Biochemistry and Physiology of Halorespiration by *Desulfitobacterium dehalogenans*

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Stellingen

1. Halorespiratie is een weinig efficiënte wijze van ademhalen.
   Dit proefschrift

2. Halorespiratie moet worden opgevat als verbreding en niet als specialisatie van het genus *Desulfitobacterium.*
   Dit proefschrift

3. Reductieve dehalogenases zijn geen nieuwe enzymen.

4. 16S-rRNA probes zijn minder geschikt voor het aantonen van specifieke metabole activiteiten in een complex ecosysteem.
   Löffler *et al.* (2000) AEM **66**: 1369; Gottschal & Krooneman (2000) Bodem **3**: 102

5. Het "twin-arginine" transportsysteem wordt niet goed genoeg begrepen om op basis van het voorkomen van het "twin-arginine" motief enzymen te lokaliseren.
   Berks *et al.* (2000) Mol. Microbiol. **35**: 260

6. Asbesthoudende bodem is niet verontreinigd.

7. Biologische groente is een pleonasme.

Stellingen behorende bij het proefschrift 'Biochemistry and physiology of halorespiration by *Desulfitobacterium dehalogenans*' van Bram A. van de Pas

Wageningen, 6 december 2000

Biochemistry and Physiology of Halorespiration by *Desulfitobacterium dehalogenans*

Bram A. van de Pas

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
dr. ir. L. Speelman,
in het openbaar te verdedigen
op woensdag 6 december 2000
des namiddags te vier uur in de Aula.

Cover: Detail of the front entrance of the Laboratory for Microbiology, Hesselink van Suchtelenweg 4, Wageningen, The Netherlands

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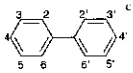
Chapter 1

General Introduction

Bram. A. van de Pas

Pollution of the environment by halogenated organic compounds is undesirable because of their impact on nature and human health. Nevertheless, modern society has produced and spilled many tons of these types of compounds, resulting in an increasing number of polluted sites. In the past, these compounds were regarded as xenobiotics, compounds that are strange (*Gk. xenos*) to life (*Gk. bios*), and therefore the compounds that were found in the environment had to be man-made. In the last 50 years, however, it has become clear that halogenated organic compounds are also produced in nature (Table 1.1), and the number of known natural organohalogens has increased from a dozen in 1954 to nearly 2400 in 1996 (Gribble, 1996). It is suggested that naturally produced organohalogens often have a chemical defensive role as antifeedant, repellant, or pesticide (Gribble, 1996). Most of these compounds are produced in small amounts when compared to the anthropogenic production, but the naturally produced organohalogens can be important locally and in some cases on global scale. For instance, *Basidiomycetes* have been found to produced chloro-aromatic compounds in concentrations that exceed the Dutch and Canadian hazardous waste norms for analogous halogenated compounds and would require mandatory remedial action (Field *et al.*, 1995). Furthermore, estimations of the amount of naturally produced halomethanes exceed the anthropogenic production by a factor 10 (Gribble, 1996).

Table 1.1: A selection of chlorinated hydrocarbons, their major applications, and natural sources

Compound	Structure	Anthropogenic source[a]	Natural source[b]
Methylchloride (MC)		reagent for methylation reactions in chemical industry	marine algae, fungi, volcanoes, forest fires
Tetrachloroethene (PCE)		solvent and degreasing agent used in industry and dry cleaning	marine algae, volcanoes
Chlorobenzenes		Intermediate of dye production *pesticide* "toilet stones"	halogen containing minerals
Chlorophenols (CP)		disinfectant, wood preservative, herbicide fungicide, insecticide	fungi, ticks, grasshoppers, forest fires, humines, microbes
polychlorinated biphenyls (PCB)		flame retardant	volcanoes

[a]: Data from (Fetzner, 1998)
[b]: Data from (Gribble, 1996)
[c]: The numbering indicates the carbon atoms that may contain chlorine atoms

Because many halogenated organic compounds were found to be toxic and recalcitrant, their removal is of utmost importance. Remediation techniques for the clean up of soil and groundwater are based on extraction, chemical conversion, or biological degradation of the contaminants (Kim & Qi, 1995; Schipper *et al.*, 1996). Similar principles apply to natural attenuation of pollutants, a complex of natural degradation processes in which microorganisms play a major role. Microorganisms are able to degrade a large number of halogenated compounds under different conditions because of their abundance, species diversity, catabolic versatility, and high metabolic activity (Alexander, 1981). The mechanisms for microbial degradation of halogenated organic compounds will be discussed here, with emphasis on halorespiration, a novel respiratory pathway in which bacteria profit from the reductive dehalogenation of halogenated organic compounds.

Microbial degradation of halogenated compounds

Microorganisms have developed different strategies to dehalogenate halogenated organic compounds. The cleavage of the carbon-halogen bond is catalyzed by specific enzymes (dehalogenases), by spontaneous dehalogenation of unstable intermediates, or by enzymes that fortuitously dehalogenate the halogenated analogs of their substrates (Fetzner & Lingens, 1994).

Microbial dehalogenation of halogenated organic compounds occurs via five different mechanisms (e.g. oxidative dehalogenation, dehydrohalogenation, substitutive dehalogenation, dehalogenation by methyl transfer, and reductive dehalogenation) (Fetzner, 1998). Oxidative dehalogenation involves the oxidation of a double bond in unsaturated organohalogens by a mono- or di-oxygenase resulting in an unstable intermediate, which is dehalogenated (Fig. 1.1, reaction 1). In dehydrohalogenation of saturated halogenated compounds, like the insecticide lindane (γ-hexachlorohexane), HCl is eliminated from the substrate by dehydrohalogenases resulting in the formation of a double bond (Fig. 1.1, reaction 2). Substitutive dehalogenation in most cases is a hydrolytic process catalyzed by halihydrolases, but the halide can also be substituted by glutathione by glutathione-S-transferases in a "thiolytic" mechanism. A third mechanism of substitutive dehalogenation involves an intramolecular substitution reaction, which is catalyzed by halohydrin hydrogen-

halide lyases (Fig. 1.1, reactions 3a, b, and c) *Dehalobacterium formicoaeticum* and *Acetobacterium dehalogenans* MC are able to dehalogenate chloromethane by methyl transfer from chloromethane onto tetrahydrofolate. This reaction is catalyzed by a chloromethane dehalogenase (Fig. 1.1, reaction 4) (Mägli *et al.*, 1996; Meßmer *et al.*, 1996). The last mechanism for dehalogenation of organohalogens is reductive dehalogenation, which in which a halogen is replaced by a hydrogen atom (Fig. 1.1, reaction 5).

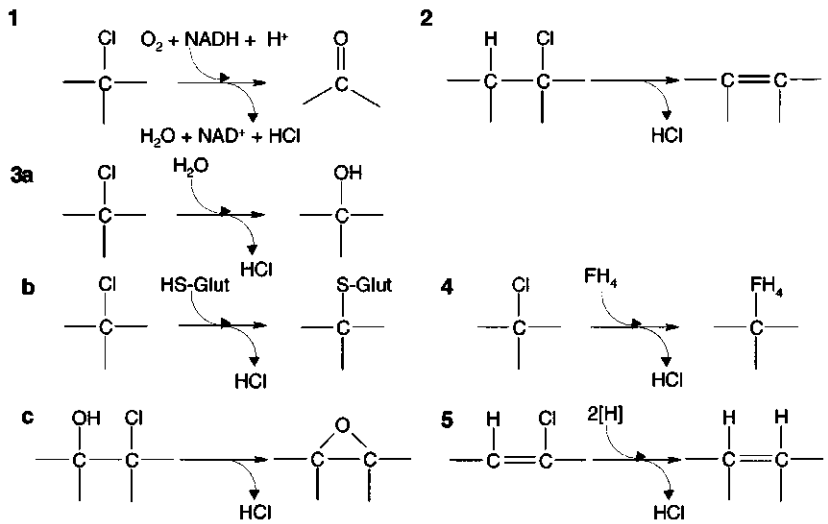


Figure 1.1: Mechanism of enzyme-catalyzed dechlorination of chlorinated compounds by microorganisms. (1) oxidative dehalogenation, (2) dehydrohalogenation, substitutive dehalogenation via (3a) hydrolytic dehalogenation, (3b) glutathione substitution, (3c) intramolecular substitution, (4) dehalogenation by methyl transfer, (5) reductive dehalogenation (Adapted from Janssen *et al.*, 1994).

While oxidative dehalogenation and substitutive dehalogenation are the most common mechanisms under aerobic conditions, reductive dehalogenation prevails under anaerobic conditions. Many anaerobic microorganisms, like methanogens, sulfate-, iron- and nitrate-reducing bacteria, are able to reductively dehalogenate haloaromatic and haloaliphatic compounds. This is a co-metabolic process, which is not coupled to energy or carbon metabolism and that is catalyzed by co-factors like F_{430}, cobalamin, and cytochromes (Holliger & Schraa, 1994). In contrast, halogenated organic compounds can be used as terminal electron acceptor for growth in the respiratory pathway by a number of anaerobic bacteria (see below and review of

Holliger *et al.*, 1999). In the literature, this process has been referred to as dehalorespiration and halorespiration (Holliger *et al.*, 1999, El Fantroussi *et al.*, 1998). The first term indicates the type of reaction involved (dehalogenation). In this thesis the term *halorespiration* is used since it refers to the type of electron acceptors which is used in this pathway (halogenated hydrodrocarbons) in analogy with other respiratory pathways e.g. fumarate respiration.

There are many review articles that deal with the different mechanisms, enzymes, and bacteria involved in microbial dehalogenation (Dolfing & Beurskens, 1995; El Fantroussi *et al.*, 1998; Fetzner, 1998; Fetzner and Lingens, 1994; Holliger and Schraa, 1994; Holliger & Schumacher, 1994; Holliger *et al.*, 1999; Janssen *et al.*, 1994; Mohn & Tiedje, 1992). For reviews that focus on the microbial degradation of haloaliphates see (Bradley, 2000; Lee *et al.*, 1998; Leisinger, 1996; Middeldorp *et al.*, 1999; Pries *et al.*, 1994; Slater *et al.*, 1997; Vogel *et al.*, 1987) and for haloaromatics see (Annachatre & Gheewala, 1996; Commandeur & Parson, 1994; Neilson, 1990; Reineke, 1988). In view of the scope of this thesis, we will focus on halorespiration.

Halorespiring bacteria

In 1984, Shelton and Tiedje described the enrichment of 3-chlorobenzoate degrading strain DCB-1 from a methanogenic consortium growing on 3 chlorobenzoate as sole carbon and energy source, which was selected from municipal digester sludge over a two-year period. The inability of this isolate to use the endproducts of dehalogenation suggested that dehalogenation could supply energy to the organism (Shelton & Tiedje, 1984). However, little progress was made towards understanding the physiology of strain DCB-1 (now designated as *Desulfomonile tiedjei*) until in 1990 a defined culture medium was described (DeWeerd *et al.*, 1990; Linkfield & Tiedje, 1990). Dolfing (1990) showed that reductive dehalogenation of 3-CB was coupled to ATP production and growth in *Desulfomonile tiedjei*, which was further substantiated by Mohn and Tiedje (1990; 1991). These results provided conclusive evidence for halorespiration in *Desulfomonile tiedjei*. At this time, *Desulfomonile tiedjei* was the only microorganism in pure culture known to perform this novel way of respiration, but the number of species able to perform halorespiration gradually increased over the years (Table 1.2). Environmental samples in which halogenated compounds could be

detected were found to be excellent inocula for enrichment of halorespiring bacteria. There is one report of the isolation of a dehalogenating bacterium from sediments containing halogenated aromatics from biological origin. This isolate, strain DSL-1, has been isolated from sediments from the burrows of the 2,6-dibromophenol-producing marine hemicordate *Balanoglossus aurantiacus* (Steward *et al.*, 1995). However, the biomass yield did not increase when brominated phenol was present in the medium, which suggests that this organism may not couple reductive dehalogenation to growth (Steward *et al.*, 1995). Halorespiring bacteria have been isolated from different polluted sources and include phylogenetically unrelated organisms (Table 1.2). In spite of this diversity, the range of halogenated substrates that is known to support growth is limited to three types of compounds, chlorobenzoates, halogenated phenols (*ortho-*, *para-* or *meta*-substituted), chloroalkenes (tetrachloroethene (PCE), trichloroethene (TCE), and dichloroethene (DCE)). *Desulfomonile tiedjei* is able to dechlorinate 3-chlorobenzoate, chlorophenols, and PCE, but it only dechlorinates PCE and chlorophenols co-metabolically together with 3-chlorobenzoate (Cole *et al.*, 1995; Mohn & Kennedy, 1992). *Dehalobacter restrictus* and *Dehalospirillum multivorans* are able to couple PCE-dechlorination to growth (Holliger *et al.*, 1993; Scholz-Muramatsu *et al.*, 1995). However, these bacteria remove two chlorines from this substrate, yielding mainly *cis*-DCE. *Dehalococcoides ethenogenes* is the only isolate that is able to convert PCE completely to ethene, but the last step in this dehalogenation sequence, vinylchloride to ethene, is not coupled to energy conservation (Maymó-Gatell *et al.*, 1997).

Figure 1.2: Reductive removal of a chlorine from the *ortho*-position of 3-chloro-4-hydroxy-phenylacetate (Cl-OHPA) as performed by *D. dehalogenans* (Utkin *et al.*, 1994)

A remarkable group is the genus *Desulfitobacterium* that comprises a large number of halorespiring bacteria. All isolates of this genus that have been described up to now are able to use halogenated compounds as terminal electron acceptor (El

Fantroussi *et al.*, 1998). The first isolate of this genus, *Desulfitobacterium dehalogenans*, was described by Utkin *et al.* (1994). This bacterium is able to reductively remove chlorine from the *ortho*-position of chlorinated phenolic compounds (Fig. 1.2). It can also use hydroxylated polychlorinated biphenyls (PCB) as electron acceptor and is reported to be able to dechlorinate PCE as well (Wiegel *et al.*, 1999). Like *Desulfomonile tiedjei*, *D. dehalogenans* cannot use PCE as terminal electron acceptor, but cells that were pregrown on pyruvate and 3-chloro-4-hydroxyphenylacetate showed a low PCE dechlorination rate (Gerritse *et al.* 1999). PCE can be used as terminal electron acceptor by the closely related *Desulfitobacterium* sp. PCE1, *Desulfitobacterium* sp. Viet 1, *Desulfitobacterium* sp. TCE1, and *Desulfitobacterium* sp. PCE-S which dechlorinate PCE either to TCE or *cis*-DCE (Gerritse *et al.*, 1999, 1996; Löffler *et al.*, 1997, 1999; Miller *et al.*, 1997). Apparently there are differences in the specificity of the reductive dehalogenases involved in dechlorination of chloroalkenes in these organisms (see Chapter 6 of this thesis). Except for strain Viet1 and strain TCE1, all *Desulfitobacterium* strains are able to dechlorinate chlorinated phenols (Table 1.2). Most often these bacteria remove a chlorine from the *ortho*-position, but dechlorination of the *meta*- and *para*-position is also observed (Table 1.2).

Next to chlorinated compounds most halorespiring bacteria are able to use other electron acceptors and several electron donors (Table 1.2). *Dehalococcoides ethenogenes* and both *Dehalobacter* species, however, are restricted to hydrogen as only known electron donor and chlorinated ethenes as electron acceptor (Table 1.2). This indicates that the latter species always have to use the chlorinated compound for their energy metabolism independent of the presence of other electron acceptors. Species that are able to use more electron acceptors must have a regulation mechanism that determines which electron acceptor is used. This mechanism is important for in situ remediation applications because other electron acceptors, like nitrate, sulfate, iron(III), and oxygen, are often present in the environment. The electron donor usually is limiting in anaerobic soils or sediments, which means that there is a competition for electrons between the dechlorinating population and other microbial populations as well as between the different respiratory pathways within the dechlorinating organism.

The competition between halorespiring bacteria and other microbial communities has not been studied with well-defined co-cultures.

Table 1.2: An overview of halorespiring bacteria

Phylogenetic group Species	Origin	Electron-donor	Electron acceptor		Fermentation substrates	Remarks	References
			Non-chlorinated	Chlorinated			
Low G+C Gram positives							
Desulfitobacterium dehalogenans	methanogenic lake sediment	formate, hydrogen, lactate, pyruvate	cysteate, fumarate, isethionate nitrate, sulfite, sulfur, thiosulfate	2,4-CP, 2,6-DCP, 2,4,6-TCP, Cl-OHPA, PCE, OH-PCB	pyruvate	*ortho*-position	Holliger *et al.*, 1999; Lie *et al.*, 1999; Utkin *et al.*, 1995; Utkin *et al.*, 1994; Wiegel *et al.*, 1999
Desulfitobacterium hafniense	municipal sludge	pyruvate	Iron(III), isethionate nitrate, sulfite, thiosulfate	2,4-DCP, 2,4,6-TCP, PCP, Cl-OHPA,	pyruvate, tryptophan	*ortho-* & *meta*-position	Christiansen & Ahring, 1996; Lie *et al.*, 1999; Madsen & Licht, 1992
Desulfitobacterium frappieri PCP-1	sewage sludge & soil	pyruvate	Nitrate, sulfite, thiosulfate	2,4,6 TCP, PCP, Cl-OHPA, OH-PCB, Cl-catechol, PCE	pyruvate	*ortho-*, *para* - & *meta*-position	Bouchard *et al.*, 1996; Dennie *et al.*, 1998
Desulfitobacterium chlororespirans	compost soil	butyrate, crotonate, formate, hydrogen, lactate, pyruvate	sulfite, sulfur, thiosulfate	2,6-DCP 2,4,6 TCP, PCP, Cl-OHPA	pyruvate	*ortho*-position	Sanford *et al.*, 1996
Desulfitobacterium sp. PCE1	polluted soil	butyrate, ethanol, formate, lactate, pyruvate, succinate	cysteate, fumarate, isethionate sulfite, thiosulfate	2, 6-DCP, 2,4,6-TCP, PCE, Cl-OHPA	pyruvate	*ortho*-position PCE is mainly converted to TCE	Gerritse *et al.*, 1996; Lie *et al.*, 1999
Desulfitobacterium sp Viet1	river sediment	hydrogen		PCE		PCE is mainly converted to TCE	Löffler *et al.*, 1997; Löffler *et al.*, 1999
Desulfitobacterium sp. PCE-S	polluted soil	formate, hydrogen, pyruvate, yeast	fumarate, sulfite	PCE, TCE, 2,4,5-TCP, PCP		*ortho-* & *meta*-position	Holliger *et al.*, 1999; Miller *et al.*, 1997
Desulfitobacterium frappieri TCE1	polluted soil	butyrate, crotonate, ethanol, formate, hydrogen, lactate,	fumarate, nitrate, sulfite, thiosulfate	PCE, TCE	pyruvate, serine		Gerritse *et al.*, 1999
Dehalobacter restrictus	river sediment	hydrogen		PCE, TCE			Holliger *et al.*, 1998; Holliger *et al.*, 1993
Strain TEA (*Dehalobacter* sp.)	anaerobic reactor	hydrogen		PCE, TCE			Wild *et al.*, 1997

Phylogenetic group Species	Origin	Electron-donor	Electron acceptor Non-chlorinated	 Chlorinated	Fermentation substrates	Remarks	References
δ Subdivision Proteobacteria							
Desulfomonile tiedjei	methanogenic consortium	formate, hydrogen, pyruvate,	sulfate, sulfite, thiosulfate	3-CB, PCE, PCP	pyruvate, methoxy-benzoates	*meta*-position, cometabolic conversion of PCE and chlorophenols	Cole *et al.*, 1995; DeWeerd *et al.*, 1990; Fathepure *et al.*, 1987; Linkfield and Tiedje, 1990; Mohn and Kennedy, 1992; Mohn & Tiedje, 1990
Desulforomonas chloroethenica	river sediment	acetate, pyruvate	fumarate, iron(III) polysulfide	PCE, TCE			Krumholz, 1997
Desulfuromonas sp. BB1	river sediment	acetate	Sulfate, sulfite, sulfur, thiosulfate	PCE, TCE			Löffler *et al.*, 1997; Löffler *et al.*, 1999
Desulfovibrio sp. TBP-1	estuarine sediments	fumarate, hydrogen, lactate,		2,6-DBP, 2,4,6 TBP		*ortho*- and *meta*-position	Boyle *et al.*, 1999
Strain 2CP-1 (*Myxobacterium* sp.)	river sediment	acetate	fumarate	2-CP, 2,6-DCP		*ortho*-position facultative anaerobe	Cole *et al.*, 1994
ε-Subdivision Proteobacteria							
Dehalospirillum multivorans	activated sludge	ethanol, formate, hydrogen, lactate, pyruvate, sodium-sulfide	arsenate, fumarate, nitrate, selenate	PCE, TCE	pyruvate		Scholz-Muramatsu *et al.*, 1995
Dehalospirillum sp. PCE-M1	polluted soil	Formate, hydrogen, lactate, pyruvate	arsenate, fumarate, nitrate, nitrite, selenate	PCE, TCE	pyruvate, fumarate		Luijten *et al.*, 2000
γ-Subdivision Proteobacteria							
Enterobacter strain MS-1	polluted sediment	acetate, amino acids, formate, glucose, lactate, pyruvate, yeast extract	nitrate, oxygen	PCE, TCE	amino acids, carbohydrates, fatty acids, purines, pyrimidines	possibly cometabolism, facultative anaerobe	Sharma & McCarty, 1996
Green non-sulfur bacteria							
Dehalococcoides ethenogenes	municipal digestersludge	hydrogen		PCE, TCE, DCE, VC; 1,1-DCE, DCA		No ATP from conversion of VC	Maymó-Gatell *et al.*, 1999; Maymó-Gatell *et al.*, 1997

¡Abbreviations: CP: chlorophenol; DCP: dichlorophenol, TCP: trichlorophenol; PCP: pentachlorophenol, Cl-OHPA: 3-chloro-4-hydroxyphenyl acetate; PCB: polychlorinated biphenyl; PCE: tetrachloroethene; TCE: trichloroethene; DCE: dichloroethene; CB: chlorobenzoate; DBP: dibromophenol; TBP: tribromophenol; VC: vinylchloride; DCA: dichloroethane

Experiments with soil-column and enrichments indicate a tendency that reductive dechlorination rates decrease with increasing redox potential (e.g. methanogenic > sulfidogenic > iron reducing > nitrate reducing conditions) (Bradley, 2000; Dolfing and Beurskens, 1995; Gerritse *et al.*, 1997; Häggblom *et al.*, 2000; Kuhlmann & Schottler, 1996; Lee *et al.*, 1997; Pavlostathis & Zhuang, 1993; Stuart *et al.*, 1999). This observation is in agreement with the Gibbs free energy values for reductive dechlorination of halogenated compounds and reduction of other electron acceptors with e.g. hydrogen as electron donor (El Fantroussi *et al.*, 1998). The importance of thermodynamics has recently been demonstrated by comparison of the hydrogen thresholds that were obtained for different halorespiring pure cultures and enrichments (Löffler *et al.*, 1999). This approach was based on the concept that the energetics of the terminal electron accepting process determines the minimum hydrogen concentration that can be used (i.e. the threshold). Hydrogen thresholds of >0.01-0.4 ppmv were obtained for the different halorespiring incubations which indicates that halorespiration competes for electrons with fumarate reduction, iron reduction, and nitrate reduction. Furthermore, this indicates that halorespiring bacteria should be able to outcompete methanogens and sulfate reducers because they are able to use hydrogen at a lower concentration. This also suggests that halorespiring bacteria may be good partners in syntrophic association with hydrogen producing bacteria (e.g. propionate-oxidizers), which would increase the competitiveness of halorespiring bacteria in the environment.

Although halorespiration is an attractive respiratory pathway from a thermodynamic point of view, additional regulatory mechanisms may determine how the electron flow is directed by the dechlorinating bacterium. Therefore it is important to determine the way other electron acceptors influence dechlorination in pure cultures. In batch cultures of *D. dehalogenans* and electron acceptor-limited continuous cultures of *Desulfitobacterium frappieri* TCE1, fumarate, nitrate and sulfite were used concomitantly with the chlorinated substrate (Gerritse *et al.*, 1999; Mackiewicz & Wiegel, 1998). In contrast, PCE dechlorination was completely inhibited when strain TCE1 was cultivated under lactate-limiting conditions (Gerritse *et al.*, 1999). These differences in effect suggest that the ratio of electron donors and acceptors is more important that the actual concentrations of these compounds. A regulation mechanism in which expression of the dehalogenase is induced by its halogenated substrate and repressed by other electron acceptors may be present. A

similar mechanism may be present for regulation of PCE dechlorination in batch cultures of *Dehalospirillum multivorans*. PCE dechlorination was found to be inhibited by fumarate and elemental sulfur (or polysulfide) when these other electron acceptors were added in 10 to 100-fold higher concentration than PCE (Neumann *et al.*, 1994). Likewise, a reduction in dehalogenating activity was demonstrated when *Desulfomonile tiedjei* was grown in the presence of both 3-chlorobenzoate (2mM) and either sulfate (5mM), sulfite (5mM), or thiosulfate (5mM) (Townsend & Suflita, 1997). In this case, the authors demonstrated that the presence of the sulfur oxyanions in the growth medium reduced the dehalogenase activity of the cell extracts made of these cultures and thus influences the regulation of this activity (Townsend and Suflita, 1997). Sulfite may well have a similar effect upon induction of dechlorination in *Desulfitobacterium* species. In addition to this indirect effect, there is a direct inhibitory effect of sulfur compounds on dechlorination by cell extracts of fully induced cells of halorespiring bacteria (DeWeerd & Suflita, 1990; Gerritse *et al.*, 1999; Löffler *et al.*, 1996; Miller *et al.*, 1997; Townsend and Suflita, 1997). The activity of purified PCE and TCE reductive dehalogenases from a co-culture containing *Dehalococcoides ethenogenes* was inhibited upon addition of 2mM sulfite, indicating that sulfite acts as an inhibitor of the enzyme (Magnuson *et al.*, 1998). These examples clearly demonstrate that the choice for an electron acceptor by dechlorinating bacteria not only depends on favorable thermodynamics.

Bioenergetics of 3-chlorobenzoate and tetrachloroethene respiration

Halorespiration has been studied in detail in *Desulfomonile tiedjei, Dehalobacter restrictus,* and *Dehalospirillum multivorans*. In general, a halorespiring chain should contain an electron donating enzyme, electron carriers, and a reductive dehalogenase as terminal reductase. In the process of electron transport from an electron donor (e.g. formate or hydrogen) to the electron acceptor (halogenated compound), a proton gradient is established across the cytoplasmic membrane which can be used for ATP synthesis by a proton-driven ATPase.

The first evidence for the formation of a proton gradient was obtained by determination of medium acidification by cell suspensions of *Desulfomonile tiedjei* during dechlorination of 3-chlorobenzoate (3-CB) with hydrogen or formate as

electron donor (Mohn and Tiedje, 1991). Under these conditions, a H^+/3-CB ratio of 2.1 was determined. Based on the enzyme localization a model was proposed for 3-chlorobenzoate respiration by *Desulfomonile tiedjei* which involves a periplasmic orientation of formate dehydrogenase and hydrogenase and a membrane-associated reductive dehalogenase which is probably oriented towards the cytoplasm (Louie & Mohn, 1999). A quinone and a cytochrome c were suggested to be involved in electron transfer from formate to 3-CB although neither the quinone nor the cytochrome c were able to function as electron donor for dechlorination in cell extracts (Louie and Mohn, 1999; Louie *et al.*, 1997). Quinone-dependent vectorial proton translocation may occur in 3-CB respiration, but the evidence is currently lacking (Fig. 1.3A).

The enzymes involved in PCE respiration by *Dehalobacter restrictus* have a similar localization (Holliger and Schumacher, 1994). Hydrogen was found to be able to reduce cytochrome b and quinone analogues and the use of quinone inhibitors has indicated that menaquinone is indeed involved in electron transfer (Holliger *et al.*, 1999; Schumacher & Holliger, 1996). However, menaquinone analogues did not act as electron donor for the reductive dehalogenase in membrane extracts, which indicates that menaquinone is not the direct electron donor for PCE reductive dehalogenase (Holliger *et al.*, 1999). Oxidant pulse experiments with cell suspensions of *Dehalobacter restrictus* generated an extrapolated H^+/e^- ratio of 1.25. This ratio is higher could be expected on basis of the suggested localization of the enzymes without the involvement of a proton pump (Holliger *et al.*, 1999). It is possible that menaquinone functions as a proton pump during electron transfer, but this is unlikely since menaquinone was shown to take up and release protons from the cytoplasmic side of the membrane (Schumacher and Holliger, 1996; Fig. 1.3 B).

A similar model has been proposed for *Dehalospirillum multivorans* although the PCE reductive dehalogenase from this bacterium has been found to be cytoplasmic (Holliger *et al.*, 1999; Miller *et al.*, 1996). In addition, a proton gradient or a membrane potential may be essential for chloroethene respiration since several ionophores have been found to inhibit dechlorination in whole cell suspensions of *Dehalospirillum multivorans* (Miller *et al.*, 1996). Involvement of menaquinone in electron transfer is doubtful, although menaquinone is present in the cells (Miller *et al.*, 1996; Scholz-Muramatsu *et al.*, 1995).

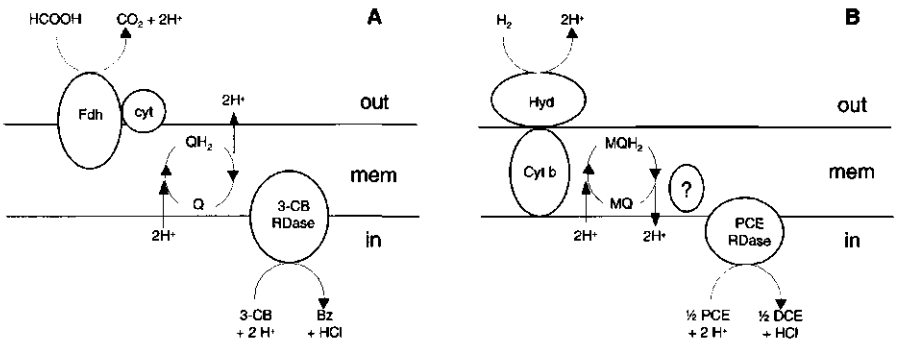


Figure 1.3: The electron transport system catalyzing the oxidation of formate or hydrogen coupled to reductive dechlorination. The right panel (A) shows a tentative model of 3-chlorobenzoate respiration with formate as electron donor in *Desulfomonile tiedjei* (adapted from Louie and Mohn, 1999). The left panel (B) shows a model for PCE respiration with hydrogen as electron donor in *Dehalobacter restrictus* (adapted from Holliger *et al.*, 1999). Fdh: formate dehydrogenase; Cyt: cytochrome; Q; quinoid; Rdase: reductive dehalogenase; Hyd. Hydrogenase; MQ: menaquinone; Mem: membrane

Little is known about chlorophenol respiration. In a yield study, Mackiewicz and Wiegel (1998) reported that a growth yield of approximately 24 gram dry weight per mol reduced electron acceptor was obtained with cultures of *D. dehalogenans* grown with pyruvate as electron donor and either nitrate, sulfite, fumarate, or 3-chloro-4-hydroxyphenyl acetate as electron acceptors. The authors assumed that the (complete) reduction of each electron acceptor yielded 1 ATP by electron transport phosphorylation. However, the growth yield decreased during growth with formate and Cl-OHPA. This suggests that the yeast extract, which was added to the medium, could be used as additional source of energy (Mackiewicz and Wiegel, 1998; van de Pas *et al.*, 2000). In continuous cultures, *D. hafniense* and *Desulfitobacterium* sp. PCE1 produced approximately 3.5 gram protein per mol Cl-OHPA reduced with lactate as electron donor and *Desulfitobacterium* sp. PCE1 produced 1.8 g protein per mol Cl-OHPA with formate as electron donor (Gerritse *et al.*, 1999). When the assumption is made that a the protein content of a cell is 50% of its dry weight, these biomass yields are 3 to 6 times lower than those reported for *D. dehalogenans*. The growth yield of strain PCE1 grown with formate and Cl-OHPA is similar to those which have been reported for cultures of *Dehalobacter restrictus* and *Desulfomonile tiedjei* grown with hydrogen and PCE or formate and 3-chlorobenzoate, respectively (Gerritse *et al.*, 1999; Holliger *et al.*, 1993; Mohn and Tiedje, 1990). Therefore, chlorophenol respiration might yield a similar amount of energy as has been reported for PCE- and 3-CB-dechlorination, which is less than 1 ATP.

Table 1.3: Characteristics of reductive dehalogenases that have been isolated from different halorespiring bacteria

Enzyme	3-CB-reductive dehalogenase	PCE-reductive dehalogenase	PCE-reductive dehalogenase	PCE-reductive dehalogenase	Cl-OHPA reductive dehalogenase	PCE-reductive dehalogenase	TCE-reductive dehalogenase
organism	*Desulfomonile tiedjei*	*Dehalospirillum multivorans*	*Dehalobacter restrictus*	*Desulfitobacterium* strain PCE-S	*Desulfitobacterium hafniense*	*Dehalococcoides ethenogenes*	*Dehalococcoides ethenogenes*
substrate	3-CB	PCE, TCE	PCE, TCE	PCE, TCE	Cl-OHPA	PCE, TCE	TCE, DCE, VC
localization	membrane	cytoplasm	membrane	membrane	membrane	membrane	membrane
Size subunits (Kda)	64, 37	57	60	65	47	51	61
Cofactors	(heme) Fe/S	1 cobalamin 8Fe 8S	1 cobalamin 8Fe 8S	1 cobalamin 8Fe 8S	1 cobalamin 12Fe 13S	cobalamin Fe/S	cobalamin Fe/S
specific activity(U/mg	0.018	158	14	39	6	21	12
N-terminal amino acid sequence	nd	GVPGANAAEK	nd	ADIVAPITESF	AETMNYVPGP	nd	nd
reference	Ni *et al.*, 1995	Neumann *et al.*, 1996	Schumacher *et al.*, 1997	Miller *et al.*, 1998	Christiansen *et al.*, 1998	Magnuson *et al.*, 1998	Magnuson *et al.*, 1998

Abbreviations: CB: chlorobenzoate; PCE: tetrachloroethene, Cl-OHPA: 3-chloro-4-hydroxyphenyl acetate; TCE: trichloroethene; DCE: dichloroethene; VC: vinylchloride; nd: not determined; U: the amount of enzyme that converts 1 µmol substrate per minute

Reductive dehalogenases

Reductive dehalogenases are the terminal reductases, which are unique for halorespiration. Reductive dehalogenases from several halorespiring bacteria have been characterized (Table 1.3). 3-chlorobenzoate reductive dehalogenase from *Desulfomonile tiedjei* was the first dehalogenase, which was (partially) purified. It is the only dehalogenase, which has been isolated as a heterologeous dimer (64 and 37 kDa). Its activity was a factor 100 lower as has been reported for other dehalogenases. Furthermore this enzyme is the only dehalogenase where a heme was suggested to be present as cofactor in the active center (Louie and Mohn, 1999; Ni *et al.*, 1995). The other dehalogenases have been isolated as monomers and contain cobalamin as cofactor (Christiansen *et al.*, 1998; Magnuson *et al.*, 1998; Miller *et al.*, 1998; Neumann *et al.*, 1996; Schumacher *et al.*, 1997). Furthermore, all reductive dehalogenases have been found to be membrane-associated, except for PCE reductive dehalogenase from *Dehalospirillum multivorans*, which was found to be located in the cytoplasm (Table 1.3). Methyl viologen ($E^{\circ\prime}$= -450mV), but not benzyl viologen ($E^{\circ\prime}$= -360mV), can be used as artificial electron donor for dechlorination, indicating that the dehalogenases involved have a low redox potential (Miller *et al.*, 1997; Neumann *et al.*, 1995; Schumacher and Holliger, 1996). In Chapters 4 and 5 of this thesis, four new reductive dehalogenases are identified, which have similar characteristics.

Different models have been postulated to explain the mechanism by which the electrons are transferred to the chlorinated substrate via cobalamin (Fig. 1.4). The models have been proposed for PCE dechlorination, but it is likely that a similar mechanism applies to both haloalkene and haloaromate dechlorination (Chapter 4, this thesis). Neumann *et al* (1996) proposed a model for PCE reductive dehalogenase of *Dehalospirillum multivorans* in which a Co(III)-chloroethene complex is assumed to be an intermediate in the dechlorination reaction since cyanide inhibits dechlorination (Fig. 1.4 A). Schumacher *et al.* (1997) proposed a mechanism for the reductive dechlorination of PCE and TCE by *Dehalobacter restrictus*, which is different from the one proposed by Neumann *et al.* (1996), and involves the formation of a chloroalkene radical (Fig. 1.4 B). The role of cobalamin in electron transfer in the catalytic center of a terminal

reductase is unique and places reductive dehalogenases as a new group of terminal reductases and of cobalt containing enzymes.

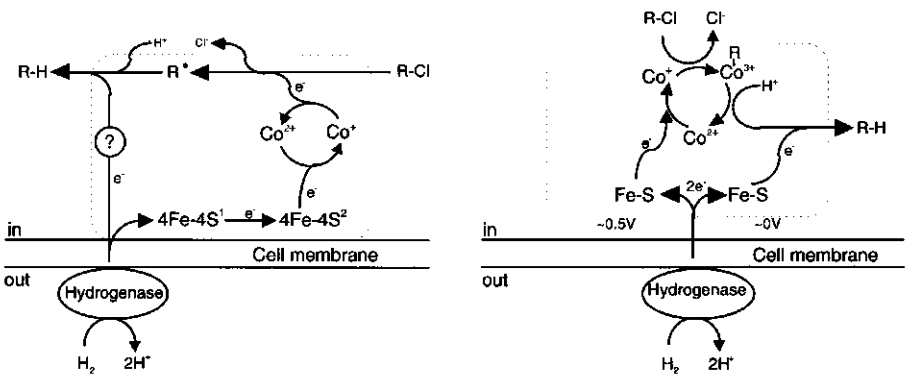


Figure 1.4: Postulated reaction mechanisms for cobalamin containing PCE reductive dehalogenases of *Dehalobacter restrictus* (left panel) and *Dehalospirillum multivorans* (right panel) (taken from Middeldorp *et al.*, 1999).

Outline of this thesis

The aim of this research was to elucidate how reductive dechlorination is coupled to ATP formation and to characterize reductive dehalogenases from halorespiring bacteria. The bioenergetics of halorespiration was studied in *Desulfitobacterium dehalogenans*, a Gram-positive, strict anaerobic bacterium that is able to use chlorophenolic compounds as terminal electron acceptor (Utkin *et al.*, 1994). We studied how this organism conserves energy via chlorophenol respiration, which resulted in two theses: this thesis which describes the biochemistry of halorespiration and the thesis of H. Smidt which will be dealing with the genetics of halorespiration.

In Chapter 2 of this thesis, the results of enzyme localization studies and a yield study are presented that were conducted with different electron donors and 3-chloro-4-hydroxyphenyl acetate (Cl-OHPA) as electron acceptor. This enabled us to determine the amount of energy that is conserved via electron transport phosphorylation during halorespiration. To gain insight into composition of this electron transport chain, the components that can be reduced by formate and oxidized by Cl-OHPA were identified by spectroscopic and HPLC analysis of cell suspensions. We compared the electron transport chain involved in halorespiration with that involved in fumarate respiration (Chapter 3). Chapter 4 describes the purification and

characterization of the chlorophenol reductive dehalogenase, which is responsible for reductive dechlorination of the halogenated compounds. The knowledge on chlorophenol respiration in *D. dehalogenans* that was obtained (Chapter 2-4) has been used to propose a model for this novel respiratory pathway. Next to chlorinated compounds, *D. dehalogenans* is able to use other compounds as electron acceptor. We investigated how other electron acceptors influence dehalogenating activity and how dehalogenase activity is induced (Chapter 5). In Chapters 6 and 7 we extended our research to other species of *Desulfitobacterium.* In Chapter 6, reductive dehalogenases are isolated and characterized from *Desulfitobacterium* strain PCE1, which is able to use both PCE and Cl-OHPA as electron acceptor, and *D. frappieri* strain TCE1, which utilizes only chloroalkenes. Furthermore, a novel strain of *Desulfitobacterium frappieri* has been isolated from human feces, which is described in Chapter 7. This strain is the first *Desulfitobacterium* species, which does not use chloroethenes or chlorophenols as terminal electron acceptor. The thesis is concluded with a summary and concluding remarks which are presented in English and in Dutch (Chapters 8 and 9)

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Chapter 2

Energy yield of chloro-aromate respiration in *Desulfitobacterium dehalogenans*

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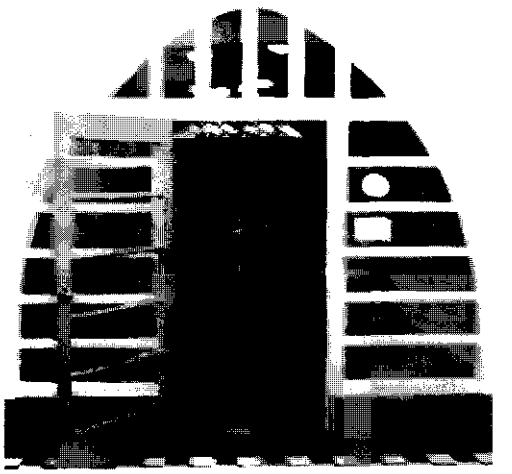

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Abstract

The amount of energy that can be conserved via halorespiration by Desulfitobacterium dehalogenans was determined by the comparison of the growth yields of cells grown with 3-chloro-4-hydroxyphenyl acetate (Cl-OHPA) and different electron donors. Cultures that were grown with lactate, pyruvate, formate, or hydrogen as electron donor and Cl-OHPA as electron acceptor yielded 3.1, 6.6, 1.6 and 1.6 g of dry weight per mole of reduction equivalents, respectively. Fermentative growth on pyruvate yielded 14 g of dry weight per mole of pyruvate oxidized. Pyruvate was not fermented stoichiometrically to acetate and lactate, but an excess of acetate was produced. ^{13}*C-labeled bicarbonate experiments showed that during pyruvate fermentation, approximately 9% of the acetate was formed from reduction of* CO_2*. Comparison of the growth yields suggests that one mole ATP is produced per mole acetate produced by substrate-level phosphorylation and that there is no contribution of electron transport phosphorylation when D. dehalogenans grows on lactate-Cl-OHPA or pyruvate-Cl-OHPA. Furthermore, the growth yields indicate that approximately 1/3 mole ATP is conserved per mole Cl-OHPA reduced in formate-Cl-OHPA and hydrogen-Cl-OHPA grown cultures. Because neither formate nor hydrogen or Cl-OHPA supports substrate-level phosphorylation, energy must be conserved through the establishment of a proton motive force. Pyruvate ferredoxin oxidoreductase, lactate dehydrogenase, formate dehydrogenase, and hydrogenase were localized by in-vitro assays with membrane impermeable electron acceptors and donors. The orientation of chlorophenol reductive dehalogenase in the cytoplasmic membrane, however, could not be determined. A model is proposed, which may explain the topology analyses as well as the results obtained in the yield study.*

Introduction

Reductive dechlorination of chlorinated compounds can be coupled to growth in a process called halorespiration (Fetzner, 1998). *Desulfomonile tiedjei* is the first described organism with this ability. It is also the first organism for which the chemiosmotic coupling of reductive dechlorination and ATP synthesis has been demonstrated (Mohn & Tiedje, 1991). Presently, several bacterial strains are known to have the ability to grow on a combination of a chlorinated compound and reduced substrates like formate and hydrogen (El Fantroussi *et al.*, 1998). Since oxidation of formate and hydrogen cannot result in ATP formation by substrate-level phosphorylation, energy conservation can only occur through formation of an electrochemical gradient across the cytoplasmic membrane. Recently, a model has been proposed for 3-chlorobenzoate respiration by *D. tiedjei*. In this organism, formate oxidation was suggested to take place at the outside of the cytoplasmic membrane whereas 3-chlorobenzoate was found to be reduced inside the cell. Coupling these two processes would result in charge separation, which can be used for ATP formation. In addition, it has been suggested that during electron transport from formate to the chlorobenzoate reductive dehalogenase, protons are actively transported across the membrane, resulting in extra energy gain by halorespiration (Louie & Mohn, 1999). However, proton translocation could not be demonstrated for PCE respiration coupled to hydrogen-oxidation in *Dehalobacter restrictus* (Schumacher *et al.*,1996).

Desulfitobacterium dehalogenans is the first described member of the genus *Desulfitobacterium* (Utkin *et al.*, 1994). All *Desulfitobacterium* isolated since have the capacity to reductively dechlorinate chloro-aromatics, chloro-alkenes, or both (El Fantroussi *et al.*, 1998). Furthermore, these organisms are able to use sulfite, fumarate, and nitrate as electron acceptors. Comparison of batch cultures of *D. dehalogenans* grown with pyruvate and different electron acceptors has indicated that comparable growth yields were obtained when 3-chloro-4-hydroxyphenylacetate (Cl-OHPA), fumarate, sulfite, or nitrate were used as electron acceptor (Mackiewicz & Wiegel, 1998). These authors proposed that chloro-aromate respiration yields 1 ATP per reduced chloro-aromate by electron transport phosphorylation.

In the present study, we have determined the amount of energy conserved through halorespiration by comparison of the growth yields obtained from batch cultures of *Desulfitobacterium dehalogenans* grown on different electron donors and Cl-OHPA as electron acceptor. The enzymes involved in electron donation and acceptation were localized, and ^{13}C-NMR was used to investigate the contribution of acetate formation by CO_2 reduction during pyruvate fermentation and halorespiration. The studies on growth yields and enzyme localization suggest that there is no electron transport phosphorylation when *D. dehalogenans* grows on lactate and Cl-OHPA or pyruvate and Cl-OHPA. When formate and hydrogen serve as electron donor for halorespiration, however, a proton motive force is established which can be used for ATP generation.

Experimental procedures

Organism and growth conditions. *Desulfitobacterium dehalogenans* strain JW/IU-DC1 (DSM 9161) was cultivated at 37°C under anaerobic conditions (100% N_2 gas phase) in a medium described previously (Van de Pas *et al.*, 1999). The medium was buffered with 40 mM bicarbonate, and amended with 0.1% peptone. The yield studies were performed in 1 l bottles containing 500 ml medium with 10 mM lactate, 20 mM pyruvate, or 20 mM formate as electron donor and 20 mM Cl-OHPA as electron acceptor. When hydrogen was used as electron donor, 10 mmol hydrogen gas was added to the bottle before autoclaving. Pyruvate fermentation was studied in one set of bottles to which only 40 mM pyruvate was added. Bottles were inoculated with 1% of an actively growing substrate-adapted culture. Cells were harvested in late log phase, which was reached after about 48 hours for cultures on pyruvate or lactate and about 72 hours for cultures that used formate or hydrogen as electron donor. These experiments were performed in triplicate. For the NMR experiment, *D. dehalogenans* was cultivated in 120-ml bottles with 25 ml medium as described above, with the exception that the bicarbonate was replaced by ^{13}C labeled bicarbonate. The bacteria were cultivated at 37°C under Cl-OHPA reducing conditions with 15 mM pyruvate and 16 mM Cl-OHPA, or were grown fermentatively on 30 mM pyruvate. The media were inoculated with 1-2% of substrate–adapted cultures.

Dry weight determination. 400 ml culture of a 500-ml incubation was concentrated by 10 minutes centrifugation at 16,000 x g. The cell pellet was resuspended in 50 ml 100 mM NaCl in MilliQ water and centrifuged. Thereafter, cells were resuspended in 2 ml MilliQ water and transferred to a dry aluminum cup of known weight. The cell suspension was dried in a stove at 95°C. The cups were weighted after 24 and 72 hours.

Nuclear Magnetic Resonance (NMR) experiments. After 46 hours, samples (1 ml) were taken from the cultures and subsequently centrifuged. Proton-decoupled ^{13}C-NMR spectra of 450 µl sample and 50 µl D_2O in a 5 mm tube (25°C) were recorded at 125.7 MHz on an AMX-500 spectrometer (Bruker GMBH, Germany), located at the Wageningen NMR-center. Approximately 50,000 transients were accumulated for one spectrum. Chemical shifts are expressed in ppm relative to the internal standard of 50 mM natural abundance succinate. The C-2,3 resonances of succinate were set at 35.1 ppm. ^1H-NMR spectra were recorded at 500 MHz of the same samples on the same spectrometer at 25°C.

Analytical methods. Organic acids were analyzed with a SpectraSystem high-performance liquid chromatograph (Thermo Separation Products, Riviera Beach, USA) as described previously (Stams *et al.*, 1993). The samples for Cl-OHPA and OHPA determination were analyzed on a SpectraSystem high-performance liquid chromatograph, with a SpectraSystem P2000 pump, an AS3000 autosampler and a UV1000 UV-detector. 20 µl of sample was injected into a Chrompack pesticide reversed–phase column (Chrompack, Middelburg, The Netherlands). The mobile phase was acetonitril-0.01M H_3PO_4 with a volume/volume ratio of 10/90. A flow rate of 1 $ml.min^{-1}$ was applied. Cl-OHPA and OHPA were quantified by their absorption at 206 nm.

Preparation of cell extracts. All handlings were performed in an anaerobic glove box with a N_2/H_2 gasphase in a 95%/5% ratio. 500-ml cultures of *D. dehalogenans* were harvested by 10 min centrifugation at 16,000 g and resuspended to a total volume of 2 ml buffer A, containing 100 mM potassium phosphate (KP_i) pH 7.5 and 1 mM dithiothreitol (DTT). A portion, set aside on ice, was used as whole cell suspension. Cells were permealized by incubation of the whole cell suspension with 0.1% cetyltrimethylammonium bromide (CTAB) for 10 minutes at 4°C. A few crystals of DNase I were added to a fraction of the whole cell suspension and the cells

were broken in 6 cycles of 30 sec sonication and 30 sec cooling on ice. Unbroken cells were removed by 5 minutes centrifugation at 20,000 x g. 500 µl of this cell extract was stored on ice for enzyme assays. The remaining part was centrifuged for 90 minutes at 140,000 x g to separate membranes and cytoplasmic fraction. The supernatant containing the soluble proteins was transferred to a glass vial and stored at 4°C. The pellet containing the membranes was resuspended in buffer A. The fractions were stored at 0°C under a 100% N_2 gasphase.

Enzyme assays. Chlorophenol reductive dehalogenase activity was determined as described previously (Van de Pas *et al.*, 1999) by measuring the reduction of methyl viologen (ε_{578}= 9.8 $mM^{-1}cm^{-1}$) in N_2-flushed cuvets at 30°C. The assay mixture was 100 mM Tris-HCl buffer pH 7.8 and contains 0.3 mM titanium citrate-reduced methyl viologen and extract. The reaction was started by addition of 10 mM Cl-OHPA. Formate dehydrogenase (FDH), hydrogenase (HYD), carbon monoxide dehydrogenase (CODH), and pyruvate ferredoxin oxidoreductase (PFO) activity were measured at 30°C in a rubber-stoppered N_2-flushed cuvet containing 100 mM Tris-HCl buffer pH 8.0 and 1 mM methyl viologen. Methyl viologen was slightly reduced with titanium citrate to an OD_{578} of 0.05. 5 to 40 µl extract was added to the reduced buffer and the OD_{578} was followed. Upon a stable signal 50 µl substrate was added and the reduction of methyl viologen was measured. Hydrogenase activity was measured by addition of 500 µl oxygen-free hydrogen. CO dehydrogenase activity was measured by addition of 500 µl carbon monoxide. 0.2 mM HSCoA was added prior to addition of pyruvate. Lactate dehydrogenase activity was measured as described by Miller *et al.* (1996). Protein concentrations of the different fractions were determined by the micro-biuret method using bovine serum albumin as standard (Goa, 1953).

Chemicals. All chemicals were obtained from commercial sources, and of the highest purity available. Yeast extract was obtained from Difco, Detroit, USA. Peptone, made from trypsin-digested casein, was obtained from Merck, Darmstadt, Germany. ^{13}C-labeled bicarbonate was purchased from Isotec. Inc., Miamisburg, USA.

Results

Biomass yield study. The amount of cell material that is produced during growth of *D. dehalogenans* on different media has been used in this study to determine the amount of energy that is conserved through halorespiration. The culture medium contained peptone, instead of yeast extract, to supply for certain growth factors, since we had found that approximately 10 mM Cl-OHPA was dechlorinated when medium with only yeast extract (0.1%) and Cl-OHPA was inoculated with 1% of a pyruvate-Cl-OHPA grown culture. Replacing yeast extract by peptone did not result in growth and dechlorination. The biomass yield was determined of cells grown fermentatively with pyruvate (40 mM) or grown with Cl-OHPA (20 mM) as electron acceptor and lactate (10 mM), pyruvate (20 mM), formate (20 mM), and hydrogen (10 mmol) as electron donor. We have compared substrate depletion, product formation and calculated amount of electrons that was transferred (Table 2.1). Determination of substrate and product concentrations showed that all Cl-OHPA converted, was recovered as OHPA in the medium. The ratio of electron donor oxidized and Cl-OHPA reduced is approximately 1 for the incubations with pyruvate, hydrogen, and formate as electron donors, indicating that the redox balance was complete. The C-balance for the oxidation of pyruvate was complete, but not for lactate where only 80% of the electron donor converted was recovered as acetate. In the latter case, 0.46 mole acetate was found to be oxidized per mole Cl-OHPA produced (Table 2.1). This indicated that there was an extra source of reduction equivalents in these incubations, which has not been taken into account. Growth on lactate and Cl-OHPA reduction yielded 3.1 g biomass per mole of reduction equivalents, which was approximately half of the biomass yield that was obtained when pyruvate oxidation was coupled to Cl-OHPA reduction (Table 2.1). The yield per mole of acetate was comparable for both growth substrates, which indicated that oxidation of lactate to acetate and the conversion of pyruvate to acetate yielded a comparable amount of ATP. Oxidation of hydrogen or formate was not coupled to substrate-level phosphorylation. Under these growth conditions the biomass yield was approximately 3.2 g biomass per mole of Cl-OHPA reduced.

Table 2.1: Biomass yield and substrate utilization ratio of *Desulfitobacterium dehalogenans* grown under different conditions with Cl-OHPA as electron acceptor. The number between brackets gives the deviation from average. Yield is expressed as gram of biomass per mole of product or reduction equivalents produced.

Growth substrate	Ratio Donor/acetate	Ratio Cl-OHPA/OHPA	Ratio acetate/Cl-OHPA	Yield g/mol Cl-OHPA	Yield g/mol acetate	Yield g/mol [H]
Lactate-Cl-OHPA	0.82 (±0.09)	1.02 (±0.05)	0.46 (±0.05)	5.54 (±0.53)	11.85 (±0.36)	3.06 (±0.05)
Pyruvate-Cl-OHPA	1.00 (±0.21)	1.02 (±0.03)	1.02 (±0.06)	13.11(±0.64)	12.75 (±0.60)	6.59 (±0.28)
Formate-Cl-OHPA		1.08 (±0.01)	1.08*(±0.01)	3.20 (±0.49)	2.97#(±0.49)	1.60 (±0.23)
H_2-Cl-OHPA		0.96 (±0.11)	1.02*(±0.21)	3.30 (±0.85)	3.50#(±1.84)	1.65 (±0.52)

*: Conversion ratio electron donor: Cl-OHPA is given since product formation has not been measured.
#: Yield is given per gram of electron donor used since the product formation from formate or hydrogen oxidation has not been determined.

The conversion ratio of pyruvate, obtained when *D. dehalogenans* was grown fermentatively on pyruvate, was different from that reported previously (Utkin *et al.*, 1994). The latter authors reported that pyruvate was fermented to equal amounts of lactate and acetate in a HEPES buffered medium, which had a low bicarbonate concentration. However, in our experiments, where *D. dehalogenans* was cultivated in medium containing 30 mM bicarbonate, 28.1 (±0.7) mM pyruvate was converted to 23.4 (±0.5) mM acetate and 10.9 (±0.6) mM lactate. Growth by pyruvate fermentation yielded 14.2 (± 0.1) g biomass per mole of acetate produced. The concentration of acetate was about twice as high as the lactate concentration. Furthermore, the total concentration of organic acids increased with approximately 5 mM. Fixation of CO_2 into acetate is a possible explanation for the increase in organic acid concentration during fermentative growth on pyruvate.

Table 2.2: Substrate conversion and product formation by *Desulfitobacterium dehalogenans* grown for 46 hours at 37°C in medium buffered with 40 mM ^{13}C-labeled bicarbonate.

	Substrate conversion and product formation mmol/liter				
Culture	Pyruvate degraded	Lactate formed	Acetate formed	Cl-OHPA degraded	OHPA formed
Pyruvate-Cl-OHPA[a]	15.3		15.0	11.5	14.5
Pyruvate[b]	29.9	8.3	23.3		

[a]: The initial concentrations were 15 mM pyruvate and 16 mM Cl-OHPA
[b]: The initial pyruvate concentration was 30 mM

CO_2-fixation. To investigate the possibility of CO_2-reduction, we carried out a ^{13}C-NMR experiment with ^{13}C-labeled bicarbonate as source of CO_2 and as buffer. HPLC analysis of the organic acids in these cultures shows a similar conversion pattern as

was obtained in the yield study (Table 2.2). The ^{13}C-NMR spectra of supernatant of cultures of *D. dehalogenans* grown for 46 hours with ^{13}C labeled bicarbonate and pyruvate as energy source (Fig. 2.1, Trace A) showed a triplet at a chemical shift of 24.2 ppm, reflecting the presence of C_2 of single- and double-labeled acetate. The C_2-doublet around the single-labeled resonance has a coupling constant of 52 Hz, being the scalar coupling constant $^{13}C_2$-$^{13}C_1$. The amount of double-labeled acetate was estimated to be 40% of the total labeled acetate, which includes natural abundant ^{13}C-labeled acetate. From the ^{1}H-NMR spectrum it was estimated that approximately 4% of the acetate was labeled at the C_2 position, exceeding the 1% natural abundance of ^{13}C (data not shown). 96% of the acetate was not enriched and contributed to the signal. Also the C_1 carboxyl group of acetate was manifested in the ^{13}C spectrum as a triplet at 182.3 ppm. The doublet again had a splitting of 52 Hz. In addition, CO dehydrogenase, a key enzyme of the acetyl-CoA pathway (Ragsdale, 1994), was detected at an activity of 1.3 μmol.min^{-1}.mg protein^{-1}.

During growth on pyruvate and Cl-OHPA, the double-labeled peaks were absent when $^{13}CO_2$ was present (Fig. 2.2, Trace B). ^{1}H proton NMR indicated that acetate was not enriched (approximately 1% was found to be labeled) and reflected only natural abundance acetate.

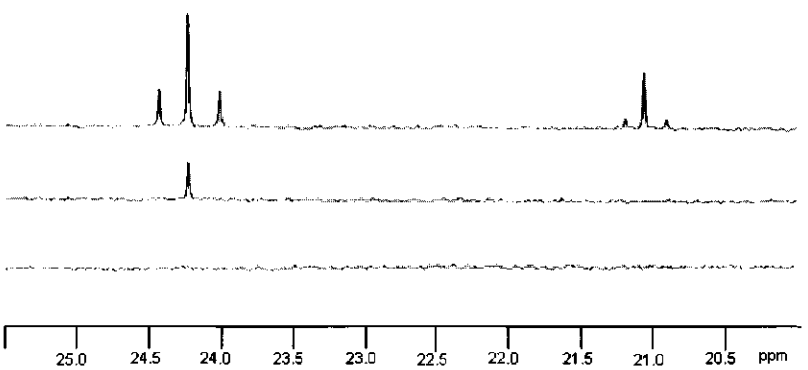

Figure 2.1: ^{13}C Nuclear magnetic resonance spectra of culture supernatants of *Desulfitobacterium dehalogenans* incubated for 46 hours with 40mM pyruvate (Trace A) or 20 mM pyruvate and 20 mM Cl-OHPA (Trace B). The spectrum given in trace C showed the supernatant of the pyruvate incubation just after inoculation (t=0).

Table 2.3: Localization of enzymes involved in respiration in *Desulfitobacterium dehalogenans* grown with Cl-OHPA as electron acceptor and different electron donors. Concentrated cells were permealized by addition of 0.1% v/v CTAB.

Enzyme	Growth-condition	Whole cells		Permealized cells		Cell free extract		Membrane fraction		Soluble fraction	
		Spec act. (mU*/m	Total act. (U)	Spec act. (mU/mg)	Total act. (U)	Spec act. (mU/mg)	Total act. (U)	Spec act. (mU/mg)	Total act. (U)	Spec act. (mU/mg)	Total act. (U)
LDH	Lactate-Cl-OHPA	74	6.05	69	5.57	142	1.70	195	1.45	356	1.87
PFO	Pyruvate-Cl-OHPA	6	0.25	270	11.11	161	4.05	0	0	107	1.08
FDH	Formate-Cl-OHPA	14	1.25	108	9.43	59	5.26	190	1.57	973	2.04
HYD	Hydrogen-Cl-OHPA	81	1.52	110	2.06	884	3.04	206	0.54	1726	0.69
CPRD	Pyruvate-Cl-OHPA	55	1.14	nd	nd	306	3.84	326	2.47	194	0.98

* :1 unit (U) is defined as the conversion of 1 µmol substrate per minute

Abbreviations: LDH: lactate dehydrogenase; PFO: pyruvate ferredoxin oxidoreductase ; FDH: formate dehydrogenase; HYD: hydrogenase; CPRD: chlorophenol reductive dehalogenase ;nd: not determined

These results demonstrate that CO_2 fixation is only important when *D. dehalogenans* is grown with pyruvate and CO_2 only and not when Cl-OHPA is present as electron acceptor. The contribution of CO_2 fixation to the acetate pool can be calculated from the recovery of organic acids. Approximately 9% of the acetate was produced by CO_2 fixation.

In addition to double-labeled acetate, double-labeled lactate was found when *D. dehalogenans* was grown fermentatively on pyruvate. The C_3 of lactate exhibits a resonance at 21.1 ppm (Fig. 2.1, Trace A). Approximately 6% of this signal was split into a doublet because of concomitant labeling at the C_2 position. The C_1 carboxyl group was found to be labeled to a higher degree. This is due to carboxylation of unlabeled acetate to pyruvate that became reduced to lactate. When these mechanisms are taken into account the mass balance on pyruvate is complete (Table 2.2).

Enzyme localization. Lactate dehydrogenase, pyruvate ferredoxin oxidoreductase, formate dehydrogenase, hydrogenase, and chlorophenol reductive dehalogenase were localized by *in-vitro* enzyme assays with methyl viologen as artificial electron donor or acceptor (Table 2.3). The pyruvate ferredoxin oxidoreductase was clearly located in the cytoplasmic fraction. Lactate dehydrogenase, formate dehydrogenase and hydrogenase activities were recovered for approximately 44% in the membrane fraction, which suggests that these activities are membrane associated. The chlorophenol reductive dehalogenase was for 71% located in the membrane fraction, which indicates that CPRD is a membrane bound enzyme.

The known inability of methyl viologen to pass through cell membranes was used for localization of the active sites of membrane bound enzymes (Kröger *et al.*, 1980). Enzyme activities were measured in concentrated cell suspensions, CTAB permealized cells, and cell extracts (Table 2.3). Pyruvate ferredoxin oxidoreductase could hardly be measured in the whole cell suspension, but the activity increased 45 times when the cells were permealized. The activity in cell extract was a little lower. This all points to a cytoplasmic location of PDH. Formate dehydrogenase activity measured in whole cells was high, but increased over 7.7 times after incubation with CTAB. The total activity of hydrogenase increased little after CTAB treatment, but increased 11 times upon sonication. Lactate dehydrogenase did not react with methyl viologen and had to be localized with phenazine methosulphate (PMS). Like methyl viologen, PMS cannot penetrate the membrane (Kröger *et al.*, 1980). High LDH activity was observed in the whole cell suspension. No increase was found when cells

were incubated with CTAB, but the specific activity did increase 2-fold upon sonication. CPRD could be assayed with whole cells, but could not be detected in cells that were permealized with CTAB. Furthermore, the CPRD activity of cell extract dropped when CTAB was added to the cuvet, indicating that CTAB has an inhibiting effect on CPRD. CPRD activity increased 5.6 times upon sonication of the whole cells.

Discussion

We have investigated the energy metabolism of *Desulfitobacterium dehalogenans* during halorespiration with different electron donors by analyzing biomass yields and enzyme localization. During growth of *D. dehalogenans* with pyruvate, formate, and hydrogen as electron donor and Cl-OHPA as electron acceptor, the oxidation of one mole pyruvate, formate, or hydrogen was coupled to the reduction of one mole Cl-OHPA. When lactate was used as electron donor, the conversion of one mole lactate was coupled to the reduction of two moles Cl-OHPA (Table 2.1). These results confirm the conversion pattern published previously (Utkin *et al.*, 1994). However, the fermentation pattern for growth with pyruvate as sole energy source was different. We did not find conversion of pyruvate into equal amounts of lactate and acetate, but instead a higher amount of acetate. The presence of double-labeled acetate and increased labeling at the C_2 position of acetate in the ^{13}C NMR spectrum shows that *Desulfitobacterium dehalogenans* is able to use CO_2 as electron acceptor. Although this is the first report which provides evidence for reduction of CO_2 by a *Desulfitobacterium* sp., other *Desulfitobacterium* strains are also thought to be able to reduce CO_2 to acetate, since acetate was the only product of fermentation of pyruvate (Bouchard *et al.*, 1996; Christiansen & Ahring, 1996; Gerritse *et al.*, 1999; Sanford *et al.*, 1996). Net ATP formation from CO_2 to acetate can only be formed via a chemiosmotic gradient across the cell membrane. The amount of energy thus conserved is not exactly clear, but ranges between 1/3 and 2/3 ATP per acetate formed (Diekert & Wohlfarth, 1994). This is less than the 1 ATP, which would be the result of substrate-level phosphorylation in pyruvate oxidation. As a result of CO_2-reduction, less energy is conserved per mole of acetate. When this effect is taken into account, the growth yield during fermentative growth is 14.6 to 15.0 g biomass per

mole ATP produced, which is comparable to the biomass yield reported for pyruvate fermentation by Mackiewicz and Wiegel (1998).

When *D. dehalogenans* was cultivated on lactate-Cl-OHPA or pyruvate-Cl-OHPA, we found in both cases a biomass yield of approximately 12.5 g per mole of acetate produced, which suggests that no electron transport phosphorylation occurred with lactate and pyruvate as electron donor. This yield is close to the amount of biomass that was formed from 1 ATP during fermentative growth, but is much lower than the biomass yield that was reported for growth on pyruvate-Cl-OHPA by Mackiewicz and Wiegel (1998). They reported a biomass yield of 24.2 g biomass per mole acetate produced, which was found to be comparable to the biomass yield for growth with pyruvate-fumarate, pyruvate-nitrate or pyruvate–sulfite. The discrepancy with our results can be due to the fact that we used a different growth medium, replaced yeast extract by peptone to supply for certain growth factors, and used smaller inocula (1% instead of approximately 10%) to limit the supply of extra growth substrates. For the related bacteria, *Desulfitobacterium sp.* PCE1 and *Desulfitobacterium hafniense*, grown in continuous culture with lactate and Cl-OHPA, growth yields of approximately 3.5 g protein per mole of electrons released have been reported (Gerritse *et al.*, 1999). When the protein contents is assumed to be 50% of the dry weight, the growth yields for *D. hafniense* and *Desulfitobacterium sp.* PCE1 are twice the growth yield we found for *D. dehalogenans*.

Formate and hydrogen were found to be poor electron donors for halorespiration, each yielding 1.6 g biomass per mole reduction equivalents. Mackiewicz and Wiegel (1998) reported the same growth yield for formate-Cl-OHPA grown cultures of *D. dehalogenans* that were harvested in late-exponential growth phase. The growth yields obtained for other halorespiring bacteria grown with formate or hydrogen range between 2.1 and 3.6 g biomass per mole chloride released. (Gerritse *et al.*, 1999; Holliger & Schumacher, 1994; Mohn & Tiedje, 1990). When comparing these biomass yields, it has to be taken into account that the amount of energy needed for maintenance of cell integrity is only constant when cells grow at the same rate. In our study, the maintenance coefficients for growth with lactate-Cl-OHPA, pyruvate-Cl-OHPA and pyruvate fermentation may be comparable whereas for growth with hydrogen or formate they may be higher since *D. dehalogenans* grows much slower with these two electron donors. A higher maintenance coefficient results in a lower biomass yield. Continuous culture studies as performed by Gerritse

et al. (1999) have the advantage that the maintenance coefficient is constant because the growth rate can be controlled. In batch cultures the growth rate is not constant which results in different yields in time. This was demonstrated by Mackiewicz and Wiegel (1998) who reported that the growth yield of *D. dehalogenans* with formate-Cl-OHPA varied between 1.6 to 5.7 g biomass per mol electrons when cells were harvested in different growth phases. Because of this effect the growth yields obtained from continuous cultures are likely to be higher than those obtained from cells that are grown in batch culture and harvested in late-exponential growth phase.

Pyruvate ferredoxin oxidoreductase was found to be located in the cytoplasm. Lactate dehydrogenase activity did not increase when cells were permealized by CTAB, which indicates that the active site of the enzyme is accessible from the outside. However, LDH activity increased 2-fold upon sonication of the cells. Furthermore LDH activity in *Desulfitobacterium* strain PCE-S was localized at the inside of the cytoplasmic membrane (Miller *et al.*, 1996). Therefore, we hypothesize that the LDH activity in *D. dehalogenans* is located at the inside of the cell membrane but that electrons can be transported over the cell membrane by an unknown mechanism and reduce PMS. Hydrogenase activity and formate dehydrogenase activity are localized both inside and outside the cell membrane, suggesting multiple enzymes to be present. For both enzyme activities, it could not be determined which activity corresponds to the respiratory enzyme. When the localization of FDH was determined in pyruvate-Cl-OHPA grown cells, little activity was found in whole cell suspension, but activity increased 22-times upon permeation (data not shown). This suggests the presence of at least two formate dehydrogenases, one facing outwards being involved in formate oxidation and one facing the cytoplasm and being involved in other processes such as CO_2 fixation. A similar situation may exist for hydrogenase. Since chlorophenol reductive dehalogenase activity increased 3.4 times after sonication of whole cell suspension, it might be argued that CPRD has its active site facing inwards. However, the CPRD activity measured in whole cells is relatively high, indicating that the active site of CPRD must be at least partly accessible for methyl viologen. The whole cell suspension did not contain lyzed cells because pyruvate ferredoxin oxidoreductase activity could not be measured in this cell suspension.

Based on the results presented in this study we propose a model for halorespiration in *Desulfitobacterium dehalogenans* in which the respiratory

hydrogenase and formate dehydrogenase activities are facing outwards analogous to the situation in *E. coli* (Sawer, 1994). Per formate or hydrogen, a gradient of 2 charges ($2q^+/2e^-$) can be established when CPRD is located inside the cell without vectorial proton translocation across the cytoplasmic membrane coupled to electron transport. There may be a loss of energy due to unknown processes (e.g. transport of substrates or products, or an activation barrier), which then explains the low growth yield of 3.2 g biomass per mole of substrate. Similar models have been proposed for 3-chlorobenzoate respiration in *Desulfomonile tiedjei* and PCE respiration in *Dehalobacter restrictus* and *Dehalospirillum multivorans* (Louie & Mohn, 1999; Miller *et al.*, 1996; Schumacher & Holliger, 1996). On the other hand, one may also argue that the CPRD is located at the outside of the cytoplasmic membrane, in which case electron transport has to be coupled to proton translocation. At present, it is not possible to discriminate between these two possibilities. On basis of these models, no proton motive force is established and no energy is conserved via electron transport phosphorylation during growth on pyruvate-Cl-OHPA and lactate-Cl-OHPA because LDH and PDH are located at the inside of the cell membrane. This is in accordance with the finding that the ATP yield per acetate is constant under different growth conditions. Cells that grow on these substrates conserve one mole ATP per mole acetate via substrate level phosphorylation of acetyl-CoA.

Our study indicates that chlorophenol respiration is not an efficient respiration pathway since only a fraction of the energy can be conserved that is theoretically possible, which is approximately 2 ATP per chloride removed with hydrogen as electron donor (El Fantroussi *et al.*, 1998). Future study of the composition of the electron transport chain involved in halorespiration may provide better insight in the halorespiration pathway.

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Chapter 3

Analysis of the halorespiratory pathway in whole cells of *Desulfitobacterium dehalogenans*

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Abstract

The respiratory electron transport chain from formate to 3-chloro-4-hydroxyphenyl acetate (Cl-OHPA) was investigated in Desulfitobacterium dehalogenans. Molybdenum, cobalt, menaquinone, cytochrome c, different iron-sulfur clusters, and a high-spin heme could be identified in concentrated cell suspensions by EPR, visible spectroscopy, and HPLC analysis of extracts from membranes. In cell suspensions, these components were reduced upon addition of formate and oxidized after addition of Cl-OHPA indicating that they are involved in halorespiration. Molybdenum is a common component of formate dehydrogenases, cobalamin is characteristic for the chlorophenol reductive dehalogenase and cytochrome c and menaquinone could be part of a connecting electron transport chain. The heme detected by EPR, may be the high-spin form of a low-spin cytochrome. Cytochrome b was detected at a low concentration in formate-Cl-OHPA grown cultures and may be involved in halorespiration. It could be demonstrated that the same menaquinone and cytochrome c are active with halorespiration and with fumarate respiration. Cytochrome b was present in a higher concentration, relative to cytochrome c, in formate-fumarate grown cultures than in formate-Cl-OHPA grown cultures and it was found to be active in fumarate respiration. This suggests that cytochrome b is involved in electron transport from menaquinone to fumarate reductase, but not in transport of electrons from MQH_2 to the reductive dehalogenase in halorespiration.

Introduction

Microorganisms in anaerobic environments can reductively dechlorinate highly chlorinated compounds like tetrachloroethene (PCE), pentachlorophenol (PCP), and polychlorinated biphenyls (PCB's). Strictly anaerobic bacteria, e.g. *Desulfomonile tiedjei*, *Dehalobacter restrictus*, *Dehalospirillum multivorans*, *Dehalococcoides ethenogenes*, and several *Desulfitobacterium* spp., are able to couple reductive dechlorination of chlorinated alkenes or aromatics to energy conservation in a respiratory process, often referred to as halorespiration (Holliger *et al.*, 1999). Formate and hydrogen can be used as electron donors in this process and since these compounds do not allow substrate-level phosphorylation, energy is most likely conserved by the formation of an electrochemical gradient across the cytoplasmic membrane (Holliger *et al.*, 1999).

In 1992, a model has been postulated for 3-chlorobenzoate respiration with formate in *Desulfomonile tiedjei* in which a formate dehydrogenase and a reductive dehalogenase are located at opposite sides of the cell membrane. Energy conservation was proposed to take place by establishment of a proton gradient without vectorial proton translocation (Mohn & Tiedje, 1992). Evidence for this model has recently been reported (Louie & Mohn, 1999). A similar model was proposed for hydrogen-PCE metabolism in *Dehalobacter restrictus* (Schumacher & Holliger, 1996). In *Desulfitobacterium dehalogenans*, the growth yield with 3-chloro-4-hydroxy-phenylacetate (Cl-OHPA) as electron acceptor and formate or hydrogen as electron donor could be explained without a mechanism of proton translocation across the cell membrane in a model analogous to that postulated for halorespiration in *D. tiedjei* (van de Pas *et al.*, 2000). In this model, the active sites of the uptake formate dehydrogenase and hydrogenase are located at the outside of the cytoplasmic membrane whereas the chlorophenol reductive dehalogenase (CPRD) faces the cytoplasm (van de Pas *et al.*, 2000). However, the orientation of the CPRD in the cytoplasmic membrane is not clear and analysis of the CPRD gene suggested localization at the outside of the cytoplasmic membrane (van de Pas *et al.*, 1999). If so, vectorial proton translocation may take place, but cannot be used for generation of ATP (van de Pas *et al.*, 2000).

In addition to *ortho*-chlorophenols, *D. dehalogenans* is able to use sulfite, thiosulfate, sulfur, nitrate, and fumarate as terminal electron acceptor (Utkin *et al.*, 1995; Utkin *et al.*, 1994). In this study, the involvement of redox active components for the transfer of electrons from formate to Cl-OHPA or fumarate was investigated. The redox state of these components was analyzed during their reduction by formate and subsequent oxidation by Cl-OHPA. The results suggest the involvement of molybdenum, cytochrome c, iron-sulfur clusters, menaquinone, cobalamin, and a high-spin heme in the chlorophenol respiration in *D. dehalogenans*.

Materials and methods

Organism and growth conditions. *D. dehalogenans* strain JW/IU-DC1 (DSM 9161) was cultivated at 37°C anaerobically under a 100% N_2 gas phase. The cultures were grown in 3-l bottles containing 2 l medium as described previously (van de Pas *et al.*, 1999). Cells were grown with 20 mM formate and 20 mM 3-chloro-4-hydroxyphenylacetate (Cl-OHPA) or with 20 mM formate and 20 mM fumarate.

Cell fractionation and enzyme assays. Late-exponential phase cultures were harvested by centrifugation and fractionated as described previously (van de Pas *et al.*, 2000). The protein concentration of the extracts was determined with the micro biuret method with bovine serum albumin as standard (Goa, 1953). Formate dehydrogenase, chlorophenol reductive dehalogenase, and fumarate reductase activities in cell extracts were determined by spectroscopic recording of changes in the reduced methyl viologen concentration at 578 nm as was described previously (van de Pas *et al.*, 2000)

Quinone-extraction. Quinones were extracted in methanol-petroleum ether and separated by HPLC as described previously (Bergen *et al.*, 1994). The wavelength was changed to 245 nm for the detection of menaquinone and quinol. The absorption spectrum of Vitamin K was used as control. Instability of (chemically prepared) menaquinol prevented reliable quantification of the quinol extinction coefficient. Therefore, the ratio between the peak areas in the HPLC chromatogram was used as indicator of the degree of quinone reduction.

UV-visible spectroscopic analysis of cell suspensions. Cultures (2 l) of *D. dehalogenans* grown on formate and fumarate or formate and Cl-OHPA were

harvested and washed anaerobically with 8 ml buffer containing 50 mM Tris-HCl pH 8.0 and 0.5 mM dithiothreitol (DTT) and resuspended in 50 mM Tris-HCl pH 8.0 to a concentration of 0.36 g wet cell weight.ml^{-1} in nitrogen-flushed vials. 90 µl of this concentrated cell suspension was resuspended in 0.9 ml 50 mM Tris-HCl pH 8.0. After incubation with reducing or oxidizing substance(s), the suspension was frozen in liquid N_2 and the spectrum (320-700 nm) was recorded at 77 K, on a DW-2a™ spectrophotometer (American Instrument Co.) as described previously (vanWielink *et al.*, 1982). After recording, the base lines were corrected for light scattering by subtracting a straight line between 540 and 580 nm. The corrected spectra were deconvoluted into two gaussian peaks of variable width, one signal (at 552 nm) representing cytochrome c and another signal (at 561 nm) representing cytochrome b. The spectra were analyzed as described previously (Krab *et al.*, 2000). The area of the peaks was taken to be proportional to the reduction level of the cytochromes. The involvement of cytochromes in electron transfer from formate to fumarate or Cl-OHPA was investigated by recording of the changes in their redox state after reduction of the cells with 1 mM formate and reoxidation by addition of 10 mM Cl-OHPA or fumarate.

Analysis of cell suspensions by EPR spectroscopy. Cells were harvested anaerobically by centrifugation, washed with nitrogen-flushed buffer containing 100 mM potassium phosphate buffer pH 7.5 and 1 mM DTT, and centrifuged again for 10 minutes at 16,000 x g. After centrifugation the cell pellet was resuspended in buffer to a total volume of 5 ml in nitrogen-flushed 20-ml vials. This concentrated cell suspension was incubated with 5 mM formate for 30 minutes at 37°C. Subsequently, Cl-OHPA was added to give a concentration of 20 mM and after 30 minutes incubation at 37°C Cl-OHPA was added to increase the concentration with 20 mM. Before each addition and 60 minutes after the last addition a sample (1 ml) was taken and concentrated in an EPR tube to approximately 150 µl by centrifugation. EPR spectroscopy was performed as described earlier (Pierik *et al.*, 1993). The supernatant was removed and stored at – 20℃ for Cl-OHPA and formate analysis by HPLC (van de Pas *et al.*, 2000). The pellet was removed from the anaerobic glove box and quickly frozen by dipping the tube in liquid nitrogen.

Results

Comparison of growth with formate and either Cl-OHPA or fumarate. The electron transport chain involved in halorespiration was investigated with formate as electron donor since formate-oxidation is well understood and formate alone does not support growth of *D. dehalogenans*. The addition of a suitable electron acceptor like fumarate or Cl-OHPA led to exponential growth with doubling times of 8 to 10 h. In the late-exponential phase, the formate-Cl-OHPA (FC) and formate-fumarate (FF) grown cultures reached an OD_{600} of 0.17 and 0.25, respectively, in medium with 20mM formate and 20 mM electron acceptor. This demonstrates that *D. dehalogenans* is able to use fumarate and Cl-OHPA as terminal electron acceptor for growth.

Topology of formate dehydrogenase, fumarate reductase and chlorophenol reductive dehalogenase. Formate dehydrogenase (FDH), chlorophenol reductive dehalogenase (CPRD), and fumarate reductase (FRD) were localized in FC and FF cells by determination of the activity of the enzymes in different fractions with in-vitro enzyme assays using the membrane impermeable artificial electron donor methyl viologen (Table 3.1). Enzyme activities were determined in concentrated cell suspensions, cells that were permealized with cetyltrimethylammonium bromide (CTAB), and cell extracts were obtained by sonication of concentrated cell suspensions. The membrane and cytoplasmic fractions were separated by ultracentrifugation of the cell extract. The protein content of the concentrated cell suspension and cell free extract was comparable in both preparations. Therefore the specific activities that are measured in concentrated cells, permealized cells and cell extracts can be compared. The membrane and cytoplasmic fractions contained 90 to 100% of the activity that was measured in the cell extracts except for CPRD where only 48% of the activity could be recovered. Furthermore, only 20% and 64% of the total protein from the cell extract was recovered from the membrane and cytoplasmic fraction of FC and FF cells, respectively. The activities that were measured in the membrane and cytoplasm fractions were used to determine in which fraction the enzyme was localized.

In a concentrated cell suspension of FC cells, an FDH activity of 14 nmol formate.min^{-1}.mg $protein^{-1}$ was measured, which increased 7.7 times after permeabilization of the cells. In FF cells, the specific FDH activity in concentrated

cells was 42% of the activity that was present in cell extract and increased 2.8 times upon permeabilization of the cells with CTAB. We found for both growth conditions that the specific activity of FDH is higher in the cytoplasmic fraction than in the membrane fraction, but over 90% of the activity was recovered from the membrane fraction. FRD was found to be strongly membrane bound and its activity increased 4-fold when cells were permealized with CTAB. The CPRD activity increased 1.7-fold upon permeation with toluene, which was used because CPRD was found to be inhibited by CTAB. However, FRD and FDH activities determined in toluene-permeabilized cells were 4 to 5 times lower than those determined in CTAB-treated cells, indicating that permeabilization with toluene was less efficient (not shown). The CPRD activity was mainly found in the membrane fraction.

Table 3.1: Specific activity of formate dehydrogenase (FDH), chlorophenol reductive dehalogenase (CPRD) and fumarate reductase (FRD) in different fractions of *Desulfitobacterium dehalogenans* cells, grown with formate as electron donor and Cl-OHPA or fumarate as electron acceptor. The total protein contents of the concentrated cells, permeable cells, cell extract, membrane fraction, and cytoplasm fraction was 174, 174, 180, 32, and 4 mg, respectively, for FC cells and 94, 94, 96, 56, and 5 mg, respectively, for FF cells. The ratio of the total activity of the cell extract that is recovered from the membrane and cytoplasm fraction is given between brackets.

Enzyme	e-acceptor	Concentrated cells Spec act[1]	Permeable cells[2] Spec act[1]	CFE Spec act[1]	Membrane fraction Spec act[1] (%)	Cytoplasm fraction Spec act[1] (%)
FDH	Cl-OHPA	14	108	59	189 (60)	973 (39)
	Fumarate	47	133	110	167 (90)	654 (32)
CPRD	Cl-OHPA	51	87[3]	89	234 (48)	7 (0)
FRD	Fumarate	37	151	294	457 (92)	0 (0)

[1]: Specific activity is expressed as nmol of substrate converted per minute per milligram of protein
[2]: Permeabilization with 0.1% CTAB
[3]: Cells were permealized with 0.04% toluene to avoid inactivation of CPRD by CTAB.

These results suggest that formate dehydrogenase is loosely membrane associated and present at both sides of the cytoplasmic membrane. Chlorophenol reductive dehalogenase activity is membrane bound and so is fumarate reductase. The latter enzyme was determined to be orientated at the cytoplasmic side of the cell membrane.

Menaquinone extraction of formate-Cl-OHPA and formate-fumarate grown cells. Quinones were extracted from formate-Cl-OHPA and formate-fumarate grown cells and their involvement in fumarate and halorespiration was investigated. The quinone, which could be extracted, exhibited an UV absorbance spectrum characteristic for menaquinones (not shown). The oxidized form of the isolated quinones showed two peaks at 245 and 270 nm and a broad absorption centered round

325 nm. The dithionite-reduced form exhibited a single peak at 245 nm and a broad absorption at 330 nm. The hydrophobic side chain of the menaquinone isolated from *D. dehalogenans* is likely to be shorter than 8 isoprenyl units because its retention time on a HPLC reversed phase column was shorter than that of MQ8 of *Escherichia coli*.

To investigate the possible involvement of this menaquinone in the fumarate and Cl-OHPA respiration, concentrated cell suspensions (FF and FC) were incubated in the presence of formate, and Cl-OHPA or fumarate (Table 3.2). After 10 minutes of incubation, the quinones were extracted from these cells. In both FF and FC grown cells the menaquinone pool was reduced with 10 mM formate. The quinone pool was also reduced upon addition of the reducing agent dithionite. The reduced quinone became oxidized when 10 mM Cl-OHPA was added to formate-reduced FC grown cells, or when 10 mM fumarate was added to formate-reduced FF grown cells. This indicates that menaquinone is involved in electron transport from formate to Cl-OHPA and fumarate.

Table 3.2: The ratio of peak areas of reduced and oxidized menaquinone extracted under different conditions from cells *of D. dehalogenans* grown with formate as electron donor and fumarate or Cl-OHPA as electron acceptor. Cells were incubated for 10 minutes with 10mM formate, Cl-OHPA or fumarate prior to quinone extraction. nd: not determined.

Conditions	Ratio MQH_2/MQ	
	Formate-fumarate grown cells	Formate-Cl-OHPA grown cells
formate	0.33	1.79
Cl-OHPA	nd	0.09
fumarate	0.01	nd
No addition	0.12	0.30

UV-visible spectroscopic studies of concentrated cell suspensions of formate-Cl-OHPA and formate-fumarate grown cultures. The absorption spectra were recorded from concentrated cell suspensions of FF and FC cultures to determine the involvement of cytochromes in fumarate respiration and halorespiration.

Cytochrome c was detected in both formate-Cl-OHPA and formate-fumarate grown cells. The dithionite-reduced minus oxygen-oxidized spectrum (at 77 K) of this cytochrome c showed optima at 419 and 552 nm (not shown). This cytochrome c could not be reduced by ascorbate, suggesting that the redox potential of this cytochrome is below +60 mV. In addition, another type of cytochrome was found to

be present in FC and FF cells. The reduced minus oxidized spectrum showed optima around 430 and 561 nm, suggesting the presence of a b-type cytochrome (not shown). This cytochrome was detected under both growth conditions, but the ratio of cytochrome b/cytochrome c was highest in the FF cells suggesting that cytochrome b is more important during growth on fumarate than during growth on Cl-OHPA. Spectral analysis of the membrane fractions of the cells indicated that both cytochromes are membrane associated. Spectra of the cytoplasmic fraction did not show any signal typical for cytochromes (not shown).

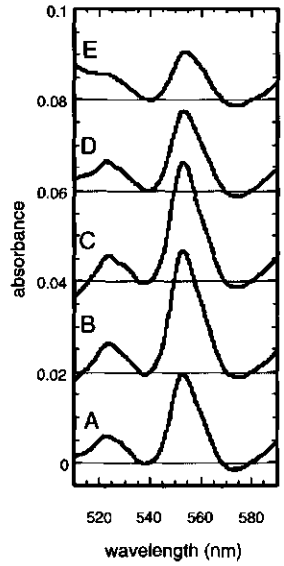

Figure 3.1: Low temperature cytochrome b, c α-band spectra of a concentrated cell suspension of *Desulfitobacterium dehalogenans* grown with formate as electron donor and Cl-OHPA as electron acceptor. Spectra were shifted upwards in steps of 0.02 absorbance units to clarify the Figure. Trace A shows the absorption spectrum from cells without additions. Trace B was recorded after incubation with 10 mM formate. Trace C was recorded after reduction of the sample with dithionite for 8 minutes. Trace D was recorded after incubation of the cells with 1 mM formate and 10 mM Cl-OHPA. Trace E was recorded after incubation of the cells with 10 mM Cl-OHPA.

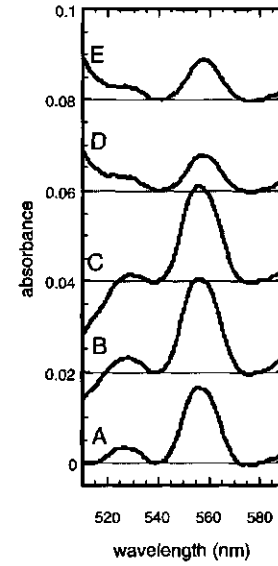

Figure 3.2: Low temperature cytochrome b, c α-band spectra of a concentrated cell suspension of *Desulfitobacterium dehalogenans* grown with formate as electron donor and fumarate as electron acceptor. For details see legend to Fig. 3.1. Trace A shows the absorption spectrum from cells without addition. Trace B was recorded after incubation with 10 mM formate. Trace C was recorded after reduction of the sample with dithionite. Trace D was recorded after incubation of the cells with 1 mM formate and 10 mM fumarate. Trace E was recorded afar incubation of the cells with 10 mM fumarate.

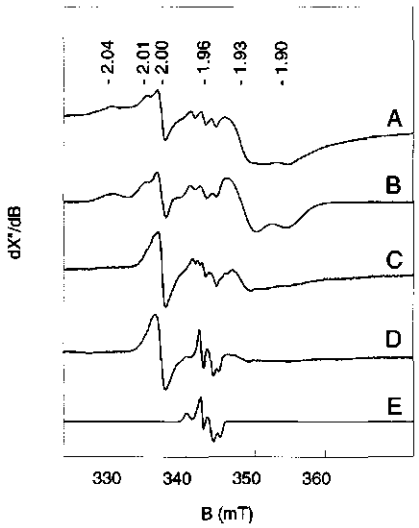

Figure 3.3: EPR of $[2Fe\text{-}2S]^{1+}$ and Mo^{5+} in whole cells of *D. dehalogenans* grown on formate-Cl-OHPA. Cells were reduced by incubation for 10 min. with formate (trace A-C) and maximally re-oxidized by incubation for 40 min. with Cl-OHPA (trace D-E). Trace A was taken at high microwave power to emphasize Fe/S signals over Mo and radical signals. Trace B is a simulation on the basis of two different [2Fe-2S] signals (g_{xy}=1.926, g_z=2.01 and g_{xy}=1.902, g_z=2.04) in a 1:1 ratio plus a small amount of Mo signal (4%) and radical signal (2%). Trace C was taken at low microwave power to de-emphasize Fe/S signals. Trace C changes into trace D upon prolonged oxidation with Cl-OHPA. Trace E is a simulation of the Mo(V) signal in trace D with g_{xyz} = 1.952, 1.961, 1.972. The signal is split by hyperfine interaction with one proton: A_{xyz} = 0.9, 1.0, 1.8 mT. EPR conditions: microwave frequency, 9410 ± 5 MHz; microwave power, 80 mW (trace A) or 0.2 mW; modulation amplitude, 0.4 mT; temperature, 40 K.

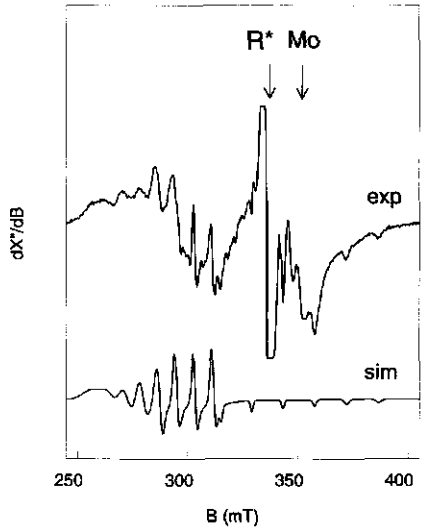

Figure 3.4: EPR of Co(II)balamin in Cl-OHPA re-oxidized cells of *D. dehalogenans* grown on formate-Cl-OHPA. The experimental spectrum (exp) is dominated by the base-off form of Co(II)balamin with simulation (sim) parameters essentially identical to those determined for the purified dehalogenase: g_{xy}=2.355, g_z=1.990; linewidth in mT is W_{xy}=7, W_z=1; cobalt hyperfine splitting in mT is A_{xy}=7.6, A_z=14.0. EPR conditions: microwave frequency, 9411 MHz, microwave power, 2 mW; modulation amplitude, 0.4 mT; temperature, 27 K.

The involvement of these cytochromes in the electron transfer from formate to fumarate or Cl-OHPA was investigated. Changes in the redox state of the cytochromes were followed by visible spectrometry at 552 and 561 nm. The concentration of cytochrome b in FC cells was too low to allow determination of changes in its redox state, but in FF cells the concentration of both cytochrome pools was sufficiently high to study their involvement in respiration during growth on formate and fumarate. Figure 3.1 shows the changes in the redox state of the cytochromes upon addition of formate or Cl-OHPA to cells grown with formate and

Cl-OHPA. Figure 3.2 shows the results of a similar experiment with cells grown with formate and fumarate. The maxima between 550 and 560 nm that were seen in the absorption spectrum of concentrated cell suspensions of FF and FC grown cultures indicated that the cytochrome pool was partly reduced (Figs. 3.1 & 3.2, trace A). Upon addition of formate to concentrated cell suspensions a rapid reduction of the cytochrome pools could be seen (Figs. 3.1 & 3.2, trace B), which is similar to the level of reduction that was obtained upon addition of dithionite (Figs, 3.1 & 3.2 Trace C). Incubation of concentrated FC cells with Cl-OHPA or concentrated FF cells with fumarate resulted in a decrease of the absorption peaks, indicating that the cytochrome pools became oxidized (Figs. 3.1 & 3.2, trace E). In other experiments, cells were first incubated in the presence of 1 mM formate and then analyzed for the effect of the electron acceptors on the redox state of the cytochromes. Upon addition of 10 mM Cl-OHPA to formate-reduced FC cells the cytochrome c became oxidized (Fig 3.1. trace D). A complete reoxidation of cytochrome c was observed neither at low nor at high Cl-OHPA concentrations. A complete re-oxidation of both cytochrome pools was observed when 10 mM fumarate was added to formate-reduced FF cells (Fig 3.2, trace D). The results obtained for FC and FF cells are similar, indicating that cytochrome c is involved in both fumarate and halorespiration. Furthermore, cytochrome b is involved in fumarate respiration.

Electron paramagnetic resonance (EPR) spectroscopy on concentrated cell suspension of formate-Cl-OHPA grown cells. Spectra were recorded of concentrated FC cells, as harvested, after addition of formate, and after addition of formate and Cl-OHPA. To obtain information on the involvement of iron sulfur-clusters and molybdenum, the samples were first analyzed at 40 K by EPR (Fig 3.3). The formate-reduced sample shows spectra resembling two [2Fe-2S] clusters and molybdo-enzymes. The molybdenum appeared to occur in multiple species, which is common for molybdenum. Upon oxidation with Cl-OHPA, the molybdenum signal apparently became a single spectral species. Furthermore a radical signal was observed in the cells. This signal was similar under formate-reduced and Cl-OHPA-oxidized conditions, suggesting that a quinone was the main source for this radical signal and not FAD (not shown).

Thereafter, the temperature was lowered to 27 K to analyze cobalt, which, in the form of cobalamin, is a cofactor of the *ortho*-chlorophenol reductive dehalogenase (van de Pas *et al.*, 1999). After 30 minutes of incubation of formate-reduced cells with

Cl-OHPA (20 mM) at 37°C, 10 mM OHPA was formed but a Co^{2+}-signal was not observed. When the cells were incubated with Cl-OHPA (40 mM) for 90 minutes at 37°C, 16 mM OHPA was formed and a base-off Co^{2+} signal was detected, which had similar characteristics as the cobalamin of the purified CPRD (Fig. 3.4).

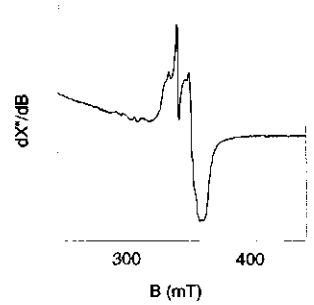

Figure 3.5: EPR of $[4Fe\text{-}4S]^{1+}$ clusters in whole cells of *D. dehalogenans*, grown on formate-Cl-OHPA, reduced with formate. The complex spectral shape is characteristic for two [4Fe-4S] cubanes in mutual dipolar interaction at a distance of approximately 1 nm. Upon increasing the temperature to 40 K the signal broadens beyond detection. EPR conditions: microwave frequency, 9410 MHz; microwave power, 5 mW; modulation amplitude, 0.63 mT; temperature, 16 K.

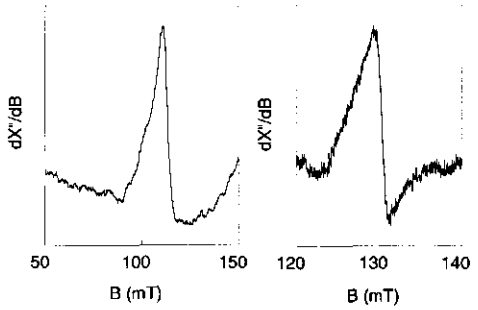

Figure 3.6: High-spin EPR signals from whole cells of *D. dehalogenans* grown on formate-Cl-OHPA. The left-hand panel is from Cl-OHPA oxidized cells and shows an S=5/2 high-spin Fe(III) heme signal with effective g-value, g^{eff}=5.89; the right-hand panel is from formate reduced cells and shows a signal of unknown origin, presumably an unusual Fe/S cluster. The effective g-value is g^{eff}=5.2. EPR conditions; microwave frequency, 9410 MHz; microwave power, 200 mW; modulation amplitude, 0.62 mT; temperature, 16 and 20 K, respectively.

To identify [4Fe-4S] and [3Fe-4S] clusters, which were previously found to be present in the purified chlorophenol reductive dehalogenase, the temperature was further decreased to 16 K (van de Pas *et al.*, 1999). Under formate-reduced conditions, a [4Fe-4S] cluster could be identified with characteristics similar to those present in the dehalogenase (Fig 3.5). Similar conditions were used in an attempt to identify low-spin hemes present in the cytochromes that were identified by optical spectroscopy. However, a broad signal was found in the low-spin heme region, possibly due to an "inorganic" precipitant. Under these conditions no low-spin cytochromes could be detected.

When the samples were analyzed at high power (200 mW) and a temperature of 16 K, a signal was found in the oxidized sample, characteristic for a high-spin heme (Fig. 3.6). This signal had a slightly asymmetrical shape and a low intensity. It could be the high-spin form of a (low-spin) heme that was optically detected.

Another signal was detected with a g-value of 5.2, which is very unusual. It is not clear if this signal is an isotropic line or part of an anisotropic spectrum. The line is reminiscent of (part of) the spectrum of the P-cluster in nitrogenase in the intermediate, P^+, redox state (Tittsworth and Hales, 1993, Spee *et al.*, 1998).

Discussion

The redox active components involved in electron transfer from formate to Cl-OHPA or fumarate were investigated by HPLC analysis and spectroscopic investigation of concentrated cell suspensions of the halorespiring anaerobic bacterium *Desulfitobacterium dehalogenans*. Since neither of these substrates support substrate-level phosphorylation, energy conservation has to be coupled to respiratory electron transport during growth on formate-Cl-OHPA or formate-fumarate. Cells that are grown with formate-fumarate reach a higher density than cells that are grown with formate-Cl-OHPA. This suggests that during halorespiration less ATP is conserved per mole of formate or hydrogen oxidized than during fumarate respiration, which is in agreement with the results reported previously (van de Pas *et al.*, 2000) and the model for fumarate respiration postulated previously (Kröger *et al.*, 1992). These models postulate that halorespiration yields 1/3 mole ATP per mole formate oxidized whereas formate oxidation coupled to fumarate reduction would yield 2/3 mole ATP.

The localization study indicates that there may be two formate dehydrogenase activities in both formate-Cl-OHPA and formate-fumarate grown cultures. This was observed in *E. coli*, where FDH-N and FDH-O are located at the outside, and FDH-H is located at the inside of the cytoplasmic membrane (Sawers, 1994). In *D. dehalogenans*, high formate dehydrogenase activity was located at the inside of the membrane. The other FDH may face the outside of the cell membrane. Fumarate reductase activity was localized at the inside of the cell membrane. Chlorophenol reductive dehalogenase activity was present in whole cells but increased upon permeabilization of the cells by toluene. The CPRD activity that was detected in permealized cells was similar to the activity that was detected in cell extracts which suggests that the dehalogenase is made accessible for methyl viologen in permealized cells. However, the increase in activity in permealized cells as well as in cell extracts

was less pronounced than for fumarate reductase activity. Therefore, a clear identification of the localization of the CPRD was not possible.

For the transport of electrons from formate to Cl-OHPA and fumarate in *D. dehalogenans*, we identified cytochrome c, cytochrome b, and menaquinone as possible components of the electron transport chain to both electron acceptors. The b-type cytochrome was present in a higher concentration in formate-fumarate grown cells than in formate-Cl-OHPA grown cells. This may indicate that this cytochrome is not involved in electron transfer from formate to Cl-OHPA. The cytochrome b could be reduced by formate and oxidized by addition of fumarate, which confirms that it is part of the electron transport chain from formate to fumarate. Previously, the involvement of cytochrome b in electron transfer from menaquinone to fumarate reductase has been reported for sulfate-reducing bacteria (Kröger *et al.*, 1992). Furthermore, cytochrome b is involved in electron transfer from formate dehydrogenase to menaquinone (Kröger *et al.*, 1992; Sawers, 1994). However, cytochrome b is not the only type of cytochrome that can accept electrons from formate dehydrogenase. Sebban-Kreuzer *et al.* (1998) found that cytochrome c_{553} accepts electrons directly from formate dehydrogenase in the sulfate-reducing *Desulfovibrio vulgaris* strain Hildenborough. This cytochrome has a low redox potential of +40 mV and is located at the periplasmic side of the cytoplasmic membrane. A similar type of cytochrome was copurified with formate dehydrogenases from other *Desulfovibrio* species (Riederer-Henderson & Peck, 1986; Costa *et al.*, 1997). The cytochrome c pool in cells from *D. dehalogenans* could be reduced by formate and oxidized by addition of either fumarate or Cl-OHPA. The two respiratory pathways apparently share this part of the electron transport chain. It also has a low redox potential (< + 60mV) and may have a similar function.

Menaquinone is the other component present in electron transport to fumarate and Cl-OHPA. The involvement of menaquinone in the halorespiratory pathway has also been found in cells of *Dehalobacter restrictus* that use PCE as electron acceptor and hydrogen as electron donor (Schumacher and Holliger, 1996). Furthermore, Louie and Mohn (1999) extracted an unknown quinone from formate-3-chlorobenzoate grown cells of *Desulfomonile tiedjei*, but its involvement in halorespiration has not been reported.

EPR analyses of formate-Cl-OHPA grown cells showed that molybdenum could be reduced by formate and oxidized upon addition of Cl-OHPA. It is likely that

a molybdenum-containing formate dehydrogenase is present in formate-Cl-OHPA grown cells. Furthermore, a high-spin heme was detected by EPR spectroscopy that can be reduced by formate and oxidized by Cl-OHPA. This high-spin heme may be the high-spin form of the (low-spin) cytochrome c, but can also be part of a nitrite reductase complex, because nitrite reductases contain high-spin hemes and nitrite reductase activity was detected in these cells (unpublished data). It is therefore possible that this high-spin heme is not directly involved in electron transfer to Cl-OHPA, but that it is part of an alternative pathway, which functions as an electron sink in the absence of its terminal electron acceptor. This sink is reduced upon oxidation of formate and may donate its electrons via the electron transport chain to Cl-OHPA. Nevertheless, the involvement of the high-spin heme in electron transport to Cl-OHPA cannot be excluded. A high-spin cytochrome c is very likely to be involved in 3-chlorobenzoate respiration in *Desulfomonile tiedjei*, because a unique high-spin cytochrome c has been reported to be co-induced with 3-chlorobenzoate dechlorination (Louie *et al.*, 1997). The EPR analyses clearly showed that cobalamin is involved in halorespiration in-vivo. This confirms earlier observations, such as inhibition of the enzyme activity by N_2O and 1-iodopropane in in-vitro assays, and the oxidation of reduced cobalamin in a purified chlorophenol reductive dehalogenase sample, upon addition of Cl-OHPA (van de Pas *et al.*, 1999).

Halorespiration in *D. dehalogenans* shares part of its electron transport chain with the fumarate respiratory pathway and possibly also with nitrate respiration (Smidt *et al.*, 1999). A unique feature of the halorespiratory pathway is the chlorophenol reductive dehalogenase. Based on the results presented in this study, the model postulated previously (van de Pas *et al.*, 2000) for the halorespiratory chain in *D. dehalogenans* with formate as electron donor can be refined. Analogous to other bacteria (e.g. *Desulfovibrio* spp., *E. coli*), formate is oxidized at the outside of the cell membrane by a molybdenum-containing formate dehydrogenase. Electrons are donated to the electron transport chain, which may consist of cytochrome c, cytochrome b, menaquinone, a high-spin heme, and an unknown iron-sulfur cluster. In what order the components of the electron transport chain should be placed and which components are essential for halorespiration is currently unknown. Comparison of the electron transport chain of halorespiration with the one that is used during fumarate respiration indicates that the first reactions are identical. This suggests that electrons are transported from formate dehydrogenase via cytochrome c or cytochrome b to

menaquinone. Finally, the electrons are used to reduce the cobalamin of the chlorophenol reductive dehalogenase and transferred onto the Cl-OHPA, which is reductively dechlorinated. The high-spin heme or the unknown iron-sulfur cluster may be involved in electron transfer from menaquinone to the dehalogenase. When formate dehydrogenase and chlorophenol reductive dehalogenase are located at different sides of the cell membrane, a proton gradient will established without proton translocation during electron transport analogous to the models postulated for 3-CB respiration in *D. tiedjei* and PCE respiration in *D. restrictus* (Mohn & Tiedje, 1992; Schumacher & Holliger, 1996). When the active side of chlorophenol reductive dehalogenase is located at the outside of the cytoplasmic membrane, the quinone pool has to be involved in proton translocation. Both models that are postulated are based on the assumption that all components that we have identified are essential for halorespiration. Future investigations such as characterization of knockout mutants and the study of halorespiration in membrane vesicles should give a conclusive answer to the position of these components in the chain.

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Chapter 4

Purification and molecular characterization of *ortho*-chlorophenol reductive dehalogenase, a key enzyme of halorespiration in *Desulfitobacterium dehalogenans*

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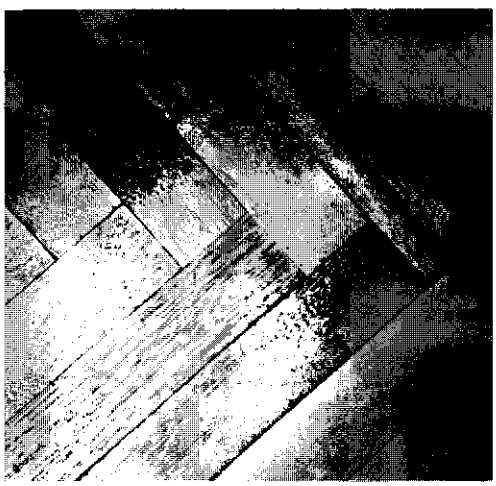

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Abstract

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 Vmax of 28 units/mg protein and a Km of 20 mM. 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 = +170 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 nonsulfur 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, *pce*B, 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). 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; 1995). Comparison of

biomass yields on pyruvate and different electron acceptors indicated that chlorophenol dechlorination in *D. dehalogenans* is an energy-yielding process (Mackiewicz and 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 dehaloge-nase) 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.

Materials and methods

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, 50 mM $NaHCO_3$, and trace elements and vitamins 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 mg/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, Heraeus Sepatech), which yielded 1.6 g of concentrated cells/liter of culture. The concentrated cells were stored at 220°C.8 g of cells was resuspended in 8 ml of buffer 1, consisting of 100 mM potassium phosphate (KPi), pH 7.5, and 2.5 mM dithiothreitol. 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 3 *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 dithiothreitol). 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 dithiothreitol) 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 $mM^{-1}.cm^{-1}$) as described by Schumacher and 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 ml of 50mM 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 1 mmol of chlorinated substrate or the oxidation of 2 mmol 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 (1976) with bovine serum albumin as a standard.

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 mM to 10 mM substrate in the cuvette.

Composition of o-CP dehalogenase. The molecular mass of the de-natured protein was determined by SDS-polyacrylamide gel electrophoresis according to Laemmli (1970). A low molecular weight marker (Bio-Rad) was used as reference. The gels were stained with Coomassie Brilliant Blue R-250. The concentration of acid labile sulfur of three individual samples was determined according to 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 inductively coupled plasma mass spectrometry samples was determined by measuring the absorbance changes in the Rose Bengal binding assay as described by Elliot and Brewer (1978) with bovine serum albumin as a standard. 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 *et al.*, 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 (1977). Deazaflavin was synthesized according to Janda and Hemmerich (1978).

N-terminal Amino Acid Sequence. Purified enzyme was transferred from a 12% SDS-polyacrylamide gel onto a polyvinylidene difluoride membrane (Immobilon polyvinylidene difluoride, Millipore Corp.) by blotting with a Trans-Blot SD semidry

transferring cell (Bio-Rad). Blotting was carried out at 14 V for 2 h 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.* (1991).

DNA Isolation, Manipulation, and Oligonucleotides. Chromosomal DNA from *D. dehalogenans* was isolated as follows. Protoplasts were prepared from 12 ml of culture (A600 5 0.4) as described by van Asseldonk *et al.* (1993), recovered at 13,000 3 *g* for 2 min, and resuspended in 100 ml of THMS buffer (30 mM Tris-HCl, pH 8.0, and 3 mM $MgCl_2$ in 25% sucrose). After the addition of 400 ml 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 the 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 and Amersham Pharmacia Biotech, respectively. Prehybridization and hybridization were performed at 65 and 50 °C, respectively. Posthybridization washeswere conducted at 40 °C.

Oligonucleotides used in this study were BG 444 (59-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-39, nucleotides 644–703), BG 458 (59-GCC GGA GCC TTG ATC GC-39, nucleotides 427–411), and BG 475 (59-GGC AGG TCT GGG AGA ATT G-39, 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/ ml. 5 ng of self-ligated DNA was used as the template in a 25- ml PCR reaction containing the following: 2 ng/ ml each primer; 2.25 mM $MgCl_2$; 200 mM 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). 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 *Xc*mI.

DNA Sequencing and Sequence Analysis. DNA sequencing was per-formed 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 Seque-nase fluorescent labeled primer cycle sequencing kit (Amersham Phar-macia Biotech). Infrared labeled oligonucleotides were purchased from MWG Biotech. Sequence similarity searches and alignments were per-formed 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 and H. B. J. Nicholas, unpublished communication), respectively.

Results

Table 4.1: 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.

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 3-chloro-4-hydroxyphenylacetate (Table 4.1). The specific activity increased 90-fold upon purification and amounted to 28 units/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 *Km* for this chlorinated substrate was determined to be 20 mM 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-CP, 2,3-dichlorophenol (2,3-DCP), 2,4-DCP, 2,6-DCP, and pentachlorophenol as substrate (Table 4.2). 3-CP, 4-CP, and 2,5-DCP were not dechlorinated. Additionally, 2-bromo-4-chlorophenol, but not 2-fluoro-4-chlorophenol, 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 4.2: 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 dehalogenase, 1 mM electron acceptor, and 50 mM Tris-HCl at pH 7.8. One unit is defined as the amount of enzyme which 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. 4.1). 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 of 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.

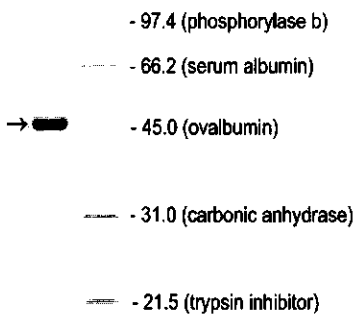

Figure 4.1: 12% SDS PAGE with the purified *ortho*-chlorophenol reductive dehalogenase of *Desulfitobacterium dehalogenans* (5µg) in lane 1. Molecular size markers are shown in lane 2. The arrow indicates the purified protein band. The gel was stained with Coomassie Brilliant Blue G-250.

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-AETMNYVPGPTNARSKLRPVH-DFA. 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-kilobase 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-kilobase fragment of pLUW910 revealed the determined N-terminal amino acids immediately down-stream 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 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. Figure 4.2 shows a restriction map of the DNA region cloned and sequenced.

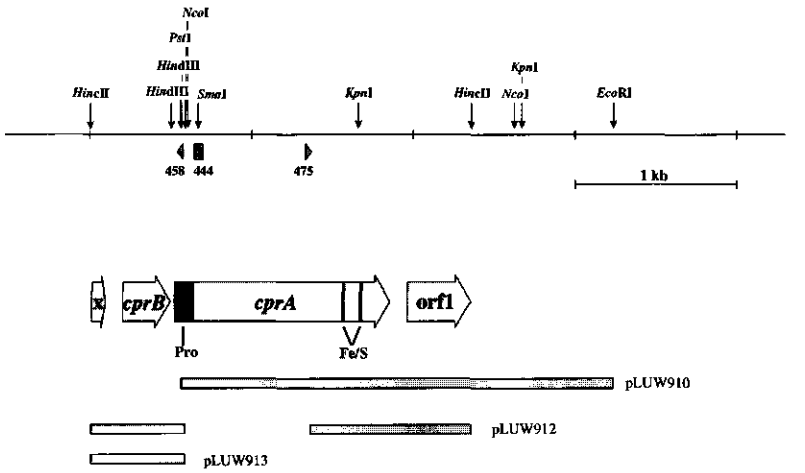

Figure 4.2: Restriction map of the *D. dehalogenans* genomic *cpr*-region. Vertical arrows mark DNA restriction sites. Horizontal bars indicate fragments, cloned either in pUC 18 or pMON38201. Horizontal arrows indicate open reading frames. Oligonucleotides used in this study are shown. The 32 C-terminal 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.

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 nucleotide 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 characteristic for a large number of mainly periplasmic proteins binding different redox cofactors (Berks, 1996). These twin arginine signal sequences (consensus (S/T)R*R*XFLK) 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 (1984). The *D. dehalogenans* CprA sequence reveals the presence of an extended cluster of cysteine

residues (Cys^{330} –Cys^{387}, Fig. 4.3). The first group of four cysteines Cys^{330} –Cys^{340} is identical to the consensus sequence of bacterial ferredoxin type clusters (*CXXCXXCXXX*CP; Bruschi and Guerlesquin, 1988), including the conserved proline at position 341. The second cluster shows the same conserved residues (Cys^{380} –Pro^{388}) but lacks the first cysteine. The B12 binding motif *DXHXX*G-(41)-SxL-(26–28)-GG, as it was determined for a subset of B12 -dependent enzymes (Ludwig and Matthews, 1997), is not present in CprA.

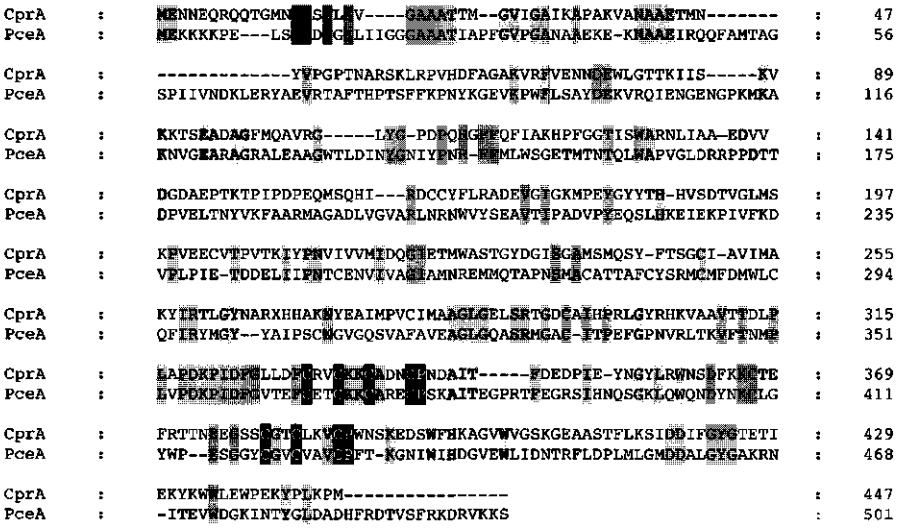

Figure 4.3: Primary sequence alignment for the *ortho*-chlorophenol reductive dehalogenase from *D. dehalogenans* (CPR) and the PCE dehalogenase from *Dehalospirillum multivorans*. The alignment was performed using the programs Clustal X and GeneDoc (Thompson *et al.*, 1997; Nicholas and Nicholas, 1997). Light grey boxes mark identical residues. Dark grey 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* (GeneBank™ accession Number AF115542), *PceA*: PCE dehalogenase from *Dehalospirillum multivorans* (GeneBank™ accession Number AF022812).

Upstream of *cpr*A, a second potential gene, *cpr*B, 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. 4.4). Following the positive-inside rule for integral membrane proteins (von Heijne and Gavel, 1988), 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. *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).

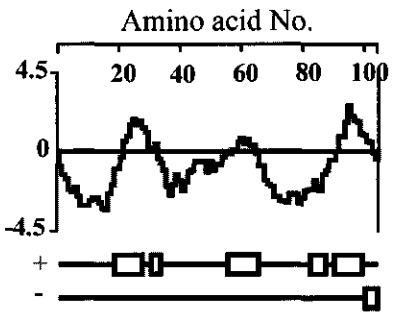

Figure 4.4: Hydrophilicity plot and charge distribution for CprB. The hydrophilicity plot was determined according to the method of Kyte and Doolittle (1982). The analysis was performed using the program Protean from the DNAstar software package.

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 of [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 that is dominated by a signal with g values of 2.05, 1.93, and 1.87, typical for reduced [2Fe-2S] or [4Fe-4S] clusters (Fig. 4.5, 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.

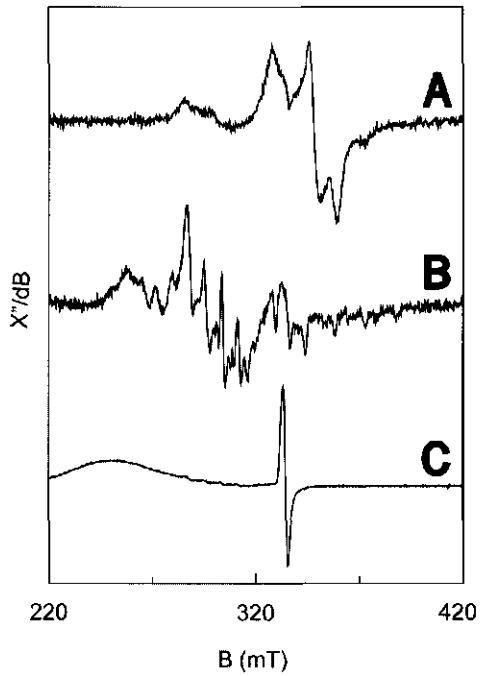

Figure 4.5: EPR spectra of *D. dehalogenans* *o*-CP dehalogenase. Trace A, the [4Fe-4S] signal from enzyme fully reduced by illumination with visible light for 50 min in the presence of 20 µM deazaflavin and 2 mM EDTA. Base-on Cob(II)alamin can be detected as a minor component in trace A. Trace B, the base-off Cob(II)alamin signal from enzyme re-oxidized 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: 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).

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. 4.5, 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. The 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 of 1.99, 2.35, and 2.35 and cobalt hyperfine (I = 7/2) values of 14, 7.5, and 7.5 millitesla. 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, since 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. 4.5, trace C). The broad peak at low field is the g_z from excess $[Fe(CN)_6]^{3+}$. 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.

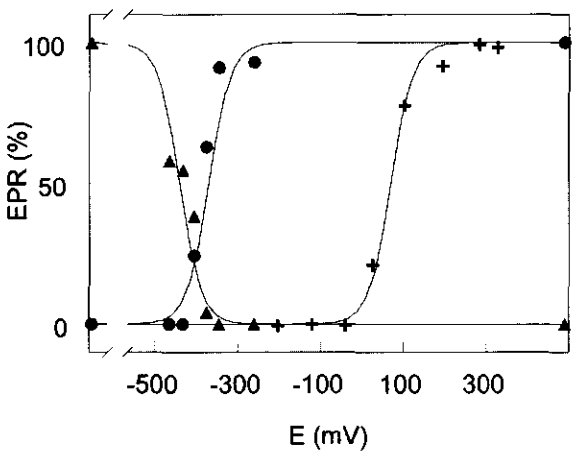

Figure 4.6: EPR-monitored redox titration of the metal centers in *D. dehalogenans* *o*-CP dehalogenase. (✚) $[3Fe\text{-}4S]^{1+}$; (●) Cob(II)alamin; (▲) $[4Fe\text{-}4S]^{1+}$. Starting from a redox potential of -130mV, the sample was reduced by substoichiometric additions of dithionite, and oxidized by substoichiometric additions of ferricyanide, both in the presence of a cocktail 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. 4.5. The solid traces are fits to the Nernst equation assuming single-electron transfer.

The signals behave as expected in reductive (dithionite) and oxidative (ferricyanide) bulk redox titrations in the presence of a mixture of redox mediators (Fig. 4.6); in an oxidative titration, the signal ascribed to a [3Fe-4S] cluster appears with an oxidation potential of $E_{m,7.8}$ = +170 mV; in a reductive titration the Co^{2+} signal disappears with a reduction potential of $E_{m7.8}$ = -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).

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.*, 1994; Table 4.2).

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. 4.3) 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* (Neumann *et al.*, 1998), revealed a rather high degree of similarity on the amino acid level in the C-terminal part of both enzymes (Fig. 4.3). 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*-dichloroethene, 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 of dechlorinating 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* Me_2SO 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 down-stream of *cprA* revealed the presence of a second potential gene, *cpr*B. 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 and 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 and 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 the 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.

The nucleotide sequence reported in this paper has been submitted to the GenBank™ /EBI Data Bank with accession number AF115542.

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Chapter 5

The influence of alternative electron acceptors on halorespiration in *Desulfitobacterium dehalogenans*

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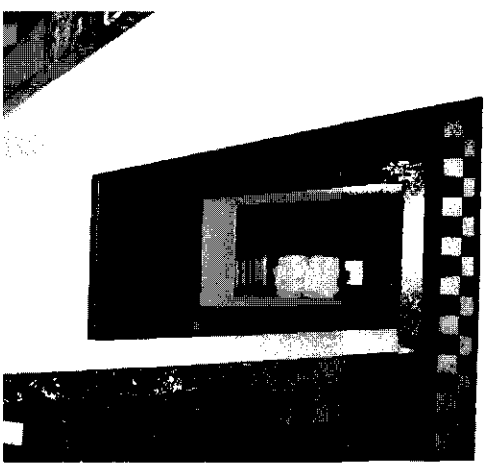

Abstract

Desulfitobacterium dehalogenans is able to use ortho-chlorinated phenolic compounds as terminal acceptor for growth, next to more conventional electron acceptors like fumarate, sulfite, thiosulfate and nitrate. We have investigated the effects of these electron acceptors on the induction of dechlorination and on dechlorinating activity in growing cultures and cell extracts. The terminal reductases associated with these electron acceptors were found to be induced by their specific substrate. A low fumarate reductase activity was induced by yeast extract and a low dehalogenase activity was detected in fumarate grown cells. Furthermore, the induction of dechlorination was found to be delayed in cells that were adapted to another electron acceptor. Dechlorination was observed 7 to 12 hours after addition of Cl-OHPA to cultures that were growing with fumarate, sulfite, thiosulfate, or nitrate as electron acceptor, when the alternative electron acceptor was still present. In contrast, dechlorination started 2 hours after addition of Cl-OHPA to a culture that grew on pyruvate in absence of an additional electron acceptor. This is not an effect of the direct inhibition of dechlorination by these compounds, because addition of sulfite, thiosulfate, or nitrate had no effect on the dechlorination rate of cells that were adapted to growth on lactate and Cl-OHPA. However, nitrate (1 mM), sulfite (1 mM), and thiosulfate inhibited the dehalogenase activity in cell extracts 1.2, 2.5, and 1.03-fold, respectively. This suggests that the enzyme is protected against these compounds in intact cells. In contrast, the dechlorination rate of a growing culture decreased 1.2 times upon addition of fumarate, while no inhibition of dehalogenase activity by this compound was detected in cell extracts. This suggests that there is a competition for reducing equivalents between fumarate reductase and Cl-OHPA reductive dehalogenase that are both present under these growth conditions.

Introduction

Biological degradation of chlorinated compounds in anaerobic environments is stimulated by addition of electron donors (Lee *et al.*, 1998). Reductive dechlorination will then become the dominant reaction mechanism for bacterial dechlorination. This dechlorination can be a cometabolic or a metabolic reaction. The latter is called halorespiration, a process in which halogenated compounds are used as terminal electron acceptor for growth (El Fantroussi *et al.*, 1998). Most bacterial strains that are able to use chlorinated compounds as terminal electron acceptor are also able to use other electron acceptors (El Fantroussi *et al.*, 1998). This may result in a competition for reducing equivalents between the different respiratory pathways within a single organism. Townsend and Suflita (1997) demonstrated that the 3-chlorobenzoate-dechlorinating activity in cells of *Desulfomonile tiedjei* is lower when sulfate, sulfite, or thiosulfate are present in the growth medium. Moreover, PCE dechlorination in lactate-limited continuous cultures of *Desulfitobacterium frappieri* strain TCE1 was found to be completely inhibited when other electron acceptors were present (Gerritse *et al.*, 1999). In addition, sulfite inhibited reductive dechlorination in PCE-limited growing cells of *D. frappieri* strain TCE1 (Gerritse *et al.*, 1999) and in cell extracts of *Desulfomonile tiedjei* (DeWeerd & Suflita, 1990; Townsend and Suflita, 1997), *Desulfitobacterium* strain PCE-S (Miller *et al.*, 1997), and *D. chlororespirans* (Löffler *et al.*, 1996). However, nitrate, fumarate, sulfite, and PCE were used concomitantly when *D. frappieri* strain TCE1 was cultivated under electron acceptor-limitation (Gerritse *et al.*, 1999). The closely related *D. dehalogenans* used nitrate, sulfite, and 3-chloro-4-hydroxyphenyl acetate (Cl-OHPA) simultaneously in batch experiments, whereas fumarate utilization increased substantially after Cl-OHPA was depleted (Mackiewicz & Wiegel, 1998). In other experiments, simultaneous sulfite reduction and Cl-OHPA dechlorination took place (Mackiewicz and Wiegel, 1998). However, the influence of these electron acceptors on the rate of dechlorination and the induction of dechlorination activity in *D. dehalogenans* is not clear. We have investigated the influence of nitrate, thiosulfate, sulfite and fumarate on dechlorination rate and induction of dechlorinating activity in growing cultures and cell extracts of *Desulfitobacterium dehalogenans*. Our results indicate that sulfite, and to a lesser extent thiosulfate and nitrate, have a direct inhibiting effect on dechlorination in cell extracts but not in cell suspensions. Furthermore, nitrate, sulfite, thiosulfate and fumarate

do not induce dehalogenase activity. These compounds inhibited induction of dechlorinating activity.

Materials and Methods

Organism and growth conditions. *Desulfitobacterium dehalogenans* strain JW/IU-DC1 (DSM 9161) was routinely cultivated anaerobically (1.2 bar N_2 gas phase) at 37°C in medium containing lactate and an electron acceptor. The basal medium contained (in grams per liter of demineralized water): Na_2SO_4 0.07, KH_2PO_4 0.20; NH_4Cl 0.25; NaCl 1.0; $MgCl_2.6H_2O$ 0.40; KCl 0.50; $CaCl_2.2H_2O$ 0.15; $NaHCO_3$, 4.2, and 0.1% peptone or yeast extract. Prior to inoculations the basal medium was supplemented with trace elements and vitamins from a stock solution to a final concentration (in milligrams per liter of liter of basal medium): EDTA 2.5; $FeCl_3.6H_2O$ 6.75; $MnCl_2.4H_2O$, 0.50; $CoCl_2.6H_2O$, 0.12; $CaCl_2.2H_2O$, 0.50; $ZnCl_2$, 0.50; $CuCl_2.2H_2O$, 0.12; H_3BO_3, 0.05; $Na_2MoO_4.2H_2O$, 0.12; NaCl, 5.0; $NiCl_2.6H_20$, 0.60; $Na_2SeO_3.5H_2O$, 0.13, biotin, 0.04; folic acid, 0.04; pyridoxine-HCl, 0.20; thiamin-HCl 0.10; riboflavin, 0.10, nicotinic acid, 0.10; Ca-pantotheate, 0.10; vitamin B_{12}, 0.10; p-aminobenzoic acid, 0.10; lipoic acid, 0.10.

Cell fractionation and enzyme assays. Late-exponential phase cultures were harvested by centrifugation and fractionated as described previously (van de Pas *et al.*, 2000).

Chlorophenol reductive dehalogenase, nitrate reductase, nitrite reductase, thiosulfate reductase, and sulfite reductase activities were determined by measuring the reduction of methyl viologen (ε_{578}= 9.8 $mM^{-1}cm^{-1}$) in N_2-flushed cuvets at 30°C. The assay mixture was 100 mM Tris-HCl buffer pH 7.8 and contained 0.3 mM titanium citrate-reduced methyl viologen and cell extract (van de Pas *et al.*, 1999). The reaction was started by addition of 10 µl from a 1 M stock solution of the electron acceptor. Fumarate reductase activity was determined as described previously (Odom & Peck, 1981). Protein concentrations of the different fractions were determined according the method described by Bradford (1976) with bovine serum albumin as standard.

Analytical methods. Organic acids were analyzed with a SpectraSystem high-performance liquid chromatograph (Thermo Separation Products, Riviera Beach, USA) as described previously (Stams *et al.*, 1993). The samples for Cl-OHPA and OHPA determination were analyzed on a SpectraSystem high-performance liquid

chromatograph, with a SpectraSystem P2000 pump, an AS3000 autosampler and a UV1000 UV-detector. 20 µl of sample was injected into a Chrompack pesticide reversed–phase column (Chrompack, Middelburg, The Netherlands). The mobile phase was acetonitril-0.01M H_3PO_4 with a volume / volume ratio of 10:90. A flow rate of 1 $ml.min^{-1}$ was applied. Cl-OHPA and OHPA were quantified by their absorption at 206 nm.

Anions were analyzed by HPLC as described previously (Scholten & Stams, 1995). Ions were separated on an IonPack AS9-SC column (Dionex, Breda, The Netherlands) with an eluent consisting of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ at a flow rate of 1 $ml.min^{-1}$ at 25 °C and they were detected by suppressed conductivity.

Results

Table 5.1. The effect of the growth substrates on the activity of terminal reductases in *D. dehalogenans* cultivated with 0.1% yeast extract, 10 mM lactate, and 20 mM Cl-OHPA, 20 mM fumarate, 5 mM nitrate, 20 mM thiosulfate, or 7 mM sulfite as electron acceptor. Enzyme activity is expressed in units per milligram of protein. One unit is defined as the amount of enzyme that oxidizes 2 nmol reduced methyl viologen per minute.

Enzyme	Lactate + Cl-OHPA	Lactate + Fumarate	Lactate + NO_3^-	Lactate + $S_2O_3^{2-}$	Lactate + SO_3^{2-}
Chlorophenol reductive dehalogenase	420	23	nd	nd	nd
Fumarate reductase	65	2610	92	85	254
Nitrate reductase	nd	nd	105	nd	nd
Thiosulfate reductase	nd	nd	nd	672	2900
Sulfite reductase	nd	nd	nd	nd	22

nd: not detected

Expression of terminal reductase activities under different growth conditions. A viologen-based enzyme assay was used to determine the activity of chlorophenol reductive dehalogenase, fumarate- nitrate-, sulfite-, and thiosulfate reductase in cell extracts of *D. dehalogenans* grown with lactate and Cl-OHPA, fumarate, sulfite, thiosulfate, and nitrate. For each growth condition, the activity of the reductase required for growth was determined (Table 5.1).

In addition, fumarate reductase activity was found to be present in cultures that had been adapted to Cl-OHPA, sulfite, or thiosulfate, and was determined to be 3 to 10% of that detected in the fumarate-induced culture. This activity was probably induced by yeast extract, which was added to the medium as supply for certain growth factors, since its replacement by peptone resulted in a complete loss of the fumarate reductase activity (data not shown).

Thiosulfate reductase activity in cells grown on sulfite was higher than the sulfite reductase activity in the same extract or thiosulfate reductase activity in thiosulfate-grown cells. In other experiments, we found that the sulfite reductase activity was higher and the thiosulfate reductase activity was constant when cells were harvested in the early exponential phase (data not shown). It is possible that sulfite was enzymatically reduced to sulfide via thiosulfate or that sulfide chemically reacted with sulfite to form thiosulfate in this culture. The thiosulfate, which was thus formed in the culture medium, could then induce the thiosulfate reductase activity.

Remarkably, a low chlorophenol reductive dehalogenase activity was detected in fumarate-grown cells. When cells were grown fermentatively on 40 mM pyruvate, chlorophenol reductive dehalogenase could not be detected.

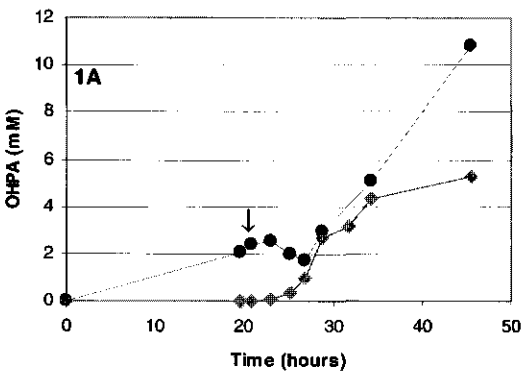

Figure 5.1A. Accumulation of OHPA after addition of 5 mM lactate and 10 mM Cl-OHPA to cultures of *D. dehalogenans,* that had been adapted to growth on pyruvate (◆) and lactate-Cl-OHPA (●). The arrow indicates the moment of addition of the extra substrates, 21 hours after inoculation of the media.

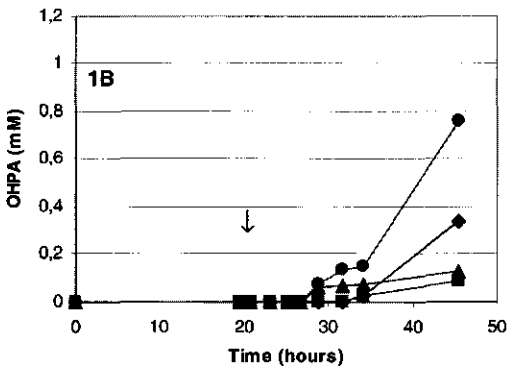

Figure 5.1B. Accumulation of OHPA after addition of 5 mM lactate and 10 mM Cl-OHPA to cultures of *D. dehalogenans,* that had been adapted to growth on lactate-thiosulfate (●), lactate-sulfite (▲), lactate-nitrate (■) and lactate-fumarate (◆). The arrow indicates the moment of addition of the extra substrates, 21 hours after inoculation of the media.

Induction of dechlorination in cell suspensions. The effect of the culture conditions on induction of dechlorination was investigated in cell suspensions that were adapted to a non-chlorinated electron acceptor or adapted to fermentative growth on pyruvate. Medium containing 0.1% peptone, lactate (40 mM), and Cl-OHPA (20 mM), NO_3^- (10mM), SO_3^{2-} (15 mM), $S_2O_3^{2-}$ (20 mM), or fumarate (20 mM) as electron acceptor were inoculated with 1% of substrate-adapted culture. Additionally, a culture was cultivated with pyruvate (40 mM) as source of carbon and energy. 21 hours after inoculation, 5mM lactate and 10 mM Cl-OHPA were added to each culture and the concentration of OHPA was followed by

HPLC (Figs 5.1A & B; Table 5.2). A control experiment where lactate and Cl-OHPA had been added from the beginning showed a non-linear dechlorination rate (Fig 5.1A). The increase of OD_{600} showed that the cells of this culture were in the early exponential growth phase after 21 hours, which explains the exponential increase of the dechlorination rate (Fig 5.1A; Table 5.2). In the culture that was adapted to fermentative growth on pyruvate, dechlorination started 2 hours after addition of Cl-OHPA.

Table 5.2. Induction of Cl-OHPA dechlorination in cultures of *D. dehalogenans* that had been adapted to fermentative growth with pyruvate or growth on lactate and different electron acceptors. 5mM lactate and 10 mM Cl-OHPA were added 21 hours after inoculation of the media.

Electron acceptor	OD_{600}[1]		electron acceptor present (mM)		OHPA produced(mM)	
	t = 21	t = 46	t = 21	t = 46	t = 21	t = 46
Cl-OHPA	0.06	0.26			2	10.8
Nitrate	0.09	0.15	0.5	0.1	0	0.1
Sulfite	0.46	0.58	3.6	0.7	0	0.1
Thiosulfate [2]	0.23	0.36	20.8	1.0	0	0.7
Fumarate	0.30	0.32	20.0 [3]	7.7 [3]	0	0.3
Pyruvate	0.45	0.61	22.5	3.0	0	5.2

[1]: At t=0 all incubations had an OD_{600} of approximately 0.05. A black precipitate of metal-sulfides that formed in the cultures with sulfite and thiosulfate prevented accurate determination of the OD_{600}.
[2]: The culture with thiosulfate was inoculated 40 hours prior to the other cultures
[3]: The fumarate concentration has been calculated from the amount of succinate in the medium.

In cultures that had been adapted to a non-chlorinated electron acceptor, OHPA was first observed 7-12 hours after addition of Cl-OHPA (Fig 5.1B, note the difference in scale with Fig 5.1A). After 25 hours, the dechlorination rates were still lower than those observed in the control and pyruvate-grown culture. It has to be noted that the first electron acceptor was still present in the incubation when Cl-OHPA was added. The majority (90%) of the initial amount of nitrate and most (73%) of the sulfite had been consumed after 21 hours of incubation. To compensate for the long lag phase with this substrate, the culture with thiosulfate was inoculated 40 hours prior to the other cultures. Nevertheless thiosulfate was not used when lactate and Cl-OHPA were added. However the thiosulfate reduction rate increased exponentially within the time of the experiment. Succinate could not be detected in the medium of the culture containing fumarate as

electron acceptor 21 hours after inoculation, although growth was observed. It is possible that succinate accumulated in the cells since fumarate reductase is located at the cytoplasmic side of the cell membrane (van de Pas *et al.*, 2000).

Table 5.3. Dechlorination rate of Cl-OHPA in growing cultures of *D. dehalogenans* before and after addition of nitrate, sulfite, thiosulfate, fumarate, or oxygen to the medium.

Additive		Dechlorination rate ($\mu M.min^{-1}$)	
		Before addition	After addition
NO_3^-	(2.5 mM)	0.48	0.51
SO_3^{2-}	(1.7 mM)	0.47	0.47
$S_2O_3^{2-}$	(2.5 mM)	0.46	0.45
Fumarate	(10 mM)	0.35	0.30
O_2	(20%)	0.22	0.002

The influence of other electron acceptors on the dechlorination rate by cell suspensions. The effect of fumarate, nitrate, sulfite, thiosulfate and oxygen on the dechlorination rate of Cl-OHPA was studied by addition of an electron acceptor and 10 mM lactate to cultures of *D. dehalogenans* that had been incubated for 21.5 hours with 10 mM Cl-OHPA and 25 mM lactate. The production of OHPA from Cl-OHPA was followed by HPLC (Table 5.3) and growth was followed by determination of the OD_{600} (data not shown). The cultures to which nitrate, sulfite and thiosulfate had been added had a similar growth and dechlorination rate before and after addition of the alternative electron acceptor. After addition of fumarate to a dechlorinating culture, the dechlorination rate decreased. Two hours after addition of fumarate, the culture showed an increased growth rate, without an increase in the dechlorination rate. Fumarate reductase activity could have been present in these cultures and fumarate may have been used simultaneously with Cl-OHPA. Growth and dechlorination of an active culture stopped immediately after addition of oxygen (20%), which is in agreement with previous observations (Utkin *et al.*, 1994).

The effect of alternative electron acceptors on chlorophenol-reductive dehalogenase activity in cell extracts was investigated by addition of 1 mM nitrate, sulfite, thiosulfate, or fumarate to chlorophenol reductive dehalogenase assay. The cell extract had a dehalogenase activity of 92 nmol Cl-OHPA.min^{-1}.mg $protein^{-1}$ ($mU.mg^{-1}$). Other

reductase activities could not be detected. Upon addition of sulfite, the dehalogenase activity became 36 $mU.mg^{-1}$. Addition of thiosulfate and nitrate to the assay also led to a decrease in the dehalogenase activity and resulted in an activity of 60 and 72 $mU.mg^{-1}$, respectively. Addition of fumarate had no effect on the rate of dechlorination.

Discussion

We have investigated the expression of chlorophenol reductive dehalogenase activity in cell suspensions and extracts of cells of *Desulfitobacterium dehalogenans* grown with Cl-OHPA, sulfite, thiosulfate, or fumarate as electron acceptor. Our results indicate that sulfite, thiosulfate, and nitrate inhibit dehalogenase activity in cell extracts and inhibit induction of dehalogenase activity in cell suspensions. Dehalogenase activity was not co-induced with the activity of other terminal reductases (Table 5.1). Moreover, we could demonstrate that induction of dechlorination was inhibited in cells that were grown with another electron acceptor (including fumarate), compared to cells that were grown fermentatively (Fig 5.1A&B). These results suggest that dehalogenase activity is induced in *D. dehalogenans* when extra energy can be gained by utilization of chlorinated compounds, because we demonstrated in a previous study that this bacterium is able to conserve more energy per mol pyruvate consumed when pyruvate oxidation is coupled to Cl-OHPA reduction than when pyruvate is fermented (van de Pas *et al.*, 2000). No extra energy gain could be achieved when *D. dehalogenans* shifted from fumarate-, sulfite-, thiosulfate- or nitrate reduction to Cl-OHPA reduction (Mackiewicz and Wiegel, 1998).
In addition, the experiments demonstrate that the induction of dechlorination was not completely blocked by the presence of other electron acceptors, since dechlorination started before the alternative electron acceptor was depleted from the medium (Table 5.2). This result extend those reported previously indicates by Mackiewicz and Wiegel (1998), who demonstrated that Cl-OHPA and other electron acceptors could be used simultaneously.

The effect of other electron acceptors on dechlorination itself was investigated as well. In actively dechlorinating cultures with Cl-OHPA present, no inhibition of dechlorination was found upon the addition of nitrate, thiosulfate or sulfite, but the dechlorination rate decreased a little upon addition of fumarate (Table 5.3). Since fumarate reductase activity was found in cells of *D. dehalogenans* grown under different

conditions (Table 5.1) and fumarate did not inhibit dehalogenase activity in the enzyme assay, we suggest that fumarate and Cl-OHPA compete for reducing equivalents, which results in a lower dechlorination rate. A similar model of competition for reducing equivalents has previously been proposed to explain the inhibition of PCE dechlorination by fumarate in cell suspensions of *Dehalospirillum multivorans* (Neumann *et al.*, 1994).

In cell suspensions, nitrate, sulfite, and thiosulfate did not inhibit dechlorination. However, we did find that these compounds inhibited dehalogenase activity in cell extracts. The dehalogenase is more accessible to the other electron acceptors in cell extracts than in cell suspensions. The mechanism by which these compounds inhibit the dehalogenase activity is unknown, but sulfite and thiosulfate have been suggested to be able to oxidize iron-sulfur clusters or to bind to the cobalamin (Magnuson *et al.*, 1998; Miller *et al.*, 1997). Cobalamin has been identified as a cofactor of reductive dehalogenases in most halorespiring bacteria (Holliger *et al.*, 1999). Sulfite also inhibited reductive dehalogenases in cell extracts of *Desulfitobacterium* sp. strain PCE-S (Miller *et al.*, 1997), *Desulfitobacterium chlororespirans* (Löffler *et al.*, 1996), *Dehalospirillum multivorans* (Neumann *et al.*, 1995), and *Desulfomonile tiedjei* (DeWeerd and Suflita, 1990; Townsend and Suflita, 1997). Moreover, addition of sulfite to a PCE-limited growing culture of *D. frappieri* TCE1 resulted in a complete suppression of dechlorination and actual washout of the culture (Gerritse *et al.*, 1999).

This study is the first report that describes the inhibition dehalogenase activity by nitrate. Addition of 1 mM nitrate to cell extracts resulted in 5-fold inhibition of the reductive dehalogenase activity. However, nitrite inhibited of PCE dechlorination in cell extracts of *Dehalospirillum multivorans* (Neumann *et al.*, 1995). Addition of 1 mM nitrite resulted in complete inhibition of PCE reductive dehalogenase activity. Complex formation of the inhibitor with a transition metal in the enzyme was suggested as a possible inhibition mechanism for both compounds.

Gerritse *et al.* (1999) demonstrated that PCE dechlorination by a continuous culture of *D. frappieri* TCE1 was not affected upon addition of a mixture of nitrate, sulfite and fumarate to a PCE-limited culture. However, dechlorination was completely blocked when a similar experiment was performed with a lactate-limited culture of *D. frappieri* TCE1. The authors suggested that the ratio of electron donors and acceptors may be more important than their actual concentrations (Gerritse *et al.*, 1999). Our finding that the addition of alternative electron acceptors had no effect on the dechlorination rate in

batches where neither the electron donor (lactate) nor the chlorinated electron acceptor (Cl-OHPA) was limiting supports with this explanation.

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Chapter 6

Two distinct enzyme systems are responsible for tetrachloroethene and chlorophenol reductive dehalogenation in *Desulfitobacterium* strain PCE1

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Abstract

Desulfitobacterium strain PCE1 is able to use chloroalkenes as well as chloroaromatics as terminal electron acceptor for growth. Cell extracts of Desulfitobacterium strain PCE1 grown with tetrachloroethene (PCE) as electron acceptor showed dehalogenase activity of 0.28 μmol PCE.min^{-1}.mg protein^{-1} in an in vitro assay with methyl viologen as artificial electron donor. Trichloroethene (TCE) was dechlorinated at a rate of 0.01 μmol TCE. min^{-1}.mg protein^{-1}. 3-Chloro-4-hydroxyphenylacetate (Cl-OHPA) and other ortho-chlorophenolic compounds were not dechlorinated by cell extract of PCE grown cultures. Cell extract of cells that were grown with Cl-OHPA as electron acceptor showed a dehalogenase activity of 0.22 μmol Cl-OHPA.min^{-1}.mg protein^{-1}. This cell extract dechlorinated PCE at 10% of the dechlorination rate of Cl-OHPA. In both cell extracts dechlorination was inhibited by addition of the cobalamin inhibitors 1-iodopropane and dinitrogen oxide. Dechlorination was completely inhibited by addition of 1 mM sulfite to the assay mixture. The enzymes responsible for PCE and Cl-OHPA dechlorination were partially purified. A 100-fold enriched fraction of chlorophenol reductive dehalogenase was obtained from Cl-OHPA-grown cells that mainly contained a protein with a subunit size of 48 kDa. The subunit size, substrate spectrum, and N-terminal amino acid sequence of this protein are similar to that of the chlorophenol reductive dehalogenase of D. dehalogenans. The specific activity of PCE reductive dehalogenase was increased 340-fold after purification. This fraction contained another protein with a subunit size of 48 kDa. The N-terminal sequence of this protein showed no homology with the chlorophenol reductive dehalogenase sequence or with the N-terminal amino acid sequence that was determined from the 59 kDa subunit of PCE/TCE reductive dehalogenase of Desulfitobacterium frappieri strain TCE1. These results provide strong evidence that two different enzymes are responsible for PCE and chlorophenol dechlorination in Desulfitobacterium sp. strain PCE1.

Introduction

In recent years several bacteria have been isolated that are able to use chlorinated substrates as terminal electron acceptor (El Fantroussi *et al.*, 1998). This group of bacteria is able to use two types of compounds: chloroaromatics and chloroalkenes. Few organisms are able to dechlorinate both. One of these bacteria, *Desulfomonile tiedjei*, is able to dechlorinate tetrachloroethene (PCE) to dichloroethene (DCE) co-metabolically when 3-chlorobenzoate is used as terminal electron acceptor (Cole *et al.*, 1995). The authors provided evidence that PCE dechlorinating activity was co-induced with 3-chlorobenzoate dechlorinating activity. Whether the same enzyme is responsible for both conversions is unknown, since PCE dechlorination by the partially purified 3-chlorobenzoate reductive dehalogenase has not yet been investigated (Ni *et al.*, 1995). A number of *Desulfitobacteria* has been reported to be able to reductively dechlorinate both PCE and 3-chloro-4-hydroxyphenyl acetate (Cl-OHPA) (Holliger *et al.*, 1999). The use of chloroalkenes and chloroaromatics in halorespiration by a single organism was first shown for *Desulfitobacterium* strain PCE1, which converts PCE mainly to TCE and produces only traces of *cis*-DCE (Gerritse *et al.*, 1996). When strain PCE1 uses chlorophenols as electron acceptor, the chlorine is removed from the *ortho* position.

The aim of the present study was to determine whether the activities in *Desulfitobacterium* strain PCE1 are co-regulated and which enzymes are involved in PCE and Cl-OHPA conversion. Dechlorinating activity of strain PCE1 grown with either of these compounds as electron acceptor was characterized in in vitro assays. The reductive dehalogenases were partially purified and compared with dehalogenases purified from *Desulfitobacterium frappieri* strain TCE1 and *Desulfitobacterium dehalogenans*. Strain TCE1 is able to dechlorinate PCE via TCE to *cis*-DCE, but does not use chlorophenols as electron acceptor (Gerritse *et al.*, 1999). *D. dehalogenans* is able to dechlorinate both chlorophenols and PCE (Holliger *et al.*, 1999), but the purified *ortho*-chlorophenol reductive dehalogenase does not dechlorinate PCE (van de Pas *et al.*, 1999). We were able to demonstrate that chloroalkene and chloroaromate reductive dehalogenation activities are not coupled in strain PCE. Characterization of partially purified PCE reductive dehalogenase indicated that this enzyme is a novel type of reductive dehalogenase.

Materials and Methods

Organisms and cultivation. *Desulfitobacterium dehalogenans* strain JW/IU-DC1 (DSM 9161) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellculturen. *Desulfitobacterium* strain PCE1 and *Desulfitobacterium frappieri* strain TCE1 were described before (Gerritse *et al.*, 1999; Gerritse *et al.*, 1996). *Desulfitobacterium dehalogenans* was cultivated in batch culture as was described previously with 10 mM lactate as electron donor and 20mM Cl-OHPA as electron acceptor (van de Pas *et al.*, 1999). *Desulfitobacterium* strain PCE1 and strain TCE1 were cultivated in continuous culture as described by Gerritse *et al.* (1999). Strain PCE1 was grown with 40 mM lactate as electron donor and 15 mM PCE as electron acceptor and with 50 mM formate and 20 mM Cl-OHPA. Strain TCE1 was grown with formate (50 mM) and PCE (15 mM).

Cell fractionation. Cells were harvested in the late exponential growth phase by centrifugation and washed with P-buffer consisting of 100 mM potassium phosphate, pH 7.5, and 2.5 mM dithiothreitol (DTT). The concentrated cells were stored at -20°C. To prepare cell extracts, cells (1 g) were resuspended in 1 ml of P- buffer and a few crystals of DNase I were added. Cells were broken by sonication (VC 40, Sonic Materials Inc., USA) under anaerobic conditions. The cell debris was removed by centrifugation for 1 min at 20,000 x g. The supernatant was 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 2 ml 10 mM MOPS pH 6.5 supplemented with 1% Triton X-100 and 20% glycerol and incubated for 30 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. *Ortho*-chlorophenol reductive dehalogenase from *D. dehalogenans* was purified as described previously (van de Pas *et al.*, 1999). PCE reductive dehalogenase activity of strain PCE1 did bind to Q-Sepharose (0.5 x 5 cm) (Amersham Pharmacia Biotech, Uppsala, Sweden) when this column was equilibrated with buffer A (10mM MOPS pH 6.5, 0.1% (w/v) Triton X-100, 20% glycerol, and 1 mM DTT). The column was eluted with a 40-ml linear gradient from 0 to 400 mM

NaCl in buffer A at a flow rate of 1.0 ml/min. Fractions containing the highest dechlorinating activity were pooled and diluted with an equal volume of buffer A. The sample was applied on a MonoQ column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer A. The dehalogenase activity eluted from the column with a 40-ml linear gradient from 0 to 400 mM NaCl in buffer A and a flow rate of 1.0 ml/min. 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 onto 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. All chromatographic steps were performed by fast protein liquid chromatography (Amersham Pharmacia Biotech, Uppsala, Sweden) in an anaerobic chamber with N_2 /H_2 (95%/5%) gas phase. The same procedure was used for purification of *ortho*-chlorophenol reductive dehalogenase from strain PCE1, and PCE/TCE reductive dehalogenase from strain TCE1. For purification of PCE/TCE reductive dehalogenase, the Q-Sepharose column was omitted since the enzyme did not bind well to this material. The purity of the protein was determined by SDS-polyacrylamide gel electrophoresis according to Laemli (1970).

Enzyme assays. Reductive dehalogenase activities were determined spectrophotometrically at 30°C in rubber-stoppered nitrogen-flushed cuvets by following the oxidation of methyl viologen as described previously (van de Pas *et al.*, 1999). Cell extract was added to 1 ml of buffer containing 50 mM Tris-HCl pH 7.8 and 0.3 mM titanium citrate-reduced methyl viologen. The assay was started by addition of 20 µl substrate from a 50 mM stock solution, which was made in ethanol. The pH optimum was determined in a 200 mM Tris-maleate buffer ranging from pH 5.5 to 9.0. Other electron donors were tested in a similar assay as described previously (Neumann *et al.*, 1995). Protein was determined according to the method of Bradford, with bovine serum albumin as standard (Bradford, 1976).

N-terminal Amino Acid Sequence. Purified enzyme was transferred from a 12% SDS-polyacrylamide gel onto a polyvinylidene difluoride membrane (Immobilon polyvinylidene difluoride, Millipore Corp., Etten-Leur, The Netherlands) by blotting with a Trans-Blot SD semidry transferring cell (BioRad, Utrecht, The Netherlands) as

described previously (van de Pas *et al.*, 1999). The N-terminal amino acid sequence of the blotted protein was determined as described previously (Schiltz *et al.*, 1991).

Results

Table 6.1. Substrate specificity profiles of cell extracts of *Desulfitobacterium dehalogenans*, and strain PCE1 grown with Cl-OHPA as electron acceptor and strain PCE1 and TCE1 grown with PCE as electron acceptor.

Substrate	*D. dehalogenans* Cl-OHPA spec. act[1]	strain PCE1 Cl-OHPA spec. act[1]	strain PCE1 PCE spec. act[1]	strain TCE1 PCE spec. act[1]
Cl-OHPA	218	100	<5[2]	<5
2-CP	<5	8	<5	nd
3-CP	<5	<5	<5	nd
4-CP	<5	<5	<5	nd
2,3-DCP	286	141	<5	<5
2,4-DCP	54	41	<5	nd
2,5-DCP	9	<5	<5	nd
2,6-DCP	31	11	<5	nd
PCE	<5	10	281	115
TCE	<5	<5	10	95
DCE	nd	<5	<5	<5

[1] specific activity is expressed in nmol.min^{-1}.mg protein^{-1}

[2] a specific activity of 5 nmol/min.mg protein is the detection limit

Abbreviations: Cl-OHPA: 3-chloro-4-hydroxyphenyl acetate; CP: chlorophenol, DCP: dichlorophenol; PCE: tetrachloroethene; TCE: trichloroethene; DCE: *cis*-dichloroethene; nd: not determined

Substrate specificity of extracts of *Desulfitobacterium* cells grown with different electron acceptors. The substrate specificity of cell extracts of *Desulfitobacterium* strain PCE1 grown with either PCE or Cl-OHPA as electron acceptor was compared with that of cell extracts of *D. dehalogenans* and *D. frappieri* strain TCE1 (Table 6.1). The chloro-aromate dehalogenating capacity of cell extracts was tested with Cl-OHPA and all congeners of *ortho*-chlorinated dichlorophenol because strain PCE1 is able to dechlorinate chlorophenols at the *ortho* position (Gerritse *et al.* 1996). Additionally, all four isomers of monochlorophenol, the products from the dechlorination of these dichlorophenols, were tested. In a similar approach, chloro-alkene dehalogenation by

cell extracts was investigated by testing PCE and its products TCE and *cis*-DCE as possible substrates.

When strain PCE1 was grown on Cl-OHPA, the substrate specificity profile of the cell extract resembled that of cell extracts of *D. dehalogenans* grown with the same electron acceptor. Besides Cl-OHPA, 2,3-dichlorophenol was dechlorinated at high rates by these cell extracts. Cell extracts of Cl-OHPA-grown cells of strain PCE1 showed little activity when PCE was tested as a possible substrate and no activity was detected with TCE.

Cell extracts of strain PCE1 grown with PCE as electron acceptor used a different set of chlorinated substrates. The dechlorination rate of PCE was 100 nmol PCE.min^{-1}.mg protein^{-1}, but none of the chlorophenols was dechlorinated. TCE was also dechlorinated, but only at 10% of the PCE dechlorination rate. In strain PCE1 reductive dechlorination of PCE and Cl-OHPA was not co-induced, which indicates that strain PCE1 contains two separate enzyme systems for chloro-alkene and chloro-aromate dehalogenation.

Cell extracts of strain TCE1 dechlorinated PCE and TCE at a rate of 115 and 95 nmol.min^{-1}.mg protein^{-1}, respectively. The cell extracts of strain TCE1 showed a higher activity with TCE as cell extracts of PCE-grown cells of strain PCE1. No activity was observed with chlorophenols in cell extracts from strain TCE1.

Table 6.2. Effect of inhibitors on PCE and Cl-OHPA dechlorinating activity in cell extracts of *Desulfitobacterium* strain PCE1 grown with PCE or Cl-OHPA, respectively. The percentage of inhibition is given between brackets

Inhibitor	PCE spec. act[1]. (%)	Cl-OHPA spec. act[1].(%)
None	62	252
N_2O (23 mM)	1 (98)	10 (96)
1-iodopropane (50 µM)	39 (37)	152 (37)
SO_3^{2-} (10 mM)	0(100)	0(100)

[1]: specific activity is expressed in nmol.min^{-1}.mg protein^{-1}

Characterization of PCE and Cl-OHPA dechlorinating activity in cell extracts of strain PCE1. Methyl viologen, benzyl viologen, $FADH_2$, $FMNH_2$, NADPH, and NADH were tested as electron donors for PCE and Cl-OHPA dechlorination in cell extracts of strain PCE1. The only artificial electron donor used for reductive dechlorination was methyl viologen, which has the lowest redox potential of the

electron donors tested (–446 mV). The inhibitors of cobalamin-containing enzymes, dinitrogen oxide and 1-iodopropane inhibited both PCE and chlorophenol dechlorinating activities in cell extracts (Table 6.2). Moreover, sulfite was found to be a strong inhibitor of both dechlorinating activities in cell extracts of strain PCE1.

Purification of chlorophenol, PCE, and PCE/TCE reductive dehalogenase. Two reductive dehalogenases with either PCE or Cl-OHPA dechlorinating activity were purified by FPLC from cell extracts of strain PCE1.

Table 6.3. Purification scheme for partial purification of PCE and Cl-OHPA reductive dehalogenase of *Desulfitobacterium* strain PCE1 and PCE/TCE reductive dehalogenase of *Desulfitobacterium frappieri* strain TCE1.

	PCE1 CPRD		PCE1 PCERD		TCE1 PCE/TCERD	
	Total act (U[1])	Spec act (U/mg)	Total act (U)	Spec act (U/mg)	Total act (U)	Spec act (U/mg)
Cell extract	6.7	0.03	10.0	0.02	3.4	0.10
Membrane fraction	7.5	0.03	3.5	0.01	2.5	0.07
Cytoplasm fraction	0.9	0.05	2.0	0.02	0	0
Solubilized fraction	5.6	0.16	3.9	0.01	15.9	0.77
Q-Sepharose	0.7	0.33	1.7	0.75	-	-
Mono-Q pH 6.5	0.1	2.17	0.4	6.27	16.2	11.41
Mono-Q pH 7.8	0.02	4.09	0.1	5.51	2.0	10.01

[1]: One unit (U) of activity is defined as the oxidation of 2 mmol of reduced methyl viologen.

Cl-OHPA reductive dehalogenase was purified 132-fold from cell extract of strain PCE1 (Table 6.3). This activity was eluted from the Q-Sepharose and MonoQ (pH 6.5) columns at a salt concentration of 0.2 M NaCl. Cl-OHPA reductive dehalogenase was eluted from the MonoQ column (pH 7.8) at a salt concentration of 0.22 M NaCl. The fraction containing the Cl-OHPA reductive dehalogenase activity showed a band of approximately 48 kDa (Fig. 6.1: lane 3), which has a similar size as the Cl-OHPA reductive dehalogenase of *D. dehalogenans* (Fig. 6.1: lane 2).

The same procedure was used to purify PCE reductive dehalogenase from strain PCE1. PCE reductive dehalogenase activity was eluted from the Q-Sepharose and MonoQ columns (pH 6.5) at a salt concentration of 0.15 M NaCl. It was eluted from the MonoQ column (pH 7.8) at a salt concentration of 0.13 M NaCl. PCE reductive dehalogenase was purified 340-fold. The PCE reductive dehalogenase activity eluted at a lower salt concentration than the Cl-OHPA reductive dehalogenase activity. On SDS-PAGE, there are bands visible of 48 kDa, a similar size as the Cl-OHPA reductive dehalogenase, and 44 kDa (Fig 6.1: lane 4). This lower band was not visible in all fractions where enzyme activity was detected (data not shown). The final yield of

PCE and Cl-OHPA reductive dehalogenase was 13 and 5 µg, respectively. The small scale of the experimental set-up resulted in a low yield, also because not all the active fractions were pooled during purification.

The Q-Sepharose column was omitted in the procedure for purification of PCE/TCE reductive dehalogenase from strain TCE1 because the enzyme did not bind to the material under the conditions used. The activity was eluted from the MonoQ column run at pH 6.5 at a salt concentration of 70 mM NaCl and when the pH was shifted to 7.8 the activity was eluted at 40 mM NaCl. The fraction that was obtained was 100-fold enriched in PCE/TCE reductive dehalogenase activity (Table 6.3). SDS PAGE revealed that this fraction contained one protein of approximately 59 kDa (Fig 6.1: lane 5). Compared to the dehalogenases from strain PCE1, the PCE/TCE reductive dehalogenase activity of strain TCE1 eluted from the column at a low salt concentration, which indicates that, under the conditions tested, this dehalogenase was less negatively charged than the other dehalogenases.

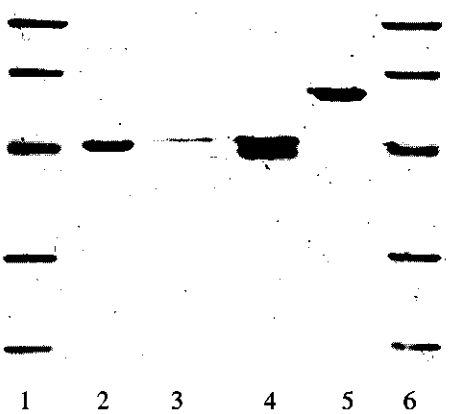

Figure 6.1: SDS page gel of purified reductive dehalogenases. Lane 2: 2 µg Chlorophenol reductive dehalogenase from *Desulfitobacterium dehalogenans*; lane 3: 0.3 µg of Cl-OHPA reductive dehalogenase from strain PCE1; Lane 4: 0.5 µg of PCE reductive dehalogenase from strain PCE1; Lane 5: 2 µg of PCE/TCE reductive dehalogenase from strain TCE1; lane 1 & 6 contain marker proteins of 97.4, 66.2, 45.0, 31.0, and 21.5 kDa. The gel was stained with Coomassie Brilliant Blue R-250.

The N-terminal amino acid sequences of the 48 kDa protein of PCE reductive dehalogenase, the 48 kDa Cl-OHPA reductive dehalogenase of strain PCE1, and the 59 kDa PCE/TCE reductive dehalogenase of strain TCE1 were determined an found to be GQESESAIVXFAVQXV, AETM, and ADIVAPITEXTEFPYPV, respectively (X is unknown).

Discussion

Desulfitobacterium strain PCE1 is the only known *Desulfitobacterium* species that uses both PCE and chlorophenols as terminal electron acceptor for growth (Gerritse *et al.*, 1996). Determination of the dehalogenase substrate specificity profiles from cell extracts of strain PCE1 grown under different conditions showed that different profiles were obtained when cells were grown with PCE or Cl-OHPA as electron acceptor. Similar results were obtained with whole-cell incubations of strain PCE1, pre-grown on PCE or Cl-OHPA, respectively (Gerritse *et al.*, 1999). This indicates that PCE and chlorophenol dechlorination is not co-induced and that different enzymes are involved in these processes. The PCE and Cl-OHPA dechlorinating activities of strain PCE1 were both inhibited by N_2O and 1-iodopropane, which are known inhibitors of cobalamin containing enzymes. The inhibiting effect of sulfite on dehalogenase activity has also been found for 3-chloro-4-hydroxybenzoate dechlorination by *Desulfitobacterium chlororespirans* (Löffler *et al.*, 1996), 3-chlorobenzoate dechlorination in *Desulfomonile tiedjei* (Townsend & Suflita, 1997) and PCE/TCE dechlorination in *Desulfitobacterium* strain PCE-S, *Desulfitobacterium frappieri* strain TCE1, *Dehalospirillum multivorans*, and *Dehalococcoides ethenogenes* (Gerritse *et al.*, 1999; Magnuson *et al.*, 1998; Miller *et al.*, 1997).

The enzymes responsible for PCE and chlorophenol dechlorination by strain PCE1 and the PCE/TCE reductive dehalogenase of strain TCE1 have been isolated. The Cl-OHPA reductive dehalogenase that was isolated from cell extract of a Cl-OHPA-grown culture of strain PCE1 has a size of 48 kDa, which is similar as has been reported for the chlorophenol reductive dehalogenases from *D. dehalogenans* and *D. hafniense* (Christiansen *et al.*, 1998; van de Pas *et al.*, 1999). The substrate spectrum of Cl-OHPA grown cells of strain PCE1 was similar to the substrate

spectrum of Cl-OHPA-grown cultures of *D. dehalogenans*. Only four amino acids could be determined of the N-terminal amino acid sequence of this enzyme, but their sequence was completely identical to that of the chlorophenol reductive dehalogenase of *D. dehalogenans* (van de Pas *et al.*, 1999). Furthermore, a gene was recently detected in strain PCE1, which is identical to the chlorophenol reductive dehalogenase gene from *D. dehalogenans* (H. Smidt, unpublished results). These results suggest that Cl-OHPA reductive dehalogenase from strain PCE1 is a member of the chlorophenol reductive dehalogenase family, which also includes the dehalogenases of *D. hafniense* and *D. dehalogenans* (Christiansen *et al.*, 1998; van de Pas *et al.*, 1999).

The PCE reductive dehalogenase of strain PCE1 has a similar size as the chlorophenol reductive dehalogenases, but its substrate spectrum, behavior on the anion exchange columns, and N-terminal amino acid sequence are completely different. The first amino acid of the N-terminal amino acid sequence of PCE reductive dehalogenase was glutamine, instead of alanine that was found for other reductive dehalogenases (Table 6.4; Holliger *et al.*, 1998). The absence of methionine at this position indicates post-translational processing. Sequencing of the structural genes for chlorophenol reductive dehalogenase of *D. dehalogenans* and PCE reductive dehalogenase of *Dehalospirillum multivorans* revealed the presence of a leader sequence which precedes the N-terminal amino acid sequence (Neumann *et al.*, 1998; van de Pas *et al.*, 1999). This would also be expected for PCE reductive dehalogenase of strain PCE1. The PCE reductive dehalogenase activity of cell extracts of strain PCE1 was 30-fold higher when PCE instead of TCE as added as substrate. This is in agreement with the finding that strain PCE1 produces mainly TCE from PCE and not *cis*-DCE, as has been reported for all other PCE-respiring strains (Gerritse *et al.*, 1996). The PCE reductive dehalogenase of 51 kDa, which has been isolated from an enrichment containing *Dehalococcoides ethenogenes*, also shows activity only with PCE and not with TCE (Magnuson *et al.*, 1998). There may be a relation between the reduced substrate spectrum and the reduced size of these PCE reductive dehalogenases, when compared to PCE/TCE reductive dehalogenases that have similar activities with PCE and TCE as substrate.

The PCE/TCE reductive dehalogenase of strain TCE1 has similar properties as the PCE reductive dehalogenase of *Desulfitobacterium* strain PCE-S, including the N-terminal sequence (Miller *et al.*, 1998). The PCE reductive dehalogenase of strain

PCE-S has a size of 65 kDa and contains a cobalamin. Based on the similarities that have been found between the dehalogenases from strain PCE-S and TCE1, we expect that the PCE/TCE reductive dehalogenase of strain TCE1 also contains a cobalamin.

Table 6.4: Comparison of reductive dehalogenases from *Desulfitobacterium* species based on their substrate specificity and N-terminal sequence.

	Ortho-chlorophenol reductive dehalogenase	PCE reductive dehalogenase	PCE/TCE reductive dehalogenase
Organisms	*Desulfitobacterium dehalogenans*[1] *Desulfitobacterium* strain PCE1 *Desulfitobacterium hafniense*[2]	*Desulfitobacterium* strain PCE1	*Desulfitobacterium frappieri* strain TCE1 *Desulfitobacterium* strain PCE-S[3]
Substrate	Cl-OHPA	PCE	PCE/TCE
Localization	membrane	membrane	membrane
Size (kDa)	48	48	60-65
Cofactors	cobalamin Fe-S	cobalamin	cobalamin Fe-S
Activity [4]	5-28	5	14-39
N-terminal sequence[5]	AETMNYVPGP	GQESESAIV	ADIVAPITESF

[1]: from van de Pas *et al.*, 1999
[2]: from Christiansen *et al.*, 1998
[3]: from Miller *et al.* (1998)
[4]: Activity is expressed in μmol substrate.min^{-1}.mg protein^{-1}
[5]: The N-terminal amino acid sequences of the strains listed together are identical

The reductive dehalogenases that have been investigated in this study are compared with reductive dehalogenases that have been isolated from other *Desulfitobacterium* species (Table 6.4). All enzymes are membrane-bound and (likely all) contain cobalamin in their catalytic center. Nevertheless, three groups of dehalogenases can be distinguished based on their substrate spectrum and N-terminal amino acid sequence. The N-terminal amino acid sequence of the *Desulfitobacterium* PCE/TCE reductive dehalogenases is completely different from the PCE/TCE reductive dehalogenase of *Dehalospirillum multivorans,* which has been reported previously (Neumann *et al.*, 1996). In addition, this enzyme was reported to be localized in the cytoplasm. The factor(s) that determine the substrate specificity of reductive dehalogenases are not yet known. No dehalogenases have yet been described that can reduce both chloroalkenes and chloroaromatics at similar rates. The binding of the substrate in the active site may determine the substrate specificity of the

enzymes, because the redox potentials for the reduction of chlorophenols and chlorinated ethenes are comparable and both reactions are catalyzed by cobalamin containing enzymes. Elucidating the reaction mechanism and determination of the 3D-structure of reductive dehalogenases may provide novel insight in this.

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van de Pas, B. A., Smidt, H., Hagen, W. R., van der Oost, J., Schraa, G., Stams, A. J. M. & de Vos, W. M. (1999). Purification and molecular characterization of *ortho*-chlorophenol reductive dehalogenase, a key enzyme of halorespiration in *Desulfitobacterium dehalogenans*. *J. Biol. Chem.* **274**, 20287-20292 (see: Chapter 4, this thesis).

Chapter 7

Isolation from human feces of *Desulfitobacterium* strain DP7, a *Desulfitobacterium* that does not dechlorinate chloroethenes or chlorophenols

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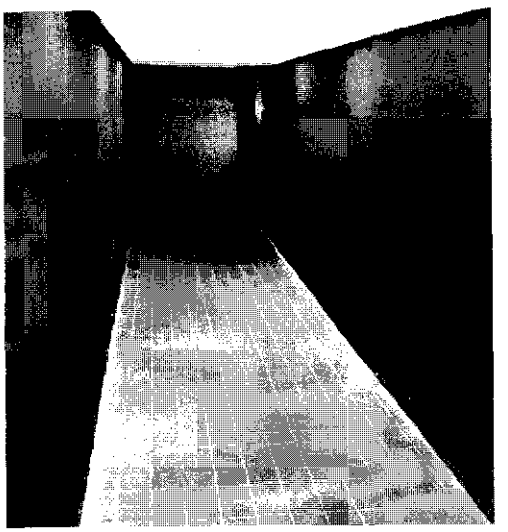

Submitted to Archives of Microbiology

Abstract

An anaerobic bacterium, strain DP7, was isolated from human feces in mineral medium with formate and 0.02% yeast extract as energy and carbon source. This rod-shaped motile bacterium can use pyruvate, lactate, formate, hydrogen, butyrate, and ethanol as electron donor for sulfite reduction. Other electron acceptors such as thiosulfate, nitrate, fumarate stimulate growth in the presence of 0.02% yeast extract and formate. Acetate is the only product during fermentative growth on pyruvate. Six moles of pyruvate are fermented to seven moles of acetate. ^{13}C *NMR labeling experiments showed homoacetogenic* ^{13}C-CO_2 *incorporation into acetate. The pH and temperature optimum for fermentative growth on pyruvate is 7.4 and 37°C, respectively. The growth rate under these conditions was approximately 0.10* h^{-1}. *Strain DP7 was identified as a new strain of Desulfitobacterium frappieri on basis of 16S rRNA sequence analysis (99% similarity) and DNA-DNA hybridization (83% homology) with Desulfitobacterium frappieri TCE1. Strain DP7 is the first Desulfitobacterium, which has not been isolated from a polluted environment and does not use chloroethenes or chlorophenols as electron acceptor.*

Introduction

Desulfitobacterium is a genus of low G+C Gram-positive bacteria. All strains within this genus have been isolated from soils, compost soil or sewage sludges polluted with chlorinated organic compounds (El Fantroussi *et al.*, 1998). *Desulfitobacterium* species are able to couple reductive dechlorination of chlorinated organic compounds to growth. Other electron acceptors that *Desulfitobacterium* species can use include nitrate, fumarate, sulfur, thiosulfate, and sulfite. Sulfate is not an electron acceptor for any known strain of *Desulfitobacterium.* Hydrogen, formate, pyruvate, and lactate have been found to serve as electron donors for respiration, while some strains are also able to use butyrate, crotonate, serine, and ethanol (Gerritse *et al.*, 1999; Sanford *et al.*, 1996). Pyruvate can be fermented to lactate and acetate by *D. dehalogenans* and to acetate only by *D. chlororespirans*, *D. hafniense,* and all known strains of *D. frappieri* (Utkin *et al.*, 1994; Sanford *et al.*, 1996; Christiansen & Ahring, 1996; Bouchard *et al.*, 1996; Gerritse *et al.*, 1999).

We obtained a pure culture of a strain of *Desulfitobacterium* (strain DP7) from a human fecal sample, which is an environment that is thought not to contain high levels of chlorinated compounds. The physiological characteristics of strain DP7 are presented and its taxonomic position within the genus *Desulfitobacterium* is discussed.

Materials and Methods

Bacterial strains, cultivation and isolation procedures. *Desulfitobacterium dehalogenans* strain JW/IU-DC1 (DSM 9161) and *Desulfitobacterium chlororespirans* Co23 (DSM 11544) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellculturen (DSMZ, Braunschweig, Germany). *Desulfitobacterium frappieri* TCE1 was a kind gift from J. Gerritse (TNO Institute of Environmental Sciences, The Netherlands).

Strain DP7 originates from a culture that was enriched from a fresh fecal sample of a healthy 28-year-old female person. A fecal sample of 0.5 g was resuspended in 125 ml of anaerobic mineral medium (Stams *et al.*, 1993). One ml of this suspension was used to inoculate a 10-fold dilution series of 10 Hungate tubes

with 9 ml mineral medium containing 40 mM formate, 0.01% yeast extract and 1% rumen fluid. The highest dilution, where growth was observed after one week of incubation at 37° C, was the 10^4 dilution. No methane was detected in this tube. One ml of this culture was transferred to 10 ml basal salt medium containing 40 mM formate and 0.01% yeast extract. This was repeated 5 times before a new 10-fold dilution series was inoculated in the same medium. After 3 weeks at 37°C, growth was observed until the 10^7 dilution. One ml from this culture was used to inoculate a 10-fold dilution series in 9 ml Hungate tubes with anaerobic mineral medium containing 40 mM formate, 10 mM $NaSO_3$, 0.01% yeast extract and 50 $\mu g.l^{-1}$ aztreonam (Bristol-Myers Squibb B.V., Woerden, The Netherlands). After 4 days of incubation at 37°C growth was observed till the 10^7 dilution. The presence of contaminating bacteria was examined by microscopic investigation and by inoculation of 10 ml Peptone Yeast Glucose (PYG) medium (Holdeman *et al.*, 1977). Little growth was observed and only the motile rod could be detected, which was designated strain DP7. A pure culture of strain DP7 was obtained from a single colony on solidified medium. This culture, designated *Desulfitobacterium frappieri* DP74, has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellculturen (DSMZ, Braunschweig, Germany) as strain DSM 13498.

Gram staining of strain DP7 was performed in a one step fluorescence assay with the LIVE *Bac*Light™ Bacterial Gram stain Kit (Molecular Probes Inc., Eugene, USA) according to the manufacturer's manual.

Growth rate and growth optima. The growth rate was calculated from the increase of the optical density at 600 nm in time in duplicate cultures of strain DP7 grown at 37 °C with 40 mM pyruvate as sole energy and carbon source. The optimum temperature for growth of strain DP7 was determined by following the increase in optical density at 600 nm of cultures, which were incubated at different temperatures with 40 mM pyruvate as sole source of energy and carbon. The incubations were inoculated with 1% of a full-grown culture of DP7. The optimal pH for growth of strain DP7 was determined by following the increase in optical density at 600 nm in incubations of strain DP7 at 37°C in media with different pH values. The pH of the bicarbonate buffered medium was adjusted after autoclaving by addition of sterile NaOH or HCl. Pyruvate was added as sole source of carbon and energy.

Substrate utilization. Growth with different substrates was determined in bicarbonate buffered medium without addition of yeast extract. All carbon sources were added from sterile stock solutions to a final concentration of 10 mM or 20 mM. Sulfite (10 mM) was added as electron acceptor. The increase in turbidity and the disappearance of the substrate were used to determine whether the carbon source was used for growth. Utilization of different electron acceptors was tested with 20 mM formate as electron donor and 10 mM electron acceptor. The increase in turbidity and the disappearance of formate were taken as measure of growth.

Utilization of chlorinated compounds. The use of chlorinated compounds as possible terminal electron acceptors was tested with both 10 mM lactate and 10 mM formate as electron donor. Chlorinated compounds were added from a sterile stock solution to a final concentration of 1 mM. Growth with 3-chloro-4-hydroxy phenyl acetate (Cl-OHPA) (10 mM) as electron acceptor was tested with 20 mM formate, lactate, and pyruvate as electron donor. Product formation from the halogenated aromatic compounds was followed by HPLC as described previously (van de Pas *et al.*, 2000). For determination of chlorophenols the mobile phase was acetonitril-0.01 M H_3PO_4 with a vol. / vol. ratio of 20:80 and a flow rate of 1 $ml.min^{-1}$. Cl-OHPA and OHPA concentrations were determined as described previously (van de Pas *et al.*, 2000). All chlorophenolic compounds were quantified by their absorption at 206 nm. The tetrachloroethene (PCE) concentration was followed by GC analysis as described previously (Kengen *et al.*, 1999).

Nuclear Magnetic Resonance (NMR) experiments. For the NMR experiment strain DP7 was cultivated in medium as described with ^{13}C labeled bicarbonate as buffer. The bacteria were grown fermentatively on 30 mM pyruvate. At t= 0 and t= 75 h., samples were prepared for proton-broad-band-decoupled ^{13}C-NMR spectra and spectra were recorded at 25 °C in a Bruker AMX-500 Fourier transform spectrophotometer (Brüker GMBH, Germany) as described previously (van de Pas *et al.*, 2000). ^{1}H-NMR spectra were recorded of the same samples at 500 MHz on the same spectrometer at 25°C.

Phylogenetic analysis and genome characteristics. For phylogenetic analysis, DNA of the strain DP7 was isolated as described (Harmsen *et al.* 1995), and the 16S rRNA-gene was amplified using universal 16S rRNA specific primers (Alm *et al.* 1996). The amplification products were cloned in pGEM®-T Easy (Promega, Leiden, The

Netherlands) according to the manufacturer's manual. One clone was sequenced using standard vector sequencing primers and universal 16s rRNA specific primers (Alm *et al.* 1996). The 16S rRNA sequences were aligned to reference sequences present in the EMBL database using the ARB software (Ludwig *et al.*,1998). A distance matrix was calculated with Jukes Cantor correction based on *E. coli* positions 116 to 1488, excluding a hypervariable region in the first part of the 16S rRNA. Similarities were calculated from this distance matrix and from the distance matrix including all sequence information. A phylogenetic tree was constructed using a neighbor joining method based on the distance matrix based on *E. coli* positions 116 to 1488 and parsimony, implemented in the ARB software.

For determination of the G+C contents and DNA-DNA hybridization, DNA was isolated and purified according to standard protocol (Marmur, 1961). The G+C content of the genomic DNA was determined by the thermal denaturation method (Owen *et al.*, 1969). DNA-DNA hybridization was performed by the optical reassociation method according to De Ley *et al.* (1970).

Chemicals. All chemicals were obtained from commercial sources, and the highest purity available (more than 98%) was used in each case. ^{13}C-labeled bicarbonate was purchased from Isotec. Inc., Miamisburg, USA.

Nucleotide sequence accession number. The 16S rRNA gene sequence of *D. frappieri* strain DP7 has been deposited in the EMBL database under accession number AJ276701

Results

Enrichment and cell morphology of strain DP7. Strain DP7 is a rod-shaped motile organism, which has been isolated from human feces. Strain DP7 is a motile rod of 4 to 6 μm long and 0.6 μm wide (Figure 7.1). Spores were not detected and no growth was observed after pasteurization of a full-grown culture. The cells positively reacted with the fluorescent probe for Gram-positive bacteria.

Fermentative growth conditions. Cultivation of strain DP7 in mineral medium with 0.01% yeast extract and 40 mM formate resulted in the formation of acetate. During fermentative growth on pyruvate, 30 mM pyruvate was degraded and about 35 mM acetate was formed. Under these growth conditions the bacterium has a specific

growth rate of 0.10 h^{-1}. The optimal temperature for growth was between 34-40°C; no growth occurred at 50°C. Strain DP7 grew optimal between pH 7.2-7.4. No growth was observed below pH 5.5 and above pH 8.5.

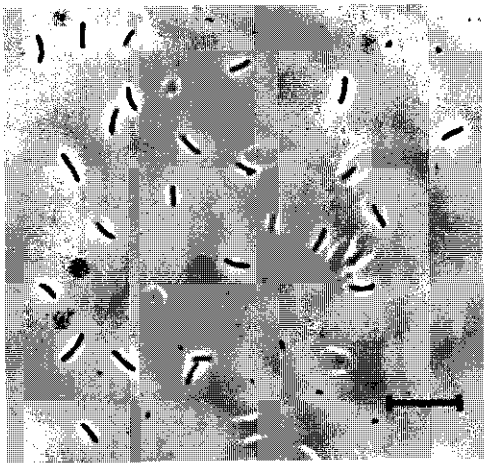

Figure 7.1. Phase-contrast light micrograph of a culture of strain DP7. A bar with a size 10 µm is included in the lower right corner.

The observation that acetate was the only product from pyruvate fermentation and the increase in the amount of organic acids suggested that strain DP7 is able to incorporate carbon dioxide into acetate. This was investigated by analyzing medium of a culture of DP7 that was cultivated in ^{13}C labeled bicarbonate buffered medium and 30 mM pyruvate as substrate. ^{13}C NMR spectra of supernatant of strain DP7, grown under these conditions, showed a triplet at a chemical shift of 24.2 ppm, reflecting the presence of C_2 of single- and double-labeled acetate (Figure 7.2, Trace A), indicating that ^{13}C-labeled CO_2 is incorporated. The coupling constant of the C_2-doublet around the single-labeled resonance was determined to be 52 Hz, which is the scalar coupling constant $^{13}C_2$-$^{13}C_1$. The amount of double-labeled acetate was estimated to be 45% of the total labeled acetate. Based on the ^1H-NMR spectrum, it was estimated that approximately 7% of the acetate was labeled at the C_2 position, exceeding the 1% natural abundance of ^{13}C (data not shown), confirming that ^{13}C-labeled bicarbonate is incorporated. At the beginning of the experiment (t=0), no labeled acetate was detected (Fig. 7.2, Trace B).

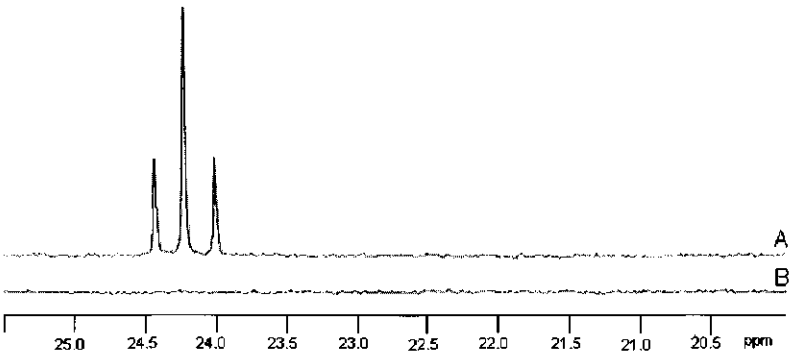

Figure 7.2: ^{13}C Nuclear magnetic resonance spectra of culture supernatants of strain DP7 incubated for 75 hours with 40mM pyruvate (Trace A). The spectrum given in trace B shows the supernatant of the pyruvate incubation just after inoculation (t=0).

Utilization of electron donors and acceptors by strain DP7. Strain DP7 uses hydrogen, formate, pyruvate, lactate, butyrate, and ethanol as electron donor in combination with sulfite (20 mM) as electron acceptor. Acetate, malate, propionate, crotonate, and glucose did not support growth in medium with 0.02% yeast extract and 20 mM sulfite as electron acceptor. Besides sulfite, thiosulfate, nitrate and fumarate, but not sulfate, were used as electron acceptors for growth of strain DP7 in medium containing 20 mM formate and 0.02% yeast extract. The dechlorinating capability of strain DP7 was tested in media containing 10 mM lactate or formate as electron donor and 1 mM chlorinated compound. After 4 months of incubation at 37°C, no growth or product formation (dechlorinated phenol or ethene) was detected with 2-chlorophenol (2-CP), 3-CP, 4-CP, 2,3-dichlorophenol (2,3-DCP), 2,4-DCP, 2,5-DCP, 2,6-DCP, pentachlorophenol, tetrachloroethene, and 3-chloro-4-hydroxyphenylacetate (Cl-OHPA). Under the conditions tested, none of the chlorinated compounds was dechlorinated by strain DP7. Moreover, dechlorination of 2,6-DCP and Cl-OHPA was also not observed in incubations where pyruvate was used as electron donor. In these cultures growth was observed, which is due to fermentation of pyruvate.

Phylogeny. The main part of the 16S ribosomal gene of strain DP7 was amplified by PCR, cloned and sequenced, resulting in a nearly complete sequence of 1644 nucleotides. A phylogenetic analysis was performed with related species (Fig 7.3). Comparison of strain DP7 with *Desulfitobacterium dehalogenans* JW/IU-DC1 (nucleotide sequence accession no. L28946), *Desulfitobacterium* sp. strain PCE1

(X81032), *D. chlororespirans* Co23 (L68528), *D. hafniense* DCB-2 (X94975), *D. frappieri* strain PCP-1 (U40078), and *D. frappieri* strain TCE1 (X95972) revealed that strain DP7 is more related to *Desulfitobacterium frappieri* strain TCE1 (99,4% similarity and only 9 mismatches, both based on the total sequences) than to the other *Desulfitobacterium* species. The sequence similarity of the 16S rRNA of strain DP7 with the other *Desulfitobacterium* strains and *Desulfosporosinus orientis* was determined (Table 7.1). This shows that the phylogenetic analysis of this group can be biased by the inclusion of a hypervariable region between *E. coli* positions 75 to 97. When this region is included in the analysis DP7 has only 95% similarity with *D. frappieri* PCP1, while it has 99% similarity with PCP1 when this region is excluded. However, DP7 and the two *D. frappieri* strains all have a long insert, unlike the other *Desulfitobacterium* strains, indicates the relationship between the *D. frappieri* strains.

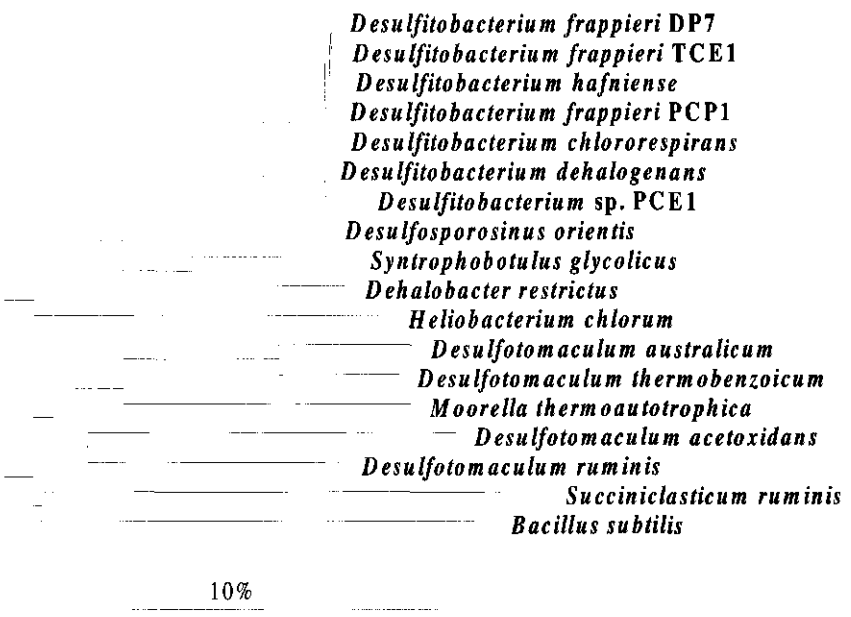

Figure 7.3: Neighbor Joining with Jukes Cantor correction of *E.coli* position 116 to 1488.

Table 7.1: Similarities (%) and comparison of insert size (nucleotides) of between 16S rRNA sequences of *Desulfitobacterium* sp. DP7 with other *Desulfitobacterium* species and *Desulfosporosinus orientis*.

Strain	Similarities including *E. coli* positions 116 to 1488	Similarities including all positions of the shortest sequence	Size of insert between *E. coli* positions 75 to 97
Desulfitobacterium sp. DP7	100.0	100.0	141
Desulfitobacterium frappieri TCE1	99.93	99.38	141
Desulfitobacterium frappieri PCP1	99.79	94.97	122
Desulfitobacterium hafniense	99.78	98.05	24
Desulfitobacterium dehalogenans	97.28	96.04	28
Desulfitobacterium sp. PCE1	96.54	96.44	ns[1]
Desulfitobacterium chlororespirans	91.24	96.73	24
Desulfosporosinus orientis	94.12	92.61	28

[1]ns is no sequence information available.

The G+C contents of the genomic DNA of strain DP7, *Desulfitobacterium dehalogenans* JW/IU-DC1, *D. frappieri* TCE1, *D. chlororespirans* Co23 were determined to be 48.3, 45.8, 47.5, and 48.8 (±0.3) mol%, respectively. DNA-DNA hybridization was performed with the same strains. The DNA-DNA homology between these strains and the type strain of *D. dehalogenans* (100 %) was found to be 37 % for strain Co23, 45% for strain TCE1, and 35% for strain DP7. DNA-DNA hybridization of strain Co23 and TCE1 with DP7 showed 71 and 83 % homology. This indicates that all these strains belong to the same species.

Discussion

We isolated strain DP7, a new member of the genus *Desulfitobacterium*. Strain DP7 is the first isolate of this genus that has not been isolated for its ability to use chlorinated compounds as terminal electron acceptor. The source from which strain DP7 has been isolated, a human fecal sample, is thought not to contain chlorinated compounds. Strain DP7 has the same morphology as other *Desulfitobacterium* species and utilizes similar electron donors (Bouchard *et al.*, 1996; Christiansen and Ahring, 1996; Gerritse *et al.*, 1999; Gerritse & Renard, 1996; Miller *et al.*, 1997; Sanford *et al.*, 1996; Utkin *et al.*, 1994). The formation of double-labeled acetate in ^{13}C-labeled bicarbonate buffered medium shows that 2 CO_2 molecules are reduced to produce acetate via the acetyl-CoA pathway. This has been described previously for

Desulfitobacterium dehalogenans (van de Pas *et al.*, 2000). How strain DP7 is able to grow in mineral medium with 0.01% of yeast extract and 40 mM formate is yet unclear. The oxidation of formate could be coupled to CO_2-fixation via the acetyl-CoA pathway since acetate was detected in formate grown cultures. Most strains of *Desulfitobacterium* can use nitrate, sulfite, thiosulfate, fumarate, and chlorophenols or tetrachloroethene as electron acceptor (Bouchard *et al.*, 1996; Christiansen and Ahring, 1996; Gerritse *et al.*, 1999; Gerritse and Renard, 1996; Miller *et al.*, 1997; Sanford *et al.*, 1996; Utkin *et al.*, 1994). Strain DP7 is able to reduce nitrate, sulfite, thiosulfate, and fumarate, but does not dechlorinate chloroethenes or –phenols.

Strain DP7 is very closely related to the PCE-dechlorinating strain *Desulfitobacterium frappieri* TCE1 and the chlorophenol-respiring *Desulfitobacterium frappieri* PCP-1, *D. chlororespirans* Co23, and *D. hafniense* DCB-2. The G+C values of strains DP7, TCE1, and Co23 were similar, whereas *D. dehalogenans* has a slightly lower G+C content. The highest homology of 16 rRNA sequence level was found with *D. frappieri* TCE1, which agrees with the results obtained by DNA-DNA hybridization. Strain DP7 is most similar to *Desulfitobacterium frappieri* TCE1 in morphological, physiological, and genetic characteristics, except for its inability to dechlorinate chloroethenes. We propose this new isolate to be designated as a new strain of *Desulfitobacterium frappieri*.

Members of the genus *Desulfitobacterium* have been isolated from different polluted environments (Sanford *et al*, 1996; Christiansen *et al.*, 1996; Bouchard *et al.*, 1996; Gerritse *et al.*, 1996 & 1999; Miller *et al.*, 1997; Utkin *et al.*, 1994). The isolation of strain DP7 from a human fecal sample shows that the presence of *Desulfitobacterium* species is not restricted to polluted ecosystems. This strain cannot use chloroethenes or chlorophenols, the usual chlorinated electron acceptors for *Desulfitobacterium* species. This finding indicates that the presence of members of the genus *Desulfitobacterium*, which can be determined with phylogenetic probes, cannot be used as an indication for the dechlorinating potential of an ecosystem.

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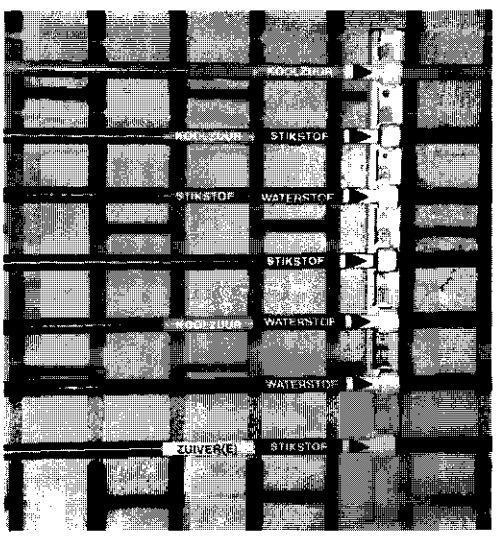
Chapter 8

Summary and Concluding Remarks

Bram A. van de Pas



Halorespiration is a novel respiratory pathway, which has been discovered as a result of the search for microorganisms that can be used in bioremediation of chlorinated compounds. Halorespiring bacteria are able to use these compounds as terminal electron acceptor for growth in anaerobic environments. These bacteria have developed enzyme systems with high dechlorination rates and low threshold values. These characteristics are important for the application of dechlorinating bacteria in bioremediation.

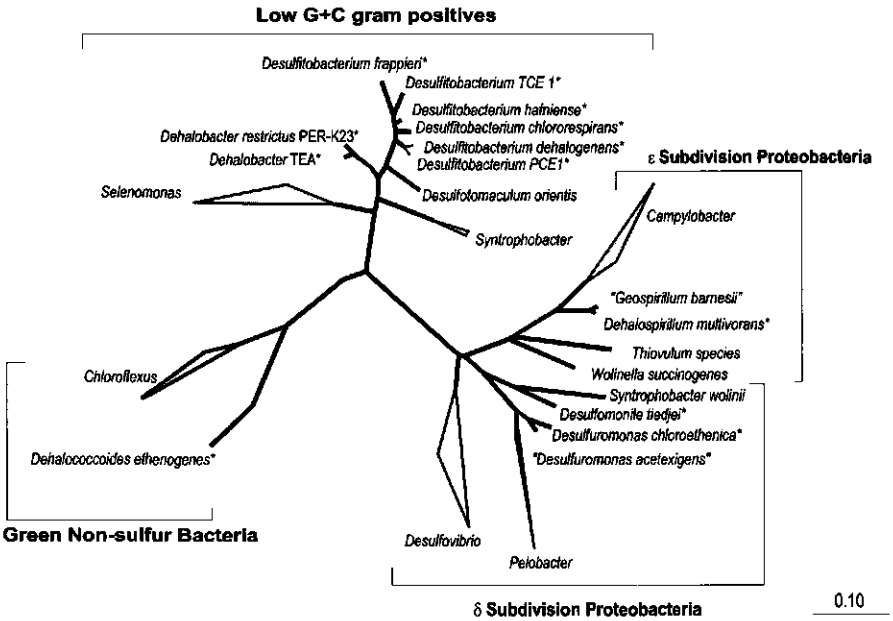


Figure 8.1. A 16S rRNA based phylogenetic tree reflecting the relationships of halorespiring bacteria (marked *) with other bacteria.

Ten years ago, a defined medium was developed for cultivation of *Desulfomonile tiedjei,* which was the only halorespiring bacterium that was grown in pure culture at the time (DeWeerd *et al.*, 1990). This discovery made it possible to study 3-chlorobenzoate respiration in more detail. In the following years, the isolation of other halorespiring bacteria indicated that halorespiration is widespread throughout the bacterial domain (Fig 8.1). Moreover, these halorespiring bacteria have been found to be present in all sorts of polluted environments (Table 1.1) Insight in the physiology and biochemistry of these bacteria is currently lacking. This study aimed to get a better comprehension of the biochemistry of halorespiration. The research has

focused on three topics: (i) elucidation of the coupling of reductive dechlorination to ATP formation in *Desulfitobacterium dehalogenans,* (ii) isolation and characterization of dehalogenases from different *Desulfitobacterium* species, and (iii) isolation and characterization of a novel *Desulfitobacterium* strain from human feces. In **Chapter 1,** an overview is given of microbial dehalogenation mechanisms with emphasis on halorespiration. The halorespiring bacteria that have been obtained in pure culture, the current models for 3-chlorobenzoate and tetrachloroethene (PCE) respiration, and the characteristics of reductive dehalogenases, are also reviewed.

Desulfitobacterium dehalogenans is an anaerobic Gram-positive bacterium that uses *ortho*-chlorinated phenolic compounds as terminal electron acceptor for growth (Utkin *et al.*, 1994). Comparison of growth yields of *D. dehalogenans* grown with pyruvate as electron donor and 3-chloro-4-hydroxyphenyl acetate (Cl-OHPA), sulfite, nitrate, or fumarate as electron acceptor has indicated that regardless of which electron acceptor was used, *D. dehalogenans* is able to conserve the same amount of energy (Mackiewicz & Wiegel, 1998). In **Chapter 2,** the growth yields of *D. dehalogenans* grown with hydrogen, formate, pyruvate, or lactate as electron donor and Cl-OHPA as electron acceptor have been compared. In addition, the activities of the different electron donating and electron accepting enzymes were localized. These results indicate that the oxidation of lactate and pyruvate coupled to the reduction of Cl-OHPA yields 1 ATP per mole of acetate produced by substrate level phosphorylation. When formate or hydrogen is used as electron donor for reductive dechlorination, the growth yield is approximately 1/3 of the growth yield with pyruvate as electron donor. Under these growth conditions, energy cannot be conserved via substrate-level phosphorylation. However, a proton motive force (PMF) may be established, which can be used by a proton-driven ATPase for ATP-formation. A model has been postulated in which the localization of the electron-donating enzyme (e.g. hydrogenase, formate dehydrogenase, lactase dehydrogenase, or pyruvate ferredoxin oxidoreductase) determines whether a PMF is established. In contrast to the electron transport by the electron transport chain (ETC) and the reduction of the chlorinated compound by the reductive dehalogenase, which do not contribute to the PMF (see also Fig. 8.2). We have investigated the composition of the ETC, which is involved in electron transport from formate to Cl-OHPA in cell suspensions and have compared it with the ETC involved in fumarate respiration with formate as electron donor (**Chapter 3**). Menaquinone, cytochrome c, and b were

components that were found to be present in cells grown with formate and either Cl-OHPA or fumarate. We have demonstrated that these components could be reduced by formate and oxidized upon addition of the induced electron acceptor. This suggests that (a part of) the halorespiratory chain is shared with fumarate respiration. Previously, it has been shown that halorespiration shares elements with nitrate respiration on basis of the analysis of halorespiration-deficient mutants of *D. dehalogenans* (Smidt *et al.*, 1999). That different respiratory pathways share common elements is not unusual and it has been reported for bacteria such as *Paracoccus denitrificans* and *E. coli* (Richardson, 2000; Unden & Bongaerts, 1997). However, the ETCs involved in halorespiration and fumarate respiration are not identical. The involvement of cytochrome b in fumarate respiration could be demonstrated while this was not possible for halorespiration. The results suggest that cytochrome b is the direct electron donor for fumarate reductase, analogous with findings for *W. succinogenes* and *E. coli* (Kröger *et al.*, 1992).

The electron transport chain from formate to Cl-OHPA has been investigated in more detail by electron paramagnetic resonance spectroscopy. In these experiments, we have shown that molybdenum, iron-sulfur clusters, cobalamin, a high spin heme and an unknown iron-sulfur cluster are components that were reduced by formate and oxidized by Cl-OHPA. This may indicate that the formate dehydrogenase which is active in halorespiration is a molybdenum and iron-sulfur containing formate dehydrogenase. This enzyme donates its electrons either to cytochrome c (analogous to *Desulfovibrio* sp.), or the electrons are transferred to cytochrome b (analogous to *E. coli* and other bacteria). Since cytochrome c is usually membrane-associated but not membrane-bound, an additional component, possibly cytochrome b, is needed to transport the electrons from the outside of the cell membrane to the cytoplasmic side of the cell membrane. The electrons may then be transferred to menaquinone which takes 2 protons from the cytoplasm and, depending on the localization of the reductive dehalogenase, the protons are released at the outside or inside of the cell, as is shown in figure 8.2 model A and B, respectively. In addition, oxidation of cobalamin, a cofactor of chlorophenol reductive dehalogenase, was observed in cell suspensions upon addition of Cl-OHPA. This observation strongly suggests that the dehalogenase, which we have characterized, is involved in in vivo halorespiration. The exact orientation of the dehalogenase in the cytoplasmic membrane could not be determined, but dehalogenases of other halorespiring bacteria have been localized at

the cytoplasmic side of the cytoplasmic membrane (Holliger *et al.*, 1999). In case the reductive dehalogenase of *D. dehalogenans* has a similar topology, the results (chapter 2 and 3) could be explained by model B presented in figure 8.2. This model is analogous to those postulated for 3-chlorobenzoate and PCE respiration in *D. tiedjei* and *D. restrictus*, respectively (Fig. 1.3).

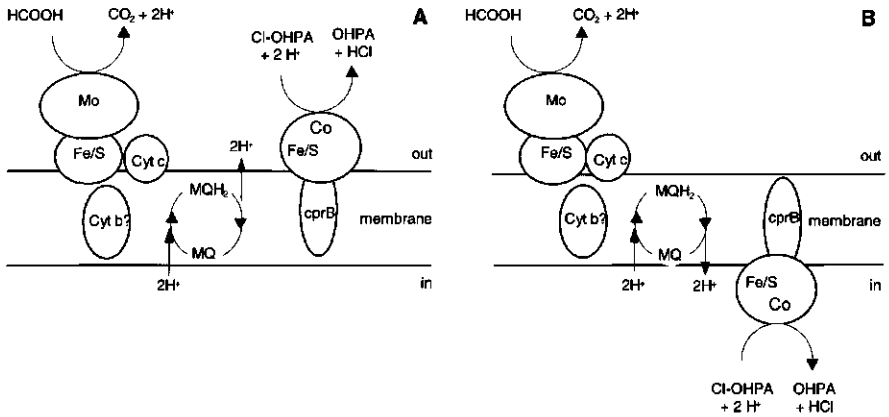


Figure 8.2: The electron transport system of *D. dehalogenans* catalyzing the oxidation of formate coupled to reductive dechlorination of 3-chloro-4-hydroxyphenyl acetate. It shows two tentative models for the generation of a proton gradient based on the localization of the *ortho*-chlorophenol reductive dehalogenase at the outer (model A) or the inner aspect (model B) of the cytoplasmic membrane.

The isolation and characterization of a chlorophenol reductive dehalogenase is described in **Chapter 4.** This enzyme was purified anaerobically from a Triton X-100 extract of the membrane fraction. The purified enzyme catalyzed the dechlorination of Cl-OHPA with a V_{max} of 28 units/mg protein and a K_m of 20 mM. In addition, the purified dehalogenase catalyzed the reductive dehalogenation of several *ortho*-chlorinated phenols and 2-bromo-4-chlorophenol with reduced methyl viologen as electron donor. The EPR analysis indicated one [4Fe-4S] cluster (midpoint redox potential (E_m = -440 mV), one [3Fe-4S] cluster (E_m = 170 mV), and one cobalamin per 48-kDa monomer. The Co^+/Co^{2+} transition had an E_m of -370 mV. The corresponding gene has been isolated, cloned, and sequenced, and revealed the presence of two closely linked genes: (i) *cprA*, encoding the o-chlorophenol reductive dehalogenase, (ii) *cprB*, coding for an integral membrane protein that could act as a membrane anchor of the dehalogenase. Moreover, *cprA* contains a twin-arginine type signal sequence that is processed in the purified enzyme. This is an indication for localization of the enzyme at the outside of the cytoplasmic membrane, because

almost all genes that contain a twin-arginine leader sequence code for proteins are located at the outside of the cytoplasmic membrane (Berks *et al.*, 2000). However, *pceA*, the gene coding for the cytoplasmic PCE reductive dehalogenase of *Dehalospirillum multivorans,* also contains a twin arginine leader sequence (Neumann *et al.*, 1998). From this it can be argued that the TAT (twin arginine transport) system is only involved in maturation of dehalogenases and not in transport of these enzymes across the cytoplasmic membrane.

Besides *ortho*-chlorinated phenols, *D. dehalogenans* is able to use other electron acceptors. Mackiewicz and Wiegel (1998) showed that several electron acceptors, including Cl-OHPA, can be used simultaneously. In **Chapter 5**, the influence of other electron acceptors on the induction of dechlorinating activity and on the dechlorinating activity in cell suspensions and cell extracts is described. Dechlorinating activity was found to be induced by its substrate (Cl-OHPA) and to a lesser extent by fumarate. However, when cells had been adapted to another electron acceptor (including fumarate), induction of dechlorination was found to be inhibited when compared to cells that were grown fermentatively on pyruvate. However, when an additional electron acceptor was added to a dechlorinating culture, the dechlorination rate decreased by 14 % when fumarate was added, but no effect was detected with nitrate, sulfite or thiosulfate. In cell extracts, the addition of fumarate had no effect and the other electron acceptors inhibited the dechlorinating activity. These results indicate that *D. dehalogenans* does not have a preferred electron acceptor in batch cultures, but it utilizes several electron acceptors simultaneously. This could be relevant for in situ bioremediation techniques because the presence of multiple electron acceptors in polluted sediments is not unusual.

While *D. dehalogenans* is able use *ortho*-chlorinated phenols as terminal electron acceptors for growth, *Desulfitobacterium* sp. strain PCE1 is able to use both chlorophenols and PCE and *Desulfitobacterium frappieri* strain TCE1 can use PCE and TCE (Gerritse *et al.*, 1999; 1996; Utkin *et al.*, 1994). We compared the substrate spectrum of the enzymes in cell extracts of these strains grown with Cl-OHPA or PCE as electron acceptors (**Chapter 6**). The results indicate that strain PCE1 contains separate enzymes for PCE and chlorophenol dechlorination. This was studied in more detail by the isolation of the chlorophenol reductive dehalogenase and the PCE reductive dehalogenase of strain PCE1 and the PCE/TCE reductive dehalogenase from strain TCE1. Based on the N-terminal sequence, size and substrate spectrum, the

chlorophenol reductive dehalogenase of strain PCE1 was found to be very similar to the dehalogenase of *D. dehalogenans*. The PCE/TCE reductive dehalogenase of strain TCE1 has similar characteristics as have been described for PCE reductive dehalogenase of strain PCE-S (Miller *et al.*, 1998). The PCE reductive dehalogenase from strain PCE1 was found to be a novel type of reductive dehalogenase. The enzyme catalyzed the reduction of PCE, and had a low activity with TCE. The purified enzyme had a subunit size of 45 kDa on SDS-PAGE. The activity of this enzyme as well as of the chlorophenol reductive dehalogenase of strain PCE1 was found to be inhibited upon addition of the cobalamin inhibitors 1-iodopropane and NO to cell extracts.

In **Chapter 7**, the isolation and characterization of a new strain of *Desulfitobacterium frappieri* is described. This isolate is the first *Desulfitobacterium* strain described that is not able to use chlorinated ethenes or phenols as terminal electron acceptor.

In summary, the research presented in this thesis aimed to give an answer to the question how halorespiring bacteria couple reductive dechlorination to energy conservation. The results obtained in chapters 2, 3 and 4 provide evidence for the mechanism, which is summarized in figure 8.2. The difference between models A and B is caused by the localization of the dehalogenase, which is an interesting topic for future investigations. Upon growth in the presence of multiple electron acceptors, *D. dehalogenans* was found to react as an "opportunist". It does not switch rapidly from using one electron acceptor to using another, but will use more electron acceptors simultaneously when neither the electron donor nor the acceptor is limiting. We have not investigated the influence of electron acceptors on dechlorination under electron donor or acceptor limited conditions. In a study of PCE dechlorination by *D. frappieri* TCE1, Gerritse *et al.* (1999) found that dechlorination was completely inhibited by other electron acceptors in cells grown under electron donor limitation, but not in cells grown under electron acceptor limitation. Since the enzymes that catalyze PCE dechlorination and chlorophenol dechlorination in different strains of *Desulfitobacterium* contain the same cofactors, the reaction and regulation mechanisms may be similar. Investigation of PCE and chlorophenol dechlorination in *Desulfitobacterium* strain PCE1 has demonstrated that the dehalogenases, which are associated with these activities, have completely different substrate specificity profiles. Dechlorination of different chlorinated compounds requires a combination of

specialized dehalogenases, which are probably not all present in a single halorespiring species. Additionally, we have isolated a strain of *Desulfitobacterium frappieri* that does not dechlorinate the usual chlorinated substrates for *Desulfitobacterium*. This demonstrates that one should be cautious in extrapolation of the presence of certain bacterial groups or species to the dechlorinating potential of a polluted environment (Löffler *et al.*, 2000).

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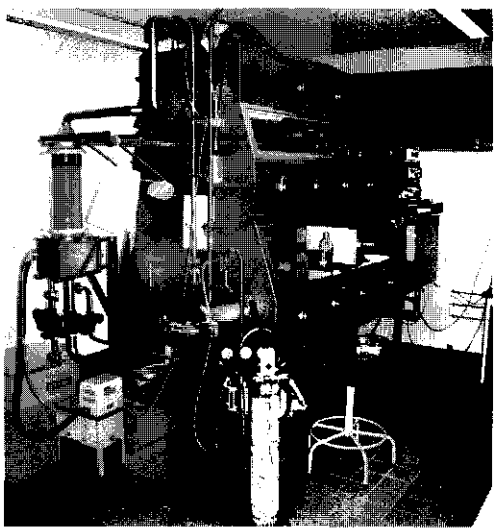
Chapter 9

Samenvatting en Conclusies

Bram A. van de Pas



Halorespiratie is een nieuwe ademhalingsroute, die ontdekt is bij micro-organismen die gebruikt kunnen worden voor de biologische afbraak van gechloreerde verbindingen. Halorespirerende bacteriën kunnen deze componenten onder anaërobe condities gebruiken als terminale elektronenacceptor voor groei. Deze bacteriën hebben hiervoor een enzymsysteem ontwikkeld met een hoge dechloreringssnelheid en een lage drempelwaarde. Deze eigenschappen zijn van belang voor de toepassing van dechlorerende bacteriën in bioremediatie.

Tien jaar geleden is er een gedefinieerd medium beschreven voor de kweek van *Desulfomonile tiedjei,* de enige halorespirerende bacterie die destijds in reincultuur werd gekweekt (DeWeerd *et al.*, 1990). Deze ontdekking maakte het mogelijk om de ademhaling op 3-chloorbenzoaat in detail te bestuderen. In de daaropvolgende jaren zijn er meer halorespirerende bacteriën geïsoleerd en kwam men tot de conclusie dat halorespiratie wijdverbreid is door het bacteriële rijk (Hoofdstuk 8, figuur 8.1). Daarbij zijn halorespirerende bacteriën teruggevonden in allerlei verontreinigde milieus (Hoofdstuk 1, tabel 1.1).

Dit proefschrift heeft de fysiologie en biochemie van deze bacteriën als onderwerp. Het onderzoek is gericht op drie onderwerpen: (i) het bestuderen van de koppeling tussen reductieve dechlorering en ATP vorming in *Desulfitobacterium dehalogenans,* (ii) de isolatie en karakterisatie van dehalogenases uit verschillende *Desulfitobacterium* soorten en (iii) de isolatie en karakterisatie van een *Desulfitobacterium* stam uit menselijke feces. In **Hoofdstuk 1** is een overzicht gegeven van de verschillende microbiële dehalogeneringsmechanismen, waarbij het accent op halorespiratie is gelegd. Daarnaast wordt een overzicht gegeven van de halorespirerende bacteriën welke in reincultuur zijn beschreven, de huidige modellen voor 3-chloorbenzoaat- en tetrachlooretheen(PCE)-ademhaling, en de karakteristieken van reductieve dehalogenases.

Desulfitobacterium dehalogenans is een anaërobe Gram-positieve bacterie, die in staat is om *ortho*-gechloreerde fenolachtige verbindingen te gebruiken als terminale elektronenacceptor (Utkin *et al.*, 1994). Een vergelijking van de opbrengsten van *D. dehalogenans* gegroeid met pyruvaat (pyrodruivenzuur) als elektronendonor en 3-chloro-4-hydroxyfenyl-acetaat (Cl-OHPA), sulfiet, nitraat, of fumaraat als elektronenacceptor geeft aan dat *D. dehalogenans* altijd dezelfde hoeveelheid energie vastlegt, onafhankelijk van de gebruikte elektronenacceptor (Mackiewicz & Wiegel, 1998). In **Hoofdstuk 2** zijn de opbrengsten van *D. dehalogenans,* gegroeid met

waterstof, formiaat (mierenzuur), pyruvaat, of lactaat (melkzuur) als elektronendonor en Cl-OHPA als elektronenacceptor, vergeleken. Daarnaast zijn de activiteiten van de verschillende elektronendonerende en –accepterende enzymen gelokaliseerd. Deze resultaten wijzen erop dat de koppeling van de oxidatie van lactaat en pyruvaat aan de reductie van Cl-OHPA één ATP per mol geproduceerde acetaat (azijnzuur) opbrengt via fosforylering op substraatniveau. Als formiaat of waterstof wordt gebruikt als elektronendonor voor reductieve dechlorering is de groeiopbrengst ongeveer 1/3 van de opbrengst met pyruvaat als elektronendonor. Onder deze groeicondities kan de energie niet via fosforylering op substraatniveau vastgelegd worden.

Formiaatdehydrogenase en hydrogenase zijn aan beide zijden van het celmembraan gelokaliseerd. Dit in tegenstelling tot pyruvaatdehydrogenase dat in het cytoplasma gelokaliseerd is en lactaatdehydrogenase dat gelokaliseerd is aan de cytoplasmatische kant van het celmembraan. Op basis van deze gegevens wordt er een model voorgesteld waarin de lokalisatie van de elektronendonerende enzymen bepaalt of er een protondrijvende kracht (PMF) is verkregen. Het elektronentransport via de elektronentransportketen (ETC) en de reductie van de gechloreerde verbinding door het reductief dehalogenase dragen niet bij aan de PMF (Hoofdstuk 8, Figuur 8.2). We hebben de samenstelling van de ETC, die betrokken is bij het elektronentransport van de elektronendonor formiaat naar Cl-OHPA, onderzocht in celsuspensies en hebben deze vergeleken met de ETC betrokken bij de fumaraatademhaling met formiaat als elektronendonor (**Hoofdstuk 3**). Menaquinon, cytochroom c en cytochroom b zijn de componenten die gevonden zijn in alle cellen die gegroeid zijn met formiaat en Cl-OHPA of fumaraat. We hebben aangetoond dat deze componenten gereduceerd kunnen worden door formiaat en geoxideerd worden na toevoeging van de geïnduceerde elektronenacceptor. De resultaten suggereren dat (een deel van) de halorespiratieketen gedeeld wordt met fumaraatademhaling. In een eerder onderzoek is geconcludeerd op basis van de analyse van mutanten van *D. dehalogenans* die deficiënt zijn in halorespiratie, dat halorespiratie waarschijnlijk componenten deelt met nitraatademhaling (Smidt *et al.,* 1999). Dat verschillende ademhalingsroutes gemeenschappelijke elementen delen is niet ongewoon en is eerder gerapporteerd voor o.a. *Paracoccus denitrificans* en *E. coli* (Richardson, 2000; Unden & Bongaerts, 1997). In het huidige onderzoek kon de betrokkenheid van cytochroom b alleen worden aangetoond voor fumaraatademhaling en niet voor halorespiratie. In cellen gegroeid met fumaraat als elektronenacceptor is de concentratie cytochroom b ook

hoger in verhouding tot het gehalte aan cytochroom c. Dit zou erop kunnen duiden dat cytochroom b de directe elektronendonor is voor fumaraatreductase. Een vergelijkbare situatie is gevonden in *W. succinogenes* en *E. coli* (Kröger *et al.*, 1992). Fumaraatreductase is gelokaliseerd aan de cytoplasmatische kant van het celmembraan.

De elektronentransportketen van formiaat naar Cl-OHPA is in meer detail onderzocht met behulp van elektron paramagnetische resonantie spectroscopie (EPR). In deze experimenten hebben we aangetoond dat molybdeen, ijzer-zwavel clusters, cobalamine, een "high-spin heme" en een onbekend ijzer-zwavel cluster, onderdelen zijn die onder invloed van formiaat worden gereduceerd en o.i.v. Cl-OHPA worden geoxideerd. Dit suggereert dat het formiaatdehydrogenase dat actief is gedurende de halorespiratie een molybdeen en ijzer-zwavel bevattend formiaatdehydrogenase is. Dit enzym doneert zijn elektronen aan cytochroom c (analoog aan *Desulfovibrio* sp.), of de elektronen worden overgedragen aan cytochroom b (analoog aan *E. coli* en andere bacteriën). