Molecular Characterization of Anaerobic Dehalogenation by *Desulfitobacterium dehalogenans*

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MOLECULAR CHARACTERIZATION OF ANAEROBIC DEHALOGENATION BY *DESULFITOBACTERIUM DEHALOGENANS*

HAUKE SMIDT

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Subject headings: anaerobic, reductive dehalogenation, halorespiration, *Desulfitobacterium dehalogenans*, chlorophenol, genetics, regulation

für meine Eltern

Stellingen

1. Chloorfenol reductief dehalogenase mRNA is een makkelijk aan te tonen marker voor actieve chloorfenol afbraak door *Desulfitobacterium* spp.

   Dit proefschrift

2. De term 'halorespiratie' behoeft geen verdere aanvulling om het proces van ademhaling met gehalogeneerde verbindingen correct weer te geven.

   Dit proefschrift

   Holliger, C., Wohlfarth, G., and Diekert, G. (1999) Reductive dechlorination in the energy metabolism of anaerobic bacteria. *FEMS Microbiol Rev* **22:** 383-398.

   Sanford, R. A., Cole, J. R., Löffler, F. E., and Tiedje, J. M. (1996) Characterization of *Desulfitobacterium chlororespirans* sp. nov., which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate. *Appl Environ Microbiol* **62:** 3800-3808.

3. Het uitblijven van methylviologeen-afhankelijke enzymactiviteit in hele cellen geeft uitsluitend informatie over de localisatie van het elektronen accepterende of donerende centrum van een oxido-reductase.

   Jones, R. W., and Garland, P. B. (1977) Sites and specificity of the reaction of bipyridylium compounds with anaerobic respiratory enzymes of *Escherichia coli*. Effects of permeability barriers imposed by the cytoplasmic membrane. *Biochem J* **164:** 199-211.

4. Vanwege een opmerkelijke microheterogeniteit tussen kopieën van het 16S ribosomaal RNA gen in het genoom is deze als fylogenetische marker voor het definiëren van soorten binnen het genus *Desulfitobacterium* niet voldoende.

   Ebersbach, H., Breitenstein, A., and Lechner, U. (2000) All species of *Desulfitobacterium* contain two types of 16S rRNA genes of different length. *Biospektrum, special issue Microbiology 2000* 15.P.13.01.

5. Veel xenobiotica zijn deze naam niet waard.

6. Hygiëne is net als een medicijn. Bij goede dosering levensreddend, maar in overmaat gebruikt slecht voor de gezondheid.

   Diaz-Sanchez, D. (2000) Pollution and the immune-response: atopic diseases - are we too dirty or too clean? *Immunology* **101:** 11-18.

7. Het wel of niet nasynchroniseren van anderstalige films heeft voornamelijk met geld te maken.

8. Concurrentie is goed, coöperatie is beter.

Stellingen behorende bij het proefschrift 'Molecular characterization of anaerobic dehalogenation by *Desulfitobacterium dehalogenans*' van Hauke Smidt

Wageningen, 16 maart 2001

ABSTRACT

Smidt, H. (2001). Molecular characterization of anaerobic dehalogenation by *Desulfitobacterium dehalogenans*. PhD thesis. Laboratory of Microbiology, Wageningen University, The Netherlands.

Haloorganics such as chlorophenols and chlorinated ethenes are among the most abundant pollutants in soil, sediments and groundwater, mainly caused by past and present industrial and agricultural activities. Due to bioaccumulation and toxicity, these compounds threaten the integrity of the environment, and human and animal health. A recently discovered, phylogenetically diverse, group of anaerobic so-called halorespiring bacteria is able to couple the reductive dehalogenation of various haloorganic compounds to energy conservation and hence to growth, significantly contributing to *in situ* dehalogenation processes in anoxic environments. The observed persistence of halogenated pollutants in untreated ecosystems and the accumulation of degradation intermediates during bioremediation, however, indicated the need for engineering of process conditions and/or augmentation with efficient degraders. This thesis describes genetic approaches towards a thorough understanding of the molecular basis of anaerobic reductive dehalogenation in order to enable the further optimization of clean up procedures for contaminated anoxic environments.

The Gram-positive *Desulfitobacterium dehalogenans*, capable of degrading *o*-chlorophenols, PCE and hydroxylated PCB's, was used as model organism throughout a major part of this study. The key enzyme *o*-chlorophenol reductive dehalogenase (CPR) was isolated and characterized at the biochemical and molecular levels, and comparison with known chlorophenol- and chloroalkene-reductive dehalogenases indicated that these enzymes constitute a yet unknown, but evolutionary ancient family of corrinoid-containing iron-sulfur proteins, which in addition share a twin-arginine signal sequence. Transcriptional analysis of the CPR-encoding gene cluster revealed that dehalogenation activity is strongly regulated at the transcriptional level. Efficient gene cloning, and random and specific gene inactivation systems were developed to enable (i) the elucidation of additional components involved in the anaerobic dehalogenation process and (ii) the study of their structure and function within the respiratory network. Halorespiration-deficient mutants were isolated following random chromosomal integration, and their characterization lead to the identification of genes encoding proteins possibly involved in structure, maturation and regulation of respiratory complexes. Reductive dehalogenase-encoding gene targeted (RT-)PCR-based molecular approaches were developed that were useful for the detection of reductive dehalogenation potential and activity in pure cultures of potentially halorespiring microorganisms.

The results obtained in this study provide valuable knowledge on the molecular basis of anaerobic reductive dehalogenation and might serve as a sound basis for the further exploitation of halorespiring bacteria as dedicated degraders in biological remediation processes.

CONTENTS

PREFACE

Solid evidence has emerged almost a decade ago that strictly anaerobic bacteria are able to conserve energy that is derived from the reductive dehalogenation of haloorganic compounds in a respiration-type of metabolism. In view of their favorable degrading capacities, i. e. high dehalogenation rate and low residual concentration of the contaminant, it has been anticipated that these halorespiring microorganisms should be of utmost significance for highly efficient biological remediation of halogenated hydrocarbons in anoxic environments. Previous studies mainly focused on the isolation of halorespiring microbes from various environments and their characterization at the (eco-)physiological level. Nevertheless, only little information was available on the structure, function and regulation of the novel respiratory pathways these organisms possess. To gain this knowledge, a study was initiated that aimed to (i) identify and characterize the key components of the halorespiratory system at the molecular level (Chapter 2-5), (ii) unravel the regulatory circuits that control halorespiration activity (Chapter 3, 5), and (iii) develop molecular tools for the further exploitation of halorespiring bacteria as dedicated degraders in biological *in situ* remediation processes (Chapter 4, 6, 7).

Throughout a major part of this study, the versatile strictly anaerobic *ortho*-chlorophenol respiring Gram-positive *Desulfitobacterium dehalogenans* was used as model organism, representing one of the most significant groups of halorespiring isolates.

In close collaboration with a complementary project that focused on the biochemistry and physiology of halorespiration by *Desulfitobacterium dehalogenans* (Bram A. van de Pas, PhD thesis, Wageningen University, 2000), the key enzyme *ortho*-chlorophenol reductive dehalogenase from *D. dehalogenans* has been characterized at the biochemical and genetic levels, revealing significant similarities with chloroethene-reductive dehalogenases at the mechanistic and structural level (**Chapter 2**). The detailed molecular analysis of the *cpr* gene cluster, including the *ortho*-chlorophenol reductive dehalogenase-encoding *cprBA* genes, showed the halorespiration-specific expression of at least seven genes, coding for proteins potentially involved in function, maturation and regulation of the reductive dehalogenase complex (**Chapter 3**).

To enable the *in vivo* study of function and regulation of chromosomal genes involved in halorespiration, a comprehensive set of genetic tools has been developed for *D. dehalogenans*. **Chapter 4** describes the development of an efficient plating, delivery and screening system based on the conjugative broad host-range transposon Tn*916*, that was instrumental for the

isolation of halorespiration-deficient mutants. The physiological and genetic characterization of the isolated mutants led to the identification of several genes that might code for (i) polypeptides playing a role in regulation and functional assembly of the respiratory network and (ii) redox complexes involved as structural components. These and other respiratory complex-encoding genes were further characterized at the molecular level and their expression was studied at the biochemical and transcriptional levels (**Chapter 5**).

Chapter 6 focuses on the development and initial application of efficient host-vector systems for *D. dehalogenans*. Gram-positive broad host-range cloning vectors could be introduced by electroporation and were stably maintained. Conditionally replicating thermosensitive vectors are demonstrated to be useful for the functional disruption of chromosomal genes. In **Chapter 7**, the development and evaluation of molecular methods for the detection of reductive dehalogenation potential (DNA) and activity (mRNA) is described. Known and novel potentially functional and cryptic reductive dehalogenase-encoding genes were isolated from the chromosome of halorespiring bacteria using a multiple PCR-approach, and their expression was analyzed by RT-PCR. Finally, **Chapter 8** summarizes our current knowledge on the molecular basis of the halorespiratory network that has been gained in this and other studies, including recent data that were obtained from the almost complete genome sequence of the halorespiring *Dehalococcoides ethenogenes*. Furthermore, implications for the optimization of biological remediation strategies and possibilities for the application of innovative metabolic engineering approaches are discussed.

1

HALORESPIRING BACTERIA - MOLECULAR CHARACTERIZATION OF KEY ENZYMES AND DETECTION

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Abstract

Halorespiring bacteria are able to couple the reductive dechlorination of halogenated aliphatic and aromatic compounds to energy conservation and hence to microbial growth. Isolation of these strains and their expected potential for application in *in situ* biodegradation of haloorganic compounds also have led to an increased interest in the molecular basis of the halorespiratory pathway. Integrated physiological, biochemical and molecular genetic approaches have provided deeper insights in the structure, function and regulation of the halorespiratory electron transfer chain. The identification of reductive dehalogenases as the key enzymes in this process was followed by their detailed molecular characterization. This revealed considerable similarities at both the mechanistic and structural level, suggesting that these enzymes constitute a novel class of corrinoid containing reductases. Our current knowledge on the phylogeny of halorespiring bacteria and on the molecular characterization of their dehalogenating systems provides a sound basis for the further exploitation of these microorganisms as dedicated degraders in polluted environments.

Introduction

Halogenated hydrocarbons are present in the environment in high quantities due to their past and present application in industry and agriculture e.g. as solvents, pesticides and preservatives, compromising environmental integrity and health (Ahlborg & Thunberg, 1980; Hileman, 1993; Jensen, 1996). However, as more than 2000 haloorganic compounds are naturally produced at considerable levels, they should not be regarded as of solely anthropogenic origin. Rather, the abundance of natural halogenated compounds has been the selective pressure that has resulted in the evolution of microbial dehalogenating populations. This might explain the unexpectedly high microbial capacity to dehalogenate different classes of xenobiotic haloorganics (Gribble, 1996; Häggblom *et al.*, 2000).

The biodegradability of halogenated hydrocarbons largely depends on their chemical structure and the environmental conditions. The degradation of lower halogenated compounds, which proceeds relatively efficiently under aerobic conditions has been studied in considerable detail at the physiological, biochemical and genetic level (Janssen *et al.*, 1994; Slater *et al.*, 1997; Fetzner, 1998). However, dehalogenating systems that depend on molecular oxygen are only suited for the attack of haloorganic compounds carrying a limited number of highly electronegative halogen-substituents, resulting in the persistence of polyhalogenated compounds in aerobic environments. Whereas e.g. the co-metabolic oxidation of partially chlorinated mono- di- and trichloroethene is fortuitously catalyzed by mono- and dioxygenases in various bacteria, the fully halogenated tetrachloroethene is not degradable under these conditions (Arp, 1995; Leisinger, 1996). During the last two decades, it has been shown for a large variety of halogenated aliphatic and aromatic compounds, that reductive dehalogenation is the crucial step, by which the degradation of these pollutants can be initiated in anoxic environments. The abiotic or co-metabolic conversion under different redox conditions by numerous anaerobic mixed and pure cultures is proposed to be catalyzed in most cases by metal ion-containing heat stable tetrapyrroles or enzymes, in which these compounds are incorporated as cofactors (Gantzer & Wackett, 1991; Holliger & Schraa, 1994; El Fantroussi *et al.*, 1998). In addition, a great number of anaerobic bacteria has recently been isolated that are able to couple the reductive dehalogenation of halogenated aliphatic and aromatic compounds to energy conservation and hence to microbial growth (El Fantroussi *et al.*, 1998; Holliger *et al.*, 1999) (see also Figs. 1.1 and 1.2). In contrast to the above-mentioned co-metabolic conversions, in these microorganisms dehalogenation is

catalyzed with high specific activity and affinity at specific enzymes. This novel respiratory process has previously been described as halorespiration (*halo*genated hydrocarbons as terminal electron acceptor in anaerobic *respiration*), (Sanford *et al.*, 1996). Although the alternative term dehalorespiration has been proposed to be more appropriate (*dehalo*genation as terminal electron accepting process in anaerobic *respiration*) (Holliger *et al.*, 1999), we refer to this process as halorespiration for priority reasons, its analogy to other respiratory processes, such as fumarate- nitrate- or iron-respiration, and because of the obvious direction of the conversion.

The different mechanisms by which bacteria are able to dehalogenate haloaliphatic and haloaromatic compounds have been recently reviewed in several excellent communications and their detailed exhaustive description goes far beyond the scope of this introduction (see Fetzner, 1998; Lee *et al.*, 1998; Holliger *et al.*, 1999; Middeldorp *et al.*, 1999; Bradley, 2000; Wiegel & Wu, 2000; and references therein).

This introduction will focus on the current knowledge on the key characteristics of halorespiring bacteria and the structure, function and regulation of halorespiratory systems that operate in these microorganisms. In addition, molecular approaches towards the detection of the reductive dehalogenation potential and activity of halorespiring microorganisms in polluted environments will be discussed.

Halorespiring Bacteria –Thermodynamic Rationale, Phylogeny and Key Characteristics

Estimation of Gibbs free energies and redox potentials have indicated that halogenated aliphatic and aromatic compounds should potentially be good electron acceptors in anaerobic environments (Dolfing & Harrison, 1992; Dolfing & Janssen, 1994). As an example, the Gibbs free energy available from the reductive dehalogenation of 2,3-dichlorophenol to 2-chlorophenol has been calculated to –147.9 kJ, whereas the standard redox potential of this couple amounts to +353 mV. This value is significantly higher than those calculated for the redox couples SO_4^{2-} / H_2S (E_0' = -217 mV) and fumarate/succinate (E_0' = +30 mV), and comparable to the redox potential of NO_3^- / NO_2^- (E_0' = +433 mV). Even when taking into account that H_2-partial pressures in anaerobic environments are orders of magnitude below

standard conditions, reductive dehalogenation seems energetically still highly competitive with other occurring anoxic terminal electron accepting processes (Thauer *et al.*, 1977; Dolfing & Harrison, 1992).

Over the past decade, a rapidly increasing number of bacteria has been isolated based on their ability to use chloroalkenes, such as tetrachloroethene (PCE) and trichloroethene (TCE), or chloroaromatic compounds like chlorophenols and chlorobenzoates as the terminal electron acceptor (Fig. 1.1). Strains have been isolated from various polluted and pristine environments, ranging from activated- and anaerobic granular sludge to freshwater- and estuarine sediments. These microorganisms have gained significant attention because of their potential in bioremediation of contaminated anoxic environments, the novel respiratory pathways they possess and the capacity of various isolates to dechlorinate both chloroaromates and chloroalkenes.

With one exception (*Dehalococcoides ethenogenes*, see below), halorespirers have been affiliated with distinct phylogenetic branches of the bacterial domain, namely the groups of the low G+C Gram-positives, δ- and ε-proteobacteria (Fig. 1.2), and their main characteristics have recently been summarized in two exhaustive reviews (El Fantroussi *et al.*, 1998; Holliger *et al.*, 1999). Most of these isolates are rather versatile with respect to their ability to use, besides fermentative growth on the expense of e.g. pyruvate, a whole range of different electron donors and acceptors for growth. However, also a small number of apparently obligate halorespiring isolates has been identified to date.

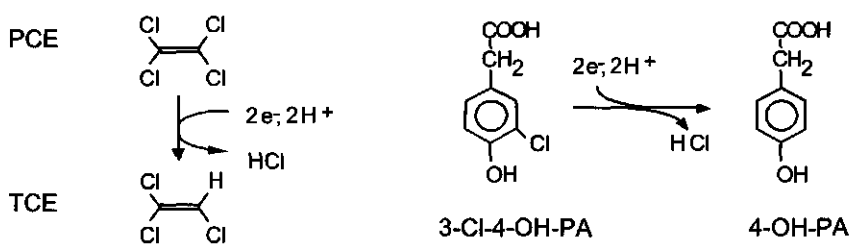

Figure 1.1 Examples of reductive dehalogenation reactions as they are performed by halorespiring microorganisms. (PCE = tetrachloroethene; TCE = trichloroethene; PA = phenylacetic acid)

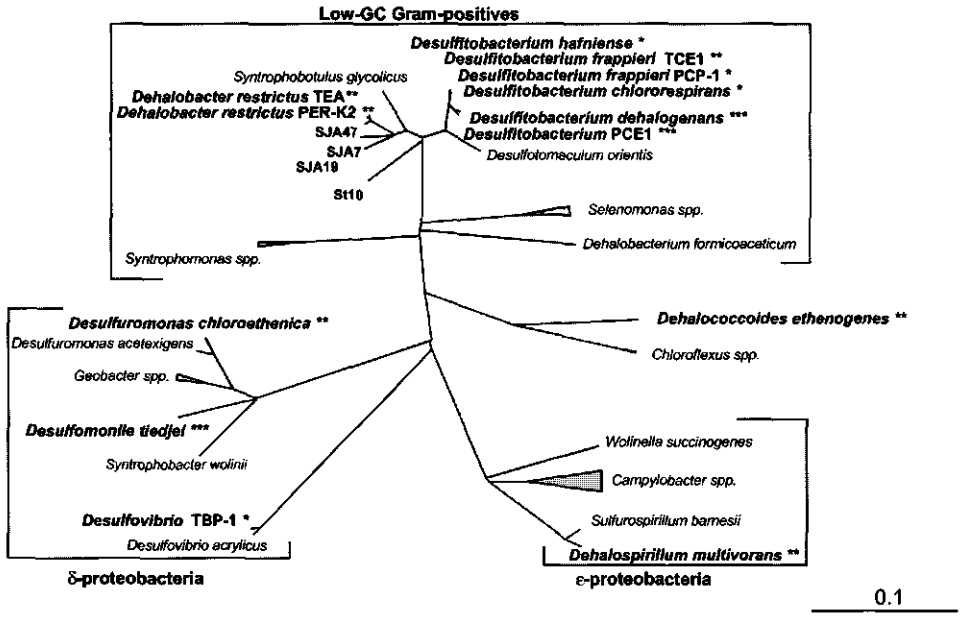

Figure 1.2 Phylogenetic tree based on bacterial SSU rRNA sequences. Halorespiring bacteria are indicated in bold. The reported capacity to dehalogenate chloroaromates (*), chloroalkenes (**) or both (***) is indicated. Alignment and phylogenetic analysis were performed with the ARB software (Strunk & Ludwig, 1995), and the tree was constructed following maximum parsimony criteria with nearest neighbor optimization. No SSU rRNA sequences were available for *Desulfitobacterium* strains PCE-S and Viet1, *Desulfuromonas* strain BB1, and strains 2-CP1 and 2-CPC. The reference bar indicates 10 nucleotide exchanges per 100 nucleotides.

The phylogenetically deeply branching hydrogenotrophic *Dehalococcoides ethenogenes* is currently the only pure culture known to completely dechlorinate and, hence, detoxify the abundant bulk contaminant PCE. While the reductive dehalogenation of PCE, TCE, cis-dichloroethene (cis-DCE), 1,1-DCE and dichloroethane (DCA) to vinylchloride (VC) is coupled to growth, the conversion of trans-DCE and VC occurs at comparably slow rates with first-order kinetics, indicating co-metabolic conversion (Maymó Gatell *et al.*, 1997; Maymó Gatell *et al.*, 1999). Two closely related isolates of the low G+C Gram-positive *Dehalobacter restrictus* have been described that are both strictly dependent on anaerobic respiration, coupling the oxidation of H_2 to the reductive dehalogenation of PCE and TCE to predominantly cis-DCE (Wild *et al.*, 1997; Holliger *et al.*, 1998). Moreover, the *Dehalobacter*-related strain ST-10 could be identified as one of two dominant species in a thermophilic (65°C) PCE-dehalogenating enrichment culture using a 16S rRNA gene-based

molecular approach (Kengen *et al.*, 1999). Using general bacterial 16S rRNA gene-specific fingerprinting techniques, amplicons have been isolated in several cases that showed highest sequence similarity with obligate halorespiring isolates. In one recent study, a clone family could be identified from an anaerobic trichlorobenzene degrading community that showed 98.8 to 99.4 % sequence identity with the 16S rRNA gene of *Dehalobacter restrictus* (SJ7, SJ19, SJ47) (von Wintzingerode *et al.*, 1999). Interestingly, a *Dehalococcoides*-like strain was shown to be specifically stimulated in an actively 2,3,5,6-tetrachlorobiphenyl-*ortho*-dechlorinating microbial consortium (Holoman *et al.*, 1998).

The first bacterial isolate, for which halorespiration was unambiguously proven, is the sulfate-reducing 3-chlorobenzoate (3-CB) degrading *Desulfomonile tiedjei* (DeWeerd *et al.*, 1990; Mohn & Tiedje, 1991). The organism is able to couple the reductive dehalogenation of 3-CB to pyruvate-, formate- and H_2-oxidation (Mohn & Tiedje, 1990; DeWeerd *et al.*, 1991). With hydrogen as the electron donor, chemiosmotic coupling of reductive dehalogenation and proton-driven ATP-synthesis could be demonstrated, as uncouplers and ionophores reduced the ATP-pool relative to dehalogenation activity (Mohn & Tiedje, 1991).

Among the halorespiring pure cultures that have been reported to date, the genus *Desulfitobacterium* comprises a major group of isolates that all belong to the Gram-positive bacteria. All of *Desulfitobacterium* spp. are rather versatile with respect to their metabolic properties. In most cases, the coupling of reductive dehalogenation to the oxidation of H_2 and/or formate could be demonstrated, indicating energy conservation via electron-transport-coupled phosphorylation (Sanford *et al.*, 1996; Löffler *et al.*, 1997; Mackiewicz & Wiegel, 1998; Gerritse *et al.*, 1999). Like other halorespiring isolates, most *Desulfitobacterium* spp. also couple the oxidation of other substrates (e.g. pyruvate, lactate) to reductive dehalogenation. However, as these substrates also support energy conservation via substrate-level phosphorylation, it can not be excluded that under these conditions reductive dehalogenation merely serves as an electron sink rather than supporting electron-transport-coupled phosphorylation (Holliger *et al.*, 1999; van de Pas *et al.*, 2001). Haloorganic compounds that are used as terminal electron acceptor by *Desulfitobacterium* spp., include chlorinated ethenes - either PCE (Gerritse *et al.*, 1996; Löffler *et al.*, 1997; Wiegel *et al.*, 1999) or PCE / TCE (Miller *et al.*, 1997; Gerritse *et al.*, 1999) - and halogenated phenolic compounds. In most cases, these are *ortho*-chlorinated compounds including several hydroxylated PCB's (Utkin *et al.*, 1994; Bouchard *et al.*, 1996; Christiansen & Ahring, 1996a; Gerritse *et al.*, 1996; Sanford *et al.*, 1996; Wiegel *et al.*, 1999), but also *meta*- and

para-substituted isomers are dehalogenated (Bouchard *et al.*, 1996). Interestingly, two of the isolates, strain PCE1 and *D. frappieri* PCP-1, were shown to have two independent activities, namely PCE- and *ortho*-chlorophenol-dechlorination and dehalogenation of chlorophenols at the *ortho*- and *meta*-/*para*-position, respectively. This is indicative for the presence of multiple enzyme systems, as it was also reported for *Dehalococcoides ethenogenes* (see below) (Bouchard *et al.*, 1996; Magnuson *et al.*, 1998; Gerritse *et al.*, 1999).

Gram-negative microorganisms that have been isolated for their halorespiring capacity include the ε-proteobacterium *Dehalospirillum multivorans*, which uses chlorinated ethenes as the terminal electron acceptor (Scholz-Muramatsu *et al.*, 1995). The study of physiology, bioenergetics and, to some extend, molecular biology of halorespiration in this organism has significantly contributed to our understanding of this process (recently reviewed in Holliger *et al.*, 1999). The first example of a halorespiring microorganism from an estuarine environment is the recently isolated *Desulfovibrio* strain TBP-1, which couples the reduction of *ortho*-and *para*-brominated phenols to the oxidation of lactate (Boyle *et al.*, 1999). The only facultative anaerobic halorespiring bacteria reported to date are the closely related strains 2-CP1 and 2-CPC, which use *ortho*-chlorinated phenols and fumarate as e-acceptors. 16S rRNA gene-based phylogenetic analysis revealed that they are most closely related to the myxobacteria within the δ-proteobacteria (Cole *et al.*, 1994; Löffler *et al.*, 1999).

Although several reports have recently emerged on strains that use acetate rather than other compounds as source of electrons for reductive dehalogenation, many halorespiring isolates are able to use hydrogen or formate as the electron donor (Krumholz *et al.*, 1996; Löffler *et al.*, 2000; Sun *et al.*, 2000). Based on thermodynamic considerations and confirmed by H_2-threshold measurements, it has been concluded that halorespiring bacteria should be able to out-compete hydrogenotrophic sulfate reducers and methanogens in environments, where hydrogen is the main source of electrons (Fennell & Gossett, 1998; Löffler *et al.*, 1999). Thus, close syntrophic interactions of hydrogenotrophic reductively dechlorinating bacteria with H_2-producing microorganisms that, due to thermodynamic limitations, depend on low H_2-partial pressures, are highly feasible. Indeed, experimental evidence has been obtained with enrichment cultures that under conditions resulting in the generation of low levels of hydrogen (application of slowly fermented substrates like propionate or the administration of very low concentrations of readily fermented compounds such as lactate), dechlorination was specifically stimulated versus methanogenesis (Smatlak *et al.*, 1996; Ballapragada *et al.*, 1997; Fennell *et al.*, 1997; Fennell & Gossett, 1998).

Table 1.1 Main characteristics of reductive dehalogenases from halorespiring microorganisms

Organism	*Desulfomonile tiedjei*	*Desulfitobacterium dehalogenans*	*Desulfitobacterium hafniense*	*Desulfitobacterium chlororespirans*	*Dehalobacter restrictus*	*Dehalospirillum multivorans*	*Desulfitobacterium PCE-S*	*Dehalococcoides ethenogenes*	
Substrates	*m*-CB, *m*-CP, PCE/TCE	*o*-CP	3-Cl-4-OH-PA	*o*-CB, *o*-CP	PCE, TCE	PCE, TCE, Cl-propenes	PCE, TCE, Cl-propenes	TCE, cis-DCE	PCE
Inducers	*m*-CB, *m*-substituted benzamides and -benzylalcohols	2-Cl,Br-4-R-phenol. compounds	3-Cl-4-OH-PA	3-Cl-4-OH-BA	ND	PCE (also constitutive and non-inducible strain isolated)	constitutive	ND	constitutive
Inhibitors	sulfite, thiosulfate	1-iodopropane[d]	ND	sulfite, thiosulfate	1-iodopropane, cyanide	1-iodopropane, Cl-methanes, EDTA sulfite, cyanide	1-iodopropane, EDTA, cyanide, sulfite, azide,	1-iodopropane, cyanide, sulfite, dithionite	iodoethane, cyanide, sulfite, dithionite
Electron donor	MV	MV	MV	MV	MV	viologens with $E_0' < -360$ mV	viologens with $E_0' < -360$ mV	MV	MV
specific activity in cell extracts (U / mg)[e]	0.15×10^{-3}	0.38	0.55	0.009	0.18	1.5	0.24	0.51	0.27
specific activity of purified protein (U / mg)	0.018	28	6	0.019 (in membranes)	14	158	39	12	21
Size (SDS-PAGE)	64 & 37 kDa	48 kDa	47 kDa	ND	60 kDa	58 kDa	65 kDa	61 kDa	51 kDa
Cofactors (mol/mol enzyme)	heme[c] Fe-S	1 cobalamin[a, b] 1 [3Fe4S][b] 1 [4Fe4S][b]	1 cobalamin[c] 12Fe/12S	ND	1 cobalamin[a, c] 2 [4Fe4S][b]	1 cobalamin[a, c] 8Fe/8S	1 cobalamin[a, c] 8Fe/8S	1 cobalamin[a] Fe-S	1 cobalamin[a] Fe-S
N-terminus	ND	AETMNYVPG	AETLNYVPG	ND	19/20 residues identical to PCE-S	GVPGANAAEK	ADIVAPITESF	ND	ND
O_2-sensitivity ($t_{1/2}$)	insensitive	= 90 min[d]	= 100 min (in membranes)	insensitive (in membranes)	280 min	120 min	50 min	ND	ND
References	(DeWeerd & Suflita,1990; Mohn & Tiedje, 1992; Ni *et al.*, 1995; Townsend & Suflita, 1997)	(Utkin *et al.*, 1995; van de Pas *et al.*, 1999)	(Christiansen *et al.*, 1998)	(Löffler *et al.*, 1996)	(Schumacher & Holliger, 1996; Schumacher *et al.*, 1997; Holliger *et al.*, 1999)	(Neumann *et al.*, 1995; Neumann *et al.*, 1996; Miller *et al.*, 1997; Neumann *et al.*, 1998)	(Miller *et al.*, 1997; Miller *et al.*, 1998)	(Magnuson *et al.*, 1998)	(Magnuson *et al.*, 1998)

[a] Indicated by photoreversible inactivation of the reduced enzyme by iodoalkanes; [b] Indicated by EPR spectroscopic analysis; [c] Indicated by optical spectroscopic analysis; [d] (van de Pas, 2000); [e] One unit (U) of enzymatic activity is defined as 1µmol Cl^- released or 2 µmol MV oxidized. Abbreviations: MV, methyl viologen; ND, not determined; CB, chlorobenzoate; CP, chlorophenol; PCE, tetrachloroethene; TCE, trichloroethene; PA, phenylacetic acid; BA, benzoic acid.

Key Components of Halorespiratory Chains

Isolation of halorespiring bacteria and their expected potential for application *in situ* biodegradation of haloorganic pollutants also led to an increased interest in the molecular basis of this novel anaerobic respiratory pathway. To date, investigations have mainly focused on reductive dehalogenase as the key enzyme in halorespiration. However, efforts have also been made to identify additional structural and regulatory components of the halorespiratory electron transport chain (see below).

It is known from physiological experiments that in several halorespiring bacteria described to date, reductive dehalogenase activity is induced in the presence of a halogenated substrate. Moreover, the influence of alternative electron acceptors on the activity of the dehalogenating system has been investigated, indicating that particularly sulfur oxyanions are potential inhibitors of halorespiration. However, insight in the regulatory circuits involved in the induction and repression of the halorespiration process is still very limited. Evidence is now emerging that at least partly, regulation takes place at the level of transcription (see below).

Reductive Dehalogenases - Enzymes and Genes

Major Characteristics of Reductive Dehalogenases

To date, several haloaryl- and haloalkyl reductive dehalogenases have been partially or completely purified and characterized at the biochemical, and in some cases, the genetic level. As one would expect for a respiratory complex, all enzymes were shown to be membrane-associated. Only the PCE reductive dehalogenase from *Dehalospirillum multivorans* was isolated from the cytoplasm (Neumann *et al.*, 1996). However, evidence is available from the molecular characterization of the encoding gene that the catalytic subunit might be anchored to the cytoplasmic membrane through an integral membrane protein (Neumann *et al.*, 1998; see below). In some cases, the electron-accepting site of the enzymes was inaccessible for reduced methyl viologen (MV) in whole cells. As MV is not able to permeate the cytoplasmic membrane, it was concluded that the enzyme is facing the cytoplasm (Jones & Garland, 1977). Nonetheless, molecular analysis revealed that the proteins are produced as pre-proteins, in which the mature polypeptides are preceded by a

twin arginine-type signal sequence characteristic for periplasmic respiratory complexes (Berks *et al.*, 2000). Moreover, experimental evidence is available that the reductive dehalogenases present in *Dehalococcoides ethenogenes* are indeed located at the outside of the cytoplasmic membrane, indicating that unequivocal elucidation of the topology of reductive dehalogenases has to await further study (Nijenhuis & Zinder, 2000). Major characteristics of all enzymes are summarized in Table 1.1.

The first dehalogenating enzyme that has been purified from a halorespiring pure culture and characterized at the biochemical level, was the inducible 3-CB reductive dehalogenase from the sulfate reducing *Desulfomonile tiedjei* (Ni *et al.*, 1995). As for all reductive dehalogenases isolated since then, the enzymatic activity could be measured *in vitro* using reduced MV (E_0' = -446 mV) rather than benzyl viologen (BV, E_0' = -360 mV) as artificial electron donor. The enzyme was purified from the membrane fraction as a heterodimer, which was insensitive to oxygen. A yellowish chromophore, proposed to be a heme, was present in the small 37-kDa subunit of the enzyme. *In vivo*, the reductive dehalogenation of *m*-chlorinated phenols and chlorinated ethenes (PCE, TCE) is co-induced by 3-CB, suggesting co-metabolic conversion of these compounds by the 3-CB reductive dehalogenase. However, no data are available for the purified enzyme (Mohn & Kennedy, 1992; Townsend & Suflita, 1996).

In contrast to the reductive dehalogenase from *Desulfomonile tiedjei,* all other proteins characterized to date have been isolated as monomers, probably containing Fe-S clusters as well as a corrinoid as cofactors, indicating a common mode of catalytic action (Table 1.1, see below).

Involvement of Transition Metal Cofactors and Fe-S Clusters in Catalysis

Photoreversible inactivation by iodoalkanes indicated that cob(I)alamin is probably involved in the reductive dehalogenation of chlorinated aliphatic and aromatic compounds in most halorespiring bacteria (Neumann *et al.*, 1995; Schumacher & Holliger, 1996; Miller *et al.*, 1997; Magnuson *et al.*, 1998; van de Pas, 2000). The 3-CB reductive dehalogenase from *Desulfomonile tiedjei* was not inhibited by 250 mM 1-iodopropane, and it has been concluded that no corrinoid is involved in its catalytic activity (Louie & Mohn, 1999). Rather, heme (yet another transition metal cofactor) seems to be present in the small subunit of this enzyme (Ni *et al.*, 1995). However, in two cases, namely the 3-chloro-4-hydroxy-phenylacetic acid (Cl-OHPA)- and PCE reductive dehalogenases from *Desulfitobacterium*

hafniense and *Dehalococcoides ethenogenes*, respectively, the involvement of a corrinoid was demonstrated despite the fact that no inhibition by 1-iodopropane was observed (Christiansen *et al.*, 1998; Magnuson *et al.*, 1998). This indicates that it can not be unambiguously excluded that a corrinoid plays a role in the *Desulfomonile tiedjei* dehalogenase. It is noteworthy that for different classes of transition metal cofactors, including corrinoids and hemes, abiotic reductive dehalogenation activity has been demonstrated, indicating their potential role as cofactors also in enzyme catalyzed dechlorination (Gantzer & Wackett, 1991; Glod *et al.*, 1997).

For two enzymes, the presence of cofactors and their involvement in catalytic activity has been demonstrated by optical and electron paramagnetic resonance (EPR) spectroscopic analysis. Studies on the purified *ortho*-chlorophenol (*o*-CP) reductive dehalogenase of *Desulfitobacterium dehalogenans* revealed the presence of a cobalamin ($Em(Co^{1+/2+})$ = -370 mV and $Em(Co^{2+/3+})$ > 150 mV), one [4Fe4S] cluster ($Em \approx$ -440 mV) and one [3Fe4S] cluster ($Em \approx$ +70 mV). The reoxidation of fully (light/deazaflavin/EDTA) reduced enzyme with Cl-OHPA yielded base-off cob(II)alamin (van de Pas *et al.*, 1999). Similar results were obtained with purified PCE reductive dehalogenase from *Dehalobacter restrictus*. However, this enzyme contains two [4Fe4S] clusters with $Em \approx$ -480 mV, rather than one high- and one low-potential cluster (Schumacher *et al.*, 1997). The observed redox potentials of the corrinoid are significantly higher than those found for other corrinoid containing enzymes, indicating that activation is probably not required prior to reduction to the Co(I)-state. Rather, this could easily be accomplished by the low-potential Fe-S centers. It was therefore suggested that a single-electron transfer occurs from a yet unknown electron donor via the two cubanes and the corrinoid to the chlorinated substrate (Schumacher *et al.*, 1997). Nevertheless, experimental proof for a single-electron transfer yielding an alkyl-radical intermediate is still lacking.

Alternatively, Wohlfarth & Diekert proposed that the dehalogenation reaction proceeds through addition of cob(I)alamin to a carbon of the halogenated substrate with subsequent β-elimination of a chlorine ion (Wohlfarth & Diekert, 1997). This model assumes the splitting of electrons into one low-potential and one high-potential electron via the two Fe-S clusters. The low-potential electron would be required for the reduction of cob(II)alamin to cob(I)alamin prior to catalysis, the high potential electron for the reduction of cob(III)alamin to cob(II)alamin after dechlorination (Wohlfarth & Diekert, 1997).

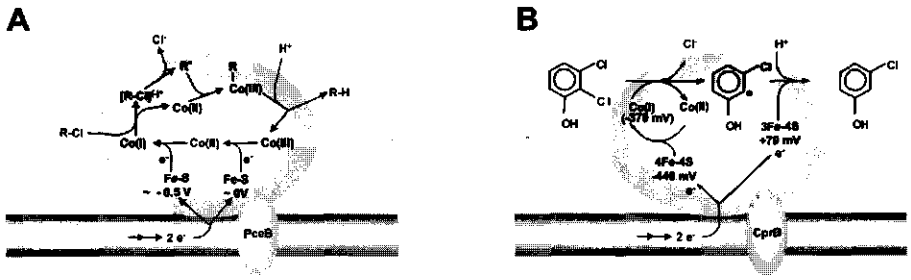

Figure 1.3 Proposed reaction mechanisms of (**A**) corrinoid-containing PCE reductive dehalogenases according to (Holliger *et al.*, 1999) and (**B**) *o*-CP reductive dehalogenase from *Desulfitobacterium dehalogenans* (van de Pas *et al.*, 1999).

However, it has recently been observed that the PCE reductive dehalogenases from both *Dehalospirillum multivorans* and *Desulfitobacterium frappieri* PCE-S also convert *trans*-1,3-dichloropropene to 1-chloropropene. This favors the involvement of a one-electron transfer mechanism as it was suggested for the *Dehalobacter restrictus* PCE reductase rather than the proposed β-elimination, since only the former would yield the actual dehalogenation product (Fig. 1.3A) (Holliger *et al.*, 1999).

Still, in both enzymes for which the different cofactors have been analyzed by EPR spectroscopic analysis, the corrinoid could not be oxidized to the cob(III)alamin form, suggesting that it might not play a role in the catalytic cycle of reductive dehalogenation. Similarly, it was proposed that cob(III)alamin is not involved in the reductive dehalogenation of PCE by free cobalamin (Glod *et al.*, 1997). Aiming at the incorporation of all experimental evidence obtained to date, yet another reaction mechanism can be proposed for the *o*-chlorophenol reductive dehalogenase from *Desulfitobacterium dehalogenans* (Fig. 1.3B) (van de Pas *et al.*, 1999). This model involves activation of the oxidized cob(II)alamin to cob(I)alamin by an electron from the low-potential [4Fe4S] cluster. The reduced cob(I)alamin might then donate one electron to the chlorinated substrate, yielding the release of a chlorine ion and an alkyl-radical intermediate, which, however, has not been unambiguously demonstrated. The dehalogenated product would then be released following the transfer of a second electron from the high-potential [3Fe4S] cluster. Future experiments have to reveal, whether both, haloalkene- and haloaryl reductive dechlorination proceed

through identical mechanisms, or whether the various enzymes catalyze the reactions along similar, albeit different pathways.

Molecular Characteristics of Reductive Dehalogenases

Using a reversed genetics approach based on the N-terminal amino acid sequences of the purified enzymes, the *o*-CP- and PCE reductive dehalogenase-encoding genes were isolated from genomic libraries of *Desulfitobacterium dehalogenans*, *Dehalospirillum multivorans* and *Desulfitobacterium* strain PCE-S, respectively, cloned and sequenced (Neumann *et al.*, 1998; Neumann *et al.*, 1999; van de Pas *et al.*, 1999). In all cases, sequence analysis revealed the presence of two closely linked genes: (i) *cprA* and *pceA*, coding for the catalytic subunit of the respective reductive dehalogenases; and (ii) *cprB*, *pceB* and orf1, encoding small integral membrane proteins of 11, 8.4 and 11 kDa, that are composed of two (PceB) or three (CprB, Orf1) transmembrane helices (Fig. 1.4). As cotranscription of both genes could be demonstrated for *Desulfitobacterium dehalogenans* and *Dehalospirillum multivorans*, it has been proposed that the integral membrane protein acts as a membrane anchor for the reductive dehalogenase (Neumann *et al.*, 1998; van de Pas *et al.*, 1999; Smidt *et al.*, 2000).

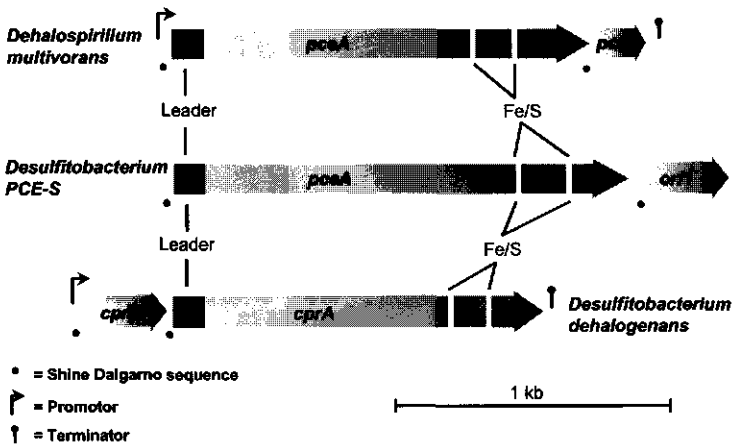

Figure 1.4 Comparison of *o*-chlorophenol- and PCE reductive dehalogenase-encoding operons from different halorespiring microorganisms (Neumann *et al.*, 1998; Neumann *et al.*, 1999; van de Pas *et al.*, 1999; Smidt *et al.*, 2000).

The smaller size and lack of a third transmembrane helix in PceB, as compared to the *Desulfitobacterium* counterparts, might explain the significantly looser attachment of the *Dehalospirillum* catalytic subunit to the cytoplasmic membrane, as it was observed during purification (Neumann *et al.*, 1996).

Furthermore, all reductive dehalogenases share a rather long twin arginine (RR) signal sequence of 30 to 42 amino acids, which is cleaved off in the purified proteins (Fig. 1.5). These signal peptides (consensus (S/T)-R-R-x-F-L-K) are thought to play a major role in the maturation and translocation of mainly periplasmic proteins binding different redox cofactors by the recently described Twin Arginine Translocation (TAT) system (Berks *et al.*, 2000). This is in obvious contradiction to a possibly cytoplasmic orientation of the reductive dehalogenases, and the only other RR-enzyme with a proposed cytoplasmic location is the *E. coli* DMSO reductase (Weiner *et al.*, 1998). It has been hypothesized that the presence of a membrane anchor might prevent translocation by the TAT-system (Neumann *et al.*, 1998). Still, the actual role of the TAT system in maturation and, possibly, translocation of reductive dehalogenases from halorespiring microorganisms deserves further study.

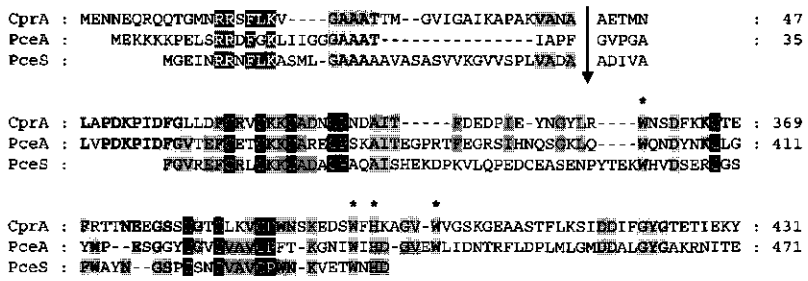

Figure 1.5 Partial primary sequence alignment of reductive dehalogenases from halorespiring bacteria. The alignment was performed using the programs Clustal X and GeneDoc (Thompson *et al.*, 1997; K. B. Nicholas & H. B. J. Nicholas, GeneDoc: a tool for editing multiple sequence alignments, 1997). The apparent sites of leader cleavage, deduced from the N-terminal sequence of the purified proteins, are indicated by the vertical arrow. Conserved residues are highlighted as follows: conserved residue; RR – motif; iron-sulfur cluster binding motif. Conserved tryptophan and histidine residues are indicated by stars. CprA, *Desulfitobacterium dehalogenans* *o*-CP reductive dehalogenase (Rdh) (acc. no. AAD44542); PceA, *Dehalospirillum multivorans* PCE-Rdh (acc. no. AAC60788); PceS, *Desulfitobacterium* PCE-S PCE-Rdh (Neumann *et al.*, 1999).

The presence of two Fe-S clusters, as determined by EPR-analysis, was confirmed by the identification of one ferredoxin-like and one truncated Fe-S cluster binding motif in the sequence of CprA (Fig. 1.5). The same two motifs are also present in the sequence of the two PCE reductive dehalogenases, indicating a conserved mode of intramolecular transfer of electrons to the enzyme's active site that contains the cobalamin. These enzymes thus might differ in Fe-S cluster contents from the PCE reductive dehalogenase of *Dehalobacter restrictus*, for which EPR analysis had indicated the presence of 2 [4Fe4S] clusters of identical midpoint potential. Similarly, 3 Fe-S clusters have been suggested for the Cl-OHPA reductive dehalogenase from *Desulfitobacterium hafniense*. Nevertheless, these differences still have to be supported by the yet unidentified primary structures of both proteins.

Although the reductive dehalogenases share highly conserved sequences in the C-terminal part of the proteins, they all lack the consensus sequence for the binding of the corrinoid cofactor, which is common to known methylcobalamin-dependent methyltransferases and mutases (Ludwig & Matthews, 1997). This is, however, not surprising as EPR analysis revealed that the active CprA enzyme contains the corrinoid in the base-off form, which is in contrast to the above mentioned proteins (van de Pas *et al.*, 1999). Of special interest is the presence of highly conserved tryptophan and histidine residues that have been shown or proposed to be involved in the stabilization of the leaving halide in hydrolytic dehalogenases and dichloromethane dehalogenases, respectively (Fig. 1.5) (Marsh & Ferguson, 1997; Damborský & Koca, 1999).

The elucidation of structure-function relations and the identification of residues involved in catalysis in this novel class of corrinoid-containing reductases will have to await the availability of a structural model as well as heterologous expression systems, which are an obligate prerequisite for site-directed- and other mutagenesis approaches. Recently, heterologous expression of the PCE reductive dehalogenase-encoding gene *pceA* from *Dehalospirillum multivorans* could be achieved in *Escherichia coli* in the presence of a helper plasmid, which supplied the expression host with the rare *E. coli* $tRNA_4^{Arg}$, correcting for differences in codon usage between *E. coli* and *Dehalospirillum multivorans*. However, expression of the full-length *pceA* led to the production of unprocessed inactive pre-protein, still containing the RR-signal peptide. Neither truncation of the signal sequence from the construct nor expression in the presence of *Dehalospirillum multivorans* corrinoids led to functional expression (Neumann *et al.*, 1998).

Multiple Activities in Halorespiring Bacteria – Involved Enzymes and Evolutionary Aspects

It is known from several halorespiring bacteria that they possess multiple dechlorinating activities. Thus, the question arises, whether this is due to single enzymes with rather broad substrate specificities or a set of highly specific isoenzymes. Support for the latter scenario has been obtained by substrate-dependent differential induction patterns in several *Desulfitobacterium* spp.

Desulfitobacterium frappieri PCP-1 has been shown to possess one specific dehalogenating system inducible for *ortho*-dehalogenation and one for *meta*- and *para*-dehalogenation (Bouchard *et al.*, 1996). Moreover, Cl-OHPA is transformed to 2-chlorophenol rather than being dehalogenated, indicating, that the *ortho*-dehalogenating enzyme system differs from the *o*-CP- and Cl-OHPA reductive dehalogenases isolated from *Desulfitobacterium dehalogenans* and *Desulfitobacterium hafniense* (Dennie *et al.*, 1998).

