DISCOVERING THE ORGANOHALIDE RESPIRING CAPACITY OF DELTAPROTEOBACTERIA IN MARINE AND ESTUARINE ENVIRONMENTS

By

JIE LIU

A dissertation submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Microbial Biology

Written under the direction of

Max M. Häggblom

And approved by

_____

_____

_____

_____

New Brunswick, New Jersey

January, 2019

ABSTRACT OF THE DISSERTATION

Discovering the organohalide respiring capacity

of *Deltaproteobacteria* in marine and estuarine environments

by JIE LIU

Dissertation Director:

Max M. Häggblom

Organohalides are widespread in the environment from both anthropogenic and natural sources. The marine environment is a major reservoir of organohalides, particularly organobromides. Organohalide respiration is the process mediated by bacteria that utilize organohalides as electron acceptors for energy conservation. This process is of importance in the degradation organohalide pollutants and the overall organohalide cycle. The objective of this study was to investigate the ecophysiology of organohalide respiring bacteria (OHRBs) with a focus on examining their utilization of organobromine compounds, evaluating their substrate range and activity under different conditions, and determining their reductive dehalogenase gene diversity and gene expression.

Investigation of *Desulfoluna spongiiphila*, a marine sponge associated

organobromide-respiring bacterium, revealed that the expression of one reductive dehalogenase gene was significantly upregulated in response to bromophenol and sponge extracts. The transcriptomic and proteomic analysis further confirmed the function of this reductive dehalogenase gene in reductive dehalogenation of bromophenol. Two bacterial strains with organobromide respiring ability were isolated from New Jersey estuarine sediments, which are representatives of a novel *Halodesulfovibrio* species. Three reductive dehalogenase genes are present in their genomes, one of which showed significant upregulation in gene expression in response to bromophenols. In addition, the overall organohalide respiring capacity of members of the Class *Deltaproteobacteria* was evaluated by surveying the genome database for the prevalence of reductive dehalogenase genes in their genomes. Result showed that approximately 10% of *Deltaproteobacteria* contain reductive dehalogenase genes in their genomes, which is an indicator for potential organohalide respiring ability. Three of these strains were experimentally confirmed to have organobromide respiring ability. In total, six organobromide respiring *Deltaproteobacteria*, all marine or estuarine origin, were investigated in this study. One common reductive dehalogenase gene cluster (namely *brp* gene) was present in all the genomes and the expression of *brpA* gene was significantly upregulated in the presence of bromophenols in all the strains. This study highlights the potential role of *Deltaproteobacteria* in the marine organohalide cycle.

Acknowledgement and Dedication

I would like to give my greatest gratitude to my advisor, Prof. Max Häggblom for all his guidance, encouragement and support for my study and future career path. He respected my research ideas, no matter they were realistic or not; he provided me suggestions and solutions when I was stuck in my project and he inspired me to think more comprehensively. I would like to extend this gratitude to my thesis committee Prof. Lee Kerkhof, Prof. Costantino Vetriani, Prof. Donna Fennell for their assistances and valuable comments on my research projects and dissertation.

I would thank Prof. Lorenz Adrian (Helmholtz Centre for Environmental Research) for his assistance in proteomic analysis and Prof. Ute Hentschel (GEOMAR Helmholtz-Zentrum für Ozeanforschung Kiel) for providing me sponge samples. I appreciate Lora McGuinness for her helps in TRFLP analysis and Dr. Young-Beom Ahn, Dr. Isabel Horna-Gray and Nora Lopez for their precedent work in sponge project.

I would like thank Microbial Biology Graduate Program committees for enrolling me as a PhD student and Woodruff Fellowship for supporting me in my first year. Also, I thank all the funding sources that have supported my travels to scientific conferences.

I would like appreciate Department of Biochemistry and Microbiology for providing me a Teaching Assistant position. Teaching is one of important parts in my graduate life. I would like to thank the Biochemistry Teaching Group, especially Dr.

Voloshchuk for her trusts in me as her TA and Dr. Kahn for his generosity to take our TAs for nice lunches.

I would like to thank people in Häggblom Lab, especially Tiffany + Toffee, Hang, Preshita + Pebbles and Michelle for their helps in and out of lab. Also, I would like to thank the administrative staff in Lipman Hall.

Thanks to my friends that I have met at Rutgers, especially Lele for her ccompany.

I would like to thank my family. Thanks to my husband Yuanjun for his support and our parents for their unconditional love.

This dissertation is dedicated to my son, Keming, who has made my last year of graduate study so special.

Preface

Chapter two has been published as Liu, J., Lopez, N., Ahn, Y., Goldberg, T., Bromberg, Y., Kerkhof, L.J., and Haggblom, M.M. (2017) Novel reductive dehalogenases from the marine sponge associated bacterium Desulfoluna spongiiphila. Environ Microbiol Rep 9: 537-549. Jie Liu was responsible for writing the manuscript. conducting experiments for Illumina genome sequencing, gene expression and data analysis.

Chapter five has been accepted by mBio as Liu J, Häggblom MM (2018) Genome guided identification of organohalide-respiring Deltaproteobacteria from the marine environment. Jie Liu was responsible for writing the manuscript and conducting the experimental work.

Table of Contents

List of Figures

List of Tables

Chapter 1 Introduction

1. Organohalides

1.1 General knowledge of organohalides

Organohalides constitute a large group of chemicals that contain one or more halogen (F, Cl, Br, or I) substituents on the carbon skeleton (Fig. 1.1). These chemicals are of importance to human society because of their beneficial applications in industry and agriculture, e.g. chlorinated solvents and biocides (Häggblom and Bossert, 2003). However, the extensive use of organohalides brings increasing concern over their adverse effects to the environment and human health (Alaee et al., 2003; Darnerud, 2003; Crinnion, 2009; Ni et al., 2010; Covaci et al., 2011). The book "Silent Spring" by Rachel Carson in 1962 brought this concern to the public for the first time, which led to a nationwide ban on the use of DDT (dichlorodiphenyltrichloroethane) in agriculture (Turusov et al., 2002). The development of more sensitive analytical techniques also reveals the widespread occurrence of industrial organohalides in the environment at trace level contaminants (Santos and Galceran, 2002). Although many organohalides are commonly considered as anthropogenic contaminants, there is evidence showing that a large amount of organohalides are from natural biogenic sources, e.g. bacteria, fungi and sponges, or geogenic sources, e.g. volcanic emissions (Teeyapant et al., 1993; Teeyapant and Proksch, 1993; Pée, 1996; Gribble, 2000; Lincoln et al., 2005; Gribble, 2010). The chemical properties and toxicity of organohalides are highly impacted by

their halogen substituents, which generally increase its lipophilicity (Häggblom and Bossert, 2003). The increase in lipophilicity causes the organohalides to absorb on to soil or sediment in the environment and decreases their bioavailability in the environment. Although many organohalides are considered persistent organic pollutants, the biodegradation of organohalides can occur under both aerobic and anaerobic condition. Reductive dehalogenation is a microbial mediated process to remove halogen substituents under anaerobic conditions, which usually facilitate the further biodegradation of dehalogenating products under aerobic conditions.

1.2 Anthropogenic sources and contamination to environment

Organohalides are released into the environment as contaminants due to their wide usage in industry, agriculture and households since the last century. Chlorinated solvents including perchloroethene (PCE), 1,1,1-trichloroethane (TCA), trichloroethene (TCE) are major halogenated contaminants that have been frequently detected in the environment (Moran et al., 2007). The use of many chlorinated pesticides, DDT as an example, has caused severe environmental concern and several of these compounds have since been banned in the US and many other countries (Turusov et al., 2002). However, the adverse effects of these chlorinated pesticides to the environment and human health are still problematic because of their resistance to biodegradation and bioaccumulation in fatty tissue (Crinnion, 2009). Brominated flame retardants (BFRs), including tetrabromobisphenol A (TBBPA), polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD) comprise another

set of industrial produced organohalides (Darnerud, 2003). Similar as with chlorinated pesticides, the use of some BFRs has been restricted due to their persistence to biodegradation. The application of emerging "novel" BFRs as substitutions for the banned BFRs requires more studies for their environmental behavior and potential risks to human health (Covaci et al., 2011).

1.3 Natural sources of organohalides

Besides anthropogenic sources, a variety of organohalides are produced by natural processes. To date, over 5,000 naturally produced organohalides have been identified, the majority of which are chlorinated and brominated compounds (Gribble, 1992, 2010). Marine environments are the greatest source for the natural organohalide production. Around 15- 20% of the newly discovered marine natural products are halogenated compounds (Gribble, 2010). Living organisms, such as sponges, algae, fungi, bacteria and insects, are biogenic sources of halogenated compounds (Teeyapant and Proksch, 1993; Lincoln et al., 2005; Gribble, 2010; Lira et al., 2011). The most abundant organohalides from natural sources are bromine-containing compounds produced by marine organisms because of the higher content of bromine in the marine than terrestrial environment (Pée, 1996; Gribble, 2000). The terrestrial organisms preferentially produce chlorinated compounds, many of which are found from fungi (Pée, 1996). Many of the biogenetically produced organohalides show antibiotic and antimicrobial activity, which may be produced by the organism as a chemical defense (Gribble, 2010).

2. Organohalide respiration

2.1 Thermodynamic consideration of organohalide respiration

Organohalide respiration is the process in which bacteria respire halogenated compounds as terminal electron acceptors for energy conservation under anaerobic conditions. During this process, the halogen substituent is replaced with a hydrogen atom by reductive dehalogenase enzymes (Fincker and Spormann, 2017) There are two kinds of organohalide respirations, hydrogenolysis and dihaloelimination (Adrian and Löffler, 2016) (Fig. 1.2). Organohalide respiration via hydrogenolysis results in the formation of one mole of HX (X is Cl, Br, F or I). Dihaloelimination requires the presence of two vicinal halogens to form an unsaturated carbon bond and forms two moles of HX (Fig. 1.2). These organohalide respiration reactions are thermodynamically favorable with Gibbs free energies of -120 to -179 kJ/Cl removed (Dolfing, 2016). As a terminal electron acceptor, the redox potential of an organohalide is about 210 - 600 mV, which is comparable to the redox potential of NO_3^- /NO_2^- and much higher than the redox potential for the SO_4^{-2}/HS^- couple (Dolfing, 2016). This means that organohalide respiration is more energetically favorable than sulfate reduction. Although the energy yield from organohalides respiration is sufficient to produce approximately 2 ATP molecules (with the assumption that 70 kJ is required for making one molecule of ATP), biomass yield per mole of chloride released for cell growth is generally low. The typical yield of is in the range of 2-4 g

cell dry weight per mole Cl (Mayer-Blackwell et al., 2016; Fincker and Spormann, 2017).

2.2 Phylogenetic diversity of organohalide-respiring bacteria

Since the first description of an organohalide-respiring bacterium, *Desulfomonile tiedjei*, was reported for dehalogenation of 3-chlorobenzoate Shelton and Tiedje, 1984; DeWeerd et al., 1990), numerous OHRB strains that are spread among distinct phyla have been isolated (Atashgahi et al., 2016). These bacterial phyla are the Firmicutes, Chloroflexi and *Proteobacteria* (Fig. 1.3). Based on their metabolic versatility, OHRBs can be classified as either obligate or facultative organohalide respirers (Maphosa et al., 2010; Hug et al., 2013). The obligate OHRBs, including Chloroflexi and *Dehalobacter* in the Firmicutes, have a restricted metabolism to organohalides. In contrast, facultative OHRBs, including *Proteobacteria* and *Desulfitobacterium* strains in the Firmicutes, can utilize electron acceptors such as sulfate and nitrate in addition to organohalides. The book "Organohalide-respiring bacteria" published in 2016 gives detailed descriptions of the physiology and substrate range of each OHRBs isolated before 2014 (Adrian and Löffler, 2016). Additionally, there are more *Dehalococcoides* sp. strains and *Dehalobacter* sp. strains isolated or enriched from diverse environments, such as *Dehalobacter* sp. UMSWDHB capable of respiring chloroform and chlorinated ethane from soil and *Dehalobacter* sp. strain TeCB1 isolated from polluted groundwater for

chlorobenzene respiration (Wong et al., 2016b; Alfán-Guzmán et al., 2017; Yohda et al., 2017).

OHRBs in the Chloroflexi includes members of the genera *Dehalococcoides*, *Dehalogenimonas*, and *Dehalobium*. Of these, *Dehalococcoides maccartyi* strains are the most well studied OHRBs and comprise the largest OHRB group (Löffler et al., 2013; Zinder, 2016). This is mainly because of their highly specialized organohalide respiring ability and great potential in organohalide bioremediation of organohalide-contaminated sites (Saiyari et al., 2018). *Desulfitobacterium* strains are also of high interest in the study of organohalide respiration because of their high growth rate and versatile metabolism (Villemur et al., 2006; Kruse et al., 2017b). OHRBs in *Deltaproteobacteria* are diverse and found in several genera, including *Anaeromyxobacter*, *Desulfuromonas*, *Geobacter*, *Desulfoluna*, *Desulfovibrio* and *Desulfomonile* (Sanford et al., 2016) (Table 1). OHRBs are also found in *Shewanella* in *Gammaproteobacteria* and *Sulfurospirillum* in *Epsilonproteobacteria*. The metabolism of *Proteobacteria* OHRBs are versatile with utilization of diverse electron acceptors including sulfate, sulfite, nitrate.

2.3 Dehalogenating range of OHRBs

Organohalides can be divided into aromatic and aliphatic organohalides based on their carbon structure. So far, the majority of OHRB were isolated for their dehalogenating ability for chloroethenes, including *Dehalococcoides* (Zinder, 2016), *Dehalobacter* (Wong et al., 2016a; Alfán-Guzmán et al., 2017), *Desulfitobacterium*,

Desulfuromonas (Sung et al., 2003), *Geobacter* (Sung et al., 2006) and *Sulfurospirillum* (Luijten et al., 2003) strains. The intensive studies on chloroethene dehalogenation are driven by the urgent demand for bioremediation of chloroethene contamination (Hendrickson et al., 2002; Bradley, 2003). Some OHRBs have the ability to respire aromatic organohalides such as chlorobenzene, chlorophenol, bromophenol and PCBs. *Anaeromyxobacter dehalogenans* and *Desulfitobacterium* strain PCP are able to respire chlorophenols (Sanford et al., 2002; Villemur, 2013) and *Desulfomonile* strains are found to respire chlorobenzoates (DeWeerd et al., 1990; Sun et al., 2001). The position of the halogen substitution affects its accessibility for OHRB. The dehalogenating activity of *Desulfomonile tiedjei* DCB-1 and *Desulfomonile limimaris* is specific to the meta-position of chlorobenzoates (DeWeerd et al., 1990; Sun et al., 2001). As we see, most of the OHRB isolates have been obtained using organochlorides. Very limited studies focused on organobromides. Although debrominating activity have been reported in anaerobic environmental samples (Häggblom and Young, 1995; Monserrate and Häggblom, 1997; Rhee et al., 2003; Chang et al., 2012; Yang et al., 2015; Zhao et al., 2018), only three OHRBs, *Desulfoluna spongiiphila*, *Desulfovibrio* sp. TBP-1 were originally isolated from an enrichment culture on organobromides (Boyle et al., 1999; Ahn et al., 2009; Chen et al., 2013). Other OHRBs, isolated originally on chlorinated compounds, including *Anaeromyxobacter dehalogenans* strains, *Dehalococcoides mccartyi* strain CBDB1, *Desulfitobacterium* sp. PCE-S and *Sulfurospirillum multicorans* are also reported with debrominating activity (Sanford et al., 2002; Ye et al., 2010; Yang et al., 2015).

3. Molecular and chemical basis or organohalide respiration

3.1 Reductive dehalogenase gene

Organohalide respiration is catalyzed by the enzyme reductive dehalogenase (referred to as RDase or Rdh in some studies) (Maphosa et al., 2010). The gene cluster encoding for reductive dehalogenase (RDase gene or *rdh* for reductive dehalogenase homologous genes) commonly contains a *rdhA*, *rdhB* and other accessory genes that may involve in transcriptional regulation and maturation (Hug et al., 2013; Fincker and Spormann, 2017). The *rdhA* genes encoding for the catalytic RDase A share conserved domains and motifs, including a twin-arginine (TAT) signal motifs (RRXFXK) at the N terminal and two iron-sulfur cluster binding motifs at the C terminal (Jugder et al., 2015) (Fig. 1.4). Putative *rdhB* genes encoding for a membrane anchoring protein for RDase A are usually associated with the *rdhA* gene. The order of *rdhA* and *rdhB* (*rdhAB* or *rdhBA*) varies in different *rdh* gene clusters. However, *rdhA* genes without an associated *rdhB* are also found in the genomes of some OHRBs, such as *Dehalogenimonas lykanthroporepellens* BL-DC-9T (Magnuson et al. 2000).

Although the expression of some *rdh* genes is inducible by organohalides, little is known about the mechanisms of *rdh* gene regulation. The available *rdh* gene cluster compositions indicate that the regulatory mechanism may vary according to the OHRB phylogeny rather than the metabolic versatility of bacterium. The *rdh* gene operon in Firmicutes OHRBs, *Desulfitobacterium* and *Dehalobacter*, are usually associated with *rdhK*, which encodes transcriptional regulators of the CRP/FNR family (Smidt et al., 2000; Joyce et al., 2006; Mazon et al., 2007; Levy et al., 2008).

OHRBs in *Dehalococcoidia* class commonly have *rdh* gene clusters associated with genes encoding for a MarR regulator or two-component signal transduction (Seshadri et al., 2005; Wagner et al., 2013; Krasper et al., 2016). The regulatory mechanisms of *rdh* genes in *Proteobacteria* have not been studied yet.

3.2 Reductive dehalogenase enzyme

Our understanding of RDases is technically limited because of the low growth rate of OHRB, sensitivity to oxygen and lack of genetic tools for heterologous expression of RDases (Jugder et al., 2016a). The presence of the TAT motif in *rdhA*, the presence of *rdhB* in its vicinity and RDase A activity found in the membrane fraction during purification provide evidence for the location of RDase in the outer side of the cytoplasmic membrane (Ni et al., 1995; Neumann et al., 1996; Maillard et al., 2003). Heterologous expression of RDases with activity is reported to be challenging (Jugder et al., 2016a). Heterologous expression of RDase A from *Dehalobacter restrictus* (Sjuts et al., 2012; Jugder et al., 2018), *Desulfitobacterium hafniense* (Mac Nelly et al., 2014), and *Dehalococcoides mccartyi* (Parthasarathy et al., 2015) have been reported. Together with RDases directly purified from OHRBs, these RDases have been characterized with different substrate ranges as summarized by Fincker and Spormann (2017). More than half of these RDases have affinity to aliphatic organohalides, including PCE, TCE, PCA and TCA. The rest are for aromatic organohalides, including chlorophenol, chlorobenzoates and chlorobenzene. Most RDases are dechlorinases with specificity for chlorinated organohalides. BhbA from

Comamonas sp. 7D-2, NpRdhA from *Nitratireductor pacificus* are known debrominases for 3,5-dibromo-4-hydroxybenzoate. NpRdhA also showed activity for dibromophenol, dichlorophenol, 3,5-dichloro-4-hydroxybenzoate and 3,5-diiodo-4-hydroxybenzoate (Payne et al., 2015). There is no correlation between the RDaseA substrate specificity and the RDaseA phylogenetic origin. RDases of similar function may come from divergent phylogenetic origins. Example for this is the RDase capable of dehalogenating PCE (PceA) were found in *Dehalococcoides* (Magnuson et al., 1998), *Desulfitobacterium* (Miller et al., 1998), *Dehalobacter* (Maillard et al., 2003) and *Sulfurospirillum* (Neumann et al., 1996) species. On the contrary, RDases with similar sequences may have different substrate specificity, as exemplified by DcrA and Cfra from two *Dehalobacter* spp. who share 95% amino acid sequence similarity. CfrA dechlorinates chloroform and 1,1,1-trichloroethane but not 1,1-dichloroethane; DcrA dechlorinates 1,1-dichloroethane but not chloroform and 1,1,1-trichloroethane (Tang and Edwards, 2013).

3.3 Cobalamin as a cofactor

Corrinoids are cofactors for three families of enzymes, including cobalamin-dependent methyltransferases, adenosylcobalamin dependent isomerases and reductive dehalogenases (Banerjee and Ragsdale, 2003). The crystal structures of NprdhA from *Nitratireductor pacificus* pht-3B and PceA from *Sulfurospirillum multivorans* reveal the involvement of the corrinoid in the active center (Bommer et

al., 2014; Payne et al., 2015). However, how corrinoids catalyze the organohalide reduction in RDases is still not clear.

A comparative genomics study showed that 76% of genome sequenced bacteria have corrinoid-dependent enzymes but only 39% of them contain the complete corrinoid biosynthesis pathway in their genomes (Zhang et al., 2009b). OHRBs require corrinoids to carry out the organohalide respiring process, however, many of them need to obtain the corrinoid from their environment instead of *de novo* biosynthesis (Moore and Escalante-Semerena, 2016). *Dehalococcoides* spp. are obligate OHRBs whose growth are dependent on organohalide respiration; however, they cannot synthesize corrinoid *de novo* but obtain it from the environment or co-cultures such as in co-culture with *Geobacter lovleyi* (Yan et al., 2012; Yi et al., 2012; Men et al., 2014). Some facultative OHRBs including *Sulfurospirillum* spp., *Geobacter lovleyi* and *Desulfitobacterium* spp. encode complete corrinoid biosynthesis pathways and are able to produce the corrinoid *de novo* (Nonaka et al., 2006; Kim et al., 2012; Choudhary et al., 2013). *Sulfurospirillum multivorans* strains was shown to be a producer for unusual cobamides (Keller et al., 2014; Schubert, 2017).

Many organisms utilize cobalamin riboswitch as regulators for corrinoid metabolism. Cobalamin riboswitch were also found and characterized in *Desulfitobacterium hafniense* strains (Choudhary et al., 2013) and *Dehalobacter redtrictus* (Rupakula et al., 2015). Studies on *Desulfitobacterium hafniense* and *Dehalobacter restrictus* show that transcription of some corrinoid biosynthesis or transport genes are upregulated upon corrinoid starvation or repressed after adding

corrinoid into corrinoid starving culture (Choudhary et al., 2013; Rupakula et al., 2015).

3.4 Electron transport chain

Organohalide respiration involves electron transfer from an electron donor to the terminal RDase and energy is preserved through oxidative phosphorylation (Wang et al., 2018). The mechanism of electron transfer in organohalide respiratory chains varies in different OHRBs, which can be classified into quinone-dependent electron and proton shuttling within the membrane vs. quinone independent electron transport chains (Schubert et al., 2018). The presence of (meta-)quinone has been confirmed in *Desulfomonile tiedjei* (Louie and Mohn, 1999), *Dehalobacter restrictus* (Schumacher and Holliger, 1996; Holliger et al., 1998; Kruse et al., 2013), *Dehalobacterium dehalogenans* (Kruse et al., 2015) and *Sulfurospirillum multivorans* (Scholz-Muramatsu et al., 1995). Their organohalide respiring activity is inhibited by the presence of the quinone antagonist 2-n-heptyl-4-hydroxyquinoline N-oxide (HQNO), which further indicates the involvement of quinones in organohalide respiratory chains in these OHRBs (Schubert et al., 2018). In contrast, studies on *Dehalococcoides* and *Dehalogenimons* species indicate that quinones appear not to be involved in electron transfer chain for organohalide respiration (Jayachandran et al., 2004; Moe et al., 2009; Schipp et al., 2013). It is hypothesized that Hup hydrogenase, complex iron-sulfur molybdoenzyme may associate with RDase for electron transfer (Kublik et al., 2016). More studies were done in order to understand this unique

electron transfer pathway in *Dehalococcoides* as summarized in Schubert, 2018 (Schubert et al., 2018).

4. Overall goals and objectives

This study focuses on the organohalide respiring bacteria with debrominating activity in the *Deltaproteobacteria* group. The overall goal of this study was to investigate the ecophysiology of OHRBs with organobromide respiring ability in terms of their dehalogenating range and activity under different conditions, and to determine their reductive dehalogenase gene diversity and gene expression. Three specific objectives are presented as below.

1. To study the metabolism related to organohalide respiration in *Desulfoluna spongiiphila* strain AA1, an organobromide-respiring *Deltaproteobacteria* isolated from a marine sponge, using both traditional molecular techniques and 'omic' techniques.

Specific aims were to 1) study its organohalide respiring range, reductive dehalogenase gene expression in response to bromophenols; 2) using transcriptomic and proteomic analysis to investigate the gene and protein expression profile during organohalide respiration and to determine possible elements involved in electron transport chain.

2. To evaluate the organohalide respiring capacities of *Deltaproteobacteria* using sequenced genomes and experimental validation.

Specific aims were to 1) to survey the prevalence of reductive dehalogenase genes in *Deltaproteobacteria* genomes as an indicator for organohalide respiring capacity using the available genome database, 2) to identify and characterize new OHRBs guided by the presence of reductive dehalogenase genes in their genomes and study their reductive dehalogenase gene expression.

3. To isolate characterize novel organobromide-respiring bacteria from estuarine sediment.

Specific aims were to 1) set up microcosms enriched by bromophenols and purify novel bacteria with debrominating activity; 2) to characterize the new isolated OHRBs and study their reductive dehalogenase gene expression.

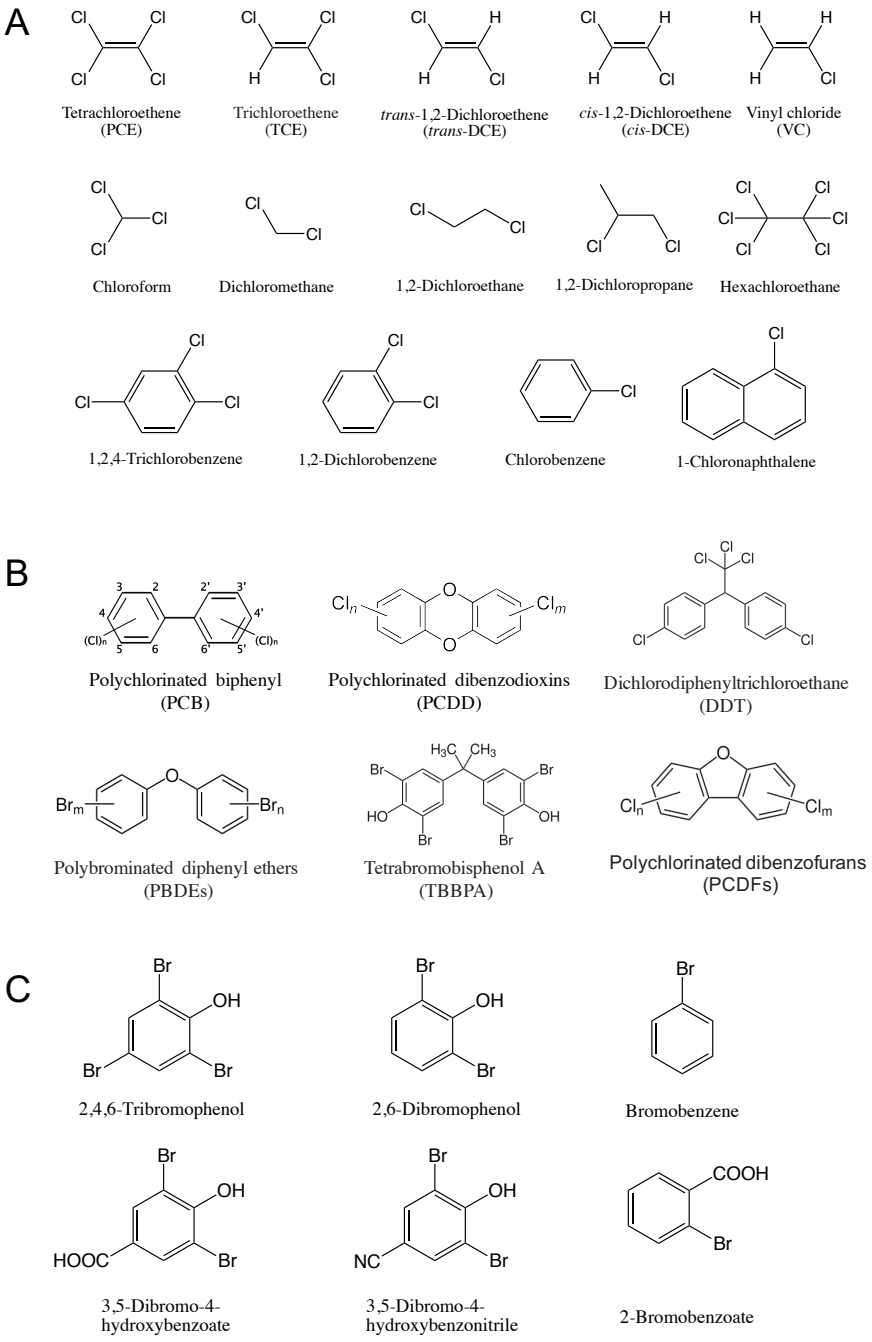
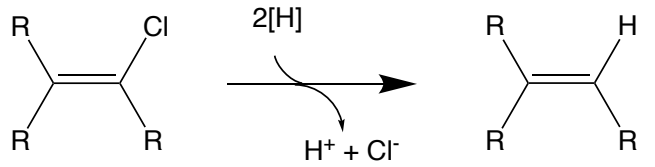


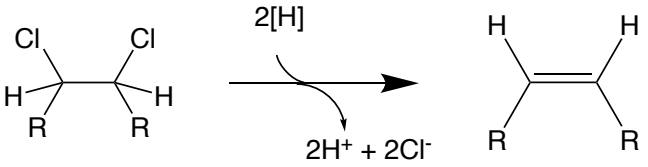
Figure 1.1 Chemical structures of chlorinated solvents commonly used in industry (A), organohalide contaminants (B), and brominated compounds utilized by known OHRBs (C).

A **Reductive dehalogenation**



$\Delta G^{0'}$ = -120 to -179 kJ/mol Cl^- removed

B **Dihaloelimination**



$\Delta G^{0'}$ = -100 to -115 kJ/mol Cl^- removed

Figure 1.2 Two kinds of organohalide respiration reactions: reductive dehalogenation (A) and dihaloeliminaton (B).

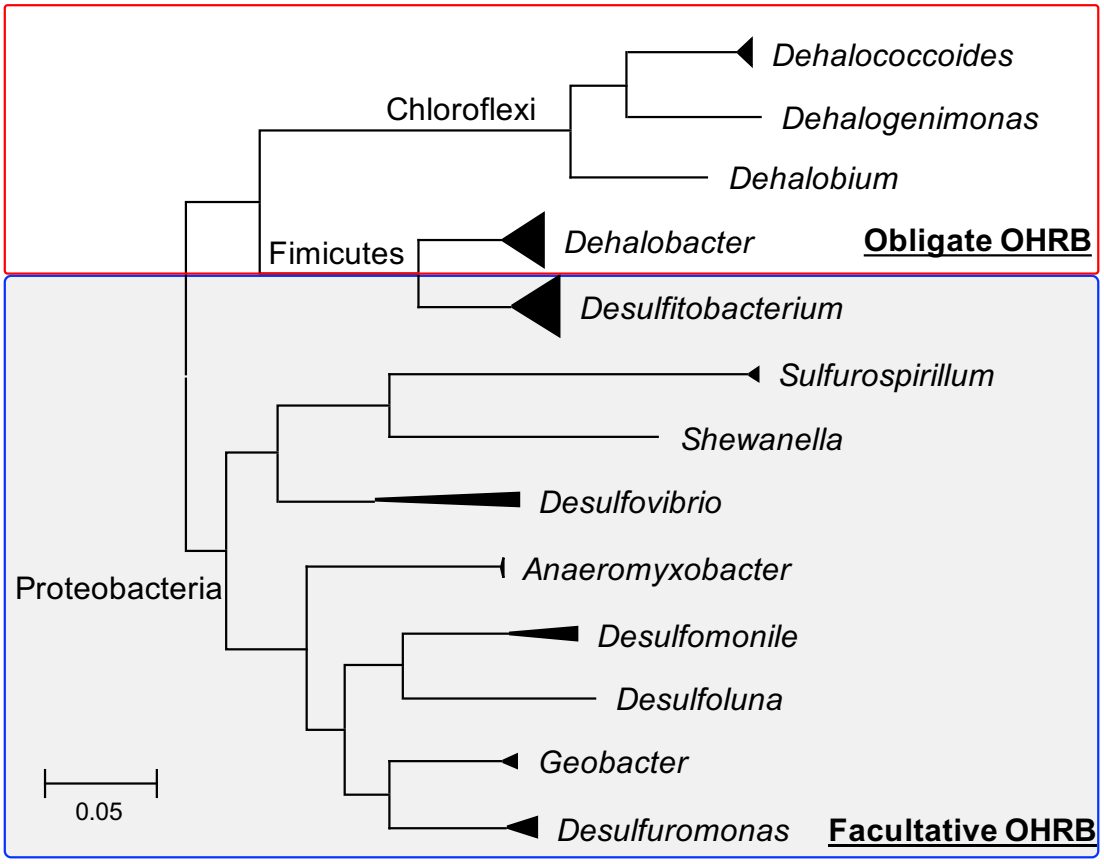


Figure 1.3 The phylogenetic relationship of genera reported to contain OHRBs based on 16S rRNA sequence. This simplified tree doesn't include all OHRBs strains but covers all known genera.

Table 1.1 The information of OHRBs belonging to the *Deltaproteobacteria* group.

Strain	Initial isolation enrichment	Dehalogenating activity	Tested but not dehalogenate	Other electron acceptors	Source	Reference
Anaeromyxobacter dehalogenans[a]	2-CP	2-CP; 2,5-DCP; 2,6-DCP; 2,4,6-TCP; 2-BP	3-CP; 4-CP; 2,3-DCP; 3-C-4-HBA; 2,4,6-TCP; 2,3,5-TCP; 2-FP; 2-IP; 3-Cl-anisaldehyde	Nitrate; oxygen; Fumarate; Fe(III)	soil or sediment	Sanford et al., 2002; He and Sanford, 2003
Desulfoluna spongiiphila AA1	2-BP	2-, 3-, 4-BP; 2,6-DBP, 2,4,6-TBP, 3,5-DB-4-HBA, 2-IP, 2-B-4-FP	2,6-DBP; BBA; 2-, 3-, 4-CP; FP isomers	sulfate; sulfite; thiosulfate	marine sponge	Ahn et al., 2003
Desulfovibrio sp. TBP-1	2,4,6-TBP	2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP	2-, 3-, 4-CP; 2,4-DBP; 2,4,6-TCP; 2-, 3-, 4-FP; 2-, 3-, 4-IP; 2-, 3-, 4-BBA; 2-, 4-CBA; 2,4-DCBA	sulfate	estuarine sediment	Boyle et al., 1999
Desulfomonile tiedjei DCB-1[b]	3-CBA	3-CBA; 3,5-DCBA; PCP	2-, 3-, 4-CP; 2,4-DCP; 2,6-DCP	sulfate; sulfite; thiosulfate	sewage sludge	DeWeerd et al.,1990; Mohn and Kennedy, 1992
Desulfomonile limimaris DCB-F	3-CBA			sulfite; sulfate; thiosulfate; nitrate; fumarate	marine sediment	Sun et al., 2001
Desulfomonile limimaris DCB-M[c]	3-CBA	3-CBA; 3-BBA; 2,3-DCBA; 2,5-DCBA; 3,5-DCBA; 2,3,5-TCBA	2-CBA; 3-C-4-HBA; 2-CP; 3-CP; 2,3-DCP; 3,4-DCBA; 2,4,6-TCBA	sulfite; sulfate; thiosulfate; nitrate; fumarate;	marine sediment	Sun et al., 2001
Desulfovibrio dechloroacetivorans SF3[d]	2-CP	2-CP; 2,6-DCP	2-BP; 3-, 4-CP; 2,3-DCP; 2,4-DCP; 2,5-DCP	sulfate; sulfite; thiosulfate; nitrate; fumarate	San Francisco Bay sediment	Sun et al., 2000
Desulfuromonas chloroethenica TT4B	TCE	PCE; TCE		polysulfide; Fe(III)	contaminated freshwater sediment	Krumholz, 1997
Desulfuromonas michiganensis BB1[e]	PCE	PCE; TCE			pristine river sediment	Sung et al., 2003
Desulfuromonas michiganensis BRS1[e]	PCE	PCE; TCE		fumarate; Fe(III)	contaminated aquifer material	Sung et al., 2003
Geobacter thiogenes K1[f]	TCA	TCA		Fe(III)	anaerobic soil	De Wever et al., 2000; Nevin et al., 2007
Geobacter lovleyi SZ[g]	PCE	PCE		nitrate; fumarate; Fe(III); malate; Mn(IV); U(VI); sulfur	creek sediment	Sung et al., 2006

n-CP for n-chlorophenol; n,n-DCP for n,n-dichlorophenol; n,n,n-TCP for n,n,n-trichlorophenol; n-BP for n-bromophenol; n,n-DBP for n,n-dibromophenol; n,n,n-TBP for n,n,n-tribromophenol; FP for fluorophenol; IP for iodophenol; 3,5-DB-4HBA for 3,5-

dibromo-4-hydroxybenzoate; 3,5-DB-4HBN for 3,5-dibromo-4-hydroxybenzonitrile; 3-C-4HBA for 3-chloro-4-hydroxybenzoate; BBA for bromobenzoate; CBA for chlorobenzoate; DCBA for dichlorobenzoate; TCBA for trichlorobenzoate; 2-B-4-FP for 2-bromo-4-fluorophenol; PCE for tetrachloroethene; TCE for trichloroethene; TCA for trichloroacetate. PCP for pentachlorophenol

a- This includes strain 2CP-1, 2CP-2, 2CP-3, 2CP-5 and 2CP-C. Substrate listed is for strain 2CP-C.

b- Dehalogenation is meta-position specific.

c- Dehalogenation is meta-position specific.

d- Only use ortho-substituted aromatic organohalide. It incompletely oxidizes lactate to acetate to the reduction of sulfate, sulfite and thiosulfate, but completely oxidize acetate when growing with 2-CP.

e- Sulfite had a strong inhibitory effect on growth and dechlorination. Alternate electron acceptors don't influence dehalogenation.

f- Dehalogenation is coupled to a novel sulfur-sulfide redox cycle.

g- Alternate electron acceptors don't affect dehalogenation.

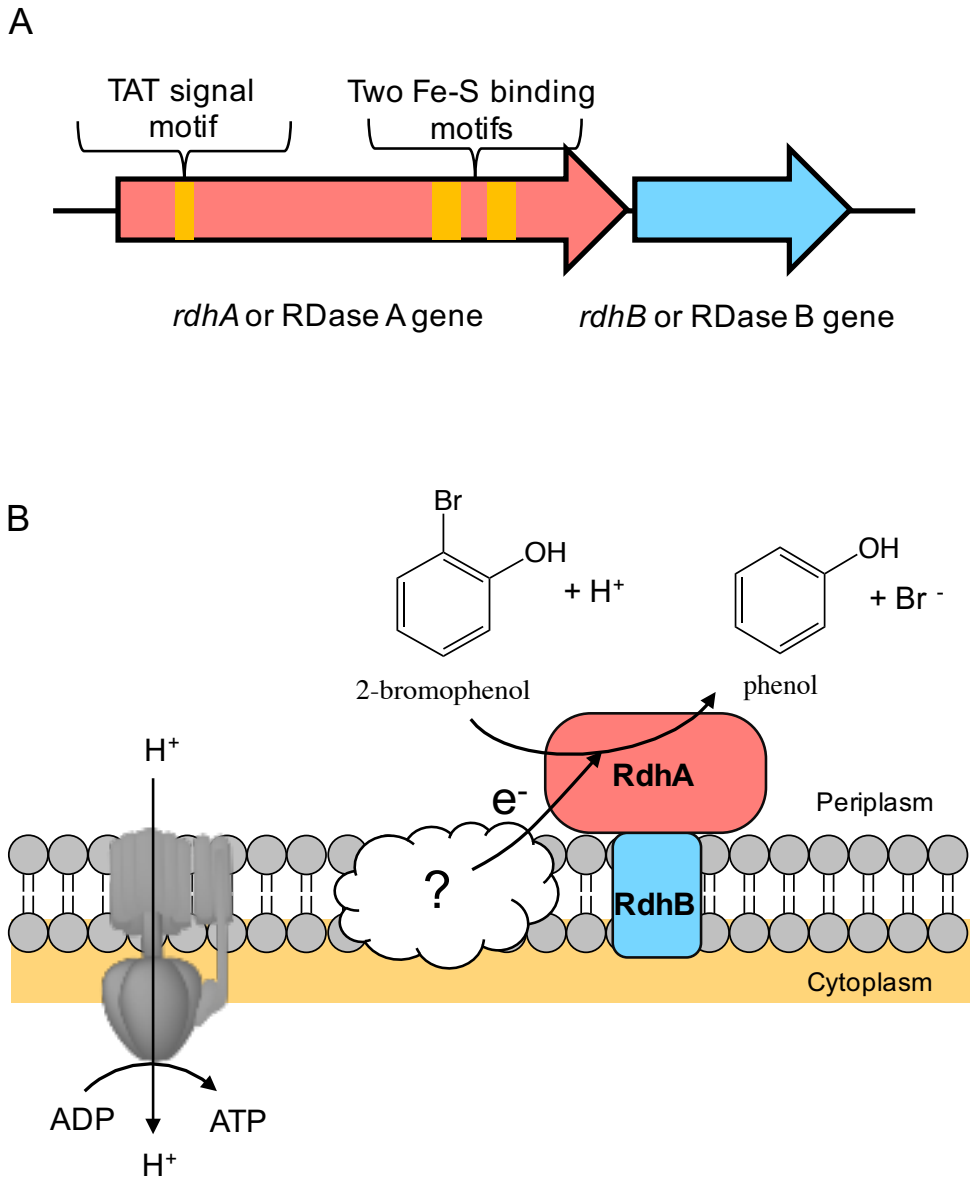


Figure 1.4 Schematic representation of the reductive dehalogenase gene (A) and respiratory reductive dehalogenation using 2-bromophenol as example.

Chapter 2 Novel reductive dehalogenases from the marine sponge associated bacterium *Desulfoluna spongiiphila*

This chapter has been published as

Liu, J., Lopez, N., Ahn, Y., Goldberg, T., Bromberg, Y., Kerkhof, L.J., and Haggblom, M.M. (2017) Novel reductive dehalogenases from the marine sponge associated bacterium *Desulfoluna spongiiphila*. Environ Microbiol Rep 9: 537-549.

Abstract

Desulfoluna spongiiphila strain AA1 is an organohalide respiring bacterium, isolated from the marine sponge *Aplysina aerophoba*, that can use brominated and iodinated phenols, in addition to sulfate and thiosulfate as terminal electron acceptors. The genome of *Desulfoluna spongiiphila* strain AA1 is approximately 6.5 Mb. Three putative reductive dehalogenase (*rdhA*) genes involved in respiratory metabolism of organohalides were identified within the sequence. Conserved motifs found in respiratory reductive dehalogenases (a twin arginine translocation signal sequence and two iron-sulfur clusters) were present in all three putative AA1 *rdhA* genes. Transcription of one of the three *rdhA* genes was significantly upregulated during respiration of 2,6-dibromophenol and sponge extracts. Strain AA1 appears to have the ability to synthesize cobalamin, the key cofactor of most characterized

reductive dehalogenase enzymes. The genome contains genes involved in cobalamin synthesis and uptake and can grow without cobalamin supplementation. Identification of this target gene associated with debromination lays the foundation for understanding how dehalogenating bacteria control the fate of organohalide compounds in sponges and their role in a symbiotic organobromine cycle. In the sponge environment, *D. spongiiphila* strain AA1 may thus take advantage of both brominated compounds and sulfate as electron acceptors for respiration.

Introduction

The marine environment is a major source of naturally occurring organohalides produced by algae, jellyfish, acorn worms and sponges (Gribble, 1998). These compounds include bromopyrroles, bromoindoles, bromophenols and brominated diphenyl ethers and are hypothesized to function as chemical defense to protect sponges from predators and biofouling (Teeyapant and Proksch, 1993; Ebel *et al*., 1997). These natural sources of brominated compounds also appear to select for dehalogenating bacteria living within the host animal (Ahn *et al*., 2003). Considering the extraordinary pumping capacity and abundant microbial communities of sponges (Hentschel *et al*., 2012), an understanding of the microbial processes that control the fate of organohalide compounds in sponges is needed in order to understand the role that these dehalogenating bacteria play within the animal and a marine organobromine cycle.

Reductive dehalogenation is a key process in the degradation of both natural and anthropogenic halogenated compounds (for reviews see Häggblom and Bossert, 2003; Adrian and Löffler, 2016). Cleavage of the carbon-halogen bond can be mediated by anaerobic bacteria that utilize the organohalide as terminal electron acceptor for respiration. These organohalide respiring bacteria are distributed among diverse phyla (Maphosa *et al*., 2010) and can be grouped into either obligate or non-obligate organohalide respirers based on their metabolic versatility (Hug *et al*., 2013). The *Deltaproteobacterium*, *Desulfoluna spongiiphila* strain AA1, isolated from the marine sponge *Aplysina aerophoba* is of interest because of its ability to reductively dehalogenate various brominated and iodinated phenolic compounds (Ahn *et al.*, 2009*). D. spongiiphila* is a non-obligate organohalide respiring bacterium; in addition to organohalide respiration it has the ability to also grow with sulfate and thiosulfate as the terminal electron acceptor.

Organohalide respiration is mediated by reductive dehalogenases (RDases), encoded by homologous *rdhA* genes. Available genomes reveal that organohalide-respiring bacteria (OHRB) can possess over 30 putative *rdhA* genes (Kube *et al*., 2005; Wang *et al*., 2014). The *rdh* operon contains *rdhA*, the gene coding for the active enzyme, and *rdhB*, the gene coding for a putative membrane-anchoring protein (Jugder *et al*., 2015). Putative regulatory genes are often found to be associated with *rdhAB*, i.e., a two-component regulatory system (RdhCD) or MarR-type regulators (RdhR) in *Dehalococcoides* spp., and CRP/FNR type regulators (CprK) in *Desulfitobacterium* spp. (Gábor *et al*., 2006; Joyce *et al*., 2006; Kemp *et al*., 2013; Krasper *et al*., 2016). Most RdhA proteins possess two conserved features, a twin-

arginine translocation (TAT) motif (RRXFXK) at the N terminus (Maillard *et al*., 2003) and two iron-sulfur cluster-binding motifs at the C terminus (CXXCXXCXXXCP and CXXCXXXCP) (Maillard *et al*., 2003; Maphosa *et al*., 2010; Hug *et al*., 2013; Bommer *et al*., 2014). In addition, most RdhA proteins depend on a corrinoid cofactor for activity (Bommer *et al*., 2014; Payne *et al*., 2015; Rupakula *et al*., 2015). The crystal structures of two RDases, PceA from *Sulfurospirillum. multivorans* and NpRdhA from *Nitratireductor pacificus* pht-3B, reveal the presence of cobalamin factors in the active site (Bommer *et al*., 2014; Payne *et al*., 2015). The sources of the corrinoid cofactor for organohalide respiring bacteria can be either *de novo* synthesis or from an exogenous vitamin B_{12} supply (Rupakula *et al*., 2015). For example, *Dehalococcoides mccartyi* strains are dependent on corrinoid salvaging from the environment (Yi *et al*., 2012; Yan *et al*., 2013; Men *et al*., 2014), while *Sulfurospirillum multivorans* and *Desulfitobacterium hafniense* are known to possess a complete set of corrinoid biosynthesis genes and are able to synthesize vitamin B_{12} *de novo* (Nonaka *et al*., 2006; Choudhary *et al*., 2013; Keller *et al*., 2014; Goris, *et al*., 2014). However, even with a nearly complete set of corrinoid biosynthesis genes, *Dehalobacter restrictus* strain PER-K23 still needs an exogenous corrinoid supply due to the truncation of one critical gene (*cbiH*) (Rupakula *et al*., 2015).

In this study, we set out to identify the reductive dehalogenase genes in the sponge-associated bacterium *D. spongiiphila* strain AA1. The genome of *D. spongiiphila* strain AA1 was sequenced and analyzed to detect the gene(s) coding for reductive dehalogenases and the cobalamin synthesis pathway. Transcript analysis was conducted to identify and characterize the expression of reductive dehalogenase

genes under different growth conditions. Most *rdhA* gene products studied to date are involved in dechlorination. The substrate specificity of *D. spongiiphila* strain AA1, however, is different from that of most well characterized reductive dehalogenating bacteria. Strain AA1 can dehalogenate bromophenols and iodophenols, but not chlorophenols (Ahn *et al*., 2009). Our findings represent an example of a respiratory debrominase and provide an avenue to explore the role of organohalide respiration in the marine halogen cycle.

Results

Genome characteristics and identification of putative respiratory reductive dehalogenases in *Desulfoluna spongiiphila* strain AA1

The draft genome sequence of *D. spongiiphila* strain AA1 consists of 52 scaffolds ranging in length from 1,069 bp to 728,457 bp. The total genome size is approximately 6.5 Mbp with a GC content of 57.2%. Based on the annotation by JGI, approximately 5,200 protein-coding genes are predicted in the genome, 74% of which are predicted with function (JGI Analysis Project ID: Ga0104423). The annotated protein sequences are also available in NCBI (BioProject Accession: PRJEB15715). The genome of *D. spongiiphila* contains genes encoding for proteins for dissimilatory sulfate reduction, including genes for sulfate adenylyltransferase (*sat*), dissimilatory adenylylsulfate reductase (*aprAB*) and dissimilatory sulfite reductase (*dsrAB*).

The presence of three putative reductive dehalogenase genes was initially uncovered from a draft genome assembled from SoLiD sequencing data (see Materials and Methods). The presence of these putative reductive dehalogenase genes was further confirmed based on the higher quality Illumina sequence data (labelled here as *rdh AA1_02299,* JGI Locus Tag: Ga0104423_102299; *rdh AA1_07176*, JGI Locus Tag: Ga0104423_107176; *rdh AA1_16032*, JGI Locus Tag: Ga0104423_11632). The *rdhA* gene labels correspond to the scaffold in which the putative *rdhA* is located followed with gene number in that scaffold. The size of each putative reductive dehalogenase gene/enzyme are as follows: *rdh AA1_02299*- 1,452 bp, 483 aa; *rdh AA1_07176*- 1,290 bp, 429 aa; and *rdh AA1_16032*- 1,683 bp, 560 aa. Clustal W pairwise alignments of the N- and C-terminal amino acid sequences of the three putative *D. spongiiphila* AA1 reductive dehalogenases (RdhA) and 20 known RDases identified the twin arginine translocation (Tat) signal motifs (RRXFXK) and two iron-sulfur cluster binding motifs (CXXCXXCXXXCP and CXXCXXXCP) (Fig. S2.1).

Pairwise alignment of the three *D. spongiiphila* strain AA1 RdhA amino acid sequences indicates that they are distinct. Rdh AA1_02299 has a sequence identity of 22.3% with Rdh AA1_16032 and 23.8% with Rdh AA1_07176. Rdh AA1_07176 has an identity of 25.8% with Rdh AA1_16032. They are located in distant branches of the phylogenetic tree of functionally characterized RdhAs (Fig. 2.1). BlastP analysis of each RdhA against the NCBI database revealed that Rdh AA1_02299 has 39% amino acid identity with an RdhA from *Dehalococcoides mccartyi* SG1 (Accession: WP_034376939), Rdh AA1_07176 has an identity of 43% with a *Dehalobacter* spp. CF RdhA (Accession: AFV06381), and Rdh AA1_16032 has an identify of 50% with an

RdhA from *Shewanella sediminis* HAW-EB3 (Accession: WP_012142447). According to the reductive dehalogenase classification system proposed by Hug *et al*. (2013), the three RdhAs from *D. spongiiphila* strain AA1 cannot be grouped with any other previously characterized RdhA groups, which are defined by a 90% PID threshold (Supporting Information Fig. S2.2).

The analysis of the region around the putative *rdh* genes of *D. spongiiphila* revealed other possible genes involved in reductive dehalogenation (Fig. 2.2). All three *rdh* gene clusters follow the *rdhABC* model together with transcriptional regulatory genes. Each *rdh* gene cluster harbors a putative membrane-anchoring gene, *rdhB*, downstream of *rdhA*. Putative *rdhC* genes are named according to *cprC* of *Desulfitobacterium dehalogenans* (Smidt *et al*, 2000) based on the domain similarity. They all contain a FMN-binding domain and Fe-S binding domains. Additional genes annotated with transcriptional regulatory functions were found in all *rdh* gene clusters. Sigma factor 54 dependent transcriptional activators, which usually assist the initiation of sigma factor 54 dependent transcription, are both found upstream of the *rdh AA1_07176* and *16032* gene clusters. The analysis of their promoter region reveals the presence of a sigma factor 54 binding site (CCGGCACGCTTTGTGCT and TTGGCACACCGCTTGCT) (Fig. 2.2). No such sigma-54 dependent protein and binding site are found in the *rdh AA1_02299* cluster and its promoter region. A LuxR family regulator is present upstream of *rdh AA1_02299* and a MarR-type transcriptional regulator is far downstream of the *rdh AA1_02299* gene cluster.

Expression of reductive dehalogenase genes

D. spongiiphila strain AA1 dehalogenates *ortho* bromo- and iodophenols, but not chlorophenols. We conducted a set of experiments to determine whether reductive debromination activity in *D. spongiiphila* was inducible. Strain AA1 was cultivated with sulfate as the electron acceptor and then transferred into fresh medium amended with 2,6-dibromophenol (2,6-DBP) and streptomycin to inhibit further protein synthesis. In the presence of streptomycin, debromination activity was minimal over 48 h. In the absence of streptomycin, debromination of 200 µM 2,6-DBP was observed after a lag phase of 6 h (Fig. 2.3) with transient formation of 2-bromophenol (2-BP) and the accumulation of phenol over the next 60-80 h, indicating that dehalogenation activity in strain AA1 is induced by 2,6-DBP.

