIT University, India.
- In a class of 120 students, Ranked in top 10 during M.Sc Microbiology degree.
- Won Merit-based Cash Scholarship under SAP (Semester Abroad Program), November 2009,by VIT University, India, for master's thesis research at EPFL University, Switzerland. http://info.vit.ac.in/smbs/studentsprojectabroad.asp
- Won 2nd prize in "Recycle Bin " creativity competition held at "greeneon 09" by Energy Club, VIT University ,India April 2009

Conferences :

- Participated in "International Conference on Biotechnological Solutions for Environmental Sustainability"
- Underwent a hands-on-training course of "Analytical Instrumentation" at Technology Business Incubator (HPLC, FTIR, AAS, Flame Photometry, Mass Spectrometry,UV)

Bachelor of Science (B.Sc.) in Microbiology — June 2005 — June 2008

NVPAS college, Sardar Patel University,Gujarat, India
http://nvpas.edu.in/
Final cGPA : 6.7/10 (66.7 %, First class)

- 1rst rank in second year, first semester (in a class of 120)
- 8th rank in second year, second semester

Experiences :

- Internship in the Quality and Control Department of Aurobindo Pharmacy,Hyderabad, India for 2 months
- Active member of Science Association & Science-portfolio of my college. I organized competitions such as Scitoon making, Science Essays, Quiz, Crossword puzzles & updated the college notice board weekly on latest science developments.
- National Cadet Coprs (NCC); Cadet in 4GUJ Girls Batallion, From june 2005 to April 2007. Attended Annual training camp where I won in inter-college competitions such as map reading, debate, dance & drama

Awards :

- Won 1rst Consolation Prize in a poster titled "Recent advances in treatment of Malignant Tumors"at a National-level Science Symposium held at Rajkot, Gujarat, India. 3rd Dec 2006
- Won 3rd prize in seminar competition of Nature Club on "coke controversy" at Nattubhai Vallabh College of pure and applied sciences 2006-2007
- Won 1rst prize at selection round for seminar competition of GIBion,organized by Gujarat State biotech mission and Dept of Sci&Tech,India, to represent Nattubhai Vallabh College of pure and applied sciences. 17th dec 2006
- Won 3rd prize in "best out waste creativity" competition of Nature Club, at Nattubhai Vallabh College of pure and applied sciences 2006-2007

Conferences :

- Prions (talk) National level conference, Ahmedabad.
- Transgenic Crops (talk) National level conference, Christ college, Rajkot, India.
- Coke controversy in India (case study & seminar)
- Is nanotechnology the last stop in science?(medical nanotechnology-seminar)
- Selected to represent my college at the MICRO-STUDY CIRCLE, nadiad, organised by DBT-India. Gave a talk on "A bio-scientist who fascinates me the most- – Sir Alexander Flemming"
- Selected to represent my college in Lions club, group discussion competition.

Senior-High school (AISSCE- CBSE) **May 2003 — May 2005**

Sanskriti Civil Services School, Chanakya Puri, New Delhi, India

http://www.sanskritischool.edu.in/

All India Secondary School Certificate Examination, Central Board of Secondary Education AISSCE-CBSE 11th and 12th class in Science stream (Maths, Biology, Physics, Chemistry, English) http://www.sanskritischool.edu.in/

- Represented my school in oral-oratory category at the American Embassy school competition.
- "Effect of antiseptics on the growth of bacteria"- (experimental project & presentation)
- Attended a 10 Day Rural Sensitization camp as a Research student, in Gadhwal district, Uttaranchal, India.
- Organized Stage- Dramas for the school Assembly
- Participated in Debates, Elocution & Essay writing competitions.

GCSE CAMBRIDGE "O" LEVELS, High school **January 2002 — December 2002**

Mpepu college, Molepolole, Botswana, Africa

http://www.localbotswana.com/company/8100/Mpepu_Private_Sec_School_eng_Med

Botswana General Certificate of Secondary Education (BGCSE) Cambridge "O" Level

- A+ GRADE in overall
- "Why do we look like we do"-(poster –research category, case study-10th class)

Awards :

- Ranked 5th throughout my school in Cambridge "O" Levels.
- Won 1rst Prize in a project "variations among humans" in Research category,representing Motswasele CJSS, at South Central Regional Science & Mathematics Fair, Botswana Science Association. 2000
- Merit award for " being the most achieving member of the maths and science club", at Mpepu senior secondary school, Molepolole, Botswana, Africa . 20th September 2002
- Merit award for position 1 in computer studies and geography, and position 2 in agriculture, at Mpepu senior secondary school, Molepolole, Botswana, Africa. 20th September 2002

All Academic-References Available at :

https://www.dropbox.com/s/6lx0j2ywdgobaf6/A.Rupakula_References.pdf?dl=0