Molecular and physiological characterization of dehalorespiring microbial communities

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Molecular and physiological characterization

of dehalorespiring microbial communities

（脱ハロゲン呼吸微生物群集の分子生物学的および生理学的特性）

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Abstract

Tetrachloroethene (PCE) and 1,1,2-trichloroethane (112-TCA) are suspected carcinogens and widespread groundwater contaminants that are reductively dechlorinated to either toxic or benign end products by organohalides respiring bacteria (OHRB). This study successfully established both PCE and 112-TCA dechlorinating consortia. The first consortium (named YN3 culture) dechlorinated PCE into non-toxic ethene (ETH) by reductive dechlorination, while the other (named KJ-TCA culture) dechlorinated 112-TCA by dichloroelimination to vinyl chloride (VC), the proven carcinogen. Illumina amplicon analysis indicated the presence of *Dehalococcoides* and *Dehalobacter* as the potential dechlorinators in YN3 and KJ-TCA cultures, respectively. YN3 culture dechlorinated up to 800 μM PCE to ETH within only 14 days. This activity indicated a potential application of YN3 culture to the bioremediation of the groundwater contaminated with PCE and other chloroethenes (CEs). YN3 metagenome analysis showed the presence of 18 *rdhA* genes (designated *YN3rdhA1–18*) encoding the catalytic subunit of reductive dehalogenase (RdhA), the key enzyme in the reductive dechlorination. Of these 18 *YN3rdhA* genes, four genes were suggested to be involved in the dechlorination of PCE to ETH, based on the significant increases in their transcription levels in response to the addition of CEs. In these four *rdhA* genes, two *rdhAs*, *YN3rdhA6* and *YN3rdhA12,* were never proved before as *rdhA* to be involved in the dechlorination CEs. The *YN3rdhA6* and *YN3rdhA12* genes showed particularly high transcription level upon the addition of VC, suggesting their involvements in the VC dechlorination as novel *rdhA* genes. The metagenome data also indicated the existence of three bacteria taxa belonging to phyla *Bacteroidetes*, *Actinobacteria* and *Firmicutes*. Moreover, analysis of YN3 metagenome indicated that

the metagenome of *Bacteroidetes* was the largest and represented by a novel species of the genus *Bacteroides*. Thereafter, the novel species of the genus *Bacteroides*, designated strain YN3PY1, was isolated from YN3 culture. The strain enhanced the dechlorination of *cis*-dichloroethene to ETH by C4C4 culture, which is a *Dehalococcoides* enriched culture obtained from YN3 culture, especially at the early stages of cultivation. However, even the enhanced dechlorination activity is still small if compared with the parent culture YN3 culture. This indicated different mechanisms or microbes enhanced the dechlorination. This study would contribute the development of bioremediation technology using *Dehalococcoides* as a dehalogenator with the enhancement by the coexisting bacteria such as *Bacteroides*. This study provides potential candidates for *in situ* bioaugmentation for remediation of sites contaminated with PCE (using YN3 culture) and 112-TCA using (using YN3 and KJ-TCA cultures).

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Contents

1. Introduction 1

    1.1 Groundwater importance and pollution 1

    1.2 Remediation of aquifers (or groundwater) contaminated with CAHs 2

    1.2.1 Chemical and physical remediation 2

    1.2.2 Bioremediation: Pathways, *ex situ* technologies and *in situ* aerobic/anaerobic technologies 4

    1.2.3 Biostimulation and bioaugmentation: Safety and regulation 5

    1.2.4 Dehalogenation reactions and microbial growth 7

    1.3 Organohalide respiration (OHR) 7

    1.3.1 Phylogenetic distribution of OHRB and extent of dehalogenation 7

    1.3.2 Biochemistry and molecular biology of reductive dehalogenases 15

    1.3.3 Physiology of OHR (energy conservation and electron transfer to reductive dehalogenase) 21

    1.4 The aim of this research 23

    1.5 Organization of the thesis 23

2. Enrichment and screening of organohalide-dehalorespiring consotia from environments for dechlorination of 1,1,2-trichloroethane and tetrachloroethene 25

    2.1 Introduction..25

    2.2 Materials and methods 26

    2.2.1 Enrichment of dechorinating consortia 26

    2.2.1.1 Enrichment of CEs dechlorinating bacteria 26

    2.2.1.2 Enrichment of 112-TCA dechlorinating bacteria 27

2.2.2 Chemical analysis 28
2.2.3 DNA extraction 28
2.2.4 Microbial composition analysis 29
2.2.5 Quantitative PCR (qPCR) 29
2.3 Results..30
2.3.1 Enrichment of PCE/cis-DCE to ETH dechlorinating culture 30
2.3.2 Dechlorination of PCE to ETH by YN3 culture 30
2.3.3 Enrichment of 112-TCA dechlorinating culture 32
2.3.4 Identification of microbial composition in YN3 and KJ-TCA cultures 34
2.3.5 qPCR 35
2.4 Discussion...37
2.5 Conclusion 38
3. Metagenomic analysis of tetrachloroethene to ethene dechlorinating *Dehalococcoides* enriched culture and transcriptional analysis of its reductive dehalogenases genes 39
3.1 Introduction...39
3.2 Materials and methods 40
3.2.1 DNA extraction 40
3.2.2 Metagnome sequencing, analysis and phylogenetic trees construction 41
3.2.3 Reverse transcription RT-qPCR 41
3.3 Results.... 44
3.3.1 Population of Dehaloccoides and non-dehalorespiring bacteria in YN3 ...44
3.3.2 *Dehalococcoides* metagenome...45
3.3.3 Non -*Dehalococcoides* metagenome 47

3.3.4 Reductive dehalogenase and asiciated genes 48
3.3.5 Reverse transcription RT-qPCR 53
3.4 Dicussion 56
3.5 Conclusion 60
4. *Bacteroides sedimenti*, a novel species of the genus *Bacteroides* isolated from chloroethenes dechlorinating consortium and its effect on the dechlorination by *Dehalococcoides* 62
4.1 Introduction 62
4.2 Materials and methods 63
4.2.1 Isolation and culture condition of strain YN3PY1T 63
4.2.2 Morphological, physiological and biochemical analyses 65
4.2.3 Genetic analysis 66
4.2.4 Chemotaxonomic analysis 66
4.2.5 Assessment of the enhancement of dechlorination activity 67
4.2.6 GenBank acession numbers 68
4.3 Results 68
4.3.1 Isolation of strain YN3PY1T 68
4.3.2 Genetic characterization 68
4.3.3 Physiological and biochemical characterization 72
4.3.4 Chemotaxonomic characterization 75
4.3.5 Dechlorination enhancement 77
4.4 Discussion 80
4.5 Conclusion 81
5. General discussion ……. 83

5.1 Significance of this study 83

5.2 Achievements/summary of this study 88

5.2.1 Enrichment of PCE and 112-TCA dechlorinating cultures 88

5.2.2 Identification of the microbial composition of YN3 culture and *rdhA* genes suggested to be involved in the PCE to ETH dechlorination. 88

5.2.3 Enhancement the dechlorination of *cis*-DCE to ETH by co-culturing of strain YN3PY1, with the dechlorinator *Dehalococcoides* 89

5.3 Suggestions for the future study 89

5.3.1 Discovery of new OHRB 89

5.3.1 Molecular-based research 90

5.3.2 Community-based research 91

References 93

List of figures

Figure 1-1	Structure of CAHs where A, chloroethenes and B, chloroethanes	2
Figure 1-2	Microbial dehalogenation pathways for CAHs where A, Dihaloelimination B, Hydrogenolysis C, Dehydrohalogenation and D, Oxidation . (MMO, Methane monooxygenase)	6
Figure 1-3	Obligate and facultative OHRB	10
Figure 1-4	PCE dechlorination extent by various OHRB	12
Figure 1-5	Reductive dehalogenase (A) Operon structure, (B) Conserved motifs.	16
Figure 1-6	Reductive dehalogenase encoding operon in *Dehalococcoides* and *Desulfitobacterium.*	20
Figure 1-7	Electron transfer in OHRB where, A, Quinone-dependent model, B, Quinone-independent model	22
Figure 2-1	Dechlorination of cis-DCE to ETH by YN3 culture	31
Figure 2-2	Dechlorination of PCE (70 μM) to ETH by YN3 culture	31
Figure 2-3	Dechlorination of PCE (200-800 μM) to ETH by YN3 culture	32
Figure 2-4	Dechlorination pathways observed in the two enrichment cultures, YN3 and KJ-TCA..	33
Figure 2-5	Population of *Dehalococcoides* and total bacteria during PCE dechlorination to ETH..	36
Figure 2-6	Population of Dehalococcoides and total bacteria during *cis*-DCE dechlorination to ETH	36
Figure 3-1	Neighbor-joining tree showing the phylogenetic position of phylotype YN3-01 and related strains of *Dehalococcoides*	44
Figure 3-2	RAST functional distribution of genes identified in YN3 metagenome	47
Figure 3-3	Gene clusters containing reductive dehalogenases in the YN3 *Dehalococcoides* metagenome	50
Figure 3-4	Neighbor-joining tree of RdhAs detected in the YN3–*Dehalococcoides* metagenome	52
Figure 3-5	Analysis of transcription level of *YN3rdhA6* (A), *YN3rdhA12* (B), *YN3rdhA16* (C), and *YN3rdhA8* (D) in YN3 spiked with PCE, TCE, *cis*-DCE, or VC	55
Figure 3-6	Comparsion of gene cluster for *YN3rdhA6* (A) and *YN3rdhA12* (B) and their related *rdhAs* gene cluster from *Dehalococcoides*	60
Figure 4-1	Neighbor–joining tree based on the 16S rRNA gene sequences of strain YN3PY1^T and the related species in the genus *Bacteroides*	70
Figure 4-2	Unrooted neighbor-joining tree based on concatenated sequences of three genes; *dnaJ*, *gyrB*, and *hsp60*, showing the phylogenetic position of strain YN3PY1^T in the *'Coprosuis'* clade of the genus *Bacteroides*………………………………………..	71
Figure 4-3	Possible pathways of glucose fermentation in strain YN3PY1^T	74
Figure 4-4	Dechlorination of *cis*-DCE in *Dehalococcoides*-enriched culture,	

C4C4, with and without strain YN3PY1^{T} 79

List of tables

Table 1-1	List of some isolated OHRB	9
Table 1-2	List of some functionally characterized RdhA units	18
Table 2-1	List of primers used in this study	29
Table 2-2	Microbial composition of YN3 culture as determined by Illumina MiSeq analysis. Potential dechlorinator was shown in bold……..…………………	35
Table 2-3	Microbial composition of KJ-TCA culture as determined by Illumina MiSeq analysis. Potential dechlorinator was shown in bold	35
Table 3-1	List of primers used in this study	43
Table 3-2	Overall characteristics of the YN3-Dehalococcoides-metagenome and comparison with genomes of other strains of Dehalococcoides	46
Table 3-3	Comparison of dechlorination activity for CEs in YN3 culture and RdhAs in the YN3-Dehalococcoidess metagenome with those of other strains or consortia of Dehalococcoides	51
Table 3-4	The change in transcription of YN3rdhA1-18 in response to spiking of CEs	54
Table 3-5	Comparison of transcribed rdhAs genes in response to CEs in Dehalococcoides-metagenome with those of other consortium or strains of Dehalococcoides	59
Table 4-1	The DNA G+C content and phenotypic characteristics of strain YN3PY1T and closely related strains of the genus Bacteroides	73
Table 4-2	Fatty acid composition of strain YN3PY1T and closely related strains of the genus Bacteroides.	77

1. Introduction

1.1 Groundwater importance and pollution

Water is the most essential element for the life on earth. Up to 70% of our planet is covered with water. Among this 70%, fresh water represents only 3%. Fresh water is furtherly divided into ice and snow, groundwater, rivers and lakes and soil moisture and swamp water. After excluding ice and snow, groundwater represents around 98% of the total freshwater available on earth and up to 50% of the world population depend on it as a source of drinking water (NASA Earth Observatory, 2003; Zehnder, 2005). In addition, 30-40% of water used in agriculture comes from groundwater (Zehnder, 2005). Thus, groundwater is a precious natural resource and should be protected against chemical contamination.

Chlorinated aliphatic hydrocarbons (CAHs), especially simple C2 chloroethenes (CEs) and chloroethanes (CAs) (Figure 1-1) are commonly detected contaminants in groundwater (known as chlorinated solvents). Chlorinated solvents were enormously produced since the beginning of 20th century and used in many industrial applications such as dry cleaning, textile processing and plastic manufacturing (Löffler et al., 2013). In 2002, the global production of chlorinated solvents was approximately 750,000 metric tons. These chemicals were selected based on their desired criteria such as chemical stability, fire safety, low price and availability (Löffler et al., 2013; Saiyari et al., 2018). The widespread of these chemicals and improper disposal and storage, and absence of regulatory rules over decades of heavy usage drove to wide contamination of groundwater with these chemicals. They are denser than water and not mixed easily with it and thus, present in a separate phase from water known as dense non-aqueous phase liquid (DNAPL) (Lee et al., 2015). These chemicals have usually longer half-lives than the other volatile organic contaminates and persist for longer time in groundwater. Public concern with the groundwater pollution with these

chemicals has raised based on their potential toxicity and carcinogenicity (Stroo et al., 2013). Once the contamination of the groundwater with CAHs is confirmed, the remediation of groundwater is required by applying one or more of the following remediation technologies (Stroo et al., 2013).

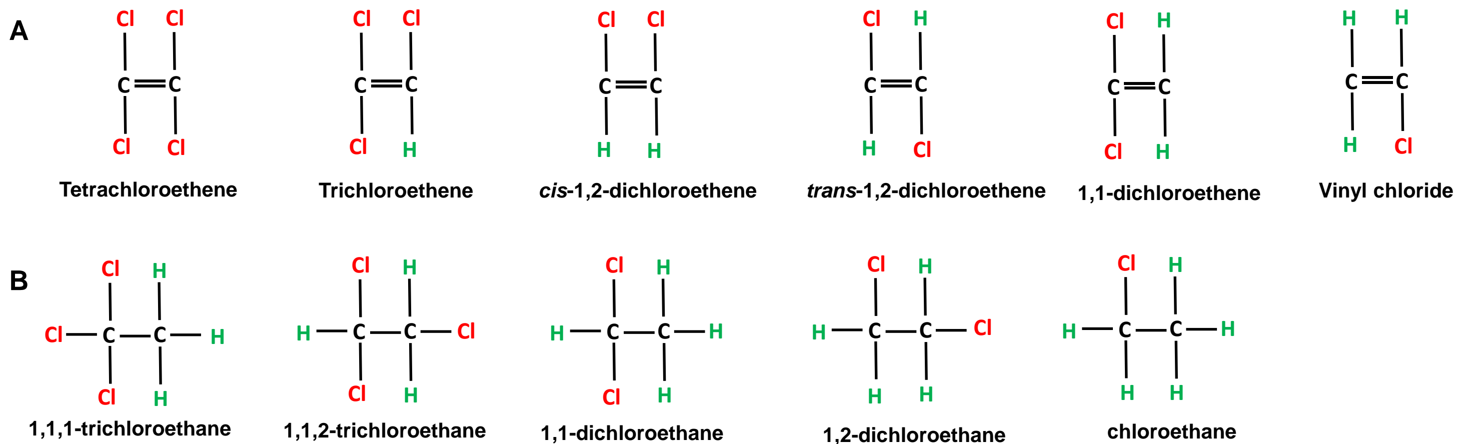

Figure 1-1 Structure of CAHs where A, chloroethenes and B, chloroethanes

1.2 Remediation of aquifers (or groundwater) contaminated with CAHs

In general, remediation methods can be divided into *in situ* and *ex situ* remediation. *In situ* remediation means in place treatment of the contaminated groundwater at the original site. The expenses of the treatment process and environmental impacts may be reduced. On the other hand, *ex situ* remediation includes excavation followed by pumping of the contaminated groundwater to the aboveground for the treatment. The *in situ* remediation technologies are of special significance since the contamination with CAHs occurs in deep aquifers because the excavation is not realistic in such cases.

1.2.1 Chemical and physical remediation

In general, the remediation technologies using chemical and/or physical methods are too expensive or unsuitable for large scale application. Several approaches are used under this category, among them (Steffan and Schaefer, 2016);

Pump and treat (*ex situ*)

In this method, the contaminated groundwater is pumped up to the aboveground where the CAHs are removed by adsorption to activated carbon. However, because of low solubility of these chemicals, large amount of contaminated water must be pumped up to the surface, and long period, often longer than 10 years, is required to achieve an efficient treatment (Steffan and Schaefer, 2016).

Air sparging (*in situ* or *ex situ*)

In this method, large amounts of air will be injected into the contaminated groundwater to expel the contaminants. The consumption high energy during the air injection is known to limit the use of this method (Steffan and Schaefer, 2016).

Permeable reactive barriers (*in situ*)

In this method, a permeable wall is placed across the path of a contaminant plume. Zero valent iron (ZVI) is widely used as the reactive material contained in the barrier. Thereafter, as the contaminated water passes through the ZVI wall, the contaminants are either removed or degraded (Geranio, 2007). The contaminates or CAHs are removed from the groundwater via an abiotic reductive dechlorination. Three pathways were suggested to be operated for the reduction of chlorinated compound by ZVI. The first one, it is likely that the dechlorination process proceeds via this pathway, includes direct electron transfer from ZVI to the chlorinated compound, $Fe^0 + RCl + H^+ \longrightarrow Fe^{2+} + RH + Cl^-$. In the second pathway, the chlorinated compound is reduced by the hydrogen produced from the corrosion of ZVI, $H_2 + RCl \longrightarrow RH + H^+ + Cl^-$, while the third

mechanism, the quite slow one, includes the reduction of chlorinated compound by Fe^{2+} produced from corrosion of ZVI, $2Fe^{2+} + RCl + H^{+} \longrightarrow 2Fe^{3+} + RH + Cl^{-}$ (Matheson and Tratnyek, 1994).

1.2.2 Bioremediation: Pathways, *ex situ* technologies and *in situ* aerobic/anaerobic technologies

Bioremediation is exploiting the metabolic activities of microorganisms to clean-up the contaminated environment. Main metabolic pathways for detoxification of CAHs can be divided into reductive dehalogenation (hydrogenolysis or dihaloelimination), dehydrohalogenation or oxidation (Figure 1-2). Hydrogenolysis is proceeded via the replacement of chlorine atom by hydrogen one while dihaloelimination involves the removal of two chlorine atoms from two neighbor carbon atoms with subsequent formation of double bond between these two carbon atoms (Dolfing, 2016). Dehydrohalogenation involves the removal of HCl with subsequent formation of double bond. Oxidation involves the transformation of the halogenated compound into CO_2, H^+ and Cl^- (Dolfing, 2016). *Ex situ* technologies are of less significance regarding bioremediation of CAHs. This is because the contamination with CAHs occurs in deep aquifers, thus, *ex situ* bioremediation requires high cost and may has a negative impact on the environment. Therefore, the *in situ* bioremediation technologies (under aerobic or anaerobic condition) are the technologies of choice to avoid the undesired effects of *ex situ* ones. The usage of aerobic or anaerobic technology depends largely on the species of CAHs to be degraded. For example, tetrachloroethene (PCE) and trichloroethene (TCE) exist in highly oxidized forms that hamper the degradation under aerobic (oxygenolytic) conditions. Several studies have demonstrated the dechlorination of TCE and dichloroethene (DCE) under aerobic condition via cometabolic reactions. However, because PCE,

TCE and DCE are present in subsurface environment, where is often anoxic, thus, the anaerobic pathways for dechlorination are more suitable (Löffler et al., 2013).

1.2.3 Biostimulation and bioaugmentation: Safety and regulation

Biostimulation is carried out by the addition of nutrients to speed up the remediation of CAHs by the indigenous microbes. Bioaugmentation means the addition of exogenous microbes to the contaminated site when indigenous microbes are unable to perform the remediation adequately. In general, there are several regulatory points should be considered when using bioaugmentation. Among these regulatory points, the proof of absence of pathogens in the bioaugmentation culture, the cost and the time taken during the treatment process (Stroo et al., 2013). Also, the impact of the bioremediation process on the quality of the treated water (secondary water quality) should be considered. For example, the reducing conditions built during the dehalogenation can lead to formation of H_2S or other compounds with subsequent undesired impact on the quality the of the treated water (Steffan and Schaefer, 2016).

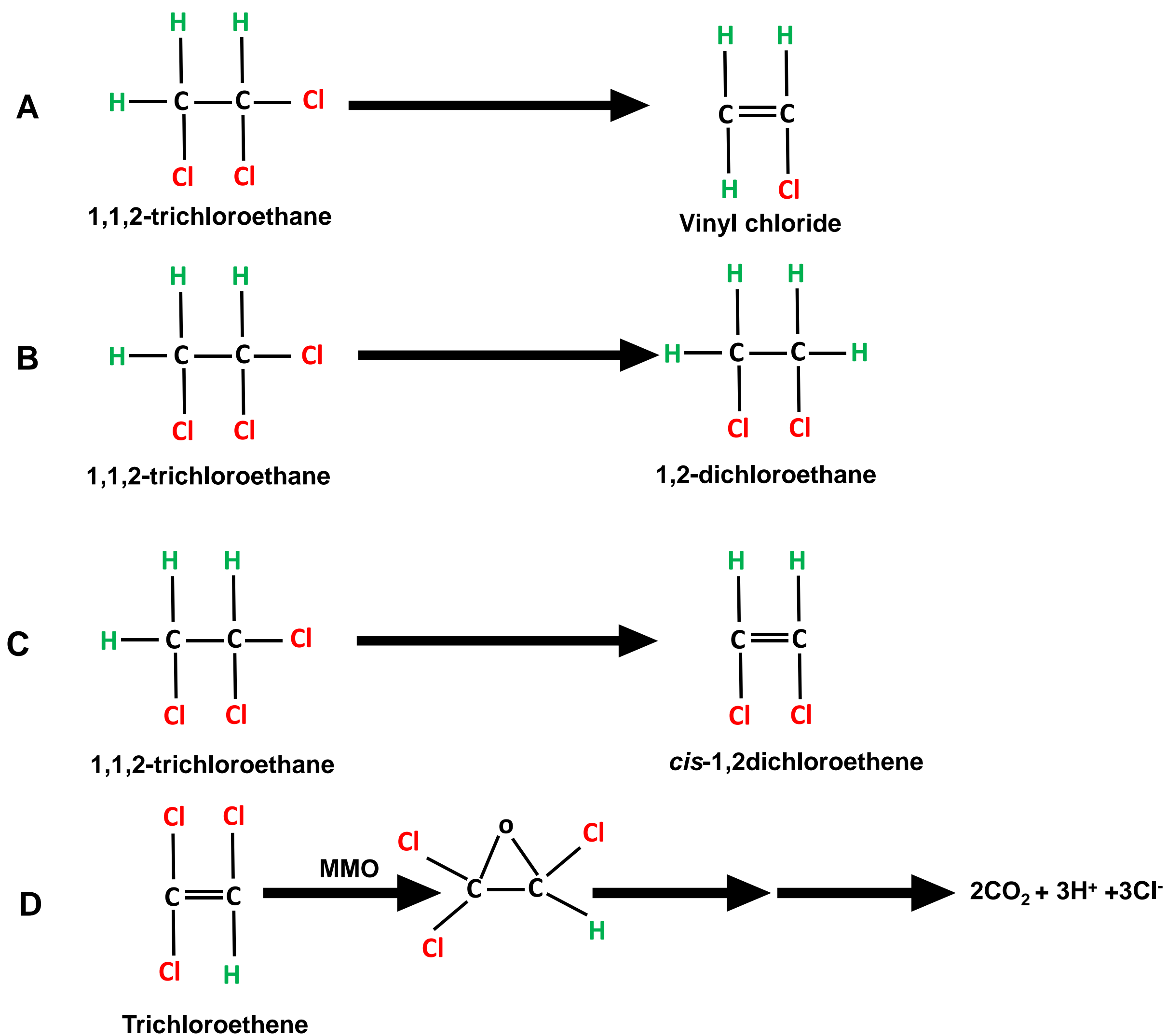

Figure 1-2 Microbial dehalogenation pathways for CAHs where A, Dihaloelimination B, Hydrogenolysis C, Dehydrohalogenation and D, Oxidation . (MMO, Methane monooxygenase)

1.2.4 Dehalogenation reactions and microbial growth

The dehalogenation reactions are classified into metabolic (linked to energy conservation and growth of the microbe) or cometabolic reactions (no energy conservation or growth). The cometabolic dehalogenation is a fortuitous or incidental dehalogenation by non-specific enzymes. Technically, cometabolic dehalogenation requires primary substrates and usually incomplete. In metabolic dehalogenation the halogenated compound serves as either an electron donor/carbon source or an electron acceptor. The latter type is a respiratory process and is mediated via reductive dehalogenation metabolic pathways (hydrogenolysis or dihaloelimination) under anaerobic conditions. The respiratory reductive dehalogenation (named organohalide respiration) is carried out by organohalide respiring bacteria (OHRB) and currently one of the most promising methods for *in situ* remediation of groundwater contaminated with CAHs (Saiyari et al., 2018). Organohalide respiration of some CAHs is the subject of study in this thesis.

1-3 Organohalide respiration (OHR)

1-3-1 Phylogenetic distribution of OHRB and extent of dehalogenation

OHRB are distributed among phylogenetically diverse bacterial phyla, *Chloroflexi*, *Firmicutes* and *Proteobacteria* (Table 1-1). OHRB can be classified into obligate (or specialists) OHRB that grow only using organohalides as electron acceptors and facultative (or generalists) OHRB that grow using organohalides or other chemicals such as nitrate or iron as electron acceptors (Figure1-3). All dehalogenating members belonging to the phylum *Chloroflexi* are obligate OHRB, while, dehalogenating members affiliated within the phyla *Firmicutes* and *Proteobacteria* are facultative OHRB. (Adrian and Löffler, 2016). General characteristics of the identified OHRB will be summarized as follow:

The genus *Dehalococcoides*

The genus *Dehalococcoides* from the phylum *Chloroflexi* is a well-studied OHRB owing to its diverse dehalogenating activity towards organohalides, especially CEs. So far, members of the genus contain Archaeal-like cell wall structure, and use only hydrogen, acetate and organohalides as an energy source, a carbon source and electron acceptors, respectively. *Dehalococcoides* converts CEs such as PCE, TCE, *trans*-dichloroethene (trans-DCE), *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC) completely into ethene (ETH). On the other hand, members of the other OHRB such as *Dehalobacter*, *Desulfitobacterium* and *Geobacter* convert CEs into toxic daughter compounds (Futagami et al., 2008). The advantage of complete CE dechlorination enables *Dehalococcoides* to play an important role in the bioremediation of CE-contaminated groundwater. *Dehalococcoides mccartyi* strain 195 (formerly *Dehalococcoides ethenogenes* 195) was the first isolated strain of *Dehalococcoides* with the ability of dechlorination of PCE into ETH. However, the last step, the conversion of VC into ETH, was slow and incomplete, i.e., cometabolic. Later, He et al. (2003) reported the first isolate of *Dehalococcoides*, strain BAV1, which dechlorinated VC completely into ETH. Subsequently, several other isolates of *Dehalococcoides* with varying dehalogenating activities were obtained (Table 1-1). The dehalogenating activities of *Dehalococcoides* are not limited to halogenated aliphatic compounds but also extend to aromatic ones such as chlorobenzene (Adrian et al., 2000) and polychlorinated biphenyls (PCB) (Wang et al., 2014).