After demonstrating induction of debromination activity, we attempted to identify which of the three putative reductive dehalogenase genes (*rdh AA1_02299, rdh AA1_07176,* and *rdh AA1_16032*) were transcribed and expressed during bromophenol respiration. After growth on sulfate, dehalogenation of 200 µM 2,6-DBP by strain AA1 was observed after a 3 h lag phase with sequential production of 2-BP and phenol as debromination products over the next 9 h of incubation (Fig. 2.4a). The expression of the three putative *rdhA* genes was analyzed by reverse transcription at time points during this incubation. Transcripts were amplified with two sets of specific primers designed for each gene and the cDNA product loaded onto an agarose gel. As shown in Fig. 2.4b, clear bands of *rdh AA1_07176* and *rdh AA1_16032* cDNA PCR products were observed, while no band was found in control cultures without 2,6-DBP amendment. For *rdh AA1_02299*, there was no difference found between

induced and control cultures. These results indicate that the expression of *rdh AA1_07176* and *rdh AA1_16032*, but not *rdh AA1_02299* was induced by the addition of 2,6-DBP.

The gene *rdh AA1_16032* exhibited a more pronounced upregulation compared to *rdh AA1_07176* with an approximately 5-fold increase in expression at the initial time point, taken 20 min after 2,6-DBP addition (Fig. 2.4c). Expression of *rdh AA1_16032* increased approximately 200-fold over the first 12 h. A lower fold increase in the expression of *rdh AA1_07176* was observed after 2,6-DBP addition. These results suggest that *rdh AA1_16032* is the major gene responsible for both 2,6-DBP and 2-BP debromination, which is upregulated in response to 2,6-DBP and 2-BP.

Extracts of an *Aplysina aerophoba* sponge also induced pronounced upregulation of *rdh AA1_16032* after 4 h of incubation compared to the other two *rdhA*s (Fig. 2.4d). This indicates that the dehalogenase encoded by *rdh AA1_16032* may be also responsible in debrominating natural sponge-derived organohalides *in vivo*.

Corrinoid biosynthesis genes in the genome of strain AA1

Most RdhA proteins depend on a corrinoid cofactor for dehalogenation activity. The source of cobalamin for dehalogenating bacteria can be from *de novo* biosynthesis or scavenging from the environment (Rupakula *et al*., 2015). *D. spongiiphila* strain AA1 has been grown in medium with approximately 50 µg/L cobalamin from the time it was isolated. Cobalamin biosynthesis and uptake genes

are present in the genome of strain AA1 (Supporting Information Table S1) and when strain AA1 was sequentially transferred and grown in cobalamin-free medium and then exposed to different cobalamin concentrations, there was no substantial difference in debrominating activity (Fig. S2.3). This indicates that strain AA1 does not require an exogenous cobalamin supply for dehalogenation. Experiments to determine whether propyl iodide, an inhibitor of corrinoid enzymes (Ghambeer *et al*., 1971), would inhibit dehalogenation activity were inconclusive (data not shown). Whether the RdhA cofactor indeed is a corrinoid or a heme, as in *Desulfomonile tiedjei* DCB-1 (Ni *et al*., 1995) will require additional confirmation.

Discussion

Because of their metabolic diversity, non-obligate organohalide respiring bacteria usually have a larger genome size and fewer reductive dehalogenase genes than obligate ORB. For example, the obligate ORB *Dehalobacter* sp. strain 12DCB1 has 39 *rdhA* homologs in its 2.9 Mb genome, while the facultative *Desulfitobacterium hafniense* TCE-1 has only one *rdhA* gene in its 5.74 Mb genome (Kruse *et al*., 2016). *D. spongiiphila* strain AA1 is a typical non-obligate ORB with a genome size of around 6.5 Mb containing three putative *rdhA* genes.

A classification for orthologous group RhdA proteins was proposed by Hug *et al*. (2013) to categorize dehalogenases based on a 90% cutoff of amino acid sequence identity. None of the three RdhAs of *D. spongiiphila* strain AA1 can be clustered with any of the current existing groups. The highest identity of the AA1 dehalogenases to

any RdhAs in the database is approximately 50%, which is much lower than the 90% cutoff (Fig. S2.2). The substrate specificity of a new RDase cannot be predicted based on its sequence similarity with characterized RDases (Jugder *et al*., 2016). The determination of substrate specificity requires the isolation and purification of the functional enzymes, which remains a major constraint in many cases. The substrates of most well characterized RDases so far are chlorinated compounds, including chlorinated ethenes, benzenes and phenols (Fig. 2.1). The substrate specificity of *D. spongiiphila*, however, is different from these characterized RDases in that strain AA1 can dehalogenate bromophenols and iodophenols, but not chlorophenols (Ahn *et al*., 2009). In addition, reductive debromination in *D. spongiiphila* AA1 occurs in the presence of sulfate (Ahn *et al.*, 2009) in contrast with many other dehalogenating bacteria and cultures, whose dehalogenation activity are inhibited by sulfate (see Zanaroli *et al.,* 2015).

Many brominated pollutants, such as brominated biphenyls and tetrabromobisphenol A are ubiquitously present in the environment and reductive dehalogenation is considered crucial in their biodegradation (e.g., Bedard and Van Dort, 1998; Voordeckers *et al*., 2002; Liu *et al*., 2013). Reductive debromination has been observed in sediment microcosms (e.g., Monserrate and Häggblom, 1997), and some bacteria are reported with reductive debromination ability, e.g., a highly enriched *Dehalococcoides* culture was able to debrominate polybrominated diphenyl ethers, tetrabromobisphenol A and other phenolic bromoaromatics (He *et al*., 2006; Lee *et al*., 2011; Yang *et al*., 2015; Cooper *et al*., 2015), and *Desulfovibrio* strains and *Desulfobacterium chlororespirans* are reported to debrominate brominated phenolics

(Boyle *et al*., 1999; Fennell *et al*., 2004; Cupples *et al*., 2005). *Sulfurospirillum multivorans* and *Desulfitobacterium hafniense* PCE-S are able to debrominate brominated ethenes (Ye *et al*., 2010). Nonetheless, compared with reductive dechlorinases, there is limited knowledge on reductive debrominases (Adrian and Löffler, 2016). Two characterized reductive debrominases (BhbA from *Comamonas* sp. 7D-2 and NpRdhA from *Nitratireductor pacificus* pht-3B) are not involved in organohalide respiration (Payne *et al*., 2015) and belong to a different dehalogenase enzyme class than the RdhAs in strain AA1 and other organohalide-respiring bacteria. Alternatively, a transcriptional approach coupled to metabolic analysis can be used to link a putative *rdhA* gene with a measured gene function.

In this study, we used known structural information about *rdh* genes and gene clusters to identify putative *rdh* gene clusters in strain AA1. For example, the *pceABCT* cluster is commonly present in the genomes of many organohalide respiring bacteria (Jugder *et al*., 2015). The *rdhB* gene encodes for a putative membrane anchoring protein while *rdhC* encodes for a putative membrane bound transcriptional regulatory protein (Smidt *et al*., 2000). The three *rdh* gene clusters of *D. spongiiphila* strain AA1 follow the *rdhABC* model. An *rdhC*-like gene encodes a FMN binding domain and 4Fe-4S binding domain-containing protein, which may function as transcriptional regulators. All three *rdh* gene clusters of strain AA1 contain this *rdhC*-like gene downstream of *rdhB*. The *rdhT* gene encodes a trigger factor-like protein, which may be involved in RdhA folding (Smidt *et al*., 2000). However, *rdhT*-like genes are not found in the *rdh* gene clusters of strain AA1.

Although the debromination of 2,6-DBP exhibited a short lag phase, the up-regulation of *rdh AA1_16032* was immediately detected after addition of 2,6-DBP, while *rdh AA1_07176* was only weakly upregulated during debromination of 2,6-DBP to 2-BP. Considering that *rdh AA1_07176* and *rdh AA1_16032* don't show the same response pattern to 2,6-DBP, their regulatory mechanisms may be distinct. These two clusters both contain a transcriptional regulatory gene belonging to the HxlR family, at a sequence identity of 50%. HxlR is a putative transcriptional regulator with a winged helix-turn-helix (wHTH) structure similar to the MarR type wHTH. It was first elucidated as a transcriptional activator of the *hxlAB* operon in *Bacillus subtilis* (Yurimoto *et al*., 2005).

The regulatory mechanisms differ among organohalide respiring bacteria of different phylogeny (Kruse *et al*., 2016). In *Desulfitobacterium* and *Dehalobacter*, the *rdh* clusters often contain an *rdhK* gene, which encodes for a transcriptional regulator belonging to the CRP/FNR family. CprK could act as a transcriptional activator and bind to a dehalobox motif in the promoter regions in the presence of substrate (Futagami *et al*., 2006; Gábor *et al*., 2006; Kemp *et al*., 2013). None of the strain AA1 *rdh* gene clusters possesses CRP/FNR family-like regulators, suggesting a different regulatory mechanism for strain AA1 *rdh* gene regulation.

Interestingly, sigma-54-dependent transcriptional regulators are found upstream of the *rdh AA1_07176* and *rdh AA1_16032* gene promoter regions. Although sharing low pairwise identity (30.8%), these two genes contain a regulatory domain, a sigma factor 54 interaction domain and a DNA binding domain. The sigma-54-depenedent regulators are also called enhancer-binding-protein (EBP), which play an

important role in sigma factor 54 dependent transcription (Bush and Dixon, 2012). Unlike sigma factor 70, sigma factor 54 recognizes a different but more conserved consensus sequence, YTGGCACGrNNNTTGCW, where binding occurs at -24 (GG) and -12 (TGC) elements (Studholme and Dixon, 2003; Bush and Dixon, 2012). The initiation of sigma factor 54 dependent transcription requires the assistance of EBP to open the closed complex. Examining the promoter region of these two *rdhA* genes, we found conserved sigma factor 54 binding sites in their promoters. Further molecular investigations are needed to confirm the function of the sigma factor 54 dependent regulators in these gene clusters.

The MarR-type regulator is known to act as a repressor in *Dehalococcoides* species, which contains a winged helix-turn-helix (wHTH) motif that involves in the interaction between regulator and palindrome site on binding DNA (Wagner *et al*., 2013; Krasper *et al*., 2016). However, most MarR-type regulatory genes in the *rdh* clusters of *Dehalococcoide* spp. are very close and in opposite orientation to the *rdhA* gene. The MarR-type regulator in the *rdh AA1_02299* cluster may not function as in *Dehalococcoides* species. Another transcriptional regulator belonging to the LuxR family is closely upstream of *rdhA* in the *rdh AA1_02299* cluster. This contains aHTH DNA binding structure, and may also be involved in *rdh AA1_02299* gene cluster regulation.

The *pceABCT* gene cluster in *D. hafniense* strain Y51 and *pceA* in strain TCE1 are not regulated at the mRNA level (Prat *et al*., 2011; Peng *et al*., 2012). However, for *D. hafniense* TCE1 a significant PceA expression difference was found at the protein level between cultures during long-term cultivation with or without PCE (Prat *et al*.,

2011). Unlike other well-studied reductive dehalogenase gene clusters, the *pceABCT* gene cluster in strains Y51 and TCE1 are located on mobile genetic elements and do not contain any obvious regulatory elements (Maillard *et al*., 2005; Futagami *et al*., 2006). The *pceABCT* gene cluster in these strains may gradually be lost in the absence of organohalides, which could explain the expression difference between mRNA and protein level in these studies (Prat *et al*., 2011).

The sponge *Aplysina aerophoba*, the host of *D. spongiiphila*, is known to produce a variety of brominated compounds (Norte *et al.,* 1988; Teeyapant *et al*., 1993; Hentschel *et al*., 2003). Because of their antimicrobial activity, these brominated metabolites e.g., aeroplysinin-1 and dienone may be produced for a defense purpose and can be toxic to some bacteria. The response of *rdh AA1_16032* to *Aplysina aerophoba* extracts reveals that this *rdhA* can be induced by natural organohalides produced in sponges (Fig. 2.4d). In the sponge environment, *D. spongiiphila* strain AA1 may thus take advantage of both brominated compounds and sulfate as electron acceptors for respiration. Interestingly, debromination by *D. spongiiphila* strain AA1 occurs concurrently with sulfate reduction (Ahn *et al.,* 2009; unpublished data) in contrast to the inhibitory effect of sulfur oxyanions observed for many dechlorinating bacteria (for review see Zanaroli *et al.,* 2015).

A corrinoid is a key cofactor found in most characterized reductive dehalogenases (Adrian and Löffler, 2016). Cobalamin or vitamin B_{12} along with cobalt is found at the center of the corroinoid enzyme (Bommer *et al*., 2014). A few OHRB have been shown to be dependent on an external source of vitamin B_{12}, while others have been shown to have *de novo* corrinoid biosynthesis pathways (Nonaka *et al*.,

2006; Yan *et al*., 2013; Rupakula *et al*., 2015). Most potential corrinoid biosynthesis and uptake proteins were found in the genome of *D. spongiiphila* strain AA1 (Supporting Information Table S1). However, corrinoid biosynthesis ability can be eliminated by a minor truncation in one single gene, as shown for *Dehalobacter restrictus* (Rupakula *et al*., 2015). Thus, a nearly complete corrinoid biosynthesis pathway from genome annotation cannot guarantee a functional metabolic pathway. Our growth and dehalogenation experiments indicated that *D. spongiiphila* does not rely on an exogenous cobalamin supply. However, experiments to determine whether propyl iodide, a light-reversible inhibitor of corrinoid enzymes (Ghambeer et al., 1971), would inhibit dehalogenation activity were inconclusive (data not shown). For comparison, the 3,5-dichlorophenol reductive dehalogenase from *Desulfitobacterium frappieri* PCP-1 was inhibited by propyl iodide, suggesting the involvement of a cobalamin cofactor in the enzyme (Thibodeau *et al*., 2004). Pure PCE-RDase of *Dehalobacter restrictus* was also inhibited by propyl iodide and reactivated once illuminated (Maillard *et al*., 2003). However, reductive dehalogenation in *Desulfomonile tiedjei* DCB-1 was not inhibited by propyl iodide (Louie and Mohn, 1999) and the RdhA cofactor is a heme (Ni et al., 1995). Further investigations are needed to identify the cofactor of *D. spongiiphila* strain AA1 dehalogenases.

Conclusions

Considering the abundant organohalide content, marine environments provide favorable habitats for organohalide respiring bacteria. The remarkable

distribution of *rdhA* homologs in marine sediments reveals that organohalide respiration is a significant process of the organohalide cycle (Futagami *et al*., 2009; Futagami *et al*., 2013). As the only pure ORB culture isolated from a marine sponge, the genomic and metabolic properties of *D. spongiiphila* strain AA1 enrich our knowledge about the organohalide cycle. Our studies uncovered three distinct reductive dehalogenase genes in strain AA1, which show low similarity with *rdhA*s from other sources. The transcriptional upregulation of these *rdhA* genes indicate their potential roles in reductive dehalogenation. The *rdh* gene clusters of *D. spongiiphila* strain AA1 contain transcriptional regulatory genes, whose function needs to be further studied. Future work will aim to unravel the *rdh* gene transcription regulation mechanism of strain AA1 and how strain AA1 responds to organohalides.

Experimental Procedures

Growth of *Desulfoluna spongiiphila* strain AA1

Strain AA1 was grown anaerobically in a minimal salts medium as described by Fennell *et al*. (2004) with minor modification at room temperature. The NaCl concentration was modified to 25 g/L, which is the optimal salinity for strain AA1 growth (Ahn *et al*., 2009). Lactate (30 mM) and sulfate (20 mM) were utilized as electron donor and acceptor.

Genome sequencing and annotation

The genome of *D. spongiiphila* strain AA1 was initially sequenced on the SOLiD™ 3 Analyzer platform (Applied Biosystems, Foster City, CA) in 2010 and later on the Illumina platform in 2015. For SOLiD sequencing, three liters of strain AA1 grown under sulfidogenic conditions was collected by filtration and total DNA was extracted using the QIAGEN QIAamp® DNA mini kit.

For Illumina sequencing, Strain AA1 grown under sulfidogenic conditions was collected by centrifugation. Genomic DNA was extracted and purified by following a DOE Joint Genomic Institute (JGI) genomic DNA phenol-chloroform extraction protocol. A 300 bp insert standard shotgun library was constructed and sequenced using the Illumina HiSeq–2000 1TB platform which generated 5,262,106 reads totaling 789.3 Mbp. All raw Illumina sequence data was filtered using BBDuk, which removes known Illumina artifacts and PhiX. Reads with more than one "N" or with quality scores (before trimming) averaging less than 8 or reads shorter than 51 bp (after trimming) were discarded. Remaining reads were mapped to masked versions of human, cat and dog references using BBMAP and discarded if identity exceeds 93%. Sequence masking was performed with BBMask. Following steps were then performed for assembly: (1) artifact filtered Illumina reads were assembled using Velvet (version 1.2.07) (Zerbino and Birney, 2008); (2) 1–3 kbp simulated paired end reads were created from Velvet contigs using wgsim (version 0.3.0); (3) Illumina reads were assembled with simulated read pairs using Allpaths–LG (version r46652) (Gnerre *et al*., 2011). The *D. spongiiphila* strain AA1 genome is available as JGI Analysis Project ID: Ga0104423 (GOLD Project ID: Gp0127298). The annotated

protein sequences are available in NCBI (BioProject Accession: PRJEB15715).

Identification of *rdhA* genes

The initial identification of *rdh* genes was conducted on SoLiD sequencing data. The scaffold sequences were analyzed through TrAnsFuSE (Harel *et al.*, 2012), a protein sequence-scanning program that targets proteins containing EC1 (transition metal-utilizing redox domains); LocTree2 (Goldberg *et al.*, 2012) to predict the subcellular localization and type of protein (membrane-bound or cytoplasmic); and Blast2GO (Conesa *et al.*, 2005) used to annotate and identify functional proteins. This process uncovered three putative reductive dehalogenase genes in the *D. spongiiphila* strain AA1 genome.

N and C-terminal amino acid sequence alignment of potential reductive dehalogenases of *D. spongiiphila* and functionally characterized reductive dehalogenases (Jugder *et al*., 2015) was done using Clustal W pairwise alignment with Cost Matrix BLOSUM in MEGA 7 (Kumar *et al*., 2016).

Phylogenetic analyses of reductive dehalogenases were conducted in MEGA7 (Kumar *et al*., 2016). Initial tree(s) of strain AA1 and characterized reductive dehalogenases for the heuristic search were obtained automatically by applying Neighbor-Joining (NJ) and advanced NJ (BioNJ) algorithms to a matrix of pairwise distances estimated using a Jones–Thornton–Taylor (JTT) model, and then selecting the topology with superior log likelihood value.

Induction and expression experiments

For analysis of *rdhA* gene expression strain AA1 was grown in sulfidogenic medium and then inoculated into fresh anaerobic medium containing 1 mM lactate as electron donor and 200 μM 2,6-dibromophenol (2,6-DBP, Aldrich Chemical Co., Milwaukee, Wis) as an electron acceptor (10% transfer for the induction experiment and 50% for the expression experiment). Debromination of 2,6-DBP was monitored via high performance liquid chromatography (HPLC) as described below. For inhibition of protein synthesis 1mg/ml streptomycin was added. In the expression experiment, the control contained 200 μM sulfate instead of 200 μM 2,6-DBP. Samples for total RNA extraction and analysis *rdhA* gene expression were taken periodically.

Expression of *rdhAs* in the presence of sponge extracts

Approximately 1.5 g (dry weight) of *Aplysina aerophoba* sponge tissue (collected in 2001 and stored at -65 ^{o}C; Ahn *et al*., 2003) was extracted with 55 ml of methanol by shaking for two days. Extracts (10 ml) was pipetted into autoclaved serum bottles and the methanol evaporated in a fume hood. A culture of *D. spongiiphila* AA1 grown on lactate and sulfate as described above was transferred to the serum bottles containing the dried extracts and examined for *rdhA* gene expression. In total, three treatments (control without sponge extracts; with sponge extracts; with both sponge extracts and 200 μM 2-BP) in duplicate were prepared and analyzed for *rdhA* gene expression.

RNA extraction, reverse transcription and qPCR

Total RNA was extracted from 5 ml of culture samples. Cells were pelleted by centrifugation and then extracted using TRIzol (Ambion, Life Technologies) reagent according to the manufacturer's instructions. The RNA pellet was dissolved in 30 µl nuclease-free water and further treated by DNA-free™ DNA removal kit (Ambion, Life Technologies) to remove gDNA contamination in RNA. The integrity and purity of RNA was validated by analysis on an agarose gel. 0.5 µl of RNA was used to synthesize cDNA in a 20 µl reaction by using the iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Inc.) according to manufacturer's instructions. The obtained cDNA was amplified with primer sets specifically designed to each *rdhA* gene and to the 16S rRNA gene using both regular PCR and qPCR (Supporting Information Table S2.2). The specificity of the *rdhA* gene primers was examined by sequencing of the PCR products and comparison to the genome. For qPCR, a serial dilution of a cDNA sample of 2,6-DBP induced cells was made to generate a standard curve of each gene. RT-qPCR was performed using an IQ™ SYBR Green Supermix (BIO-RAD) in a 20 µl reaction. Thermocycling conditions for RT-qPCR were as follows: 2 min at 90 ^{o}C, followed by 40 cycles of 30 s at 94 ^{o}C, 30 s at 59 ^{o}C and 70 s at 72 ^{o}C. The melting curve data collection started from 100 ^{o}C for 10 s with a decreasing rate of 0.5 ^{o}C/cycle for 130 cycles.

Transcription levels of the *rdhA* genes were calculated by relative quantitation using relative standard curve methods normalized to the 16S rRNA gene. Normalized expression in cultures treated with 2,6-DBP was divided by normalized expression in control cultures to indicate the expression level.

Effect of exogenous cobalamin on debromination

D. spongiiphila strain AA1 was previously grown in medium containing 50 µg/l of cobalamin. To remove cobalamin from the culture, strain AA1 culture was successively transferred into cobalamin-free sulfidogenic medium. The transfers represented a 1:3000 dilution, yielding a cobalamin concentration below 0.02 µg/L. The culture was then exposed to different cobalamin concentrations (0, 1, 10, 50, 200 µg/L) in medium amended with 2,6-DBP to determine debrominating activity.

Analytical methods

The concentration of 2,6-DBP, 2-BP and phenol was measured via High Performance Liquid Chromatography (HPLC) on a Shimadzu system equipped with an auto injector (SIL-10A, Shimadzu), a system controller (SCK-10A, Shimadzu), a diode array detector (SPD-M10A, Shimadzu). A Sphereclone C-18 column (250 mm × 4.6 mm, particle size 5 µm; Phenomenex) was used with a mobile phase consisting of methanol:water:acetic acid (methanol varied from 70 to 80% depending on sample batch; 1% acetic acid) at an isocratic flow rate of 1 ml/min. The detection wavelength was 280 nm.

Acknowledgements

This work was funded in part by a grant from the National Science Foundation (OCE-451708) and the New Jersey Agricultural Experiment Station.

The strain AA1 genome was sequenced as part of the "Genomic Encyclopedia of Archaeal and Bacterial Type Strains, Phase II (KMG-II): from individual species to whole genera" study. The work conducted by the U.S. Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.

We thank Randall Kerstetter, Mark Diamond, Ariella S. Sasson, Anirvan M. Sengupta and Joachim Messing (Waksman Institute of Microbiology, Rutgers University) for the initial sequencing of the *D. spongiiphila* strain AA1 genome on the SoLiD platform.

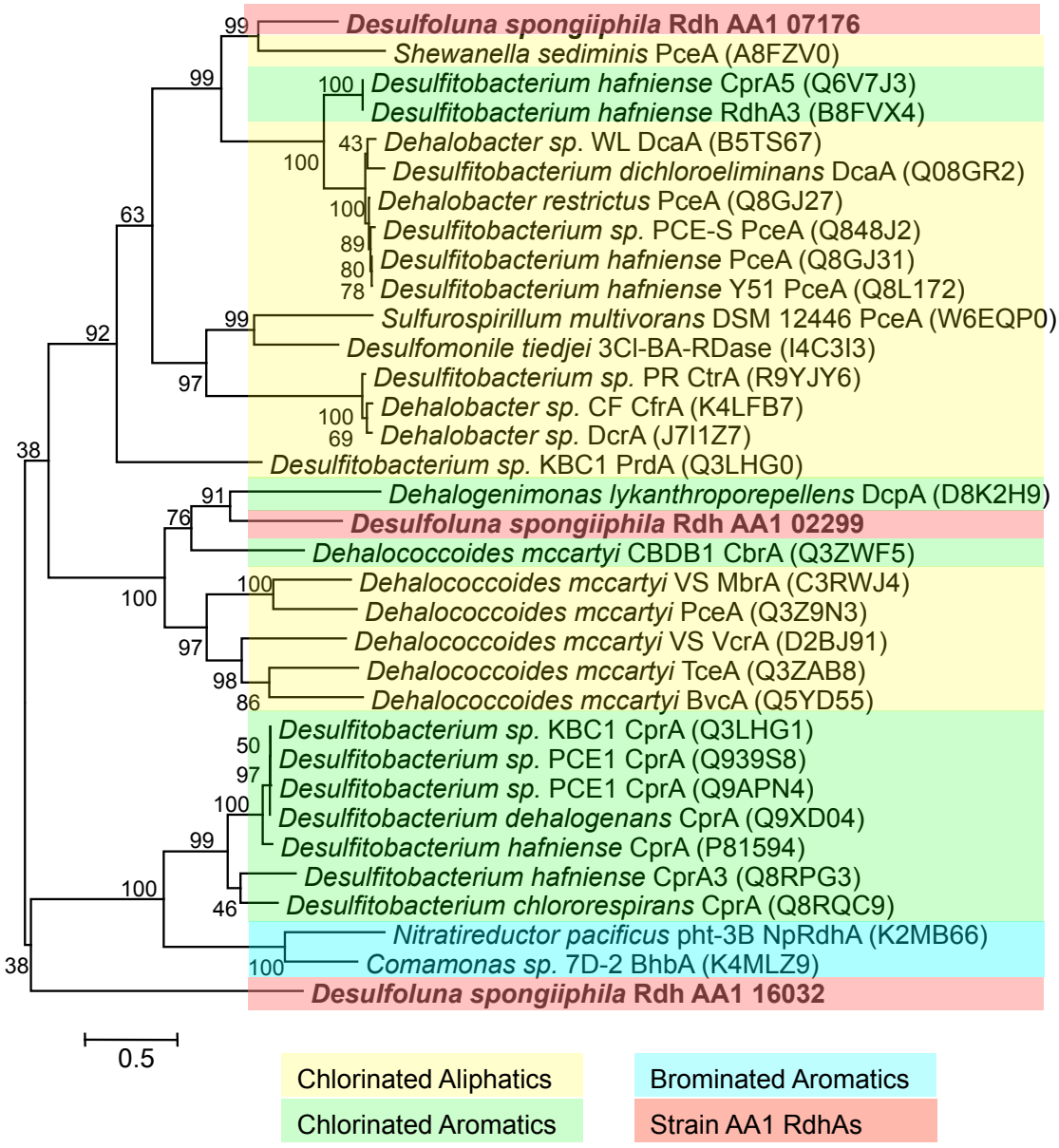


Figure 2.1 Phylogeny of strain AA1 and characterized reductive dehalogenases. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining (NJ) and advanced NJ (BioNJ) algorithms to a matrix of pairwise distances estimated using a Jones–Thornton–Taylor (JTT) model, and then selecting the topology with superior log likelihood value. The Maximum Likelihood tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The

analysis involved 34 amino acid sequences. All positions with less than 60% site coverage were eliminated, i.e., fewer than 40% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 479 positions in the final dataset. The characterized RdhAs are highlighted with different colors based on their substrates. *D. spongiiphila* AA1 RdhAs are highlighted in red.

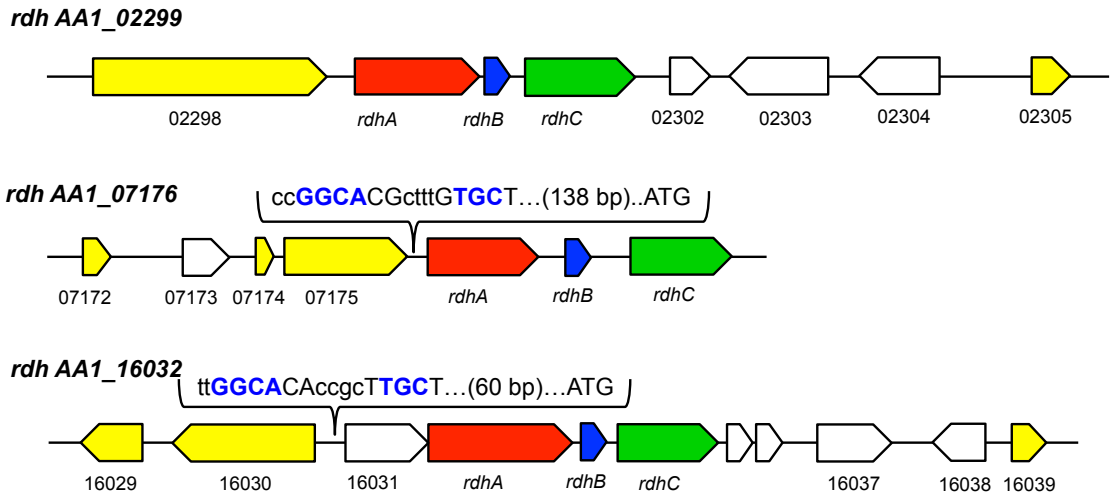


Figure 2.2 Reductive dehalogenase gene clusters in strain AA1. The putative *rdhA* is in red, putative membrane anchoring gene *rdhB* is in blue, and the putative *rdhC*-like gene is in green. The genes annotated with potential transcriptional regulation function are colored in yellow. The genes with other functions or hypothetic proteins are colored in white. The sequence of sigma factor 54 binding site in promoter region (-12 and -24 element) is indicated for *rdh AA1_07176* and *rdh AA1_16032*. Detailed annotation is as follows: *rdh AA1_02299*: 02298 – LuxR family regulatory protein; 02305 – MarR family transcriptional regulator. *rdh AA1_07176*: 07172 – HxlR family transcriptional regulator; 174 – Putative transcription regulatory; 175 - Sigma-54-dependent transcriptional regulator. *rdh AA1_16032*: 01629 - TetR family transcriptional regulator; 16030 - Sigma-54-dependent transcriptional regulator; 16031 -Tetratricopeptide repeat containing protein; 16037 - Transporter; 16039 - HxlR family transcriptional regulator.

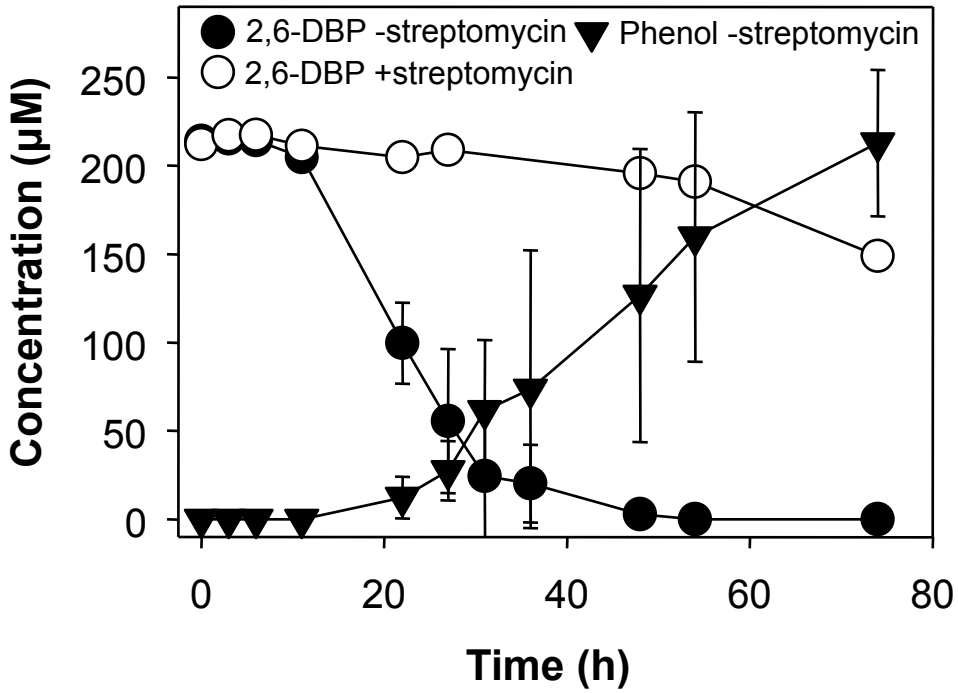


Figure 2.3 Induction of debromination activity. The concentration of 2,6-DBP in *D. spongiiphila* strain AA1 culture with or without streptomycin (1 mg/ml) to inhibit protein synthesis. Data points are the means of triplicate cultures.

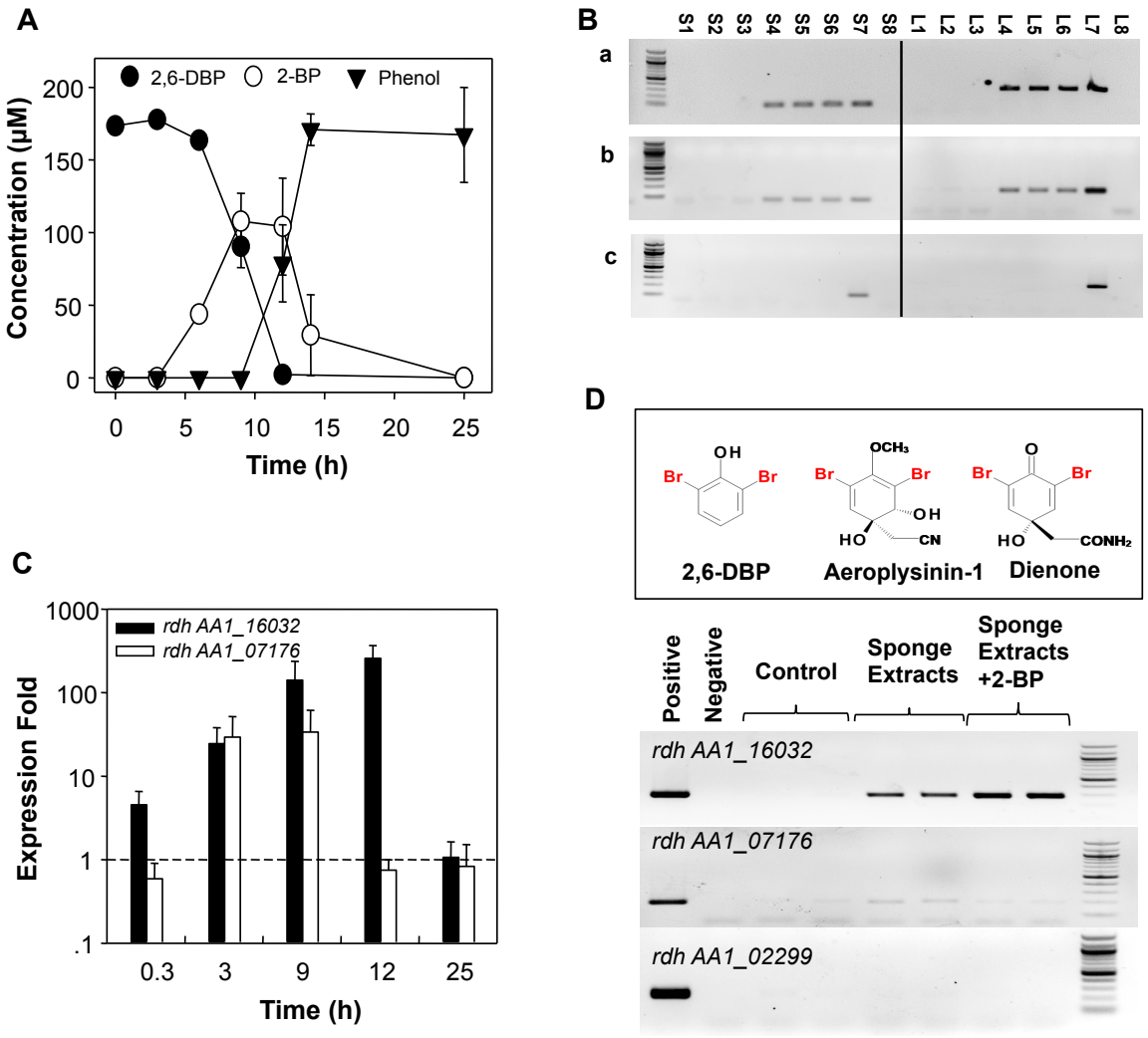

A
2,6-DBP
2-BP
Phenol
Concentration (μM)
Time (h)
B
S1 S2 S3 S4 S5 S6 S7 S8 L1 L2 L3 L4 L5 L6 L7 L8
a
b
c
C
rdh AA1_16032
rdh AA1_07176
Expression Fold
Time (h)
D
2,6-DBP
Aeroplysinin-1
Dienone
Positive
Negative
Control
Sponge Extracts
Sponge Extracts +2-BP
rdh AA1_16032
rdh AA1_07176
rdh AA1_02299

Figure 2.4 Debromination activity in 2,6-DBP induced *D. spongiiphila* AA1 culture over time and the expression of each *rdhA* gene. (A) Concentration of 2,6-DBP and its debrominating product, 2-bromophenol and phenol in induced cultures over time. Points and error bars are means and standard deviations of biological triplicates. (B) PCR amplification of cDNA from strain AA1 culture after 6 h incubation with primers targeting *rdh AA1_16032* (a), *rdh AA1_07176* (b) and *rdh AA1_02299* (c). For each *rdh* gene, two primer sets (S and L) were used in order to confirm the results. Lanes S/L1-3: Control culture triplicates; Lane S/L 4-6: Induced culture triplicates; S/L 7: Positive control; S/L 8: Negative control. 100 bp DNA ladder (New England Biolabs) was loaded on the left of the gel. (C) Relative expression of *rdh AA1_16032 and rdh AA1_07176* over incubation time. The expression level of *the* genes was normalized to the expression of the 16S rRNA gene. The y-axis indicates the expression fold of 2,6-DBP induced cultures compared to untreated controls. Error bars represent the standard deviation of three biological triplicates, each of which contains two or three RT-PCR reaction replicates. (D) Structure of 2,6-DBP, aeroplysinin-1 and dienone, two examples of brominated compounds found in *Aplysina aerophoba* sponges (Teeyapant *et al*., 1993) and expression of strain AA1 *rdhA*s in the presence of sponge extracts after 4 h incubation.

Supplementary Information

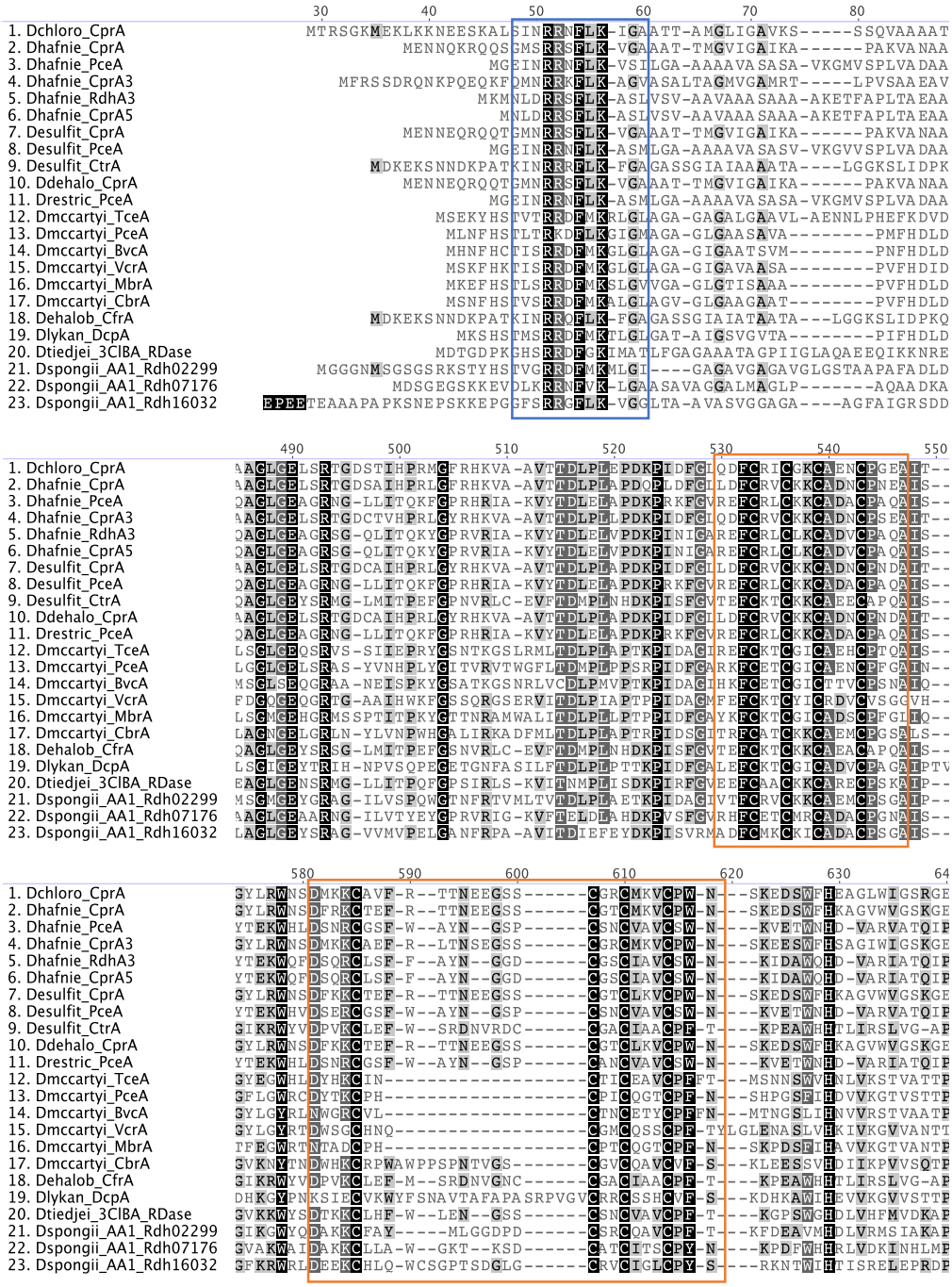


Figure S2.1 N and C-terminal amino acid sequence alignment of potential reductive dehalogenases of *D. spongiiphila* and functionally characterized reductive dehalogenases whose functions have been identified using proteomic techniques (Jugder *et al.,* 2015). The conserved twin arginine signal sequence motifs (RRXFXK) are boxed in blue and the two iron-sulfur cluster motifs (CXXCXXCXXXCP and CXXCXXXCP) are boxed in red. The twin arginine translocation (Tat) signal sequence motif found in the reductive dehalogenase genes indicates the presence of the RdhA at the cell membrane. The role of the Fe-S clusters in the enzyme is proposed in electron transfer (Schumacher *et al*., 1997). Uniprot entries for the organohalide respiring bacteria and their reductive dehalogenases are as follows: Dchloro_CprA: *Desulfitobacterium chlororespirans*, Q8RQC9; Dhafnie_CprA: *Desulfitobacterium hafniense*, P81594; Dhafnie_PceA: *Desulfitobacterium hafniense* strain Y51, Q8L172; Dhafnie_CprA3: *Desulfitobacterium hafniense,* Q8RPG3; Dhafnie_RdhA3: *Desulfitobacterium hafniense*, B8FVX4; Dhafnie_CprA5: *Desulfitobacterium hafniense*, Q6V7J3; Desulfit_CprA: *Desulfitobacterium* sp. PCE1, Q9APN4; Desulfit_PceA: *Desulfitobacterium* sp. PCE-S, Q848J2; Desulfit_CtrA: *Desulfitobacterium* sp. PR, R9YJY6; Ddehalo_CprA: *Desulfitobacterium dehalogenans*, Q9XD04; Drestric_PceA: *Dehalobacter restrictus*, Q8GJ27; Dmccartyi_TceA: *Dehalococcoides mccartyi*, Q3ZAB8; Dmccartyi_PceA: *Dehalococcoides mccartyi,* Q3Z9N3; Dmccartyi_BvcA: *Dehalococcoides mccartyi*, Q5YD55; Dmccartyi_VcrA: *Dehalococcoides mccartyi*, D2BJ91; Dmccartyi_MbrA: *Dehalococcoides* mccartyi MB, C3RWJ4; Dmccartyi_CbrA: *Dehalococcoides* mccartyi CBDB1, Q3ZWF5; Dehalob_CfrA: *Dehalobacter* sp. CF, K4LFB7; Dlykan_DcpA: *Dehalogenimonas lykanthroporepellens*, D8K2H9; Dtiedjei_3ClBA_RDase:

Desulfomonile tiedjei strain, I4C3I3; Dspongii_AA1_Rdh02299: *Desulfoluna spongiiphila* strain AA1; Dspongii_AA1_Rdh07176: *Desulfoluna spongiiphila* strain AA1; Dspongii_AA1_Rdh16032: *Desulfoluna spongiiphila* strain AA1.

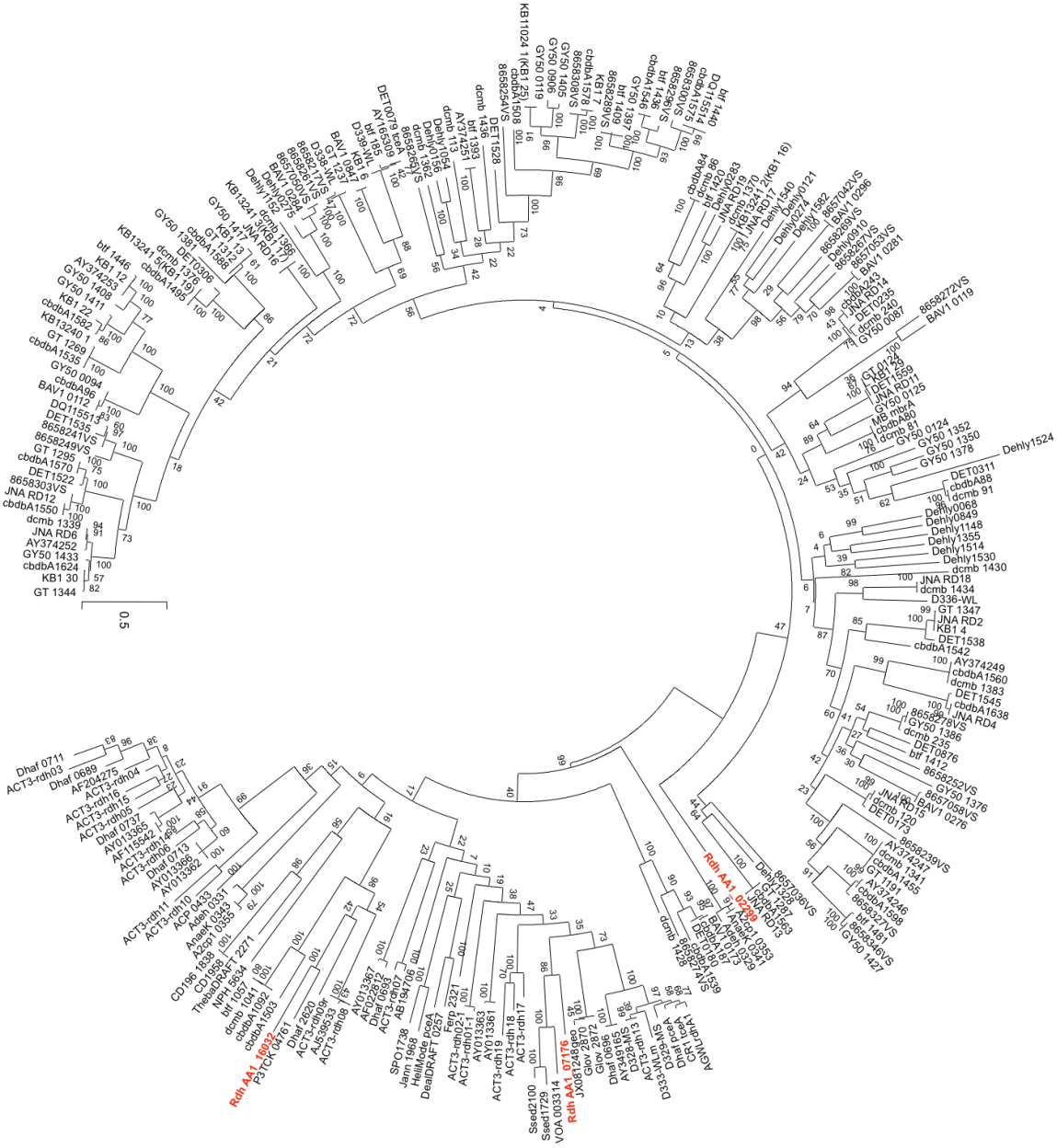

0.5
Rdh AA1 16032
Rdh AA1 07176
Rdh AA1 07238

Figure S2.2 Phylogenetic tree of reductive dehalogenases. Maximum Likelihood tree of reductive dehalogenases and translated *rdhA* genes based on amino acid alignment. The amino acid sequences for reductive dehalogenases (other than *D. spongiiphila* RdhAs) were obtained from the public link: docs.google.com/folder/d/0BwCzK8wzlz8ON1o2Z3FTbHFPYXc/edit provided by Hug et al., 2013. The original file contains 355 reductive dehalogenase sequences, which are classified into 57 ortholog groups base on a threshold of 90% identity in amino acid alignments. In order to reduce the tree complexity, only 3 sequences were used from ortholog groups containing more than 2 members. The multiple sequence alignment was processed using GUIDANCE2 with the MAFFT algorithm. Unreliable columns were removed based on column score given by GUIDANCE2 results. The phylogenetic tree was constructed using the Maximum Likelihood method based on the JTT matrix based mode in MEGA7 (Kumar *et al*., 2016). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. In total, 254 sequences and 953 positions were analyzed to construct the tree. *D. spongiiphila* Strain AA1 RdhAs are colored in red on tree. The branch name and its corresponding organism are listed in Supplemental Table S2.3.

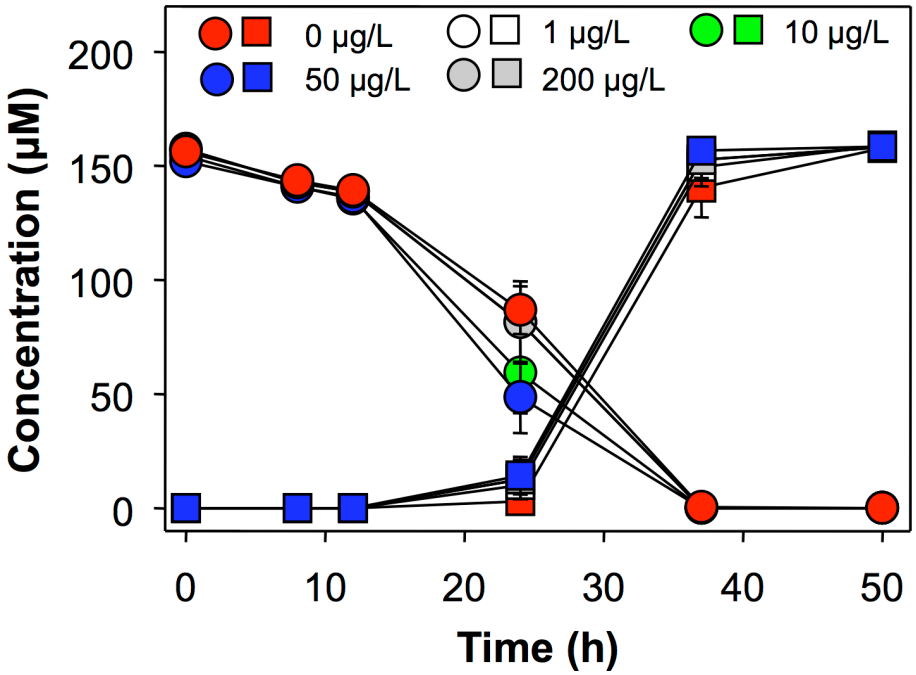


Figure S2.3 Influence of exogenous cobalamin on *D. spongiiphila* strain AA1 debrominating activity. Circles indicate the concentration of 2,6-DBP and square the concentration of phenol at different concentrations of cobalamin. Data points are the means of triplicate cultures.

Table S2.1 Potential corrinoid genes associated with *Desulfoluna spongiiphila*. Potential genes associated with *de novo* corrinoid biosynthesis pathway and corrinoid uptake.

Scaffold	Gene Abbrev.	Functional Role
1	CobT	Nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase
	CblY	Displacement for Alpha-ribazole-5'-phosphate phosphatase
2	CbiO	Cobalt ABC transporter ATPase component
3	HemC	Porphobilinogen deaminase
	CobA	Uroporphyrinogen-III methyltransferase
5	BtuR	Cob(I)alamin adenosyltransferase
	CobC	Alpha-ribazole-5'-phosphate phosphatase
6	HemL	Glutamate-1-semialdehyde aminotransferase
	BtuB	Outer membrane vitamin B12 receptor
8	BtuD	Vitamin B12 ABC transporter, ATPase component
	BtuC	Vitamin B12 ABC transporter, permease component
	BtuF ⋆	Vitamin B12 ABC transporter, B12-binding component
	CblY	Displacement for Alpha-ribazole-5'-phosphate phosphatase
	CbiK	Sirohydrochlorin cobaltochelatase
	BtuB	Outer membrane vitamin B12 receptor
11	-	Glutamyl-tRNA synthetase
12	HemL	Glutamate-1-semialdehyde aminotransferase
	BtuB	Outer membrane vitamin B12 receptor
20	HemL	Glutamate-1-semialdehyde aminotransferase

25	CbiA	Cobyrinic acid A,C-diamide synthase
28	CbiK	Sirohydrochlorin cobaltochelatase
	BtuC	Vitamin B12 ABC transporter, permease component
	BtuD	Vitamin B12 ABC transporter, ATPase component
	BtuF	Vitamin B12 ABC transporter, B12-binding component
	CbiA	Cobyrinic acid A,C-diamide synthase
	CbiC	Cobalt-precorrin-8x methylmutase
	CbiL	Cobalt-precorrin-2 C20-methyltransferase
	CblY	Displacement for Alpha-ribazole-5'-phosphate phosphatase
	CbiDJ	Cobalt-precorrin-6 synthase
	CbiET	Cobalt-precorrin-6y C5-methyltransferase
	CbiF	Cobalt-precorrin-4 C11-methyltransferase
	CbiG	Cobalamin biosynthesis protein
	CbiH	Cobalt-precorrin-3b C17-methyltransferase
	BtuR	Cob(I)alamin adenosyltransferase
	CbiP	Cobyric acid synthase
	CobU	Adenosylcobinamide-phosphate guanylyltransferase
	CobS	Cobalamin synthase
	CobD	L-threonine 3-O-phosphate decarboxylase
	CbiB	Adenosylcobinamide-phosphate synthase
29	CysG	Siroheme synthase / Precorrin-2 oxidase
	HemA	Glutamyl-tRNA reductase
30	HemB	Porphobilinogen synthase
34	PduS ⋆	Cob(II)alamin reductase

*: Two genes with annotated function are present in this scaffold.