Desulfitobacterium PCE1 is able to reductively dehalogenate as well *o*-chlorinated phenolic compounds as well as PCE. However, cells grown in the presence of Cl-OHPA as the electron acceptor showed only 0.25% of the PCE reductive dehalogenation activity of cells that used PCE as electron acceptor, indicating that two distinct enzyme systems might be involved in both activities (Gerritse *et al.*, 1999). As the *o*-chlorophenol reductive dehalogenase of the closely related *Desulfitobacterium dehalogenans* does not convert PCE, it is tempting to speculate that the low PCE dechlorinating activity present in this organism could be due to a similar protein as it is active in *Desulfitobacterium* PCE1, of which either catalytic functionality or induction have been drastically impaired (Wiegel *et al.*, 1999). Similarly, the 3,5-DCP *meta*-dechlorinating activity of *Desulfitobacterium hafniense* could be possibly due to a distinct enzyme, which might resemble the highly active catalyst in the closely related *Desulfitobacterium frappieri* PCP-1 (Christiansen & Ahring, 1996a).

From the PCE-detoxifying *Dehalococcoides ethenogenes*, two distinct enzyme systems have been partially purified, a 51-kDa PCE reductive dehalogenase and a 61-kDa TCE/cis-DCE reductive dehalogenase (Magnuson *et al.*, 1998). Recently, the partial genome sequence of *Dehalococcoides ethenogenes* has been released, and sequence comparison led to the identification of at least sixteen non-identical copies of potentially reductive dehalogenase-encoding genes. Moreover, these were all closely linked to genes, coding for CprB/PceB like small hydrophobic proteins of ~10 kDa (Preliminary sequence data was obtained from The Institute for Genomic Research website at http://www.tigr.org). The presence of these

multiple alleles in the genome of *Dehalococcoides ethenogenes* might reflect a common strategy that should enable the organism to relatively fast acquire novel degradation capacities upon exposure to an environmental trigger such as the anthropogenic release of non-natural halogenated hydrocarbons. It has been suggested that the cryptification of dehalogenase genes and their decryptification by environmentally directed mutations might play an important role in adaptive evolution (Thomas *et al.*, 1992; Hill *et al.*, 1999). Hill and co-workers used degenerated primers to PCR-amplify halocarboxylic acid dehalogenase-encoding genes (*deh*) from a wide variety of bacterial isolates. Expression studies on the identified genes allowed for the discrimination between cryptic or silent, as well as active *deh* genes, and yielded the identification of several cryptic genes that either encoded non-functional gene products or had been silenced by the absence of an active promoter (Hill *et al.*, 1999). It is tempting to speculate that e.g. the high PCE reductive dehalogenase activity in *Desulfitobacterium* PCE1 and its hardly detectable presence in the closely related *Desulfitobacterium dehalogenans* might be another example of such a silencing / unsilencing process.

The Halorespiratory Chain

Physiological, biochemical and molecular genetic approaches have been used to unravel structure, function and regulation of the halorespiratory electron transfer chain. Studies have mainly focused on halorespiratory chains in which either hydrogen or formate serves as the electron donor, as in these cases energy can only be conserved by electron-transport-coupled phosphorylation. For several organisms, additional evidence for energy conservation via a chemiosmotic coupling mechanism has now arisen from the application of uncouplers, protonophores and oxidant pulses (Mohn & Tiedje, 1991; Miller *et al.*, 1996; Miller *et al.*, 1997). Based on these and other results that have been collected to date and which are summarized below, basic models for structure and function of halorespiratory chains have been proposed (Fig. 1.6A-C) (Holliger *et al.*, 1999; Louie & Mohn, 1999). An essential assumption for these models is the cytoplasmic location of the dehalogenating enzyme. In case this is located at the outside of the cell membrane, as is suggested based on the above-mentioned evidence, these models do not apply. An alternative model is proposed, assuming an extracytoplasmic orientation of the reductive dehalogenase (Fig. 1.6D).

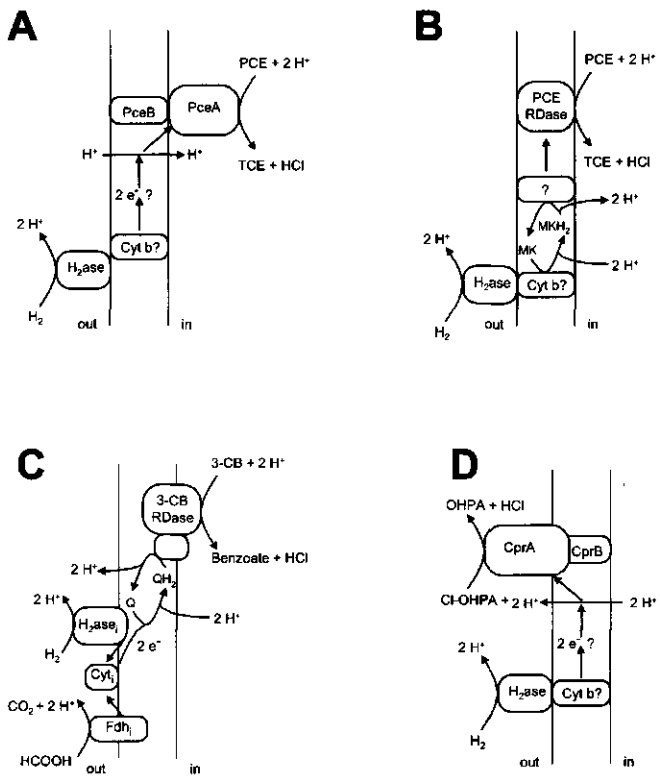

Figure 1.6 Proposed models of the halorespiratory chains in *Dehalospirillum multivorans* (**A**, adapted from Holliger *et al.*, 1999), *Dehalobacter restrictus* (**B**, adapted from Holliger *et al.*, 1999), *Desulfomonile tiedjei* (**C**, adapted from Louie *et al.*, 1999) and *Desulfitobacterium dehalogenans* (**D**). Abbreviations: $H_2ase_{(i)}$, (inducible) hydrogenase; Fdh_i, inducible formate dehydrogenase; Cyt_i, inducible cytochrome; Cyt b, b-type cytochrome; RDase, reductive dehalogenase; MK, menaquinone; Q, quinone; out/in, outer/inner face of the cytoplasmic membrane.

Topology of the Halorespiratory Chain

The enzymes catalyzing the electron donating process have been characterized from different halorespiring strains, indicating an extra-cytoplasmic location in all cases. In *Desulfitobacterium* strain PCE-S and *Dehalospirillum multivorans*, membrane-associated extra-cytoplasmic hydrogenases and formate dehydrogenases have been identified (Miller *et al.*, 1996). Similarly, hydrogenase activity was localized on the outside of the cytoplasmic membrane of *Desulfomonile tiedjei* and *Dehalobacter restrictus*, as MV-dependent activity was similar in intact and lysed cells. Moreover, proton liberation from H_2 as the electron

donor was inhibited by membrane impermeable Cu^{2+} (Holliger & Schumacher, 1994; Schumacher & Holliger, 1996; Louie & Mohn, 1999). A periplasmic formate dehydrogenase was induced in *Desulfomonile tiedjei* when grown in the presence of formate as the electron donor (Louie & Mohn, 1999).

Based on the apparent extra-cytoplasmic location of the electron donating process and the proposed cytoplasmic location of the terminal reductive dehalogenase, a scalar mechanism of proton translocation was suggested, which would theoretically yield an H^+/e^- ratio of 1. However, from yield studies on *Dehalospirillum multivorans* cells grown with H_2 / PCE and acetate as the carbon source, it was calculated that only 0.4 mol ATP were formed per mol Cl^- released, suggesting an H^+/e^- ratio of 0.5 (Scholz-Muramatsu *et al.*, 1995; Miller *et al.*, 1996). In view of the proposed reaction mechanism for PCE reductive dehalogenase, which requires the endergonic transfer of a high-potential electron for the reduction of cob(III)alamin to cob(II)alamin, the results point towards the involvement of a reversed electron flow, driven by the electrochemical proton potential (Fig. 1.3). As reductive dehalogenation was still observed in the presence of protonophores in *Dehalobacter restrictus* and *Desulfomonile tiedjei*, whereas proton liberation was abolished, reverse electron flow does not seem to be required for *in vivo* reductive dehalogenase activity in these systems (Mohn & Tiedje, 1991; Schumacher & Holliger, 1996). Contrarily, fast proton liberation during electron transport from H_2 to PCE and TCE in *Dehalobacter restrictus* resulted in an H^+/e^- ratio of 1.25 ± 0.2. Similarly, an H^+/3-CB ratio of 2.1 was observed during halorespiration in *Desulfomonile tiedjei*, indicating that additional vectorial proton translocation cannot be excluded in these systems. However, experimental proof is still missing.

Electron Mediating Components of the Halorespiratory Chain

Membrane-bound menaquinone (MK), which could possibly act as proton pump, was shown to be directly involved in electron mediation from hydrogenase to PCE reductase in *Dehalobacter restrictus*. Nevertheless, oxidant pulse experiments with the MK/MKH_2 analogues DMN/$DMNH_2$ indicated that MK is not involved in vectorial proton translocation (Schumacher & Holliger, 1996). Furthermore, MK is presumably not the direct physiological electron donor for the reductive dehalogenase, suggesting the presence of an additional electron-mediating component, which remains to be identified (Holliger *et al.*, 1999). In contrast, it was assumed that MK is not involved in electron transfer in *Dehalospirillum*

multivorans, as PCE reduction was not effected by 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO), which is an inhibitor of many quinone-dependent redox reactions. However, the insensitivity of normally MK-dependent fumarate reduction might suggest that the inhibition experiment should be interpreted with caution (Miller *et al.*, 1996). Neither ubi- nor menaquinone could be detected in *Desulfomonile tiedjei*. However, a yet unidentified quinoid could be extracted from the organism. An essential function of this component in halorespiration was proposed based on the observation that reductive dehalogenation in whole cells of *Desulfomonile tiedjei* is severely inhibited by HOQNO. Moreover, reductive dehalogenation of this organism is dependent on 1,4-naphtoquinone, which might be an essential quinoid-precursor. However, the isolated quinoid failed to replace MV in *in vitro* activity measurements, indicating that it is not the direct electron donor for reductive dehalogenation (Louie & Mohn, 1999).

Yet another class of potent electron-mediating components are the cytochromes. Both b- and c-type cytochromes have been isolated from virtually all halorespiring bacteria. Nevertheless, involvement in halorespiratory electron transport has not been investigated. Interestingly, a high spin c-type cytochrome was co-induced with dehalogenation activity in *Desulfomonile tiedjei*, and the corresponding gene has been cloned and sequenced (Louie *et al.*, 1997). The 50-kDa gene product exhibited two conserved c-type heme binding motifs (CxxCH), but substantially differed from known cytochromes and also no homologues could be identified from the *Dehalococcoides ethenogenes* partial genome sequence. However, as the above-mentioned quinoid, also the induced cytochrome presumably is not the physiological electron donor for the dehalogenating system (Louie & Mohn, 1999).

Regulation of Halorespiration

It is known from physiological experiments that in several halorespiring bacteria described to date, reductive dehalogenase activity is induced by the presence of a halogenated substrate (Table 1.1). In most cases, the inducer is also substrate for the dehalogenating system, as might be expected. Occasionally, as it was observed for the *meta*-dehalogenating enzyme in *Desulfomonile tiedjei*, reductive dehalogenase activity is not only inducible by the substrate, but by a whole variety of also non-halogenated compounds (DeWeerd & Suflita, 1990; Mohn & Tiedje, 1992).

In strains of *Desulfitobacterium* that contain multiple dehalogenating activities, independent induction of the various enzyme systems has been observed (Gerritse *et al.*, 1999). In at least *Desulfitobacterium dehalogenans* and *Desulfitobacterium frappieri* PCP-1, induction is dependent on *de novo* protein biosynthesis, as dehalogenating activity did not develop in case chloroamphenicol had been added prior to the inducer (Utkin *et al.*, 1995; Dennie *et al.*, 1998). Only for a few strains, constitutively present dehalogenating activity has been described to date (Neumann *et al.*, 1994; Miller *et al.*, 1997; Gerritse *et al.*, 1999; Maymó Gatell *et al.*, 1999). One of these is the versatile *Dehalospirillum multivorans*, for which the isolation of three different variants has been reported: one of the isolates was found to be inducible for its dehalogenating activity, one expressed this system constitutively and one isolate could not be induced by the addition of the chlorinated substrate (Neumann *et al.*, 1994).

A better understanding of the regulatory circuits that are involved not only in the induction, but also the occasional repression of halorespiration activity by various environmental factors, is believed to be highly relevant for the application of halorespiring microorganisms for the *in situ* bioremediation of contaminated environments and the optimization of such processes (Gerritse *et al.*, 1999). Being one of the relevant parameters, the influence of alternative electron acceptors on the activity of the dehalogenating system has been investigated for a few isolates, indicating that particularly sulfur oxyanions are potential inhibitors of halorespiration (DeWeerd & Suflita, 1990; Townsend & Suflita, 1997; Gerritse *et al.*, 1999). Extensive chemostat studies on the PCE dehalogenating *Desulfitobacterium frappieri* TCE1 showed that dehalogenation was especially sensitive to the presence of various alternative electron acceptors under electron donor-limiting conditions, whereas only sulfite significantly inhibited PCE reduction when the culture was PCE-limited (Gerritse *et al.*, 1999). Contrarily, the presence of equimolar concentrations of sulfite did not inhibit Cl-OHPA dechlorination in non-acclimated cultures of *Desulfitobacterium dehalogenans* (Mackiewicz & Wiegel, 1998).

Detection of Halorespiration Potential and Activity

In situ biological remediation strategies that exploit the reductive dehalogenation capacity of anaerobic microorganisms, are considered to be of great importance for the efficient removal of chlorinated pollutants such as PCE, as they potentially yield the complete dechlorination in a single one-phasic process (Lee *et al.*, 1998). Such approaches may include the stimulation of the intrinsic dehalogenating microbiota or the augmentation with specific degrading populations at sites, where such organisms are not present in sufficient numbers. In order to identify and maintain the optimal conditions for such processes, both, natural attenuation and bioaugmentation strategies require reliable and efficient methods to follow fate, distribution and activity of the relevant dehalogenating microorganisms (Vogel, 1996; Stapleton *et al.*, 1998). As it is widely accepted that current microbiological culture techniques often capture only a minor fraction of the bacteria present in the environment and, in addition, require extended incubation periods, increasing efforts are currently being made to develop novel molecular tools for the monitoring of bioremediation processes, and the identification and isolation of yet unknown dehalogenating microorganisms.

Ahring *et al.* used an immunofluorescence-based approach to follow fate and localization of *Desulfomonile tiedjei* added to unsterile anaerobic granular sludge. The authors could show that the bacteria had formed microcolonies inside the granules (Ahring *et al.*, 1992). Taking a similar approach, the formation of uniformly distributed net-like structures of *Desulfitobacterium hafniense* that had been added to sterile sludge granules was demonstrated, suggesting that ecological parameters inside a granule, such as structure and viability of the present microbial community, might have a major impact on the colonization performance of *de novo* added specific strains (Christiansen & Ahring, 1996b). Comparable results were obtained in studies on the performance and distribution of cells of the PCE-dechlorinating *Dehalospirillum multivorans* that had been added to sterile and non-sterile granular sludges. However, in this case, the organism was organized in microcolonies within the sterile granules, whereas net-like structures were observed on the surface of the living granules, indicating that the localization within the ecosystem also largely depends on the specific characteristics of the *de novo* introduced cells (Horber *et al.*, 1998). Cells of *Dehalospirillum multivorans* could be specifically detected and quantified from a PCE-dechlorinating mixed-culture biofilm from a fluidized-bed reactor using an enzyme-linked immunosorbent assay (ELISA), confirming that immunofluorescence-based methods are not

only highly suited for the detection, but to a certain extent also for the quantification of specific degraders in microbial ecosystems of high cell density and complexity (Bauer-Kreisel *et al.*, 1996).

In a yet different approach targeting specific biomolecules, the detection of signature lipid biomarkers (lipopolysaccharide branched 3-hydroxy fatty acids) has been used for the detection of *Desulfomonile tiedjei* cells that had been added to aquifer sediment microcosms (50 g scale). With this method, the detection of > 4×10^6 cells / g of soil was possible, indicating its usefulness in the monitoring of the fate of dehalogenating microorganisms during bioaugmentation (Ringelberg *et al.*, 1994).

Although the above-mentioned approaches proved to be instrumental for the specific detection of various halorespiring strains in different dehalogenating systems, rather high cell densities of the target organism were required for reliable results. However, microbial communities present in e.g. polluted soils and sediments are often rather low in cell numbers, indicating that more sensitive methods have to be available for the application in such highly relevant ecosystems.

Several 16S rRNA gene targeted PCR approaches have been reported that significantly improved the detection threshold. In a nested PCR approach, as little as 10^3 and 10^5 cells per gram of soil could be detected after introduction of *Desulfitobacterium dehalogenans* and *Desulfomonile tiedjei*, respectively, into soil slurry microcosms (El Fantroussi *et al.*, 1997). Recently, *Desulfomonile tiedjei* was used for bioaugmentation at a more realistic pilot scale (0.5 m^3), and the previously designed nested PCR approach was instrumental for monitoring the fate of the added strain (El Fantroussi *et al.*, 1999). Similarly, nested PCR was used to detect cells of *Desulfitobacterium frappieri* PCP-1 in DNA extracted from soil that was amended with different concentrations of the organism, enabling for the detection of 100 cells / g soil (Levesque *et al.*, 1997). This method was subsequently further optimized for the development of a competitive PCR protocol to quantify *Desulfitobacterium frappieri* PCP-1 in mixed bacterial populations and inoculated in soil (Levesque *et al.*, 1998). The approach was applied to monitor strain PCP-1 in bioaugmented PCP-contaminated soil as well as in anaerobic upflow sludge bed reactors, to which it had been added to improve the PCP-degradation performance (Beaudet *et al.*, 1998; Tartakovsky *et al.*, 1999).

Taking a more physiological rather than a molecular approach, Löffler *et al.* could show that a combination of measurements of H_2-consumption threshold values, and determination of the fraction of electrons used for reductive dechlorination was highly useful to indicate

whether or not halorespiration was occurring in both pure and mixed cultures (Löffler *et al.*, 1999).

Very interesting results were obtained in a recent study that combined the 16S rRNA gene-based detection of acetate-oxidizing *Desulfuromonas* and hydrogenotrophic *Dehalococcoides* species with the monitoring of dechlorination activity in microcosms. In samples, for which the molecular approach indicated the presence of dechlorinating species of *Desulfuromonas*, acetate-dependent dechlorination was found in the microcosm experiments. Similarly, *Dehalococcoides*-specific amplicons were obtained from those sediments, in which dehalogenation was detected when H_2 was added as the electron donor, indicating that such molecular approaches can indeed be a powerful tool for the prediction of halorespiring potential in polluted environments (Löffler *et al.*, 2000).

Conclusions

It is now widely accepted that anaerobic halorespiring bacteria are among the key players in biological dehalogenation processes in anoxic environments. Our rapidly increasing knowledge on the phylogeny and molecular characteristics of these microorganisms has brought us to a better understanding of the novel respiratory chains they possess. The detailed characterization of chlorophenol- and chloroethene reductive dehalogenases from phylogenetically distinct halorespiring bacteria at the biochemical and genetic levels has revealed significant similarities in structure and function, suggesting that they comprise a novel class of corrinoid-containing enzymes.

The development and optimization of culture-independent molecular approaches now allows for the *in situ* detection of reductive dehalogenation potential and activity. The integrated application of different halorespiration-specific molecular markers will be helpful in further improvement of biological remediation strategies, and will stimulate the exploitation of halorespiring bacteria as dedicated degraders for the clean-up of polluted sites.

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2

PURIFICATION AND MOLECULAR CHARACTERIZATION OF *ORTHO*-CHLOROPHENOL REDUCTIVE DEHALOGENASE, A KEY ENZYME OF HALORESPIRATION IN *DESULFITOBACTERIUM DEHALOGENANS*

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Abstract

ortho-Chlorophenol reductive dehalogenase of the halorespiring Gram-positive *Desulfitobacterium dehalogenans* was purified 90-fold to apparent homogeneity. The purified dehalogenase catalyzed the reductive removal of a halogen atom from the *ortho* position of 3-chloro-4-hydroxyphenylacetate, 2-chlorophenol, 2,3-dichlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol, pentachlorophenol, and 2-bromo-4-chlorophenol with reduced methyl viologen as electron donor. The dechlorination of 3-chloro-4-hydroxyphenylacetate was catalyzed by the enzyme at a V_{max} of 28 U/mg protein and a K_m of 20 µM. The pH and temperature optimum were 8.2 and 52°C, respectively. EPR analysis indicated one [4Fe-4S] cluster (midpoint redox potential (E_m) = -440 mV), one [3Fe-4S] cluster (E_m = +70 mV), and one cobalamin per 48-kDa monomer. The Co(I)/Co(II) transition had an E_m of -370 mV. Via a reversed genetic approach based on the N-terminal sequence, the corresponding gene was isolated from a *D. dehalogenans* genomic library, cloned, and sequenced. This revealed the presence of two closely linked genes: (i) *cprA*, encoding the *o*-chlorophenol reductive dehalogenase, which contains a twin-arginine type signal sequence that is processed in the purified enzyme; (ii) *cprB*, coding for an integral membrane protein that could act as a membrane anchor of the dehalogenase. This first biochemical and molecular characterization of a chlorophenol reductive dehalogenase has revealed structural resemblance with haloalkene reductive dehalogenases.

Introduction

Anaerobic bacteria that are able to conserve metabolic energy from the dechlorination of chlorinated compounds have gained a lot of attention because of their role in bioremediation of contaminated sites and the novel respiration pathways they possess (El Fantroussi *et al.*, 1998). Halorespiring bacteria have been found within the groups of low G+C Gram-positives, green non-sulfur bacteria, and δ- and ε- proteobacteria. These bacteria can use chloroalkenes, e.g. tetrachloroethene (PCE) and trichloroethene (TCE) or chloroaromatic compounds such as chlorophenols or 3-chlorobenzoate as the terminal electron acceptor.

The halorespiratory pathway of anaerobic PCE degradation has been studied in some detail. A key enzyme in this respiratory pathway is the PCE reductive dehalogenase, which catalyzes the reductive removal of a chlorine atom from PCE and TCE. A 58-kDa PCE reductive dehalogenase was purified from *Dehalospirillum multivorans*, which contains cobalamin and probably two iron-sulfur clusters (Neumann *et al.*, 1996). Cloning and sequencing of the corresponding *pceA* gene revealed the presence of an additional open reading frame, *pceB*, being cotranscribed with *pceA* and coding for an 8-kDa membrane-spanning protein (Neumann *et al.*, 1998). The PCE reductive dehalogenases isolated from *Dehalobacter restrictus* (60 kDa) and *Desulfitobacterium frappieri* strain PCE-S (65 kDa) resemble the enzyme from *Dehalospirillum multivorans* with respect to cofactor content and catalytic properties (Schumacher *et al.*, 1997; Miller *et al.*, 1998). Electron paramagnetic resonance (EPR) analysis of the *D. restrictus* enzyme confirmed the presence of cobalamin and two [4Fe-4S] clusters. All chloroalkene reductive dehalogenases characterized up to now are monomeric and either membrane-bound or membrane-associated.

Enzymes involved in chloroaryl respiration have been studied in *Desulfomonile tiedjei* and *Desulfitobacterium* species (Ni *et al.*, 1995; Löffler *et al.*, 1996; Christiansen *et al.*, 1998). However, no further molecular characterization of these enzymes was reported.

We investigated *ortho*-chlorophenol dechlorination in *Desulfitobacterium dehalogenans*. This organism is able to couple the reductive dechlorination of different *ortho*-chlorinated phenolic compounds to growth with lactate, pyruvate, formate, or hydrogen as electron donor (Utkin *et al.*, 1994; Utkin *et al.*, 1995). Comparison of biomass yields on pyruvate and different electron acceptors indicated that chlorophenol dechlorination in *D. dehalogenans* is an energy-yielding process (Mackiewicz & Wiegel, 1998). This study for the first time describes the purification and characterization of the catalytic subunit of the *ortho*-

chlorophenol reductive dehalogenase (*o*-CP dehalogenase) from *Desulfitobacterium dehalogenans*. Its redox properties were studied by EPR spectroscopy, and the corresponding *cprA* gene was cloned and characterized revealing structural resemblance with haloalkene reductive dehalogenases.

Experimental Procedures

Bacterial Strains, Plasmids, and Growth Conditions

D. dehalogenans strain JW/IU-DC1 (DSM 9161) was cultivated under anaerobic conditions (100% N_2 gas phase) in 25-liter vessels containing 20 liters of basal medium as described by Neumann *et al.* (Neumann *et al.*, 1994), supplemented with 0.2% yeast extract, 20 mM lactate sodium salt, 20 mM 3-chloro-4-hydroxyphenyl acetate (Cl-OHPA), 50 mM $NaHCO_3$, and trace elements and vitamin solution as recommended by the German Collection of Microorganisms. The 20-liter cultures were incubated at 37°C for 2 days. After 1 day of incubation, 250 ml of 2 M NaOH was added to the culture to avoid acidification of the medium.

Escherichia coli XL1-Blue (Stratagene) was used as a host for cloning vectors. The strain was grown in Luria Bertani medium at 37°C, and ampicillin was added at 100 µg/ml when appropriate. The cloning vectors pUC18 and pUC19 were purchased from Amersham Pharmacia Biotech and pMON38201 was obtained from Monsanto.

Preparation of Cell Extracts

Late-exponential-phase cultures of *D. dehalogenans* were harvested by continuous flow centrifugation at 16,000 x g (Biofuge 28RS, Heraus Sepatech) which yielded 1.6 g of concentrated cells per liter of culture. The concentrated cells were stored at -20°C. 8 g cells was resuspended in 8 ml of buffer 1, consisting of 100 mM potassium phosphate (KPi), pH 7.5, and 2.5 mM dithiothreitol (DTT). A few crystals of DNase I were added to the cell suspension. Cells were broken by sonication (Vibra, Sonic Materials Inc.) under anaerobic conditions. The cell debris was removed by centrifugation for 5 min at 20,000 x g. The supernatant was incubated for 10 min in the presence of 0.5 M KCl and 0.02% Triton X-100 and then separated into a membrane fraction and a soluble fraction by centrifugation for 90 min at 140,000 x g and 4°C. The membrane fraction was resuspended in 8 ml of buffer 1

supplemented with 1% Triton X-100 and 20% glycerol and incubated for 60 min under anaerobic conditions at 4°C. The insoluble fraction was removed from this preparation by centrifugation for 60 min at 140,000 x g and 4°C. The solubilized enzyme fraction was stored under a N_2 gas phase at 4°C.

Column Chromatography

All chromatographic steps were performed by fast protein liquid chromatography (Amersham Pharmacia Biotech) in an anaerobic chamber with N_2/H_2 (95/5%) gas phase. The Triton X-100 concentration of the sample was raised to 3% before it was applied to a column to prevent protein aggregation. The solubilized enzyme preparation was loaded on a Q-Sepharose column (2.2 x 8.9 cm) (Amersham Pharmacia Biotech) equilibrated with buffer A (50 mM KPi pH 6.0, 0.1% w/v Triton X-100, 20% glycerol, and 1 mM DTT). The column was eluted with a 75-ml linear gradient from 0 to 300 mM NaCl in buffer A at a flow of 2.5 ml/min. The *o*-CP dehalogenase activity was eluted at a NaCl concentration of approximately 180 mM. Fractions containing the highest dechlorinating activity were pooled and diluted with an equal volume of buffer A. The sample was applied on a Mono Q column (Amersham Pharmacia Biotech) equilibrated with buffer A. The enzyme was eluted with a 40-ml linear gradient from 0 to 400 mM NaCl in buffer A and a flow rate of 1.0 ml/min at a NaCl concentration of 180 mM.

Combined fractions containing dechlorinating activity were mixed with an equal volume of buffer B (50 mM Tris-HCl, pH 7.8, 0.1% w/v Triton X-100, 20% glycerol, and 1 mM DTT) and applied on a Mono Q column equilibrated with the same buffer. The enzyme activity was eluted with a 40-ml linear gradient from 0 to 400 mM NaCl in buffer B, and a flow rate of 1.0 ml/min at a NaCl concentration of 280 mM.

Enzyme Assay

Chlorophenol reductive dehalogenase activity was assayed in stoppered 1-cm cuvettes at 30°C and pH 7.8 by photometric recording of the oxidation of titanium(III)citrate reduced methyl viologen at 578 nm (ε_{578} = 9.7 $mM^{-1} \times cm^{-1}$) as described by Schumacher and Holliger (Schumacher & Holliger, 1996). The assay mixture contained 0.3 mM methyl viologen, and had an initial absorption at 578 nm of 2.6. The assay was started by the addition of 20 µl of 50 mM Cl-OHPA to give a final concentration of 1 mM Cl-OHPA. One unit is defined as the amount of enzyme that catalyzed the reduction of one µmol of chlorinated

substrate or the oxidation of two µmol of reduced methyl viologen per minute. The same specific activity was obtained whether methyl viologen oxidation, Cl-OHPA disappearance, or 4-hydroxyphenyl acetate appearance was followed. The protein content of the samples was determined according to Bradford with bovine serum albumin as standard (Bradford, 1976).

Kinetic Parameters

The pH optimum was determined in a 200 mM Tris-maleate buffer ranging from pH 5.5 to 9.0. Michaelis-Menten constants were determined from Lineweaver-Burk representations of data obtained by determining the initial rate of Cl-OHPA reduction under the assay conditions described above and using 5 µM to 10 mM substrate in the cuvette.

Composition of *o*-CP Dehalogenase

The molecular mass of the denatured protein was determined by SDS-polyacrylamide gel electrophoresis according to Laemmli (Laemmli, 1970). A low molecular weight marker (Biorad) was used as reference. The gels were stained with Coomassie Brilliant Blue R50. The concentration of acid labile sulfur of three individual samples was determined according to Rabinowitz (Rabinowitz, 1978). The iron and cobalt content of three independent enzyme preparations was measured by inductively coupled plasma mass-spectrometry (Elan 6000, Perkin-Elmer). The protein concentration of the samples was determined by measuring the absorbance changes in the Rose Bengal binding assay as described by Elliot & Brewer with bovine serum albumin as standard (Elliott & Brewer, 1978). A correction factor was determined with purified *o*-CP dehalogenase to compare the Rose Bengal protein determination and the Bradford protein determination. A correction factor of 1.10 was applied for the Rose Bengal determinations.

Cobalamin and Iron-Sulfur Cluster Analysis by EPR

EPR spectra were recorded on a Bruker 200 D spectrometer with cryogenics, peripheral equipment, and data acquisition as described previously (Pierik & Hagen, 1991). The protein concentration of the EPR samples was 0.4 mg/ml in buffer B. The enzyme was completely reduced in 45 min by deazaflavin/EDTA mediated light reduction as described by Massey and Hemmerich (Massey & Hemmerich, 1977). Deazaflavin was synthesized according to Janda and Hemmerich (Janda & Hemmerich, 1976).

N-Terminal Amino Acid Sequence

Purified enzyme was transferred from a 12% SDS polyacrylamide gel onto a polyvinylidene difluoride membrane (Immobilon polyvinylidene difluoride, Milipore Corporation) by blotting with a Trans-Blot SD semidry transferring cell (Biorad). Blotting was carried out at 14 V for 2 hours using a transfer buffer containing 48 mM Tris, 39 mM glycine, and 20% methanol, pH 9.1. The transferred protein was stained with Coomassie Brilliant Blue R-250. The N-terminal amino acid sequence of the blotted protein was determined as described by Schiltz *et al.* (Schiltz *et al.*, 1991).

DNA Isolation, Manipulation and Oligonucleotides

Chromosomal DNA from *D. dehalogenans* was isolated as follows. Protoplasts were prepared from 12 ml of culture (A_{600} = 0. 4) as described by van Asseldonk *et al.*, recovered at 13,000 g for 2 min, and resuspended in 100 µl of THMS buffer (30 mM Tris-HCl, pH 8.0, and 3 mM $MgCl_2$ in 25 % sucrose) (van Asseldonk *et al.*, 1993). After the addition of 400 µl of 50 mM Tris-HCl (pH 8.0), containing 5 mM EDTA, 50 mM NaCl and 0.5 % SDS, chromosomal DNA was purified through successive steps of phenol/chloroform extraction and recovered by ethanol precipitation.

Plasmid DNA was isolated from *E. coli* by using the alkaline lysis method, and standard DNA manipulations were performed according to established procedures (Sambrook *et al.*, 1989) and manufacturers instructions. Enzymes were purchased from Life Technologies Inc, Roche Molecular Biochemicals, or New England Biolabs. Oligonucleotides and [α-^{32}P]dATP were obtained from Life Technologies Inc., and Amersham Pharmacia Biotech, respectively. Prehybridization and hybridization were performed at 65°C and 50°C, respectively. Posthybridization washes were conducted at 40°C.

Oligonucleotides used in this study were BG 444 (5' GCI GA(A/G) ACI ATG AA(C/T) TA(C/T) GTI CCI GGI CCI ACI AA(C/T) GCI GCI (A/T)(C/G)I AA(A/G) (C/T)TI GGI CCI GT 3', nucleotides 644 - 703), BG 458 (5' GCC GGA GCC TTG ATC GC 3', nucleotides 427 - 411) and BG 475 (5' GGC AGG TCT GGG AGA ATT G 3', nucleotides 1366 - 1384). In order to restrict the extent of degeneration for BG 444, inosine (I) was used at 3- or 4-fold degenerated positions.

DNA Amplification by Inverse PCR

Inverse PCR (Triglia *et al.*, 1988) was performed as follows. Chromosomal DNA was digested with *Hin*cII and ligated at a concentration of 0.5 ng/µl. 5 ng of self-ligated DNA was used as the template in a 25-µl PCR reaction, containing the following: 2 ng/µl each primer; 2.25 mM $MgCl_2$; 200 µM dATP, dCTP, dGTP, and dTTP; and 1 unit of Expand Long Template enzyme mixture (Roche Molecular Biochemicals). The DNA was amplified using the GeneAmp PCR System 2400 (Perkin Elmer Cetus). After preheating to 94°C for 2 min, 35 cycles were performed, consisting of: denaturation at 94°C for 20 s, primer annealing at 50°C for 30 s, and elongation at 68°C for 3 min. After 10 cycles, the elongation time was extended with 20 s/cycle. A final extension of 7 min at 68°C was included. PCR products were purified from agarose gel by Gene Clean (Bio 101) and cloned into pMON38201, cut with *Xcm*I.

DNA Sequencing and Sequence Analysis

DNA sequencing was performed using a Li-Cor DNA sequencer 4000L. Plasmid DNA used for sequencing reactions was purified with the QIAprep Spin Miniprep kit (Qiagen GmbH). Reactions were performed using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech). Infrared labeled oligonucleotides were purchased from MWG Biotech. Sequence similarity searches and alignments were performed using the BLAST 2.0 program (Altschul *et al.*, 1997) (NCBI) and the programs Clustal X and GeneDoc (Thompson *et al.*, 1997; K. B. Nicholas & H. B. J. Nicholas, GeneDoc: a tool for editing multiple sequence alignments, 1997), respectively.

Nucleotide Sequence Accession Number

The nucleotide sequence of the *Hin*cII fragment carrying the *cprA* locus has been deposited in the GenBank database under GenBank Accession Number AF115542.

Table 2.1 Purification scheme for *ortho*-chlorophenol reductive dehalogenase of *D. dehalogenans*. *o*-CP dehalogenase activity was monitored throughout the fractionations of the cell extract of *D. dehalogenans*.

Sample	Protein mg	Activity U*	Yield %	Specific activity mU/mg	Purification factor
Cell free extract	419.0	129	100	308	1.0
Membrane fraction	240.0	101	78	423	1.4
Solubilized fraction	91.0	109	84	1210	4.0
Q-sepharose fraction	21.1	101	78	4786	15.0
MonoQ, pH 6.0, fraction	4.4	64	50	14612	47.0
MonoQ, pH 7.8, fraction	2.1	59	46	27872	90.0

*One unit (U) of activity is defined as the oxidation of 2 μmol of reduced methyl viologen.

Results

Purification and Characterization of *o*-CP Dehalogenase

o-Chlorophenol reductive dehalogenase was purified under strict anaerobic conditions from the membrane fraction of *D. dehalogenans* grown on lactate and Cl-OHPA (Table 2.1). The specific activity increased 90-fold upon purification and amounted to 28 U/mg protein with reduced methyl viologen as an artificial electron donor. The purified enzyme had a pH and temperature optimum of 8.1 and 52°C, respectively. At 30°C, the enzyme showed Michaelis-Menten kinetics for Cl-OHPA. The K_m for this chlorinated substrate was determined to be 20 μM at a methyl viologen concentration of 0.3 mM. Cl-OHPA showed no inhibitory effect up to 10 mM, which was the highest concentration used. Several halogenated compounds were tested as possible alternative substrates for *o*-CP dehalogenase. Activity of *o*-CP dehalogenase was observed with 2-chlorophenol (2-CP), 2,3-dichlorophenol (2,3-DCP), 2,4-DCP and 2,6-DCP, and pentachlorophenol (PCP) as substrate (Table 2.2).

3-CP, 4-CP, and 2,5-DCP were not dechlorinated. Additionally, 2-bromo-4-chlorophenol (2Br-4CP), but not 2-fluoro-4-chlorophenol (4Cl-2FP), could be dehalogenated. This confirms that reductive dehalogenation is the reaction mechanism of *o*-CP dehalogenase, since bromide and chloride are more readily reductively removed than fluoride. No activity was measured with PCE or TCE, indicating that chlorinated aliphatics do not serve as a substrate for the *o*-CP dehalogenase.

Table 2.2 Substrate specificity profile of purified *o*-CP dehalogenase. The rate of methyl viologen oxidation catalyzed by *o*-CP dehalogenase in the presence of different possible electron acceptors was spectrophotometrically followed at 30°C. The reaction mixture contained 0.3 mM methyl viologen, 7 µg of dehalogenase, 1 mM electron acceptor, and 50 mM Tris-HCl at pH 7.8. One unit is defined as the amount of enzyme that catalyzed the oxidation of 2 µmol of reduced methyl viologen per min. 3-CP, 4-CP, 2,5-DCP, 4Cl-2FP, PCE, and TCE were dechlorinated at a rate below the detection limit (0.12 U/mg).

Substrate	Specific activity (U/mg)	Ratio % (compared to Cl-OHPA)
Cl-OHPA	12.0	100
2-Br-4-CP	24.3	202
2,3-DCP	15.5	129
2,4-DCP	4.2	35
2,6-DCP	0.8	7
PCP	0.2	2
2-CP	0.2	2

SDS-polyacrylamide gel electrophoresis analysis of the purified enzyme preparation revealed one band of approximately 48 kDa (Fig. 2.1). An accurate determination of the native size of the enzyme was not possible due to the high concentration of detergent needed to prevent protein aggregation (data not shown).

The analysis of metals revealed the presence of 0.7 ± 0.1 mol cobalt and 7 ± 1.4 mol of iron atoms per mol of monomer. Acid labile sulfur analysis showed 9.9 ± 1.2 mol of sulfur atoms / mol of monomer. We conclude from these results and the EPR data (see below) that 1 cobalamin and 2 iron-sulfur clusters are present per mol of enzyme.

N-Terminal Sequence, Cloning and Sequencing of the *cprA* Locus

The N-terminal amino acid sequence of the *o*-CP dehalogenase purified from *D. dehalogenans* was determined and revealed the sequence NH_2-AETMNYVPGPTNARSKLRPVHDFA. A 59-bp 256-fold degenerated oligonucleotide (BG 444) was designed based on the sequence of the first 20 N - terminal amino acids. Southern blot analysis of *Eco*RI-*Hin*dIII digested chromosomal DNA of *D. dehalogenans* revealed a 2.7-kb fragment that hybridized strongly to radiolabeled BG 444. This fragment was cloned in *E.coli* using *Eco*RI - *Hin*dIII digested pUC18, resulting in pLUW910. Sequence analysis of the *Hin*dIII – *Hin*cII 1.8-kb fragment of pLUW910 revealed the determined N–terminal amino acids immediately downstream of the *Hin*dIII site, indicating that pLUW 910 lacks the

translation start of the gene of interest. Therefore, the divergent primer pair BG 458 / BG 475 was used to specifically amplify the pLUW910 upstream flanking fragment in an inverse PCR reaction from *Hin*cII digested chromosomal DNA. To ensure determination of the correct nucleotide sequence, three independently obtained PCR products were cloned yielding pLUW912a-c. From these, *Hin*cII deletion clones were prepared, giving the corresponding pLUW913a-c. Fig. 2.2 shows a restriction map of the DNA region cloned and sequenced.

Organization of the *cprA* Locus

Sequence analysis revealed the presence of two closely linked open reading frames, namely *cprB* (nucleotides 194 - 505) and *cprA* (nucleotides 518 - 1861). A third open reading frame, ORF1, starts at nucleotides 1958. Preceding each of the three open reading frames, potential Shine Dalgarno sequences were found (data not shown).

The predicted gene product of *cprA* is a polypeptide of 447 amino acids with a molecular mass of 49,720 Da. The first 42 N-terminal residues of CprA comprise a leader sequence that is cleaved off upon maturation of the protein, leaving a mature 405-amino acid polypeptide with a calculated molecular mass of 45,305 Da. The leader sequence contains an RR motif being characteristic for a large number of mainly periplasmic proteins binding different redox cofactors (Berks, 1996). These twin arginine signal sequences (consensus (S/T)RR*X*FLK) are thought to play a major role in the maturation and translocation of such proteins. As all twin arginine signal sequences, the CprA leader sequence shows the structural characteristics of standard Sec signal sequences. Furthermore, the established cleavage site ~VANA↓AETM~ follows the '-1/-3 - rule' of von Heijne (von Heijne, 1984).

The *D. dehalogenans* CprA sequence reveals the presence of an extended cluster of cysteine residues (Cys^{330} - Cys^{387}, Fig. 2.3). The first group of four cysteines Cys^{330} - Cys^{340} is identical to the consensus sequence of bacterial ferredoxin type clusters (C*XX*C*XX*C*XXX*CP), including the conserved proline at position 341 (Bruschi & Guerlesquin, 1988). The second cluster shows the same conserved residues (Cys^{380} - Pro^{388}) but lacks the first cysteine. The B_{12} binding motif D*X*H*XX*G-(41)-S*X*L-(26-28)-GG, as it was determined for a subset of B_{12}-dependent enzymes, is not present in CprA (Ludwig & Matthews, 1997).

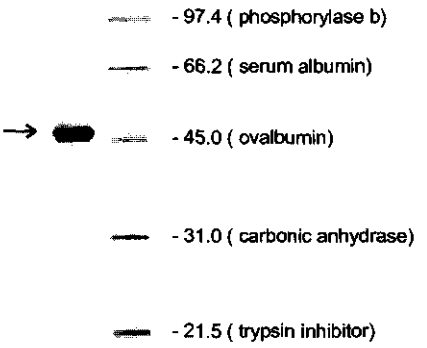

Figure 2.1 12% SDS polyacrylamide gel electrophoresis with the purified *ortho*-chlorophenol reductive dehalogenase of *D. dehalogenans* (5µg) in lane 1. Molecular size markers are shown in lane 2. The arrow indicates the purified protein band. The gel was stained with Coomassie Brilliant Blue R-250.

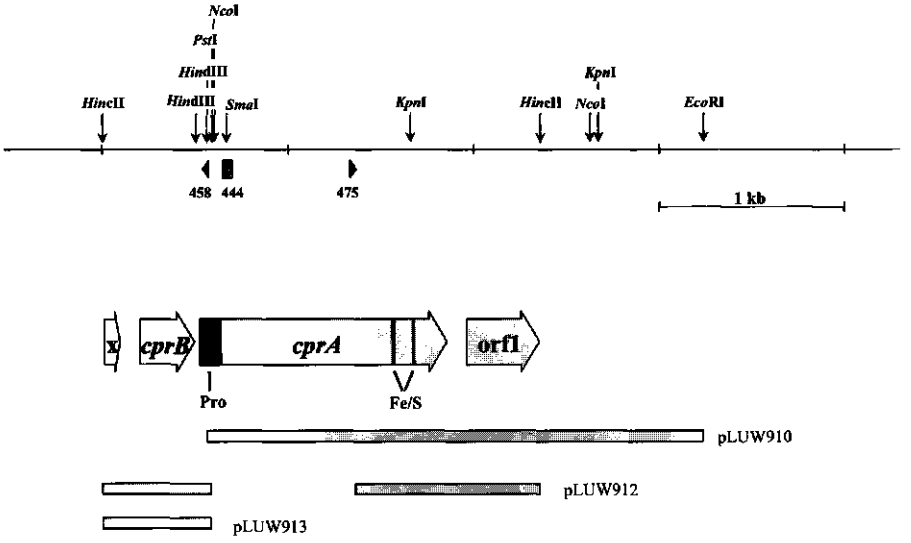

Figure 2.2 Restriction map of the *D. dehalogenans* genomic *cpr* - region. The vertical arrows mark DNA restriction sites. The horizontal bars indicate fragments, cloned either in pUC18 or pMON38201. The horizontal arrows indicate open reading frames. Oligonucleotides used in this study are shown. The 32 C-terminal amino acids of ORF X show some similarity with the C-terminal part of GroEL - type chaperonines. ORF1 exhibits no significant similarities with known proteins.

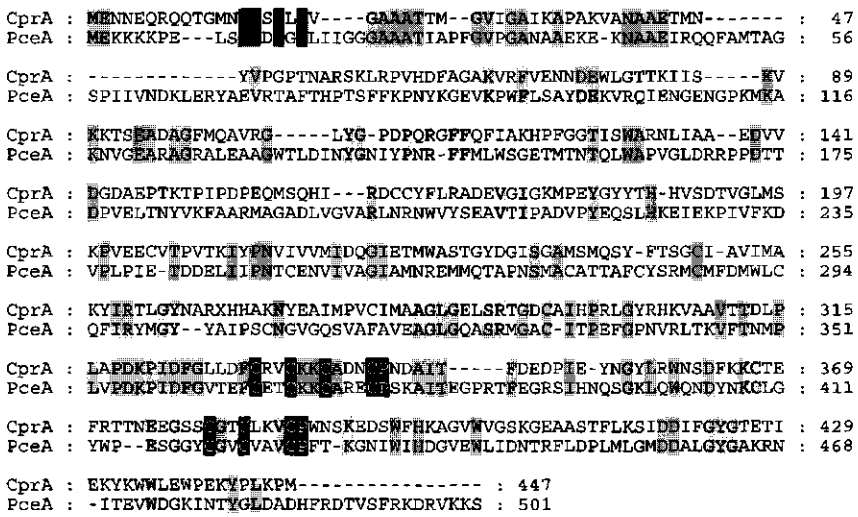

Figure 2.3 Primary sequence alignment for the *ortho*-chlorophenol reductive dehalogenase from *D. dehalogenans* (CPR) and the PCE dehalogenase from *Dehalospirillum multivorans*. The alignment was performed using the programs Clustal X and GeneDoc (Thompson *et al.*, 1997; K. B. Nicholas & H. B. J. Nicholas, GeneDoc: a tool for editing multiple sequence alignments, 1997). The light gray boxes mark identical residues. The dark gray boxes show residues from the twin arginine consensus motif. Residues highlighted in black indicate the conserved iron-sulfur cluster binding motifs. CprA: *ortho*-chlorophenol reductase from *D. dehalogenans* (GenBank accession Number AF115542); PceA: PCE dehalogenase from *D. multivorans* (GenBank accession Number AF022812).

Upstream of *cprA*, a second potential gene, *cprB*, was found, that could encode a 103 amino acid polypeptide with a calculated molecular mass of 11,517 Da. The predicted *cprB* gene product does not exhibit significant similarities with any known proteins present in the data bases. A hydrophilicity plot indicates the presence of three membrane spanning helices (Fig. 2.4). Following the positive-inside rule for integral membrane proteins, the N-terminus of this polypeptide is predicted to point outward, whereas the C-terminal part is located at the cytoplasmic face of the membrane (von Heijne & Gavel, 1988). *cprB* and *cprA* are separated by only 12 nucleotides. Neither transcription termination nor initiation signals are present between the two genes. Preliminary experiments suggest co-transcription of both genes (data not shown).

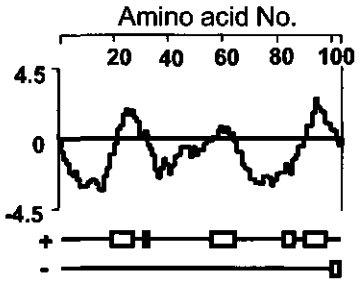

Figure 2.4 Hydrophilicity plot and charge distribution for CprB. The hydrophilicity plot was determined according to the method of Kyte and Doolittle (Kyte & Doolittle, 1982). The analysis was performed using the program Protean from the DNAstar software package.