Table 1-1 List of some isolated OHRB

Strain	**Electron acceptor**	**Product**	**References**
Dehalococcoides mccartyi 195	PCE	VC, ETH	(Maymo-Gatell et al., 1997)
Dehalococcoides mccartyi VS	VC	ETH	(Rosner et al., 1997)
Dehalococcoides mccartyi IBARAKI	*cis*-DCE , VC	ETH	(Yohda et al., 2015)
Dehalococcoides mccartyi CG1	PCE	TCE	(Wang et al., 2014)
Dehalococcoides mccartyi CG4	PCE	TCE	(Wang et al., 2014)
Dehalococcoides mccartyi CG5	PCE	TCE	(Wang et al., 2014)
Dehalococcoides mccartyi UCH007	TCE, *cis*-DCE , VC	ETH	(Uchino et al., 2015)
Dehalococcoides mccartyi MB	PCE, TCE	*trans*-DCE, *cis*-DCE	(Cheng and He, 2009)
Dehalococcoides mccartyi BAV1	*trans*-DCE, *cis*-DCE	ETH	(He et al., 2003)
Dehalococcoides mccartyi FL2	TCE, *trans*-DCE, *cis*-DCE	VC, ETH	(He et al., 2005)
Dehalogenimonas lykanthroporepellens	1,2-dichloroethane and 1,2-dichloropropane	ETH and propene	(Moe et al., 2009)
Dehalogenimonas alkenigignens	1,2-dichloroethane and 1,2-dichloropropane	ETH and propene	(Bowman et al., 2013)
Dehalobacter restrictus	PCE, TCE	*cis*-DCE	(Holliger et al., 1998)
Desulfitobacterium hafniense TCE1	PCE, TCE	*cis*-DCE	(Gerritse et al., 1999)
Desulfitobacterium hafniense Y51	PCE, TCE	*cis*-DCE	(Suyama et al., 2001)
Desulfitobacterium hafniense JH1	PCE, TCE	*cis*-DCE	(Fletcher et al., 2008)
Desulfuromonas chloroethenica	PCE, TCE	*cis*-DCE	(Krumholz et al., 1996); Krumholz, 1997)
Desulfuromonas michiganensis	PCE, TCE	*cis*-DCE	(Sung et al., 2003)
Sulfurospirillum multivorans	PCE, TCE	*cis*-DCE	(Luijten et al., 2003);
Sulfurospirillum halorespirans	PCE, TCE	*cis*-DCE	(Luijten et al., 2003)
Geobacter lovleyi	PCE, TCE	*cis*-DCE	(Sung et al., 2006)

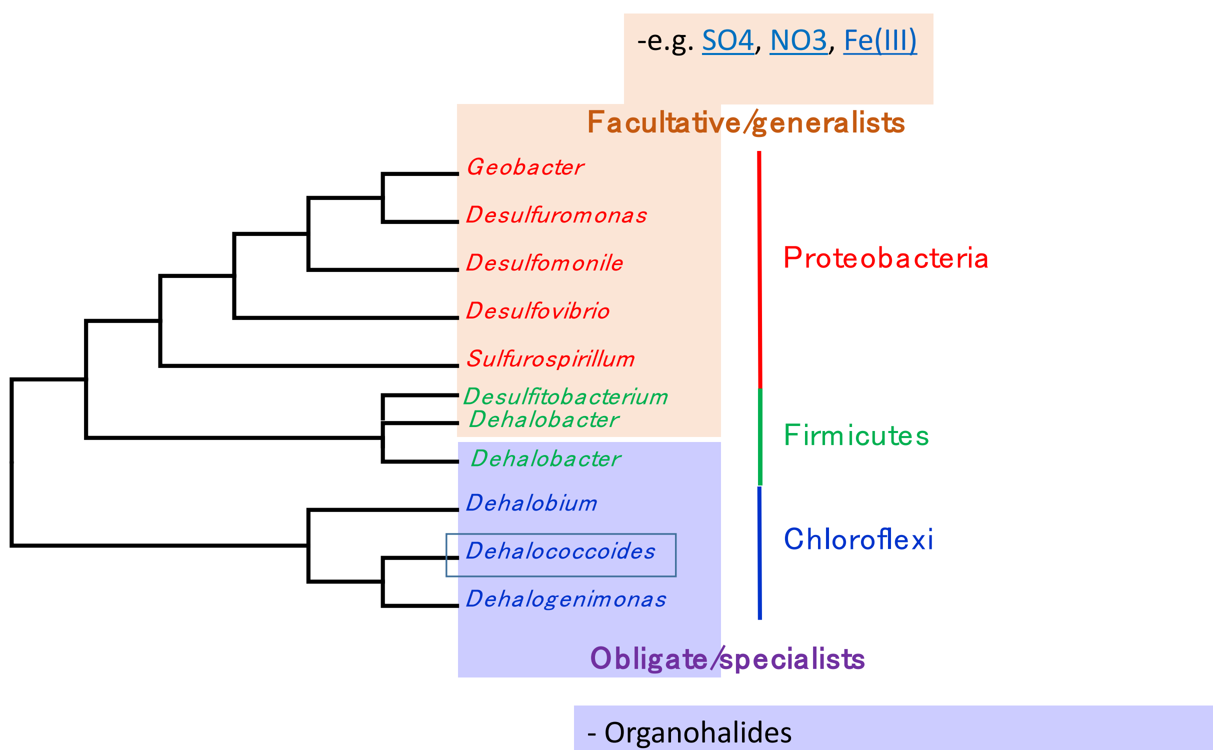

Figure 1-3 Obligate and facultative OHRB

Several studies have reported that *Dehalococcoides* achieves better growth and dechlorination activity in mixed culture together with coexisting bacteria. Coexisting bacteria helped *Dehalococcoides* by supplementation with essential substances for growth such as acetate, hydrogen and vitamin B_{12} (He et al., 2007; Men et al., 2012). Also, coexisting bacteria eliminated the toxic substances which may exert negative effects on *Dehalococcoides* such as oxygen and carbon monoxide (Zhuang et al., 2014; Liu et al., 2017)

In general, members of the genus *Dehalococcoides* contain small-sized genome of around 1.5 MB. These genomes are characterized by multiple non-identical *rdhA* genes, (up to 10-38 gene per genome), encoding the active unit of reductive dehalogenase (RdhA). However, the substrate specificity has been rarely assigned for the genes (Hug et al., 2013).

Members of the genus *Dehalococcoides* showed more than 98% of sequence similarity with respect to 16S rRNA gene and thus are comprised into single species *Dehalococcoides mccartyi*. Hendrickason et al. (2002) subdivided the genus into three subgroups Cornell, Pinellas and Victoria based on small differences in the 16S rRNA gene sequences.

Dehalococcoides represents the key player included in the commercially available bioaugmentation cultures, e.g., KB-1 consortium which has been distributed commercially by SiREM (Guelph, Ontario, Canada), for clean up of CAHs-contaminated sites.

The genus *Dehalogenimonas*

The genus resides in the phylum *Chloroflexi* and represents the second member of the obligate OHRB. The genus is strict anaerobe and only use hydrogen and organohalides as an electron donor and electron acceptors, respectively. Available genomes are relatively larger than that of *Dehalococcoides* and contain up to 25 *rdhA* genes per genome. Notably, many *rdhA* genes are exist without the adjacent *rdhB* genes (encoding RdhB units with a putative anchoring role for RdhA unit) (Siddaramappa et al., 2012; Key et al., 2016). Recently, it was reported that *Dehalogenimonas*-enriched culture can respire VC, and completely convert it into ETH (Yang et al., 2017). This confirmed the link between the extent of the dechlorination activity and the taxonomic position since all the complete CEs-dechlorinating OHRB, i.e., dechlorinate CEs completely into ETH, reside in the phylum *Chloroflexi* (Figure 1-4).

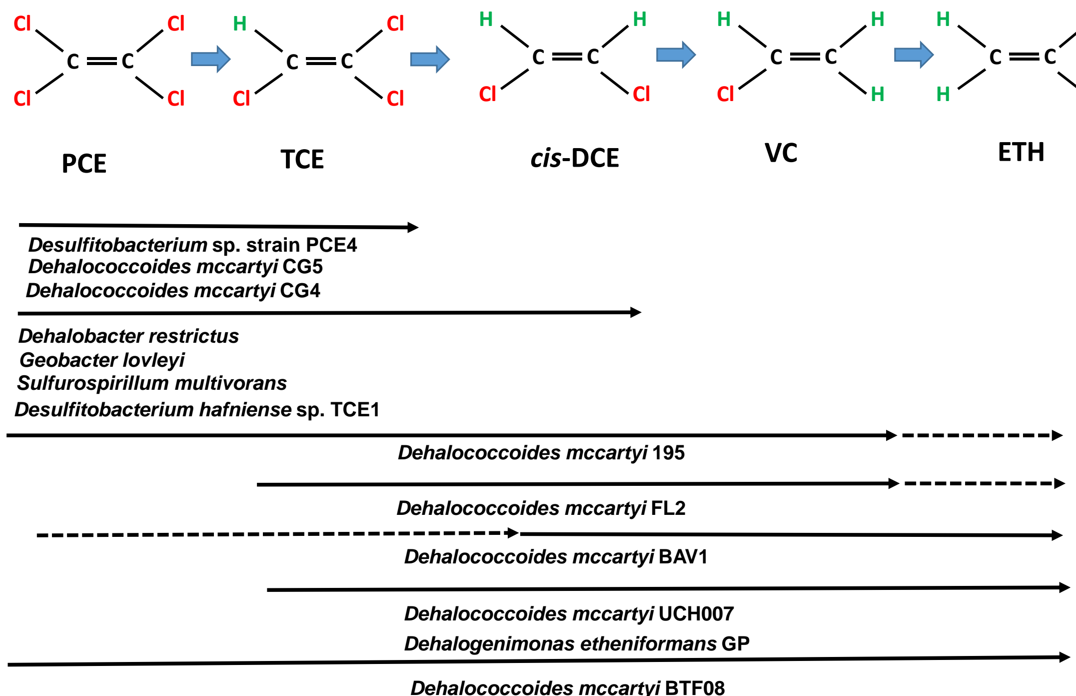

Figure 1-4 PCE dechlorination extent by various OHRB

The genus *Dehalobium*

The genus also belonging to the phylum *Chloroflexi* and not validated officially as a genus. Unlike *Dehalococcoides* and *Dehalogenimonas*, *Dehalobium* uses formate as an energy source besides hydrogen. The microbe showed dechlorination activity toward PCB, chlorinated benzenes, PCE and TCE. The genus dechlorinates PCE and TCE into higher amount of *trans*-DCE and lesser amount of *cis*-DCE, unlike the majority of OHRB that dechlorinate PCE and TCE into only *cis*-DCE. Until now there is no available information regarding the genomes and Rdh operated in this microbe (Wu et al., 2002; May et al., 2016).

The genus *Dehalobacter*

Members of the genus are affiliated within the phylum *Firmicutes* and mostly use hydrogen as an electron donor. Lee et al. (2012), reported the fermentation of chloromethane by some

members of *Dehalobacter*. Members of *Dehalobacter* have relatively small-sized genomes around 2.6-3.0 Mb, with up to 36 *rdhA* genes per genome. The genus can use a wide variety of organohalides as electron acceptors including PCE, TCE, chlorinated phenols, chlorinated phthalide, chlorinated benzenes and PCBs (Maillard and Holliger, 2016).

The genus *Desulfitobacterium*

The genus belongs to the phylum *Firmicutes* and uses sulfite as an electron acceptor beside organohalides. Available genomic information revealed the presence of 1-7 *rdhA* genes per genome and the possession of all genes required for the biosynthesis of the essential corrinoid. The genus has been playing a vital role as a model OHRB owing to its ability for dechlorination of diverse organohalides and relatively high growth rate. As a result, diverse RdhA units were purified and characterized from this genus (Futagami and Furukawa, 2016).

The genus *Anaeromyxobacter*

The microbe belongs to class *Deltaproteobacteria* of phylum *Proteobacteria* and can grow using *ortho*-substituted halophenols as electron acceptors. In addition, the microbe also can use several other compounds as electron acceptors such as nitrate, fumarate, ferric iron and even oxygen. Acetate, hydrogen, formate, lactate, pyruvate and succinate have been identified as electron donors for the microbe (Sanfdord et al., 2002).

The Genus *Desulfuromonas*

The genus affiliates within the class *Deltaproteobacteria* of the phylum *Proteobacteria.* Members of the genus use PCE and TCE as electron acceptors, as well as Fe (III)-nitriloacetate and

fumarate as alternative electron acceptors (Adrian and Löffler, 2016).

The genus *Geobacter*

The genus is placed in the class *Deltaproteobacteria* of the phylum *Proteobacteria* and some members, e.g., *Geobacter lovleyi*, can respire PCE and TCE, converting them into *cis*-DCE. *Geobacter lovleyi* can also use in addition to organohalides, Fe(III), Mn (IV) and fumarate as an alternative electron acceptors. *Geobacter thiogenes* represents another species within the genus which dechlorinates trichloroacetate into dichloroacetate (Adrian and Löffler, 2016).

The genus *Desulfomonile*

The genus belongs to the class *Deltaproteobacteria* of the phylum *Proteobacteria*. The genus comprises two dehalogenating species *Desulfomonile limimaris* and *Desulfomonile tiedjei*. *Desulfomonile tiedjei* uses *meta*-halobenzoates, sulphate, thiosulphate and sulfite as electron acceptors in the presence of pyruvate, acetate, formate or hydrogen as an electron donor. Fermentation of pyruvate has been demonstrated to occur by this microbe with addition of pyruvate as a sole carbon source. *Desulfomonile limimaris* has been isolated from marine sediments thus NaCl is required for its growth. The organism dechlorinates 3-chlorobenzoate into benzoate with hydrogen and formate as an energy source. Nitrate, fumarate, sulphate, thiosulphate and sulfite represent the alternative electron acceptors for the microbe, while lactate, pyruvate, propionate and butyrate represent the alternative electron donors (Sun et al., 2001).

The genus *Desulfovibrio*

The genus belongs to the class *Deltaproteobacteria* of the phylum *Proteobacteria*. Two

isolates from this genus have shown dehalogenation activity. *Desulfovibrio* sp. strain TBP-1 reduces 2,4,6-tribromophenol into phenol in the presence of hydrogen and acetate as an electron donor and carbon source, respectively (Boy et al., 1999). Also, the organism grew using sulfate as an alternative electron acceptor. *Desulfovibrio dechloroacetivorans*, respires using 2-chlorophenol as an electron acceptor, and also can use sulfate, sulfite, thiosulfate, fumarate and nitrate as alternative electron acceptors (Sun et al., 2000).

The genus *Sulfurospirillum*

The genus affiliates within the class *Epsilonproteobacteria* of the phylum *Proteobacteria. Sulfurospirillum multivorans* (formerly, *Dehalospirillum multivorans*), is a well-studied OHRB from this genus. The organism dechlorinates PCE into *cis*-DCE with pyruvate as an electron donor. The organism can use also sulfur and nitrate as alternative electron acceptors, where nitrate is reduced to nitrite only. Special type of corrinoid, norpseudo-B_{12}, has been found to be synthesized by the organism and incorporated as a cofactor with RdhA units (Goris and Diekert, 2016).

The genus *Comamonas*

The genus is affiliates within the class *Betaproteobacteria* of the phylum *Proteobacteria.* Comamonas sp. strain 7D-2 is strict aerobe and the only dehalogenating microbe in this genus, and dehalogenates 3,5-dibromo-4-hydroxybenzoate into 4-hydroxybenzoate. The *rdhA* gene in this strain has been found to lie on a plasmid and flanked by transposes genes, indicating its horizontal transfer from another bacterium (Adrian and Löffler, 2016).

1-3-2 Biochemistry and molecular biology of reductive dehalogenases

***rdhA/rdhB* genes**

Reductive dehalogenase (Rdh) is the terminal reductase and the key enzyme which catalyzes the OHR in OHRB. Rdh-encoding genes are typically exist in an operon of at least two genes, *rdhA* and *rdhB* genes (Figure 1-5A). The *rdhA* gene encodes the catalytic subunit of Rdh (known as RdhA or RDase if the functionally characterized). The second gene, *rdhB* gene, encodes a putative anchoring subunit for RdhA, RdhB. The RdhA subunit shows some conserved features as two Fe-S clusters in the C-terminus, either one 4Fe-4S and one 3Fe-4S or two 4Fe-4S, and one twin-arginine translocation (Tat) signal peptide sequences in the N-terminus (Figure 1-5B).

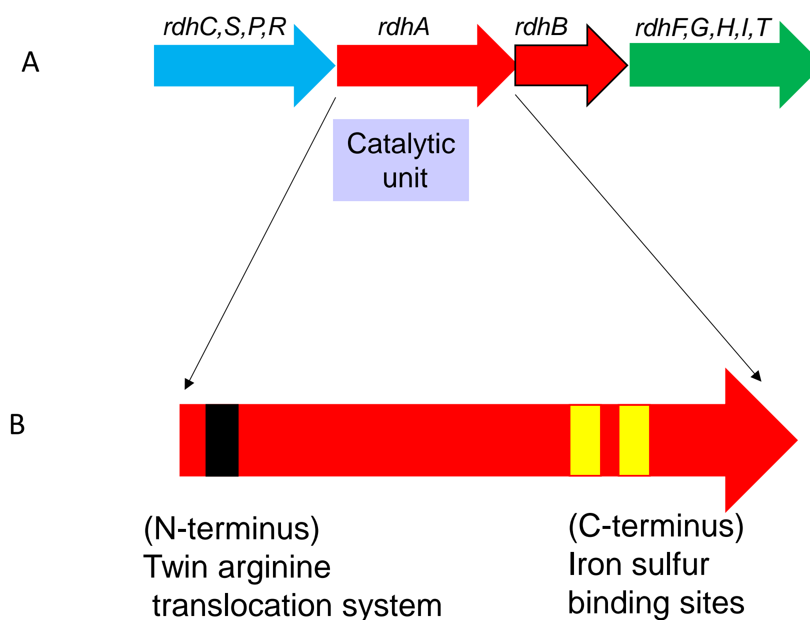

Figure 1-5 Reductive dehalogenase (A) Operon structure, (B) Conserved motifs.

Furthermore, the activity of Rdh is dependent on a corrinoid cofactor (vitamin B_{12} derivative). It has been reported the loss of the dehalogenation activity by addition of propyl iodide, a corrinoid antagonist (Numan et al., 1995). The gene encodes 3-chlorobenozote dehalogenase in *Desulfomonile tiedjei* was the firstly identified *rdhA* gene via the amino acid sequencing of the purified RdhA unit (Ni et al., 1995). Later, several hundred *rdhA* gene sequences have been

deposited into different databases based on the sequence similarity to the functionally identified *rdhA* genes. However, the substrate specificity has been assigned for only very few of them (Table 1-2) by direct protein purification, transcription analysis or in- native gel assay of the RdhA units (Hug et al., 2014). In general, the slow growth rate of OHRB and high sensitivity of Rdhs to oxygen are known to aggravate the difficulties encountered during the isolation and purification of the RdhA unit. The last success in two trials for heterologous expression of *rdhA* genes in non-OHRB, fast growing and less oxygen-sensitive microbes, opened the door for the extensive and easier characterization of RdhA units. In the first trial, *vcrA* (involved in the dechlorination of VC) gene from *Dehalococcoides* was expressed in *E. coli* followed by the in vitro refolding of the expressed protein in the presence of iron-sulfur and corrinoid cofactors (Parthasarathy et al., 2015). In the second study, a corrinoid cofactor-producing bacterium, *Shimwellia blattae*, was used directly for the expression of *pceA* gene (involved in the dechlorination of PCE) obtained from *Desulfitobacterium* (Mac Nelly et al., 2014). So far, the two *rdhA* genes *vcrA* and *bvcA* are existed exclusively in members of *Dehalococcoides* and were proved to be involved in the VC to ETH dechlorination step (Stroo et al., 2013). These genes appeared to be recently acquired by horizontal gene transfer within the genomes of *Dehalococcoides*. This supports the possibility of introducing these genes independently to the VC-contaminated sites after carrying on a bacteriophage or any other mobile elements, i.e., bioaugmentation with functional genes rather than the whole microbe (Stroo et al., 2013).

In addition, one or more of OHR-related genes, *rdhCEFGHIJKMNOPRSTZ*, are frequently detected with *rdhAB* genes or operon. With few exceptions, the exact contribution of these genes to OHR is still unknown and generally can be divided into two main categories, regulatory and accessory genes.

Table 1-2 List of some functionally characterized RdhA units

Name of RdhA unit	Electron acceptor	Characterization method	Organism	Reference
PceA	PCE	Direct protein purification	*Dehalococcoides mccartyi*	(Magnuson et al., 1998)
VcrA	VC	Direct protein purification	*Dehalococcoides mccartyi*	(Muller et al., 2004)
BvcA	VC	In-gel activity	*Dehalococcoides mccartyi*	McMurdie et al., 2009
PcbA1	PCB/PCE	In-gel activity	*Dehalococcoides mccartyi*	(Wang et al., 2014)
PcbA4	PCB/PCE	In-gel activity	*Dehalococcoides mccartyi*	(Wang et al., 2014)
PcbA5	PCB/PCE	In-gel activity	*Dehalococcoides mccartyi*	(Wang et al., 2014)
PceA	PCE	Protein purification	*Dehalobacter restrictus*	(Schumacher et al., 1997)
PceA	PCE	Direct protein purification	*Desulfitobacterium hafniense* TCE1	(van de Pas BA et al., 2001)
PceA	PCE	Direct protein purification	*Sulfurosprillium multivorans*	(Neumann et al., 1997)
PceA	PCE	Direct protein purification	*Desulfitobacterium hafniense* Y51	(Suyama et al., 2002)

Regulatory genes

The *rdhC* gene encodes a putative NirR/NosR-type regulator and found in OHRB belonging to all phyla and its actual role has not been clarified. The *rdhS* and *rdhP* genes are assumed to encode the putative proteins of the two component regulatory system, sensory histidine kinase and response regulator, respectively. The genes have been detected in OHRB belonging to phylum *Chloroflexi* and its actual contribution in OHR has yet to be proven experimentally. The *rdhR* gene encodes MarR-type transcriptional regulator. The gene was identified in OHRB members of phyla *Firmicutes* and *Chloroflexi* and its contribution in OHR as a repressor for *rdhA*/*rdhB* operon has been confirmed experimentally (Krasper et al., 2016). The *rdhK* gene encodes a regulator of CRP (cAMP receptor protein)/FNR (fumarate and nitrate reductase regulatory protein)-type and has been found in the OHRB members belonging to phylum *Firmicutes*. In 2000, the activity of the *rdhK* gene encoded protein as an activator for *rdhA*/*rdhB* operon was experimentally confirmed (Smidt et al., 2000)

Accessory genes

The *rdhE* gene is considered to encode a protein of high similarity to GroEL and its co-chaperone GroES that is well characterized two components chaperonin system identified in *E. coli*, which assist in the folding. The *rdhF* gene encodes a putative protein which may participate in the proper incorporation of corrinoid into RdhA units. The *rdhG* gene encodes a putative protein with a speculated role in the maturation of Rdhs or a proteolytic activity. The *rdhH* encodes a hypothetical protein which may be included in the corrinoid modification and the correct incorporation of corrinoid into RdhA units. The gene *rdhI* encodes a hypothetical protein with speculated radial sterile alpha motif (SAM) domain and hence, play a role in the incorporation and modification of Rdh-related cofactors. The *rdhM* and *rdhN* encode proteins which may play a role in the electron

transferring from menaquinones to RdhA unit. The *rdhT* gene has been experimentally proven to encode a chaperone protein which integrates in the correct folding of RdhAs units. RdhT delays the translocation RdhA units to the cell membrane and thus allows enough time for cofactors incorporation into RdhA unit. The *rdhZ* encodes a putative protein, which may acts as a chaperone for *rdhA*/*rdhB* operon (Adrian and Löffler, 2016).

Finally, it is worth to mention that not all regulatory and accessory genes are exist in single OHRB. For example, the regulatory genes are exist as *rdhS* and *rdhP* genes in *Dehalococcoides*, and as *rdhC* gene in *Desulfitobacterium* (Figure 1-6). For OHR activity, the requirements for *rdhA* (Hug et al., 2013), *rdhK* (Smidt et al., 2000), *rdhR* (Krasper et al., 2016) and *rdhT* (Morita et al., 2009) genes were confirmed experimentally. However, follow-up studies are needed to judge whether other genes are essential to OHR activity.

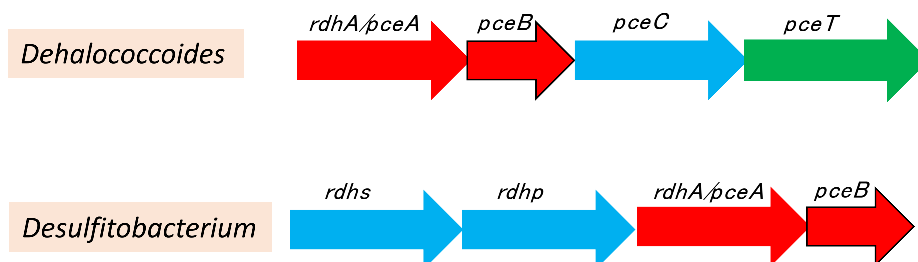

Figure 1-6 Reductive dehalogenase encoding operon in *Dehalococcoides* and *Desulfitobacterium.*

1-3-3 Physiology of OHR (energy conservation and electron transfer to reductive dehalogenase)

Organohalide respiration is an anaerobic respiratory process via reductive dehalogenation in which the microbe uses the halogenated compound as an electron acceptor. The microbe conserves the energy required for the growth from this reaction. In OHRB, two models for electron transfer to reductive dehalogenases has been proposed, quinone-dependent and independent models (Adrian and Löffler, 2016). In the quinone-dependent model (Figure 1-7A), the electrons firstly are acquired from hydrogen oxidation by hydrogenase. The electrons then are transferred through menaquinones (MK) and membrane associated oxidoreductases to the terminal reductases, reductive dehalogenases. The transfer of electron across the membrane creates proton motive force with subsequent generation of ATP (Fincher and Spormann, 2017). The quinone-independent model, without MK involvement, was suggested specially to explain the electron transfer in *Dehalococcoides* and *Dehalogenimonas*. The model has been suggested based on the lack of the genes required for MK biosynthesis in the available genomes of *Dehalococcoides* and *Dehalogenimonas*. Also, the model has been suggested based on the fact that the addition of MK inhibitors did not affect the dechlorination activity in *Dehalococcoides* strains CBDB1 and 195 (Fincker and Spormann, 2017). The quinone-independent model is similar to the first model except that the complex iron-sulfur molybdoenzymes (CISM) substitute the presence and electron transferring function of MK (Figure 1-7B) (Wang et al., 2018).