Table S2.2 Oligonucleotides and plasmids used in this study.

Primer	**Sequence (5'-3')**	**Target Gene**	**Target Length (bp)**
S_422f	AGGCTCTTGGGTCGTAAAGC	*16s rRNA*	97
S_519r	GTTAGCCGGTGCTTCCTTCA		
S_1029f	AGACCTTCCTCTGGCTGAAA	*rdh AA1_02299*	64
S_1093r	CACATTTCAGCGCATTTCTT		
L_1071f	TGTGACGTTTTGCAGGGTCT		224
L_1295r	ACGGCTTCATCGAACTTGGT		
S_770f	GCTGGACCTACGCCTCTTTT	*rdh AA1_07176*	74
S_844r	ATTTGGGGGTAAAGGGCAGG		
L_669f	CCGCTACGATTTTGCATCCC		187
L_856r	TGACGATGACCGATTTGGGG		
S_1061f	AGAGCATCATCGTCTTCGG	*rdh AA1_16032*	64
S_1125r	AATAGGCGTCAAAGGAGGTG		
L_121f	GTCCACAGGATGGAAGGAGC		285
L_406r	AACCTGTGTAGGCATCGTCG		

Table S2.3 Reductive dehalogenase homologous genes shown in Figure S2.2.

Name on Tree	ORGANISM	KEY	Source	Phylogy (Class)
ACP_0433	*Acidobacterium capsulatum*	ACP_0433	JGI locus tag	Acidobacteria
Jann_1968	*Jannaschia* sp. CCS1	3934419	NCBI Gene ID	Alphaproteobacteria
SPO1738	*Ruegeria pomeroyi* DSS-3	3192799	NCBI Gene ID	Alphaproteobacteria
Ferp_2321	*Ferroglobus placidus* DSM 10642	8779861	NCBI Gene ID	Archaeoglobi
8657036VS	*Dehalococcoides mccartyi* VS	8657036	NCBI Gene ID	Chloroflexi
8657042VS	*Dehalococcoides mccartyi* VS	8657042	NCBI Gene ID	Chloroflexi
8657050VS	*Dehalococcoides mccartyi* VS	8657050	NCBI Gene ID	Chloroflexi
8657053VS	*Dehalococcoides mccartyi* VS	8657053	NCBI Gene ID	Chloroflexi
8657058VS	*Dehalococcoides mccartyi* VS	8657058	NCBI Gene ID	Chloroflexi
8658217VS	*Dehalococcoides mccartyi* VS (*vcrA*)	8658217	NCBI Gene ID	Chloroflexi
8658239VS	*Dehalococcoides mccartyi* VS	8658239	NCBI Gene ID	Chloroflexi
8658241VS	*Dehalococcoides mccartyi* VS	8658241	NCBI Gene ID	Chloroflexi
8658249VS	*Dehalococcoides mccartyi* VS	8658249	NCBI Gene ID	Chloroflexi
8658252VS	*Dehalococcoides mccartyi* VS	8658252	NCBI Gene ID	Chloroflexi
8658254VS	*Dehalococcoides mccartyi* VS	8658254	NCBI Gene ID	Chloroflexi
8658261VS	*Dehalococcoides mccartyi* VS	8658261	NCBI Gene ID	Chloroflexi
8658265VS	*Dehalococcoides mccartyi* VS	8658265	NCBI Gene ID	Chloroflexi
8658267VS	*Dehalococcoides mccartyi* VS	8658267	NCBI Gene ID	Chloroflexi

8658269VS	*Dehalococcoides mccartyi* VS	8658269	NCBI Gene ID	Chloroflexi
8658272VS	*Dehalococcoides mccartyi* VS	8658272	NCBI Gene ID	Chloroflexi
8658274VS	*Dehalococcoides mccartyi* VS	8658274	NCBI Gene ID	Chloroflexi
8658278VS	*Dehalococcoides mccartyi* VS	8658278	NCBI Gene ID	Chloroflexi
8658289VS	*Dehalococcoides mccartyi* VS	8658289	NCBI Gene ID	Chloroflexi
8658296VS	*Dehalococcoides mccartyi* VS	8658296	NCBI Gene ID	Chloroflexi
8658300VS	*Dehalococcoides mccartyi* VS	8658300	NCBI Gene ID	Chloroflexi
8658303VS	*Dehalococcoides mccartyi* VS	8658303	NCBI Gene ID	Chloroflexi
8658308VS	*Dehalococcoides mccartyi* VS	8658308	NCBI Gene ID	Chloroflexi
8658327VS	*Dehalococcoides mccartyi* VS	8658327	NCBI Gene ID	Chloroflexi
8658346VS	*Dehalococcoides mccartyi* VS	8658346	NCBI Gene ID	Chloroflexi
AY165309	*Dehalococcoides mccartyi* FL2	AY165309	NCBI Accession	Chloroflexi
AY374246	*Dehalococcoides mccartyi* FL2	AY374246	NCBI Accession	Chloroflexi
AY374247	*Dehalococcoides mccartyi* FL2	AY374247	NCBI Accession	Chloroflexi
AY374249	*Dehalococcoides mccartyi* FL2	AY374249	NCBI Accession	Chloroflexi
AY374251	*Dehalococcoides mccartyi* FL2	AY374251	NCBI Accession	Chloroflexi
AY374252	*Dehalococcoides mccartyi* FL2	AY374252	NCBI Accession	Chloroflexi
AY374253	*Dehalococcoides mccartyi* FL2	AY374253	NCBI Accession	Chloroflexi
BAV1_0112	*Dehalococcoides mccartyi* BAV1	5132438	NCBI Gene ID	Chloroflexi
BAV1_0119	*Dehalococcoides mccartyi* BAV1	5131444	NCBI Gene ID	Chloroflexi
BAV1_0173	*Dehalococcoides mccartyi* BAV1	5132211	NCBI Gene ID	Chloroflexi

BAV1_0276	*Dehalococcoides mccartyi* BAV1 (*bvcA*)	5132094	NCBI Gene ID	Chloroflexi
BAV1_0281	*Dehalococcoides mccartyi* BAV1	5132381	NCBI Gene ID	Chloroflexi
BAV1_0296	*Dehalococcoides mccartyi* BAV1	5132361	NCBI Gene ID	Chloroflexi
BAV1_0284	*Dehalococcoides mccartyi* BAV1	5132431	NCBI Gene ID	Chloroflexi
BAV1_0847	*Dehalococcoides mccartyi* BAV1	5131305	NCBI Gene ID	Chloroflexi
btf_1057	*Dehalococcoides mccartyi* BTF08	452112403	NCBI Gene ID	Chloroflexi
btf_1393	*Dehalococcoides mccartyi* BTF08	452112727	NCBI Gene ID	Chloroflexi
btf_1409	*Dehalococcoides mccartyi* BTF08	452112743	NCBI Gene ID	Chloroflexi
btf_1412	*Dehalococcoides mccartyi* BTF08	452112746	NCBI Gene ID	Chloroflexi
btf_1420	*Dehalococcoides mccartyi* BTF08	452112754	NCBI Gene ID	Chloroflexi
btf_1436	*Dehalococcoides mccartyi* BTF08	452112770	NCBI Gene ID	Chloroflexi
btf_1440	*Dehalococcoides mccartyi* BTF08	452112774	NCBI Gene ID	Chloroflexi
btf_1446	*Dehalococcoides mccartyi* BTF08	452112780	NCBI Gene ID	Chloroflexi
btf_1481	*Dehalococcoides mccartyi* BTF08	452112815	NCBI Gene ID	Chloroflexi
btf_185	*Dehalococcoides mccartyi* BTF08	452111563	NCBI Gene ID	Chloroflexi
cbdbA1092	*Dehalococcoides mccartyi* CBDB1	3623213	NCBI Gene ID	Chloroflexi
cbdbA1455	*Dehalococcoides mccartyi* CBDB1	3623215	NCBI Gene ID	Chloroflexi
cbdbA1495	*Dehalococcoides mccartyi* CBDB1	3623217	NCBI Gene ID	Chloroflexi
cbdbA1503	*Dehalococcoides mccartyi* CBDB1	3623218	NCBI Gene ID	Chloroflexi
cbdbA1508	*Dehalococcoides mccartyi* CBDB1	3623219	NCBI Gene ID	Chloroflexi
cbdbA1535	*Dehalococcoides mccartyi* CBDB1	3623220	NCBI Gene ID	Chloroflexi

cbdbA1539	*Dehalococcoides mccartyi* CBDB1	3623221	NCBI Gene ID	Chloroflexi
cbdbA1542	*Dehalococcoides mccartyi* CBDB1	3623222	NCBI Gene ID	Chloroflexi
cbdbA1546	*Dehalococcoides mccartyi* CBDB1	3623223	NCBI Gene ID	Chloroflexi
cbdbA1550	*Dehalococcoides mccartyi* CBDB1	3623444	NCBI Gene ID	Chloroflexi
cbdbA1560	*Dehalococcoides mccartyi* CBDB1	3623445	NCBI Gene ID	Chloroflexi
cbdbA1563	*Dehalococcoides mccartyi* CBDB1	3623446	NCBI Gene ID	Chloroflexi
cbdbA1570	*Dehalococcoides mccartyi* CBDB1	3623447	NCBI Gene ID	Chloroflexi
cbdbA1575	*Dehalococcoides mccartyi* CBDB1	3623448	NCBI Gene ID	Chloroflexi
cbdbA1578	*Dehalococcoides mccartyi* CBDB1	3623468	NCBI Gene ID	Chloroflexi
cbdbA1582	*Dehalococcoides mccartyi* CBDB1	3623469	NCBI Gene ID	Chloroflexi
cbdbA1588	*Dehalococcoides mccartyi* CBDB1	3623470	NCBI Gene ID	Chloroflexi
cbdbA1598	*Dehalococcoides mccartyi* CBDB1	3623472	NCBI Gene ID	Chloroflexi
cbdbA1624	*Dehalococcoides mccartyi* CBDB1	3623474	NCBI Gene ID	Chloroflexi
cbdbA1638	*Dehalococcoides mccartyi* CBDB1	3623476	NCBI Gene ID	Chloroflexi
cbdbA187	*Dehalococcoides mccartyi* CBDB1	3623477	NCBI Gene ID	Chloroflexi
cbdbA243	*Dehalococcoides mccartyi* CBDB1	3623479	NCBI Gene ID	Chloroflexi
cbdbA80	*Dehalococcoides mccartyi* CBDB1	3623480	NCBI Gene ID	Chloroflexi
cbdbA84	*Dehalococcoides mccartyi* CBDB1 *(cbrA*)	3623481	NCBI Gene ID	Chloroflexi
cbdbA88	*Dehalococcoides mccartyi* CBDB1	3623482	NCBI Gene ID	Chloroflexi
cbdbA96	*Dehalococcoides mccartyi* CBDB1	3623483	NCBI Gene ID	Chloroflexi
D336-WL	*Dehalococcoides mccartyi* WL	(WL_*Dhc*_01)	in house name	Chloroflexi

D338-WL	*Dehalococcoides mccartyi* WL		in house name	Chloroflexi
D339-WL	*Dehalococcoides mccartyi* WL		in house name	Chloroflexi
dcmb_1041	*Dehalococcoides mccartyi* DCMB5	452110910	NCBI Gene ID	Chloroflexi
dcmb_113	*Dehalococcoides mccartyi* DCMB5	452110014	NCBI Gene ID	Chloroflexi
dcmb_120	*Dehalococcoides mccartyi* DCMB5	452110021	NCBI Gene ID	Chloroflexi
dcmb_1339	*Dehalococcoides mccartyi* DCMB5	452111197	NCBI Gene ID	Chloroflexi
dcmb_1341	*Dehalococcoides mccartyi* DCMB5	452111199	NCBI Gene ID	Chloroflexi
dcmb_1362	*Dehalococcoides mccartyi* DCMB5	452111220	NCBI Gene ID	Chloroflexi
dcmb_1366	*Dehalococcoides mccartyi* DCMB5	452111224	NCBI Gene ID	Chloroflexi
dcmb_1370	*Dehalococcoides mccartyi* DCMB5	452111228	NCBI Gene ID	Chloroflexi
dcmb_1376	*Dehalococcoides mccartyi* DCMB5	452111234	NCBI Gene ID	Chloroflexi
dcmb_1383	*Dehalococcoides mccartyi* DCMB5	452111241	NCBI Gene ID	Chloroflexi
dcmb_1428	*Dehalococcoides mccartyi* DCMB5	452111285	NCBI Gene ID	Chloroflexi
dcmb_1430	*Dehalococcoides mccartyi* DCMB5	452111287	NCBI Gene ID	Chloroflexi
dcmb_1434	*Dehalococcoides mccartyi* DCMB5	452111291	NCBI Gene ID	Chloroflexi
dcmb_1436	*Dehalococcoides mccartyi* DCMB5	452111293	NCBI Gene ID	Chloroflexi
dcmb_235	*Dehalococcoides mccartyi* DCMB5	452110134	NCBI Gene ID	Chloroflexi
dcmb_240	*Dehalococcoides mccartyi* DCMB5	452110139	NCBI Gene ID	Chloroflexi
dcmb_81	*Dehalococcoides mccartyi* DCMB5	452109982	NCBI Gene ID	Chloroflexi
dcmb_86	*Dehalococcoides mccartyi* DCMB5	452109987	NCBI Gene ID	Chloroflexi
dcmb_91	*Dehalococcoides mccartyi* DCMB5	452109992	NCBI Gene ID	Chloroflexi

DET0079_tceA	*Dehalococcoides mccartyi* 195 (*tceA*)	3229017	NCBI Gene ID	Chloroflexi
DET0173	*Dehalococcoides mccartyi* 195	3230555	NCBI Gene ID	Chloroflexi
DET0180	*Dehalococcoides mccartyi* 195	3230541	NCBI Gene ID	Chloroflexi
DET0235	*Dehalococcoides mccartyi* 195	3230432	NCBI Gene ID	Chloroflexi
DET0306	*Dehalococcoides mccartyi* 195	3230325	NCBI Gene ID	Chloroflexi
DET0311	*Dehalococcoides mccartyi* 195	3230320	NCBI Gene ID	Chloroflexi
DET0876	*Dehalococcoides mccartyi* 195	3229805	NCBI Gene ID	Chloroflexi
DET1522	*Dehalococcoides mccartyi* 195	3229220	NCBI Gene ID	Chloroflexi
DET1528	*Dehalococcoides mccartyi* 195	3229214	NCBI Gene ID	Chloroflexi
DET1535	*Dehalococcoides mccartyi* 195	3229198	NCBI Gene ID	Chloroflexi
DET1538	*Dehalococcoides mccartyi* 195	3229187	NCBI Gene ID	Chloroflexi
DET1545	*Dehalococcoides mccartyi* 195	3229172	NCBI Gene ID	Chloroflexi
DET1559	*Dehalococcoides mccartyi* 195	3229173	NCBI Gene ID	Chloroflexi
DQ115513	*Dehalococcoides mccartyi* FL2	73913555	NCBI Gene ID	Chloroflexi
DQ115514	*Dehalococcoides mccartyi* FL2	73913558	NCBI Gene ID	Chloroflexi
GT_0241	*Dehalococcoides mccartyi* GT	8808689	NCBI Gene ID	Chloroflexi
GT_1191	*Dehalococcoides mccartyi* GT	8809641	NCBI Gene ID	Chloroflexi
GT_1237	*Dehalococcoides mccartyi* GT	8809688	NCBI Gene ID	Chloroflexi
GT_1269	*Dehalococcoides mccartyi* GT	8809720	NCBI Gene ID	Chloroflexi
GT_1287	*Dehalococcoides mccartyi* GT	8809738	NCBI Gene ID	Chloroflexi
GT_1295	*Dehalococcoides mccartyi* GT	8809746	NCBI Gene ID	Chloroflexi

GT_1312	*Dehalococcoides mccartyi* GT	8809763	NCBI Gene ID	Chloroflexi
GT_1344	*Dehalococcoides mccartyi* GT	8809795	NCBI Gene ID	Chloroflexi
GT_1347	*Dehalococcoides mccartyi* GT	8809798	NCBI Gene ID	Chloroflexi
GY50_0087	*Dehalococcoides mccartyi* BTF08	559771361	NCBI Gene ID	Chloroflexi
GY50_0094	*Dehalococcoides mccartyi* BTF08	559771368	NCBI Gene ID	Chloroflexi
GY50_0119	*Dehalococcoides mccartyi* BTF08	559771393	NCBI Gene ID	Chloroflexi
GY50_0124	*Dehalococcoides mccartyi* BTF08	559771398	NCBI Gene ID	Chloroflexi
GY50_0125	*Dehalococcoides mccartyi* BTF08	559771399	NCBI Gene ID	Chloroflexi
GY50_0906	*Dehalococcoides mccartyi* BTF08	559772169	NCBI Gene ID	Chloroflexi
GY50_1350	*Dehalococcoides mccartyi* BTF08	559772609	NCBI Gene ID	Chloroflexi
GY50_1352	*Dehalococcoides mccartyi* BTF08	559772611	NCBI Gene ID	Chloroflexi
GY50_1376	*Dehalococcoides mccartyi* BTF08	559772634	NCBI Gene ID	Chloroflexi
GY50_1378	*Dehalococcoides mccartyi* BTF08	559772636	NCBI Gene ID	Chloroflexi
GY50_1381	*Dehalococcoides mccartyi* BTF08	559772639	NCBI Gene ID	Chloroflexi
GY50_1386	*Dehalococcoides mccartyi* BTF08	559772643	NCBI Gene ID	Chloroflexi
GY50_1397	*Dehalococcoides mccartyi* BTF08	559772654	NCBI Gene ID	Chloroflexi
GY50_1405	*Dehalococcoides mccartyi* BTF08	559772662	NCBI Gene ID	Chloroflexi
GY50_1408	*Dehalococcoides mccartyi* BTF08	559772665	NCBI Gene ID	Chloroflexi
GY50_1411	*Dehalococcoides mccartyi* BTF08	559772668	NCBI Gene ID	Chloroflexi
GY50_1417	*Dehalococcoides mccartyi* BTF08	559772674	NCBI Gene ID	Chloroflexi
GY50_1427	*Dehalococcoides mccartyi* BTF08	559772684	NCBI Gene ID	Chloroflexi

GY50_1433	*Dehalococcoides mccartyi* BTF08	559772690	NCBI Gene ID	Chloroflexi
JNA_RD11	*Dehalococcoides mccartyi* JNA	KJ580609	NCBI Accession	Chloroflexi
JNA_RD12	*Dehalococcoides mccartyi* JNA	KJ5806010	NCBI Accession	Chloroflexi
JNA_RD13	*Dehalococcoides mccartyi* JNA	KJ5806011	NCBI Accession	Chloroflexi
JNA_RD14	*Dehalococcoides mccartyi* JNA	KJ5806012	NCBI Accession	Chloroflexi
JNA_RD15	*Dehalococcoides mccartyi* JNA	KJ5806013	NCBI Accession	Chloroflexi
JNA_RD16	*Dehalococcoides mccartyi* JNA	KJ5806014	NCBI Accession	Chloroflexi
JNA_RD17	*Dehalococcoides mccartyi* JNA	KJ5806015	NCBI Accession	Chloroflexi
JNA_RD18	*Dehalococcoides mccartyi* JNA	KJ5806016	NCBI Accession	Chloroflexi
JNA_RD19	Dehalococcoides mccartyi JNA	KJ5806017	NCBI Accession	Chloroflexi
JNA_RD2	Dehalococcoides mccartyi JNA	KJ580600	NCBI Accession	Chloroflexi
JNA_RD4	*Dehalococcoides mccartyi* JNA	KJ580602	NCBI Accession	Chloroflexi
JNA_RD6	*Dehalococcoides mccartyi* JNA	KJ580604	NCBI Accession	Chloroflexi
JX081248geo	*Dehalococcoides* sp. KB-1 consortium	JX081248	NCBI Gene ID	Chloroflexi
KB1_12	*Dehalococcoides* sp. KB-1 consortium	77176880	NCBI Gene ID	Chloroflexi
KB1_13	*Dehalococcoides* sp. KB-1 consortium	77176883	NCBI Gene ID	Chloroflexi
KB1_22	*Dehalococcoides* sp. KB-1 consortium	JX081249	NCBI Accession	Chloroflexi
KB1_29	*Dehalococcoides* sp. KB-1 consortium	733372969	NCBI Gene ID	Chloroflexi
KB1_30	*Dehalococcoides* sp. KB-1 consortium	733372971	NCBI Gene ID	Chloroflexi
KB1_4	*Dehalococcoides* sp. KB-1 consortium	77176856	NCBI Gene ID	Chloroflexi
KB1_6	*Dehalococcoides* sp. KB-1 consortium	77176862	NCBI Gene ID	Chloroflexi

KB1_7	*Dehalococcoides* sp. KB-1 consortium	77176865	NCBI Gene ID	Chloroflexi
KB11024_1/KB1_25	*Dehalococcoides* sp. KB-1 consortium	733372957	NCBI Gene ID	Chloroflexi
KB13240_1	*Dehalococcoides* sp. KB-1 consortium	DCKB1_114790	JGI locus tag	Chloroflexi
KB13241_2/KB1_16	*Dehalococcoides* sp. KB-1 consortium	733372933	NCBI Gene ID	Chloroflexi
KB13241_3KB1_17	*Dehalococcoides* sp. KB-1 consortium	733372936	NCBI Gene ID	Chloroflexi
KB13241_5/KB1_19	*Dehalococcoides* sp. KB-1 consortium	733372942	NCBI Gene ID	Chloroflexi
MB_mbrA	*Dehalococcoides mccartyi* MB (mbrA)	GU120391	NCBI Accession	Chloroflexi
Dehly0068	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9389745	NCBI Gene ID	Chloroflexi
Dehly0121	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9389797	NCBI Gene ID	Chloroflexi
Dehly0156	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9389835	NCBI Gene ID	Chloroflexi
Dehly0274	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9389954	NCBI Gene ID	Chloroflexi
Dehly0275	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9389955	NCBI Gene ID	Chloroflexi
Dehly0283	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9389963	NCBI Gene ID	Chloroflexi
Dehly0849	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9390556	NCBI Gene ID	Chloroflexi
Dehly0910	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9390618	NCBI Gene ID	Chloroflexi
Dehly1054	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9390763	NCBI Gene ID	Chloroflexi
Dehly1148	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9390857	NCBI Gene ID	Chloroflexi
Dehly1152	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9390861	NCBI Gene ID	Chloroflexi
Dehly1328	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9391042	NCBI Gene ID	Chloroflexi
Dehly1355	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9391069	NCBI Gene ID	Chloroflexi
Dehly1514	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9391237	NCBI Gene ID	Chloroflexi

Dehly1524	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9391247	NCBI Gene ID	Chloroflexi
Dehly1530	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9391253	NCBI Gene ID	Chloroflexi
Dehly1540	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9391263	NCBI Gene ID	Chloroflexi
Dehly1582	*Dehalogenimonas lykanthroporepellens* BL-DC-9	9391305	NCBI Gene ID	Chloroflexi
A2cp1_0353	*Anaeromyxobacter dehalogenans* 2CP-1	A2cp1_0353	JGI locus tag	Deltaproteobacteria
A2cp1_0355	*Anaeromyxobacter dehalogenans* 2CP-1	A2cp1_0355	JGI locus tag	Deltaproteobacteria
Rdh AA1_02299	*Desulfoluna spongiiphila* AA1		This study	Deltaproteobacteria
Rdh AA1_07176	*Desulfoluna spongiiphila* AA1		This study	Deltaproteobacteria
Rdh AA1_16032	*Desulfoluna spongiiphila* AA1		This study	Deltaproteobacteria
Adeh_0329	*Anaeromyxobacter dehalogenans* 2CP-C	3886157	NCBI Gene ID	Deltaproteobacteria
Adeh_0331	*Anaeromyxobacter dehalogenans* 2CP-C	3886159	NCBI Gene ID	Deltaproteobacteria
AnaeK_0341	*Anaeromyxobacter dehalogenans* K	6786349	NCBI Gene ID	Deltaproteobacteria
AnaeK_0343	*Anaeromyxobacter dehalogenans* K	6786351	NCBI Gene ID	Deltaproteobacteria
Glov_2870	*Geobacter lovleyi* SZ	6369093	NCBI Gene ID	Deltaproteobacteria
Glov_2872	*Geobacter lovleyi* SZ	6366908	NCBI Gene ID	Deltaproteobacteria
NPH_5634	delta proteobacterium NaphS2	301058510	NCBI Gene ID	Deltaproteobacteria
AF022812	*Sulfurospirillum multivorans*	AF022812	NCBI Accession	Epsilonproteobacteria
AY013367	*Sulfurospirillum halorespirans* PCE-M2	AY013367	NCBI Accession	Epsilonproteobacteria
ACT3-rdh01-1	*Dehalobacter* CF (*cfrA*)	AFV05253	NCBI Accession	Firmicutes
ACT3-rdh02-1	*Dehalobacter* DCA (*dcrA*)	AFV02209	NCBI Accession	Firmicutes
ACT3-rdh03	*Dehalobacter* CF	AFV06361	NCBI Accession	Firmicutes

ACT3-rdh04	*Dehalobacter* CF	AFV06212	NCBI Accession	Firmicutes
ACT3-rdh05	*Dehalobacter* CF	AFV06391	NCBI Accession	Firmicutes
ACT3-rdh06	*Dehalobacter* CF	AFV05217	NCBI Accession	Firmicutes
ACT3-rdh07	*Dehalobacter* CF	AFV05214	NCBI Accession	Firmicutes
ACT3-rdh08	*Dehalobacter* CF	AFV05205	NCBI Accession	Firmicutes
ACT3-rdh09r	*Dehalobacter* DCA	AFV02160	NCBI Accession	Firmicutes
ACT3-rdh10	*Dehalobacter* CF	AFV05983	NCBI Accession	Firmicutes
ACT3-rdh11	*Dehalobacter* CF	AFV05981	NCBI Accession	Firmicutes
ACT3-rdh13	*Dehalobacter* CF	AFV05674	NCBI Accession	Firmicutes
ACT3-rdh14	*Dehalobacter* CF	AFV04610	NCBI Accession	Firmicutes
ACT3-rdh15	*Dehalobacter* CF	AFV06370	NCBI Accession	Firmicutes
ACT3-rdh16	*Dehalobacter* CF	AFV06369	NCBI Accession	Firmicutes
ACT3-rdh17	*Dehalobacter* CF	AFV06381	NCBI Accession	Firmicutes
ACT3-rdh18	*Dehalobacter* CF	AFV06387	NCBI Accession	Firmicutes
ACT3-rdh19	*Dehalobacter* CF	AFV06377	NCBI Accession	Firmicutes
AGWLrdhA1	*Dehalobacter* WL (*dcrA*)	FJ010189	NCBI Accession	Firmicutes
AJ539533	*Dehalobacter restrictus* DSM 9455	AJ539533	NCBI Accession	Firmicutes
D328-MS	*Dehalobacter* MS (MS_rdhA1)	FJ010192	NCBI Accession	Firmicutes
D329-MS	*Dehalobacter* MS (MS_rdhA2)	FJ010193	NCBI Accession	Firmicutes
D333-WLm	*Dehalobacter* WL (WL_rdhA2)	FJ010190	NCBI Accession	Firmicutes
AB194706	*Desulfitobacterium* sp. KBC1 (*prdA*)	AB194706	NCBI Accession	Firmicutes

AF115542	*Desulfitobacterium dehalogenans* ATCC 51507 (*cprA*)	AF115542	NCBI Accession	Firmicutes
AF204275	*Desulfitobacterium chlororespirans*	AF204275	NCBI Accession	Firmicutes
AY013361	*Desulfitobacterium* sp. PCE1	AY013361	NCBI Accession	Firmicutes
AY013362	*Desulfitobacterium hafniense* TCE1	AY013362	NCBI Accession	Firmicutes
AY013363	*Desulfitobacterium dehalogenans*	AY013363	NCBI Accession	Firmicutes
AY013365	*Desulfitobacterium hafniense* DCB-2	AY013365	NCBI Accession	Firmicutes
AY013366	*Desulfitobacterium hafniense* DCB-2	AY013366	NCBI Accession	Firmicutes
AY349165	*Desulfitobacterium hafniense* PCP-1 (*cprA*)	AY349165	NCBI Accession	Firmicutes
Dhaf_0693	*Desulfitobacterium hafniense* DCB-2	7257660	NCBI Gene ID	Firmicutes
Dhaf_0696	*Desulfitobacterium hafniense* DCB-2	7257663	NCBI Gene ID	Firmicutes
Dhaf_0711	*Desulfitobacterium hafniense* DCB-2	7257678	NCBI Gene ID	Firmicutes
Dhaf_0713	*Desulfitobacterium hafniense* DCB-2	7257680	NCBI Gene ID	Firmicutes
Dhaf_0737	*Desulfitobacterium hafniense* DCB-2	7257704	NCBI Gene ID	Firmicutes
Dhaf_2620	*Desulfitobacterium hafniense* DCB-2	7259622	NCBI Gene ID	Firmicutes
Dhaf_pceA	*Desulfitobacterium hafniense* Y51	89332194	NCBI Gene ID	Firmicutes
CR1_tceA	*Desulfitobacterium* sp. CR1	145860191	NCBI Gene ID	Firmicutes
Dhaf_0689	*Desulfitobacterium hafniense* DCB-2	7257656	NCBI Gene ID	Firmicutes
CD1958	*Clostridium difficile* 630	115249003	NCBI Gene ID	Firmicutes
CD196_1838	*Clostridium difficile* R20291	260211391	NCBI Gene ID	Firmicutes
DealDRAFT_0257	*Dethiobacter alkaliphilus* AHT 1	DealDRAFT_0257	JGI locus tag	Firmicutes
HeliMode_pceA	*Heliobacterium modesticaldum* Ice1	171696369	NCBI Gene ID	Firmicutes

P3TCK_04761	*Photobacterium profundum* 3TCK	90409568	NCBI Gene ID	Gammaproteobacteria
Ssed1729	*Shewanella sediminis* HAW-EB3	5610108	NCBI Gene ID	Gammaproteobacteria
Ssed2100	*Shewanella sediminis* HAW-EB3	5612296	NCBI Gene ID	Gammaproteobacteria
VOA_003314	*Vibrio* sp. RC586	262401591	NCBI Gene ID	Gammaproteobacteria
ThebaDRAFT_2271	*Thermotogales* bacterium mesG1.Ag.4.2	NZ_AEDC01000006.1	NCBI genome	Thermotogae

Chapter 3 Transcriptomic and proteomic analysis reveals the metabolism of *Desulfoluna spongiiphila*, a marine sponge associated dehalogenating bacterium

Abstract

Organohalide respiration is an important process for the global halogen cycle and bioremediation. In this study, we compared the global transcriptome and proteome of *Desulfoluna spongiiphila*, an organohalide respiring *Deltaproteobacteria* isolated from a marine sponge, grown under conditions with or without 2,6-dibromophneol. The most significant difference revealed by the transcriptomic analysis was the expression of one reductive dehalogenase gene cluster (*rdh16032*), which was significantly upregulated by 2.6-dibromophenol. The corresponding protein RdhA16032 was also exclusively detected in the proteome of the 2,6-dibromophenol amended cultures. There was no significant difference in genes for corrinoid biosynthesis between the two treatments, indicating that the production of corrinoid in *D. spongiiphila* is not specific for organohalide respiration. A metabolic scheme of *D. spongiiphila* is proposed based on the transcriptomic and proteomic results showing the metabolic versatility of *D. spongiiphila*.

Introduction

Many organohalides are released into the environment as environmental contaminants from industrial and agricultural sources (Alaee et al., 2003; Häggblom and Bossert, 2003; Covaci et al., 2011). Nevertheless, the widespread distribution of organohalides in the environment is not caused by recent release from human activities. Around 5,000 halogenated compounds produced from natural sources have been identified to date (Gribble, 1992, 2010). Microbial activities play important roles in the global halogen cycle as well as in the biodegradation of organohalide contaminants. Organohalide respiration is the process that microorganisms utilize halogenated compounds as electron acceptors for energy generation (Adrian and Löffler, 2016). Through this process, the dehalogenated metabolites are more vulnerable to further degradation, which may facilitate the complete removal of halogenated contaminants (Liu et al., 2013).

Organohalide-respiring bacteria (OHRBs) of diverse phyla have been identified from various environments (Hug et al., 2013). The key enzyme for organohalide respiration, reductive dehalogenase, consists of the active subunit (RdhA) and a putative membrane anchor, RdhB. Biochemical inhibitor studies and the crystal structure of a reductive dehalogenase confirmed the involvement of a corrinoid cofactor in the active center (Bommer et al., 2014). The biochemistry underlying organohalide respiration were recently reviewed (Fincker and Spormann, 2017; Schubert et al., 2018). Generally, our understanding of the biochemistry of organohalide respiration, such as electron transfer chains and gene regulatory

systems, are limited due to the experimental difficulties. The slow growth rate, oxygen sensitivity and limited genetic manipulation system of OHRBs are major reasons for the difficulties in traditional biochemical approaches. Much of our knowledge about organohalide respiration is thus based on genomic, transcriptomic and proteomic studies. The application of "omic" analyses in organohalide respiration have been reviewed recently (Türkowsky et al., 2018). These "omic" studies allow us to understand the metabolic potentials, global gene expression and protein production of OHRBs during organohalide respiration.

Desulfoluna spongiiphila strain AA1, a member of the *Desulfobacteraceae* in the *Deltaproteobacteria*, is an organohalide-respiring bacterium that was originally enriched and isolated from the marine sponge *Aplysina aerophoba* (Ahn et al., 2009). A number of sponge species are known to produce a variety of brominated compounds, mainly bromophenols and brompyrroles, which can account for over 10 percent of the sponge dry weight (Teeyapant et al., 1993). Isolated from such a brominated compound rich environment, the dehalogenating range of *D. spongiiphila* is specific to brominated compounds rather than chlorinated compounds (Ahn et al., 2003; Ahn et al., 2009). The debrominating activity is independent of exogenous cobalamin (Liu et al., 2017). As a facultative OHRB, *D. spongiiphila* can also utilize sulfate, sulfite and thiosulfate as electron acceptors for respiration. Debromination and sulfate reduction could occur simultaneously when lactate was used as electron acceptor (Ahn et al., 2009). However, lactate, pyruvate, fumarate and malate cannot be utilized by *D. spongiiphila* without an electron acceptor (Ahn et al., 2009).

D. spongiiphila has a 6.5 Mbp genome containing over 5,000 protein coding genes (Liu et al., 2017). Three distinct putative reductive dehalogenase genes and genes encoding for a complete cobalamin biosynthesis pathway were identified in the genome. The three reductive dehalogenase genes share low similarity to each other (< 26%) and to other functional characterized RdhAs (<30%) (Liu et al., 2017). The expression of one of the reductive dehalogenases, *Dsp_16032*, was found to be induced by the addition of 2,6-dibromophenol using a qPCR approach (Liu et al., 2017). Nevertheless, an understanding of the overall gene expression and the protein production profile in response to organohalides is needed to identify the full complement of genes or enzymes involved in organohalide respiration.

Thus, in this study, we applied RNA sequencing and proteomic analysis techniques to investigate the global gene expression and protein production of *D. spongiiphila* under conditions with and without 2,6-dibromophenol as electron acceptor. Differences in mRNA and protein profiles were expected to be observed under the two growth conditions to reveal the potential genes or enzymes that are play a role in respiratory reductive dehalogenation.

Results

General features of transcriptomic and proteomic analysis

In total, six samples (two treatments in triplicates) were used for RNA sequencing and proteomic analyses. RNA sequencing analysis generated 5,825,305 - 6,888,536 raw reads and 4,449,353 – 5,232,222 trimmed reads for each sample.

Around 75 - 78% of trimmed reads could be mapped back to the genome, covering 5,029 of the 5,238 protein coding genes of *D. spongiiphila*. The expression level of 402 genes was statistically different between the sulfate and 2,6-dibromophenol treatments ($p =< 0.05$). In total, 150 genes were significantly upregulated and 252 genes were downregulated. The proteomic analysis detected 793 proteins in total, 7 of which were exclusively detected in the sulfate treatment and 5 were exclusively detected in the 2,6-dibromophenol treatment. The proteins in each treatment were ranked based on the average abundance and the ranks compared between the two treatments. In total, 180 proteins had a rank difference equal or more than 20 between the two treatments.

In the genome of *D. spongiiphila*, there are 3,236 protein coding genes that can be classified into 24 Clusters of Orthologous Groups (COGs) based on their annotated functions. Of the 402 statistically differently expressed genes, 268 genes were assigned in COGs with their distribution shown in Fig. 3.1A. Compared to sulfate-amended cultures, 36 of 40 genes involved in energy production and conversion were downregulated, indicating that 2,6-dibromophenol may be not as favorable as sulfate to support cell growth.

The genome annotation indicated the presence of 1,444 protein coding genes for transmembrane proteins, 126 of which were detected in the proteome. Over 80% of the proteins detected in the proteome could be assigned into COGs, which include 11% for amino acid transport and metabolism, 10% for energy production and conversion and 12% for translation, ribosomal structure and biogenesis (Fig. 3.1B).

Reductive dehalogenation

The composition of the three reductive dehalogenase gene clusters in *D. spongiiphila* are shown in Fig. 3.2. The expression of the *rdh16* gene cluster was inducible by the addition of 2,6-dibromophenol because the genes in this cluster were upregulated to various extent within 12 h after the addition of 2,6-dibromophenol. The *rdhA* gene was upregulated to the highest level, which is up to 260-fold. The accessory genes, encoding a tetratricopeptide repeat containing protein (Dsp_16031), RdhBC (Dsp_16033-34) and hypothetic proteins (Dsp_16035-36), were upregulated to 25 - 109-fold. The reductive dehalogenase encoded by Dsp_16032 together with the tetratricopeptide repeat containing protein (Dsp_16031) were exclusively detected in the proteome of all 2,6-dibromophenol induced culture triplicates. Other accessory proteins were not found in the proteome even though their transcription was upregulated to a high level in 2,6-dibromophenol cultures.

The second reductive dehalogenase gene cluster *rdh07* showed a constitutive expression pattern as the addition of 2,6-dibromophenol did not significantly change the expression of *rdh07176* at either the transcriptional or translational level. The transcript level of this *rdhA* was equivalent with and without 2,6-dibromophenol, which was at a relatively high level. Further supported by the proteomic data, this reductive dehalogenase was detected in the proteome under both conditions with no significant rank difference. Also, the protein abundance rank of *rdh17076* in either

treatment (rank 173 and 183) was higher than *rdhA16* in 2,6-dibromophenol induced cultures (rank 230).

For *rdh02299*, the transcript level was very low and no protein was found in the proteome in the three replicates of either treatments, indicating that this *rdhA* gene was almost mute even with 2,6-dibromophenol as electron acceptor.

Corrinoid transport and biosynthesis

D. spongiiphila does not require an exogenous cobalamin supply for reductive dehalogenation activity and growth. The genome of *D. spongiiphila* encodes for a complete cobalamin biosynthesis pathway (Liu et al., 2017). These genes are located in several gene clusters with cobalamin riboswitches predicted to be present before the gene cluster (Fig. 3.3). RNA sequencing revealed that all cobalamin biosynthesis related genes were transcribed with various transcripts detected under both conditions. However, none of these genes showed significant expression changes in response to the addition of 2,6-dibromophenol. The constant levels of the transcripts of these genes indicate that their expression was not induced by 2,6-dibromophenol. The proteomic analyses were not consistent with RNA sequencing results. In spite of the presence of gene transcripts, for most of genes, the encoded proteins were not detected by proteomic analyses. However, for the detected proteins, there were no differences in the protein abundance rank.

Sulfate reduction

D. spongiiphila was pre-grown on sulfate as electron acceptor before the addition of 2,6-dibromophenol. The transcripts of genes directly involved in sulfate reduction, including ATP sulfurylase (*sat*), APS reductase (*aprAB*) and dissimilatory sulfite reductase(*dsrAB*) were abundant in cultures only grown on sulfate (Table 3.1). The corresponding proteins were also detected in the proteome with top abundance (Table 3.1). Sulfate permease genes were expressed with relatively low transcript numbers, and the corresponding proteins were not detected. Like the majority of sulfate-reducing bacteria, *D. spongiiphila* contains *qmoABC* genes encoding for a quinone-interacting membrane-bound oxidoreductase complex. These *qmoABC* genes are located in the same gene locus as *sat* and *aprAB*, which were transcribed in high abundance and the corresponding proteins were detected in the proteome. The QmoABC proteins are proposed to transfer electrons from the quinone pool to AprAB, which is necessary for sulfate reduction (Pereira et al., 2011). Most of the dissimilatory sulfite reductase associated proteins were also detected in the *D. spongiiphila* proteome. DsrD, a DNA binding protein with unspecified role in sulfite reduction, was also detected in the proteome (Mizuno et al., 2003). DsrMK proteins were detected under both conditions, and may be involved in the menaquinol oxidation and reduction of DsrC in the cytoplasm. The DsrJOP module is proposed to be involved in electron transfer between menaquinol and a periplasmic component (Rabus et al., 2015), but only the DsrO protein was detected. In our experimental setup, the 2,6-dibromophenol treatment cultures contained residual sulfate, and hence relevant gene transcripts and proteins for sulfate reduction were also detected.

Some of such genes connected to sulfate reduction were slightly downregulated compared to the sulfate treatment, but were still in high abundance at the RNA and protein level (Table 3.1).

Lactate Oxidation

D. spongiiphila utilizes lactate as electron donor and carbon source in the presence of electron acceptors. Lactate permease (Dsp_11048) allows permeation of lactate into the cell which is then oxidized to pyruvate by lactate dehydrogenase. Two sets of L-lactate utilization proteins (Lut) are encoded in the genome of strain AA1 (Dsp_11155-Dsp_11156 and Dsp_19035-Dsp_19037). The lactate utilization proteins are membrane-associated proteins that are thought to directly transfer electrons to the quinone pool (Venceslau et al., 2014). Only one set of LutBC (Dsp_11155-Dsp_11156) proteins together with a lactate permease and a pyruvate-ferredoxin oxidoreductase (Dsp_11153) in close vicinity in the genome were detected in the proteome (Table 3.1). The produced pyruvate is further oxidized by pyruvate: ferredoxin dehydrogenase (Dsp_11153 and Dsp_01306) to acetyl-CoA and CO_2. Two pyruvate: ferredoxin dehydrogenases were highly expressed in RNA and detected in proteome under both conditions. Phosphate acetyltransferase (Dsp_09040) and acetate kinase (Dsp_09039) were also detected in proteome, which results in substrate level ATP synthesis. With both electron acceptors, sulfate and 2,6-dibromophenol, lactate is used as the sole electron donor. As expected, there was no

difference in RNA and protein level between two treatments in terms of lactate metabolism.

Hydrogenases, formate dehydrogenases and Wood-Ljungdahl pathway

Hydrogen and formate cycling models have been proposed as possible energy-providing pathways for sulfate reducing bacteria grown on lactate plus sulfate (Odom and Peck, 1981). The *D. spongiiphila* genome encodes a periplasmic [FeFe] hydrogenase complex (HydABEFG, Dsp_10097-102). Although the transcripts of hydrogenase maturation genes *hydEFG* were at a relatively high level, the transcripts for *hydAB* were very low. No subunit of this hydrogenase complex was detected in the proteome. A cytoplasmic NAD(P)-dependent hydrogenase complex (HndABCD, Dsp_02400-404) and two [FeFe] hydrogenases are encoded in the genome of *D. spongiiphila*. Three proteins of the HndABCD complex were detected in the proteome. HndABCD complex is a type of [Fe] hydrogenase of *Desulfovibrio* that can carry out hydrogen-driven NADP-reduction and methyl-viologen reduction (Dermoun et al., 2002).

Formate can be used as an electron donor by *D. spongiiphila* (Ahn et al., 2009). Three gene clusters containing formate dehydrogenases genes were found in the genome of *D. spongiiphila* but their transcripts are distinct. Transcripts of one formate dehydrogenase (Dsp_19025) gene located in the vicinity of NADH-quinone oxidoreductase genes were found at low abundance. In contrast, the transcripts of the other two formate dehydrogenases (Dsp_05146-Dsp_05147 and Dsp_10045) were at

very high levels. Even with abundant transcripts, no translated proteins were detected in the proteome.

The genome of *D. spongiiphila* encodes for a complete Wood-Ljungdahl (WL) pathway. The proteomic analyses detected most of the proteins involved in the WL pathway under both growth conditions except for formate dehydrogenase (Fdh), although the transcripts of *fdh* genes were high. The Fdh protein is the key enzyme for the last reaction of the WL pathway in the oxidative direction. The absence of this protein may result in the lack of complete oxidation of acetyl-CoA to CO_2.

Energy metabolism proteins

The genome of *D. spongiiphila* encodes a complete alternative menaquinone biosynthesis pathway (MqnABCDE*,* Dsp_15087-Dsp_15092) as in *Streptomyces coelicolor*, rather than the pathway in *E. coli* (MenABCDEFGH). MqnCE and two dehypoxanthine futalosine cyclases were detected in the proteome under both conditions. The expression of MqnACE was significantly upregulated in the 2,6-dibromophenol cultures.

Like most sulfate-reducing bacteria, the genome of strain AA1 also encodes for multiple cytochrome c oxidases and cytochrome bd-I ubiquinol oxidases. One cytochrome c3 (Dsp_25023) was detected in the proteome, which may play a role in electron shuttling from the electron donor to cytochrome-containing membrane complexes (Pereira et al., 2011). Cytochrome containing membrane complexes are also encoded in the genome of strain AA1. The Qrc complexes (Dsp_02122-

Dsp_02127) are the molybdopterin containing oxidoreductases with cytochrome c. Two proteins of the Qrc complexes were detected in the proteome. The membrane complexes TmcABCD (Dsp_23058-Dsp_23061) containing a tryptophan-rich protein, iron-sulfur protein, integral membrane cytochrome b and cytochrome c3 are present in the genome like many of other sulfate-reducing bacteria. However, none of the proteins was found in the proteome. Two proteins of another molybdopterin containing oxidoreductase (MopABCD, Dsp_02124–Dsp_02127) were detected in the proteome, which is proposed to be involved in H_2 oxidation in *Desulfovibrio desulfuricans* G20 (Li et al., 2009). One gene cluster (Dsp_15013-Dsp_15018) containing two sets of iron-sulfur oxidoreductases, a NarG-like protein and a cytochrome c is present in the genome of *D. spongiiphila*, which encode an uncharacterized membrane complexes. The transcripts of this gene cluster were high and the corresponding proteins were detected in the proteome.

The membrane complex RnfABCDEG (Na^+ translocating ferredoxin: NAD^+ oxidoreductase) is encoded in the genome (Dsp_11002 and Dsp_11006-Dso_11011) and expressed in high transcript level. Except for RnfB which shows a significant downregulation, no significant changes were found between the two treatments. Four proteins of these complexes were detected in the proteome. Two sets of NADH:quinone oxidoreductase (Nqr and Nuo) genes, encoding for proteins for the transfer of electrons from NADH to quinones were found in the genome of *D. spongiiphila*. The transcripts of NqrABCDEF (Dsp_10046-Dsp_10051) were detectable, although there was no difference in expression of these gene sets between the two treatments. The Nuo complex lacks the NAD(H) redox module NuoEFG

encoded in vicinity. The *nuoEF*-like genes were found in the formate dehydrogenase cluster. The transcripts of the *nuo* gene cluster (Dsp_10115-Dsp_10116 and Dsp_10120-Dsp_10128) are low or even non-detectable. None of these two complexes was detected in proteomic analysis.

Heterodisulfide reductase (Hdr)-like proteins are encoded in the genome of strain AA1. One HdrA-containing cluster (Dsp_08006-Dsp_08011) contains genes encoding NADH:flavin oxidoreductase, two 4Fe-4S domain containing protein, Fe-S oxidoreductase and one coenzyme F420 reducing hydrogenase. All the proteins encoded by this gene cluster were detected in the proteome, indicating their unknown roles in metabolism. One HdrA/Mvh and related proteins (Dsp_01293-Dsp01299) are encoded in the same cluster with DsrC, which is a key enzyme for dissimilatory sulfite reduction. Most of the proteins were detected in the proteome, indicating their possible role in sulfite reduction.

Multidrug efflux pumps, Type VI secretion system and antiporter

There are 12 sets of RND (Resistance-Nodulation Division) family multidrug efflux pumps encoded in the genome of *D. spongiiphila*, with different levels of transcription. The transcriptional expression of three multidrug efflux pumps was significantly downregulated and four efflux pumps were partially or completely detected in the proteome. Proteins of one multidrug efflux pump (Dsp_20083-Dsp_20085) were detected in the proteome under both conditions. The transcription of this efflux pump gene cluster was significantly downregulated in the 2,6-

dibromophenol treatment. Another ArcB protein (Dsp_01130) was exclusively detected in the proteome of 2,6-dibromophenol cultures. A type VI secretion system gene cluster (Dsp_05100–Dsp05114) was present in the genome of strain AA1 and transcription was very high under both conditions. Eight of type VI secretion related proteins were detected in the proteome. Although few proteins were detected in the proteome, the gene cluster of multisubunit sodium/proton antiporter was significantly downregulated in the presence of 2,6-dibromophenol.

Discussion

The transcriptome analyses revealed that the three *rdh* gene clusters in *D. spongiiphila* have distinct expression patterns in response to 2,6-dibromophenol (inducible for *rdh16*, constitutive for *rdh07* and mute for *rdh02*). The transcripts of *rdh16032* were significantly upregulated from almost muted to 264-fold and the protein was exclusively produced during 2,6-dibromophenol dehalogenating conditions. In contrast, the transcripts of *rdh07176* were detected under both sulfate-reducing and dehalogenating conditions with a relative low transcript abundance. However, the protein abundance of Rdh07176 was higher than that of Rdh16032 regardless the transcript abundance. This incongruency may be because of the time needed for strain AA1 to produce and accumulate Rdh16032 after gene induction, which is consistent with the observed lag phase for debromination activity (Liu et al., 2017). Although the expression of the gene cluster of *rdh16032* was upregulated, no putative Rdh accessory proteins were identified in the proteome except for the

tetratricopeptide repeat-containing protein (Dsp_16031). The tetratricopeptide repeating proteins are associated with multiprotein complexes and the tetratricopeptide repeat motif is considered to be involved in protein-protein interactions (Blatch and Lassle, 1999). Except for the proteomic study of *D. mccarty*i and *Sulfurospirillum multivorans*, the membrane anchoring protein RdhB is commonly not identified in proteomic analyses because of its small size and integration in the membrane (Goris et al., 2015; Kublik et al., 2016). The RdhC-like protein contains an FMN binding and two 4Fe-4S binding domains, which resembles the characteristics of RdhC in *Desulfitobacterium hafniense* DCB-2 RdhA6 cluster (Dhaf_0738) (Kim et al., 2012). The RdhC-like protein in strain AA1 contains a transmembrane helix, and may act as a membrane associated regulatory protein. We previously hypothesized that the transcription of *rdh16032* is initiated by sigma factor 54 rather than sigma factor 70 (Liu et al., 2017). The sigma 54 dependent transcriptional regulator (Dsp_16030) was expressed with constant transcript amounts under the two conditions although no corresponding proteins were detected in the proteome.

Most reductive dehalogenases contain corrinoids in their active center (Moore and Escalante-Semerena, 2016; Sanford et al., 2016). *D. spongiiphila* has the complete corrinoid biosynthesis pathway genes in its genome, which are expressed at the transcriptional level. The corresponding proteins may be produced in low abundance as only some of them were detected in the proteome. The expression of these genes is not induced in contrast to the *rdh16032* gene cluster, indicating that the cobalamin biosynthesis related proteins in *D. spongiiphila* were not specifically produced for

reductive dehalogenation. Cobalamin riboswitches were found in the major gene clusters of corrinoid biosynthesis, which may regulate corrinoid metabolism in *D. spongiiphila* (Choudhary *et al*., 2013).

Biochemical studies have revealed that electron transfer to the reductive dehalogenase can be via quinone-dependent electron transport, as in facultative OHRBs, or via quinone-independent electron transport, as in *Dehalococcoides* (Fincker and Spormann, 2017; Schubert et al., 2018). Menaquinones have been extracted from *S. multivorans*, *D. restrictus* and *D. dehalogenans* (Scholz-Muramatsu et al., 1995; Holliger et al., 1998; Kruse et al., 2015). The addition of a menaquinone analog enhanced PCE reduction in *D. restrictus* (Schumacher and Holliger, 1996). The genome of *D. spongiiphila* encodes for a complete futalosine-dependent menaquinone biosynthesis pathway. The expression of three genes in this gene cluster was upregulated up to 2 fold in the presence of 2,6-dibromophenol, however, two of the corresponding proteins were downregulated in the 2,6-dibromophenol treatment. A proposed quinone-dependent electron transport chain to the reductive dehalogenase involves components linking the quinol pool to reductive dehalogenase in order to overcome the energy barrier from quinol to the cobalamin cofactor (Fincker and Spormann, 2017). From the transcriptomic and proteomic analysis, we did not find such potential components whose expression was upregulated or exclusively present under 2,6-dibromophenol dehalogenating conditions. Considering that sulfate reduction is also involved in the electron transfer from a quinol pool to the terminal reductase, the reductive dehalogenation process may share electron transport components with sulfate reduction. Most of the proteins predicted to be involved in

sulfate reduction were detected in the proteome regardless of the treatment, most likely because residual sulfate from the pre-grown culture was present in the 2,6-dibromophenol treatments. Membrane complexes that have the potential for electron transport are illustrated in Fig. 3.4 based on their presence in the proteome. Biochemical and physiological studies are needed to provide direct evidence of their function in the metabolism of *D. spongiiphila*.

