MOLECULAR CHARACTERIZATION OF KEY ENZYMES INVOLVED IN DEHALORESPIRATION WITH TETRACHLOROETHENE

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Overview

Version abrégée

Depuis 1920, les chloroéthènes, et plus particulièrement le tetra- (PCE) et le trichloroéthène (TCE) sont devenus des polluants majeurs dans les eaux souterraines de par leur utilisation intensive en tant que solvants dans l'industrie. La forte électronégativité des atomes de chlore leur confère une grande stabilité en conditions aérobies. Cependant, en conditions anaérobies, la biodégradation des chloroéthènes par des microorganismes s'est avérée être une technique de dépollution prometteuse pour favoriser leur élimination au sein des sites contaminés. A ce jour, une quinzaine de souches bactériennes ont été isolées avec la capacité d'utiliser les chloroéthènes comme accepteur final d'électrons au cours d'un processus appelé déhalorespiration. Alors que la plupart des souches isolées, appartenant aux phyla *Firmicutes*, *Protéobactéries* δ et ε, sont capables de déchlorurer le PCE et le TCE principalement jusqu'au *cis*-1,2-dichloroéthène (*cis*-1,2-DCE), quelques représentants du genre *Dehalococcoides*, affiliés au phylum *Chloroflexi*, peuvent en outre déchlorurer le *cis*-1,2-DCE et le chlorure de vinyl (VC), rejetant ainsi de l'éthène, considéré comme non-toxique, dans l'environnement. Certaines bactéries, telles que *Dehalobacter restrictus*, ainsi que les membres du genre *Dehalococcoides*, sont même totalement dépendantes des chloroéthènes pour leur croissance, ce qui donna naissance à des questions d'ordre évolutif. L'identification des enzymes-clés de la réaction de déchloruration, appelées déhalogénases réductrices, a révélé une nouvelle classe d'enzymes contenant un corrinoïde et deux centres fer-soufre comme cofacteurs. Neuf membres de cette nouvelle classe d'enzymes étaient caractérisés sur le plan biochimique au début de cette thèse, et très peu d'information était disponible au plan moléculaire. Ainsi le but principal de cette thèse était de caractériser sur le plan moléculaire les déhalogénases réductrices impliquées dans la déhalorespiration du tetrachloroéthène, et d'obtenir des indications concernant l'évolution de ce nouveau processus de respiration anaérobie.

A partir de la séquence N-terminale de la PCE déhalogénase réductrice (PceA) de *Dehalobacter restrictus*, et d'une séquence de huit acides aminés conservée dans deux autres déhalogénases réductrices, une approche de type PCR dégénérée a permis l'isolement du gène *pceA* de *D. restrictus*. La comparaison de cette nouvelle séquence, encore partielle, avec une séquence similaire de *Desulfitobacterium* sp. PCE-S a révélé une identité proche de 100%. Avec l'aide de cette dernière, la séquence totale des gènes *pceAB* a été isolée à partir de *Dehalobacter restrictus*, mais également de *Desulfitobacterium hafniense* TCE1. L'analyse de cette nouvelle séquence confirma la présence d'un peptide signal de type Tat (pour Twin-Arginine Translocation), connu pour être impliqué dans l'incorporation de l'enzyme dans la membrane cytoplasmique de la bactérie. Une analyse détaillée des motifs de liaison des centres fer-soufre présents dans les séquences de la PCE déhalogénase réductrice de *Dehalobacter restrictus* et de la chlorophénol (CP) déhalogénase réductrice de *Desulfitobacterium dehalogenans* a mis à jour des différences structurales dans le deuxième motif. Ces différences corroborent les résultats obtenus par le passé au moyen de la spectroscopie RPE (résonance paramagnétique de l'électron). En effet, la présence de deux centres [4Fe-4S] dans l'enzyme de *D. restrictus* et celle d'un centre [4Fe-4S] et d'un centre [3Fe-4S] dans l'enzyme de *D. dehalogenans* peut être expliquée par la présence dans la première enzyme, et non dans la dernière, de résidus glycine et proline aux extrémités du peptide séparant les résidus cystéine, responsables de lier les atomes de fer. Cette structure primaire de la chaîne polypeptidique permet très probablement la formation d'une boucle dans la structure tertiaire ainsi que la participation du premier résidu cystéine à la formation du deuxième centre [4Fe-4S]. En outre, le gène *pceB* code pour une petite protéine à caractère hydrophobe,

contenant trois hélices α structuralement conservées, indiquant un possible rôle dans l'ancrage dans la membrane de la sous-unité catalytique de la déhalogénase réductrice (PceA).

L'identité parfaite de séquence des déhalogénases réductrices isolées à partir de *Dehalobacter restrictus* et de *Desulfitobacterium hafniense* TCE1 a soulevé la question d'un possible transfert horizontal de gène entre ces deux souches. C'est pourquoi les régions voisines des gènes *pceAB* ont fait l'objet d'une nouvelle investigation. Cette étude a révélé la présence dans le génome de *D. hafniense* TCE1 d'un transposon composite (appelé *Tn*-Dha1), bordé par deux séquences d'insertion identiques (*IS*Dha1, incluant le gène pour la transposase, *tnpA*1) et englobant six autres gènes : les gènes *pceAB*, préalablement caractérisés ; deux gènes (*pceCT*) montrant une homologie avec des membres d'un groupe de gènes impliqués dans la synthèse de la CP déhalogénase réductrice de *D. dehalogenans* ; et finalement deux autres gènes potentiellement tronqués avec une homologie envers une autre transposase (*tnpA*2) et une sous-unité de la machinerie Tat (*tatA*), respectivement. Chez *Dehalobacter restrictus*, par contre, seul le groupe de gène *pceABCT* a été observé (c'est-à-dire sans la présence de la structure du transposon et des deux autres gènes), indiquant que les gènes responsables pour les enzymes-clés impliquées dans l'activité déchlorurante sont stables dans le génome de *D. restrictus*. Une étude détaillée de *Tn*-Dha1 au moyen de la PCR et de l'analyse par Southern blot a montré que le transposon peut former différentes molécules circulaires, attestant de l'activité de cet élément génétique mobile. Sur la base des résultats obtenus ici, un modèle de transposition de *Tn*-Dha1 a été proposé, selon lequel le transposon peut s'exciser du chromosome et former une molécule circulaire, contribuant ainsi à la formation d'une structure instable avec deux *IS*Dha1 côte à côte. En effet, le fort promoteur créé par la jonction des deux *IS* doit conduire à une surexpression de la transposase, qui à son tour réagit avec la structure circulaire soit en la réintégrant dans le chromosome, soit en y excisant un, voire les deux *IS*. Les structures résultant de ce phénomène seraient des *IS* isolées, un tandem d'*IS*, ainsi que des molécules circulaires contenant un ou aucun *IS*, ces dernières pouvant être considérées comme des déchets du processus de transposition.

L'hypothèse selon laquelle les bactéries déhalorespirantes s'échangeraient les gènes-clés de la déhalorespiration par transfert de gène horizontal a également été testée au moyen d'une approche de type génomique. Les données issues du projet de séquençage du génome de *Dehalococcoides ethenogenes* (établie par « The Institute for Genome Research », TIGR), une bactérie capable de déchlorurer le PCE jusqu'à l'éthène et dont il a été montré qu'elle contenait jusqu'à 18 copies différentes de gènes codant potentiellement pour des déhalogénases réductrices, ont été analysées au moyen d'un outil bioinformatique permettant d'attribuer une signature génomique à l'ensemble de la séquence et d'en étudier les variations tout au long du génome. Pour ce faire, la fréquence des mots d'ADN de 4 lettres a été calculée au fil du génome et représentée graphiquement. Ainsi des perturbations locales de la signature génomique ont été mises en évidence dans certains segments du génome, appelés régions originales, correspondant à de l'ADN présumé avoir été acquis par transfert horizontal. Cette analyse révéla que des 18 copies de déhalogénases réductrices présentes dans le génome de *D. ethenogenes*, 15 d'entre elles sont localisées à l'intérieur de ces régions originales. De plus, de nombreux gènes codant pour des recombinases (transposase, integrase) ont été trouvés à l'intérieur de ces mêmes régions, corroborant l'hypothèse d'un transfert horizontal de gène.

Pour aucune bactérie déhalorespirante, ni la chaîne de transport d'électrons menant les électrons à la déhalogénase réductrice, ni le donneur direct d'électrons pour aucune déhalogénase réductrice n'ont été elucidés. C'est pourquoi la présence de cytochromes au sein des cellules de *Desulfitobacterium hafniense* TCE1 a été investiguée en fonction de la présence ou absence de

PCE dans le milieu de culture. Ainsi la détection des cytochromes au moyen d'une méthode sensible basée sur la chémiluminescence a révélé un signal fortement amplifié dans la fraction membranaire de cellules cultivées sur PCE comme accepteur final d'électrons, en comparaison de la même fraction à partir de cellules cultivées sur acide fumarique. Une analyse de type Western blot a mis en évidence une protéine d'environ 45 kDa dans la fraction membranaire, correspondant très probablement à un cytochrome de type *c*. Une analyse des mêmes échantillons au moyen de la spectroscopie UV-visible a confirmé ces résultats. Bien que cette étude requiert d'autres analyses, une forte indication selon laquelle un cytochrome de type *c* serait impliqué dans le transfert direct d'électrons vers la PCE déhalogénase réductrice de *D. hafniense* TCE1 a été apportée.

Pendant la durée de cette thèse, de très nombreuses séquences de déhalogénases réductrices ont été déposées dans les banques de données, attestant de l'intérêt de la communauté scientifique pour cette nouvelle forme de respiration anaérobie. Ainsi plusieurs études basées sur une approche de PCR dégénérée ont abouti à l'identification de 22 gènes de déhalogénases réductrices, pour la plupart partiels. Parallèlement, l'analyse des séquences préliminaires des génomes de *Dehalococcoides ethenogenes* et de *Desulfitobacterium hafniense* DCB-2 a révélé la présence de 18 et de 6 nouvelles séquences, respectivement. L'alignement de toutes ces séquences (66 au total), ainsi que l'analyse de leur homologie a révélé un classement en quatre groupes principaux, deux correspondant aux déhalogénases réductrices de type chlorophénols et chloroéthènes présentes au sein du phylum *Firmicutes*, un autre contenant les séquences isolées à partir des *Protéobactéries* de type ε, le dernier enfin correspondant à l'ensemble des gènes isolés du genre *Dehalococcoides*. Ainsi les déhalogénases réductrices semblent être conservées au sein des groupes phylogénétiques, formant une classe d'enzymes relativement ancienne. De plus, certaines caractéristiques présentes chez les déhalogénases réductrices, tels que le peptide signal de type Tat, ainsi que les centres fer-soufre, se retrouvent très fréquemment dans l'ensemble des réductases. Cependant, la présence d'un corrinoïde dans le site actif et la présence de plusieurs peptides spécifiques aux déhalogénases réductrices font de ces enzymes une classe à part. Finalement les fortes variations dans la topologie et la composition de la chaîne de transport d'électrons observées parmi les bactéries déhalorespirantes indiqueraient que les déhalogénases réductrices ont très probablement été intégrées à des chaînes de respiration existantes, et non pas que le processus de déhalorespiration a évolué en tant qu'entité distincte.

Summary

Chloroethenes, and most particularly tetra- (PCE) and trichloroethene (TCE) are major groundwater pollutants due to their extensive industrial use as solvents since the 1920s. The strong electronegativity of the chlorines renders them very stable under aerobic conditions. However, biodegradation of chloroethenes under anaerobic conditions has been shown to be a promising strategy for remediation of chloroethene-contaminated sites. To date around fifteen bacterial strains have been isolated with the property of using chloroethenes as terminal electron acceptor in a process called dehalorespiration. Anaerobic dehalorespiring bacteria show an unequal chloroethene substrate range and an unequal extent of dechlorination. While most of the dehalorespiring bacteria dechlorinate PCE and TCE to *cis*-1,2-dichloroethene (*cis*-1,2-DCE) and belong to the phyla *Firmicutes*, δ- and ε-*Proteobacteria*, a few strains of the genus *Dehalococcoides* affiliated with the phylum *Chloroflexi* and are able to dechlorinate *cis*-1,2-DCE and vinyl chloride (VC) to the non-toxic ethene. *Dehalobacter restrictus* and *Dehalococcoides* isolates were found to be completely restricted to dehalorespiration which gave rise to some basic evolutionary questions. Identification of the key enzyme in the dechlorination reaction, the reductive dehalogenase, has revealed a new class of enzymes containing a corrinoid and two iron-sulfur clusters as cofactors. At the beginning of this thesis, nine chloroethene reductive dehalogenases have been characterized on biochemical level, while only little information was available on molecular level. Therefore, the overall goal of this thesis was to characterize on a molecular level the reductive dehalogenases involved in tetrachloroethene dehalorespiration and to get some indications on the evolution of this novel anaerobic respiration process.

Starting from the N-terminal sequence of the PCE reductive dehalogenase (PceA) of *Dehalobacter restrictus* and from a conserved amino acid stretch found in two already sequenced reductive dehalogenases, a degenerate PCR approach allowed the isolation of the gene encoding PceA. Comparison with unpublised data from *Desulfitobacterium* sp. strain PCE-S showed 100% sequence identity. The full sequence of the *pceAB* gene of strain PCE-S helped to isolate the corresponding gene cluster from *D. restrictus* and *Desulfitobacterium hafniense* strain TCE1, which has also been shown to contain an identical N-terminal sequence. Sequence analysis confirmed the presence of a Twin-Arginine Translocation (Tat) signal peptide, which is involved in the incorporation of the reductive dehalogense into the cytoplasmic membrane. Detailed analysis of the iron-sulfur cluster binding motifs present in PceA of *D. restrictus* and the chlorophenol reductive dehalogenase (CprA) of *Desulfitobacterium dehalogenans* revealed differences in the second motif, which may explain results obtained by EPR spectroscopy, namely the presence of two [4Fe-4S] clusters in the former enzyme and the presence of one [3Fe-4S] and one [4Fe-4S] cluster in the latter one. Structure breaking residues such as glycine and proline are present at the two extremities of the ten amino acid stretch separating the first and second iron-binding cysteine residues of the second motif in PceA, but not in CprA. This primary structure probably allows the formation of a loop in the tertiary structure and the participation of the first cyteine as a ligand in a [4Fe-4S] cluster. In both new sequences, the presence of a short gene (*pceB*) encoding a hydrophobic protein with three conserved trans-membrane α-helices was confirmed, indicating a possible role in anchoring the catalytic unit of the reductive dehalogenase into the membrane.

The complete sequence identity observed in the newly isolated reductive dehalogenases raised the question of a possible horizontal gene transfer between *Dehalobacter restrictus* and *Desulfitobacterium hafniense* strain TCE1. Therefore, the flanking regions of the reductive dehalogenase genes (*pceAB*) in *Desulfitobacterium hafniense* strain TCE1 and *Dehalobacter restrictus* were investigated. This study revealed the presence of a composite transposon (named *Tn*-Dha1) in strain TCE1 bordered with two identical insertion sequences (*IS*Dha1, including the transposase gene *tnpA*1) and containing six open reading frames: the already characterized *pceAB*, two genes (*pceCT*) related to members of the *o*-chlorophenol reductive dehalogenase gene cluster of *Desulfitobacterium dehalogenans*, and two possibly truncated genes with homology to another transposase (*tnpA*2) and to a subunit of the Tat machinery (*tatA*), respectively. In contrast, only the *pceABCT* gene cluster (i.e. without the transposon structure and the other two genes) was present in *Dehalobacter restrictus*, indicating that the genes encoding the key enzymes for the dechlorination activity are stably integrated into the genome. A detailed investigation of *Tn*-Dha1 by PCR and Southern blot analysis indicated that *Tn*-Dha1 may form various circular molecules, an indication for an active mobile genetic element. A model for the transposition of *Tn*-Dha1 was proposed, in which the transposon may excise from the chromosome and circularize, forming an unstable structure with two abutted *IS*Dha1. The strong promoter formed by the junction of both *IS* would lead to high expression of the transposase, which in turn reacts with the circular element by either re-integrating it in the chromosome or excising one or both *IS*Dha1 from that element. The resulting structures would be single *IS*, *IS* tandems and circular molecules with one or no remaining *IS*, both latter structures being dead-end products of the transposition event.

The hypothesis of mobile reductive dehalogenase genes was also investigated using a genomic approach in preliminary sequence data (released by The Institute for Genome Research, TIGR) of the genome of *Dehalococcoides ethenogenes*, a dehalorespiring bacterium capable to completely dechlorinate PCE to ethene. The genome was shown to contain the extraordinary number of eighteen different copies of reductive dehalogenase genes, including the well characterized *tceA*. A genomic signature of *D. ethenogenes* was obtained by calculating the frequency of 4-letter DNA words along the genome and was graphically represented. Local disruptions of the genomic signature in certain segments of the genome were highlighted, corresponding to DNA, which may have been acquired by horizontal gene transfer, so-called original regions. It revealed that from the eighteen putative reductive dehalogenase genes present in the genome of *D. ethenogenes*, fifteen were located in original regions. Moreover, several genes encoding for recombinases (transposase, integrase) were found within these original regions, strongly indicating that these may have been acquired horizontally.

The complete electron transport chain leading the electrons to the reductive dehalogenase has not yet been characterized for any of dehalorespiring bacteria and the direct electron-donor has not yet been elucidated for any of reductive dehalogenases. Therefore, the presence of cytochromes in cells of *Desulfitobacterium hafniense* strain TCE1 was investigated with regard to the presence or absence of PCE in the growth medium. Detection of cytochromes using a sensitive detection method based on chemiluminescence revealed a strongly enhanced signal in the membrane fractions of strain TCE1 cells grown on PCE instead of fumarate as terminal electron acceptor. Western blot analysis revealed the presence of a 45 kDa protein in membrane fraction, corresponding most probably to a *c*-type cytochrome. UV-visible spectroscopy confirmed the

presence of *c*-type cytochromes in membrane fractions. This study, although further investigations are needed, indicated that a *c*-type cytochrome may be involved in the direct electron transfer to the PCE reductive dehalogenase of *D. hafniense* strain TCE1.

At present, numerous sequences of reductive dehalogenase genes have been reported and deposited on sequence databases, revealing the great interest shown for this new anaerobic respiration pathway. While several degenerate PCR approaches have led to the isolation of 22 mostly partial genes, analysis of preliminary genome sequence data from *Dehalococcoides ethenogenes* and *Desulfitobacterium hafniense* strain DCB-2 has revealed 18 and 6 sequences, respectively. Sequence alignment and homology analysis of the 66 reductive dehalogenases genes available in August 2004 revealed four main clusters, two corresponding to chlorophenol and chloroethene reductive dehalogenases found in the phylum *Firmicutes*, one with sequences mostly isolated from *ε-Proteobacteria*, and one containing most of the genes isolated from the genus *Dehalococcoides*. Hence, the reductive dehalogenases appear to be rather conserved wihtin phylogenetic groups, indicating a relatively ancient enzyme class. Reductive dehalogenases show some features such as the presence of a Tat signal peptide and iron-sulfur clusters that are common to most of terminal reductases. However, the presence of a corrinoid at the catalytic center and of several specific conserved amino acid stretches makes them a new class of anaerobic reductases. Finally, the strong variation in the topology of the dehalorespiration chain and the variable presence and involvement of different electron transferring components such as quinones and cytochromes in dehalorespiring bacteria indicate that reductive dehalogenases may have been integrated into existing respiration chains rather than that dehalorespiration has evolved as a whole.

List of abbreviations

Chlorinated compounds

CBe	chlorobenzene
CE	chloroethene
Cl-OHPA	3-chloro-4-hydroxyphenyl acetic acid
(*o*-)CP	(*ortho*-)chlorophenol
CPa	chloropropane
DCA	dichloroethane
DCB	dichlorobenzene
DCBa	dichlorobutane
DCE	dichloroethene
DCP	dichlorophenol
DCPa	dichloropropane
ETH	ethene
HCA	hexachloroethane
PCA	pentachloroethane
PCE	tetrachloroethene (= perchloroethene)
PCP	pentachlorophenol
PCPe	pentachloropropene
TCA	trichloroethane
TCB	trichlorobenzene
TCE	trichloroethene
TCP	trichlorophenol
TeCA	tetrachloroethane
TeCB	tetrachlorobenzene
TeCP	tetrachlorophenol
VC	vinyl chloride

Other abbreviations

cprA, CprA	chlorophenol reductive dehalogenase subunit A (*gene*, protein)
DNA	desoxyribonucleic acid
DMSO	dimethylsulfoxide
EPR	electron paramagnetic resonance
FAD	flavin adenine dinucleotide
FLOE	fluorescently labeled oligonucleotide extension
GC	guanosine + cytosine
HGT	horizontal gene transfer
iP	inverse PCR
IR	inverted repeats
IS	insertion sequence
MGEs	mobile genetic elements
ORF	open reading frame
pceA, PceA	tetrachloroethene reductive dehalogenase (*gene*, protein)
(RT-)PCR	(reverse transcriptase-) polymerase chain reaction
RDase	reductive dehalogenase (enzymatic activity)
rdhA, RdhA	putative reductive dehalogenase subunit A (*gene*, protein)
RNA	ribonucleic acid
TMAO	trimethylamine-*N*-oxide
Tn	transposon

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CHAPTER

1

General introduction

1.1 Chloroethenes in the environment

Tetrachloroethene (PCE) belongs together with tri- (TCE), dichloroethene (DCE) and vinyl chloride (VC) to a group of chlorinated compounds, generally called chloroethenes (CEs), that are most widely distributed groundwater pollutants. Although these compounds have been reported to be naturally produced by marine algae and fungi or present in volcanic emissions (1, 36, 48), their presence in the environment drastically increased since the 1920s due to their industrial production and widespread use. Their high stability and low flammability render them very attractive in industrial and commercial applications as metal degreasers, reagents in dry cleaning and intermediates in chemical synthesis. Due to inappropriate handling and storage, large amounts of chloroethenes contaminated soils, sediments and aquifers forming there dense non-aqueous phase liquid (DNAPL) (22, 31, 66). Their toxicity, carcinogenicity, and persistence in the environment represent a threat to the biosphere and human health (3). For this reason, PCE and TCE are among the thirty-six priority hazardous substances listed in Annex 1A of the Hague Declaration (http://odin.dep.no/md/nsc/declaration/bn.html). Bioremediation is one possibility to decontaminate sites polluted with these compounds (70).

1.2 Biodegradation of chloroethenes

Due to the electronegativity of their chlorine substituents, chloroethenes are oxidized compounds and susceptible to serve as electron acceptors in reduction-oxidation reactions. PCE is the strongest oxidant, with exception of O_2, among all possible electron-accepting species found in groundwater (101). Therefore, the biodegradation of chloroethenes in the environment has been systematically related to the presence or absence of oxygen.

1.2.1 Aerobic degradation

While under aerobic conditions highly chlorinated ethenes (PCE, TCE) are mainly transformed via co-metabolic pathways (without gain of energy or carbon for the bacteria), the less chlorinated ones (DCEs and VC) may as well be used as carbon and energy source. In the case of co-metabolism, mono- and dioxygenases that are involved in metabolic degradation of compounds such as methane, ammonia, phenol, and toluene, are due to their large substrate spectrum responsible for unspecific oxidation of chloroethenes, resulting in the formation of epoxides which spontaneously decompose (37, 46, 94, 103, 106). Several studies have reported the mineralization of DCE and VC under aerobic conditions, and pure cultures of bacteria have been described that

use these as sole source of carbon and energy (11, 38, 50, 64, 81, 100). However, only little is known about the enzymes involved in such reactions.

1.2.2 Anaerobic degradation

In the early eighties, different studies have described the degradation of PCE to less chlorinated ethenes under methanogenic and denitrifying conditions (8, 9, 102). Although anaerobic oxidation of VC and DCE has been observed in the presence of strong oxidants such as Fe(III)-oxides (12), the most relevant anaerobic biodegradation pathway of chloroethenes is reductive dechlorination. Indeed it has been shown that due to the relative high oxidation state, PCE was likely to be reduced under strict anaerobic conditions. Several studies have reported the reductive dechlorination of chloroethenes by microorganisms either in co-metabolic processes, or in an energy-conserving process, called dehalorespiration (see (43) for a review), where bacteria use the chlorinated compounds as terminal electron acceptors.

1.3 Dehalorespiration

During the last decade research on the biodegradation of chlorinated compounds has made considerable progress due to the isolation of anaerobic bacteria able to use these compounds as source of terminal electron acceptors in an energy-conserving manner. This new metabolic pathway, called dehalorespiration, represents today a considerable source of investigation in various fields such as energy conservation, enzymology, bacterial adaptation and evolution, as well as biotechnological applications.

1.3.1 Phylogenetic diversity of dehalorespiring bacteria

Bacteria with reductive dechlorinating activity (Table 1.1, including references) belong to the phyla Firmicutes, δ- and ε-Proteobacteria, and a group distantly related to Chloroflexi (Figure 1.1). The dominant group (Firmicutes) is made of numerous species of the genus *Desulfitobacterium*, *Dehalobacter restrictus* strain PER-K23 and its close relative isolate TEA, and *Clostridium bifermentans* strain DPH-1. The genus *Desulfitobacterium*, whose name comes from the ability to grow on sulfite as electron acceptor, is the most versatile with regard to electron donors and acceptors. Several if not all *Desulfitobacteria* contain two 16S rRNA operons with different length and secondary structures, as evidenced for *D. hafniense* strain TCE1 (Figure 1.1, (30)). It is important to note that the nomenclature of members of this genus has been recently updated, in which strains of *D. frappieri* have been renamed and included in the species *D. hafniense* (for details, see the DSMZ website at www.dsmz.de). Dehalorespiring bacteria from the

Table 1.1. Phylogenetic affiliation of and electron donors and acceptors used by dehalorespiring bacteria.

Isolates	Electron donors	Chlorinated electron acceptors [2]	Dechlorination products	Non-chlorinated electron acceptors	16S rRNA accession N°	References
Firmicutes						
Dehalobacter restrictus strains PER-K23 and isolate TEA	H_2	PCE, TCE	*cis*-1,2-DCE	none	U84497 Y10164	(42, 105)
Desulfitobacterium hafniense strain TCE1	H_2, formate, pyruvate, lactate, butyrate, crotonate, ethanol, serine	PCE, TCE	*cis*-1,2-DCE	nitrate, fumarate, sulfite, thiosulfate	X95742 X95972	(32)
Desulfitobacterium sp. strain PCE-S	H_2, formate, pyruvate, yeast extract	PCE, TCE	*cis*-1,2-DCE	fumarate, sulfite	AJ512772	(35, 67)
Desulfitobacterium sp. strain Y51	pyruvate, lactate, formate	PCE, TCE, HCA, PCA, TeCAs, hepta-CPa	*cis*-1,2-DCE, 1,1-DCE, PCPe	fumarate, nitrate, sulfate	AB049340	(90)
Desulfitobacterium sp. strain PCE1	formate, pyruvate, lactate, butyrate, crotonate, succinate, ethanol, serine	PCE, Cl-OHPA, 2,4,6-TCP, 2,4-DCP, 2-CP	TCE, OHPA, phenol, 4-CP	fumarate, sulfite, thiosulfate, cysteate, isethionate	X81032	(33)
Desulfitobacterium hafniense strain PCP-1	pyruvate	PCP, TeCPs, TCPs, DCPs, 2-CP	DCPs, CPs	nitrate, sulfite, thiosulfate	U40078	(7)
Desulfitobacterium dehalogenans strain JW/IU-DC1	H_2, formate, lactate, pyruvate, butyrate, ethanol	Cl-OHPA, PCP, TeCPs, TCPs, DCPs	OHPA, TCPs, DCPs, CPs	nitrate, fumarate, sulfite, sulfur, thiosulfate, cysteate, isethionate	L28946	(95)
Desulfitobacterium hafniense strain DCB-2	formate, pyruvate, lactate, butyrate, crotonate, ethanol, serine	Cl-OHPA, PCP, TCPs, DCPs	OHPA, 4-CP	nitrate, iron(III), isethionate, sulfite, thiosulfate	X94975	(21)
Desulfitobacterium hafniense strain TCP-A	H_2, formate, pyruvate, lactate, alanine, butyrate, glutamate	2,3,5,6-TeCP, TCPs, DCPs, 2-CP	CPs, phenol	nitrate, fumarate, sulfite, thiosulfate	AJ404686	(13)
Desulfitobacterium chlororespirans strain Co23	H_2, formate, pyruvate, lactate, butyrate, crotonate	Cl-OHPA, 3-Cl-4-OH-benzoate, 2,4,6-TCP, DCPs	OHPA, 4-OH-benzoate, CPs	sulfite, sulfur, thiosulfate	U68528	(82)
Desulfitobacterium sp. strain Viet-1	n.d. [1]	PCE, CPs	TCE, phenols	n.d. [1]	AF357919	(Davis & Tiedje, unpubl.)
Desulfitobacterium dichloroeliminans strain DCA1	H_2, formate, lactate	1,2-DCA, 1,1,2-TCA, vicinal DCPa & DCBa	ethene, VC, corresponding alkenes	nitrate, sulfite, thiosulfate	AJ565938	(25)
Clostridium bifermentans strain DPH-1	n.d. [1]	PCE, TCE	*cis*-1,2-DCE	n.d. [1]	Y18787	(19)

Isolates	Electron donors	Chlorinated electron acceptors [2]	Dechlorination products	Non-chlorinated electron acceptors	16S rDNA accession N°	References
δ-Proteobacteria						
Desulfomonile tiedjei strain DCB-1	H_2, formate, pyruvate	3-CBe	benzoate	sulfate, sulfite, thiosulfate	M26635	(26)
Desulfuromonas chloroethenica strain TT4B	acetate, pyruvate	PCE, TCE	*cis*-1,2-DCE	fumarate, iron(III), sulfur	U49748	(49)
Desulfuromonas michiganensis strains BB1 and BRS1	n.d. [1]	PCE, TCE	cis-1,2-DCE	fumarate, malate, iron(III), sulfur	AF357915 AF357914	(89)
ε-Proteobacteria						
Sulfurospirillum multivorans	H_2, formate, pyruvate, lactate, ethanol, glycerol, sulfide	PCE, TCE	*cis*-1,2-DCE	nitrate, fumarate, arsenate, selenate	X82931	(83)
Sulfurospirillum halorespirans strain PCE-M2	H_2, formate, lactate, pyruvate	PCE, TCE	*cis*-1,2-DCE	fumarate	AF218076	(58)
Green nonsulfur bacteria						
Dehalococcoides ethenogenes strain 195	H_2	PCE, TCE, *cis*-DCE, 1,1-DCE, 1,2-DCA, VC*	ethene	none	AF004928	(65)
Dehalococcoides sp. strain CBDB1	H_2	TeCBs, TCBs	1,3,5-TCB, DCBs	none	AF230641	(2)
Dehalococcoides sp. strain BAV1	H_2	cis-DCE, trans-DCE, 1,1-DCE, VC, 1,2-DCA, VB	ethene	none	AY165308	(39)
Dehalococcoides sp. strain VS	H_2	1,1-DCE, VC	ethene	n.d. [1]	AY323233	(74)

[1] n.d. = not determined.