Cobalamin Involved in Electron Transfer in *o*-CP Dehalogenase

Cobalt in biological systems occurs in oxidation states 3+, 2+, and 1+. Only the Co^{2+} $3d^7$-system is half-integer spin and, therefore, readily detectable in EPR spectroscopy. In cobalamin, the Co^{2+} is low spin S = 1/2. The EPR of *D. dehalogenans* *o*-CP dehalogenase, as isolated, exhibits a signal characteristic for Cob(II)alamin in the base-off form and a weak, near-isotropic, S = 1/2 signal around g = 2 indicative for [3Fe-4S] (see below).

Previously, it was found that full chemical reduction of another reductive dehalogenase, the PCE reductase from *D. restrictus*, could not be achieved with dithionite (Schumacher *et al.*, 1997). Therefore, we used the light-induced strongly reducing system of deazaflavin plus EDTA. Prolonged illumination resulted in a clear EPR spectrum which is dominated by a signal with g-values 2.05, 1.93, and 1.87, typical for reduced [2Fe-2S] or [4Fe-4S] clusters (Fig. 2.5, trace A). The signal rapidly broadens above 20 K, which indicates that its origin is a $[4Fe\text{-}4S]^{1+}$ cluster. Cob(II)alamin in the base-on form is present as a minor component in trace A, while the base-off form of Cob(II)alamin is fully reduced.

When the enzyme is anaerobically hand-mixed with the substrate Cl-OHPA and immediately frozen in liquid nitrogen (i.e. a reaction time of ≈ 0.5 min), another spectrum is obtained (Fig. 2.5, trace B). This is the signal of the base-off form of Cob(II)alamin (Schumacher *et al.*, 1997). The signal is essentially identical to that obtained from enzyme as isolated. In a control experiment where water, flushed with nitrogen gas, was added to a reduced *o*-CP dehalogenase sample no base-off cobalamin signal developed. Addition of an excess of ferricyanide did not affect the signal, and this indicated an unusually high oxidation

potential for the Co(II/III) couple, as previously found for the *D. restrictus* dehalogenase (Schumacher *et al.*, 1997). Estimation of the spin-Hamiltonian parameters by simulation gives g-values 1.99, 2.35, and 2.35 and cobalt hyperfine (I = 7/2) values of 14, 7.5, and 7.5 mT. These values are close to those found for the dehalogenase from *D. restrictus* (Schumacher *et al.*, 1997). The simulation indicates furthermore that the spectrum contains a minor second component, namely a base-on form of Cob(II)alamin; this form is also detectable as a minor component in trace A.

Upon incubation with excess potassium ferricyanide, the Co^{2+} signal is still present at maximal amplitude, but it is now hardly discernible, as the gain has been reduced for the observation of a near isotropic signal around g = 2 typical for a $[3Fe\text{-}4S]^{1+}$ cluster (Fig. 2.5, trace C). The broad peak at low field is the g_z from excess $[Fe(CN)_6]^{3+}$.

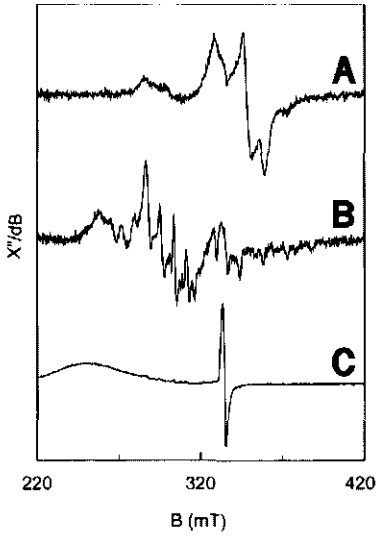

Figure 2.5 EPR spectra of *D. dehalogenans o*-CP dehalogenase. **Trace A**, the [4Fe-4S] signal from enzyme fully reduced by illumination with visible light for 50 min in the presence of 20 μM deazaflavin and 2 mM EDTA. Base-on Cob(II)alamin can be detected as a minor component in trace A. **Trace B**, the base-off Cob(II)alamin signal from enzyme reoxidized by 0.5 min anaerobic incubation with 2 mM Cl-OHPA. **Trace C**, the [3Fe-4S] signal from enzyme fully oxidized by anaerobic incubation with 2 mM potassium ferricyanide for 5 min. EPR conditions were as follows: microwave frequency, 9.41 GHz; microwave power 5 mW (trace A: 0.8 mW); modulation frequency, 100 kHz; modulation amplitude, 0.63 mT; temperature, 9.5 K (trace A), 30 K (trace B), 15 K (trace C).

All three signals, the $[4Fe\text{-}4S]^{1+}$ signal, the Cob(II)alamin signal, and the $[3Fe\text{-}4S]^{1+}$ signal integrate to approximately the same value corresponding to a spin count close to 1 spin per 48-kDa monomer.

The signals behave as expected in reductive (dithionite) and oxidative (ferricyanide) bulk redox titrations in the presence of a mixture of redox mediators (Fig. 2.6): in an oxidative titration, the signal ascribed to a [3Fe-4S] cluster appears with an oxidation potential of $E_{m,7.8}$ = +70 mV; in a reductive titration the Co^{2+} signal disappears with a reduction potential of $E_{m,7.8}$ = -370 mV; and the signal from the [4Fe-4S] cluster appears with $E_{m,7.8}$ = -440 mV. The E_m values for Co(II) and [4Fe-4S] are similar to those found for the *D. restrictus* dehalogenase. However, that enzyme contains two [4Fe-4S] clusters (Schumacher *et al.*, 1997). The EPR of the present *D. dehalogenans* enzyme strongly suggests the presence of one [4Fe-4S] and one [3Fe-4S] cluster, consistent with sequence analysis (see above).

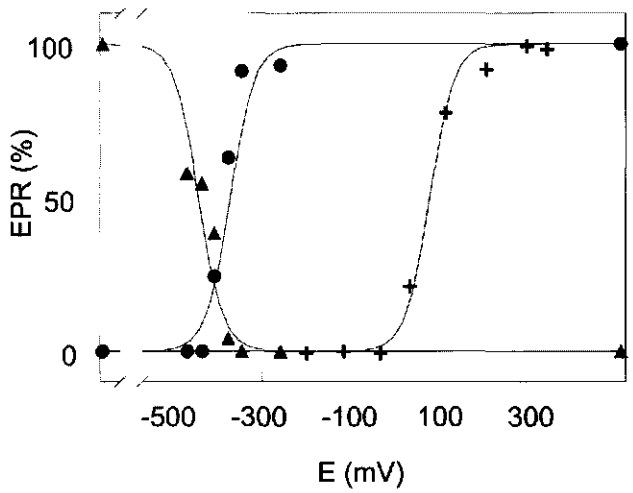

Figure 2.6 EPR-monitored redox titration of the metal centers in *D. dehalogenans* *o*-CP dehalogenase. (+) $[3Fe\text{-}4S]^{1+}$; (●) Cob(II)alamin; (▲) $[4Fe\text{-}4S]^{1+}$. Starting from a redox potential of -130mV, the sample was reduced by substoichiometric additions of dithionite, and oxidized by substoichiometric additions of ferricyanide, both in the presence of a mixture of redox mediators covering the full potential axis. Amplitudes are given as a percentage of maximal signal intensities. The latter correspond to enzyme fully oxidized by excess ferricyanide or enzyme fully reduced by light/deazaflavin/EDTA. These extreme forms have undefined potentials and are presented in the figure as points on the vertical borders. EPR conditions were as in Fig. 2.5. The solid traces are fits to the Nernst equation assuming single electron transfer.

Discussion

ortho-chlorophenol reductive dehalogenase is the terminal reductase involved in the halorespiratory chain of *D. dehalogenans*. Here we describe the purification and molecular characterization of this key enzyme and its gene *cprA*. This membrane-associated enzyme mediates the electron transfer from a yet unidentified electron donor to the halogenated substrate. The substrate spectrum of the purified enzyme was similar to that reported for resting cells, indicating that a single enzyme is involved in dehalogenation of *ortho*-halogenated phenols (Utkin *et al.*, 1995) (Table 2.2).

The purified *o*-CP dehalogenase contains one [4Fe-4S] cluster, one [3Fe-4S] cluster, and one cobalamin per monomer. The presence of two iron-sulfur clusters was confirmed by the identification of one ferredoxin-like and one truncated iron-sulfur cluster binding motif (Fig. 2.3) in the sequence of CprA. These iron-sulfur clusters might be involved in the electron transfer to the active site that contains the cobalamin. The primary sequence alignment of CprA with PceA, the PCE reductive dehalogenase of *D. multivorans* , revealed a rather high degree of similarity on the amino acid level in the C-terminal part of both enzymes (Fig. 2.3) (Neumann *et al.*, 1998). In PceA, the same two iron-sulfur cluster binding motifs are present, indicating a conserved mode of intramolecular transport of electrons. Both reductive dehalogenases probably differ in iron-sulfur cluster contents from the PCE reductase isolated from *D. restrictus*, where two [4Fe-4S] clusters were identified (Schumacher *et al.*, 1997). In the case of the 47-kDa Cl-OHPA reductive dehalogenase of the closely related *Desulfitobacterium hafniense*, the presence of three iron-sulfur clusters has been reported (Christiansen *et al.*, 1998). However, more sequence information on both the enzymes from *D. restrictus* and *D. hafniense* is not yet available.

The formation of Co(II) in base-off conformation upon the addition of Cl-OHPA to light-reduced *o*-CP dehalogenase confirms the involvement of the cobalamin in the dechlorination reaction. PCE reductase from *D. restrictus*, which converts PCE via TCE to 1,2-*cis*-DCE, also contains cobalamin (E_m = –350 mV) in its base-off conformation (Schumacher *et al.*, 1997). A similar mechanism could account for both chlorophenol and PCE dechlorination, although PCE is not a substrate for *o*-CP dehalogenase and *D. restrictus* is not capable to dechlorinate chlorophenols.

The *cprA* gene encodes a proprotein, in which the mature polypeptide is proceeded by a *twin arginine*-type signal sequence characteristic for periplasmic enzymes containing

complex redox cofactors. A similar leader sequence is present in the *pceA* gene product. For both dehalogenases, it has been proposed by dye-mediated activity measurements in intact and broken cells that the dehalogenating activities are located at the inner face of the cytoplasmic membrane (data not shown; Neumann *et al.*, 1998). The only other *twin arginine* enzyme with similar contradictory results concerns the *E. coli* DMSO reductase (Berks, 1996; Weiner *et al.*, 1998). Additional experiments will be required to solve the topology of these enzymes.

Elucidation of the nucleotide sequences upstream and downstream of *cprA* revealed the presence of a second potential gene, *cprB*. The hydrophobic gene product, CprB, might have a role in anchoring the catalytic subunit of the *o*-CP reductive dehalogenase to the cytoplasmic membrane. A similar function has been proposed for PceB in *D. multivorans* (Neumann *et al.*, 1998).

Although CprA and PceA exhibit highly conserved boxes, both primary sequences lack the consensus sequence for the binding of the corrinoid cofactor conserved among several methylcobalamin dependent methyltransferases and mutases (Ludwig & Matthews, 1997).

The role of cobalamin in the reductive dehalogenases from chlorophenol and PCE degrading organisms is of special interest, since it does not mediate the "usual rearrangement" or alkyl transfer but an elimination reaction (Ludwig & Matthews, 1997). Two models have been proposed for the reaction mechanism of PCE reductive dehalogenation. One model involves the formation of a Co(III)-chloroethene carbon-metal bond (Neumann *et al.*, 1996), whereas the second model postulates the formation of a chloroethene radical (Schumacher *et al.*, 1997). However, neither of these intermediates has been demonstrated unequivocally for PCE reductive dehalogenases. Based on our data, it is not possible to determine which model applies for *ortho*-chlorophenol reductive dehalogenase from *D. dehalogenans*. On one hand, an essential intermediate in the first model, Cob(III)alamin, was not formed upon oxidation of the enzyme. On the other hand, there was no radical formation upon addition of substrate to the reduced enzyme. The latter could be due to the slow reaction time, which makes it difficult to detect a reactive compound such as a phenol radical. Additional experiments are required in which the supposed radical would be stabilized.

The similarities between the *o*-chlorophenol reductive dehalogenase of *D. dehalogenans* and the PCE reductive dehalogenases of *Dehalospirillum multivorans* and *Dehalobacter*

restrictus on both mechanistic and structural properties as well as their primary sequences suggest that these enzymes constitute a novel class of corrinoid containing reductases.

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3

TRANSCRIPTIONAL REGULATION OF THE *CPR* GENE CLUSTER IN *ORTHO*-CHLOROPHENOL RESPIRING *DESULFITOBACTERIUM DEHALOGENANS*

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Abstract

To characterize the expression and possible regulation of reductive dehalogenation in halorespiring bacteria, a 11.5-kb genomic fragment containing the *o*-chlorophenol reductive dehalogenase-encoding *cprBA* genes of the Gram-positive bacterium *Desulfitobacterium dehalogenans* was subjected to detailed molecular characterization. Sequence analysis revealed the presence of eight designated genes with the order *cprTKZEBACD*, and with the same polarity except for *cprT*. The deduced *cprC* and *cprK* gene products belong to the NirI/NosR- and CRP-FNR families of transcription regulatory proteins, respectively. CprD and CprE are predicted to be molecular chaperones of the GroEL type, whereas *cprT* may encode a homologue of the trigger factor folding catalysts. Northern blot analysis, reverse transcriptase PCR, and primer extension analysis were used to elucidate the transcriptional organization and regulation of the *cpr* gene cluster. Results indicated halorespiration-specific transcriptional induction of the monocistronic *cprT* gene and the biscistronic *cprBA* and *cprZE* genes. Occasional read through at *cprC* gives rise to a tetracistronic *cprBACD* transcript. Transcription of *cprBA* was induced 15-fold upon addition of the *o*-chlorophenolic substrate 3-chloro-4-hydroxyphenylacetic acid within 30 min with concomitant induction of dehalogenation activity. Putative regulatory protein binding motifs that to some extent resemble the FNR box, were identified in the *cprT-cprK* and *cprK-cprZ* intergenic regions and the promoter at *cprB*, suggesting a role of the FNR-like CprK in the control of expression of the *cprTKZEBACD* genes.

Introduction

Halorespiring bacteria have received increasing attention during the last decade, as they are able to couple the reductive dehalogenation of a large variety of halogenated aromatic and aliphatic hydrocarbons to energy conservation and hence to microbial growth. These compounds are present in the environment as a consequence of their past and present application in industry and agriculture and due to natural production, compromising environmental integrity and health (Hileman, 1993; Gribble, 1996). Halorespiring bacteria are believed to play an important role in the *in situ* bioremediation of soil and groundwater polluted with halogenated hydrocarbons. The ability to perform halorespiration appears to be widespread throughout the Bacteria, as halorespiring bacteria have been found in the groups of low G+C-content Gram-positive bacteria, green non-sulfur bacteria, and δ- and ε-proteobacteria (El Fantroussi *et al.*, 1998). Among these, the Gram-positive genus *Desulfitobacterium* comprises a major group of isolates. The versatile *Desulfitobacterium dehalogenans* has been isolated because of its ability to use *o*-halogenated phenolic compounds as terminal electron acceptors in an anaerobic respiratory chain with lactate, pyruvate, formate, and molecular hydrogen as electron donors (Utkin *et al.*, 1994). Recently, the reductive dehalogenation of tetrachloroethene and hydroxylated polychlorinated biphenyls by the halorespirational system of *D. dehalogenans* has been reported (Wiegel *et al.*, 1999).

In order to understand the molecular basis of this novel respiratory system, efforts have focused not only on the reductive dehalogenases as the central enzymes in halorespiration (Neumann *et al.*, 1998; Holliger *et al.*, 1999; van de Pas *et al.*, 1999), but also on the identification of additional structural and regulatory components of the halorespiratory electron transport chain. An efficient conjugation system has been used for the integration of the conjugative transposon Tn*916* into the chromosome of *D. dehalogenans*, leading to the isolation of a number of halorespiration-deficient mutants, which were characterized at the physiological, biochemical and genetic levels (Smidt *et al.*, 1999).

It is known from physiological experiments that halorespiration is induced by the presence of a halogenated substrate in most halorespiring bacteria described to date. For two halorespiring strains, *Desulfomonile tiedjei* and *Desulfitobacterium frappieri* TCE1, the influence of alternative electron acceptors on the activity of the dehalogenating system has been described, indicating that particularly sulfur oxyanions are potential inhibitors of halorespiration (Townsend & Suflita, 1997; Gerritse *et al.*, 1999). In contrast, expression of

halorespiration by 3-chloro-4-hydroxyphenylacetic acid (Cl-OHPA) in non-acclimated cultures of *D. dehalogenans* was not affected by the presence of equimolar amounts of sulfite (Mackiewicz & Wiegel, 1998). However, the level at which regulation takes place, the control mechanisms involved, and the inducing signal remain to be elucidated.

This study addresses the molecular analysis of the regulation of reductive dehalogenation in a halorespiring bacterium. Chromosomal fragments flanking the *o*-chlorophenol reductive dehalogenase-encoding gene *cprA* in *D. dehalogenans* were cloned and characterized, revealing the presence of open reading frames that encode polypeptides possibly involved in regulation and maturation of the dehalogenating system. The expression of the different genes identified in the *cpr* gene cluster was studied under various growth conditions and found to be tightly controlled at the transcriptional level.

Materials and Methods

Materials

Cl-OHPA was purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands) and filtered prior to use. All gasses were obtained from Hoek Loos (Schiedam, The Netherlands). When appropriate, experiments were carried out in an anaerobic glove box (Coy Laboratories Products, Grass Lake, Mich.) under an atmosphere of 96% N_2 and 4% H_2. The oxygen concentration was kept low with the palladium catalyst RO-20 provided by BASF (Arnhem, The Netherlands).

Bacterial Strains, Plasmids, Growth and Induction Conditions

D. dehalogenans strain JW/IU-DC1 (DSM 9161) (Utkin *et al.*, 1994) was routinely grown under anaerobic conditions (100% N_2 gas phase) at 37° C in rubber stoppered serum bottles containing basal mineral medium as described by Neumann *et al.* (Neumann *et al.*, 1994), supplemented with 0.1% peptone, 30 mM $NaHCO_3$, and trace elements and vitamin solution as recommended by the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). An electron donor and acceptor were added to a concentration of 20 mM from anaerobic stock solutions. Growth was monitored spectrophotometrically by determining the optical density at 600 nm (A_{600}). The concentrations of Cl-OHPA and OHPA during growth in the presence of Cl-OHPA as the electron acceptor were determined by

HPLC analysis on a SpectraSystem high-performance liquid chromatograph, with a SpectraSystem P2000 pump, an AS3000 autosampler and a UV1000 UV-detector (ThermoQuest, Austin, Tex). The sample (20 µl) was injected into a Chrompack pesticide reversed-phase column (Chrompack, Middelburg, The Netherlands). The mobile phase was acetonitrile-0.01 M H_3PO_4 (10:90 [vol/vol]). A flow rate of 1 ml min^{-1} was applied, and Cl-OHPA and OHPA were quantified by their absorption at 206 nm. For the induction with Cl-OHPA, cells were grown with pyruvate to A_{600} of 0.1 and supplemented with 5 mM of Cl-OHPA. Samples were taken before and after induction and stored on ice prior to further processing.

Escherichia coli XL1-Blue (Stratagene, La Jolla, Calif.) was used as a host for cloning vectors. The strain was grown in Luria Bertani medium at 37°C (Sambrook *et al.*, 1989) and ampicillin was added at 100 µg/ml when appropriate. The cloning vectors pUC18 and pUC19 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden) and the PCR product cloning vectors pGEM-T and pMON38201 (Borovkov & Rivkin, 1997) were obtained from Promega (Madison, Wisc.) and Monsanto (St. Louis, Mo.), respectively.

DNA Isolation and Manipulation

Chromosomal DNA of *D. dehalogenans* was isolated as described previously (van de Pas *et al.*, 1999). Plasmid DNA was isolated from *E.coli* by using the alkaline lysis method and standard DNA manipulations were performed according to established procedures (Sambrook *et al.*, 1989) and manufacturers instructions. Enzymes were purchased from Life Technologies B.V. (Breda, The Netherlands), Roche Molecular Biochemicals (Mannheim, Germany), and New England Biolabs (Beverly, Mass.). Oligonucleotides were obtained from Eurogentec (Seraing, Belgium), Life Technologies Inc., and MWG Biotech (Ebersberg, Germany). PCR products were purified prior to subsequent manipulation using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). A Hybond-N+ nylon transfer membrane (Amersham Pharmacia Biotech) was used for Southern blot analysis. Probes for hybridization experiments were labeled by nick translation in the presence of [α-^{32}P]-dATP (Amersham Pharmacia Biotech).

Sequence Analysis of the *cpr* Gene Cluster

In order to extend the sequence downstream of the *cprA* locus, as it was determined previously from pLUW910 and pLUW913 (van de Pas *et al.*, 1999) (Fig. 3.1), a 0.9-kb

*Hin*cII-*Eco*RI restriction fragment of pLUW910 was subcloned in pUC19, yielding pLUW910EH_2, and sequenced. Subsequently, Southern blot analysis of *Hin*cII-*Hin*dIII-digested chromosomal DNA of *D. dehalogenans* revealed a 1.9-kb fragment that strongly hybridized with the aforementioned radiolabeled 0.9-kb fragment. The 1.9-kb fragment was cloned in *E.coli* using *Hin*cII-*Hin*dIII-digested pUC19, resulting in pLUW911. pLUW916 was obtained by inverse PCR, which was performed as described previously from *Nco*I-digested and self-ligated chromosomal DNA of *D. dehalogenans* with the divergent primer-pair BG580-BG581 (BG580, positions 9023 to 9002, and BG581, positions 9671 to 9692 of the *cpr* gene cluster; Fig. 3.1) (Triglia *et al.*, 1988; van de Pas *et al.*, 1999). The resulting 1.8-kb PCR-product was cloned in *E.coli* using *Xcm*I-digested pMON38201. Subsequently, PCR was performed with chromosomal DNA of *D. dehalogenans* with primers BG581 and HS22 (positions 10260 to 10240) and HS23 and HS27 (HS23, positions 10237 to 10257, and HS27, positions 11117 to 11097). Both PCR-products were cloned in *E.coli* using *XcmI*-digested pMON38201, yielding pLUW921 and pLUW922, respectively.

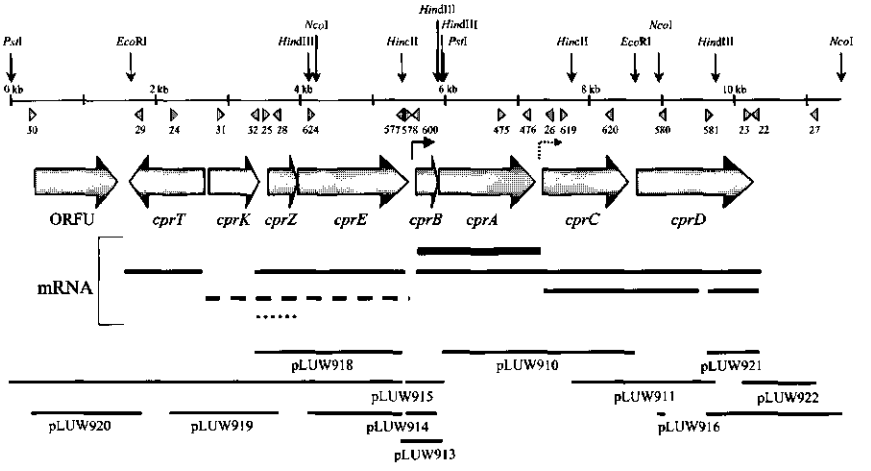

Figure 3.1 Physical map of the *cpr* gene locus in *D. dehalogenans*. Horizontal arrows, open reading frames; triangles, oligonucleotides used in this study; vertical arrows, DNA restriction sites, which were relevant for the construction of clones (bars); mRNA: solid bars, apparent halorespiration-specific transcription products; dashed and dotted lines, apparent constitutive and halorespiration-repressed transcripts, respectively.

To elucidate the sequence upstream of *cprB*, inverse PCR products were obtained from *Hin*dIII- or *Pst*I-digested and self-ligated chromosomal DNA using the divergent primer pair BG577-BG578 (BG577, positions 5443 to 5423, and BG578, positions 5459 to 5485), resulting in pLUW914 and pLUW915, respectively. pLUW915*EN* was obtained by subcloning a 2.3-kb *Eco*RI-*Nco*I-fragment of pLUW915 in *E.coli* using *Eco*RI-*Nco*I-digested pMON38201. Finally, pLUW918, pLUW919 and pLUW920 were obtained after PCR using chromosomal DNA as the template and primers BG577 and HS25, HS24 and HS28 or HS29 and HS30 (HS24, positions 2244 to 2267; HS25, positions 3521 to 3545; HS28, positions 3628 to 3605; HS29, positions 1768 to 1748; HS30, positions 266 to 284), respectively.

Using the above-mentioned set of clones, the almost-complete double-stranded *cpr* gene cluster nucleotide sequence could be elucidated. Where the sequence was only single-stranded, sequence analysis of multiple, independently obtained PCR-products was used to obtain an unambiguous result.

Amplification, Cloning and Sequencing of rRNA Genes

The 16S rRNA-encoding gene was amplified from chromosomal DNA of *D. dehalogenans* with the universal primer pair 7f-1510r (Lane, 1991). Primers 1492f (Lane, 1991) and 23InsR (Roller *et al.*, 1992) were used for the amplification of the 3'- and 5'-ends of the 16S and 23S rRNA genes, respectively, and the 16S-23S intergenic spacer. PCR products were cloned in the pGEM-T vector, yielding pLUW900 (16S) and pLUW901 (16S-23S) and their authenticity was verified by nucleotide sequence analysis.

DNA Sequencing and DNA and Protein Sequence Analysis

DNA sequencing was performed using a Li-Cor DNA sequencer 4000L (LiCor, Lincoln, Nebr.). Plasmid DNA used for sequencing reactions was purified with the QIAprep Spin Miniprep kit (Qiagen GmbH). Reactions were performed using the Thermo Sequenase fluorescently labeled primer cycle sequencing kit (Amersham Pharmacia Biotech). Fluorescently (IRD 800) labeled universal sequencing primers were purchased from MWG Biotech. Sequence similarity searches and alignments were performed using the BLAST, version 2.0, program (Altschul *et al.*, 1997) (National Center for Biotechnology Information, Bethesda, Md.), and the programs Clustal X, GeneDoc (Thompson *et al.*, 1997; K. B. Nicholas & H. B. J. Nicholas, GeneDoc: a tool for editing multiple sequence alignments, 1997) and the DNAstar package (DNASTAR Inc., Madison, Wis.), respectively. Protein

secondary structure and helical transmembrane region predictions were performed with the profile network systems PHDsec and PHDhtm, respectively (Rost & Sander, 1993; Rost & Sander, 1994; Rost *et al.*, 1995). Prediction of helix-turn-helix (H-T-H) DNA-binding motifs was performed using the method of Dodd and Egan (Dodd & Egan, 1990).

Isolation of Total RNA, Northern Analysis, RT-PCR and Primer Extension

Total RNA was isolated from exponentially growing cultures of *D. dehalogenans* by the Macaloid method described by Kuipers *et al.* (Kuipers *et al.*, 1993). For Northern blot analysis, 7 µg RNA was separated on a formaldehyde-1% agarose gel, transferred to Hybond-N+ nylon transfer membrane (Amersham Pharmacia Biotech) by downwards capillary transfer as described by Chomczynski with 5 × SSC, 10 mM NaOH as transfer liquid and immobilized by UV-crosslinking (Chomczynski, 1992). Prehybridization and hybridization were performed in ULTRAhyb – hybridization buffer (Ambion, Austin, Tex.) as recommended by the manufacturer.

A *cprA*-specific probe was generated by PCR amplification from chromosomal DNA of *D. dehalogenans* with the primer pair BG475-BG476 (BG475, positions 6778 to 6797; BG476, positions 7213 to 7192). As probes specific for *cprC*, *cprD* and *cprE*, PCR products that were obtained after PCR amplification with primer pairs BG619-BG620 (BG619, positions 7632 to 7658, and BG620, positions 8282 to 8255), BG581-HS22 and BG624-BG577 (BG624, positions 4147 to 4171), respectively, were used. For the detection of transcription products of ORFU, *cprT*, *cprK* and *cprZE*, probes were generated by PCR with primer pairs HS29-HS30, HS24-HS28, HS31-HS32 (HS31, positions 2906 to 2927, and HS32, positions 3440 to 3419) and HS25-BG577, respectively.

Reverse transcriptase PCR (RT-PCR) was performed to analyze the transcriptional organization of the genes in the *cpr* gene cluster. 500 ng of DNAse-treated RNA (RQ1 RNAse-free DNAse, Promega) was used in a 25-µl reaction, containing the following: 25 pmol of each primer, 200 µM dATP, dCTP, dGTP, and dTTP, 1.7 mM $MgSO_4$, 5 µl of AMV/*Tfl* 5× Reaction buffer and 2.5 U of AMV RT and *Tfl* polymerase (Access RT-PCR system, Promega). In negative controls, AMV RT was omitted and chromosomal DNA of *D. dehalogenans* was added to positive-control reaction mixtures. cDNA synthesis and subsequent PCR amplification were performed using the GeneAmp PCR System 9700 (Perkin Elmer Cetus, Norwalk, Conn.). The reaction mixture was incubated at 48°C for 45 min. After the mixture was preheated to 94° C for 2 min, 40 amplification cycles, consisting

of: denaturation at 94° C for 20 s, primer annealing at 50° C for 30 s and elongation at 68° C for 1 min 30 s were performed. A final extension of 7 min at 68° C was performed.

Primer extension analysis was performed to determine the transcription start sites of the *cprBA* and *cprCD* transcripts. For this purpose, 10 or 30 µg of RNA and 4 pmol of the fluorescently (IRD 800) labeled oligonucleotides BG600 (positions 5670 to 5648) and HS26 (positions 7476 to 7456), respectively, were dissolved in 10 µl 1× RT buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM dithiothreitol), incubated at 70°C for 5 min, and slowly cooled to room temperature. After addition of 10 µl 1× RT buffer containing 2 mM of dATP, dCTP, dGTP, and dTTP, 10 U of RNAsin, and 200 U of Superscript II RT (Life Technologies), the sample was incubated at 48°C for 30 min. RNase (0.2 mg/ml) was added, and the sample was precipitated with ethanol and washed once with 70% ethanol. The pellet was dried, dissolved in 2 µl formamide loading buffer, and separated on a Li-Cor 4000L DNA sequencer.

Nucleotide Sequence Accession Numbers

The nucleotide sequence of the *cpr* gene cluster described here has been deposited in the GenBank database under accession no. AF115542.

Results

Genetic Organization of the *cpr* Gene Cluster

Previously we analyzed the nucleotide sequence of the *cprBA* genes that encode the catalytic subunit and putative membrane anchor of the *o*-chlorophenol-reductive dehalogenase in *D. dehalogenans* (van de Pas *et al.*, 1999). Here we report the transcriptional organization of the *cprBA* genes. The chromosomal regions flanking the cluster were characterized by sequence and transcriptional analysis, revealing the presence of six additional transcribed genes, tentatively designated *cprC*, *cprD*, *cprE*, *cprK*, *cprT* and *cprZ* and one untranscribed open reading frame ORFU (Fig. 3.1; see below). With the exception of *cprT*, all genes are transcribed in the same direction as *cprBA*. In front of each of the genes potential Shine-Dalgarno sequences are present; these sequences are complementary to the 3'-end of the *D. dehalogenans* 16S rRNA (3'-AGAAUCUUUCCUCCA-5'; see below). The *cprC* gene, previously designated orf1 (van de Pas *et al.*, 1999), is located downstream of the structural

gene for the *o*-chlorophenol-reductive dehalogenase (*cprA*), and is predicted to encode a polypeptide of 395 amino acids with a molecular mass of 43,867 Da. Secondary structure prediction suggests the presence of six transmembrane helices (Fig. 3.2). Within the C-terminal cytoplasmic domains, two cysteine-rich signatures of the type CXXXCP were identified. For the C-terminal part of the predicted protein CprC, significant similarity was observed with membrane-bound regulators of the NosR/NirI type, which have been shown to play a role in a signal transduction pathway that eventually controls the transcription of the nitrous oxide (*nos*) and nitrite reductase (*nir*) gene clusters of *Pseudomonas stutzeri* and *Paracoccus denitrificans*, respectively (Cuypers *et al.*, 1992; Saunders *et al.*, 1999). This similarity was most pronounced in the vicinity of the cysteine-rich motifs (Fig. 3.2).

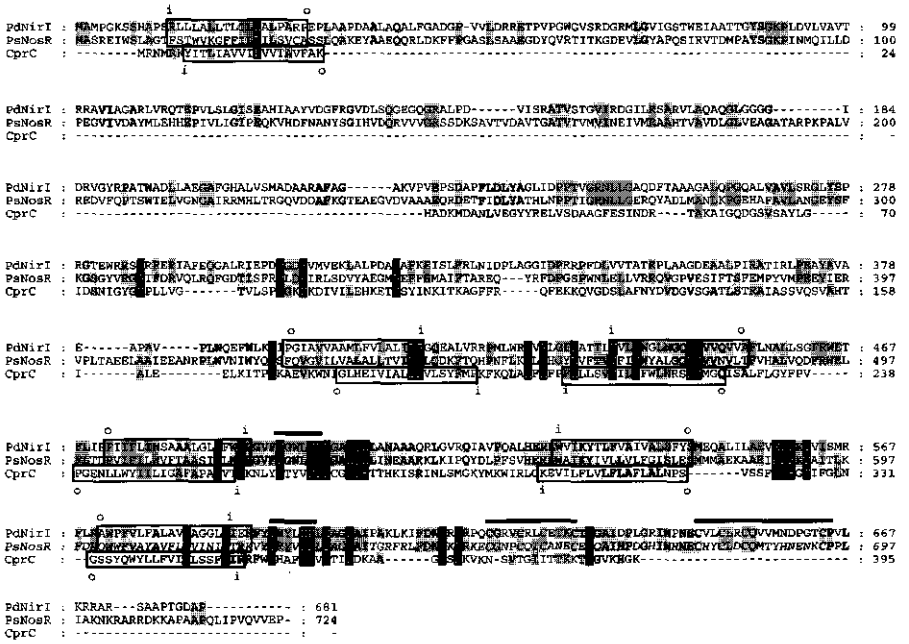

Figure 3.2 Amino acid sequence alignment of CprC from *D. dehalogenans* with NosR from *Pseudomonas stutzeri* (accession no. Q00790; PsNosR) and NirI from *Paracoccus denitrificans* (accession no. AJ001308; PdNirI). Residues conserved between two or all sequences are highlighted in gray and black, respectively. Horizontal bars, cysteine-rich motifs; boxes, predicted transmembrane helices; i and o, intra- and extracytoplasmic domains, respectively.

Figure 3.3 Alignment of CprK with proteins of the CRP-FNR family of regulatory proteins (Lv-PrfA, *Listeria ivanovii* listeriolysin regulatory protein PrfA, accession no. CAA51231; Dr, *Deinococcus radiodurans* putative transcriptional regulator, accession no. AAF11910; Ec-FNR, *Escherichia coli* FNR, accession no. P03019; Rm-FixK, *Rhizobium meliloti* FixK, accession no. S04122; Rs-NNR, *Rhodobacter sphaeroides* NNR-like protein, accession no. AAD27624; Pd-NNR, *Paracoccus denitrificans* Fnr-like transcriptional activator, accession no. AAA69977.1). Cysteine residues and residues conserved between three or more sequences are highlighted in black and gray, respectively. Asterisks, cysteine residues essential for activity of the *E.coli* FNR protein. Predicted H-T-H DNA-binding motifs are indicated.

However, CprC is significantly smaller than known proteins of the NosR/NirI family, due to a much shorter N-terminal extracytoplasmic loop (approximately 170 amino acids compared to 350) and the lack of a C-terminal cytoplasmic domain containing two additional ferredoxin-like motifs binding either [2Fe-2S] or [4Fe-4S] clusters in NosR and NirI (Bruschi & Guerlesquin, 1988).

Downstream of *cprC* and upstream of *cprB*, two other genes, *cprD* and *cprE* (the latter was previously designated orfX; van de Pas *et al.*, 1999), were identified, both potentially coding for chaperonins of the GroEL type (Fig. 3.1). The predicted gene products of *cprD* and *cprE* are polypeptides of 537 and 516 amino acids with calculated molecular masses of 58,002 Da and 54,632 Da, respectively. The two proteins share a sequence identity of 34 % on the amino acid level. Highest values of sequence similarity were observed with proteins from *Thermus thermophilus* (P45746; 45 % identity on the protein level for CprD and 40 % for CprE) and *Clostridium thermocellum* (P48212; 44 % identity for CprD and 39 % for CprE).

Upstream of *cprE*, the *cprK*, *cprT* and *cprZ* genes and the open reading frame ORFU were identified (Fig. 3.1). ORFU potentially encodes a polypeptide of 388 amino acids with a calculated molecular mass of 43,887 Da with no homologue in the databases. The predicted

gene product of *cprT* is a polypeptide of 311 amino acids with a molecular mass of 35,667 Da. CprT exhibits significant similarity to the trigger factor, a peptidyl prolyl isomerase that is considered to act as a protein folding catalyst (Fink, 1999). Highest similarities were observed with RopA from *Streptococcus pyogenes* (AAC82391, 15 % identity on the amino acid level) and the trigger factor from *Bacillus subtilis* (P80698, 14 % identity). Relatively low values of sequence identity are mainly caused by the fact that CprT lacks an approximately 110-amino acids N-terminal region which is present in known trigger factor homologues. CprT, however, still contains the complete FKBP-domain, which is associated with the peptidyl prolyl isomerase activity of known Trigger factors (Fink, 1999). Downstream of *cprT* is the location of *cprK*, which could encode a polypeptide of 233 residues with a calculated molecular mass of 26,646 Da. CprK revealed low, but significant, sequence similarity with known members of the CRP-FNR family of transcriptional regulators (Fig. 3.3). Preliminary results indicate that CprK deeply branches within subclass III (NtcA) of the CPR-FNR family (Vollack *et al.*, 1999). By applying the method of Dodd and Egan, an H-T-H motif could be predicted with 71 % probability, aligning with the H-T-H motif that is conserved among members of the CRP-FNR family (Dodd & Egan, 1990). However, the sequence E--SR, which is conserved in the recognition helix of nearly all FNR-like proteins, is only partially conserved in CprK (Fig. 3.3). This suggests that the recognition motif for CprK binding might be different from the common FNR box TTGAT-N_4-ATCAA (Fig. 3.3) (Zumft, 1997). *cprZ* is located downstream of *cprK*, overlapping with *cprE* over 4 nucleotides, and may code for a polypeptide of 138 amino acids with a calculated molecular mass of 15,546 Da. Significant sequence similarity of 24 % identity on the protein level was observed only with a hypothetical protein from *Synechocystis* sp. (BAA17004). Substantial conservation of the CprZ N-terminus and a well-defined ribosome binding site suggested an alternative translation start codon (GTG) for *cprZ*.

Transcriptional Analysis of the *cpr* Gene Cluster

The observation that the *cprBA* genes for the *o*-chlorophenol-reductive dehalogenase are flanked by five genes that could encode proteins which can be expected to play a role in regulation, maturation or action of CprA, prompted us to investigate the transcription of these genes under different conditions. Northern blot analysis was performed on total RNA isolated from cells grown with pyruvate as the electron donor and either Cl-OHPA, fumarate, nitrate or pyruvate as the electron acceptor.

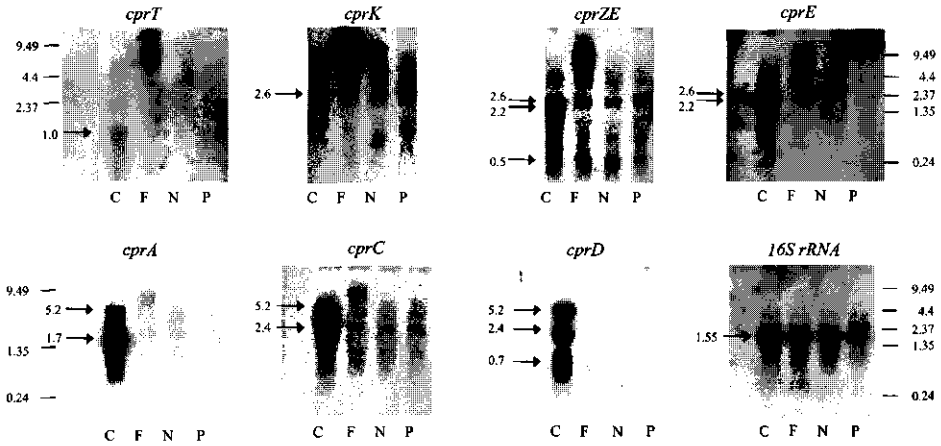

Figure 3.4 Northern blot analysis of total RNA extracted from cells of *D. dehalogenans* grown with pyruvate as the electron donor and various electron acceptors (C, Cl-OHPA; F, fumarate; N, nitrate; P, pyruvate). ^{32}P-labeled probes that were specific for genes present at the *cpr* gene locus and the 16S rRNA-encoding gene of *D. dehalogenans* were applied. RNA size markers are in kilobases. Arrows, specific hybridizing signals that were obtained after 3- to 48-h exposures. The high-molecular weight hybridization signals obtained with RNA isolated from fumarate-grown cells are due to residual amounts of chromosomal DNA.

The sizes of the transcripts were estimated by comparison with RNA molecular weight markers. Hybridization with a ^{32}P-labeled *cprA*-specific probe revealed the presence of two transcripts of approximately 1.7 and 5.2 kb, which were solely detectable in RNA isolated from cells grown by halorespiration (Fig. 3.4). The major hybridizing transcript of approximately 1.7 kb indicated co-transcription of the structural gene *cprA* with *cprB*, as was anticipated because of the fact that both genes are only 12 nucleotides apart (van de Pas *et al.*, 1999) (Fig. 3.4). Hybridization with probes specific for genes downstream of *cprA* unveiled transcripts of approximately 2.4 and 5.2 kb for *cprC* and 0.7, 2.4 and 5.2 kb for *cprD*. All transcripts were only observed in RNA obtained from cells grown with Cl-OHPA as the electron acceptor (Fig. 3.4). The presence of a large transcript of about 5.2 kb hybridizing with probes specific for *cprA*, *cprC* and *cprD*, and its concentration relative to that of the major 1.7-kb *cprBA* transcript, indicate occasional read-through after *cprA*. This would imply that the *cprBACD* genes are cotranscribed from the promoter preceding *cprB* (see below). The smaller transcripts (2.4 and 0.7 kb) detected with the *cprC*- and *cprD*-specific probes could be products of posttranscriptional processing of either the large 5.2-kb polycistronic *cprBACD*

mRNA or a 3-kb biscistronic *cprCD* transcript transcribed from a promoter preceding *cprC*. Hybridization with probes specific for *cprE*, *cprZE* and *cprT* indicated a biscistronic transcript of *cprE* and *cprZ* and monocistronic transcription of *cprT* specifically induced under halorespiring conditions (Fig. 3.4). A small 0.5-kb transcript that was detected with the *cprZE*-specific probe in RNA from cells grown on pyruvate, fumarate or nitrate as the electron acceptor, was absent from halorespiring cells, indicating that halorespiration induced read-through between *cprZ* and *cprE*. A transcript of approximately 2.6 kb could be detected with a probe specific for *cprK*, which was constitutively produced at very low levels. The same transcript was also detected with probes specific for *cprE* and *cprZE*, indicating constitutive transcription of a tricistronic *cprKZE* transcript (Fig. 3.4). Transcription of ORFU could not be detected by Northern blot analysis.

To verify the transcriptional organization of the *cpr* gene cluster as proposed from the results of the Northern blot analysis, RT-PCR was performed using primer pairs that were designed to detect (i) transcription of each single gene and (ii) cotranscription of two neighboring genes. The results was in perfect agreement with the Northern blot analysis, i.e., cotranscription of *cprB-cprA*, *cprA-cprC*, *cprC-cprD* and *cprK-cprZ* could be demonstrated, whereas no RT-PCR product was obtained for *cprE-cprB* (data not shown).

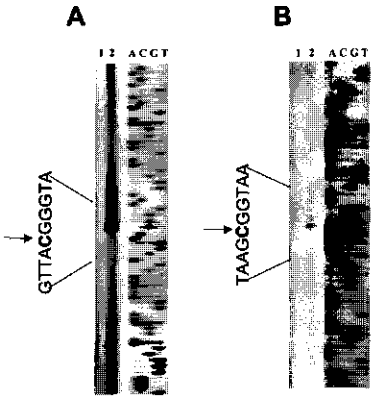

Figure 3.5 Analysis of the transcription initiation sites (arrows) at the *cprB* (**A**) and *cprC* (**B**) promoters by primer extension. Primer extension was performed with RNA isolated from cells grown on pyruvate (lane 1) or pyruvate and Cl-OHPA (lane 2). Primer extension products were electrophoresed in parallel with a sequence ladder (lanes A, C, G, and T) generated with the same primer on the noncoding strands of *cprB* and *cprC*, respectively.

Transcription Initiation from Putative *cprB* and *cprC* Promoters

The results obtained by Northern blot analysis and RT-PCR indicated transcription initiation under halorespiring conditions from a promoter preceding *cprB*. The start site of Cl-OHPA-specific transcription from the *cprB* promoter could be identified 43 nucleotides upstream of the translation start site by primer extension using total RNA extracted from cells of *D. dehalogenans* grown by halorespiration with pyruvate as the electron donor and Cl-OHPA as the electron acceptor (Fig. 3.5A).

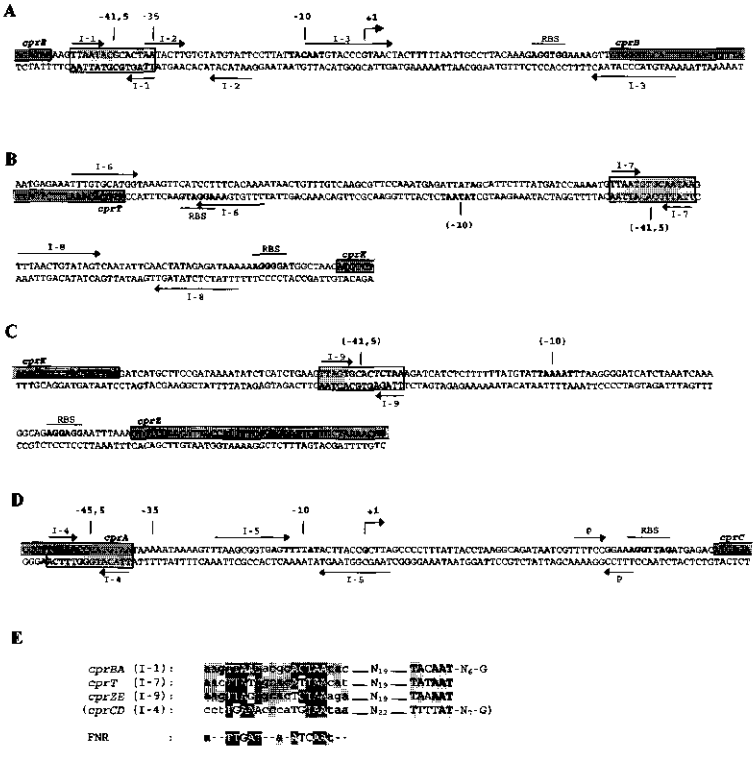

Figure 3.6 Detailed analysis of the *cprE-cprB-* **(A)**, *cprT-cprK-* **(B)**, *cprK-cprZ-* **(C)**, and *cprA-cprC-* **(D)** intergenic regions. Bent arrows (+1), apparent transcription initiation sites; boldface, apparent and putative -10 regions and ribosome binding sites (RBS); horizontal arrows, palindromic sequence (P) and inverted repeats (I-1 to I-9); parentheses, hypothetical elements of putative promoters preceding the *cprT* and *cprZ* genes; dark and light gray boxes, protein-encoding sequences and FNR box-like motifs, respectively. **(E)** Alignment of putative CprK-recognition motifs with the *E.coli* FNR recognition consensus motif (Spiro & Guest, 1990). Conserved residues within the recognition motifs, and at apparent and putative –10 consensus motifs are indicated in gray.

