Chapter 4
Genetic System of Organohalide-Respiring Bacteria

Taiki Futagami, Masatoshi Goto, and Kensuke Furukawa

Abstract Organohalide-respiring bacteria (OHRB) utilize halogenated organic compounds as terminal electron acceptors and are considered to be significantly important from both viewpoints of bioremediation and natural halogen cycle. Growth-linked bioremediation using OHRB has been successfully applied to removal of chlorinated solvents, e.g., tetrachloroethene is successively converted to trichloroethene, dichloroethenes, vinyl chloride, and nontoxic ethene. From OHRB, versatile reductive dehalogenases (RDases), which catalyze the reductive dehalogenation reaction, were purified and their corresponding genes have been identified. In this chapter, we present an overview of current understanding of organohalide respiration, showing the RDase genes and their associated genes are highly conserved in phylogenetically diverse OHRB.

Keywords Chlorinated solvent • Chloroethene • Halogen cycle • Halogenated organic compound • Organohalide • Organohalide respiration • Organohalide-respiring bacteria • Reductive dehalogenase • Reductive dehalogenation

Abbreviations

3Cl4OHPA	3-Chloro-4-hydroxyphenylacetate
4-OHPA	4-Hydroxyphenylacetate
CARD-FISH	Catalyzed reporter deposition-fluorescent in situ hybridization

T. Futagami (✉) • M. Goto
Department of Bioscience and Biotechnology, Kyushu University,
6-10-1, Hakozaki, Fukuoka 812-8581, Japan
e-mail: futagami@brs.kyushu-u.ac.jp

K. Furukawa
Department of Food and Bioscience, Faculty of Food Science and Nutrition,
Beppu University, Kitaishigaki 82, Beppu, Oita 874–8501, Japan

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CD	Carbon dichloride
CF	Chloroform
cis-DCE	*cis*-1,2-dichloroethene
CP	Chlorophenol
DMSO	Dimethyl sulfoxide
EPR	Electron paramagnetic resonance
ETH	Ethene
IS	Insertion sequence
OHRB	Organohalide-respiring bacteria
PCBs	Polychlorinated biphenyls
PCE	Tetrachloroethene
RDase	Reductive dehalogenase
Tat	Twin-arginine translocation
TCB	Trichlorobenzene
TCE	Trichloroethene
TeCB	Tetrachlorobenzene
trans-DCE	*trans*-1,2-dichloroethene
VC	Vinyl chloride

4.1 Introduction

Organohalide-respiring bacteria (OHRB) utilize halogenated organic compounds as terminal electron acceptors and have been successfully applied to the detoxification of soil and groundwater contaminated with chlorinated ethenes (Fig. 4.1a). Growth-linked bioremediation using OHRB is a powerful technology for the removal of chlorinated solvents such as tetrachloroethene [perchloroethene (PCE)] and trichloroethene (TCE) in anaerobic environments. In contrast to aerobic microbial degradation processes, the reductive processes associated with organohalide respiration favor highly halogenated organic substrates. This is advantageous for degradation of compounds such as PCE, as removal of PCE through reductive dehalogenation is generally much more effective than oxygenative degradation.

In this chapter, we present an overview of current understanding of organohalide respiration by anaerobic bacteria. The ability to perform organohalide respiration is widespread among bacteria and to date has been described in the phyla *Chloroflexi* and *Firmicutes*, as well as the epsilon and gamma subdivisions of the phylum *Proteobacteria*. However, the genetic system controlling organohalide respiration is well conserved among the versatile OHRB. The key enzyme, reductive dehalogenase (RDase), contains an N-terminal Tat (twin-arginine translocation) signal sequence and two Fe-S cluster-binding motifs. In addition, biochemical studies have revealed that a corrinoid cofactor plays a significant role in the RDase-mediated redox reaction. The gene encoding RDase is found in a gene cluster that also contains a gene encoding a membrane-spanning protein that is believed to act as membrane anchor for RDase. Genes encoding a transcriptional regulator, chaperone,

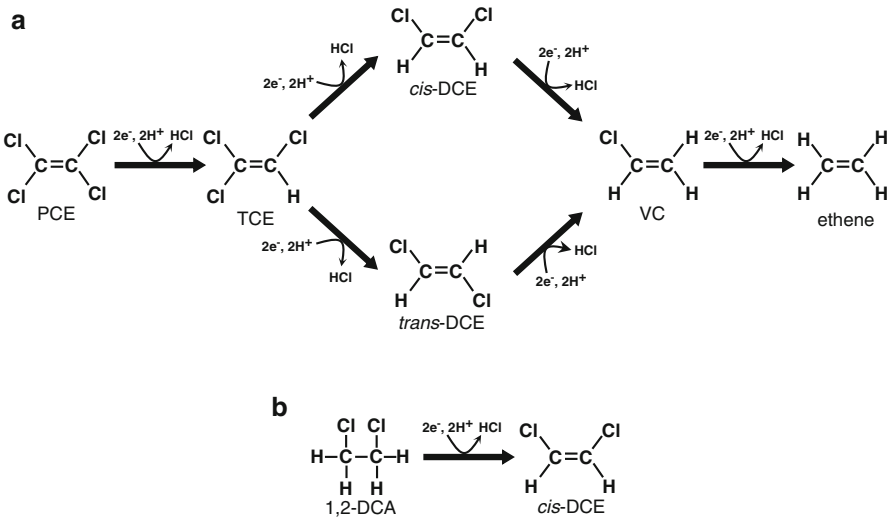


Fig. 4.1 Reductive dechlorination during organohalide respiration. Sequential dechlorination of chlorinated ethenes by hydrogenolytic dechlorination (**a**). Dichloroelimination of 1,2-trichloroethane (**b**). *PCE* tetrachloroethene, *TCE* trichloroethene, *DCE* dichloroethene, *VC* vinyl chloride, *1,2-DCA* 1,2-dichloroethane

transposase, and phage integrase are also frequently found near the RDase gene. Consequently, the transcriptional regulation and genetic rearrangement of these gene clusters and chaperone-associated maturation of RDase have been studied. Genetic events such as mutation and horizontal gene transfer were likely involved in the evolution of the RDase gene, as well as in the evolution of other degradation genes. An increasing number of studies have revealed that OHRB and RDase genes are present even in pristine environments, indicating that they also play a significant role in the dehalogenation of naturally produced organohalides, and not just artificial compounds.