For obligate OHRBs, hydrogen is the only utilizable electron donor and hydrogenase complexes can be detected in proteomic analyses (Schiffmann et al., 2014; Jugder et al., 2016b). Four hydrogenase gene clusters are encoded in the genome of *D. mccartyi*, all of which are identified in high abundance in the proteome (Türkowsky et al., 2018). A periplasmically oriented hydrogenase complex is present in all *Dehalobacter restrictus* strains (Rupakula et al., 2013). The non-obligate OHRB *S. multivorans* contains four gene clusters encoding a NiFe hydrogenase in its genome, which enables it to grow with hydrogen. However, transcript and biochemical analysis indicated that only one hydrogenase is involved in the organohalide respiratory chain (Kruse et al., 2017a). The genome of *D. spongiiphila* encodes one periplasmic hydrogenase cluster (Dsp_10097-Dsp_10102), a cytoplasmic hydrogenase cluster (HydABEFG, Dsp_02400-Dsp_02404) and two [FeFe] hydrogenase genes, of which only the cytoplasmic hydrogenase could be detected in the proteome. Almost all genomes of sulfate-reducing bacteria contain 1-4 periplasmic hydrogenases (Rabus et al., 2015). Hydrogen cycling was proposed to be a way to gain an energy benefit for sulfate-reducing bacteria by coupling the reduction of sulfate to the incomplete oxidation of lactate (Odom and Peck, 1981). A

periplasmic hydrogenase is not always necessary for the growth of sulfate-reducing bacteria with lactate/sulfate. In a study of *Desulfovibrio gigas*, a deletion experiment indicated that neither of its hydrogenases was required for the growth under lactate/sulfate but the periplasmic hydrogenase was required for growth with hydrogen/sulfate and pyruvate fermentation (Morais-Silva et al., 2013). The only periplasmic hydrogenase (HydABEFG) encoded in *D. spongiiphila* has an unusual transcript pattern in which the maturation genes hydEFG transcript are high level (>500) but the hydrogenase catalytic units are in very low abundance (2-36). The low transcription of this hydrogenase catalytic units indicates that this hydrogenase may not be functional under lactate/sulfate growth conditions. Additional experiments will be needed to determine whether hydrogen can be used as a sole energy source for *D. spongiiphila*, and we are not yet able to draw a clear picture about the hydrogen cycle in *D. spongiiphila* in terms of energy generation.

The Wood-Ljungdahl (WL) pathway is found in a broad range of bacteria in both the oxidative and reductive directions (Ragsdale and Pierce, 2008). The reductive direction is used for CO_2 fixation and assimilation of CO_2 into acetyl-CoA. It does not appear that WL is used by *D. spongiiphila* for CO_2 fixation, as lactate was provided as the carbon source. In some sulfate reducing bacteria, the WL pathway runs in the reverse direction in order to completely oxidize acetyl-CoA. For example, proteomic analysis of *Desulfobacula toluolica* Tol2 revealed a complete WL pathway indicating a complete oxidation of acetyl-CoA (Wöhlbrand et al., 2013). All proteins except formate dehydrogenase in the WL pathway were detected in the proteome of *D. spongiiphila.* A previous study indicated that formate can be used as an electron

donor to support the growth of *D. spongiiphila* (Ahn et al., 2009). As high RNA transcripts of formate dehydrogenase were detected, the missing of formate dehydrogenase in the proteome may be due to the low protein amount below the detect limit for proteomic analysis.

RND family multidrug efflux pumps are thought to be relevant to antibiotic resistance in Gram-negative microorganisms (Alvarez-Ortega et al., 2013). The typical model of RND family members is composed of three proteins that span across the inner and outer membranes, e.g. AcrAB-TolC efflux pump in *E. coli* (Blanco et al., 2016). Efflux pumps are considered as antibiotic resistance determinants but their evolution is driven by their physiological and ecological functions in bacterial virulence, host-bacteria interactions, and detoxification (Alvarez-Ortega et al., 2013; Blanco et al., 2016). For example, bacterial phytopathogens with efflux pumps are proposed to have an important role in resistance against antibacterial toxins produced by the plant, which facilitates the colonization of host plant (Burse et al., 2004). We examined the genome of 18-sulfate reducing bacteria for the presence of RND family multidrug efflux pumps, showing that their genomes contain 2 to 9 RND family multidrug efflux pumps. *D. spongiiphila* contains 12 multidrug efflux pumps which is more than other sulfate-reducing bacteria. It is interesting to note that sponge microbiota produces antimicrobial products as chemical defense (Weiss et al., 1996) and the multidrug efflux pumps in *D. spongiiphila* may thus be an important survival strategy for a sponge symbiont in an environment rich in antimicrobial products and other secondary metabolites. Our RNA sequencing results show that three sets of highly expressed efflux pump gene clusters undergo a significant

downregulation under 2,6-dibromophenol dehalogenating compared to sulfate-reducing conditions (0.24-0.57 fold). The expression of efflux pump genes is usually tightly regulated in response to the right effectors (Blanco et al., 2016). Since strain AA1 was grown without sponge derived products, it is not clear what effectors will activate these efflux pumps. The downregulation may be caused by the uncoupling effect of bromophenol and phenol, which would result in a reduction of metabolic activity (Hiraishi and Kawagishi, 2002). Substituted phenols, e. g. chlorophenol and nitrophenol compounds, are used as protonophores, which could uncouple the link between respiratory electron transport and protein translocation (Heytler, 1980). A downregulation of multiunit antiporter gene cluster was also observed in *D. spongiiphila* in the presence of 2,6-dibromophenol, which may also be due to the uncoupling effects caused by bromophenol or phenol.

The presence of type VI secretion system (T6SS) proteins in the proteome reveals a unique secretion system of *D. spongiiphila*. Type VI secretion is a newly described protein transport mechanism in which proteins are transported from the donor cell across the cellular envelope into the target cell (Bingle et al., 2008). It has been found in 100 different bacterial species, which are mainly pathogenic or symbiotic (Filloux et al., 2008). Although our knowledge about the function of Type VI secretion system is very limited, this system appears to be important in bacterial pathogenesis and bacteria-host interaction (Filloux et al., 2008). The presence of a Type VI secretion system does not appear to be a common feature for sulfate reducing bacteria. Of 18 other sulfate-reducing bacteria, only one strain, *Desulfovibrio salexigens* DSM 2638, encodes for the type VI secretion system in its genome. in *D.*

spongiiphila, the type VI secretion system may be important for its survival in the sponge environment.

As a facultative organohalide respiring bacterium, *D. spongiiphila* has a more versatile and complicated metabolism than most OHRBs, adding to the challenges of elucidating the metabolic role of organohalide respiration. The application of transcriptomic and proteomic analysis is a good start to explore the overall metabolism of novel microorganisms. To date, global transcriptomic studies of anaerobic organohalide-respiring bacteria have been only conducted with *D. mccartyi* and *D. hafniense*, most of which were based on microarray approaches rather than RNA sequencing (Türkowsky et al., 2018). Compared to microarrays, RNA sequencing is more sensitive allowing for the detection of transcripts in low abundance. Transcriptomic and proteomic data often do not correspond with each other. High transcripts don't always indicate a high protein level abundance. For example, the formate dehydrogenases (Dsp_05146-Dsp_05147 and Dsp_10045) and cytochrome bd-I ubiquinol oxidases of strain AA1 were in high transcript abundance (1200-6000), but the encoded proteins were not detected in the proteome. In contrast, the NAD(P)-dependent hydrogenase HndACD (Dsp_02401-03) were detected in the proteome with the transcripts ranged from 300 to 900. These discrepancies between transcriptomic and proteomic analysis are attributed to both the nature of metabolism and the methodological sensitivity and bias (Haider and Pal, 2013). Post-transcriptional regulation, translation efficiency of difference operons, and codon bias can cause inconsistency in transcript and protein abundance. The cellular location of the proteins and sample preparation efficiency will influence the detection

of low abundance proteins in proteome (Haider and Pal, 2013). The RNA sequencing of *D. spongiiphila* detected transcription of over 5,000 genes, which almost covers all protein coding genes in the genome. The proteomic analysis detected approximately 15% of the coding proteins, which is lower than the 70% coverage obtained for *D. mccartyi* (Schiffmann et al., 2014). The high coverage of *D. mccartyi* could likely be explained by it compact genome and restricted metabolism. The highest proteome coverage for *D. restrictus*, which is also an obligate OHRB but has a genome twice the size of *D. mccartyi*, was around 35% (Rupakula et al., 2013). *D. spongiiphila* has an even larger genome which encodes a more flexible metabolism. The relative low coverage may be because many genes are not translated into protein or translated into low abundance under the growth conditions with lactate as carbon sources and sulfate or 2,6-dibromophenol as electron acceptor. Moreover, some membrane integral proteins are closely associated to the membrane, which make it difficult to identify these in the proteome.

Methods and Materials

Cultivation

D. spongiiphila strain AA1 was pre-grown anaerobically in medium with 30 mM lactate as electron donor and 20 mM sulfate as electron acceptor for 3 days yielding a turbid culture (~10^7 cells/ml). Then, 300 ml of this pre-grown culture (without washing off remaining sulfate) was added to 300 ml of fresh sulfate-free medium containing 2 mM lactate. For the organohalide treatment, 200 μM 2,6-

dibromophenol was added as electron acceptor, while 200 μM sulfate was used as electron acceptor in the control treatment. The two treatments were established in triplicate. The cultures were incubated at room temperature and sampled periodically to monitor for debromination of 2,6-dibromophenol via high performance liquid chromatography (HPLC). After 12 h, the 300 ml culture was centrifuged at 10,000 ×g for 10 min to collect the cell biomass. The cell pellets were stored at -80 ^{o}C prior to RNA and protein extraction.

RNA Extraction and Library Preparation

Strain AA1 pellets were resuspended in Trizol reagent (Life Technologies) and beaten on TissueLyser II (Qiagen) with a 7mm stainless steel bead for 3 times in a cycle of 2 min 30 sec beating and 2 min resting on ice. Chloroform was then added into clarified lysate and centrifuged at maximum speed for 15 min at 4 ^{o}C. The upper aqueous layer was transferred into new tubes, and an equal volume of 70% ethanol was added. The mixture containing RNA was then purified using RNeasy Mini Kit (Qiagen) by following the manufacturer's protocol, including the on-column DNase step. RNA quantity and quality was measured on a Nanodrop, Qubit (RNA Broad Range) (Life Technologies), and Agilent Bioanalyzer 2100 with RNA Nano chip.

For rRNA depletion, the RiboZero rRNA Removal Kit (Illumina) was used according to the manufacturer's protocol. Final RNA cleanup was performed using the Zymo Research RNA Clean & Concentrator-5 kit according to the manufacturer's protocol. The quantification of rRNA-depleted samples was measured on Qubit (RNA

Broad Range). Five µl of rRNA-depleted samples (26-49 ng) were used to prepare libraries using the Illumina TruSeq RNA Library Prep Kit v2, according to the manufacturer's protocol. Final libraries were measured on Qubit (dsDNA Broad Range) and run on the Agilent Bioanalyzer 2100 (DNA High Sensitivity chip). All 6 libraries were pooled in equimolar fashion and loaded at a final concentration of 20 pM onto the Illumina MiSeq benchtop sequencer with a MiSeq Reagent Kit v3 150-cycle kit.

RNA Sequence Analysis

Raw RNA sequencing reads from the 6 samples were trimmed to remove sequencing adapters and low-quality base-pairs, the trimmed reads were then mapped to the predicted gene regions of *Desulfoluna spongiiphila*'s genome assembly (GenBank accession GCA_900101345.1) while applying a 95% identity threshold. Trimming and mapping were done using CLC Genomic Workbench 8.5.1 (https://www.qiagenbioinformatics.com/). After mapping, the number of uniquely mapped reads (a read that maps to only one locus) per gene for each sample were gathered and used as input to DESeq2 (Love et al., 2014), an R package for differential gene expression analysis. Genes were considered differentially expressed if their adjusted *p*-value (False Detective Rate) was smaller than 0.05.

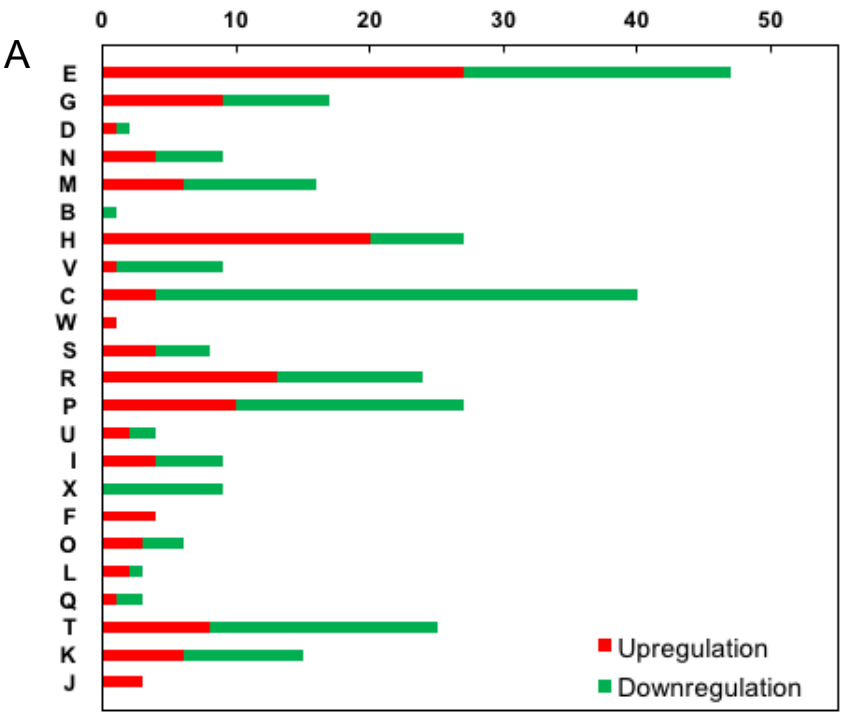
Protein Extraction

Cells were harvested by centrifugation (10,000 × g, 30 min) and washed once with Ambic buffer (100 mM ammonium bicarbonate, pH 7.0). Then cells were disrupted by three cycles of freezing and thawing, and sonicated for 30 s. After a short centrifugation step to remove cell debris, GAP-DH of *Staphylococcus aureus* was added to each sample as an internal standard. Then, disulfide bridges were reduced with 62.5 mM dithiothreitol for 1 h at 30°C, and SH-groups were derivatized with 128 mM iodoacetamide for 1 h at room temperature in the dark. A total amount of 0.63 µg trypsin was added to each sample and incubated overnight on a shaker at 37°C. After stopping the trypsin digest with 1 µl of 100% formic acid and removal of precipitates at 13,000 rpm for 10 min the peptides in the supernatant were treated with ZipTips to remove salts, dried and resolved in 15 µl 0.1% formic acid (Seidel et al., 2018).

Mass spectrometry and protein identification

Separation of peptides on a nano-column, Electron-Spray-Ionisation and tandem mass spectrometry was done on an Ultimate 3000 nanoRSLC instrument (Thermo Scientific, Germering, Germany) coupled to an Orbitrap Fusion mass spectrometer (Thermo Scientific, San Jose, CA, USA) via a TriVersa NanoMate (Advion, Ltd., Harlow, UK) as described previously (Seidel et al., 2018). Peptides were identified with ProteomeDiscoverer version 2.2 and SequestHT as a search engine using coding sequences annotated from the *D. spongiiphila* genome as a database. The peptide identification threshold was set to a false discovery rate of 0.01 based on the

q-values comparing hits to target and decoy databases. ‘Minora Feature Detector’, ‘Feature Mapper’ and ‘Precursor Ions Quantifier’ nodes in ProteomeDiscoverer were applied for label-free quantification (2 ppm mass precision for precursor ions) and using the GAP-DH from *Staphylococcus aureus* as an internal standard. Relative abundance ranks of proteins were computed in Microsoft Excel on the basis of the label-free quantification output.


A
0
10
20
30
40
50
E
G
D
N
M
B
H
V
C
W
S
R
P
U
I
X
F
O
L
Q
T
K
J
Upregulation
Downregulation

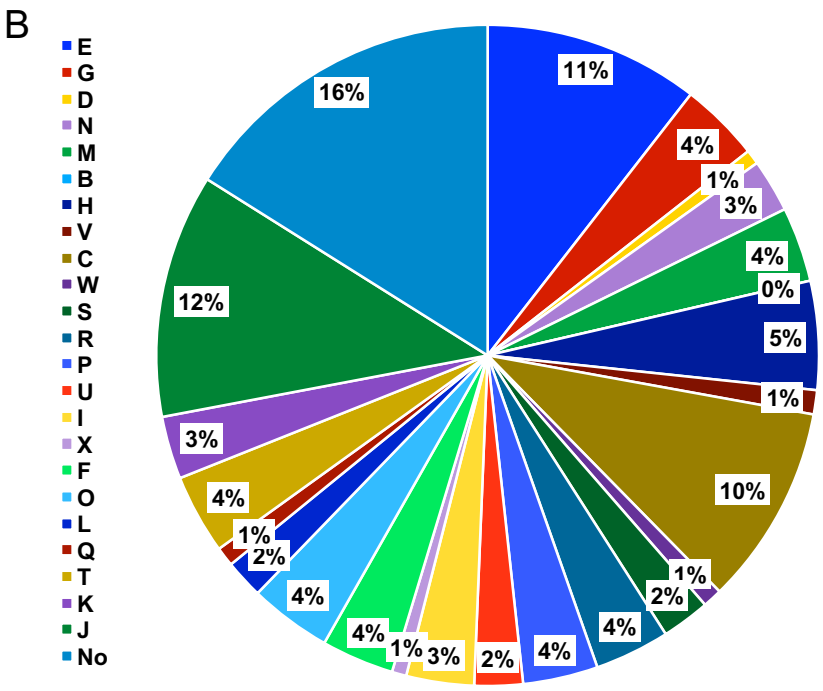

B
E
G
D
N
M
B
H
V
C
W
S
R
P
U
I
X
F
O
L
Q
T
K
J
No
11%
4%
1%
3%
4%
0%
5%
1%
10%
1%
2%
4%
4%
2%
4%
3%
1%
4%
4%
2%
1%
4%
3%
4%
12%
16%

Figure 3.1 Distribution of upregulated and downregulated genes in *D. spongiiphila* strain AA1 grown with 2,6-dibromophenol vs. sulfate as electron acceptor for the COG functional categories presenting significant changes ($p < 0.05$) (**A**) and COG functional categories of proteins detected in proteomic analysis (**B**). The letter abbreviations of COG category indicate: **E**-Amino acid transport and metabolism; **G**-Carbohydrate transport and metabolism; **D**-Cell cycle control, cell division, chromosome partitioning; **N**-Cell motility; **M**-Cell wall/membrane/envelope biogenesis; **B**-Chromatin structure and dynamics; **H**-Coenzyme transport and metabolism; **V**-Defense mechanisms; **C**-Energy production and conversion; **W**-Extracellular structures; **S**-Function unknown; **R**-General function prediction only; **P**-Inorganic ion transport and metabolism; **U**-Intracellular trafficking, secretion, and vesicular transport; **I**-Lipid transport and metabolism; **X**-Mobilome: prophages, transposons; **F**-Nucleotide transport and metabolism; **O**-Posttranslational modification, protein turnover, chaperones; **L**-Replication, recombination and repair; **Q**-Secondary metabolites biosynthesis, transport and catabolism; **T**-Signal transduction mechanisms; **K**-Transcription; **J**-Translation, ribosomal structure and biogenesis; **No**-Not in COG.

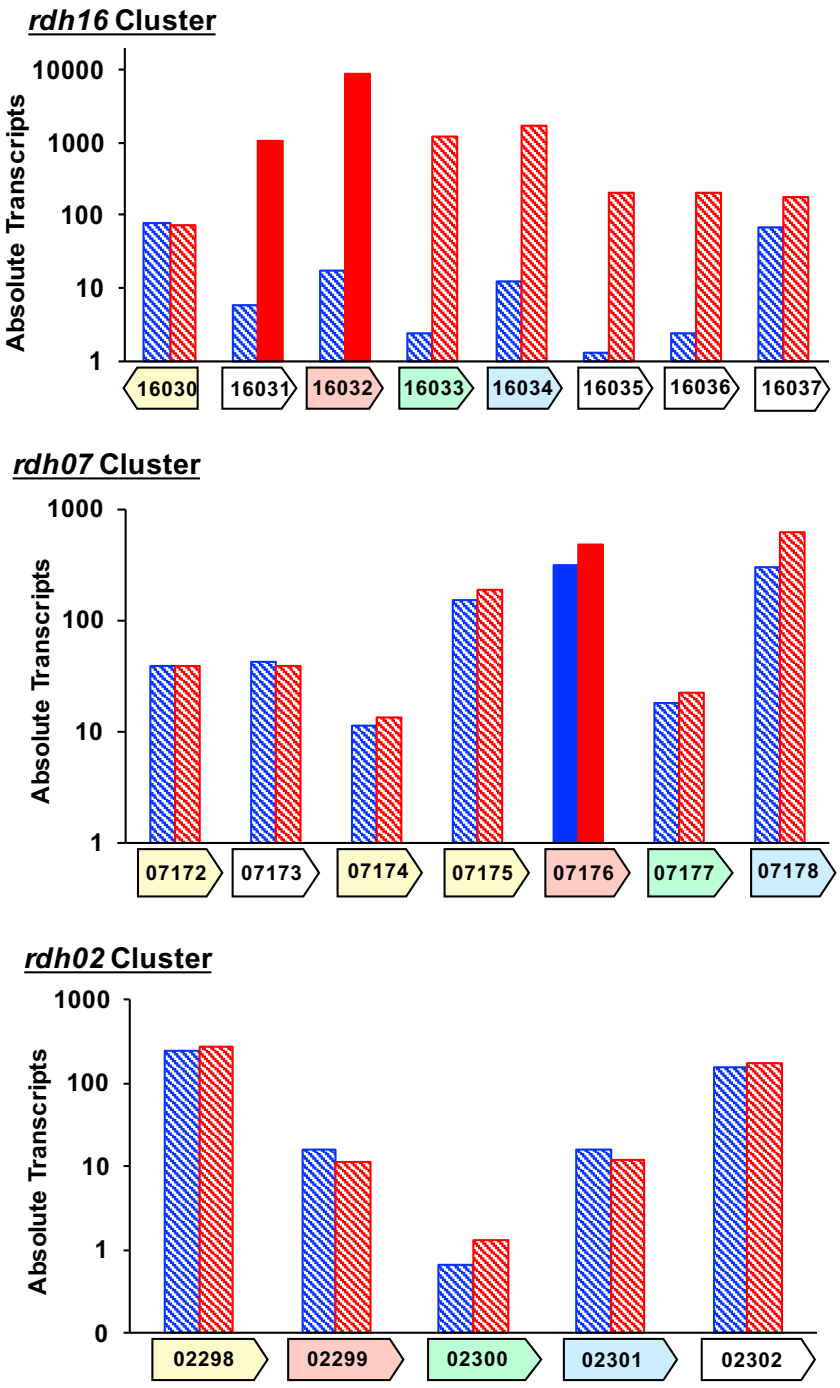

rdh16 Cluster
Absolute Transcripts
10000
1000
100
10
1
16030
16031
16032
16033
16034
16035
16036
16037
rdh07 Cluster
Absolute Transcripts
1000
100
10
1
07172
07173
07174
07175
07176
07177
07178
rdh02 Cluster
Absolute Transcripts
1000
100
10
1
0
02298
02299
02300
02301
02302

Figure 3.2 The absolute transcripts of three putative *rdh* gene clusters detected by RNA-sequencing analysis. Blue bars indicate the transcripts in sulfate-reducing conditions and red bars indicate the transcripts in the presence of 2,6-dibromophenol. The bars in solid color indicate that the encoded proteins were also detected in the proteome. Clusters are shown by arrows with their locus tags. The red arrow indicates *rdhA*, green for *rdhB*, blue for *rdhC*-like, yellow for a putative regulatory gene and white for genes with other functions. The arrow sizes are not to scale with gene size. The detailed annotations for the given genes are: *rdh16* cluster: Dsp_16030- sigma-54-dependent transcriptional regulator; Dsp_16031- tetratricopeptide repeat-containing protein; Dsp_16035- hypothetic protein; Dsp_16036- DoxX-like family protein; Dsp_16037- metabolite transporter. rdh07 cluster: Dsp_07172- HxlR family transcriptional regulator; Dsp_07173- unknown function protein; Dsp_07174- putative regulatory gene; Dsp_07175- sigma-54-dependent transcriptional regulator. rdh02 cluster: 02298- LuxR family transcriptional regulator; Dsp_02302- hypothetical protein.

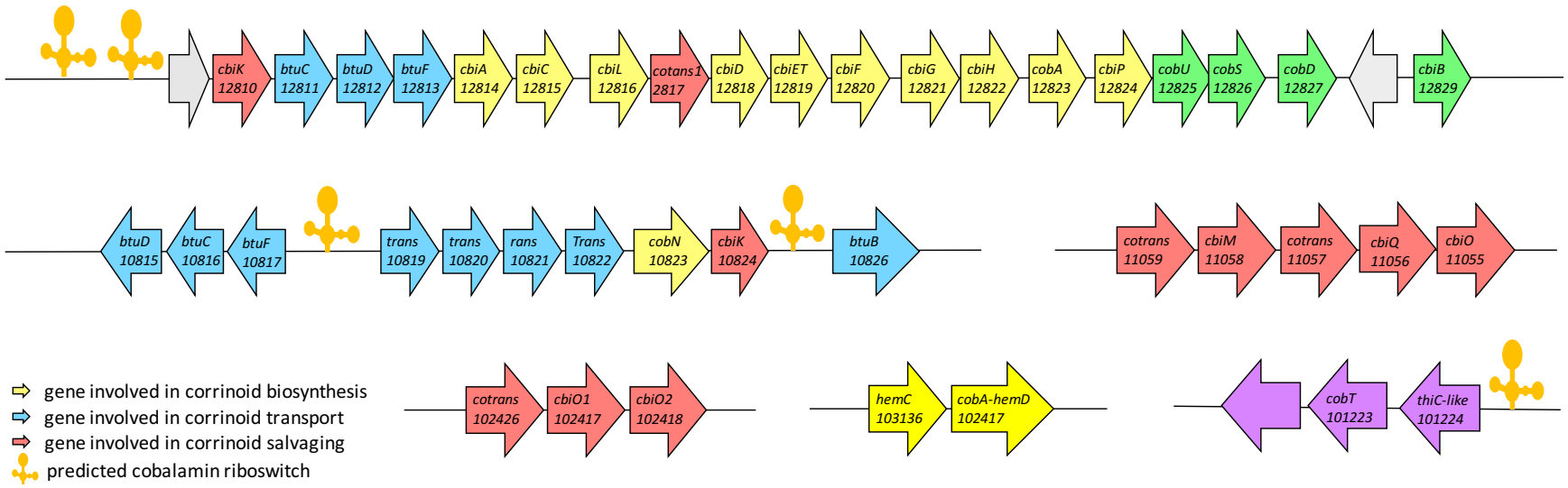


Figure 3.3 Corrinoid biosynthesis gene clusters of *D. spongiiphila* with gene name and JGI gene ID indicated. The arrow direction indicates the gene orientation and the arrow is not true to gene size.

Table 3.1 Transcripts and proteins quantified by RNA sequencing and mass spectrometry, respectively, in cultures grown with lactate as electron donor and sulfate as electron acceptor.

Locus Tag	Protein Name	Annotation	Transcriptomic					Proteomic		
			S_T[a]	DBP_T[b]	Log2	Fold	*p-V*	S_P[c]	DBP_P[d]	R_D[e]
Lactate Oxidation										
Dsp_11048		Lactate permease	9394	5529	-0.8	0.6	1.00E-03	280	217	63
Dsp_11153	PFOR	Pyruvate-ferredoxin/flavodoxin oxidoreductase	14272	12052	-0.3	0.8	1.79E-01	2	1	1
Dsp_11155	LutB	L-lactate utilization protein	14907	14697	-0.1	0.9	7.82E-01	10	9	0
Dsp_11156	LutC		3780	4012	0.0	1.0	9.89E-01	34	32	2
Dsp_01306	PFOR	Pyruvate-ferredoxin/flavodoxin oxidoreductase	21830	14960	-0.6	0.6	4.00E-03	150	155	6
Dsp_09039	ACK	Acetate Kinase	4750	4296	-0.2	0.8	4.36E-01	49	46	2
Dsp_09040	PTA	Phosphate Acetyltransferase	8690	8106	-0.2	0.9	6.22E-01	28	30	2
Sulfate Reduction										
Dsp_11045	AprA	Dissimilatory adenylylsulfate reductase	46009	32059	-0.6	0.7	4.67E-02	3	4	1
Dsp_11044	AprB		10665	7551	-0.5	0.7	1.15E-01	13	16	3
Dsp_11046	QmoA	Adenylylsulfate reductase-associated electron transfer proteins	4556	2891	-0.7	0.6	4.98E-04	59	70	11
Dsp_11047	QmoB		4934	3122	-0.7	0.6	1.10E-03	18	20	2
Dsp_11048	QmoC		2826	1649	-0.8	0.6	2.82E-04	190	206	16
Dsp_11049	Sat	Sulfate adenylyltransferase	78836	56291	-0.6	0.7	8.31E-03	1	2	1
Dsp_25030	DsrA	Dissimilatory sulfite reductase	14141	9912	-0.6	0.7	8.18E-03	23	24	1
Dsp_25029	DsrB		14458	9934	-0.6	0.7	8.77E-03	18	18	1
Dsp_25028	DsrD	Putative sulfite reductase-associated electron transfer proteins	4333	4227	-0.1	0.9	8.16E-01	80	86	6
Dsp_18097	DsrM		2122	1840	-0.3	0.8	2.87E-01	261	281	20
Dsp_18098	DsrK		2175	1418	-0.7	0.6	2.38E-04	294	294	0
Dsp_18099	DsrJ		479	333	-0.6	0.7	1.97E-02			
Dsp_18100	DsrO		1338	914	-0.6	0.7	2.51E-03	280	332	52
Dsp_18101	DsrP		3431	2576	-0.5	0.7	2.10E-02			
Dsp_01300	DsrC		4763	5729	0.2	1.1	6.89E-01	22	21	1
Dsp_04190		Sulfate permease, SulP family	77	92	0.2	1.1	8.38E-01			
Dsp_11127			54	64	0.1	1.1	9.33E-01			
Dsp_23064			27	83	1.3	2.5	7.82E-06			

[a] S_T indicates the absolute number of transcripts with sulfate as electron acceptor; [b] DBP_T indicates the absolute number of transcripts with 2,6-dibromophenol as electron acceptor. [c] S_P indicates the protein rank with sulfate as electron acceptor; [d] DBP_P indicates the protein rank with 2,6-dibromophenol as electron acceptor; [e] R_D indicates the rank difference.

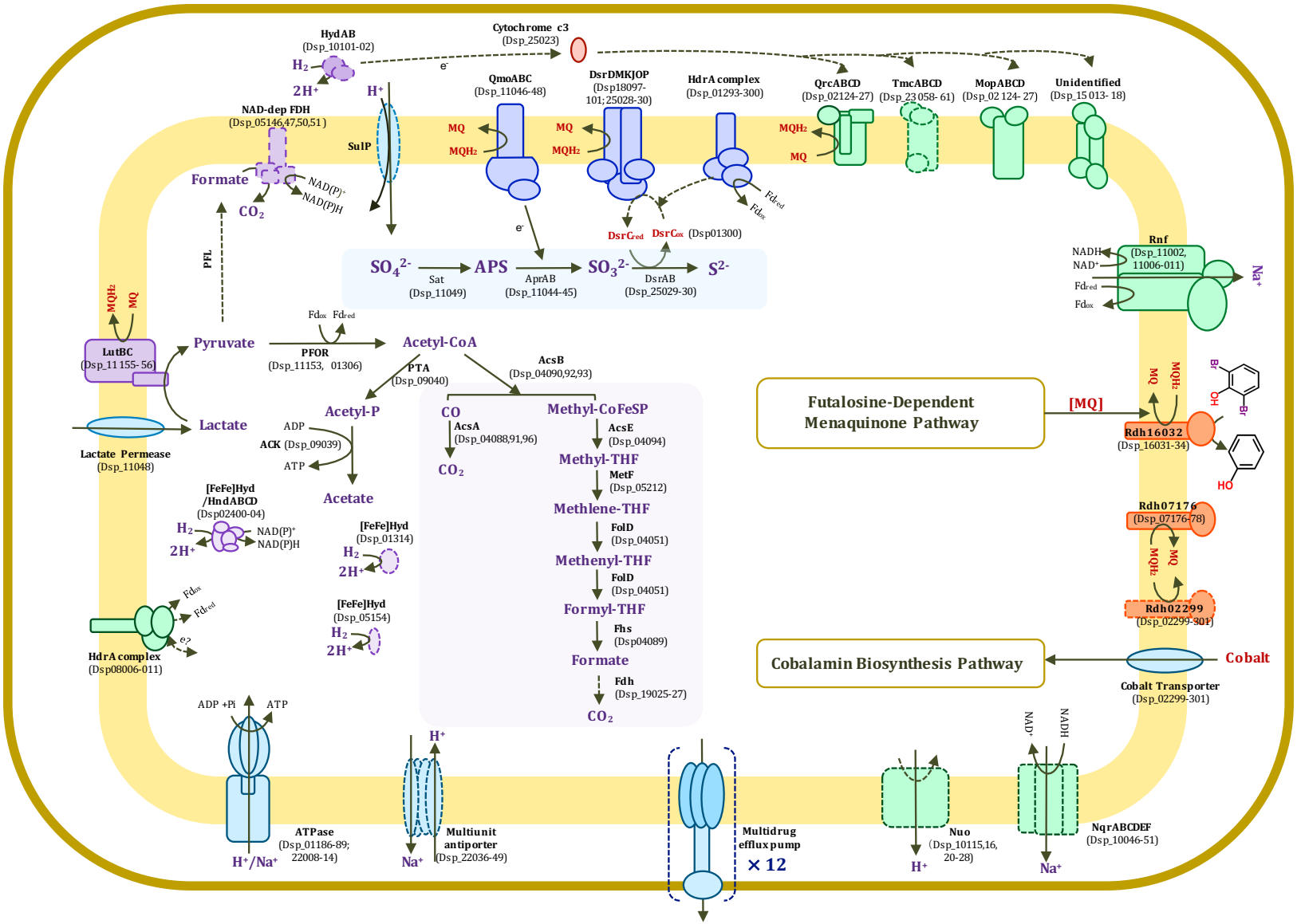

HydAB
(Dsp_10101-02)
H_2
$2H^+$
H^+
e⁻
Cytochrome c3
(Dsp_25023)
QmoABC
(Dsp_11046-48)
DsrDMKJOP
(Dsp18097-101;25028-30)
HdrA complex
(Dsp_01293-300)
QrcABCD
(Dsp_02124-27)
TmcABCD
(Dsp_23058-61)
MopABCD
(Dsp_02124-27)
Unidentified
(Dsp_15013-18)
MQ
MQH_2
NAD-dep FDH
(Dsp_05146,47,50,51)
Formate
CO_2
$NAD(P)^+$
$NAD(P)H$
SulP
PFL
$DsrC_{red}$
$DsrC_{ox}$ (Dsp01300)
Fd_{red}
Fd_{ox}
SO_4^{2-}
Sat
(Dsp_11049)
APS
AprAB
(Dsp_11044-45)
SO_3^{2-}
DsrAB
(Dsp_25029-30)
S^{2-}
Rnf
(Dsp_11002, 11006-011)
NADH
NAD^+
Na^+
LutBC
(Dsp_11155-56)
Pyruvate
PFOR
(Dsp_11153, 01306)
Acetyl-CoA
PTA
(Dsp_09040)
AcsB
(Dsp_04090,92,93)
Lactate
Lactate Permease
(Dsp_11048)
Acetyl-P
ADP
ACK (Dsp_09039)
ATP
Acetate
CO
AcsA
(Dsp_04088,91,96)
Methyl-CoFeSP
AcsE
(Dsp_04094)
Methyl-THF
MetF
(Dsp_05212)
Methlene-THF
FolD
(Dsp_04051)
Methenyl-THF
FolD
(Dsp_04051)
Formyl-THF
Fhs
(Dsp04089)
Formate
Fdh
(Dsp_19025-27)
[FeFe]Hyd
/HndABCD
(Dsp02400-04)
[FeFe]Hyd
(Dsp_01314)
[FeFe]Hyd
(Dsp_05154)
HdrA complex
(Dsp08006-011)
Futalosine-Dependent
Menaquinone Pathway
[MQ]
Rdh16032
(Dsp_16031-34)
Rdh07176
(Dsp_07176-78)
Rdh02299
(Dsp_02299-301)
Cobalamin Biosynthesis Pathway
Cobalt
Cobalt Transporter
(Dsp_02299-301)
ADP +Pi
ATP
ATPase
(Dsp_01186-89; 22008-14)
H^+/Na^+
Multiunit
antiporter
(Dsp_22036-49)
Multidrug
efflux pump
× 12
Nuo
(Dsp_10115,16, 20-28)
NqrABCDEF
(Dsp_10046-51)

Figure 3.4 Proposed metabolic scheme of *D. spongiiphila* based on genomic, transcriptomic and proteomic analysis. The cytoplasmic membrane is in yellow, the periplasmic membrane is in gold. Protein complexes that were partially or completely detected by mass spectrometry are indicated with a solid outline. Proteins or pathways that were not detected by mass spectrometry are drawn with a dashed outline or line.

Chapter 4 Two organohalide-respiring *Deltaproteobacteria* representing novel *Halodesulfovibiro* species, isolated from New Jersey river sediments

Abstract

Organohalide-respiring bacteria, which utilize organohalides as electron acceptors for energy conservation, play an important role in the global halogen cycle. In this study, we isolated two anaerobic bacteria with dehalogenating ability, strain AK and strain HS, from estuarine sediments in New Jersey, USA. These bacterial strains are sulfate reducing *Deltaproteobacteria* that can dehalogenate a variety of brominated compounds. Their growth is supported by lactate and 2,6-dibromophenol, but not lactate alone, indicating that respiratory reductive dehalogenation is used for energy generation. Their debrominating activity is not influenced by the presence of sulfate or exogenous cobalamin. Phylogenetic analysis indicates that these two strains belong to the genus *Halodesulfovibrio*. Whole genome comparison revealed that these two strains are highly similar with an average nucleotide identity (ANI) of 97.8%, but share low similarity (ANI < 90%) with other *Halodesulfovibrio* species (*H. marinisediminis*, *H. aestuarii* and *H. spirochaetisodalis*). The physiological comparison with *H. marinisediminis* also shows difference in salinity tolerance and dehalogenation range. Combined, this provides evidence that

these two strains represent a novel species, for which we propose the name *Halodesulfovibrio debrominans* with strain HS is the type strain. Three reductive dehalogenase genes and the genes encoding for a complete corrinoid biosynthesis are present in their genomes. Transcript analysis shows that the expression of one reductive dehalogenase gene in each strain is induced by 2,6-dibromophenol. This study expands our knowledge about the organohalide respiring potential of marine and estuarine *Deltaproteobacteria*.

Introduction

Many organohalides, such as trichloroethylene (TCE), tetrachloroethylene (PCE), polychlorinated biphenyls (PCBs) and brominated flame retardants, are released into the environment via anthropogenic sources and are problematic environmental contaminants (Doherty, 2000; Jo et al., 2010; Yu et al., 2016). Nevertheless, natural production of organohalides is also a major source and contributes to the wide distribution of organohalides in the environment (Gribble, 1998; Vetter, 2006; Gribble, 2010). To date, there are over 5000 naturally produced halogenated compounds detected in the environment from both biogenic and geogenic sources (Gribble, 2010).

Organohalides can be utilized for organohalide respiration under anaerobic conditions, a process in which microorganisms utilize organohalides as electron acceptors for energy generation through reductive dehalogenation (Mohn and Tiedje, 1992; Zanaroli et al., 2015; Adrian and Löffler, 2016). During this process, the halogen

substituents are partially or completely removed from the carbon skeleton. This process is of importance for overall biodegradation of organohalide pollutants because the dehalogenated metabolites are often more vulnerable to further degradation under aerobic conditions (Liu et al., 2013). Organohalide-respiring bacteria (OHRB) are now recognized to be widespread among bacteria phyla (Maphosa et al., 2010; Hug et al., 2013). The so-called obligate OHRBs, including *Chloroflexi* and some *Firmicutes*, are restricted to organohalide respiration, while non-obligate OHRBs, including *Proteobacteria* and some *Firmicutes*, are more versatile in their metabolism and are able to use electron acceptors other than organohalides (Maphosa et al., 2010; Hug et al., 2013). OHRBs have be isolated from diverse environments including contaminated locations, marine sediments, marine sponges and sewage sludge (DeWeerd et al., 1990; Sun et al., 2000; Sun et al., 2001; Ahn et al., 2009; Atashgahi et al., 2018). A reductive dehalogenase is the key enzyme catalyzing this process, whose structure was revealed to have a corrinoid cofactor in the active center (Bommer et al., 2014; Payne et al., 2015). OHRBs are either able to biosynthesize the required corrinoid *de novo* or by can obtain it from the environment, produced by other bacteria (Yi et al., 2012; Men et al., 2014; Rupakula et al., 2015; Liu et al., 2017). The reductive dehalogenase gene clusters usually contain a *rdhA* gene encoding for the catalytic enzyme, a *rdhB* gene encoding for the membrane anchoring protein and some other accessory genes that may be involved in gene regulation and protein maturation (Hug and Edwards, 2013; Jugder et al., 2016a). The presence of reductive dehalogenase genes in the environment could be an indicator for the

dehalogenating potential, nevertheless, the functionality of encoded reductive dehalogenases still need to be tested experimentally.

Organohalide-respiring bacteria have been isolated after enrichment on organochlorine contaminants, such as the extensively studied *Dehalococcoides mccartyi* and *Dehalobacter* spp. for their potential in bioremediation of organochlorine contaminated sites. Comparatively, there are fewer OHRBs isolated and studied for their debrominating ability. Organobromides, such as brominated flame retardants, have gained widespread use, and are now common anthropogenic contaminants of aquatic sediments. The marine environment is also a rich source of natural organobromides. It is therefore of interest to characterize debrominating bacteria in estuarine and marine habitats. In this study, we enriched estuarine sediments with bromophenol and isolated two organohalide-respiring bacteria. The physiology and dehalogenation range of these two bacteria were investigated and genomes were sequenced and analyzed for their metabolic potentials. The genes relevant to organohalide respiration were further studied to extend our knowledge on the diversity of reductive dehalogenase genes.

Results and Discussion

Isolation of stains AK and HS

Anaerobic enrichment cultures were established with estuarine sediment samples from the New York-New Jersey Harbor estuary. Two pure culture with debrominating activity for 2,6-dibromophenol were isolated from each enrichment

culture, namely strain AK from the Authur Kill source and strain HS from the Hackensack River source.

Genomic features and phylogeny of strain AK and HS

Draft genomes were obtained for both strain AK and strain HS. The genome size of strain AK is 3,734,830 bp with a GC content of 44.72% in 63 scaffolds. The genome of strain HS is 3,743,617 bp with a GC content of 44.83%, consisting of 42 scaffolds. The genome was annotated in JGI-IMG, which removed scaffolds below 1000 bp and left 24 scaffolds for strain AK and 14 scaffolds for strain HS. 3,278 protein coding genes were identified in strain AK, of which 2,130 genes are in Clusters of Orthologous Groups (COGs) while 3,363 protein coding genes and 2,169 genes in COGs were identified in strain HS (Table 4.1). The functional comparison of these two strains indicates that around 3,000 protein coding genes are homologs in these two genomes with an identity range from 60 - 100%, indicating their similarity in function. It appears that strain HS contains a nitrogenase gene cluster involved in nitrogen fixation (HS_103253 - 103267), which is absent in the strain AK genome. However, neither of these two strains can grow with nitrogen fixation based on our experimental results.

The phylogenetic analysis indicates that these two strains are members of the *Halodesulfovibrio* genus (Fig. 4.1). The BLAST results show that the 16S rRNA gene of strain AK shares highest similarity to *Halodesulfovibrio marinisediminis* DSM17456^{T} (99.7%, NR_041631) while strain HS shares highest 16S rRNA gene similarity to

Halodesulfovibrio spirochaetisodalis JC271^T (99.7%, LN614381). *Halodesulfovibrio* was recently proposed as a novel genus because of its overall difference from other *Desulfovibrionaceae* genera (Shivani et al., 2017). This newly proposed genus includes four species, which are *H. spirochaetisodalis*, *H. aestuarii*, *H. marinisediminis* and *H. oceani*. *Halodesulfovibrio* strains are obligate anaerobes capable of sulfate reduction, with high salt tolerance, and originate from marine environments (Shivani et al., 2017). The 16S rRNA genes of *Halodesulfovibrio* species share >98.7% similarity, which is higher than the typical species cutoff. Other housekeeping genes, e.g. *gyrB* and *rpoB*, have been used to provide additional resolution for bacterial species classification (Wang et al., 2007; Adékambi et al., 2008). The similarity of *gyrB* and *rpoB* gene in strain HS and AK are 99.5% and 99.8%, which further proved their phylogenetic closeness. However, their *gyrB* and *rpoB* genes share similarity lower than 87.8% and 93.7%, respectively, with other *Halodesulfovibrio* species. When comparing overall genome similarity with the other *Halodesulfovibrio* species, the ANI ranged from 82% to 86%, which is below the species cutoff of 95% - 96% (Richter and Rosselló-Móra, 2009). Strains AK and HS are distinct from other *Halodesulfovibrio* species and are closely related; they share 99.9% 16S rRNA gene identity and have high genome similarity with 97.8% average nucleotide identity (ANI) and 98.0% average amino acid identity (AAI) (Fig. 4.2). We thus conclude that they represent a new *Halodesulfovibrio* species.

The salinity tolerance of strain AK and HS was compared with *H. marinisediminis* to further distinguish these strains (Fig. S4.1). For strain AK and strain HS, NaCl is not necessary for growth and the salinity tolerance range is 0 – 50

g/L, although growth at 0 g/L and 50g/L was delayed compared to other salinity levels. For *H. marinisediminis* which is a marine isolate, no significant growth was observed at 0 g/L over 21 days incubation and the salinity range for growth was 5 – 35 g/L NaCl. This is consistent with the previous report that the salinity tolerance of *H. marinisediminis* is 0.5 -35 g/L (Shivani et al., 2017). Previous reports also indicate that NaCl is necessary for all other *Halodesulfovibrio* strains isolated from marine environments, with a minimum concentration of 0.5 to 10 g/L (Shivani et al., 2017). In contrast, strain AK and strain HS are unique as they don't require NaCl for growth although they are from estuarine sediment.

Organohalide respiring activity of strains AK and HS

Strains AK and HS were selectively isolated for their debrominating activity on 2,6-dibromophenol (2,6-DBP). The two strains debrominated 200 μM 2,6-DBP via 2-bromophenol with phenol accumulating as the end products. The consumption of 2,6-DBP and formation of phenol was stoichiometric. Strains AK and HS grew with 2,6-DBP as the electron acceptor and lactate as carbon source and electron donor, with protein yields of 1.96 and 2.32 mg/L (0.50 mmol and 0.68 mmol protein per mmol electron utilized), respectively (Fig. 4.3). The growth of each strain on 5 mM lactate alone was minimal, with a protein yield of only 0.27 and 0.17 mg/L for strains AK and HS, respectively, after 24 days of incubation, indicating that 2,6-DBP was respired to support the growth of strains AK and HS.

In addition to 2,6-DBP, strains AK and HS can also debrominate 2,4,6-tribromophenol, 4-bromophenol, 3,5-dibromo-4-hydroxybenzoate and 3,5-dibromo-4-hydroxybenzonitrile to phenol, 4-hydroxybenzoate and 4-hydroxybenzonitrile. Neither of them are able to dechlorinate 2,6-dichlorophenol or debrominate 3-bromophenol, 2-bromobenzoate, 3-bromobenzoate or 4-bromobenzaote (200 μM) in 18 days (Table S4.2). A similar dehalogenation range was also observed for *H. marinisediminis*, except that 2,4,6-tribromophenol was only debrominated to 4-bromphenol as the end product. Debrominating activity in estuarine sediments has been studied previously, including the Arthur Kill site. A *Desulfovibrio* sp. strain TBP-1 was isolated on 2,4,6-tribromphenol from Arthur Kill sediment (Boyle et al., 1999). In another study when Arthur Kill sediments were enriched on 2-bromophenol, a co-culture consisting of two *Desulfovibrio* species was obtained, one of which (strain 2BP-48) was a debrominating bacterium (Fennell et al., 2004). Phylogenetic analysis showed that neither of these two *Desulfovibrio* strains were closely related to strain TBP-1. These debrominating isolates indicate that the presence of debrominating bacteria are not rare in estuarine environments and these debrominating bacteria are likely to be also sulfate reducing bacteria. Our two strains enriched on 2,6-DBP, although from two different estuarine locations, share a relatively high phylogenetic similarity (99.7%,) to *Desulfovibrio* sp. strain TBP-1 (AF090830), which would also need to be reclassified as a member of *Halodesulfovibrio*. The report on *Desulfovibrio* strain TBP-1 indicated that it is tolerant to a higher salinity (75 g/L NaCl) but does not grow in the absence of NaCl (Boyle et al., 1999), which is different from strains

AK and HS. Unfortunately, further comparison with *Desulfovibrio sp*. TBP-1 cannot be done because that strain has been lost during storage.

Reductive dehalogenase gene feature and expression

Reductive dehalogenase genes (*rdhA*s) encode the catalytic enzyme for reductive dehalogenation. *rdhA* genes contain a TAT box and two Fe-S conserved motifs. Usually, a membrane anchoring gene (*rdhB*) is associated in the close vicinity to *rdhA*. Based on these conserved motifs, three putative *rdhA* genes were found in the genomes of strains AK and HS (Fig. 4.4). These three RdhAs share very low identity to each other (< 30%), and are located in different clades of the RdhA phylogenetic tree (Fig. 4.5). In a Blast search against the NCBI database, RdhA3-1 of strain AK and RdhA2-1 of strain HS shared the highest similarity (90.9 - 92.3%) to *H. marinisediminis* RdhA (SIN79729), whose expression is found to be upregulated by 2,6-dibromophenol. The strain AK *rdh3-1* and *rdh2-1* gene cluster compositions are similar; both contain a sigma factor 54 dependent transcriptional regulator, a tetratricopeptide repeating protein coding gene and *rdhABC*. RdhA3-2 of strain AK and RdhA2-2 of strain HS share highest similarity (58 - 59%) with the sole RdhA of *H. spirochaetisodalis* (WP_082936245). The gene cluster composition of strain AK *rdh3-2* and strain HS *rdh2-2* contain a sigma factor 54 dependent transcriptional regulatory and the *rdhBCA*, which is also found in the genome of *H. spirocheatisodalis*. *H. spirochaetisodalis* was reported as an antibiotic-producing bacterium and the dehalogenating ability of this strain has not been explored (Shivani et al., 2017). The

presence of the *rdh* gene cluster indicates the potential for reductive dehalogenation. *rdhA5* of strain AK is located at the beginning of scaffold 5, which results in a missing TAT box and only two conserved Fe-S motifs were annotated. Strain AK RdhA5 and strain HS RdhA4 are closest to RdhA3 in *Desulfovibrio bizertensis*, which is also an organohalide respiring bacterium. But the expression of *rdhA3* in *Desulfovibrio bizertensis* is not upregulated by 2,6-dibromphenol in our previous study (Liu and Häggblom, in press; Chapter 5).

With specific primers designed to amplify each of the *rdhA*s in strain AK and HS, the expression of each *rdhA* in response to 2,6-dibromophenol was studied (Fig. 4.6). After addition of 2,6-DBP, debrominating activity was observed within 5 hours and completed in 25 hours. The expression of strain AK *rdhA3-1* and *rdhA3-2* of strain HS exhibited a similar trend over time. The expression of these two *rdhA*s was upregulated immediately at the time of 2,6-DBP addition. The expression level increased along with 2,6-DBP debromination to 2-BP and then decreased back to the initial level when all of 2,6-DBP and 2-BP had been transformed to phenol. In contrast, the expression of *rdhA3-2* and *rdhA5* of strain AK and *rdhA2-1* and *rdhA4* of strain HS did not show a clear trend during the timecourse of debromination.

The phylogenetic analysis with other functionally characterized RdhAs indicates that the strain AK RdhA3-1 and strain HS RdhA2-1 are in the RdhA clade upregulated by bromophenols, including *H. marinisediminis* RdhA, *Desulfoluna spongiiphila* RdhA16032, *Desulfovibrio bizertensis* RdhA18 and *Desulfuromusa kysingii* RdhA10. All these *rdhA*s are found to be upregulated by 2,6-DBP and their gene clusters all contain a sigma-54-dependent transcriptional regulator, a

tetratricopeptide repeat protein and *rdhABC*. Although strain AK *rdhA3-2* and HS *rdhA2-2* are not upregulated by 2,6-DBP, their gene clusters seem to be integral in function which also contain a sigma-54-dependent transcriptional regulator and *rdhABC* genes. These *rdh* gene clusters may be able to respond to organohalides other than 2,6-DBP. For strain AK *rdh5* and HS *rdh4* gene cluster, *rdhA*s were alone without other accessory genes, suggesting that they may not encode functional reductive dehalogenases.

Influence of sulfate and cobalamin on reductive dehalogenation

The high concentration of sulfate in marine and estuarine environments has been reported to delay the dehalogenation process of PCBs and other organohalides (Zanaroli et al., 2006; Zanaroli et al., 2015; Sohn and Häggblom, 2016). This inhibition was proposed to be due to the competition for available electron donors between sulfate reducing bacteria with the dehalogenator. Moreover, sulfate completely inhibits the PCB dehalogenation mediated by *Dehalobium chlorocoercia* DF-1, a marine obligate OHRB (May et al., 2008). However, for OHRBs isolated from marine or estuarine environments which are also sulfate reducers, including *Desulfovibrio* sp. TBP-1 (Boyle et al., 1999), *Desulfoluna spongiiphila* (Ahn et al., 2009), *Desulfovibrio dechloracetivorans* SF3 (Sun et al., 2000), and *Desulfomonile liminaris* DCB-M (Sun et al., 2001), the presence of sulfate does not inhibit reductive dehalogenation. Strain AK and strain HS are also sulfate reducers isolated from estuarine environments. Our results indicate that the presence of 20 mM sulfate does not influence the

dehalogenation of 2,6-DBP by these strains. This is consistent with other sulfate reducing OHRBs.