No primer extension product was obtained with RNA isolated from cells grown by pyruvate fermentation. Upstream of the transcription initiation site, the consensus sequence for a –10 region could be detected, but no consensus –35 region was observed (Fig. 3.6A). Northern blot analysis also suggested posttranscriptional processing of a polycistronic mRNA or another transcription initiation at a site preceding *cprC*. A halorespiration-specific primer extension product indicated that this site is localized 58 nucleotides upstream of the translation start of *cprC* (Fig. 3.5B).

Kinetics of Induction of the *cprBA* Operon Expression

To investigate the induction kinetics of *cprBA* expression under halorespiring conditions, cells grown with pyruvate as the sole carbon and energy source were amended with Cl-OHPA during exponential growth, and *cprBA* transcription and dechlorination of Cl-OHPA to OHPA were determined. Normalized by comparison to the 16S rRNA levels, transcription of *cprBA* was already induced 15-fold 30 min after induction, whereas significant amounts of the dechlorination product OHPA (5.8 % of Cl-OHPA added) were detected after 2 h (Fig. 3.7). Considering a specific growth rate of approximately 0.2 h^{-1} (generation time [t_D] ≈ 3 h), the massive induction of halorespiration-specific transcription within 0.15 × t_D is fast. Maximal induction of 18-fold was observed 3 h after addition of Cl-OHPA.

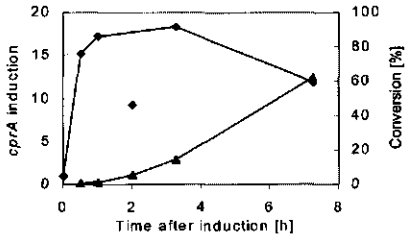

Figure 3.7 *Kinetics of induction of cprBA transcription and dechlorination of Cl-OHPA.* Northern blot analysis was performed with total RNA extracted from *D. dehalogenans* cells grown on pyruvate that were amended with Cl-OHPA during exponential growth. Relative transcription values were obtained after quantification of hybridization signals with ^{32}P-labeled probes that were specific for *cprA* and the 16S rRNA-encoding gene of *D. dehalogenans* (◆). The unusual ratio obtained after 2 h of induction may be due to degradation of the *cprBA* transcript compared to the 16S rRNA. Concentrations of Cl-OHPA and OHPA were determined by reverse-phase high-performance liquid chromatography analysis. The degree of conversion of Cl-OHPA to OHPA was calculated as [OHPA] × 100% / ([Cl-OHPA] + [OHPA]) (▲).

Discussion

Halorespiring bacteria have been demonstrated to be actively involved in the reductive dehalogenation of chlorinated aliphatic and aromatic compounds of natural and anthropogenic origin. Hence they contribute significantly to the detoxification of these contaminants in the environment (Fetzner, 1998). The reductively dehalogenating enzymes from a limited number of halorespiring bacteria have been characterized at the biochemical and genetic levels, indicating similar modes of catalytic action (Holliger *et al.*, 1999). However, insight in the regulatory circuits involved in the induction and repression of the halorespiration process is still very limited. For the first time, we here present the molecular analysis of the control of halorespiration-specific gene expression.

We have cloned and sequenced extended chromosomal fragments up- and downstream of the *ortho*-chlorophenol-reductive dehalogenase-encoding *cprBA* gene cluster in halorespiring *D. dehalogenans*. Previously, we showed that *cprA* codes for a pre-protein containing a twin arginine (RR) signal sequence (van de Pas *et al.*, 1999). This signal peptide is cleaved off in the mature protein and is thought to play a major role in the maturation and translocation of mainly periplasmic proteins binding different redox cofactors by the recently described twin arginine translocation system (Berks *et al.*, 2000). Putative functions of the newly detected open reading frames could in most cases be assigned by similarity to proteins present in the databases. CprC and CprK are potentially involved in regulation of transcription at different levels, whereas CprD, CprE and CprT share significant similarity with molecular chaperones being involved in the correct folding, processing and assembly of proteins. However, no function could be assigned to the predicted gene products of ORFU and *cprZ*.

CprD and CprE belong to the family of GroEL chaperonins, which are found in prokaryotes, chloroplasts and mitochondria. GroEL chaperonins are tetradecameric proteins that are involved in preventing protein aggregation, and facilitating protein folding and assembly (Fink, 1999). In addition, it has been suggested that accessory proteins like GroEL might play a role in correct assembly and cofactor insertion during the maturation of RR signal peptide containing proteins, such as the reductive dehalogenases from halorespiring bacteria (Santini *et al.*, 1998; Berks *et al.*, 2000). CprT has significant similarity to the trigger factor, a prolyl peptidyl isomerase that catalyzes proline *cis-trans* isomerization, a potential rate-limiting step in protein folding (Fink, 1999). Studies on trigger factor from *E.coli* showed its association with nascent polypeptide chains and the 50S ribosome, suggesting a role in

protein folding (Stoller *et al.*, 1995; Valent *et al.*, 1995). Interestingly, complexes between trigger factor and GroEL formed *in vivo* have been reported to have a much higher affinity for partially folded polypeptides than GroEL alone (Kandror *et al.*, 1997). Moreover, overexpression of trigger factor together with GroEL and GroES significantly improved the solubility of recombinant proteins, normally prone to aggregation into inclusion bodies (Nishihara *et al.*, 2000). Unlike known trigger factors, CprT lacks part of the N-terminal domain, which is non-catalytic but which appears to be required for full activity in protein folding (Zarnt *et al.*, 1997). Nonetheless, the specific coordinated expression of *cprD*, *cprE* and *cprT* under halorespiring conditions suggests a synergistic role of these molecular chaperones in the maturation of the dehalogenating complex.

Northern blot analysis and RT-PCR revealed that the transcription of almost all genes identified in the *cpr* gene cluster is induced under halorespiring growth conditions, whereas no or significantly less-abundant transcription was observed under pyruvate-fermenting and fumarate- or nitrate-respiring conditions. We could reveal the transcriptional organization of the locus, i.e. two biscistronic units *cprZE* and *cprBA*, with occasional read through at *cprC*, yielding expression of the polycistronic *cprBACD* genes. Possibly, transcription of a third biscistronic unit, *cprCD*, might be initiated at a promoter preceding *cprC*. *cprT*, encoding a trigger factor, is obviously transcribed into a monocistronic mRNA (Fig. 3.4). Low-level constitutive transcription under all tested conditions was solely observed for tricistronic transcript *cprKZE*. Transcription from the *cprB* promoter was strongly induced within 30 min upon the addition of Cl-OHPA to cells growing by fermentation of pyruvate with concomitant dehalogenation of Cl-OHPA to OHPA, indicating *o*-chlorophenol-reductive dehalogenase activity. This is in agreement with the earlier result that dehalogenation can not be induced in the presence of chloroamphenicol, showing that activation of dehalogenation requires *de novo* protein synthesis (Utkin *et al.*, 1995).

Sequence analysis revealed the presence of a gene *cprK*, constitutively expressed at a low level, encoding a potential transcription regulatory protein. The observed tight control of the expression of the structural and putative accessory *cpr* genes might imply a direct involvement of CprK in the functionality of the *D. dehalogenans* halorespirational system. CprK has significant similarity to FNR- and FixK-like regulators, which are important *trans*-acting factors in regulatory networks of anaerobic assimilation and dissimilation. Like FixK, CprK lacks the N-terminal cysteine cluster, characteristic for FNR, which is involved in binding of an Fe/S center, and as such in redox-sensing (Zumft, 1997). However, CprK does

show an unusually high content of five cysteine residues, among which is the conserved internal cysteine residue Cys^{105}. In the *E.coli* FNR protein, the corresponding Cys^{122} has been shown to be essential for Fe-binding, disulfide bond formation and covalent modification (Green *et al.*, 1993). The FNR- and FixK-like regulatory proteins share a common conserved DNA-binding motif in their C-terminal recognition helices, which is complementary to a palindromic recognition motif, the so-called FNR or anaerobox in the promoter of target genes (TTGAT-N_4-ATCAA) (Spiro & Guest, 1990). In positively regulated promoters, this FNR binding motif is preferentially centered at a distance of 41.5 nucleotides upstream of the transcription start (Spiro & Guest, 1990). Inspection of the mapped halorespiration-inducible *cprB* promoter showed that it lacks the -35 consensus motif of strong constitutive promoters but does contain an anaerobox-like palindromic structure (I-1, TTAAT-N_4-ACTAA). This putative regulatory protein binding motif is centered 41.5 nucleotides upstream of the apparent transcription start, suggesting positive regulation of transcription by an FNR-like factor (Fig. 3.6A). Another interesting feature of the *cprB* promoter is the presence and position of an additional long inverted repeat (I-3) that overlaps with the transcription start site, suggesting a function in control of transcription initiation. Northern blot analysis revealed that expression from putative promoters preceding *cprT* and *cprZ* was also stimulated under halorespiring conditions. FNR box-like motifs centered 87.5 bp and 77.5 bp upstream of *cprT* and *cprZ*, respectively (I-7 and I-9; Figs. 3.6B and C), could be identified. Moreover, in both cases conserved -10 consensus motifs were found at the same distance (19 bp) downstream of the FNR box-like motifs as in the mapped *cprB* promoter (Figs. 3.6B, C and E). Both, the lower degree of conservation of the proposed FNR box-like motif at *cprC* (Figs. 3.6D and E) and the small difference in spacing to the transcription start (45.5 bp instead of 41.5 bp) and a less well conserved –10 motif favor the idea that the mapped start site of the *cprCD* mRNA is the site of processing of the larger tetracistronic unit *cprBACD* rather than of transcription initiation.

In conclusion, it is tempting to speculate that the FNR-homologue CprK is the factor binding to the different anaerobox-like motifs preceding at least the 3 halorespiration-inducible promoters present in the *cpr* gene cluster. An alignment of the identified motifs revealed a consensus sequence for all analyzed promoters that only slightly differs from the FNR box (Fig. 3.6E), which could reflect corresponding differences in the recognition helix of CprK (Fig. 3.3). CprK might be activated by the addition of a halogenated substrate, either directly or via an *o*-chlorophenol-sensor, such as the two component regulatory system that

was previously detected from the detailed analysis of halorespiration-deficient mutants (Smidt *et al.*, 1999). If so, active CprK then induces transcription from the apparent *cprB* promoter and the putative *cprT* and *cprZ* promoters, generating the set of polypeptides required to obtain a functional dehalogenating complex. Such a model suggests a similar regulatory loop as has been proposed recently for nitrite reductase- and nitrous oxide reductase-encoding gene clusters from denitrifying bacteria. There, the expression of both the structural genes, *nirS* and *nosZ*, and of the membrane-bound regulator, encoded by *nirI* and *nosR*, are under the control of FNR-like regulatory proteins NNR and FnrD, respectively (Cuypers *et al.*, 1995; Saunders *et al.*, 1999). However, the exact function and mode of action of the NirI- NosR- and CprC regulatory proteins remain unknown. Interestingly, the analysis of the partially available genome sequence of the halorespiring *Dehalococcoides ethenogenes* has revealed the occasionally close linkage of reductive dehalogenase-encoding genes with *cprC* and *cprK* homologues, as well as with genes, potentially coding for two-component regulatory systems (Preliminary sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org). This might serve as an additional, although only indirect, indication for the involvement of such regulatory proteins in the regulation of expression of reductive dehalogenases.

The molecular analysis of the *cpr* gene cluster reported here, for the first time provides insight in the molecular basis of regulation and maturation of the halorespiratory system and suggests regulatory circuits which are similar to those proposed for respiratory complexes present in denitrifying bacteria. The remarkably fast induction of halorespiration-specific gene expression and its relative insensitivity towards the presence of alternative electron acceptors indicate the potential of *D. dehalogenans* as a dedicated degrader in contaminated environments (Mackiewicz & Wiegel, 1998; Smidt et al., unpublished results).

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4

RANDOM TRANSPOSITION BY TN*916* IN *DESULFITOBACTERIUM DEHALOGENANS* ALLOWS FOR ISOLATION AND CHARACTERIZATION OF HALORESPIRATION-DEFICIENT MUTANTS

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Abstract

To allow for the molecular analysis of halorespiration by the strictly anaerobic Gram-positive bacterium *Desulfitobacterium dehalogenans*, halorespiration-deficient mutants were selected and characterized following insertional mutagenesis by the conjugative transposon Tn*916*. To facilitate rapid screening of transconjugants, a highly efficient method for the growth of single colonies on solidified medium has been developed. A streptomycin-resistant mutant of *D. dehalogenans* was isolated and mated with *Enterococcus faecalis* JH2-2 carrying Tn*916*. Insertion of one or two copies of Tn*916* into the chromosome of *D. dehalogenans* was observed. From a total of 2500 transconjugants, 24 halorespiration-deficient mutants were selected upon their inability to use 4-chloro-3-hydroxyphenylacetic acid as electron acceptor. Physiological characterization led to the definition of three phenotypic classes of mutants that differed in their ability to use the additional terminal electron acceptors nitrate and fumarate. The activities of hydrogenase and formate dehydrogenase were determined, and the transposon insertion sites in selected mutants representing the different classes were analyzed on the sequence level following amplification by inverse PCR. The results of the molecular characterization as well as the pleiotropic phenotype of most mutants indicate that genes coding for common elements shared by the different respiratory chains present in the versatile *D. dehalogenans* have been disrupted.

Introduction

Halogenated hydrocarbons are present in the environment in high quantities due to their past and present application in industry and agriculture, e.g. as solvents, pesticides and preservatives, and to natural production, compromising environmental integrity and health (Gribble, 1996. The biodegradability of these compounds under different environmental conditions has been studied extensively (Fetzner, 1998). In particular, higher halogenated hydrocarbons can often be degraded solely under anaerobic conditions by reductive dehalogenation. In contrast to aerobic degradation processes, however, only limited knowledge exists on reductive dehalogenation at the molecular level (Mohn & Tiedje, 1992). Recently, a rapidly expanding group of anaerobic bacteria has been isolated that are able to couple the reductive dehalogenation to energy conservation and hence to growth (El Fantroussi *et al.*, 1998; Holliger *et al.*, 1999). These bacteria can use chloroalkenes, e.g. tetra- and trichloroethene, or chloroaromatic compounds, such as chlorophenols or 3-chlorobenzoate, as the terminal electron acceptor. Among these, the Gram-positive genus *Desulfitobacterium* comprises a major group of isolates, including *D. dehalogenans*, which is able to couple the reductive dechlorination of different *ortho*-chlorinated phenolic compounds to growth with lactate, pyruvate, formate or hydrogen as the electron donor. In addition, pyruvate is also used for fermentative growth (Utkin *et al.*, 1994).

Isolation of these strains and their expected potential for application in *in situ* biodegradation of haloorganic pollutants also led to an increased interest in the molecular bases of this novel anaerobic respiratory pathway. To date, efforts have mainly focused on the reductive dehalogenase as the key enzyme in halorespiration. The inducible *ortho*-chlorophenol reductive dehalogenase was purified from *D. dehalogenans* and characterized at the biochemical and genetic level (van de Pas *et al.*, 1999). A comparison with other chlorophenol- and tetrachloroethene-reductive dehalogenases shows that all enzymes are either membrane-bound or membrane-associated, contain Fe-S clusters and, with one exception, a corrinoid as redox centra (for a recent review, see Holliger *et al.*, 1999). The presence of two iron-sulfur clusters as determined by electron paramagnetic resonance analysis was confirmed by the identification of one ferredoxin-like motif and one truncated iron-sulfur cluster binding motif in the deduced primary sequence. Similar results were obtained for the tetrachloroethene-reductive dehalogenase of the Gram-negative

Dehalospirillum multivorans (Neumann *et al.*, 1998). Furthermore, both reductive dehalogenases share a twin arginine (RR) leader sequence, which is cleaved off in the mature proteins. These RR – leader sequences are thought to play a major role in the maturation and translocation of mainly periplasmic proteins binding different redox cofactors (Berks, 1996).

In order to enable a molecular characterization of additional components involved in halorespiratory electron transport, as well as in folding, targeting and regulation of the reductive dehalogenase in *D. dehalogenans*, we tested the use of the conjugative transposon Tn*916* for the isolation of halorespiration-deficient (HRD) mutants. Tn*916* was the first conjugative transposon to be identified, and members of the Tn*916*-Tn*1545* family have been found in or introduced into more than 50 different Gram-positive and -negative species (Clewell *et al.*, 1995).

We report here the development of an efficient conjugation system for the integration of Tn*916* in the chromosome of the newly described halorespiring *D. dehalogenans*. This allowed for the isolation of HRD mutants that were characterized by analyzing their respiratory and biochemical properties as well as by sequence analysis of the insertion sites.

Table 4.1 Bacterial strains used in this study.

Strain	Relevant characteristics[a]	Reference or source
Enterococcus faecalis JH2-2	Rif^r, Fus^r, Tn*916* (Tet^r)	(Jacob & Hobbs, 1974)
Escherichia coli XL-1 Blue	*recA1, endA1, gyrA96, thi hsdR17, supE44, relA1*, [F'.*lacI*^{q}ZM15, Tn*10* (Tet^r)]	Stratagene
Desulfitobacterium dehalogenans		
JW/IU-DC1 (DSM 9161)	wild-type strain	DSM[b]
HSS1	Str^r	This study
HRD0	Str^r, Tn*916* (Tet^r)	This study
HRD2 (Class I)	Str^r, Tn*916* (Tet^r), HRD	This study
HRD1, 3-5, 7-24 (Class II)	Str^r, Tn*916* (Tet^r), HRD, Nar$^-$	This study
HRD6 (Class III)	Str^r, Tn*916* (Tet^r), HRD, Nar$^-$, Ffr$^-$	This study

[a] Rif^r, rifampicin resistant; Fus^r, fusidic acid resistant; Tet^r, tetracycline resistant; Str^r, streptomycin resistant; HRD, no growth with Cl-OHPA plus formate or lactate; Nar$^-$, no growth with nitrate plus formate or lactate; Ffr$^-$, no growth with fumarate plus formate. [b] DSM, Deutsche Sammlung von Mikroorganismen.

Materials and Methods

Materials

4-chloro-3-hydroxyphenylacetic acid (Cl-OHPA) was purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands) and filtered prior to use. All gasses were obtained from Hoek Loos (Schiedam, The Netherlands). When appropriate, experiments were carried out in an anaerobic glove box (Coy Laboratories Products, Grass Lake, Mich.) under an atmosphere of 96% N_2 and 4% H_2. The oxygen concentration was kept low with the palladium catalyst RO-20 provided by BASF (Arnhem, The Netherlands).

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains used in this study are listed in Table 4.1. Strains of *Escherichia coli* were grown in Luria Bertani medium at 37°C (Sambrook *et al.*, 1989). *Enterococcus faecalis* was cultivated at 30°C in M17 broth (Oxoid, Basingstoke, United Kingdom), supplemented with 0.5 g of glucose/liter. Strains of *D. dehalogenans* were routinely grown anaerobically (100% N_2 gas phase) at 37°C in a phosphate-buffered medium (pH 7.5) with a low chloride concentration. The basal medium contained (in grams per liter demineralized water): $Na_2HPO_4 \cdot 2H_2O$, 3.56; K_2HPO_4, 3.31; KH_2PO_4, 0.87; $(NH_4)_2HPO_4$, 0.61; $MgCl_2 \cdot 6H_2O$, 0.22; $CaCl_2 \cdot 2\ H_2O$, 0.03; resazurin, 0.0005. Prior to inoculation, the basal medium was supplemented with 0.04 g of Na_2S/liter, 0.04 g of cysteine-HCl/liter, 1g of yeast extract/liter, and trace elements and vitamin solution as recommended by the German Collection of Microorganisms. The electron donor and acceptor were added to the desired concentrations. If appropriate, media were amended with ampicillin (100 μg/ml), streptomycin (2,000 μg/ml) and tetracycline (5 μg/ml). For the long term storage of cultures of *D. dehalogenans* at -80°C, prior to freezing them, glycerol was added to a final concentration of 20 % from an anoxic stock solution inside the glove box.

The cloning vectors pUC18 and pUC19 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden) and the PCR product cloning vectors pGEM-T and pMON38201 were obtained from Promega (Madison, Wis.) and Monsanto (St. Louis, Mo.) respectively.

Plating of *D. dehalogenans*

Basal medium was autoclaved in serum bottles in the presence of agar. After addition of vitamins, trace elements, 0.1 g of yeast extract/liter, 20 mM electron donor, and -acceptor and,

if necessary the appropriate antibiotics, the medium was reduced with 0.04 g of both Na_2S and cysteine-HCl/liter. Plates were poured in the glove box and stored there for 1 day. Cells from the exponential growth phase were diluted appropriately into reduced basal medium and distributed on the solidified medium in aliquots of 100 µl with sterile glass beads. The plates were incubated at 37°C upside down under N_2 in a gas-tight jar. Application of 0.8% BBL agar purified (Becton Dickinson, Meylan, France) resulted in an average efficiency of plating (EOP, equal to CFU / number of cells counted) of 0.85 ± 0.14, compared to 0.15 ± 0.05 in the case of agar of normal purity (Difco Laboratories, Detroit, Mich.). An agar concentration of 1% or higher resulted in a 2.7-fold decrease in efficiency. For all subsequent experiments, 0.8% of BBL agar purified was routinely used for the plating of *D. dehalogenans*.

For further subcultivation, colonies were picked inside the glove box, resuspended in 0.5 ml of reduced basal medium, and transferred to 120-ml serum bottles with 20 ml anaerobic medium, containing the appropriate substrates and antibiotics.

Isolation of *D. dehalogenans* HSS1

Spontaneous streptomycin-resistant mutants of *D. dehalogenans* were isolated, following growth in liquid culture in the presence of 20 mM pyruvate and 10 µg of streptomycin/ml. In subsequent cultures, the concentration of streptomycin was raised stepwise to 150 µg/ml. Strain *D. dehalogenans* HSS1 was finally isolated from a single colony grown on a plate containing 20 mM lactate, 20 mM Cl-OHPA, and 200 µg of streptomycin/ml.

Filter Mating, Selection of Transconjugants, and Screening for Halorespiration Deficiency

For the conjugation experiments, exponentially growing cultures of *E. faecalis* JH 2-2 and *D. dehalogenans* HSS1 were diluted into fresh medium containing the appropriate antibiotics and grown to an A_{660} of 0.4 to 0.5 and 0.2 to 0.3, respectively. *E. faecalis* JH 2-2 was cultivated at 30 or 37°C under either aerobic or anaerobic conditions (optimized protocol, 37°C and anaerobic). Subsequently, the cultures were transferred to a glove box, washed twice and resuspended in reduced basal medium. Donor and recipient cultures were mixed in different ratios and filtered on a 0.45 µm HA - filter (Millipore, Bedford, Mass.). The membrane was placed with the bacterium side up on an agar plate containing 20 mM pyruvate, and incubated at 30 or 37°C in an anaerobic jar under N_2 for 6 to 16 h (optimized protocol, 37°C for 16 h). The cells were resuspended from the membrane in reduced basal

medium, and transconjugants were selected on plates containing 20 mM pyruvate, 2000 μg of streptomycin/ml and 5 μg of tetracycline/ml. To determine the influence of the mating procedure on the viability of *D. dehalogenans* HSS1, plates without tetracycline were inoculated in parallel. Screening for HRD mutants of *D. dehalogenans* HSS1 was performed by streaking colonies of transconjugants on plates containing 2000 μg of streptomycin/ml, 5 μg of tetracycline/ml, and either 20 mM pyruvate or 20 mM lactate and 20 mM Cl-OHPA.

DNA Isolation and Manipulation

Chromosomal DNA of *D. dehalogenans* was isolated as described previously (van de Pas *et al.*, 1999).

Plasmid DNA was isolated from *E. coli* by using the alkaline lysis method, and standard DNA manipulations were performed according to established procedures (Sambrook *et al.*, 1989) and manufacturers instructions. Enzymes were purchased from Life Technologies B.V. (Breda, The Netherlands), Boehringer Mannheim GmbH (Mannheim, Germany) or New England Biolabs (Beverly, Mass.). Oligonucleotides were obtained from Life Technologies B.V.. Hybond-N+ nylon transfer membrane (Amersham Life Science, Little Chalfont, United Kingdom) was used for Southern blot analysis. Probes for hybridization experiments were labeled by nick translation in the presence of [α-^{32}P] dATP (Amersham Pharmacia Biotech). As specific probe for the tetracycline resistance determinant *tetM*, a 4.2-kb *Hin*cII restriction fragment of the Tn*919 tetM*- gene from pCI182 was used (Hill *et al.*, 1988).

Characterization of Tn*916* Insertion Sites

Chromosomal fragments flanking the sites of Tn*916* insertion were amplified by inverse PCR (Triglia *et al.*, 1988). The divergent primer pairs BG 285-BG 287 (BG 285, 5' GAC CTT GAT AAA GTG TGA TAA GTC C 3', nucleotides (nt) 62 to 38; BG 287, 5' GGA GTT TTA GCT CAT GTT GAT GC 3', nt 12141 to 12163) and BG 286-BG 288 (BG 286, 5' CTC GAA AGC ACA TAG AAT AAG GC 3', nt 17956 to 17978; BG 288, 5' CCA CGC TTC CTA ATT CTG TAA TCG 3', nt 12231 to 12208) were used to specifically amplify the upstream and downstream flanking fragments, respectively. The sequence of the primers was based on the nucleotide sequence of Tn*916* (GenBank accession no. U09422) (Flannagan *et al.*, 1994). Chromosomal DNA was digested with *Hin*dIII and ligated at a concentration of 0.5 ng/μl. Five nanograms of self-ligated DNA was used as the template in a 25-μl PCR reaction mixture containing 2 ng of each primer, 2 mM $MgCl_2$, 200 μM of dATP, dCTP, dGTP, and

dTTP; and 1 U of Expand Long Template enzyme mixture (Boehringer Mannheim GmbH). The DNA was amplified with the GeneAmp PCR System 2400 (Perkin Elmer Cetus, Norwalk, Conn.). After the mixture was preheated to 94°C for 2 min, 35 amplification cycles were performed, consisting of denaturation at 94°C for 20 s, primer annealing at 55°C for 30 s and elongation at 68°C for 3 min. From cycle 6 onwards, the elongation was extended with 20 s per cycle to increase the yield. A final extension of 7 min at 68°C was performed. The PCR products were purified from agarose gel by Gene Clean (Bio 101, La Jolla, Calif.) and cloned into *E. coli* using pGEM-T or pMON38201.

For the further elucidation of the *D. dehalogenans hyd* locus identified in HRD mutant 6 (HRD6), the divergent primer pair BG 345-BG 346 (BG 345, 5'-CCA TTC GAT ACC ATG AGA CC-3', nt 1235 to 1254; BG 346, 5'-GTA CTA ATG ATT CGA TAC TGG G-3', nt 1222 to 1201) was used for inverse PCR from *Pst*I-digested chromosomal DNA of the parental strain. The reaction conditions were as described above, and the PCR product was cloned and sequenced. For additional information on the *hyf-hyc* locus (HRD4), a 2.5-kb *Eco*RI – *Bam*HI fragment was isolated from a *D. dehalogenans* genomic library, cloned, and sequenced.

DNA Sequencing

DNA sequencing was performed using a Li-Cor (Lincoln, Nebr.) DNA sequencer 4000L. Plasmid DNA used for sequencing reactions was purified with the QIAprep Spin Miniprep kit (Qiagen GmbH, Hilden, Germany). Reactions were performed using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham Pharmacia Biotech). Infrared-labeled universal sequencing primers were purchased from MWG Biotech (Ebersberg, Germany). Sequence similarity searches and alignments were performed with the BLAST 2.0 program (Altschul *et al.*, 1997) (National Center for Biotechnology Information, Bethesda, Md.) and the DNAstar package (DNASTAR Inc., Madison, Wis.), respectively.

Enzyme and Protein Assays

Harvesting of cells and preparation of cell extracts by sonication under anoxic conditions were performed as described previously (van de Pas *et al.*, 1999). Enzyme activities in cell extracts were determined spectrophotometrically at 30°C in rubber stoppered N_2-flushed cuvettes by following the oxidation or reduction of benzylviologen or methylviologen at 578 nm (ε_{578} = 9.2 $mM^{-1} \times cm^{-1}$ or 9.7 $mM^{-1} \times cm^{-1}$, respectively). Formate dehydrogenase and

hydrogenase were assayed in 1 ml 100 mM Tris-HCl (pH 8.0) containing 1 mM benzylviologen and 10 mM formate or 1 ml H_2, respectively. Fumarate reductase activity was determined in 1 ml 100 mM sodium phosphate buffer (pH 7.5) containing 5 mM titanium(III)citrate-reduced benzylviologen and 10 mM fumarate. For all assays, one U of enzyme activity corresponds to the amount of enzyme catalyzing the conversion of 1 µmol substrate or 2 µmol of benzyl- or methylviologen per minute. For the semiquantitative determination of formate-H_2 lyase activity, 200 µl cell extract was added to 1.8 ml of 100 mM Tris-HCl (pH 7.8) buffer containing 10 mM formate and incubated at 30°C. The formation of hydrogen was followed by gas chromatography. Protein was determined according to the method of Bradford, with bovine serum albumin as the standard (Bradford, 1976).

Nucleotide Sequences

The nucleotide sequences of the sites of Tn*916* insertion in the different mutants have been deposited in the GenBank database under GenBank Accession Numbers AF157637 (HRD2 upstream), AF157638 (HRD2 downstream), AF157642 (HRD22), AF176224 (HRD4 - 1), AF157639 (HRD4 - 2 upstream), AF157640 (HRD4 – 2 downstream) and AF157641 (HRD6).

Results

Development of a Tn*916*-Based Transposition System for *D. dehalogenans*

To allow for the genetic analysis of halorespiration by *D. dehalogenans*, a transposition system was designed and optimized based on the broad host-range conjugative transposon Tn*916* (Clewell *et al.*, 1995). Previously, it had been reported that *D. dehalogenans* showed a poor EOP on agar plates (Utkin *et al.*, 1994). However, application of strict anaerobic conditions, an optimized agar concentration (0.8 %), and an appropriate source of agar (BBL agar purified), resulted in a highly efficient plating system (EOP, 0.85 ± 0.14). On plates containing 20 mM pyruvate, colonies that were translucent, white and spherical with a diameter of 2 - 3 mm appeared after 3 days of incubation. Similar results were obtained with 20 mM of pyruvate or lactate as the electron donor and nitrate, fumarate, or Cl-OHPA as the electron acceptor (data not shown).

Table 4.2 Conjugation frequencies for the mating of *E. faecalis* JH2-2 and *D. dehalogenans* HSS1.

Preincubation *E.faecalis*	Mating conditions	Donor / recipient ratio	conjugation frequency (10^{-7})[a]
aerobic, 37°C	37°C, 16 h	0.5	1.1
aerobic, 30°C	30°C, 6 h	10.0	4.0
aerobic, 37°C	37°C, 6 h	6.0	6.7
anaerobic, 37°C	37°C, 16 h	4.0	30.0

[a] Conjugation frequencies were calculated as number of transconjugants per recipient.

To enable counterselection following conjugation, a streptomycin-resistant mutant, strain HSS1, was isolated, which showed an EOP of 0.85 ± 0.1 on plates containing up to 4000 µg of streptomycin/ml, while for the parental strain, *D. dehalogenans* DSM 9161, the EOP was below 10^{-8} on plates containing 50 µg of streptomycin/ml. No resistance to other antibiotics (tetracycline, chloroamphenicol or rifampicin) was observed in strain HSS1 (data not shown).

The conjugative transposon Tn*916* was introduced into *D. dehalogenans* strain HSS1 by filter mating with the streptomycin-sensitive *E. faecalis* JH2-2, which carries a single copy of Tn*916* on its chromosome and is widely used as Tn*916* donor in matings of Gram-positive bacteria (Clewell *et al.*, 1995; de Vos *et al.*, 1997). After 5 days of incubation on plates containing streptomycin and tetracycline, transconjugant colonies of *D. dehalogenans* developed. Subsequently, conjugal transfer was optimized by varying growth of the donor, mating conditions and the donor / recipient ratio (Table 4.2). The highest conjugation frequencies were observed when the *E. faecalis* donor was grown at 37°C in the absence of oxygen and mated in a four fold excess over the recipient *D. dehalogenans* HSS1 for 16 h at 37°C, resulting in 3×10^{-6} transconjugants per recipient.

The insertion of Tn*916* in the chromosome of *D. dehalogenans* HSS1 was confirmed by Southern blot analysis with a *tetM*-specific probe and revealed many single copy insertions, occasional insertion of two copies and an apparently random distribution (data not shown) (see below).

HRD Mutants and their Phenotypic Classification

A total of 2500 tetracycline-resistant transconjugants obtained from several independent matings were analyzed for halorespiration deficiency. Transconjugants, which showed growth

on pyruvate but not on lactate - Cl-OHPA within 7 days, were considered HRD. After rescreening, a total of 24 HRD mutants was isolated and subsequently subjected to a series of 20-ml batch incubations in the presence of 20 mM lactate or formate as the electron donor and fumarate, nitrate, or Cl-OHPA as the electron acceptor. As a control, fermentative growth with 20 mM pyruvate was followed. Furthermore, a transconjugant, which had not lost the ability to grow upon halorespiration (HRD0), was used as a control strain. Deficiency in using a certain combination of electron donor and acceptor was defined by a lag phase increased more than two-fold in growth compared to strain HRD0. This criterion was chosen because continuous incubation in the medium resulted in the growth of tetracycline-resistant revertants in which the transposon probably had moved from the original site of integration to another site. This was confirmed by Southern analysis with a *tetM*-specific probe, as the specific hybridizing signals progressively disappeared with successive cultivation under selective conditions (results not shown). Based on the results, the strains could be grouped into 3 major classes, which differed in their capacities to utilize additional electron acceptors (Table 4.1). The first class contained only one mutant, HRD2, that was found to be solely deficient in halorespiration with lactate or formate as the electron donor. Class II was the major group, comprising 22 HRD mutants, which had lost the ability to grow by both halorespiration and nitrate respiration with lactate or formate as the electron donor. The third phenotypic group again included only a single mutant, strain HRD6, which was not only impaired in the use of Cl-OHPA and nitrate, but also fumarate as electron acceptor. The pleiotropic phenotype of most mutants indicates the disruption of genes that encode common elements shared by the different respiratory chains present in this organism.

Genetic Characterization of HRD Mutants

Chromosomal DNA was isolated from HRD transconjugants and digested with *Hin*dIII. Tn*916* contains one single *Hin*dIII site, yielding left end right arms of 12.2 and 5.8 kb, respectively (Flannagan *et al.*, 1994). The fragments were separated by agarose gel electrophoresis and transferred to membranes that were hybridized to a 4.2-kb *Hin*cII restriction fragment of the Tn*919 tetM* gene (Fig. 4.1). While no DNA fragments from the recipient *D. dehalogenans* strain HSS1 hybridized with the *tetM* probe, most of the mutant strains exhibited 2 hybridizing fragments, indicating chromosomal integration of a single copy of Tn*916*. Only HRD1, -3 and -4 showed more than 2 hybridizing bands, suggesting multiple transposon insertions. The difference in size of the hybridizing fragments in the

mutants indicates that the transposon inserted in an apparently random manner into the chromosome of *D. dehalogenans*. An additional 18-kb hybridizing fragment was found with different intensities in all mutants, suggesting the presence of a circular intermediate of Tn*916* (Gawron-Burke & Clewell, 1984).

Characterization of the Tn*916* Insertion Sites in Different HRD Mutants

Inverse PCR was used to specifically amplify the Tn*916* - flanking fragments from the chromosomal DNA of representative HRD mutants. The identity of relevant PCR products was verified by Southern blot analysis of *Hin*dIII-digested chromosomal DNA isolated from the different mutants. Fragments hybridizing with the different PCR products also hybridized strongly with a radiolabelled *tetM* probe, confirming that the primers used specifically yielded the amplification of Tn*916* - flanking fragments (data not shown).

Characterization of the Tn*916* insertion sites indicated that 4 of the 24 HRD mutants isolated carried the transposon inserted at the same site but in different orientations (HRD5 - HRD24 and HRD9 - HRD10). Interestingly, all these mutants arose from independent mating experiments. Similarly, Tn*916* insertion sites were identical for HRD3, HRD20, and HRD22, with HRD20 carrying the transposon in the inverse orientation compared to those in HRD3 and HRD22.

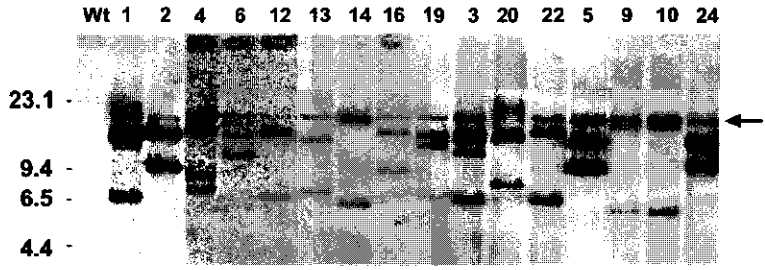

Figure 4.1 Hybridization of *Hin*dIII-digested chromosomal DNA from wild-type (wt) and tetracycline-resistant HRD mutants of *D. dehalogenans* with a probe for *tetM* (obtained from pCI182) (Hill *et al.*, 1988). The autoradiograph was digitally corrected for differences in DNA concentration. Lanes: 1, strain HSS1; 2 to 17, mutant strains HRD1, -2, -4, -6, -12, -13, -14, -16, -19, -3, -20, -22, -5, -9, -10 and -24, respectively. DNA size markers are in kilobase pairs. Fragments smaller than 4 kb were not found to hybridize with the *tetM* probe. The arrow indicates the possible 18-kb circular intermediate of Tn*916*.

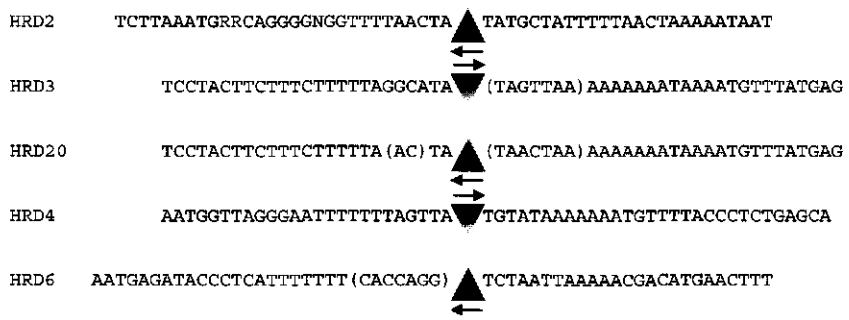

Figure 4.2 Nucleotide sequences of the Tn*916* insertion sites in different HRD mutants of *D. dehalogenans*. The triangles indicate the site and orientation of Tn*916* insertion (nt 1 to 18032). The bases given in parentheses for HRD3, -6 and -20 were not present in the wild-type sequence.

Nucleotide sequence analysis of the flanking chromosomal regions showed that the target sites are generally AT rich (Fig. 4.2). In two cases, the wild-type sequence of the insertion site was determined, revealing the presence of additional nucleotides after the insertion event, as shown for HRD3, HRD20, and HRD6. These short so-called coupling sequences are a common feature of Tn*916* insertion sites and are a result of the integration event (Clewell *et al.*, 1995). The insertions were located in the vicinity (i.e. within potential promoter and terminator sequences) of open reading frames that encode regulatory proteins or enzymes of anaerobic respiratory pathways present in *D. dehalogenans* (Fig. 4.3).

Four representatives of class I, II, and III mutants were analyzed in detail. In the class I mutant HRD2, Tn*916* had inserted downstream of *hrd2-1*, which shows limited similarity (20 % identity at the amino acid level) with *cheX*, encoding a chemotaxis - related protein in different strains of *Treponema*. It is located upstream of *hkhA*, revealing significant similarity (22 % identity at the protein level) with a gene coding for a sensory transduction histidine kinase in *Methanobacterium thermoautotrophicum* (GenPept accession no. AAB84866).

In the class II mutant HRD22, the transposon inserted in the *hrd22-1* gene, which could encode a 30-kDa protein of unknown function. This gene is followed by *hkhB*, which may encode a member of the two-component sensor histidine kinases (28 % identity at the protein level with YwpD from *Bacillus subtilis*; GenPept accession no. CAB05945). One kilobase downstream of the transposon insertion site in HRD22 is the *hemN* gene, which shows strong similarity with genes coding for oxygen-independent coproporphyrinogen III oxidases that are involved in porphyrin biosynthesis (Fig. 4.3).

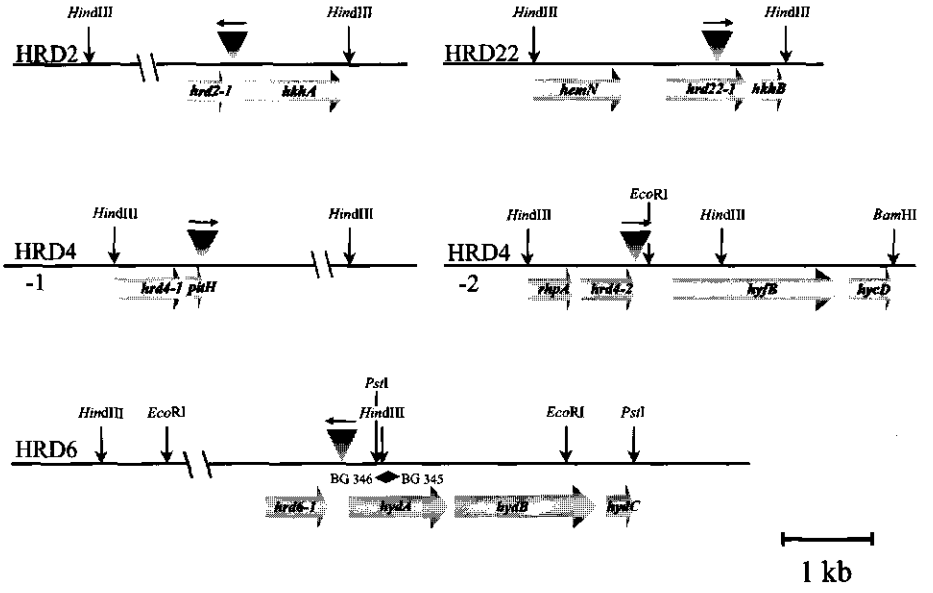

Figure 4.3 Physical map of the Tn*916* insertion sites in different class I, II and III HRD mutants of *D. dehalogenans*. Triangles indicate the sites and orientations of transposon integration. The horizontal arrows show identified open reading frames. Restriction sites and oligonucleotides are indicated by vertical arrows and by arrowheads, respectively.

The class II mutant HRD4 contains two Tn*916* insertions (Fig 4.3). One copy of the transposon inserted in the *pitH* gene showing significant similarity with genes encoding low-affinity inorganic phosphate transporters (60% identity at the protein level with YkaB from *B. subtilis*, GenPept accession no. CAB13141). A second copy of Tn*916* had inserted downstream of *hrd4-2*, coding for a hypothetical protein, and *rhpA*, exhibiting significant similarity with genes encoding putative regulatory proteins (44% identity at the protein level with the hypothetical protein YwhH from *B. subtilis*, GenPept accession no. CAB15775; and 25% with EbsC from *E. faecalis*, GenPept accession no. AAC36853). The putative proteins encoded by the two genes identified downstream of the insertion, *hyfB* and *hycD*, show the highest similarity to the gene products of *hyfB* (31 % identity) and *hycD* (25 % identity), respectively, which are integral membrane subunits of two formate-hydrogen lyase complexes present in *E. coli*, and to the proteins encoded by genes from an unidentified *Mycobacterium tuberculosis* operon (Andrews *et al.*, 1997; GenBank accession no. Z74410).

In the class III mutant strain HRD6, Tn*916* had inserted downstream of an open reading frame *hrd6-1*, encoding a protein of unknown function, and in the presumed promoter-region 200 bp upstream of a potential hydrogenase-encoding gene cluster (Fig. 4.3). The predicted gene products of *hydA*, *hydB* and *hydC* show significant homology with the small, large and B-type cytochrome subunits of membrane bound periplasmic quinone-reactive hydrogenases of different bacteria.

Biochemical Characterization of HRD Mutants

The results of both the physiological and the genetic characterization of the isolated HRD mutants indicated the disruption of either structural or regulatory components shared by different respiratory chains present in *D. dehalogenans*. Key respiratory enzyme activities were determined for the class II mutants HRD4 and HRD22, as well as for HRD6 (class III), and compared to those of a transconjugant strain showing wild-type phenotype with respect to its halorespiring ability (HRD0). The class I mutant HRD2 revealed a rather high degree of instability, making a more detailed biochemical characterization impossible. Formate dehydrogenase, hydrogenase, and fumarate reductase activities were determined in cell extracts prepared from cells grown by fermentation on 20 mM pyruvate. These experiments indicated that the class II mutant strain HRD4 had strongly reduced activity in both formate dehydrogenase (50 U/mg) and hydrogenase activity (60 U/mg) in contrast to the fumarate activity (1910 U/mg), which was even higher than that found in the wild-type strain (520, 640, and 720 U/mg in HRD0 for the above mentioned enzymes, respectively). Semiquantitative determination of H_2 formation upon formate addition indicated no significant differences in formate-H_2 lyase activity in HRD0 and HRD4. Similar results were obtained for the class III mutant, HRD6. Remarkably, the class II mutant HRD22 was affected only in formate dehydrogenase activity (40 U/mg). The results obtained from the activity measurements could be qualitatively confirmed by hydrogenase and formate dehydrogenase activity staining experiments. After polyacrylamide gel electrophoresis of cell extracts under non-denaturing conditions, the experiments were performed with benzylviologen as the electron acceptor and showed single bands for each enzyme although some material did not enter the gel (data not shown).

Discussion

Halorespiration is a recently discovered mode of anaerobic respiration carried out by a rather wide range of Gram-positive and -negative bacteria. Nevertheless, our current knowledge on the architecture, bioenergetics and control of this respiratory pathway at the molecular level is very limited and is restricted to the molecular characterization of reductive dehalogenases (Neumann *et al.*, 1998; van de Pas *et al.*, 1999). Here we describe the development of a plating, delivery and screening system that allowed integration of the conjugative transposon Tn*916* in the chromosome of the newly described *o*-chlorophenol respiring Gram-positive bacterium *D. dehalogenans*. Subsequently, HRD mutants that were genetically and physiologically characterized and found to be deficient in the biosynthesis of one or more of the components of the halorespiratory electron transfer chain were isolated.

Using *E. faecalis* JH2-2 as a donor in anaerobic filter matings, we were able to accomplish Tn*916* integration into the chromosome of genetically marked *Desulfitobacterium dehalogenans* HSS1 with a frequency of 3×10^{-6} transconjugants per recipient (0.8×10^{-6} per donor). This transposition frequency is within the range commonly observed for Tn*916*, which varies from $<10^{-9}$ to $>10^{-4}$ per donor (Clewell *et al.*, 1995). Based on their inability to use Cl-OHPA as electron acceptor, we isolated a total of 24 HRD mutants. Based on subsequent physiological characterization, the mutants could be grouped in three major phenotypic classes. Only one mutant was deficient solely in halorespiration (class I). The rest of the mutants (22 class II- and 1 class III mutant) were all impaired in both halorespiration and nitrate respiration. This suggests that the nitrate respiratory and halorespiratory chains share common components, the function of which has been affected by the insertion of the transposon.

Southern blot analysis revealed that Tn*916* integrated in single copy, but occasionally two copies, into the chromosome of *D. dehalogenans*. Furthermore, the presence of a large 18-kb Tn*916* – specific band suggests there is a circular intermediate of Tn*916* in virtually all mutants, indicating that the transposon is still mobile in *D. dehalogenans* (Fig. 4.1) (Gawron-Burke & Clewell, 1984). Both the occurrence of multiple insertions and the remaining mobility of the transposon in transconjugants of *D. dehalogenans* might explain the relative instability of the mutants, which was most pronounced in the single class I mutant HRD2.

Table 4.3 Characterization and putative function of selected HRD mutants

Clone	Class	additional phenotype[a]	Putative disruption	Putative function
HRD2	I		upstream *hkhA* (regulator)	regulation, Cl-OHPA sensing
HRD22	II	Nar$^-$	downstream *hemN* (maturation), upstream *hkhB* (regulator)	regulation or maturation
HRD4	II	Nar$^-$	upstream *hyf* - *hyc*, downstream *rhpA* (regulator)	formate-hydrogen lyase (respiration)
HRD6	III	Nar$^-$, Ffr$^-$	upstream *hydABC*	Uptake hydrogenase (respiration)

[a] All HRD mutants are deficient in growth with Cl-OHPA plus formate or lactate; Nar$^-$, no growth with nitrate plus formate or lactate; Ffr$^-$, no growth with fumarate plus formate.