[2] CBe: chlorobenzoate; Cl-OHPA: 3-chloro-4-hydroxyphenyl acetate; CP: chlorophenol; CPa: chloropropane; DCA: dichloroethane; DCB: dichlorobenzene; DCBa: dichlorobutane; DCE: dichloroethene; DCP: dichlorophenol; DCPa: dichloropropane; HCA: hexachloroethane; PCA: pentachloroethane; PCE: tetrachloroethene; PCP: pentachlorophenol; PCPe: pentachloropropene; TCA: trichloroethane; TCB: trichlorobenzene; TCE: trichloroethene; TCP: trichlorophenol; TeCA: tetrachloroethane; TeCB: tetrachlorobenzene; TeCP: tetrachlorophenol; VC: vinyl chloride.

Proteobacteria δ- and ε-subgroups were isolated mainly on PCE, with the exception of *Desulfomonile tiedjei*, the first dehalorespirer to be isolated, which grows with 3-chlorobenzoate. *Dehalococcoides ethenogenes* was the first isolated strain of a genus, which shows promissing degradation capacities. Although the phylogeny of this genus is not clear, it appears to be most closely related to the Chloroflexi (green non-sulfur bacteria). Whereas *D. ethenogenes* is the only bacterium able to completely dechlorinate PCE to ethene, several strains have recently been isolated that specifically dechlorinate lower chlorinated ethenes (*Dehalococcoides* sp. strains BAV1 and VS) or chlorobenzenes (*Dehalococcoides* sp. strain CBDB1).

1.3.2 Metabolic diversity of dehalorespiring bacteria

Within the twenty species listed in Table 1.1, there is quite diversity in the use of electron donors and acceptors. Electron donors have been shown to play an important role in the efficiency and extent of the reduction of chlorinated compounds for pure cultures (24, 32, 51, 59) as well as for enrichments (27, 40, 55, 56, 92). Hydrogen and formate are used as source of electrons by most dehalorespiring bacteria and are the only substrates with which a dehalorespiration process has been unequivocally demonstrated (28, 51, 72). *Desulfitobacterium* spp. and *Sulfurospirillum* spp. often use additionally lactate and pyruvate, the latter compound being also fermented. Members of the genera *Dehalobacter* and *Dehalococcoides* are restricted to hydrogen as electron donor (23, 39, 42, 47). One isolate, *Desulfuromonas chloroethenica*, has been shown to use only acetate as electron donor (49). Furthermore, some volatile fatty acids, ethanol, and phenyl methyl ethers (75) can also serve as electron donors for some dehalorespiring bacteria.

Two major groups of chlorinated compounds are commonly used as terminal electron acceptor in the dehalorespiration process: chlorinated aromatics and aliphatics. While the former group includes a large variety of compounds such as chlorinated phenols, benzoates, benzenes, and phenoxyacetates, the latter contains chloroethenes and some chloroethanes (Table 1.1). Chlorophenols are exclusively dechlorinated by members of the versatile genus *Desulfitobacterium*. Whereas chlorobenzoates and chlorobenzenes serve as electron donors for only a few bacteria (*D. chlororespirans* and *D. tiedjei*, and *Dehalococcoides* strain sp. CBDB1, respectively), PCE and TCE are used by organisms of all four phylogenetic groups containing dehalorespiring bacteria. Dehalorespiring bacteria show a variable spectrum of chlorinated compounds to be used as electron acceptors. For example, *Sulfurospirillum* strains only dechlorinate PCE and TCE, whereas *Desulfitobacterium* sp. strain PCE1 is able to use PCE, Cl-OHPA and various chlorinated phenols. Also the extent of dechlorination can vary. *D. ethenogenes*, *D. restrictus* and *Desulfitobacterium* sp. strain PCE1 all use PCE as electron

acceptor, but with different final dechlorination products. They produce either ethene, *cis*-1,2-DCE, and TCE, respectively, possibly the consequence of the presence of different dehalogenases in different strains (61, 97).

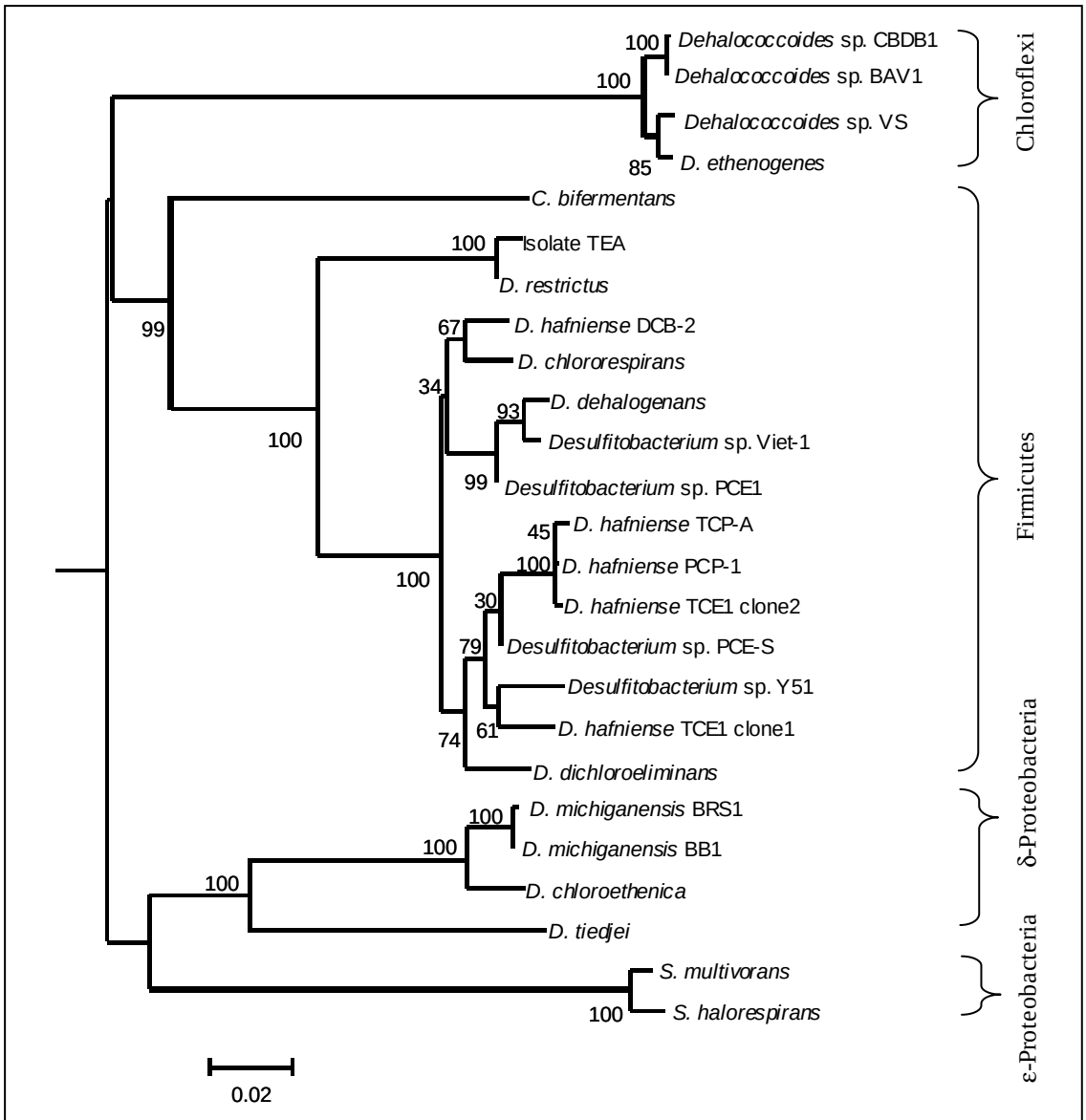

Figure 1.1. Unrooted phylogenetic tree of 16S rRNA gene sequences of dehalorespiring bacteria. The sequences were aligned using ClustalX and graphically represented with TreeExplorer version 2.12.

Alternative non-chlorinated electron acceptors were also reported for dehalorespiring bacteria (Table 1.1). Nitrate, fumarate, sulfite and thiosulfate are the most common ones, again mainly used by members of the versatile genus *Desulfitobacterium*. *S. multivorans* has been shown to use also arsenate and selenate as electron acceptors. Inhibition of the dechlorination activity has been found in the presence of sulfite and thiosulfate for a few strains (26, 82, 96). However, it is not always clear whether the non-chlorinated electron acceptor is preferentially used or whether the reduced dechlorination activity is due to direct inhibition of the key enzymes involved in the

dehalorespiration process by the alternative electron acceptor. No alternative non-chlorinated electron acceptor has been found for members of the genera *Dehalobacter* and *Dehalococcoides*, raising the question of the evolution of such specialized strains.

1.3.3 Topology of the dehalorespiration chain

Some aspects of the electron transport chain involved in dehalorespiration have been studied for only a few dehalorespiring bacteria. There are different possibilities how the topology of the dehalorespiration chain could look like. With hydrogen as electron donor, the hydrogenase and the reductive dehalogenase can be located either both on the outside or inside of the cytoplasmic membrane, or the hydrogenase on the outside and the dehalogenase on the inside (84). Whereas the scalar production of protons upon hydrogen oxidation in the latter configuration would be sufficient to create a proton motive force, vectorial proton translocation during electron transfer is required for the other two. It is now well established that the electron-donating enzymes (hydrogenase or formate dehydrogenase) are membrane-bound. Experiments with intact cells and the artifical membrane-impermeable electron acceptor methyl viologen and the membrane-impermeable hydrogenase inhibitor Cu^{2+} have demonstrated that hydrogenase and formate dehydrogenase are located on the outside of the cytoplasmic membrane (53, 84). The terminal electron-accepting enzyme in the dehalorespiration process, the reductive dehalogenase (RDase), has been found to be associated with the membrane, with two exceptions where the dehalogenase activity has mainly been detected in the soluble fraction (Table 1.2). It is still controversial whether the reductive dehalogenase is facing the inside or the outside of the cytoplasmic membrane. For some dehalorespiring bacteria, an increase of the dechlorination activity has been observed with permeabilized cells using membrane-impermeable methyl viologen as electron donor (67, 69, 84, 96), indicating that the RDase is facing the inside of the membrane. However, the same experimental approach done with *D. ethenogenes* did not show a change of the dechlorination rate (45, 79). In addition, immunoblotting with antibodies against a soluble PceA fusion protein indicated the presence of this enzyme in the periplasmic protein fraction of *Desulfitobacterium* sp. strain Y51 (91). Finally, it is also possible that the topology of the dehalorespiration chain is different in different organisms.

1.3.4 Other components of the electron transport chain

In *D. restrictus*, menaquinones have been shown to mediate the transfer of electrons within the cytoplasmic membrane from the electron-donating hydrogenase to the terminal reductive dehalogenase. This has been shown by optical difference spectra, and by the use of the

menaquinone-analogue 2,3-dimethyl-1,4-naphthoquinone and the specific inhibitor 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (84). Menaquinones and other unidentified quinones have been detected in *S. multivorans* (83), *D. dehalogenans* (96), and *D. tiedjei* (53), but no indication for their involvement in electron transfer has been presented. Cytochromes, other common electron-mediating components of anaerobic respiration chains, have also been detected in different dehalorespiring organisms. *B*-type cytochromes have been found in *S. multivorans* (83) , *D. restrictus* (42) and *D. dehalogenans* (96). Evidence of their involvement in the electron transport has been obtained by optical difference spectra for the two latter bacteria. Membrane-bound *c*-type cytochromes were found in *D. tiedjei* (54), *D. hafniense* strains DCB-2 (21) and TCE1 (32), *D. dehalogenans* (96), *Desulfitobacterium* sp. strain PCE1 (33) and *Desulfuromonas chloroethenica* (49). While biochemical evidence has been reported for the involvement of *c*-type cytochromes in *D. dehalogenans* (96), it has been shown that the cytochrome *c* of *D. tiedjei* is co-induced with the dechlorination activity (54). However, no evidence has been presented for this cytochrome to be the physiological electron donor of the RDase.

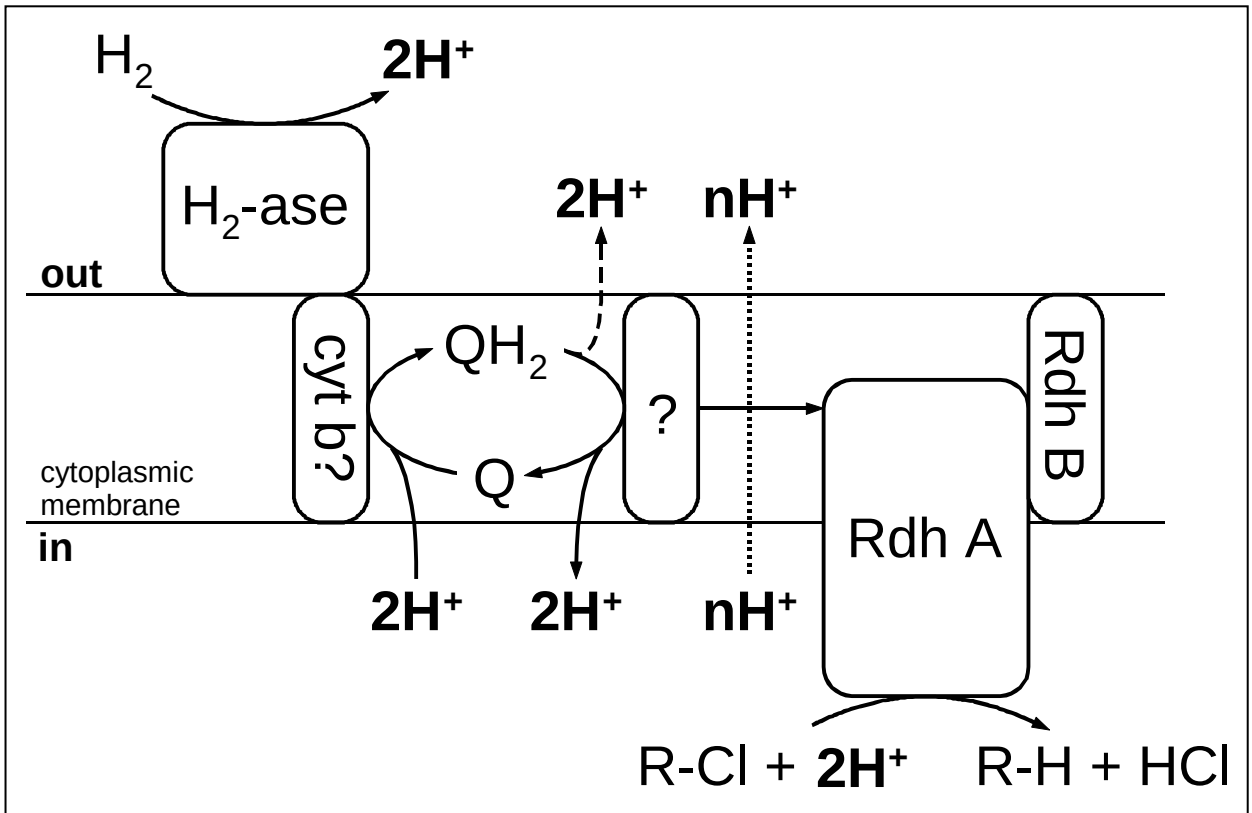

Figure 1.2. Tentative model for the respiration chain of *Dehalobacter restrictus* (from (43)). Electrons are transferred from hydrogen to the chlorinated compound via the hydrogenase, a cytochrome *b*, the quinone pool, an unknown component, and the reductive dehalogenase. A proton gradient is established by a scalar mechanism. Additional proton translocation may be occur via the quinone pool (dashed arrow) or another mechanism (dotted arrow).

1.3.5 Tentative model of the dehalorespiration chain

The establishment of a proton motive force is the ultimate goal of a respiration process, since it serves as trigger for the synthesis of energy-rich ATP. Investigation of the effect of uncouplers on H^+/e^- ratios of cell suspensions by oxidant pulse experiments has revealed a chemiosmotic coupling of reductive dechlorination and ATP synthesis in *D. tiedjei* (71) and *D. restrictus* (84). However, it is not yet fully understood how the proton gradient across the membrane is established and whether the reductive dehalogenase is facing the cytoplasm or the periplasm. Therefore, several models have been proposed for the electron transport chain of dehalorespiring bacteria (43, 44, 53, 69, 86). Assuming a cytoplasm-facing reductive dehalogenase (86), which suggests a scalar mechanism of proton formation and a H^+/e^- ratio of 1, the model proposed for *D. restrictus* is depicted in Figure 1.2. As mentioned above, other organizations of the dehalorespiration chain cannot be excluded.

1.4 Reductive dehalogenases

1.4.1 Biochemical properties

All biochemically characterized reductive dehalogenases with the exception of three enzymes form a homogeneous class, harboring the same features. They are monomers of a molecular size ranging from 47 to 65 kDa and contain a corrinoid and two iron-sulfur [Fe-S] clusters as cofactors. The involvement of the corrinoid in the reductive dechlorination was evidenced by photoreversible inactivation with iodopropane. Cobalt content analysis and corrinoid extraction by cyanolysis have revealed stoichiometrical amount of corrinoid in various RDases (20, 68, 76, 85, 98). The presence of iron-sulfur clusters was indicated by the analysis of the iron and acid-labile sulfide content of the purified enzymes. When tested, RDases were found to be oxygen-sensitive with half-time inactivation of 50 to 330 min. Their specific activity varies between 3 and 2640 nkat/mg protein (43). Reductive dehalogenases were usually observed in the membrane, although evidence was given that the PCE RDase of *S. multivorans* is located in the cytoplasm (69) and indications that the PCE RDase of *Desulfitobacterium* sp. Y51 is in the periplasmic space (91) (Table 1.2, for the chloroethene RDases). The three atypical RDases are the 3-CB RDase of *D. tiedjei* (78), the PCE RDase of *C. bifermentans* (80) and the 2,4,6-TCP RDase of *D. hafniense* strain PCP-1 (10). The first two enzymes are dimeric proteins, whereas the involvement of corrinoid has been shown for the latter two. Some indications have been presented for the presence of a heme instead of a corrinoid in the 3-CB RDase of *D. tiedjei* (78).

Electron Paramagnetic Resonance (EPR) spectroscopy has been applied to the purified PCE RDase of *D. restrictus* (85) and *o*-CP RDase of *D. dehalogenans* (98). Redox potentials of the Co(I/II) and Co(II/III) transitions have been measured at -350mV and >150mV, respectively, which are relatively high compared to other corrinoid-dependent enzymes. However, only the Co(I/II) couple may have physiological significance in the reductive dechlorination (85). Furthermore, the Co(II) form has been observed in base-off/His-off configuration, which is quite unusual in corrinoid-dependent enzymes. EPR spectroscopy of the predicted iron-sulfur clusters has revealed two different situations in the enzymes of *D. restrictus* and *D. dehalogenans*. While the signal corresponded to two [4Fe-4S] with redox potential of -480mV in the former enzyme (85), one [4Fe-4S] and one [3Fe-4S] clusters with redox potential of -440mV and 70mV, respectively, were detected in the latter one (98). Although various reaction mechanisms have been proposed that take these differences into account (see (43) for details), not much is known yet on enzymatic level. A study implying work with the free chemically reduced corrinoid cofactor and PCE and TCE has indicated a mechanism involving the formation of radicals (34).

1.4.2 Molecular properties

In January 2001 the sequence of three biochemically characterized reductive dehalogenases were present in databases: the PCE RDase of *Sulfurospirillum multivorans* (77), the *o*-CP RDase of *Desulfitobacterium dehalogenans* (98) and the TCE RDase of *Dehalococcoides ethenogenes* (60). Percentage of sequence identity among these three RDases ranged between 26 and 32% (43). Figure 1.3 depicts the sequence alignment and the main conserved regions among these RDases. The N-terminus presents the consensus sequence of the Twin-Arginine Translocation (Tat) system dedicated to the incorporation or export of complex holoenzymes in or across the cytoplasmic membrane (5). Since most of the Tat-dependent enzymes are transported to the periplasmic space of bacteria, the presence of the Tat signal peptide on the cytoplasm-oriented RDases may appear as a contradiction. However, another example of a cytoplasmic-facing membrane-bound Tat-dependent enzyme has been reported, namely the DMSO reductase of *E. coli* (104).

Two iron-sulfur cluster binding motifs are present in the C-terminal part of RDases (Figure 1.3). While the first motif (CxxCxxCxxxCP) is strictly conserved among the three enzymes and corresponds exactly to ferredoxin-type iron-sulfur cluster (as referred in the PROSITE database under domain PS00198, www.expasy.org/prosite/), the second one shows some variation in the number of residues between the first two cysteines. Whereas the second motif of the TCE RDase

Table 1.2. Biochemical properties of chloroethene reductive dehalogenases.

Strain	Enzyme	Substrate [1]	Localization	Molecular mass (kDa)	Cofactors	Spec. activity (nkat/mg protein)	References
D. restrictus	PCE RDase	PCE, TCE	membrane	60	1 corrinoid [4,5,6] 2 [4Fe-4S] [4]	234	(63, 85)
D. hafniense strain TCE1	PCE RDase	PCE, TCE	membrane	59	corrinoid [6]	167	(97)
Desulfitobacterium sp. PCE-S	PCE RDase	PCE, TCE	membrane	65	1 corrinoid [5,6] 8Fe/8S [7]	650	(68)
Desulfitobacterium sp. Y51	PCE RDase	PCE, TCE	soluble [2]	58	corrinoid [6]	3	(91)
Desulfitobacterium sp. PCE1	PCE RDase	PCE	membrane	48	corrinoid [6]	92	(97)
S. multivorans	PCE RDase	PCE, TCE	soluble [3]	57	1 corrinoid [5,6] 8Fe/8S [7]	2640	(77)
D. ethenogenes	PCE RDase	PCE	membrane	51	corrinoid [6] Fe/S	350	(61)
D. ethenogenes	TCE RDase	TCE, cis-DCE, VC	membrane	61	corrinoid [6] Fe/S	350	(61)
Clostridium bifermentans	PCE RDase	PCE, TCE, DCEs	membrane	70 (2x35)	corrinoid [6]	1.2	(80)

[1] DCE: dichloroethene; PCE: tetrachloroethene; TCE: trichloroethene; VC: vinyl chloride.
[2] Some indications have been given that the PCE RDase of *Desulfitobacterium* sp. strain Y51 is located in the periplasmic space (91).
[3] Clearly evidenced in the cytoplasm.
[4] Determined by EPR spectroscopy.
[5] Quantified by analysis of cobalt content and by extraction of corrinoid from purified enzyme followed by spectroscopic analysis.
[6] Indicated by photo-reversible inhibition of the reduced enzyme by iodo-alkanes.
[7] Quantified by atomic absorption spectroscopy and by standard procedures for acid-labile sulfide.

of *D. ethenogenes* is identical to the first one (with two residues between C_1 and C_2), the two other enzymes harbor a stretch of 10 and 12 residues in between. However it has been postulated for proteins harboring two $C_1xxC_2xxC_3xxxC_4P$ motifs, that the two [4Fe-4S] clusters are not bound by the individual motifs, but that the cysteines C_1, C_2 and C_3 of one motif together with C_4 of the other motif are the effective ligands of the four irons in a single [4Fe-4S] cluster (Figure 1.4). This binding mode seems to stabilize the tertiary structure of the protein and to bring both iron-sulfur clusters into close vicinity and therefore help in transferring the electrons from one cluster to the other (88). The *o*-CP RDase of *D. dehalogenans* is the only RDase so far, which allows a direct sequence-structure comparison of the iron-sulfur clusters. Although the presence of one [3Fe-4S] and one [4Fe-4S] cluster has been clearly observed by EPR spectroscopy (98), the cysteine motifs

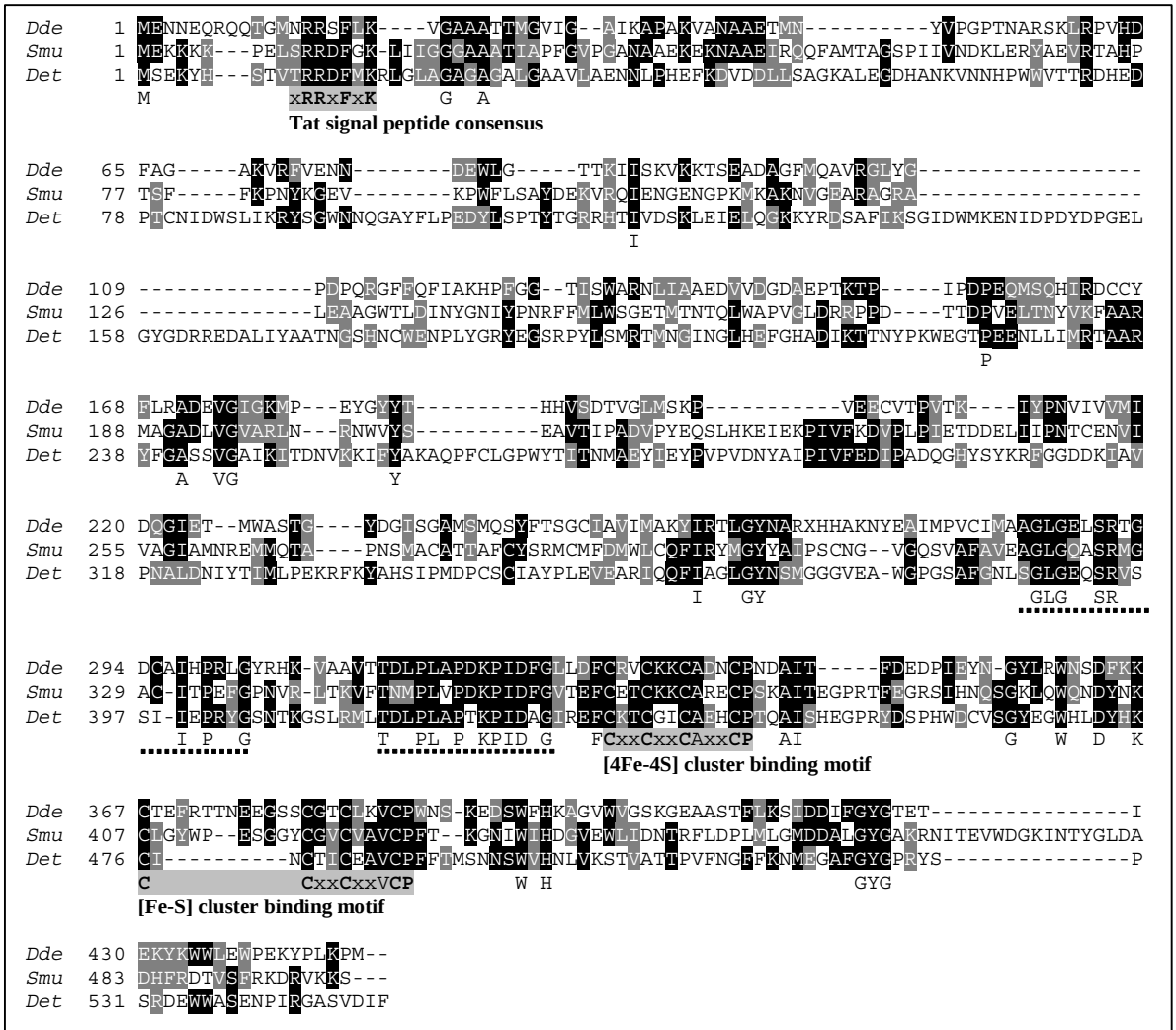

Figure 1.3. Sequence alignment of three biochemically well characterized reductive dehalogenases. Fully conserved residues are indicated below the alignment. Conserved motifs with predicted function are indicated by grey boxes, in which the conserved residues are in bold. Dotted lines indicate other conserved stretches with unknown function. *Dde*: *o*-CP RDase of *Desulfitobacterium dehalogenans*; *Smu*: PCE RDase of *Sulfurospirillum multivorans*; *Det*: TCE RDase of *Dehalococcoides ethenogenes*.

do not resemble those found in proteins with similar structure (14, 73), but are rather like two [4Fe-4S] cluster binding motifs. However the 12 amino acid stretch between the two first cysteines of the second motif may lead to the loss of one cysteine ligand, forming the [3Fe-4S] cluster (W.R. Hagen, personal communication).

The B_{12}-binding motif that has been defined in a subset of B_{12}-dependent enzymes (**D**x**H**xx**G**, (57)) was not found in RDases. This may be correlated to the base-off/His-off form observed for the Co(II)-state of the corrinoid in the *o*-CP RDase of *D. dehalogenans* (98). Similar observation has been made for the PCE RDase of *D. restrictus* (85), from which however no sequence was available. Two further amino acid stretches are well conserved among the three RDases (Figure 1.3, dotted lines). However, no function could be assigned.

Another conserved feature of RDases is the presence of a short ORF in the direct vicinity of the gene coding for the catalytic unit of RDases (named *rdhA*). The predicted proteins (PceB of *S. multivorans*, CprB of *D. dehalogenans* and TceB of *D. ethenogenes*) show weak sequence identity with each other. However, the reductive dehalogenase B subunits (RdhB) seem to be conserved on a structural basis, since they show two to three hydrophobic α-helices, indicating that they may be located in the cytoplasmic membrane and play a role in anchoring the catalytic unit RdhA of the RDase (77, 98). Co-expression of the *rdhA* and *rdhB* genes has confirmed the importance of the second ORF (77, 87).

