Chapter 6

*Dehalobacter* – a genus of anaerobic bacteria dedicated to organohalide degradation

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Abstract

The genus *Dehalobacter* embraces bacterial populations that seem to exclusively degrade organohalides. All isolates in pure culture and highly enriched strains are obligate organohalide-respiring bacteria that use hydrogen as energy and electron source, acetate as carbon source, and an organohalide as terminal electron acceptor. Depending on the strain, they are restricted to the use of only one or two organohalides from the same chemical group (i.e. aliphatic or aromatic organohalides), a few strains however can use several compounds and from different groups. Organohalides used by *Dehalobacter* are chlorinated methanes, ethanes, ethenes, cyclohexanes, benzenes, phenols, and phthalides. However, two enrichments dominated by *Dehalobacter* spp. indicate another metabolic pathway with a specific organohalide, namely fermentation of dichloromethane. No particular habitat can be defined for this bacterial genus since the different strains have been enriched and isolated from various matrices such as sediments, aquifers, and anaerobic sludge from waste treatment processes. The small motile rods (0.5 μm in diameter, 2-3 μm long) usually stain Gram-negative, contain, however, peptidoglycan features of Gram-positives, menaquinones, and cytochrome *b*, and are surrounded by proteinaceous S-layer. Phylogenetically *Dehalobacter* is affiliated to low GC Gram-positive *Firmicutes*. Recently available genome sequences revealed that *Dehalobacter* spp. harbour an unexpected large number of putative reductive dehalogenase genes (10-27 paralogs) showing a relatively high diversity, several hydrogenases of different types, an 11-subunit respiration complex I, all necessary genes for the Wood-Ljungdahl pathway and the biosynthesis pathway of corrinoids, and seemed to confirm that *Dehalobacter* spp. cannot carry out any other respiration process than organohalide respiration. Hence, the hydrogen and carbon metabolisms seem to be more complex than anticipated, and also the observed restriction to few organohalides as electron acceptor is perhaps not reflecting the real dechlorination capabilities of *Dehalobacter* strains with the numerous putative reductive dehalogenase genes in their genomes.

6.1. Enrichment and Isolation of *Dehalobacter* spp.

The first *Dehalobacter* population has been enriched from a packed-bed column that was filled with a mixture of river Rhine sediment sampled near Wageningen, The Netherlands, and with ground anaerobic granular sludge from a wastewater treatment plant of a sugar refinery ([Holliger et al. 1993](#_ENREF_12)). The column was operated under anaerobic conditions and percolated with a mineral medium containing lactate and tetrachloroethene (PCE). PCE was completely reduced to ethane with trichloroethene (TCE), *cis*-1,2-dichloroethene (cDCE), vinyl chloride (VC) and ethene as intermediates ([De Bruin et al. 1992](#_ENREF_3)). Initial enrichments in batch cultures containing a mineral medium low in chloride amended with lactate or H2-CO2 formed all PCE dechlorination products found in the column effluent, but not ethane. To provide high amounts of the electron acceptor PCE at non-toxic concentration in the water phase, a two-phase system has been used where PCE has been dissolved in hexadecane ([Holliger et al. 1993](#_ENREF_12)). These enrichments were transferred on H2-CO2 only in the presence of 2-bromoethanesulfonate (BES) in order to inhibit methanogens. Adding fermented yeast extract as source of potentially needed growth factors, omitting selenium and tungsten in the trace element solution, and using dilution series to extinction led to the loss of the homoacetogenic activity. Finally, after further dilution series to extinction, a microscopically homogeneous culture of thin rods staining gram-negatively was obtained. Due to a high sensitivity of this bacterium to temperatures above 35°C, cultivation in solidified medium was difficult but finally succeeded. Single colonies have been transferred to liquid medium which allowed isolating a pure culture and better characterizing this bacterium respiring with PCE while using hydrogen as electron donor. The organism was named *Dehalobacter restrictus* strain PER-K23 and will in the following be simply referred to as *Dehalobacter restrictus* ([Holliger et al. 1998](#_ENREF_11)).

A very close relative of *D. restrictus* has been enriched from a laboratory fixed-bed reactor that was inoculated with material from a full-scale anaerobic charcoal reactor treating groundwater contaminated with dichloromethane (DCM) and traces of TCE and PCE ([Wild et al. 1996](#_ENREF_51)). Several dilution series on medium with TCE as electron acceptor resulted in a culture containing two morphologically distinct bacteria, a small rod and a motile vibrio. The latter could be subsequently eliminated by cultivating this enrichment in the presence of sodium molybdate, a selective inhibitor of sulfate-reducing bacteria. Three additional dilution series to extinction resulted in a pure culture of the small rod named strain TEA. The sequence of the 16S rRNA gene cloned was for 99.7% identical with the sequence of *Dehalobacter restrictus*.

Only four additional *Dehalobacter* strains have since then been obtained in pure culture. Strain TCA1 has been isolated from upper Hudson River sediment enrichments fed with lactate or H2-acetate and with 1,1,1-trichloroethane (1,1,1-TCA) as electron acceptor, and incubated at 25°C ([Sun et al. 2002](#_ENREF_43)). With dilution series in deep agarose shake cultures containing low-melting agarose white faint colonies have been obtained within three weeks in tubes where dechlorination activity was observed. *Dehalobacter* sp. strain TCP1 has been enriched and isolated from cultures dechlorinating 2,4,6-trichlorophenol and inoculated with digester sludge from an industrial wastewater treatment plant in Singapore by using serial dilutions and agar shakes ([Wang et al. 2014](#_ENREF_50)). Finally, two strains have been obtained in pure culture using dichlorobenzenes as electron acceptor, strain 12DCB1 dechlorinates 1,2-dichlorobenzene only, whereas strain 13DCB1 can use 1,2- and 1,3-dichlorobenzene ([Nelson et al. 2014](#_ENREF_32)). Although a detailed description of the isolation of these strains has not yet been published, the enrichment procedure involved the use of BES or mevinolin to inhibit methanogenic activity, and butyrate as substrate to select for organisms with a high affinity for hydrogen ([Nelson et al. 2011](#_ENREF_31)). In addition, the two-phase system with hexadecane was used to maximize the amount of dichlorobenzene added and the amendment of additional organics has been minimized by adding 20 mg/L casamino acids.