4.2 Diversity of Organohalide-Respiring Bacteria

After the 3-chlorobenzoate-respiring bacterium *Desulfomonile tiedjei* DCB-1 was first isolated in 1984 (Shelton and Tiedje 1984; Deweerd and Suflita 1990), other versatile OHRB belonging to a wide range of bacterial phyla have been identified, including members of the *Firmicutes* (low G+C Gram-positive bacteria), the epsilon and gamma subdivisions of the phylum *Proteobacteria*, and the *Chloroflexi* (Fig. 4.2). Most OHRB are obligate anaerobic bacteria that require reducing conditions, the exception being the organohalide-respiring *Anaeromyxobacter dehalogenans* strains, which are facultative anaerobes (Cole et al. 1994; Sanford et al. 2002).

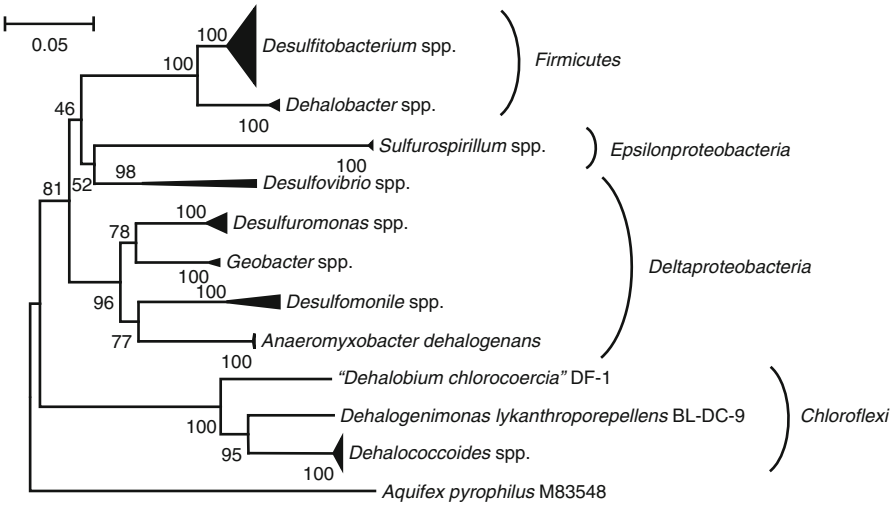


Fig. 4.2 Phylogenetic tree of organohalide-respiring bacteria. The neighbor joining (NJ) method was used to construct a phylogenetic tree of the 16S rRNA gene sequences obtained from the Hierarchy Browser of the Ribosomal Database Project (http://rdp.cme.msu.edu/). The 16S rRNA gene sequence of *Aquifex pyrophilus* was used as the out group. The *scale bar* represents 0.02 substitutions per nucleotide sequence

With respect to their auxotrophic character, the OHRB can be classified roughly into two types: (1) obligate OHRB and (2) OHRB capable of utilizing a range of electron acceptors. The former are able to grow only by organohalide respiration, whereas the latter can grow using electron acceptors other than organohalides. All of the isolates from the phylum *Chloroflexi*, including the genera *Dehalococcoides*, "*Dehalobium*," and *Dehalogenimonas*, are obligate OHRB (Fig. 4.2). In addition, isolates from the genus *Dehalobacter* are also obligate OHRB, but they are classified into the phylum *Firmicutes* and the genus *Desulfitobacterium*, members of which are able to utilize a range of electron acceptors, including fumarate, thiosulfate, sulfite, nitrate, nitrite, dimethyl sulfoxide (DMSO), sulfonate, trimethylamine *N*-oxide, As(V), Mn(IV), Fe(III), U(VI), Se(VI), and anthraquinone-2,6-disulfonate (a humic acid analog) (reviewed in Villemur et al. 2006). Non-dechlorinating *Desulfitobacterium* spp. strains have also been identified (van de Pas et al. 2001).

4.3 The Genus *Dehalococcoides*: A Key Bacterial Group for DCE- and VC-Reductive Dechlorination

The genus *Dehalococcoides* is an important member of the OHRB because these bacteria are able to completely detoxify chlorinated ethenes by converting them to ethene. In this process, PCE is successively converted to TCE, dichloroethenes [*cis-1*,2-dichloroethene (*cis*-DCE) or *trans*-1,2-dichloroethene (*trans*-DCE)],

vinyl chloride (VC), and nontoxic ethene through a process of hydrogenolytic dehalogenation (Fig. 4.1a).

A number of OHRB genera are capable of respiring with PCE and TCE, including *Dehalobacter*, "*Dehalobium*," *Dehalococcoides*, *Desulfitobacterium*, *Desulfuromonas*, *Geobacter*, and *Sulfurospirillum*. In contrast, DCE and VC respirers have thus far been found solely in the genus *Dehalococcoides* (reviewed in Smidt and de Vos 2004; Löffler and Edwards 2006; Hiraishi 2008; Tiehm and Schmidt 2011). Because VC is a well-known carcinogen and causes liver cancer (Kielhorn et al. 2000), the accumulation of VC during the degradation process is of serious concern. The dechlorination of PCE by OHRB is also important because oxygenative degradation of PCE is generally difficult and thus rarely reported. Some examples of aerobic degradation of PCE are available, such as that mediated by toluene-*o*-xylene monooxygenase of *Pseudomonas stutzeri* OX1 and the cytochrome P450 system of the white-rod fungus *Trametes versicolor* (Ryoo et al. 2000; Marco-Urrea et al. 2006, 2009).

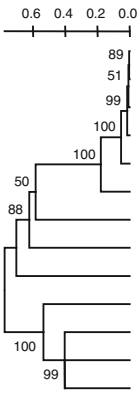
The first member of the genus *Dehalococcoides* to be isolated was *Dehalococcoides mccartyi* 195 (formerly "*Dehalococcoides ethenogenes*" 195), which was described in 1997 (Maymó-Gatell et al. 1997; Löffler et al. 2012). *D. mccartyi* strains CBDB1, FL2, BAV1, VS, GT, and MB have been isolated since that time. Strains 195 and FL2 dechlorinate PCE and TCE, respectively, to ethene (Maymó-Gatell et al. 1997; Löffler et al. 2000; He et al. 2005). However, these strains are unable to use VC as a growth-supporting electron acceptor, and the dechlorination of VC to ethene is thus a cometabolic process (Maymó-Gatell et al. 1999). In contrast, three other *D. mccartyi* strains, BAV1, VS, and GT, can use VC as an electron acceptor and thereby efficiently dechlorinate VC to ethene (He et al. 2003; Cupples et al. 2003; Müller et al. 2004; Sung et al. 2006). The strains CBDB1 and MB predominantly dechlorinate PCE to *trans*-DCE (Cheng and He 2009; Marco-Urrea et al. 2011). Strain CBDB1 was isolated based upon its ability to respire with chlorobenzenes and dioxins, such as 1,2,3-trichlorobenzene, 1,2,4-trichlorobenzene, 2,3-dichloro-*p*-dibenzodioxin, and 2,3,7,8-tetrachloro-p-dibenzodioxin (Adrian et al. 2000; Bunge et al. 2003).