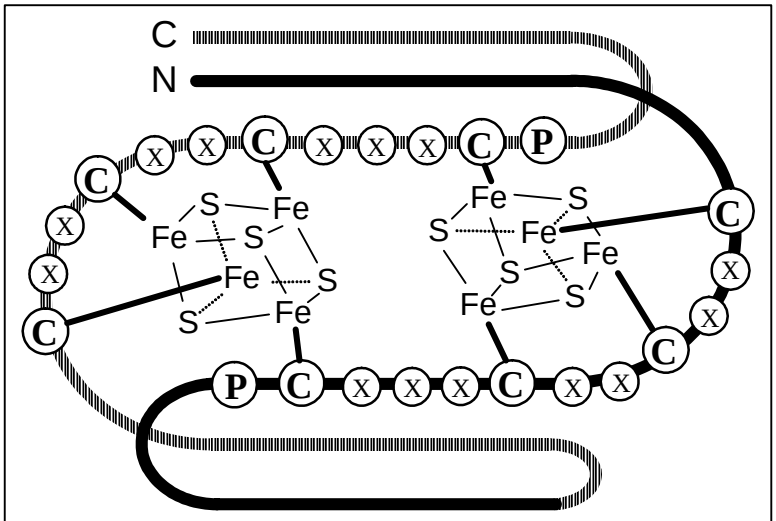

Figure 1.4. Typical binding mode of two [4Fe-4S] clusters. Each iron-sulfur cluster is bound by the first three cysteines of one motif and the fourth one of the other. Adapted from (17).

The *cpr* locus of *D. dehalogenans*, including *cprA* coding for the catalytic unit of the *o*-CP RDase, has been more extensively investigated (87). The *cpr* locus contains eight genes (including *cprA* and *cprB*) in a cluster defined as *cprTKZEBACD*. With the exception of *cprT*, all genes are transcribed in the same direction. Whereas two subunits (CprK, -C) seem to be dedicated to the transcription regulation of the RDase catalytic unit, three subunits (CprT, -E, -D) may play a role in protein folding. It is quite obvious from the complexity of RDases, that these enzymes would need several helper proteins to achieve their correct structure and be finally exported by the Tat machinery towards the cytoplasmic membrane.

1.5 Evolution mechanisms in bacteria

Thanks to the haploid nature of their chromosomes, bacteria are subjected to a relative rapid evolution compared to eukaryotes. It makes them a very good substrate to study the effect of environmental constraints (as taken in a broad sense) (4). Several fine-tuned, but undirected DNA modification strategies have been developed in the prokaryotic world to insure a prompt and efficient response to environmental stress. Several modification levels with variable consequences can be defined: nucleotide substitutions, DNA rearrangements within a genome, and DNA acquisition by horizontal gene transfer. While positive nucleotide substitutions are selected over a large number of generations (vertical modifications) and lead slowly to an improved activity of the affected gene product, DNA rearrangements in form of inversions or small deletions/insertions may lead to strong changes in the expression of the flanking genes, therefore improving the fitness of an already present metabolic activity of the bacteria. More significantly DNA acquisition of specific genes may confer new metabolic capabilities to the affected bacteria (horizontal modifications). Three mechanisms enable bacteria to acquire new DNA: natural transformation, transduction, and conjugation. Natural transformation is characterized by the fortuitous integration of DNA present in the surrounding environment of the bacterial cell (29, 52). Transduction is mediated by the specific machinery of bacteriophages, by which the phage DNA is injected into the bacterial cell (6, 18). Finally conjugation consists of a unique mechanism in which a bacterial cell establishes a direct contact with a recipient cell. This direct cell-cell contact is possible through a specific machinery usually encoded by plasmids or conjugative transposons (15). Next to the classical plasmids and bacteriophages, new structures mediating horizontal gene transfer, so-called mobile genetic elements (MGEs), have been recently discovered. The relatively chaotic nomenclature of MGEs reflects the rapid development of this field. Nowadays certain authors show a clear will of simplification and it will be referred to them in the next section (15, 41, 62).

Insertion sequences (*IS*) represent the smallest unit able to mediate their own mobility. It consists of DNA segments of around 1500 base pairs containing the gene encoding a transposase, the key enzyme in the transposition activity, flanked by two short inverted repeats (for a review, see (62)). *IS* can jump from one location of the bacterial genome to another, thus conferring certain plasticity to the genome. The consequences of the insertion of an *IS* are gene disruption and subsequently loss of the corresponding activity, or regulation of the expression of the surrounding genes. Most often indeed, inverted repeats of *IS* contain partial promoter sequences. *IS* are very often present within structurally different MGEs (see below) and therefore also transferred from one cell to another.

Transposons (*Tn*) are MGEs structurally related to *IS*. Next to the transposase gene, they contain other genes conferring new metabolic activities to the bacteria such as resistance to antibiotics and heavy metals, and degradation of toxic compounds. Three classes of *Tn* have been defined with regard to their structure and mode of transposition.

Integrative and conjugative elements (ICE) regroup several types of MGEs with similar structure and reactivity, conjugative transposons, integrons and genomic islands. Although the size of ICE may strongly vary (10-500kb), they are made of three modules responsible for their maintenance, dissemination and regulation (16). A site-specific recombinase (often named integrase, *Int*), part of the maintenance module, enables the ICE to be excised at the left and right extremities (*attL*, *attR*) and to be circularized. Genes encoding the conjugation machinery are part of the dissemination module. Usually the ICE is transferred to the recipient cell as single strand DNA, where the second strand is synthesized. Re-integration of the ICE in the genome occurs at more or less specific locations and is mediated by the integrase. Typical target locations are 3'-end of tRNAs or A+T-rich regions. The most striking feature of ICE is that they can confer to the host a large variety of metabolic activities, including antibiotic resistance, heavy metal resistance, degradation of toxic aromatic compounds, biosynthesis of antimicrobial compounds, error-prone DNA repair system and symbiosis (16, 41, 93, 99).

1.6 Aims and outline of the thesis

In the last few years, several anaerobic bacteria have been isolated showing metabolic activity towards chlorinated aromatic and aliphatic compounds. A new anaerobic respiration pathway, dehalorespiration, has been discovered involving the chlorinated compounds as terminal electron acceptors. Detailed investigations of some of these strains have revealed the key role of a new class of corrinoid and iron-sulfur clusters containing enzymes, the reductive dehalogenases. At the start time of this thesis, the sequences of only three reductive dehalogenases were available in general databases and almost nothing was known about the natural direct electron donor of this class of enzymes. The overall goals of this thesis were (i) to characterize on a molecular level biochemically well characterized tetrachloroethene reductive dehalogenases, (ii) to investigate the possible involvement of horizontal gene transfer in the evolution of the intriguing dehalorespiration process, and (iii) to identify components of the electron transport chain directing the electrons onto the terminal reductive dehalogenase.

Chapter 2 presents the degenerate PCR approach used to isolate the two tetrachloroethene reductive dehalogenases genes (*pceAB*) of *Dehalobacter restrictus* and *Desulfitobacterium hafniense* strain TCE1 and discusses their homology to other reductive dehalogenases. In **Chapter 3**, the flanking regions of the *pceAB* genes were further investigated, revealing the presence of a composite transposon containing the reductive dehalogenase genes in *D. hafniense* strain TCE1, and the absence of the same in *Dehalobacter restrictus*. **Chapter 4** presents the analysis of the genome of the dehalorespiring bacterium *Dehalococcoides ethenogenes* strain 195 indicating that fifteen of the surprising number of eighteen different copies of putative reductive dehalogenases genes were possibly acquired by horizontal gene transfer. The investigation of different cell fractions of *D. hafniense* strain TCE1 cells grown with PCE or fumarate as electron acceptor showed an enhanced expression of a *c*-type cytochrome upon growth with PCE (**Chapter 5)**. Finally, the results obtained in this thesis are discussed taking into account the numerous sequences of putative reductive dehalogenase genes that have been reported in the time frame of the thesis (**Chapter 6**).

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CHAPTER 2

Isolation and characterization of the genes encoding the tetrachloroethene reductive dehalogenases of *Dehalobacter restrictus* and *Desulfitobacterium hafniense* strain TCE1

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2.1 Abstract

Starting from the N-terminal sequence of the tetrachloroethene (PCE) reductive dehalogenase of Dehalobacter restrictus *and a conserved peptide of eight amino acids present in both PCE and o-chlorophenol (CP) reductive dehalogenases of* Sulfurospirillum multivorans *and* Desulfitobacterium dehalogenans*, respectively, a partial* pceA *gene was isolated by degenerate PCR from* D. restrictus*. Analysis revealed an almost identical sequence as the* pceA *gene present in* Desulfitobacterium *sp. strain PCE-S, which finally helped in isolating the complete* pceAB *gene cluster from* D. restrictus*. The same gene cluster was also isolated from* Desulfitobacterium hafniense *strain TCE1, another strain with a PCE reductive dehalogenase harboring a very similar N-terminal sequence. A detailed sequence analysis of PceA revealed the presence of a Twin-Arginine Translocation (Tat) signal peptide, of two iron-sulfur cluster binding motifs, but no consensual binding motif for the corrinoid. While the first iron-sulfur cluster binding motif is like typical ferrodoxin motifs ($Cx_2Cx_2Cx_3CP$), the second one shows a stretch of ten residues between the first and the second cysteine ($Cx_{10}Cx_2Cx_3CS$), which is in apparent contradiction with the presence of two [4Fe-4S] clusters as determined by EPR spectroscopy. However, in contrast to the CP reductive dehalogenase of* D. dehalogenans*, the extremities of the amino acids stretch are structure-breaking residues, which allow the formation a second [4Fe-4S] cluster. A further conserved feature of PCE reductive dehalogenases seems to be the relative position of the* A-B *genes, in contrast to* B-A *in CP reductive dehalogenases.*

2.2 Introduction

The PCE reductive dehalogenase (PCE RDase) of *Dehalobacter restrictus* has been purified and thoroughly characterized, revealing a 60 kDa protein with one corrinoid and two [4Fe-4S] clusters as cofactors (8, 13), similarly to the PCE reductive dehalogenase of *Sulfurospirillum multivorans* (10) and *Desufitobacterium* sp. strain PCE-S (9), and the CP RDase of *Desulfitobacterium dehalogenans* (18). While the full-length sequence of the corresponding genes have only been obtained for the enzyme of *S. multivorans* (11) and *D. dehalogenans* (18), the N-terminal sequences of the proteins have been reported for the reductive dehalogenases of *D. restrictus* (8), *Desulfitobacterium* sp. strain PCE-S (9) and *Desulfitobacterium hafniense* strain TCE1 (17). They appeared to be almost identical (*D. restrictus*: ADIVA PITET SEFPY KVDAK). Starting from this sequence information, this chapter reports on the isolation of the PCE RDase sequences (designated as RdhA, to distinguish with the biochemical activity) of *D. restrictus* and *D. hafniense* strain TCE1, and discusses the conserved protein features of this new corrinoid-dependent class of enzymes.

2.3 Materials & Methods

DNA Isolation. Cells were harvested by centrifgution at 5000 rpm for 10 min, resuspended in a lysis buffer containing 20 mM Tris (pH 8.0), 10 mM NaCl, 1 mM EDTA, 100 µg/ml Proteinase K and 0.5% SDS, and incubated 6 h at 50°C. One volume of Phenol/Chloroform/Isoamylalcohol 25:24:1 was added. After incubation for 10 min at room temperature and centrifugation at 6000 x g and 10°C for 20 min, the aqueous phase was transferred in a fresh tube and again extracted with one volume of Phenol/Chloroform/Isoamylalcohol. The sample was mixed and incubated on ice for 5 min and finally centrifuged at 18000 x g and 4°C for 15 min. The aqueous phase was again transferred and remaining phenol was extracted with one volume of diethylether. Finally the DNA was precipitated with ethanol, washed and dried in a vacuum centrifuge. Plasmid DNA isolation was performed using the QIAprep Spin Miniprep Kit (Qiagen AG, Basel, Switzerland).

DNA Amplification. Oligonucleotides (Microsynth GmbH, Balgach, Switzerland) used in this study were DR3f 5'-GA(C/T) ATI GTI GCI CCI ATI AC-3', DR4r 5'-CC(A/G) AA(A/G) TCI ATI GG(C/T) TT(A/G) TCI GG-3', PCE1f 5'-ATG CAA TTA TTA TTA AGG AGG AAG-3', PCE2r 5'-CTA AGC AGA AAT AGT ATC CGA ACT-3', T7 promoter primer 5'-TAA TAC GAC TCA CTA TAG GG-3' and SP6 promoter primer 5'-ATT TAG GTG ACA CTA TAG-3'. In

order to avoid too high level of degeneration of primers DR3f and DR4r, inosine (I) was inserted for 3-4 fold degenerate bases.

Degenerate PCR was performed under the following conditions: a 50 µl PCR mixture contained 5 µl of Taq DNA Polymerase 10 x Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 5 µM each degenerate primer DR3f and DR4r, 2.5 units of Taq DNA Polymerase (Promega, Catalys AG, Wallisellen, Switzerland). 50 ng of *D. restrictus* genomic DNA was used as template. The DNA was amplified in a T3 Thermocycler (Biometra, Biolabo Scientific Instruments, Châtel-St-Denis, Switzerland) with the following program: 3 min preheating at 94°C, 36 cycles of 30 s denaturation at 94°C, 1 min of primer annealing at 50°C, 2 min of elongation at 72°C. A final extension step of 10 min at 72°C was included. Specific PCR using the primers PCE1f and PCE2r was performed under the following condition: a 50 µl PCR mixture contains 5 µl of Pfu DNA Polymerase 10 x Buffer, 0.2 mM dNTPs, 1 µM each primer, 5 units of proofreading Pfu DNA Polymerase (Promega, Catalys AG, Wallisellen, Switzerland). A PCR reaction was performed in 30 cycles of 30 s denaturation at 94°C, 1 min primer annealing at 56°C and 2 min elongation at 72°C. A final 10 min extension step at 72°C was included.

PCR products purification, cloning and selection of clones. PCR products were analyzed through agarose gel elelctrophoresis and purified using the PCR Purification Minelute Kit (Qiagen AG, Basel, Switzerland), eluted in 10 µl final volume. Previous to ligation 7 µl of purified PCR products amplified with Pfu DNA Polymerase were incubated 30 min at 70°C with 1 µl of Taq DNA Polymerase 10 x Buffer, 0.2 mM dATP and 5 units of Taq DNA Polymerase. For cloning, PCR products were ligated into pGEM-T easy vector (Promega, Catalys AG, Wallisellen, Switzerland) according to the manufacturer's instructions. Ligated products were transformed into $CaCl_2$ competent *E. coli* DH5α cells following the standard heat shock protocol. Transformed cells were incubated 1 h at 37°C on a rotary shaker at 200 rpm before plating onto LB plates containing 100 µg/ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal (Blue/white selection). White *E. coli* colonies were resuspended in 10 µl dH_2O, lyzed for 10 min at 95°C and subsequently briefly centrifuged. One µl of supernatant was used as template in a 10 µl PCR reaction using 1 µM of T7 and SP6 promoter primers. The PCR products were analyzed through agarose gel electrophoresis. Clones containing DNA fragments of expected length were selected.

DNA Sequencing and Sequence Analysis. Cycle sequencing reactions were performed using the BigDye Terminator v3.0 Ready Reaction Kit (Applied Biosystems, Rotkreuz, Switzerland) according to the manufacturer's instructions. Following primers were used: T7 and SP6 promoter

primers and *pceAB* specific internal primers. Samples were run in the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Rotkreuz, Switzerland).

Sequence alignment was performed using a local version of T-coffee Mocca (12). Comparison with databanks was done with Blast (1).

Nucleotide sequence accession number. The *pceAB* genes of *D. restrictus* and *D. hafniense* strain TCE1 have been submitted to EMBL and got the accession numbers AJ439607 and AJ439608, respectively. The *pceAB* genes of *Desulfitobacterium* sp. strain PCE-S has been submitted to GenBank and obtained the accession number AY216592 (G. Diekert, personal communication).

2.4 Results and Discussion

2.4.1 PCR amplification, cloning and sequencing of the *pceAB* genes of *D. restrictus* and *D. hafniense* strain TCE1

The forward primer DR3f was designed according to part of the N-terminal sequence (DIVAPIT) of the purified PCE reductive dehalogenase (PceA) of *D. restrictus* (8), resulting in 2-fold degeneration containing 5 neutral bases (inosine). The reverse primer DR4r was designed based on a conserved 8 amino acid stretch (PDKPIDFG) found in the only two RdhA sequences reported at that time in databases: PceA of *S. multivorans* and in the CprA of *D. dehalogenans* (11, 18). This resulted in a 16-fold degenerate primer containing 3 inosines. Using genomic DNA of *D. restrictus* as template, the degenerate PCR resulted in a product of approximately 1100 bp which was purified, cloned (giving the plasmid pDR1), and sequenced. Sequence analysis revealed an 1126 bp DNA fragment. Compared with the sequence of PceA from *Desulfitobacterium* sp. strain PCE-S, obtained from G. Diekert prior to publication, the PCR product matched at 97% identity on protein level. Therefore, new primers specific to the begin of the *pceA* gene and to the end of the *pceB* gene of *Desulfitobacterium* sp. strain PCE-S were designed, which allowed the isolation of the *pceAB* gene clusters from *D. restrictus*. Since the N-terminal sequence of the PceA of *D. hafniense* strain TCE1 was found to be very similar to the previous ones (17), the same pair of specific primers was used to isolate the *pceAB* genes from this strain. The resulting plasmids were called pDR2 and pTCE, respectively. Both plasmids were completely sequenced in both directions.

2.4.2 Isolation of the *pceAB* genes

Both plasmids pDR2 and pTCE carried two ORFs of 1656 and 318 bp named *pceA* and *pceB* in analogy to the *pceA* and *B* genes found in *S. multivorans* (11). As already indicated by the very similar N-terminal sequence of the proteins, the sequence of the *pceAB* gene clusters of *D.*

restrictus and *D. hafniense* strain TCE1 were found to be very close to each other (13 of 551 amino acids are different) and almost identical to the *pceAB* sequences of *Desulfitobacterium* sp. strain PCE-S and of the recently isolated *Desulfitobacterium* sp. strain Y51 (15). These four sequences originating from four different species within two genera form a very conserved cluster, named here *Dehalobacter-Desulfitobacterium pceAB* group.

2.4.3 Physical map of the *pceAB* gene cluster

2.4.3.1 The Twin-Arginine signal peptide of PceA

The physical map of the *pceA* and *pceB* genes of the four above mentioned bacterial strains is depicted in Figure 2.1. The *pceA* gene is 1656 bp long and codes for a 551 amino acids protein which has a theoretical molecular weight of 61,299 Da in its unprocessed form. As observed for all other chloroethene RdhA sequences, this PceA also contains a leader sequence that is 39 amino-acids long and contained a twin-arginine motif (RRxFLK) usually found in proteins that are exported to the periplasm and contain redox cofactors (21). However, the residue in front of the two arginines is not a serine or threonine as it has been speculated to be typical for this motif (2), but an asparagine which is found quite frequently in RdhA sequences. In fact, only TceA of *Dehalococcoides ethenogenes* and PceA of *S. multivorans* (7, 11) respect fully the twin-arginine motif proposed by Berks (2), all the others have an asparagine instead. The signal peptide cleavage site (ADA↓ADIVA) respects the "-1/-3" rule as defined by von Heijne (19). The theoretical molecular mass of the processed PceA is 57,372 Da. Taking into account the eight iron and sulfur atoms and the cobalamin cofactor the molecular mass sums up to 59,426 Da which is close to the 60.1 kDa estimated from the purified PceA of *D. restrictus* on a SDS gel (8).

2.4.3.2 The iron-sulfur cluster binding motifs of PceA

A motif very similar to the binding motifs for two [4Fe-4S] iron-sulfur clusters (3) was present towards the C-terminal part of the PceA (Fig. 7A). The motif for the first four cysteines ($Cx_2Cx_2Cx_3CP$) is found among all RdhAs. They contain residues in between the cysteines that are quite conserved leading to the consensus sequence CRxCKKCADxCP that seems to be very specific for RdhAs using the pattern searching software against general protein databases (PATTERNp (4)). The most variations to that consensus were found in TceA of *D. ethenogenes* and PceA of *S. multivorans* (7, 11). The second half of the two [4Fe-4S] iron-sulfur cluster binding motif is less conserved among RdhAs. In the four PceA sequences discussed here it can be defined as $Cx_{10}Cx_2Cx_3C$ missing a proline at the end of the motif and having the first two cysteines

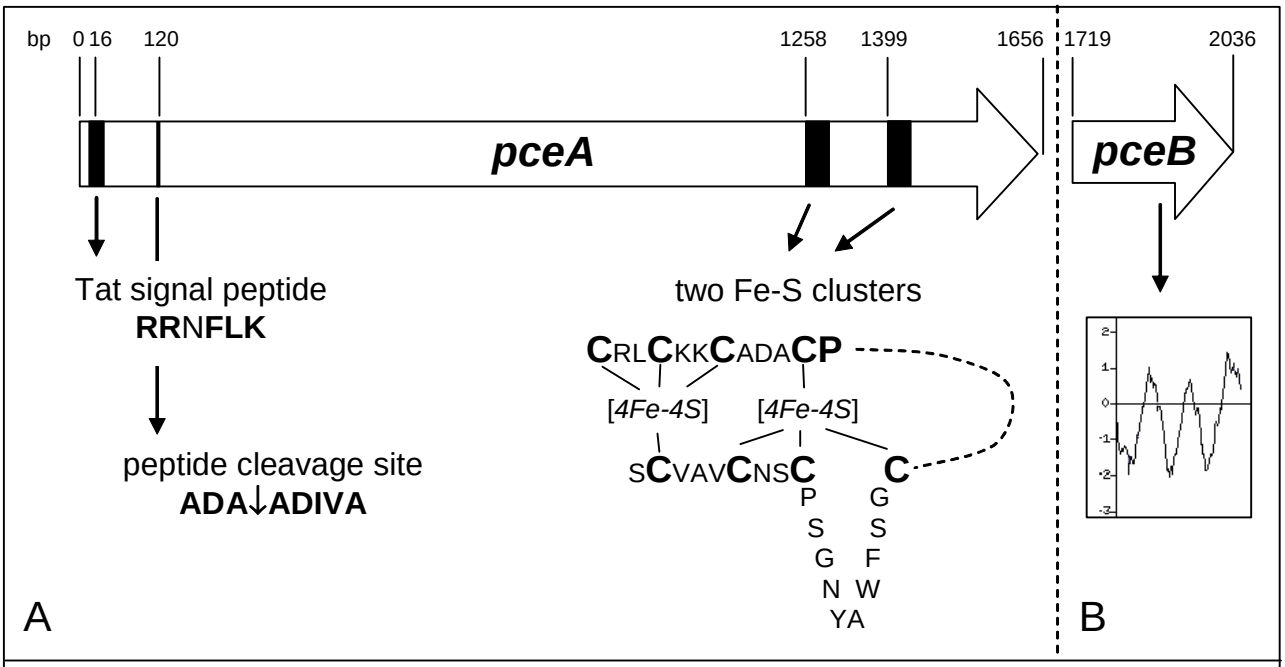

Figure 2.1. Physical representation of the PceA and putative PceB anchor protein of the *Dehalobacter-Desulfitobacterium* group. (A) PceA and its features: the Tat signal peptide with a conserved RRxFLK signature, the peptide cleavage site, and two [4Fe-4S] clusters towards the C-terminal end. (B) PceB putative protein and a hydrophobicity plot indicating the presence of three transmembrane α-helices with the N-terminus pointing towards outside (20). The Kyle-Doolittle hydrophobicity plot was obtained by using the software Protein Hydrophilicity/Hydrophobicity Search (Bioinformatics Unit, Weizmann Institute of Science, Rehovot, Israel).

separated by ten instead of two amino acids. In other RdhAs, the proline is present and the first two cysteines are separated by variable stretches of 2 residues for TceA of *D. ethenogenes* leading to an identical motif as found for Fe_8S_8 ferredoxins (7), 10 residues for PceA of *S. multivorans* (11), and 12 residues for all CprA's ((18) and Genbank AY013365 and AF204275, unpublished data). It is therefore not possible to deduce a general consensus sequence for the second iron-sulfur cluster motif for all RdhAs. EPR measurements with the purified enzymes allowed clearly defining the kind of iron-sulfur clusters present in CprA of *D. dehalogenans* (18) and PceA of *D. restrictus* (13). The enzyme of *D. dehalogenans* contains one [4Fe-4S] and one [3Fe-4S] cluster, the one of *D. restrictus* two [4Fe-4S] clusters. In the amino acid sequence of both enzymes the first cysteine of the second motif seems to be missing being replaced by a glycine. However, both RdhAs contain a cysteine further upstream, 12 amino acids for *D. dehalogenans*, and 10 amino acids for *D. restrictus*. The ten amino acid stretch of the latter enzyme starts with a glycine and ends with a proline, two structure breaking residues, indicating the formation of a loop in the tertiary structure allowing the participation of the cysteine that is ten amino acids upstream as a ligand in a [4Fe-4S] cluster. The 12 amino acid stretch of CprA of *D. dehalogenans* does not start or end with structure breaker amino acid, indicating that this cysteine may not be involved in iron-sulfur cluster binding. The presence of a [3Fe-4S] cluster in this enzyme corroborates with this hypothesis.

2.4.3.3 Unknown cobalamin binding site in PceA

Since the cobalt in cob(II)alamin of the PceA of *D. restrictus* was not coordinated by a fifth ligand according to EPR spectroscopy (13), it was not surprising that the PceA does not contain a corrinoid binding motif (DxHxxG) as found in vitamin B_{12}-dependent mutases and methionine synthases, where the histidine is responsible for the binding of the corrinoid (6). This suggests that in RdhAs another binding motif is responsible for the insertion of the corrinoid cofactor.

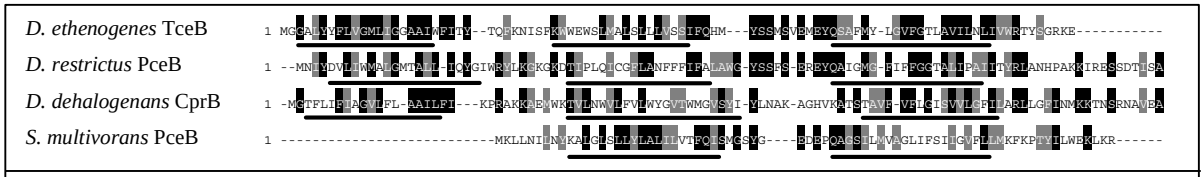

Figure 2.2. Sequence alignment of the ORF B of biochemically characterized RDases. The sequences were first aligned using T-coffee, then the alignment was manually improved regarding not only the residues, but also the relative position of the predicted α-helices (black bars).

2.4.3.4 The conserved structure of PceB

A spacer of 62 bp separates genes *pceA* and *pceB* of *D. restrictus* (Fig. 7). Despite the very low sequence homology of putative RdhB proteins (Figure 2.2), a common feature is their conserved secondary structure. It always appears as a stretch of three hydrophobic transmembrane α-helices, with exception of the putative PceB of *S. multivorans* where only two helices are observed (11). Hence, these putative RdhB proteins seem to be functionally conserved. It has been speculated that the putative RdhB protein is active in anchoring RdhA in or to the membrane but there is no biochemical evidence presented so far. The only indication for a functional RdhB protein is the co-expression of the *rdhA* and *B* genes that has been shown for *cprBA* of *D. dehalogenans* (14) and *pceAB* of *S. multivorans* (11). In contrast to the *pceAB* gene clusters, all the *cprB* genes are located in the direct upstream region of their *cprA* counterpart (*Desulfitobacterium dehalogenans* (18), *Desulfitobacterium chlororespirans* Genbank AF204275, *Desulfitobacterium* sp. strain PCE1 and strain *Desulfitobacterium* sp. strain Viet-1 AY013360 and AF259791, *Desulfitobacterium hafniense* strain DCB-2 AY013365 and AF403180 to AF403185).

2.4.4 Sequence homology among reductive dehalogenases

Figure 2.3 shows the position of the two newly isolated PceA within the sequences of biochemically characterized RDases. As already mentioned before, the PceA of *D. restrictus* and *D. hafniense* strain TCE1 form together with the RdhAs of *Desulfitobacterium* sp. strain PCE-S and *Desulfitobacterium* sp. strain Y51 a coherent and extremely conserved group with at least 97% identity on DNA as well as on protein level. Comparing the *pceAB* genes of the *Dehalobacter-*

Desulfitobacterium group with the genome data of *D. hafniense* strain DCB-2 (available at the Joint Genome Institute, www.jgi.doe.gov) revealed ORFs with 65% identity on one contig (contig2389, already submitted as clone2977 by Davies & Tiedje, Genbank AF403185). Two overlapping ORFs (ORF1 and ORF2) are matching with PceA, a third one (ORF3) with PceB. Suspecting a frame-shift in the sequence data of the JGI, a fragment covering this region was amplified by PCR from genomic DNA of *D. hafniense* strain DCB-2, cloned and sequenced (data not shown). In this new sequence an additional cytosine was observed at position 1410 of the inverse-complementary sequence of clone2977 (position 309 of ORF1). Once the data corrected, the stop codon of ORF1 (at position 457) does not exist anymore leading to one single 1647 bp ORF instead of two overlapping ones. It encodes a 548 aa putative protein (named pRdhA5) with 66% identity to the PceA of the *Dehalobacter-Desulfitobacterium* group. The homology level is clearly above the typical chlorophenol versus PCE RdhA level (~30% only). In addition, ORF3 which has high homology with *pceB* genes is located directly downstream of the ORF corresponding to the *pceA* gene.