Two cultures containing a dechlorinating *Dehalobacter* population have been described as rather defined co-cultures harboring another non-dechlorinating bacterium which has not been possible to eliminate. A co-culture of a *Dehalobacter* and a *Sedimentibacter* population that dechlorinated β-hexachlorocyclohexane (-HCH) to monochlorobenzene and benzene, has been obtained from a contaminated sandy soil ([van Doesburg et al. 2005](#_ENREF_46)). Use of BES allowed eliminating methanogenic activity after seven transfers and obtaining a culture of four morphologically distinct microorganisms. They were separated on a Percoll gradient resulting in a pure culture of a *Sedimentibacter* strain. The Percoll gradient band containing predominantly the short rod of *Dehalobacter* resulted always in a culture of two bacteria when transferred in liquid medium with H2 and β-HCH. However, specific metagenomic approaches allowed obtaining the genome sequence of *Dehalobacter* sp.strain E1 from this co-culture ([Maphosa et al. 2012](#_ENREF_28)).

A defined co-culture of a *Dehalobacter* and a *Acetobacterium* population has been obtained from an enrichment fed with ethanol as substrate and 1,2-dichloroethane (1,2-DCA) as electron acceptor ([Grostern and Edwards 2009b](#_ENREF_10)). Switching to H2 as electron donor and acetate as carbon source, transferring regularly, and carrying out dilution series resulted in the loss of methanogenic activity first followed by the loss of the earlier detected population of *Dehalococcoides*. No colony formation was observed in roll tubes amended with H2 and 1,2-DCA despite the formation of ethene. Also the use of a medium lacking selenium and tungsten did not lead to the elimination of the homoacetogen as it was the case in the enrichment of *Dehalobacter restrictus* ([Grostern and Edwards 2009b](#_ENREF_10)). However, it has been shown that the *Acetobacterium* population was unable to dechlorinate 1,2-DCA and that growth of *Dehalobacter* correlated with dechlorination of the chlorinated compound.

Finally, different enrichments dominated by *Dehalobacter* populations have been described but not as pure or defined co-cultures. An enrichment culture designated KFL contained two phylotypes named FTH1 and FTH2 that were affiliated with *Dehalobacter* and that increased to 30% cell abundance upon dechlorination of 4,5,6,7-tetrachlorophthalide as determined by qPCR ([Yoshida et al. 2009](#_ENREF_52)). The KFL culture has been enriched from paddy soil with lactate as substrate and the rather insoluble chlorinated compound as thin film on the walls of the incubation glass vials. The KFL culture dechlorinated 4,5,6,7-tetrachlorophtalide with H2 as the electron donor in the presence of 0.01% peptone.

An enrichment first referred to as MS culture ([Grostern and Edwards 2006](#_ENREF_8)) and later named ‘*Dhb*-TCA’ ([Grostern et al. 2010](#_ENREF_7)), and containing over 60% *Dehalobacter*, has been obtained from groundwater and solids of a site contaminated with 1,1,1-TCA and TCE as inoculum. A mixture of methanol, ethanol, acetate, and lactate (MEAL) was used as electron donor. Dechlorination products were 1,1-dichloroethane (1,1-DCA) and chloroethane (CA) and methanogenesis was only observed during 1,1-DCA dechlorination. Transfers with H2 as electron donor and acetate as carbon source sustained 1,1,1-TCA dechlorination but dechlorination of 1,1-DCA was lost after three transfers. Monitoring *Dehalobacter* 16S rRNA gene copy numbers by qPCR in cultures dechlorinating 1,1,1-TCA and 1,1-DCA showed a ten-fold increase during dechlorination ([Grostern and Edwards 2006](#_ENREF_8)). Subsequent sub-culturing under different conditions indicated that at least two different *Dehalobacter* populations were present in *Dhb*-TCA ([Grostern et al. 2010](#_ENREF_7)). Transfers of *Dhb*-TCA with CF as electron acceptor led to a sub-culture designated *Dhb*-CF that was only able to dechlorinate CF and 1,1,1-TCA but not 1,1-DCA ([Grostern et al. 2010](#_ENREF_7)). This is the first report of an OHRB that can use a chlorinated methane as electron acceptor. A sub-culture maintained for months on 1,1-DCA only dechlorinated 1,1-DCA but not CF and 1,1,1-TCA. A metagenomic analysis of these enrichments allowed obtaining the complete genome of both *Dehalobacter* sp. strains CF and DCA ([Tang et al. 2012](#_ENREF_44)).

Two enrichments selected on a medium containing dichloromethane revealed a metabolic feature of *Dehalobacter* not described previously, namely the fermentation of a chlorinated compound ([Justicia-Leon et al. 2012](#_ENREF_14); [Lee et al. 2012](#_ENREF_19)). In the study of Lee et al., initial cultures inoculated with samples of a core retrieved from an aquifer (state of contamination unknown) and fed with emulsified vegetable oil as electron donor transformed CF into DCM that disappeared as well ([Lee et al. 2012](#_ENREF_19)). Subsequent transfers with H2 and acetate dechlorinated CF to DCM but the latter was only depleted when H2 was removed from the cultures at day 182 ([Lee et al. 2012](#_ENREF_19)). Pyrosequencing of 16S rRNA gene amplicons showed the presence of *Dehalobacter* and *Dehalococcoides*. However, in cultures with H2 where DCM was not degraded only *Dehalobacter* could be quantified by qPCR.Fresh cultures supplied with DCM as the sole organic carbon and energy source showed methane production with *Dehalobacter, Geobacter,* an unclassified *Synergistaceae* and *Methanoculleus* as the dominant microbial community members. Subsequent transfers showed the proliferation of *Dehalobacter* by qPCR quantification along with the consumption of DCM. At the same time, another enrichment was described to ferment DCM and to contain a *Dehalobacter* population that increased in abundance upon DCM consumption ([Justicia-Leon et al. 2012](#_ENREF_14)). H2-fed cultures showed acetate formation but lost the ability to degrade DCM and bicarbonate was needed in sediment-free cultures. This enrichment was unable to dechlorinate CF to DCM.

6.2. Morphology and cytological characteristics of *Dehalobacter* spp.

All reports on the morphology of *Dehalobacter* strains describe them as motile, short rods with a diameter of about 0.5 μm and a length around 2-3 μm ([Wild et al. 1996](#_ENREF_51); [Holliger et al. 1998](#_ENREF_11); [Sun et al. 2002](#_ENREF_43); [Wang et al. 2014](#_ENREF_50)). The Gram staining was mostly negative except in one case where cells staining Gram-positive have been reported ([Grostern and Edwards 2009a](#_ENREF_9)). However, thin-sections of strain PER-K23 cells did not indicate the presence of an outer membrane that is typical for the cell envelope of Gram-negative bacteria. The cell envelope of PER-K23 cells was rather composed of a cytoplasmic membrane, peptidoglycan, and a surface layer that was probably composed of proteins in a hexagonal arrangement and that could potentially hinder the Gram stain to reach the peptidoglycan layer ([Holliger et al. 1998](#_ENREF_11)). The cell wall of PER-K23 contains the peptidoglycan type A3γ as found in certain groups of unequivocally Gram-positive bacteria which indicates a phylogenetic relationship between *Dehalobacter* and Gram-positive bacteria ([Pickett et al. 1994](#_ENREF_35)). The peptidoglycan type A3γ contains LL-diaminopimelic acid that has also been detected in *Desulfitobacterium* sp. strain PCE1 that stains Gram-positive. However, the peptidoglycan type has not been identified in the latter. PER-K23 cells contain *b*-type cytochromes and menaquinones, in particular MQ-7 and MQ-8, but also some MQ-6 and MQ-9.

6.3. Habitat of *Dehalobacter* spp.

The type strain *Dehalobacter restrictus* PER-K23 has been isolated from a packed-bed column that was filled with a mixture of river Rhine sediment and ground anaerobic granular sludge, two sources of environmental microorganisms that can perhaps not be considered as particularly pristine but also not as environments specifically contaminated with chlorinated compounds ([Holliger et al. 1993](#_ENREF_12)). Also *Dehalobacter* sp. strain TCA1 has been isolated from samples of a polluted, but not specifically organohalide-contaminated river sediment ([Sun et al. 2002](#_ENREF_43)) whereas *Dehalobacter restrictus* strain TEA has been isolated from an activated carbon reactor that was colonized by bacteria originating from an aquifer contaminated with DCM ([Wild et al. 1996](#_ENREF_51)). The most recent isolated *Dehalobacter* strain has been enriched from digester sludge of an industrial wastewater treatment plant probably purifying wastewater with a rather undefined mixture of different contaminants ([Wang et al. 2014](#_ENREF_50)). Finally, the strains 12DCB1 and 13DCB1 have also been isolated from samples of an environment that was probably not specifically contaminated with organohalides, namely from sediment of a water-saturated drainage ditch of a chemical industry site.

Different enrichment cultures containing *Dehalobacter* and dechlorinating an organohalide have been obtained from material of sites contaminated with specific chlorinated compounds, mainly contaminated soils and aquifers ([Grostern and Edwards 2006](#_ENREF_8); [Lacroix et al. 2014](#_ENREF_18); [Lee et al. 2012](#_ENREF_19); [Rouzeau-Szynalski et al. 2011](#_ENREF_38); [van Doesburg et al. 2005](#_ENREF_46)) whereas others enrichments have been inoculated with material that was not specifically contaminated with the organohalide used as the electron acceptor in the culture medium ([Griffin et al. 2004](#_ENREF_6); [Yoshida et al. 2009](#_ENREF_52); [Zhang et al. 2012](#_ENREF_53); [Li et al. 2013a](#_ENREF_20); [Li et al. 2013b](#_ENREF_21); [Wang and He 2013](#_ENREF_49); [Zhang et al. 2013](#_ENREF_54)). *Dehalobacter* has also been detected in several aquifers at sites contaminated with 1,1,1-TCA and TCE ([Lowe et al. 2002](#_ENREF_24); [Lima et al. 2012](#_ENREF_22); [Damgaard et al. 2013](#_ENREF_2)).

The above shows that *Dehalobacter* has been enriched from many different sources of inoculum which renders difficult the definition of a typical habitat for this bacterial genus. Since *Dehalobacter* has mainly been characterized as a bacterium that respires chlorinated compounds as electron acceptor, it has been enriched from contaminated soils and aquifers, from sediments of polluted rivers and from sludge of wastewater treatment plants. Although *Dehalobacter* seems to be widespread, all the samples used as inoculum for enrichments originate from environments where *Dehalobacter* has probably already been “naturally” enriched due to exposure to organohalides. With the help of next generation sequencing it would be interesting to know how frequently *Dehalobacter* can be found in the so-called rare microbial biosphere in order to get an idea how widespread *Dehalobacter* really is.

6.4. Nutritional requirements and growth conditions of *Dehalobacter* spp.

The first *Dehalobacter* strain isolated in pure culture has been enriched with fermented yeast extract as source of growth factors in addition to a mixture of vitamins. Fermented yeast extract amendment could be subsequently replaced by peptone if acetate was provided as carbon source ([Holliger et al. 1998](#_ENREF_11)). Vitamins have also been added to the culture media in all other studies where *Dehalobacter* populations have been enriched and peptone or another oligopeptide/amino acid has often been added as well. A detailed analysis of the growth factor requirements of *Dehalobacter restrictus* has shown that it depends on the amendment of the two vitamins thiamine and cyanocobalamin, and the three amino acids arginine, histidine, and threonine ([Holliger et al. 1998](#_ENREF_11)). No information is available on the growth factor requirements of other *Dehalobacter* isolates but as some strains could only be maintained in co-culture indicates that those strains depend on growth factors produced by partner organisms, e.g. *Sedimentibacter* ([van Doesburg et al. 2005](#_ENREF_46)), *Acetobacterium* ([Grostern and Edwards 2009b](#_ENREF_10)), and *Desulfovibrio* ([Grostern and Edwards 2006](#_ENREF_8)).

From the limited available information it can be concluded that *Dehalobacter* spp. are mesophilic bacteria that have an optimal growth at 25-30°C and that are very sensitive to temperatures above 35°C ([Holliger et al. 1993](#_ENREF_12); [Sun et al. 2002](#_ENREF_43)). Whereas *Dehalobacter restrictus* was not able to grow at pH <6.5 ([Holliger et al. 1993](#_ENREF_12)), a *Dehalobacter* population in consortium AQ-5 dechlorinated PCE to *cis*-1,2-DCE well at pH 6.5 ([Lacroix et al. 2014](#_ENREF_18)). Since pH sensitivity is an important parameter for application of organohalide-respiring bacteria (OHRB) in bioremediation of organohalide-contaminated sites, especially when treating source zones, it would be interesting to know more about the range of pH at which different *Dehalobacter* strains are active. A characterization of different PCE-dechlorinating consortia has shown that different *Dehalococcoides* spp. can have quite distinct sensitivities to lower pH values ([Lacroix et al. 2014](#_ENREF_18)).

6.5. Physiology and biochemistry of *Dehalobacter* spp.

*Dehalobacter* spp. can only use molecular hydrogen as electron donor and source of energy, with the exception of *Dehalobacter* sp. strain TCA1 that can also use formate ([Sun et al. 2002](#_ENREF_43)). The majority of highly enriched and pure cultures of *Dehalobacter* cannot ferment organic compounds and can only use organohalides as electron acceptors. Most *Dehalobacter* strains can use at most two different organohalides of the same chemical group, either aliphatic or aromatic organohalides. Strains PER-K23 and TEA use PCE and TCE as electron acceptor and produce *cis*-1,2-DCE ([Holliger et al. 1993](#_ENREF_12); [Wild et al. 1996](#_ENREF_51); [Holliger et al. 1998](#_ENREF_11)), and strain TCA1 uses 1,1,1-TCA and 1,1-DCA and produces CA ([Sun et al. 2002](#_ENREF_43)). The *Dehalobacter* population in the β-HCH dechlorinating co-culture also dechlorinated α- and γ-HCH, the former at the same rates as β-HCH whereas the latter was dechlorinated at much slower rates ([van Doesburg et al. 2005](#_ENREF_46)). This co-culture did not dechlorinate 1,2-DCA and PCE. The *Dehalobacter* population in the 1,2-DCA dechlorinating co-culture did only dechlorinate this organohalide among all the different ones tested (PCE, TCE, *cis*-1,2-DCE, VC, 1,1,2-TCA, 1,1,1-TCA and 1,1-DCA ([Grostern and Edwards 2009a](#_ENREF_9))). The exceptions of this rather general *Dehalobacter* ability are the most recently isolated strains that dechlorinate aromatic as well as alkene organohalides. Strain TCP1 dechlorinates besides chlorophenols also PCE and TCE that are transformed into *cis*-1,2-DCE and *trans*-1,2,-DCE in a ratio of 5.6:1 ([Wang et al. 2014](#_ENREF_50)), and strains 12DCB1 and 13DCB1 dechlorinate a multitude of chlorobenzenes as well as PCE and TCE to *cis*-1,2-DCE ([Nelson et al. 2014](#_ENREF_32)). These findings are not surprising looking at the numerous reductive dehalogenase genes found in *Dehalobacter* genomes (see below), and invite the different groups working with *Dehalobacter* to reassess the dechlorination potential of their strains.

Two reports provide strong evidence that certain *Dehalobacter* strains can ferment DCM and produce acetate from this chlorinated compound, a process that requires a syntrophic relationship with a hydrogen consumer ([Justicia-Leon et al. 2012](#_ENREF_14); [Lee et al. 2012](#_ENREF_19)). In a culture degrading CF completely, CF was first dechlorinated by an organohalide respiration (OHR) process to DCM which then was fermented to acetate and hydrogen ([Lee et al. 2012](#_ENREF_19)). Although *Dehalobacter* was involved in both processes, it was not possible to determine whether two distinct *Dehalobacter* populations were responsible for the two metabolic reactions or only one. In either case, these results indicate that growth of *Dehalobacter* is not restricted to OHR only.

In contrast to hydrogenophilic homoacetogens and methanogens, *Dehalobacter* spp. are not able to grow autotrophically, i.e. they need acetate as carbon source. Succinate cannot replace acetate and one third of the carbon in newly formed biomass is coming from inorganic carbon by heterotrophic carbon fixation ([Holliger et al. 1993](#_ENREF_12); [Holliger et al. 1998](#_ENREF_11)). *Dehalobacter* has this metabolic feature, being a chemolithotroph and a heterotroph, called mixotrophy, in common with *Dehalococcoides*, the other genus of obligate organohalide-respiring bacteria ([Löffler et al. 2013](#_ENREF_23)).

A detailed study of the creation of a proton gradient upon hydrogen oxidation and reductive dechlorination of PCE indicated a H+/e- ratio of 1.25 ± 0.2 which suggests that besides formation of protons due to hydrogen oxidation on the outside of the cytoplasmic membrane, vectorial translocation of protons from the inside to the outside could also occur ([Schumacher and Holliger 1996](#_ENREF_40)). In addition, this study showed that menaquinones are involved in electron transfer from the hydrogenase to the reductive dehalogenase and that the reductive dehalogenase could be photoreversibly inactivated by 1-iodopropane, an inhibitor of corrinoid-mediated reactions.

The PCE reductive dehalogenase of *Dehalobacter restrictus*, in the following referred to as PceA, indeed contains a corrinoid that is present in the protein in base-off form ([Schumacher et al. 1997](#_ENREF_41)). In addition, PceA contains two 4Fe-4S clusters with very low redox potentials. Although initially characterized as membrane-associated protein that is cytoplasmically oriented ([Schumacher and Holliger 1996](#_ENREF_40)), recent investigations with protoplasts and proteinase K treatment suggested that PceA is facing the periplasm (unpublished results). This topology of PceA is in agreement with the sequence information obtained some years after the first sequence of a reductive dehalogenase was published (PceA of *Sulfurospirillum multivorans*, ([Neumann et al. 1998](#_ENREF_33))). The sequence of PceA of *Dehalobacter restrictus* was obtained using a degenerate PCR approach that targeted a conserved amino acid stretch of PceA of *Sulfurospirillum multivorans* and CprA of *Desulfitobacterium dehalogenans* ([von Wintzingerode et al. 2001](#_ENREF_48)) and the N-terminal sequence of PceA from *Dehalobacter restrictus* ([Maillard et al. 2003](#_ENREF_27)). The sequence of PceAof *Dehalobacter restrictus* had the same features as the one of *Sulfurospirillum multivorans*, namely the absence of a corrinoid binding motif, the presence of consensus sequences for binding two 4Fe-4S clusters, and the presence of a twin-arginine motif that is usually found in proteins that contain redox cofactors and are exported across the cytoplasmic membrane in a folded conformation. This indicates that PceA should indeed rather be located at the outside of the cytoplasmic membrane.

The characterization of the genetic context around *pceA* resulted in the identification of the *pceABCT* gene cluster that has also been found in *Desulfitobacterium hafniense* strain TCE1 with 99% sequence identity ([Duret et al. 2012](#_ENREF_5); [Maillard et al. 2005](#_ENREF_26)). In the latter organism, this gene cluster is part of a composite transposon but not in *Dehalobacter restrictus*. The product of *pceB* is predicted to be a protein with three transmembrane helices and is therefore, as it is the case for many other sequenced reductive dehalogenases such as PceA of *S. multivorans* ([Neumann et al. 1998](#_ENREF_33)) and CprA of *D. dehalogenans* ([Smidt et al. 2000](#_ENREF_42)), proposed to be a membrane anchor for PceA. The additional genes *pceC* and *pceT* were named according to homologous genes identified in the chlorophenol reductive dehalogenase (*cpr*) gene cluster of *Desulfitobacterium dehalogenans* ([Smidt et al. 2000](#_ENREF_42)). In *Desulfitobacterium hafniense* strain TCE1, the role of PceT has been identified to be a trigger factor-like protein that seems to function as dedicated chaperone for PceA and that specifically interacts with the twin-arginine signal peptide of PceA ([Maillard et al. 2011](#_ENREF_25)). PceC could, according to the characterization of the gene cluster of *Desulfitobacterium dehalogenans*, be a membrane-bound regulatory protein. However, this protein contains a typical FMN binding site ([Rupakula et al. 2013](#_ENREF_39)) and could therefore also be involved in electron transfer.

Based on physiological data and sequence information of the *pce* gene cluster, a refined version of a previously published model of the respiration chain of *Dehalobacter restrictus* ([Schumacher and Holliger 1996](#_ENREF_40)) is presented in **Figure 6.1**. This model results in a theoretical H+/e- ratio of 1.5 which is in the range of the one experimentally determined earlier ([Schumacher and Holliger 1996](#_ENREF_40)). Assuming the need of three protons for the formation of one molecule of ATP, about one mole of ATP would be formed per mole of chloride released. The published growth yields for *Dehalobacter* spp. range between 3.3 and 5.6 g dry weight per mole of chloride released ([Holliger et al. 1998](#_ENREF_11); [Sun et al. 2002](#_ENREF_43); [Grostern and Edwards 2009b](#_ENREF_10); [Wang et al. 2014](#_ENREF_50)). When assuming a biomass yield of 5-10 g dry weight per mole of ATP formed during catabolism, the respiration chain of *Dehalobacter* produces about half to one mole of ATP per mole of chloride released which is in agreement with the proposed model in **Figure 6.1**.



**Fig.6.1.** Tentative model of the respiration chain of *Dehalobacter restrictus* involving hydrogen oxidation by a Ni-Fe hydrogenase, transfer of electrons via menaquinones from the cytochrome *b* subunit of the hydrogenase to PceC, and finally to PceA that reductively dechlorinates PCE to *cis*-1,2-DCE.

6.6. Phylogeny of the genus *Dehalobacter*

*Dehalobacter* spp. belong to the low GC Gram-positive *Firmicutes*. An analysis of the phylogenetic position of *Dehalobacter* spp. within OHRB is presented in Chapter 5 of this book and will not be addressed here. However, a detailed analysis of the 16S rRNA genes within the genus *Dehalobacter* reveals a relatively high heterogeneity both between and within strains. The genome sequences of *Dehalobacter restrictus* ([Kruse et al. 2013](#_ENREF_17)), *Dehalobacter* sp. strains CF, DCA ([Tang et al. 2012](#_ENREF_44)), and TCP1 ([Wang et al. 2014](#_ENREF_50)), all contain between 3 and 5 copies of the 16S rRNA gene. Multiple rRNA operons have already been recognized as a property of other Gram-positive OHRB, namely *Desulfitobacterium* spp. ([Villemur et al. 2006](#_ENREF_47)). **Figure 6.2** shows the phylogenetic tree of the *Dehalobacter* strains.



**Fig.6.2**. Phylogenetic analysis of the 16S rRNA genes of *Dehalobacter* spp. For some strains, multiple 16S rRNA genes are present in the genome (noted as # followed by a number). The long branches of *Dhb*-12DCB1-#2 and *Dhb*-TCP1-#2 are due to extended V1 variable region. Legend: *Dhb*: *Dehalobacter* sp.; *Dre*: *Dehalobacter restrictus*. The abbreviation is followed by the name of the strain. The gene index (GI) reference number is given in parentheses. Notes: the 16S rRNA gene sequence of *Dehalobacter* sp. strain 13DCB1 was excluded as it is not complete and does not contain the variable V1 region. For *Dehalobacter* sp. strain E1, only one copy of 16S rRNA gene sequence is available in databases, although 3 distinct copies have been reported ([Maphosa et al. 2012](#_ENREF_28)).

A closer look at the alignment of *Dehalobacter* 16S rRNA gene sequences revealed that the high diversity is due to the large degree of variability in the V1 region. Indeed this region ranges from 34 to 188 nucleotides (nt) in length and varies also in sequence. *Dehalobacter* sp. strain TCP1 harbours five 16S rRNA genes, four of them being very similar with a V1 variable region of 140 nt and one displaying a 188-nt long V1 region. In contrary, *Dehalobacter restrictus* harbours four almost identical copies of the 16S rRNA gene (with a 34-nt long V1 region). The topology of the tree does not allow making any correlation between 16S rRNA sequence and the halogenated compounds that are reduced by these strains.

6.7. Metabolic features deduced from the genome of *Dehalobacter restrictus* and ‘omics’ studies

The almost 3-Mb long genome of *Dehalobacter restrictus* PER-K23 contains 2826 protein-coding and 82 RNA genes. For a total of 76.7% of the protein-coding genes a putative function could be identified whereas 781 genes could not be associated with any of the general COG functional categories (cluster of orthologous groups, www.ncbi.nlm.nih.gov/COG/) ([Kruse et al. 2013](#_ENREF_17)). Numerous genes that probably play a role in ORH with H2 as electron donor and PCE and TCE as electron acceptor have been identified. However, no functional gene for any other known respiration metabolism has been found, confirming the cultivation attempts that did not show growth with alternative electron donors and acceptors. Nevertheless, it cannot be excluded that the numerous unidentified genes encode for unsuspected metabolic pathways that are yet unknown and have not been tested in cultivation experiments.

Eight different hydrogenases are present on the genome of *Dehalobacter restrictus* which underscores the central role of hydrogen in its metabolism. One of the three membrane-bound Ni/Fe uptake hydrogenases (Hup) has also been detected in the proteome of cells harvested during different growth phases, suggesting a major role of this hydrogenase in the core metabolism ([Rupakula et al. 2013](#_ENREF_39)). In addition to this Hup, two of the three Fe-only hydrogenases (Hym), identified on the genome and lacking the typical membrane-associated components, have also been detected. They might be involved in generating reducing equivalents needed in anabolism in the form of NADH and FADH, or they might work with the 11-subunit respiration complex I to generate a proton motive force. Indeed, an 11-subunit respiration complex I is present in the genome and its cytoplasm-oriented subunits NuoBCD were detected in the proteome. This 11-subunit version of complex I is widely distributed, both in the archaeal and the eubacterial kingdoms, and has been proposed to be capable to function with various electron donor and acceptor proteins ([Moparthi and Hägerhäll 2011](#_ENREF_30)). Finally, also the two large membrane-bound putatively proton-translocating hydrogenase complexes Hyc and Ech have been expressed during growth of *Dehalobacter restrictus* which illustrates the complex nature of hydrogen metabolism in this bacterium and possibly also the energy metabolism involving the build-up of a proton motive force.

On the electron acceptor side, a total of 25 genes predicted to encode catalytic subunits of reductive dehalogenases (*rdhA*) have been found in the genome of *Dehalobacter restrictus* and a total of 86 genes potentially associated with reductive dehalogenase expression and maturation such as membrane anchors (*rdhB*), transcriptional regulators (*rdhK*), and chaperones (*rdhT*) ([Kruse et al. 2013](#_ENREF_17)). All four proteins encoded by the *pceABCT* gene cluster have been identified in the proteomeand they seemed constitutively expressed. In addition, also RdhA14 has been detected, a reductive dehalogenase with unknown substrate spectrum. A more detailed discussion of the functional diversity of the different *rdhA* genes is presented below.

The genome of *Dehalobacter restrictus* encodes an intact Wood-Ljungdahl pathway that has also been reported for other OHRB such as the closely related *Desulfitobacterium hafniense* strains Y51 ([Nonaka et al. 2006](#_ENREF_34)), TCE1 ([Prat et al. 2011](#_ENREF_36)) and DCB-2 ([Kim et al. 2012](#_ENREF_15)) and the more distantly related *Dehalococcoides mccartyi* strains ([Tang et al. 2009](#_ENREF_45); [Kruse et al. 2013](#_ENREF_17)). Furthermore, the genome of *Dehalobacter restrictus* contains several homologues of pyruvate synthase, an enzyme that could be involved in heterotrophic CO2 fixation ([Kruse et al. 2013](#_ENREF_17)). For *Dehalococcoides mccartyi* strain 195, it has been shown that CO2 is assimilated via two reactions, conversion of acetyl-coenzyme A to pyruvate catalyzed by pyruvate synthase and pyruvate conversion to oxaloacetate via pyruvate carboxylase and that the Wood-Ljungdahl pathway is not involved in CO2 fixation ([Tang et al. 2009](#_ENREF_45)). In *Desulfitobacterium hafniense* strains, components of the Wood-Ljungdahl pathway have been shown to participate in the use of phenyl methyl esters as electron donor ([Kreher et al. 2008](#_ENREF_16)). Although enzymes belonging to the Wood-Ljungdahl pathway and products of pyruvate synthase genes have been detected in the proteome of *Dehalobacter restrictus*, it is not known at present how heterotrophic CO2 fixation is achieved and what the role of the Wood-Ljungdahl pathway enzymes is. Cultivation attempts of *Dehalobacter restrictus* with vanillate as carbon source and electron donor were so far not successful (unpublished results).

In addition to *D. restrictus*, the genome of *Dehalobacter* sp. E1 has been deduced from a metagenomic analysis of a coculture with Sedimentibacter sp. ([Maphosa et al. 2012](#_ENREF_28)). At the time it was compared to the genome of *Dehalococcoides* spp. revealing an overall richer arsenal in the metabolism of amino acids, energy and cofactor biosynthesis. Two formate dehydrogenases and one uptake hydrogenase have been also identified. Ten reductive dehalogenases have been identified in *Dehalobacter* sp. E1 (see below).

6.8. Functional diversity of reductive dehalogenases in the *Dehalobacter* genus

At the time when the sequence information of PceA was retrieved with classical molecular approaches ([Maillard et al. 2003](#_ENREF_27)), two additional however partial *rdhA* genes have been identified from *Dehalobacter restrictus* by a more extensive degenerate PCR approach ([Regeard et al. 2004](#_ENREF_37)), suggesting that *Dehalobacter restrictus* was harbouring several *rdhA* genes, although its capability of reducing organohalides is restricted to PCE and TCE. High levels of sequence conservation of the *pceABCT* gene cluster of *Dehalobacter restrictus* with sequences found in several *Desulfitobacterium* strains including the 1,2-DCA reductive dehalogenase of *Desulfitobacterium dichloroeliminans* ([Marzorati et al. 2007](#_ENREF_29)) have been found suggesting that horizontal gene transfer and adaptation to other organohalides occurred here ([Duret et al. 2012](#_ENREF_5)). This trend was further illustrated by the identification of three *rdhA* genes from an enrichment culture dechlorinating 1,2-DCA that was dominated by *Dehalobacter* sp. strain WL. All three genes showed high sequence homology with *pceA* of *Dehalobacter restrictus*, while the product of one of them (RdhA1, GI: 198404178) was proposed to be involved in 1,2-DCA dechlorination ([Grostern and Edwards 2009b](#_ENREF_10)).

Several recently published genomes and metagenomic analyses targeting pure and mixed cultures of *Dehalobacter* spp. revealed a much larger diversity of *rdhA* genes ([Deshpande et al. 2013](#_ENREF_4); [Kruse et al. 2013](#_ENREF_17); [Maphosa et al. 2012](#_ENREF_28); [Tang et al. 2012](#_ENREF_44)), analogous to what was observed in the genus *Dehalococcoides* (see ([Löffler et al. 2013](#_ENREF_23)) for a review). Ten *rdhA* genes were identified from a metagenomic analysis of *Dehalobacter* sp. strain E1 in a co-culture dechlorinating β-HCH ([Maphosa et al. 2012](#_ENREF_28)). A common set of 17 *rdhA* genes was identified in the genome of the two *Dehalobacter* sp. strains CF and DCA dechlorinating chloroform and chloroethanes ([Tang et al. 2012](#_ENREF_44)). *Dehalobacter* sp. strain UNSWDHB dechlorinating chloroform displays 17 *rdhA* genes ([Deshpande et al. 2013](#_ENREF_4)), 14 of them identical to those found in strains CF and DCA. The genome of *Dehalobacter restrictus* revealed the presence of 25 *rdhA* genes, including the well-characterized *pceABCT* gene cluster ([Kruse et al. 2013](#_ENREF_17)). Recently, a new genome was deposited in databases (as part of the sequencing project coordinated by H. Smidt and the JGI) belonging to *Dehalobacter* sp. strain FTH1, which was isolated from a culture dechlorinating 4,5,6,7-tetrachlorophtalide ([Yoshida et al. 2009](#_ENREF_52)). This genome harbors the highest number of *rdhA* genes in *Dehalobacter* spp. with 27 analogs.

The corresponding amino acid sequences of all the *rdhA* genes identified in *Dehalobacter* spp. were aligned and are depicted in **Figure 6.3**. The RdhA sequences belonging to individual *Dehalobacter* strains appear to be relatively well distributed over the overall diversity. Most RdhA sequences are present at least in two members of the genus, while only a few sequences are exclusively found in one specific strain. Interestingly, only one RdhA sequence seems to be conserved in all strains considered here (indicated by an asterisk).



**Fig.6.3**. Protein sequence likelihood tree analysis of all putative reductive dehalogenases identified in *Dehalobacter* spp. Each sequence is given by its gene index (GI) reference number and an abbreviation for the species and strain. A colour code is used to distinguish the strains: red: *Dehalobacter restrictus*; orange: *Dehalobacter* sp. strain E1; yellow: *Dehalobacter* sp. strain UNSWDHB; light blue: *Dehalobacter* sp. strain CF; dark blue: *Dehalobacter* sp. strain DCA; green: *Dehalobacter* sp. strain FTH1; grey: *Dehalobacter* sp. in coculture or enrichment cultures. Legend: *Dre*: *Dehalobacter restrictus*; *Dhb*: *Dehalobacter* sp.; *Dde*: *Desulfitobacterium dehalogenans*; *Ddi*: *Desulfitobacterium dichloroeliminans*; *Dha*: *Desulfitobacterium hafniense*; *Dmc*: *Dehalococcoides mccartyi*; *Smu*: *Sulfurospirillum multivorans*.

From the topology of the tree, one could consider two classes of *Dehalobacter* RdhA sequences, one that is relatively conserved and homologous to CprA of *Desulfitobacterium dehalogenans* (left side of the tree), and another class which contains many diverse RdhA sequences (right side of the tree). This distinction is further validated when considering the genetic structure of *rdh* operons in *Dehalobacter restrictus* ([Rupakula et al. 2013](#_ENREF_39)). Indeed, a variety of gene clusters was identified there, with the *cprA*-like genes being embedded in a simple *rdhBA* operon structure, while the other ones are part of *rdhBAC*, *rdhABC* or *rdhABCT* structures. This suggests that the numerous *cprA*-like genes in *Dehalobacter* are likely the result of relatively recent gene duplications.

As for other RdhA in general (see ([Hug et al. 2013](#_ENREF_13)) and Chapter 16), the sequence-substrate relationships in *Dehalobacter* RdhAs is impossible to establish. Too scarce knowledge is available not allowing any clear correlation between sequence features and substrate specificity. As an example, in the group of the best defined and closely related RdhA enzymes in *Dehalobacter* (indicated by a double asterisk), two substrates were identified, PCE (and TCE) and 1,2-DCA.

6.9. Corrinoid metabolism in *Dehalobacter* spp.

As already stated above, *Dehalobacter restrictus* has been characterized as a corrinoid auxotrophic bacterium ([Holliger et al. 1998](#_ENREF_11)). The elucidation of the genome sequence of *Dehalobacter restrictus* ([Kruse et al. 2013](#_ENREF_17)), but also other *Dehalobacter* spp. ([Deshpande et al. 2013](#_ENREF_4); [Maphosa et al. 2012](#_ENREF_28); [Tang et al. 2012](#_ENREF_44)) gave new insights in the corrinoid metabolism and more specifically in the corrinoid biosynthesis pathway. At the very first look it was surprising to identify the complete corrinoid biosynthetic pathway in *Dehalobacter restrictus*. However, a deletion mutation affecting the *cbiH* gene was then proposed to be responsible for its corrinoid auxotrophy ([Kruse et al. 2013](#_ENREF_17); [Rupakula et al. 2013](#_ENREF_39)). In a proteomic study, several proteins of this pathway, including CbiH, were not detected in the proteome of *Dehalobacter restrictus* cultivated in standard growth conditions (i.e. in presence of 250 g/L of cobalamin in the medium) ([Rupakula et al. 2013](#_ENREF_39)). A functional genomic study was then conducted to investigate the effect of corrinoid limitation on corrinoid metabolism (Rupakula *et al*., paper submitted). Five distinct operons were characterized in *Dehalobacter restrictus* and two major differences were observed in the genomes of other *Dehalobacter* spp., namely the presence of an intact copy of *cbiH* and the lack of operon-2, which encodes several corrinoid transport proteins and proteins involved in corrinoid salvaging. All five operons are regulated by cobalamin riboswitches (Rupakula *et al*., paper submitted), baring similarity to *Desulfitobacterium hafniense* ([Choudhary et al. 2013](#_ENREF_1)). The comparison of the proteome from *Dehalobacter restrictus* cells cultivated in the presence of 250, 50 and 10 g/L of cobalamin in the medium revealed a strong up-regulation of proteins encoded in operon-2, suggesting that *Dehalobacter restrictus* exploits an enhanced capacity of corrinoid transport and salvaging under corrinoid limitation. Sequence analysis further indicated that operon-2 of *Dehalobacter restrictus* shows strong homology to an operon present in *Acetobacterium woodii*, suggesting that horizontal gene transfer may have occurred (Rupakula *et al*., paper submitted). So far, scarce information is available on corrinoid metabolism at the physiological level in other *Dehalobacter* strains. It was suggested that *Dehalobacter* sp. strain E1 might benefit from corrinoids provided by *Sedimentibacter* sp. present in the co-culture ([Maphosa et al. 2012](#_ENREF_28)). When indicated, cultures of *Dehalobacter* spp. were always cultivated in the presence of cobalamin. Hence, further work is needed to test the ability of *Dehalobacter* strains that have an intact *cbiH* gene to synthesize corrinoids *de novo*.

6.10. Concluding remarks

*Dehalobacter* in pure culture or enrichments seem to be dedicated to organohalide degradation, either by reduction in organohalide respiration or by fermentation as in the case of DCM. For the fermentative strains it is however not known whether they have additional metabolic capabilities not involving an organohalide. The recently available genome sequences and the recently isolated new strains provide new avenues of research for this bacterial genus. The increasing number of scientific publications dealing with *Dehalobacter* populations since 2009, about 70 in total, indicates that we will learn a lot more about the *Dehalobacter* genus in the near future.

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