The involvement of a corrinoid in reductive dehalogenases has been confirmed from the crystal structure of PceA in *Sulfurospirillum multivorans* and NprdhA in *Nitratireductor pacificus* (Bommer et al., 2014; Payne et al., 2015). As the key cofactor of reductive dehalogenases, the corrinoid can be synthesized *de novo* or obtained from the environment. For *Dehalococcoides* and *Chloroflexi*, the absence of an exogenous cobamide supply retards dehalogenating activity (Yi et al., 2012; Men et al., 2014; Rupakula et al., 2015). Our study revealed that the debromination of 2,6-DBP by strain AK and strain HS occurred independent of exogenous cobalamin supplement. The debromination rate of 2,6-DBP was not influenced by the concentration of exogenous cobalamin (0 to 50 μg/L). The genomes of strain AK and HS encode for genes for a complete anaerobic biosynthesis pathway of the corrinoid, except for CbiJ, cobalt-precorrin-6A reductase (Fig. 4.7 and Table S4.2). The absence of *cbiJ* appears to be a common trait for *Deltaproteobacteria* as the study on a suite of anaerobic metal-reducing *Deltaproteobacteria* genome indicated that *cbiJ* is commonly missing, except for *Desulfuromonas* spp. (Rodionov et al., 2004). The genomes of other *Halodesulfovibrio* strains (*H. marinisediminis*, *H. aestuarii* and *H. spirochaetisodalis*) also contain corrinoid anaerobic biosynthesis genes without *cbiJ* even though *H. aesturii* does not contain a reductive dehalogenase gene in its genome. It is hypothesized that *cbiJ* may not be required or is replaced by other enzymes supported by the evidence of cobalamin *de novo* synthesis in *Desulfovibrio vulgaris* even without CbiJ (Lobo et al., 2008).

Deltaproteobacteria are found to contain many cobalt utilizing proteins that are not restricted to reductive dehalogenases (Zhang et al., 2009a). A cobalamin riboswitch is found before the anaerobic ribonucleotide triphosphate reductase gene in all *Halodesulfovibrio* strains, indicating that ribonucleotide triphosphate reductase is a corrinoid dependent enzyme (Matthews, 2009). This is consistent with the finding that most of the corrinoid biosynthesis genes are conserved in a similar gene neighborhood in all *Halodesulfovibrio* strains regardless of reductive dehalogenation ability (Fig. 4.7 and Table S4.3). All *Halodesulfovibrio* strains contain a common gene cluster with a predicted cobalamin riboswitch between *cbtX* and *cbiK*. Interestingly, a gene cluster involved in corrinoid salvaging is found exclusively in strains AK and HS, which includes a gene for adenosylcobinamide amidohydrolase (*cbiZ*), corrinoid transport genes (*btuBFC*) and a set of genes encoding unknown transport proteins (Fig. 4.7). A cobalamin riboswitch is present between *cbiZ* and transporter genes in this gene cluster. Corrinoid salvaging is a strategy for bacteria to obtain exogenous corrinoids from the environment, which is usually utilized by corrinoid autotrophs, e.g. *Dehalococcoides mccartyi* and *Dehalobacter restrictus* (Yi et al., 2012; Men et al., 2014; Men et al., 2015; Rupakula et al., 2015). *Dehalobacter restrictus* has an operon encoding for a corrinoid transporter and salvaging enzymes, whose expression is controlled by the upstream cobalamin riboswitch in response to the corrinoid condition (Rupakula et al., 2015). *Dehalococcoides mccartyi* is able to salvage corrinoids and remodel the exogenous phenolic corrinoid into cobalamin to support reductive dehalogenation (Men et al., 2014). Since strains AK and HS are capable of synthesizing the corrinoid *de novo*, the existence of corrinoid salvaging genes seems

not as important as for corrinoid autotrophs. However, salvaging corrinoids exogenously and remodeling them to the appropriate form may be an energy saving strategy. The presence of the riboswitch in this corrinoid salvaging gene cluster indicates that its expression may be affected by the corrinoid condition of the culture. More studies are needed to elucidate this hypothesis.

Description of *Halodesulfovibrio debrominans* sp. nov.

Halodesulfovibrio debrominans (for ability to dehalogenate organobromine compounds).

Gram negative, anaerobic, sulfate reducing and organohalide respiring bacterium. Non-fermentive. Uses sulfate and 2,6-dibromophenol as electron acceptor with lactate as electron donor and carbon source. Other organohalides, e.g. 2,4,6-tribromophenol, 3,5-dibromo-4-hydroxybenzoate and 3,5-dibromo-4-hydroxybenzonitrile, can also be dehalogenated. Grows at a salinity range of 0 – 50 g/L NaCl with an optimum of 15-35 g/L. The genome size is 3.7 Mb with a GC content of 44.8%. The Type strain is HS which was isolated from Hackensack River sediment.

Conclusion

Marine and estuarine environments are a major reservoir for organohalides from both anthropogenic release and natural production. The prevalence of *rdhA* genes in marine environments indicates that organohalide respiring potential is

widely distributed. However, the high salinity and high sulfate contents in these environments could adversely affect the functionality of these reductive dehalogenases. The verification of organohalide respiring ability of strain AK and strain HS under different conditions provide evidence for their organohalide respiring ability in marine environments, regardless of the high salinity and sulfate content. The isolation of two novel *Halodesulfovibrio* from estuarine environments expands our knowledge about the diversity of *Deltaproteobacteria* OHRBs and provide more evidence in the role of organohalide respiring *Deltaproteobacteria* in halogen cycling in marine environment.

Materials and Methods

Bacterial isolation and culturing

The anaerobic sediments from the Arthur Kill (40°35'33"N 74°12'13"W) and Hackensack River (40°44'24"N 74° 4'47"W) were sampled and stored at 4 ^{o}C before used for establishment of enrichment cultures (Sohn and Häggblom, 2016). 10% of the sediment was used to set up the initial enrichment cultures in 50 mL serum bottles with either sulfate-free or sulfidogenic anaerobic minimum medium (Fennell et al., 2004) with the modification of salinity to 25 g/L NaCl. A mixture of short chain organic acids (lactate, propionate and acetate, 500 μM each) was added as carbon sources and electron donors and 100 μM of 2,6-dibromophenol (2,6-DBP) was provided as electron acceptor to enrich for debrominating bacteria. The debrominating activity of the culture was monitored and when 2,6-DBP had been

depleted, 10% of the culture was transferred to fresh medium and amended with 2,6-DBP and the short chain organic acid mixture. After 5 transfers, the cultures had high debrominating activity and sediment debris was minimal. Semi-solid agar shake tubes were made with 0.8% Noble Agar in minimum medium with short chain organic acids and 2,6-DBP to isolate anaerobic debrominating bacteria following the method of Ahn et al. (2009). After incubation at 30 ^{o}C, individual colonies were extracted using a syringe under anaerobic conditions and transferred into liquid anaerobic medium with lactate and 2,6-DBP. The selected colonies that showed debrominating activity were grown in larger volume and DNA was extracted for purity determination. Multiple rounds of agar shake tubes and single colony isolation were done until pure cultures, designated strain AK and HS, respectively, were obtained.

Halodesulfovibrio marinisediminis DSM17456 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) in freeze-dried form. The culture was revived by inoculating into anaerobic medium with lactate and sulfate under oxygen-free condition.

Physiological analysis

To test the salinity tolerance range, strain AK, strain HS and *H. marinisediminis* cultures pregrown on sulfidogenic medium were washed to remove residual NaCl and inoculated into sulfidogenic medium (30 mM lactate and 20 mM sulfate) with different NaCl concentrations (0 - 70 g/L). To test for growth on nitrogen gas (N_2) as the sole nitrogen source, strain AK, strain HS and *H. marinisediminis* cultures were

washed to remove residual NH_4Cl and inoculated into NH_4Cl-free sulfidogenic medium under a headspace of N_2. The culture was shaken at 200 rpm and the headspace was replenished with N_2 every two days. Growth was determined by measurement of OD_{600} using a spectrophotometer.

Characterization of organohalide respiring ability

To prove that the growth of strain AK and strain HS could be supported by organohalide respiration, strains AK and HS were pre-grown in sulfate-free medium with lactate and 2,6-DBP. When 2,6-DBP was completely debrominated to phenol, 10% of the culture was inoculated into medium with lactate (5 mM) alone and medium with lactate (5 mM) plus 2,6-DBP (200-300 μM). The later culture was refed with 2,6-DBP (260-460 μM each time) for 4 times after each round of debromination was complete. In total, approximately 1.6 mM and 1.8 mM of 2,6-DBP was utilized by strain AK and strain HS, respectively. The cultures were then collected via centrifugation and the cellular protein concentrations were determined using the Bradford assay. Briefly, 40 ml of the culture was collected and rinsed with phosphate-buffered saline (PBS, pH 7.4) to remove any medium residue. The cell pellets were resuspended with 0.5 ml PBS and 0.5 ml 2N NaOH and incubated at 70 ^{o}C for 45 min. Then, samples were then centrifuged at 13,000g for 3 min. The supernatant was neutralized with HCl and measured using the Quick Start™ Bradford protein assay (Biorad Laboratories, Inc.). Biological duplicates for the lactate alone control and lactate with 2,6-DBP were done for protein determination.

In order to test the organohalide substrate range of strains AK and HS and *H. marinisediminis*, 200 μM 2,4,6-tribromophenol, 2,6-dibromophenol, 2-bromophenol, 3-bromophenol, 4-bromophenol, 2-bromobenzoate, 3-bromobenzoate, 4-bromobenzoate, 3,5-dibromo-4-hydroxybenzoate, 3,5-dibromo-4-hydroxybenzonitrile or 2,6-dichlorophenol were provided as electron acceptors with lactate as carbon source and electron donor. Cultures were sampled and analyzed by HPLC over an incubation of 18 days or longer. To test the influence of cobamide on the dehalogenating activity, exogenous cobamide (0 to 50 μg/L) was added to a washed cell culture in cobamide-free medium with lactate and 2,6-DBP. The dehalogenating activity was compared under different cobamide concentrations. To determine the influence of sulfate on dehalogenating activity 20 mM sulfate was added to the cultures with lactate and 2,6-DBP.

Analytical methods

The concentrations of halogenated compounds and their dehalogenated products were measured via high-performance liquid chromatography (HPLC) on a Shimadzu system equipped with an auto injector (SIL-10A, Shimadzu), a system controller (SCK- 10A, Shimadzu), and a diode array detector (SPD-M10A, Shimadzu). A Sphereclone C-18 column (250 mm × 4.6 mm, particle size 5 μm; Phenomenex) was used with a mobile phase consisting of methanol: water: acetic acid (methanol varied from 55% to 70% depending on the compound; 1% acetic acid) at an isocratic flow rate of 1 ml/min. The UV absorbance detection wavelength was 230 nm or 280 nm.

Genome sequencing, annotation and phylogenetic analysis

Genomic DNA was extracted from cultures grown on 20 mM sulfate and 30 mM lactate using a phenol-chloroform based method (JGI cTAB Method). Genome sequencing was performed on the Illumina Miseq platform using 2*250bp pair-end sequencing protocols. The reads were trimmed using Trimmomatic and the quality was assessed using in-house scripts combined with Samtools, BedTools and BWA-mem. *De novo* assembly was conducted with SPAdes. JGI-IMG and RAST platform were used to annotate and visualize the genomes (Aziz et al., 2008; Markowitz et al., 2012). BRIG was used to compare the genomes of two strains and other close related bacteria by using strain HS as reference (Alikhan et al., 2011). The Average Nucleotide Identity (ANI) was determined using reciprocal best hits as calculated by Goris et al. (2007) (Goris et al., 2007).

Phylogenetic analysis of 16S rRNA, *rpoB* and *gyrB* genes, and reductive dehalogenase genes were conducted in MEGA7 (Kumar et al., 2016). Alignments were performed using ClustalW with Cost Matrix Connet. Phylogenetic trees were constructed by applying Neighbor-Joining and advanced NJ algorithms to a matrix of pairwise distances estimated using a Jones-Thornton-Taylor model, and then selecting the topology with superior log likelihood value.

Transcriptional analysis of *rdhA* genes

Strain AK and strain HS were initially grown on 30 mM lactate and 20 mM sulfate to a large biomass. Then, 50 ml of the pre-grown culture was inoculated into 50 ml of fresh medium with 2 mM lactate. The cultures were amended with 200 μM of 2,6-DBP to induce the expression of *rdhA* genes. For the control treatment, 200 μM of sulfate was added as electron acceptor. The concentration of 2,6-DBP and its products were monitored by HPLC. At each time point, 5 ml of culture was centrifuged to collect a cell pellet for RNA extraction. Collected pellets were stored at -80 ^{o}C prior to analysis.

RNA was extracted using the Direct-Zol kit by following the manufacturer's manual (Zymo Research). DNA was removed using Turbo-DNase Removal Kit (Ambion, Life Technologies). RNA was quantified using Nanodrop and 100 ng of RNA was used for the reverse transcription. cDNA was generated using the iScript TM Reverse Transcription Supermix (Bio-Rad Laboratories, Inc) according to the manufacturer's instructions. The obtained cDNA was amplified with primers designed for *rdhA* genes and 16S rRNA genes using RT-qPCR (Table S4.1), which was performed using an IQ SYBR Green Supermix (BIO-RAD) as described in Liu et al. (2017). Transcription levels of each *rdhA* genes were determined using relative standard curve method and normalized to 16S rRNA gene. Expression level was indicated by comparing the transcription in 2,6-DBP treatment to control treatment.

Acknowledgements

Genome sequencing was provided by MicrobesNG (http://www.microbesng.uk), which is supported by the BBSRC (grant number BB/L024209/1

Table 4.1 Genome information and annotation of strain AK and strain HS compared to *H. marinisediminis* (*H.m*), *H. aestuarii* (*H.a*) and *H. spirochaetisodalis* (*H.s*).

	AK	**HS**	***H.m***	***H.a***	***H.s***
Genome Size (Mbp)	3.73	3.74	3.71	3.40	3.61
G+C Content (%)	44.72	44.83	45.02	46.19	44.95
Scaffold	63	42	13	15	55
Genes total number	3397	3363	3071	3295	3310
Protein Coding Genes	3278	3244	2965	3152	3199
Genes Coding transmembrane proteins	879	888	782	846	879
Reductive dehalogenase (*rdhA*)	3	3	1	0	1
Protein Coding Genes with COGs	2130	2169	2014	2106	2130
- Amino acid transport and metabolism	258	265	243	262	250
- Carbohydrate transport and metabolism	111	113	95	113	109
- Cell cycle control, cell division, chromosome partitioning	28	26	27	29	27
- Cell motility	90	88	80	98	95
- Cell wall/membrane/envelope biogenesis	142	148	138	150	141
- Chromatin structure and dynamics	1	1	1	1	1
- Coenzyme transport and metabolism	146	144	140	135	139
- Cytoskeleton	1	1	1	1	1
- Defense mechanisms	45	54	44	61	56
- Energy production and conversion	220	222	206	196	218
- Extracellular structures	22	20	17	8	25
- Function unknown	101	107	96	93	99
- General function prediction only	158	156	154	163	161
- Inorganic ion transport and metabolism	122	131	119	122	123
- Intracellular trafficking, secretion, and vesicular transport	46	57	60	42	64
- Lipid transport and metabolism	66	58	67	66	71
- Mobilome: prophages, transposons	34	36	25	18	30
- Nucleotide transport and metabolism	63	63	64	65	67
- Posttranslational modification, protein turnover, chaperones	99	98	92	97	98
- Replication, recombination and repair	98	90	89	88	99
- Secondary metabolites biosynthesis, transport and catabolism	33	25	37	41	33
- Signal transduction mechanisms	195	207	173	184	183
- Transcription	119	122	100	131	115
- Translation, ribosomal structure and biogenesis	190	194	189	188	187
Not in COG	1267	1194	1057	1189	1180

The genome of *h.m, h.a* and *h.s* are obtained from JGI.

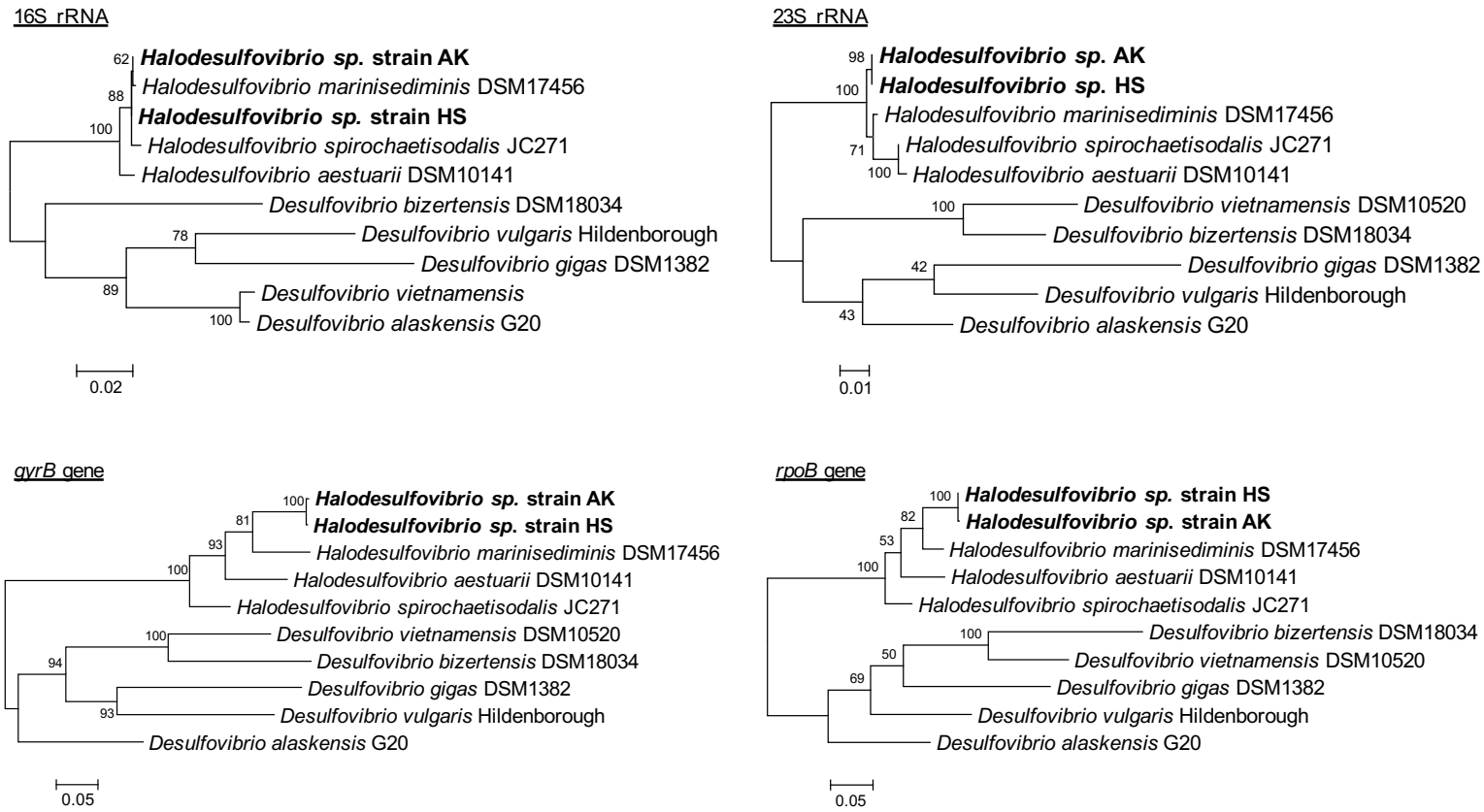


Figure 4.1 Phylogenetic trees of 16S rRNA, 23S rRNA, *gyrB* and *rpoB* genes of strains HS and AK compared to representative *Halodesulfovibrio* and *Desulfovibrio* species. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The percentage of trees in which the associated taxa clustered together is shown next to the

branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 10 nucleotide sequences. All positions with less than 100% site coverage were eliminated. There were a total of 1405 positions for 16S rRNA, 1258 positions for 23S rRNA, 2365 positions for *gyrB* and 4087 positions for *rpoB* in the final dataset. Evolutionary analyses were conducted in MEGA7.

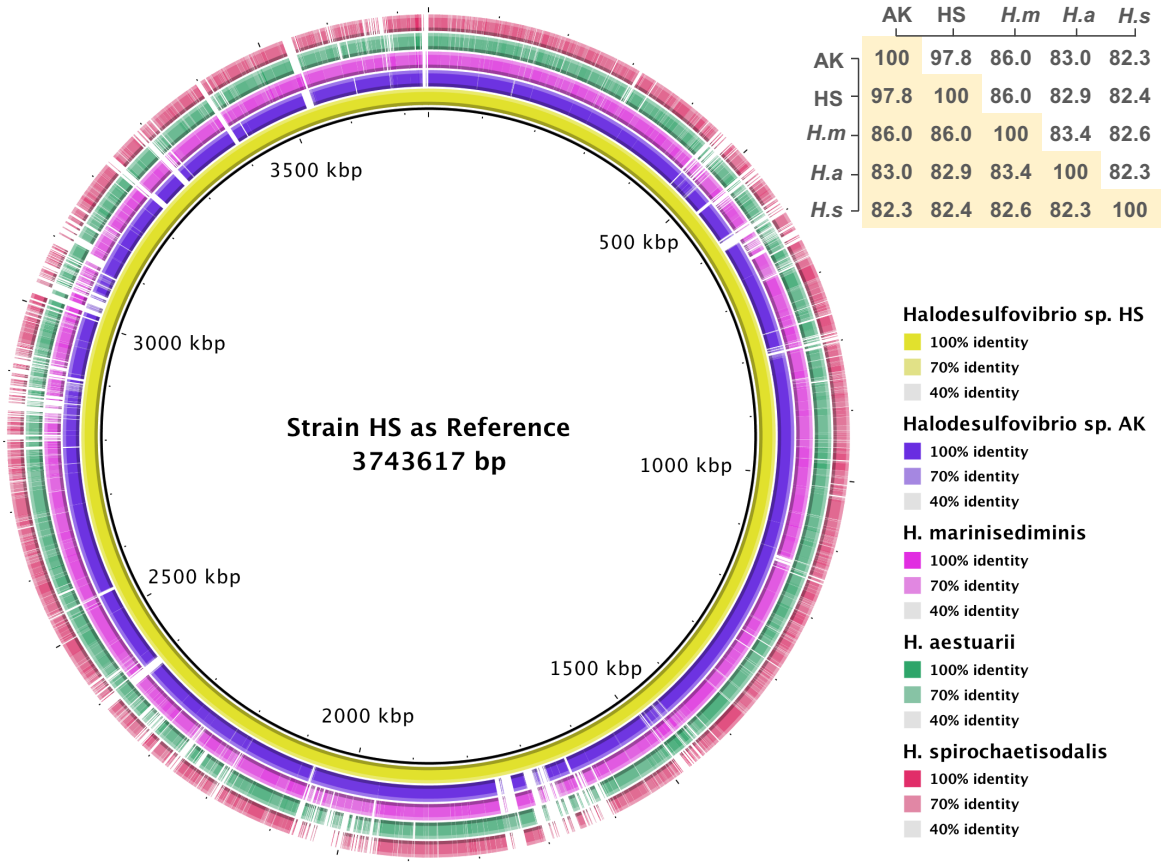


Figure 4.2 The whole genome comparison of strain HS and AK with *H. marinisediminis, H. aestuarii* and *H. spirochaetisodalis*. The genome of strain HS was used as the reference genome for comparison to other genomes. The ANI matrix between these genomes are shown in the right corner.

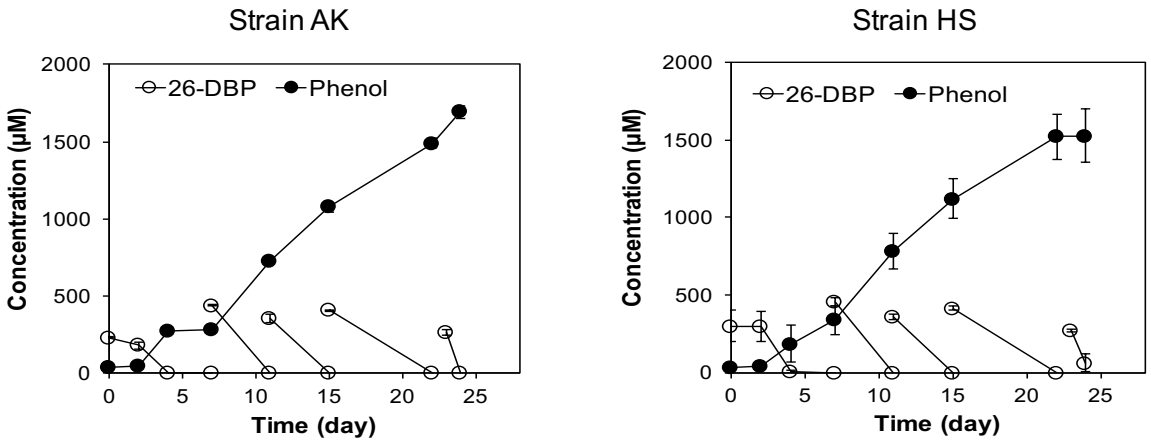


Figure 4.3 Debromination of 2,6-DBP and accumulation of phenol in strain AK and strain HS. Standard deviations are from biological duplicates.

Table 4.2 The protein yield of strain AK and strain HS grown under lactate only and lactate with 2,6-dibromophenol culture. Utilized electrons were calculated from the concentration of phenol and 2-BP produced from debromination of 2,6-DBP, based on two electrons provided per bromine removed. Protein was determined at 24 day shown in Figure 4.3.

Strain	Electron donor	Electron acceptor	Electron used for reduction (mmol in 1L)	Produced protein (mg in 1L)	Yield (mg of protein per mmol electron)
AK	Lactate	None	NA	0.27 ± 0.22	NA
	Lactate	2,6-DBP	3.36 ± 0.09	1.96 ± 0.11	0.50 ± 0.15
HS	Lactate	None	NA	0.17 ± 0.08	NA
	Lactate	2,6-DBP	3.16 ± 0.35	2.32 ± 0.20	0.68 ± 0.15

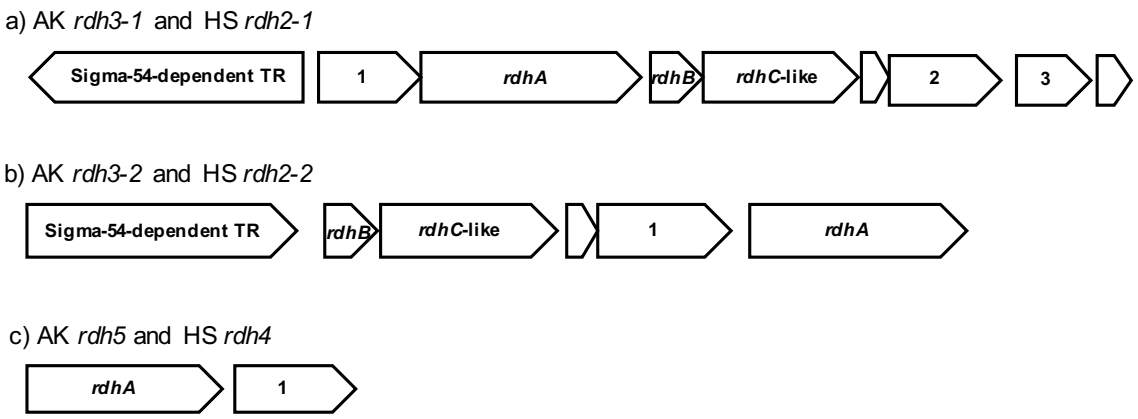


Figure 4.4 Reductive dehalogenase gene clusters in strain AK and HS. *rdhA* is for putative reductive dehalogenases, *rdhB* is for putative membrane anchoring gene, *rdhC*-like is for gene encoding FMN binding domain and 4Fe-4S binding domain-containing protein and sigma-54-dependent TR is for sigma factor 54 dependent transcriptional regulatory gene. Detailed annotation of other genes is a) 1- Tetratricopeptide repeat containing protein; 2- Thiamine biosynthesis lipoprotein ApbE; 3- Inner membrane proteins; b) 1- Thiamine biosynthesis lipoprotein ApbE; c) 1- Class III cytochrome C family protein. The white arrows with no number marker are hypothetical proteins.

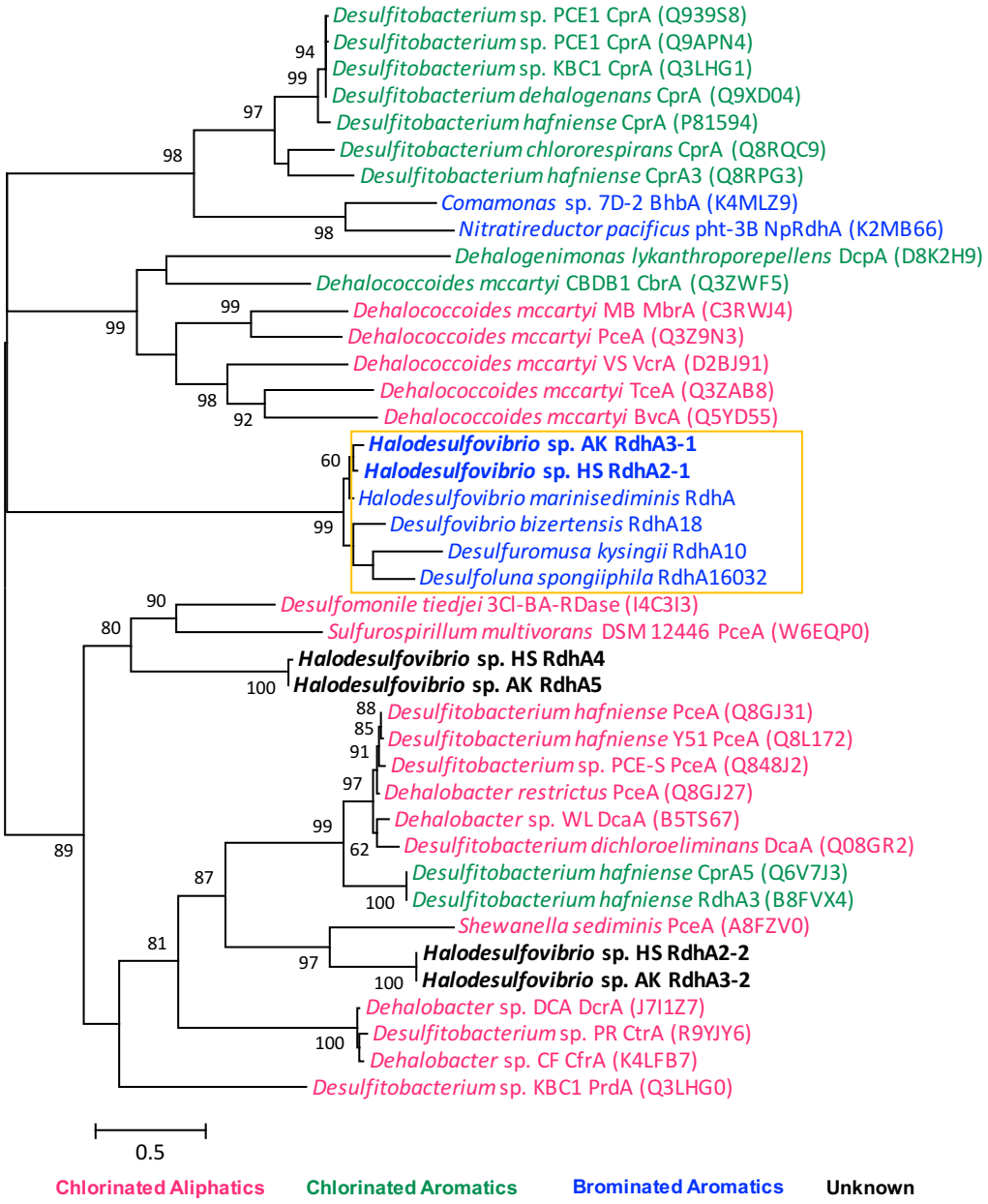


Figure 4.5 Phylogenetic tree of strain AK and strain HS RdhAs compared to other functionally characterized RdhAs. The clade of 2,6-dbiromophenol dehalogenating RdhAs are boxed in yellow. The observed functions of the RdhAs are color-coded as shown in the figure.

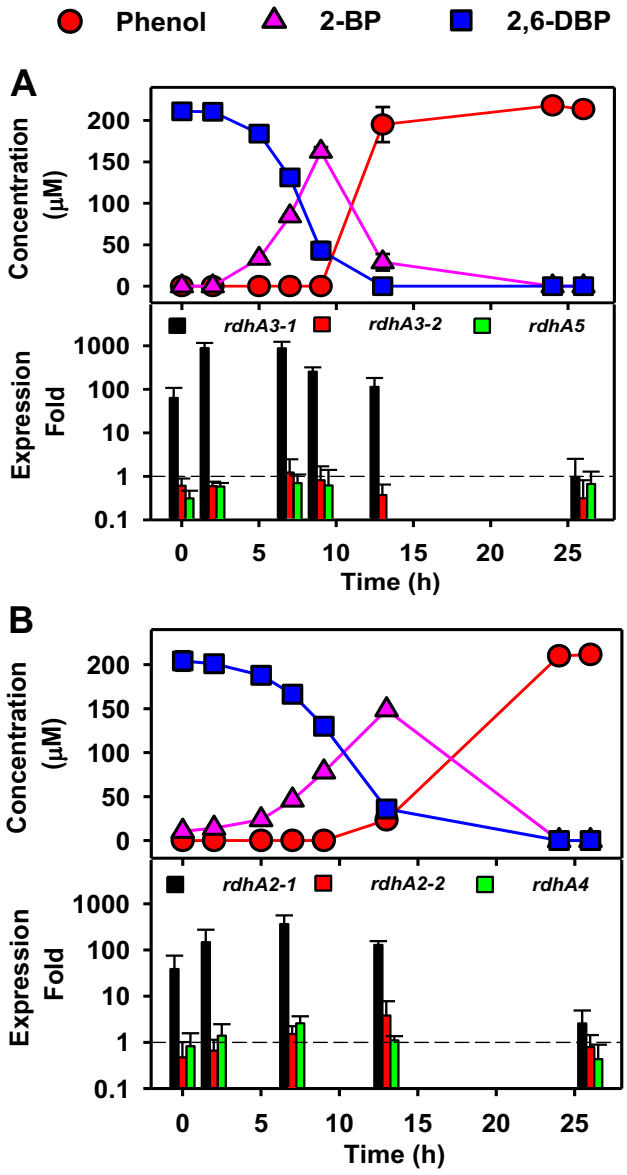


Figure 4.6 Dehalogenation of 2,6-dibromophenol by strain AK and strain HS in the presence of lactate (upper) and the expression of rdhA genes along with debromination with the 16S rRNA gene used as control for normalization (B).

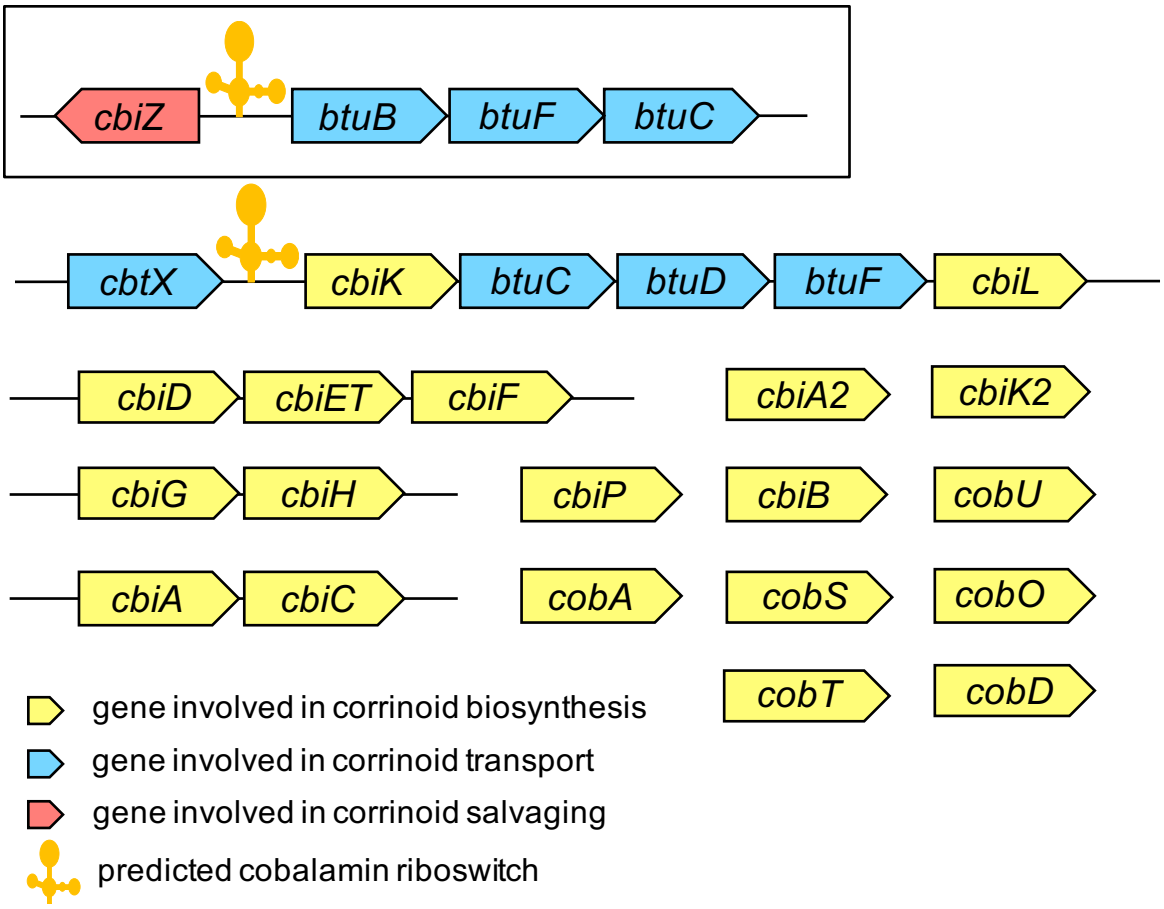


Figure 4.7 Arrangement of genes involved in corrinoid biosynthesis pathway in strain AK, strain HS and other *Halodesulfovibrio* strains (*H. marinisediminis*, *H. aestuarii* and *H. spirochaetisodalis).* The gene cluster highlighted in the box is exclusive in the genomes of strain AK and strain HS. Details genome locus of the genes are shown in Table S4.3.

Supplementary Information

Table S4.1 Primers used in this study for regular PCR and qRT-PCR.

Name	Sequence (5'- 3')	Target	Length (bp)
27F	AGAGTTTGATCMTGGCTCAG	Bacterial 16S rRNA universal	~1070
1100R	AGGGTTGCGCTCGTTG		
16SrRNA_1428F	AAACCAGTGAGCTAACCGGC	Strain AK and HS 16S rRNA	87
16SrRNA_1515R	TCCCCTACGGCTACCTTGTT		
AK_Rdh3_1_1507F	CTTCAGGCTTGCTGGCAATG	Strain AK *rdhA3-1*	53
AK_Rdh3_1_1560R	GTAGTTTGCTTCCTTGCCGC		
AK_Rdh3_2_1146F	CGGTGGTGAGTGTGCTAACT	Strain AK *rdhA3-2*	72
AK_Rdh3_2_1218R	GACTAAGCGATGGTGCCAGA		
AK_Rdh5_917F	CTGTATCTGTTCCGCTGGCA	Strain AK *rdhA5*	90
AK_Rdh5_1007R	CTGATATGCGGACCGAACCA		
HS_Rdh2_1_290F	ACCGTGAACCATTCCGTACC	Strain HS *rdhA2-1*	70
HS_Rdh2_1_360R	CCATTCTGGACGCTCAACCT		
HS_Rdh2_2_683F	CTTTCTGGACTGCACAGGGT	Strain HS *rdhA2-2*	74
HS_Rdh2_2_758R	AATGAAGCAACGCAGGATGC		
HS_Rdh4_567F	AGTAGGTGCCGATCGTGTTG	Strain HS *rdhA4*	75
HS_Rdh4_642R	GTACATGTTTCGGCAGGTGC		

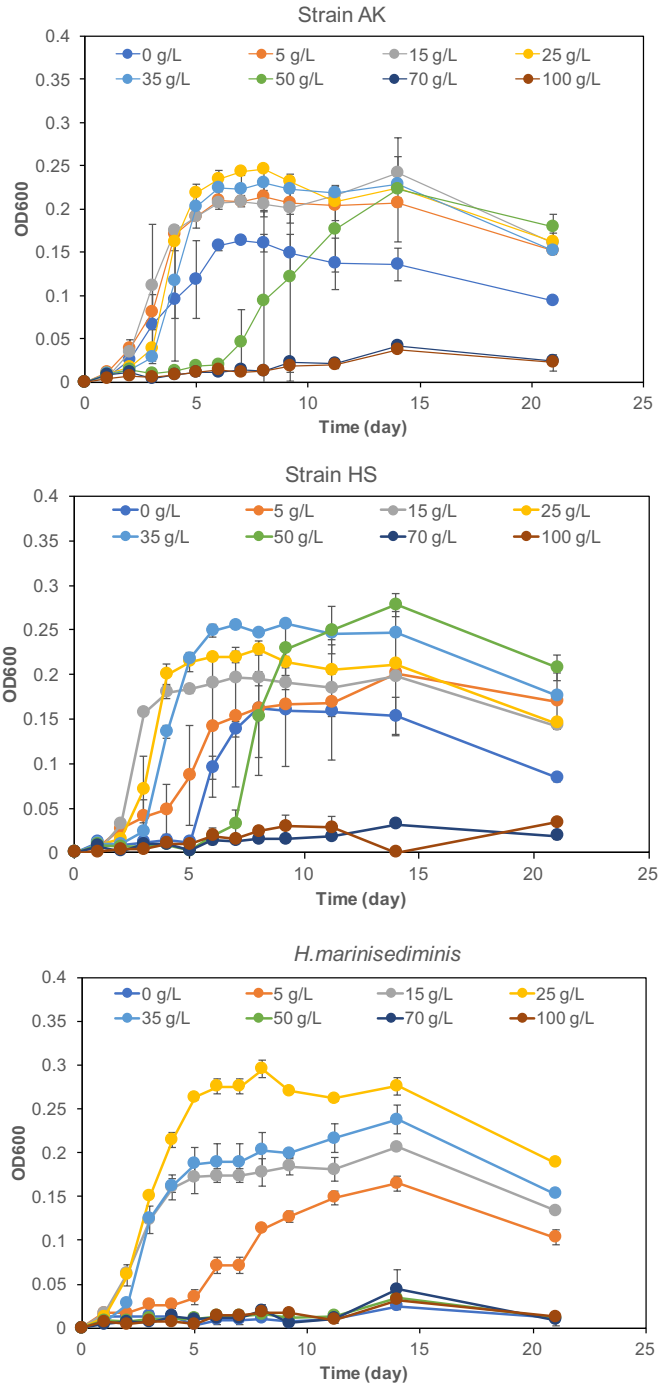


Figure S4.1 The optical density (OD600) of strain AK, strain HS and *H. marinisediminis* grown on lactate and sulfate under different salinity condition (0 – 100 g/L NaCl). Error bars represent the standard deviations of biological triplicates.

Table S4.2 Summary of dehalogenating range of strain AK, strain HS and *H. marinisediminis*.

	Strain HS	**Strain AK**	***H. marinisediminis***
2,4,6-Tribromophenol	Phenol	Phenol	4-Bromophenol
2,6-Dibromophenol	Phenol	Phenol	Phenol
2-Bromophenol	Phenol	Phenol	Phenol
3-Bromophenol	-	-	-
4-Bromophenol	Phenol	Phenol	-
2-Bromobenzoate	-	-	-
3-Bromobenzoate	-	-	-
4-Bromobenzoate	-	-	-
3,5-Dibromo-4-hydroxybenzoate	4-Hydroxybenzoate	4-Hydroxybenzoate	4-Hydroxybenzoate
3,5-Dibromo-4-hydroxybenzonitrile	4-Hydroxybenzonitrile	4-Hydroxybenzonitrile	4-Hydroxybenzonitrile
2,6-Dichlorophenol	-	-	-

Dehalogenation capability determined after 18 days of incubation or longer

Table S4.3 The detailed JGI information of genes involved in corrinoid biosynthesis in strain AK, strain HS, *H. marinisediminis, H. aestuarii* and *H. spirochaetisodalis* shown in Figure 4.6.

Abbrev.	Functional Role	AK	HS	*H.m*	*H.a*	*H.s*
BtuC	Vitamin B12 ABC transporter, permease component	101517	101465			
BtuF	Vitamin B12 ABC transporter, B12-binding component	101518	101466			
BtuB	Outer membrane vitamin B12 receptor	101519	101467			
CbiZ	Adenosylcobinamide amidohydrolase (EC 3.5.1.90)	101521	101469			
CbiL	Cobalt-precorrin-2 C20-methyltransferase	10296	101620	1958	2203	12213
BtuF	Vitamin B12 ABC transporter, B12-binding component	10297	101621	1959	2204	12212
BtuD	Vitamin B12 ABC transporter, ATPase component	10298	101622	1960	2205	12211
BtuC	Vitamin B12 ABC transporter, permease component	10299	101623	1961	2206	12210
CbiK	Sirohydrochlorin cobaltochelatase	102100	101624	1962	2207	12209
CbtX	Predicted cobalt transporter in sulfate-reducing delta-proteobacteria	102102	101625	1963	2208	12208
CbiG	Cobalamin biosynthesis protein	101136	101137	1076	1319	10641
CbiH	Cobalt-precorrin-3b C17-methyltransferase	101137	101138	1077	1320	10640
CbiC	Cobalt-precorrin-8x methylmutase	101178	101176	1096	1281	1062
CbiA	Cobyrinic acid A,C-diamide synthase	101179	101177	1097	1280	1061
CbiF	Cobalt-precorrin-4 C11-methyltransferase	102252	101764	2091	2344	12137
CbiET	Cobalt-precorrin-6y C5-methyltransferase	102253	101765	2092	2345	12138
CbiD	Cobalt-precorrin-6 synthase	102254	101766	2093	2346	12139
CobD	L-threonine 3-O-phosphate decarboxylase	11232	103558	2998	2690	109126
CobU	Adenosylcobinamide-phosphate guanylyltransferase	101268	101261	1187	1233	12610
CobT	Nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase	101268	101261	1187	1233	12610
CbiA2	Cobyrinic acid A,C-diamide synthase	101291	101284	1205		110102
CobO	Cob(I)alamin adenosyltransferase	103409	102486	0477	0323	104161
CbiK2	Sirohydrochlorin cobaltochelatase	10437	102411	0405	0394	101204
CbiB	Adenosylcobinamide-phosphate synthase	104365	10286	0082	0714	11414
CobS	Cobalamin synthase	10694	103372	1618	0818	11660
HemD	Uroporphyrinogen-III methyltransferase	10629	103307	1153	0885	102199
CbiP	Cobyric acid synthase	106194	103472	2913	2604	10937

Chapter 5 Genome guided identification of organohalide-respiring *Deltaproteobacteria* from the marine environment

This chapter is in press in mBio as "Liu J, Häggblom MM (2018) Genome guided identification of organohalide-respiring *Deltaproteobacteria* from the marine environment."

Abstract

Organohalide compounds are widespread in the environment as a result of both anthropogenic activities and natural production. The marine environment, in particular, is a major reservoir of organohalides, and reductive dehalogenation is thought to be an important process in the overall cycling of these compounds. *Deltaproteobacteria* are important members of the marine microbiota with diverse metabolic capacities and reductive dehalogenation has been observed in some *Deltaproteobacteria*. In this study, a comprehensive survey of *Deltaproteobacteria* genomes revealed that approximately 10% contain reductive dehalogenase (RDase) genes, which are found within a common gene neighborhood. The dehalogenating potential of select RDase A-containing *Deltaproteobacteria* and their gene expression were experimentally verified. Three *Deltaproteobacteria* strains isolated from marine environments representing diverse species, *Halodesulfovibrio marinisediminis, Desulfuromusa kysingii* and *Desulfovibrio bizertensis*, were shown to reductively

dehalogenate bromophenols and utilize them as terminal electron acceptors in organohalide respiration. Their debrominating activity was not inhibited by sulfate or elemental sulfur and these species are either sulfate or sulfur reducing bacteria. The analysis of RDase A gene transcripts indicated significant upregulation induced by 2,6-dibromophenol. This study extends our knowledge of the phylogenetic diversity of organohalide-respiring bacteria and their functional RDase A gene diversity. The identification of reductive dehalogenase genes in diverse *Deltaproteobacteria* and confirmation of their organohalide-respiring capability suggests that *Deltaproteobacteria* play an important role in natural organohalide cycling.

Importance

The marine environment is a major reservoir for both anthropogenic and natural organohalides, and reductive dehalogenation is thought to be an important process in the overall cycling of these compounds. Here we demonstrate that the capacity of organohalide respiration appears to be widely distributed in members of marine *Deltaproteobacteria*. The identification of reductive dehalogenase genes in diverse *Deltaproteobacteria*, and the confirmation of their dehalogenating activity through functional assays and transcript analysis in select isolates extends our knowledge of organohalide-respiring *Deltaproteobacteria* diversity. The presence of functional reductive dehalogenase genes in diverse *Deltaproteobacteria* implies they may play an important role in organohalide respiration in the environment.

Introduction

Organohalides are widespread in the environment as a result of both anthropogenic and natural sources. Many organohalides, for example trichloroethene (TCE), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and brominated flame retardants (BFRs), have been widely used in industry, households, and/or agriculture and are problematic environmental pollutants (Häggblom and Bossert, 2003). Over 5,000 naturally produced organohalides have also been identified, originating from diverse biogenic sources and geogenic activities (Gribble, 2010). Microbes are now recognized to play a key role in the cycling of these organohalides through both halogenation and dehalogention processes (Pée, 1996; Häggblom and Bossert, 2003; Adrian and Löffler, 2016). Of particular interest is the process of respiratory reductive dehalogenation in which bacteria utilize organohalides as electron acceptors for energy generation. Through this process, the halogen substituent is removed and the dehalogenation products are usually more amenable to further biodegradation and are reduced in toxicity (Häggblom and Bossert, 2003; Futagami et al., 2008; Adrian and Löffler, 2016; Jugder et al., 2016a). This process is crucial for not only the removal of organohalide pollutants from contaminated environments but also in the cycling of natural organohalides as part of a global halogen cycle (Smidt and de Vos, 2004; Adrian and Löffler, 2016).

Organohalide-respiring bacteria (OHRBs) have been isolated from diverse environments, including organohalide-contaminated soils and sediments as well as

pristine sites (Hug and Edwards, 2013; Atashgahi et al., 2018). Based on their metabolic versatility they can be classified into facultative vs. obligate OHRBs (Maphosa et al., 2010). The growth of obligate OHRBs, including *Dehalobacter* (in the Firmicutes) and *Dehalococcoides* and *Dehalogenimonas* (in the Chloroflexi), is restricted to organohalide respiration, while facultative OHRBs, including *Desulfitobacterium* (Firmicutes) and various *Proteobacteria,* are more versatile in their metabolism and can utilize diverse electron acceptors other than organohalides. Although an increasing number of OHRBs have been isolated, it is apparent that their diversity and distribution is even more extensive in the environment considering that dehalogenation activities mediated by indigenous bacteria are reported in diverse environments (Zanaroli et al., 2015; Atashgahi et al., 2018). Reductive dehalogenase (RDase) genes encode for the key enzymes for organohalide respiration. Typically, the respiratory RDase gene operon consists of a RDase A gene encoding the catalytic unit (RDase A), a RDase B gene encoding a putative membrane-anchoring protein, and other accessory genes involved in regulation and maturation (Hug et al., 2013). The sequence of the RDase A gene (also indicated as reductive dehalogenase homologous A, *rdhA,* in many publications) commonly contains a conserved arginine translocation (Tat) signal motif (RRXFXK) and two iron-sulfur cluster binding motifs (CXXCXXCXXXCP and CXXCXXXCP motifs) (10, 30). In addition to respiratory reductive dehalogenases in anaerobes, some RDases are metabolic and not involved in energy conservation, e.g. *Nitratireductor pacificus* pht-3B (NprdhA) and *Comamonas* sp. 7D-2 (BhbA) (Chen et al., 2013; Payne et al., 2015). The crystal

structures of both kinds of reductive dehalogenases indicated the presence of a corrinoid cofactor in the active center (Bommer et al., 2014; Payne et al., 2015).

RDase A genes have been detected in marine sediments using specific PCR-primers and metagenomics analyses (Rhee et al., 2003; Futagami et al., 2009; Futagami et al., 2013; Hug and Edwards, 2013; Kawai et al., 2014; Zinke et al., 2017; Marshall et al., 2018). The prevalence of RDase A genes in pristine marine environments suggests a widespread distribution of OHRBs and organohalide respiration may be an important energy-yielding metabolic pathway for anaerobic marine bacteria, which makes it important to study OHRBs from diverse species to gain a better understanding of the role of OHRBs in the global cycling of organohalides. *Deltaproteobacteria* have diverse metabolisms and are ubiquitously present in the environment. Based on an earlier survey of RDase A genes in 208 *Deltaproteobacteria* genomes, approximately 10% of the sequenced *Deltaproteobacteria* contained RDase A genes in their genomes, suggesting their potential ability for organohalide respiration (Sanford et al., 2016). Metagenomic analysis data also indicated that *Deltaproteobacteria* were dominant members of debrominating enrichment cultures derived from deep ocean sediments (Futagami et al., 2013). The presence of putative RDase A genes is an indicator of potential organohalide respiration, however, the dehalogenating activity and gene functionality should be experimentally verified.