Many molecular ecological studies have shown that the *Dehalococcoides*-like *Chloroflexi* and their close relatives inhabit a wide range of anaerobic terrestrial and marine ecosystems. The "*Dehalobium*" and *Dehalogenimonas* belong to the class "*Dehalococcoidetes*" (subphylum II) of the phylum *Chloroflexi*, as does *Dehalococcoides* (Hugenholtz and Stackebrandt 2004; Yamada et al. 2006) (Fig. 4.2). "*Dehalobium chlorocoercia*" DF-1 predominantly dechlorinates PCE and TCE to *trans*-DCE rather than to *cis*-DCE (Miller et al. 2005). Strain DF-1 was isolated based upon its ability to dechlorinate chlorobenzenes as well as polychlorinated biphenyls (PCBs). For example, cultures containing DF-1 are able to dechlorinate hexachlorobenzene, pentachlorobenzene, 1,2,3,5-tetrachlorobenzene, and 1,3,5-trichloro benzene (Wu et al. 2002; May et al. 2008). On the other hand, *Dehalogenimonas lykanthroporepellens* strains BL-DC-8 and BL-DC-9 dechlorinate polychlorinated aliphatic alkanes, including 1,2,3-trichloropropane, 1,2-dichloropropane, 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane, and 1,2-dichloroethane (Moe et al. 2009; Yan et al. 2009).

4.4 The Substrate Specificity of Reductive Dehalogenase

Reductive dehalogenase is a key enzyme in the organohalide respiratory chain, acting as a terminal reductase to catalyze the dehalogenation reaction. RDases from a number of genera, including *Desulfitobacterium*, *Dehalobacter*, *Dehalococcoides*, and *Sulfurospirillum*, have been purified and functionally characterized (Fig. 4.3a). Importantly, the substrate spectrum of each OHRB is not dependent on the bacterial strain but rather is more likely dependent upon the type of RDase. Studies of RDases

a



Protein name	Host organism	Enzymatic reaction	Accession number	Reference
PceA	*Desulfitobacterium hafniense* Y51	PCE→TCE→*cis*-DCE	AAW80323	Suyama et al. 2002
PceA	*Desulfitobacterium hafniense* TCE1	PCE→TCE→*cis*-DCE	CAD28792	Maillard et al. 2003
PceA	*Dehalobacter restrictus*PER-K23	PCE→TCE→*cis*-DCE	CAD28790	Maillard et al. 2003
PceA	*Desulfitobacterium hafniense* PCE-S	PCE→TCE→*cis*-DCE	AAO60101	Miller et al. 1997, Maillard et al. 2003
DcaA	*Desulfitobacterium dichloroeliminans* DCA1	PCE→*cis*-DCE	CAJ75430	Marzorati et al. 2007
CprA5	*Desulfitobacterium hafniense* PCP-1	3,5-dichlorophenol→3-CP	AAQ54585	Thibodeau et al. 2004
PrdA	*Desulfitobacterium* sp. KBC1	PCE→TCE	BAE45338	Tsukagoshi et al. 2006
PceA	*Sulfurospirillum multivorans*	PCE→TCE→*cis*-DCE	AAC60788	Neumann et al. 1998
CprA	*Desulfitobacterium dehalogenans*JW/IU-DC1	3Cl4OHPA→4-OHPA	AAD44542	Van de Pas et al. 1999
CbrA	*Dehalococcoides mccartyi* CBDB1	1,2,3,4-TeCB→1,2,4-TCB	CAI82345	Adrian et al. 2007
TceA	*Dehalococcoides mccartyi* 195	TCE→*cis*-DCE→VC→ETH	AAW39060	Magnuson et al. 2000
BvcA	*Dehalococcoides mccartyi* BAV1	*cis*-DCE→VC→ETH	AAT64888	Krajmalnik-Brown et al. 2004
VcrA	*Dehalococcoides mccartyi* VS	*cis*-DCE→VC→ETH	AAQ94119	Müller et al. 2004

b

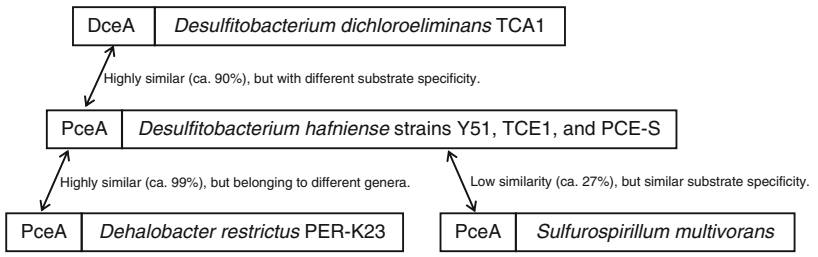


Fig. 4.3 Phylogenetic comparison of amino acid sequences of the functionally characterized reductive dehalogenases (**a**). The NJ method was used to build the phylogenetic tree. The substrates shown in the "Enzymatic reaction" column that utilize chloroethenes were confirmed not to dechlorinate the other chloroethenes. However, it should be noted that the catalytic reactions shown here are representative examples, e.g., the PceA RDases from *Sulfurospirillum multivorans* and *Desulfitobacterium hafniense* PCE-S also debrominate brominated ethenes (Ye et al. 2010). In addition, CprA and CbrA are known as *ortho*-chlorophenol dehalogenase and chlorobenzene dehalogenase, respectively. *PCE* tetrachloroethene, *TCE* trichloroethene, *DCE* dichloroethene, *VC* vinyl chloride, *ETH* ethene, *CP* chlorophenol, *3Cl4OHPA* 3-chloro-4-hydroxyphenylacetate, *4-OHPA* 4-hydroxyphenylacetate, *TeCB* tetrachlorobenzene, *TCB* trichlorobenzene. Schematic relationships among the RDases from *Desulfitobacterium*, *Dehalobacter*, and *Sulfurospirillum* (**b**). Three relationships were found: highly similar (ca. 90 %), but with different substrate specificity; highly similar (ca. 99 %), but belonging to different genera; low similarity (ca. 27 %), but similar substrate specificity

isolated from *Desulfitobacterium*, *Dehalobacter*, and *Sulfurospirillum* illustrate the relationship between the RDase and OHRB (Fig. 4.3b).