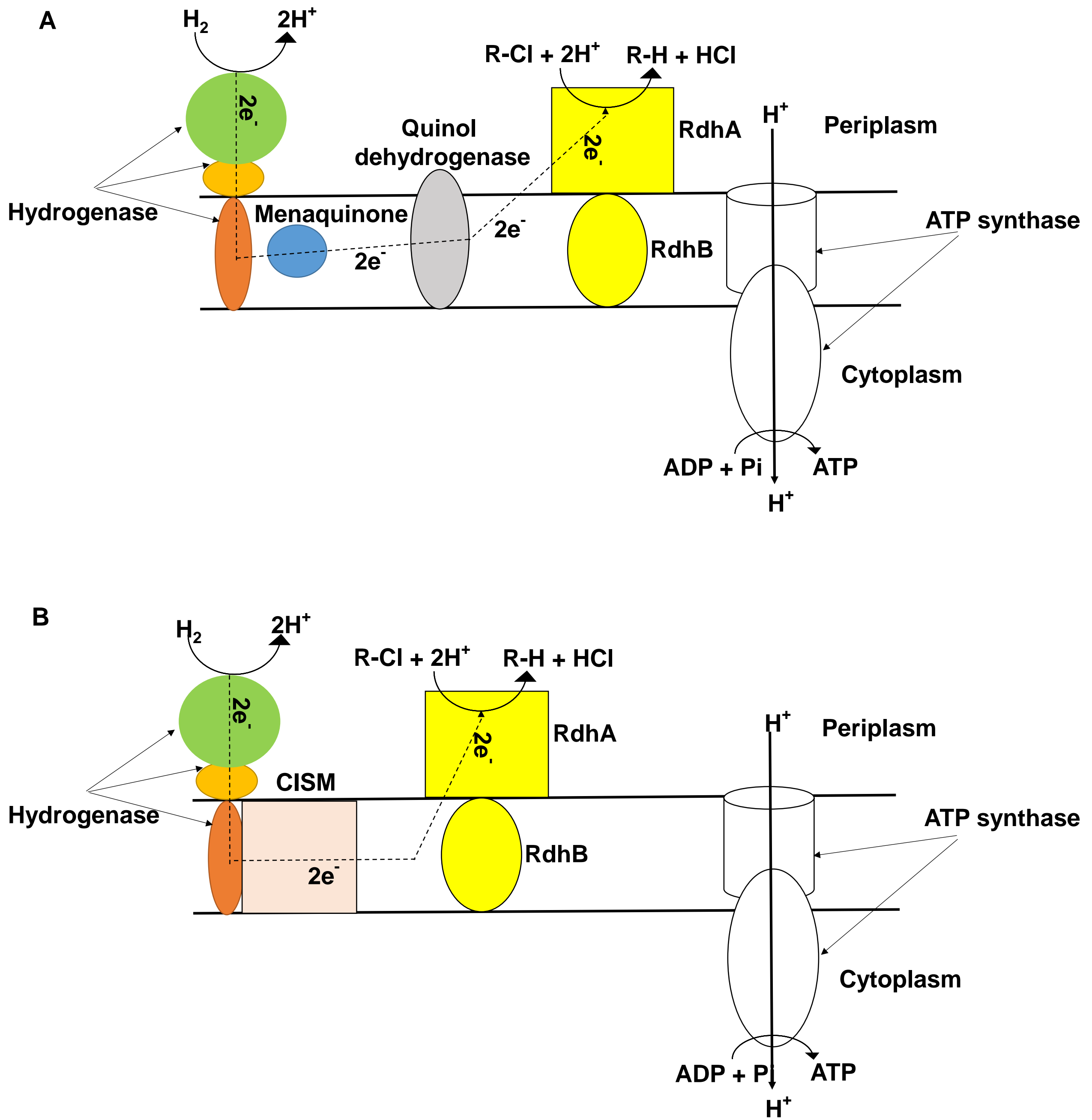

Figure 1-7 Electron transfer in OHRB where, A, Quinone-dependent model, B, Quinone-independent model

1-4 The aim of this research

The main objective of this study is to develop and characterize dehalogenating microbial consortia for detoxification/bioremediation of certain organohalides, which pose serious risks to the human health and the total environment. The study also aimed to identification of the *rdhA* genes involved in the dehalogenation (detoxification/bioremediation) of the organohalides of concern and elucidating the interactions between the dehalogenating and non-dehalogenating members (microbes) inside the dechlorinating culture for evolving an efficient bioremediation technology.

1-5 Organization of the thesis

The thesis consisted of five chapters.

Chapter 1 – described the general introduction, containing the background and the literature survey in the study on the OHRBs and the dehalogenating genes, and the aim of this study.

Chapter 2 –describes the enrichment of microbial consortia with dechlorinating activity against the two toxic and suspected carcinogens, PCE and 112-TCA. Also, this chapter describes the determination of the microbial compositions of these enriched consortia, and the final metabolites were determined in order to judge the applicability of using these cultures for environmental remediation purposes

Chapter 3 –describes the phylogenetic characterization of the PCE to ETH dechlorinating culture, YN3 culture, enriched in chapter 2. The chapter also describes the identification of the different metagenomes detected in YN3 culture and the *rdhA* genes suggested to be involved in the dechlorination of PCE to ETH.

Chapter 4 –describes the characterization of the morphology, physiology and phylogeny of strain

YN3PY1^T, isolated from YN3 culture which enriched and characterized in chapters 2 and 3. This chapter also describes the contribution of the strain YN3PY1^T to the dechlorination activity of *Dehalococcoides*.

Chapter 5 – gives the general discussion on this study, and then describes the significant achievements and the future prospects.

2. Enrichment and screening of organohalide dehalorespiring consortia from environments for dechlorination of tetrachloroethene and 1,1,2-trichloroethane

2.1 Introduction

Chloroethenes (CEs), particularly tetrachloroethene (PCE), and chloroethanes, particularly 1,1,2-trichloroethane (112-TCA), were extensively manufactured and used especially at the beginning of the past century (Löffler et al., 2013). The widespread production and usage of these chemicals were made based on their desired properties such as chemical stability, low-price, good solvent and inflammability (Saiyari et al., 2018). PCE was extensively used in dry cleaning, degreasing and paper manufacture while, 112-TCA being used in degreasing and paint removal (Löffler et al., 2013). As a result of extensive production and usage, accidental spilling, and improper storage and handling, these chemicals were encountered as widespread pollutants for environments, especially aquifer (groundwater). Public concern with respect to the pollution with these chemicals has been raised based on hazardous effects of these chemicals, especially on human health (e.g. suspected carcinogens). Several, chemical and physical methods have been developed in order to decompose these pollutants. However, they are often unsuitable for remediating large scale contaminated aquifer as they are expensive or inefficient, especially for the deep subsurface. On the other hand, biological methods, especially microbial reductive dechlorinationunder anaerobic conditions, represent an efficient and popular method for clean up against these chemicals. Microbial reductive dechlorination is a process carried out by special kind of microbes, the organohalide respiring bacteria (OHRB). OHRB are able to respire and conserve energy for growth upon using these chemicals as electron acceptors. For PCE, several OHRB have been reported with

the incomplete dechlorination activity, i.e., production of toxic (e.g., *cis*-dichloroethene (*cis*-DCE)) or even carcinogenic intermediates (e.g., vinyl chloride (VC)) (Yoshikawa et al., 2017). Fortunately, members of the genus *Dehalococcoides* from the phylum *Chloroflexi*, have been reported with the activity dechlorinating PCE completely into innocuous ethene (ETH) (Saiyari et al., 2018). For the 112-TCA, fewer cultures have been developed with activity of the dechlorination. In most cases, the proven carcinogen VC has been reported as the end product of the dechlorination. In less cases, one or more metabolites of 1,2-dichloroethane (12-DCA), chloroethane (CA) and trace amount of ETH have been detected (Fathepure and Tiedje, 1994; Grostern and Edwards, 2006; Maness et al., 2012; Zhao et al., 2015).

In this chapter, microbial consortia were enriched with dechlorinating activity against the two toxic and suspected carcinogens, PCE and 112-TCA. In addition, the microbial compositions of these enriched consortia were determined with emphasis on the potential dechlorinators or OHRB. The pathways of dechlorination and the final metabolites were determined in order to judge the applicability of using these cultures for environmental remediation purposes.

2.2 Materials and methods

2.2.1 Enrichment of dechlorinating consortia

2.2.1.1 Enrichment of PCE dechlorinating bacteria

A sediment sample collected from Arako River (Nagoya city, Aichi prefecture, Japan) was used as a microbial source for the enrichment of PCE dechlorinating bacteria. In brief, around 10 grams of the sediment were placed in 60-mL serum bottles filled with 20 mL sterile distilled water and supplied with 1 mg/L resazurin as a redox indicator dye. The bottles were then flushed with nitrogen gas for 15 min to create anaerobic condition and sealed with rubber stoppers and aluminum

caps. Finally, the headspace gas of the bottles was exchanged with a mixture of H_2 and CO_2 (4:1, v/v) and then supplied with 20 mM acetate as C source and either 1 mM of PCE or 500 μM of *cis*-DCE as an electron acceptor and incubated for 1 month at 28°C. During the incubation period, 100 μL from the cultures headspace were withdrawn periodically for chemical analysis to judge the presence and extent of the dechlorination activity as described below. After the incubation, 1 mL of the microcosm, which showed PCE or *cis*-DCE dechlorination activity, was transferred to 60-mL serum bottle filled will 20 mL of DHB-CO_3 medium. DHB-CO_3 medium is a mineral medium consisting of (per liter): 1g NaCl; 2.5g $NaHCO_3$; 0.5g KCl; 0.5g NH_4Cl; 0.5 g $CaCl_2 \cdot 2H_2O$; 0.1g $MgCl_2 \cdot 6H_2O$; 0.2g KH_2PO_4; 1 mL of 1 mg/L resazurin solution; 1 mL of trace element solution SL10 (Widdel et al., 1983); 10 mL of vitamin solution (Holliger et al., 1998); 0.5 mL of Se/W solution (Widdel et al., 1983); and 10 mL of titanium (III) trinitriloacetic acid solution (Moench et al., 1983). The medium was prepared under anaerobic condition with flashing of a mixture of N_2 and CO_2 (4:1, v/v), as described previously (Lovley and Phillips, 1986). Prior to the inoculation, the headspace was exchanged aseptically with a gas mixture of H_2 and CO_2 (4: 1, v/v). The transferred cultures were repeatedly incubated and transferred every month after observation of the dechlorination activity. The enriched cultures spiked with PCE initially were not transferred further after the 3rd transfer because the dechlorination activity became weak. On the other hand, the cultures spiked with *cis*-DCE were continuously transferred for the enrichment as the stable dechlorination activity was observed.

2.2.1.2 Enrichment of **112-TCA** *dechlorinating bacteria*

Around 25 grams of soil sample, which have been collected from Kamajima (Aichi, Japan), were inoculated directly into 120-mL serum bottles containing 50-mL DHB-CO_3 medium and

supplemented with 20 mM lactate and 500 μM of 112-TCA. As described above, the samples were purged with nitrogen gas, followed by the incubation at 28°C for one month, and analyzed for the activity of the dechlorination. One mL of the culture was transferred to the fresh medium containing acetate as C source when the dechlorination activity was detected. Prior to the inoculation, the headspace of the bottles was exchanged with mixture of H_2 and CO_2 (4:1, v/v), and then spiked with 500 μM of 112-TCA.

2.2.2 Chemical analysis

PCE, 112-TCA, their chlorinated metabolites and ETH were measured by withdrawing 100 μL-sample from the bottle headspace using gastight glass syringe with subsequent manual injection into a gas chromatograph (GC) (GC-2014, Shimadzu, Japan) equipped with a flame ionization detector (FID) and a Porapak Q column (80/100 mesh, 0.3 mm in diameter and 2 min length). Nitrogen was used as a carrier gas at a flow rate of 35 mL/min. Temperature of the column was set at 200°C, and the injection and detection temperatures were also set at 200°C.

2.2.3 DNA extraction

The cell pellet for DNA extraction was obtained by centrifugation at 15, 000 xg for 15 minutes. The DNA was extracted according to He. (2011). The cell pellet was re-suspended in a lysis buffer (consisted of 9.34 ml TE buffer, 600 μL of 10% SDS, 60 μL of 20 mg/mL proteinase K and 60 μL of 10 mg/ml lysozyme) and incubated at 37ºC for one hour. After incubation, the lysate was extracted with phenol/chloroform/isoamyl alcohol, and the extracted DNA was precipitated using ethyl alcohol (95%) and 3 M sodium acetate. The precipitated DNA was washed with ethyl alcohol (80%) and then dissolved into 50 μL water.

2.2.4 Microbial composition analysis

The microbial composition of the enriched culture was analyzed by PCR amplification of V4 region of their 16S rRNA genes using the primer pair 515F and 806R (Table 2-1). The primer pair, which is universal for both bacteria and archaea, was modified by adding Illumina adapter sequences according to Caporaso et al. (2011). PCR products was subjected to the second PCR amplification to add Illumina barcode sequences and then sequenced on Illumina MiSeq platform with paired-end sequencing mode at Bioengineering Lab. Co., Ltd. (Kanagawa, Japan). Obtained sequences were then passed through quality filtering using Sickle software version 1.33 (Joshi and Fass, 2011). Chimera sequences were identified and removed using the UCHIME algorithm in USEARCH. Finally, the 16S rRNA operational taxonomic units (OTUs) were grouped with 97% similarity as threshold, through the Quantitative Insights Into Microbial Ecology (QIIME) pipeline software (Caporaso et al., 2010).

Table 2-1 List of primers used in this study

Primer	Target	Direction	Sequence (5′ to 3′)	Reference
515F	16S rRNA gene of bacteria (V4 region)	Forward	GTGCCAGCMGCCGCGGTAA	(Muyzer, et al, 1993)
806R		Reverse	GGACTACHVGGGTWTCTAAT	
DhcF50	16S rRNA gene of *Dehalococcoides*	Forward	CCTTATGCATGCAAGTCGAA	This study
DhcR155		Reverse	ACCACATGCGGTATTACCTTC	
341F	Bacterial 16S rRNA gene (V3 region)	Forward	CCTACGGGAGGCAGCAG	(Muyzer, et al, 1993)
518R		Reverse	ATTACCGCGGCTGGCTGG	

2.2.5 Quantitative PCR (qPCR)

The 16S rRNA gene copy numbers of *Dehalococcoides* and total bacteria were quantified by qPCR using specific and universal primer sets, respectively (Table 2-1). Primers were designed using GenScript online tool (www.genscript.com). The qPCR reaction was conducted on a

LightCycler system (Roche Diagnostics, Germany) using FastStart essential DNA Green Master kit (Roche Diagnostics, Germany).

2.3 Results

2.3.1 Enrichment of PCE/*cis*-DCE to ETH dechlorinating culture

A serial transfer culture established from chloroethene-contaminated river sediment, using 1 mM PCE and 500 μM *cis*-DCE, yielded two enrichment cultures: an unstable PCE-dechlorinating culture and the stable *cis*-DCE to ETH-dechlorinating culture (designated YN3 culture) (Figure 2-1). YN3 culture maintained activity to dechlorinate 500 μM *cis*-DCE to ETH within 15 days even after 50 transfers over five years. In contrast, the PCE dechlorinating culture lost its dechlorination activity after the third transfer. Next, YN3 culture was tested for dechlorination of PCE at a low concentration (70 μM) and showed stable PCE to ETH dechlorination activity.

2.3.2 Dechlorination of PCE to ETH by YN3 culture

Figure 2-2 shows the concentration changes of PCE and its chlorinated intermediates over the incubation time in YN3 culture, initially spiked with 70 µM of PCE. At the sixth day, YN3 culture initiated the PCE to trichloroethene (TCE) dechlorination. At day 11, the culture started the dechlorination of TCE to ETH with metabolite formation of *cis*-DCE and VC. At day 25, the production of ETH reached its maximum concentration (54 ± 2 μM) which represented 77% of the initially spiked PCE. These results indicated the capability of YN3 culture to dechlorinate PCE completely into ETH, one chlorine by one chlorine, via the dechlorinated intermediates TCE, *cis*-DCE and VC. In addition, the culture completely dechlorinated the re-spiked PCE at 200- 800 µM

within 14 days (Figure 2-3). YN3 culture also dechlorinated 200 μM of 1,1-dichloroethene (11-DCE) completely into ETH but showed no dechlorination activity toward trans-dichloroethene (*trans*-DCE).

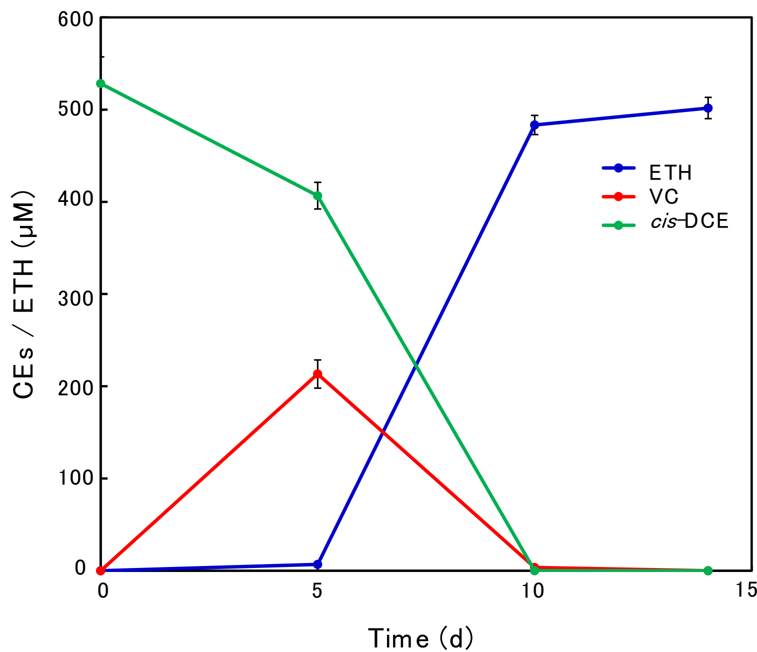

Figure 2-1 Dechlorination of *cis*-DCE to ETH by YN3 culture

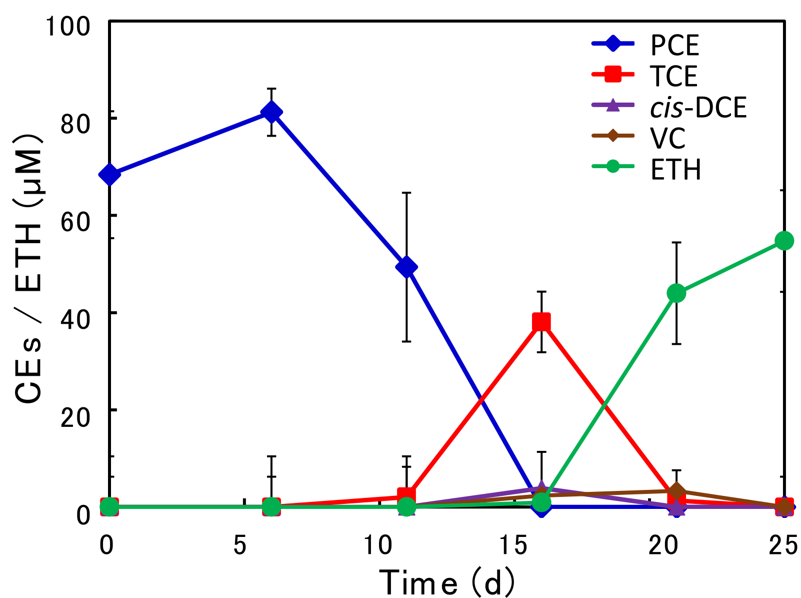

Figure 2-2 Dechlorination of PCE (70 μM) to ETH by YN3 culture

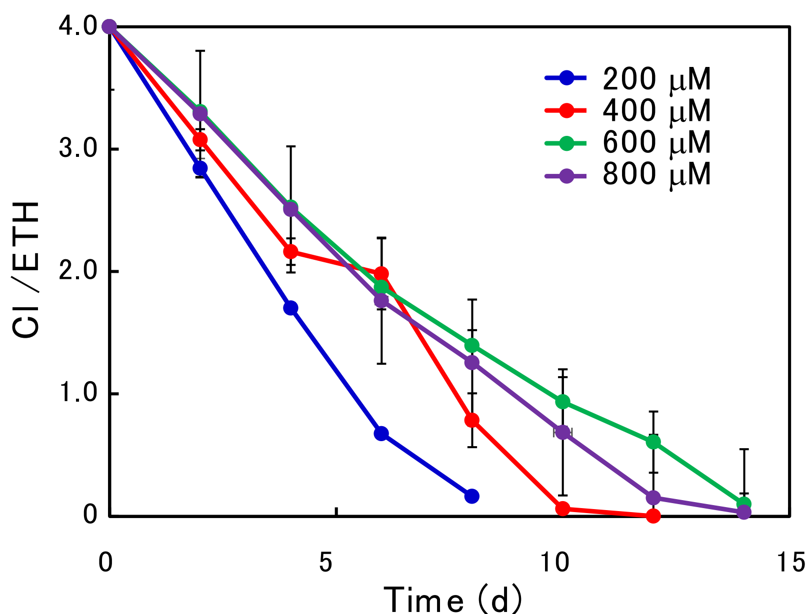

Figure 2-3 Dechlorination of PCE (200-800 μM) to ETH by YN3 culture

2.3.3 Enrichment of 112-TCA dechlorinating culture

The microcosm of paddy soil slurry (Kamajima) spiked with 20 mM lactate and 500 μM of 112-TCA dechlorinated 112-TCA to VC completely within a month. Then a portion of the microcosm was transferred to fresh medium supplemented with 20% H_2 in headspace, 5 mM acetate and 500 μM of 112-TCA as electron donor, C source and electron acceptor, respectively. The serial transferring cultivation using H_2 and acetate and 112-TCA has been performed periodically over around 2.5 years. The obtained culture (designated KJ-TCA culture) stably showed dechlorination of 112-TCA to VC without any other intermediate, suggesting dichloroelimination type of reductive dechlorination. Figure 2-4 shows the overall dechlorination pathways and metabolites observed in the two enrichment cultures, YN3 and KJ-TCA.

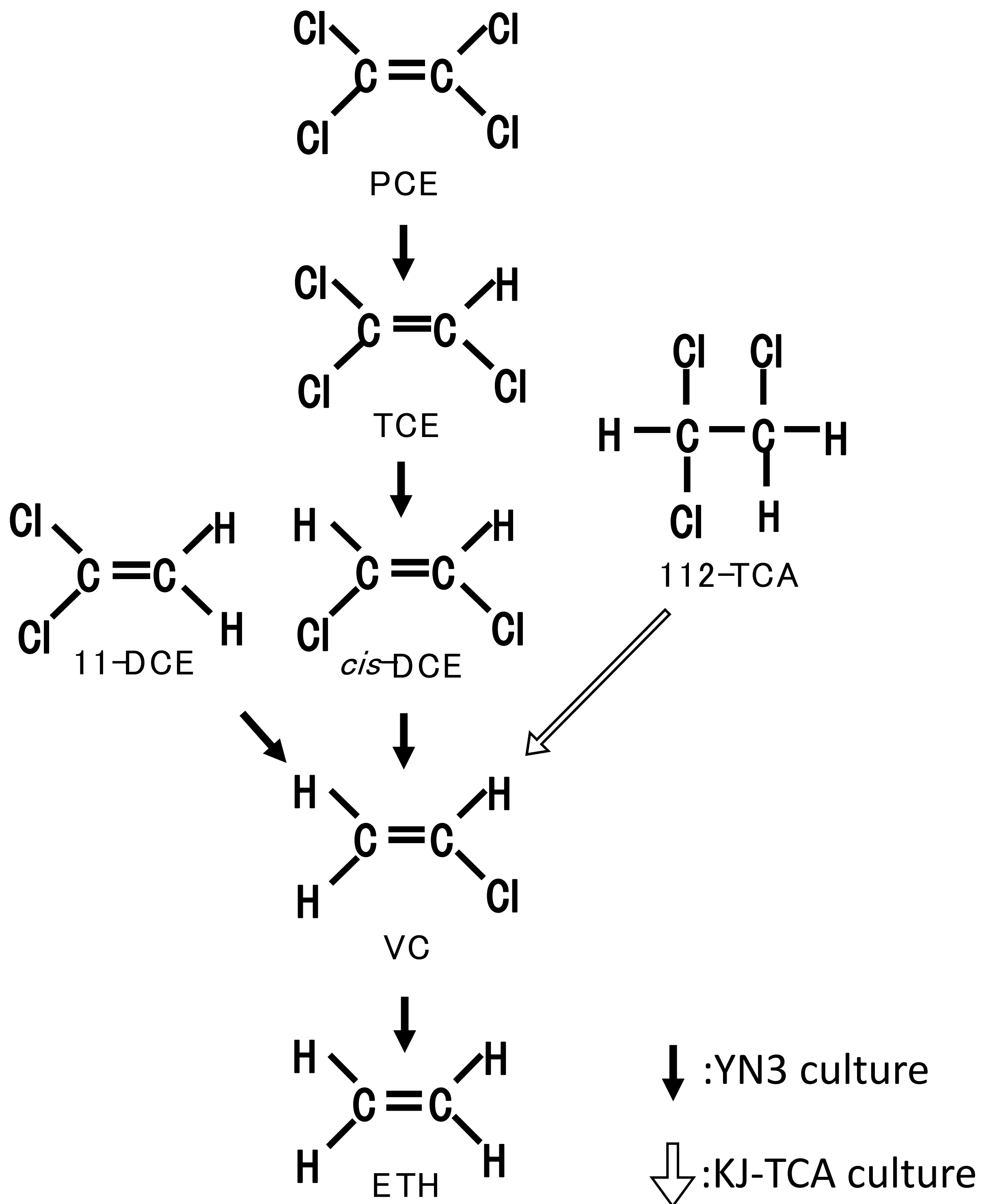

Figure 2-4 Dechlorination pathways observed in the two enrichment cultures, YN3 and KJ-TCA

2.3.4 Identification of microbial composition in YN3 and KJ-TCA cultures

In order to identify the microbial compositions of YN3 and KJ-TCA cultures Illumina MiSeq analysis was carried out. The microbial composition of YN3 culture showed the dominance of bacteria affiliated to genera *Bacteroides* (42%), *Tisserella* (32%) and *Dehalococcoides* (25%) (Table 2-2). Among these bacteria, members of *Dehalococcoides* are only the bacterium that has been reported as OHRB. *Dehalococcoides* has been reported as an obligate OHRB that cannot grow without organohalides. It was suggested that the detected *Dehalococcoides* was involved in the dechlorination of CEs in the YN3 culture. The sequence of OTU affiliated to genus *Dehalococcoides* has around 250 bp of length and has 100% of sequence similarity with *Dehalococcoides* strains CG5, IBARAKI and CBDB1.