In this study, we analyzed the frequency and diversity of RDase A genes in 556 published *Deltaproteobacteria* genomes and examined the common features of the reductive dehalogenase gene clusters identified in their genomes. Three

representative *Deltaproteobacteria*, not previously recognized with organohalide respiration ability, were investigated for their dehalogenating ability. Transcript analysis was also conducted to confirm the expression of RDase A in these *Deltaproteobacteria* in response to organobromides.

Results

A survey of reductive dehalogenases in *Deltaproteobacteria*

In a search of the JGI Integrated Microbial Genomes & Microbiome Samples (IMG/MER) genome database (May, 2017), 556 annotated genomes were grouped in the *Deltaproteobacteria*. When searching for genes annotated with (potential) reductive dehalogenase function, 9% of these genomes (50 of 556) were found to contain at least one putative RDase A gene and a total of 80 putative RDase A genes were found in these different *Deltaproteobacteria* genomes. The similarities among these RDase A amino acid sequences are in the range of 10%-100%. The prevalence of RDase genes in members of the *Deltaproteobacteria* was higher than that of other *Proteobacteria*, e.g., 7.8% in *Alphaproteobacteria*, 1.4% in *Betaproteobacteria*, 0.14% in *Epsilonproteobacteria*, and 0.4% in *Gammaproteobacteria.* In order to distinguish potential organohalide-respiring bacteria among characterized *Deltaproteobacteria*, 35 RDases from 19 pure culture isolates were chosen for comparison with functionally characterized RDases from non-*Deltaproteobacteria* (the details of their genome information is shown in Table S5.1 and Table S5.2). A tree with all 80 RDases

from both isolates and metagenomics data is also shown in the supplementary information (Fig. S5.1).

According to the RDase A classification system proposed by Hug et al. (12), the *Deltaproteobacteria* RDase As could not be assigned to existing groups with a cutoff of 90% identity except for six RDase As (*Geobacter lovleyi* SZ RDase A1 and RDase A2 in ortholog group 41, *Anaeromyxobacter dehalogenans* 2CP-1 RDase A and *Anaeromyxobacter* sp. K RDase A in ortholog group 42, *Deltaproteobacterium* NaphS2 RDase A and *Anaeromyxobacter dehalogenans* 2CP-C RDase A in an unassigned group) that were already included in their most recent database (April, 2018). Here, we constructed phylogenetic trees of characterized and putative *Deltaproteobacteria* RDase As as shown in Fig. 5.1A. The RDase As can be divided into nine clades based on the tree nodes rather than a fixed sequence identity cutoff. Most of the functionally characterized RDase As are from the *Firmicutes* (Clades 2 and 9) and *Chloroflexi* (Clade 3). RDase As from the *Chloroflexi* are grouped together in Clade 3 with an identity range of 19.5 to 46.5%, while RDase As from *Firmicutes* are grouped in Clade 2 (24.5 to 99.8% identity) and Clade 9 (60.2 to 99.8% identity). Most of the putative RDase As from the *Deltaproteobacteria* grouped together with other *Proteobacteria* and were separated into several clades (Clade 1, 4, 5, 6, 7 and 8). *Desulfoluna spongiiphila (Dlu. spongiiphila)* RDase A02299 shared the highest identity with RDase As in the *Chloroflexi* clade (Clade 3), while *Desulfobacula phenolica* RDase A6 shared an identity of 40% with the RDase As in the *Firmicutes* clade (Clade 9). RDase A07176 from *Dlu. spongiiphila* and RDase As from *Geobacter lovleyi* SZ grouped within *Firmicutes* Clade 2.

Reductive dehalogenase gene clusters in *Deltaproteobacteria*

The reductive dehalogenase gene clusters of *Deltaproteobacteria* were explored for their common features (Fig. 5.1B). The conserved arginine translocation (Tat) signal was found in the N terminal region 16 of 35 putative RDase A genes. The lack of the Tat motif indicates that these RDase As may be cytoplasmic rather than periplasmic (Palmer and Berks, 2012). The RDase A gene is usually associated with RDase B, that encodes for a putative membrane anchoring protein. In total, 26 of 35 *Deltaproteobacteria* RDase A genes have a RDase B gene in close association, indicating that the encoded reductive dehalogenase is likely a membrane associated protein. The RDase B gene is located downstream of the RDase A gene with two exceptions, *Desulfovibrio bizertensis* RDase A3 gene and *Desulfobacula phenolica* RDase A6 gene which have RDase B upstream. Genes encoding for sigma factor (σ)-54-dependent transcriptional activators (also called bacterial enhancer-binding protein, bEBP) were frequently found to be present near the *Deltaproteobacteria* RDase gene clusters (Fig. 5.1B), unlike what has been reported for *Dehalococcoides* and *Dehalobacter*. In total, 22 bEBP genes were found in 31 RDase gene clusters. These findings indicate that the transcription of these RDase gene operons in the *Deltaproteobacteria* may be σ^{54}-dependent rather than σ^{70}-dependent. In contrast to the σ^{70} holoenzyme ($E\sigma^{70}$) that recognizes and binds to conserved -10 and -35 promoter elements, $E\sigma^{54}$ binds to -12 and -24 conserved elements (YTGGCACGrNNNTTGCW) and the initiation of transcription requires the assistance of bEBPs (Bush and Dixon, 2012). The potential $E\sigma^{54}$ binding sites were identified in

these RDase gene operon promoter regions (Fig. 5.2), providing further evidence that the transcription of these RDase gene operons in *Deltaproteobacteria* may be σ^{54}-dependent.

Certain RDase gene clusters were found to be conserved in some *Deltaproteobacteria* species. The three RDase As of *Desulfobacula phenolica* are not only nearly identical (>99% identity) to those of *Desulfobacula toluolica*, but are also highly similar in gene cluster components. Moreover, the RDase gene clusters in Clade 5 have similar components with high RDase A gene identity (>68%) as shown in Fig. 5.1C. Although from different species, all these gene clusters contain a σ^{54}-dependent transcriptional regulatory gene, a tetratricopeptide repeat containing protein gene and reductive dehalogenase genes. These similarities in operon components and sequence may indicate that these gene clusters have been obtained through horizontal gene transfer. The expression of genes in this clade was studied in further detail.

Phylogeny of RDase A-containing *Deltaproteobacteria* isolates

Six *Deltaproteobacteria* genera have previously been reported to contain members with dehalogenating ability, namely *Anaeromyxobacter*, *Geobacter*, *Desulfomonas*, *Desulfomonile*, *Desulfoluna* and *Desulfovibrio* (Adrian and Löffler, 2016). Of these organohalide-respiring *Deltaproteobacteria*, six strains have available genomes in JGI and are included in our survey. The remaining 13 RDase A-containing

Deltaproteobacteria species cover 11 genera, 10 of which have never been reported to contain organohalide-respiring members (Fig. 5.4 and Table S5.3).

Reductive dehalogenation properties of selected *Deltaproteobacteria*

Three *Deltaproteobacteria* species, *Desulfovibrio bizertensis* (*Dvi. bizertensis*), *Halodesulfovibrio marinisediminis* (*H. marinisediminis*) and *Desulfuromusa kysingii* (*Dur. kysingii*), were chosen for examination of their dehalogenating activity because: 1) these species represent diverse genera and have been isolated from the marine environment but from different geographic locations; and 2) they all contain a complete structurally similar RDase gene cluster with RDase accessory genes but variable in sequence (Clade 5). None of these species were previously reported to show dehalogenating activity (Gribble, 2000; Lincoln et al., 2005; Sim et al., 2009).

The physiological properties reported in the original species descriptions, the genome information, protein yield and their dehalogenating activity tested in this study are summarized in Table 5.1. *Dvi. bizertensis* and *H. marinisediminis* are sulfate reducing bacteria that can grow with lactate as the carbon source. *Dur. kysingii* is a sulfur reducing bacterium, and is phylogenetically and physiologically distinct from *Dvi. bizertensis* and *H. marinisediminis*. Their genomes are between 3-4 Mbp, which is smaller than *Dlu. spongiiphila* AA1.

Bromophenolic compounds are widespread in marine environments from natural sources as well as anthropogenic input (Gribble, 2000; Lincoln et al., 2005; Lira et al., 2011; Agarwal et al., 2017) and debromination has previously been

demonstrated in marine and estuarine sediment microcosms (Häggblom and Young, 1995; Monserrate and Häggblom, 1997) as well as isolates (Boyle et al., 1999; Ahn et al., 2009). We therefore tested the dehalogenating abilities of the three selected marine *Deltaproteobacteria* species using 2,4,6-tribromophenol (2,4,6-TBP), 2,6-bromophenol (2,6-DBP), 2-bromophenol (2-BP) as electron acceptors (Table 5.1). The dechlorinating ability was also tested with 2,6-dichlorophenol (2,6-DCP). All three strains were able to debrominate 2,6-DBP and 2-BP to phenol, but they did not dechlorinate 2,6-DCP. This specificity to bromophenol rather than chlorophenol is similar to what was shown for *Dlu. spongiiphila* (Ahn et al., 2009; Liu et al., 2017). *Dvi. bizertensis* and *H. marinisediminis* are also capable of debrominating 2,4,6-TBP to 2,4-DBP and 4-bromophenol (4-BP). However, there was no further debromination of 4-BP to phenol even after extended incubation (Data not shown). *Dur. kysingii* showed no 2,4,6-TBP debrominating activity over 18 days. In contrast, *Dlu, spongiiphila* strain AA1 was able to completely dehalogenate 2,4,6-TBP to 2,4-DBP, 4-BP and phenol. The growth of *Dvi. bizertensis*, *H. marinisediminis* and *Dur. kysingii* strains could be supported by lactate with 2,6-DBP as the sole electron acceptor. Lactate alone does not support the growth of these strains as no protein yield was detectable after 24 days of incubation. In contrast, the protein yields of three strains grown on lactate and 2,6-DBP was between 0.54 to 0.80 mg per mmol electron utilized, with 2,6-DBP debrominated with the stoichiometric accumulation of phenol (Table 5.1 and Fig. S5.2).

We additionally examined the debrominating activity of the three strains under different growth conditions using 2,6-DBP as the electron acceptor (Table 5.1).

The debromination rate of *Dvi. bizertensis* and *H. marinisediminis* cultures was identical with or without exogenous cobamide (Vitamin B12, cyanocobalamin) (Fig. S5.3). However, the debrominating activity of *Dur. kysingii* was dependent on a supply of exogenous cobamide. Compared to the debrominating activity under cobamide-rich conditions (50 μg/l), the debrominating activity in the absence of cobamide was minimal over 80 h, indicating that exogenous cobamide is necessary for dehalogenation by *Dur. kysingii*. However, these results are not necessarily consistent with the genome annotations (Table S5.4). Although the debromination of *Dur. kysingii* requires exogenous cobamide, *Dur. kysingii* possesses a nearly complete cobamide biosynthesis pathway as was also the case for *Dvi. bizertensis* and *H. marinisediminis*. However, the cobamide synthesis pathway in *Dur. kysingii* is apparently not fully functional.

The presence of sulfate did not significantly influence the debromination rate of *H. marinisediminis* (Fig. S5.3A). For *Dvi. bizertensis*, sulfate did not affect the rate of 2,6-DBP debromination, but the rate of phenol formation in the culture with sulfate was more rapid, indicating that the subsequent debromination of 2-BP to phenol was stimulated by sulfate (Fig. S5.3B). This stimulation may be attributed to the biomass increase supported by sulfate reduction over a longer incubation time. For *Dur. kysingii*, elemental sulfur did not affect the 2,6-DBP debromination rate when cobamide was provided in the culture (Fig. S5.3C). These results indicate that the debrominating activity of the tested sulfate-reducing and sulfur-reducing strains are not inhibited by the presence of other available electron acceptors.

Expression of reductive dehalogenase genes induced by 2,6-DBP in selected *Deltaproteobacteria*

To study whether the expression of the RDase A gene is inducible, streptomycin was applied to sulfate/sulfur-grown cultures as a protein synthesis inhibitor and then spiked with 2,6-DBP (Fig. 5.3). The debrominating activity of *H. marinisediminis* and *Dur. kysingii* was almost completely inhibited by streptomycin, indicating that the expression of the RDase A gene in these two strains is downregulated during growth with sulfate or sulfur, respectively, as the terminal electron acceptor. In the *Dvi. bizertensis* culture, 2,6-DBP was debrominated even in the presence of streptomycin, but at a lower rate compared with the control. This indicates that at least some upregulation of reductive dehalogenase enzymes when 2,6-DBP was amended to the culture.

The genome of *H. marinisediminis* contains one putative RDase A gene, whose expression was upregulated immediately after addition of 2,6-DBP (Fig. 5.3A). The expression level increased 50-fold in the first half hour prior to detection of debromination activity. Along with debromination activity, the expression level of this RDase A gene increased up to 400-fold and after 30 h then returned to the same level as the non-amended control.

The two RDase A genes of *Dur. kysingii* are located on different scaffolds of the (draft) genome and are phylogenetically distinct. The expression of the RDase A5 gene showed no difference between the control and the 2,6-DBP amended culture, indicating that the RDase A5 gene was not induced by 2,6-DBP (Fig. 5.3B). In contrast,

the expression of the RDase A 10 gene was induced by 2,6-DBP and the expression level increased up to 400-fold.

Dvi. bizertensis has four RDase A genes in its genome, which responded differently to 2,6-DBP (Fig 5.3C). The genes RDase A2-1 and RDase A2-2 are located in close vicinity of each other in the genome and share 70% identity. The upregulation of RDase A2-1 and RDase A2-2 genes, to 5 and 20-fold respectively, was detected from the onset of debromination and until 9 h of incubation. Then the expression of these two RDase A genes decreased to background levels at 13 h. The expression of RDase A18 gene was upregulated significantly ($p < 0.05$), over 600-fold, indicating that the dehalogenase encoded by the RDase A18 gene may be the major one responsible for 2,6-DBP debromination. The expression of RDase A18 gene significantly decreased to 5-fold at 13 h, although there was still around 11 μM 2-BP remaining in the culture. The concentration of remaining 2-BP in culture may be not sufficient to maintain the expression of RDase A18 gene at a high level. The expression of RDase A3 gene in *Dvi. bizertensis* was not induced by 2,6-DBP. Interestingly, the RDase A3 gene cluster has a different gene order from most other RDase gene clusters, in that its RDase B gene is located upstream of RDase A gene. Whether this might affect the functionality of the RDase A3 gene in organohalide respiration is not known.

Discussion

Since the first OHRB, *Desulfomonile tiedjei* DCB-1, was named in 1990 (DeWeerd et al., 1990), several others have been isolated from diverse environments

(Adrian and Löffler, 2016). To date, approximately 20 organohalide-respiring *Deltaproteobacteria* isolates have been identified with the ability to dehalogenate chlorophenols, bromophenols and/or chlorinated ethenes (Fig. 5.4). Our genomic survey of *Deltaproteobacteria* expanded to 556 genomes confirms and extends the earlier analysis (Sanford et al., 2016), indicating that a diverse group of *Deltaproteobacteria* have potential reductive dehalogenating ability encoded in their genomes. Of the RDaseA-containing *Deltaproteobacteria* species identified in our survey, 12 of 19 are from marine environments (Fig. 5.4). As a major source and reservoir of natural organohalides, the marine environment appears to select for abundant and diverse organohalide-respiring microorganisms and a suite of RDase A genes (for reviews, see (Zanaroli et al., 2015; Atashgahi et al., 2018)). Although *Deltaproteobacteria* are frequently found in marine dehalogenating enrichment cultures, their function in the marine halogen cycle has been overlooked (14). Of the *Deltaproteobacteria* isolates with genomes in JGI, approximately 20% were from geographically diverse marine environments (50 of 255), and one fourth of these marine *Deltaproteobacteria* contain one or more RDase A genes in their genomes (12 of 50), indicating that these are particularly prevalent in marine *Deltaproteobacteria*.

The application of metagenomics allows us to evaluate the dehalogenating potential in environments by screening for putative RDase A3 genes and eliminating the time-consuming and often difficult pure culture isolation process (Kawai et al., 2014; Yang et al., 2017). However, pure cultures of OHRBs are of significance in order to determine their physiological features and correlate with dehalogenating activity observed in environmental samples. Instead of isolating OHRBs from the

environmental matrix, which is a challenge because of their low growth rates on organohalides and sensitivity to oxygen, the existing genome database can also serve as a guide for screening and identifying new OHRBs. An arginine translocation (Tat) signal motif (RRXFXK) and two iron-sulfur cluster binding motifs (CXXCXXCXXXCP and CXXCXXXCP motifs) are commonly conserved in RDase A genes (Maillard et al., 2003; Maphosa et al., 2010). Even though annotated as putative RDase A genes, their sequence identity can be lower than 10% due to the sequence diversity. The annotation accuracy of the putative RDase A genes with low identity to other known RDase A geness can be questionable and misleading for predicting RDase A gene functionality. In this case, the RDase gene cluster composition and presence of accessory genes such as an RDase B gene and potential regulatory genes should be used as additional indicators to predict the dehalogenating functionality of the bacteria.

Three *Deltaproteobacteria* were selected as promising OHRBs for more detailed study because they contain complete RDase gene clusters similar to the known functional *Dlu. spongiiphila* RDase16032 (Liu et al., 2017). These three *Deltaproteobacteria* were not originally isolated for their dehalogenating activity (Haouari et al., 2006; Takii et al., 2008). *H. marinisediminis* shares high phylogenetic similarity to *Desulfovibrio* sp. strain TBP-1, which is a bromophenol respiring bacterium isolated from estuarine sediment (Boyle et al., 1999). *H. marinisediminis* was first reported to have no debrominating activity on 2,4,6-TBP (Takii et al., 2008), which is contradictory to our results. The negative result from the original report may be due to different growth or medium conditions. *Dur. kysingii* is an elemental sulfur

reducing bacterium isolated from marine sediment (Liesack and Finster, 1994), but has not been previously reported to have dehalogenating ability. However, in a study of 2,4,6-TBP and 2,4,6-triiodophenol (2,4,6-TIP) dehalogenating sediments, bacteria belonging to *Desulfuromusa* were enriched and became dominant members of the bacterial community compared to control sediments (Futagami et al., 2013), suggesting their role in dehalogenation. Although the *Deltaproteobacteria* tested in this study are from different geographic locations, their common RDase gene cluster organization and similar expression profiles during debromination of 2,6-DBP indicates that this RDase gene cluster appears to be conserved in some *Deltaproteobacteria* and may be important for their metabolism.

The majority of RDase As identified in the *Deltaproteobacteria* are distinct from previously functionally characterized RDase As, which are mainly dechlorinases from the genera *Desulfitobacterium*, *Dehalococcoides* and *Dehalobacter* (Fig. 5.1). Also, most organohalide-respiring *Deltaproteobacteria* were previously studied for their dechlorinating abilities (Fig. 5.4). Brominated compounds however are widespread especially in marine environment (Gribble, 2010). *Anaeromyxobacter* strains and *Desulfomonile limimaris* DCB-M were found to dehalogenate brominated aromatics (Sun et al., 2001; Sanford et al., 2002). *Dlu. spongiiphila* strain AA1 and *Desulfovibrio* sp. TBP-1 are able to dehalogenate a variety of brominated compounds rather than chlorinated compounds (Boyle et al., 1999; Ahn et al., 2009). The three marine *Deltaproteobacteria* tested here are functional for brominated rather than chlorinated phenols.

The analysis of the RDase gene clusters in *Deltaproteobacteria* genomes revealed features different from those in *Dehalococcoides* and *Desulfitobacterium* species. The regulatory mechanisms of reductive dehalogenation have been reported for *Dehalobacter*, *Desulfitobacterium* and *Dehalcoccoides* strains. The *rdhK* genes encoding for CRP/FNR transcriptional regulators are commonly present in *Desulfitobacterium* and *Dehalobacter* RDase gene clusters, and appear to function as transcriptional activators (Futagami et al., 2006; Gabor, 2006; Gábor et al., 2008; Kemp et al., 2013). In contrast, a *marR* regulatory gene in *Dehalococcoides mccartyi* is found to be associated with RDase gene clusters and acts as a negative regulator (Wagner et al., 2013; Krasper et al., 2016). Except for *Dvi. bizertensis* RDase3 with a *marR* gene, neither *CRP/FNR* nor *marR* genes were found in the RDase gene clusters of *Deltaproteobacteria*. Our previous analysis of *Dlu. spongiiphila* revealed the presence of σ^{54}-dependent activator (bEBPs) near the two 2,6-DBP induced RDase gene cluster (Liu et al., 2017). Similar bEBPs were also found close to the RDase A gene either directly next to or in the vicinity of the RDase As in most of the *Deltaproteobacteria* RDase gene clusters. The prevalence of bEBPs near the RDase gene promoter region in *Deltaproteobacteria* suggests that *Deltaproteobacteria* RDase gene operons may be regulated by a mechanism different from the *marR* or CPR/FNR systems. Sigma factor 54 initiated transcription is widespread in bacteria (60% of bacterial genomes) to coordinate many metabolic processes (Francke et al., 2011). Anaerobic *Deltaproteobacteria* isolated from soil and aquatic habitats contain the highest relative number of bEBPs (normalized by genome size) among all sequenced bacterial species (Kazakov et al., 2015). In the genomes of RDase A-

containing *Deltaproteobacteria*, the number of bEBPs are in the range of 27 to 88, indicating the prevalence of σ^{54}-mediated transcription (Table S5.1). There are few to no bEBPs in the genomes of dehalogenating *Dehalococcoides* and *Dehalobacter* spp., while none of the identified bEBPs in *Desulfitobacterium* spp. were close to the RDase gene clusters, suggesting that bEBP associated RDase genes are unique to the *Deltaproteobacteria*. Whether the RDase gene operon transcription is σ^{54} initiated and organohalides are substrates for these bEBPs in modulating the expression warrants further investigation.

Most characterized reductive dehalogenases contain a corrinoid co-factor, which is essential for reductive dehalogenation (Adrian and Löffler, 2016; Fincker and Spormann, 2017). The crystal structure of the RDase from *Sulfurospirillum multivorans* and *Nitratireductor pacificus* pht-3B reveals the involvement of a corrinoid cofactor in the active center (Bommer et al., 2014; Payne et al., 2015). OHRBs that are not capable of *de novo* cobamide synthesis need to obtain the cobamide through exogenous sources (Loffler et al., 2013; Rupakula et al., 2015). For example, *Dehalococcoides* strains which are not able to biosynthesize cobamide *de novo* were found to utilize the cobamide produced by other species grown in co-culture, such as *Geobacter lovleyi*, which is also an organohalide-respiring *Deltaproteobacteria* (Yan et al., 2012; Men et al., 2013; Yan et al., 2013). Two of our tested *Deltaproteobacteria* strains do not require exogenous cobamide for debrominating activity, indicating that they may be able to synthesize cobamide *de novo*. Although most of the needed cobamide biosynthesis genes were found in the genomes of the tested dehalogenating *Deltaproteobacteria*, it does not necessarily

indicate a functional cobamide biosynthesis pathway. A truncation of even a single gene involved in cobamide biosynthesis could result in a loss of cobamide biosynthesis ability (Rupakula et al., 2015). Similarly, the genome of *Dur. kysingii* contains a near complete cobamide biosynthesis pathway, however its debrominating activity is dependent on exogenous cobamide supplementation.

Sulfate is abundant in marine and estuarine environments, which can be utilized as electron acceptor with production of sulfite and hydrogen sulfide. Sulfate has been found to inhibit reductive dehalogenation in enrichment cultures due to the competition between sulfate reducing and dehalogenating bacteria (Aulenta et al., 2008; Heimann et al., 2005). Sulfide produced from sulfate reduction inhibited the growth and reductive dehalogenation of *Dehalococcoides mccartyi* 195 (Mao et al., 2017), while sulfite and thiosulfate negatively influenced the dehalogenation of *Desulfomonile tiedjei* in both culture and cell extracts (DeWeerd et al., 1991; Townsend et al., 1997). Our results indicated that the debrominating activity of the three tested marine *Deltaproteobacteria* was not inhibited by sulfate or sulfur. These findings are consistent with previous studies showing that the dehalogenating activities of sulfate-reducing OHRBs, *Desulfovibrio* sp. TBP-1, *Desulfomonile liminaris* DCB-M and *Desulfoluna spongiiphila* AA1 of marine/estuarine origin were not inhibited by sulfate (Boyle et al., 1999; Sun et al., 2001; Ahn et al., 2009). In our survey, 11 of 12 RDase A-containing *Deltaproteobacteria* of marine origin are sulfate or sulfur-reducing bacteria. Since the marine environment is a major reservoir for sulfate, sulfur and organohalides, these OHRBs would be able to take advantage of

both sulfate/sulfur reduction and respiratory dehalogenation in their natural habitats for growth.

Conclusions

In order to link the dehalogenating activity to functional bacterial species, it is necessary to have a better understanding of OHRB diversity. As a result of the exploration of available bacterial genomes for RDase A genes, their presence in a wide range of *Deltaproteobacteria* was confirmed. The marine environment is the greatest reservoir of organohalides from both anthropogenic and natural sources and metagenomic analysis of marine sediment samples has uncovered abundant reductive dehalogenases (Kawai et al., 2014; Zinke et al., 2017; Marshall et al., 2018). Three selected *Deltaproteobacteria* were confirmed to grow by organohalide respiration and examination of their features extend our knowledge about OHRBs and RDase A gene diversity. *Deltaproteobacteria* are widespread in the environment and are involved in diverse global chemical process, e.g., the sulfur and carbon cycles. The presence of RDase A genes in diverse *Deltaproteobacteria*, especially in those of marine origin and the confirmation of their dehalogenating activity implies they may play an important role in organohalide respiration and the cycling of organohalides in the marine environment.

Materials and Methods

Survey of putative RDase A genes in *Proteobacteria* genomes

The Joint Genome Institute Integrated Microbial Genomes & Microbiome Samples (JGI-IMG/MER) database was used to survey the occurrence of putative RDase A genes in genomes of annotated *Deltaproteobacteria*. Genes annotated as "pfam13486", contain the putative reductive dehalogenase subunit domain, was used as the "Pfam Domain Search (list)*" filter to search for RDase A genes in the database of "All Finished, Permanent Draft and Draft" *Deltaproteobacteria* genomes through the JGI "Find Genes" function. In total, 556 *Deltaproteobacteria* genomes were chosen, from which 80 putative reductive dehalogenases were found (May, 2017). These putative RDase A genes hits were from 50 samples that include genomes of bacterial isolates, metagenomic sequences and single cell genome sequences. In a similar way, the other *Proteobacteria* Classes, and the Chlorofexi and Firmicutes were also surveyed for the presence of putative reductive dehalogenase genes.

The amino acid sequences encoded by RDase A genes from *Deltaproteobacteria* isolates together with functionally characterized RDase As whose sequences were obtained from Uniprot were used to construct phylogenetic trees. The genes surrounding these RDase A genes were also examined to explore the potential regulatory genes. The amino acid sequence alignment of RDase A and the alignment of 16S rRNA gene sequences were performed using Clustal W alignment with Cost Matrix Gonnet in MEGA 7 (Kumar et al., 2016).

Identification of sigma factor 54-related genes and binding sites

The genomes of the selected *Deltaproteobacteria* were downloaded from JGI-IMG/EMR with annotations. The surrounding RDase gene regions were examined for potential MarR and sigma factor-54 dependent transcriptional regulators. For the RDase genes with a sigma factor 54-dependent transcriptional regulator, the binding site was searched in the promoter regions for the YTGGCACGRNNNTTGC motif in Geneious. Obtained potential binding sites were further manually examined.

Bacterial strains and growth conditions

Desulfovibrio bizertensis DSM18034, *Halodesulfovibrio marinisediminis* DSM17456 and *Desulfuromusa kysingii* DSM7343 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) in freeze-dried form. The cultures were revived by inoculating into anaerobic medium under oxygen-free condition and incubated at 27 ^{o}C under a headspace of N_2 until visible growth was observed. The anaerobic growth medium contained minimal salts, reductant (0.5 g/L sodium sulfide nonahydrate) and 25 g/L NaCl as described previously (Liu et al., 2017). For *Dvi. bizertensis* and *H. marinisediminis*, 30 mM lactate was utilized as carbon source and electron donor with 20 mM sulfate as electron acceptor. For *Dur. kysingii,* 10 mM fumarate was added to support growth with 1 g/L elemental sulfur as electron acceptor. The growth of the cultures occurred at either room temperature or 27 ^{o}C. Elemental sulfur was prepared using fine, homogeneous

sulfur powered in anaerobic medium. The sulfur was not dissolved but the slurry was thoroughly shaken before addition to the growth medium.

The dehalogenating activity of tested strains under different conditions

The *Deltaproteobacteria* strains were initially pre-grown on lactate and sulfate/sulfur in anaerobic medium containing 50 µg/l of cobamide (in form of vitamin B12). In order to remove cobamide or sulfate from the cultures, 30 ml of *Dvi. bizertensis* and *H. marinisediminis* culture pre-grown on sulfate and lactate were centrifuged for 5 min at 8,000 x g to collect the cell pellets, these were washed twice and resuspended into cobamide-free medium. In order to reduce cobamide and sulfur in the *Dur. kysingii* culture to a low level, successive transfers were made into cobamide-free and sulfur-free medium. These cultures were then used to set up the experiments to test for the influence of cobamide and an additional electron acceptor (sulfate or sulfur) on dehalogenating activity.

Dvi. bizertensis, H. marinisediminis and *Dur. kysingii* cells were inoculated into medium containing lactate and 2,6-DBP. The consumption of 2,6-DBP and production of 2-BP and phenol were used as indicator of viability. When 2,6-DBP was depleted, another 200 µM of 2,6-DBP was refed to support growth. The cultures were transferred into fresh medium after 5 refeedings of 2,6-DBP for a total of three culture transfers. To prove that the reductive dehalogenation supports the culture growth as a respiratory process, 10 ml of these stock cultures after depletion of 2,6-DBP were inoculated into 90 ml medium containing 5 mM lactate with or without 2,6-DBP as

electron acceptor (370 μM). The culture with lactate and 2,6-DBP were refed twice with 2,6-DBP. After the second refeeding, 40 ml of culture was centrifuged for 15 min at 10,000 x g for protein analysis as described previously with modifications (Boyle et al., 1999). Briefly, the collected culture was rinsed with phosphate-buffered saline (PBS, pH 7.4) to remove any medium residue. The samples were resuspended with 0.5 ml PBS and 0.5 ml 2N NaOH and incubated at 70 ^{o}C for 45 min. Then, samples were centrifuged at 13,000 x g for 3 min. Supernatants were neutralized with HCl and measured using the Quick Start™ Bradford protein assay (Biorad Laboratories, Inc.). Biological culture duplicates for lactate only controls and biological culture triplicates for lactate with 2,6-DBP were analyzed. Triplicates assays were done for each sample to determine the protein concentration.

To test for dehalogenating activity, 2 ml of washed *Dvi. bizertensis* or *H. marinisediminis* culture was inoculated into 18 ml of cobamide-free anaerobic medium containing 2 mM lactate and about 200 μM 2,6-dibromophenol, 2,4,6-tribromophenol or 2,6-dichlorophenol with 50 g/L cobamide. For *Dur. kysingii*, 4 ml of culture was inoculated into 16 ml of cobamide-free anaerobic medium containing 2 mM fumarate and about 200 μM 2,6-DBP, 2,4,6-TBP or 2,6-DCP with 50 g/L cobamide.

To test the influence of cobamide and other electron acceptors, three treatments were conducted in biological triplicates for each species. The control treatment contained no additional cobamide, one treatment contained 50 μg/l cobamide and the third treatment contained 20 mM sulfate or 2 mM sulfur slurry

together with cobamide. Samples were taken periodically and measured by HPLC to determine the concentration of organohalides and their dehalogenation products.

Induction and expression of RDase A genes

Cultures pre-grown in 2,6-DBP free medium were inoculated into 20-50 ml fresh anaerobic medium containing 1 mM lactate for *Dvi. bizertensis* and *H. marinisediminis* as electron donor and 1 mM fumarate for *Dur. kysingii*. One treatment was amended with 200 μM 2,6-DBP to induce the expression of RDase A genes, while 1 mg/ml streptomycin was added to the other 2,6-DBP amended treatment to inhibit protein synthesis. The control treatment contained an equivalent concentration of sulfate or sulfur (200 μM) as electron acceptor. In total, triplicate treatments for each species were incubated at 27 ^{o}C. The cultures were sampled periodically for 2,6-DBP concentration analysis and RNA extraction.

RNA extraction, reverse transcription and qPCR

Total RNA was extracted from 4 ml of culture samples using TRIzol (Ambion, Life Technologies) reagent according to the manufacturer's instructions. The obtained RNA was treated as described previously or the downstream applications (Liu et al., 2017). DNA-free™ DNA removal kit (Ambion, Life Technologies) was applied to remove gDNA contamination in RNA before reverse transcription. 1 μl of RNA was used to synthesize cDNA in a 10 μl reaction by using the iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Inc.). The obtained cDNA was

amplified using an IQ™ SYBR Green Supermix (Bio-Rad) in a 10 µl reaction on a iCycler Real-Time PCR Detection System (Bio-Rad). The design and specificity examination of primers used for RT-qPCR were performed in Geneious Primer 3 (Table S5.5). Thermal cycling conditions for RT-qPCR were as described previously (Liu et al., 2017).

The expression levels of the RDase A genes were calculated using a relative standard curve method. A serial dilution of gDNA of each strain was made to generate the standard curve for each gene. The transcription levels of each RDase A gene was normalized to the 16S rRNA gene. To obtain the relative expression levels the normalized expression in 2,6-DBP induced cultures was divided by normalized expression in control culture.

Analytical methods

The concentration of 2,6-DBP, 2,6-DCP, 2-BP, 2,4,6-TBP, 2,4-DBP, 4-BP and phenol was measured by HPLC using a Sphereclone C-18 column (250 mm × 4.6 mm, particle size 5 µm; Phenomenex) on a Shimadzu system as described previously (Liu et al., 2017). The mobile phase consisted of methanol: water: acetic acid (vol: vol: vol, 70: 29: 1) was used at an isocratic flow rate of 1 ml/min with UV absorbance detection at 280 nm.

Acknowledgements

The work was supported in part by the USDA National Institute of Food and Agriculture Hatch project accession number 1012785 through the New Jersey Agricultural Experiment Station (Hatch Project NJ01160).

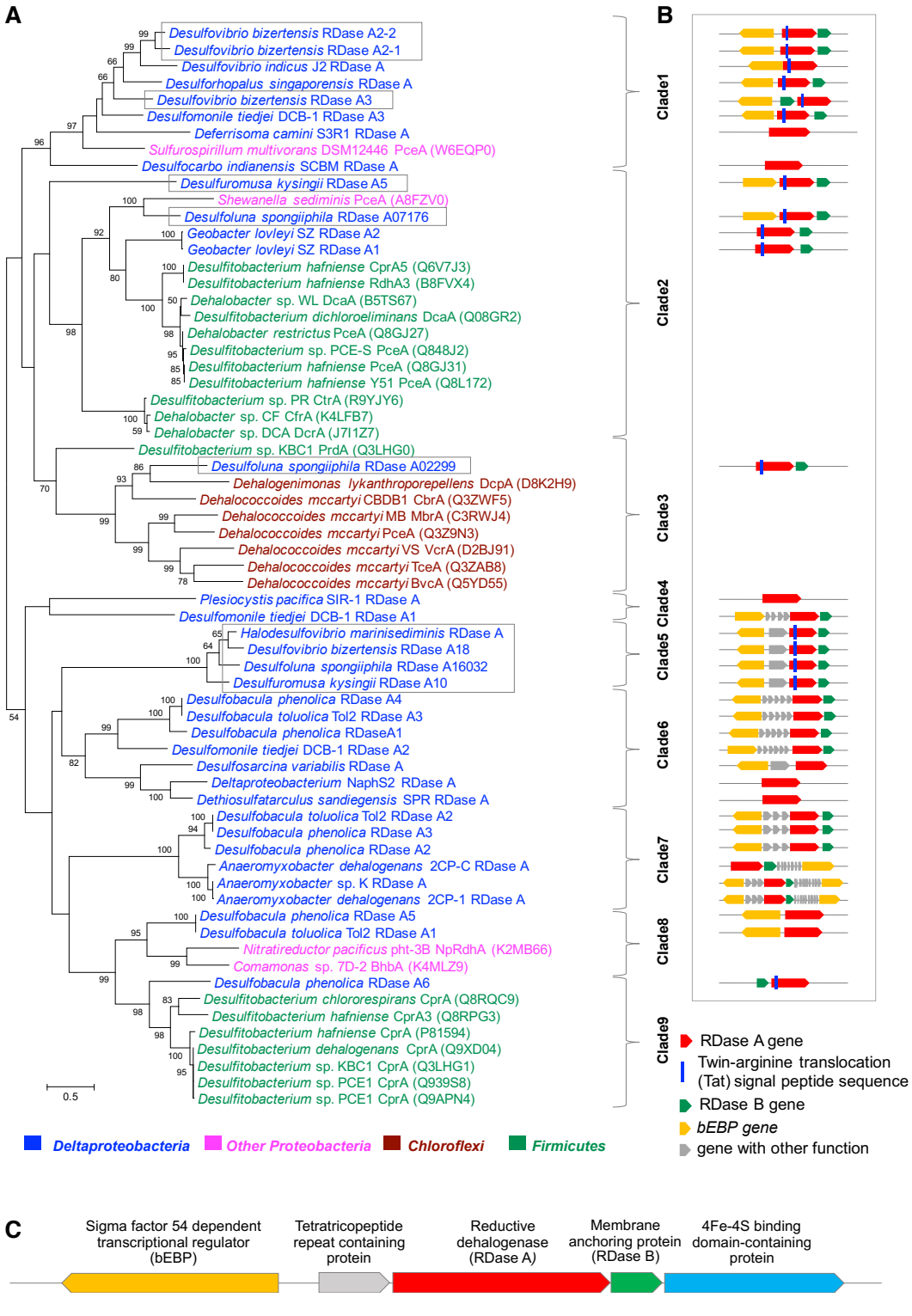

A
Desulfovibrio bizertensis RDase A2-2
Desulfovibrio bizertensis RDase A2-1
Desulfovibrio indicus J2 RDase A
Desulforhopalus singaporensis RDase A
Desulfovibrio bizertensis RDase A3
Desulfomonile tiedjei DCB-1 RDase A3
Deferrisoma camini S3R1 RDase A
Sulfurospirillum multivorans DSM12446 PceA (W6EQP0)
Desulfocarbo indianensis SCBM RDase A
Desulfuromusa kysingii RDase A5
Shewanella sediminis PceA (A8FZV0)
Desulfoluna spongiiphila RDase A07176
Geobacter lovleyi SZ RDase A2
Geobacter lovleyi SZ RDase A1
Desulfitobacterium hafniense CprA5 (Q6V7J3)
Desulfitobacterium hafniense RdhA3 (B8FVX4)
Dehalobacter sp. WL DcaA (B5TS67)
Desulfitobacterium dichloroeliminans DcaA (Q08GR2)
Dehalobacter restrictus PceA (Q8GJ27)
Desulfitobacterium sp. PCE-S PceA (Q848J2)
Desulfitobacterium hafniense PceA (Q8GJ31)
Desulfitobacterium hafniense Y51 PceA (Q8L172)
Desulfitobacterium sp. PR CtrA (R9YJY6)
Dehalobacter sp. CF CfrA (K4LFB7)
Dehalobacter sp. DCA DcrA (J7I1Z7)
Desulfitobacterium sp. KBC1 PrdA (Q3LHG0)
Desulfoluna spongiiphila RDase A02299
Dehalogenimonas lykanthroporepellens DcpA (D8K2H9)
Dehalococcoides mccartyi CBDB1 CbrA (Q3ZWF5)
Dehalococcoides mccartyi MB MbrA (C3RWJ4)
Dehalococcoides mccartyi PceA (Q3Z9N3)
Dehalococcoides mccartyi VS VcrA (D2BJ91)
Dehalococcoides mccartyi TceA (Q3ZAB8)
Dehalococcoides mccartyi BvcA (Q5YD55)
Plesiocystis pacifica SIR-1 RDase A
Desulfomonile tiedjei DCB-1 RDase A1
Halodesulfovibrio marinisediminis RDase A
Desulfovibrio bizertensis RDase A18
Desulfoluna spongiiphila RDase A16032
Desulfuromusa kysingii RDase A10
Desulfobacula phenolica RDase A4
Desulfobacula toluolica Tol2 RDase A3
Desulfobacula phenolica RDaseA1
Desulfomonile tiedjei DCB-1 RDase A2
Desulfosarcina variabilis RDase A
Deltaproteobacterium NaphS2 RDase A
Dethiosulfatarculus sandiegensis SPR RDase A
Desulfobacula toluolica Tol2 RDase A2
Desulfobacula phenolica RDase A3
Desulfobacula phenolica RDase A2
Anaeromyxobacter dehalogenans 2CP-C RDase A
Anaeromyxobacter sp. K RDase A
Anaeromyxobacter dehalogenans 2CP-1 RDase A
Desulfobacula phenolica RDase A5
Desulfobacula toluolica Tol2 RDase A1
Nitratireductor pacificus pht-3B NpRdhA (K2MB66)
Comamonas sp. 7D-2 BhbA (K4MLZ9)
Desulfobacula phenolica RDase A6
Desulfitobacterium chlororespirans CprA (Q8RQC9)
Desulfitobacterium hafniense CprA3 (Q8RPG3)
Desulfitobacterium hafniense CprA (P81594)
Desulfitobacterium dehalogenans CprA (Q9XD04)
Desulfitobacterium sp. KBC1 CprA (Q3LHG1)
Desulfitobacterium sp. PCE1 CprA (Q939S8)
Desulfitobacterium sp. PCE1 CprA (Q9APN4)
0.5
Deltaproteobacteria
Other Proteobacteria
Chloroflexi
Firmicutes
B
Clade1
Clade2
Clade3
Clade4
Clade5
Clade6
Clade7
Clade8
Clade9
RDase A gene
Twin-arginine translocation (Tat) signal peptide sequence
RDase B gene
bEBP gene
gene with other function
C
Sigma factor 54 dependent transcriptional regulator (bEBP)
Tetratricopeptide repeat containing protein
Reductive dehalogenase (RDase A)
Membrane anchoring protein (RDase B)
4Fe-4S binding domain-containing protein

Figure 5.1 Phylogeny of putative RDase A amino acid sequences of 19 *Deltaproteobacteria* isolates and characterized reductive dehalogenases (A), the structure of *Deltaproteobacteria* reductive dehalogenase gene clusters (B) and detailed annotation of Clade 5 RDase gene clusters (C). Initial tree(s) for the heuristic search were obtained by applying Neighbor-Joining (NJ) and advanced NJ (BioNJ) algorithms to a matrix of pairwise distances estimated using a Jones–Thornton–Taylor (JTT) model, and then selecting the topology with superior log likelihood value. The Maximum Likelihood tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 65 amino acid sequences. All positions with less than 60% site coverage were eliminated, i.e., fewer than 40% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 489 aa positions in the final dataset. The RDase As are highlighted with different colors based on the phyla. The clades were designated based on the tree node rather than a fixed sequence cutoff. For the reductive dehalogenase gene cluster structure (B), the arrows indicate the gene functions and orientation. The size of those symbols are not true to real gene length. RDase A genes examined in more detail in this study and our previous study on *Dlu. spongiiphila* are highlighted in a box.

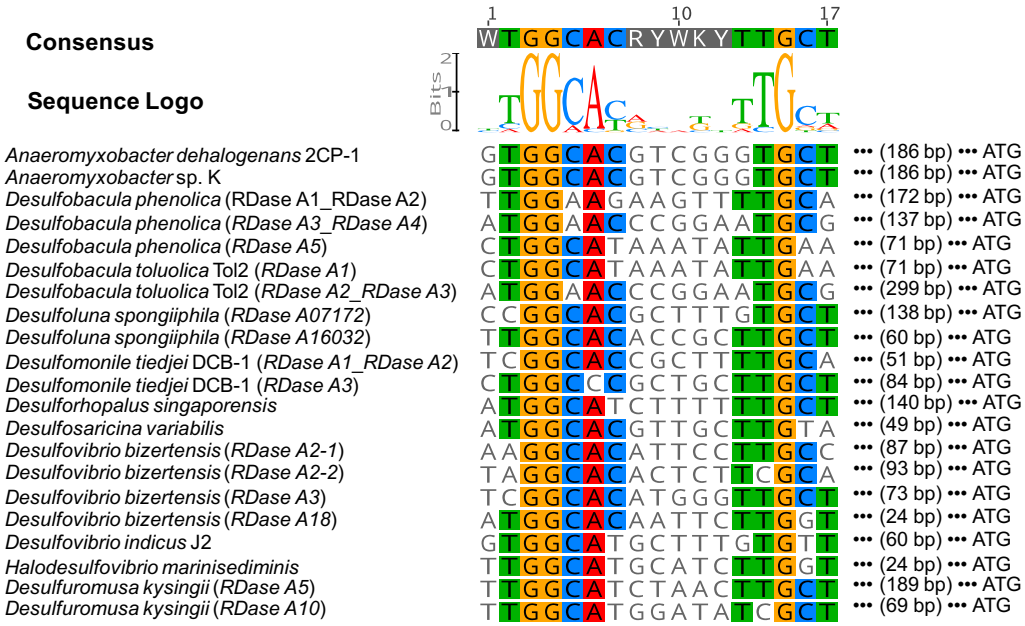


Figure 5.2 Potential sigma factor 54 holoenzyme binding sites in the promoter region of putative reductive dehalogenases with a sigma factor 54-dependent transcriptional activator gene nearby as shown in Figure 5.1. The potential binding sites were identified based on the binding motif in the potential promoter region.

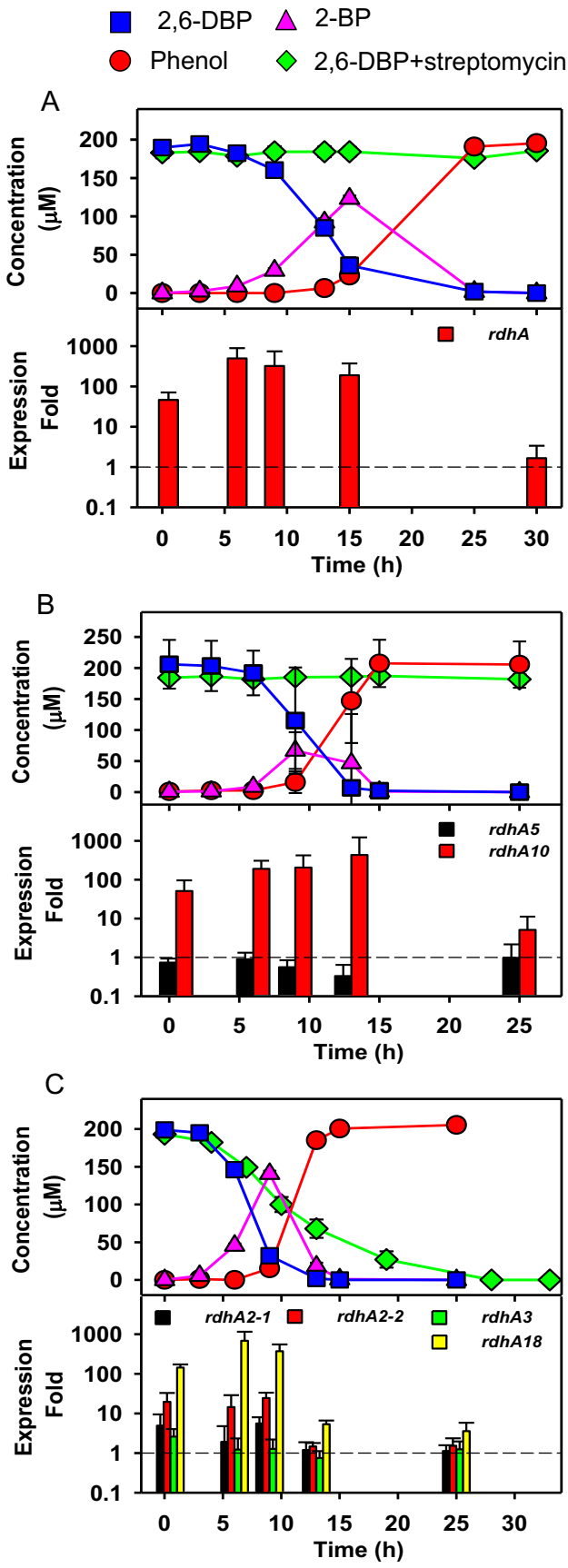

2,6-DBP
2-BP
Phenol
2,6-DBP+streptomycin
A
Concentration (μM)
Expression Fold
rdhA
Time (h)
B
rdhA5
rdhA10
C
rdhA2-1
rdhA2-2
rdhA3
rdhA18

Figure 5.3 Debromination activity in 2,6-dibromophenol induced cultures (upper panel) and the expression of RDaseA genes (lower panel) of *H. marinisediminis* (A), *Dur. kysingii* (B) and *Dvi. bizertensis* (C) over time. The upper panel for each strain shows the concentration of 2,6-DBP and its debromination products, 2-BP and phenol, without or with 1 mg/ml streptomycin. Error bars when larger than the symbols indicate the means and standard deviation of biological triplicates. The lower panel for each strain indicates the relative expression of RDaseA genes induced by 2,6-DBP, was normalized to the expression of the 16S rRNA gene. The y-axis indicates the expression fold of 2,6-DBP induced cultures compared to untreated controls. Error bars represent the standard deviation of three biological triplicates, each with RT-PCR reactions performed in duplicate.

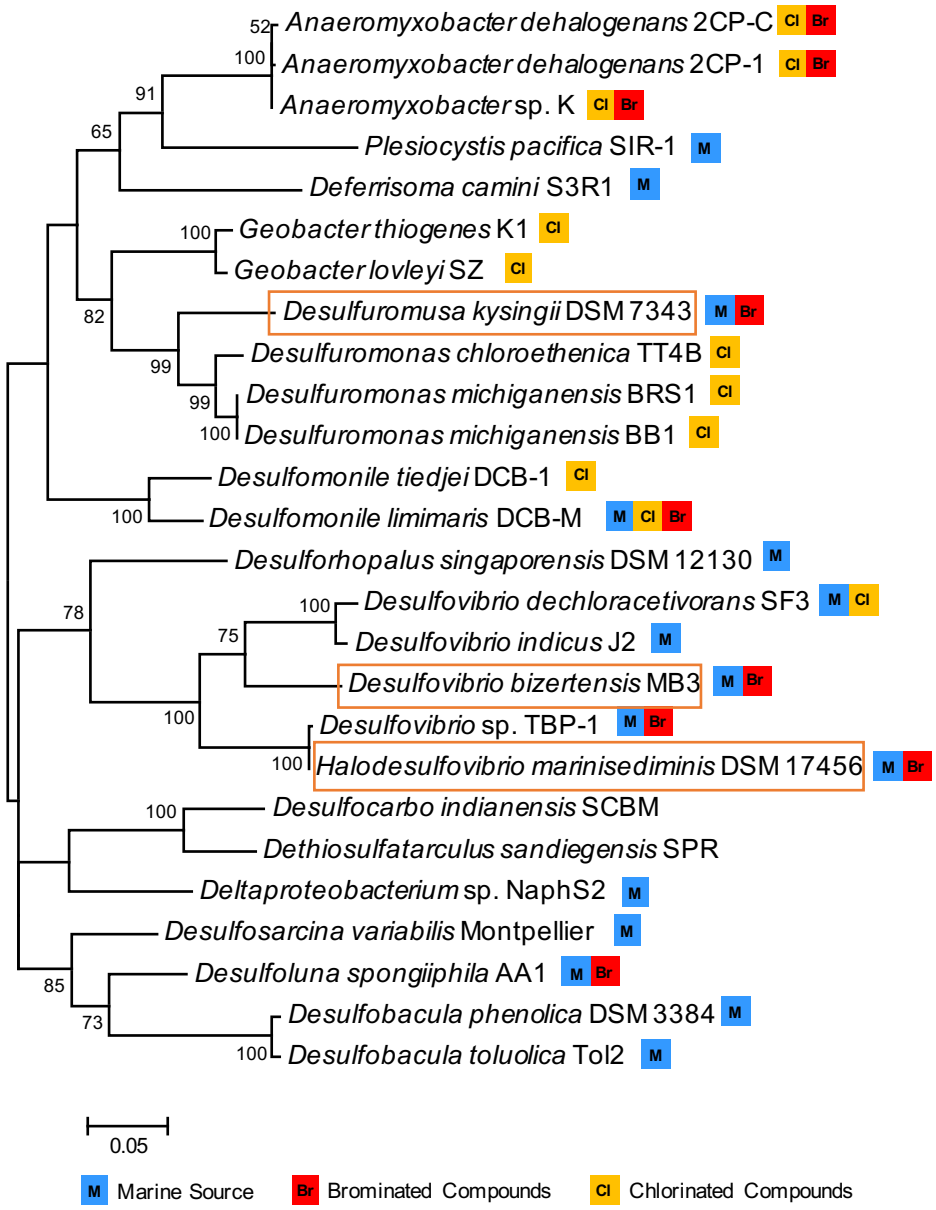


Figure 5.4 16S rRNA gene phylogenetic tree of the *Deltaproteobacteria* with dehalogenating activity or containing putative RDase A genes. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. Initial tree(s) for the heuristic search were obtained by applying Neighbor-Joining and advanced NJ (BioNJ) algorithms to a matrix of pairwise distances estimated using a Jones–Thornton–Taylor (JTT) model, and then selecting the

topology with superior log likelihood value. In total, 26 sequences were used to build the tree. For organisms with multiple 16S rRNA gene copies, only one representative 16S rRNA was chosen. The detail sequence information is listed in Table S5.3. All positions with less than 60% site coverage were eliminated, i.e., fewer than 40% alignment gaps, missing data, and ambiguous bases were allowed at any position. There was a total of 1532 positions in the final dataset. The marine *Deltaproteobacteria* and the ability to dehalogenate chlorinated or brominated compounds are marked with symbols as indicated. Microorganisms studied in this study are highlighted in a box.