The repeated isolation of identical integrants from independent conjugation experiments suggests saturation mutagenesis. Another possible explanation would be the preferential integration of Tn*916* at specific sites, i.e. the occurrence of hot spots of mutation. Sequence analysis revealed that the insertion sites are very AT rich, ranging from 75 to 91 %, compared to an average AT content of the *D. dehalogenans* genome of 55% (Utkin *et al.*, 1994). In most cases, insertions were situated in the immediate vicinity of potential promoter and terminator sequences. This is a frequently observed feature of transposons belonging to the Tn*916* – Tn*1545* family and it was proposed that the special conformation of DNA carrying those sequences, like strong bending or supercoiling, could possibly be recognized by the integrase (Scott *et al.*, 1994; Renault *et al.*, 1995).

The results of the genetic characterization of the Tn*916* insertion site in HRD2 indicate that regulatory functions might be impaired in this class I mutant (Table 4.3). Considering that halorespiration is induced in *D. dehalogenans* by the presence of different *o*-chlorophenols, it is tempting to speculate that the *hkhA* gene product may be the *o*-chlorophenol sensing part of a two component regulatory system (Utkin *et al.*, 1995). Unfortunately, high instability made a more detailed physiological and biochemical investigation of this interesting mutant impossible.

In the class II mutant HRD22, the site of Tn*916* integration was located 0.5 kb upstream of a second putative sensor histidine kinase-encoding gene, *hkhB*, and downstream of *hemN*, a gene encoding a protein involved in porphyrin biosynthesis. This suggests that regulatory

functions involved in maturation of redox complexes might be disrupted in this mutant (Table 4.3).

The results from both molecular and biochemical analysis of different class II and III HRD mutants indicate an involvement of respiratory complexes in halorespiration in *D. dehalogenans* (Table 4.3). In HRD6, Tn*916* inserted upstream of a hydrogenase-encoding operon. HRD4 carried two copies of the transposon, of which one had inserted in a gene encoding a putative phosphate transporter. However, the second copy was found upstream of genes encoding subunits of a putative formate-H_2 lyase, suggesting that this insertion is responsible for the observed respiration-deficient phenotype. As HRD6 can not utilize any of the tested electron acceptors, the presumed *hyd* gene product seems to play a central role in the different respiratory chains. The hydrogenase might play an essential role in electron transfer to the terminal reductases. For a long time, it has been assumed that formate-H_2 lyase (Hyc-complex) complex is only involved in fermentation in the extensively studied facultative anaerobe *E. coli* (Sawers, 1994). However, it was recently proposed that the formate-H_2 lyase, which is likely to be encoded by the *E. coli hyf* operon, might be proton-translocating and thereby involved in respiratory metabolism (Andrews *et al.*, 1997). According to this hypothesis, the hydrogen evolved by Hyf would be oxidized by an electron transport chain involving a menaquinone-reducing uptake hydrogenase and a terminal reductase (Andrews *et al.*, 1997). Such a respiratory chain may be impaired in HRD4 and HRD6, suggesting that it may indeed be an essential part of both the halo- and nitrate respiratory chain in *D. dehalogenans*. In both mutants, hydrogenase and formate dehydrogenase activities were severely affected, suggesting coordinated regulation of the different enzyme complexes within the proposed respiratory chains. Inactivation of the hydrogenase thus also affects formate dehydrogenase activity (which might be part of the formate-hydrogen lyase complex), and vice versa. In the latter case, the lack of hydrogen formation in a formate-hydrogen lyase deficient mutant might affect the induction of the hydrogenase.

A priori, it was expected that the HRD mutants obtained would be impaired in the key enzyme *o*-chlorophenol reductive dehalogenase (CprA), additional structural components of the halorespiratory chain, and enzymes involved in processing and targeting of respiratory complexes or regulatory functions. However, the characterization of the 24 HRD mutants isolated revealed that none of the first type was obtained. A mutation in *cprA* is not expected to be lethal, as the organism is rather versatile in its ability to use alternative electron acceptors. The possibility of multiple copies of *cprA* can also be ruled out based on results

obtained by Southern blot analysis (van de Pas *et al.*, 1999). The detailed analysis of the isolated mutants at the physiological, biochemical and genetic levels reported here complements our earlier identification of the *cpr*-operon, provides insight into the complexity of halorespiration, and indicates that the halorespiratory chain and other electron transport chains are integrated.

Acknowledgments

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5

MOLECULAR CHARACTERIZATION OF REDOX COMPLEX-ENCODING GENES IN HALORESPIRING *DESULFITOBACTERIUM DEHALOGENANS*

Hauke Smidt, John van der Oost and Willem M. de Vos

A modified version of this chapter has been submitted for publication.

Abstract

To gain insight in the composition and regulation of the halorespiratory network in the low G+C gram-positive *Desulfitobacterium dehalogenans*, several gene clusters potentially encoding respiratory redox complexes were characterized at the molecular level and their expression under different physiological conditions was studied by biochemical and Northern analyses.

The capacity to couple the reductive dehalogenation of chlorinated aliphatic and aromatic compounds to growth in a novel type of anaerobic respiration has led to the identification and isolation of an increasing number of halorespiring bacteria from various bacterial phyla (El Fantroussi *et al.*, 1998; Fetzner, 1998; Holliger *et al.*, 1999). The high metabolic activity and apparent ubiquitous occurrence not only in polluted but also in pristine environments, suggest a significant contribution of these bacteria to dehalogenation in anoxic environments. These findings have highly stimulated efforts to unravel structure, function and regulation of the halorespiratory network (Holliger *et al.*, 1999; Louie & Mohn, 1999; Smidt *et al.*, 2001a). Several studies have focused on the versatile low G+C Gram-positive *Desulfitobacterium dehalogenans*, which couples the oxidation of pyruvate, lactate, formate or H_2 to the reduction of, among others, *ortho*-chlorinated phenols (*o*-CP), fumarate and nitrate (Utkin *et al.*, 1994). Recently, the key enzyme, *ortho*-chlorophenol reductive dehalogenase, has been characterized and shown to belong to a novel family of corrinoid-containing Fe-S proteins (van de Pas *et al.*, 1999). Furthermore, it has been shown that reductive dehalogenation is strongly induced at the transcriptional level (Smidt *et al.*, 2000). In yet another approach, random integration of the conjugative transposon Tn*916* into the chromosome of *D. dehalogenans* resulted in the isolation of halorespiration-deficient (HRD) mutants, which had lost the ability to use 3-chloro-4-hydroxyphenylacetic acid (Cl-OHPA) as electron acceptor, whereas fermentative growth with pyruvate was unaffected (Smidt *et al.*, 1999). The molecular analysis of these mutants revealed that the transposon had inserted adjacent to several genes that are predicted to code for respiratory complexes. To study whether the genes in the proximity of the Tn*916* insertion site in 3 distinct HRD mutants are indeed functional components of a halorespiratory network, we analyzed their expression under different growth conditions at the transcriptional and enzymatic levels.

Characterization of Redox Complex-Encoding Genes of *D. dehalogenans*

Halorespiration-deficient mutant HRD4 contains a copy of Tn*916* inserted upstream of the *hyfB* and *hycD* genes that could encode formate-H_2 lyase integral membrane subunits (Smidt *et al.*, 1999). To further characterize this chromosomal locus, sequences downstream from the *hycD* gene were amplified by inverse PCR with divergent primer pair BG358-BG359 from *Eco*RI-digested and self-ligated *D. dehalogenans* chromosomal DNA isolated as described previously (Fig. 5.1) (van de Pas *et al.*, 1999). Analysis of the *hycD*-downstream region revealed the presence of an additional open reading frame (*hyfF*) that could code for a protein

with highest similarity (44% identity on the amino acid level) to HyfF, another integral membrane subunit of the *E. coli* formate-H_2 lyase complex (Andrews *et al.*, 1997).

In HRD6, the transposon was found inserted immediately upstream of the uptake hydrogenase-encoding *hydABC* gene cluster, and inverse PCR with oligonucleotide primers BG564-BG565 was used to amplify *hydC*-downsstream sequences from *Eco*RI-digested and self-ligated *D. dehalogenans* chromosomal DNA. A fourth gene was identified downstream of *hydC*, namely *hydD*, that could encode a polypeptide with significant similarity (36% identity on the amino acid level with *Desulfovibrio gigas* HynC) with members of the HupD/HyaD family of hydrogenase processing proteins (Sawers, 1994) (Fig. 5.1).

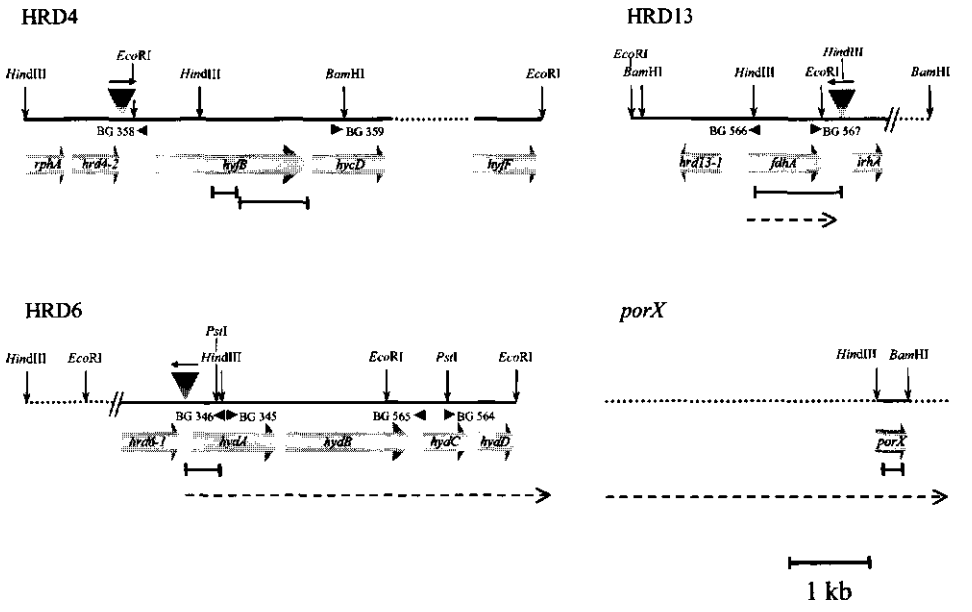

Figure 5.1 Physical map of analyzed putative redox-complex encoding gene clusters of *D. dehalogenans*. Triangles, site and orientation of Tn*916* integration in HRD mutants (Smidt *et al.*, 1999); dotted line, sequence not determined; horizontal arrows, identified open reading frames; downwards pointing arrows and arrowheads, relevant restriction sites and oligonucleotides (BG358, position 241 to 221 and BG565, position 2614 to 2635 of acc. no. AF157640; BG564, position 3999 to 4021 and BG565, position 3548 to 3525 of acc. no. AF157641; BG566, position 1623 to 1603 and BG567, position 2271 to 2293 of acc. no. AF299114); solid bars, gene-specific probes used for Northern analysis; dashed arrows, apparent transcription products.

The detection of an approximately 4.3-kb polycistronic transcript hybridizing with a *hydA*-specific probe (see below) and the presence of highly AT-rich putative promoter sequences immediately upstream of *hydA* suggests that the *hyd* operon might contain one additional small open reading frame downstream *hydD*, as it is known from other hydrogenase-encoding gene clusters (Smidt *et al.*, 1999).

In order to screen for the presence of additional redox enzyme-encoding genes with a possible function in the halorespiratory network, the Tn*916* insertion sites of another available HRD mutant, HRD13, was analyzed at the sequence level. A 3.3-kb region around the transposon insertion site in HRD13 was amplified by inverse PCR with Tn*916*-specific divergent primer pair BG286-BG288 and divergent primer pair BG566-BG567 from *Eco*RI- or *Bam*HI-digested and self-ligated chromosomal DNA from *D. dehalogenans* (Fig. 5.1) (Smidt *et al.*, 1999).

Sequence analysis revealed the presence of three open reading frames, designated *hrd13-1*, *fdhA* and *irhA*. The *hrd13-1* and *irhA* genes potentially code for polypeptides that exhibit highest similarity with CDP-alcohol phosphatidyltransferase class I proteins and iron-responsive repressor proteins, respectively. The predicted *fdhA* gene product is a polypeptide of 311 amino acids with a calculated molecular weight of 34,343 Da. It exhibits significant similarity to the N-terminal domain of the catalytic subunit of $NAD(P)^+$-dependent formate dehydrogenases and hydrogenases, and NuoG of the *E.coli* NADH-ubiquinone oxido-reductase (40% identity on the amino acid level with the NH_2-terminal domain of *Moorella thermoacetica* FdhA). This domain is thought to be part of the diaphorase (NADH dehydrogenase) entity of these complexes and contains 5 conserved ferredoxin-like Fe-S cluster binding motifs involved in intramolecular electron transfer (Oh & Bowien, 1998). Although the *D. dehalogenans* FdhA lacks the major part of the catalytic C-terminal domain, similar functional splitting of the ferredoxin- and the catalytic domain into two subunits has also been observed for the NAD^+-linked hydrogenase from *Ralstonia eutrophus* and a putative respiratory chain oxido-reductase-encoding operon in *Streptomyces coelicolor* (Tran-Betcke *et al.*, 1990; acc. no. AL034443).

Finally, the 0.4-kb partial sequence of an open reading frame, designated *porX*, was by coincidence detected upstream of a cryptic reductive dehalogenase-encoding gene homologue (Chapter 7 of this thesis). The predicted *porX* gene product shows high similarity to the C-terminus of the 1175-amino acid *Clostridium pasteurianum* pyruvate ferredoxin oxido-reductase (45 % identity on the amino acid level) (Fig. 5.1).

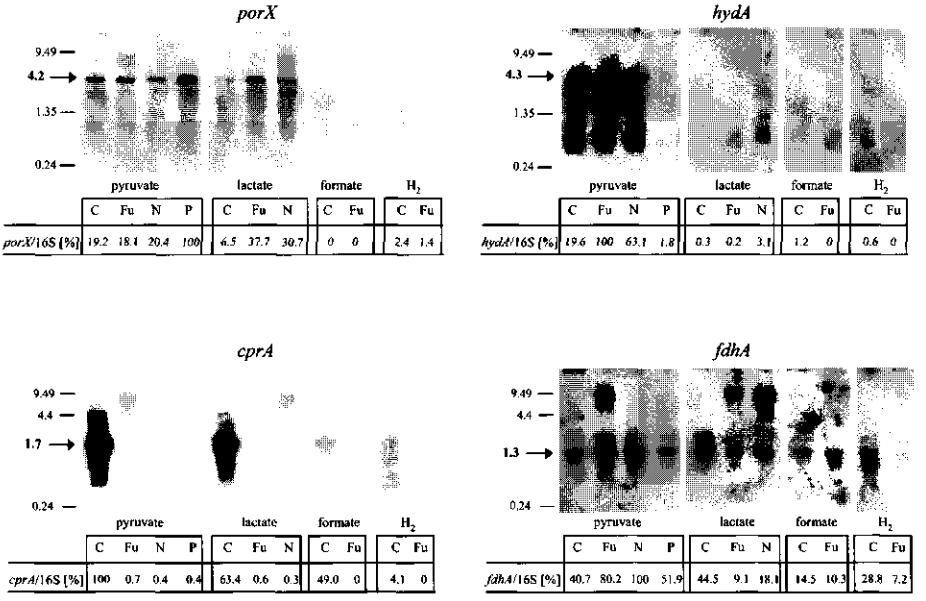

Figure 5.2 Northern blot analysis of total RNA extracted from cells of *D. dehalogenans* grown with pyruvate, lactate, formate or H_2 as the electron donor and various electron acceptors (C, Cl-OHPA; Fu, fumarate; N, nitrate; P, pyruvate). [^{32}P]-labeled probes were applied that were specific for the various redox-complex encoding gene clusters and the 16S rRNA-encoding gene of *D. dehalogenans*, respectively. RNA size markers are in kilobase. Arrows indicate specific hybridizing signals that were obtained after 8 to 68 h exposures. The occasionally obtained high molecular weight hybridization signals are due to residual amounts of chromosomal DNA. No hybridization was observed with probes specific for *hyfB* (data not shown). Relative transcription values were obtained after quantification of hybridization signals using the ImageQuant software package (Molecular Dynamics, Sunnyvale, Calif.) and were calculated as $\{([mRNA] / [16S\ rRNA])_n / ([mRNA] / [16S\ rRNA])_{max}\} \times 100\%$.

Isolation of Total RNA, Northern Analysis, and Enzyme Assays

To gain insight in the function of the characterized redox genes, their transcription was determined in parallel with the activity of the redox enzymes in cultures of *D. dehalogenans* grown in the presence of different electron donors (pyruvate, lactate, formate, and H_2) and acceptors (Cl-OHPA, fumarate, nitrate, and pyruvate) (Fig. 5.2 and Table 5.1). The anaerobic conditions for growth have been described previously (Smidt *et al.*, 2000), and for growth with H_2 as electron donor, the N_2 - headspace was replaced by H_2. Enzyme activities in cell extracts (van de Pas *et al.*, 1999) were determined at 30°C by following the oxidation or reduction of benzyl viologen at 578 nm ($\varepsilon_{578} = 9.2\ mM^{-1}.cm^{-1}$).

Table 5.1 Oxido-reductase activities in cell extracts of *D. dehalogenans* grown with pyruvate, lactate, formate or H_2 as the electron donor and Cl-OHPA (C), fumarate (F), nitrate (N) or pyruvate (P) as the electron acceptor.

Donor	pyruvate				lactate			formate		H_2	
Acceptor	C	Fu	N	P	C	Fu	N	C	Fu	C	Fu
H_2ase[a]	0.21	0.81	0.73	0.10	0	0.04	0.05	1.20	1.59	1.32	1.26
Fdh[a]	1.43	1.17	1.46	1.15	1.09	0.35	0.40	3.83	5.09	2.37	2.79
Por[a,b]	0.29	0.85	0.61	0.50	-0.49	0.20	0.19	-1.40	-3.33	-1.26	-1.31

[a]Enzyme activities are given in [U / mg of protein]. For all assays, one unit (U) of enzyme activity corresponds to the amount of enzyme catalyzing the conversion of 1 µmol substrate or 2 µmol of benzyl viologen per minute. [b]Negative values indicate HS-CoA independent reduction of pyruvate with Ti(III)citrate-reduced benzyl viologen as the electron donor.

Formate dehydrogenase, hydrogenase and pyruvate ferredoxin oxido-reductase were assayed in 1 ml of 100 mM Tris-HCl (pH 8.0) containing 1 mM benzyl viologen and 10 mM formate sodium salt, 1 ml H_2 or 10 mM pyruvate sodium salt and traces of HS-coenzyme A, respectively. Cl-OHPA reductive dehalogenase activity could not be determined in cell extracts prepared from mid-exponential phase cultures due to high background methyl viologen-oxidizing activity. However, dehalogenase activity was confirmed in Cl-OHPA-respiring cultures by following the release of Cl^- ions (data not shown). Protein was determined according to the method of Bradford, with bovine serum albumin as the standard (Bradford, 1976).

Total RNA was isolated from *D. dehalogenans* as described previously and used for Northern analysis with gene-specific probes and, after deprobing, with a probe for the 16S rRNA which served as an internal standard (Smidt *et al.*, 2000) (Fig. 5.2). The halorespiration-specific expression of the *o*-chlorophenol reductive dehalogenase-encoding *cprBA* bis-cistronic operon, as previously reported for cultures grown with pyruvate as the electron donor, could be confirmed with all electron donors tested (Smidt *et al.*, 2000) (Fig. 5.2). Similarly, halorespiration-specific transcription was also observed for the other transcripts of the *cprTKZEBACD* gene cluster (data not shown) (Smidt *et al.*, 2000). Hybridization with a *porX*-specific probe revealed the presence of a 4.2-kb transcript, probably reflecting the monocistronic transcription of the complete *porX* gene, which in analogy with known pyruvate ferredoxin oxido-reductases is expected to have a size of approximately 3.5 kb. The gene was solely expressed under pyruvate- and lactate-oxidizing

conditions, which coincided with significant HS-CoA dependent oxidation of pyruvate (Table 5.1, Fig. 5.2). In contrast, under growth conditions during which no *porX* transcription could be detected, only HS-CoA independent reduction of pyruvate was observed.

The genes and gene clusters, which were identified in the vicinity of the Tn*916* insertion site in HRD mutants have previously been proposed to be essential for expression of a functional halorespiratory chain (Smidt *et al.*, 1999). Transcriptional analysis of *fdhA* revealed the constitutive presence of a 1.3-kb transcript, indicating monocistronic transcription. Consensus promoter sequences were present immediately preceding *fdhA*. Expression was specifically induced when pyruvate or lactate was used as the electron donor (Fig. 5.2). The transcription pattern corresponds to the formate dehydrogenase activities determined in pyruvate- and lactate-grown cells, suggesting that the *fdhA* gene product indeed is part of a formate dehydrogenase complex. The *fdhA* expression profile, however, does not correspond to the strongly induced formate dehydrogenase activity in formate- and H_2-grown cells (Table 5.1).

A similar discrepancy was obtained for hydrogenase activity and *hydA* expression. High hydrogenase activity was observed under hydrogen- and formate-oxidizing conditions and moderate (2-20 fold reduced) activity in cultures grown with pyruvate as the electron donor; no hydrogenase activity was found in lactate-grown cells. Hybridization with a *hydA*-specific probe, however, revealed measurable transcription of an approximately 4.3-kb polycistronic messenger solely in pyruvate-grown cultures (Fig. 5.2). These results indicate the presence of at least two distinct hydrogenases and formate dehydrogenases expressed under different physiological conditions, as it is also known e.g. for *E. coli* (Sawers, 1994). Indeed, additional experiments revealed that the formate-dehydrogenase activity induced in the presence of formate is localized at the outer face of the cytoplasmic membrane, whereas cytoplasmic activity was constitutively present under all conditions tested (data not shown). This is in complete agreement with earlier results, obtained with cells of *D. dehalogenans* grown under formate-oxidizing conditions with either fumarate or Cl-OHPA as the electron acceptor, that indicate the presence of both cytoplasmic and extracytoplasmic formate dehydrogenase activity (van de Pas, 2000). In addition, only the cytoplasmic formate dehydrogenase showed activity with oxidized cytochrome C partially purified from *Syntrophobacter fumaroxidans* as an electron acceptor (data not shown; de Bok, personal communication).

Interestingly, enzyme activity measurements in cell extracts of the 3-chlorobenzoate-respiring *Desulfomonile tiedjei* similarly indicated the specific induction of a periplasmic

hydrogenase in the presence of pyruvate, and the presence of two differentially expressed formate dehydrogenases, one of which was induced in formate-grown cells (Louie & Mohn, 1999). The *fdhA* gene product might be involved in acetate production via the reductive acetyl-coA pathway, as significant CO_2-fixation could be shown for *D. dehalogenans* during pyruvate fermentation (Ragsdale, 1991; van de Pas, 2000). A similar function has been suggested for a cytoplasmic formate dehydrogenase that was induced under pyruvate-fermenting conditions in *D. tiedjei* (Louie & Mohn, 1999).

The results presented here indicate that the *porX*-, *fdhA*- and *hydABCD*-encoded oxido-reductases are all transcribed and hence may indeed play a role in anaerobic respiratory processes by *D. dehalogenans*. Although the detailed transcriptional analysis indicated that neither of these genes is specifically expressed under halorespiring conditions, their involvement in the respiratory network was demonstrated. Nevertheless, as expression of the *hydABCD* gene cluster could only be observed in cultures grown with pyruvate as the electron donor, an essential role in the halorespiratory network can be ruled out for the corresponding enzyme. This also might be the case for the predicted formate-H_2 lyase encoding gene cluster, for which no measurable transcription could be detected under any of the conditions tested (not shown). It remains to be elucidated whether the disruption of additional genes in the vicinity of the inserted transposon, i.e. proteins involved either in maturation of respiratory complexes or regulation (as *irhA* in HRD13), or other polar effects of the insertion are the cause for the halorespiration-deficient phenotype of the mutant strains.

The specific induction of oxido-reductases during respiration by *D. dehalogenans* and their detailed characterization at the sequence and transcription level presented here, extend our knowledge of the molecular basis of the complex respiratory network present in this *ortho*-chlorophenol-respiring organism. In addition, the results indicate the need for the specific gene inactivation systems that have recently been developed for *D. dehalogenans* (Smidt *et al.*, 2001b) and are expected to be instrumental in further elucidating the structure and function of its halorespiratory network.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of the different genes and operons described have been deposited in the GenBank database under accession numbers AF157640 (*hyfB-hycD*), AF157641 (*hydABCD*), AF299114 (*fdhA*), AF299115 (*hyfF*), and AF299116 (*porX*).

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6

DEVELOPMENT OF A GENE CLONING AND INACTIVATION SYSTEM FOR THE HALORESPIRING *DESULFITOBACTERIUM DEHALOGENANS*

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Abstract

Efficient host-vector systems have been developed for the versatile strictly anaerobic halo- and fumarate-respiring Gram-positive bacterium *Desulfitobacterium dehalogenans*. An electroporation-based transformation procedure resulting in approximately 10^3 to 10^4 transformants per μg of the cloning vector pIL253 was developed and validated. The broad host-range vector pG^+host9 was shown to replicate at a permissive temperature of 30°C, whereas the replicon was not functional at 40°C. The *D. dehalogenans frdCAB* operon, predicted to encode a fumarate reductase, was cloned, characterized, and targeted for insertional inactivation by pG^+host9 carrying a 0.6-kb internal *frdA* fragment. Single-crossover integration at the *frdA* locus occurred at a frequency of 3.3×10^{-4} per cell and resulted in partially impaired fumarate reductase activity. The gene cloning and inactivation systems described here provide a solid basis for the further elucidation of the halorespiratory network in *D. dehalogenans* and allow for its further exploitation as a dedicated degrader.

Introduction

It has been shown for a wide range of haloorganic compounds that reductive dechlorination is the first crucial step in the degradation of such pollutants (Mohn & Tiedje, 1992; Fetzner, 1998). Halorespiring bacteria have received increasing attention during the past decade due to a significant contribution to reductive dehalogenation processes occurring in anoxic polluted environments such as soils, aquifers, and sediments (El Fantroussi *et al.*, 1998; Middeldorp *et al.*, 1999). In contrast to the co-metabolic reductive dehalogenation catalyzed by various metal-containing tetrapyrrol cofactors in a variety of anaerobic bacteria, this reaction is catalyzed at much higher rates by specific enzymes in halorespiring microbes, where it is coupled to energy conservation by electron transport-coupled phosphorylation (El Fantroussi *et al.*, 1998; Holliger *et al.*, 1999; Smidt *et al.*, 2001). One of these strains is the versatile low G+C Gram-positive bacterium *Desulfitobacterium dehalogenans,* which is able to link the oxidation of several electron donors such as hydrogen, formate, lactate, and pyruvate to the reduction of various organic and inorganic acceptors, including *ortho*-chlorinated phenols (*o*-CP), fumarate, and nitrate (Utkin *et al.*, 1994). Recently, the *o*-CP-reductive dehalogenase (CPR) from *D. dehalogenans* has been purified and characterized at the biochemical and genetic levels (van de Pas *et al.*, 1999; Smidt *et al.*, 2000). Comparison with other chloroalkene- and haloaromate-reductive dehalogenases isolated and characterized from various phylogenetically distinct halorespiring bacteria indicated that these enzymes share significant similarities in both structural and functional properties, suggesting that they constitute a novel class of corrinoid-containing reductases (for a recent review, see Holliger *et al.*, 1999; Smidt *et al.*, 2001).

The detailed molecular analysis of the *cpr*-gene cluster in *D. dehalogenans* led to the identification of genes encoding putative regulatory proteins and protein folding catalysts, the transcription of which was specifically induced under halorespiring conditions. From these results, their potential involvement in regulation and maturation of the reductive dehalogenase complex has been suggested (Smidt *et al.*, 2000). Additional genomic loci that appear essential for functional *o*-CP respiration of *D. dehalogenans* have been identified by means of random chromosomal integration of the conjugational transposon Tn*916* (Smidt *et al.*, 1999). Nevertheless, detailed structural and functional analysis of these proteins has been hampered by the absence of genetic techniques for *D. dehalogenans,* including transformation, gene

cloning, and specific gene disruption and insertion. Moreover, the development of such genetic modification tools would also enable the design of strains with improved performance in the bioremediation of polluted environments (Keasling & Bang, 1998; Timmis & Pieper, 1999).

Host - vector systems that allow for the genetic, metabolic, and protein engineering of low G+C Gram-positive bacteria (LGB) have been developed and optimized mainly for industrially applied strains of lactic acid bacteria, for bacilli and, to a lesser extent, clostridia (for reviews see de Vos & Simons, 1994; de Vos *et al.*, 1997; de Vos, 1999; Young *et al.*, 1999). It has been shown that vectors based on the theta replicon of the broad host-range conjugative plasmid pAMβ1 (Bruand *et al.*, 1991), among which are the cloning vectors pIL252 and pIL253, are functional in all genera of LGB studied, indicating their potential use for halorespiring genera of LGB, such as *Desulfitobacterium* and *Dehalobacter* (Simon & Chopin, 1988; de Vos & Simons, 1994). Similarly, vectors of the pG$^+$host series of thermosensitive derivatives of yet another broad host-range plasmid, pWV01, have been proven to be instrumental for high-efficiency gene inactivation, replacement, and insertional mutagenesis, especially in poorly transformable LGB (Maguin *et al.*, 1992; Biswas *et al.*, 1993; de Vos & Simons, 1994; Maguin *et al.*, 1996).

The main objectives of this study were (i) to develop an efficient protocol for the transformation of *D. dehalogenans*, (ii) to investigate the suitability of gene transfer systems previously developed for other LGB, (iii) to confirm temperature-sensitive replication of pG$^+$host9 in *D. dehalogenans,* and (iv) to demonstrate its applicability for specific gene disruption using the putative fumarate reductase-encoding *frdA* gene as a model target.

Materials and Methods

Materials

All gases were obtained from Hoek Loos (Schiedam, The Netherlands). When appropriate, experiments were carried out in an anaerobic glove box (Coy Laboratory Products, Grass Lake, Mich.) under an atmosphere of 96% N_2 and 4% H_2. The oxygen concentration was kept low with the palladium catalyst RO-20 provided by BASF (Arnhem, The Netherlands).

Bacterial Strains, Plasmids, and Culture Conditions

Desulfitobacterium dehalogenans strain JW/IU-DC1 (DSM 9161) (Utkin *et al.*, 1994) was routinely grown under anaerobic conditions (gas phase, 100% N_2) at 37°C in basal mineral medium as described by Neumann *et al.* (Neumann *et al.*, 1994), supplemented with 0.1% peptone, 30 mM $NaHCO_3$, and trace elements and vitamin solution as recommended by the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). An electron donor and an electron acceptor were added to the appropriate concentrations from sterile anaerobic stock solutions.

Strains of *Escherichia coli* were grown in Luria Bertani medium at 37°C (Sambrook *et al.*, 1989). *E. coli* XL1-Blue (Stratagene, La Jolla, Calif.) was generally used as a host for cloning vectors. As a host for the rolling-circle pG$^+$host vectors, *E. coli* MC1061 was used (Casadaban & Cohen, 1980). *Lactococcus lactis* MG1614 (Gasson, 1983) was grown at 30°C in M17 broth (Difco, Detroit, Mich.) supplemented with 0.5% glucose (GM17). Where appropriate, media were amended with ampicillin (100 μg/ml) or erythromycin (150 μg/ml for *E. coli*, 10 μg/ml for *L. lactis* and 5 μg/ml for *D. dehalogenans*). The MIC of erythromycin for *D. dehalogenans* was determined on plates containing 0 to 5 μg of erythromycin/ml, and 20 mM lactate and fumarate as the electron donor and electron acceptor, respectively.

The cloning vectors pUC18 and pUC19 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), and the PCR product cloning vectors pGEM-T and pMON38201 (Borovkov & Rivkin, 1997) were obtained from Promega (Madison, Wis.) and Monsanto (St. Louis, Mo.), respectively. Plasmids pIL253 (Simon & Chopin, 1988) and pG$^+$host9 (Maguin *et al.*, 1996) were kindly provided by Richard van Kranenburg (NIZO Food Research, Ede, The Netherlands) and Emmanuelle Maguin (Laboratoire de Génetique Microbienne, Institut National de la Recherche Agronomique, Jouy en Josas Cedex, France).

DNA Isolation and Manipulation

Total DNA of *D. dehalogenans* was isolated as described previously (van de Pas *et al.*, 1999). Plasmid DNA was isolated from *E. coli* by using the alkaline lysis method, and standard DNA manipulations were performed according to established procedures (Sambrook *et al.*, 1989) and manufacturers' instructions. Isolation of plasmid DNA from *L. lactis* was performed as described previously (de Vos *et al.*, 1989). *L. lactis* was transformed according to the method of Wells *et al.* (Wells *et al.*, 1993). Large-scale preparations of plasmid DNA

(pIL253, pG$^+$host9) were purified by CsCl density gradient centrifugation (Sambrook *et al.*, 1989).

Enzymes were purchased from Life Technologies B.V. (Breda, The Netherlands), Roche Molecular Biochemicals (Mannheim, Germany), or New England Biolabs (Beverly, Mass.). Oligonucleotides were obtained from Eurogentec (Seraing, Belgium), Life Technologies Inc., and MWG Biotech (Ebersberg, Germany). PCR products were purified prior to subsequent manipulation using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany).

Transformation Procedure and Competence of *D. dehalogenans*

For the transformation of *D. dehalogenans*, the electroporation-based method described by Wells *et al.* was modified and optimized for use with anaerobic bacteria (Wells *et al.*, 1993). Cells of *D. dehalogenans* were grown in the presence of 40 mM glycine with 20 mM lactate as the electron donor and 20 mM fumarate as the electron acceptor. Unless otherwise indicated, all subsequent steps were carried out in the anaerobic glove box. Exponentially growing cells were harvested at an A_{600} of approximately 0.2 by centrifugation at 2,600 × g for 10 min at 4°C and then resuspended in 0.15 volume of ice-cold anaerobic washing buffer (0.5 M sucrose - 10% glycerol). Cells were recovered by centrifugation at 4,000 × g for 10 min at 4°C, washed with 0.05 volume of washing buffer, recentrifuged, and finally resuspended in 0.001 volume of washing buffer. For electroporation, DNA was added in 0.5 to 1.0 µl of deionized water to 40 µl of concentrated cell suspension and transferred to precooled 0.2-cm electroporation cuvettes. A single pulse was applied outside the glove box at different settings (field strength, 12.5 kV × cm^{-1}; capacitance, 25 µF; resistance, 200 to 800 Ω) using a Gene Pulser (Bio-Rad, Hercules, Calif.). Immediately after electroporation, cells were moved back into the anaerobic glove box, mixed with 0.96 ml of recovery medium (growth medium containing 20 mM lactate and fumarate and 0.5 M sucrose), and incubated at 37°C for 5 h. To determine the influence of the transformation procedure on the viability of *D. dehalogenans*, appropriate dilutions were inoculated onto plates without erythromycin containing 20 mM of lactate and fumarate as described previously (Smidt *et al.*, 1999). Transformants were selected on plates containing 20 mM lactate and fumarate, and 5 µg of erythromycin/ml. Further subcultivation of single colonies in liquid medium was performed as described previously (Smidt *et al.*, 1999). Plasmid DNA was isolated from *D. dehalogenans* using a protocol modified from (de Vos *et al.*, 1989). Briefly, protoplasts were prepared from 12 ml of early stationary phase culture in 250 µl of THMS buffer (30 mM Tris-

HCl [pH 8.0] and 3 mM $MgCl_2$ in 25% sucrose), containing 20 mg of lysozyme/ml (van Asseldonk *et al.*, 1993). Subsequently, plasmid DNA was purified by alkaline lysis and recovered by isopropanol precipitation. Agarose gel electrophoresis and Southern blot analysis were used to check for the presence of plasmids.

Thermosensitivity of pG$^+$host9 in *D. dehalogenans*

To determine the segregational stability of the thermosensitive vector pG$^+$host9 in *D. dehalogenans*, an early-stationary-phase culture of plasmid-carrying *D. dehalogenans* that was grown in the presence of 40 mM pyruvate and 5 µg of erythromycin/ml at 30°C was diluted 40-fold into medium without antibiotics and incubated at 30°C to stationary phase (0 h). This culture was then diluted 100-fold into fresh medium without antibiotics and incubated at 30, 37, and 40°C. Appropriate dilutions were inoculated onto plates with or without erythromycin (5 µg/ml) at 0.1, 16, and 40 h after dilution and were incubated at 30°C. After 40 h of growth, all cultures had reached stationary phase. Cultures were again diluted 10-fold and kept for an additional 24 h at the respective temperatures until stationary phase was reached (68 h). Total DNA was isolated from samples taken at 0, 40, and 68 h, digested with *Eco*RI, and analyzed by Southern blot analysis. Linearized pG$^+$host9 was used as a plasmid-specific probe for hybridization. Hybridization with a probe specific for the *D. dehalogenans frdAC* genes was used as an internal standard. This probe was a PCR-product obtained with primers BG355 (positions 942 to 974 of the *frd* gene cluster) and IK04 [5'-(A/G)TG NGC NCC NC(G/T) NS(A/T) (C/T)TC-3'; positions 3157 to 3140] (see below and Fig. 6.3). A Hybond-N+ nylon transfer membrane (Amersham Life Science, Little Chalfont, United Kingdom) was used for Southern blot analysis, and probes for hybridization experiments were labeled by nick translation in the presence of [α-^{32}P]-dATP (Amersham Pharmacia Biotech).

Cloning of a Putative Fumarate Reductase-Encoding Operon

The degenerated primers IK01 [5'-GA(A/G) (A/G/T)(G/C)N (G/T)(G/C)N A/C)GN GGN GAN GGN GG-3', positions 2312 to 2334] and IK04, which were designed based on an amino acid sequence alignment of known bacterial fumarate reductases, were used to PCR amplify a fragment of a putative fumarate reductase-encoding operon from the chromosomal DNA of *D. dehalogenans*. The resulting 0.85-kb PCR product was cloned in *E. coli* using *Xcm*I-digested pMON38201, yielding pLUW902. Subsequently, Southern blot analysis of *Pst*I-*Eco*RI-digested chromosomal DNA of *D. dehalogenans* revealed a 3-kb fragment that

strongly hybridized with the radiolabelled 0.85-kb PCR product. The 3-kb fragment was cloned in *E. coli* using *Pst*I-*Eco*RI-digested pUC18, resulting in pLUW903. pLUW904 was obtained by inverse PCR (Triglia *et al.*, 1988) that was performed as described previously (van de Pas *et al.*, 1999) with *Bam*HI-digested and self-ligated chromosomal DNA of *D. dehalogenans* by using the divergent primer pair BG283 and BG284 (positions 2456 to 2477 and positions 2356 to 2335, respectively).

Plasmid Constructions and Single-Crossover Integration into the *D. dehalogenans* Chromosome

A 578-bp *Apa*I-*Eco*RI internal fragment of the *D. dehalogenans frdA* gene was cloned in *E. coli* MC1061 using *Apa*I-*Eco*RI - digested pG$^+$host9, yielding pLUW906. Subsequently, electrocompetent cells of *D. dehalogenans* were transformed with plasmid DNA isolated from *E. coli* MC1061 using the QIAprep Spin Miniprep kit (Qiagen GmbH). Recovery after electroporation and cultivation on selective plates were performed at 30°C. Erythromycin-resistant colonies that appeared within 5 days were transferred to liquid selective medium containing 40 mM pyruvate and were incubated at 30°C. Cultures were diluted 20-fold in the same medium, grown at 30°C for 8 h to reach log phase, and then shifted to 40°C for 16 h (3 to 5 generations). Appropriate dilutions were incubated on plates in the presence of 20 mM pyruvate and erythromycin at 40°C in order to detect integration events and on nonselective plates at 40°C for the determination of viable cell counts. The ratio of the two counts was used to determine the frequency of integration per cell as described by Biswas *et al.* (Biswas *et al.*, 1993). Integrants that were isolated at 40°C were subsequently routinely maintained in selective medium containing 20 to 40 mM pyruvate. Southern blot analysis of *Hin*cII-digested chromosomal and plasmid DNA and preparation of pG$^+$host9- and *D. dehalogenans frdAC*-specific probes were performed as described above.

DNA Sequencing and Sequence Analysis

DNA sequencing was performed using a LiCor (Lincoln, Nebr.) DNA sequencer 4000L. Plasmid DNA used for sequencing reactions was purified with the QIAprep Spin Miniprep kit (Qiagen GmbH). Reactions were performed using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham Pharmacia Biotech). Fluorescently (IRD 800) labeled universal sequencing primers were purchased from MWG Biotech. Sequence similarity searches and alignments were performed using the BLAST 2.0 program (Altschul *et al.*,

1997) (National Center for Biotechnology Information, Bethesda, Md.) and the Clustal X and GeneDoc programs (Thompson *et al.*, 1997; K. B. Nicholas & H. B. J. Nicholas, GeneDoc: a tool for editing multiple sequence alignments, 1997) and the DNAstar package (DNASTAR Inc., Madison, Wis.), respectively.

Enzyme and Protein Assays

Harvesting of cells and preparation of cell extracts by sonication under anoxic conditions were performed as described previously (van de Pas *et al.*, 1999). Fumarate reductase activities were determined spectrophotometrically at 30°C in 1 ml of 100 mM Tris-HCl (pH 8.0) as described previously (Smidt *et al.*, 1999). One unit of enzyme activity corresponds to the amount of enzyme catalyzing the conversion of 1 μmol of substrate or 2 μmol of benzyl viologen per min. Succinate dehydrogenase activity was measured with 2,6-dichlorophenolindophenol and phenazine methosulfate as an artificial electron acceptor as described by Schirawski and Unden (Schirawski & Unden, 1995). Protein was determined according to the method of Bradford, with bovine serum albumin as the standard (Bradford, 1976).

Nucleotide Sequence Accession Number

The nucleotide sequence of the putative fumarate reductase-encoding operon has been deposited in GenBank under accession no. AF299117.

Results

Development of an Electroporation-Based Transformation Protocol for *D. dehalogenans*

To allow for the application of plasmid vector systems for genetic manipulation of the strict anaerobe *D. dehalogenans*, an electroporation-based transformation protocol for this bacterium was designed and optimized using the promiscuous plasmid pIL253 (Simon & Chopin, 1988). Because this cloning vector, which is a derivative of the broad host-range theta-replicating plasmid pAMβ1, carries an erythromycin resistance marker, we checked *D. dehalogenans* for its sensitivity to this antibiotic. On plates that contained 0.1 μg of erythromycin/ml, 6.5×10^6 CFU/ml was obtained, compared to 4×10^7 CFU/ml on plates

without any antibiotic. At erythromycin concentrations of 0.25 and 0.5 µg/ml, micro-colonies appeared after 4 days, whereas no colonies developed at concentrations of ≥ 1 µg of erythromycin/ml, indicating that the frequency of spontaneous resistance to erythromycin is below 2.5×10^{-7} per CFU. Subsequently, a concentration of 5 µg of erythromycin/ml was used in solid and liquid media for the selection of strains of *D. dehalogenans*, carrying the erythromycin resistance marker.

Electrocompetent cells of *D. dehalogenans* were prepared from exponential-phase cells that had grown in the presence of the cell wall-weakening agent glycine as described by Wells *et al.* (Wells *et al.*, 1993). Cells were washed and finally concentrated approximately 1,000-fold in ice-cold anaerobic washing buffer. On average, approximately 70% of the cells could be recovered as viable CFU on nonselective plates after the cell collection and washing procedure. Electroporation of 40-µl aliquots of concentrated cell suspension in the presence or absence of different amounts of plasmid DNA was performed outside the anaerobic chamber at a field strength of 12.5 kV × cm^{-1}, a capacitance of 25 µF and a resistance of 200 of 800 Ω. After a subsequent incubation of 5 h in the presence of 0.5 M sucrose, cells were inoculated onto plates with or without 5 µg of erythromycin/ml.

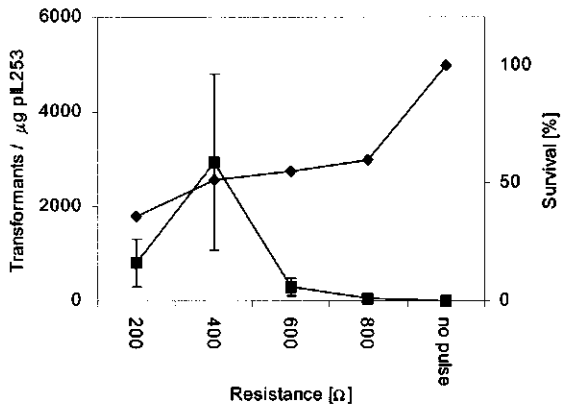

Figure 6.1 Survival and transformation efficiency of cells of *D. dehalogenans* after electroporation. Survival was defined as CFU per milliliter of cell suspension on non-selective plates (◆). Efficiency of transformation of *D. dehalogenans* was determined on plates containing 5 µg of erythromycin/ml and calculated as CFU per microgram of CsCl-purified pIL253 (■).

After 3 days of incubation at 37°C, colonies were counted to determine survival and transformation efficiency. The pulse resulted in a 40 to 65% decrease in CFU on nonselective plates with decreasing resistance compared to the survival of an aliquot of concentrated cells that was kept inside the anaerobic chamber and was not subjected to a pulse (Fig. 6.1). The highest numbers of transformants were obtained at a resistance of 400 Ω, resulting in a pulse time constant of approximately 7.5 ms. Both shorter and longer pulse times (200 Ω, 4.7 ms; 600 Ω, 12.8 ms; 800 Ω, 16.4 ms) resulted in significantly lower numbers of transformants (Fig. 6.1). Routinely, 3,000 ± 1,900 transformants (maximal value, 6.6×10^3) were obtained per μg of CsCl-purified pIL253, independent of the amount of plasmid DNA (ranging from 50 to 800 ng) used in the electrotransformation. In order to check the transformants for the presence and concentration of plasmid, erythromycin-resistant colonies were transferred to liquid selective medium containing 20 mM lactate and fumarate, and plasmid DNA was isolated from early-stationary-phase-cultures (A_{600} = 0.25). Plasmid DNA was detectable by agarose gel electrophoresis, and quantification indicated a concentration of 5 ng of pIL253/ml of culture, corresponding to approximately 10 copies per cell (data not shown).

Segregational Stability and Thermosensitivity of pG$^+$host9 in *D. dehalogenans*

The thermosensitive broad host-range pG$^+$host vector family has been shown to be instrumental for high-efficiency gene inactivation and replacement in Gram-positive bacteria (Biswas *et al.*, 1993). In order to study the applicability of this system in the halorespiring bacterium *D. dehalogenans*, electrocompetent cells were transformed with CsCl-purified pG$^+$host9. To ensure functional replication, post-transformation incubation and cultivation on selective media were performed at 30°C. Transformation yielded, on average, 600 transformants per μg of plasmid DNA. Colonies that appeared on selective plates were transferred to liquid medium. Plasmid DNA was isolated from early-stationary-phase cultures and could be detected by agarose gel electrophoresis (data not shown). In order to determine the permissive and nonpermissive temperatures for the replication of pG$^+$host9 in *D. dehalogenans*, the segregational stability of the plasmid at nonselective concentrations of erythromycin was analyzed at different temperatures. A culture of *D. dehalogenans* containing the plasmid was diluted into fresh medium without any antibiotic and incubated at 30, 37, and 40°C. The ratio of the CFU on selective plates to the CFU on nonselective plates at 30°C was determined at 0, 16, and 40 h after dilution.

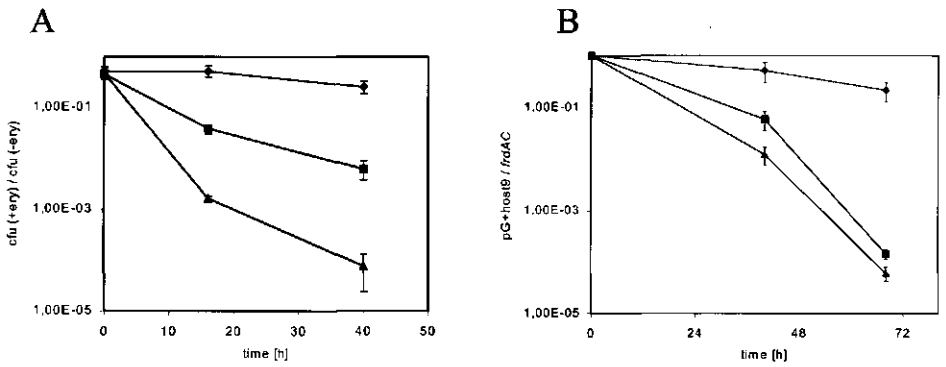

Figure 6.2 Segregational stability and thermosensitivity of pG$^+$host9 in *D. dehalogenans* under nonselective culture conditions at 30°C (◆), 37°C (■) and 40°C (▲). **A)** Ratio of CFU on selective and nonselective plates. **B)** Normalized ratio of hybridization signal intensities obtained with probes specific for pG$^+$host9 and *frdAC*.