The members comprising the KJ-TCA culture belonged to genera *Acetobacterium* (51%), *Bacteroides* (13%), *Dehalobacter* (9.9%), *Sedimentibacter* (9.2%), *Lutispora* (3.1%) and Order *Bacteroidales* (9.6%) (Table 2-3). *Dehalobacter* is only known OHRB detected in the KJ-TCA culture, and the detection suggested the involvement of the bacterium corresponding to *Dehalobacter*-OTU in the dechlorination of TCA to VC in the KJ-TCA culture. The sequence of OTU affiliated to genus *Dehalobacter* has around 250 bp of length and has 99% of sequence similarity with *Dehalobacter* strain TCP.

Table 2-2 Microbial composition of YN3 culture as determined by Illumina MiSeq analysis. *Dehalococcoides* as the potential dechlorinator was shown in bold.

OTU/taxonomic order	Ratio (%)
Genus *Bacteroides*	41.8
Genus *Tisserella*	32.4
Genus ***Dehalococcoides***	**24.8**
Genus others (<0.05% each)	1.0

Table 2-3 Microbial composition of KJ-TCA culture as determined by Illumina MiSeq analysis. *Dehalobacter* as the potential dechlorinator was shown in bold.

OTU/taxonomic order	Ratio (%)
Genus *Acetobacterium*	50.6
Genus *Bacteroides*	13
Genus ***Dehalobacter***	**9.9**
Order Bacteroidales	9.6
Genus *Sedimentibacter*	9.2
Genus *Lutispora*	3.1
others (<0.05% each)	4.6

2.3.5 qPCR

To confirm the involvement of *Dehalococcoides* in the dechlorination of PCE to ETH dechlorination, qPCR was conducted targeting the 16S rRNA genes of *Dehalococcoides* and total bacteria. The qPCR showed an increase in the 16S rRNA gene copy number of *Dehalococcoides* from $0.86 \pm 1.5 \times 10^7$ copies/mL at day 0 to $1.6 \pm 0.69 \times 10^8$ copies/mL at day 21, the day at which PCE is completely dechlorinated to ETH (Figure 2-5). Also, the 16S rRNA gene copy number of the total bacteria increased from $3.0 \pm 5.2 \times 10^7$ copies/mL to $6.7 \pm 4.0 \times 10^8$ copies/mL at the same period of time from day 0 to day 21. This suggested that during the dechlorination, the population of *Dehalococcoides* occupied 15–28% of the total bacterial population. The results also showed the population growth of *Dehalococcoides* and total bacteria in YN3 culture during the *cis*-DCE to ETH dechlorination and

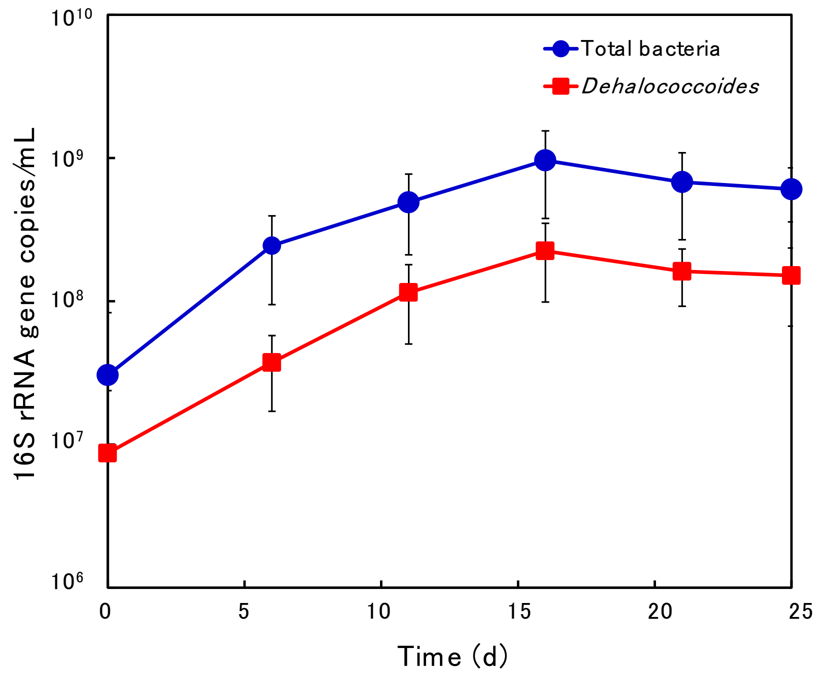

Figure 2-5 Changes in the 16S rRNA gene copy numbers of *Dehalococcoides* and total bacteria during PCE dechlorination to ETH

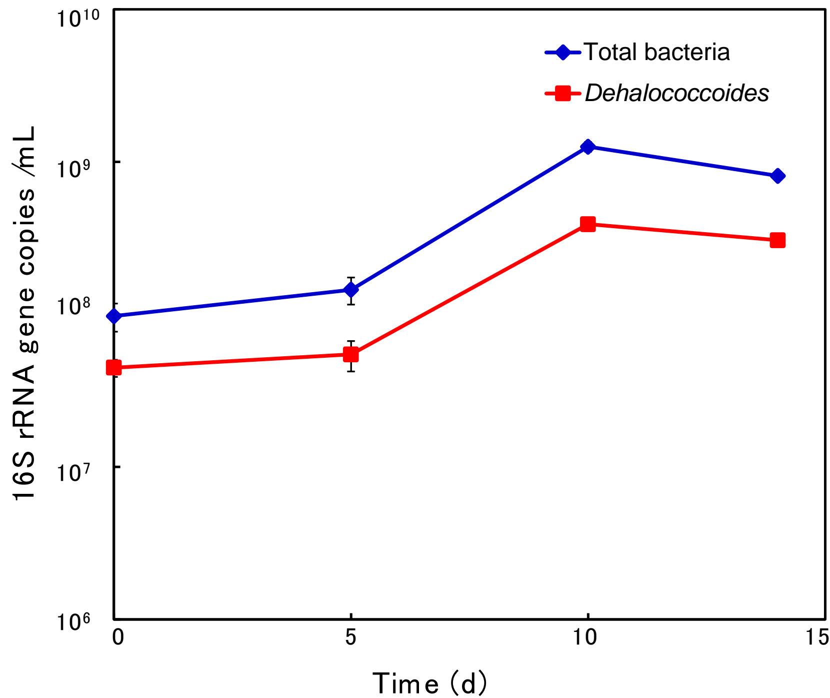

Figure 2-6 Changes in the 16S rRNA gene copy numbers of *Dehalococcoides* and total bacteria during *cis*-DCE dechlorination to ETH

2.4 Discussion

In this study, two enriched cultures that dechlorinate PCE and 112-TCA were successfully obtained and designed as YN3 and KJ-TCA cultures, respectively. The YN3 culture showed stable activity for the dechlorination of up to 800 μM PCE into ETH and included *Dehalococcoides* as candidate bacteria involved in the dechlorination. Several studies have indicated the ability of very few cultures of *Dehalococcoides* either in pure or enriched form to do the complete dechlorination of PCE to ETH. Additionally, the PCE to ETH dechlorinating activity of the YN3 culture appears to be superior in the dechlorination rate than the other studies. They are the KB-1/PCE culture which dechlorinates 100-300 μM PCE to ETH within two weeks (Duhamel et al., 2002), BTF08, a highly enriched culture containing with only single strain of *Dehalococcoides* as a dechlorinator, and dechlorinating up to 500 μM in approximately 100 days (Cichocka et al., 2010), and finally the AMEC-4P culture which dechlorinated up to 2 mM PCE to ETH within 143 days (Kim et al., 2010).

In the KJ-TCA culture, we observed the dichloroelimination of 112-TCA to VC and presence of *Dehalobacter* as known OHRB. Dechlorination of 112-TCA has been reported before in several pure and enriched cultures with representative two pathways as follows. One is the dichloroelimination of 112-TCA to VC as the sole end product or as the major end products, and this pathway has been observed in the culture containing *Dehalobacter, Desulfitobacterium, Dehalogenimonas* as dehalorespiring bacteria (De Wildeman et al., 2003; Aulenta et al., 2006; Grostern et al., 2006; Maness et al., 2012). The another pathway is the dechlorination of 112-TCA to 1,2-DCA. This second pathway was firstly observed in the culture of *Desulfomonile tiediei* strain DCB-1 (Chen et al., 1996) and then observed also in the culture of *Desulfitobacterium* sp. strain PR that dechlorinates 112-TCA to 12-DCA and CA as the major end products with trace amounts of VC and ETH (Zhao et al., 2015).

Besides dehalorespiring bacteria, members of the genus *Bacteroides* were coexisted in the both cultures. Genus *Bacteroides* have been frequently detected in the dehalorespiring microbial community (Miura et al., 2015; Duhamel et al., 2006; Freeborn et al., 2005). The non-dechlorinating bacteria in the two cultures also suggested the possible contribution to the enhancement of the dechlorination activity of *Dehalococcoides* and *Dehalobacter*.

2.5 Conclusion

Two enrichment cultures of OHRB were newly constructed in this study: YN3 culture including *Dehalococcoides* as known OHRB that dechlorinates PCE to ETH, KJ-TCA culture containing *Dehalobacter* as candidate dehalorespiring bacteria that dechlorinates 112-TCA to VC. *Bacteroides* co-existed in both enrichment cultures, indicating a possible contribution to support the dehalorespiring bacteria in the two cultures.

3. Metagenomic analysis of tetrachloroethene to ethene dechlorinating *Dehalococcoides* enriched culture and transcriptional analysis of its reductive dehalogenases genes

3.1 Introduction

The suspected carcinogen tetrachloroethene (PCE) was extensively produced and used in diverse industrial purposes particularly in the developed countries. As a result of improper disposal, PCE was commonly detected as a groundwater and aquifers pollutant (Stroo et al., 2013). Several organohalide respiring bacteria (OHRB) have been reported during the last three decades with the ability to dechlorinate PCE (Yoshikawa et al., 2017). However, the dechlorination process usually proceeds as incomplete and resulted in the formation of more toxic or carcinogenic metabolites such as *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC) (Adrian and Löffler, 2016). Fortunately, several members of the genus *Dehalococcoides* (Löffler et al., 2013) and single member of the genus *Dehalogenimonas* (Yang et al., 2017) can dechlorinate *cis*-DCE and VC into innocuous ethene (ETH). In addition, very few *Dehalococcoides* enriched cultures can dechlorinate PCE completely into ETH (Duhamel et al., 2002; Cichocka et al., 2010; Kim et al., 2010; Lee and Lee, 2015). Several studies have reported that *Dehalococcoides* grew more robustly in mixed culture with other bacteria, the coexisting bacteria (He et al., 2007; Men et al., 2012). The coexisting bacteria help *Dehalococcoides* by supplementation with nutrients, cofactors, or elimination of toxic substances (Distefano et al., 1992; He et al., 2007; Men et al., 2012; Zhuang et al., 2014; Liu et al., 2017). To date, genome sequences for several strains of *Dehalococcoides* have been completed and released into public databases. The genomes of *Dehalococcoides* are small in size and contained multiple *rdhA* genes, the gene encodes the catalytic unit (RdhA) of the reductive dehalogenase

(Rdh) (Löffler et al., 2013). Several hundred of *rdhA* genes were deposited into GenBank, however, only very few of them have been functionally characterized (Hug et al., 2014). For example: *pceA* gene which involved in the dechlorination of PCE to TCE, *tceA* which involved in the dechlorination of TCE and *cis*-DCE to VC, and *vcrA* which involved in the dechlorination of *cis*-DCE and VC into ETH (Hug et al., 2013). The identification of these genes were carried out based on reverse genetics using primers designed from the classically and partially purified RdhAs units. In general, the low biomass obtained is known to hinder the efforts for functional characterization of RdhAs units in *Dehalococcoides*. Nowadays, other promising and easier methods were successfully employed to overcome the low biomass obtained by *Dehalococcoides* such as the RT-qPCR and native gel assay (Chow et al., 2010; Wang et al., 2014). The *bvcA* gene which dechlorinates *cis*-DCE and VC into ETH was typically identified using RT-qPCR at first and later by native gel assay (Hug et al., 2013).

The aims of this chapter were to phylogenetically characterize the PCE to ETH dechlorinating YN3 culture, enriched in chapter 2, and to identify metagenome of the dechlorinator as well as, the metagenomes of other bacteria beside the dechlorinator in YN3 culture, then finally to identify the *rdhA* genes suggested to be involved in the dechlorination of PCE to ETH in YN3 culture.

3.2 Materials and methods

3.2.1 DNA extraction

After complete dechlorination of CEs to ETH, cells from YN3 culture were harvested by centrifugation at 15,000 ×g for 15 minutes. The cells were then stored at −20°C until the DNA extraction. DNA was extracted as mentioned in chapter 2.

3.2.2 Metagenome sequencing, analysis and phylogenetic trees construction

The total DNA obtained was directly sequenced using Illumina HiSeq platform at Hokkaido System Science Co., Ltd. (Hokkaido, Japan) after addition of Illumina adaptor sequences. Trimming of the Illumina adaptor sequences followed by sequences assembly were carried out using Cutadapt (version 1.1) (Martin, 2011) and Velvet (version. 1.2.08) (Zerbino and Birney, 2008) softwares, respectively. Gene prediction and classification into functional categories were carried out using the online servers Microbial Genome Annotation Pipeline (MiGAP) (Sugawara et al., 2009) and Rapid Annotation using Subsystems Technology (RAST) (Aziz et al., 2008). The contigs obtained from the metagenomic reads were taxonomically classified based on DNA sequence similarity of the entire genes by NCBI BLASTN (https://blast.ncbi.nlm.nih.gov). In this study, contigs having at least 90% sequence similarity to a known bacterial sequence were classified at the genus level (Jung et al., 2011; Maphosa et al., 2012). Contigs showing poor similarity with partial sequences in the known genome were classified based on similarity of the translated sequences of individual genes presenting in the contigs with genes in the published genome by BLASTP. Construction of the phylogenetic trees based on sequences of 16S rRNA genes and predicted RdhAs were carried out using MEGA6 software (Tamura et al., 2013).

3.2.3 Reverse transcription-quantitative PCR (RT-qPCR)

To identify which *rdhA* genes from YN3 culture, (designated as *YN3rdhA*), potentially involved in the PCE to ETH dechlorination, transcripts or mRNA of *YN3rdhA* genes were targeted and quantified using specific primers and RT-qPCR in YN3 culture spiked with different chloroethenes (CEs), (Table 3-1). The YN3 culture was firstly spiked with either PCE or *cis*-DCE

and incubated for around 1 month to ensure the complete dechlorination of PCE or *cis*-DCE to ETH. Subsequently, the culture bottle was purged with a filter-sterilized nitrogen gas to eliminate the remained CEs or ETH. The YN3 culture was then incubated for additional 5 days as starvation period without electron donor or acceptor to eliminate any residual mRNA from the cells. From the starved culture, 20 ml aliquots were then withdrawn and transferred into 6o-ml serum bottles and spiked ith either PCE, TCE, *cis*- DCE or VC at concentrations 100 μM, 200 μM, 500 μM and 100 μM, respectively. In parallel, aliquots from the starved culture were withdrawn and kept without addition of any CEs to be used as negative control. For RNA and DNA extraction, cells from the spiked and negative control samples were collected by centrifugation after 9–15 hours. Furthermore, cells specified for RNA extraction were spiked with luciferase mRNA as an internal control (Promega, Fitchburg, WI, USA) prior to RNA extraction to quantify the RNA loss during the different preparation steps. RNA extraction was conducted using ISOGEN II kit (Nippon Gene, Tokyo, Japan), while quantifications of *rdhA* transcripts and luciferase mRNA were carried out using the One Step SYBR PrimeScript RT-PCR Kit II (Takara Bio Inc., Japan). Recovery percentages for the added luciferase mRNA were calculated, and then the mRNA of the *rdhA* genes were normalized to these values. In our experiment the recovery rates of the luciferase mRNA were in the range of 2–17%. Finally, the transcription level of the each *rdhA* gene was calculated by dividing the mRNA copy number of the gene (after normalization to luciferase mRNA) by its gene copy number which quantified by the qPCR in parallel.

Table 3-1 List of primers used in this study

Primer	Target	Direction	Sequence (5´ to 3´)	Reference
F640	*YN3rdhA1*	Forward	TATACTACCGGTCACGCCAA	This study
R741		Reverse	CGGTAAGTGCATTCAGGGTA	
F117	*YN3rdhA2*	Forward	AGCTGTCTCTGCGCCTGTAG	This study
R219		Reverse	TCCACGAATTGTCCGAACGC	
F3	*YN3rdhA3*	Forward	GCAAGGATTCCATTCTGCTT	This study
R107		Reverse	GGAAGCACCTTCATCCAAAT	
F1143	*YN3rdhA4*	Forward	CCAGAGCTGGGCTGATGACA	This study
R1239		Reverse	CGCTGGCACAAGGCGTAAAT	
F926	*YN3rdhA5*	Forward	TGGTTACTGACCTGCCGCTT	This study
R1010		Reverse	ACACTGGGCGGGACATTCAT	
F1050	*YN3rdhA6*	Forward	TGAGCCTACTCCGCCCATTG	This study
R1136		Reverse	GGGCTCATGGTCAGTGGGAA	
F743	*YN3rdhA7*	Forward	ACGTAGACGAAGCGGCTGAA	This study
R828		Reverse	GGCGTCTGGTGGACTCGTAA	
F600	*YN3rdhA8*	Forward	GGGTACTCCCGAAGATAACCT	This study
R706		Reverse	CCGTACTGGTCAACGGTAAA	
F1323	*YN3rdhA9*	Forward	CCTTTCCACCACCGGCATTT	This study
R1417		Reverse	AAGCCGTATCTGGGCAGGTC	
F616	*YN3rdhA10*	Forward	CAGGATGTTGGTTGTGCCGAA	This study
R705		Reverse	CAATCTGTTTGCCGCCTGCT	
F373	*YN3rdhA11*	Forward	TATGGCTGGCAGGCAGGTTT	This study
R460		Reverse	CTGCCATTCCAAGCAGGCAA	
F1248	*YN3rdhA12*	Forward	AGGCTTCTGGCCTGACATGG	This study
R1345		Reverse	TGCATCATGGCGGCTTTGTC	
F116	*YN3rdhA13*	Forward	ACTCTGTGGGCGGCATACAT	This study
R200		Reverse	ATAGCCGACTGCCGGGTTAC	
F381	*YN3rdhA14*	Forward	GGGTACTTCTCCCGGTTGGG	This study
R480		Reverse	CCGCCCTGAGCATACGTGAA	
F128	*YN3rdhA15*	Forward	CGCCTAAGGCTGAATGGAAACG	This study
R220		Reverse	CTGTCTGACGGGCATCCCA	
F459	*YN3rdhA16*	Forward	GCACGGACTATTCCAACCTT	This study
R565		Reverse	TCTGGAGTACCCTCCCATTT	
F55	*YN3rdhA17*	Forward	TTAGCAGGCGCGGGAGTAG	This study
R148		Reverse	TGCTTAACCCACCAGGGCAT	
F2	*YN3rdhA18*	Forward	TGAAGGGACTGGGTCTGGCT	This study
R93		Reverse	GGGCGACTAAGTGTTGCCTGA	

3.3 Results

3.3.1 Identification of the OHRB in YN3 culture

Analysis of the metagenome of YN3 culture (designated as YN3 metagenome) indicated the presences of a phylotype belonging to the genus *Dehalococcoides* (designated as phylotype YN3-01), which was considered as the sole dechlorinating bacterium in YN3 culture. The 16S rRNA gene sequence of the phylotype YN3-01 agreed completely (100% similarity) with those of *Dehalococcoides* strains CBDB1, CG5, DCMB5 and IBARAKI. All these strains are affiliated within in the Pinellas subgroup of *Dehalococcoides* (Figure 3-1). Subgrouping the members of *Dehalococcoides* into three subgroups Pinellas, Cornell and Victoria was suggested based on the detection of small differences in the 16S rRNA gene sequences between the three subgroups (Hendrickson et al., 2002). Notably, strains CBDB1, CG5, and DCMB5, which harboring 16S rRNA genes identical to this of phylotype YN3-01, were unable to dechlorinate neither DCE nor VC. This indicated that the strains with identical 16S rRNA gene sequences were not necessarily to have the same dechlorination spectrum as suggested previously (Loffler et al., 2013; Portiz et al., 2015; Wang et al., 2014).

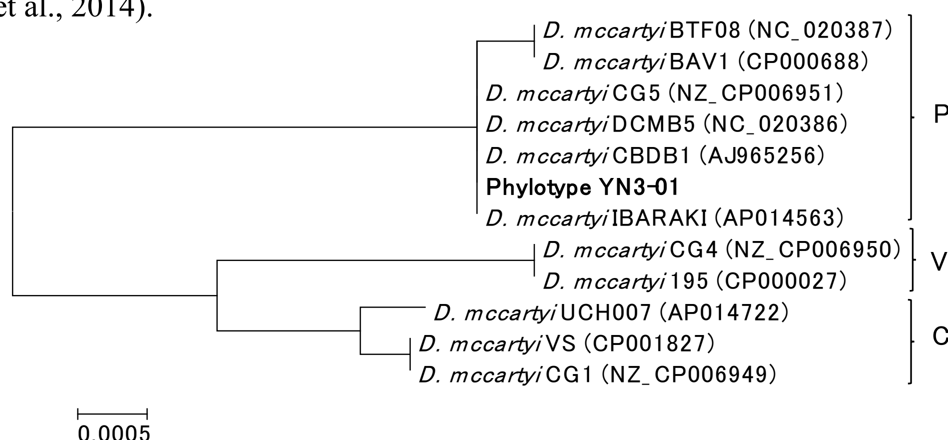

Figure 3-1 Neighbor-joining tree showing the phylogenetic position of phylotype YN3-01 and related strains of the genus *Dehalococcoides*. P, V, and C represent taxonomic subgroups of *Dehalococcoides*, where (P) = Pinellas, (C) = Cornell, and (V) = Victoria. The GenBank accession numbers are shown in parentheses. The bar represents 0.05 substitutions per 100 bp.

3.3.2 *Dehalococcoides*-metagenome

Analysis of YN3 metagenome indicated that 65 contigs were assigned to *Dehalococcoides*-metagenome (the contigs had a total length of 1.3Mbp). The number of protein coding sequences (CDSs) and size of the *Dehalococcoides*-metagenome are similar to genomes published before for the other strains of *Dehalococcoides* (Table 3-2). Among the *Dehalococcoides*-metagenome, 48 contigs (104 to 202,438 bp in length) showed 92-100% similarity to the genomes of strains DCMB5 and CG5 which were phylogenetically affiliated within the Pinellas subgroup of *Dehalococcoides*. The remained 17 contigs of *Dehalococcoides* metagenome (129–2,468 bp in length) showed 91–99%similarity to the genome sequence of *Dehalococcoides* strain VS which belonging to Victoria subgroup of *Dehalococcoides*. Because of the limited sequencing information as well as the high similarity between the genomes of different strains of *Dehalococcoides*, it was unable to determine whether the contigs of *Dehalococcoides*-metagenome represented a single strain of *Dehalococcoides* or not. The overall comparison of the *Dehalococcoides* metagenome with those of strains CG5, CBDB1, and DCMB5 (the closest strains with *Dehalococcoides* in the YN3 culture with identical 16S rRNA gene) revealed that both CDSs number (1420) and size (1.34 Mbp) of *Dehalococcoides* metagenome in the YN3 culture were slightly lower than those of the closest strains, which are 1413–1477 CDSs and 1.39–1.43 M bp, respectively. With very few exception, RAST-functional categorizations of the genes of *Dehalococcoides*-metagenome and genomes of related strains were very similar (Figure 3-2(A)).