Table 5.1 Summary of physiological and genomic properties of OHRB tested in this study and *Dlu. spongiiphila* strain AA1

	***Halodesulfovibrio marinisediminis* DSM 17456**	***Desulfuromusa kysingii* DSM7343**	***Desulfovibrio bizertensis* DSM 18034**	***Desulfoluna spongiiphila* strain AA1**
Dehalogenation activity [a]				
2,4,6-TBP	Yes, to 4-BP	No	Yes, to 4-BP	Yes, to Phenol
2,6-DBP	Yes, to Phenol	Yes, to Phenol	Yes, to Phenol	Yes, to Phenol
2,6-DCP	No	No	No	No
Protein yield (mg per mmol e^-) [b]	0.69 ± 0.25	0.80 ± 0.19	0.54 ± 0.34	NA [c]
Exogenous cobalamin dependency	No	Yes	No	No
Inhibition by sulfate or sulfur [d]	No	No	No	No
Genome size (Mbp)	3.71	3.74	3.23	6.54
GC%	44.95	46.63	52.09	57.20
Scaffolds in draft genome	13	27	27	52
RDase A genes in genome	1	2	4	3
σ^{54}-regulatory gene in vicinity	Yes	Yes for both	Yes for all	Yes for 2
Potential σ^{54}-binding site	Yes	Yes for both	Yes for all	Yes for 2
Total σ^{54} activators in genome	35	33	29	70
Source	Marine sediment, Tokyo Bay, Japan	Mud, Kysing Fjord south of Århus	Marine sediment, Tunisia	*Aplysina aerophoba* sponge, France
Reference for isolation	39	40	38	32

a – 2,4,6-TBP for 2,4,6-tribromophenol; 2,6-DBP for 2,6-dibromophenol; 2,6-DCP for 2,6-dichlorophenol.
b – Protein yield assay was conducted on lactate and 2,6-DBP. Utilized electrons were calculated from concentration of phenol and 2-BP present in culture based on two electrons provided per bromine removed.
c – NA for Not Available
d –Sulfur for *Dur. kysingii* and sulfate for others.

Supplementary Information

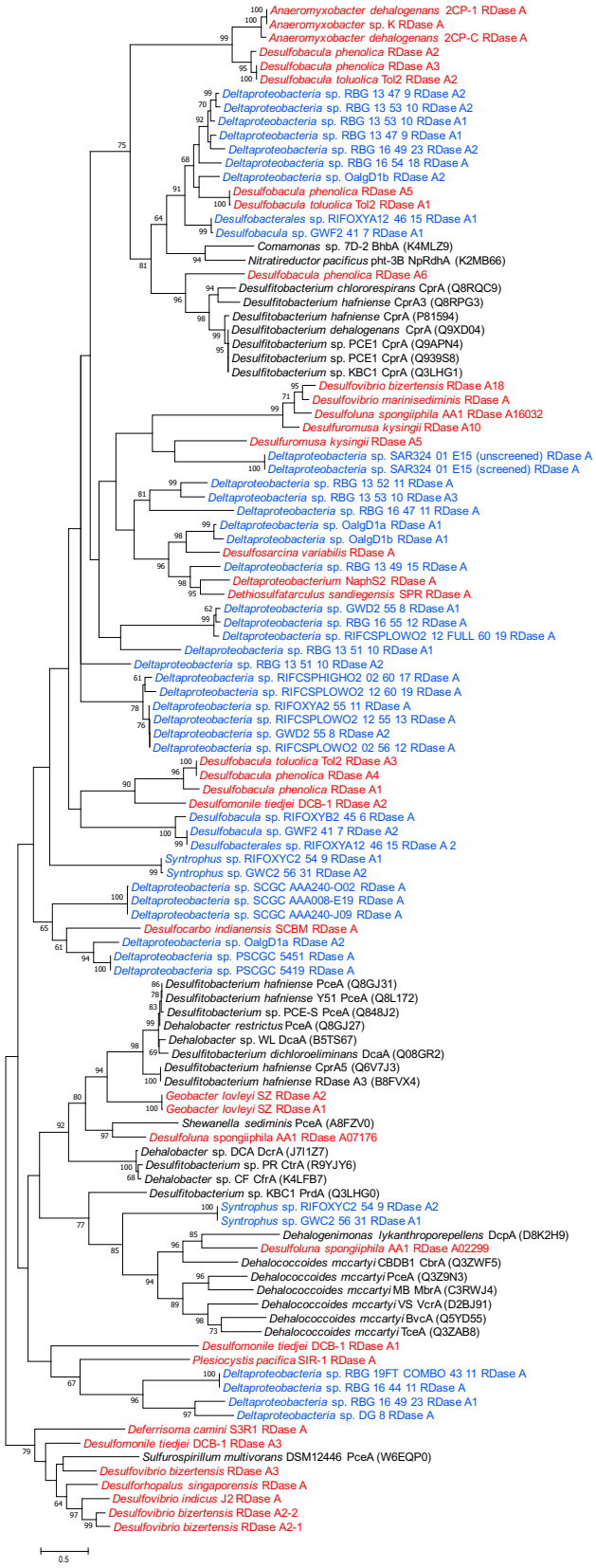

Anaeromyxobacter dehalogenans 2CP-1 RDase A
Anaeromyxobacter sp. K RDase A
Anaeromyxobacter dehalogenans 2CP-C RDase A
Desulfobacula phenolica RDase A2
Desulfobacula phenolica RDase A3
Desulfobacula toluolica Tol2 RDase A2
Deltaproteobacteria sp. RBG 13 47 9 RDase A2
Deltaproteobacteria sp. RBG 13 53 10 RDase A2
Deltaproteobacteria sp. RBG 13 53 10 RDase A1
Deltaproteobacteria sp. RBG 13 47 9 RDase A1
Deltaproteobacteria sp. RBG 16 49 23 RDase A2
Deltaproteobacteria sp. RBG 16 54 18 RDase A
Deltaproteobacteria sp. OalgD1b RDase A2
Desulfobacula phenolica RDase A5
Desulfobacula toluolica Tol2 RDase A1
Desulfobacterales sp. RIFOXYA12 46 15 RDase A1
Desulfobacula sp. GWF2 41 7 RDase A1
Comamonas sp. 7D-2 BhbA (K4MLZ9)
Nitratireductor pacificus pht-3B NpRdhA (K2MB66)
Desulfobacula phenolica RDase A6
Desulfitobacterium chlororespirans CprA (Q8RQC9)
Desulfitobacterium hafniense CprA3 (Q8RPG3)
Desulfitobacterium hafniense CprA (P81594)
Desulfitobacterium dehalogenans CprA (Q9XD04)
Desulfitobacterium sp. PCE1 CprA (Q9APN4)
Desulfitobacterium sp. PCE1 CprA (Q939S8)
Desulfitobacterium sp. KBC1 CprA (Q3LHG1)
Desulfovibrio bizertensis RDase A18
Desulfovibrio marinisediminis RDase A
Desulfoluna spongiiphila AA1 RDase A16032
Desulfuromusa kysingii RDase A10
Desulfuromusa kysingii RDase A5
Deltaproteobacteria sp. SAR324 01 E15 (unscreened) RDase A
Deltaproteobacteria sp. SAR324 01 E15 (screened) RDase A
Deltaproteobacteria sp. RBG 13 52 11 RDase A
Deltaproteobacteria sp. RBG 13 53 10 RDase A3
Deltaproteobacteria sp. RBG 16 47 11 RDase A
Deltaproteobacteria sp. OalgD1a RDase A1
Deltaproteobacteria sp. OalgD1b RDase A1
Desulfosarcina variabilis RDase A
Deltaproteobacteria sp. RBG 13 49 15 RDase A
Deltaproteobacterium NaphS2 RDase A
Dethiosulfatarculus sandiegensis SPR RDase A
Deltaproteobacteria sp. GWD2 55 8 RDase A1
Deltaproteobacteria sp. RBG 16 55 12 RDase A
Deltaproteobacteria sp. RIFCSPLOWO2 12 FULL 60 19 RDase A
Deltaproteobacteria sp. RBG 13 51 10 RDase A1
Deltaproteobacteria sp. RBG 13 51 10 RDase A2
Deltaproteobacteria sp. RIFCSPHIGHO2 02 60 17 RDase A
Deltaproteobacteria sp. RIFCSPLOWO2 12 60 19 RDase A
Deltaproteobacteria sp. RIFOXYA2 55 11 RDase A
Deltaproteobacteria sp. RIFCSPLOWO2 12 55 13 RDase A
Deltaproteobacteria sp. GWD2 55 8 RDase A2
Deltaproteobacteria sp. RIFCSPLOWO2 02 56 12 RDase A
Desulfobacula toluolica Tol2 RDase A3
Desulfobacula phenolica RDase A4
Desulfobacula phenolica RDase A1
Desulfomonile tiedjei DCB-1 RDase A2
Desulfobacula sp. RIFOXYB2 45 6 RDase A
Desulfobacula sp. GWF2 41 7 RDase A2
Desulfobacterales sp. RIFOXYA12 46 15 RDase A 2
Syntrophus sp. RIFOXYC2 54 9 RDase A1
Syntrophus sp. GWC2 56 31 RDase A2
Deltaproteobacteria sp. SCGC AAA240-O02 RDase A
Deltaproteobacteria sp. SCGC AAA008-E19 RDase A
Deltaproteobacteria sp. SCGC AAA240-J09 RDase A
Desulfocarbo indianensis SCBM RDase A
Deltaproteobacteria sp. OalgD1a RDase A2
Deltaproteobacteria sp. PSCGC 5451 RDase A
Deltaproteobacteria sp. PSCGC 5419 RDase A
Desulfitobacterium hafniense PceA (Q8GJ31)
Desulfitobacterium hafniense Y51 PceA (Q8L172)
Desulfitobacterium sp. PCE-S PceA (Q848J2)
Dehalobacter restrictus PceA (Q8GJ27)
Dehalobacter sp. WL DcaA (B5TS67)
Desulfitobacterium dichloroeliminans DcaA (Q08GR2)
Desulfitobacterium hafniense CprA5 (Q6V7J3)
Desulfitobacterium hafniense RDase A3 (B8FVX4)
Geobacter lovleyi SZ RDase A2
Geobacter lovleyi SZ RDase A1
Shewanella sediminis PceA (A8FZV0)
Desulfoluna spongiiphila AA1 RDase A07176
Dehalobacter sp. DCA DcrA (J7I1Z7)
Desulfitobacterium sp. PR CtrA (R9YJY6)
Dehalobacter sp. CF CfrA (K4LFB7)
Desulfitobacterium sp. KBC1 PrdA (Q3LHG0)
Syntrophus sp. RIFOXYC2 54 9 RDase A2
Syntrophus sp. GWC2 56 31 RDase A1
Dehalogenimonas lykanthroporepellens DcpA (D8K2H9)
Desulfoluna spongiiphila AA1 RDase A02299
Dehalococcoides mccartyi CBDB1 CbrA (Q3ZWF5)
Dehalococcoides mccartyi PceA (Q3Z9N3)
Dehalococcoides mccartyi MB MbrA (C3RWJ4)
Dehalococcoides mccartyi VS VcrA (D2BJ91)
Dehalococcoides mccartyi BvcA (Q5YD55)
Dehalococcoides mccartyi TceA (Q3ZAB8)
Desulfomonile tiedjei DCB-1 RDase A1
Plesiocystis pacifica SIR-1 RDase A
Deltaproteobacteria sp. RBG 19FT COMBO 43 11 RDase A
Deltaproteobacteria sp. RBG 16 44 11 RDase A
Deltaproteobacteria sp. RBG 16 49 23 RDase A1
Deltaproteobacteria sp. DG 8 RDase A
Deferrisoma camini S3R1 RDase A
Desulfomonile tiedjei DCB-1 RDase A3
Sulfurospirillum multivorans DSM12446 PceA (W6EQP0)
Desulfovibrio bizertensis RDase A3
Desulforhopalus singaporensis RDase A
Desulfovibrio indicus J2 RDase A
Desulfovibrio bizertensis RDase A2-2
Desulfovibrio bizertensis RDase A2-1
0.5

Figure S5.1 Phylogenetic tree of 80 RDases from D*eltaproteobacteria* isolates and metagenomics in JGI with functionally characterized RDase As. The tree construction method is the same as Figure 5.1. The RDases in *Deltaproteobacteria* isolates are in red, which are included in Figure 5.1. RDases in *Deltaproteobacteria* metagenomics data are in blue, which are not included in Figure 5.1.

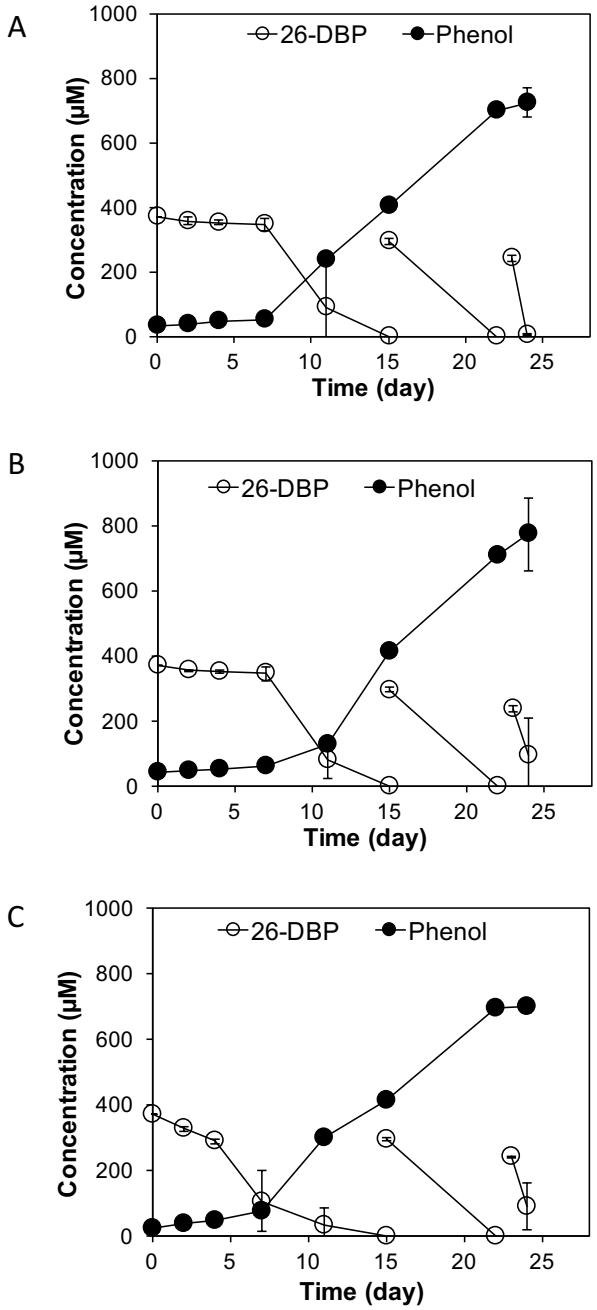


Figure S5.2 Debromination of 2,6-DBP and accumulation of phenol in *Halodesulfovibrio marinisediminis* (A), *Desulfuromusa kysingii* (B) and *Desulfovibrio bizertensis* (C) culture. The cultures were collected for an end point protein assay.

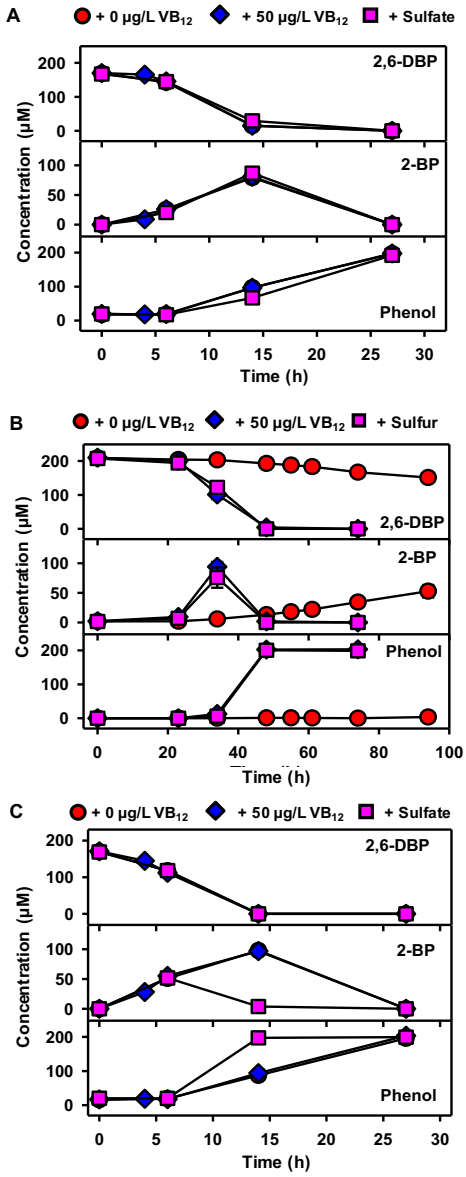


Figure S5.3 Dehalogenation of 2,6-DBP by *Halodesulfovibrio marinisediminis* (A), *Desulfuromusa kysingii* (B) and *Desulfovibrio bizertensis* (C) in the absence or presence of cobalamin (vitamin B12) and sulfate/sulfur as electron acceptor. For each figure, the upper panel indicates the concentration of 2,6-DBP in each treatment; the middle panel indicates the concentration of 2-BP and lower panel indicates the concentration of phenol. Data points indicate the means and standard deviations of triplicate cultures.

Table S5.1 The genomic information of *Deltaproteobacteria* examined in this study and details of their reductive dehalogenase genes.

Name	GOLD Analysis Project Id	NCBI Taxon ID	Source	Genome Size (Mb)	GC Content (%)	σ^{54} - Dependent Activator	RDase A gene	Gene ID	Length (bp)
Desulfovibrio bizertensis DSM18034	Ga0006634	1121442	Marine sediment, Tunisia	3.23	52.09	29	RDase A1-2	2568539639	1443
							RDase A2-2	2568539642	1440
							RDase A3	2568540098	1440
							RDase A18	2568541747	1665
Desulfovibrio indicus J2	Ga0133372	1716143	Serpentinized peridotite, Indian Ocean	3.97	63.49	27	RDase A	2688215644	1455
Desulforhopalus singaporensis DSM 12130	Ga0056957	91360	Black marine mud, Singapore	5.0	50.55	74	RDase A	2596708091	1416
Desulfoluna spongiiphila	Ga0104423	2654587882	Marine sponge	6.54	57.2	70	RDase A02299	2656130309	1449
							RDase A07176	2656131703	1287
							RDase A16032	2656133146	1680
Desulfomonile tiedjei DCB-1	Ga0025025	706587	Sludge, Wastewater, USA	6.52	50.09	71	RDase A1	2509738725	1680
							RDase A2	2509738727	1056
							RDase A3	2509739354	1416
Deferrisoma camini S3R1	Ga0026649	1125863	Deep-sea hydrothermal chimney, Pacific Ocean	4.23	70.08	29	RDase A	2517271284	1098
Geobacter lovleyi SZ	Ga0028907	398767	Freshwater sediment, South Korea	3.99	54.74	38	RDase A1	642678287	1545
							RDase A2	642678289	1545
Desulfobacula phenolica DSM 3384	Ga0066824	90732	Marine mud, Venice, Italy	4.87	41.33	55	RDase A1	2616662663	1404
							RDase A2	2616662665	1905
							RDase A3	2616662682	1920
							RDase A4	2616662684	1404
							RDase A5	2616666133	1245
							RDase A6	2616666538	1392

Table S5.1 Continued

Name	GOLD Analysis Project Id	NCBI Taxon ID	Source	Genome Size (Mb)	GC Content (%)	σ^{54} - Dependent Activator	RDase A gene	Gene ID	Length (bp)
Desulfobacula toluolica Tol2	Ga0012828	651182	Marine mud, USA	5.2	41.45	51	RDase A1	2524508258	1245
							RDase A2	2524509456	1920
							RDase A3	2524509458	1404
Anaeromyxobacter dehalogenans 2CP-1	Ga0027433	455488	Freshwater sediment, Michigan	5.03	74.72	38	RDase A	643593148	1923
Anaeromyxobacter dehalogenans 2CP-C	Ga0027434	290397	Tropical soil, Cameroon	5.01	74.91	40	RDase A	637861846	1923
Anaeromyxobacter sp. K	Ga0027436	447217	Soil	5.06	74.84	43	RDase A	642761794	1923
Delta-Proteobacterium NaphS2	Ga0031586	88274	Marine sediment, Germany	6.55	49.82	38	RDase A	648669933	1437
Desulfosarcina variabilis Montpellier	Ga0010238	859321	Marine mud, Montpellier, France	9.42	51.32	88	RDase A	2502438632	1440
Desulfuromusa kysingii DSM 7343	Ga0056096	37625	Marine mud, Germany	3.74	46.63	33	RDase A5	2599543621	1356
							RDase A10	2599544492	1668
Halodesulfovibrio marinisediminis DSM 1745	Ga0008187	1121457	Marine sediment	3.71	44.95	35	RDase A	2587790293	1677
Desulfocarbo indianensis SCBM	Ga0081924	1348163	Coal bed, Indiana, USA	4.91	63.06	39	RDase A	2654894503	1188
Plesiocystis pacifica SIR-1	Ga0029812	391625	Marine sandy beach, Japan	10.59	70.66	27	RDase A	641166146	1005
Dethiosulfatarculus sandiegensis SPR	Ga0077793	1429043	Methanogenic consortium, paraffin-degrading enrichment	5.93	52.06	53	RDase A	2629332177	1410

Table S5.2 The genome information of the organohalide-respiring bacteria represented in Figure 5.1.

Name	GOLD Analysis Project Id	NCBI Taxon ID	Source	Genome Size (Mb)	GC content (%)	σ^{54} - Dependent activator	RDase A
Desulfitobacterium chlororespirans DSM 11544	Ga0008181	1121395	Compost pile, USA	5.61	47.29	40	2
Desulfitobacterium dehalogenans JW/IU-DC1	Ga0024867	756499	Freshwater pond sediment, Georgia USA	4.32	44.97	21	6
Desulfitobacterium dichloroeliminans LMG P-21439	Ga0024953	871963	Soil polluted with 1,2-DCA	3.62	44.22	12	1
Desulfitobacterium hafniense DCB-2	Ga0028500	272564	Municipal sludge, Denmark	5.28	47.54	34	7
Desulfitobacterium hafniense Y51	Ga0028501	138119	Soil contaminated with tetrachloroethene, Japan	5.73	47.36	38	1
Dehalococcoides mccartyi BAV1	Ga0028484	216389	Fresh water, Michigan	1.34	47.17	0	12
Dehalococcoides mccartyi CBDB1	Ga0028485	255470	Anoxic river sediment	1.4	47.03	0	31
Dehalococcoides mccartyi 195	Ga0028483	243164	Anaerobic sewage digestor	1.47	48.85	0	18
Dehalobacter restrictus DSM 9455	Ga0025056	871738	PCE-dechlorinating packed-bed column, Netherlands	2.94	44.56	3	24
Dehalobacter sp. CF	Ga0026847	1131462	1,1,1-TCA and TCE contaminated site	3.09	44.31	1	17
Nitratireductor pacificus pht-3B	Ga0012014	391937	Sediment, Indian Ocean	4.47	65.51	5	3
Sulfurospirillum multivorans DSM 12446	Ga0023093	1150621	Activated sludge, Germany	3.18	40.9	4	2
Dehalogenimonas lykanthroporepellens BL-DC-9	Ga0028488	552811	Waste recovery well at the Petro-Processors of Louisiana, Inc. Superfund Site	1.69	55.04	0	22
Shewanella sediminis HAW-EB3	Ga0030285	425104	Sediment at depth of 215m from an unexploded-ordinance-dumping site at Halifax	5.52	46.08	18	5

Table S5.3 The 16S rRNA gene database source of OHRBs in Figure 5.4 and their substrate range.

Name	Substrate Range	ID	Reference
Anaeromyxobacter dehalogenans 2CP-1	2,6-Dichorophenol; 2,5-Dichlorophenol; 2-Chlorophenol; 2-Bromophenol	643594130	Sanford et al., 2002
Anaeromyxobacter dehalogenans 2CP-C		640712235	
Anaeromyxobacter sp. K		642762693	
Deferrisoma camini S3R1	Not Studied	2517270461	-
Desulfobacula phenolica DSM 3384	Not Studied	2616666760	-
Desulfobacula toluolica Tol2	Not Studied	2524508170	-
Desulfomonile tiedjei DCB-1	3-Chlorobenzoate; 2,5-Dichlorobenzoate	2509740724	DeWeerd et al., 1990
Desulforhopalus singaporensis DSM 12130	Not Studied	2596710058	-
Desulfosarcina variabilis Montpellier	Not Studied	2502441539	-
Desulfovibrio indicus J2	Not Studied	2688214528	-
Dethiosulfatarculus sandiegensis SPR	Not Studied	2629331604	-
Geobacter lovleyi SZ	Tetrachloroethene; Trichloroethene	642676148	Sung et al., 2006; Wagner et al., 2012
Plesiocystis pacifica SIR-1	Not Studied	641164571	-
Deltaproteobacterium sp. NaphS2	Not Studied	AJ132804.1	-
Desulfocarbo indianensis SCBM	Not Studied	NR_126285.1	-
Desulfoluna spongiiphila AA1	2,6-Dibromophenol; 2-Bromophneol; 4-Bromophenol; 2,4-Dibromophenol; 2,4,6-Tribromophenol; 2-Iodophenol; 3-Iodophenol; 2-Bromo-4-fluorophenol; 3,5-Dibromo-4-hydroxybenzoate; 3,5-Dibromo-4-hydroxybenzonitrile	NR_115979.1	Ahn et al., 2009; Liu et al., 2017
Desulfuromonas chloroethenica TT4B	Tetrachloroethene; Trichloroethene	NR_026012.1	Krumhlz, 1997
Desulfomonile limimaris DCB-M	3-Chlorobenzoate; 3-Bromobenzoate; 2,3-Dibromobenzoate; 2,5-Dibromobenzoate; 3,5-Dichlorobenzoate	NR_025079.1	Sun et al., 2001
Desulfuromonas michiganensis BB1	Tetrachloroethene; Trichloroethene	AF357915.2	Sung et al., 2003
Desulfuromonas michiganensis BRS1	Tetrachloroethene; Trichloroethene	AF357914.2	Sung et al., 2003
Desulfovibrio bizertensis MB3	2,6-Dibromophenol; 2-Bromophenol; 2,4,6-Tribromophenol; 2,4-Dibromophenol	NR_043808.1	This study
Halodesulfovibrio marinisediminis DSM 17456	2,6-Dibromophenol; 2-Bromophenol; 2,4,6-Tribromophenol; 2,4-Dibromophenol	NR_041631.1	This study
Desulfuromusa kysingii DSM 7343	2,6-Dibromophenol; 2-Bromophenol; 2,4,6-Tribromophenol; 2,4-Dibromophenol	2599544949	This study
Desulfovibrio sp. TBP-1	2,6-Dibromophenol; 2-Bromophenol; 2,4,6-Tribromophenol; 2,4-Dibromophenol; 4-Bromophenol	AF090830.1	Boyle et al., 1999
Desulfovibrio dechloracetivorans SF3	2,6-Dichlorophenol; 2-Chlorophenol	NR_025078.1	Sun et al., 2000
Geobacter thiogenes K1[a]	Trichloroacetate	NR_028775.1	De Wever et al., 2000

a-Reductive dehalogenation of *Geobacter thiogenes* K1 may involve a sulfur-sulfide cycle.

Table S5.4 Putative genes involved in cobalamin biosynthesis in the genomes of organohalide-respiring bacteria tested in this study and *Desulfoluna spongiiphila* strain AA1.

Gene Abbrev.	Functional Role	*H. marinisediminis* Gene ID	*Dur. kysingii* Gene ID	*Dvi. bizertensis* Gene ID	*Dlu. spongiiphila* Gene ID
CbiK	Sirohydrochlorin cobaltochelatase	2587791438 2587789883	2599543416	2568540274 2568540624	2656131771 2656134271
CbiL	Cobalt-precorrin-2 C20-methyltransferase	2587791434	2599543414 2599543411	2568540270	2656134277
CbiH	Cobalt-precorrin-3b C17-methyltransferase	2587790554	2599543408	2568541449	2656134283
CbiG	cobalt-precorrin 5a hydrolase	2587790553	2599543409	2568541450	2656134282
CbiF	Cobalt-precorrin-4 C11-methyltransferase	2587791567	2599543410	2568541455	2656134281
CbiD	Cobalt-precorrin-6 synthase	2587791569	2599543413	2568540852	2656134279
CbiJ	Cobalt-precorrin-6x reductase	-	2599543407	-	-
CbiET	Cobalt-precorrin-6y C5-methyltransferase	2587791568	2599543412 2599544127	2568540853	2656134280
CbiC	Cobalt-precorrin-8x methylmutase	2587790573	2599543417	2568538875	2656134276
CbiA	Cobyrinic acid a,c-diamide synthase	2587790574 2587790682	2599543418	2568540851 2568541122	2656134105 2656134275
CobA	Uroporphyrinogen-III methyltransferase	2587791030	2599543419 2599542566	2568541128	2656130579
CbiP	Cobyric acid synthase	2587792387	2599542341	2568541166	2656134285
CobD	L-threonine 3-O-phosphate decarboxylase	2587792472	-	-	2656134288
CbiB	Adenosylcobinamide-phosphate synthase	2587789560	2599542342	2568541021	2656134290
CobU	Adenosylcobinamide-phosphate guanylyltransferase	2587790455	2599542338	2568541742	2656134286
CobC/CbiY	Alpha-ribazole-5'-phosphate phosphatase	2587791323	2599544128	2568539480	2656131162
CobT	Nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase	2587790664	2599542339	2568541109	2656129606
CobS	Cobalamin synthase	2587791095 2587792173	2599542340	2568540769 2568540067 2568539740	2656134287
-	Glutamyl-tRNA synthetase	2587792331 2587790117	2599544587	2568538920 2568540059	2656132446 2656133606
HemA	Glutamyl-tRNA reductase	2587790085	2599542568	2568540320	2656134349
HemB	Porphobilinogen synthase	2587791660	2599542565	2568539875	2656134423
HemC	Porphobilinogen deaminase	2587792401	2599542567	2568541682	2656130578
HemE	Uroporphyrinogen decarboxylase	-	2599542026	2568539509	2656134671 2656131981 2656129945 2656133015
HemL	Glutamate-1-semialdehyde aminotransferase	2587790552	2599544468	2568541451	2656131384 2656132659 2656133626
HemN	Oxygen-independent coproporphyrinogen-3 oxidase	2587790636	2599541825 2599544729	2568539183	2656130302 2656132734 2656132719
HemY	Protoporphyrinogen oxidase	-	2599542035	-	-
CbiM,N,O,Q	Cobalt/nickel transport system protein	2587791554 2587792083 2587792081 2587792082	2599541849	2568539455 2568540623 2568540622 2568541056	2656132238 2656132234 2656130427 2656132235 2656132237
BtuBCDF	Vitamin B12/Fe3+ ABC transporter, permease component	2587791435 2587791436 2587791437	2599542344 2599542345 2599542346 2599542347	2568539097 2568539554 2568540272 2568539555 2568539098 2568540273 2568539556 2568539099 2568540271	2656131763 2656134273 2656131826 2656131825 2656131764 2656134272 2656131765 2656131828 2656134274
BtuR	Cob(I)alamin adenosyltransferase	2587789955	2599542343	-	2656131122
CysG	Siroheme synthase / Precorrin-2 oxidase	2587790087	-	2568540318	2656134347

Table S5.5 Nucleotide primer sequences used for RT-PCR in this study.

Strain	Primer	Sequence (5'-3')	Target Gene	Product Length (bp)
***Halodesulfovibrio marinisediminis* DSM 17456**	653F	GAGGGAAGTGGAATTTCTGGTGTAG	16S rRNA	94
	747R	CAGTGTCAGTAATAGTCCAGGAAGT		
	725F	AGCCTATGGAGTTCAAATCACCTAA	RDase A gene	99
	824R	GGGTCAAATTTAGCAATACCAACCA		
***Desulfuromusa kysingii* DSM 7343**	887F	CGCAAGGCTAAAACTCAAAGGAA	16S rRNA	88
	975R	GGTAAGGTTCTTCGCGTTGC		
	650F	TCGTTGATAATGGGCGCGATA	RDase A5 gene	98
	748R	CGACATTTGTTTTCAGCCCGT		
	621F	TTCCACCATTCCAGAAGATCCAG	RDase A10 gene	70
	691R	CCCATTTTTCTGGAGGTTGTTCC		
***Desulfovibrio bizertensis* DSM 18034**	209F	CATGCTTTCACTTCTAGATGAGTCC	16S rRNA	91
	300R	CCTCTTAGACCAGTTATCCATCGTC		
	993F	GATTCTGACCAACATGCCGC	RDase A1-2 gene	90
	1083R	CTCGCGGGCACACTTTTTAC		
	367F	CTGTATGGCATGGAACGGATGAA	RDase A2-2 gene	84
	451R	CATCATTCCAGCTCAAAGGTCCC		
	213F	CTACCAGCGTTTTTCTACAGCAAAT	RDase A3 gene	96
	309R	CTTGATTAAATTTCTGCCATACGGT		
	1012F	CAATTCCTGAAAGAACTGGGCTATC	RDase A18 gene	66
	1078R	TCATGATTTCGTAGTTGTGACCG		

Chapter 6 Conclusions

In this study, we examined the organohalide respiring capability of members of the *Deltaproteobacteria* group, investigating their ecophysiology in terms of their dehalogenating range and activity under different conditions, and determining their reductive dehalogenase gene diversity and gene expression. We took advantage of the available genome database to evaluate the dehalogenating capacity of *Deltaproteobacteria*, revealing that approximately 10% of the *Deltaproteobacteria* with their genomes sequenced contain reductive dehalogenase genes in their genomes. In total, six organohalide respiring *Deltaproteobacteria* were studied in detail for their physiologies related to organohalide respiration, including two newly isolated novel *Halodesulfovibrio debrominans* strains, three newly identified OHRBs based on their genomes, and one previously isolated OHRB (Fig. 6.1). The description of new OHRBs in *Deltaproteobacteria* extends our knowledge about the phylogenetic diversity of OHRBs. Two genera (*Halodesulfovibrio* and *Desulfuromusa*) are for the first time reported to contain OHRBs.

The reductive dehalogenase gene clusters in *Deltaproteobacteria* group were examined for their common features. Two conserved iron-sulfur cluster binding motifs are found in all these RdhAs and a TAT signal motif is present in most of the RdhA N-terminal regions. Although many of these *rdhA*-containing *Deltaproteobacteria* were not isolated as known OHRBs, the presence of an r*dhA* gene indicates that they may have organohalide respiring ability. The reductive dehalogenase gene cluster composition in *Deltaproteobacteria* contains the catalytic

rdhA gene and most of them contain *rdhB* gene encoding for the membrane anchoring protein. In addition, a gene encoding for a sigma factor 54 dependent transcriptional regulator (bEBP) is commonly found in the near vicinity of the reductive dehalogenase gene cluster. This is unique from other OHRBs groups such as *Dehalococcoides* spp. that commonly have *marR* genes and *Desulfitobacterium* spp. that have a CRP/FNR family regulator. Sigma factor 54 initiated transcription was initially identified for its role in nitrogen assimilation, but then found to be in 60% of bacterial genomes to coordinate many metabolic processes (Francke et al., 2011). Different from sigma factor 70 initiated transcription, sigma factor 54 initiated transcription recognizes -24 and -12 conserved elements with a consensus sequence of YTGGCACGrNNNTTGCW. The initiation of sigma factor 54 dependent transcription requires the assistance of bEBP to open the closed complex. The bEBP typically binds to a site 80 to 150 bp upstream of the promoter region and interacts with the RNA polymerase holoenzyme by bending the DNA to a loop. Ligand binding is one of the regulatory mechanisms for bEBP, which substrates can bind to the regulatory region of bEBP to modulate the activity of bEBP and eventually affect the expression of related sigma factor 54 dependent operons (Shingler, 1996; Bush and Dixon, 2012). The prevalence of bEBPs in the vicinity of *rdh* gene operons and the presence of predicted sigma factor 54 binding sites in promoter regions of *rdh* gene operons in *Deltaproteobacteria* indicate that the transcription of these *rdh* gene operons may be sigma factor 54 dependent. However, this hypothesis and corresponding transcription regulatory system need further investigation.

The six organohalide respiring *Deltaproteobacteria* that were studied in detail have some common features (Table S6.1), which are: 1) they were all isolated from marine or estuarine environments; 2) they are sulfate or sulfur reducing bacteria but the presence of sulfate or sulfur does not influence the dehalogenation rate; 3) their dehalogenating activity is specific to bromophenol rather than chlorophenol; and 4) they all have one common *rdhA* gene (namely *brpA* for bromophenol reductase gene) in a similar gene cluster composition, whose expression is significantly upregulated by bromophenol.

Desulfuromusa kysingii is a sulfur reducing bacterium which is distinct from the other five sulfate reducing bacteria in some aspects. The dehalogenating activity of *Desulfuromusa kysingii* is dependent on exogenous cobalamin, while the other five strains could dehalogenate at same rate even without an exogenous cobalamin supply. However, all six genomes contain a near complete corrinoid biosynthesis pathway (with one missing gene, *cbiJ*) with a cobalamin riboswitch found around the corrinoid biosynthesis and transport gene clusters. *Desulfuromusa kysingii* cannot dehalogenate 2,4,6-tribromophenol, while *Desulfovibrio bizertensis* can partially dehalogenate 2,4,6-tribrmophenol to 4-bromophenol and *Desulfoluna spongiiphila*, *Halodesulfovibrio debrominans* AK and HS can completely dehalogenate 2,4,6-tribromophenol to phenol.

The *Deltaproteobacteria* strains contain various numbers of reductive dehalogenase genes in their genomes. One *rdh* gene cluster (designated a *brp* gene cluster for it role in bromophenol respiration) which shares similar gene composition is common to all of the strains (Fig. 6.2). The common gene cluster contains genes

coding for a sigma factor 54 dependent regulator (*sigma54*), a tetratricopeptide repeat containing protein (*brpX*), a reductive dehalogenase catalytic protein (*brpA*), a membrane anchoring protein (*brpB*) and an iron-sulfur domain containing protein (*brpC*). The transcript analysis indicates that the *brpA* in these gene clusters is the ones responding to 2,6-dibromophenol. The expression of *brpA* in each strain is significantly upregulated in the presence of 2,6-dibromophenol. These BrpAs share relatively high sequence similarity and cluster together in the RdhA phylogenetic trees to form a clade of a reductive debrominase (Fig. 6.3). Most functionally characterized RdhAs so far are specific for chlorinated compounds. Two debrominases were reported in *Comamonas* sp. 7D-2 BhbA and *Nitratireductor pacificus* pht-3B NaRdhA, which are catabolic rather than respiratory reductive dehalogenases. The BrpA clade for bromophenol reported in this study expands the diversity the known RdhA with debrominase activity.

Organohalide respiring bacteria may acquire reductive dehalogenase genes through horizontal gene transfer. The presence of putative transposable elements near the *rdh* gene cluster and the location of *rdh* genes in atypical regions in the genomes of *Dehalococcoides* spp. are evidence for horizontal gene transfer of *rdh* genes (Regeard et al., 2005; Krajmalnik-Brown et al., 2007). However, the *brp* gene cluster in *the Deltaproteobacteria* appear to be acquired through vertical gene transfer. When comparing the phylogeny of each gene in the *brp* gene cluster with housekeeping genes, they show the same phylogenetic tree branch structure (Fig. 6.2). This indicates that the *brp* gene cluster is conserved in the genomes of these six bacteria and shares similar evolutionary history as the housekeeping genes.

The transcriptomic and proteomic analysis of *Desulfoluna spongiiphila* revealed the overall metabolism of organohalide respiring *Deltaproteobacteria* in response to bromophenol. The upregulation of *brpXABC* genes together with other genes in the same operon confirms the role of *brpA* in organohalide respiration. The detection of BrpX and BrpA exclusively in the 2,6-DBP treatment proteome provides additional evidence for the role of BrpA in bromophenol respiration. No electron transfer element was exclusively found in the proteome of 2,6-DBP amended treatment, indicating that organohalide respiration in *Desulfoluna spongiiphila* may share the electron transfer elements with other respiratory processes, i.e. sulfate reduction. Future work could try to determine what these elements are. One experiment would be to add bromophenol into starved culture to induce the production of necessary proteins. By comparing the proteome before and after bromophenol addition, the elements rather than reductive dehalogenase involved in organohalide respiration could be identified. However, as organohalide respiration is slow and low in yield, the challenge part will be to get enough biomass for proteomic analysis.

The organohalide respiring *Deltaproteobacteria* are diverse in phylogeny, and make up important members of OHRB groups. Previous studies show that *Deltaproteobacteria* are widely distributed in the marine subsurface (Inagaki et al., 2003; Inagaki et al., 2006) and *Deltaproteobacteria* can become dominant in marine sediment enrichments with 2,5,6-tribromophenol (Futagami et al., 2013). Our results indicate that many of the *rdhA*-containing *Deltaproteobacteria* isolated from marine environments of different geographical locations are organobromide respirers. As the

marine environment is a major source and reservoir for organohalides especially organobromides, this study highlights the potential roles of organohalide respiring *Deltaproteobacteria* in the marine halogen cycle.

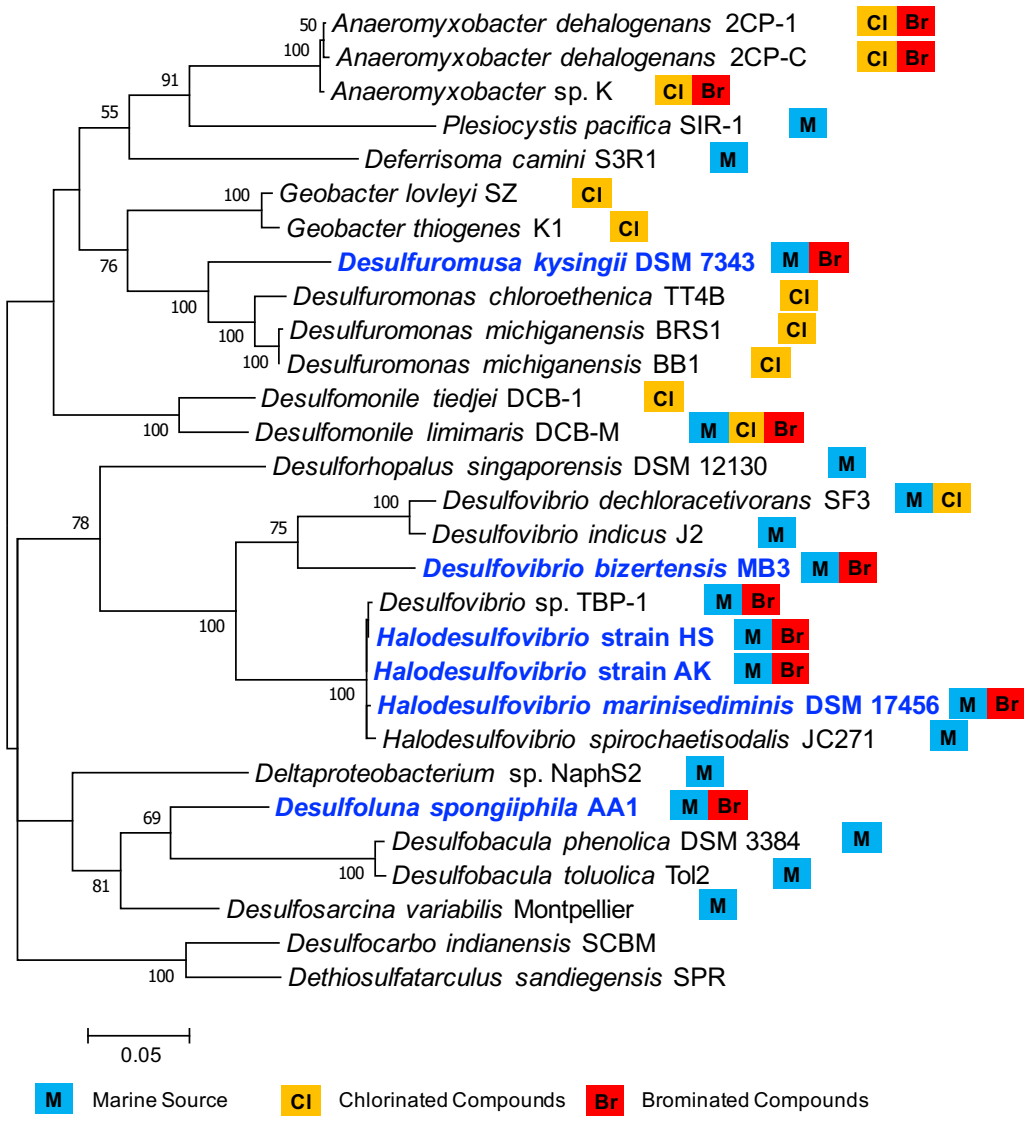


Figure 6.1 The 16S rRNA phylogenetic tree of *rdhA*-containing *Deltaproteobacteria*. Bacteria highlighted in blue are strains studied in this thesis. Except for *Desulfoluna spongiiphila*, all other studied strains were either not previously known as OHRBs or were newly isolated in this study.

Table 6.1 The comparison of physiological and genomic features of six OHRBs studied in this thesis.

	***Desulfoluna spongiiphila* strain AA1**	***Halodesulfovibrio debrominans* strain AK**	***Halodesulfovibrio debrominans* strain HS**	***Halodesulfovibrio marinisediminis* DSM17456**	***Desulfuromusa kysingii* DSM7343**	***Desulfovibrio bizertensis* DSM18034**
Dehalogenation activity [a]						
2,4,6-TBP	Phenol	Phenol	Phenol	4-BP	No	4-BP
2,6-DBP	Phenol	Phenol	Phenol	Phenol	Phenol	Phenol
2,6-DCP	No	No	No	No	No	No
2-BP	Phenol	Phenol	Phenol	Phenol	Phenol	Phenol
3-BP	Phenol (very slow)	No	No	No	nt	nt
4-BP	Phenol	Phenol	Phenol	No	No	No
2-,3-,4-BBA	No	No	No	No	nt	nt
3,5-DB-4HBA	4-HBA	4-HBA	4-HBA	4-HBA	nt	nt.
3,5-DB-4HBN	4-HBN	4-HBN	4-HBN	4-HBN	nt	nt
Protein yield (mg per mmol e^-) [b]	nt	0.50 ± 0.15	0.68 ± 0.15	0.69 ± 0.25	0.80 ± 0.19	0.54 ± 0.34
Exogenous cobalamin dependency	No	No	No	No	Yes	No
Inhibition by sulfate or sulfur [c]	No	No	No	No	No	No
Genome size (Mbp)	6.54	3.73	3.74	3.71	3.74	3.23
GC%	57.2	44.72	44.83	44.95	46.63	52.09
***rdhA* genes in genome**	3	3	3	1	2	4
Source	*Aplysina aerophoba* sponge, France	Authr kill sediment, NJ, US	Hackensack river sediment, NJ, US	Marine sediment, Tokyo Bay, Japan	Mud, Kysing Fjord south of Århus	Marine sediment, Tunisia
Reference for isolation	Ahn *et al*., 2009	This study	This study	Takii, *et al*., 2008	Liesack and Finster, 1995	Haouari *et al*., 2006

a – shown as dehalogenating end product. 2,4,6-TBP for 2,4,6-tribromophenol; 2,6-DBP for 2,6-dibromophenol; 2,6-DCP for 2,6-dichlorophenol; 2-BP for 2-bromophenol; 3-BP for 3-bromophenol; 4-BP for 4-bromophenol; 2-,3-,4-BBA for 2-,3-,4-

bromobenzoate; 3,5-DB-4-HBA for 3,5-dibromo-4-hydroxybenzoate; 4-HBA for 4-hydroxybenzoate; 3,5-DB-4-HBN for 3,5-dibromo-4-hydroxybenzonitrile and 4-HBN for 4-hydroxybenzonitrile.
b – Protein yield assay was conducted on lactate and 2,6-DBP. Utilized electrons were calculated from concentration of phenol and 2-BP present in culture based on two electrons provided per bromine removed.
c –Sulfur for *Dur. kysingii* and sulfate for others
nt- Not Tested

Gene	Protein	AA sequence identity
sigma54	Sigma factor 54 dependent transcriptional regulator	46.1-95.1%
brpX	Tetratricopepetide repeat containing protein	27.4-91.1%
brpA	Reductive dehalogenase catalytic protein	62.7-92.7%
brpB	Membrane anchoring protein	54.9-94.3%
brpC	4Fe-4S binding domain-containing protein	43.2-97.1%

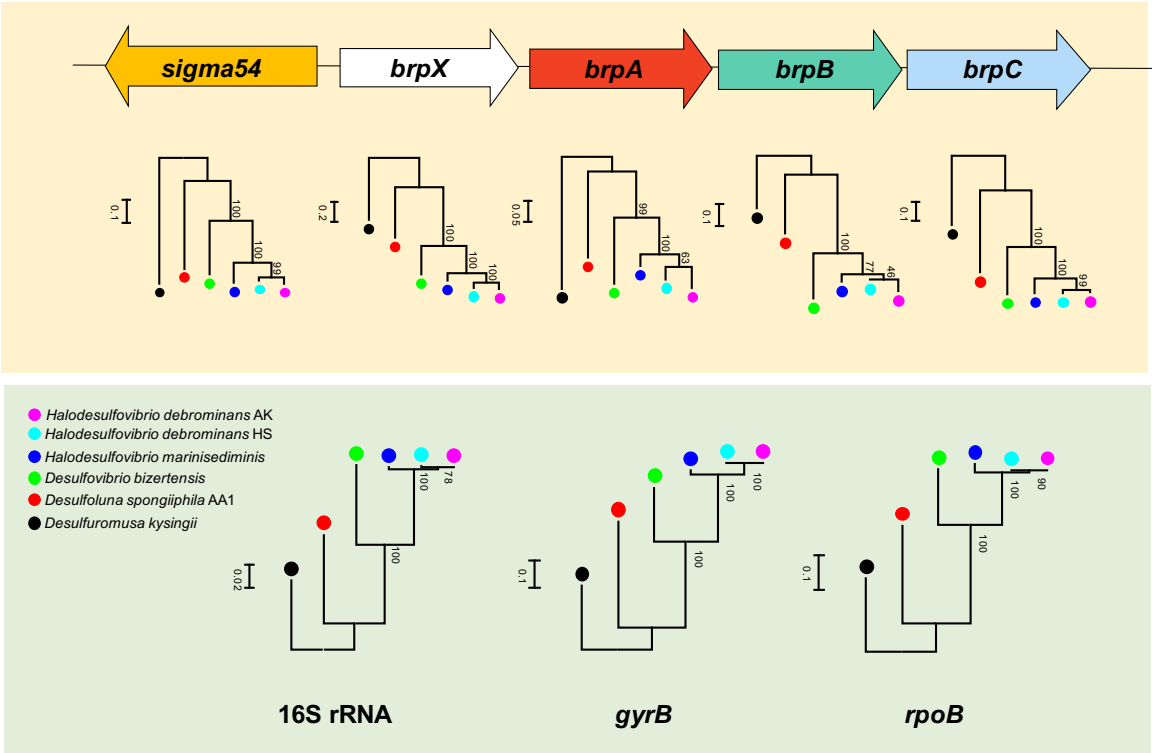


Figure 6.2 The phylogenetic trees of *brp* genes and housekeeping genes of six bromophenol-respiring *Deltaproteobacteria*. The AA sequence identity is the identity range of all six strains for each gene coded amino acid.

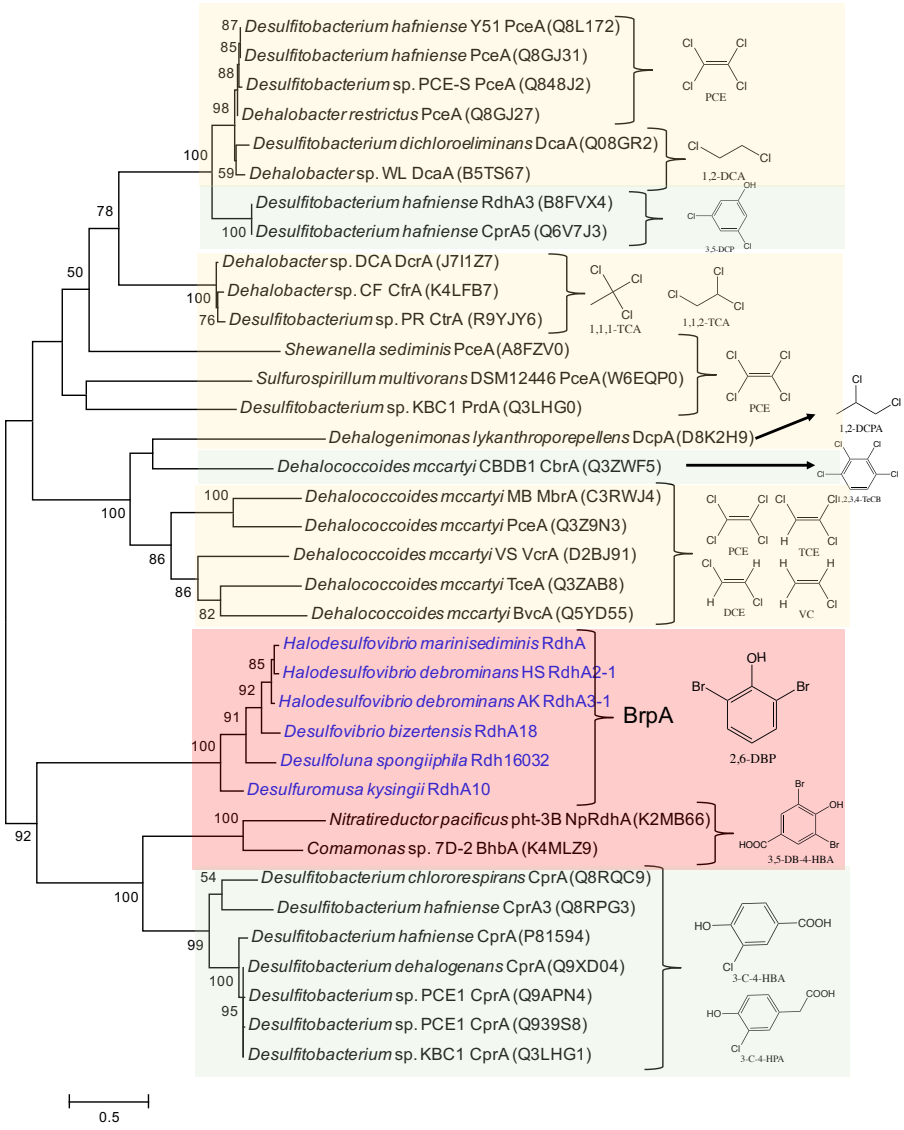


Figure 6.3 The phylogenetic tree of functionally characterized RdhAs with the chemical structure of the substrate. For RdhAs tested with more than one substrate, only the primary one is indicated in this figure. The tree is constructed in the same manner as described in other chapters.