The genus *Desulfitobacterium* is ubiquitous at contaminated sites (reviewed in Villemur et al. 2006). Most of the isolates are capable of reductively dechlorinating chloroethenes and/or chlorophenols, and the corresponding RDase enzymes have been identified and characterized. *Desulfitobacterium hafniense* strains Y51, TCE1, PCE-S, and JH1 dechlorinate PCE to *cis*-DCE via TCE, but do not dechlorinate chlorophenols (Suyama et al. 2001; Miller et al. 1997; Gerritse et al. 1999; Fletcher et al. 2008). These strains produce PCE/TCE RDases (PceA) that are nearly 99 % similar based on amino acid sequences (Fig. 4.3a). In contrast, *Desulfitobacterium dehalogenans* IW/IU-DC1 and *D. hafniense* DCB-2 dechlorinate chlorophenols but not chloroethenes (Madsen and Licht 1992; Utkin et al. 1994; Christiansen and Ahring 1996). Strains IW/IU-DC1 and DCB-2 produce an *ortho*-chlorophenol RDase (CprA) (Fig. 4.3a).

Dehalobacter restrictus strain PER-K23 dechlorinates PCE to *cis*-DCE via TCE in a reaction mediated by a PceA enzyme that is highly similar (99 %) to the enzyme produced by *Desulfitobacterium* (Maillard et al. 2003) (Fig. 4.3a, b). As is the case with *Desulfitobacterium*, *Dehalobacter* is classified in the phylum *Firmicutes*, but *Dehalobacter* is phylogenetically distant from the genus *Desulfitobacterium* (Fig. 4.1), indicating that horizontal gene transfer might have occurred between species of these two genera (see Sect. 4.8).

Sulfurospirillum multivorans also dechlorinates PCE to *cis*-DCE via TCE using PceA (Fig. 4.3a, b) (Scholz-Muramatsu et al. 1995). However, the PceA produced by *Sulfurospirillum multivorans* shows only 27 % sequence identity to the PceA enzymes produced by *Desulfitobacterium* and *Dehalobacter* (Neumann et al. 1996, 1998). In contrast, DcaA, which is produced by *Desulfitobacterium dichloroeliminans* DCA1 and was identified from a 1,2-dichloroethane (1,2-DCA)-contaminated enrichment culture, shows a higher similarity (90 %) to the PceA enzymes from *Desulfitobacterium* and *Dehalobacter* than to the PceA from *S. multivorans* (Marzorati et al. 2007). The DcaA enzyme catalyzes a different reaction, however, dechlorinating 1,2-DCA to *cis*-DCE through a reductive dehalogenation reaction called dichloroelimination that simultaneously removes the adjacent chlorine atoms (De Wildeman et al. 2003; Marzorati et al. 2007) (Fig. 4.1b). An analysis of evolution rates using the method of Nei and Gojobori (Nei and Gojobori 1986) indicated that DcaA evolved due to positive selection. Both the enrichment culture and *D. dichloroeliminans* DCA1 were isolated from a site that had been contaminated with 1,2-DCA for more than 30 years (De Wildeman et al. 2003; Marzorati et al. 2006, 2007).

The functions of four *Dehalococcoides* spp. RDases (TceA, VcrA, BvcA, and CbrA) have been characterized to date (Fig. 4.3a). TceA catalyzes the dechlorination of TCE to ethene (Magnuson et al. 1998). The VcrA and BvcA dehalogenases isolated from strains VS and BAV1, respectively, catalyze the dechlorination of *cis-DCE* to ethene via VC (Müller et al. 2004; Krajmalnik-Brown et al. 2004). Alternatively, CbrA, identified from strain CBDB1, catalyzes the reductive dechlorination of chlorobenzenes such as 1,2,3-trichlorobenzene (1,2,3-TCB) (Adrian et al. 2007).

Most of the OHRB RDases thus far characterized reductively dechlorinate chlorinated aliphatic or aromatic compounds, such as chloroethenes, chlorophenols, and chlorobenzenes. The fact that the known substrates for these enzymes consist primarily of chlorinated organic compounds may be due to study bias. It should be noted that OHRB are also able to utilize brominated and iodinated compounds. The PceA enzymes were first identified as PCE- and TCE-reductive dehalogenases. However, further study revealed that the PceA enzymes from *S. multivorans* and *D. hafniense* PCE-S also catalyze the reductive debromination of brominated ethenes (Ye et al. 2010).

Because the type of RDase has a significant influence on the dehalogenation capability of OHRB, detecting the specific RDase genes rather than the 16S rRNA gene is necessary for assessing and monitoring bioremediation potential in a given environment. Detection of both RDase and 16S rRNA genes of OHRB using techniques such as real-time PCR is now well established (reviewed in Cupples 2008). Active *Dehalococcoides* populations can be detected also through fluorescent in situ hybridization (FISH) and catalyzed reporter deposition (CARD)-FISH of 16S rRNA (Aulenta et al. 2004; Dijk et al. 2008; Fazi et al. 2008). Recent advances now enable researchers to detect and monitor OHRB using various omics technologies as well (reviewed in Maphosa et al. 2010), for monitoring not only the expression of the RDase gene but also the expression of other key genes. For example, DNA-chip technology is no longer limited to the whole-genome microarray analysis of OHRB, but can be used to detect the hydrogenase genes expressed by hydrogen-producing and hydrogen-consuming microbes (Marshall et al. 2012). This is a significant advance since hydrogen is a key electron donor for obligate hydrogenotrophic OHRB such as *Dehalococcoides* and *Dehalobacter*.

4.5 Biochemical Properties of Reductive Dehalogenase

Corrinoid, which is a derivative of vitamin B_{12} (cobalamin), is a cofactor located at the catalytic center of the RDase enzyme. Involvement of the corrinoid cofactor in the dehalogenation reaction was predicted through studies in which corrinoid was reversibly inactivated with propyl iodides (Neumann et al. 1994). The importance of corrinoid to the activity of *S. multivorans* PceA was also confirmed by the discovery that mutants incapable of dechlorinating PCE cannot synthesize corrinoid (Siebert et al. 2002). The corrinoid cofactor of *S. multivorans* PceA was purified and structurally identified as norpseudovitamin B_{12} (Kräutler et al. 2003). Furthermore, the activity of the PceA from *S. multivorans* was found to be enhanced by an estimated 4,800-fold over the nonenzymatic cofactor-dependent reaction (Glod et al. 1997; Neumann et al. 2002; McCauley et al. 2005). The corrinoid cofactor of *Dehalobacter restrictus* PER-K23 PceA has also been isolated, and its properties were found to be the same as those of commercially available cobalamin (Maillard et al. 2003). A cobalamin-binding domain "DXHXXG…SXL…GG," which is found in subsets of cobalamin-dependent methyltransferases and isomerases, has also been found in

seven RDase homologues from *Dehalococcoides* strains and the CbrA enzyme produced by strain CBDB1 has a truncated cobalamin-binding domain; however, in many cases no cobalamin-binding domain has been identified (Ludwig and Matthews 1997; Hölscher et al. 2004; Adrian et al. 2007).

Schemes involving one corrinoid and two Fe-S clusters have been proposed as feasible reaction mechanisms for reductive dechlorination by OHRB. For instance, PCE is reduced through one-electron transfer from the Co(I) corrinoid, producing the trichlorovinyl radical (Neumann et al. 1996; Holliger et al. 1998, 2003; Banerjee and Ragsdale 2003; McCauley et al. 2005; Diekert et al. 2005). All of the functionally characterized RDase sequences contain two highly conserved Fe-S cluster-binding motifs. These two Fe-S clusters are hypothesized to be involved in the redox activation of the corrinoid cofactor. The presence of Fe-S clusters in the PceA produced by *D. restrictus* PER-K23 and in the CprA produced by *D. dehalogenans* IW/IU-DC1 was experimentally confirmed through electron paramagnetic resonance (EPR) analyses, which demonstrated that the former enzyme contains two [4Fe-4S] clusters and that the latter contains one [4Fe-4S] and one [3Fe-4S] cluster (Schumacher et al. 1997; van de Pas et al. 1999; Maillard et al. 2003).

4.6 The Reductive Dehalogenase Gene Cluster

RDase-encoding genes are always organized in an operon with at least one gene encoding a protein containing a 2- or 3-transmembrane domain (Fig. 4.4). Suyama et al. (2002) reported that the mature PceA RDase of *D. hafniense* Y51, from which the N-terminal Tat sequence has been cleaved, is localized in the periplasmic space, while the unprocessed protein with the Tat sequence present is found in the cytoplasmic fraction. Moreover, the authors reported that the RDase did not possess any membrane-associated domain structures. Given these facts, B proteins (e.g., PceB; Fig. 4.5) are believed to act as membrane anchors for RDases. The localization of the PceA of *S. multivorans* was characterized microscopically using the freeze-fracture replica immunogold labeling technique (John et al. 2006). The results of that study indicated that localization of PceA depends upon the electron acceptor, though the mechanism remains unknown. When *S. multivorans* is grown with fumarate as the electron acceptor, PceA localizes in the cytoplasm or associates with the membrane side facing the cytoplasm. In contrast, when the same strain is grown with either PCE or TCE as an electron acceptor, most of the PceA localizes along the periplasmic side of the cytoplasmic membrane.

The constitution and order of the *pceA*, *pceB*, *pceC*, and *pceT* genes is highly conserved among the *Desulfitobacterium* and *Dehalobacter* strains that dechlorinate PCE to *cis*-DCE (Fig. 4.4). The PceC protein has not been functionally characterized, but is similar to the NirI/NosR family of membrane-binding transcriptional regulators that are known to be involved in nitrous oxide respiration (Cuypers et al. 1992) (Fig. 4.5). NosR has also been characterized as an electron-donating protein (Wunsch and Zumft 2005). The PceC protein is composed of a 6-transmembrane

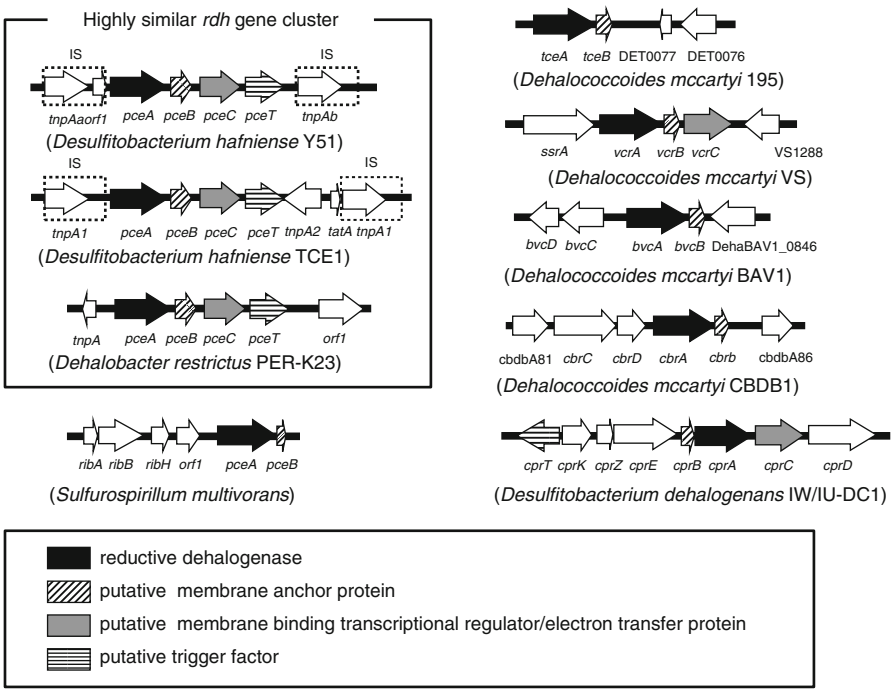
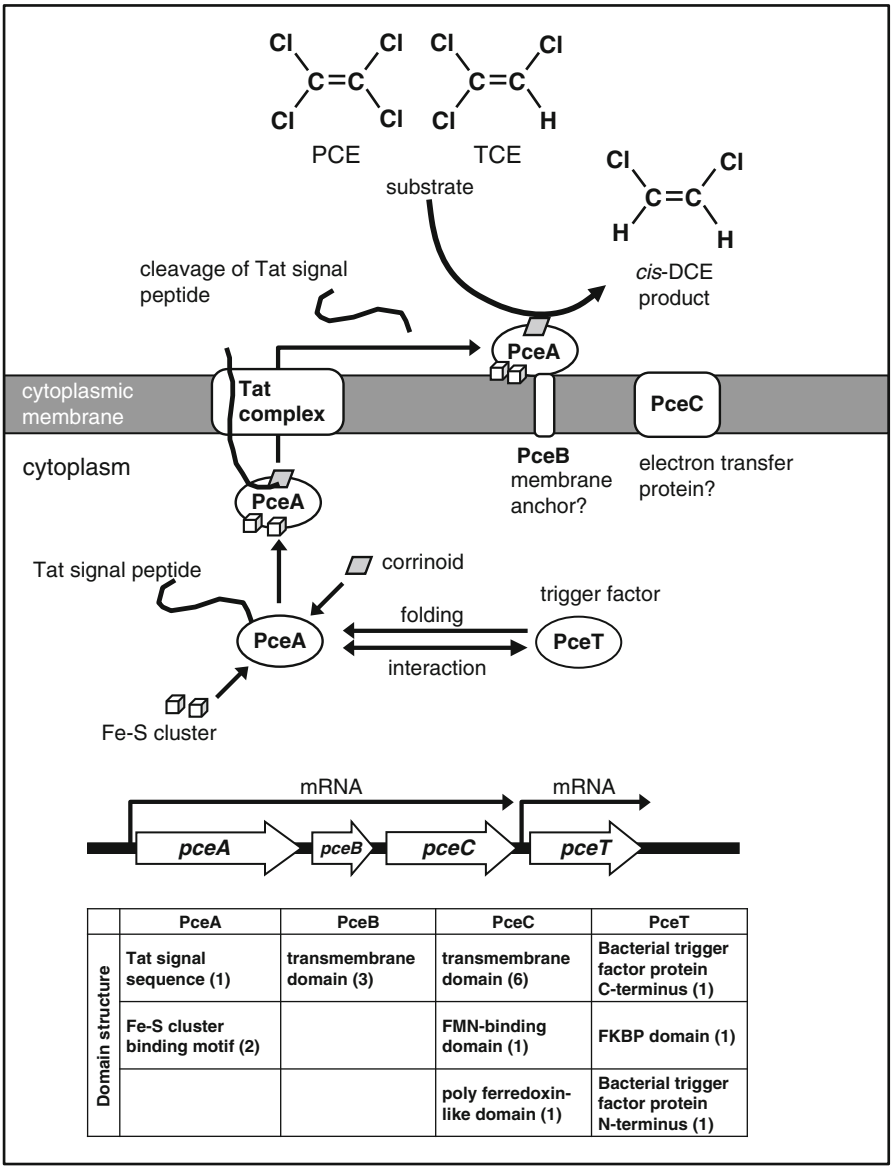


Fig. 4.4 Comparison of reductive dehalogenase gene clusters. An identical pattern indicates similar genes, as described in the *inset*. GenBank accession numbers are as follows. *Desulfitobacterium hafniense* Y51 *pce* genes: AY706985; *Desulfitobacterium hafniense* TCE1 *pce* genes: AJ439608; *Desulfitobacterium hafniense* PCE-S *pce* genes: AY216592; *Dehalobacter restrictus* PER-K23 *pce* genes: AJ439607; *Sulfurospirillum multivorans pce* genes: AF022812; *Dehalococcoides mccartyi* 195 *tce* genes: CP000027; *Dehalococcoides mccartyi* VS *vcr* genes: AY322364; *Dehalococcoides mccartyi* CBDB1 *cbr* genes: AJ965256; *Desulfitobacterium dehalogenans strain* IW/IU-DC1 *cpr* genes: AF115542. The genome information for *Dehalococcoides mccartyi* strains VS, BAV1, and CBDB1 was also obtained from the Joint Genome Institute (http://jgi.doe.gov/)

domain, a flavin mononucleotide (FMN)-binding domain, and a poly ferredoxin-like domain (Fig. 4.5), implying that PceC localizes in the cell membrane in order to donate electrons to PceA. The importance of PceC for organohalide respiration is supported by the fact that the protein is returned as a reciprocal "best hit" in comparisons of the genome sequences of *D. hafniense* Y51 and *D. mccartyi* 195 (Nonaka et al. 2006). In addition, the NirI/NosR-like protein-encoding genes are conserved, as is the *cprC* gene in *D. dehalogenans* IW/IU-DC1 and the *vcrC* gene in *D. mccartyi* VS (Smidt et al. 2000; Müller et al. 2004). Moreover, the *pceABC* genes of strain Y51 and the *vcrABC* genes of strain VS are cotranscribed (Müller et al. 2004; Furukawa et al. 2005; Futagami et al. 2006a, b), indicating that the C proteins might play a role in organohalide respiration.

On the other hand, the *pceT* gene is not cotranscribed in strain Y51 (Furukawa et al. 2005; Futagami et al. 2006b), but might be involved in the maturation of PceA as a trigger factor (Fig. 4.5). The RDase contains a conserved Tat signal sequence in the N-terminal region, indicating that the enzyme is localized across



	PceA	PceB	PceC	PceT
Domain structure	Tat signal sequence (1)	transmembrane domain (3)	transmembrane domain (6)	Bacterial trigger factor protein C-terminus (1)
	Fe-S cluster binding motif (2)		FMN-binding domain (1)	FKBP domain (1)
			poly ferredoxin-like domain (1)	Bacterial trigger factor protein N-terminus (1)

Fig. 4.5 Schematic representation of the maturation and localization of PCE-/TCE-reductive dehalogenase (PceA) in *Desulfitobacterium hafniense* Y51. The domain constitution of each protein is summarized in the *inset*. The number of each domain is shown in *parentheses*. *Tat* twin-arginine translocation system, *PCE* tetrachloroethene, *TCE* trichloroethene, *cis-DCE cis*-1,2-dichloroethene

the plasma membrane. Due to this Tat system, PceA should localize correctly only when the PceA precursor has been previously folded properly with its cofactors (reviewed in Palmer and Berks 2012). Thus, PceT may contribute to the correct folding of the PceA precursor protein during Tat-mediated secretion.

Recombinant PceT has peptidyl-prolyl *cis–trans* isomerase and chaperone activity, and co-immunoprecipitation assay results showed that PceT interacts with the Tat signal sequence of PceA in strain Y51, indicating that PceT helps mediate correct folding of the precursor PceA (Morita et al. 2009). The PceT enzyme from *D. hafniense* TCE1 has also been characterized and was shown to efficiently aid in the solubilization of PceA during heterologous expression using an *Escherichia coli* strain lacking both the trigger factor and DnaK chaperones (Maillard et al. 2011). These results confirm that PceT is involved in mediating the correct folding of the precursor of PceA.

The larger components of the RDase gene cluster have been identified in chlorophenol-respiring *Desulfitobacterium* strains. For example, *D. dehalogenans* IW/IU-DC1 possesses an *ortho*-chlorophenol RDase gene cluster containing eight genes: *cprT*, *cprK*, *cprZ*, *cprE*, *cprB*, *cprA*, *cprC*, and *cprD* (Fig. 4.4) (Smidt et al. 2000). CprK is a CRP-FNR (cAMP-binding protein/fumarate nitrate reduction regulatory protein) family transcriptional regulator, whereas CprD and CprE are putative GroEL-type molecular chaperones.

4.7 Regulation of Reductive Dehalogenase Gene Expression

Transcription of the *ortho*-chlorophenol RDase-encoding *cprA* gene in *Desulfitobacterium* is regulated by CprK, which is similar to CRP-FNR family proteins. The *cpr* gene cluster responds at the transcription level to the presence of chlorophenols (Smidt et al. 2000; Gábor et al. 2008; Bisaillon et al. 2011). Both in vivo and in vitro studies have revealed that high-affinity interaction between chlorinated aromatic compounds and a CprK effector domain triggers binding of CprK to an upstream target DNA sequence called a dehalobox "TTAAT-N4-ATTAA," which closely resembles the FNR box (Pop et al. 2004, 2006; Gábor et al. 2006, 2008; Joyce et al. 2006; Mazon et al. 2007). Joyce et al. (2006) determined X-ray crystal structures of the oxidized form of *D. hafniense* DCB-2 CprK bound to a 3-chloro-4-hydroxyphenylacetate ligand and of the reduced form of *D. dehalogenans* IW/IU-DC1 CprK (both proteins are 89 % identical) without the ligand, thus enabling identification of the allosteric changes induced by ligand binding.

Long-term regulation of the *pceA* gene in *S. multivorans* has also been demonstrated (John et al. 2009). The authors of that study reported that transcription of *pceA* decreases during subcultivation. After 35 subcultivations (approximately 105 generations), no *pceA* transcripts, PceA protein, or PceA activity could be detected. Biosynthesis of catalytically active PceA could be restored to a level before the subcultivation within about 50 h (approximately three generations) by the addition of PCE or TCE to the culture medium. These results indicated that a novel type of long-term regulation of *pceA* gene expression exists in *S. multivorans*.

Expression of the *Dehalococcoides* RDase genes *bvcA* and *cbrA* has been detected in cells cultivated in the presence of VC and 1,2,3-TCB, respectively (Krajmalnik-Brown et al. 2004; Adrian et al. 2007). These genes are located near by the putative

transcriptional regulator genes. Within the *bvc* and *cbr* gene clusters of *D. mccartyi* strains BAV1 and CBDB1, the *bvcC-bvcD* and *cbrC-cbrD* genes encode a putative two-component system consisting of a sensor signal transduction histidine kinase and a response regulator (Fig. 4.4) (Adrian et al. 2007; McMurdie et al. 2009).

Expression of RDase gene is not always regulated, however (e.g., the *pceA* gene of *D. hafniense* Y51 is constitutively transcribed in cultures grown in media containing various electron acceptors such as fumarate, TCE, nitrate, or DMSO) (Peng et al. 2012). The level of PceA expression in populations of *D. hafniense* Y51 is affected by the emergence of non-dechlorinating variants that have lost the *pceABCT* genes or *pceABC* promoter region through genetic rearrangements (Futagami et al. 2006a, b) (see Sect. 4.8).

4.8 Genetic Rearrangement of the Reductive Dehalogenase Gene Cluster

Degradative genes are frequently found on mobile DNA elements (reviewed in van der Meer et al. 1992; Tsuda et al. 1999; Liang et al. 2012). The RDase gene cluster is no exception, and rearrangements involving mobile genetic elements, such as gene duplication, the formation of chimeric genes, and gene transfer, are believed to have played a role in the evolution of organohalide respiration.

Comparative sequence analyses have revealed vestiges of chromosomal rearrangement. The *pceABCT* gene cluster of *D. hafniense* strains Y51 and TCE1 is surrounded by two nearly identical copies of an insertion sequence (IS) element that include a gene encoding the IS*256* type transposase (Fig. 4.4). The *pceABCT* genes of strain TCE1 share a 99.7 % identity with those of strain Y51. The direct repeat sequences "CTGAACCA" and "TTTTTATA" are found just upstream of the first IS and downstream of the second IS in strains Y51 and TCE1, respectively (Maillard et al. 2005; Futagami et al. 2006a). Thus, the *pceABCT* gene cluster seems to be inserted into the chromosome as a composite transposon in these two strains. In addition, a circular molecule carrying an entire *pceABCT* gene cluster and two terminal IS copies has been described, indicating that the catabolic transposon can still function and be excised from the chromosome. The *pceABCT* gene cluster, including the intergenic regions, is highly conserved among *Desulfitobacterium* and *Dehalobacter* sp., suggesting horizontal transfer between these genera. The recent acquisition of the *pceABCT* gene cluster was supported by proteomic analyses of *D. hafniense* TCE1, the results of which revealed that the expression of proteins involved in stress responses and associated regulation pathways increases in the presence of PCE, suggesting that strain TCE1 is still incompletely adapted to PCE respiration and that this strain is thus not fully suited to PCE respiration (Prat et al. 2011).

During subculturing, strain Y51 was also found to spontaneously give rise to two types of non-PCE dechlorinating variants (Futagami et al. 2006a). One variant was generated from deletion of the left IS, which contains a promoter region of the *pceABC* gene

Table 4.1 Comparison of the genomes of organohalide dehalorespiring bacteria

Name	Size (Mbp)	G+C (%)	rRNA operon	Predicted CDS	RDase gene	Plasmid
Desulfitobacterium hafniense Y51	5.7	47	6	5,060	2	0
Desulfitobacterium hafniense DCB-2	5.3	48	5	5,042	7	0
Anaeromyxobacter dehalogenans 2CP-C	5.0	75	6	4,346	2	0
Geobacter lovleyi SZ	3.9	55	2	3,476	2	1
Dehalococcoides mccartyi 195	1.5	49	1	1,590	17	0
Dehalococcoides mccartyi CBDB1	1.4	47	1	1,385	32	0
Dehalococcoides mccartyi BAV1	1.3	47	1	1,371	11	0
Dehalococcoides mccartyi VS	1.4	47	1	2,096	36	0
Dehalogenimonas lykanthroporepellens BL-DC-9	1.7	55	1	1,720	17	0

The data were summarized from Kube et al. (2005); Seshadri et al. (2005); Nonaka et al. (2006); Thomas et al. (2008); McMurdie et al. (2009); Kim et al. (2012), and Wagner et al. (2012). *Dehalogenimonas lykanthroporepellens* BL-DC-9 sequence information was obtained from the Joint Genome Institute (http://www.jgi.doe.gov/)

cluster (Fig. 4.4). Transcription of the *pceABC* genes was thus abolished in this variant, and accordingly the PCE-dechlorination capability. The other variant arose from homologous recombination between the left IS and right IS, resulting in excision of the entire *pceABCT* gene cluster. Thus, in the absence of chloroethenes, several modes of genetic rearrangement occur around the *pceABCT* gene cluster in strain Y51.

The PCE non-dechlorinating variants of strain Y51 predominate in the presence of chloroform (CF) because CF significantly inhibits the growth of wild type strain Y51 but not the non-dechlorinating variants (Futagami et al. 2006b). Moreover, CF-mediated inhibition of dechlorination by *Sulfurospirillum* and *Dehalococcoides* has also been reported (Neumann et al. 1996; Maymó-Gatell et al. 2001; Duhamel et al. 2002). On the other hand, studies have shown that CF can act as an electron acceptor. Growth-linked dechlorination of CF to carbon dichloride (CD) was observed in the enrichment culture containing *Dehalobacter* (Grostern et al. 2010). Dechlorination of CF to CD and fermentation of CD to acetate, hydrogen, and carbon dioxide has also been observed in *Dehalobacter*-dominated cultures (Lee et al. 2012).

Large numbers of putative RDase genes have been identified in the genome sequences of OHRB (Table 4.1). From genomic information of *Dehalococcoides* strains, recombination appears to have taken place between two RDase genes (DhcVS1399 and DhcVS1427) of *D. mccartyi* strain VS, resulting in formation of the apparently chimeric gene, DET1535 of strain 195 (McMurdie et al. 2009). Analyses of codon usage in the *vcrA* and *bvcA* genes of *D. mccartyi* strains VS and BAV1, respectively, showed that these genes are highly unusual and are characterized by a low G+C content at the third position (McMurdie et al. 2007). The comparatively high degree of abnormal codon usage in the *vcrA* and *bvcA* genes suggests that the evolutionary history of these genes is quite different than that of most other *Dehalococcoides* genes. These data also suggest that mobile elements played an important role in the arrangement and consequently the evolution of the RDase genes in *Dehalococcoides*.

The *tceAB* gene cluster of *D. mccartyi* 195 is located in a putative integrated element (Seshadri et al. 2005). The gene DET0076, which is located downstream of the *tceAB* genes, encodes a protein that is highly similar to resolvase (Fig. 4.4). The *vcrABC* genes of *D. mccartyi* VS are also embedded in a horizontally acquired genomic island (McMurdie et al. 2011). The *ssrA* gene encodes a site-specific recombinase (Müller et al. 2004) (Fig. 4.4). The *vcrABC*-containing genomic islands obtained from *Dehalococcoides* enrichment cultures have been sequenced. Using available *Dehalococcoides* phylogenomic data, it can be estimated that these *ssrA*-specific genomic islands are at least as old as the *Dehalococcoides* group itself, which in turn far predates human civilization, indicating that it took place before emergence of anthropogenic chemicals. The *vcrABC*-containing genomic islands represent a recently acquired subset of a diverse collection of *ssrA*-specific mobile elements that are a major contributor to strain-level diversity in *Dehalococcoides*, and may have been throughout its evolution. The high degree of similarity between *vcrABC* sequences is quantitatively consistent with recent horizontal acquisition driven by ~100 years of industrial pollution with chloroethenes (McMurdie et al. 2011). Moreover, transcriptional analysis of a *Dehalococcoides-containing* microbial consortium uncovered evidence of prophage activation (Waller et al. 2012).

D. mccartyi FL2 was isolated from a pristine environment, suggesting that OHRB might be able to survive on naturally occurring organohalides (Löffler et al. 2000). In fact, more than 4,500 different natural organohalides have been identified in samples from biotic and abiotic sources (Gribble 2003, 2010). RDase homologous genes were detected in marine subsurface environment using a degenerate primer set designed based on *Dehalococcoides* RDase genes (Krajmalnik-Brown et al. 2004; Futagami et al. 2009). The deepest sediment in which RDase homologous genes were detected was formed ca. 460,000 years ago, suggesting that OHRB existed before industrial activity (Aoike 2007; Futagami et al. 2009). Molecular ecological studies have shown that the density of *Dehalococcoides*-like *Chloroflexi* in terrestrial pristine environments is proportional to the quantity of natural organochlorines, suggesting that these bacteria play a significant role in natural halogen cycles (Krzmarzick et al. 2012).

4.9 Conclusions and Future Perspectives

Organohalide respiration has received considerable attention because of its important role in the remediation of environments polluted with chlorinated organic chemicals. Investigations of the physiology of OHRB and the key RDase enzymes have provided crucial background information for the establishment of OHRB bioremediation technologies. An increasing number of studies have also begun to focus on the evolutionary history of organohalide respiration. Genetic events such as mutation and the gene transfer, as well as the selective force imparted by anthropogenic organohalides released into the environment, likely have played a significant role in the evolution of microbial organohalide respiration.

Recent progress in genomic technologies has enabled researchers to undertake experiments using global approaches in order to move toward a more comprehensive understanding of organohalide respiration. Currently, the complete genome sequences of the genera *Anaeromyxobacter*, *Desulfitobacterium*, *Dehalococcoides*, *Dehalogenimonas*, and *Geobacter* are available (Table 4.1). However, more in-depth studies of OHRB (e.g., determination of the substrates of the large number of uncharacterized RDase homologues) require the establishment and application of genetic engineering tools tailored to the study of organohalide respiration. Several such tools, such as gene recombination using thermosensitive plasmids, gene disruption using transposon, and in vitro expression of active RDases, have been described (Smidt et al. 1999, 2001; Kimoto et al. 2010).

For those interested in obtaining a deeper understanding of organohalide respiration, we strongly recommend several additional resources, including the excellent volume edited by Häggblom and Bossert (2003), and a number of recent review articles (Holliger et al. 1998; Smidt and de Vos 2004; Villemur et al. 2006; Löffler and Edwards 2008; Hiraishi 2008; Futagami et al. 2008; Maphosa et al. 2010; Tiehm and Schmidt 2011).

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