Table 3-2 Characteristics of the *Dehalococcoides*-metagenome and comparison with genomes of other strains of *Dehalococcoides*

Strain or consortium	**Size**	**% G+C**	**CDS**	**rRNAs**	**tRNAs**	**Predicted *rdhAs***	**Reference**
YN3 culture	1,346,295	47.2	1,420	3	46	18	This study
CBDB1	1,395,502	47.0	1,458	3	47	32	(Kube et al., 2005)
DCMB5	1,431,902	47.1	1,477	3	46	23	(Portiz et al., 2013)
CG5	1,362,151	47.2	1,413	3	47	26	(Wang et al., 2014)
BTF08	1,452,335	47.3	1,529	3	46	20	(Portiz et al., 2013)
BAV1	1,341,892	47.2	1,371	3	48	11	(McMurdie et al., 2009)
CG1	1,486,678	46,9	1,557	3	49	35	(Wang et al., 2014)
VS	1,413,462	47.3	1,442	3	50	36	(McMurdie et al., 2009)
IBARAKI	1,451,062	47.0	1,516	3	47	28	(Yohda et al., 2015)
UCH007	1,473,548	46.9	1,509	3	47	29	(Uchino et al., 2015)
CG4	1,382,308	48.7	1,421	3	47	15	(Wang et al., 2014)
195	1,469,720	48.9	1,591	3	46	17	(Seshadri et al., 2005)

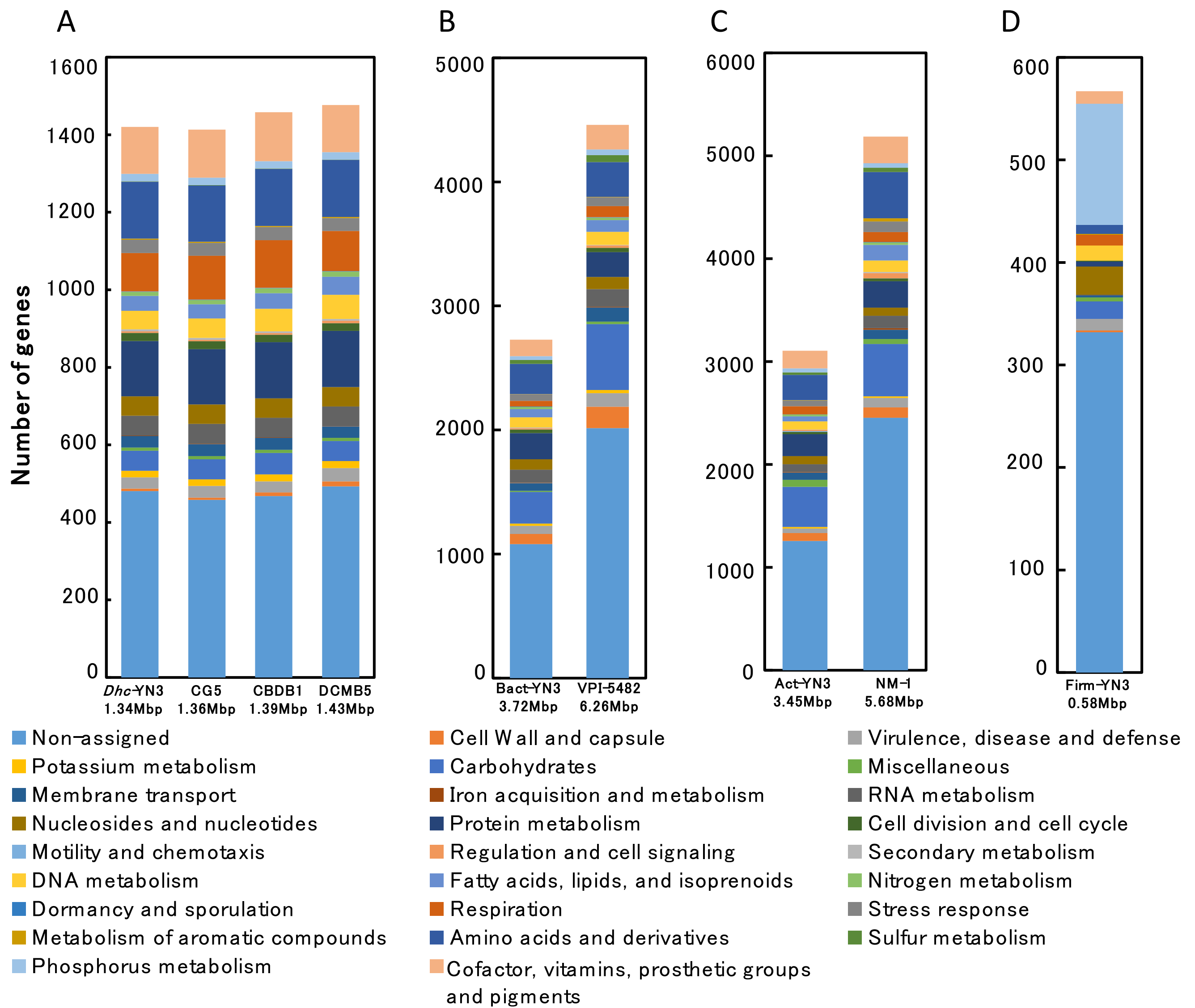

Figure 3-2 RAST functional distribution of genes identified in YN3 metagenome. (A) YN3 *Dehalococcoides*-metagenome (Dhc-YN3) and *Dehalococcoides* strains CBDB1, CG5 and DCMB5, (B) *Bacteroidetes*-metagenome (Bact-YN3) and *Bacteroides thetaiotaomicron* VPI-5482, (C) *Actinobacteria*-metagenome (Act-YN3) and *Microlunatus phosphovorus* NM-1, and (D) *Firmicutes*-metagenome (Firm-YN3).

3.3.3 Non *Dehalococcoides*-metagenomes

After extracting the contigs belonged to *Dehalococcoides*, the other contigs of YN3 metagenome were further assigned into metagenomes belonged to phyla *Bacteroidetes, Actinobacteria* and *Firmicutes*. Fortunately, we detected the housekeeping genes, as well as 16S

rRNA gene, among the *Bacteroidetes*-metagenome which showed 97.1 % similarity with 16S rRNA gene of *Bacteroides thetaiotaomicron* VPI-5482. Also, we detected the housekeeping gene, *rpoB* gene, among the *Actinobacteria*-metagenome which showed 86% similarity with *rpoB* gene of *Microlunatus phosphovorus* NM-1. Thus, we were able to compare the metagenomes assigned for to those phyla with the closest strains (Figure 3-2 (B and C)). On the other side, we were unable to compare the *Firmcutes*-metagenome to any published genomes due to its very small size and absence of any housekeeping genes (Figure 3-2(D)). In comparison with genomes of their closest relatives (Figure 3-2(B and C)), *Bacteroidetes*-metagenomes and *Actinobacteria*-metagenomes showed smaller-sized metagenomes and overall lower number of genes assigned for RAST-functional categories

3.3.4 Reductive dehalogenase and associated genes

Analysis of *Dehalococcoides*-metagenome indicated the presence of full length 18 *YN3rdhA* genes (designated as *YN3rdhA1-18*), which encoded the catalytic subunits of Rdh. Each *YN3rdhA* gene was found with accompanying *rdhB* gene which encodes the putative anchoring unit of Rdh (Figure 3-3). All the predicted RdhAs, designated YN3RdhA1-18, contained Tat peptide signal at the N-terminus and two iron binding motifs at the C-terminus except YN3RdhA10 which lacked the peptide sequence. Most of the *YN3rdhA* genes identified were found in the vicinity of other genes which assumed to encode proteins participated in either regulation of *rdhA* gene transcription (*rdhC, rdhS/P* and *rdhR* genes), or RdhAs maturation and assembling (*rdhF*, *rdhG*, *rdhH*, and *rdhI* genes) (Adrian and Loffler, 2016). Out of the predicted 18 YN3RdhAs, only YN3RdhA8 and YN3RdhA16 showed significant amino acid sequence identity, >90%, with RdhAs which were experimentally identified as a CEs dechlorinating proteins (Table 3-3). The predicted YN3RdhA8

showed 94% identity with PceA of *Dehalococcoides* strain 195, the protein which was partially purified and proved to dechlorinates PCE to TCE (Magnuson et al., 1998). In the phylogenetic tree (Figure 3-4), YN3RdhA8 formed an independent cluster with PceA of strain 195 in addition to other RdhAs from PCE-dechlorinating strains (195, CBDB1, CG1 and BTF08) and non-PCE dechlorinating strains VS, UCH007 and IBARAKI. Similarly, YN3RdhA16 showed 97% identity (Table 3-3) with VcrA of strain VS, the protein which was partially purified and proved to dechlorinates TCE, *cis*-DCE and VC to ETH. However, the results demonstrated that TCE was dechlorinated at lower rate (Muller et al., 2004). In the phylogenetic tree, (Figure 3-4) YN3RdhA16 was clustered with VcrA and RdhAs from strain BTF08 (which dechlorinates PCE to ETH) and strains UCH007 and IBARAKI (which dechlorinate *cis*-DCE to ETH).

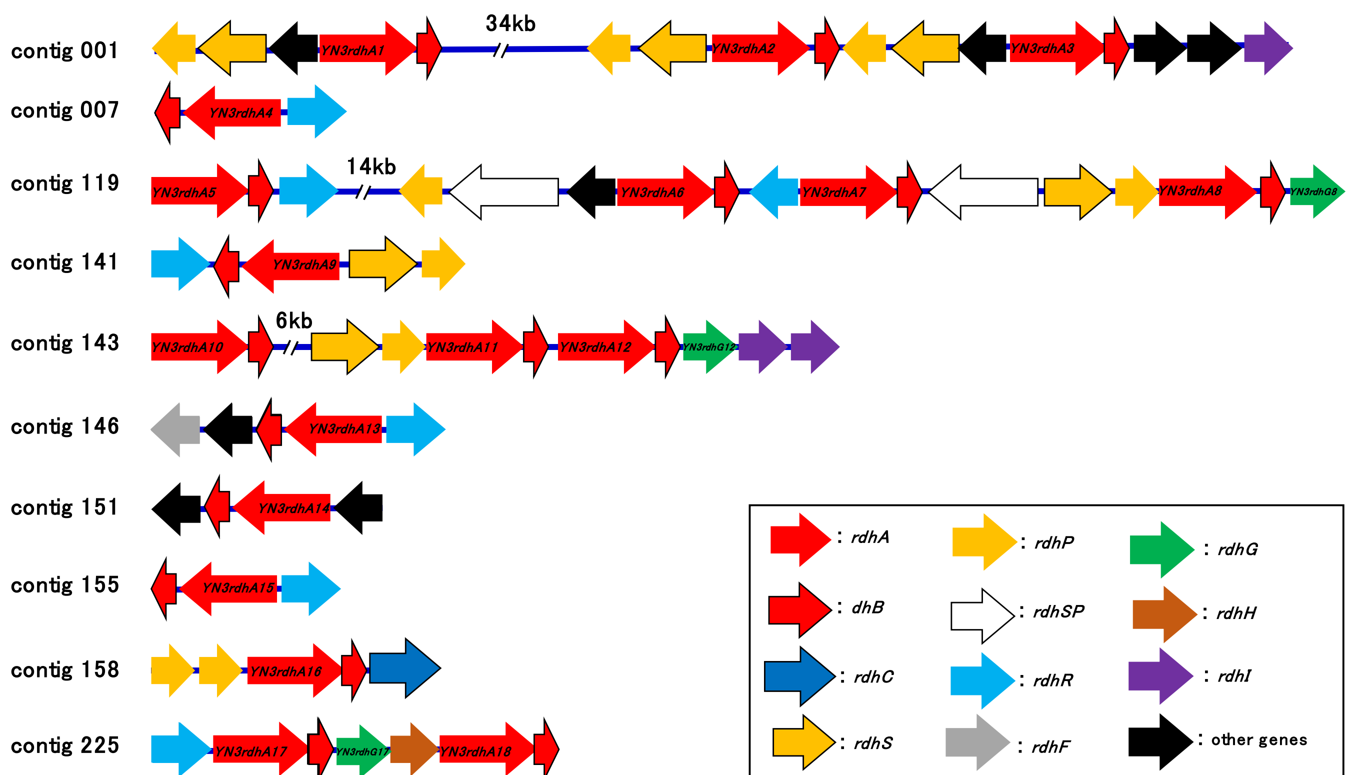

Figure 3-3 Gene clusters containing *rdhA* and *rdhB* and other genes identified in *Dehalococcoides*-metagenome. The predicted encoded proteins are as follows: *rdhA*: catalytic subunit of Rdh, *rdhB*: membrane anchor protein of Rdh, *rdhC*: protein that has a putative function in regulation or electron transport, *rdhS* and *rdhP*: sensor histidine kinase and response regulator of the two-component regulatory system, respectively, *rdhSP*: hybrid *rdhS* and *rdhP*, *rdhR*: multiple resistance regulator (MarR) regulator, *rdhF*: corrinoid-synthetizing protein, *rdhG*: Rdh-modifying proteolytic protein, *rdhH*: hypothetical protein of unknown function, and *rdhI*: corrinoid-modifying protein.

Table 3-3 Comparison of dechlorination activity for CEs in YN3 culture and RdhAs in the *Dehalococcoides*-metagenome with those of other strains or consortia of *Dehalococcoides*.

Strain or consortium	YN3	IBARAKI	UCH007	BTF08	DCMB5	BAV1	VS	CBDB1	195	CG1	CG4	CG5
Dechlorinated CEs	PCE TCE *cis*-DCE VC	*cis*-DCE VC	TCE *cis*-DCE VC	PCE TCE DCE VC	PCE	(PCE)[a] (TCE)[a] *cis*-DCE VC	TCE *cis*-DCE VC	PCE TCE	PCE TCE *cis*-DCE (VC)[a]	PCE TCE	PCE TCE	PCE TCE
CEs are not dechlorinated					TCE DCE VC		PCE	*cis*-DCE VC		*cis*-DCE VC	*cis*-DCE VC	*cis*-DCE VC
RdhAs showed ≥85 % similarity to those in *Dehalococcoides*-metagenome of YN3 (similarity, %)	YN3RdhA1	IBK_0226 (99)	UCH007_02120 (95)	btf_121 (99)	dcmb_184 (99)	DehaBAV1_0173 (99)	DhcVS_169 (96)	cbdbA187 (100)	DET0180 (95)	RD5 (96)	RD4 (95)	RD3 (100)
	YN3RdhA2	IBK_0275 (99)	UCH007_13410 (96)		dcmb_235 (99)	DehaBAV1_0121 (99)	DhcVS_1353 (96)	cbdbA238 (99)	DET0302 (95)			RD4 (100)
	YN3RdhA3	IBK_0283 (99)			dcmb_240 (99)			cbdbA243 (89)	DET0235 (94)	RD1 (93)	RD5 (86)	RD5 (99)
	YN3RdhA4	IBK_1434 (99)	UCH007_13710 (88)	btf_1440 (99)			DhcVS_1375 (87)	cbdbA1575 (100)	DET1519 (92)	RD5 (87)		RD17 (100)
	YN3RdhA5	IBK_1468 (99)	UCH007_14130 (95)	btf_1481 (99)			DhcVS_1421 (95)	cbdbA1618 (99)		RD32 (95)		RD23 (100)
	YN3RdhA6[c]	IBK_1448 (99)		btf_1463 (99)			DhcVS_1402 (94)	cbdbA1598 (100)		RD31 (95)		RD22 (100)
	YN3RdhA7	IBK_1445 (99)	UCH007_13940 (97)	btf_1460 (97)			DhcVS_1316 (94)	cbdbA1595 (99)	DET1535 (93)	RD22 (94)	RD11 (94)	RD21 (99)
							DhcVS_1399 (96)			RD30 (96)		
	YN3RdhA8[c]	IBK_1439 (100)	UCH007_13880 (96)	btf_1454 (100)			DhcVS_1393 (96)	cbdbA1588 (100)	DET0318 (PceA)[b] (94)	RD29 (96)		RD20 (100)
	YN3RdhA9	IBK_1477 (100)	UCH007_14220 (98)	btf_1491 (100)	dcmb_1438 (99)		DhcVS_1430 (98)	cbdbA1627 (99)	DET1538 (87)	RD34 (98)	RD14 (87)	RD25 (100)
	YN3RdhA10		UCH007_13740 (93)					cbdbA1570 (99)	DET1522 (94)	RD26 (93)		RD16 (99)
	YN3RdhA11	IBK_1383 (100)			dcmb_1385 (100)			cbdbA1563 (100)				RD11 (100)
	YN3RdhA12[c]	IBK_1381 (100)			dcmb_1383 (100)			cbdbA1560 (100)				RD14 (100)
	YN3RdhA13	IBK_0998 (99)	UCH007_13480 (96)	btf_1057 (99)	dcmb_1041 (99)	DehaBAV1_0988 (99)		cbdbA1092 (100)	DET1171 (95)		RD10 (95)	RD6 (99)
	YN3RdhA14	IBK_1483 (99)	UCH007_14280 (99)	btf_1497 (99)	dcmb_1444 (99)		DhcVS_1436 (98)	cbdbA1638 (99)	DET1545 (94)	RD35 (98)	RD15 (94)	RD26 (100)
	YN3RdhA15	IBK_1313 (100)	UCH007_12960 (97)		dcmb_134 (100)		DhcVS_1263 (90)	cbdbA1455 (99)		RD8 (90)		RdhA8 (100)
	YN3RdhA16[c]	IBK_1342 (98)	UCH007_13830 (95)	btf_1407 (97)			DhcVS_1291 (VcrA)[b] (97)					
	YN3RdhA17		UCH007_13640 (92)	btf_1449 (99)			DhcVS_1387 (95)	cbdbA1582 (100)		RD28 (95)		RD19 (100)
	YN3RdhA18		UCH007_13790 (94)	btf_1443 (99)			DhcVS_1383 (94)	cbdbA1578 (99)		RD27 (95)		RD18 (100)
RdhAs showed <85 % similarity to those in *Dehalococcoides*-metagenome of YN3		IBK_0161 IBK_1311 IBK_1358 IBK_1368 IBK_1374 IBK_1409 IBK_1411 IBK_1415 IBK_1418 IBK_1420 IBK_1423 IBK_1427 IBK_1434 IBK_1474	UCH007_00760 UCH007_00810 UCH007_08570 UCH007_09870 UCH007_09900 UCH007_09930 UCH007_12300 UCH007_12320 UCH007_12670 UCH007_13520 UCH007_13580 UCH007_13610 UCH007_13670 UCH007_13970 UCH007_14190	btf_1393 btf_1420 btf_1412 btf_1436 btf_1446 btf_1488 btf_1497	dcmb_81 dcmb_86 dcmb_91 dcmb_113 dcmb_120 dcmb_1041 dcmb_1339 dcmb_1362 dcmb_1366 dcmb_1370 dcmb_1376 dcmb_1428 dcmb_1430 dcmb_1434	DehaBAV1_0104 DehaBAV1_0112 DehaBAV1_0119 DehaBAV1_0276 DehaBAV1_0281 DehaBAV1_0284 DehaBAV1_0296 DehaBAV1_0847- (BvcA)[b]	DhcVS_1427 DhcVS_1260- DhcVS_1324 DhcVS_1316- DhcVS_1336 DhcVS_96 DhcVS_1340 DhcVS_1329 DhcVS_1320 DhcVS_99 DhcVS_1342 DhcVS_1344 DhcVS_88 DhcVS_82 DhcVS_104 DhcVS_1327 DhcVS_1314 DhcVS_1360 DhcVS_1349 DhcVS_1378	cbdbA1453 cbdbA1624 cbdbA1550 cbdbA1495 cbdbA1508 cbdbA96 cbdbA1535 cbdbA1546 cbdbA1542 cbdbA1491 cbdbA88 cbdbA80 cbdbA1503 cbdbA1539 cbdbA84 (CbrA)[b]	DET0079 (TceA)[b] DET0173 DET0306 DET0311 DET0876- DET1528 DET1559	RD2 , RD3 RD4 RD6 RD7 RD9 RD10 RD11 RD12 RD13 RD14 RD15 RD16 RD17 (PcbA1)[b] RD18 RD19 RD20 RD21 RD23 RD24 RD33	RD1 (PcbA4)[b] RD2 RD3 RD6 RD7 RD8 RD9 RD12 RD13	RD1 (PcbA5)[b] RD2 RD7 RD9 RD10 RD12 RD13 RD15 RD24

[a]:CEs dechlorinated through cometabolic reaction are shown in parentheses, [b]: The functionally identified RdhAs are shown in blue, [d]: RdhA expecto

be involved in the PCE-to-ETH dechlorination in YN3are shown in red.

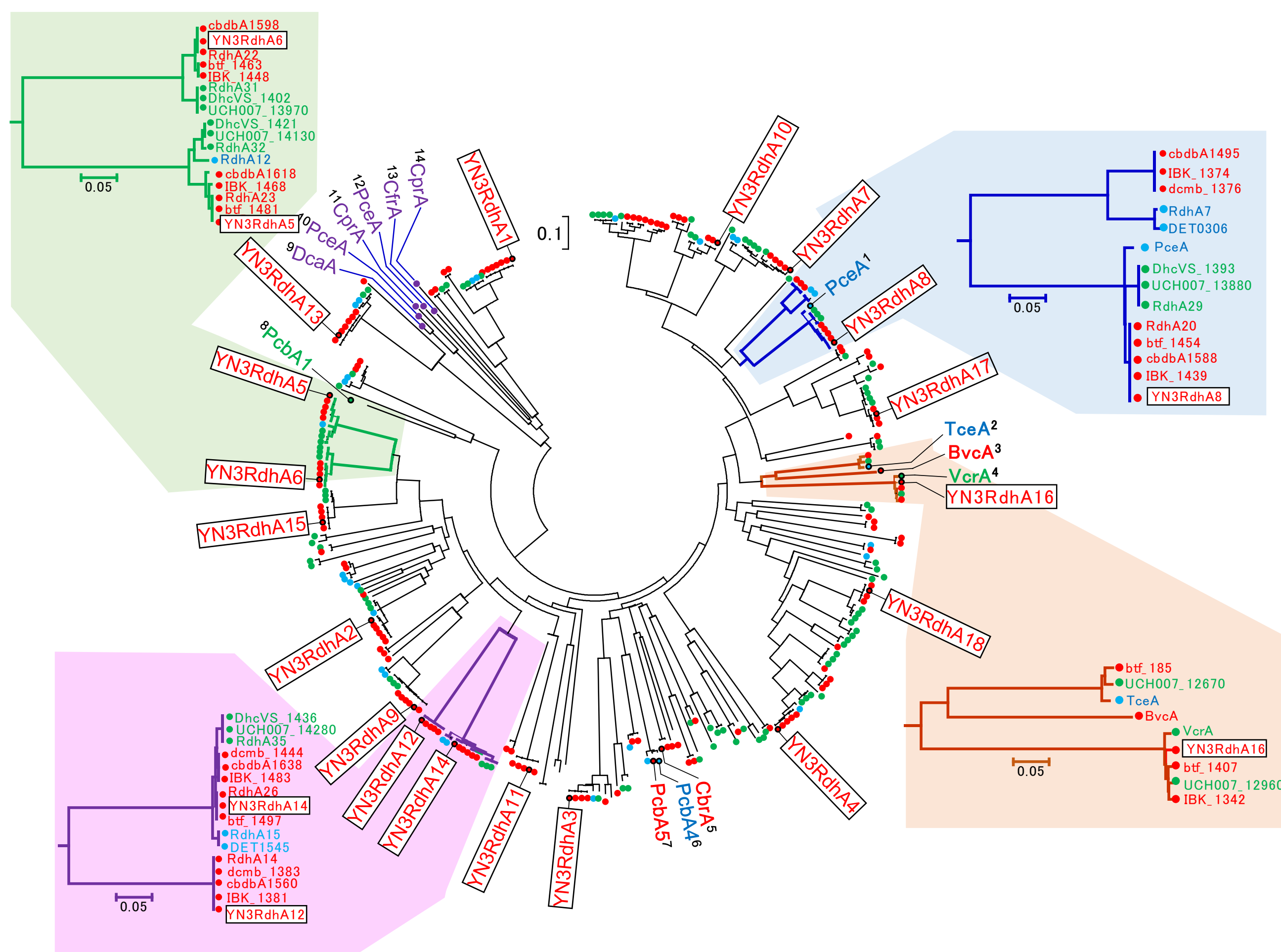

Figure 3-4 Neighbor-joining tree of RdhAs detected in the *Dehalococcoides*-metagenome. The tree showing the phylogeny of RdhAs detected in *Dehalococcoides*-metagenome (inside boxes) and other RdhAs from other strains of *Dehalococcoides* and non-*Dehalococcoides* OHRB. Red, green, and blue and solid circles indicate RdhAs in the Pinellas, Victoria, Cornell and subgroups of genus *Dehalococcoides*, respectively, while violet indicates those of non-*Dehalococcoides* OHRB. Superscript numbers 1–14 indicate biochemically characterized RdhAs and their substrate(s) as follows: [1]PceA (AAW40342): RdhA for PCE; [2]TceA (AAW39060): RdhA for TCE, *cis*-DCE, 1,1-DCE, 1,2-DCA, and 1,2-dibromoethane; [3]BvcA (ABQ17429): RdhA for *cis*-DCE, *trans*-DCE, 1,1-DCE, 1,2-DCA, and VC; [4]VcrA (AAQ94119): RdhA for TCE, *cis*-DCE, *trans*-DCE, 1,1-DCE, and VC; [5]CbrA (CAI82345): RdhA for 1,2,3,4-tetrachlorobenzene, 1,2,3-tetrachlorobenzene and pentachlorobenzene; [6]PcbA4 (WP_041340852): RdhA for PCB and PCE; [7]PcbA5 (AII60305): RdhA for PCB and PCE; [8]PcbA1 (AII58466): RdhA for PCB and PCE; [9]DcaA (CAJ75430): RdhA for 1,2-DCA; [10]PceA (CAD28790): RdhA for PCE and TCE; [11]CprA (AAQ54585): RdhA for 3,5-dichlorophenol, 2,3,5-trichlorophenol, PCP, 2,3,4,5-tetrachlorophenol, 3,4,5-trichlorophenol, 2,4,6-trichlorophenol, 2,4,5-trichlorophenol, and 2,4-dichlorophenol; [12]PceA (AAC60788): RdhA for PCE and TCE; [13]CfrA (AFQ20272): RdhA for 1,1,1-trichloroethane and chloroform; [14]CprA (AAG49543): RdhA for orthochlorophenols.

3.3.5 Transcriptional analysis of *YN3rdhA* genes in response to CEs

RT-qPCR targeting and quantifying the mRNAs of all *YN3rdhA* genes identified in *Dehalococcoides*-metagenome was conducted to determine which *YN3rdhA* genes involved in the PCE to ETH dechlorination. In this experiment, the *rdhA* genes showing the increase by more than twice in its transcription level by single determination in comparison with the control condition without spiking CEs were selected as *rdhAs* for CEs dechlorination. For *cis*-DCE, the firstly conducted RT-qPCR suggested three *YN3rdhA* genes to be involved in CEs dechlorination (Table 3-4), that is *YN3rdhA6*, *YN3rdhA12*, and *YN3rdhA16*. The *YN3rdhA16* gene is closely related to *vcrA* gene, previously identified to be involved in the *cis*-DCE/VC dechlorination to ETH (Müller et al., 2004). Notably in, the *YN3rdhA8* gene, closely related to *pceA* gene, previously identified to be involved in the PCE dechlorination to TCE (Magnuson et al., 1998), showed no significant increase in its transcription level. Thus, a second RT-qPCR was conducted for the *YN3rdhA8* gene, and *YN3rdhA6*, *YN3rdhA12* and *YN3rdhA16* genes (selected from the first RT-qPCR) in three determinations to confirm their involvement in the CEs dechlorination. The results confirmed the significant increase in the transcription levels of *YN3rdhA6*, *YN3rdhA12*, and *YN3rdhA16* genes, while *YN3rdhA8* gene showed no significant increase in its transcription level. Specifically, the transcription level of *YN3rdhA6* gene showed significant increase in response to spiked VC (75 ± 38 mRNA copies per gene (Figure 3-5(A)) and slight transcription level increase in response to other spiked CEs. Similarly, *YN3rdhA12* gene was transcribed significantly with VC (16 ± 8.6 mRNA copies per gene) and slightly with other spiked CEs **(**Figure 3-5(B)). The *YN3rdhA16* gene showed transcriptional response with different CEs as follows; VC (4.0±2.9 mRNA copies per gene), *cis*-DCE (7.4 ± 2.7 mRNA copies per gene), and TCE (3.4 ± 1.4 mRNA copies per gene) (Figure 3-5(C)). The insignificant increase in the transcription level of *YN3rdhA8* gene as well as, the very

small increase in the transcription levels of *YN3rdhA6* and *YN3rdhA12* genes with spiked PCE motivated us to re-examine the transcription level using YN3 culture grown with PCE as an electron acceptor. The results indicated increase in the transcription level of *YN3rdhA8* gene in response to spiked PCE by 2.6±1.8 mRNA copies per gene (Table 3-4 and Figure 3-5(D)). Overall, the results suggested the involvement of four *rdhA* genes in different steps of PCE to ETH dechlorination. The *YN3rdhA8* gene is suggested to be involved in PCE to TCE dechlorination step, especially under PCE-growing condition. The *YN3rdhA16* gene is suggested to be involved in the TCE to ETH dechlorination. Besides these two known *rdhA* genes, *YN3rdhA6* and *YN3rdhA12* genes were suggested to be involved in VC to ETH dechlorination step.