References

Adékambi, T., Shinnick, T.M., Raoult, D., and Drancourt, M. (2008) Complete *rpoB* gene sequencing as a suitable supplement to DNA–DNA hybridization for bacterial species and genus delineation. International Journal of Systematic and Evolutionary Microbiology 58: 1807-1814.

Adrian, L. and Löffler, F.E. (Editors) (2016) Organohalide-Respiring Bacteria. Springer, Berlin, 10.1007/978-3-662-49875-0

Agarwal, V., Blanton, J.M., Podell, S., Taton, A., Schorn, M.A., Busch, J. et al. (2017) Metagenomic discovery of polybrominated diphenyl ether biosynthesis by marine sponges. Nature Chemical Biology 13: 537-543.

Ahn, Y-B., Kerkhof, L.J. & Häggblom, M.M. (2009) *Desulfoluna spongiiphila* sp. nov., a dehalogenating bacterium in the *Desulfobacteraceae* from the marine sponge *Aplysina aerophoba*. International Journal of Systematic and Evolutionary Microbiology 59: 2133-2139.

Ahn, Y-B., Rhee, S-K., Fennell, D.E., Kerkhof, L.J., Hentschel, U. & Häggblom, M.M. (2003) Reductive dehalogenation of brominated phenolic compounds by microorganisms associated with the marine sponge *Aplysina aerophoba.* Applied and Environmental Microbiology 69: 4159-4166.

Alaee, M., Arias, P., Sjödin, A. & Bergman, Å. (2003) An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release. Environment International 29: 683-689.

Alfán-Guzmán, R., Ertan, H., Manefield, M. & Lee, M. (2017) Isolation and characterization of *Dehalobacter* sp. strain TeCB1 including identification of TcbA: A novel tetra- and trichlorobenzene reductive dehalogenase. Frontiers in Microbiology 8: 558.

Alikhan, N-F., Petty, N.K., Ben Zakour, N.L. & Beatson, S.A. (2011) BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics 12: 402.

Alvarez-Ortega, C., Olivares, J., and Martinez, J. (2013) RND multidrug efflux pumps: what are they good for? Frontiers in Microbiology 4: 7.

Atashgahi, S., Haggblom, M.M., and Smidt, H. (2018) Organohalide respiration in pristine environments: implications for the natural halogen cycle. Environmental Microbiology 20: 934-948.

Atashgahi, S., Lu, Y., and Smidt, H. (2016) Overview of known organohalide-respiring bacteria—phylogenetic diversity and environmental distribution. In Organohalide-Respiring Bacteria. Adrian, L., and Löffler, F.E. (eds). Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 63-105.

Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A. et al. (2008) The RAST server: rapid annotations using subsystems technology. BMC Genomics 9: 75.

Banerjee, R., and Ragsdale, S.W. (2003) The many faces of vitamin b12: catalysis by cobalamin-dependent enzymes. Annual Review of Biochemistry 72: 209-247.

Bedard, D.L., and Van Dort, H.M. (1998) Complete reductive dehalogenation of brominated biphenyls by anaerobic microorganisms in sediment. Applied and Environmental Microbiology 64: 940-947.

Bhatt, P., Kumar, M.S., Mudliar, S. & Chakrabarti, T. (2007) Biodegradation of chlorinated compounds—a review. Critical Reviews in Environmental Science and Technology 37: 165-198.

Bingle, L.E.H., Bailey, C.M., and Pallen, M.J. (2008) Type VI secretion: a beginner's guide. Current Opinion in Microbiology 11: 3-8.

Blanco, P., Hernando-Amado, S., Reales-Calderon, J.A., Corona, F., Lira, F., Alcalde-Rico, M. et al. (2016) Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants. Microorganisms 4: 14.

Blatch, G.L., and Lassle, M. (1999) The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. Bioessays 21: 932-939.

Bommer, M., Kunze, C., Fesseler, J., Schubert, T., Diekert, G., and Dobbek, H. (2014) Structural basis for organohalide respiration. Science 346: 455-458.

Boyle, A.W., Phelps, C.D., and Young, L.Y. (1999) Isolation from estuarine sediments of a *Desulfovibrio* strain which can grow on lactate coupled to the reductive dehalogenation of 2,4,6-tribromophenol. Applied and Environmental Microbiology 65: 1133-1140.

Bradley, P.M. (2003) History and ecology of chloroethene biodegradation: a review. Bioremediation Journal 7: 81-109.

Burse, A., Weingart, H., and Ullrich, M.S. (2004) The phytoalexin-inducible multidrug efflux pump acrab contributes to virulence in the fire blight pathogen, *Erwinia amylovora*. Molecular Plant-Microbe Interactions 17: 43-54.

Bush, M., and Dixon, R. (2012) The role of bacterial enhancer binding proteins as specialized activators of σ54-dependent transcription. Microbiology and Molecular Biology Reviews 76: 497-529.

Chang, B.-V., Yuan, S.-Y., and Ren, Y.-L. (2012) Anaerobic degradation of tetrabromobisphenol-A in river sediment. Ecological Engineering 49: 73-76.

Chen, K., Huang, L., Xu, C., Liu, X., He, J., Zinder, S.H., Li, S. & Jiang, J. (2013) Molecular characterization of the enzymes involved in the degradation of a brominated aromatic herbicide. Molecular Microbiology 89: 1121-1139.

Choudhary, P.K., Duret, A., Rohrbach-Brandt, E., Holliger, C., Sigel, R.K.O., and Maillard, J. (2013) Diversity of cobalamin riboswitches in the corrinoid-producing

organohalide respirer *Desulfitobacterium hafniense*. Journal of Bacteriology 195: 5186-5195.

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M., and Robles, M. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21: 3674-3676.

Cooper, M., Wagner, A., Wondrousch, D., Sonntag, F., Sonnabend, A., Brehm, M. et al. (2015) Anaerobic microbial transformation of halogenated aromatics and fate prediction using electron density modeling. Environmental Science & Technology 49: 6018-6028.

Covaci, A., Harrad, S., Abdallah, M.A.E., Ali, N., Law, R.J., Herzke, D., and de Wit, C.A. (2011) Novel brominated flame retardants: A review of their analysis, environmental fate and behaviour. Environment International 37: 532-556.

Crinnion, W.J. (2009) Chlorinated pesticides: threats to health and importance of detection. Alternative Medicine Review 14: 347-359.

Cupples, A.M., Sanford, R.A., and Sims, G.K. (2005) Dehalogenation of the herbicides bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and ioxynil (3,5-diiodino-4-hydroxybenzonitrile) by *Desulfitobacterium chlororespirans*. Applied and Environmental Microbiology 71: 3741-3746.

Darnerud, P.O. (2003) Toxic effects of brominated flame retardants in man and in wildlife. Environment international 29: 841-853.

De Wever, H., Cole, J.R., Fettig, M.R., Hogan, D.A., Tiedje, J.M. (2000) Reductive dehalogenation of trichloroacetic acid by *Trichlorobacter thiogenes* gen. nov., sp. nov. Applied and Environmental Microbiology 66:2297-2301.

Dermoun, Z., De Luca, G., Asso, M., Bertrand, P., Guerlesquin, F. & Guigliarelli, B. (2002) The NADP-reducing hydrogenase from *Desulfovibrio fructosovorans*: functional interaction between the C-terminal region of HndA and the N-terminal region of HndD subunits. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1556: 217-225.

DeWeerd, K., Mandelco, L., Tanner, R., Woese, C. & Suflita, J. (1990) *Desulfomonile tiedjei* gen. nov. and sp. nov., a novel anaerobic, dehalogenating, sulfate-reducing bacterium. Archives of Microbiology 154: 23-30.

Doherty, R.E. (2000) A history of the production and use of carbon tetrachloride, tetrachloroethylene, trichloroethylene and 1,1,1-trichloroethane in the United States: part 2-trichloroethylene and 1,1,1-trichloroethane. Environmental Forensics 1: 83-93.

Dolfing, J. (2016) Energetic Considerations in Organohalide Respiration. In Organohalide-Respiring Bacteria. Adrian, L., and Löffler, F.E. (eds). Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 31-48.

Ebel, R., Brenzinger, M., Kunze, A., Gross, H.J., and Proksch, P. (1997) Wound activation of protoxins in marine sponge *Aplysina aerophoba*. Journal of Chemical Ecology 23: 1451-1462.

Fennell, D.E., Rhee, S.-K., Ahn, Y.-B., Häggblom, M.M., and Kerkhof, L.J. (2004) Detection and characterization of a dehalogenating microorganism by terminal restriction fragment length polymorphism fingerprinting of 16S rRNA in a sulfidogenic, 2-bromophenol-utilizing enrichment. Applied and Environmental Microbiology 70: 1169-1175.

Filloux, A., Hachani, A. & Bleves, S. (2008) The bacterial type VI secretion machine: yet another player for protein transport across membranes. Microbiology 154: 1570-1583.

Fincker, M., and Spormann, A.M. (2017) Biochemistry of catabolic reductive dehalogenation. Annual Review of Biochemistry 86: 357-386.

Francke, C., Groot Kormelink, T., Hagemeijer, Y., Overmars, L., Sluijter, V., Moezelaar, R., and Siezen, R.J. (2011) Comparative analyses imply that the enigmatic sigma factor 54 is a central controller of the bacterial exterior. BMC Genomics 12: 385.

Futagami, T., Goto, M., and Furukawa, K. (2008) Biochemical and genetic bases of dehalorespiration. Chemical Record 8: 1-12.

Futagami, T., Morono, Y., Terada, T., Kaksonen, A.H., and Inagaki, F. (2009) Dehalogenation activities and distribution of reductive dehalogenase homologous genes in marine subsurface sediments. Applied and Environmental Microbiology 75: 6905-6909.

Futagami, T., Morono, Y., Terada, T., Kaksonen, A.H., and Inagaki, F. (2013) Distribution of dehalogenation activity in subseafloor sediments of the Nankai Trough subduction zone. Philosophical Transactions of the Royal Society B: Biological Sciences 368: 20120249.

Futagami, T., Yamaguchi, T., Nakayama, S.-i., Goto, M., and Furukawa, K. (2006) Effects of chloromethanes on growth of and deletion of the *pce* gene cluster in dehalorespiring *Desulfitobacterium hafniense* Strain Y51. Applied and Environmental Microbiology 72: 5998-6003.

Gabor, K. (2006). Molecular analysis of halorespiration in *Desulfitobacterium* spp. Doctoral, Wageningen University.

Gábor, K., Hailesellasse Sene, K., Smidt, H., de Vos, W.M., and van der Oost, J. (2008) Divergent roles of CprK paralogues from *Desulfitobacterium hafniense* in activating gene expression. Microbiology 154: 3686-3696.

Gábor, K., Veríssimo, C.S., Cyran, B.C., ter Horst, P., Meijer, N.P., Smidt, H. et al. (2006) Characterization of CprK1, a CRP/FNR-type transcriptional regulator of halorespiration from *Desulfitobacterium hafniense*. Journal of Bacteriology 188: 2604-2613.

Ghambeer, R.K., Wood, H.G., Schulman, M., and Ljungdahl, L. (1971) Total synthesis of acetate from CO_2. 3. Inhibition by alkylhalides of the synthesis from CO_2, methyltetrahydrofolate, and methyl-B12 by *Clostridium thermoaceticum*. Archives of Biochemistry and Biophysics 143: 471-484.

Gnerre, S., MacCallum, I., Przybylski, D., Ribeiro, F.J., Burton, J.N., Walker, B.J. et al. (2011) High-quality draft assemblies of mammalian genomes from massively parallel sequence data. Proceedings of the National Academy of Sciences 108: 1513-1518.

Goldberg, T., Hamp, T., and Rost, B. (2012) LocTree2 predicts localization for all domains of life. Bioinformatics 28: i458-i465.

Goris, J., Konstantinidis, K.T., Klappenbach, J.A., Coenye, T., Vandamme, P. & Tiedje, J.M. (2007) DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. International Journal of Systematic and Evolutionary Microbiology 57: 81-91.

Goris, T., Schiffmann, C.L., Gadkari. J., Schubert. T., Seifert. J., Jehmlich. N., von Bergen, M. & Diekert. G. (2015) Proteomics of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* adapted to tetrachloroethene and other energy substrates. Scientific Reports 5: 13794.

Goris, T., Schubert, T., Gadkari, J., Wubet, T., Tarkka, M., Buscot, F., Adrian, L., Diekert, G. (2014) Insights into organohalide respiration and the versatile catabolism of *Sulfurospirillum multivorans* gained from comparative genomics and physiological studies. Environmental Microbiology 16: 3562-3580.

Gribble GW (1998) Naturally occurring organohalogen compounds. Accounts of Chemical Research 31: 141-152.

Gribble, G. (2000) The natural production of organobromine compounds. Environmental Science and Pollution Research 7: 37-49.

Gribble, G.W. (1992) Naturally occurring organohalogen compounds - A survey. Journal of Natural Products 55: 1353-1395.

Gribble, G.W. (2010) Naturally 0ccurring organohalogen compounds - A comprehensive update: Springer-Verlag Wien.

Häggblom, M.M. & Bossert, I.D. (2003) Microbial Processes and Environmental Applications. Springer.

Häggblom, M.M., and Young, L.Y. (1995) Anaerobic degradation of halogenated phenols by sulfate-reducing consortia. Applied and Environmental Microbiology 61: 1546-1550.

Haider, S. & Pal, R.(2013) Integrated analysis of transcriptomic and proteomic data. Current Genomics 14: 91-110.

Haouari, O., Fardeau, M.-L., Casalot, L., Tholozan, J.-L., Hamdi, M., and Ollivier, B. (2006) Isolation of sulfate-reducing bacteria from Tunisian marine sediments and

description of *Desulfovibrio bizertensis* sp. nov. International Journal of Systematic and Evolutionary Microbiology 56: 2909-2913.

Harel, A., Falkowski, P., and Bromberg, Y. (2012) TrAnsFuSE refines the search for protein function: oxidoreductases. Integrative Biology 4: 765-777.

He, J.Z., Robrock, K.R., and Alvarez-Cohen, L. (2006) Microbial reductive debromination of polybrominated diphenyl ethers (PBDEs). Environmental Science & Technology 40: 4429-4434.

Hendrickson, E.R., Payne, J.A., Young, R.M., Starr, M.G., Perry, M.P., Fahnestock, S., Ellis, D.E. & Ebersole, R.C. (2002) Molecular analysis of *Dehalococcoides* 16S ribosomal DNA from chloroethene-contaminated sites throughout North America and Europe. Applied and Environmental Microbiology 68: 485-495.

Hentschel, U., Fieseler, L., Wehrl, M., Gernert, C., Steinert, M., Hacker, J., and Horn, M. (2003) Microbial Diversity of Marine Sponges. In Sponges (Porifera). Müller, W.G. (ed): Springer Berlin Heidelberg, pp. 59-88.

Hentschel, U., Piel, J., Degnan, S.M., and Taylor, M.W. (2012) Genomic insights into the marine sponge microbiome. Nature Reviews Microbiology 10: 641-U675.

Heytler, P.G. (1980) Uncouplers of oxidative phosphorylation. Pharmacology & Therapeutics 10: 461-472.

Hiraishi, A. & Kawagishi, T. (2002) Effects of chemical uncouplers on microbial biomass production, metabolic activity, and community structure in an activated sludge system. Microbes and Environments 17: 197-204.

Holliger, C., Hahn, D., Harmsen, H., Ludwig, W., Schumacher, W., Tindall, B. et al. (1998) *Dehalobacter restrictus* gen. nov. and sp. nov., a strictly anaerobic bacterium that reductively dechlorinates tetra- and trichloroethene in an anaerobic respiration. Archives of Microbiology 169: 313-321.

Hug, L.A., and Edwards, E.A. (2013) Diversity of reductive dehalogenase genes from environmental samples and enrichment cultures identified with degenerate primer PCR screens. Frontiers in Microbiology 4: 341.

Hug, L.A., Maphosa, F., Leys, D., Löffler, F.E., Smidt, H., Edwards, E.A., and Adrian, L. (2013) Overview of organohalide-respiring bacteria and a proposal for a classification system for reductive dehalogenases. Philosophical Transactions of the Royal Society B: Biological Sciences 368: 20120322.

Inagaki, F., Nunoura, T., Nakagawa, S., Teske, A., Lever, M., Lauer, A. et al. (2006) Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin. Proceedings of the National Academy of Sciences 103: 2815-2820.

Inagaki, F., Suzuki, M., Takai, K., Oida, H., Sakamoto, T., Aoki, K. et al. (2003) Microbial communities associated with geological horizons in coastal subseafloor sediments from the sea of Okhotsk. Applied and Environmental Microbiology 69: 7224-7235.

Jayachandran, G., Görisch, H. & Adrian, L. (2004) Studies on hydrogenase activity and chlorobenzene respiration in *Dehalococcoides* sp. strain CBDB1. Archives of Microbiology 182: 498-504.

Jo, Y.J., Lee, J.Y., Yi, M.J., Kim, H.S. & Lee, K.K. (2010) Soil contamination with TCE in an industrial complex: contamination levels and implication for groundwater contamination. Geosciences Journal 14: 313-320.

Joyce, M.G., Levy, C., Gábor, K., Pop, S.M., Biehl, B.D., Doukov, T.I. et al. (2006) CprK crystal structures reveal mechanism for transcriptional control of halorespiration. Journal of Biological Chemistry 281: 28318-28325.

Jugder, B.-E., Ertan, H., Bohl, S., Lee, M., Marquis, C.P. & Manefield. M. (2016a) Organohalide respiring bacteria and reductive dehalogenases: key tools in organohalide bioremediation. Frontiers in Microbiology 7: 249.

Jugder, B.-E., Ertan, H., Lee, M., Manefield, M., and Marquis, C.P. (2015) Reductive dehalogenases come of age in biological destruction of organohalides. Trends in Biotechnology 33: 595-610.

Jugder, B.-E., Ertan, H., Wong, Y.K., Braidy, N., Manefield, M., Marquis, C.P., and Lee, M. (2016b) Genomic, transcriptomic and proteomic analyses of *Dehalobacter* UNSWDHB in response to chloroform. Environmental Microbiology Reports 8: 814-824.

Jugder, B.-E., Payne, K.A.P., Fisher, K., Bohl, S., Lebhar, H., Manefield, M. et al. (2018) Heterologous production and purification of a functional chloroform reductive dehalogenase. ACS Chemical Biology 13: 548-552.

Kawai, M., Futagami, T., Toyoda, A., Takaki, Y., Nishi, S., Hori, S. et al. (2014) High frequency of phylogenetically diverse reductive dehalogenase-homologous genes in deep subseafloor sedimentary metagenomes. Frontiers in Microbiology 5: 80.

Kazakov, A.E., Rajeev, L., Chen, A., Luning, E.G., Dubchak, I., Mukhopadhyay, A., and Novichkov, P.S. (2015) σ(54)-dependent regulome in *Desulfovibrio vulgaris* Hildenborough. BMC Genomics 16: 919.

Keller, S., Ruetz, M., Kunze, C., Kräutler, B., Diekert, G., and Schubert, T. (2014) Exogenous 5,6-dimethylbenzimidazole caused production of a non-functional tetrachloroethene reductive dehalogenase in *Sulfurospirillum multivorans*. Environmental Microbiology 16: 3361-3369.

Kemp, L.R., Dunstan, M.S., Fisher, K., Warwicker, J., and Leys, D. (2013) The transcriptional regulator CprK detects chlorination by combining direct and indirect readout mechanisms. Philosophical Transactions of the Royal Society of London B: Biological Sciences 368: 20120323.

Kim, S.-H., Harzman, C., Davis, J.K., Hutcheson, R., Broderick, J.B., Marsh. T.L. & Tiedje, J.M. (2012) Genome sequence of *Desulfitobacterium hafniense* DCB-2, a Gram-positive anaerobe capable of dehalogenation and metal reduction. BMC Microbiology 12: 1-20.

Krajmalnik-Brown, R., Sung, Y., Ritalahti, K.M., Michael Saunders, F., and Löffler, F.E. (2007) Environmental distribution of the trichloroethene reductive dehalogenase gene (*tceA*) suggests lateral gene transfer among *Dehalococcoides*. FEMS Microbiology Ecology 59: 206-214.

Krasper, L., Lilie, H., Kublik, A., Adrian, L., Golbik, R., and Lechner, U. (2016) The MarR-type regulator Rdh2R regulates rdh gene transcription in *Dehalococcoides mccartyi* strain CBDB1. Journal of Bacteriology198: 3130-3141.

Krumholz, L.R. 1997. *Desulfuromonas chloroethenica* sp. nov. uses tetrachloroethylene and trichloroethylene as electron acceptors. International Journal of Systematic and Evolutionary Microbiology 47:1262-1263.

Kruse, S., Goris, T., Wolf, M., Wei, X., and Diekert, G. (2017) The NiFe hydrogenases of the tetrachloroethene-respiring Epsilonproteobacterium *Sulfurospirillum multivorans*: biochemical studies and transcription analysis. Frontiers in Microbiology 8: 444.

Kruse, T., Goris, T., Maillard, J., Woyke, T., Lechner, U., de Vos, W., and Smidt, H. (2017) Comparative genomics of the genus *Desulfitobacterium*. FEMS Microbiology Ecology 93: fix135.

Kruse, T., Maillard, J., Goodwin, L., Woyke, T., Teshima, H., Bruce, D. et al. (2013) Complete genome sequence of *Dehalobacter restrictus* PER-K23T. Standards in Genomic Sciences 8: 375-388.

Kruse, T., Smidt, H., and Lechner, U. (2016) Comparative genomics and transcriptomics of organohalide- respiring bacteria and regulation of *rdh* gene transcription. In Organohalide-Respiring Bacteria. Adrian, L. and Löffler, F.E. (eds). Springer-Verlag Berlin Heidelberg, pp 345-376.

Kruse, T., van de Pas, B.A., Atteia, A., Krab, K., Hagen, W.R., Goodwin, L. et al. (2015) Genomic, proteomic, and biochemical analysis of the organohalide respiratory pathway in *Desulfitobacterium dehalogenans*. Journal of Bacteriology 197: 893-904.

Kube, M., Beck, A., Zinder, S.H., Kuhl, H., Reinhardt, R., and Adrian, L. (2005) Genome sequence of the chlorinated compound-respiring bacterium *Dehalococcoides species* strain CBDB1. Nature Biotechnology 23: 1269-1273.

Kublik, A., Deobald, D., Hartwig, S., Schiffmann, C.L., Andrades, A., von Bergen, M., Sawers, R.G. & Adrian, L. (2016) Identification of a multi-protein reductive dehalogenase complex in *Dehalococcoides mccartyi* strain CBDB1 suggests a protein-dependent respiratory electron transport chain obviating quinone involvement. Environmental Microbiology 18:3044-3056.

Kumar, S., Stecher, G., and Tamura, K. (2016) MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33: 1870-1874.

Lee, L.K., Ding, C., Yang, K.-L., and He, J. (2011) Complete debromination of tetra- and penta-brominated diphenyl ethers by a coculture consisting of

Dehalococcoides and *Desulfovibrio* species. Environmental Science & Technology45: 8475-8482.

Levy, C., Pike, K., Heyes, D.J., Joyce, M.G., Gabor, K., Smidt, H., van der Oost, J. & Leys, D. (2008) Molecular basis of halorespiration control by CprK, a CRP-FNR type transcriptional regulator. Molecular Microbiology 70: 151-167.

Li, X., Luo, Q., Wofford, N.Q., Keller, K.L., McInerney, M.J., Wall, J.D. & Krumholz, L.R. (2009) A molybdopterin oxidoreductase is involved in H2 oxidation in *Desulfovibrio desulfuricans* G20. Journal of Bacteriology 191: 2675-2682.

Liesack, W., and Finster, K. (1994) Phylogenetic analysis of five strains of gram-negative, obligately anaerobic, sulfur-reducing bacteria and description of *Desulfuromusa* gen. nov., including *Desulfuromusa kysingii* sp. nov., *Desulfuromusa bakii* sp. nov., and *Desulfuromusa succinoxidans* sp. nov. International Journal of Systematic Bacteriology 44: 753-758.

Lincoln, D.E., Fielman, K.T., Marinelli, R.L., and Woodin, S.A. (2005) Bromophenol accumulation and sediment contamination by the marine annelids *Notomastus lobatus* and *Thelepus crispus*. Biochemical Systematics and Ecology 33: 559-570.

Lira, N.S., Montes, R.C., Tavares, J.F., Silva, M.S.d., Cunha, E.V.L.d., Athayde-Filho, P.F.d. et al. (2011) Brominated compounds from marine sponges of the genus *aplysina* and a compilation of their 13C NMR spectral data. Marine Drugs 9: 2316.

Liu, J., Lopez, N., Ahn, Y., Goldberg, T., Bromberg, Y., Kerkhof, L.J., and Haggblom, M.M. (2017) Novel reductive dehalogenases from the marine sponge associated bacterium *Desulfoluna spongiiphila*. Environmental Microbiology Reports 9: 537-549.

Liu, J., Wang, Y., Jiang, B., Wang, L., Chen, J., Guo, H., and Ji, R. (2013) Degradation, metabolism, and bound-residue formation and release of tetrabromobisphenol A in soil during sequential anoxic–oxic incubation. Environmental Science & Technology 47: 8348-8354.

Lobo, S.A.L., Brindley, A.A., Romão, C.V., Leech, H.K., Warren, M.J., and Saraiva, L.M. (2008) Two distinct roles for two functional cobaltochelatases (CbiK) in *Desulfovibrio vulgaris* Hildenborough. Biochemistry 47: 5851-5857.

Löffler, F.E., Yan, J., Ritalahti, K.M., Adrian, L., Edwards, E.A., Konstantinidis, K.T. et al. (2013) *Dehalococcoides mccartyi* gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, *Dehalococcoidia* classis nov., order *Dehalococcoidales* ord. nov. and family *Dehalococcoidaceae* fam. nov., within the phylum Chloroflexi. International Journal of Systematic Bacteriology 63: 625-635.

Louie, T.M., and Mohn, W.W. (1999) Evidence for a chemiosmotic model of dehalorespiration in *Desulfomonile tiedjei* DCB-1. Journal of Bacteriology 181: 40-46.

Love, M.I., Huber, W., and Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15: 550.

Luijten, M.L.G.C., de Weert, J., Smidt, H., Boschker, H.T.S., de Vos, W.M., Schraa, G. & Stams, A.J.M. (2003) Description *of Sulfurospirillum halorespirans* sp. nov., an anaerobic, tetrachloroethene-respiring bacterium, and transfer of *Dehalospirillum multivorans* to the genus *Sulfurospirillum* as *Sulfurospirillum multivorans* comb. nov. International Journal of Systematic Bacteriology 53: 787-793.

Mac Nelly, A., Kai, M., Svatoš, A., Diekert, G. & Schubert, T. (2014) Functional heterologous production of reductive dehalogenases from *Desulfitobacterium hafniense* strains. Applied and Environmental Microbiology 80: 4313-4322.

Magnuson, J.K., Stern, R.V., Gossett, J.M., Zinder, S.H., and Burris, D.R. (1998) Reductive dechlorination of tetrachloroethene to ethene by a two-component enzyme pathway. Applied and Environmental Microbiology 64: 1270-1275.

Maillard, J., Schumacher, W., Vazquez, F., Regeard, C., Hagen, W.R. & Holliger, C. (2003) Characterization of the corrinoid iron-sulfur protein tetrachloroethene reductive dehalogenase of *Dehalobacter restrictus*. Applied and Environmental Microbiology 69: 4628-4638.

Maillard, J., Regeard, C., and Holliger, C. (2005) Isolation and characterization of Tn-Dha1, a transposon containing the tetrachloroethene reductive dehalogenase of *Desulfitobacterium hafniense* strain TCE1. Environmental Microbiology 7: 107-117.

Mao, X., Polasko, A., and Alvarez-Cohen, L. (2017) Effects of sulfate reduction on trichloroethene dechlorination by Dehalococcoides-containing microbial communities. Applied and Environmental Microbiology 83: e03384-16

Maphosa, F., de Vos, W.M., and Smidt, H. (2010) Exploiting the ecogenomics toolbox for environmental diagnostics of organohalide-respiring bacteria. Trends in Biotechnology 28: 308-316.

Markowitz, V.M., Chen, I.M.A., Palaniappan, K., Chu, K., Szeto, E., Grechkin, Y. et al. (2012) IMG: the integrated microbial genomes database and comparative analysis system. Nucleic Acids Research 40: D115-D122.

Marshall, I.P.G., Karst, S.M., Nielsen, P.H., and Jørgensen, B.B. (2018) Metagenomes from deep Baltic Sea sediments reveal how past and present environmental conditions determine microbial community composition. Marine Genomics 37: 58-68.

Matthews, R.G. (2009) Cobalamin-and corrinoid-dependent enzymes. Metal Ions in Life Sciences 6: 53.

May, H.D., Miller, G.S., Kjellerup, B.V. & Sowers, K.R. (2008) Dehalorespiration with polychlorinated biphenyls by an anaerobic ultramicrobacterium. Applied and Environmental Microbiology 74: 2089-2094.

Mayer-Blackwell, K., Sewell, H., Fincker, M., and Spormann, A.M. (2016) comparative physiology of organohalide-respiring bacteria. In Organohalide-Respiring Bacteria. Adrian, L., and Löffler, F.E. (eds). Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 259-280.

Mazon, H., Gábor, K., Leys, D., Heck, A.J.R., van der Oost, J., and van den Heuvel, R.H.H. (2007) Transcriptional activation by CprK1 is regulated by protein structural changes induced by effector binding and redox state. Journal of Biological Chemistry 282: 11281-11290.

Men, Y., Lee, P.K.H., Harding, K.C., and Alvarez-Cohen, L. (2013) Characterization of four TCE-dechlorinating microbial enrichments grown with different cobalamin stress and methanogenic conditions. Applied Microbiology and Biotechnology 97: 6439-6450.

Men, Y., Seth, E.C., Yi, S., Allen, R.H., Taga, M.E., and Alvarez-Cohen, L. (2014) Sustainable growth of *Dehalococcoides mccartyi* 195 by corrinoid salvaging and remodeling in defined lactate-fermenting consortia. Applied and Environmental Microbiology 80: 2133-2141.

Men, Y., Seth, E.C., Yi, S., Crofts, T.S., Allen, R.H., Taga, M.E., and Alvarez-Cohen, L. (2015) Identification of specific corrinoids reveals corrinoid modification in dechlorinating microbial communities. Environmental Microbiology 17: 4873-4884.

Miller, E., Wohlfarth, G., and Diekert, G. (1998) Purification and characterization of the tetrachloroethene reductive dehalogenase of strain PCE-S. Archives Microbiology 169: 497-502.

Mizuno, N., Voordouw, G., Miki, K., Sarai, A., and Higuchi, Y. (2003) Crystal structure of dissimilatory sulfite reductase D (DsrD) protein—possible interaction with B- and Z-DNA by its winged-helix motif. Structure 11: 1133-1140.

Moe, W.M., Yan, J., Nobre, M.F., da Costa, M.S., and Rainey, F.A. (2009) *Dehalogenimonas lykanthroporepellens* gen. nov., sp. nov., a reductively dehalogenating bacterium isolated from chlorinated solvent-contaminated groundwater. International Journal of Systematic Bacteriology 59: 2692-2697.

Mohn, W.W., and Tiedje, J.M. (1992) Microbial reductive dehalogenation. Microbiological Reviews 56: 482-507.

Monserrate, E., and Häggblom, M.M. (1997) Dehalogenation and biodegradation of brominated phenols and benzoic acids under iron-reducing, sulfidogenic, and methanogenic conditions. Applied and Environmental Microbiology 63: 3911-3915.

Moore, T.C., and Escalante-Semerena, J.C. (2016) corrinoid metabolism in dehalogenating pure cultures and microbial communities. In Organohalide-Respiring Bacteria. Adrian, L., and Löffler, F.E. (eds). Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 455-484.

Morais-Silva, F.O., Santos, C.I., Rodrigues, R., Pereira, I.A.C., and Rodrigues-Pousada, C. (2013) Roles of HynAB and Ech, the only two hydrogenases found in the model sulfate reducer *Desulfovibrio gigas*. Journal of Bacteriology 195: 4753-4760.

Moran, M.J., Zogorski. J.S. & Squillace, P.J. (2007) Chlorinated solvents in groundwater of the United States. Environmental Science & Technology 41: 74-81.

Neumann, A., Wohlfarth, G. & Diekert, G. (1996) Purification and characterization of tetrachloroethene reductive dehalogenase from *Dehalospirillum multivorans*. Journal of Biological Chemistry 271: 16515-16519.

Ni, H.-G., Zeng, H., Tao, S., and Zeng, E.Y. (2010) Environmental and human exposure to persistent halogenated compounds derived from e-waste in China. Environmental Toxicology and Chemistry 29: 1237-1247.

Ni, S., Fredrickson, J.K., and Xun, L. (1995) Purification and characterization of a novel 3-chlorobenzoate-reductive dehalogenase from the cytoplasmic membrane of *Desulfomonile tiedjei* DCB-1. Journal of Bacteriology 177: 5135-5139.

Nonaka, H., Keresztes, G., Shinoda, Y., Ikenaga, Y., Abe, M., Naito, K. et al. (2006) Complete genome sequence of the dehalorespiring bacterium *Desulfitobacterium hafniense* Y51 and comparison with *Dehalococcoides ethenogenes* 195. Journal of Bacteriology 188: 2262-2274.

Norte, M., Rodriguez, L., Fernandez, J. J., Eguren, L. and Estrada, D. M. (1988) Aplysinadiene and (r,r) 5 [3:5-dibromo-4-[(2-oxo-5-oxazolidinyl)] methoxyphenyl]-2-oxazolidinone, two novel metabolites from *Aplysina aerophoba*. Synthesis of Aplysindiene. Tetrahedron 44:4973–4980.

Odom, J.M., and Peck, H.D. (1981) Hydrogen cycling as a general mechanism for energy coupling in the sulfate-reducing bacteria, *Desulfovibrio* sp. FEMS Microbiology Letters 12: 47-50.

Palmer, T., and Berks, B.C. (2012) The twin-arginine translocation (Tat) protein export pathway. Nature Reviews Microbiology 10: 483.

Parthasarathy, A., Stich, T.A., Lohner, S.T., Lesnefsky, A., Britt, R.D. & Spormann, A.M. (2015) Biochemical and EPR-spectroscopic investigation into heterologously expressed vinyl chloride reductive dehalogenase (VcrA) from *Dehalococcoides mccartyi* strain VS. Journal of the American Chemical Society 137: 3525-3532.

Payne, K.A., Quezada, C.P., Fisher, K., Dunstan, M.S., Collins, F.A., Sjuts, H. et al. (2015) Reductive dehalogenase structure suggests a mechanism for B12-dependent dehalogenation. Nature 517: 513-516.

Pée, K.-H.v. (1996) Biosynthesis of halogenated metabolites by bacteria. Annual Review of Microbiology 50: 375-399.

Peng, X., Yamamoto, S., Vertès, A., Keresztes, G., Inatomi, K.-i., Inui, M., and Yukawa, H. (2012) Global transcriptome analysis of the tetrachloroethene-dechlorinating bacterium *Desulfitobacterium hafniense* Y51 in the presence of various electron donors and terminal electron acceptors. Journal of Industrial Microbiology & Biotechnology 39: 255-268.

Pereira, I.A.C., Ramos, A.R., Grein, F., Marques, M.C., da Silva, S.M. & Venceslau, S.S. (2011) A comparative genomic analysis of energy metabolism in sulfate reducing bacteria and archaea. Frontiers in Microbiology 2: 69.

Prat, L., Maillard, J., Grimaud, R., and Holliger, C. (2011) Physiological adaptation of *Desulfitobacterium hafniense* strain TCE1 to tetrachloroethene respiration. Applied and Environmental Microbiology 77: 3853-3859.

Rabus, R., Venceslau, S.S., Wöhlbrand, L., Voordouw, G., Wall, J.D., and Pereira, I.A.C. (2015) Chapter Two - A post-genomic view of the ecophysiology, catabolism and biotechnological relevance of sulphate-reducing prokaryotes. In Advances in Microbial Physiology. Poole, R.K. (ed): Academic Press, pp. 55-321.

Ragsdale, S.W. & Pierce, E. (2008) Acetogenesis and the Wood-Ljungdahl pathway of CO(2) fixation. Biochimica et biophysica acta 1784: 1873-1898.

Regeard, C., Maillard, J., Dufraigne, C., Deschavanne, P., and Holliger, C. (2005) Indications for acquisition of reductive dehalogenase genes through horizontal gene transfer by *Dehalococcoides ethenogenes* strain 195. Applied and Environmental Microbiology 71: 2955-2961.

Rhee, S.-K., Fennell, D.E., Häggblom, M.M., and Kerkhof, L.J. (2003) Detection by PCR of reductive dehalogenase motifs in a sulfidogenic 2-bromophenol-degrading consortium enriched from estuarine sediment. FEMS Microbiology Ecology 43: 317-324.

Richter, M. & Rosselló-Móra, R. (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proceedings of the National Academy of Sciences 106: 19126-19131.

Rodionov, D.A., Dubchak, I., Arkin, A., Alm, E. & Gelfand, M.S. (2004) Reconstruction of regulatory and metabolic pathways in metal-reducing δ-proteobacteria. Genome Biology 5: R90.

Rupakula, A., Kruse, T., Boeren, S., Holliger, C., Smidt, H., and Maillard, J. (2013) The restricted metabolism of the obligate organohalide respiring bacterium *Dehalobacter restrictus*: lessons from tiered functional genomics. Philosophical Transactions of the Royal Society of London B: Biological Sciences 368: 20120325.

Rupakula, A., Lu, Y., Kruse, T., Boeren, S., Holliger, C., Smidt, H., and Maillard, J. (2015) Functional genomics of corrinoid starvation in the organohalide-respiring bacterium Dehalobacter restrictus strain PER-K23. Frontiers in Microbiology 5:751.

Saiyari, D.M., Chuang, H.-P., Senoro, D.B., Lin, T.-F., Whang, L.-M., Chiu, Y.-T., and Chen, Y.-H. (2018) A review in the current developments of genus *Dehalococcoides*, its consortia and kinetics for bioremediation options of contaminated groundwater. Sustainable Environment Research 28: 149-157.

Sanford RA, Cole JR, Tiedje JM. 2002. Characterization and description of *Anaeromyxobacter dehalogenans* gen. nov., sp. nov., an aryl-halorespiring facultative anaerobic *Myxobacterium*. Applied and Environmental Microbiology 68:893-900.

Sanford, R. A., J. Chowdhary and F. E. Löffler (2016). Organohalide-Respiring Deltaproteobacteria. Organohalide-Respiring Bacteria. L. Adrian and F. E. Löffler. Berlin, Heidelberg, Springer Berlin Heidelberg: 235-258.

Santos, F.J., and Galceran, M.T. (2002) The application of gas chromatography to environmental analysis. TrAC Trends in Analytical Chemistry 21: 672-685.

Schiffmann, C.L., Jehmlich, N., Otto, W., Hansen, R., Nielsen, P.H., Adrian, L., Seifert, J. & von Bergen, M. (2014) Proteome profile and proteogenomics of the organohalide-respiring bacterium *Dehalococcoides mccartyi* strain CBDB1 grown on hexachlorobenzene as electron acceptor. Journal of Proteomics 98: 59-64.

Schipp, C.J., Marco-Urrea, E., Kublik, A., Seifert, J., and Adrian, L. (2013) Organic cofactors in the metabolism of *Dehalococcoides mccartyi* strains. Philosophical Transactions of the Royal Society B: Biological Sciences 368: 20120321.

Scholz-Muramatsu, H., Neumann, A., Meßmer, M., Moore, E., and Diekert, G. (1995) Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. Archives of Microbiology 163: 48-56.

Schubert, T., Adrian, L., Sawers, R.G. & Diekert, G. (2018) Organohalide respiratory chains: composition, topology and key enzymes. FEMS Microbiology Ecology 94: fiy035.

Schubert, T. (2017) The organohalide-respiring bacterium *Sulfurospirillum multivorans*: a natural source for unusual cobamides. World Journal of Microbiology and Biotechnology 33: 93.

Schumacher, W., and Holliger, C. (1996) The proton/electron ration of the menaquinone-dependent electron transport from dihydrogen to tetrachloroethene in "*Dehalobacter restrictus*". Journal of Bacteriology 178: 2328-2333.

Schumacher, W., Holliger, C., Zehnder, A.J.B., and Hagen, W.R. (1997) Redox chemistry of cobalamin and iron-sulfur cofactors in the tetrachloroethene reductase of *Dehalobacter restrictus*. FEBS Letters 409: 421-425.

Seidel, K., Kühnert, J. & Adrian, L. (2018) The complexome of *Dehalococcoides mccartyi* reveals its organohalide respiration-complex is modular. Frontiers in Microbiology 9:1130.

Seshadri, R., Adrian, L., Fouts, D.E., Eisen, J.A., Phillippy, A.M., Methe, B.A. et al. (2005) Genome sequence of the pce-dechlorinating bacterium *Dehalococcoides ethenogenes*. Science 307: 105-108.

Shelton, D.R., and Tiedje, J.M. (1984) Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. Applied and Environmental Microbiology 48: 840-848.

Shingler, V. (1996) Signal sensing by sigma54-dependent regulators: depression as a control mechanism. Molecular Microbiology 19: 409-416.

Shivani, Y., Subhash, Y., Sasikala, C. & Ramana, C.V. (2017) *Halodesulfovibrio spirochaetisodalis* gen. nov. sp. nov. and reclassification of four *Desulfovibrio* spp. International Journal of Systematic and Evolutionary Microbiology 67: 87-93.

Sim, W.-J., Lee, S.-H., Lee, I.-S., Choi, S.-D., and Oh, J.-E. (2009) Distribution and formation of chlorophenols and bromophenols in marine and riverine environments. Chemosphere 77: 552-558.

Sjuts, H., Fisher, K., Dunstan, M.S., Rigby, S.E. & Leys, D. (2012) Heterologous expression, purification and cofactor reconstitution of the reductive dehalogenase PceA from *Dehalobacter restrictus*. Protein Expression and Purification 85: 224-229.

Smidt, H., van Leest, M., van der Oost, J. & de Vos, W.M. (2000) Transcriptional regulation of the *cpr* gene cluster in *ortho*-chlorophenol-respiring *Desulfitobacterium dehalogenans*. Journal of Bacteriology 182: 5683-5691.

Smidt, H., and de Vos, W.M. (2004) Anaerobic microbial dehalogenation. Annual Review of Microbiology 58: 43-73.

Sohn, S.Y. & Häggblom, M.M. (2016) Reductive dehalogenation activity of indigenous microorganism in sediments of the Hackensack River, New Jersey. Environmental Pollution 214: 374-383.

Studholme, D.J., and Dixon, R. (2003) Domain architectures of σ54-dependent transcriptional activators. Journal of Bacteriology 185: 1757-1767.

Sun, B., Cole, J.R., and Tiedje, J.M. (2001) *Desulfomonile limimaris* sp. nov., an anaerobic dehalogenating bacterium from marine sediments. International Journal of Systematic and Evolutionary Microbiology 51: 365-371.

Sun, B., Cole, J.R., Sanford, R.A., and Tiedje, J.M. (2000) Isolation and characterization of *Desulfovibrio dechloracetivorans* sp. nov., a marine dechlorinating bacterium growing by coupling the oxidation of acetate to the reductive dechlorination of 2-chlorophenol. Applied and Environmental Microbiology 66: 2408-2413.

Sung, Y., Fletcher, K.E., Ritalahti, K.M., Apkarian, R.P., Ramos-Hernández, N., Sanford, R.A. et al. (2006) *Geobacter lovleyi* sp. nov. strain SZ, a novel metal-reducing and tetrachloroethene-dechlorinating bacterium. Applied Environmental Microbiology 72: 2775-2782.

Sung, Y., Ritalahti, K.M., Sanford, R.A., Urbance, J.W., Flynn, S.J., Tiedje, J.M., and Löffler, F.E. (2003) Characterization of two tetrachloroethene-reducing, acetate-oxidizing anaerobic bacteria and their description as *Desulfuromonas michiganensis* sp. nov. Applied and Environmental Microbiology 69: 2964-2974.

Takii, S., Hanada, S., Hase, Y., Tamaki, H., Uyeno, Y., Sekiguchi, Y., and Matsuura, K. (2008) *Desulfovibrio marinisediminis* sp. nov., a novel sulfate-reducing bacterium isolated from coastal marine sediment via enrichment with casamino acids. International Journal of Systematic and Evolutionary Microbiology 58: 2433-2438.

Tang, S. & Edwards, E.A. (2013) Identification of *Dehalobacter* reductive dehalogenases that catalyse dechlorination of chloroform, 1,1,1-trichloroethane and 1,1-dichloroethane. Philosophical Transactions of the Royal Society B: Biological Sciences 368: 20120318.

Teeyapant, R. & Proksch, P. (1993) Biotransformation of brominated compounds in the marine sponge *Verongia aerophoba* — Evidence for an induced chemical defense? Naturwissenschaften 80: 369-370.

Teeyapant, R., Kreis, P., Wray, V., Witte, L., and Proksch, P. (1993) Brominated secondary compounds from the marine sponge *Verongia aerophoba* and the sponge feeding gastropod *Tylodina perversa*. Zeitschrift für Naturforschung C 48: 640.

Thibodeau, J., Gauthier, A., Duguay, M., Villemur, R., Lépine, F., Juteau, P., and Beaudet, R. (2004) Purification, cloning, and sequencing of a 3,5-dichlorophenol reductive dehalogenase from *Desulfitobacterium frappieri* PCP-1. Applied and Environmental Microbiology 70: 4532-4537.

Türkowsky, D., Jehmlich, N., Diekert, G., Adrian, L., von Bergen, M., and Goris, T. (2018) An integrative overview of genomic, transcriptomic and proteomic analyses in organohalide respiration research. FEMS Microbiology Ecology: fiy013.

Turusov, V., Rakitsky, V. & Tomatis, L. (2002) Dichlorodiphenyltrichloroethane (DDT): ubiquity, persistence, and risks. Environmental Health Perspectives 110: 125-128.

Venceslau, S.S., Stockdreher, Y., Dahl, C. & Pereira, I.A.C. (2014) The "bacterial heterodisulfide" DsrC is a key protein in dissimilatory sulfur metabolism. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1837: 1148-1164.

Vetter, W. (2006) Marine halogenated natural products of environmental relevance. In Reviews of Environmental Contamination and Toxicology. Ware, G., Whitacre, D., Albert, L., de Voogt, P., Gerba, C., Hutzinger, O. et al. (eds): Springer New York, pp. 1-57.

Villemur, R. (2013) The pentachlorophenol-dehalogenating *Desulfitobacterium hafniense* strain PCP-1. Philosophical Transactions of the Royal Society B: Biological Sciences 368: 20120319.

Villemur, R., Lanthier, M., Beaudet, R., and Lépine, F. (2006) The *Desulfitobacterium* genus. FEMS Microbiology Reviews 30: 706-733.

Voordeckers, J.W., Fennell, D.E., Jones, K., and Häggblom, M.M. (2002) Anaerobic biotransformation of tetrabromobisphenol A, tetrachlorobisphenol A, and bisphenol A in estuarine sediments. Environmental Science & Technology 36: 696-701.

Wagner, A., Segler, L., Kleinsteuber, S., Sawers, G., Smidt, H. & Lechner, U. (2013) Regulation of reductive dehalogenase gene transcription in *Dehalococcoides mccartyi*. Philosophical Transactions of the Royal Society of London B: Biological Sciences 368: 20120317.

Wagner, D.D., Hug, L.A., Hatt, J.K., Spitzmiller, M.R., Padilla-Crespo, E., Ritalahti, K.M., Edwards, E.A., Konstantinidis, K.T., Löffler, F.E.. (2012). Genomic determinants of organohalide-respiration in *Geobacter lovleyi*, an unusual member of the *Geobacteraceae*. BMC Genomics 13:200.

Wang, L.-T., Lee, F.-L., Tai, C.-J. & Kasai, H. (2007) Comparison of *gyrB* gene sequences, 16S rRNA gene sequences and DNA–DNA hybridization in the *Bacillus subtilis* group. International Journal of Systematic and Evolutionary Microbiology 57: 1846-1850.

Wang, S., Qiu, L., Liu, X., et al. (2018) Electron transport chains in organohalide-respiring bacteria and bioremediation implications. Biotechnology Advances 36: 1194-1206.

Wang, S., Chng, K.R., Wilm, A., Zhao, S., Yang, K.-L., Nagarajan, N., and He, J. (2014) Genomic characterization of three unique Dehalococcoides that respire on persistent polychlorinated biphenyls. Proceedings of the National Academy of Sciences 111: 12103-12108.

Weiss, B., Ebel, R., Elbrächter, M., Kirchner, M. & Proksch, P. (1996) Defense metabolites from the marine sponge *Verongia aerophoba*. Biochemical Systematics and Ecology 24: 1-12.

Wöhlbrand, L., Jacob, J.H., Kube, M., Mussmann, M., Jarling, R., Beck, A., Amann, R., Wilkes, H., Reinhardt. R. & Rabus, R. (2013) Complete genome, catabolic sub-proteomes and key-metabolites of *Desulfobacula toluolica* Tol2, a marine, aromatic compound-degrading, sulfate-reducing bacterium. Environmental Microbiology 15: 1334-1355.

Wong, Y.K., Holland, S.I., Ertan, H., Manefield, M., and Lee, M. (2016) Isolation and characterization of *Dehalobacter* sp. strain UNSWDHB capable of chloroform and chlorinated ethane respiration. Environmental Microbiology 18: 3092-3105.

Yan, J., Ritalahti, K.M., Wagner, D.D. & Löffler, F.E. (2012) Unexpected specificity of interspecies cobamide transfer from *Geobacter* spp. to organohalide-respiring *Dehalococcoides mccartyi* strains. Applied and Environmental Microbiology 78: 6630-6636.

Yan, J., Im, J., Yang, Y., and Löffler, F.E. (2013) Guided cobalamin biosynthesis supports *Dehalococcoides mccartyi* reductive dechlorination activity. Philosophical Transactions of the Royal Society of London B: Biological Sciences 368: 20120320.

Yang, C., Kublik, A., Weidauer, C., Seiwert, B. & Adrian, L. (2015) Reductive dehalogenation of oligocyclic phenolic bromoaromatics by *Dehalococcoides mccartyi* strain CBDB1. Environmental Science & Technology 49: 8497-8505.

Yang, Y., Higgins, S.A., Yan, J., Şimşir, B., Chourey, K., Iyer, R. et al. (2017) Grape pomace compost harbors organohalide-respiring *Dehalogenimonas* species with novel reductive dehalogenase genes. The ISME Journal 11: 2767.

Ye, L., Schilhabel, A., Bartram, S., Boland, W. & Diekert, G. (2010) Reductive dehalogenation of brominated ethenes by *Sulfurospirillum multivorans* and *Desulfitobacterium hafniense* PCE-S. Environmental Microbiology 12: 501-509.

Yi, S., Seth, E.C., Men, Y.-J., Stabler, S.P., Allen, R.H., Alvarez-Cohen, L., and Taga, M.E. (2012) Versatility in corrinoid salvaging and remodeling pathways supports

corrinoid-dependent metabolism in *Dehalococcoides mccartyi*. Applied and Environmental Microbiology 78: 7745-7752.

Yohda, M., Ikegami, K., Aita, Y., Kitajima, M., Takechi, A., Iwamoto, M. et al. (2017) Isolation and genomic characterization of a *Dehalococcoides* strain suggests genomic rearrangement during culture. Scientific Reports 7: 2230.

Yu G, Bu Q, Cao Z, Du X, Xia J, Wu M & Huang J (2016) Brominated flame retardants (BFRs): A review on environmental contamination in China. Chemosphere 150: 479-490.

Yurimoto, H., Hirai, R., Matsuno, N., Yasueda, H., Kato, N., and Sakai, Y. (2005) HxlR, a member of the DUF24 protein family, is a DNA-binding protein that acts as a positive regulator of the formaldehyde-inducible *hxlAB* operon in *Bacillus subtilis*. Molecular Microbiology 57: 511-519.

Zanaroli, G., Negroni, A., Häggblom, M.M. & Fava, F. (2015) Microbial dehalogenation of organohalides in marine and estuarine environments. Current Opinion in Biotechnology 33: 287-295.

Zanaroli, G., Pérez-Jiménez, J.R., Young, L.Y., Marchetti, L. & Fava, F. (2006) Microbial reductive dechlorination of weathered and exogenous co-planar polychlorinated biphenyls (PCBs) in an anaerobic sediment of Venice Lagoon. Biodegradation 17: 121-129.

Zerbino, D.R., and Birney, E. (2008) Velvet: Algorithms for *de novo* short read assembly using de Bruijn graphs. Genome Research 18: 821-829.

Zhang, Y., Rodionov, D.A., Gelfand, M.S. & Gladyshev, V.N. (2009) Comparative genomic analyses of nickel, cobalt and vitamin B12 utilization. BMC Genomics 10: 78.

Zhao, S., Rogers, M.J., Ding, C. & He, J. (2018) Reductive debromination of polybrominated diphenyl ethers - microbes, processes and dehalogenases. Frontiers in Microbiology 9: 1292.

Zinder, S.H. (2016) The Genus *Dehalococcoides*. Organohalide-Respiring Bacteria (Adrian L & Löffler FE, eds.), pp. 107-136. Springer Berlin Heidelberg, Berlin, Heidelberg.

Zinke, L.A., Mullis, M.M., Bird, J.T., Marshall. I.P.G., Jørgensen. B.B., Lloyd. K.G., Amend, J.P. & Kiel, R.B. (2017). Thriving or surviving? Evaluating active microbial guilds in Baltic Sea sediment. Environmental Microbiology Reports 9: 528-536.