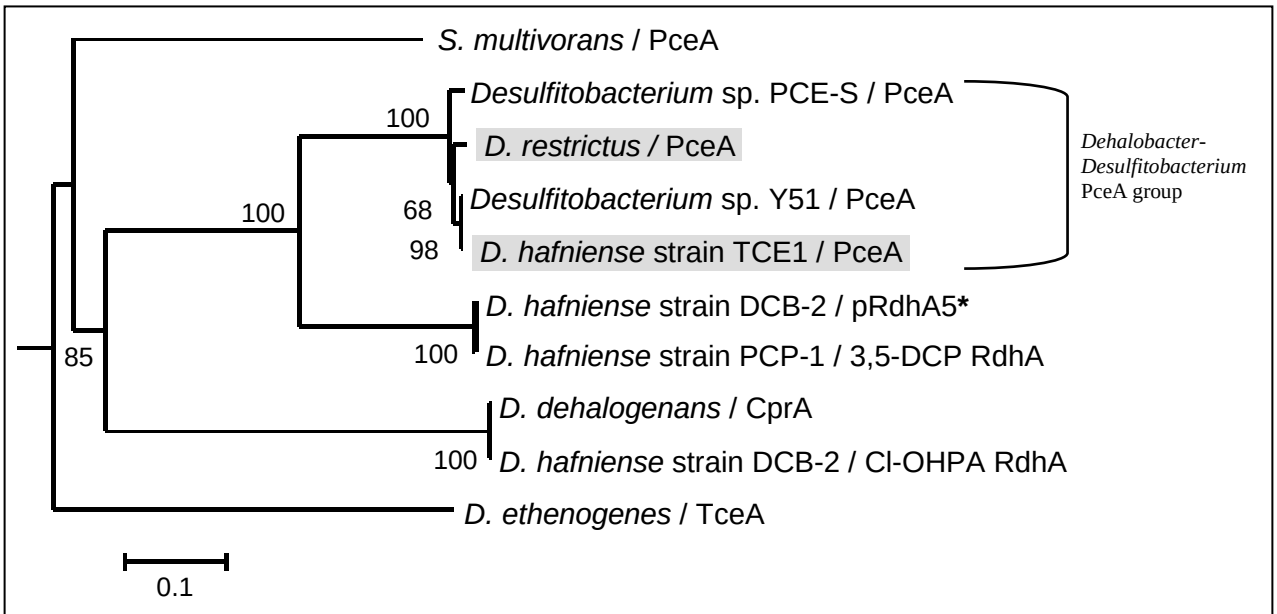

Figure 2.3. Position of the new isolated PceA of *D. restrictus* and *D. hafniense* strain TCE1 within the biochemically characterized RDases. Both new sequences match perfectly with the two PceA of *Desulfitobacterium* sp. strain PCE-S and strain Y51 forming the *Dehalobacter-Desulfitobacterium* PceA group. The star indicates that the sequence of *D. hafniense* strain DCB-2 pRdhA5 was not biochemically characterized, but retrieved from preliminary genome sequence data.

Although already annotated as putative chlorophenol *rdhA* genes, and although *D. hafniense* strain DCB-2 does not show PCE reductive dehalogenase activity (5), the homology level and the relative position of the A and B genes make that these ORFs should rather be named putative chloroethene *rdhA* genes. Interestingly the 3,5-DCP RdhA (specific name: CprA5) of *D. hafniense* strain PCP-1 is 100% identical to pRdhA5 of *D. hafniense* strain DCB-2 and has been shown to mainly dechlorinate chlorophenols with high rates. However, some weak activity towards PCE has been found too ((16), and R. Villemur, personal communication). Although it is tempting to classify the RdhAs in two classes based on sequence homology, the chloroethene and the chlorophenol RdhAs, it is difficult to compare them on a biochemical level since this characterization has not been done rigorously enough in all cases.

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CHAPTER 3

Isolation and characterization of *Tn*-Dha1, a transposon containing the tetrachloroethene reductive dehalogenase of *Desulfitobacterium hafniense* strain TCE1

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3.1 Abstract

A new 9.9 kb catabolic transposon, Tn-*Dha1, containing the gene responsible for the tetrachloroethene (PCE) reductive dechlorination activity, was isolated from* Desulfitobacterium hafniense *strain TCE1. Two fully identical copies of the insertion sequence* IS*Dha1, a new member of the* IS*256 family, surround the gene cluster* pceABCT, *a truncated gene for another transposase and a short open reading frame with homology to a member of the twin-arginine transport system (*tatA*). Evidence was obtained by Southern blot hybridization for an alternative form of the transposon element as a circular molecule containing only one copy of* IS*Dha1. This latter structure represents most probably a dead-end product of the transposition of* Tn-*Dha1. Strong indications for the transposition activity of* IS*Dha1 was given by PCR amplification and sequencing of the intervening sequence located between both inverted repeats of* IS*Dha1 (IR junction). A stable genomic* IS*Dha1 tandem was excluded by quantitative real-time PCR. Promoter mapping of the* pceA *gene, encoding the reductive dehalogenase, revealed the presence of a strong promoter partially encoded in the right inverted repeat of* IS*Dha1. A sequence comparison with* pce *gene clusters from* Desulfitobacterium *sp. strains PCE-S and Y51 and from* Dehalobacter restrictus, *all of which show 100% identity for the* pceAB *genes, indicated that both* Desulfitobacterium *strains seem to possess the same transposon structure, whereas only the* pceABCT *gene cluster is conserved in* D. restrictus.

3.2 Introduction

Four independently purified PCE reductive dehalogenases from four different isolates, namely *Dehalobacter restrictus* (15), *Desulfitobacterium hafniense* strain TCE1 (10), and both *Desulfitobacteria* sp. strains PCE-S and Y51 (21, 33), were found to have an identical N-terminal sequence. The isolation of the corresponding genes (*pceA*) revealed an almost 100% identity at the DNA level (Chapter 2). This raised the question whether horizontal gene transfer could have been involved in the acquisition of this gene.

Transposons and more specifically catabolic transposons are the subject of increasing interest for biodegradation due to the variety of their structure, their mode of action and of course due to the bioremediation potential that they confer to bacteria. Examples of class I composite catabolic transposons are the chlorobenzoate degradative *Tn*5271 and *Tn*5707 from *Alcaligenes* sp. BR60 (22) and *Alcaligenes eutrophus* strain NH9 (25) respectively, the chlorobenzene degradative *Tn*5280 from *Pseudomonas* sp. P51 (38), the benzene degradative *Tn*5542 from *Pseudomonas putida* strain ML2 (9), the toluene sulfate degradative transposon from *Comamonas testosterone* strain T-2 (36), the α-halocarboxylic acid degradative DEH element from *Pseudomonas putida* PP3 (39) and the haloacetate degradative *Tn*Had1 from *Delftia acidovorans* strain B (32). Several catabolic transposons of class II-type or of an undefined type have also been reported (for a review see (34)). Although no clear evidence has been shown for most of them that such composite transposons are able to mediate their own transfer to another location, some structural elements may indicate whether the transposon could be active or whether it is the result of a random distribution of a given insertion sequence around some specific genes. The presence and location of direct repeats (DR) around the whole transposon entity is one of these structural elements. Another is the presence of transposition intermediates such as circular transient forms of the transposon. These latter forms have been observed for several insertion sequences and transposons (2, 4, 5, 12, 17-19, 26-28, 37). Whereas most of these studies report the formation of circular mobile elements from artificial plasmid constructs, few of them were shown to be naturally occurring in a circular form, despite the fact that their low copy number renders their observation by classical methods more difficult.

Here we report the isolation of a composite transposon made of two directly repeated copies of a new insertion sequence, *IS*Dha1, flanking the PCE reductive dehalogenase encoding gene *pceA* together with several other open reading frames, from a PCE-dechlorinating anaerobic bacterium, *Desulfitobacterium hafniense* strain TCE1. Evidence is given for a circular form of this transposon

and several elements are presented to indicate the presence of transposition events. Finally a comparative analysis of the closely related *pce* gene clusters from strains already showing 100% identity for the reductive dehalogenase gene is discussed.

3.3 Material & Methods

Bacterial strains, plasmids, growth conditions. *Desulfitobacterium hafniense* strain TCE1 (DSMZ 12704, former *Desulfitobacterium frappieri* strain TCE1) was cultivated under anaerobic conditions in a medium described before (11) with addition of PCE. The culture was incubated at 30°C at 120 rpm. *E. coli* DH5α (*deo*R, *end*A1, *gyr*A96, *hsd*R17(r_k^- m_k^+), *rec*A1, *rel*A1, *sup*E44, *thi*-1, Δ(*lac*ZYA-*arg*FV169), ϕ80*lac*ZΔM15, F^-,(13)) was used as host for cloning vectors. The strain was cultivated in Luria-Bertani liquid medium and plates containing 100 µg/ml of ampicillin or 50 µg/ml of kanamycin at 37°C. The pGEM-T easy cloning vector (Promega, Catalys AG, Wallisellen, Switzerland) was used as in the manufacterer's instructions.

DNA Isolation and Quantification. Total DNA was isolated as described previously (Chapter 2). DNA samples were quantified using the PicoGreen dsDNA Quantitation kit (Eugene, Oregon, USA) on a TD-700 Fluorometer (Turner Designs, Witec AG, Littau, Switzerland) following the manufacturer's instructions.

Oligonucleotides and DNA Amplification. Oligonucleotides (Microsynth GmbH, Balgach, Switzerland) used in this study are listed in Table 3.1. Standard PCR reactions were performed with the following 50 µl reaction mixture: 5 µl of Taq DNA Polymerase 10 x Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM each primer, 2.5 units of Taq DNA Polymerase (Promega). The DNA was amplified in a T3 Thermocycler (Biometra, Biolabo Scientific Instruments, Châtel-St-Denis, Switzerland) with the following program: 3 min preheating at 94°C, 30 cycles of 45 s denaturation at 94°C, 1 min of primer annealing at 55°C (when not specified), 1-4 min of elongation at 72°C. A final extension step of 10 min at 72°C was included.

Inverse PCR. An aliquot of 150 ng of total DNA was digested with single restriction enzymes (Promega) for 1 h at 37°C in a 25 µl reaction mixture containing: 2.5 µl of 10 x Restriction Buffer, 0.1 mM BSA and 5-10 units of the restriction enzyme. After digestion, 75 µl of water were added, the mixture was extracted with one volume of Phenol/Chloroform/Isoamylalcohol (25:24:1), incubated 10 min on ice and centrifuged for 15 min at 13000 rpm at 4°C. The aqueous phase was transferred to a clean tube and the digested DNA was precipitated by ethanol. The DNA pellet was dissolved in 40 µl of water, to which 5 µl of T4 DNA ligation buffer 10 x, 5 µl 10 mM ATP and 3 units of T4 DNA ligase (Promega) were added. After overnight ligation at 16°C, the re-ligated

DNA was again ethanol precipitated, resuspended in 50 µl of water and used as template for the inverse PCR. DNA amplification of circularized DNA molecules was done with selected primers for inverse PCR (Table1) looking outwards of each other. 5 µl of religated DNA were used as template in 50 µl PCR reaction containing 5 µl of Taq DNA Polymerase 10 x Buffer, 2.5 mM $MgCl_2$, 0.3 mM dNTPs, 1 µM each primer and 5 units of Taq DNA Polymerase (Promega). The PCR program was identical to the one described above with a systematic elongation time of 4 min.

Table 3.1. Oligonulceotides used in this study.

Name	Sequence (5'→3')	Position (bp) on EMBL AJ439608
L1f	AAGCAAAATCAGGCATAGAAGG	84
IS1r	TGCTTCAGCACTTACCTTTTCGG	213 / 8086
IS2r	TGACATGGAGTCAATGCCTAAGG	629 / 8502
IS3f	CGTAGAGCCCATGCCAGAAG	827 / 8700
IS4r	CGACCGGATATATGAGCGAGG	1033 / 8906
IS5f	CACTTCGATTGAGCAGGAACG	1394 / 9267
IS6r	GCTAGCGCCGAGCGTCATGC	1444 / 9346
IS7f	ACATAAAACATTTAGCCTTAAGGG	1647 / 9520
T1r	ACAGCAGCTGCAGCTGCTCC	1833
P1r-FAM	CTTCACCGCAGATGCCGAG	1855
T2f	AAGTTTCATTACGACGATGTTTCC	2018
T2r	GGAAACATCGTCGTAATGAAACTT	2041
T3f	ATCCAACCCGGTCCAGCCTG	2196
T4r	GCGCTGCATAATAGCCAAGC	2846
T5f	GCAAGTTTGATGAGTGGTTCGG	3303
T5r	CCGAACCACTCATCAAACTTGC	3324
T6f	TTTGGATGGCACTGGGTATG	3509
T6r	CATACCCAGTGCCATCCAAA	3528
T7r	CTGGCCTCATTAACCCTATGG	3841
T8f	TAATTGCGTTTGCCTCAGCC	4555
T9r	AGGACAAATCCAAGCGCAATAC	4796
T10f	AGCGTTGGTGGGGAAGCG	5672
T11r	CCAGAGAATCCTTCTGGATGCC	6075
T12f	CCTTGGAATGGCCATATTCG	6638
T13r	AACTTGACGACCTGATTCGCTC	7129
T14f	TTTATTTGGGCCTGAAGAGTTG	7732
T15f	ACATTCGGTTTCTCCGCGTG	7876
R1r	TGTCCTCTCAGTTTCCTAAGCC	9648

PCR products purification, cloning and selection of clones. PCR products were analyzed through agarose gel electrophoresis and purified as previously described (Chapter 2). For cloning, PCR products were ligated into the pGEM-T easy vector (Promega) according to the manufacturer's instructions. Transformation into *E. coli* DH5α and selection of transformants by PCR on colony were done as previously described (Chapter 2).

DNA Sequencing. Cycle sequencing reactions were performed using the BigDye Terminator v3.0 Ready Reaction Kit (Applied Biosystems, Rotkreuz, Switzerland) according to the manufacturer's

instructions. Vector-specific primers T7 and SP6 promoter primers and insert-specific ones were used as sequencing primers. Samples were run on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Sequence Analysis. Sequence analysis was done using the Lasergene software from the DNASTAR package (DNASTAR Inc., Madison, Wisconsin). Comparisons with databases were performed using BLAST at NCBI. Promoter prediction was performed using the BDGP Neural Network Promoter Prediction on-line software for prokaryotes (www.fruitfly.org/seq_tools/promoter.html).

Southern blot hybridization. One µg of *D. hafniense* strain TCE1 total DNA was digested overnight in 50 µl reaction volume with selected restriction enzymes according to manufacturer's instructions (Promega). 5-10 µl of digested products were loaded together with the DNA molecular weight marker II, digoxigenin-labeled (Roche Diagnostics AG, Rotkreuz, Switzerland) onto a 1% TAE agarose gel and electrophoresis run at 10 V/cm. DNA was then transferred to positively charged nylon membrane (Roche) using the alkaline transfer method (30). The DNA was fixed on the membrane by baking it at 120°C for 30 min. Hybridization was performed as described in (1). Probes were labeled using the PCR DIG Probe synthesis kit (Roche) according to manufacturer's instructions. Detection of the probe was done with 1:5000 diluted Anti-Digoxigenin-AP antibody (Roche) and then colorimetrically with the NBT/BCIP reagent (Roche).

RT-PCR. A 50 ml culture of *D. hafniense* strain TCE1 in exponential phase (OD_{600}: 0.2) were harvested, the pellet was resuspended in 150 µl of buffer QRL1 (Qiagen RNA/DNA Mini Kit, Qiagen AG, Basel, Switzerland) and mixed with 200 mg of zirconium beads. The cells were lysed by 3 x 10 s of bead-beating (Mikro-Dismembrator S, B.Braun Biotech GmbH, Meisungen, Germany) cooled on ice between each run. Then the RNA was obtained as indicated by the manufacturer's instructions (Qiagen). Finally the RNA was eluted in 50 µl of RNase-free water. 20 µl of RNA extract was incubated 30 min at 37°C with 2.5 µl of RQ1 RNase-free DNase (Promega) in RQ1 buffer to remove all traces of contaminating DNA. 2.5 µl of RQ1 DNase Stop Solution was added and the sample was incubated at 65°C for 10 min. Reverse transcription was performed as following: 3 µl of the RNA extract and 0.5 µl of 10 µM reverse primer on the target gene were incubated together 5 min at 70°C, then cooled on ice. Then 5 µl of AMV RT 5 x reaction buffer (Promega), 2.5 µl of 10 mM dNTPs, 1 µl of RNasin Inhibitor (Promega), 10 µl of RNase-free water and 3 µl of AMV Reverse Transcriptase (30 units) were added. The reaction mixture was incubated 1 h at 42°C, then the enzyme denaturated by 15 min at 70°C. Standard PCR conditions (as described above) were used to amplify the cDNA. Positive (genomic DNA) and negative

controls (no template, DNase-treated RNA) were included. The results of RT-PCR were visualized on 1% agarose gel.

Promoter mapping by FLOE. RNA was isolated from a culture in its exponential phase using the SV Total RNA Isolation System (Promega). Fluorescently labeled oligonucleotide extension (FLOE) method was used as reported by (8). The RNA was reversely transcribed to cDNA using a reaction mixture of 25 µl containing 2.5 µM of a primer labeled at 5'-end with FAM, 1 µM of dNTPs, 5 µl of AMV 5 x reaction buffer and 3 µl of AMV Reverse Transcriptase (Promega). RT products were purified with Minelute PCR Purification kit (Promega) and concentrated to 10 µl. Half of the sample was mixed with 4.5 µl of formamide and 0.5 µl of the GeneScan-500 ROX Size Standard (Applied Biosystems) and run onto the 3100 Genetic Analyzer. The ABI file was then analyzed and exported using Chromagna, a software developed by M.J. Miller at NIH (8).

Nucleotide sequence accession number. The sequence of the integrated transposon element of *Desulfitobacterium hafniense* strain TCE1 was deposited in EMBL under an updated version of accession number AJ439608, whereas AJ581127 describes the circular form of the transposon containing only one *IS*Dha1 copy. EMBL AJ439607 describes the *pceAB* flanking regions of *Dehalobacter restrictus*.

3.4 Results

3.4.1 DNA isolation of the composite transposon *Tn*-Dha1

A one-liter culture of *D. hafniense* strain TCE1, growing on lactate as electron donor and a combination of fumarate and PCE as electron acceptors, was harvested and total DNA was extracted and aliquoted. Most of the DNA-based experiments were done from the same DNA pool. Starting from the *pceAB* fragment isolated from *D. hafniense* strain TCE1 DNA (Chapter 2), a combination of several inverse PCRs and a direct PCR led to the isolation of a complete composite transposon containing the PCE reductive dehalogenase genes.

Table 3.2. Inverse and direct PCRs for the isolation of *Tn*-Dha1

PCR	Restriction Enzyme	Primers	PCR Product (bp)
iP1	*Kpn*I	T2r + T3f	1733
iP2	*Hha*I	IS2r + IS5f	850
iP3	*Pst*I	T5r + T6f	1538
iP4	*Hind*III	T4r + T8f	1340
P5	-	T10f + IS1r	2415
iP6	*Hha*I	IS1r + IS5f	288
iP7	*Rsa*I	IS6r + IS7f	879

The DNA fragments obtained are illustrated in Figure 3.1 (iP1 to iP4, P5, iP6 and iP7) and details on the reactions are given in Table 3.2. Sequence analysis of iP1 revealed the presence of a transposase (*tnpA*1) in the direct upstream region of *pceA* and therefore motivated further investigations. The second inverse PCR (iP2), originally performed to isolate the start region of the transposase, led to the complete sequence of *IS*Dha1, a new insertion sequence containing *tnpA*1, and to its direct upstream sequence. Fragments iP3 and iP4 were then isolated and sequenced, revealing new DNA sequence downstream of *pceAB*. The hypothesis of a composite transposon (the presence of another *IS*Dha1 copy downstream of *pceAB*) was successfully tested by a direct PCR (P5). Surprisingly the last 616bp of the fragment P5 were identical to the start of iP2, which suggested two possible explanations: either iP2 was not located directly upstream of *pceA*, but downstream of P5 (as represented in Figure 3.1), or iP2 was the link between P5 and *pceA* in a circular molecule containing one *IS*Dha1 copy. Additional inverse PCR (iP6 and iP7) revealed the chromosomal insertion site of a composite transposon, harboring one *IS*Dha1 copy at each end. However a direct PCR product with a reverse primer at the beginning of *pceA* and a forward one at the end of P5 led to the conclusion that both a linear chromosomal and a circular structure were plausible. Verification of the sequences isolated by inverse PCR was done by several direct PCRs and PCR products sequencing. The result was in total agreement with the sequence isolated by inverse PCRs.

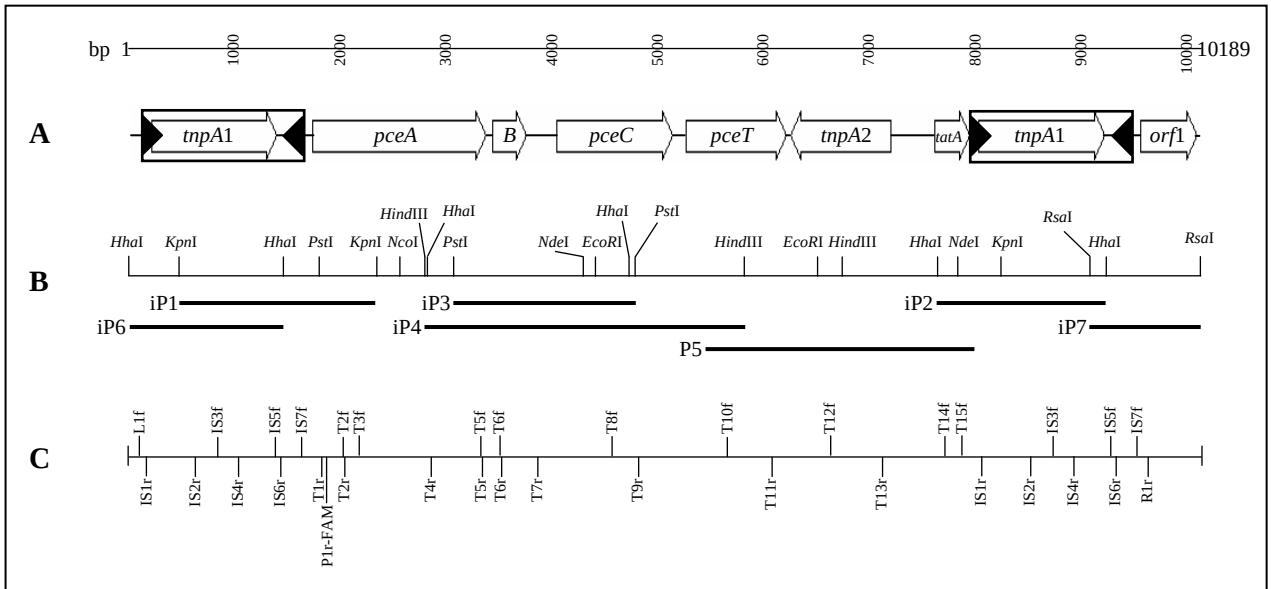

Figure 3.1. Isolation of the transposon *Tn*-Dha1. Starting from the *pceAB* genes, DNA of *Tn*-Dha1 was isolated by six successive inverse PCRs (iP1 to iP4, iP6 and iP7) and a direct PCR (P5). (**A**) The complete map of the chromosomal integrated *Tn*-Dha1 along a base pair scale. Both copies of *IS*Dha1 are depicted as ( ). (**B**) The results of the inverse and direct PCRs are indicated as black bars together with a partial restriction map. Details on the single PCRs are given in Table 3.2. (**C**) The position of the primers used in this study is indicated, forward primers being drawn above the line, reverse ones below.

3.4.2 Description of *Tn*-Dha1 and its circular form

The 9443 bp long composite transposon *Tn*-Dha1 is comprised in the 10189 bp DNA sequence of the updated EMBL AJ439608. Its structure is depicted in Figure 3.1. *Tn*-Dha1 is delimited by two identical copies of the 1570 bp long insertion sequence *IS*Dha1 (positions 138-1707 and 8011-9580, respectively). Direct repeats of 8 bp (TTTTTATA) were found directly upstream and downstream of *Tn*-Dha1, whereas this direct repeat is not conserved on the left and right sides of each *IS* (Figure 3.2 A,B). *IS*Dha1 consists of inverted repeats of 26 bp from which 19 bases are identical (Fig. 3.2 D) and a 1194 bp ORF (*tnpA*1), encoding a 397 aa putative transposase that shows 40% identity with transposases of the *IS*256 family. The *pceA* gene encoding the tetrachloroethene reductive dehalogenase starts at position 1769. Promoter prediction of this region revealed that the most probable promoter (positions 1692-1741, score 0.97 by BDGP Neural Network Promoter Prediction) is overlapping the right end of *IS*Dha1 (-35 region) and its direct flanking region (-10 region). The sequences of the *pceAB* genes have already been described (Chapter 2). New ORFs were isolated in the direct downstream region of *pceB*: *pceC* (positions 4095 to 5198), *pceT* (5326 to 6276), a second putative and probably truncated transposase gene *tnpA*2 (7274 to 6324 on complementary strand) and a putative *tatA* gene (7688 to 8026). The genes *pceC* and *pceT* were named according to the similarity (28 and 24% identity, respectively) to some genes found in the direct vicinity of the chlorophenol reductive dehalogenase (*cprA*) of *D. dehalogenans* (AF115542). Whereas CprC is thought to be a membrane-bound regulatory protein, CprT may play the role of a trigger factor, helping the folding of proteins (31). The putative 951 bp long *tnpA*2 gene was considered to be truncated since it shows 91 and 90% identity with the N-terminal part of putative transposases found in the genome of *Desulfitobacterium hafniense* strain DCB-2 (Contigs 1043 and 1087 of whole genome shotgun sequencing project at www.jgi.doe.gov, respectively). The alignment of both copies from the genome of strain *D. hafniense* strain DCB-2 allowed to identify the inverted repeats of the putative *IS* (data not shown). By similarity the localization of the putative left inverted repeat of the truncated insertion sequence found in *D. hafniense* strain TCE1 was possible (7452-7440 on complementary strand). A last ORF was localized directly upstream of the second *IS*Dha1 copy showing 25% identity with the twin-arginine translocation pathway protein TatA of *E. coli*. Tat proteins are known in *E. coli* (3) to be responsible for the export of proteins harboring a twin-arginine signal peptide, a feature that is present in many reductive dehalogenases (16) and also in *pceA* of *D. hafniense* strain TCE1 (Chapter 2). The putative *tatA* gene isolated here may be truncated, since its stop codon is located in the left inverted repeat of the second *IS*Dha1 copy. Downstream of *Tn*-Dha1 another ORF was

identified (9654 to the end) showing no relevant similarity to any sequence of the EMBL/GenBank databases.

The 7873 bp circular form of the transposon contains only one copy of *IS*Dha1. Interestingly the left flanking region of this *IS* is identical to the DNA sequence downstream of the first copy of *IS*Dha1 in *Tn*-Dha1 (ACAAGA, Fig. 3.2 C), whereas its right flanking region is identical to the sequence upstream of the second *IS*Dha1 copy (AATAGA, Fig. 3.2 C). It may indicate that this circular structure could be a dead-end product of the *Tn*-Dha1 transposition after the excision of one of the two *IS* copies.

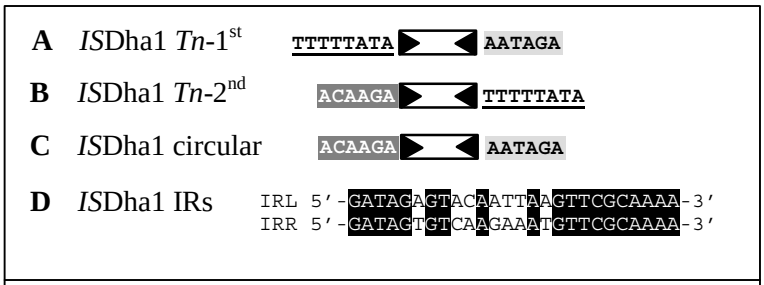

Figure 3.2. Direct flanking regions of *IS*Dha1 copies. (▶◀). **(A)** Structure of the first *IS*Dha1 copy of the integrated composite transposon. **(B)** Structure of the second *IS*Dha1 copy. **(C)** Structure of the single *IS*Dha1 copy present in the 7873bp circular form. **(D)** Inverted repeats (IRL, IRR) of *IS*Dha1.

3.4.3 Indication for a circular form not containing *IS*Dha1

A PCR with primers T14f and T2r targeting the upstream and downstream regions of the *IS*Dha1 copy in the 8 kb circular element resulted surprisingly in two distinct PCR products of about 0.6 and 2.2 kb, respectively. After cloning and sequencing, the large fragment (2184 bp) represented the expected product from the 8 kb circle, whereas the small fragment (609 bp) contained the same DNA except *IS*Dha1. The sequence at the excision point was ACAATAGA, which can be explained by an excision process where *IS*Dha1 co-excises 4 base pairs from its flanking regions, possibly 4, 3 or 2 from its left side and 0, 1 or 2 from its right side, respectively.

3.4.4 The IR junction of *IS*Dha1 forms a strong promoter

Insertion sequences were found to adopt various structures during transposition including *IS* tandems $(IS)_2$ and circular *IS* (for a review see (20)). In both cases the right and left inverted repeats are directly connected to each other, forming the so-called IR junction. Using outward directed primers IS2r and IS5f on *D. hafniense* strain TCE1 DNA targeting a possible IR junction of *IS*Dha1, an 812 bp PCR product was obtained. The downstream and upstream parts of *IS*Dha1 were connected via a short 6 bp intervening sequence. Promoter prediction on this region revealed

a very strong promoter formed by the junction of the right IR with the left IR, joining a clear -35 and -10 hexanucleotide (TTGACA and TACAAT, respectively). To study the variety of intervening sequences at the IR junction, a PCR with IR-specific primers (IS1r and IS7f) was performed on *D. hafniense* strain TCE1 DNA. The sequencing of 42 positive clones revealed the presence of five different intervening sequences: AATAGA (35 clones), TTTTTA (3), GATAGA (2), AACAGA (1) and ACAAGA (1).

To exclude the presence of a stable IR junction in form of an *IS*Dha1 tandem on the chromosome real-time PCR was performed on total DNA from *D. hafniense* strain TCE1 cells harvested during exponential growth. The IR junction on the one hand and the transposase gene *tnpA*1 on the other hand were quantified. One microgram DNA contained $6.7 \cdot 10^6$ *tnpA*1 gene copies whereas only $3.0 \cdot 10^2$ copies of the IR junction were present. The presence of 20.000-fold more transposase gene copies compared to IR junctions strongly indicated that no stable *IS*Dha1 tandem existed on the chromosome and that the IR junctions isolated by PCR represent individual excision events of *IS*Dha1 and/or *Tn*-Dha1.

3.4.5 Evidence of the 7873 bp circular element by hybridization

Aliquots of total *D. hafniense* strain TCE1 DNA were digested with the restriction enzymes *Eco*RI, *Hha*I, *Nco*I, *Nde*I and *Pst*I, digests run on an agarose gel, transferred to a nylon membrane, and hybridized with PCR-amplified DIG-labeled probes of the transposase gene *tnpA*1 (primers IS3f and IS6r) and of *pceB* (primers T5f and T7r). The results are depicted in Figure 3.3, where white arrows indicate the expected fragments of the 7873bp circular transposon element containing one copy of *IS*Dha1. According to the restriction map (Fig. 3.3 A), the fragments of the expected length containing *tnpA*1 and *pceB* were observed clearly confirming the presence of the circular form of the transposon element in *D. hafniense* strain TCE1 DNA (Fig. 3.3 B). The intensity of the fragments derived from the 7873 bp circular element is very similar to the fragments derived from genomic DNA. The stronger intensity of the indicated *Hha*I fragment detected by the *tnpA*1 probe is explained by the fact that *Hha*I produces a 1615 bp fragment containing *tnpA*1 from both the circular element and the second *tnpA*1 copy of the genomic composite transposon. Double *pceB* fragments have been observed for the *Eco*RI, *Nco*I and *Nde*I digests, which is in good correlation with the hypothesis of the presence of both a free circular and a genomic integrated copy of the transposon element in *D. hafniense* strain TCE1. The single *pceB* fragment detected for *Hha*I and *Pst*I digests may be easily explained by the presence of several restriction sites of these enzymes within the *Tn*-Dha1 structure. *Nco*I cleaves the 8 kb circle only once, which produces a linearized form, detected by both the *tnpA*1 and *pceB* probes. The other *tnpA*1 fragments observed here are

probably copies of *IS*Dha1, which are not in the direct vicinity of the reductive dehalogenase genes.

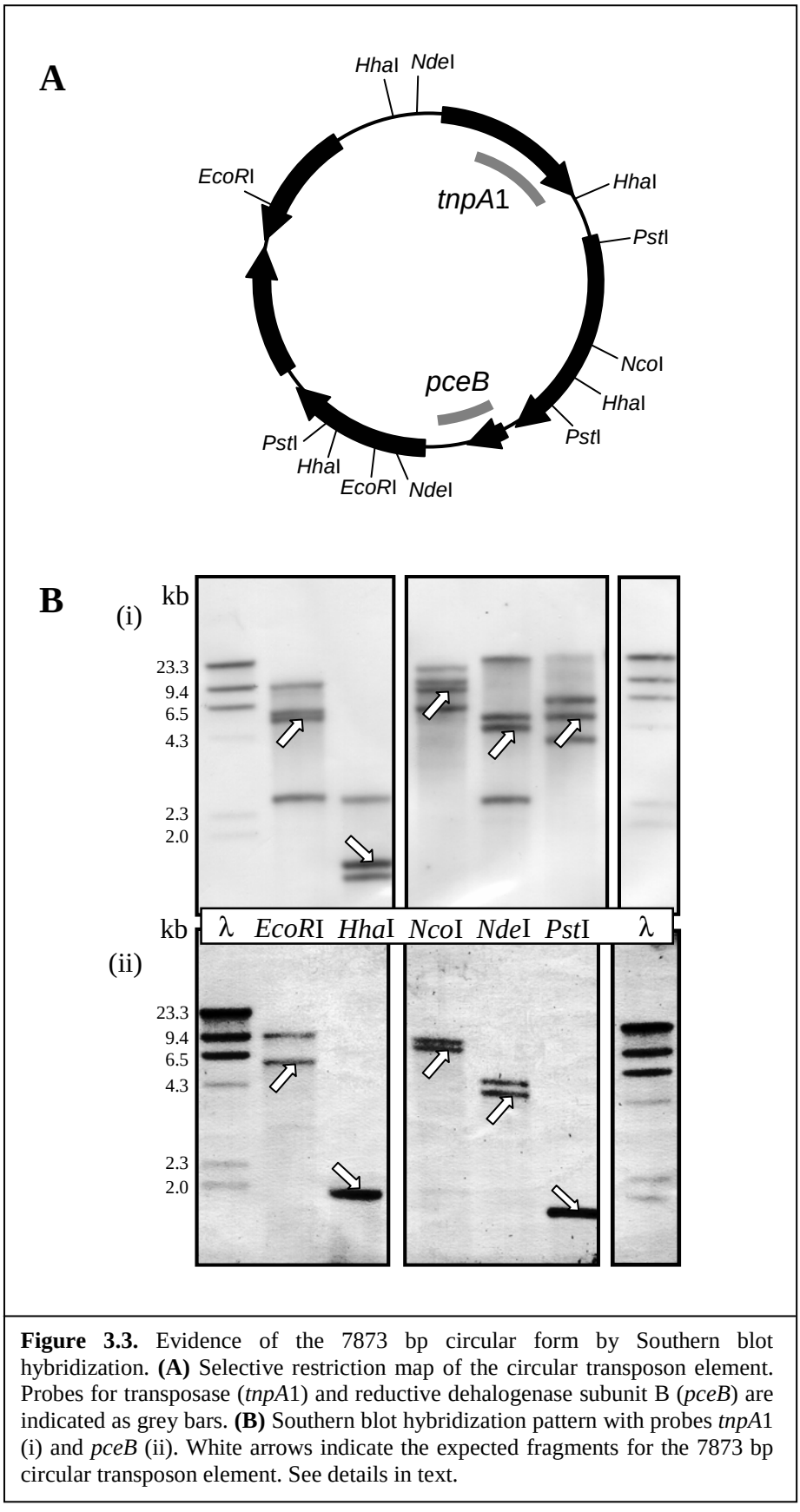

Figure 3.3. Evidence of the 7873 bp circular form by Southern blot hybridization. **(A)** Selective restriction map of the circular transposon element. Probes for transposase (*tnpA*1) and reductive dehalogenase subunit B (*pceB*) are indicated as grey bars. **(B)** Southern blot hybridization pattern with probes *tnpA*1 (i) and *pceB* (ii). White arrows indicate the expected fragments for the 7873 bp circular transposon element. See details in text.

3.4.6 Expression of the newly isolated genes

The expression of the genes present on the transposon element was studied with a RT-PCR experiment. The following primer pairs were used: IS3f/IS4r for *tnpA*1, T2f/T3r for *pceA*, T5f/T6r for the *pceAB* co-expression, T8f/T9r for *pceC*, T10f/T11r for *pceT* and T12f/T13r for *tnpA*2. The results showed that all genes except *pceT* were found to be expressed with PCE as electron acceptor and that *pceA* and *pceB* were co-expressed (data not shown), as it was demonstrated for *cprB* and *cprA* from *D. dehalogenans* (31) and for *pceA* and *B* from *S. multivorans* (24).

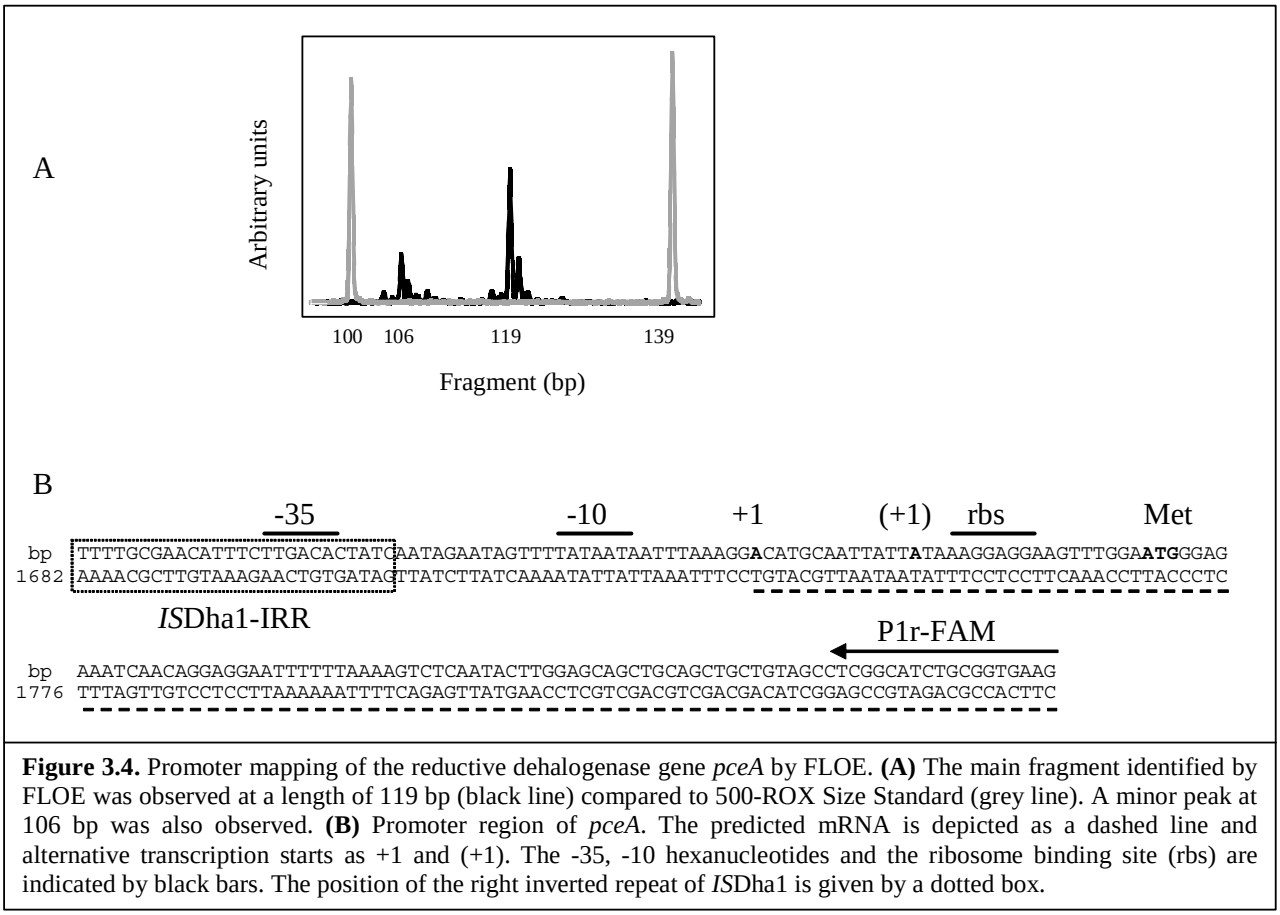

Figure 3.4. Promoter mapping of the reductive dehalogenase gene *pceA* by FLOE. **(A)** The main fragment identified by FLOE was observed at a length of 119 bp (black line) compared to 500-ROX Size Standard (grey line). A minor peak at 106 bp was also observed. **(B)** Promoter region of *pceA*. The predicted mRNA is depicted as a dashed line and alternative transcription starts as +1 and (+1). The -35, -10 hexanucleotides and the ribosome binding site (rbs) are indicated by black bars. The position of the right inverted repeat of *IS*Dha1 is given by a dotted box.

3.4.7 Identification of the transcription start of *pceA* by FLOE

The fluorescent labeled oligonucleotide extension (FLOE) method developed for DNA sequencer based on capillary system (like the 3100 Genetic Analyzer from Applied Biosystems) was used for promoter mapping. Here 20 µg of total RNA were involved in a RT step using FAM-labeled reverse primer P1r located 87 bp downstream of the start codon of the reductive dehalogenase gene *pceA*. Two signals of 119 and 106 bp of unequal intensity were identified (Figure 3.4 A), corresponding to potential transcription starts. The main transcription start is in good accordance

with the predicted promoter for *pceA* gene. Both -35 and -10 hexanucleotides are identical to *E. coli* consensus sequences and the ribosome binding site is clearly recognizable (Fig. 3.4 B). Most interestingly, the -35 region is located within the right inverted repeat of *IS*Dha1, which emphasizes the importance of *IS*Dha1 for the expression of the PCE reductive dehalogenase.

3.4.8 Comparative sequence analysis

Since the *pceAB* genes of *D. hafniense* strain TCE1 have been shown to be 100% identical to those found in *Desulfitobacterium* sp. strain PCE-S, *Desulfitobacterium* sp. strain Y51 and *D. restrictus* and 65% identical to two ORFs from the genome of *D. hafniense* strain DCB-2 (Chapter 2), the flanking regions of *pceAB* in these various strains was further investigated. In the strains PCE-S and Y51, although only few upstream and downstream information is available, the *Tn*-Dha1 structure is probably also present since the partial sequence of *tnpA*1 and *pceC* as well as the intergenic regions (on the *pceAB*-upstream and downstream sequences respectively) are conserved with 100% identity (Figure 3.5). Interestingly in *D. restrictus*, where the flanking regions of *pceAB* were isolated (data not shown), only the gene cluster *pceABCT* is fully conserved (Fig. 3.5). The sequence identity between *Tn*-Dha1 and the *pceABCT* gene cluster of *D. restrictus* starts directly after the right inverted repeat of the first *IS*Dha1 copy and ends a few bp after the stop codon of *pceT*. Whereas the structure of *Tn*-Dha1 is not conserved in *D. restrictus*, a truncated transposase-like gene, not related to any *tnpA* found in *D. hafniense* strain TCE1, is present on the

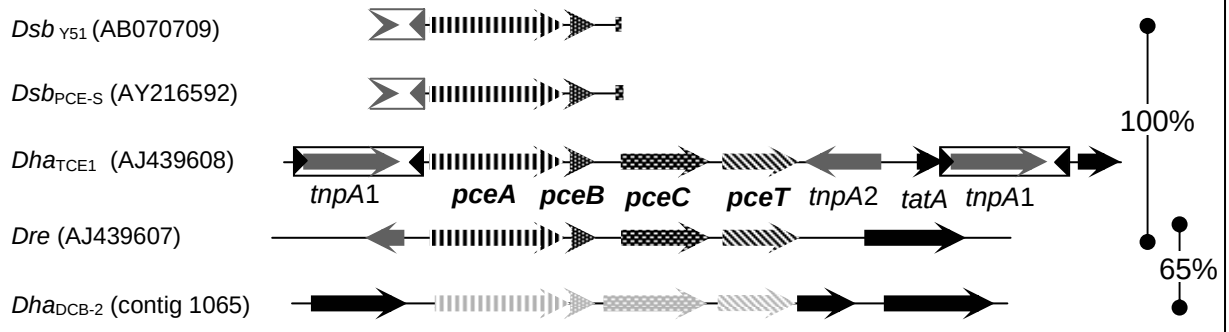

Figure 3.5. Comparative analysis of reductive dehalogenase gene clusters from *D. hafniense* strain TCE1 related strains. The genetic structure of *Tn*-Dha1 is compared to the sequence of five other strains. Whereas the transposon structure seems to be conserved in the first three, only the *pceABCT* gene cluster show 100% and 65% sequence identity with *D. restrictus* and *D. hafniense* strain DCB-2, respectively. Grey arrows represent transposase or transposase-like genes, black arrows genes of unknown function or not related to reductive dehalogenase clusters. Black boxes delimit the *IS*Dha1 structure in *D. hafniense* strain TCE1. The light grey shading for the *pceABCT* cluster of *D. hafniense* strain DCB-2 indicates its lower homology the other clusters. Dsb_{Y51}: *Desulfitobacterium* sp. strain Y51; $Dsb_{PCE\text{-}S}$: *Desulfitobacterium* sp. strain PCE-S; Dha_{TCE1}: *D. hafniense* strain TCE1; *Dre*: *D. restrictus*; $Dha_{DCB\text{-}2}$: *D. hafniense* strain DCB-2.

complementary strand of *pceA* direct upstream region. In the genome of *D. hafniense* strain DCB-2 (www.jgi.doe.gov), a gene cluster on contig 1065 (already submitted as AF403184 and AF403185 in GenBank), show 65% identity with the *pceABCT* cluster of *D. hafniense* strain TCE1. However, this high sequence similarity is also limited to those four genes (Fig. 3.5).

3.5 Discussion

It has been shown in this study that in *Desulfitobacterium hafniense* strain TCE1, the tetrachloroethene reductive dehalogenase gene is located on a composite transposon made of two identical directly repeated copies of a new insertion sequence, *IS*Dha1, and six ORFs in between, from which four of them show similarity to members of other reductive dehalogenase gene clusters (*pceABCT*) (31). Two additional ORFs, *tnpA*2 and *tatA*, are truncated possibly due to various genetic rearrangements. *IS*Dha1 has typical features of the *IS*256 family: a unique ORF encoding a putative transposase with 40% identity at the protein level to *IS*256 members, including the catalytic motif D-(65)-D-(106)-E showing only a very slight variation to the *IS*256 consensus motif as defined by Haren et al. (14). Direct repeats of 8 bp were found up- and downstream of the composite transposon, but not around single *IS*Dha1 copies, which indicates that the structure observed here is not due to random integration of two *IS*Dha1 copies, but due to the integration of the complete composite transposon. Therefore it is the first evidence that a PCE reductive dehalogenase gene is part of a catabolic transposon. Next to *D. hafniense* strain TCE1, this transposon structure is most probably also present in DNA isolated from *Desulfitobacterium* sp. strain PCE-S and *Desulfitobacterium* sp. strain Y51 (23, 33) as indicated in Figure 3.5. In contrast, the *pceABCT* cluster of *D. restrictus*, which shows almost 100% identity with the *pceABCT* gene cluster of *D. hafniense* strain TCE1 does not contain *IS*Dha1 and shows different flanking regions upstream of *pceA* and downstream of *pceT*. The same is also valid for the sequence taken from the unfinished genome of *D. hafniense* strain DCB-2 showing around 65% identity over the *pceABCT* gene cluster, but no *IS*Dha1 was found. In strain DCB-2, however, this gene cluster does not seem to be functional since no dechlorinating activity towards PCE has been observed (10). Interestingly this latter bacterium contains several copies of an insertion sequence sharing 90% identity with the truncated second transposase found in *Tn*-Dha1 (*tnpA*2).

If *IS*Dha1 may be of great importance for the mobility of the reductive dehalogenase gene cluster, it is probably also involved in the expression of *pceA*, the gene encoding the key enzyme in PCE reductive dechlorination. The analysis of the transcription start of *pceA* by FLOE revealed two signals, from which the major one is in perfect agreement with the predicted promoter that shows

perfect consensus sequences for the -35 and -10 hexanucleotides as well as for the ribosome binding site. It is well known that some insertion sequences affect the expression of neighboring genes once integrated (for a review see (20)). *IS*Dha1, which harbors a consensual -35 region in its right inverted repeat, may be part of them and may influence the expression of the reductive dehalogenase.

A circular form of about 8 kb comprising the same genes but with only one *IS*Dha1 copy was also observed in total *D. hafniense* strain TCE1 DNA. Since both the genomic and circular form gave similar band intensity on Southern blot hybridization (Fig. 3.3), it is thought that this circular form may be a quite stable structure. Looking more closely at the structure of the genomic and the circular copies (Fig. 3.2), it is possible that the circular element containing one *IS*Dha1 copy is the result of either a RecA-dependent homologous recombination over *IS*Dha1 or a transposase-dependent excision of the complete composite transposon followed by its circularization. The recombination over *IS*Dha1 would yield the circular form observed and a single *IS*Dha1 copy at the integration site. A 1.7 kb PCR product with primers L1f and R1r containing *IS*Dha1 was not observed, indicating that homologous recombination is not the major process occurring here. The second possible mechanism would yield a 9.5 kb circular element containing an *IS*Dha1 tandem. A similar form has been postulated for the composite transposon *Tn*5565 (5). This study mainly based on PCR has shown that *Tn*5565 may undergo excision from its integrated form and circularize. It has been demonstrated that the circular form harboring an *IS* tandem is very unstable and undergoes further excision of one *IS* leading to a circular transposon element containing only one *IS* copy and a single *IS*, which may be by itself circularized (5).

Based on the results obtained in this study, a tentative model of the formation of circular elements of *Tn*-Dha1 is illustrated in Figure 3.6. Through the action of the transposase, one *IS*Dha1 copy of the genomic integrated composite *Tn*-Dha1 (Fig. 3.6A) may undergo attack by the other copy producing a circular transposition intermediate. *IS* tandems are very reactive molecules since a very strong promoter is formed at the IR junction (Fig. 3.6), as shown for *IS*911 (6, 7, 29, 35). Indeed, the strongly expressed downstream located transposase would find a very good substrate in structure B (Fig. 3.6), rendering it very unstable and leading to several events, that are the re-integration of *Tn*-Dha1 into the genome (reverse reaction) and the excision (and maybe re-circularization) of one *IS*Dha1 or of the complete *IS*Dha1 tandem from the circular molecule. The remaining circular elements C and D are much less reactive and may accumulate in the bacterial population as dead-end products of aberrant transposition events. Whereas structure C was observed by Southern blot hybridization in substantial amounts (Fig. 3.3), structure D was

probably a minor product, since only PCR could detect it. This latter structure may also be the result of the excision of the remaining *IS* copy from structure C.

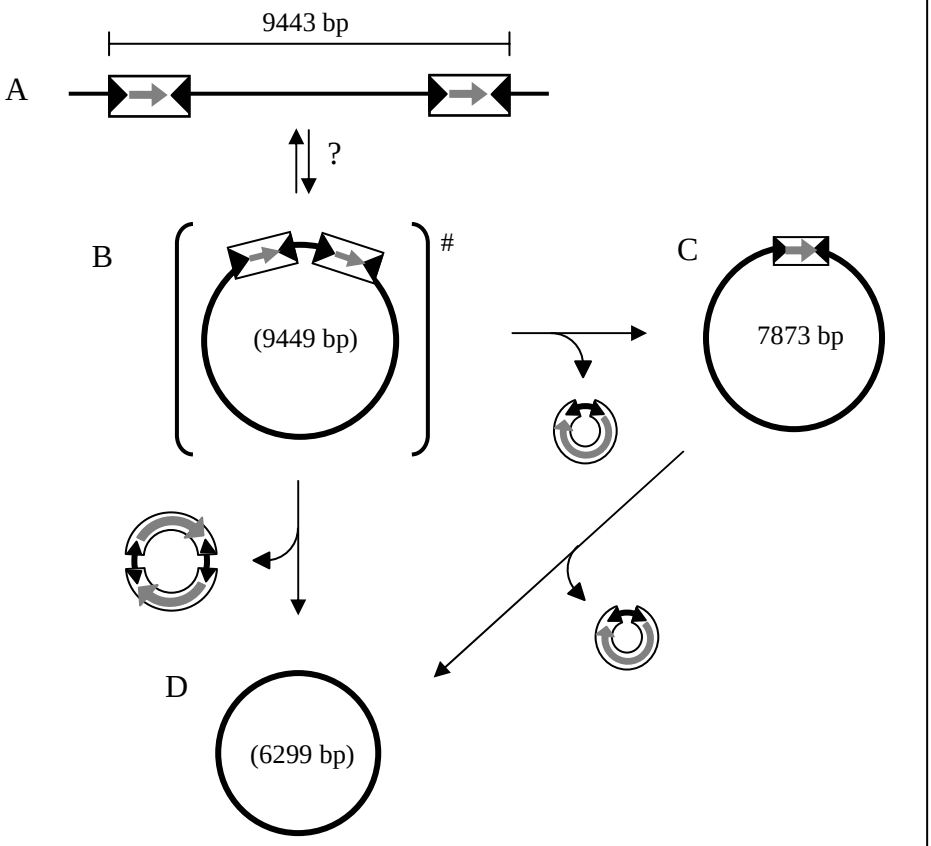

Figure 3.6. Tentative model of transposition events of *Tn*-Dha1 leading to putative dead-end products. **(A)** Genomic integrated composite transposon *Tn*-Dha1. **(B)** Putative unstable circular intermediate of the transposon element containing an *IS*Dha1 tandem. This may be the result of an excision of *Tn*-Dha1. **(C)** Circular transposon element harboring one *IS*Dha1 copy, which may be derived from the excision of an *IS*Dha1 copy from the structure depicted in (B). The *IS* released here may be circularized. **(D)** Putative circular form of *Tn*-Dha1 after the loss of the remaining *IS*Dha1 or *IS*Dha1 tandem. The *IS* and *IS* tandem released may be as well circularized. The structures depicted in (A) and (C) were experimentally observed, whereas for (B) and (D) indications were obtained by PCR.

Different PCR products corroborate the tentative model. From the five different intervening sequences of IR junctions of *IS*Dha1, three were identical to DNA directly contiguous to *IS*Dha1 copies in *Tn*-Dha1 (Fig. 3.2 A, B): AATAGA, downstream of the first *IS*Dha1 copy; ACAAGA, upstream of the second copy and TTTTTA, downstream of the second copy. This indicated that IR junctions are possible products of the excision and circularization of the composite *Tn*-Dha1 to the circular element containing the *IS* tandem (TTTTTA) and of single *IS*Dha1 copies (AATAGA and ACAAGA) from either the integrated transposon, or its circular elements harboring one *IS* or the *IS* tandem. The latter process would lead to a circular form, where both *IS*Dha1 copies are excised,

a species that is very likely to occur, since a PCR product of 609 bp was observed with primers T14f and T2r. The analysis of the DNA sequence after excision of the second copy (ACAATAGA) indicates that *IS*Dha1, when excising, takes with it four bases from the flanking regions.

The comparative analysis of reductive dehalogenase gene clusters in the *Desulfitobacteria* strains TCE1, Y51, PCE-S and DCB-2 and in *D. restrictus* revealed two classes: all members of the first class (TCE1, Y51 and PCE-S) seem to contain the transposon structure, whereas the second class (last two strains) show homology with the first class only over the *pceABCT* cluster (Figure 3.5). The diversity of transposases in complete or truncated forms around the cluster in these strains indicates multiple rearrangements and/or transfers during evolution, which finally does not allow a clear interpretation.

The well characterized chlorophenol reductive dehalogenase gene cluster from *D. dehalogenans* (31) contains next to the genes for the subunits CprABCT, a few more genes whose products seem to be involved in the regulation of the dehalorespiration as putative transcriptional regulator (CprK), and putative chaperones of the GroEL type (CprD, CprE). As these additional genes are not present in the direct vicinity of *pceABCT*, nor in *D. hafniense* strain TCE1 and neither in *D. restrictus*, it is possible that either these genes are located somewhere else in the genome or that other non-specific regulators and chaperones are involved. In this latter case, the *pceABCT* gene cluster, fully encoded on *Tn*-Dha1, would be sufficient to ensure the dechlorination activity.

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CHAPTER 4

Evidence of the acquisition of reductive dehalogenase genes through horizontal gene transfer by *Dehalococcoides ethenogenes* strain 195

Manuscript submitted for publication.

4.1 Abstract

The genome of Dehalococcoides ethenogenes *strain 195, an anaerobic dehalorespiring bacterium, contains eighteen copies of putative reductive dehalogenase genes, including the well characterized* tceA *gene, whose gene product functions as the key enzyme in the environmentally important dehalorespiration process. The genome of* D. ethenogenes *was analyzed using a bioinformatic tool based on the frequency of oligonucleotides. The results in the form of a genomic signature revealed several local disruptions of the host signature along the genome sequence. These fractures represent DNA segments of potentially foreign origin, so-called original regions, which may have been acquired by an ancestor through horizontal gene transfer. Most interestingly, fifteen of the eighteen reductive dehalogenase genes including the* tceA *gene were found to be located in these regions, strongly indicating the foreign nature of the dehalorespiration activity. The GC content and the presence of recombinase genes within some of these regions corroborate this hypothesis. A hierarchical classification of the original regions containing the reductive dehalogenase genes indicated that these regions were probably acquired by several gene transfer events.*

4.2 Introduction

The increasing knowledge of gene and genome sequences provides strong evidence that horizontal gene transfer (HGT) plays a major role in the evolution of prokaryotic genomes. Primarily evoked due to the emergence of multi-drug resistant patterns (4, 6), it is now well accepted that acquisition and integration of new DNA in prokaryote chromosomes is a dynamic process allowing rapid adaptation to new ecological niches (10). Sequenced bacterial genomes have revealed that a significant part of the genomes originates from HGT (38). For *Escherichia coli,* it has been estimated that 24% of the entire genome has been acquired by HGT (27). In contrast to the core gene pool, the horizontal gene pool (48), also called the flexible gene pool (14), is mainly composed of operational genes rather than informative genes (42). The exchange of the horizontal gene pool between bacteria sharing a common ecological niche is mediated by transmissible mobile genetic elements (MGEs). Numerous phages, plasmids, transposons, integrons, and genomic islands have been described participating in DNA transfer and can be considered as a mosaic continuum (39).

In the last few decades the environment has been polluted by numerous synthetic chemicals that are foreign, and potentially dangerous, to life either by their structure or by their unnaturally high concentrations. Despite their xenobiotic character, many of these compounds have been found to be biodegradable. However, in some environments it can take weeks, months and even years for significant biodegradation to be observed, which has led to speculations on rapid evolution of catabolic pathways. Current knowledge on the role of MGEs in bacterial evolution has been recently reviewed (49, 51, 52). Different types of experiments have revealed the existence of the metabolic horizontal gene pool. Transmissible MGEs containing degradation genes have been found for toluene-xylene (45), naphtalene (15), and chlorobenzene degradation (33), to only name a few. Direct evidence of natural spreading of MGEs in the ecosystem has been obtained when bacteria with MGEs have been used for bioremediation of polluted sites by bioaugmentation (40, 50). Indirect evidence for HGT has often been illustrated by incongruities in phylogenetic trees, as demonstrated for dissimilatory sulfite reductase genes of sulfate-reducing bacteria (23), and ferredoxins, nitroreductases, NADH oxidases, and alcohol dehydrogenases of anaerobic prokaryotes and microaerophillic protists (36).

When orthologous sequence comparisons have not been possible, other approaches based on atypical features of a sequence in its genomic context, such as nucleotide composition (26), relative frequencies of dinucleotides (22), codon usage biases (32), and Markov chain pattern

analyses (13) have been developed. These approaches revealed indications of HGT of a glycosyl hydrolase between bacteria and fungi in the rumen (11) and of UDP-glucose dehydrogenase between *Streptococcus* sp. and *Escherichia coli* (34). More recently a new tool allowing an easy screening of HGT in genomes has been developed based on signatures of sequences (8). The genomic signature concept shows that oligonucleotide usage varies less along a genome than between genomes, and is therefore species-specific (7, 21, 43). Using this concept, local variations in the genomic signature of a particular genome revealed regions that contain "original" DNA and could originate from HGT (9).

The aim of this work was to study the genome of *Dehalococcoides ethenogenes* strain 195 (TIGR Database) by analyzing variation of the genomic signature along the genome and using other bioinformatic tools in order to obtain indication of HGT events. *D. ethenogenes* strain 195 is a bacterium that strictly depends on chloroethenes as electron acceptor and only uses hydrogen as an electron donor (31). *D. ethenogenes* strain 195 is the first bacterium described in pure culture able to completely dechlorinate tetrachloroethene (PCE) to ethene in a process called dehalorespiration (16). At least two reductive dehalogenases are involved in the dechlorination to ethene, one dechlorinating PCE to trichloroethene (TCE) and the other dechlorinating TCE to ethene (29). For the latter, the gene *tceA* has been cloned and sequenced (28) showing the presence of a Tat signal peptide in the N-terminal region and motifs for the binding of two iron-sulfur clusters in the C-terminal region. In the genome of *D. ethenogenes* strain 195, sixteen to eighteen putative reductive dehalogenase genes (*rdhA*) have been identified (46, 54), respectively. The present study, examining the variation of the genomic signature in the genome of *D. ethenogenes* strain 195, indicates the foreign nature of nearly all of these putative reductive dehalogenases. Open reading frames (ORFs) at the borders of the detected foreign genome regions (referred to as original regions) showed good similarity with recombinase genes (a generic term used here that includes transposases, *IS* elements, integrases, and recombinases involved in DNA mobility (37)). The comparison of the genomic signature of the different original regions indicates that different HGT events have occurred leading to this large pool of putative *rdh* genes.

4.3 Material & Methods

Genome DNA sequence data. Preliminary sequence data of the genome of *D. ethenogenes* strain 195 was obtained from The Institute for Genomic Research (TIGR) through the website at www.tigr.org. The genome version used in this study was the one of September 2002 (contig6871). As pointed out by TIGR, it is not excluded that several sequencing errors exist in this version and

will be corrected for the final publication of this genome. However, these possible errors have little consequence for the results presented in this study.

Genomic signature determination. Genomic signatures were obtained as described recently (8). Briefly, the fast algorithm Chaos Game Representation (20) (a program developed with C++ and Matlab^{TM}) allows treating 1 MB sequences in a few seconds on a laptop computer, and calculating the whole set of frequencies of short oligonucleotides found in a sequence. Signatures are then visualized as square images where the color of each pixel represents the frequency of a given oligonucleotide (= word).

Detection of original regions. The detection of original regions, possibly originating from HGT, is based on the determination of differences between local signatures and the signature of the genome as a whole (9). The genome of *D. ethenogenes* was analyzed for local signatures using the following optimal parameters: sampling of the genome by a sliding window of 5kb (with steps of 2.5 or 0.5 kb) in which the frequency of 4-letter words is determined. The strand asymmetry bias was ruled out using double strand DNA for the analysis. The distinction between the intrinsic or host signature of a bacterium, which contains all the vertically inherited genes, and the signature of original regions, required a precise analysis of the signatures obtained along the genome. For that purpose, the preponderant signature which may represent the host signature is determined with the K-means clustering algorithm. The host signature cluster is the one that has the largest sample size and is independent of the number of cluster seeds. The comparison between the signature of a window and the host signature was obtained by an Euclidian metric accounting for differences in word usage (9).

Analysis of original regions. The gene content of detected original regions was studied with the following strategy: (i) the DNA corresponding to the original region was separated from the rest of the genome;(ii) the program ORF finder (www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to obtain a map with all possible ORFs present in the six existing frames; (iii) ORFs with a length of ≥500 bp were searched for sequence homology with either Cognitor (www.ncbi.nlm.nih.gov/gorf/orfig.cgi) that allows comparison with COG (Cluster of Orthologous Groups of proteins) or, when a protein family could not be attributed, with BLASTp (2). The GC content of each studied original region was determined by the GeeCee program (bioweb.pasteur.fr/seqanal/interfaces/geecee.html).

Comparison of genomic signatures. The signatures consisting of the frequency matrices of all 4-letter words were compared with each other using a hierarchical clustering (JMP software, SAS

Institute Inc.). Briefly, this clustering first groups the closest signatures together, calculates their mean values and iterates the process for all signatures.

4.4 Results

4.4.1 Detection of original regions in the genome of *D. ethenogenes*

The version of the *D. ethenogenes* strain 195 genome used here consisted of a major contig (numbered 6871) of a size of 1470272 nucleotides. The GC content of this small genome is around 50%. In order to investigate the possibility that this bacterium has acquired the reductive dehalogenase genes by horizontal gene transfer, the local genomic signature along the genome was calculated as described in Materials & Methods. By reading the genome with small sliding windows (5 kb) from the beginning to the end of the contig, local differences in genomic signature were detected (Figure 4.1A). By visual analysis, it was possible to recognize that at different places in the genome the apparent homogeneity of the picture was disrupted. For a more accurate analysis of the positions of these fractures, a clustering in four classes was performed (Fig. 4.1B). The most populated class, referred to as class I, accounts for 78 % of the genome and represents the intrinsic or host signature of *D. ethenogenes* strain 195. The three other classes that account for 22% of the genome are good candidates for DNA acquired by HGT. Classes II, III, and IV account for 17%, 4.9% and 0.1% of the genome, respectively. It is important to note that the different classes have no real genetic signification, they only represent parts of the genomic DNA that fall in the same cluster of signature.

4.4.2 Original regions analysis

The gene content analysis of the original regions of class III indicated that a putative temperate bacteriophage is present in the genome of *D. ethenogenes* strain 195. The large original region located around 550-600kb (Fig. 4.1B) contains only genes that are related to phage DNA. The GC content of this putative phage is identical to the GC content of its host. This shows the usefulness of signature analysis for the detection of foreign DNA, since a simple GC content analysis would not have revealed the putative prophage. The genes present in the second half of the original regions A and H (designated A2 and H2, respectively) that cluster with class III also display significant homology with the phage protein family. As previously described (9), the local signature also allows the detection of ribosomal RNA genes. In the *D. ethenogenes* strain 195 genome, the gene encoding the 16S rRNA (accession number AF004928) was localized in an original region around position 642700. The gene encoding the 23S rRNA was localized around

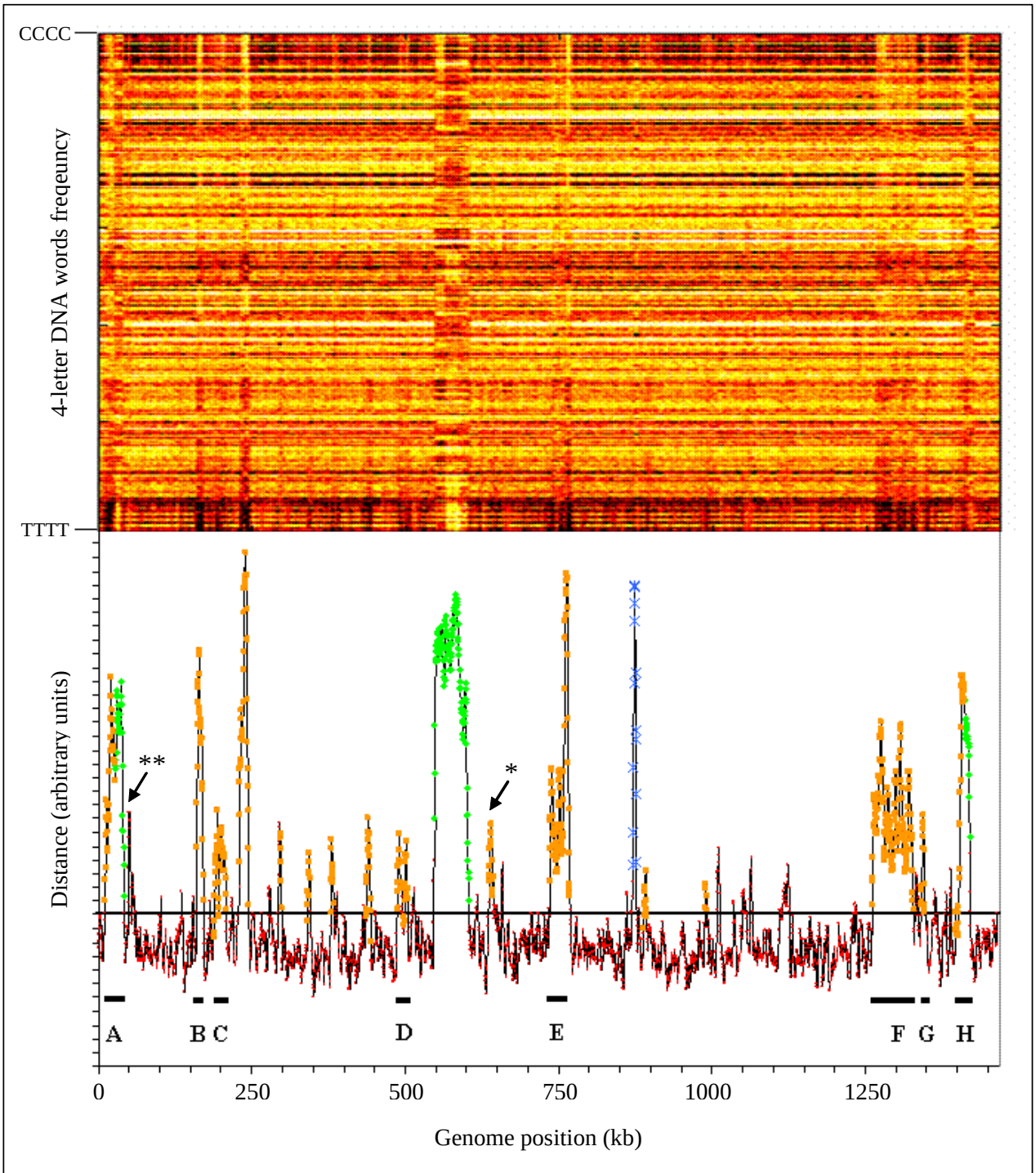

Figure 4.1. Variations of genomic signature along the genome of *Dehalococcoides ethenogenes* strain 195. (**A**) Signatures obtained by analysis of the 4-letters word frequency in 5 kb sliding windows. Each horizontal line represents the frequency of one word along the genome. Each vertical line represents the signature of a window. (**B**) Distances of local signatures to the host signature (arbitrary units). Clustering of signatures resulted in four signature classes: Class I (red dots) represents the host signature, original regions belong to class II (orange squares), class III (green diamonds), and class IV (blue crosses). The position of original regions containing *rdh* genes is indicated by letters A to H. The location of 16S rRNA and 23S rRNA genes is indicated by one or two asterisks, respectively.

position 52000 in a small original region with a signature different from the host signature. The class IV original region which accounts for only 0.1% of the genome and showed the largest distance from the host signature, corresponds to an atypical region containing ORFs without clear function but with high alanine content. This region can be qualified as a low complexity region probably due to a high number of short repetitions.

4.4.3 Identification and analysis of the putative reductive dehalogenase genes

A BLAST sequence comparison (TBlastn) of the genome of *D. ethenogenes* strain 195 with the protein sequence of the characterized reductive dehalogenase TceA as a query retrieved seventeen genes with significant homology (26-32%). The characteristics of the putative reductive dehalogenase genes (referred to as *Det-rdhA*1 to *Det-rdhA*17) are summarized in Table 4.1. The N-terminal sequences are given to allow a clear identification of each gene after annotation of the genome by TIGR. A BLAST2 sequence analysis revealed that homology between all 18 *rdhA* gene products ranged between 24 and 54% identity (data not shown). This strongly suggests that duplication phenomena and evolution by point mutation can be excluded as explanations for the high number of putative reductive dehalogenase genes. The 18 *rdhA* genes possess the typical features of reductive dehalogenases, e.g. a Tat signal peptide in the N-terminal part and two iron-sulfur clusters binding motifs in the C-terminal part. The only exception is *Det-rdhA*17 that contains the iron-sulfur cluster motifs, but lacks the signal peptide in the N-terminal part. A typical feature of the genetic structure of all well characterized reductive dehalogenases is the presence of a second ORF (referred to as *rdhB*) in the direct vicinity of the reductive dehalogenase gene, encoding a small hydrophobic protein with two or three transmembrane helices (35, 46). A membrane anchor function has been proposed for this protein, and co-transcription of the two genes has been shown by RT-PCR (35, 46). A *rdhB* gene was found downstream of the *rdhA* gene for all putative reductive dehalogenase genes of *D. ethenogenes* strain 195. The orientation of the *rdhA* and *rdhB* genes seems to be conserved among the two main classes of reductive dehalogenases. While they appear as *rdhAB* cluster in chloroethene reductive dehalogenases, the opposite orientation (*rdhBA*) is found in chlorophenol reductive dehalogenases clusters (see Chapter 2 and (54)).

Table 4.1. Properties and location in the genome of the putative reductive dehalogenase genes of *Dehalococcoides ethenogenes* strain 195.

Reductive dehalogenase gene	N-terminal amino acid sequence	Length of deduced protein (aa)	Position in genome[1]	Location in original region[2]
Det-tceA	MSEKYHSTVTRR	554	23945	A
*Det-rdhA*1	MSSFHSIVSRRD	482	166788	B
Det-rdhA2	MSKFHSMVSRRD	500	178825	-[3]
*Det-rdhA*3	MNKFHTSLSRRD	492	184414	
*Det-rdhA*4	MKEFHSTLSRRD	494	186975	C
*Det-rdhA*5	MHSFHSTVSRRD	469	191688	
*Det-rdhA*6	MSKLHSTLSRRD	507	196819	
*Det-rdhA*7	MNQFHSTVSRRD	505	199588	
*Det-rdhA*8	MTEVNRRDFLKA	532	503166	D
*Det-rdhA*9	MSNFHSTVSRRD	510	767390	E
*Det-rdhA*10	MLNFHSTLTRKD	495	1266422	F
*Det-rdhA*11	MDKFHSTLSRRD	515	1272322	
*Det-rdhA*12	MSKQHSTVSRRD	505	1276843	
*Det-rdhA*13	MNKFHSIVSRRD	514	1280982	
*Det-rdhA*14	MQNFHSTLSRRD	490	1344813	G
*Det-rdhA*15	MDGKINRRDFVK	455	1397826	-[3]
*Det-rdhA*16	MSKFHSAVTRRD	510	1403184	H
*Det-rdhA*17	MRDGVIAPQEGY	350	1413295	

[1] The position of the first base of each *rdhA* gene is given in accordance to the genome version used in this study.
[2] The location of the original regions in the genome is depicted in Figure 4.1.
[3] The genes *Det-rdhA*2, *Det-rdhA*3 are located between original regions B and C; *Det-rdhA*15 is located between regions G and H.

4.4.4 Localization of the putative reductive dehalogenase genes

The location of the *rdhA* genes was matched with the location of the original regions detected by the signature analysis. Fifteen of the eighteen *rdhA* genes were found in original regions. Five genes, one of which is *tceA*, are located alone in original regions A, B, D, E, and G, respectively (Figure 4.2). The genes *Det-rdhA*4 to *Det-rdhA*7, *Det-rdhA*10 to *Det-rdhA*13, and *Det-rdhA*16 to *Det-rdhA*17 are grouped and located in the original regions C, F, and H, respectively. Interestingly, *Det-rdhA*17, lacking the N-terminal signal peptide, is located in H2. Genes *Det-rdhA*2 and *Det-rdhA*3 and gene *Det-rdhA*15 are found in regions exhibiting the host signature, between original regions B and C and between G and H, respectively. Original regions A to H have a GC content (<50%) different from that of the host (Fig. 4.2).

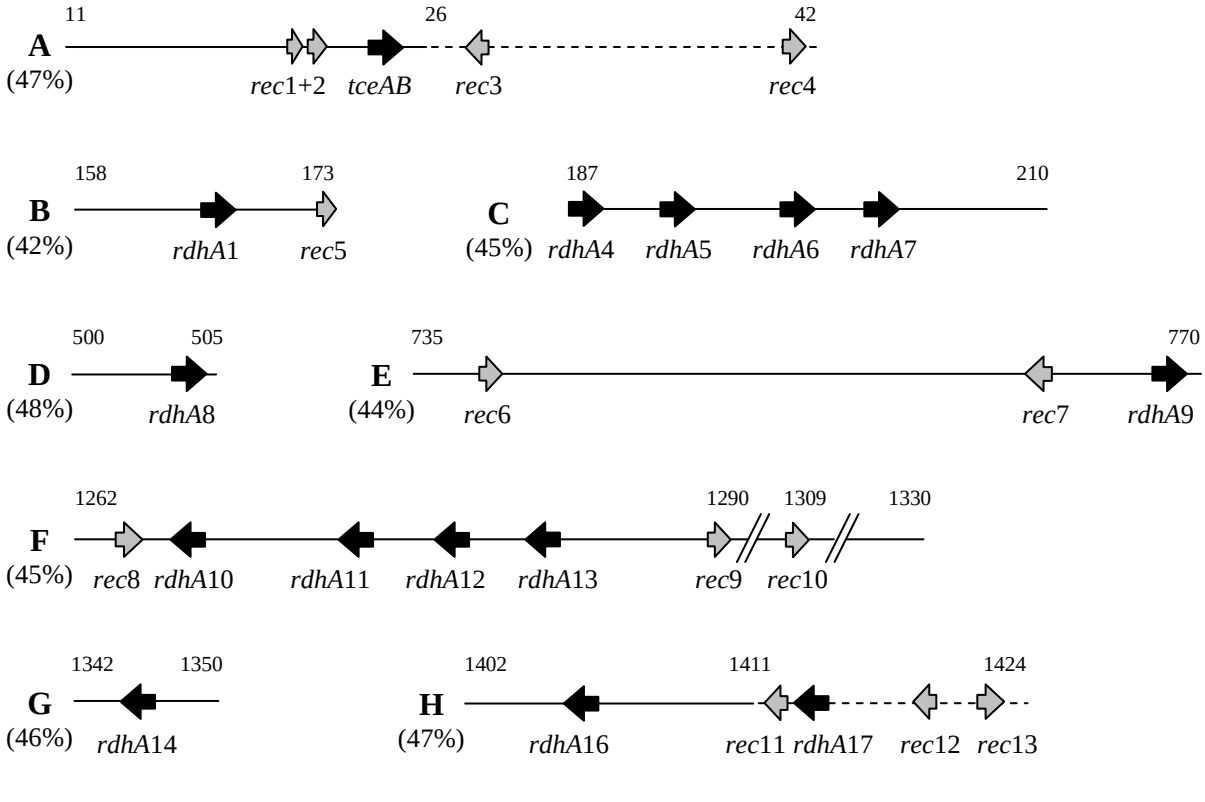

Figure 4.2. Location of *rdh* and recombinase genes in original regions A to H. Black arrows represent *rdhAB* genes and illustrate the direction of transcription in the original region. Grey arrows represent recombinase genes. Straight lines represent original regions belonging to class II, broken lines represent original region belonging to class III. Numbers show the positions (kb) of original regions in the genome. The GC content of the original region is given in parentheses.

4.4.5 Genetic characterization of the original regions

A detailed analysis of the up- and downstream regions of the *cprBA* gene cluster of *Desulfitobacterium dehalogenans*, an *o*-chlorophenol dehalorespiring bacterium, revealed several genes encoding proteins that are possibly involved in either the regulation of transcription of *cprBA* (CprC and CprK) or in the correct folding, processing and assembly of the reductive dehalogenase (CprD, CprE and CprT) (46). By sequence comparison, the genome of *D. ethenogenes* strain 195 was screened for the presence of homologous genes. Four genes putatively encoding CprC-like proteins were identified. Two of these putative *rdhC* genes have no apparent link with *rdhAB* genes, whereas the other two are located in original region F between *Det-rdhA*11 and -12 and in original region G directly upstream of *Det-rdhA*14, respectively. These putative *rdhC* genes have the same transcription direction as the *rdhAB* genes. Three genes that can potentially encode proteins homologous to CprE, CprD and CprT, respectively, have been found, but all three are neither present in an original region nor in the vicinity of a *rdhAB* cluster. Finally, one gene possibly encoding a CprK-like protein was localized in the original region F directly

downstream of *Det-rdhA*13 (data not shown). In order to obtain additional evidence that the original regions A to H are foreign DNA acquired by HGT, they were analyzed for the presence of genes encoding putative recombinases typically present in transmissible MGEs. With the exception of the original regions C, D and G, all other original regions contain recombinase-like genes (indicated as grey arrow in Fig. 4.2). In original region H, consisting of two parts with class II and III signatures, the recombinase-like genes are located in H2 belonging to class III (resembling the phage signature). Interestingly all *rdh* genes located in the first half of contig6871 (original regions A to E) have the same transcription direction. The same pattern was observed for the second half however in the opposite direction. This suggests that the origin of replication is located somewhere between original regions A and H. Therefore the low-complexity region associated with the class IV signature may represent the terminus of replication of *D. ethenogenes* strain 195 genome (Fig. 4.1B).

4.4.6 Signature comparison of the original regions

Since the original regions were clustered in an arbitrarily defined number of classes, their relationship to each other was further considered. The signatures of the original regions, together with those of two short host regions (60-70kb; 1150-1200kb) and the integrated prophage, were subjected to a hierarchical clustering. First, the result confirmed the clustering in four classes, one (class IV) being not included in the clustering (Figure 4.3). Both regions A2 and H2 were similar to the phage region, all of these forming the most distant branch. Both host signatures clustered nicely together. The original regions were found to be variably related to each other. Whereas regions E and F, and also C and G were closely related despite their wide distribution over the genome, the other regions were found to be more distantly related to each other.

4.5 Discussion

Horizontal Gene Transfer (HGT) is considered as a major factor for the rapid adaptation of bacteria to new ecological niches and environmental stresses (10, 24, 38). Several key findings based on the phylogeny of catabolic genes have evidenced the important role of transmissible MGEs in the acquisition of new catabolic properties (49). The intriguing copy number of putative reductive dehalogenase genes in *Dehalococcoides ethenogenes* strain 195 (46, 54) and the quite recent accumulation of chlorinated xenobiotics in the environment, raised the question of the evolution of these genes. Starting with unfinished genome data from the TIGR and prior to annotation, the global genome organization of *D. ethenogenes* was analyzed using the local variations of genomic signatures. The genomic signature (Fig. 4.1A) was obtained by plotting the

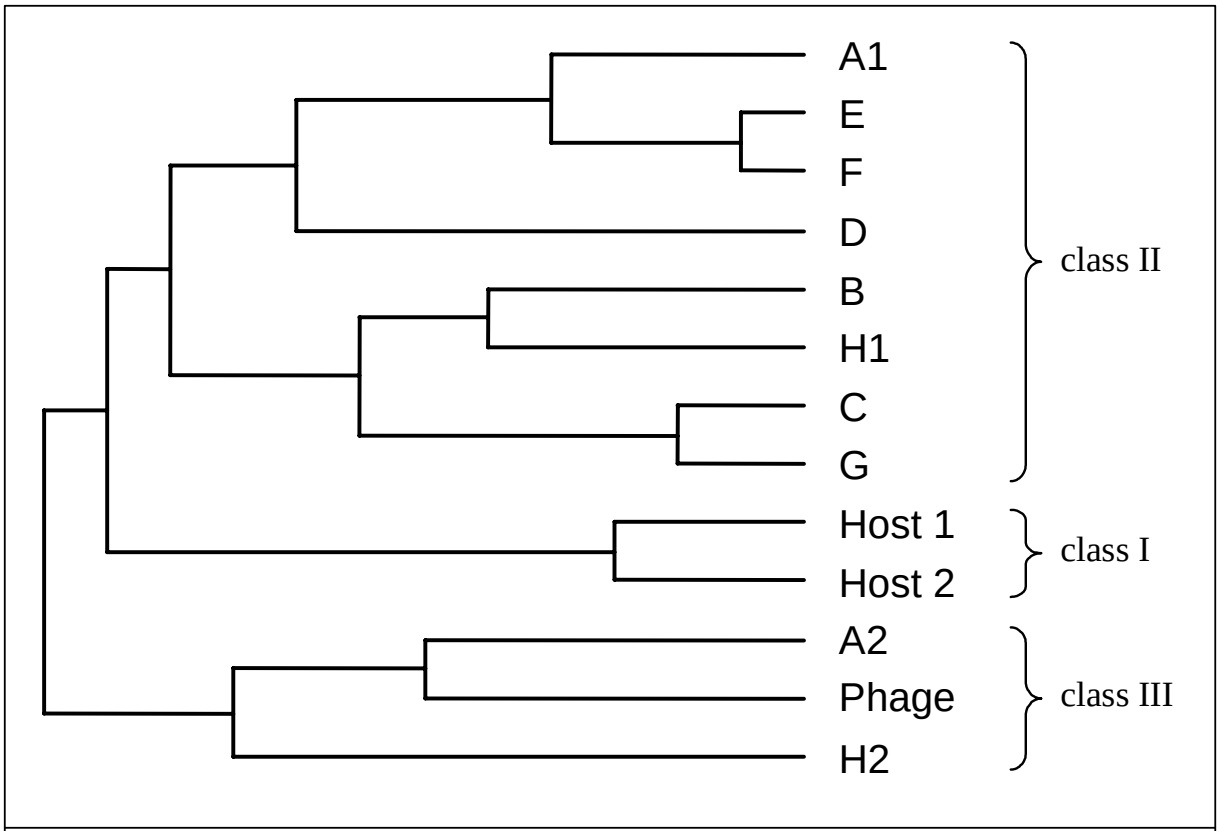

Figure 4.3. Signature comparison of original regions containing *rdhAB* genes in *D. ethenogenes* strain 195 genome. The original regions are reported as A to H, and regions A and H are split into two parts (A1/A2 and H1/H2). The signatures of two small host regions and of the detected integrated prophage were included for comparison. Corresponding clustering classes are also indicated.

frequency of all 4-letter words of 5kb sliding windows along the genome. The clustering in an arbitrarily defined number of 4 classes revealed the presence of at least as many significantly different signatures (Fig. 4.1B). While the most abundant class represents host DNA (class I, 78% of the total genome), two other classes may be considered as DNA of foreign nature, so-called original regions. Whereas class II signature (17%) is widely distributed over the whole genome, class III (4.9%) is dominated by a large DNA fragment probably consisting of an integrated prophage genome. Class IV DNA may represent the genomic terminus of replication. Interestingly original regions A and H (Fig. 4.1B) are composed of a mixture of class II and III signatures (A1, H1 and A2, H2, respectively). Looking for the exact location of the reductive dehalogenase genes, 83% of them, including the well characterized functional *tceA* gene, were found in original regions, fourteen in class II and only one (*Det-rdhA*17, a truncated copy) in class III. Only three copies (*Det-rdhA*2, -3 and -15) are localized in the host DNA. The presence of various recombinases in most original regions and the lower GC content corroborate the hypothesis that these regions may have been acquired by horizontal gene transfer by an ancestor of *D. ethenogenes*

strain 195. The recombinase-like genes identified here were shown to have variable homology to ISDatabase sequences (www-is.biotoul.fr) ranging from 26 (*rec8*) to 61% (*rec1* and -*2*) identity. Interestingly a full insertion sequence was identified directly upstream of *tceA* comprising the *rec1* and *rec2* genes (Fig. 4.2). This *IS* has strong homology to members of the *IS*3 family (such as *IS*Rso14 from *Ralstonia solanacearum* and *IS*511 from *Caulobacter crescentus*).

Signature comparisons of the original regions (Fig. 4.3) confirmed the result obtained by the clustering and clearly indicated that regions of class II and III are different from class I (host signature) and that A2 and H2 are closely related to the phage signature. Speculating about the origin of the original regions, at least three distinct events occurred for the integration of regions A1, E, F and D in one horizontal gene transfer; regions B, H1, C and G in a second; and the phage cluster in a third. Further rearrangements of DNA within the genome may explain the distribution and signature variations of these regions.

Several bacterial dehalogenases have already been reported to be associated with recombinases, most often insertion sequences. Among them, the best characterized are the haloalkane dehalogenase of *Rhodococcus rhodochrous* (25, 41), the haloacetate dehalogenases of *Xanthobacter autotrophicus* (53) and *Delftia acidovorans* (47) and the dichloromethane dehalogenase in methylotrophic bacteria (44). More closely related to the numerous putative reductive dehalogenases of *Dehalococcoides ethenogenes* strain 195, a recent study revealed that the tetrachloroethene reductive dehalogenase genes (*pceAB*) of *Desulfitobacterium hafniense* strain TCE1 was embedded in a composite transposon, *Tn*-Dha1, and that this transposon is probably also present in two other *Desulfitobacterium* strains (see Chapter 3). A fourth species, *Dehalobacter restrictus*, has the same *pce* gene cluster as the three *Desulfitobacterium* strains but it is not embedded in a transposon (Chapter 3). *Desulfitobacterium hafniense* strain DCB-2, another dechlorinating bacterium, whose genome is currently sequenced (the Joint Genome Institute, www.jgi.doe.gov) also possesses several copies of reductive dehalogenase genes (54). Here, six copies were identified sharing homology levels between 26% and 60% identity. The high copy number of reductive dehalogenase genes within a single genome may reflect the complex evolution of the dehalorespiring bacteria towards the large variety of halogenated substrates that they are susceptible to use as a final electron acceptor. Several studies have reported the reductive dehalogenation by *Dehalococcoides*-related bacteria of various halogenated compounds such as chloroethenes and -ethanes (30), chlorobenzenes (1, 18, 19), vinyl bromide (12), polychlorinated biphenyls (5, 17) and polychlorinated dibenzodioxins and dibenzofurans (3). This set of genes may function as a flexible reservoir, most probably inherited by horizontal gene transfer that allows the bacteria to react to dramatic and sudden changes in their environment.

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CHAPTER 5

Enhanced expression of a *c*-type cytochrome by *Desulfitobacterium hafniense* strain TCE1 with tetrachloroethene as terminal electron acceptor

5.1 Abstract

Desulfitobacterium hafniense *strain TCE1 was grown with different combinations of electron donors and acceptors. Hydrogen or lactate was supplied as electron donor, while tetrachloroethene (PCE) or fumarate served as terminal electron acceptors. Cultures growing on lactate and fumarate showed a growth rate twice as high as the other combinations, indicating that mass transfer either from the gaseous or the organic phase to the aqueous phase may be a growth limiting factor in the case of hydrogen and PCE, respectively. Chemiluminescence detection of heme proteins in various cell fractions revealed the presence of a strongly induced heme protein of approximatively 45 kDa, most probably a* c*-type cytochrome, in the membrane fractions of cells grown with PCE as electron acceptor. The* c*-type character was confirmed by optical difference spectra. The* c*-type cytochrome was almost absent when* D. hafniense *strain TCE1 grew with fumarate indicating a possible role of the* c*-type cytochrome in the electron transfer towards the PCE reductive dehalogenase during dehalorespiration.*

5.2 Introduction

Anaerobic respiration implies the establishment of a structured electron transport system within the cytoplasmic membrane enabling energy conservation via a proton motive force. Usually the electrons come from the oxidation of molecular hydrogen or organic compounds, involving a hydrogenase or a compound-specific dehydrogenase, respectively. Free-moving hydrophobic redox elements like quinones serve then as shuttle between the hydrogenase complex and the terminal reductase complex, by which the electrons are transferred onto the terminal electron acceptor. Dehalorespiring bacteria that use various chlorinated compounds as terminal electron acceptor must have a similar protein arrangement in the membrane. While some hydrogenase complexes (13, 18, 19, 21, 27, 31) and several reductive dehalogenases (see Chapter 1) have been studied, very little information about the intermediary elements is available so far. Menaquinones are involved in the respiratory electron transport in *Dehalobacter restrictus* (21), but not in *Sulfurospirillum multivorans* (13). Cytochrome analysis by UV-visible spectrophotometry has shown the presence of *b*-type cytochromes in *D. restrictus* (6) and *c*-type cytochromes in *Desulfomonile tiedjei* (9), *Desulfitobacterium sp*. strain PCE1 (5) and *Desulfitobacterium hafniense* strain TCE1 (4). Whereas *b*-type cytochromes are part of most hydrogenase complexes, transferring the electrons from hydrogen to the quinone pool, *c*-type cytochromes are possible candidates for the transfer of electrons from the quinone pool to the terminal reductase complexes in various anaerobic respiration pathways (1, 10, 23, 24, 26).

Biochemical methods based on the peroxidase activity of hemes have been developed for the detection of cytochromes in protein samples (2, 3). Whereas the colorimetric heme detection with 3,3',5,5'-tetramethylbenzidine was commonly used, a higher sensitivity has been obtained with chemiluminescence using luminol and hydrogen peroxide as reagents. In this chapter, the presence of cytochromes in the different fractions of *D. hafniense* strain TCE1 cells grown on various combinations of electron donors and acceptors was studied using this sensitive chemiluminescence detection and UV-visible spectrophotometry.

5.3 Materials & Methods

***Desulfitobacterium hafniense* strain TCE1 growth conditions**. *D. hafniense* strain TCE1 was grown in medium 717 (according to DSMZ, Braunschweig, Germany) with slight modifications. The trace elements solution was taken from the *Dehalobacter restrictus* medium (DSMZ, solution SL-10, medium 320) and both vitamins solutions (DSMZ, media 141 and 503) were combined and

the concentration of vitamin B_{12} was 2.5-fold increased. Hydrogen (95% H_2, 5% CO_2) or lactate (40 mM) was amended as electron donor, and fumarate (40 mM) or PCE (1 ml/100 ml medium from a 1.96 M stock solution in hexadecane, final nominal concentration 18 mM) as electron acceptor. All four possible combinations of electron donors and acceptors were applied and the codes HF (hydrogen-fumarate), HP (hydrogen-PCE), LF (lactate-fumarate) and LP (lactate-PCE) were used for the different cultivation conditions. Bacterial growth was followed by measuring the absorbance at 600 nm.

Analytical methods. Chloride production of the dechlorination reaction was determined amperometrically with a silver electrode (Chlor-o-Counter, Flohr Instruments, Nieuwegein, The Netherlands) as previously described (21). Chloroethenes were analyzed by gas chromatography (Varian Star 3400CX, Varian AG, Zug, Switzerland) as described elsewhere (28). Protein samples were routinely assayed with the Bradford method with BSA as standard.

Cell harvest and fractionation. *D. hafniense* strain TCE1 cultures in late exponential phase were harvested by centrifugation at 5000 x g at 4°C for 20 min. The cell pellet was washed in 50 mM Tris-HCl buffer (pH 8.0) and again centrifuged for 15 min in a 50 ml tarred Beckman tube. The wet weight of the cell pellet was finally determined. The cells were resuspended in an equal volume (1 ml for 1 g wet weight) of the same buffer and stored at -80°C. Cell disruption was performed by 10 cycles of 30 s sonication at 15 W (Vibra Cell 72405, Bioblock Scientific, Illkirch, France) with 5-10 min steps on ice after each cycle. Cell debris was removed by centrifugation at 4000 x g at 4°C for 10 min. The supernatant was considered as cell extract (abbreviated CE). Soluble (SF) and membrane fractions (MF) were obtained by ultracentrifugation (200'000 x g at 4°C for 1 h) in a Centrikon T-1065 centrifuge using a TST60.4 rotor (Kontron Instruments, Milton Keynes, UK). Extraction of the membrane proteins was performed by a 30 min incubation under stirring of the membrane fraction in 20 mM Tris-HCl (pH 8.0) containing 1% Triton-X-100, followed by a second run of ultracentrifugation, the supernatant being the membrane extract (ME).

SDS PAGE and transfer to PVDF membrane. Gels containing 10% SDS were prepared according to Sambrook *et al*. (17). The samples (up to 15 µl) were mixed with 3 µl of 6 x DTT-free loading buffer (200 mM Tris-HCl, pH 6.8, 6% SDS, 20% glycerol, 0.1% Bromphenol blue) and loaded without heat denaturation onto the gel. Electrophoresis was run for 2 h at 80-100 V. For direct revelation of the proteins, the gel was stained with Comassie for 30 min, then destained in 8% acetic acid, 5% methanol solution. For heme detection, the proteins in the gel were first transferred onto a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA) using the Mini-

PROTEAN 3 apparatus at 100 V, 4°C for 1 h (BIO-RAD, Reinach, Switzerland). The membrane was shortly rinsed in ultrapure water (MilliQ, Millipore S.A., Molsheim, France).

Dot blot analysis. Using the Dot Blot 96 apparatus (Biometra, Göttingen, Germany), a piece of PVDF membrane, prepared as for Western blot, was first rinsed with transfer solution (25 mM Tris, 200 mM glycin, 0.02% SDS, 20% methanol). The samples previously diluted 20 to 100-fold in transfer solution were loaded by applying a vacuum of 150 mbar. The wells were finally rinsed with transfer solution. The membrane was shortly rinsed in ultrapure water before heme detection.

Heme detection by chemiluminescence. Both reagents of the ECL Western Blotting Detection Kit (Amersham Biosciences, Little Chalfont, UK) were mixed at a 1:1 ratio and applied (6 ml for 50 cm^2) onto the PVDF membrane disposed within a plastic foil. Excess of liquid was removed after 5 min of incubation and the plastic foil sealed. In the dark room, a sheet of Hyperfilm ECL (Amersham Biosciences) was placed onto the membrane and incubated between 5 and 30 min before development on a Curix 60 apparatus (AGFA-Gewaert N.V., Belgium).

Photometrical analysis of hemoproteins. Aerobically prepared membrane extracts were diluted (maximally 2 to 3-fold) in 20 mM Tris-HCl buffer (pH 8.0) and scanned over a wide wavelength range at room temperature on a HP 8453 Diode Array spectrophotometer. Tris buffer was used as blank. The extracts were then reduced in gas-tight cuvettes with aliquots from a 200 mM sodium dithionite solution in Tris buffer. New scans were performed at different time intervals. Dithionite-reduced minus air-oxidized difference spectra were calculated. Cytochrome *c* present in the extracts was quantified from the absorbance difference between 552 nm and 538 nm using an extinction coefficient of 17.3 $mM^{-1}cm^{-1}$ (5).

5.4 Results

5.4.1 Growth of *D. hafniense* strain TCE1 with different electron donor – acceptor conditions

Growth of of *D. hafniense* strain TCE1 in batch cultures containing the electron donor - acceptor combinations HF, HP, LF, and LP (see Materials & Methods) were followed over time. Growth rates were estimated at 0.022, 0.023, 0.057 and 0.028 h^{-1}, respectively. These values may be explained by the limiting mass transfer of hydrogen (for HF and HP) and of PCE (for HP and LP) from the gaseous phase and organic phase to the aqueous medium, respectively.

5.4.2 Total heme content of *D. hafniense* strain TCE1 cells

Starting from *D. hafniense* strain TCE1 cells grown on the four combinations of electron donors and acceptors, cell fractionation was performed from cell extracts (CE) yielding soluble fractions (SF), membrane extracts (ME) and as control for the extraction efficiency, membrane rests (MR). This was done on cells from two or three independent batch cultures of each combination. The total heme content of the different protein fractions was determined by Dot blot and chemiluminescence analysis. Large differences in signal intensity were observed among the different fractions. Much stronger signals were in general obtained with cell fractions of HP cultures (Figure 5.1A, lines 5-8) than with cells of HF cultures (lines 1-4). It was estimated that the signal of cell extracts of HP cells was approximately 10-times stronger than the signal of HF cells. Moreover the distribution of heme proteins among the fractions of HF and HP cells was different. Whereas most of the heme proteins of HF cells were found in the soluble fraction (Fig. 5.1A, line 2), heme proteins were strongly enriched in the membrane extract of HP cells (line 7), indicating that the content of heme proteins in the membrane increases, if *D. hafniense* strain TCE1 grows on PCE instead of fumarate as electron acceptor. The results obtained for *D. hafniense* strain TCE1 cells grown on LF and LP showed similar trends (Fig. 5.1B). However, total heme content of LF membrane extracts was found to be higher than the HF counterpart (Fig. 5.1B, line 3).

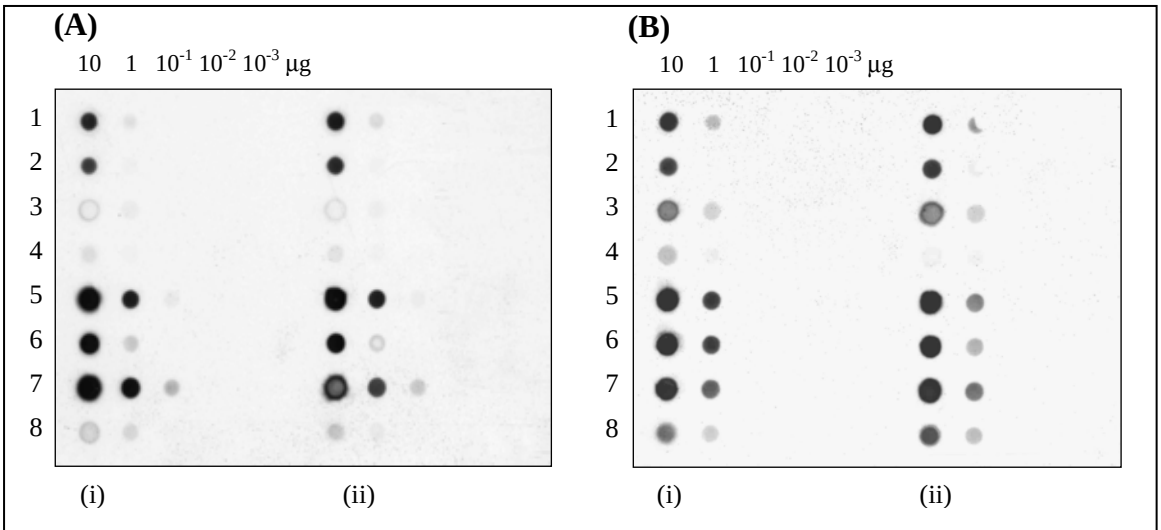

Figure 5.1. Heme detection by Dot blot analysis in protein fractions from (**A**) HF and HP grown and (**B**) LF and LP grown *D. hafniense* strain TCE1 cells. The analysis was performed from two completely independent batch cultures of each electron donor/acceptor combination (i and ii), giving very reproducible results. Protein fractions (10 µg) of cells grown on HF (A, lines 1-4), HP (A, lines 5-8), LF (B, lines 1-4) and LP (B, lines 5-8) were serially diluted to 10^{-3}µg. The following fractions were analyzed: cell extracts (lines 1 and 5), soluble fractions (lines 2 and 6), membrane extracts (lines 3 and 7) and membrane rests (line 4 and 8).

5.4.3 *C*-type cytochrome detection

Cytochromes of the *c*-type are the only cytochromes with the heme groups covalently bound by two cysteinyl residues of the polypeptide (29). Therefore, an easy way to distinguish *c*-type cytochromes from the pool of hemoproteins is to perform heme detection after running the protein samples on a SDS gel (3). Western blot-like analysis was performed with the protein fractions already analyzed by Dot blot analysis. Ten micrograms of each sample were separated on a 10% SDS gel and transferred onto a PVDF membrane. *C*-type cytochromes detection by chemiluminescence (Figure 5.2) showed the presence of only one weak band around 30 kDa in membrane extracts of HF cells (lane 3), but a very strong band around 45 kDa in membrane extracts of HP cells (lane 7). Despite the fact that several parameters may influence the quality of the detection (transfer efficiency, signal to noise ratio, signal saturation), it was quite obvious that the 45 kDa band was massively enriched in the membrane extract compared to the cell extract (lane 5). Indeed this signal was almost invisible in the CE fraction. The strong 45 kDa *c*-type cytochrome signal of the HP membrane extract fraction was probably responsible for most of the signal observed in the same fraction by the total heme detection (Fig. 5.1A, line 7). The analysis of fractions of LF and LP cells gave very similar results (data not shown).

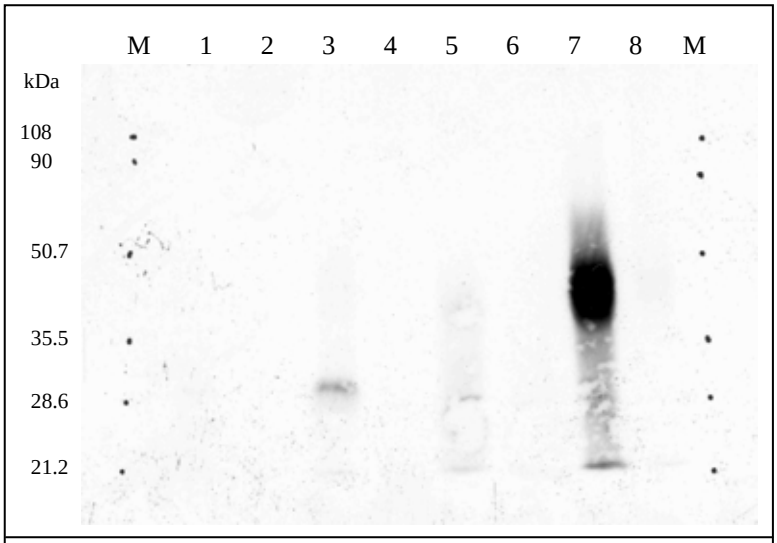

Figure 5.2. Heme detection of SDS PAGE separated protein fractions from HF (lane 1-4) and HP (lane 5-8) cells. Ten micrograms of cell extract (lane 1 and 5), soluble fraction (lane 2 and 6), membrane extract (lane 3 and 7) and membrane rest (lane 4 and 8) were analyzed. The prestained SDS PAGE Standards Low Range protein marker (BIORAD) was reported as dots on the developed film. See text for details.

5.4.4 Optical difference spectra of membrane extracts

Difference spectra of dithionite-reduced and air-oxidized membrane extracts of HF (0.7mg/ml), HP (1.3mg/ml), LF (2.6mg/ml) and LP (2.5mg/ml) cells were recorded in order to confirm the presence and type of cytochromes in these fractions by an additional method. Ferricyanide was also used as oxidant, but no further oxidation was observed. While the spectra of HP, LF and LP membrane extracts clearly indicate the presence of *c*-type cytochromes (Soret band around 426nm, α- and β-band at 552 and 523 nm, respectively), the absorbance obtained with HF membrane extract was much weaker with a Soret band around 420nm (Figure 5.3). The shoulder around 560 nm present in the α-band of LF membrane extracts indicated in addition the presence of *b*-type cytochromes in this fraction. Using an extinction coefficient of 17.3 $mM^{-1}cm^{-1}$ for to the absorbance difference between 552 and 538 nm, which corresponds to the amplitude of the α-band, cytochrome *c* contents of 1.1, 6.5, 1.8 and 3.0 μmol $(g\ protein)^{-1}$ were calculated for the membrane extracts of HF, HP, LF and LP cells, respectively. This quantification confirmed the results of the chemiluminescence detection of hemes in HF and HP cell fractions where approximately 10-times more hemes were found in cells grown with PCE as electron acceptor. Here, the *c*-type cytochrome content was about 6-times higher in HP cells. In the case of LF and LP cells, the increase of the *c*-type cytochrome content was lower than a factor of two. This may be partially explained by an overestimation of the *c*-type cytochrome content of LF cells due to the presence of *b*-type cytochromes, but also by the presence of a higher heme content in the membrane extract of LF cells compared to the corresponding fraction of HF cells, as already illustrated by Dot blot analysis (Fig. 5.1B).

5.5 Discussion

Cytochromes have been shown to play a key role in various anaerobic respiratory processes. They usually act as an electron carrier between the electron-donating enzyme complex, such as hydrogenases or formate dehydrogenases, and the quinone pool, or between the quinone pool and the terminal electron-accepting enzyme complex such as nitrate or fumarate reductases (for a review, see (29)). In dehalorespiring bacteria, which can use chlorinated compounds as terminal electron acceptors, the presence of *b*- and *c*-type cytochromes has been reported (6, 20, 30). In the work presented here, an enhanced expression of a 45 kDa *c*-type cytochrome present in the membrane fraction was evidenced in cells of *Desulfitobacterium hafniense* strain TCE1 grown with PCE as terminal electron acceptor compared with cells grown with fumarate. This difference

was most evident in membrane extracts of cells grown with hydrogen as electron donor (Figure 5.2). Dot blot analysis (Figure 5.1B) and optical difference spectroscopy (Figure 5.3B) indicated that in presence of lactate *D. hafniense* strain TCE1 forms additional cytochromes. Whether the 45 kDa *c*-type cytochrome is directly involved in dehalorespiration with PCE or whether it is just induced in the presence of PCE needs further investigations.

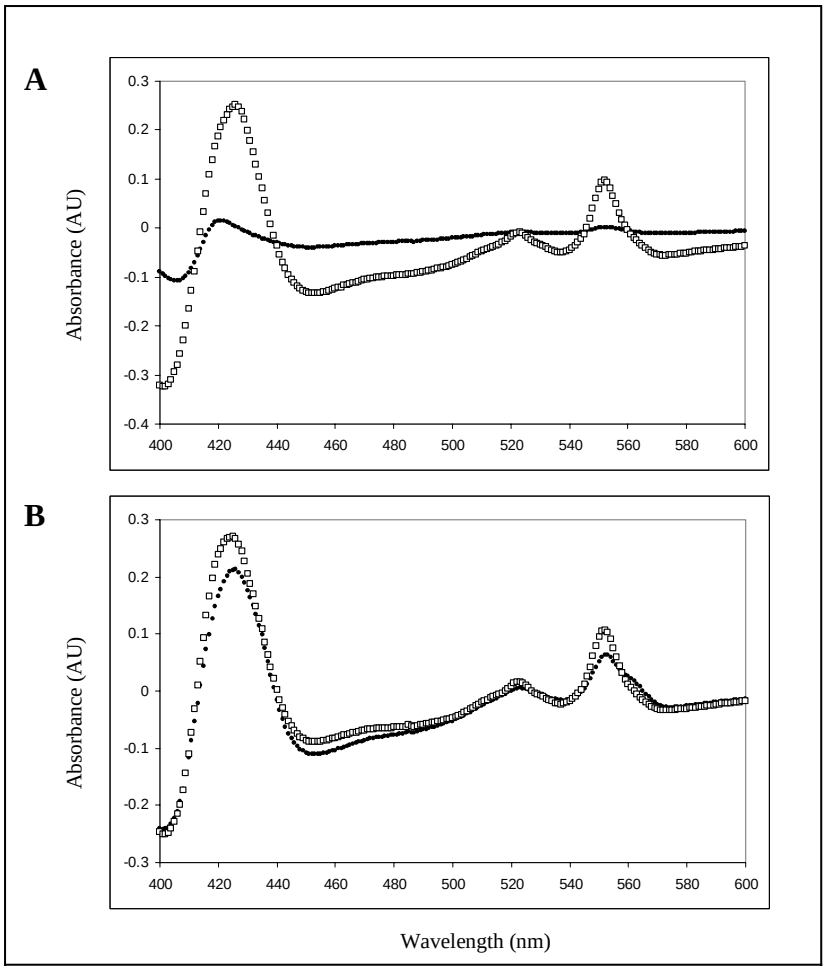

Figure 5.3. Optical difference spectra of dithionite-reduced minus air-oxidized membrane extracts from (**A**) HF (black cirlces) and HP cells (open square) and from (**B**) LF (black circles) and LP cells (open square).

An example for the latter possibility, the induction of cytochromes without clear evidence for its involvement in the anaerobic respiration pathway, has been reported for the DMSO respiration in *E. coli* . A cytochrome *c*-556 has been expressed at high levels in *E. coli* cells grown on DMSO, but a mutant defective in DMSO reductase has shown similar results. Moreover, there is no evidence for the involvement of cytochromes in the DMSO respiration of this bacterium (33). In the case of *D. tiedjei*, high level of expression of a 50 kDa cytochrome has been reported for cells grown in presence of 3-chlorobenzoate. The purification and isolation of the corresponding gene

has revealed a diheme *c*-type cytochrome with a redox potential of -342 mV (9). It has been concluded that this cytochrome is not a suitable direct electron donor for the reductive dehalogenase due to the low redox potential compared to the potential of the 3-chlorobenzoate/benzoate half reaction (E_0' = +0.297 mV) (9). However, no biochemical evidence has been presented that excluded the involvement of this cytochrome in respiration with 3-chlorobenzoate. In addition, the determination of redox potentials of iron-sulfur clusters and corrinoids present in reductive dehalogenases by EPR spectroscopy has suggested that an electron donor with relatively low redox potential is needed to reduce the enzyme, although the half reaction potential of the terminal electron acceptor is high (22, 32). The need of artificial low redox potential electron donors in *in vitro* enzyme assays for reductive dehalogenases corroborate this hypothesis (12, 13, 22). Hence, it is not certain that the induction of the *c*-type cytochrome in *D. tiedjei* upon growth with 3-chlorobenzoate is indeed a similar case as the cytochrome induction linked to DMSO respiration by *E. coli*.

There are only few indications for the involvement of cytochromes in dehalorespiration in other dechlorinating bacteria. UV-visible spectroscopic analysis of membrane-bound cytochromes in cell suspensions of *D. dehalogenans* has revealed the reduction and oxidation of *c*-type cytochromes upon addition of formate and Cl-OHPA, respectively. This indicated that the cytochromes were involved in transferring the electrons from formate to the chlorinated compound (30). In *Desulfitobacterium* sp. strain PCE1 cells *c*-type cytochromes were also observed with pyruvate as substrate, however independent of the presence or absence of PCE (5). Cytochromes have been detected in *S. multivorans*, however only in cells grown on pyruvate and fumarate (20). It is not known whether *S. multivorans* also contains cytochromes when cultivated with PCE as electron acceptor. Although *b*-type cytochromes have been observed in *D. restrictus* cells grown on hydrogen and PCE (6) and *c*-type cytochromes in *D. hafniense* strain TCE1 cells grown on lactate and PCE (4), no clear involvement in the dehalorespiration has been presented so far.

Both *b*- and *c*-type cytochromes are involved in the electron transfer from the quinone pool to diverse terminal reductases (29) and there seems to be a general rule concerning the topology of the latter. *C*-type cytochromes are often involved in electron transfer to reductases located at the periplasmic side of the membrane, as shown for the TMAO reductase of *E. coli* (11), the periplasmic nitrate reductase of *E. coli* (15) and *Rhodobacter sphaeroides* (8), and the periplasmic DMSO reductase of *Rhodobacter capsulatus* (25). On the contrary, *b*-type cytochromes mostly transfer electrons to cytoplasm-oriented terminal reductases. Well known examples are the fumarate reductase of *Wolinella succinogenes* (7) or the membrane-bound nitrate reductase of *E. coli* (16). No consensual topology can be deduced from the results presented so far for

dehalorespiration pathways. While the PCE reductive dehalogenase of *D. restrictus* and *S. multivorans* seems to be located on the cytoplasmic side of the membrane (13, 21), the TCE reductive dehalogenase of *D. ethenogenes* seems to be periplasm-oriented (14). The topology of the respiration chain in *D. restrictus* would be in agreement with the general rule presented above due to the presence of *b*-type and the absence of *c*-type cytochromes. However, as already mentioned, it is not known whether *b*-type cytochromes are involved on both side of the electron transport chain of *D. restrictus* as reported for fumarate respiration in *W. succinogenes* (7), or whether another redox element is responsible for the electron transfer to the reductive dehalogenase (as suggested in Fig. 1.2). No cytochrome analysis has been reported for *D. ethenogenes* and the Cl-OHPA reductive dehalogenase of *D. dehalogenans* that seems to be reduced by a *c*-type cytochrome could not be clearly localized (30). If the *c*-type cytochrome expressed in *D. hafniense* strain TCE1 with PCE as electron acceptor is involved in electron transfer to the reductive dehalogenase, this terminal reductase would by analogy to the general rule be located on the periplasmic side of the membrane. Unfortunately no experimental evidence is available for the location of PceA of this dehalorespiring bacterium. Futher investigations are necessary to elucidate the role of the 45 kDa *c*-type cytochrome present in the membrane of *D. hafniense* strain TCE1, and the topology of the electron transport chain involved in dehalorespiration.

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CHAPTER 6

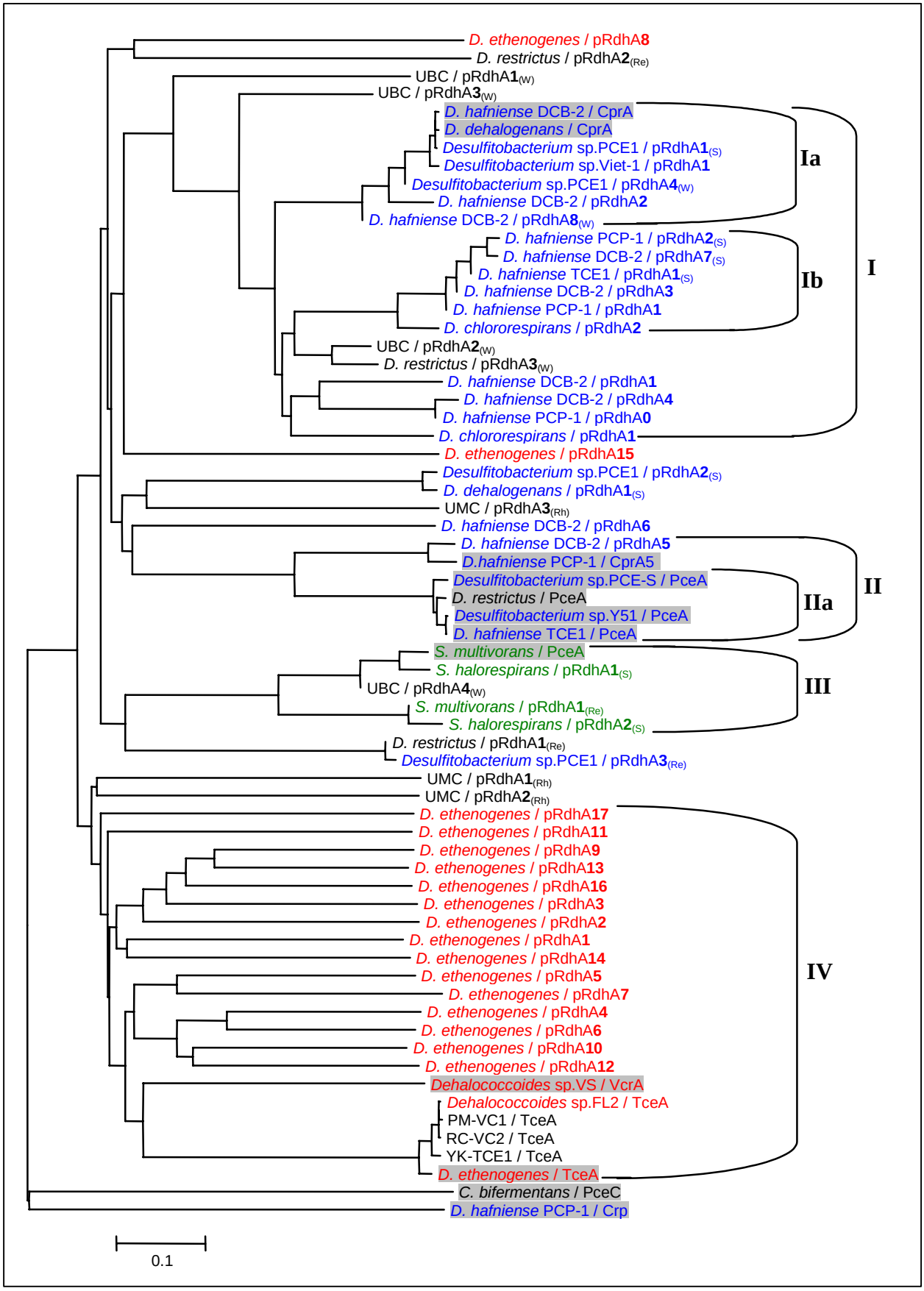
Concluding remarks and Outlook

6.1 Evolutionary considations of the dehalorespiration process

6.1.1 Emergence of reductive dehalogenase sequences

The first sequence of a reductive dehalogenase (i.e. of the new class of corrinoid and iron-sulfur containing enzymes) in general databases was the PceA of *Sulfurospirillum multivorans* reported by Neumann and co-workers in 1998 (20). In the next couple of years only two more sequences were added, CprA of *Desulfitobacterium dehalogenans* (29) and TceA of *Dehalococcoides ethenogenes* (15). Making use of a conserved region present in the first two sequences reported (29), the *pceA* genes of *Dehalobacter restrictus* and *Desulfitobacterium hafniense* strain TCE1 were isolated (Chapter 2). Both enzymes were found to be almost identical as well on amino acid as on nucleotide sequence level, sharing this identity with the sequence of the PceA of *Desulfitobacterium* sp. strains Y51 (27) and PCE-S (G. Diekert, unpublished results). During the time course of the thesis, several published and unpublished studies reported on the isolation of mainly partial gene sequences of additional members of this enzyme class. Only for a few of these sequences deposited on sequence databases the gene product has also been biochemically characterized and hence, the primary substrate is known. An extensive list of full length and partial RdhA sequences is presented in Table 6.1. Next to the classical protein-to-gene approach, several authors have developed degenerate PCR strategies for the detection of known and new *rdhA* gene sequences from pure cultures and various environmental samples (22-24, 31), revealing about 20 new sequences. Moreover, the genome sequencing projects of the two dehalorespiring bacteria *Dehalococcoides ethenogenes* (The Institute for Genome Research, www.tigr.org) and *Desulfitobacterium hafniense* strain DCB-2 (the Department of Energy Joint Genome Institute, www.jgi.doe.gov) have revealed the presence of 18 and 6 reductive dehalogenase gene copies, respectively. Taken all together, 59 sequences were isolated from 19 bacterial strains, while 7 additional sequences were obtained from bacterial consortia (Table 1). It is particularly interesting that dehalorespiring bacteria seem to have accumulated multiple copies of reductive dehalogenase genes during evolution. The most striking example is *D. ethenogenes* with 18 copies (Chapter 4).

Figure 6.1 (next page). Phylogenetic tree of the reductive dehalogenases. The alignment of all sequences was performed with ClustalX. The tree was obtained with the Neighbour-Joining algorithm and drawn using TreeExplorer. The tree was rooted with the branching point of both atypical reductive dehalogenases PceC of *C. bifermentans* and Crp of *D. hafniense* strain PCP-1. Biochemically characterized reductive dehalogenases are indicated by grey shading. *Desulfitobacterium* reductive dehalogenenases are depicted in blue, *Sulfurospirillum* in green and *Dehalococcoides* in red. An exhaustive list of reductive dehalogenases, including references, is given in Table 6.1. UBC = uncultured bacterial consortium; UMC = uncultured marine consortium. PceA = PCE reductive dehalogenase; TceA = TCE reductive dehalogenase; CprA = chlorophenol reductive dehalogenase; VcrA = vinyl chloride reductive dehalogenase; pRdhA = putative reductive dehalogenase; PceC = PCE reductase (atypical); Crp = chlorophenol reductase precursor (atypical).


D. ethenogenes / pRdhA8
D. restrictus / pRdhA2(Re)
UBC / pRdhA1(W)
UBC / pRdhA3(W)
D. hafniense DCB-2 / CprA
D. dehalogenans / CprA
Desulfitobacterium sp.PCE1 / pRdhA1(S)
Desulfitobacterium sp.Viet-1 / pRdhA1
Desulfitobacterium sp.PCE1 / pRdhA4(W)
D. hafniense DCB-2 / pRdhA2
D. hafniense DCB-2 / pRdhA8(W)
Ia
D. hafniense PCP-1 / pRdhA2(S)
D. hafniense DCB-2 / pRdhA7(S)
D. hafniense TCE1 / pRdhA1(S)
D. hafniense DCB-2 / pRdhA3
D. hafniense PCP-1 / pRdhA1
D. chlororespirans / pRdhA2
Ib
I
UBC / pRdhA2(W)
D. restrictus / pRdhA3(W)
D. hafniense DCB-2 / pRdhA1
D. hafniense DCB-2 / pRdhA4
D. hafniense PCP-1 / pRdhA0
D. chlororespirans / pRdhA1
D. ethenogenes / pRdhA15
Desulfitobacterium sp.PCE1 / pRdhA2(S)
D. dehalogenans / pRdhA1(S)
UMC / pRdhA3(Rh)
D. hafniense DCB-2 / pRdhA6
D. hafniense DCB-2 / pRdhA5
D.hafniense PCP-1 / CprA5
Desulfitobacterium sp.PCE-S / PceA
D. restrictus / PceA
Desulfitobacterium sp.Y51 / PceA
D. hafniense TCE1 / PceA
IIa
II
S. multivorans / PceA
S. halorespirans / pRdhA1(S)
UBC / pRdhA4(W)
S. multivorans / pRdhA1(Re)
S. halorespirans / pRdhA2(S)
III
D. restrictus / pRdhA1(Re)
Desulfitobacterium sp.PCE1 / pRdhA3(Re)
UMC / pRdhA1(Rh)
UMC / pRdhA2(Rh)
D. ethenogenes / pRdhA17
D. ethenogenes / pRdhA11
D. ethenogenes / pRdhA9
D. ethenogenes / pRdhA13
D. ethenogenes / pRdhA16
D. ethenogenes / pRdhA3
D. ethenogenes / pRdhA2
D. ethenogenes / pRdhA1
D. ethenogenes / pRdhA14
D. ethenogenes / pRdhA5
D. ethenogenes / pRdhA7
D. ethenogenes / pRdhA4
D. ethenogenes / pRdhA6
D. ethenogenes / pRdhA10
D. ethenogenes / pRdhA12
Dehalococcoides sp.VS / VcrA
Dehalococcoides sp.FL2 / TceA
PM-VC1 / TceA
RC-VC2 / TceA
YK-TCE1 / TceA
D. ethenogenes / TceA
IV
C. bifermentans / PceC
D. hafniense PCP-1 / Crp
0.1

6.1.2 Sequence homology among reductive dehalogenases

The 66 reductive dehalogenase sequences isolated so far were aligned (by ClustalX and a few manual corrections) and compared using the Neighbour-Joining algorithm in a tree-like structure (Figure 6.1). Although predicted protein sequences of reductive dehalogenase show a large range of similarity between 25 and 99%, the combination of a Tat signal peptide, two iron-sulfur cluster binding motifs and the presence of a short ORF with structural conserved features in the direct vicinity of the gene encoding the catalytic unit makes them easily distinguishable from other proteins. The overall homology, as depicted in Figure 6.1, allows a classification of reductive dehalogenases in at least 4 clusters with three subclusters. Subclusters (Ia) and (Ib) represent two individual sequences so far only detected in *Desulfitobacterium* strains. While subcluster (Ia) contains the sequences of the biochemically well characterized CprA of *D. dehalogenans* (29) and *D. hafniense* strain DCB-2 (24) that have dechlorinating activity towards *o*-chlorophenol and Cl-OHPA, respectively, there are no indications which substrate may be transformed by members of subcluster (Ib). Nevertheless, it is tempting to attribute chlorophenol dechlorinating activity to the relatively well conserved cluster (I), which contains both subclusters (Ia) and (Ib) as well as several slightly more divergent sequences. However, two members of cluster (I) have been isolated from exclusively chloroethene dehalorespiring bacteria, namely *Dehalobacter restrictus* and *Desulfitobacterium hafniense* strain TCE1 (22, 31). One common feature of the reductive dehalogenase genes of cluster (I) is the organization of the genes *rdhA* and *B*. They all are in the order *rdhBA* in cluster (I) whereas they are in the order *rdhAB* in the other clusters. Subcluster (IIa) groups the sequences of four highly conserved PCE reductive dehalogenases, among which three were obtained from three different strains of the genus *Desulfitobacterium* and one from *Dehalobacter restrictus* (Chapter 2). Two homologous sequences found in *D. hafniense* strains PCP-1 and DCB-2 show 65% identity with subcluster (IIa), forming cluster (II). While the sequence of strain DCB-2 was retrieved from an unfinished genome project and for which no biochemical data is available, evidence has been recently given that the CprA5 of strain PCP-1 shows strong dechlorination activity with tri- and dichlorophenols (28), but only weak activity with PCE (R. Villemur, personal communication). Sequences with homology to the PCE reductive dehalogenase of the Gram-negative *S. multivorans* were defined as cluster (III). A completely homologous sequence to PceA of *S. multivorans* has been found in *S. halorespirans* (24). Another pair of homologous sequences was also identified from both *Sulfurospirillum*

species that group in cluster (III), indicating that this genus has developed its own set of reductive dehalogenases.

6.1.3 The special case of *Dehalococcoides ethenogenes*

All reductive dehalogenase sequences related to the genus *Dehalococcoides*, with the exception of the *rdhA* gene copies 8 and 15 from the genome of *D. ethenogenes*, fall into cluster (IV) (Figure 6.1). Biochemical evidence of the dechlorinating activity has only been shown for TceA of *Dehalococcoides ethenogenes* (15, 16) and very recently for VcrA of *Dehalococcoides* sp. strain VS (19). VcrA is the first described reductive dehalogenase dechlorinating dichloroethenes and vinyl chloride but not PCE and TCE. Despite the relatively low sequence identities among members of cluster (IV), one can clearly distinguish them from the reductive dehalogenase sequences of phylogenetically unrelated bacteria.

6.1.4 Horizontal gene transfer of reductive dehalogenases

6.1.4.1 *Tn*-Dha1, a *pce* gene cluster composite transposon

The isolation of identical reductive dehalogenase genes from two different, though phylogenetically related bacterial genera, *Dehalobacter* and *Desulfitobacterium*, raised the question of possible horizontal gene transfer and evolution of these genes (Chapter 2). Further investigation of the flanking regions of the *pceA* gene in *Desulfitobacterium hafniense* strain TCE1 showed the location of this gene on a mobile genetic element (MGE). The composite transposon *Tn*-Dha1 described in Chapter 3 contains the *pce* gene cluster *pceABCT* and is flanked by two identical copies of *IS*Dha1, a new insertion sequence of the *IS*256 family. Next to the already described *pceAB* genes, five different ORFs were found on *Tn*-Dha1: *tnpA*1, the transposase encoded by *IS*Dha1, *pceC* and *pceT*, two ORFs related to genes from the *cpr* gene cluster of *D. dehalogenans*, responsible for the *o*-CP dechlorinating activity, and *tnpA*2 and *tatA*, two ORFs showing homology to another transposase and to TatA, a subunit of the twin-arginine translocation machinery, respectively. The flanking regions of *pceAB* in *D. restrictus* were also investigated showing that the *pceABCT* gene cluster was fully conserved in this bacterium (Chapter 3). However, both the insertion sequences of the transposon and the additional genes *tnpA*2 and *tatA* were missing in *D. restrictus*, indicating a possibly stable incorporation of the *pce* gene cluster in the chromosome of that strain. It is tempting to speculate about a possible direct horizontal transfer of the *pceABCT* gene cluster between *D. restrictus* and *D. hafniense* strain TCE1, two strains that have been isolated from

environmental samples taken at sites separated by a distance of less than 100 km (9, 11). Since the transposon structure seems to be also conserved in both *Desulfitobacterium* sp. strains PCE-S and Y51 (Chapter 3), two strains isolated from Germany (10) and Japan (26), respectively, the simplest event that could have occurred is the transfer from *D. hafniense* strain TCE1 to *D. restrictus* with subsequent loss of the transposon structure in *D. restrictus*. This would have led to a concomitant loss of the mobility of the *pceABCT* cluster, which is in good accordance with the dependence of *D. restrictus* on PCE dehalorespiration for growth (11).

6.1.4.2 The intriguing origin and function of the multiple reductive dehalogenase genes in *Dehalococcoides ethenogenes*

Preliminary sequence data from the genome of *Dehalococcoides ethenogenes* strain 195 (The Institute for Genome Research, TIGR) has revealed the presence of 17 copies of putative reductive dehalogenase genes (24, 30), next to the well characterized *tceA* (15). In an attempt to evaluate the chance that the *rdhA* genes could have been acquired by *D. ethenogenes* through horizontal gene transfer, a bioinformatic approach was applied to the genome sequence (Chapter 4). A method was used that is based on the assumption that the ordering and usage of the four nucleotides in DNA is conserved within a genome mainly because of the constraints imposed by the species-specific DNA replication, recombination and repair systems (13, 14). Local disruptions of the obtained genomic signature are most likely to correspond to DNA segments which have been acquired during evolution through horizontal gene transfer mechanisms (6, 7). This analysis revealed the presence of 15 (including *tceA*) out of the 18 reductive dehalogenase genes in original regions, i.e. DNA regions with a different genomic signature than the host signature (Chapter 4). This was the first indication that the dechlorinating activity may have been acquired by an ancestor of *D. ethenogenes* and that this ancestor should have had another energy metabolism. However, no alternative energy metabolism could be identified for *D. ethenogenes*, which would indicate that this latter organism has lost the ability to grow on alternative electron acceptors. The hypothesis of the foreign nature of the reductive dehalogenase genes is corroborated by the finding of several ORFs with homology to the recombinase family within the original regions. Furthermore the GC content of these regions was in general lower than of the host DNA.

Although all reductive dehalogenases present in the genome of *D. ethenogenes* show a relatively low degree of conservation among themselves, they form a clear separate cluster when compared to the reductive dehalogenases of phylogenetically unrelated strains (Fig. 6.1,

cluster (IV)). This may indicate that the origin of *D. ethenogenes* reductive dehalogenases is not to be found in yet identified phylogenetic groups containing dehalorespiring organisms. Furthermore, one could imagine two possible scenarios for the multiple presence of reductive dehalogenases: either multiple acquisition events occurred, a possibility that could not be clearly shown by comparing the genomic signature of the different original regions (Chapter 4) or only a few individual genes were acquired, followed by repeated gene duplication and high mutation rates.

The presence of multiple reductive dehalogenase genes in the genome of *D. ethenogenes* may be considered as a reservoir, whose redundancy enables the bacterium to react promptly to changes in the composition of chlorinated (or more generally halogenated) organic compounds present in the environment. A recent study revealed that, although *D. ethenogenes* was thought to dechlorinate only chloroethenes, several other chlorinated compounds such as highly chlorinated dibenzo-*p*-dioxins, dibenzofurans, biphenyls, naphthalenes and benzenes have been reduced by *D. ethenogenes* (8). This extended substrate spectrum may be correlated to the presence of multiple gene copies. However, some expression studies and purification of substrate-specific reductive dehalogenases would be necessary to validate this hypothesis.

6.1.5 On the origin of the dehalorespiration chain

The presence of rather conserved patterns among the quite diverse reductive dehalogenases suggests that they evolved from a common ancestor. Their apparent sequence conservation within given bacterial phyla (Fig. 6.1) would indicate that horizontal gene transfer is not responsible for their distribution, although individual events may have occurred as evidenced by the composite transposon *Tn*-Dha1 isolated from *D. hafniense* strain TCE1 (Chapter 3). The question of the origin of the reductive dehalogenase remains at present unanswered. By taking into account the conserved features of reductive dehalogenases and by comparing their sequences to general sequence and protein domain databases, it is not possible to get indications on the origin of this class of enzyme. On the one hand the clearly recognized protein domains such as the Tat signal peptide and the iron-sulfur clusters binding motifs are much too widely distributed in proteins targeted to the membrane (2), and involved in electron transfer (1), respectively, to make a link between reductive dehalogenases and another specific enzyme class. Other conserved unassigned amino acid stretches, on the other hand, are only detected among reductive dehalogenase sequences. Therefore, the possible function of the common ancestor remains so far unresolved. Nevertheless, the presence of a Tat signal peptide, of electron-transferring iron-sulfur clusters, and of a metal atom at the catalytic center are very

common features of terminal reductases indicating a kind of co-evolution of different terminal reductases. It is mainly the nature of the metal-containing cofactor, the cobalt-containing corrinoid that distinguishes reductive dehalogenases from other terminal reductases. The binding mode of the corrinoid, although not yet resolved, and the type of reaction catalyzed are different from other corrinoid-dependent enzymes which separates reductive dehalogenases also from these classes of enzymes (Chapter 2).

The complete dehalorespiration chain has not yet been elucidated for any of the dehalorespiring bacteria but significant differences in the topology of the electron transport chain have been reported among dehalorespiring bacteria (Chapter 5). In addition, dehalorespiring bacteria do not contain at all or do not contain the same types of cytochromes, and for the few organisms where the involvement of menaquinones has been investigated contradictory results have been obtained (12). All this indicates that the dehalorespiration chains might be the result of the incorporation of reductive dehalogenases into existing respiration chains rather than the evolution of a specific electron transport chain. Only the complete elucidation of different dehalorespiration chains will allow verifying this hypothesis.

6.2 Outlook

6.2.1 Genetics of *Desulfitobacterium hafniense* strain TCE1

The presence of a composite transposon, *Tn*-Dha1, containing the tetrachloroethene reductive dehalogenase gene cluster *pceABCT*, was reported in Chapter 3. Indications for its transposition activity were given by PCR and Southern blot analyses. However, an unequivocal demonstration of the dead-end circular molecules should be given either by $CsCl_2$ density gradient centrifugation or pulse-field electrophoresis. It would allow confirming the structure and relative amount of the elements detected so far. The question of the transposition activity should be addressed in heterologous systems by looking at the activity of the transposase in trans on a genetic engineered copy of the insertion sequence *IS*Dha1, in which the transposase gene would be replaced by a selective marker gene. A similar approach could be applied to simulate the transposition of the composite transposon. The importance of the promoter located in the right inverted repeat of *IS*Dha1 for the expression of the reductive dehalogenase gene should be evaluated by studying its strength using standard reporter genes. Similarly, the strength of the promoter encoded in the inverted repeats junction of circularized *IS*Dha1 or *Tn*-Dha1 should be evaluated and compared to the promoter region of linear forms of *IS*Dha1.

6.2.2 Functional analysis of the *pceABCT* gene cluster

Biochemical evidence has only been given so far for the catalytic unit of the reductive dehalogenase (PceA). Sequence homology allowed identifying the predicted role of other proteins encoded by the *pceABCT* gene cluster, but no clear evidence has been reported yet. Making use of the host-vector system developed by Smidt and co-workers for *Desulfitobacterium dehalogenans* (25), the *pceB*, *pceC* and *pceT* genes could be inactivated and the effect on the dehalorespiration activity measured. However, next to the numerous technical difficulties of performing genetics on strictly anaerobic bacteria, the tendency of the dehalorespiring bacteria to contain multiple reductive dehalogenase gene clusters may attenuate, if not suppress completely, the effect of single gene inactivation.

6.2.3 The dehalorespiration chain of *D. hafniense* strain TCE1

To better understand how the electrons are transferred towards the chlorinated compounds in *D. hafniense* strain TCE1, the topology and composition of the dehalorespiration chain has to be elucidated. The presence and type of free-moving electron-transferring membrane

components such as quinones should be evidenced and their involvement in mediating the electrons to the reductive dehalogenase shown using suitable analogues and inhibitors in *in-vitro* studies.

The presence of *b*- and *c*-type cytochromes in the membrane of *D. hafniense* strain TCE1 cells grown under various electron donor/acceptor conditions should be confirmed by the pyridine ferrohemochrome method (3). The question of the involvement of the 45 kDa *c*-type cytochrome in the dehalorespiration chain should be addressed by UV-visible spectroscopy analysis of cytochromes in membrane fractions upon addition of the physiological electron donors (hydrogen, lactate) and electron acceptors (PCE, fumarate). The 45 kDa *c*-type cytochrome should finally be purified in order to determine its N-terminal amino acid sequence, whose corresponding gene could be searched in preliminary sequence data of the genome of *D. hafniense* strain DCB-2, a close relative to strain TCE1. A degenerate PCR approach should be used in the case of not finding it on the genome sequence of strain DCB-2.

To investigate the topology of the key enzymes involved in dehalorespiration in *D. hafniense* strain TCE1, localization of the PCE reductive dehalogenase should be done first by simply measuring enzyme activities with whole cells and cell extracts using membrane impermeable electron donors such as methyl viologen. Additional evidence could be obtained by raising antibodies against the reductive dehalogenase *pceA* and performing immunogold-labeling and electron microscopy.

Based on this information, a model of the dehalorespiration chain of *D. hafniense* strain TCE1 could be proposed and validated by performing a reconstitution of the electron transport chain (or part of it) by incorporating all necessary enzymes and electron-transferring components into liposomes.

Table 6.1. All reported reductive dehalogenases.

Strain	Protein name	Length (aa)	Original gene name	(Sub-) Cluster	EMBL	Ref.
Desulfitobacterium sp. strain PCE-S	PceA	551	*pceA*	IIa	AY216592	(18)
Desulfitobacterium sp. strain Y51	PceA	551	*pceA*	IIa	AB070709	(27)
Desulfitobacterium hafniense strain TCE1	PceA	551	*pceA*	IIa	AJ439608	this thesis, (17)
	pRdhA1$_{(S)}$	310 [a]	*rdfA*	Ib	AY013362	(24)
Dehalobacter restrictus	PceA	551	*pceA*	IIa	AJ439607	this thesis, (17)
	pRdhA1$_{(Re)}$	181 [a]	*rdhA*1	-	AJ539532	(22)
	pRdhA2$_{(Re)}$	397 [a]	*rdhA*2	-	AJ539533	(22)
	pRdhA3$_{(W)}$	189 [a]	*cprA*	I	AJ290227	(31)
Sulfurospirillum multivorans	PceA	501	*pceA*	III	AF022812	(20)
	pRdhA1$_{(Re)}$	223 [a]	*rdhA*	III	AJ539530	(22)
Sulfurospirillum halorespirans strain PCE-M2	pRdhA1$_{(S)}$	501	*pceA*	III	AY013367	(24)
	pRdhA2$_{(S)}$	154 [a]	*rdmA*	III	AY013368	(24)
Clostridium bifermentans strain DPH-1	PceC	366	*pceC*	outgroup	AJ277528	(21)
Desulfitobacterium dehalogenans strain JW/IU-DC1	CprA	447	*cprA*	Ia	AF115542	(29)
	pRdhA1$_{(S)}$	487[b]	*rddA*	-	AY013363	(24)
Desulfitobacterium hafniense strain PCP-1	Crp (2,4,6-TCP)	327	*crp*	outgroup	AY043467	(4)
	CprA5 (3,5-DCP)	548	*cprA*5	II	AY349165	(28)
	pRdhA0	81[a]	*cprA*	I	AF321226 (Q93PQ2) [e]	(30)
	pRdhA1	380 [a]	*cprA*	Ib	AF321226 (Q9ANS1) [e]	(30)
	pRdhA2$_{(S)}$	316 [a]	*rdfA*	Ib	AY013364	(24)
Desulfitobacterium sp. strain Viet-1	pRdhA1	447	*cprA*	Ia	AF259791	(unpubl.) [f]
Desulfitobacterium sp. strain PCE-1	pRdhA1$_{(S)}$	447	*cprA*	Ia	AF259790 AY013360	(unpubl.) [f] (24)
	pRdhA2$_{(S)}$	354 [a]	*rddA*	-	AY013361	(24)
	pRdhA3$_{(Re)}$	219 [a]	*rdhA*	-	AJ539531	(22)
	pRdhA4$_{(W)}$	198 [a]	*cprA*	Ia	AY290225	(31)
Desulfitobacterium chlororespirans strain Co23	pRdhA1	456	*cprA*	I	AF204275 (Q8RQC9) [e]	(unpubl.) [f]
	pRdhA2	456	*cprA*	Ib	AF204275 (Q9F0U7) [e]	(unpubl.) [f]

Table 6.1. continued

Strain	Protein name	Length (aa)	Original gene name	(Sub-) Cluster	EMBL	References
Desulfitobacterium hafniense strain DCB-2	CprA	447	*cprA*	Ia	AY013365	(5), (24)
	pRdhA1	463	*rdhA*1 [c]	I	AF403183	(unpubl.) [f]
	pRdhA2	445	*rdhA*2 [c]	Ia	AF403182	(unpubl.) [f]
	pRdhA3	458	*rdhA*3 [c]	Ib	AF403184 (Q8RPG3) [e]	(unpubl.) [f]
	pRdhA4	488	*rdhA*4 [c]	I	AF403184 (Q8RPG4) [e]	(unpubl.) [f]
	pRdhA5	548	*rdhA*5 [c, d]	II	AF403185	(unpubl.) [f]
	pRdhA6	352 [a]	*rdhA*6 [c]	-	AF403180	(unpubl.) [f]
	$pRdhA7_{(S)}$	316 [a]	*rdfA*	Ib	AY013366	(24)
	$pRdhA8_{(W)}$	140 [a]	*cprA*	Ia	AJ290229	(31)
Dehalococcoides ethenogenes strain 195	TceA	554	*tceA*	IV	AF228507	(15)
	pRdhA1	482	*rdhA*-De1	IV	-	TIGR
	pRdhA2	500	*rdhA*-De2	IV	-	TIGR
	pRdhA3	492	*rdhA*-De3	IV	-	TIGR
	pRdhA4	494	*rdhA*-De4	IV	-	TIGR
	pRdhA5	469	*rdhA*-De5	IV	-	TIGR
	pRdhA6	507	*rdhA*-De6	IV	-	TIGR
	pRdhA7	505	*rdhA*-De7	IV	-	TIGR
	pRdhA8	532	*rdhA*-De8	-	-	TIGR
	pRdhA9	510	*rdhA*-De9	IV	-	TIGR
	pRdhA10	495	*rdhA*-De10	IV	-	TIGR
	pRdhA11	515	*rdhA*-De11	IV	-	TIGR
	pRdhA12	505	*rdhA*-De12	IV	-	TIGR
	pRdhA13	514	*rdhA*-De13	IV	-	TIGR
	pRdhA14	490	*rdhA*-De14	IV	-	TIGR
	pRdhA15	455	*rdhA*-De15	-	-	TIGR
	pRdhA16	510	*rdhA*-De16	IV	-	TIGR
	pRdhA17	350	*rdhA*-De17	IV	-	TIGR
Dehalococcoides sp. strain FL2	TceA	554	*tceA*	IV	AY165309	(unpubl.) [g]
Bacterium YK-TCE1	TceA	554	*tceA*	IV	AY165310	(unpubl.) [g]
Bacterium PM-VC1	TceA	554	*tceA*	IV	AY165311	(unpubl.) [g]
Bacterium RC-VC2	TceA	554	*tceA*	IV	AY165312	(unpubl.) [g]
Dehalococcoides sp. strain VS	VcrA	519	*vcrA*	IV	AY322364	(19)

Table 6.1. continued

Strain	Protein name	Length (aa)	Original gene name	(Sub-) Cluster	EMBL	References
Uncultured bacterial consortium (UBC)	pRdhA1$_{(W)}$	156 [a]	*cprA*	-	AJ290236	(31)
	pRdhA2$_{(W)}$	156 [a]	*cprA*	I	AJ290239	(31)
	pRdhA3$_{(W)}$	156 [a]	*cprA*	-	AJ290243	(31)
	pRdhA4$_{(W)}$	152 [a]	*pceA*	III	AJ290249	(31)
Uncultured marine consortium (UMC)	pRdhA1$_{(Rh)}$	334 [a]	*rdh61A*	-	AF462231	(23)
	pRdhA2$_{(Rh)}$	438 [a]	*rdh63A*	-	AF462232	(23)
	pRdhA3$_{(Rh)}$	374 [a]	*Rdh81A*	-	AF462233	(23)

[a] Partial RdhA sequences.
[b] *Dde* pRdhA shows an atypical structure with a truncated N-terminal domain and an extended C-terminal domain.
[c] Putative *rdhA*1 to *rdhA*6 genes retrieved from the genome database of *Dha*$_{DCB-2}$ correspond to the DNA contigs 1022 (*rdhA*1) and 1065 (*rdhA*2 to -6), but were already submitted to by Davis & Tiedje (unpublished results).
[d] The sequence of *rdhA*5 gene of *Dha*$_{DCB-2}$ was taken from the updated version of the genome sequence, since a mistake of reading frame present in EMBL AF403185 was corrected.
[e] The corresponding protein ID number is indicated in brackets, since these two *rdhA* genes are located on a DNA fragment with a unique accession number.
[f] Unpublished data by Davis, J.K. & Tiedjei, J.M.
[g] Unpublished data by Krajmalnik-Brown *et al*.
$_{(Re)}$-index designates sequences isolated by Regeard and co-workers in a degenerate PCR approach (22).
$_{(Rh)}$-index designates sequences isolated by Rhee and co-workers in a degenerate PCR approach (23).
$_{(S)}$-index designates sequences isolated by Smidt and co-workers in a degenerate PCR approach (24).
$_{(W)}$-index designates sequences isolated by von Wintzingerode and co-workers in a degenerate PCR approach (31).

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Table of content

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Conferences

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2003 Oral presentation for the SWIMM Workshop (Swiss Society for Molecular Microbiology), June 23-25, 2003 in Cartigny (Geneva), Switzerland. *Genetic structure and possible rearrangement around the tetrachloroethene reductive dehalogenase gene in* Desulfitobacterium hafniense *strain TCE1*.

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A. Papers in peer reviewed journals

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B. Papers submitted

1. Regeard, C., **J. Maillard**, C. Dufraigne, P. Deschavanne and C. Holliger. Evidence for the acquisition of reductive dehalogenase genes through horizontal gene transfer by *Dehalococcoides ethenogenes* strain 195. Submitted.