Table 3-4 Change in the transcription level of *YN3rdhA1-18* genes in response to spiking of CEs

	YN3 grown with *cis*-DCE				**YN3 grown with PCE**
Spiked CEs	**VC**	***cis*-DCE**	**TCE**	**PCE**	**PCE**
YN3rdhA1	-	-	-	-	-
YN3rdhA2	-	-	-	-	-
YN3rdhA3	-	-	-	-	-
YN3rdhA4	-	-	-	-	-
YN3rdhA5	-	-	-	-	-
YN3rdhA6	+	+	+	-	-
YN3rdhA7	-	-	-	-	-
YN3rdhA8	-	-	-	-	+
YN3rdhA9	-	-	-	-	-
YN3rdhA10	-	-	-	-	-
YN3rdhA11	-	-	-	-	-
YN3rdhA12	+	+	+	-	-
YN3rdhA13	-	-	-	-	-
YN3rdhA14	-	-	-	-	-
YN3rdhA15	-	-	-	-	-
YN3rdhA16	+	+	+	-	-
YN3rdhA17	-	-	-	-	-
YN3rdhA18	-	-	-	-	-

+ indicates more than 2 fold increase in transcription of *rdhA* to that without spiking

- indicates transcription of *rdhA* less than 2 fold to that without spiking

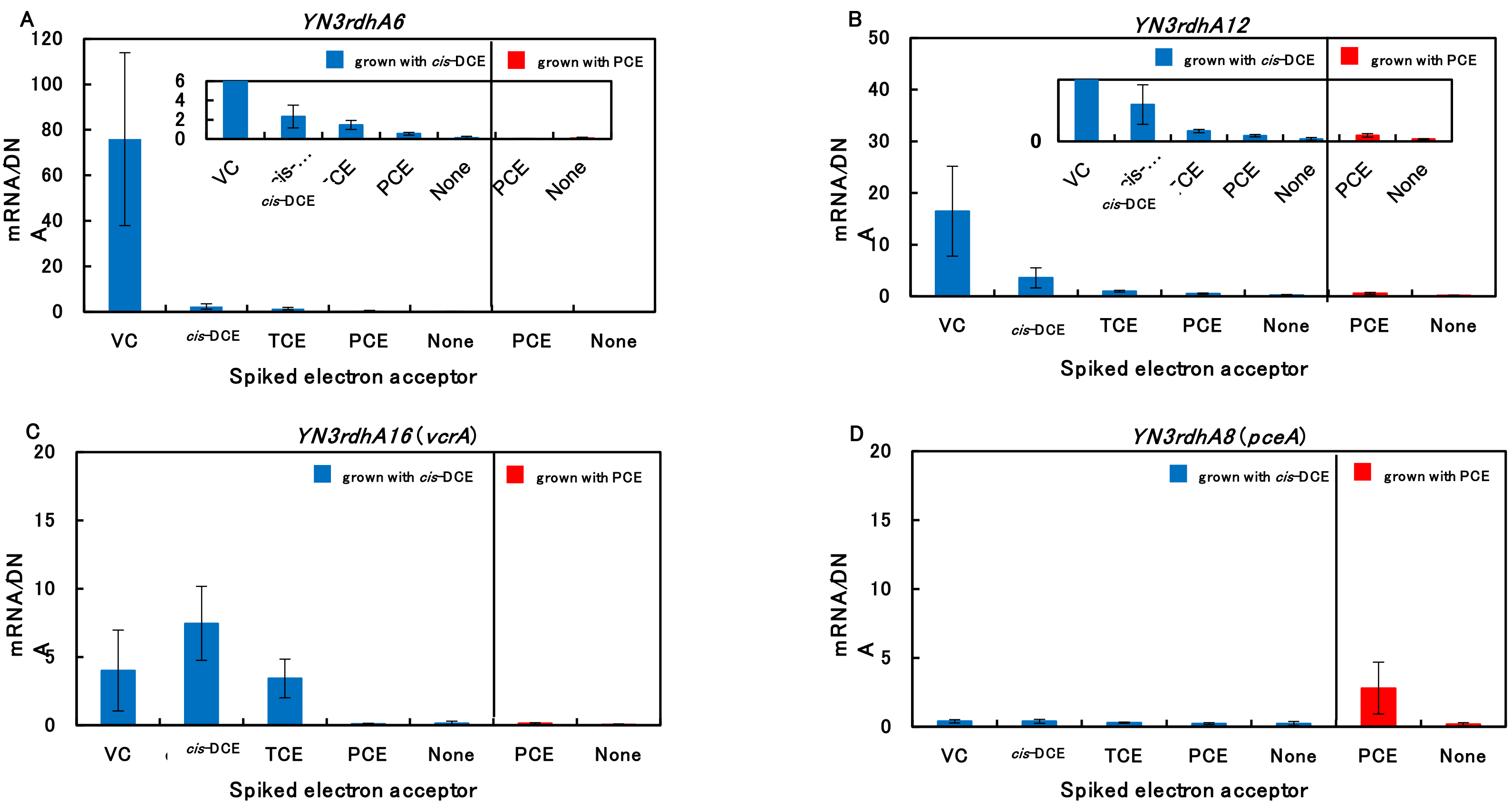

Figure 3-5 Analysis of transcription level of *YN3rdhA6* (A), *YN3rdhA12* (B), *YN3rdhA16* (C), and *YN3rdhA8* genes (D) in YN3 culture spiked with PCE, TCE, *cis*-DCE, or VC. Error bars represent standard deviations ($n = 3$). Inserted figures in panels (A) and (B) are enlarged figures. Blue and red represent the transcription level of *YN3rdhAs* in YN3 culture grown with *cis*-DCE and PCE, respectively.

3.4 Discussion

In this chapter, YN3 culture, enriched in chapter 2, was further characterized by the metagenome analysis, and four *YN3rdhA* genes were suggested to be involved in the PCE to ETH by the transcriptional analysis. Two out of the four genes, *YN3rdhA6* and *YN3rdhA12* were found in this study as new *rdhA* genes strongly transcribed in response to CEs particularly VC. Up to now, only two *rdhA* genes, *vcrA* and *bvcA*, have been identified as the genes involved in the VC dechlorination based on protein analysis (Müller et al., 2004; Tang et al., 2013). In 2004, the *vcrA* gene (Müller et al., 2004) was identified by direct PCR using primers designed from the partially purified and sequenced RdhA (or VcrA) which showed dechlorinating activity towards VC, while the identification of *bvcA* gene (Krajmalnik-Brown et al., 2004) was conducted based on the high transcriptional response against VC addition, and the suggestion which was confirmed later using native gel assay (Tang et al., 2013).

Thus, multiple *rdhA* genes were suggested to be involved in the VC to ETH dechlorination step in this study. Notably, the operon containing *YN3rdhA6* gene is very similar to other published genomes of *Dehalococcoides*. (Figure 3-6(A)). In addition, the predicted YN3RdhA6 showed high sequence identity (>94%) to those of other strains of the genus *Dehalococcoides*, some of them are unable to dechlorinate VC (Tables 3-3 and 3-5). This indicated that further experiments should be conducted at the protein level to confirm the involvement of *YN3rdhA6* in the VC to ETH dechlorination step. The *YN3rdhA12* is the second new gene found in this study, which showed significant increase in the transcription level especially with VC spiking, also showed a very similar gene cluster to those of other *Dehalococcoides* (Figure 3-6(B)). The transcription analysis of *rdhA* genes similar to *YN3rdhA12* was carried out in the pure culture of *Dehalococcoides* strain CG5 (Wang et al., 2014) and in the mixed culture of *Dehalococcoides*, TUT2264 culture (Futamata

et al., 2009) (Table 3-5). The *YN3rdhA12*-related *rdhA* genes of *Dehalococcoides* strain CG5 showed lower transcription level than other *rdhA*s genes in the culture, and consequently, this gene has been ignored as a *rdhA* to be involved in the CEs dechlorination as reported by Wang et al. (2014). While Futamata et al. (2009) reported that three *YN3rdhA12*-related *rdhA* genes of TUT2264 showed remarkable (>20-fold) increases in transcription level in response to spiking of CEs. However, the specific CE affecting transcription was different among *YN3rdhA12* and the three *rdhA*s in TUT2264 culture. Specifically, like *YN3rdhA12,* one of the *YN3rdhA12*-related *rdhA*s of TUT2264 was increasingly transcribed by addition of VC, while the two other genes were particularly transcribed in the presence of other CEs, but not VC. These results indicated that *YN3rdhA12* gene and related three *rdhA*s genes in TUT2264 culture can be suggested to be involved in the dechlorination of CEs, although further analysis at the protein level is required to confirm this suggestion.

YN3 culture showed markedly higher dechlorination activity, compared to other PCE to ETH dechlorinating *Dehalococcoides* enriched cultures (Duhamel et al., 2002; Cichocka et al., 2010; Kim et al., 2010), however, the rate of dechlorination rates in these cultures are variable depending on the culture conditions, . However, Analysis of YN3 metagenome indicated no apparent difference from the previously characterized genomes of other strains of the genus *Dehalococcoides*. Specifically, the 16S rRNA gene showed 100% similarity with those of strains IBARAKI, CG5, DCMB5 and CBDB1 of *Dehalococcoides* (Figure 3-1). In addition, all predicted RdhAs from YN3 metagenome showed 98–100% identity with some of the RdhAs predicted from genomes of different strains of *Dehalococcoides* (Table 3-3). Thus, different underlying reason might be stand behind the dechlorination activity shown by YN3 culture which plausibly could be attributed to the coexisting bacteria. Metagenomic analysis indicated that YN3 culture containing

metagenomes belonged to diverse bacterial taxa *Firmicutes, Actinobacteria* and *Bacteroidetes* (Figure 3-2), which may support for the dechlorination activity of *Dehalococcoides* in YN3 culture. Special attention will be given to the *Bacteroidetes*-metagenome as it represents the largest assembled metagenome in YN3 culture and a novel species of the genus *Bacteroides*. Also, members of the genus *Bacteroides* have been frequently detected as the coexisting bacteria of *Dehalococcoides* dechlorinating enriched consortia (Macbeth *et al*., 2004; Duhamel and Edwards, 2006; Miura *et al.,* 2015). It has been reported that coexisting bacteria support *Dehalococcoides* either by providing essential substances or removal toxic ones (He *et al.,* 2007; Men *et al.,* 2012; Zhuang *et al.,* 2014; Liu *et al.,* 2017).

Table 3-5 Comparison of transcribed *rdhAs* genes in response to CEs in *Dehalococcoides*-metagenome with those of other consortium or strains of *Dehalococcoides*.

Strain or consortium	YN3	TUT2264	KB1	BAV1	DCMB5	VS	CBDB1	IBARAKI	BTF08	195	CG1	CG5
RdhAs showed ≥85 % similarity to those in *Dehalococcoides*-metagenome of YN3 (similarity, %)[b]	YN3RdhA1			DehaBAV1_0173 (99)	dcmb_184 (99)	DhcVS_169 (96)	cbdbA187 (100)	IBK_0226 (99)	btf_121 (99)	DET0180 (95)	RD5 (96)	RD3 (100)
	YN3RdhA22			DehaBAV1_0121 (99)	dcmb_235 (99)	DhcVS_1353 (96)	cbdbA238 (99)	IBK_0275 (99)		DET0302 (95)		RD4 (100)
	YN3RdhA3				dcmb_240 (99)		cbdbA243 (89)	IBK_0283 (99)		DET0235 (94	RD1 (93)	RD5 (99)
	YN3RdhA4					DhcVS_1375 (87)	cbdbA1575 (100)	IBK_1434 (99)	btf_1440 (99)	DET1519 (92)	RD25 (87)	RD17 (100)
	YN3RdhA5	RdhA8 (99)				DhcVS_1421 (95)	cbdbA1618 (99)	IBK_1468 (99)	btf_1481 (99)		RdD32 (95)	RD23 (100)
	YN3RdhA6		RdhA2(99)			DhcVS_1402 (94)	cbdbA1598 (100)	IBK_1448 (99)	btf_1463 (99)		RD31 (95)	RD22 (100)
	YN3RdhA7		RdhA10(99)			DhcVS_1316 (94) DhcVS_1399 (96)	cbdbA1595 (99)	IBK_1445 (99)	btf_1460 (97)	DET1535 (93)	RD22 (94) RD30 (96)	RD21 (99)
	YN3RdhA8		RdhA13(100)			DhcVS_1393 (96)	cbdbA1588 (100)	IBK_1439 (100)	btf_1454 (100)	DET0318 (PceA)[c] (94)	RD29 (96)	RD20 (100)
	YN3RdhA9		RdhA4(99)		dcmb_1438(99)	DhcVS_1430 (98)	cbdbA1627 (99)	IBK_1477 (100)	btf_1491 (100)	DET1538 (87)	RD34 (98)	RD25 (100)
	YN3RdhA10						cbdbA1570 (99)			DET1522 (94)	RD26 (93)	RD16 (99)
	YN3RdhA11				dcmb_1385(100)		cbdbA1563 (100)	IBK_1383 (100)				RD11 (100)
	YN3RdhA12	RdhA3(98) RdhA4(100) RdhA5(100)			dcmb_1383(100)		cbdbA1560 (100)	IBK_1381 (100)				RD14 (100)
	YN3RdhA13			DehaBAV1_0988 (99)	dcmb_1041(99)		cbdbA1092 (100)	IBK_0998 (99)	btf_1057 (99)	DET1171 (95)		RD6 (99)
	YN3RdhA14	RdhA2(99)	RdhA5(99)		dcmb_1444(99)	DhcVS_1436 (98)	cbdbA1638 (99)	IBK_1483 (99)	btf_1497 (99)	DET1545 (94)	RD35 (98)	RD26 (100)
	YN3RdhA15	RdhA6(97) RdhA7(99)	RdhA13(99)		dcmb_134 (100)	DhcVS_1263 (90)	cbdbA1455 (99)	IBK_1313 (100)			RD8 (90)	RD8 (100)
	YN3RdhA16		RdhA14(98)			DhcVS_1291 (VcrA)[c] (97)		IBK_1342 (98)	btf_1407 (97)			
	YN3RdhA17					DhcVS_1387 (95)	cbdbA1582 (100)		btf_1449 (99)		RD28 (95)	RD19 (100)
	YN3RdhA18	RdhA1(100)	RdhA7(99)			DhcVS_1383 (94)	cbdbA1578 (99)		btf_1443 (99)		RD27 (95)	RD18 (100)
RdhAs showed <85 % similarity to those in *Dehalococcoides*-metagenome of YN3			RdhA1 RdhA3 RdhA6 RdhA8 RdhA9 RdhA11 RdhA12	DehaBAV1_0104 DehaBAV1_0112 DehaBAV1_0119 DehaBAV1_0276 DehaBAV1_0281 DehaBAV1_0284 DehaBAV1_0296 DehaBAV1_0847-(BvcA)[c]	dcmb_81 dcmb_86 dcmb_91 dcmb_113 dcmb_120 dcmb_1041 dcmb_1339 dcmb_1362 dcmb_1366 dcmb_1370 dcmb_1376 dcmb_1428 dcmb_1430 dcmb_1434	DhcVS_1427 DhcVS_1260 DhcVS_1324 DhcVS_1316 DhcVS_1336 DhcVS_96 DhcVS_1340 DhcVS_1329 DhcVS_1320 DhcVS_99 DhcVS_1342 DhcVS_1344 DhcVS_88 DhcVS_82 DhcVS_104 DhcVS_1327 DhcVS_1314 DhcVS_1360 DhcVS_1349 DhcVS_1378	cbdbA1453 cbdbA1624 cbdbA1550 cbdbA1495 cbdbA1508 cbdbA96 cbdbA1535 cbdbA1546 cbdbA1542 cbdbA1491 cbdbA88 cbdbA80 cbdbA1503 cbdbA1539 cbdbA84 (CbrA)[c]	IBK_0161 IBK_1311 IBK_1358 IBK_1368 IBK_1374 IBK_1409 IBK_1411 IBK_1415 IBK_1418 IBK_1420 IBK_1423 IBK_1427 IBK_1434 IBK_1474	btf_1393 btf_1420 btf_1412 btf_1436 btf_1446 btf_1488 btf_1497	DET0079 (TceA)[c] DET0173 DET0306 DET0311 DET0876- DET1528 DET1559	RD2 , RD3 RD4 RD6 RD7 RD9 RD10 RD11 RD12 RD13 RD14 RD15 RD16 RD17 (PcbA1)[c] RD18 RD19 RD20 RD21 RD23 RD24 RD33	RD1 (PcbA5)[c] RD2 RD7 RD9 RD10 RD12 RD13 RD15 RD24

Transcibed with (PCE or TCE)   Transcibed with (*cis*-DCE)   Transcibed with (PCE)

Not transcibed with any VC   Not transcibed with any CEs

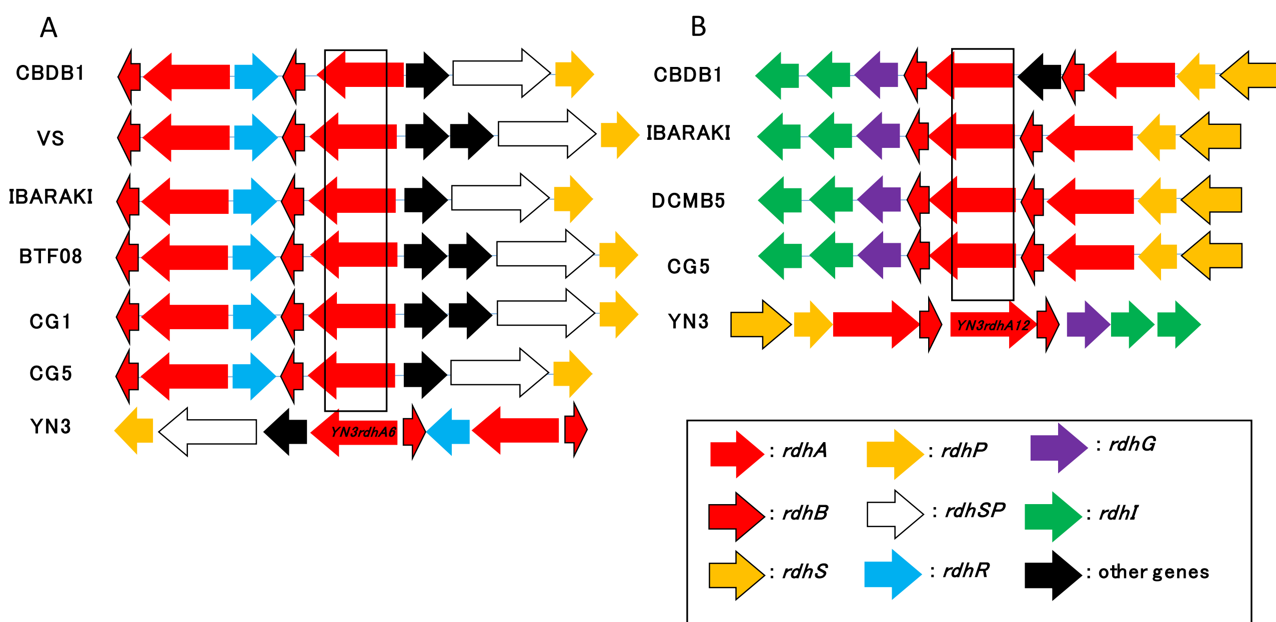

Figure 3-6 Comparsion of gene cluster for *YN3rdhA6* (A) and *YN3rdhA12* (B) and their related *rdhAs* gene cluster from *Dehalococcoides*. Rectangles show the *rdhAs* related to *YN3rdhA6* or *YN3rdhA12*. The predicted encoded proteins for all genes have been described previously, and are as follows; *rdhA*: catalytic subunit of Rdh, *rdhB*: membrane anchor protein of Rdh, *rdhS* and *rdhP*: sensor histidine kinase and response regulator of the two component regulatory system, respectively, *rdhSP*: hybrid *rdhS* and *rdhP*, *rdhR*: multiple resistance regulator (MarR) regulator, *rdhG*: Rdh-modifying proteolytic protein and *rdhI*: corrinoid-modifying protein.

3.5 Conclusion

A PCE to ETH dechlorinating *Dehalococcoides* enriched culture was further characterized by metagenome and transcription analyses. The coexisting bacteria present beside the dechlorinator, *Dehalococcoides,* were characterized and were found to be affiliated to phylogenetically diverse bacterial phyla *Bacteroidetes*, *Actinobacteria* and *Firmicutes*. Metagenomic analysis suggested 18 *rdhA* genes, *YN3rdhA1 -18*. Four *YN3rdhA* genes were suggested to be involved in the PCE to ETH dechlorination by the transcriptional analysis using RT-qPCR. Among these four *YN3rdhA* genes, two genes, *YN3rdhA8* and *YN3rdhA16,* were closely related to the previously identified genes *pceA* and *vcrA*, respectively. The other two genes, *YN3rdhA6* and *YN3rdhA12,* were new genes suggested

to be involved in the CEs dechlorination, especially the VC to ETH dechlorination step.\

4. *Bacteroides sedimenti*, a novel species of the genus *Bacteroides* isolated from chloroethenes dechlorinating consortium and its effect on the dechlorination by *Dehalococcoides*

4.1 Introduction

So far, members of the genus *Bacteroides* are characterized as being Gram-staining negative, rod-shaped, anaerobic and non-motile cells (Shah, 1992). The genus *Bacteroides* conserves the energy by fermentation and usually contains anteiso-$C_{15:0}$ as the major fatty acid, in addition to menaquinone-10 (MK-10) and MK-11 as the major respiratory quinones (Miyagawa et al., 1979; Shah, 1992). At present, members of the genus were sub-grouped into ten phylogenetic clades based on molecular analysis of concatenated sequences of 6 or 3 housekeeping genes (Sakamoto and Ohkuma, 2011).

Until now, all isolated species of the genus have an animal-related origin, i.e., isolated from blood, gut or feces (Lan et al., 2006; Watanabe et al., 2010; Kitahara et al., 2012). *Bacteroides* spp. account for around 25% of the anaerobes existed in animal gut as friendly symbionts where they degrade the complex organic matters, usually polysaccharides, into simple ones to be used as energy source by the host (Wexler, 2007). However, in some instances some species of the genus *Bacteroides* (e.g., *Bacteroides fragilis*) turned into opportunistic pathogens and cause serious diseases (Wexler, 2007). Culture-independent techniques (e.g., PCR) indicated the existence of *Bacteroides* in a wide variety of environments, some of these environments have no evidence or history for fecal contamination (Bower et al., 2005; van der Wielen and Medema, 2010; Vierheilig et al., 2012). Whitman et al. (2014) suggested that the free living members of *Bacteroides* are phylogenetically distant from the members of animal origins. Notably, members of the genus

Bacteroides have been detected among the coexisting bacteria in the dehalorespiring consortia, especially, the *Dehalococcoides* containing consortia (Macbeth et al., 2004; Duhamel and Edwards, 2006; Miura et al., 2015). However, their existence beside *Dehalococcoides* got no attention and has never been investigated.

Dehalococcoides is the most significant organohalide respiring bacterium due to its activity to dechlorinate chloroethenes (CEs) completely into innocuous ethene (ETH). The genus *Dehalococcoides* spp. harbors a small-sized genome which lacks several genes essential for free-living or independent lifestyle (e.g., the complete set of genes required for the synthesis of corrinoid cofactor). Concurrently, several studies have reported that members of the genus *Dehalococcoides* achieve better growth rate and dechlorination activity when present in mixed culture with other bacteria. Inside mixed communities, *Dehalococcoides* spp. will get benefits and compensates for the absence of some essential genes from their genome (He et al., 2007; Men et al., 2012; Zhuang et al., 2014). Considering the fact that *Bacteroides* spp. have been frequently detected with *Dehalococcoides*, it is therefore rational to assume that *Bacteroides* spp. contribute positively to the dechlorination activity of *Dehalococcoides*.

The aim of this chapter was to characterize the morphology, physiology and phylogeny of strain YN3PY1^T as a novel species belonging to the genus *Bacteroides*. The strain YN3PY1^T was isolated from YN3 culture, the tetrachloroethene (PCE) to ETH dechlorinating consortium, enriched and characterized in chapters 2 and 3. This chapter also aimed to evaluate the contribution of the strain YN3PY1^T to the dechlorination activity of *Dehalococcoides*.

4.2 Materials and Methods

4.2.1 Isolation and culture condition of the strain YN3PY1^T

Strain YN3PY1^T characterized in this chapter was isolated in pure form from YN3 culture, a PCE to ETH dechlorinating culture, which had been enriched and characterized in chapters 2 and 3. The isolation was carried out using a medium containing peptone-yeast extract, glucose, and titanium (designated as PYG-T medium). The PYG-T medium was slightly modified from the PYG medium, originally developed by Akasaka et al. (2003), by using titanium (III)-trinitriloacetic acid (0.25 mM) (Moench and Zeikus, 1983) and $Na_2S \cdot 9H_2O$ (0.15 mM) as reducing agents instead of L-cysteine·HCl·H_2O. PYG-T medium was prepared under anaerobic condition by flushing the headspace of the serum vials with 80:20 N_2-CO_2. For the isolation step, 500 μL of YN3 culture was withdrawn under anaerobic condition by a plastic syringe and injected into 30-mL serum vials containing 10-mL PYG-T medium. The vials then serially diluted (10^{-2}–10^{-7}), supplemented with 0.5% agarose and incubated for around 7-15 days. After incubation, visible and well-separated colonies were picked up randomly using sterile Pasteur pipettes, transferred into fresh PYG-T medium and incubated for up to 7 days. Thereafter, cultures with uniform cell morphology were screened for the presence of the strain YN3PY1^T by direct sequencing of their 16S rRNA genes as described below (section 4.2.3). After getting a pure culture of the strain YN3PY1^T (as indicated from the observation of the uniform cell morphology and 16S rRNA gene sequencing), subsequent morphological, physiological, biochemical and chemotaxonomic analyses were carried out. The strain obtained in this study was preserved at the Korean Collection for Type Cultures (KCTC) and the Japanese NITE Biological Resource Center (NBRC) under accession numbers KCTC 15656^T and NBRC 113168^T, respectively.