Whereas this ratio decreased only 50% for the culture that was incubated at 30°C (0.51 at 0 h and 0.26 at 40 h), it dropped 72- and 48,000-fold at 37 and 40°C, respectively, within 7 generations (Fig. 6.2A). No influence of the incubation temperature on segregational stability was observed in the case of the non-thermosensitive plasmid pIL253 (data not shown). Similar results were obtained by Southern blot analysis of total DNA that was isolated before and 7 (40 h) and 10 generations (68 h) after the shifting to nonselective conditions, respectively (Fig. 6.2B). The amount of plasmid-derived sequences detected following growth for 10 generations at 37 or 40°C was found to be more than 1,000-fold lower than that detected in cells grown at 30°C.

Cloning and Sequence Analysis of a Putative Fumarate Reductase-Encoding *frdBAC* Gene Cluster

The versatile Gram-positive anaerobe *D. dehalogenans* has the ability to utilize fumarate as the terminal electron acceptor for anaerobic respiration with H_2, formate, lactate, or pyruvate as the electron donor. High fumarate reductase activity is readily detectable in cell extracts of *D. dehalogenans* grown in the presence of fumarate or yeast extract (Utkin *et al.*, 1994; Smidt *et al.*, 1999). In order to provide an easy-to-screen target gene for the development of genetic modification approaches, we amplified a 0.85-kb fragment from the chromosome of *D. dehalogenans* using degenerated primers that were designed based on a

primary sequence alignment of known succinate:quinone oxido-reductases (Hägerhäll, 1997). Sequence analysis indicated significant similarity with the flavoproteins of fumarate reductases and succinate dehydrogenases present in the databases.

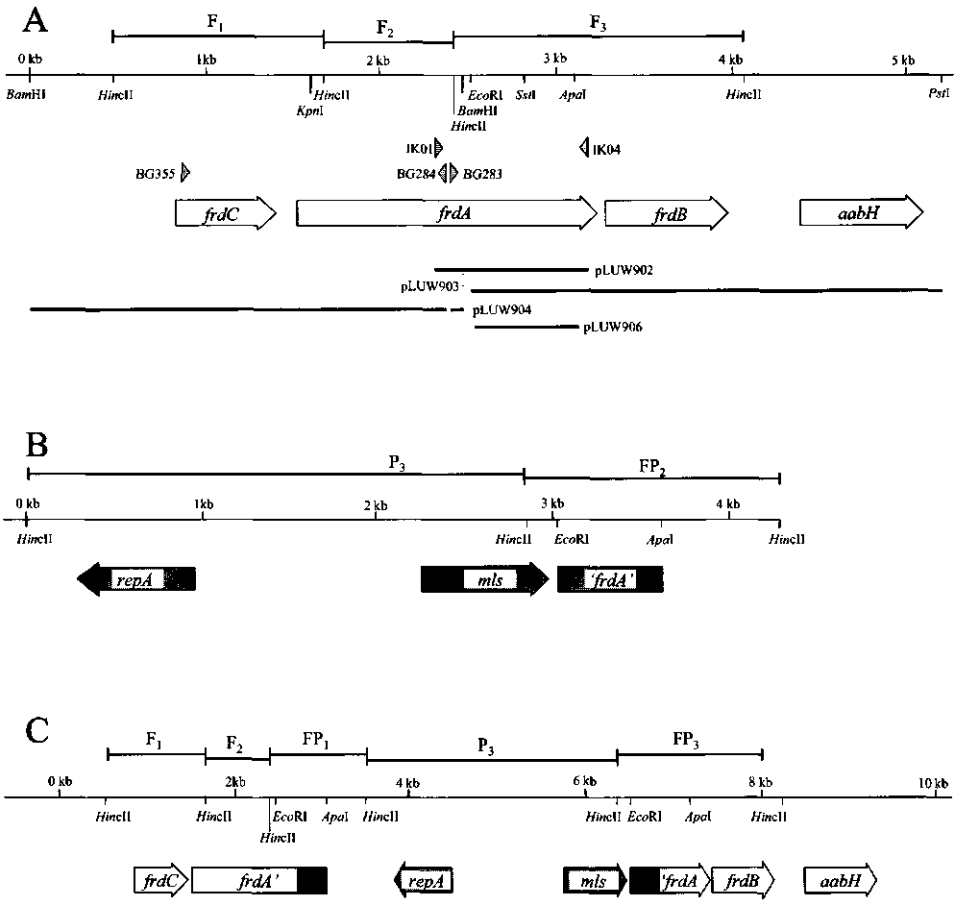

Figure 6.3 Physical maps of the *D. dehalogenans frd* gene locus (**A**), pLUW906 (linearized) (**B**) and the *frd* gene locus after recombination (**C**). Horizontal arrows, open reading frames; triangles, oligonucleotides used in this study; horizontal bars; clones, constructs and hybridizing fragments (F_1 to F_3, fragments hybridizing solely with the *frdAC*-specific probe; P_1 to P_3, fragments hybridizing solely with the pG$^+$host9 -specific probe; FP_1 to FP_3, fragments hybridizing with both probes). DNA restriction sites, which were relevant for the construction of clones and constructs, are indicated.

The subsequent isolation and analysis of a 5.3-kb *Pst*I-*Bam*HI chromosomal fragment from *D. dehalogenans* revealed the presence of three closely linked genes, *frdCAB*, and a fourth open reading frame *aabH*, potentially encoding a polypeptide with significant similarity to ATP-binding cassette transporter binding proteins. The three genes *frdC*, *frdA*, and *frdB* potentially code for polypeptides of 208, 578, and 251 amino acids with calculated molecular masses of 23,728, 64,441, and 28,043 Da, respectively (Fig. 6.3A). The predicted gene products exhibit significant similarities with the type B membrane anchor, flavoprotein, and iron-sulfur-protein subunits of known succinate:quinone oxido-reductases, respectively (Hägerhäll, 1997). The highest similarities were found with the succinate dehydrogenases of *Bacillus subtilis* and *Paenibacillus macerans* (identities on the amino acid level, 72 and 74 % for FrdA; 58 and 59 % for FrdB; and 42 and 29 % for FrdC, respectively). Upstream of each of the genes, potential Shine Dalgarno sequences that are complementary to the 3' end of the *D. dehalogenans* 16S rRNA could be identified (not shown) (Smidt *et al.*, 2000).

Gene Specific Single-Crossover Integration in the *D. dehalogenans* Chromosome

An internal 0.6-kb fragment of the *D. dehalogenans frdA* gene was cloned into pG$^+$host9 in *E. coli* MC1061. The resulting plasmid, pLUW906 (Fig. 6.3B), was introduced by transformation into *D. dehalogenans*, where it was stably maintained at 30°C. Subsequently, cultures of *D. dehalogenans* containing either pLUW906 or pG$^+$host9 were shifted to 40°C to induce single-crossover or spontaneous chromosomal integration, respectively. Single-crossover integration at the *frdA* locus would result in the generation of two chromosomal copies of the *frdA* gene, truncated either at the 3' or the 5' end, and interrupted by the vector (Fig. 6.3C). Integrants were selected as erythromycin-resistant colonies appearing at 40°C, and integration of pLUW906 occurred at a frequency of $3.3 \times 10^{-4} \pm 6.6 \times 10^{-5}$ per cell compared to $4.8 \times 10^{-6} \pm 6.9 \times 10^{-6}$ per cell for pG$^+$host9.

In order to investigate whether the significantly higher number of integration events was due to specific chromosomal integration into the *frdA* gene, pLUW906 integrants were further analyzed at the physiological, biochemical and genetic levels. Southern blot analysis of *Hin*cII-digested total DNA from pLUW906 integrants with radiolabelled *frdAC*- and pG$^+$host9-specific probes revealed the loss of a 1.7-kb wild-type genomic *frdBA* fragment (F_3), whereas two fragments (FP_1 and FP_3) appeared in the integrant DNA, which also hybridized with the pG$^+$host9 probe, as would be expected in the case of specific integration of pLUW906 into the *frdA* gene of *D. dehalogenans* (Figs. 6.3C and 6.4). Furthermore, the

1.4-kb pLUW906 fragment (FP_2) hybridizing with both probes was absent from integrant DNA, indicating the lack of free plasmid (Fig. 6.4). Similar results were obtained by PCR analysis with primers IK04 and BG355, as the 2-kb wild-type amplification product shifted to a distinct integrant-specific 6-kb fragment (data not shown).

Whereas fumarate-dependent growth was not significantly impaired in the pLUW906 integrants grown with lactate as the electron donor and fumarate as the electron acceptor, fumarate reductase activity was reduced, although not completely diminished (0.17 ± 0.01 and 0.09 ± 0.06 U/mg in two independently obtained pLUW906 integrants, compared to 0.36 ± 0.11 U/mg in a pG^+host9 integrant control strain).

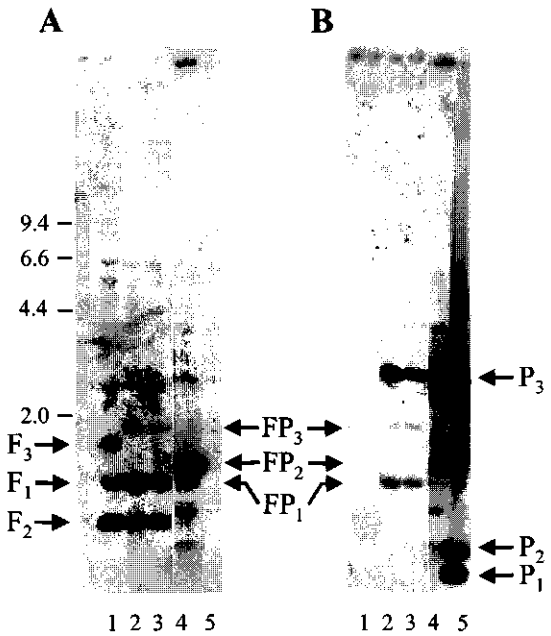

Figure 6.4 Hybridization of *Hin*cII-digested total DNA from wild-type *D. dehalogenans* and pLUW906 integrants of *D. dehalogenans* with probes specific for *frdAC* (**A**) and pG^+host9 (**B**). pG^+host9 and pLUW906 plasmid DNAs was used as controls. The autoradiograph was digitally corrected for differences in signal intensities between lanes containing total DNA and lanes containing plasmid DNA. Lanes: 1, wild-type; 2 and 3, pLUW906 integrants; 4, pLUW906; 5, pG^+host9. DNA size markers are in kilobase pairs. F_1 to F_3, fragments hybridizing solely with the *frdAC*-specific probe; P_1 to P_3, fragments hybridizing solely with the pG^+host9 - specific probe (P_1 and P_2 originate from an additional *Hin*cII-site between the *Eco*RI- and *Apa*I-sites in pG^+host9); FP_1 to FP_3, fragments hybridizing with both probes.

Discussion

The recent detailed molecular analysis of the halorespiratory system in the *o*-CP-respiring Gram-positive bacterium *D. dehalogenans* has brought us to a deeper understanding of structure, function, and control of this novel respiratory pathway (Smidt *et al.*, 1999; van de Pas *et al.*, 1999; Smidt *et al.*, 2000). Previously, we described the development of an efficient plating, delivery, and screening system that has been useful for the isolation of halorespiration-deficient mutants following the chromosomal integration of the conjugative transposon Tn*916* (Smidt *et al.*, 1999). These mutants have been instrumental in the identification of genes potentially encoding polypeptides, which might be involved as structural components of the halorespiratory network or might play a role in their control and functional assembly. However, the instability of some of these mutants and the occurrence of preferential integration has to some extent hampered their further physiological and biochemical characterization. Here we report on the development and validation of host – vector systems for the genetic modification of the environmentally important, strictly anaerobic, low G+C Gram-positive bacterium *D. dehalogenans*.

An efficient electroporation-based transformation procedure was designed using a protocol that had previously been optimized for the high-frequency electrotransformation of *L. lactis* (Wells *et al.*, 1993). Routinely, we obtained 1.0×10^3 to 6.6×10^3 erythromycin resistant transformants per μg of plasmid DNA from the 4.8-kb theta-replicating pAMβ1 derivative pIL253. These values observed for *D. dehalogenans* are in the same range as or higher than transformation frequencies obtained for several other LGB, such as *Clostridium* spp., but are lower than those obtained in genetic model strains of *L. lactis* (Chen *et al.*, 1996; Klapatch *et al.*, 1996; Buckley *et al.*, 1999; Young *et al.*, 1999). Although pIL253 was maintained in *D. dehalogenans* at only moderate copy numbers of approximately 10 copies per cell, compared with 45 to 85 copies for *L. lactis* (Simon & Chopin, 1988; de Vos & Simons, 1994), the stable replication of the vector indicates its potential use as a cloning vector in *D. dehalogenans*.

Plasmids based on the thermosensitive replicon pG$^+$host were previously shown to conditionally replicate in various LGB as well as in *E. coli* (Maguin *et al.*, 1992; Maguin *et al.*, 1996). One of these, the 3.8-kb rolling-circle-replicating, thermosensitive pWV01 derivative pG$^+$host9, was used for the development of a system for specific gene disruption in *D. dehalogenans*. Transformation efficiencies for pG$^+$host9 were on average 1 order of magnitude lower (6×10^2) than those for pIL253. These differences in frequency of

transformation might be due to the difference in the mode of replication, as was previously reported for various other strains of LGB, but could also be caused by differences in marker gene expression (Kiewit *et al.*, 1993; de Vos & Simons, 1994; O'Sullivan & Fitzgerald, 1999). We were able to confirm thermosensitive replication, which was essentially absent at 40°C in *D. dehalogenans*. Although moderate segregational instability was also observed at the permissive temperature of 30°C, the relative number of viable cells able to grow on selective plates was reduced to 2×10^{-5} at 40°C within 7 generations. The nonpermissive temperature that we found for *D. dehalogenans* is somewhat higher than that reported for *L. lactis* (Maguin *et al.*, 1992). However, as *D. dehalogenans* is still growing at almost maximum growth rates at 40°C, this does not affect the applicability of the pG$^+$host system (Utkin *et al.*, 1994).

In order to provide a model target to test the thermosensitive vector pG$^+$host9 for its applicability for specific gene disruption, we cloned and sequenced the putative fumarate reductase-encoding *frdCAB* operon from *D. dehalogenans*. A pG$^+$host9 derivative containing a 0.6-kb internal *frdA* fragment was successfully introduced and maintained in *D. dehalogenans* under permissive conditions. Chromosomal integration at nonpermissive temperatures was significantly more efficient in the case of pLUW906 compared to the empty vector, and the observed integration frequencies were similar to those found for *L. lactis* (Biswas *et al.*, 1993). However, although stable site-specific chromosomal integration of pLUW906 into the *frdA* gene could be unambiguously demonstrated by Southern blot analysis and PCR analysis, the fumarate reductase activity was only partly reduced and no changes in growth with fumarate were observed compared to the growth of *D. dehalogenans* containing pG$^+$host9. One possible explanation could be that at least one of the truncated *frdA* genes present in the pLUW906 integrant is still coding for a (partially) active fumarate reductase enzyme due to a polar effect from the inserted vector sequences. This, however, is rather unlikely, since both the 3'- and the 5'-truncated *frdA* copies lack several conserved residues that are probably essential for fumarate reductase activity (Bourgeron *et al.*, 1995; Hägerhäll, 1997). Another possibility could be that the *frdCAB* operon actually encodes a succinate dehydrogenase. Nevertheless, no significant succinate dehydrogenase activity could be detected in cell extracts of *D. dehalogenans*. Northern analysis of total RNA isolated from cultures of *D. dehalogenans* grown with different electron donors and 3-chloro-4-hydroxy-phenylacetic acid, nitrate, or fumarate as the electron acceptor indicated that transcription of the *frdCAB* operon is constitutive rather than being induced in the presence of fumarate. This,

however, is not in agreement with the highly induced fumarate reductase activity that has been measured in fumarate-grown cells of *D. dehalogenans* (H. Smidt *et al.*, unpublished data). This suggests that the *frdCAB* operon only partially codes for the fumarate reductase activity, which is measured with benzyl viologen as an artificial electron donor. If so, this strongly supports the presence of at least one additional fumarate reductase-encoding gene cluster.

The development of the various gene transfer systems reported here is the first example of a genetic system for a halorespiring microbe. It has significantly improved our possibilities for studying the function and regulation of chromosomal genes in *D. dehalogenans*, including those relevant for the novel halorespiratory pathway this organism possesses. Moreover, the present set of genetic tools will enable the further exploitation of *D. dehalogenans* and related strains as dedicated degraders of recalcitrant environmental pollutants.

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7

DIVERSITY AND EXPRESSION OF REDUCTIVE DEHALOGENASE-ENCODING GENES FROM HALORESPIRING BACTERIA

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A modified version of this chapter has been submitted for publication.

Abstract

A multiple PCR approach was developed for the specific amplification of reductive dehalogenase-encoding genes from anaerobic halorespiring cultures. Multiple sets of specific and highly degenerate oligonucleotide primers were designed based on conserved sequence motifs of known *ortho*-chlorophenol- and chloroethene reductive dehalogenases. Their application yielded the amplification of novel putative reductive dehalogenase-encoding gene homologues from the chromosome of various Gram-positive and -negative halorespiring bacteria from the genera *Desulfitobacterium* and *Dehalospirillum*. RT-PCR analysis of total RNA isolated from *Desulfitobacterium* spp. showed halorespiration-dependent differential expression of the various genes in the presence of different halogenated substrates, indicating that the current approach provides a valuable basis for the molecular monitoring of halorespiration potential and reductive dehalogenase activity.

Introduction

Several anaerobic halorespiring bacteria have been isolated during the last decade from numerous polluted and pristine ecosystems that have the ability to couple the reductive dehalogenation of chlorinated aliphatic and aromatic compounds to energy conservation by electron-transport-coupled phosphorylation and hence to growth (El Fantroussi *et al.*, 1998; Holliger *et al.*, 1999; Smidt *et al.*, 2000a; Smidt *et al.*, 2001a). Such halogenated compounds have been and are still being released into the environment at significant levels due to natural and anthropogenic activities, and their persistence, toxicity and bioaccumulation is of major concern (Hileman, 1993; Gribble, 1996). Because of their high metabolic activities and almost ubiquitous occurrence, it is now widely accepted that halorespiring microorganisms play an important role in the bioremediation of anaerobic environments polluted with halogenated hydrocarbons. The identification and maintenance of optimal process conditions, however, requires the availability of fast and accurate methods for the assessment of microbial degradation potential and activity (Stapleton *et al.*, 1998). As classical microbial cultivation techniques fail to give a complete picture of composition, properties and *in situ* metabolic activity of complex microbial ecosystems, various molecular microbial ecological methods have been developed to comprehensively address these issues (von Wintzingerode *et al.*, 1997; Head *et al.*, 1998). Among these, polymerase chain reaction (PCR) based approaches, targeting not only phylogenetic (rRNA-encoding genes) but also metabolic markers (e.g. degradative enzyme-encoding genes), have proven to be powerful tools to assess the biodegradation potential within a polluted site (Power *et al.*, 1998). Where actual degradation is reflected by the transcription of specific genes, the combination with a reverse transcriptase (RT) reaction can be used for the sensitive detection of mRNA's to monitor activity (Selveratnam *et al.*, 1995; Stapleton *et al.*, 1998; Wilson *et al.*, 1999). Nevertheless, although anoxic processes are often crucial steps in the bioremediation of polluted sites, most work has focused on aerobic pathways (Power *et al.*, 1998).

The potential of halorespiring bacteria for the clean-up of contaminated environments as soils, aquifers and sediments and the novelty of their respiratory pathways has stimulated the unraveling of the (eco-)physiological properties of these strains and triggered efforts to elucidate the molecular basis of halorespiration. This lead to the identification of a novel class of corrinoid-containing reductive dehalogenases as the key enzymes in halorespiration (recently reviewed by Holliger *et al.*, 1999; Smidt *et al.*, 2001a). The detailed biochemical and

molecular characterization of the proteins and their encoding genes has revealed significant similarities at the structural and functional levels between chlorophenol- and chloroethene reductive dehalogenases from phylogenetically distinct microorganisms. For two enzymes, the presence of Fe-S clusters was shown by EPR-spectroscopic analysis (Schumacher *et al.*, 1997; van de Pas *et al.*, 1999). These results could be confirmed in case of the *ortho*-chlorophenol reductive dehalogenase (CprA) from *Desulfitobacterium dehalogenans* by the detection of two highly conserved Fe-S binding motifs in the corresponding primary structure (van de Pas *et al.*, 1999). An almost identical signature was identified in the sequence of the PCE reductive dehalogenase (PceA) from *Dehalospirillum multivorans*, suggesting a similar mechanism of intramolecular electron transport (Neumann *et al.*, 1998). Moreover, both sequences share the presence of twin-arginine (RR) signal peptides, which are characteristic for periplasmic proteins containing complex redox cofactors, and which are cleaved off in the mature proteins (Neumann *et al.*, 1998; van de Pas *et al.*, 1999; Berks *et al.*, 2000).

This study aims at the development of a PCR-based approach for the specific amplification of putative reductive dehalogenase-encoding genes, and the detection of the expression of these genes by RT-PCR in order to provide a basis for the direct molecular assessment of halorespiring potential and activity at polluted sites. Moreover, the gained knowledge on novel sequences will be helpful for the elucidation of structure-function relations within this novel class of environmentally highly relevant biocatalysts, and might provide insight into the evolutionary history of reductive dehalogenases.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

Desulfitobacterium spp. were routinely grown under anaerobic conditions (100% N_2 gas phase) at 37° C in rubber stoppered serum bottles as described previously (Smidt *et al.*, 2000b). Namely, the following strains were used in this study: *D. dehalogenans*, *Desulfitobacterium* PCE1, *D. hafniense*, and *D. frappieri* strains PCP-1, TCE1 and DP7 (Utkin *et al.*, 1994; Bouchard *et al.*, 1996; Christiansen & Ahring, 1996a; Gerritse *et al.*, 1996; Gerritse *et al.*, 1999; van de Pas *et al.*, 2001b). Electron donor and acceptor were added to the appropriate concentration from sterile anaerobic stock solutions. Unless otherwise stated, strains were grown in the presence 20 - 40 mM of pyruvate. *Dehalospirillum*

multivorans strain PCE-M2 was grown in a previously described low-chloride mineral medium, from which yeast extract was omitted, with hydrogen as the electron donor and PCE as the electron acceptor (Holliger *et al.*, 1993; Luijten *et al.*, 2001). All gasses were obtained from Hoek Loos (Schiedam, The Netherlands). 3-chloro-4-hydroxyphenylacetic acid (Cl-OHPA) was purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands) and filtered prior to use. Growth was monitored spectrophotometrically by determining the optical density at 600 nm (A_{600}), and reductive dehalogenase activity in dechlorinating cultures was confirmed by following the release of chloride-ions (Chlor-o-Counter, Marius Instrumenten, Utrecht, The Netherlands).

Table 7.1 Reductive dehalogenase-specific oligonucleotide primers used in this study.

Primer	nucleotide sequence	sense (s) / antisense (as)		target (residue number)
Gene-specific primers				
PS1	5'-GCATTGCTTGGATCCTTTTGACTACGG-3'	s		*cpr* (5469-5485)[a]
PS2	5'-CCGGAATTCGATTATCTGCCTTAGG-3'	as		*cpr* (7353-7329)[a]
PS3	5'-ATGGAAAAGAAAAAAAAGCCTGAACTC-3'	s		*pce* (3272-3298)[a]
PS4	5'-CTTCATCAGCGCTTTGAATTCACTCC-3'	as		*pce* (5146-5121)[a]
PS5	5'-CATCCCCTATAATCAGTGTCTACCG-3'	as		*Dd-rddA* (1002-978)[a]
PS6	5'-CTCATTACCCCAGAATATGGCCCG-3'	s		*Dd-rddA* (1196-1219)[a]
8f	5'-CACGGATCCAGAGTTTGAT(C/T)(A/C)TGGCTCAG-3'	s		16S rRNA (8-27)[b]
1510r	5'-GTGAAGCTTACGG(C/T)TACCTTGTTACGACTT-3'	as		16S rRNA (1513-1492)[b]
Degenerate primers				
D1	5'-AA(C/T)(A/C)G(A/C/G)(A/C)G(A/C/G)AA(C/T)TT(C/T)CT(C/G/T)AA-3'	s	RR	NRRNFLK (13-19)[c]
D2	5'-GCIGA(C/T)AT(A/C/T)GTIGCICC-3'	s		ADIVAP[d]
D3	5'-CA(A/G)GA(A/G)(A/T)(C/G)(C/T)GA(A/G) (A/T)(C/G)(C/T)GCNAT(C/T)GT-3'	s		QESESAIV[d]
D4	5'-GGIGCIGA(C/T)(C/T)TIGTIGG-3'	s	C 1	GADLVG (170-175)[c]
D5	5'-CT(C/G/T)GCNCC(C/G/T)GA(C/T)AA(G/A)CC-3'	s	C 4	LAPDKP (316-321)[c]
D6	5'-GC(A/G)T(A/T)(A/G)TA NCC(A/C/G)AG-3'	as	C 2	LGY(Y/N)A (261-265)[c]
D7	5'-ATIGG(C/T)TT(A/G)TCIGG-3'	as	C 4	PDKPI (318-322)[c]
D8	5'-GC(A/G)CA(C/T)TTYTT(A/G)CA-3'	as	FS 1	CKKCA (333-337)[c]
D9	5'-TCCA(A/C/G)GG(A/G)CANAC-3'	as	FS 2	VCPWN (386-390)[c]

[a]Residue numbers of specific primers are taken from the *D. dehalogenans cpr* cluster- and the *D. multivorans pce* cluster nucleotide sequences (GenBank acc. no. AF115542 and AF022812, respectively) and the *D. dehalogenans rddA* locus. See also Fig. 7.1 for genetic architecture. [b]16S rRNA-targeted primers are numbered according to the *E.coli* sequence; [c]residue numbers of degenerate primer target sequences are taken from the *D. dehalogenans* CprA sequence (GenPept acc. no. AAD44542); [d]primer D2 and D3 are based on the N-terminal sequences of the PCE-reductive dehalogenases purified from *Desulfitobacterium frappieri* TCE1 and *Desulfitobacterium* PCE1, respectively (van de Pas *et al.*, 2001a).

Escherichia coli strains XL1-Blue (Stratagene, La Jolla, Calif.) and JM-109 (Promega, Madison, Wisc.) were used as a host for cloning vectors and were grown in Luria Bertani medium at 37°C (Sambrook *et al.*, 1989). Where appropriate, media were amended with ampicillin (100 µg/ml). The PCR product cloning vectors pGEM-T and pMON38201 (Borovkov & Rivkin, 1997) were obtained from Promega and Monsanto (St. Louis, Mo.) respectively.

DNA Isolation and Manipulation

Total DNA was isolated from *Desulfitobacterium* spp. and *Dehalospirillum multivorans* as described previously (van de Pas *et al.*, 1999). Plasmid DNA was purified from *E. coli* with the QIAprep Spin Miniprep kit (Qiagen GmbH, Hilden, Germany), and standard DNA manipulations were performed according to established procedures (Sambrook *et al.*, 1989) and manufacturers instructions. Enzymes were purchased from Life Technologies B.V. (Breda, The Netherlands), Roche Molecular Biochemicals (Mannheim, Germany) or New England Biolabs (Beverly, Mass.). Oligonucleotides were obtained from Life Technologies B.V. and MWG Biotech (Ebersberg, Germany).

PCR Amplification of Potential Reductive Dehalogenase-Encoding Genes

Primers used for the specific amplification of putative reductive dehalogenase-encoding genes are listed in Table 7.1. PCR amplification was performed from total DNA isolated from the various strains in a 10 to 50-µl PCR reaction mixture containing 0.2 µM of each primer, 2 mM of $MgCl_2$; 200 µM of dATP, dCTP, dGTP, and dTTP; and 1 U of Taq polymerase (Life Technologies B.V.). The DNA was amplified with an UnoII- or T-gradient-Thermocycler (Biometra, Göttingen, Germany). After the mixture was preheated to 94°C for 2 min, 35 amplification cycles were performed, consisting of denaturation at 94°C for 20 s, primer annealing at different temperatures (20°C - 50°C) for 30 s and elongation at 68°C for 1 min. A final extension of 7 min at 68°C was performed. Usually, an annealing temperature of 35°C - 40°C gave optimal amplification of specific products when degenerate primers were used (50°C for specific primers). The PCR products were purified either directly from the reaction mixture or from agarose gel using the QIAquick PCR purification kit (Qiagen GmbH) and the Concert-Matrix gel-extraction kit (Life Technologies B. V.), respectively, and cloned into *E. coli* using pGEM-T or *Xcm*I-digested pMON38201.

To further characterize the *rddA* loci identified in *Desulfitobacterium dehalogenans* and *Desulfitobacterium* PCE1, fragments flanking *rddA* were amplified by inverse PCR with the divergent primer pair PS5 / PS6 (Table 7.1) from *Eco*RI- or *Hin*dIII-digested and re-ligated chromosomal DNA as described previously (Triglia *et al.*, 1988; van de Pas *et al.*, 1999).

RNA Isolation and RT-PCR Amplification of Expressed Reductive Dehalogenase-Encoding Genes

Total RNA was isolated from exponential-phase cultures of *Desulfitobacterium* spp. as described previously (Smidt *et al.*, 2000b). RT-PCR was used to analyze expression of reductive dehalogenase-encoding genes upon growth in the presence or absence of different chlorinated electron acceptors. 50-150 ng of DNAse-treated RNA (RQ1 RNAse-free DNAse, Promega) was used in a 25-µl reaction, containing the following: 5 pmol of each primer, 200 µM dATP, dCTP, dGTP, and dTTP, 1.7 mM $MgSO_4$, 5 µl of AMV/*Tfl* 5× Reaction buffer and 2.5 U of AMV RT and *Tfl* polymerase (Access RT-PCR system, Promega). 16S rRNA-targeted universal primers 8f and 1510r were used in positive and negative control reactions (in negative controls, AMV RT was omitted) (Lane, 1991; Table 7.1). cDNA synthesis and subsequent PCR amplification were performed using a T-gradient-Thermocycler (Biometra). The reaction mixture was incubated at 35°C for 45 min. After the mixture was preheated to 94° C for 2 min, 40 amplification cycles, consisting of: denaturation at 94° C for 30 s, primer annealing at 35° C for 1 min and elongation at 68° C for 2 min were performed. A final extension of 7 min at 68° C was performed. The RT-PCR products were purified from the reaction mixture using the QIAquick PCR purification kit (Qiagen GmbH) and cloned into *E. coli* using *Xcm*I-digested pMON38201.

DNA Sequencing and Sequence Analysis

DNA sequencing was performed using a Li-Cor DNA sequencer 4000L (LiCor, Lincoln, Nebr.). Reactions were performed using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham Pharmacia Biotech.). Fluorescently (IRD 800) labeled universal sequencing primers were purchased from MWG Biotech. Sequence similarity searches and alignments were performed using the BLAST 2.1 program at [http://www.ncbi.nlm.nih.gov/blast/blast.cgi] (Altschul *et al.*, 1997) (National Center for Biotechnology Information, Bethesda, Md.) and the programs Clustal X, GeneDoc (Thompson *et al.*, 1997; K. B. Nicholas & H. B. J. Nicholas, GeneDoc: a tool for editing

multiple sequence alignments, 1997) and the DNAstar package (DNASTAR Inc., Madison, Wis.), respectively.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of the putative reductive dehalogenase-encoding gene loci have been deposited in the GenBank database under accession numbers AY013363 (*D. dehalogenans rddA*), AY013361 (*Desulfitobacterium* PCE1 *rddA*), AY013360 (*Desulfitobacterium* PCE1 *cprBA*), AY013365 (*D. hafniense cprBA*), AY013362 (*D. frappieri* TCE1 *rdfA*), AY013364 (*D. frappieri* PCP-1 *rdfA*), AY013366 (*D. hafniense rdfA*), AY013367 (*Dehalospirillum multivorans* PCE-M2 *pceAB*), and AY013368 (*Dehalospirillum multivorans* PCE-M2 *rdmA*).

Results and Discussion

Detection of Potential Reductive Dehalogenase-Encoding Genes

To date, only two reductive dehalogenases have been characterized at the molecular level, namely the *o*-chlorophenol- and PCE-dechlorinating enzymes from *Desulfitobacterium dehalogenans* and *Dehalospirillum multivorans*, respectively (Neumann *et al.*, 1998; van de Pas *et al.*, 1999). In addition, a partial amino acid sequence has been reported for the *Desulfitobacterium* PCE-S PCE-reductive dehalogenase, the N-terminus of which is almost identical to the *Dehalobacter restrictus* PCE-reductive dehalogenase, but different from that of the *D. multivorans* enzyme (Holliger *et al.*, 1999; Neumann *et al.*, 1999). The comparison of the different known chlorophenol- and chloroalkene-reductive dehalogenase-encoding genes revealed significant similarities in gene architecture, which is reflected by the presence of sequence motifs that are highly conserved at the amino acid level. These include a twin-arginine signal sequence (RR), one ferredoxin-type and one truncated Fe-S cluster (FS1, FS2) and 5 additional highly conserved motifs (C1-C5) (Figs. 7.1A and 7.2). Assuming that the characteristic gene architecture is also conserved in other reductive dehalogenases not yet characterized at the molecular level, degenerate oligonucleotide primers were designed targeting the various conserved motifs to allow for the detection and characterization of yet unknown reductive dehalogenase-encoding genes (Table 7.1, Fig. 7.1A). In addition, we used specific oligonucleotide primers to screen for the presence of *cprBA*- and *pceAB* homologues

in strains of *Desulfitobacterium* spp. and *Dehalospirillum multivorans* that show dechlorinating activities similar to those catalyzed by CprA and PceA (*cprBA*, PS1/PS2; *pceAB*, PS3/PS4; Table 7.1 and Fig. 7.1A).

The application of *cprBA*- specific primers yielded PCR-products of the expected size from the chromosome of Cl-OHPA-respiring *D. dehalogenans*, *Desulfitobacterium* PCE1 and *D. hafniense* DCB-2, whereas a *pceAB*-specific product was obtained for *Dehalospirillum multivorans* PCE-M2 (Table 7.2).

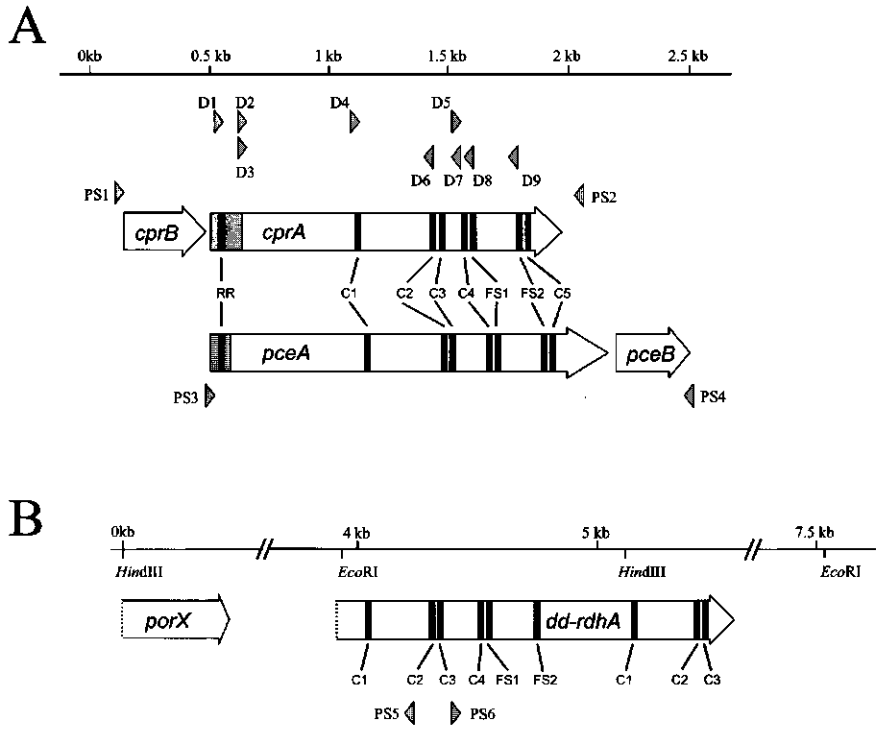

Figure 7.1 A) Sequence motifs conserved among known reductive dehalogenases and position of specific and degenerate oligonucleotide primers used in this study. The architecture is shown of the *D. dehalogenans cprBA* genes (*o*-chlorophenol reductive dehalogenase; van de Pas *et al.*, 1999) and the *D. multivorans pceAB* genes (PCE reductive dehalogenase; Neumann *et al.*, 1998). RR, twin-arginine consensus sequence motif; FS, F-S cluster binding motif; C, conserved sequence motif; PS, gene-specific primers; D, degenerate primers; arrowheads, position and orientation of oligonucleotide primers. **B)** Physical map of *D. dehalogenans* / *Desulfitobacterium* PCE1 *rddA* locus. Restriction sites used for inverse PCR are indicated. *porX*, partial (C-terminal) sequence of putative pyruvate ferredoxin oxidoreductase-encoding gene (Smidt *et al.*, 2001b).

Table 7.2 PCR amplification of partial reductive dehalogenase-encoding genes and their designations from halorespiring bacteria.

Strain	PS1 PS2	PS3 PS4	D4 D7	D4 D8	D2 D7	D1 D8
D. dehalogenans	+(*cprA*)	-	+(*rddA*)	+(*rddA*)	-	+(*cprA*)
D. PCE1	+(*cprA*)	nd	+(*rddA*)	+(*rddA*)	-	+(*cprA*)
D. hafniense	+(*cprA*)	nd	-	-	+ (*rdfA*)	nd
D. frappieri PCP-1	nd	nd	-	-	+ (*rdfA*)	nd
D. frappieri DP7	nd	nd	-	-	-	nd
D. frappieri TCE1	nd	nd	-	-	+ (*rdfA*)	-
D. multivorans PCE-M2	nd	+(*pceA*)	+(*rdmA*)	+(*pceA*)	-	nd

nd, not determined; +, specific PCR-product; -, no PCR-product.

Amplification by PCR using different pairs of degenerate primers in combination with chromosomal DNA of halorespiring strains of *Desulfitobacterium* spp. and *Dehalospirillum multivorans* PCE-M2 resulted in specific products (Table 7.2). However, not all potential primer combinations gave rise to specific PCR amplification products, and combinations that included a C3-targeting degenerate primer did not yield a product for any of the tested strains (data not shown, Table 7.2). No PCR-product was obtained in case of *D. frappieri* DP7, a non-dehalogenating isolate that was recently obtained from human faeces, indicating that its inability to perform halorespiration is caused by a deficiency in dehalogenase-related sequences (van de Pas *et al.*, 2001b).

Molecular Characterization of Reductive Dehalogenase-Specific Amplification Products

Cloning and sequence analysis revealed that *cprBA*-specific amplification products obtained from total DNA isolated from *Desulfitobacterium* strain PCE1 and *Desulfitobacterium hafniense* were almost identical (> 99% on the amino acid level for CprA and CprB) to those of *D. dehalogenans* (Figs. 7.2 and 7.3). Similarly, the PS3/PS4-amplicon obtained from the chromosome of the newly isolated *Dehalospirillum multivorans* strain PCE-M2 was found to be highly similar to the type-strain genes (PceB, > 99% identity on the amino acid level; PceA, 92% identity).

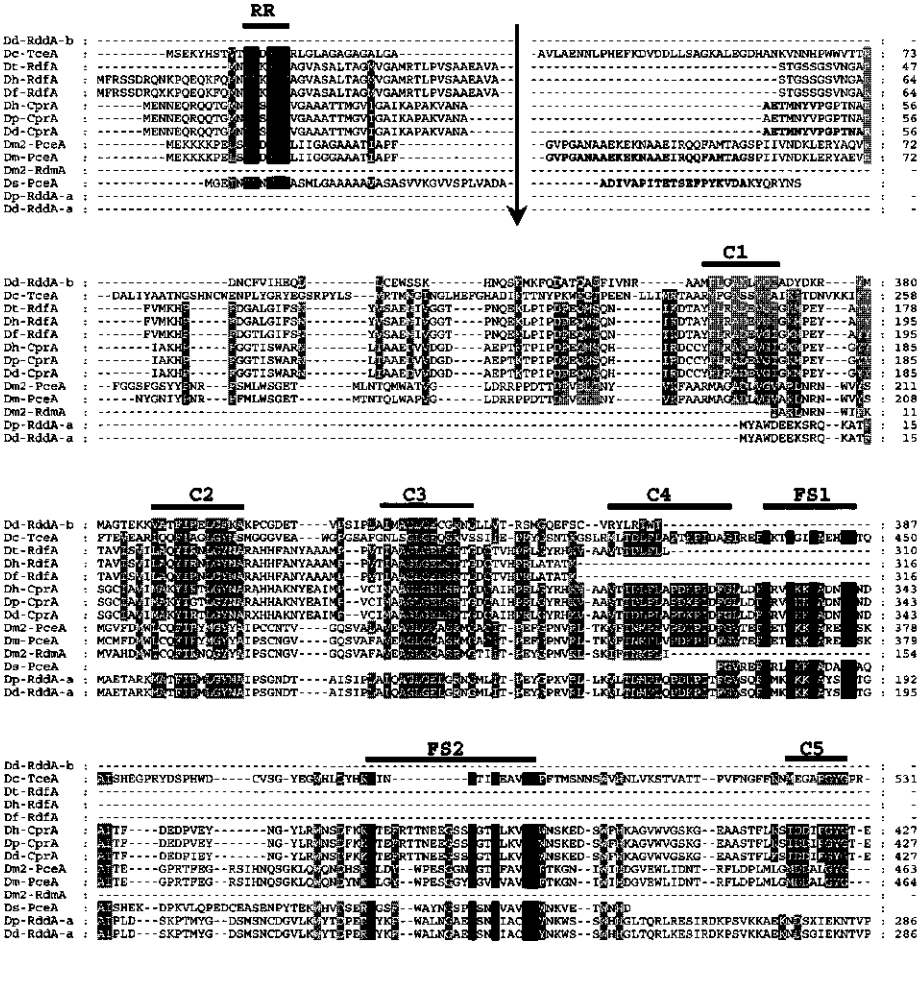

Figure 7.2 Partial amino acid sequence alignment of known and putative reductive dehalogenases. Black boxes, consensus residues of twin arginine leader peptide and Fe-S cluster binding motifs; gray boxes, conserved residues; vertical arrow, apparent and predicted leader-peptide cleavage sites; bold face, N-terminal amino acid sequences as determined from the purified proteins. Prediction of cleavage sites was performed with the SignalP V1.1 program at [http://www.cbs.dtu.dk/services/SignalP/] (Nielsen *et al.*, 1997). Conserved sequence motifs (C1-C5, FS1, FS2 and RR) are indicated by horizontal bars. Dd-CprA, *D. dehalogenans o*-CP reductive dehalogenase (Rdh) (acc. no. AAD44542); Dh-CprA, *D. hafniense* CprA-homologue; Dp-CprA, *Desulfitobacterium* PCE1 CprA-homologue; Dc-TceA, *Dehalococcoides ethenogenes* TCE-Rdh (acc. no. AAF73916); Ds-PceA, *Desulfitobacterium* PCE-S PCE-Rdh partial sequence (Neumann *et al.*, 1999); Dm-PceA, *Dehalospirillum multivorans* PCE-Rdh (acc. no. AAC60788); Dm2-PceA, *Dehalospirillum multivorans* PCE-M2 PceA-homologue; Dm2-RdmA, *Dehalospirillum multivorans* PCE-M2 Rdh homologue; Dd-RddA-a, b, *D. dehalogenans*, Rdh homologue, N- and C-terminal homologous domain; Df-RdfA, *D. frappieri* PCP-1, Rdf homologue; Dh-RdfA, *D. hafniense*, Rdh homologue; Dp-RddA, *Desulfitobacterium* PCE1, Rdh homologue; Dt-RdfA, *D. frappieri* TCE1, Rdh homologue.

The very high level of sequence identity of the newly characterized operons to the respective genes from *D. dehalogenans* and *D. multivorans* is not surprising, as it reflects highly similar *in vivo* dehalogenating activities of the organisms (Christiansen & Ahring, 1996a; Gerritse *et al.*, 1996; Luijten *et al.*, 2001). Unexpectedly, the *D. hafniense cprA* gene product exhibits the same two conserved Fe-S cluster binding motifs as does CprA from *D. dehalogenans* and *Desulfitobacterium* PCE1, which contradicts the previously assumed presence of three such clusters in the *D. hafniense* Cl-OHPA reductive dehalogenase (Christiansen *et al.*, 1998). It is unlikely that the *D. hafniense cprBA* gene cluster described here encodes a yet different protein, as the N-terminus of the deduced CprA is almost identical to that reported for the purified enzyme (only 5 / 24 mismatches, Fig. 7.2).

Sequence analysis of PCR-products obtained from the application of degenerate primers revealed that the chosen set of oligonucleotides yielded the amplification of diverse known and novel potential reductive dehalogenase (Rd)-encoding genes from phylogenetically distinct halorespiring microorganisms (Figs. 7.2 and 7.3).

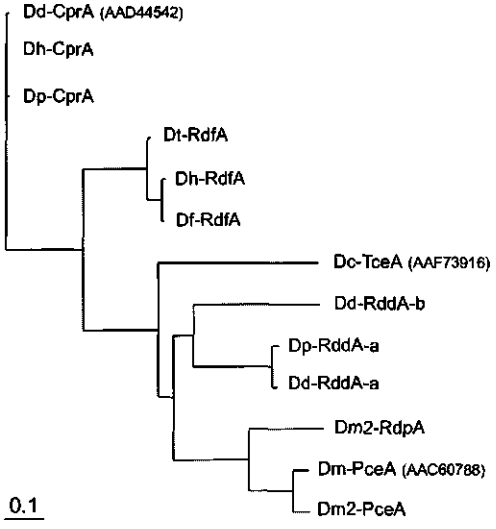

Figure 7.3 Phylogenetic tree of deduced partial amino acid sequences of reductive dehalogenases. The tree was constructed with sequences between conserved motifs C1 and C4 (see also Figs. 7.1 and 7.2). Multiple sequence alignment and construction of the phylogenetic tree using the neighbor-joining method (Saitou & Nei, 1987) were performed using the Clustal X (Thompson *et al.*, 1997) and TreeView (Page, 1996) programs. The reference bar indicates 10 amino acid exchanges per 100 amino acids. For names of sequences, see Fig. 7.2.

Novel genes, for which no unambiguous evidence on their function could be obtained, were designated here according to their source (i.e. *rdfA*, *D. frappieri* / *hafniense* cluster; *rddA*, *D. dehalogenans* / PCE1 cluster; *rdmA*, *Dehalospirillum multivorans*). Moreover, in several cases, the molecular characterization of the obtained PCR-products unveiled the presence of more than one potential reductive dehalogenase-encoding gene homologue on the chromosome of halorespiring bacteria. Amplification with primer-pairs D4/D7 and D4/D8 in combination with chromosomal DNA of the PCE-dehalogenating *Dehalospirillum multivorans* PCE-M2 resulted in two, non-identical, amplicons, namely the above-described *pceA* - homologue, Dm2-*pceA*, and a novel reductive dehalogenase homologue, referred to as Dm2-*rdmA*, that only shares 66 % identity with PceA on the amino acid level (Figs. 7.2 and 7.3).

The same primer pairs (D4/D7 and D4/D8) unexpectedly not resulted in the amplification of the *cprA* gene from the chromosome of *D. dehalogenans* and *Desulfitobacterium* PCE1, but rather a novel reductive dehalogenase gene homologue (*rddA*), which revealed highest levels of sequence identity with the PCE-reductive dehalogenase from *D. multivorans* (34% on the amino acid level for the region from C1 and C4; Figs. 7.2 and 7.3). C1-upstream- and C4-downstream sequences were cloned and sequenced following their amplification by inverse PCR. This revealed the presence of a 1.5-kb open reading frame, assuming a TTG as alternative start-codon. Remarkably, the deduced 54-kDa polypeptide is composed of two domains, RddA-a and RddA-b, both sharing significant similarity with known reductive dehalogenases (Figs. 7.1B and 7.2). It is of interest to note that PCR with the various reverse primers in combination with D3, based on the N-terminal amino acid sequence of a PCE-reductive dehalogenase isolated from *Desulfitobacterium* PCE1, did not yield any amplification products from *Desulfitobacterium* PCE1 genomic DNA (van de Pas *et al.*, 2001a).

Several strains of the *D. frappieri* / *D. hafniense* cluster have been isolated that either reductively dehalogenate chlorinated alkenes (strain TCE1), chlorinated phenolic compounds (strains PCP-1 and DCB-2) or are not able to grow by halorespiration (strain DP7) (Bouchard *et al.*, 1996; Christiansen & Ahring, 1996a; Gerritse *et al.*, 1999; van de Pas *et al.*, 2001b). With genomic DNA from strains TCE1, PCP-1 and DCB-2, only PCR with the degenerate primers D7 or D8 in combination with D2, designed based on the N-terminal amino acid sequence of the previously purified *D. frappieri* TCE1 PCE reductive dehalogenase, yielded the amplification of a novel reductive dehalogenase-encoding gene homologue, termed *rdfA*.

However, no product was obtained for the closely related non-dehalogenating *D. frappieri* strain DP7. Sequence analysis of the obtained PCR-products revealed that the D2-primer sequence could not be retrieved from the amplicons, and PCR with D7 or D8 alone revealed the amplification of the same products as were obtained in the presence of D2. However, all 3 almost identical *rdfA* genes did contain a RR-signal sequence, and well-conserved potential ribosome binding sites could be identified upstream of the respective ATG-start codon of the PCP-1 and DCB-2 genes (data not shown). Highest sequence similarity was observed with *ortho*-chlorophenol reductive dehalogenase-encoding *cprA* (63% on the amino acid level for C1-C4, Fig. 7.3). It is tempting to speculate that the *D. frappieri rdfA* gene codes for the *meta-/para*-chlorophenol reductive dehalogenase, which is functional in *D. frappieri* PCP-1 and, probably to a lesser extent, might also be active in *D. hafniense* (Christiansen & Ahring, 1996b; Dennie *et al.*, 1998). Interestingly, the *D. frappieri* TCE1 *rdfA* gene product was found to be N-terminally truncated by 17 amino acids due to a frame-shift, also resulting in the lack of a well-conserved ribosome binding site (not shown, Fig. 7.2). This might be the reason for lack of chlorophenol-dehalogenating activity in this organism (Gerritse *et al.*, 1999).

Detection of Reductive Dehalogenase-Gene Expression by RT-PCR

It has previously been shown by Northern blot- and primer extension analysis, that activity of the *ortho*-chlorophenol reductive dehalogenase is induced at the transcriptional level in *D. dehalogenans* (Smidt *et al.*, 2000b). Moreover, enzyme activity measurements indicated, that *o*-chlorophenol- and PCE-reducing activities are differentially expressed in cultures of *Desulfitobacterium* PCE1 grown in the presence of the respective halogenated substrates (van de Pas *et al.*, 2001a). Similarly, induction studies on the pentachlorophenol-degrading *D. frappieri* PCP-1 revealed that the differential induction of *ortho-* and *meta/para*-dehalogenating systems was dependent on *de novo* protein biosynthesis, as no induction was observed in the presence of chloroamphenicol (Dennie *et al.*, 1998). To enable not only the detection of potential reductive dehalogenase-encoding genes, but also their expression, and, hence, activity of the produced enzymes, we tested the applicability of the previously developed multiple sets of degenerate primers in a reverse transcriptase-PCR (RT-PCR) based approach. The closely related strains *D. dehalogenans* and *Desulfitobacterium* PCE1, which only differ in their ability to utilize PCE efficiently as electron acceptor, were grown either by fermentation of pyruvate (20 mM) or under halorespiring conditions with 20 mM of lactate as

the electron donor and 20 mM of Cl-OHPA as the electron acceptor. *Desulfitobacterium* PCE1 was in addition also grown in the presence of 10 mM of PCE as the electron acceptor. RT-PCR amplification with degenerate primers D5 and D9 revealed the differential halorespiration-specific expression of reductive dehalogenase-encoding genes in the Cl-OHPA-respiring cultures of *D. dehalogenans* and *Desulfitobacterium* PCE1, whereas no amplification product was obtained from total RNA isolated from pyruvate-fermenting and PCE-respiring cultures (Fig. 7.4, left panel). Cloning and sequence analysis of the respective amplicons confirmed the specific expression of *cprA* in both strains in the presence of the chlorophenolic substrate Cl-OHPA, as it was already demonstrated for *D. dehalogenans* by detailed transcriptional analysis (Smidt *et al.*, 2000b).

Application of degenerate primer pair D4 / D7 yielded a specific RT-PCR product from total RNA isolated from PCE-respiring cultures of *Desulfitobacterium* PCE1, whereas only background-amounts of amplification product were obtained from Cl-OHPA-respiring and pyruvate-fermenting cultures (Fig. 7.4, right panel). Molecular analysis of the PCE-respiration specific amplicon unveiled that the *Desulfitobacterium* PCE1 *rddA* gene was specifically expressed under PCE-respiring conditions. Additional experiments that aim at the determination of the start-site of *rddA* transcription are currently on their way and might help us to further understand the mechanisms involved in halorespiration-specific gene expression.

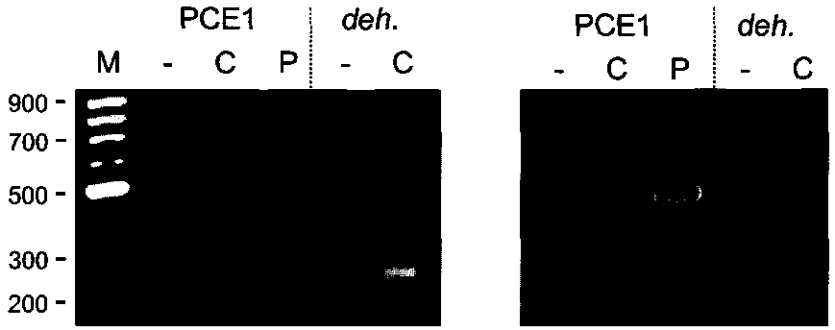

Figure 7.4 RT-PCR analysis of reductive dehalogenase-gene expression in *Desulfitobacterium* PCE1 and *D. dehalogenans* (*deh.*) under fermentative and halorespiring conditions. Left panel, primers D5/D9; right panel, primers D4/D7. **M**, DNA-size marker (in basepairs); **-**, growth by pyruvate fermentation; **C**, lactate + Cl-OHPA; **P**, lactate + PCE.

Conclusions

We here for the first time report on a reductive dehalogenase-encoding gene-targeted PCR / RT-PCR-based approach that allows for the detection of halorespiration potential and activity. We could show that the application of multiple pairs of highly degenerate primers designed based on sequence motifs conserved among known reductive dehalogenases yielded the PCR-amplification of yet unknown potential reductive dehalogenase-encoding genes from halorespiring cultures. Experiments are currently on their ways that aim at the application of the developed array of degenerate primers to reductively dehalogenating consortia and environments. The halorespiration-specific differential expression of known and yet undescribed genes could be shown by RT-PCR using the same set of degenerate primers, indicating that this approach will be useful for the monitoring of fate and activity of halorespiring microorganisms during biological *in situ* remediation processes of anoxic environments polluted with chlorinated compounds. In addition, the gained knowledge provides a solid basis for the further elucidation of structure, function and regulation of this novel family of reductive dehalogenases and will help us to understand evolution of halorespiration.

Acknowledgments

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8

General Discussion

Ecophysiological and molecular ecological studies of the past decade have indicated that halorespiring bacteria are the key players in the reductively dehalogenation process in anoxic microbial ecosystems. They have been isolated from different polluted and pristine anaerobic environments, such as granular sludges, estuarine- and freshwater sediments. Based on thermodynamic considerations, hydrogenotrophic halorespiring bacteria should be able to out-compete hydrogenotrophic sulfate reducers and methanogens in environments where hydrogen is the main source of electrons (Fennell & Gossett, 1998; Löffler *et al.*, 1999). Hence, tight syntrophic interactions of hydrogenotrophic reductively dechlorinating bacteria with H_2-producing microorganisms (that, due to thermodynamic limitations, depend on low H_2-partial pressures) are highly feasible. Indeed, at low concentrations of hydrogen enrichment cultures have been obtained, in which dechlorination was specifically stimulated on the expense of reduced methanogenesis (Smatlak *et al.*, 1996; Ballapragada *et al.*, 1997; Fennell *et al.*, 1997; Fennell & Gossett, 1998). Moreover, the recent discovery of acetotrophic halorespiring bacteria in estuarine and river sediments has once more indicated the environmental relevance of these microorganisms in diverse ecosystems (Krumholz *et al.*, 1996; Löffler *et al.*, 2000; Sun *et al.*, 2000).

This thesis describes molecular approaches aiming to gain insight in the composition, function and regulation of the halorespiratory network in the versatile *ortho*-chlorophenol degrading strictly anaerobic Gram-positive *Desulfitobacterium dehalogenans*. In addition, it describes the development of molecular tools for the further exploitation of halorespiring microorganisms as dedicated degraders in biological remediation approaches.

Molecular Characteristics of Reductive Dehalogenases

Reductive dehalogenases are key enzymes in the respiratory network of dehalogenating microbes. The *ortho*-chlorophenol reductive dehalogenase from *D. dehalogenans* is a membrane-associated corrinoid- and Fe-S containing protein, which is encoded by the *cprBA* operon. The catalytic subunit of the enzyme, CprA, is produced as a pre-protein, containing a twin arginine signal sequence that is characteristic for extracytoplasmic redox-complexes. The predicted function of the hydrophobic *cprB* gene product is that of a membrane anchor for the catalytic subunit. Activity of the enzyme is strongly induced at the transcriptional level by the addition of 3-chloro-4-hydroxyphenylacetate (van de Pas *et al.*, 1999; Smidt *et al.*, 2000). The characterization of different chlorophenol- and chloroalkene reductive dehalogenase-encoding genes has revealed the presence of several highly conserved sequence motifs, reflecting mechanistic and structural similarities between dehalogenating enzymes from phylogenetically distinct bacteria. The similarities include a twin arginine signal sequence, one ferredoxin-type and one truncated iron-sulfur cluster-binding motif. In addition, several residues are highly conserved, among which various histidine and tryptophan residues that might play a role in binding of the corrinoid cofactor or stabilization of the leaving halide, respectively. As only a few hypothetical proteins with unknown function to some extend share these sequence similarities, it has been suggested that reductive dehalogenases comprise a novel class of corrinoid-containing Fe-S proteins (see Chapter 1 of this thesis). Additional support for this hypothesis has recently been obtained from the analysis of the complete genome of the chloroaliphate-respiring *Dehalococcoides ethenogenes* (Preliminary sequence data was obtained from The Institute for Genomic Research website at http://www.tigr.org). This revealed the presence of at least 17 putative reductive dehalogenase-encoding gene homologues (*rdhAB*; Fig. 8.1). One of these is *tceAB*, encoding the trichloroethene reductive dehalogenase that has previously been characterized at the biochemical level (Magnuson *et al.*, 1998; AAF73916). The identified multiple paralogs are organized in at least two major clusters of six and four *rdhAB*-operons, respectively (Fig. 8.1). The vast majority of the above-mentioned conserved sequence motifs and residues are also present in the *D. ethenogenes* homologues, confirming their potential importance for structure and function of these enzymes.

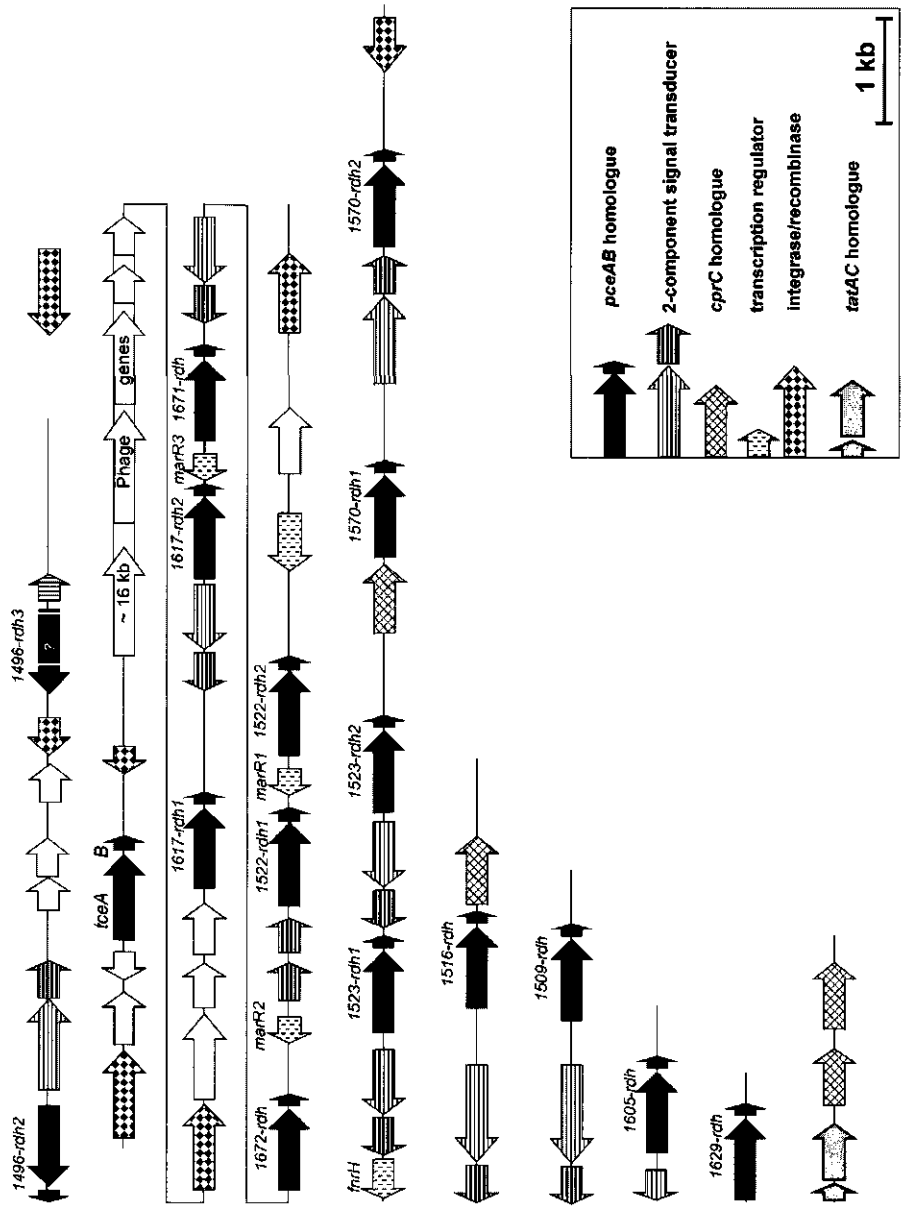

Figure 8.1 Putative reductive dehalogenase-encoding gene containing genomic loci in *Dehalococcoides ethenogenes* (Preliminary sequence data was obtained from The Institute for Genomic Research website at http://www.tigr.org). Putative open reading frames were identified using the DNAstar package (DNASTAR Inc., Madison, Wis.), and sequence similarity searches were performed using the BLAST, version 2.0, program (Altschul *et al.*, 1997) (TIGR Blast search engine for unfinished microbial genomes; and National Center for Biotechnology Information, Bethesda, Md.).

In contrast to the significant sequence similarities between the different reductive dehalogenase-encoding genes from phylogenetically distinct halorespiring bacteria, the genetic context of the genes is not conserved, indicating that recent horizontal transfer between species is rather unlikely (see below).

In order to allow for the further elucidation of structure-function relations in reductive dehalogenases, heterologous expression systems will be required to enable (i) the production of sufficient quantities of active enzyme for the determination of the 3-dimensional structure, and (ii) the analysis of modified enzymes that have been altered by site-directed and random mutagenesis approaches. Earlier attempts to use *Escherichia coli* as expression host failed, probably due to non-functional assembly of the overexpressed polypeptide (Neumann *et al.*, 1998). However, the development of host-vector systems for *D. dehalogenans* now provides a promising basis for a heterologous expression system for enzymes from other, closely related low G+C Gram-positive *Desulfitobacterium* and *Dehalobacter* species (Chapter 6 of this thesis). Moreover, the analysis of codon usage in *Dehalococcoides ethenogenes* suggests that functional expression of *D. ethenogenes* genes in *Desulfitobacterium* should be feasible. In recent experiments, an extrachromosomal copy of the *D. dehalogenans cprBA* could be introduced and stably maintained in *D. dehalogenans* using the broad host-range cloning vector pIL253. Nevertheless, overexpression could not yet be unambiguously demonstrated, and efforts to clone the *D. multivorans* PCE-M2 *pceAB*-operon in *D. dehalogenans* did not succeed. In this case, however, toxic effects of the construct could not be excluded, as also cloning of this operon in *Lactococcus lactis* MG1614 appeared not to be possible (Smidt *et al.*, unpublished results). These findings indicate that future cloning strategies should include alternative (shuttle-) vectors and well-characterized, inducible indigenous promoters.

Topology of the Reductive Dehalogenase Complex

The molecular analysis of the *D. dehalogenans cpr* gene cluster and the *Dehalospirillum multivorans pceAB* operon revealed cotranscription of the catalytic subunit-encoding genes *cprA* and *pceA* with the *cprB* and *pceB* genes, respectively, which code for small hydrophobic integral membrane proteins. This led to the assumption that the hydrophobic polypeptide might act as a membrane anchor for the reductive dehalogenase (Neumann *et al.*, 1998; van de Pas *et al.*, 1999; Smidt *et al.*, 2000). Remarkably, all putative reductive dehalogenase-encoding genes identified from the *Dehalococcoides ethenogenes* genome show the same close physical linkage with genes that code for hydrophobic proteins (Fig. 8.1). As CprB, they

all are predicted to contain three transmembrane helices, whereas the *Dehalospirillum multivorans* PceB is composed of only two such helices, which might explain the observed differences in the degree of membrane association (Smidt *et al.*, 2001). Furthermore, it has been speculated that association with the membrane anchor PceB might prevent Sec-independent translocation of the RR-protein PceA by the twin-arginine translocon (TAT), which then would solely play a role in enzyme maturation (Neumann *et al.*, 1998). However, reductive dehalogenase activity could recently be detected in whole cells with the membrane-impermeable artificial electron acceptor methyl viologen for *D. dehalogenans* *o*-chlorophenol reductive dehalogenase as well as for *D. ethenogenes* PCE- and TCE reductive dehalogenases (Nijenhuis & Zinder, 2000; van de Pas *et al.*, 2001b). This is indicative for an at least partial accessibility of the enzymes' electron-accepting site at the extracytoplasmic face of the membrane, suggesting that the TAT-system is also involved in enzyme translocation (see below; Figs. 8.3 and 8.4). The use of export reporters, such as the pFUN vector based on the *Staphylococcus aureus* secreted nuclease as a reporter for Gram-positive bacteria might be helpful in the further elucidation of the enzymes' topology (Poquet *et al.*, 1998).

Yet another approach is the identification and functional characterization of components of the TAT-system present in halorespiring bacteria. Interestingly, one single cluster of *tatA*- and *tatC*-homologues is present in the *D. ethenogenes* genome, where it is located upstream of two genes, encoding for proteins with significant similarity to the putative membrane bound regulatory protein CprC from *D. dehalogenans* (Fig. 8.1, see below). Efforts to amplify *tatA/E* and *tatC*-homologuous genes from the chromosome of *D. dehalogenans* with degenerate primers were not yet successful. A functional TAT-system should however be present in *D. dehalogenans* since not only the reductive dehalogenase catalytic subunit is produced with a RR-signal sequence, but also the small subunit of a periplasmic Fe/Ni-hydrogenase (HydA) (Smidt *et al.*, 1999). It has been shown in various bacteria that the presence of a TAT-system is essential for translocation of a functional H_2ase-complex (Sargent *et al.*, 1998; Gross *et al.*, 1999; Bernhard *et al.*, 2000).

Evolution of Reductive Dehalogenases

The remarkable phylogenetic diversity of halorespiring microorganisms has brought up the question of the evolutionary origin of the unique ability to respire haloorganic compounds. The molecular characterization of the key enzymes of this process, the reductive dehalogenases, indicated the presence of highly conserved potentially function-related

sequence motifs. It is thus most likely that halorespiration is an evolutionary ancient process involved in the degradation of naturally produced chlorinated compounds, rather than a recent development triggered by anthropogenic release of halogenated hydrocarbons into the environment. The phylogenetic analysis of the deduced primary structures of the different *Dehalococcoides ethenogenes* reductive dehalogenase encoding gene homologues (*rdhAB*) revealed that multiple alleles of *rdhAB*, which share only a moderate degree of identity at the amino acid sequence level (≤ 53% for RdhA and ≤ 71% for RdhB), are grouped in two major clusters (Fig. 8.2). These results suggest that rather ancient recombination events, including gene duplications, are probably responsible for the presence of multiple genes. Furthermore, spreading by lateral gene transfer between closely related species might also be an explanation for the occurrence of multiple copies.

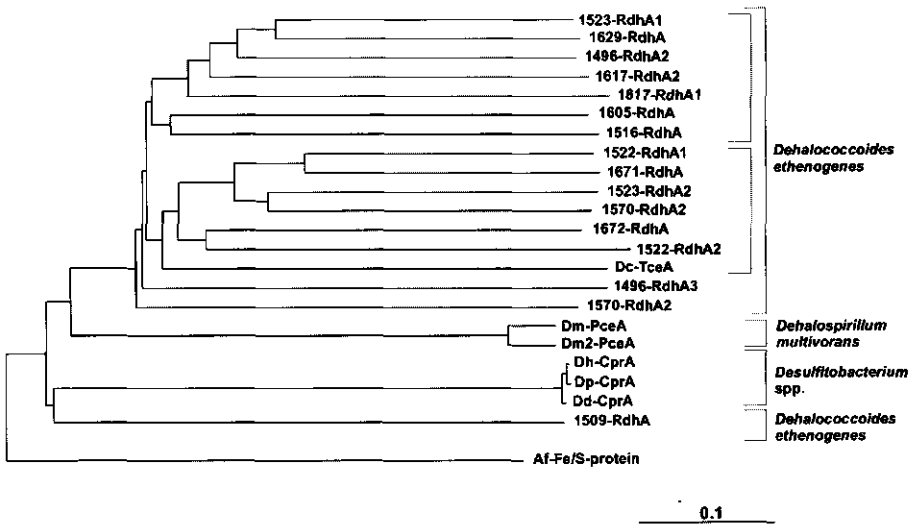

Figure 8.2 Phylogenetic tree of deduced amino acid sequences of apparent and putative reductive dehalogenase (Rdh)-encoding genes from halorespiring bacteria. Multiple sequence alignment and construction of the phylogenetic tree using the neighbor-joining method (Saitou & Nei, 1987) were performed using the Clustal X (Thompson *et al.*, 1997) and TreeView (Page, 1996) programs. Dd-CprA, *Desulfitobacterium dehalogenans ortho*-chlorophenol-Rdh (AF115542); Dh-CprA, *D. hafniense* CprA-homologue; Dp-CprA, *D.* PCE-1 *ortho*-chlorophenol-Rdh; Dm-PceA, *Dehalospirillum multivorans* PCE-Rdh (AF022812); Dm2-PceA, *Dehalospirillum multivorans* PCE-M2 PceA-homologue; Dc-TceA, *Dehalococcoides ethenogenes* TCE-Rdh (AAF73916); RdhA, putative Rdh deduced from the *D. ethenogenes* genome; Af-Fe/S-protein, *Archaeoglobus fulgidus* putative Fe-S cluster binding protein (AAB91256). The Af-Fe/S-protein sequence was used as an outgroup for tree-rooting. The reference bar indicates 10 amino acid exchanges per 100 amino acids.

Several phenotypes that have been under recent evolutionary pressure, like dehalogenation in aerobic microorganisms, antibiotic resistance and heavy metal resistance, are encoded on mobile genetic elements including plasmids and transposable elements (Ochman *et al.*, 2000). Such a mechanism of lateral gene transfer can not be excluded for halorespiration. It might be interesting to note that several of the *Dehalococcoides ethenogenes* putative reductive dehalogenase-encoding gene clusters are flanked by sequences with some similarity to bacterial integrase- and transposase-encoding genes (Fig. 8.1). The relatively low similarity with proteins from other halorespiring bacteria, namely *Dehalospirillum multivorans* PceAB (≤ 23% for PceA and ≤ 18% for PceB) and *Desulfitobacterium dehalogenans* CprBA (≤ 20% for CprA and ≤ 18% for CprB) suggests that recent acquisition by lateral gene transfer from phylogenetically distinct genera is not very likely.

Regulation of Halorespiration

The detailed molecular analysis of the *D. dehalogenans cpr* gene cluster as well as the characterization of halorespiration-deficient mutants suggested the involvement of transcriptional regulators and environmental sensors in the regulation of halorespiration activity. Moreover, the halorespiration-specific expression of genes, encoding molecular folding catalysts, such as GroEL-like chaperonins (CprD, CprE) and Trigger factor (CprT), was observed (Smidt *et al.*, 2000). The expected role of the various proteins in *D. dehalogenans* has been summarized in a working model for the mechanisms of regulation and functional assembly of the reductive dehalogenase complex (Fig. 8.3).

Comparison of three apparent and putative halorespiration-induced promoters indicated that these might be under the control of an FNR-like transcriptional regulator, possibly encoded by the *cprK* gene (Fig. 8.3) (Smidt *et al.*, 2000). Similarly, putative transcriptional regulator-encoding genes were also identified in the vicinity of reductive dehalogenase-encoding alleles of *Dehalococcoides ethenogenes*, including *fnrH*, encoding the only FNR-like transcriptional regulator-gene present in the entire genome of this halorespiring bacterium (Fig. 8.1). In addition, three genes (*marR1-3*) were found, which potentially code for proteins of the MarR-family of phenolic compound-sensing transcriptional regulators (Sulavik *et al.*, 1995). It might be interesting to note that recent studies revealed the involvement of two FNR-CRP-like proteins (AadR, HbaR) and one MarR-type regulator (BadR) in the regulation

of anaerobic 4-hydroxybenzoate –and benzoate degradation in *Rhodopseudomonas palustris* (Egland & Harwood, 1999; Egland & Harwood, 2000).

Halorespiration-specific expression in *D. dehalogenans* was also observed for *cprC*, coding for a protein with significant similarity to putative membrane-bound transcriptional regulators of the NirI / NosR-family (Smidt *et al.*, 2000). A striking analogy of NirI / NosR / CprC containing gene clusters is the presence of genes, which are predicted to code for RR-proteins. Like the reductive dehalogenase catalytic subunit (CprA), also the nitrous oxide reductase catalytic subunit NosZ is produced with a RR-signal sequence, and functional assembly was impaired in a translocation-deficient NosZ mutant (R20D) (Glockner & Zumft, 1996). Moreover, *nirI* and *nosR* are cotranscribed with the *nirX* and *nosX* genes, respectively, probably encoding RR-proteins that might be involved in functional assembly of the redox-centers of the respective catalytic subunits, NirS and NosZ (Saunders *et al.*, 2000). Finally, *napH*, encoding a topological homologue of CprC, is clustered with the *napA* gene that is coding for the RR-leader-containing periplasmic nitrate reductase in *Escherichia coli* (Berks *et al.*, 1995).

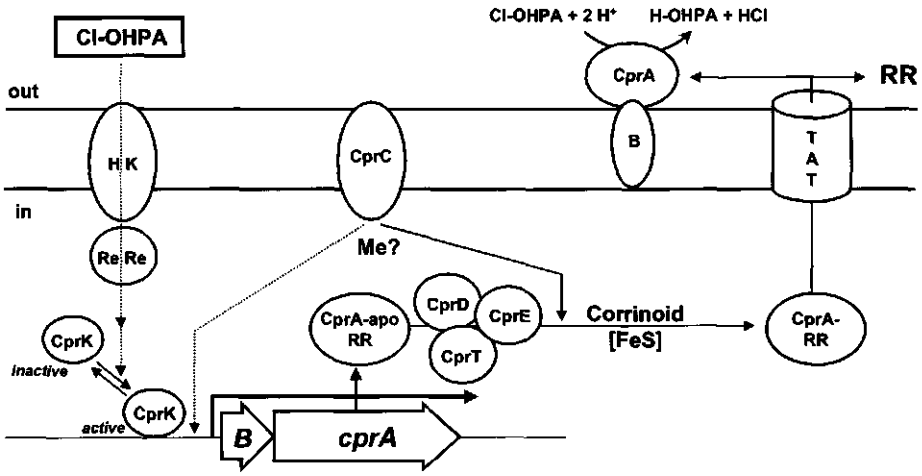

Figure 8.3 Model for the regulation and functional assembly of *ortho*-chlorophenol reductive dehalogenase complex in *D. dehalogenans*. Abbreviations: Me, metal-ions that may be involved in control of gene expression and are incorporated in the mature enzyme complex; HK, histidine kinase; ReRe, response regulator; RR, twin arginine signal sequence; TAT, twin arginine-specific translocon. Dotted lines indicate anticipated signal transduction.

A total of four CprC-like proteins are encoded by open reading frames present in the genome of *Dehalococcoides ethenogenes*, two of which are closely linked with *rdhAB*-clusters. Surprisingly, the other two genes were identified immediately downstream of the only *tatAC* cluster present in the genome (Fig. 8.1). It is thus tempting to speculate that such CprC-like proteins indeed play a role in regulation and/or maturation of RR-redox complexes containing metal-cofactors, such as corrinoids (Co, reductive dehalogenases), Cu-centers (nitrous oxide reductase) and hemes (nitrite reductase) (Fig. 8.3).

In two halorespiration-deficient mutants of *D. dehalogenans* (HRD2, HRD22), the conjugative transposon Tn*916* had inserted upstream of putative histidine kinase-encoding genes. This led to the suggestion that the corresponding two-component signal transduction systems (TCS) might play a role in regulation of halorespiration, i.e. in sensing of the chlorinated substrate (Fig. 8.3) (Smidt *et al.*, 1999). Strong support for this assumption has been gained from the identification of at least 10 clusters of putative histidine kinase- and response regulator-encoding genes upstream of *rdhAB* clusters in the genome of *Dehalococcoides ethenogenes* (Fig. 8.1).

The Halorespiratory Network

Besides fermentative growth with pyruvate as the sole carbon and energy source, *Desulfitobacterium dehalogenans* is able to use various electron donors (hydrogen, formate, lactate and pyruvate) and electron acceptors (*o*-chlorinated phenolic compounds, nitrate, fumarate and several sulfuric compounds) for growth by anaerobic respiration. Biochemical and genetic studies have shown that the halorespiratory network of *D. dehalogenans* is partly integrated in the fumarate- and nitrate-respiratory chains (Chapter 4 and 5 of this thesis; van de Pas, 2000).

Integration of the results obtained by (i) the physiological, biochemical and molecular characterization of halorespiration in *Desulfitobacterium dehalogenans*, and (ii) the analysis of halorespiration-deficient (HRD) mutants (Chapter 2-5 of this thesis, and van de Pas, 2000), has resulted in a working model of the halorespiratory network present in *D. dehalogenans* (Fig. 8.4). In this model, an extracytoplasmic orientation of the key enzyme, the *o*-chlorophenol reductive dehalogenase, is assumed (see above). Because *D. dehalogenans* can grow with either H_2 or formate as electron donor, and since these substrates do not give rise to

substrate-level phosphorylation, an extracytoplasmic location of CprA requires the generation of a membrane potential at the level of the menaquinone (MK)-pool (Fig. 8.4, left panel). In contrast, no significant contribution of electron transport-coupled phosphorylation could be determined for lactate and pyruvate as the electron donor (van de Pas *et al.*, 2001b) (Fig. 8.4, right panel). In these cases, however, growth is supported by substrate-level phosphorylation via acetate production.

Surprisingly, enzyme activity- and transcription analysis revealed the differential expression of two periplasmic uptake hydrogenases under hydrogenotrophic and pyruvate-oxidizing conditions, respectively. The presence of the latter complex might be indicative for so-called intraspecies hydrogen cycling (Chapter 5 of this thesis).

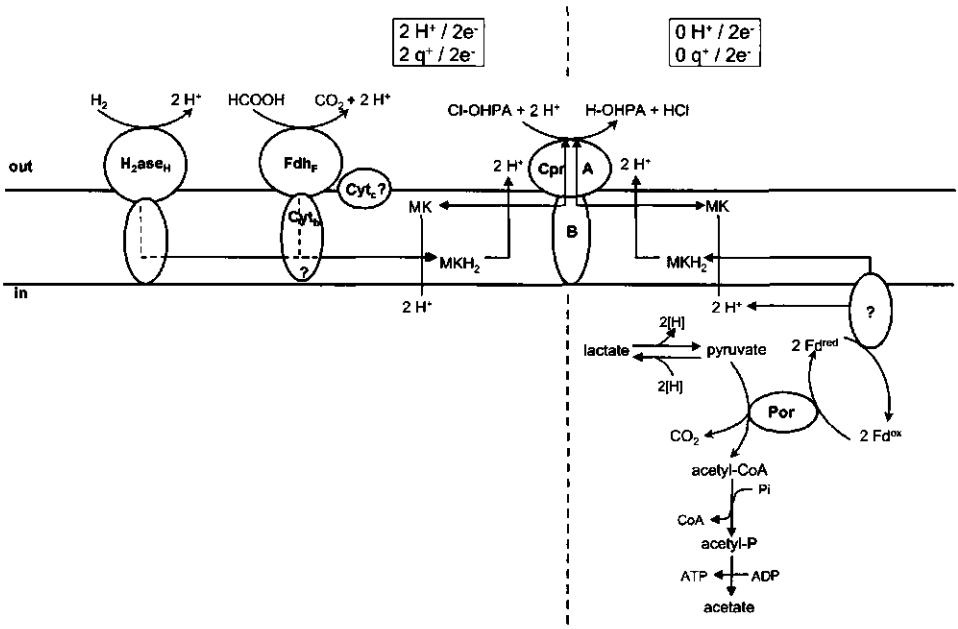

Figure 8.4 Working model for structure and function of the *D. dehalogenans* halorespirational network. Abbreviations: CprBA, *ortho*-chlorophenol reductive dehalogenase; Por, pyruvate ferredoxin oxidoreductase; H_2ase_H, respiratory (H_2-induced) uptake hydrogenase; Fdh_F, respiratory (formate-induced) formate dehydrogenase; $MK(H_2)$, (reduced) menaquinone; $Fd^{red\ /\ ox}$, reduced / oxidized ferredoxin; Cyt_b, b-type cytochrome; Cyt_c, c-type cytochrome. See text for further explanation on the charge separation boxes.

Although no significant hydrogen-production could be determined in pyruvate-fermenting cultures of *D. dehalogenans*, intermediate evolution of low amounts of hydrogen could occur along different, albeit similar pathways. Firstly, it could be the result of a combined activity of pyruvate-formate lyase and formate-hydrogen lyase, as has been proposed for *E.coli*. This was supported by the presence of putative formate-hydrogen lyase subunit-encoding genes in the direct vicinity of the transposon-insertion site in one of the analyzed HRD mutants (Andrews *et al.*, 1997; Smidt *et al.*, 1999). Nonetheless, transcriptional analysis revealed that these genes were neither expressed during fermentative nor during respiratory growth of *D. dehalogenans* (Chapter 5 of this thesis). Secondly, a hydrogen-producing, ferredoxin-dependent hydrogenase might be similarly involved in the regeneration of reduction equivalents obtained from the oxidation of low-potential electron donors, as it has been proposed for sulfate-reducing *Desulfovibrio* and methanogenic *Methanosarcina* species (Odom & Peck, 1981; Meuer *et al.*, 1999). Finally, it can not be excluded that either pyruvate-ferredoxin oxidoreductase (Por) or CO-dehydrogenase, both expressed in *D. dehalogenans* (Chapter 5 of this thesis; and van de Pas *et al.*, 2001b), exhibit hydrogen production activity, as it was observed for the respective enzymes in *Clostridium thermoaceticum*. It was proposed that pyruvate- or CO-dependent proton reduction might help to maintain a physiological redox balance of the cell under electron-acceptor (e.g. ferredoxin) limiting conditions (Menon & Ragsdale, 1996).

Additional experiments will be required to further elucidate the structure and function of the complex respiratory network functional in *D. dehalogenans*, and to reveal the relative contribution of the various pathways to the generation of membrane potential and redox balance-maintenance.

Molecular Ecology of Halorespiring Bacteria

During the past decade, halorespiring bacteria have been isolated from various pristine and polluted environments, indicating an almost ubiquitous occurrence of these microorganisms, which belong to various distinct phylogenetic branches of the eubacterial kingdom of life. In most cases, new genera have been defined, consisting solely of halorespiring microbes. The only exceptions are strains from the δ-proteobacterial genera *Desulfuromonas* and *Desulfovibrio* (Chapter 1 of this thesis). Additional evidence for this observed tendency of

halorespiration-specific phylogenetic groups has recently been provided by a comparison of bacterial community structure in different reductively dehalogenating anaerobic microbial consortia. This revealed the presence of four monophyletic bacterial clusters, which to date have only been detected in anaerobic dehalogenating freshwater ecosystems (Schlötelburg *et al.*, 2000). These findings indicate that group-specific 16S rRNA-targeted molecular fingerprinting analyses might be instrumental for the detection of organisms that are indicative for reductive dehalogenation potential in environmental samples. Indeed, such approaches have already been proven useful for the molecular monitoring of bioaugmentation plots and the discrimination between hydrogenotrophic and acetotrophic halorespiration potential (for a summary, see Chapter 1 of this thesis).

However, the isolation of non-halorespiring strains from the halorespiring *Desulfitobacterium frappieri* and *Dehalospirillum multivorans* (Neumann *et al.*, 1994; van de Pas *et al.*, 2001a) has indicated that rather than the purely phylogenetic ribosomal target sequences, monitoring approaches should focus on functional markers, such as reductive dehalogenase-encoding genes and the corresponding transcription products (Chapter 7 of this thesis) (Stapleton *et al.*, 1998). Moreover, the recent development of host-vector systems for *D. dehalogenans* now also enables the application of halorespiration-specific promoter-based reporter strains in anaerobic environments, which might be useful to (i) determine optimal process conditions for microbial remediation processes, and (ii) deepen the insight in aspects of bioavailability and toxicity of recalcitrant contaminants (Power *et al.*, 1998). Other promising approaches towards the unraveling of structure and function of halorespiring microbial consortia include ecosystem-metagenome analysis (Rondon *et al.*, 2000) and meta-transcriptome-analysis of whole ecosystems by differential display- and microarray techniques.

Outlook

We now know that both biological and non-biological factors contribute to the persistence of biohazardous compounds in the environment. There is obviously an urgent need not only for optimized *in situ* bioremediation approaches, but also for newly engineered degradation pathways (Keasling & Bang, 1998; Timmis & Pieper, 1999). The present knowledge on the phylogeny of halorespiring bacteria and the molecular characteristics of their reductive

dehalogenases has enabled the development of culture-independent molecular tools for the detection of halorespiring potential and actual activity, and will enable the fine-tuning of current *in situ* bioremediation strategies. Moreover, it will allow for the application of metabolic engineering strategies aiming at enhanced performance of halorespiring bacteria with improved and / or novel metabolic capacities as dedicated degraders in polluted anaerobic environments.

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Summary

Breathing halogenated hydrocarbons in the absence of molecular oxygen is a common feature among anaerobic so-called halorespiring bacteria. The isolation of an increasing number of phylogenetically distinct halorespirers from various pristine and polluted sites and their study by ecophysiological and molecular approaches have indicated that these microorganisms significantly contribute to biological dehalogenation in anoxic environments. Nevertheless, the often observed persistence of halogenated pollutants in natural ecosystems suggests that either prevailing environmental conditions do not support complete dechlorination by the indigenous microbiota or no potential degraders are present. Optimization of such processes, however, i.e. engineering of process parameters such as substrate availability and physicochemical conditions, and addition of efficient degraders (bioaugmentation), requires a thorough understanding of halorespiration at the physiological and molecular levels. This thesis describes molecular approaches aiming at the unraveling of the molecular basis of the halorespiration process and the development of genetic tools to enable the monitoring and further exploitation of halorespiring bacteria as dedicated degraders in biological remediation processes.

Chapter 1 provides a comprehensive overview of our current knowledge on ecology, physiology, biochemistry and molecular biology of halorespiring microorganisms, including some of the results presented in subsequent chapters of this thesis.

Chapter 2 describes the purification, biochemical and molecular characterization of *ortho*-chlorophenol reductive dehalogenase, key enzyme in the chlorophenol-respiratory pathway of the halorespiring model organism used throughout major parts of this thesis, the strictly anaerobic versatile Gram-positive *Desulfitobacterium dehalogenans*. Cloning and sequence analysis revealed the presence of two closely linked genes, one of which (*cprA*) encodes the reductive dehalogenase as a pre-protein, containing a twin-arginine signal sequence that is cleaved off in the mature protein. Such signal sequences are characteristic for extracytoplasmic respiratory enzymes containing complex redox cofactors. The *cprB* gene codes for a small hydrophobic protein, which might function as a membrane anchor coupling the catalytic subunit to the membrane-associated respiratory network. Comparison with known haloalkene reductive dehalogenases revealed significant structural and functional resemblance, suggesting that these enzymes constitute a novel family of corrinoid-containing iron-sulfur proteins.

Earlier experiments had shown that reductive dehalogenation activity is inducible in most halorespiring bacteria isolated to date. In order to get insight in the mechanisms involved in

the functional expression of *o*-chlorophenol reductive dehalogenase in *D. dehalogenans*, a large genomic fragment containing the *cprBA* genes was cloned and sequenced, revealing the presence of eight closely linked genes within the *cprTKZEBACD* cluster. Transcriptional analysis revealed the halorespiration-specific expression of seven out of eight genes, including not only the *o*-chlorophenol reductive dehalogenase-encoding *cprBA* genes, but also genes that code for molecular protein folding catalysts, possibly involved in functional assembly of the dehalogenase complex. Furthermore, two putative transcription regulator-encoding genes were identified, one of which also being transcribed solely under halorespiring conditions. Comparison of three halorespiration-specific promoters present in the *cpr* gene cluster suggested that the constitutively expressed FNR-like *cprK* gene product might be involved in control of halorespiration-dependent expression. Transcription of the *cprBA* operon was 15-fold induced within 30 minutes after addition of the *o*-chlorophenolic substrate 3-chloro-4-hydroxy-phenylacetic acid to a fermentatively growing culture with concomitant development of dehalogenating activity, indicating that regulation occurs exclusively at the transcriptional level (Chapter 3).

To further elucidate components involved in structure and function of the halorespiratory network in *D. dehalogenans*, we developed a protocol for the efficient random chromosomal integration by the conjugative transposon Tn*916*. A total of 24 halorespiration-deficient mutants could be isolated based on their inability to utilize 3-chloro-4-hydroxy-phenylacetic acid, and their characterization at the physiological, biochemical and genetic levels indicated the at least partial integration of the different respiratory pathways present in this versatile organism. In most cases, a single copy of the transposon was found to be inserted in the immediate vicinity (i.e. putative promoter regions) of genes encoding for (i) proteins that might be involved in regulation or functional assembly of respiratory complexes, and (ii) structural components of respiratory complexes, such as hydrogenase, formate dehydrogenase and formate-hydrogen lyase (Chapter 4). The analysis of gene expression and activity of these and other respiratory complexes during anaerobic respiration and fermentation revealed that they might indeed be involved in respiratory processes in *D. dehalogenans*, although not specific for halorespiration (Chapter 5).

To enable the application of metabolic engineering approaches aiming at an optimized exploitation of *D. dehalogenans* and related strains as dedicated degraders of recalcitrant pollutants, as well as to improve the possibilities to study function and regulation of chromosomal genes in this organism, efficient host-vector systems were developed for *D.*

dehalogenans (Chapter 6). An electroporation-based transformation procedure was optimized for the application under strict anaerobic conditions and the promiscuous Gram-positive broad host-range cloning vector pIL253 was introduced and stably maintained at moderate copy numbers in *D. dehalogenans*. Moreover, the conditionally replicating temperature-sensitive pGh^{+}host9 vector was instrumental for the specific disruption of the gene for a fumarate reductase, resulting in partially impaired fumarate reduction activity of *D. dehalogenans*.

Both the optimization of bioremediation process conditions and the introduction of specialized degrading strains require sensitive methods to follow the fate and activity of the dehalogenating population. The isolation of non-dehalogenating strains from halorespiring species has reinforced the notion that purely phylogenetic markers such as ribosomal RNA molecules do not necessarily reflect metabolic capacity and *in situ* activity of an organism. Therefore, we aimed at the development of PCR-based molecular monitoring approaches using reductive dehalogenase-encoding genes as a functional marker (Chapter 7). By applying degenerate oligonucleotide primers based on potentially function-related sequence motifs, highly conserved among known chloroaromate- and chloroalkene-converting enzymes, putative functional and cryptic reductive dehalogenase-encoding genes could be retrieved from various halorespiring cultures. Moreover, the halorespiration-specific expression of known and novel genes was demonstrated by Reverse Transcriptase-PCR, indicating the potential of such approaches to estimate the metabolic characteristics of reductively dehalogenating microbial systems.

In chapter 8, the results of this thesis are discussed in the light of recent findings obtained in related studies, with special emphasis on the data that has been gained from the analysis of the only recently released complete genome sequence of the hydrogenotrophic PCE-degrading *Dehalococcoides ethenogenes*. It is suggested that halorespiration is an evolutionary rather ancient capacity, being probably regulated by similar control mechanisms in the phylogenetically distinct bacteria *Desulfitobacterium dehalogenans* and *Dehalococcoides ethenogenes*. In addition, a working model of the (halo)-respiratory network of *D. dehalogenans* is presented that integrates the findings described here with the results of a complementary study which focused on the biochemistry and physiology of halorespiration in *Desulfitobacterium dehalogenans* (Bram A. van de Pas, 2000. PhD thesis. Wageningen University, The Netherlands). Finally, perspectives are outlined for the application of halorespirers as dedicated degraders of recalcitrant halogenated pollutants, including the use of metabolic engineering approaches.

Samenvatting

Vaak is een van de eerste vragen, die een onderzoeker te horen krijgt:

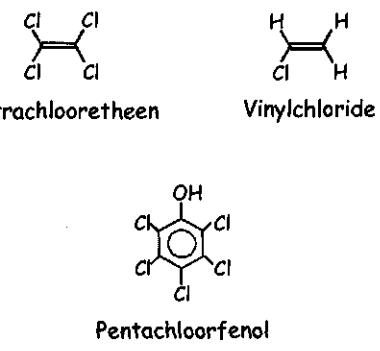
Leuk, hoor, je onderzoek...en waar is dat nou goed voor?

Deze samenvatting is bedoeld om op deze en andere vragen een antwoord te geven.

Met het onderzoek dat in dit proefschrift beschreven staat hebben we in samenwerking met het onderzoeksproject van Bram van de Pas binnen het Laboratorium voor Microbiologie geprobeerd te begrijpen, hoe bacteriën op een efficiënte manier van milieuverontreinigende verbindingen, namelijk chloorkoolwaterstoffen (CKW's), kunnen leven, en ons op deze wijze een handje kunnen helpen bij het opruimen van deze schadelijke stoffen.

Om wat voor stoffen gaat het, en waarom willen / moeten we ervan af?

Chloorkoolwaterstoffen zijn organische moleculen, waaraan een of meer chlooratomen gebonden zijn (zie Figuur 1). Een bekend voorbeeld is tetrachlooretheen (PCE; ook wel PER genoemd) dat als oplosmiddel in de chemische wasserij en als ontvettingsmiddel in de industrie wordt gebruikt. Een ander voorbeeld is vinylchloride, een grondstof voor de productie van plastic PVC. En ook chloorfenolen kom je in het dagelijks leven tegen. Zo werd pentachloorfenol tot kort geleden als conserveringsmiddel aan houtverf toegevoegd. De meeste van deze verbindingen zijn op de één of andere manier giftig, of omdat ze zelf een schadelijk effect hebben, of omdat ze in levende wezens worden omgezet tot giftige afbraakproducten. Het is algemeen bekend dat de afgelopen decennia grote hoeveelheden van deze verbindingen in het milieu zijn terechtgekomen. Ze vormen hier niet alleen een bedreiging voor de gezondheid van levende wezens, maar ze hebben ook een negatieve invloed op het functioneren van hele ecosystemen.



Figuur 1 Voorbeelden van chloorkoolwaterstoffen

....en wat kunnen wij (of eigenlijk: de micro-organismen) hieraan doen?

Onderzoekers hebben kunnen aantonen, dat micro-organismen, schimmels en bacteriën, in staat zijn, deze giftige verbindingen om te zetten in producten die veel minder giftig of zelfs onschadelijk zijn. Micro-organismen zijn dus niet alleen verantwoordelijk voor enge ziektes, voetschimmel en zwarte aanslag op de badkamermuur. De meeste zijn onmisbaar voor een goed functionerend ecosysteem. Het zou meer dan een heel proefschrift vergen, om hier uitputtend op in te gaan, maar bekende voorbeelden zijn onder andere het menselijk maagdarmkanaal, de composthoop en een waterzuiveringsinstallatie. Natuurlijk maken micro-organismen zich ook nog erg nuttig in tal van toepassingen in de industrie, bijvoorbeeld bij de productie en het houdbaar maken van voedsel zoals kaas, salami en bier.

Bij het onschadelijk maken van chloorverbindingen is het verwijderen van de chlooratomen meestal de kritische stap. Soms gebeurt dit "per ongeluk", doordat enzymen (eiwitten die chemische reacties in organismen katalyseren) toevallig ook de gechloreerde koolwaterstoffen kunnen afbreken. Maar er zijn ook bacteriën, die CKW's als voedselbron kunnen gebruiken. Daarbij worden deze in een aantal kleine stapjes door enzymen afgebroken en met zuurstof verbrand, net zoals wij dat met ons voedsel doen. Er blijft dan in de meeste gevallen ook niet meer achter dan kooldioxide (CO_2), zoutzuur (HCl) en water (H_2O). De energie die bij deze afbraak vrij komt, kan de bacterie dan gebruiken voor zijn eigen instandhouding en voor de aanmaak van nieuwe cellen (bacteriën vermeerderen zich alleen door middel van verdubbeling). De bio-afbreekbaarheid van de CKW's hangt echter wel voor een groot deel af van de heersende milieuomstandigheden en het type CKW. Hierbij speelt vooral de hoeveelheid zuurstof in de lucht een belangrijke rol. Zo kan bijvoorbeeld vinylchloride wel in de aanwezigheid van zuurstof worden afgebroken, terwijl daar in het geval van PCE geen sprake van is. De verzadiging van dit molecuul met 4 chlooratomen zorgt ervoor, dat er geen reactie met zuurstof meer mogelijk is.

We weten dat zuurstof, voor ons van levensbelang om de energie uit ons voedsel te kunnen halen, niet overal op de aarde in dezelfde concentratie voorkomt. In de sedimenten van rivieren en meren bijvoorbeeld is, afgezien van in het bovenste laagje, geen spoor van zuurstof te bekennen. Deze milieus worden dan ook anoxisch of anaëroob (vrij van zuurstof) genoemd. De micro-organismen die hier leven, **anaërobe micro-organismen** genoemd, gebruiken in plaats van zuurstof andere stoffen, om hun voedsel te verbranden. En hier komen we bij de eigenlijke hoofdrolspelers van dit proefschrift. Er zijn recent bacteriën ontdekt die in staat zijn gechloreerde verbindingen zoals tetrachlooretheen in te ademen. Dan halen ze er een

of meerdere chlooratomen vanaf en ademen het gedechloreerde product plus zoutzuur weer uit. Dit proces – ook wel **chlooradembaling (halorespiratie)** genoemd – verloopt snel en efficiënt. Vooral als je het vergelijkt met de boven genoemde afbraak die min of meer "per ongeluk" verloopt. Daarom zijn deze bacteriën zeer belangrijk voor een gedegen schoonmaak van verontreinigde zuurstofloze milieus, zoals onze bodem en grondwater.

Er is echter regelmatig gebleken, dat het opruimen van vervuild grond en grondwater door bacteriën vaak niet zo voorspoedig verloopt als we zouden willen. Regelmatig vallen de afbraaksnelheden erg tegen, en vaak ademen de bacteriën ook tussenproducten uit, die minstens zo schadelijk zijn als de oorspronkelijke vervuiling; zo is bijvoorbeeld het kankerverwekkende vinylchloride een van de tussenproducten bij de PCE-afbraak. Daarom is het belangrijk om te snappen hoe de chlooradembaling eigenlijk werkt, en onder welke milieuomstandigheden de betreffende bacteriën dit het beste doen.

Chlooradembaling - Wie doet het en hoe werkt het?

De volgende vraag: wat voor bacteriën kunnen het, en hoe doen ze dat? Wat voor voedsel vinden ze het lekkerst? Dat te weten is wel erg belangrijk, om de schoonmaak optimaal te laten verlopen.

Het eerste dat ons opviel, was dat er niet één, maar meer dan tien verschillende soorten bacteriën in staat bleken te zijn om door middel van chlooradembaling te groeien. En er worden nog steeds regelmatig nieuwe soorten ontdekt! Sommige van deze micro-organismen bleken erg beperkt in hun voedselkeuze. Ze konden alleen maar leven met een combinatie van CKW's, waterstof als substraat en een koolstofbron, bijvoorbeeld azijnzuur. Weer andere daarentegen bleken zeer veelzijdig; naast waterstof vonden deze bacteriën ook andere substraten erg lekker, zoals mierenzuur, melkzuur en soms zelfs ethanol en boterzuur. Eén van deze bacteriën, ***Desulfitobacterium dehalogenans***, hebben wij uitgekozen om achter het mechanisme van de dechlorering te komen. Deze bacterie gebruikt chloorfenolen voor zijn ademhaling en groeit met waterstof, mierenzuur en melkzuur als substraat.

In de eerste plaats hebben we het eiwit dat voor de dechlorering van chloorfenolen verantwoordelijk is uit de cel geïsoleerd. Dit om eigenschappen en werkingsmechanisme ervan te kunnen bestuderen. Verder hebben we gekeken, hoe en onder welke omstandigheden het eiwit (het **chloorfenol dehalogenase**) door de cel wordt aangemaakt. Met andere woorden: onder welke omstandigheden vindt er daadwerkelijk afbraak van de verontreiniging

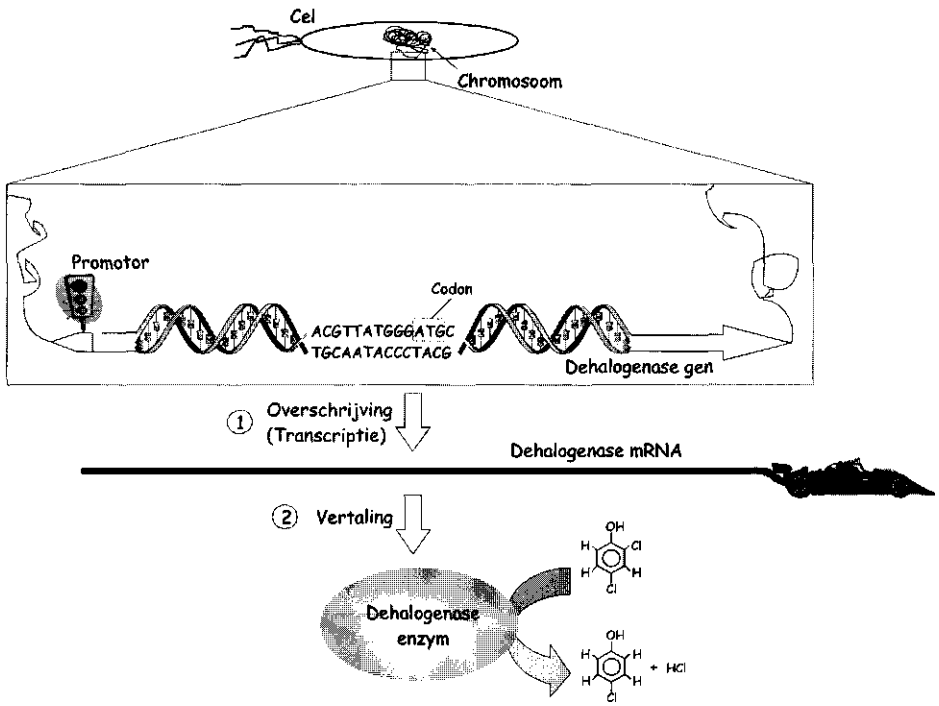
plaats. De resultaten hiervan kunnen we het beste bekijken tijdens een reis door het binnenste van de cel van *Desulfitobacterium dehalogenans* (zie Figuur 2).

EEN KORTE REIS DOOR EEN BACTERIECEL

Laten we onze reis beginnen bij de erfelijke eigenschappen van onze bacterie. Deze erfelijke eigenschappen, de **genen**, bevinden zich bij bacteriën op één enkel groot molecuul, het **chromosoom**, dat zoals bij alle organismen gemaakt is van **DNA**. Een menselijk cel heeft 46 chromosomen; een bacterie slechts 1. Een gen bevat alle informatie die nodig is om een eiwit, bijvoorbeeld het chloorfenol dehalogenase, te produceren. Deze informatie is in een speciale code geschreven en is ten eerste de volgorde van de bouwstenen in het eiwit, de aminozuren (zie Figuur 2 voor de details). Ten tweede is er ook nog informatie beschikbaar die aangeeft onder welke omstandigheden het eiwit moet worden aangemaakt en hoeveel er dan nodig is. In de eerste stap wordt het gen overgeschreven naar een **mRNA** molecuul. De machine, die hiervoor verantwoordelijk is, kan je het beste met een Formule 1 raceauto vergelijken dat tijdens de rit op het gen één mRNA molecuul aanmaakt. Voor aan het gen ligt de **promotor**, een soort startteken op de gen-racebaan. Dit stoplicht moet aangeven, wanneer de raceauto's mogen starten, en hoeveel er de baan op mogen. Met andere woorden: de promotor geeft aan, of en hoe vaak een gen daadwerkelijk via het mRNA molecuul overgeschreven en vertaald wordt naar een actief eiwit (zie Figuur 2).

...WEER TERUG BIJ *DESULFITOBACTERIUM*

Nadat we het chloorfenol dehalogenase in zuivere vorm in handen hadden gekregen, hebben we hiervan een klein stukje van de aminozuurvolgorde kunnen bepalen. Deze informatie is dan gebruikt, om het bijbehorende **chloorfenol dehalogenase gen** en nog een aantal andere genen, waarvan we dachten, dat ze ook belangrijk zouden kunnen zijn voor chlooradembaling, in kaart te brengen. Door het gen verder te analyseren, en de afgeleide aminozuur-sequentie te vergelijken met reeds bekende dehalogenases van andere chlooradembalende bacteriën, zijn we erachter gekomen dat al deze eiwitten min of meer dezelfde opbouw hebben. Het blijkt dan ook dat alle tot nu toe bekende dehalogenases uit deze bacteriën volgens een vergelijkbaar mechanisme lijken te werken.



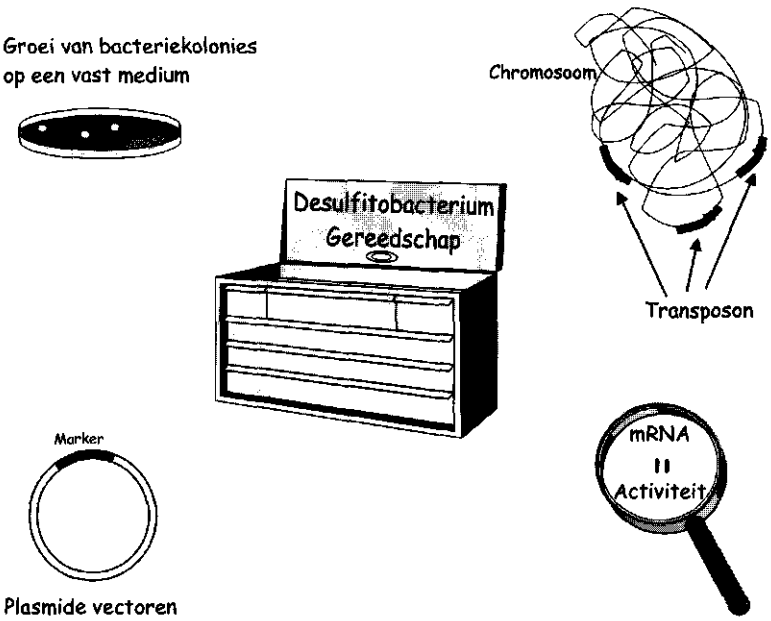
Figuur 2 Korte reis door een cel van *Desulfitobacterium dehalogenans*. Het erfelijk materiaal van alle levende wezens is **DNA**, een lange ketting die is opgebouwd uit 4 verschillende bouwstenen, de basen adenine (A), cytosine (C), guanine (G) en thymine (T). Telkens zijn twee strengen DNA aan elkaar gebonden in een spiraalvormige structuur, die gestabiliseerd wordt door zijn waterstofbruggen tussen twee tegenover elkaar liggende basen (A - T en C - G). In een bacterie bevinden zich de meeste erfelijke eigenschappen (de **genen**) op één groot DNA-molecuul, het **chromosoom**. De volgorde (sequentie) van de basen in een gen bepaalt de volgorde van de 20 verschillende aminozuur-bouwstenen in een eiwit. Aan de hand van een code worden daarbij telkens drie opeenvolgende basen (het **codon**, bijvoorbeeld ATG) 'overgeschreven' en 'vertaald' naar één aminozuur. In het geval van ATG is dat methionine. Een gen dat codeert voor een eiwit van 100 aminozuren heeft dus een lengte van 300 basen. Maar de vertaling van gen naar eiwit vind niet rechtstreeks plaats. Het DNA van een gen moet hiervoor eerst door een speciale machine worden overgeschreven naar een ander molecuul, het zogenoemd boodschapper RNA (messenger RNA, afgekort met **mRNA**). Dit proces heet **transcriptie**, en de machine is het beste met een raceauto te vergelijken dat tijdens zijn rit op het gen één mRNA molecuul aanmaakt. Het mRNA molecuul dient dan als matrijs voor de eiwit synthese (de **vertaling**) aan de ribosomen, de eiwitfabriekjes van een cel. Niet altijd worden alle eiwitten aangemaakt, omdat dat een grote verspilling van energie en grondstoffen zou zijn. In veel gevallen wordt met de eiwitsynthese pas begonnen, als het eiwit echt nodig is. Vaak wordt deze *go / no go* beslissing vóór het overschrijven van DNA naar mRNA genomen. Direct voor een gen bevindt zich een soort 'transcriptie-stoplicht' (de **promotor**), dat aangeeft of en hoeveel raceauto's de gen-racebaan opmogen. In het geval van eiwitten die altijd nodig zijn staat het stoplicht permanent op groen; in andere gevallen moet er eerst op het knopje gedrukt worden. Dat laatste is het geval bij de chloorademhaling in *Desulfitobacterium dehalogenans*. Voor de aanmaak van het **chloorfenol dehalogenase** eiwit wordt dit signaal door een chloorfenol molecuul gegeven.

Vervolgens hebben wij onderzocht, onder welke omstandigheden het chloorfenol dehalogenase gen in **chloorfenol dehalogenase mRNA** wordt overgeschreven en in actief dehalogenase wordt vertaald. Hierdoor is het gelukt, standplaats en werkingswijze van het 'promotor-stoplicht' op te helderen. Het bleek, dat het stoplicht normaal gesproken op rood staat, en overschrijving (**transcriptie**) van DNA naar mRNA alleen plaats vindt als er ook daadwerkelijk een passend CKW in de buurt aanwezig is. Daarnaast hebben we ook kunnen aantonen, dat er verder geen stoplichten te bekennen zijn op de weg tussen het gen en de aanwezigheid van een actief dehalogenase. Met andere woorden: als er dehalogenase mRNA aanwezig is in de cel, dan kan hieruit direct de aanwezigheid van actief eiwit en dus afbraak van de verontreiniging afgeleid worden.

We hebben gezien dat de aanmaak van actief eiwit in het geval van de dehalogenases gelijk is aan de productie van dehalogenase mRNA. Van deze kennis hebben we geprofiteerd bij de ontwikkeling van een methode, waarmee je door mRNA te meten snel en betrouwbaar dehalogenase activiteit (en dus actieve afbraak van de verontreiniging) aan zou kunnen tonen. Zo een methode zou erg nuttig zijn, om de milieuomstandigheden tijdens een sanering zo in te stellen, dat er optimale afbraakactiviteit resulteert. Hierbij volgen we de normale weg van DNA naar eiwit in omgekeerde richting. Met behulp van een bijzondere reactie in de reageerbuis is het mogelijk, mRNA weer over te schrijven naar het bijbehorende gen, dus naar een DNA-molecuul. Dit DNA-molecuul kan dan in een tweede stap opgespoord worden door middel van de zogenoemde PCR reactie (PCR staat voor Polymerase Ketting Reactie). Hierbij wordt een specifiek stuk DNA, in dit geval het dehalogenase gen, in een reageerbuis vermenigvuldigd tot een hoeveelheid die te meten of zelfs zichtbaar te maken is. Tot nu toe hebben we deze methode alleen uitgeprobeerd in het laboratorium bij geïsoleerde bacteriën. We denken dat we binnenkort ook in staat zullen zijn, onze test toe te passen op verontreinigd grond en grondwater, waar we met mengsels van verschillende micro-organismen te maken hebben.

Een Moleculaire Gereedschapskist voor *Desulfitobacterium*

Een belangrijk deel van dit proefschrift gaat over het inrichten van een moleculaire gereedschapskist voor *Desulfitobacterium dehalogenans*. Wat zit er nou precies in en waar kunnen die verschillende gereedschappen dan voor gebruikt worden? In het kort gezegd, het grootste deel van de inhoud van onze gereedschapskist kan worden gebruikt om veranderingen aan te brengen in de erfelijke eigenschappen van de bacterie. Een soort bouwdoos dus (zie Figuur 3).



Figuur 3 Een moleculaire gereedschapskist voor *Desulfitobacterium dehalogenans*. **Transposons** kunnen worden gebruikt voor het ongericht uitschakelen van genen, terwijl **plasmide vectoren** het gerichte manipuleren (toevoegen, uitschakelen of veranderen) van erfelijke eigenschappen mogelijk maken. De meeste van deze moleculaire technieken resulteren wel in een mengsel van bacteriën die in hun erfelijke eigenschappen een klein beetje verschillen (bijvoorbeeld kunnen verschillende genen zijn uitgeschakeld). Om deze verschillende cellen één voor één te kunnen bestuderen, is het nodig om dat kakelbonte celmengsel in enkele cellen te scheiden. Als je nu de cellen uit dat mengsel voldoende verdund op een vast medium laat groeien, ontstaan na een dag of drie kleine celhoopjes, zogenoemde **bacteriekolonies**. Elke bacteriekolonie ontstaat uiteindelijk uit één enkele cel; alle cellen binnen een kolonie hebben dan ook dezelfde erfelijke eigenschappen en worden ook wel klonen genoemd. Alleen op deze manier kunnen verschillende cellen onafhankelijk van elkaar kunnen worden bestudeerd. Tenslotte is er dan nog de mogelijkheid, om met behulp van een aantal reactiestappen in de reageerbuis de aanwezigheid van **chloorfenol dehalogenase mRNA** en dus afbraakactiviteit aan te tonen.

Een van de gereedschappen, een zogenoemd **transposon**, is zelf een mobiel stukje DNA met daarop alle nodige informatie om op een willekeurige plek het chromosoom van een bacterie in te springen. Daarbij komt het transposon natuurlijk ook in genen terecht, waardoor deze onderbroken worden, en dan dus niet meer functioneren. Het bijbehorende eiwit kan niet meer op de goede manier worden aangemaakt. Zodoende hebben wij een aantal genen op kunnen sporen, die een rol bleken te spelen bij een goed functionerende chloorademhaling.

Een tweede set van instrumenten zijn de **plasmide vectoren**. Dit zijn eveneens stukjes erfelijk materiaal, die zich in een bacteriecel onafhankelijk van het chromosoom kunnen vermenigvuldigen. De plasmide vectoren kunnen gebruikt worden om de functie van bestaande genen te achterhalen door deze genen gericht uit te schakelen. Dat gaat min of meer op de manier zoals die beschreven is voor transposons.

Maar er kunnen ook veranderingen aangebracht worden die tot een gewijzigde functie leiden. Zo zou bijvoorbeeld het 'stoplicht' voor een gen anders afgesteld kunnen worden. Je zou er dan, bij wijze van spreken, voor kunnen zorgen dat het dehalogenase eiwit altijd wordt aangemaakt. Tenslotte kunnen via een plasmide vector ook geheel nieuwe genen aan de erfelijke eigenschappen van een bacterie worden toegevoegd. Een voorbeeld zou zijn, om de bacterie in staat te stellen, een ander substraat te gebruiken, dat bijvoorbeeld goedkoper is.

De Moraal van het Verhaal

Micro-organismen, CKW's, leven zonder zuurstof, eiwitten, genen, veranderen van erfelijke eigenschappen,

.....*maar ook:* schoonmaken van verontreinigde grond.

We hebben geprobeerd, een nieuwe bacterie te karakteriseren en de eigenschappen ervan aan het licht te brengen. Met behulp van wat we daarbij geleerd hebben, kunnen we deze bacteriën nu misschien nog beter voor ons laten werken bij het verwijderen van CKW's uit grond en grondwater.

....Het blijkt dat de kloof tussen fundamenteel wetenschappelijk onderzoek en toepassing vaak véél kleiner is dan wordt aangenomen.

Zusammenfassung

Oft ist eine der ersten Fragen, die einem Wissenschaftler gestellt werden:

Ist ja sehr interessant....und was bringt uns das?

Diese Zusammenfassung versucht, auf diese und weitere Fragen eine Antwort zu geben.

Mit den Experimenten, die in dieser Dissertation beschrieben sind, haben wir in enger Zusammenarbeit mit dem Forschungsprojekt von Bram van de Pas am Institut für Mikrobiologie versucht zu verstehen, wie Bakterien auf effiziente Weise von umweltbelastenden Verbindungen, genauer gesagt von Chlorkohlenwasserstoffen (CKW's), leben und uns so bei der Beseitigung dieser Schadstoffe nützlich sein können.

Um welche Stoffe geht es, und warum wollen wir / müssen wir sie beseitigen?

Chlorierte Kohlenwasserstoffe sind organische Moleküle, an die ein oder mehrere Chloratome gebunden sind (siehe Abbildung 1). Ein bekanntes Beispiel ist Tetrachlorethen (PCE; früher auch **Perch**lorethylen genannt), welches als Lösungsmittel bei der chemischen Textilreinigung und als Entfettungsmittel in der Industrie verwendet wird. Ein weiteres Beispiel ist Vinylchlorid, der Grundstoff für die PVC-Produktion. Aber auch Chlorphenolen begegnet man im täglichen Leben. So wurde Pentachlorphenol bis vor kurzem Holzschutzfarben als Konservierungsmittel zugefügt. Die meisten dieser chlorierten Verbindungen sind auf die eine oder andere Art und Weise giftig; entweder aufgrund toxischer Effekte einer Substanz selbst oder durch Umsetzung in giftige Abbauprodukte in Lebewesen. Es ist allgemein bekannt, dass während der vergangenen Jahrzehnte wiederholt große Mengen dieser Schadstoffe in die Umwelt geraten sind, wo diese nicht nur die Gesundheit von Lebewesen bedrohen, sondern auch das Funktionieren ganzer Ökosysteme beeinträchtigen.

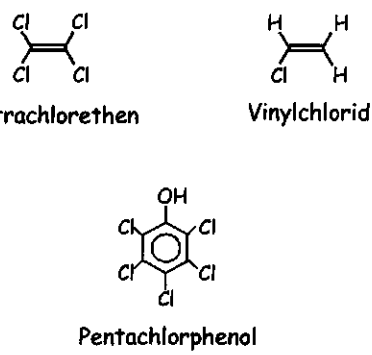


Abbildung 1 Beispiele chlorierter Kohlenwasserstoffe

....und was können wir (bzw. eigentlich die Mikroorganismen) tun?

Wissenschaftler konnten zeigen, dass Mikroorganismen, Bakterien und Pilze, in der Lage sind, die hier genannten giftigen Stoffe in weniger giftige oder selbst in unschädliche Produkte umzusetzen. Mikroben sind also nicht nur für gefährliche Krankheiten, Fußpilz und Schimmelbelag auf Badezimmerwänden verantwortlich. In den meisten Fällen sind Mikroorganismen unentbehrlich für ein funktionsfähiges Ökosystem. Es würde mehr als eine komplette Doktorarbeit erfordern, um hierauf erschöpfend einzugehen, aber bekannte Beispiele sind der menschliche Magen-Darm-Kanal, der Komposthaufen und die Kläranlage. Natürlich machen sich Mikroorganismen auch in der Industrie, z. B. bei der Produktion und Konservierung von Lebensmitteln wie Käse, Salami und Bier, sehr nützlich.

Für das Unschädlichmachen chlorierter Kohlenwasserstoffe ist die Entfernung der Chloratome normalerweise der kritische Schritt. Dies kann aus Versehen passieren, wenn Enzyme, d.h. Eiweiße, welche chemische Reaktionen in Lebewesen katalysieren, zufällig auch diese CKW's umsetzen können. Es gibt aber auch Bakterien, welche chlorierte Kohlenwasserstoffe als Nahrungsquelle verwenden können. Dabei werden diese Stoffe Schritt für Schritt durch Enzyme abgebaut und mit Sauerstoff verbrannt - genau so wie wir das auch mit unserer Nahrung machen. Oft ist das Einzige, was dann übrig bleibt, Kohlendioxid (CO_2), Salzsäure (HCl) und Wasser (H_2O). Die Energie, die bei diesem Abbau freigesetzt wird, kann die Bakterie dann für seine eigene Erhaltung sowie die Erzeugung neuer Zellen verwenden (Bakterien vermehren sich ausschließlich durch Verdopplung). Der biologische Abbau von CKW's hängt zu einem großen Teil von den Umweltbedingungen ab. Dabei spielt vor allem der Sauerstoffgehalt in der Luft eine entscheidende Rolle. So wird zum Beispiel Vinylchlorid in der Gegenwart von Sauerstoff effizient abgebaut, wogegen PCE unter diesen Umständen nicht angegriffen wird. Aufgrund der Sättigung dieses Moleküls mit 4 Chloratomen ist ein Abbau mit Sauerstoff nicht mehr möglich.

Wir wissen, dass Sauerstoff, welcher für uns lebenswichtig ist, um die Energie aus unserer Nahrung zu holen, nicht überall auf der Erde in der gleichen Konzentration vorkommt. In Fluss- und Seesedimenten ist Sauerstoff zum Beispiel nur in der obersten dünnen Schicht anzutreffen. Diese Milieus werden darum auch anoxisch oder anaerob (frei von Sauerstoff) genannt. Die Mikroorganismen, die hier leben, auch als **anaerobe Mikroorganismen** bezeichnet, verwenden statt Sauerstoff andere Verbindungen, um ihre Nahrung zu verbrennen. Und damit sind wir bei den Hauptdarstellern dieser Arbeit angelangt. Unlängst wurden nämlich Bakterien entdeckt, die in der Lage sind, statt Sauerstoff chlorierte

Verbindungen wie z. B. PCE einzuatmen. Einmal eingeatmet, entfernen sie ein oder mehrere Chloratome des PCE's und atmen schließlich das dechlorierte Produkt und Salzsäure wieder aus. Dieser Prozess – auch **Chloratmung (Halorespiration)** genannt – verläuft schnell und effektiv - vor allem, wenn man ihn mit dem oben genannten zufälligen Abbau vergleicht. Darum sind diese Bakterien sehr wichtig für eine gründliche Sanierung verunreinigter sauerstofffreier Milieus, worunter unsere Böden und Grundwässer fallen.

Allerdings hat sich gezeigt, dass der biologische Abbau der Schadstoffe oftmals nicht im wünschenswerten Ausmaß stattfindet. Regelmäßig bleiben die Abbaugeschwindigkeiten weit hinter den Erwartungen zurück. Häufig atmen die Bakterien auch unerwünschte Zwischenprodukte aus, welche nicht selten giftiger sind als die Ausgangssubstanz selbst; zum Beispiel wird das krebserregende Vinylchlorid oft als Endprodukt des PCE-Abbaus angetroffen. Darum ist es sehr wichtig zu verstehen, wie Chloratmung funktioniert, und unter welchen Umweltbedingungen dieser Prozess optimal verläuft.

Chloratmung – Wer kann's und wie funktioniert es?

Die nächste Frage: Welche Bakterien sind hierzu in der Lage, und wie machen sie das? Welche Nahrung bevorzugen diese Mikroorganismen? Dies zu wissen, ist sehr wichtig, um eine Umweltsanierung so effizient wie möglich verlaufen zu lassen.

Das Erste, was uns auffiel, war die Tatsache, dass nicht eine, sondern mehr als zehn verschiedene Arten von Bakterien in der Lage waren, mittels Chloratmung zu wachsen. Und noch immer werden regelmäßig neue Arten entdeckt! Einige dieser Bakterien entpuppten sich als sehr wählerisch. Das Einzige, was diese Bakterien zu schätzen wissen, ist eine Kombination von CKW's, Wasserstoff als Substrat und einer Kohlenstoff-Quelle, z. B. Essigsäure. Andere haben hingegen einen umfangreichen Speisezettel; neben Wasserstoff wachsen sie auch mit Ameisensäure und Milchsäure, und manche selbst mit Ethanol und Buttersäure. Eine dieser Bakterien, ***Desulfitobacterium dehalogenans***, haben wir ausgewählt, um hinter den Mechanismus von Chloratmung zu kommen. Dieses Bakterium ist in der Lage, Chlorphenole einzuatmen und unter anderem Wasserstoff, Ameisen- und Milchsäure als Substrat zu verwenden.

Zunächst haben wir das Eiweiß, welches für die Dechlorierung verantwortlich ist, aus der Zelle gereinigt, um dessen Eigenschaften und Wirkungsweise genauer untersuchen zu können. Weiterhin haben wir uns mit der Frage beschäftigt, wie und unter welchen Bedingungen dieses Enzym (die **Chlorphenoldehalogenase**) von der Zelle produziert wird;

mit anderen Worten: Unter welchen Bedingungen findet tatsächlich Schadstoffabbau statt. Die Ergebnisse können wir uns am besten während einer kurzen Reise durch das Innere einer Zelle von *Desulfitobacterium dehalogenans* vor Augen führen (siehe Abbildung 2).

Eine Kurze Reise durch eine Bakterienzelle

Wir beginnen unsere Reise bei den erblichen Eigenschaften unseres Bakteriums. Träger dieser erblichen Eigenschaften sind die **Gene.** Diese befinden sich bei Bakterien auf einem einzelnen großen Molekül, dem **Chromosom**, welches, wie in allen Lebewesen, aus **DNA** besteht. Eine menschliche Zelle hat 46 Chromosomen, ein Bakterium besitzt dahingegen nur 1 Chromosom. Ein Gen enthält alle Informationen, die notwendig sind, um ein Eiweiß (wie unsere Chlorphenoldehalogenase) zu synthetisieren. Zum einen ist dies die Reihenfolge der Bausteine im Eiweiß, den Aminosäuren. Diese Information ist in einem speziellen Code verschlüsselt (siehe Abbildung 2). Zum anderen wird angegeben, unter welchen Bedingungen wieviel Eiweiß produziert werden muss. Im ersten Schritt wird das Gen in ein **mRNA**-Molekül übertragen. Die Maschine, die für die mRNA-Produktion verantwortlich ist, kann man am besten mit einem Formel 1 Rennwagen vergleichen, der bei jeder Fahrt auf der Genrennstrecke jeweils ein mRNA-Molekül produziert. Unmittelbar vor dem Gen befindet sich der **Promotor**, eine Art Startampel am Beginn der Strecke. Diese Ampel zeigt an, wann die Rennwagen starten dürfen und wieviel Autos losgelassen werden. Mit anderen Worten: Der Promotor entscheidet, ob und wie häufig ein Gen tatsächlich via ein mRNA-Molekül in aktives Eiweiß übertragen und übersetzt wird (siehe Abbildung 2).

...wieder zurück zum *Desulfitobacterium*

Nachdem wir die Chlorphenoldehalogenase gereinigt hatten, haben wir die Reihenfolge der ersten Aminosäuren bestimmt. Mit Hilfe dieser Information haben wir dann das dazugehörige **Chlorphenoldehalogenase-Gen** sowie eine Reihe anderer Gene, wovon wir annahmen, das sie ebenfalls eine wichtige Rolle bei Chloratmung spielen könnten, kartiert und näher untersucht.

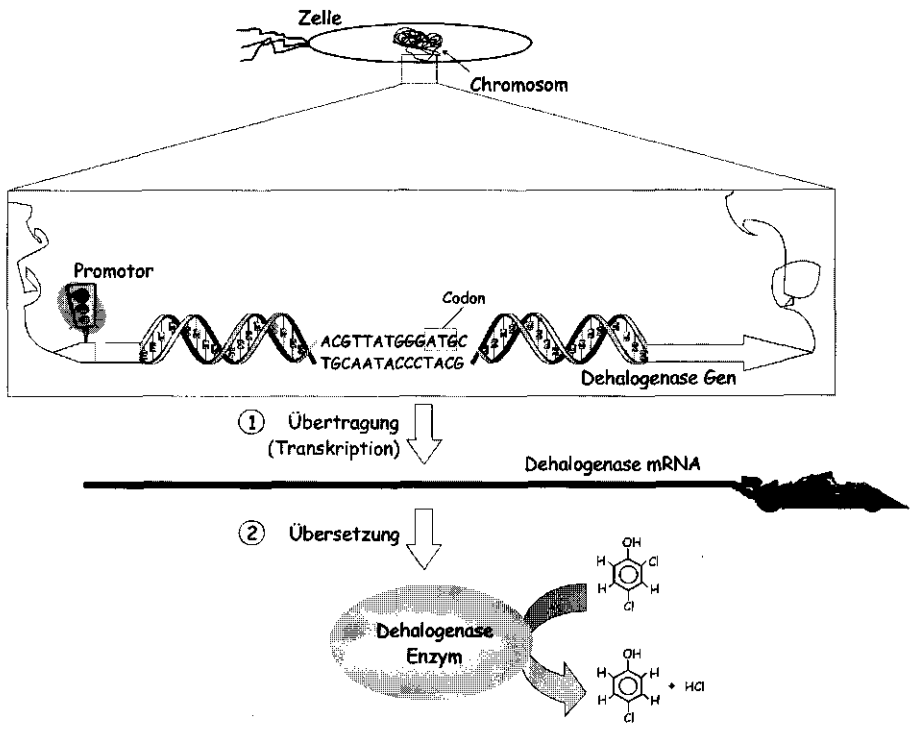


Abbildung 2 Kurze Reise durch eine Zelle von *Desulfitobacterium dehalogenans*. Das erbliche Material aller Lebewesen auf dieser Erde besteht aus **DNA**, einer langen Kette, die aus 4 unterschiedlichen Bausteinen besteht, den Basen Adenin (A), Guanin (G), Cytosin (C) und Thymin (T). Jeweils zwei DNA-Stränge sind in einer spiralförmigen Struktur aneinander gebunden, die durch sogenannte Wasserstoffbrücken zwischen zwei gegenüberliegenden Basen (A-T und G-C) stabilisiert wird. In einer Bakterienzelle befinden sich die meisten erblichen Eigenschaften, die wir **Gene** nennen, auf einem großen DNA-Molekül, dem **Chromosom**. Die Reihenfolge (Sequenz) der Basen in einem Gen bestimmt die Reihenfolge der 20 verschiedenen Aminosäurebausteine in einem Eiweißmolekül. Anhand eines Codes werden dabei jeweils drei aufeinanderfolgende Basen (das **Codon**, z.B. ATG) in eine Aminosäure übertragen und übersetzt. Das ATG wird z.B. in die Aminosäure Methionin übersetzt. Ein Gen, dass für ein Eiweiß von 100 Aminosäuren codiert, hat also eine Länge von 300 Basen. Allerdings findet die Übersetzung vom Gen zum Eiweiß nicht auf direktem Wege statt. Die DNA eines Gens muss dafür erst durch eine besondere Maschine in ein anderes verwandtes Molekül, die sogenannte Boten RNA (messenger RNA, abgekürzt mit **mRNA**), übertragen werden. Dieser Vorgang wird auch **Transkription** genannt. Die Maschine lässt sich am besten mit einem Formel 1 Rennwagen vergleichen, der während jeder Fahrt auf der Genrennstrecke ein mRNA-Molekül produziert. Die mRNA dient dann als Matrize für die Eiweißsynthese (die **Übersetzung**) an den Ribosomen, den Eiweißfabriken der Zelle. Es werden jedoch nicht zu jedem Zeitpunkt alle möglichen Eiweiße produziert; dies wäre reine Verschwendung von Energie und Rohstoffen. Zumeist wird mit der Eiweißsynthese erst begonnen, wenn das Eiweiß auch wirklich benötigt wird. Oft wird diese *go / no go* Entscheidung getroffen, bevor die Übertragung in mRNA stattfindet. Unmittelbar vor einem Gen befindet sich eine Art Transkriptionsampel (der **Promotor**), die entscheidet, wieviel Rennwagen auf die Strecke dürfen. Im Fall derjenigen Eiweiße, die permanent gebraucht werden, steht diese Ampel dauerhaft auf grün; in anderen Fällen muß erst jemand auf den Knopf drücken. Dies gilt auch für die Chloratmung in *Desulfitobacterium dehalogenans*. Für die Produktion der **Chlorphenoldehalogenase** wird dieses Signal von einem Chlorphenolmolekül gegeben.

Durch den Vergleich der abgeleiteten Aminosäuresequenz der Chlorphenoldehalogenase mit der bereits bekannter Dehalogenasen anderer Chloratmer haben wir herausgefunden, dass alle diese Eiweiße eine mehr oder weniger vergleichbare Struktur haben. Anscheinend verläuft die Dechlorierung mit allen bisher bekannten Dehalogenasen, die aus chloratmenden Bakterien gereinigt wurden, nach dem gleichen Reaktionsmechanismus.

Anschließend haben wir untersucht, unter welchen Bedingungen das Chlorphenoldehalogenase-Gen in **Chlorphenoldehalogenase-mRNA** übertragen und in aktive Dehalogenase übersetzt wird. Es ist uns gelungen, Standort und Wirkungsweise der Promotor-Ampel herauszufinden. Dabei hat sich herausgestellt, dass diese normalerweise auf Rot steht, und Übertragung (**Transkription**) nur stattfindet, wenn auch wirklich ein passender CKW in der Nähe ist. Weiterhin haben wir zeigen können, dass auf dem Weg zwischen Gen und der Anwesenheit aktiver Chlorphenoldehalogenase keine weiteren Ampeln stehen. Mit anderen Worten heißt das: Wenn sich Dehalogenase mRNA in der Zelle befindet, kann daraus direkt auf die Anwesenheit aktiver Dehalogenase, und somit auf aktiven Schadstoffabbau geschlossen werden.

Wir haben gesehen, dass die Produktion aktiven Eiweißes im Falle der Chlorphenoldehalogenase gleichbedeutend ist mit der Produktion von Dehalogenase mRNA. Von diesem Wissen haben wir bei der Entwicklung neuer Methoden profitiert, mit denen wir in der Lage sind, Dehalogenaseaktivität (also aktiven Abbau) durch Messen der Dehalogenase mRNA schnell und zuverlässig nachzuweisen. So eine Methode wäre sehr nützlich, um die Umweltbedingungen während einer Schadstoffsanierung maßgeschneidert auf optimale Abbauleistung einstellen zu können. Bei dem gewählten Ansatz gehen wir eigentlich den normalen Weg vom Gen zum Eiweiß in umgekehrter Richtung. Mit Hilfe einer besonderen Reaktion im Reagenzglas ist es möglich, ein mRNA-Molekül zurück in das dazugehörige Gen zu übertragen, also in ein DNA-Molekül. Dieses DNA-Molekül kann dann in einem zweiten Schritt mit Hilfe einer sogenannten PCR-Reaktion aufgespürt werden (PCR steht für Polymerasekettenreaktion). Dabei wird ein bestimmtes DNA-Molekül, in diesem Fall also das Dehalogenase-Gen, solange selektiv vervielfältigt, bis es nachweisbar oder sogar sichtbar gemacht werden kann. Bisher haben wir diese Methode nur mit Bakterien geprüft, die im Laboratorium isoliert wurden. Wir denken aber, dass wir demnächst auch in der Lage sein werden, unseren Test auf verunreinigte Böden und Grundwässer anzuwenden, wo wir es mit Mischungen verschiedenster Bakterien zu tun haben.

Ein Molekularer Werkzeugkasten für *Desulfitobacterium*

Ein wesentlicher Teil dieser Arbeit beschäftigt sich mit dem Einrichten eines molekularen Werkzeugkastens für *Desulfitobacterium dehalogenans*. Was befindet sich nun genau in diesem Kasten, und wofür können die unterschiedlichen Werkzeuge benutzt werden? Der größte Teil des Inhalts unseres Werkzeugkastens kann verwendet werden, um die erblichen Eigenschaften des Bakteriums zu verändern.....der Werkzeugkasten ist also eigentlich ein Baukasten (siehe Abbildung 3).

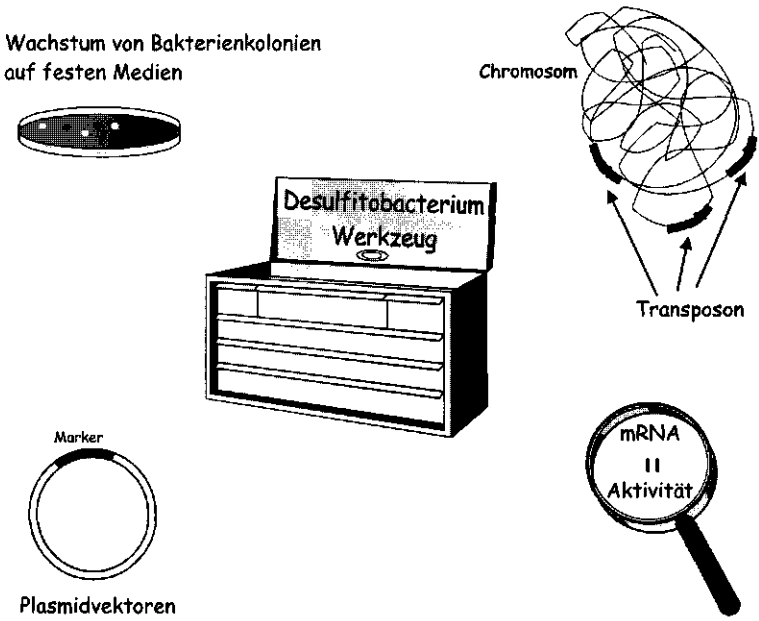


Abbildung 3 Ein molekularer Werkzeugkasten für *Desulfitobacterium dehalogenans*. Ein **Transposon** kann benutzt werden, um Gene unspezifisch auszuschalten, wogegen **Plasmidvektoren** verwendet werden können, um die erblichen Eigenschaften einer Bakterienzelle gezielt zu manipulieren (verändern, ausschalten und hinzufügen). Die meisten dieser molekularen Techniken resultieren allerdings in einer Mischung von Bakterienzellen, die jeweils kleine Unterschiede in ihren genetischen Eigenschaften haben. Will man diese Unterschiede untersuchen, muss man in der Lage sein, die einzelnen Zellen dieses Gemisches voneinander zu trennen. Lässt man die Bakterien in diesem Gemisch nun ausreichend verdünnt auf einem festen Medium wachsen, entstehen nach einiger Zeit kleine Zellhaufen, sogenannte **Bakterienkolonien**. Jede dieser Bakterienkolonien entsteht letztendlich aus einer einzelnen Zelle; alle Zellen innerhalb einer Kolonie besitzen dann dieselben erblichen Eigenschaften und werden auch Klone genannt. Nur so können die Eigenschaften unterschiedlicher Zellen unabhängig voneinander untersucht werden. Schließlich haben wir noch eine Methode entwickelt, um mit Hilfe einiger Reaktionsschritte im Reagenzglas die Anwesenheit von **Chlorphenoldehalogenase-mRNA** und somit aktiven Schadstoffabbau nachzuweisen.

Eines der Werkzeuge, ein sogenanntes **Transposon**, ist selbst ein mobiles Stückchen DNA, welches alle nötigen Informationen enthält, um an einer willkürlichen Stelle in das Chromosom einer Bakterienzelle zu springen. Dabei kann es natürlich auch passieren, dass das Transposon mitten in ein Gen springt, welches dann unterbrochen wird und nicht mehr funktioniert. Das bedeutet, dass von diesem Gen kein aktives Eiweiß mehr produziert werden kann. Auf diese Art und Weise ist es uns gelungen, eine Reihe von Genen aufzuspüren, die vermutlich eine wichtige Rolle in der Chloratmung spielen.

Eine zweite Gruppe von Instrumenten ist die der **Plasmidvektoren**. Auch dies sind DNA-Moleküle, welche sich in einer Bakterienzelle unabhängig vom Chromosom vervielfältigen können. Plasmidvektoren können verwandt werden, um die Funktion unbekannter Gene aufzuklären, indem diese gezielt ausgeschaltet werden. Das funktioniert ungefähr auf die gleiche Weise wie es gerade für ein Transposon erklärt wurde. Allerdings können wir Plasmidvektoren auch benutzen, um die Funktion eines Gens zu verändern, aber nicht auszuschalten. Zum Beispiel ist es möglich, die Ampel vor einem Gen so einzustellen, dass das entsprechende Eiweiß permanent produziert wird. Schließlich ist es auch denkbar, den erblichen Eigenschaften des Bakteriums neue Gene hinzuzufügen. So könnte man das Bakterium in die Lage versetzen, ein anderes Substrat zu verwenden, das z. B. billiger wäre.

Die Moral von der Geschichte

Mikroorganismen, CKW's, Leben ohne Sauerstoff, Eiweiße, Gene, Veränderung genetischer Eigenschaften,

....*aber eben auch:* Sanierung verschmutzter Böden und Grundwässer.

Wir haben versucht, eine kürzlich entdeckte Gruppe von Bakterien zu charakterisieren und ihre Eigenschaften ans Licht zu bringen. Unsere Ergebnisse können uns jetzt helfen, diese Bakterien noch effizienter für den Abbau chlorierter Kohlenwasserstoffe einzuspannen.

...Womit wieder mal bewiesen wäre, dass die Kluft zwischen Grundlagenforschung und Anwendung oft kleiner ist, als häufig angenommen wird.

NAWOORD

Bijna precies zes jaar geleden heb ik voor het eerst de Wageningse Berg beklommen, om aan boord van "het Schip van Blaauw" aan te monsteren voor een ontdekkingsreis over de wereldzeeën van het microbiologisch onderzoek. Al snel kwam ik erachter, dat ook op deze reis goed en slecht weer elkaar afwisselden. Er waren perioden met zon; maar ook fases met zwaar onweer. Soms kwam ik ook in dikke mist terecht – je ziet niets, en je hebt het gevoel, wekenlang géén millimeter vooruit te komen. Bijzonder opwindend waren de momenten, waarop totnogtoe onbekende eilandjes plotseling aan de horizon verrezen. Deze reis nadert nu zijn einde, en ik schrijf op een zonnige zondagmiddag met vioolconcerten van J. S. Bach op de achtergrond de laatste regels van deze reisverhalen – mijn proefschrift. Ik wil dan ook op deze plaats iedereen bedanken die het mogelijk gemaakt heeft, dat dit boekje tot stand is gekomen – binnen het laboratorium, maar ook daarbuiten. Jullie bijdrage is nauwelijks in woorden te beschrijven, maar toch veel te belangrijk, om het hierbij te laten!

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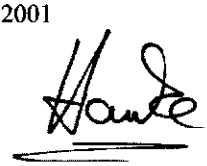
Wageningen, 21 januari 2001



About the Author

Hauke Smidt was born on November 23, 1967, in Göttingen, Germany. After moving to Mariensee at the age of eight, he completed secondary school and received his Abitur in 1987 at the Gymnasium Neustadt am Rübenberge. He then spent his military service in Celle before he started in 1988 with the study "Biotechnology" at the Technical University of Braunschweig. He graduated as a Diplom-Biotechnologe in 1995. From 1992 to 1993 he stayed in Kyoto, Japan, where he followed an intensive Japanese language course at the Kyoto English Center and carried out a short-term research project on the *Cloning and overexpression of L-ornithine-transaminase from the thermophilic Bacillus sp. YM-2 in E. coli* in the group of Prof. Kenji Soda (Laboratory of Microbial Chemistry, Kyoto University Institute for Chemical Research). In 1994, he joined the Institute of Technical Biochemistry, University of Stuttgart (Prof. Dr. Rolf D. Schmid), to study the *Preparation of optically pure chiral amines by lipase-catalyzed enantioselective hydrolysis of N-acyl-amines*. From 1995 to 2001 he worked as a PhD student at the Laboratory of Microbiology at the Wageningen University under the supervision of Dr. John van der Oost, Dr. Antoon D. L. Akkermans and Prof. Dr. Willem M. de Vos. As described in this thesis, his main interests were focused on the molecular characterization of reductive dehalogenation in halorespiring bacteria. From May 2001, he will move to Seattle, USA, where he will join the Microbial Ecology Research group of Prof. Dr. Dave A. Stahl, Department of Civil and Environmental Engineering at the University of Washington.

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