4.2.2 Morphological, physiological, and biochemical analyses

Morphological characteristics of the strain YN3PY1^T and the spore formation were observed by an Olympus BX53 phase contrast microscopy (Olympus, Tokyo, Japan). Gram-staining and motility slides were prepared using conventional Gram-staining technique (Snyder 1970) and hanging drop method (Skerman, 1967), respectively. Growth in the presence of oxygen was checked using serum vials containing PYG-T medium without adding any reducing agent and without replacing the headspace gas. Growth at different concentration of NaCl was examined using concentrations of NaCl (0 and 0.1–5.0% at intervals of 0.5% [wt/vol]). Effect of temperature on the growth of the strain was examined using temperatures 4.0, 10, 15, 20, 25, 28, 30, 35, 40, and 45 °C and DHB-CO_3 medium as described in chapter 2. Finally, the effect of pH on the growth of the strain YN3PY1^T was checked at values 5.2, 5.4, 6.0, 6.6, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, and 10. Effect of either NaCl, temperature or pH was evaluated by measuring the microbial turbidity using spectrophotometer at 660 nm. Experiment for determination of the metabolites or the end products from glucose fermentation was carried out by growing the strain YN3PY1^T in 60 mL-serum vials containing 20-mL DHB-CO_3 medium with 10 mM glucose as the sole carbon source. After around 30 days of the incubation, aliquots were withdrawn from the vials, filtered using a 0.22 µm Millipore filter and analyzed as a cell-free filtrate using two high performance liquid chromatography (HPLC) systems (Shimadzu, Japan). The first system contained an ODS column and an UV–Vis detector and employed mobile phase of 20 mM phosphoric acid and acetonitrile (95:5, v/v), whereas the second system equipped with a Shim-pack IC-A3 column (Shimadzu, Japan) and used a mixture of 3.2 mM bis-tris, 8 mM p-hydroxybenzoic acid and 50 mM boric acid as the mobile phase. Several other miscellaneous enzymatic activities for the strain YN3PY1^T were examined using API rapid ID 32A kit (bioMérieux, France) according to the

manufacturer's instruction.

4.2.3 Genetic analysis

Genomic DNAs of the strain YN3PY1^T and other colonies picked up as indicated above were extracted as described in chapter 1, with subsequent amplification of the full-length 16S rRNA gene using the universal bacterial primers 27F and 1492R (Weisburg et al., 1991). The amplified 16S rRNA genes were then directly sequenced at FASMAC Co., Ltd. (Kanagawa, Japan), using 518R, 805R, 928F and 1100R universal primers. Search for the closest relatives of the sequenced 16S rRNA genes was conducted using the online EzBiocloud pipeline (http://www.ezbiocloud.net, Yoon et al., 2017). Multiple sequence alignment of 16S rRNA gene of the strain YN3PY1^T with the closely related sequences was carried out using ClustalW algorithm (Thompson et al., 1994) included in MEGA7 software (Kumar et al., 2016). Additionally, MEGA7 was used for construction of the phylogenetic tree of 16S rRNA genes of the strain YN3PY1 ^{T} and the closely related ones. The genomic G+C content (mol%) analysis of the strain YN3PY1^T was performed at Techno Suruga Co. (Shizuoka, Japan), using a HPLC method, as described by Katayama-Fujimura *et al.* (1984).

4.2.4 Chemotaxonomic analyses

Contents of fatty acids and respiratory quinones in the strain YN3PY1^T were identified at Techno Suruga Co. (Shizuoka, Japan). Fatty acids analysis was performed according to the procedure of MIDI Sherlock Microbial Identification (Version 6.0) and the anaerobic bacteria database (MOORE6), while analysis of respiratory quinones was conducted using a HPLC method (Tamaoka et al., 1983).

4.2.5 Assessment of the enhancement of dechlorination activity

In order to evaluate the effect of the strain YN3PY1^T on the dechlorination activity of *Dehalococcoides*, the strain YN3PY1^T was co-inoculated with C4C4 culture. C4C4 culture was a *Dehalococcoides* enriched culture obtained from YN3 culture, which was obtained and characterized in chapters 2 and 3. First, YN3 culture was inoculated into freshly prepared DHB-CO_3 medium (described in chapter 2) and supplemented with 0.5% agarose, 20 mM acetate, H_2 and CO_2 (4 : 1, v/v), vitamin B_{12} (Holliger et al., 1998) and 500 µM *cis*-dichloroethene (*cis*-DCE) as a solidifying agent, a carbon source, an energy source, an enzymatic cofactor and an electron acceptor, respectively. Then after six months of incubation, separate colonies were picked up by sterile plastic syringe and inoculated into freshly prepared DHB-CO_3 medium and supplemented with acetate, hydrogen and *cis*-DCE as described above. Finally, C4C4 culture was selected using GC-FID, as described in chapter 2, based on its dechlorination positive activity, i.e., dechlorination of *cis*-DCE into ETH.

The experiment, for assessing the effect of the strain YN3PY1^T on the dechlorination activity of C4C4 culture, was carried out in 2 lines using DHB-CO_3 medium. The first line is obtained by inoculation of C4C4 culture alone into serum vials (5 vials), while the second one by inoculation of C4C4 culture into serum vials (5 vials) followed by co-inoculation with strain YN3PY1^T (C4C4+ YN3PY1^T). Thereafter, vials of both lines were supplemented with acetate, hydrogen, vitamin B_{12} and *cis*-DCE as described above, and incubated at 28ºC for 191 days. Identification and quantification of CEs and ETH was performed using the GC-FID . After incubation, the microbial composition in one vial containing C4C4 culture and the strain YN3PY1^T was identified using Illumina-based microbial composition analysis targeting the V4 region of bacterial and archaeal as described in chapter 2.

4.2.6 GenBank accession number

Sequence of the 16S rRNA gene for the strain YN3PY1^T obtained in this study, and the sequences for *DnaJ*, *gyrB* and *hsp60* genes extracted from *Bacteroides*-metagenome were deposited in GenBank/DDJB databases under accession numbers LC377263, LC379007, LC379008 and LC379009, respectively.

4.3 Results

4.3.1 Isolation and selection of the strain YN3PY1^T

Several colonies were obtained from the agarose supplemented PYG-T medium, inoculated with YN3 culture and incubated at 28 °C for 7-15 days at 10^6–10^7 dilution, and inoculated into freshly prepared, liquid PYG-T medium. After the incubation for up to 7days, the pure culture containing the strain YN3PY1^T was selected based on the result of uniform cell morphology and 16S rRNA gene sequence. The results of 16S rRNA gene sequencing indicated the presence of taxa belonging to genera *Propionicimonas* and *Tisserella* in addition to the strain YN3PY1^T. Morphological observation showed that the cells of the strain YN3PY1^T were Gram-negative, non-motile, rod with around 1.1-7.1 μm length and 0.7-0.8 μm width.

4.3.2 Genetic characterization

According to the EZ-Biocloud pipeline (Yoon et al., 2017), the 16S rRNA gene of the YN3PY1^T showed the highest sequence similarity, 97.1%, with *B. luti* strain JCM 19020^T among the isolated *Bacteroides* spp. This similarity value is lower than the cutoff values, 98.6% (Kim et al., 2014) and 98.2-99.0% (Meier–Kolthoff et al., 2013), suggested previously for bacterial species demarcation without performing the laborious DNA-DNA hybridization. Thus, the strain YN3PY1^T

is proposed as a novel species in the genus *Bacteroides*. Also, the strain showed the highest similarity, 99.9%, with uncultured phylotypes of the genus *Bacteroides*, identified from dehalogenating mixed cultures (Zhou et al., 2015; Liang and Wang, 2017). In particular, the strain was affiliated within the '*Coprosuis'* clade of the genus *Bacteroides*, in addition to *B. luti, B. graminisolvens*, *B. propionifaciens*, *B. neonate* and *B. Coprosuis* (Figure 4-1). The strain was also grouped within the '*Coprosuis'* clade (Figure 4-2) using the concatenated sequences of three housekeeping genes, extracted from the *Bacteroides*-metagenome identified in chapter 2. In general, members of the genus *Bacteroides* were phylogenetically subdivided into ten clades (Sakamoto and Ohkuma, 2011) based on joined sequences of the 3 housekeeping genes *dnaJ*, *gyrB*, and *hsp60.* The genomic G+C content of the strain YN3PY1^{T} was 37.5 mol%, which was in the range recorded previously in clade '*Coprosuis'* of the genus *Bacteroides* (Shah, 1992; Whitehead et al., 2005). Combining all, the strain YN3PY1^{T} was presented here as a novel species within the clade '*Coprosuis'* of the genus *Bacteroides*, and the five strains of the clade '*Coprosuis'* were chosen for further comparative analyses with the strain.

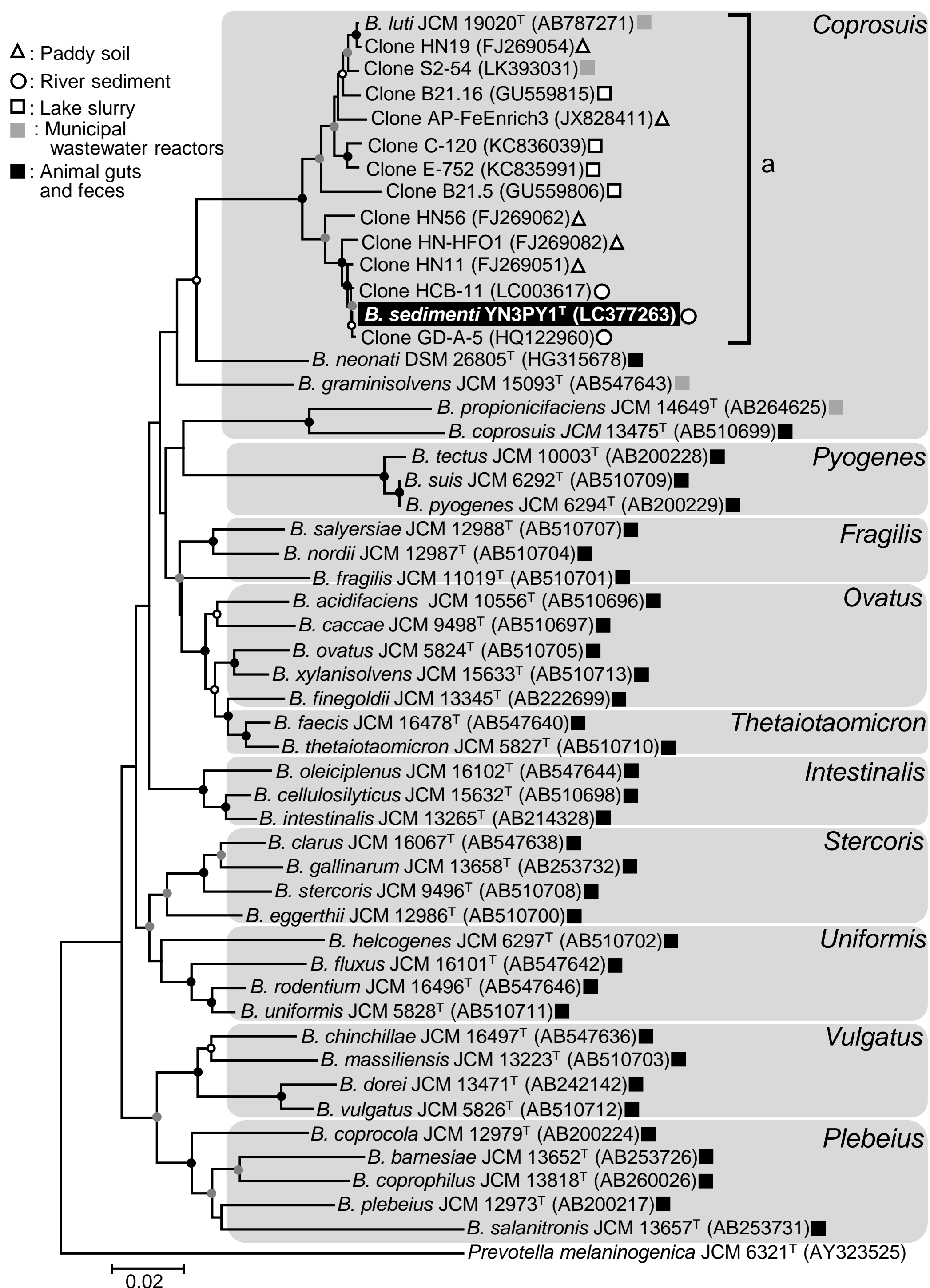

Figure 4-1 Neighbor–joining phylogenetic tree, based on 16S rRNA gene sequences, of strain YN3PY1^{T} and related species of the genus *Bacteroides*. The gray-colored highlighted rectangles are The phylogenetic clades of the genus *Bacteroides* (Sakamoto and Ohkuma, 2011). Phylotypes obtained from animal-independent environments are enclosed by Cluster "a" .Circles above branches indicate >50% bootstrap support from 1,000 replicates. Circles filled in black and gray indicate ≥ 90 and ≥70 % bootstrap support, respectively, and the opened circles indicate ≥50 % of that. *Prevotella melaninogenica* was used as an out-group. The bar represents 0.02 substitutions per nucleotide position

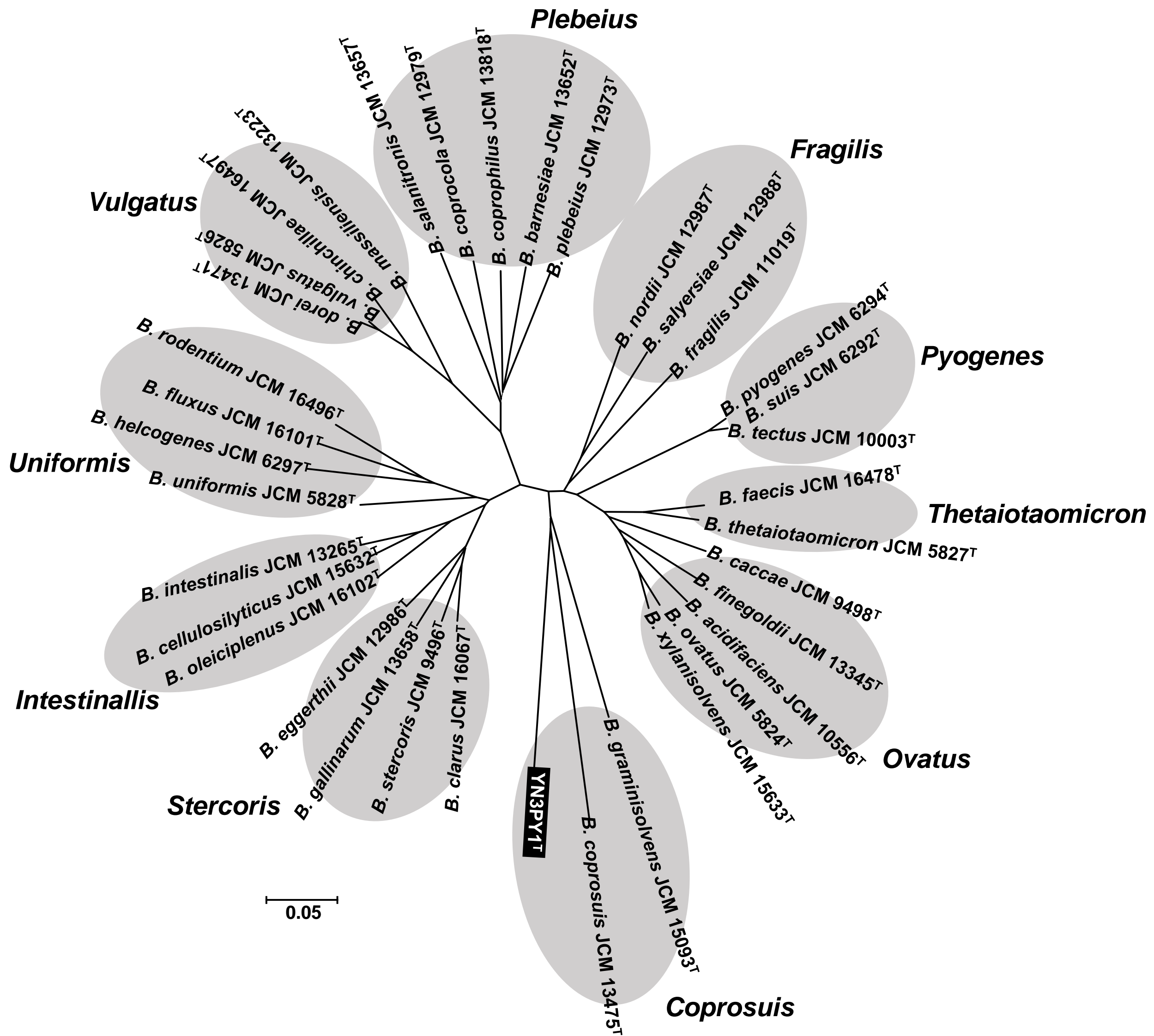

Figure 4-2 Unrooted neighbor-joining tree based on concatenated sequences of three genes; *dnaJ*, *gyrB*, and *hsp60*, showing the phylogenetic position of the strain YN3PY1^T in the *'Coprosuis'* clade of the genus *Bacteroides*. The sequences data of the strain YN3PY1^T were screened from YN3 metagenome described in chapter 3. The ten clades of the genus *Bacteroides* (Sakamoto and Ohkuma, M, 2011) were highlighted by gray-colored oval shapes. Bar indicates 0.05 nucleotide substitutions per nucleotide position.

4.3.3 Physiological and biochemical characterization

The strain YN3PY1^T was capable to grow at temperature range of 10-40 °C with optimum at 28 °C, at pH range of 5.4-8.5 with optimum at 7.0 and at NaCl 0-3.5% with optimum at 1.0%. Notably, the optimum temperature for the growth of the strain YN3PY1^T is comparatively lower than those recorded previously for the other species of the genus *Bacteroides* (Table 4-1).

Regarding the glucose utilization by the strain YN3PY1^T, our data as confirmed by two HPLC analyses indicated that lactate and malate were the end products of glucose utilization by the strain YN3PY1^T. Specifically, the strain was able to produce 15 mM lactate and 6.8 mM malate in the culture provided with 10 mM glucose. The pathway for the glucose utilization in the strain YN3PY1^T was suggested in Figure 4-3, based on the data extracted from *Bacteroides*-metagenome described in chapter 3. The production of malate as an end product of glucose fermentation is unique among members of the genus *Bacteroides* and has not been recorded before. Generally, the end products of glucose utilization in *Bacteroides* spp. have been recorded to be succinate, acetate, and propionate (Whitehead et al., 2005; Ueki et al., 2008; Nishiyama et al., 2009; Hatamoto et al., 2014) and less frequently lactate and formate (Ueki et al., 2008; Watanabe et al., 2010). The end products of glucose utilization in *Bacteroides* spp. varied according to the constituents of the employed nutritional media as reported in some instances (Ueki et al., 2008).

Table 4-1 The DNA G+C content and phenotypic characteristics of the strain YN3PY1^T and closely related strains of the genus *Bacteroides*. 1, the strain YN3PY1^T; 2, *B. luti* JCM 19020^T (Hatamoto et al., 2014); 3, *B. graminisolvens* JCM 15093^T (Nishiyama et al., 2009); 4, *B. coprosuis* JCM 13475^T (Whitehead et al., 2005); 5, *B. propionicifaciens* JCM 14649^T (Ueki et al., 2008); 6, *B. neonati* DSM 26805^T (Cassir et al., 2014). Symbols or abbreviations are used with the following meanings: +, positive; w, weakly positive; -, negative; nd., not-determined; A, Acetate; F, Formate; L, Lactate; M, Malate; P, Propionate; S, Succinate.

Characteristic	1	2	3	4	5	6
DNA G+C content (mol%)	37.5	44.4	38	36.4	46.2–47.5	43.5
Optimum pH	7.0	6.5–7.0	7.2	nd.	7.9	nd.
Optimum temperature (°C)	28	37–40	30–35	37	30	37
Optimum NaCl (%)	1.0	nd.	0	nd.	1.0	nd.
End-products from glucose fermentation	L, M	A, F, P, S	A, L, P, S	A, P, S	A, L, P, S	nd.
Rapid ID 32A kit						
α–Galactosidase	+	–	nd.	w	nd.	+
β–Galactosidase	+	+	nd.	+	nd.	–
β–Glucosidase	+	+	nd.	+	nd.	–
α–Arabinosidase	–	+	nd.	–	nd.	–
β–Glucuronidase	–	+	nd.	–	nd.	–
Mannose	–	+	+	+	+	–
Raffinose	–	+	+	–	–	–
Indole production	+	+	–	–	–	–
Alkaline phosphatase	+	+	nd.	+	nd.	–
Arginine arylamidase	–	–	nd.	+	nd.	–
Proline arylamidase	–	–	nd.	–	nd.	–
Leucyl glycine arylamidase	+	+	nd.	+	nd.	–
Alanine arylamidase	+	+	nd.	+	nd.	–
Glutamyl glutamic acid arylamidase	+	–	nd.	+	nd.	–

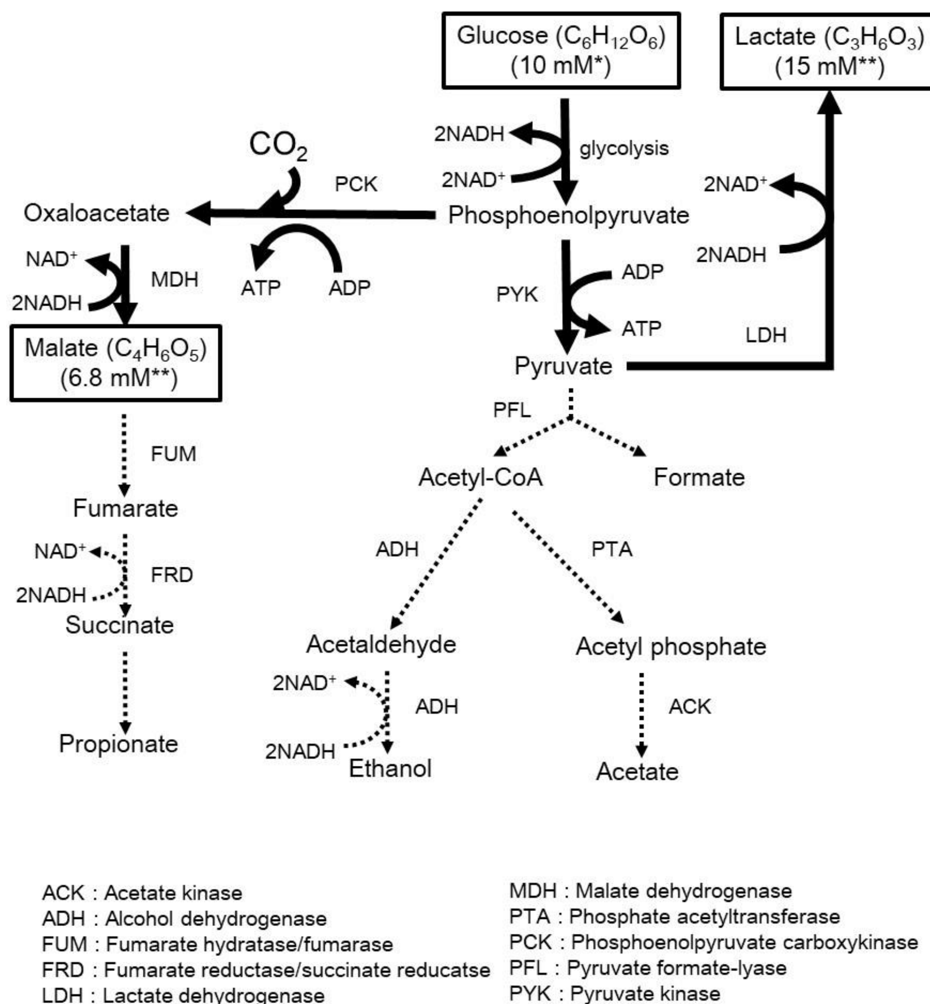

Figure 4-3 Predicted glucose fermentation pathways in the strain YN3PY1^T as suggested from *Bacteroides*-metagenome described in chapter 3. Possibly active and inactive pathways are shown in solid and dashed lines, respectively. *Added concentration of glucose was 10 mM. **15 mM lactate and 6.8 mM malate were detected by HPLC. In this figure, all enzymes shown are expected to exist in *Bacteroides*-metagenome which binned from YN3 metagenome as described in chapter 3.

Based on the data obtained from the API Rapid ID 32A kit (Table 4-1), the strain YN3PY1^T was able to hydrolyze terminal α- and β- glycosidic bonds via the production of α-galactosidase and β-galactosidase, respectively. In a similar way, activities for the production of α- glucosidase, β-glucosidase, α-fucosidase and N-acetyl-ß-glucosaminidase were demonstrated in the strain YN3PY1^T. Moreover, hydrolysis of the terminal peptide units contained leucyl glycine, alanine, and glutamyl glutamic acid showed the activities of leucyl glycine arylamidase, alanine arylamidase, and glutamyl glutamic acid arylamidase, respectively. Finally, activities demonstrated the production of alkaline phosphatase and tryptophanase were observed by the production of phosphate and indole, respectively.

4.3.4 Chemotaxonomic characterization

As shown in Table 4-2, cellular fatty acid analysis in the strain YN3PY1^T indicated the presence of anteiso-$C_{15:0}$ as the major fatty acid in the strain YN3PY1^T at 30%, which also detected as the major fatty acids in most species of the genus *Bacteroides* (Miyagawa et al., 1979; Shah, 1992). Next to anteiso-$C_{15:0}$, $C_{15:0}$, $C_{16:0}$, iso-$C_{16:0}$ and anteiso-$C_{17:0}$ were detected as the major fatty acids, > 5% of the total fatty acids, at 11%, 8.1%, 6.8% and 5.4%. respectively. The strain YN3PY1^T had MK-11 and MK-12 as the major menaquinones at 50% and 45%, respectively. In addition, MK-10 was detected as the minor menaquinone at 5.0%. So far, MK-10 and MK-11 were recorded as the major menaquinones in most *Bacteroides* spp. (Shah, 1992). Mk-12 was observed before in a such significant level, at 43%, in *B. salanitronis* which belonged to the '*Plebius'* clade of the genus *Bacteroides* (Lan et al., 2006). In comparison to other members of the '*Coprosuis'* clade, *B. luti* didn't show any menaquinones (Hatamoto et al., 2014). *B. graminisolvens* showed only MK-10 as the major menaquinone (Nishiyama et al., 2009), and *B. propionicifaciens* showed

MK-8 and MK-9 as the major menaquinones and MK-10 as the minor one (Ueki et al., 2008). Unfortunately, the quinone composition analyses in strains *B. coprosuis* and *B. neonati* were not performed. Therefore, the data indicated that members of the '*Coprosuis*' clade showed no common features with regard to the quinone composition.

Table 4-2 Fatty acid composition of the strain YN3PY1^T and closely related strains of the genus *Bacteroides*. 1, the strain YN3PY1^T; 2, *B. luti* JCM 19020^T (Hatamoto et al., 2014); 3, *B. graminisolvens* JCM 15093^T (Nishiyama et al., 2009); 4, *B. coprosuis* JCM 13475^T (Whitehead et al., 2005); 5, *B. propionicifaciens* JCM 14649^T (Ueki et al., 2008). Only values ≥1% of the total fatty acid content are shown. The abbreviation "nd." indicates "not-determined."

Characteristic	1	2	3	4	5
Fatty acid composition					
Saturated					
$C_{14:0}$	–	17.0	1.9	–	–
$C_{15:0}$	11.0	2.2	4.0	7.6	2.8
$C_{16:0}$	8.1	13.0	3.0	3.5	2.5
$C_{17:0}$	2.1	–	–	2.2	1.6
$C_{18:0}$	–	3.5	–	1.4	–
Unsaturated					
$C_{16:1}\omega7c$	2.2	2.3	–	–	–
$C_{18:1}\omega9c$	–	3.4	–	2.4	–
Hydroxy acids					
$C_{16:0}$ 3–OH	1.1	11	1.3	–	–
iso–$C_{17:0}$ 3–OH	–	–	20	17	–
anteiso–$C_{17:0}$ 3–OH	3.8	1.7	3.6	–	–
Branched					
iso–$C_{12:0}$	–	–	–	–	1.9
iso–$C_{13:0}$	1.1	3.2	4.9	1.1	–
iso–$C_{14:0}$	2.1	–	2.0	–	–
iso–$C_{15:0}$	1.4	4.3	8.9	8.2	6.3
iso–$C_{16:0}$	6.8	–	1.5	1.3	2.3
iso–$C_{17:0}$	–	–	–	10	16
anteiso–$C_{11:0}$	1.9	–	–	–	–
anteiso–$C_{13:0}$	3.9	3.4	7.0	–	1.7
anteiso–$C_{15:0}$	30	16	33	31	41
anteiso–$C_{17:0}$	5.4	–	1.2	3.2	14
anteiso– $C_{17:1}\omega9c$	–	–	–	2.2	–
Summed features*					
1	1.0	4.4	–	–	–
3	5.2	2.1	–	–	–
5	–	–	–	6.4	–
8	1.4	–	–	–	–
9	1.5	–	–	–	–
11	4.0	4.5	–	–	–

*Summed features represent groups of two or three fatty acids that cannot be separated by the Sherlock MIS system. Summed feature 1 comprises $C_{13:1}$, $C_{14:0}$ aldehyde and/or $C_{11:1}$ 2-OH, summed feature 3 comprises iso-$C_{15:0}$ aldehyde and/or unknown fatty acid ECL 13.570, summed feature 5 comprises $C_{14:0}$ 3-OH and/or $C_{15:0}$ dimethylacetal, summed feature 8 comprises $C_{17:1}$ 1ω8c and/or $C_{17:2}$, summed feature 9 comprises iso-$C_{16:0}$ 3-OH and/or unknown fatty acid ECL 17.157 dimethylacetal, summed feature 11 comprises iso-$C_{17:0}$ 3-OH and/or $C_{18:2}$ dimethylacetal.

4.3.5 Dechlorination enhancement

The potential contribution of the strain YN3PY1^T for the dechlorination by *Dehalococcoides* was assessed by the co-inoculation of the strain YN3PY1^T with C4C4 culture. The serum vials

contained C4C4 culture co-inoculated with the strain YN3PY1^T showed higher production of ETH than C4C4 culture alone (Figure 4-4 (A and B)). Also, the serum vials contained C4C4 culture co-inoculated with the strain YN3PY1^T showed higher release of the calculated Cl^- production when compared to the C4C4 culture alone especially at days 0-63 (Figure 4-4 (C)). The microbial composition of one vial contained C4C4 culture and the strain YN3PY1^T indicated the presence of *Dehalococcoides* at 67%, the strain YN3PY1^T at 22%, *Propionicimonas* at 5.2% and others (< 0.05% each) at 5.8%. The enhanced dechlorination of C4C4 culture co-inoculated with the strain YN3PY1^T is still significantly lower than this recorded in chapter 2 for YN3 culture, which dechlorinates 500 µM *cis*-DCE to ETH within 15 days only.

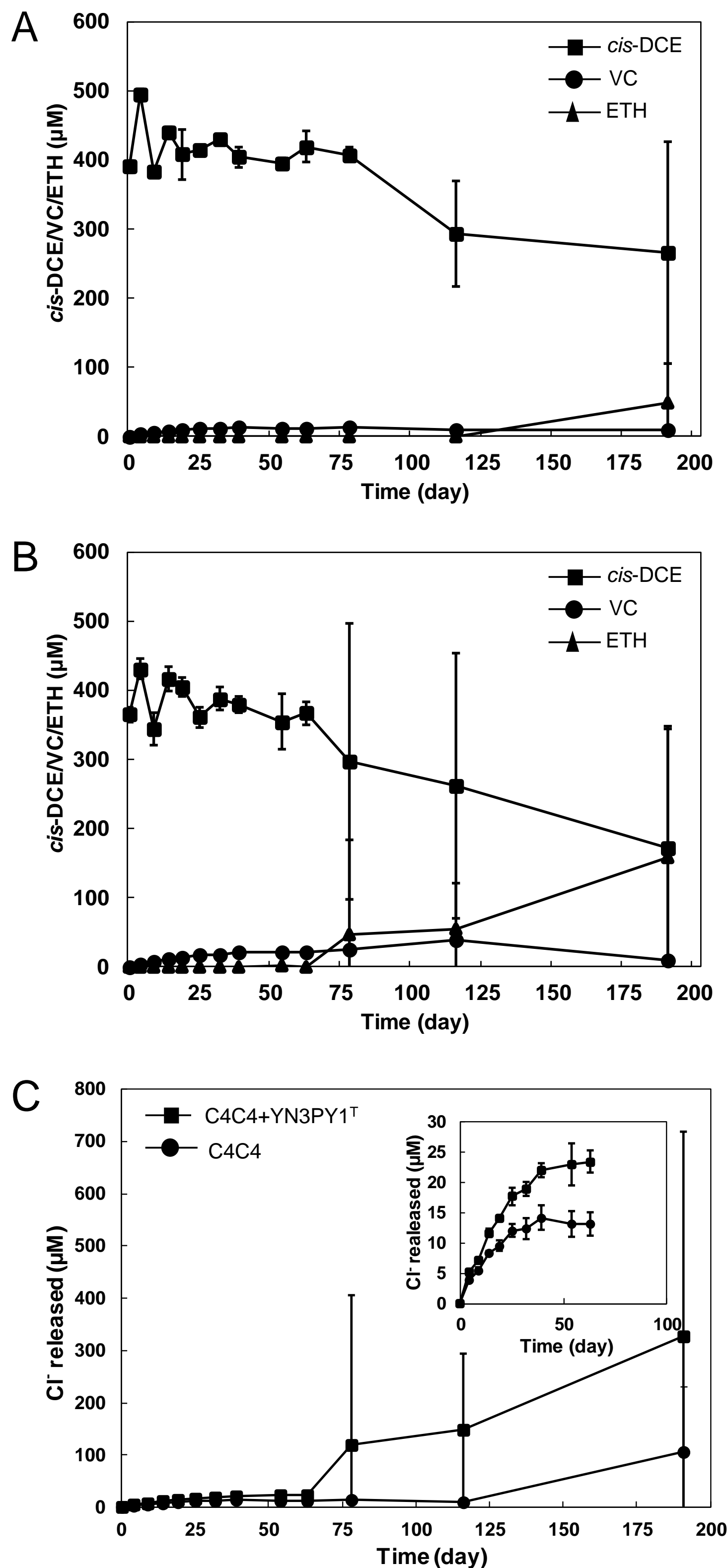

Figure 4-4 Dechlorination of *cis*-DCE A: By C4C4 culture only, B: By C4C4 culture co-inoculated with strain YN3PY1^T, and C: Released Cl^- from both C4C4 cultures with and without the strain YN3PY1^T. Error bars represent standard deviations (n =5). Dechlorination up to day 63 is represented by inset figure in panel (C).

4.4 Discussion

In this chapter, a novel species of the genus *Bacteroides* was isolated from YN3 culture and characterized using a polyphasic approach. The strain YN3PY1^T was the first pure isolate of the genus *Bacteroides* isolated from animal-independent environments. The strain was placed in an independent phylogenetic cluster (cluster a, Figure 4-1) together with uncultured phylotypes of *Bacteroides*. These uncultured phylotypes were identified from diverse animal-unrelated environments, such as paddy soil, lake slurry and river sediment (Xing et al., 2011; Zhou et al., 2015; Liang and Wang, 2017). As proved in this study and also suggested before by Whitman et al. (2014), this phylogenetic cluster is assumed to represent the free-living linage (or animal unrelated linage) within the genus *Bacteroides* (cluster a, Figure 4-1). This free-living cluster is distantly related to other *Bacteroides* spp. obtained from animal-dependent environments. Therefore, the strain YN3PY1^T represents the first member of the *Bacteroides* isolated from an animal-unrelated environment (i.e., free-living). Although, a strain of *B. luti* (Hatamoto et al., 2014) and clone S2-54 (Faust et al., 2015), contained within the free-living cluster, were identified from environments with evidence for fecal contamination.

The results obtained in this chapter showed an enhancement in the dechlorination of *cis*-DCE to ETH by C4C4 culture in serum bottles co-inoculated with the strain YN3PY1^T. So far, several studies have demonstrated that *Dehalococcoides* perform a better dechlorination activity and growth rate in mixed culture than pure one (Distefano et al., 1992; He et al., 2007; men et al., 2012). In general, coexisting bacteria support *Dehalococcoides* as follows: to provide with hydrogen and acetate which were identified as the only energy source and carbon source for *Dehalococcoides* (Distefano et al., 1992; He et al., 2007; men et al., 2012); to provide with vitamin B_{12} which strictly required for the activity of reductive dehalogenase (He et al., 2007; men et al.,

2012); and finally, to remove coexisting carbon monoxide and oxygen which well-known to negatively affect the growth and activity of *Dehalococcoides* (Zhuang et al., 2014; Liu et al., 2017). In this chapter, C4C4 culture co-inoculated with the strain YN3PY1^T was already provided with acetate, hydrogen, vitamin B_{12} and strictly anaerobic conditions. In addition, genes required for CO detoxification were not identified within the available genomes of *Bacteroides* spp. This suggested a different mechanism by which the strain YN3PY1^T supports the dechlorination by *Dehalococcoides* contained in C4C4 culture. However, the enhanced dechlorination activity of C4C4 culture co-inoculated with the strain YN3PY1^T was significantly lower than that of YN3 culture, although both C4C4 culture and the strain YN3PY1^T were obtained from YN3 culture. As reported in chapter 2, YN3 culture dechlorinated *cis*-DCE to ETH completely within two weeks. This result leads to the consideration that other coexisting microbe(s) in YN3 culture (e.g., members of the phyla *Actinobacteria* and/or *Firmicutes* described in chapter 3) is required for the dechlorination activity of YN3 culture.

4.5 Conclusion

In this chapter, the strain YN3PY1^T was isolated in pure form from a PCE to ETH dechlorinating consortium, YN3 culture, which had been enriched and characterized in chapters 2 and 3. The strain was characterized by a polyphasic approach and found as a novel species of the '*Coprosuis'* clade, the genus *Bacteroides*. In particular, the strain was the first isolate with free-living lifestyle in the genus *Bacteroides*. The strain enhanced the dechlorination of *cis*-DCE to ETH by *Dehalococcoides* contained in C4C4 culture by yet-to-be identified mechanism. The enhanced dechlorination activity shown by C4C4 was still lower than that of YN3 culture (from which both C4C4 culture and the strain YN3PY1^T were isolated), suggesting that another microbe(s) was

needed in the culture.

5. General discussion

5.1 Significance of this study

This study aimed to establish tetrachloroethene (PCE) and 1,1,2-trichloroethane (112-TCA) dechlorinating enriched consortia. PCE and 112-TCA, posing potential health risk as suspected carcinogens, were used extensively in many industrial purposes, and thus are commonly detected as groundwater contaminants in developed countries. The study also aimed to identify the *rdhA* genes, encode the catalytic subunit of reductive dehalogenase, suggested to be involved in the dechlorination of PCE into ethene (ETH), and to evaluate the contribution of a novel coexisting bacterium to the dechlorination PCE into ETH activity.

In this study, the assembled *Dehalococcoides*-metagenome showed the presence of 18 *rdhA* genes, designated as *YN3rdhA*, 4 of which were suggested to be involved in the PCE to ETH dechlorination, while the other 14 *YN3rdhA* genes remained unidentified. Among these 4 *YN3rdhA* genes, two were newly suggested in this study to be involved in the chloroethenes (CEs) dechlorination especially the vinyl chloride (VC) to ETH dechlorination step. These two new *YN3rdhA* genes are also suggested to serve as biomarkers to predict the contamination with specific contaminants (e.g., VC) and to predict the dechlorination of VC to ETH at a given VC-contaminated site. The *vcrA* and *bvcA* genes have been used previously as biomarkers to predict the complete dechlorination of CEs into ETH (Löffler et al., 2013). The unidentified 14 *YN3rdhA* genes reported in this study are considered to play a role in dehalogenation of other organohalides. As reported before (Adrian and Löffler, 2016) there are more than 2000 *rdhA* genes of unknown function and await the assignments of their substrates from the available ~5000 organohalides (of natural origin) and ~several hundreds organohalides (of anthropogenic origin). As a strategy, the presence of multiple non-identical *rdhA* genes in *Dehalococcoides* is compatible with its lifestyle as

an obligate OHRB. In other words, *Dehalococcoides* is considered to have evolved multiple *rdhA* genes to overcome the shortage of inability to use other non-halogenated chemicals as electron acceptors (Löffler et al., 2013).

In this study, the metagenomic analysis indicated the presence of bacterial taxa belonging to the genus *Dehalococcoides* and phyla *Actinobacteria*, *Bacteroidetes* and *Firmicutes.* Experiments to evaluate the effect of the isolate, strain YN3PY1 (from *Bacteroidetes*) as a coexisting bacterium, on the dechlorination by C4C4 culture was conducted and indicated that the dechlorination of *cis*-dichloroethene (*cis*-DCE) into ETH was slightly enhanced. This result suggested that coexisting microbe(s) in YN3 culture enhance the dechlorination activity, however, the strong activity of YN3 culture was not explained enough by only the slight enhancement by strain YN3PY1 under the given conditions. There would be a yet to be known enhancing mechanism in the dechlorination by *Dehalococcoides* in YN3 culture. Additional experiments are required to identify such mechanism(s).

So far, coexisting bacteria have been identified to help *Dehalococcoides* either by supplementation with essential substance (e.g., hydrogen, acetate or corrinoid (vitamin B_{12})) or elimination of toxic ones (e.g., carbon monoxide or oxygen) (He et al., 2007; Men et al., 2012; Zhuang et al., 2014; Liu et al., 2017). In this study, analysis of *Dehalococcoides*-metagenome indicated the absence of essential genes, *cbiC, cbiD, cbiF, cbiJ, cbiK,* and *cbiL*, which required for *de novo* synthesis of corrinoid cofactor. These genes have been also absent from all available genomes of the genus *Dehalococcoides* (Moore and Escalante-Semerena, 2016). Also, these genes were found to be absent from the other assembled genomes in this study. In addition, metagenome analysis indicated the absence of *acsA* gene, which involved in the carbon monoxide detoxification,

from all genomes assembled this study. This indicated that inside YN3 culture, the contribution of coexisting bacteria to *Dehalococcoides* either by supplying with corrinoid or detoxifying of carbon monoxide did not likely occur. However, it should be kept in mind that in this study the assembled genomes for the coexisting bacteria are significantly incomplete.

In this study, YN3 culture was already supplemented with hydrogen, acetate, and vitamin B_{12} at sufficient levels and kept under in proper anaerobic conditions. This suggested the possibility that, coexisting bacteria helped *Dehalococcoides* in YN3 culture via a different mechanism. In general, it is difficult to untangle metabolic exchanges or interactions in mixed microbial communities, due to the dynamic nature of these interactions and complexity of mixed microbial communities (Ponomarova and Patil, 2015). For YN3 cultures, resolving of metabolic interaction can be done using constricted or synthetic consortia approach. In this approach, coexisting bacteria, in addition to the strain YN3PY1, shall be isolated in pure form. Then, co-culturing of C4C4 culture (or any commercially available *Dehalococcoides* in pure form) with one or more of these isolated strains, of coexisting bacteria, is carried out until we arrive to the dechlorination rate as in YN3 culture. Concurrently, one or more of the following approaches can be applied to identify the interactions or metabolite cross feeding during these experiments.

Meta-omics approaches

The multi-omics approaches are culture independent-technologies which include in addition to the already performed metagenome analysis, metatranscriptomics (to characterize the gene expression of the total community), metaproteomics (to characterize the proteins of the total community), metabolomics (to characterize the metabolic intermediates and end products of the total community) analyses. The omics approaches can give us a comprehensive insight about the structure, function, activity, metabolites and interactions inside YN3 culture, and thus deep

understanding on the role of each microbe can be attained. In addition, once the interactions and metabolites inside YN3 culture are identified, isolation of *Dehalococcoides* alone, in pure form, is likely to be workable (Ponomarova and Patil, 2015).

Tracing of the isotope labelled substrates

Tracing of substrates labelled with radioactive isotopes is a standard method for metabolic pathways discovery and untangle the flow of metabolites in a given microbial consortium (Ponomarova and Patil, 2015).

Imaging of the structures formed inside the consortium

These types of analyses refer to the imaging, e.g., microscopic observation after applying fluorescent dyes, of special structures or aggregates, e.g., pili, which formed between the microbes to facilitate the metabolite exchange between them. Identification of such structures or aggregates is of value in the assessing of the general metabolic state of the consortium members (Ponomarova and Patil, 2015).

The marked PCE to ETH dechlorination activity of YN3 culture making it a potential candidate for the *in situ* bioaugmentation to cleanup the CEs-contaminated site of groundwater/aquifer. The culture safeness, regarding presence of potential pathogenic microorganisms, is one of the most important points required to be checked before the regulatory authorities allow the usage of the culture for bioaugmentation purposes. Analysis of YN3 metagenome indicated the apparent absence of pathogens from YN3 culture however, the metagenome assembled for some members are incomplete as shown in chapter 3. This indicated incomplete coverage for the all members exist in YN3 culture and thus assays to check the presence of certain pathogens should be performed to ensure the complete absence of pathogens from YN3

culture as previously recommended (Steffan and Vainberg, 2013).

The success of the bioremediation process using YN3 culture is depending largely on the providing of the appropriate niche for OHRB. The bioaugmentation with YN3 culture is likely to be successful as the dechlorination of the contaminant is metabolic (the contaminant, e.g., PCE, induce the growth of the dehalogenating microbe, *Dehalococcoides* in YN3 culture). The dehalogenating microbe is an obligate contaminant-dehalogenator, and the contaminant is electron acceptor (fewer alternatives of electron acceptors are available if compared to the electron donors). However and as a general rule, the success of the bioremediation process is largely dependent on the avoiding of some inhibitors or inhibitory conditions. Acidic pH (e.g., pH below 6), high contaminant concentrations (e.g., PCE at 540 μM) and presence of inhibitory co-contaminant (e.g., chloroform) were identified inhibitory for PCE bioremediation by OHRB (Steffan and Vainberg, 2013).

In order to achieve the improved and sustained dechlorination activity using YN3 culture during the future-suggested *in situ* bioaugmentation processes, sticking to the laboratory conditions used in this study is recommended. Applying YN3 culture to aquifers with contaminant (PCE) concentration 800 μM at maximum and pH around 7 are among the optimum conditions for improved YN3 culture activity. Also, an inoculum with initial copy number ~10^7 of *Dehalococcoides*-16S rRNA gene is also recommended. We recommend to use of YN3 culture, as much as possible, for cleaning up sites contaminated with only CEs, since certain co-contaminants (e.g., chloroform) can stop the dechlorination of the targeted contaminants (e.g., PCE) as reported before (Steffan and Vainberg, 2013). However, further studies are required to confirm or deny this assumption. As described in chapter 4, the dechlorination activity of C4C4 culture, which was obtained from YN3 culture, was significantly lower than that of the parent culture, YN3 culture. Thus and for bioremediation purposes, we recommend the usage of intact YN3 culture without any

modifications until the all metabolites and pathways inside YN3 culture are elucidated clearly.

5.2 Achievements/summary of this study

5.2.1 Enrichment of PCE and 112-TCA dechlorinating cultures

In chapter 2, two dechlorinating cultures were obtained, YN3 culture (which dechlorinates PCE completely into ETH) and KJ-TCA culture (which dechlorinates 112-TCA completely into VC). YN3 culture dechlorinates up to 800 μM of PCE dechlorinated completely into ETH within only two weeks. YN3 culture is a potential candidate for bioaugmentation technologies because of its marked CEs dechlorination activities. For KJ-TCA culture, the product of 112-TCA dechlorination is the proven carcinogen VC, this problem can be overcome, however, by combing both YN3 culture with KJ-TCA culture. Thus, impact of this thesis in environmental cleanup is not restricted to PCE–contaminated sites but also can be extended to 112-TCA contaminated ones, or sites contaminated with both. As a general, Hug et al.(2013) indicated that combing *Dehalococcoides* and *Dehalobacter* enriched cultures will not only resulted in a culture with wider contaminates range, PCE and 112-TCA, but also in a culture with accelerated dechlorination activity due to alleviation of cross inhibition between CEs and CAs.

5.2.2 Identification of the microbial composition of YN3 culture and *rdhA* genes suggested to be involved in the PCE to ETH dechlorination.

In chapter 3, the identification of the microbes comprising the YN3 cultures were characterized in this chapter using metagenome analysis. The microbes comprising YN3 culture were found to be belonging to phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Dehalococcoides*. Also in this chapter, two *YN3rdhA* genes, from the 4 *YN3rdhA* genes functionally

characterized in this chapter, were newly introduced as a genes suggested to be involved in the CEs dechlorination and can serve as a markers for the predict VC to ETH dechlorination in a given contaminated site. A further analysis at protein level is required to confirm the integration of these two genes in the dechlorination process.

5.2.3 Enhancement the dechlorination of *cis*-DCE to ETH by co-culturing of strain YN3PY1, with the dechlorinator *Dehalococcoides*

In chapter 4, the experiments proved the enhancement of the dechlorination of *cis*-DCE to ETH by co-culturing of strain YN3PY1 (a novel species of the genus *Bacteroides*) beside *Dehalococcoides* enriched culture, C4C4 culture. However, even with this enhancement the overall dechlorination speed was observed to be slow if compared to that of YN3 culture. This indicated that other experiments are required to judge the role of the other bacteria coexist in YN3 culture

5.3 Suggestions for the future study

5.3.1 Discovery of new OHRB

OHRBs have been identified in only three phyla of the domain Bacteria; in addition, only one putative *rdhA* gene was identified in the domain Archaea. Also, the majority of bacteria are unculturable to date, the organohalide respiring capability of available cultures has not been explored enough, and diverse dehalogenation reactions that had been detected in the initial microcosms were often lost during the subsequent culturing steps. Thus, it is likely that the majority of new OHRB await discovery (Adrian and löffler, 2016). In order to increase the chances to obtain new OHRB, new methods and conditions would be required. The emphasize on the identification of new species especially from the phylum *Chloroflexi* is of special significant as it is expected to

yield industrially relevant OHRB with the potential to degrade recalcitrant contaminants (Hug et al., 2013). In addition to *Dehalococcoides*, *Dehalogenimonas* was recently reported to dechlorinate TCE completely into innocuous ETH which indicates the high specialization and wide spectrum of contaminants to be degraded by the microbes from this phylum (Yang et al., 2017). Discovery of new OHRB from other phyla (non-*Chloroflexi*), are relatively easy to manipulate organisms, is also important by providing a system for examination of dehalogenation activities. With identifying new OHRB, it is likely to find a dehalogenating microbe with new or stronger dehalogenating activity, or with degrading ability toward multiple pollutants at the contaminated site (e.g., chlorinated aliphatic hydrocarbons and heavy metals). Finally, this new dehalogenating activity should be exploited for the environmental biotechnology purposes, e.g., bioaugmentation. The future of bioaugmentation is dependent on the discovery of novel organisms with novel pathways which can be found using varied culture conditions.

5.3.2 Molecular-based research

With more than putative 2000 *rdhA* gene identified sequences, only very few are functionally characterized, the majority of them were identified from *Dehalococcoides* and *Dehalobacter* and *Dehalogenimonas* (Adrian and Löffler, 2016). Because RdhA units are oxygen sensitive and slow growth rate of OHRB, the yield of the purified RdhA units obtained by direct purification techniques are insufficient for proper biochemical characterization. Alternatively, other relatively easy methods can be used, e.g., transcriptomic and proteomic analyses, and non-denaturing polyacrylamide gel electrophoresis (Hug et al., 2013). The last success in heterologous expression of active two *rdhA* genes is expected to open up the way for functional characterization of many RdhA units (Mac Nelly et al., 20104; Parthasarathy et al., 2015). In addition, heterologous

expression can be used for the characterizing cofactor requirements and structural properties of the RdhA units. Also, the RdhA-related proteins, e.g., regulatory protein, accessory protein and protein associated with electron transport across the membrane are also expected to be characterized by this system (Adrian and Löffler, 2016).

With more functional and structural characterization of RdhA units, it is expected via comparative analyses to identify function-structure relationships and to predict the substrate range of a given RdhA. Once the *rdhA* gene is identified to be involved in the dechlorination of certain contaminant, the gene can be used as a biomarker for tracking the contaminant or the detoxification/dehalogenation of this contaminant at a given contaminated site. The functional identification of a given *rdhA* gene also can help in the usage of the specialized culture (containing the specialized gene) for better and more efficient remediation process at contaminated sites. Finally, the gene itself can be introduced alone after carrying on a mobile element, e.g., bacteriophage, in case that applying the whole culture is unsuitable (e.g., containing pathogenic microbes beside the dehalogenating ones).

5.3.3 Community-based research

Inside a given dehalogenating microbial community, knowing the metabolic interaction between OHRB and non-OHRB is essential to deepen understanding of the factors affecting growth and dechlorination activity of OHRB in their environment (Hug et al., 2013). Regarding bioaugmentation experiments, understanding of the metabolic interaction of the dehalogenating culture to be introduced is critical to predict the metabolic advantage of this culture over indigenous population. This understanding will also influence the dechlorination process. Such kind of detailed knowledge is essential to refine the bioremediation technologies to desired results (Adrian and

Löffler, 2016).

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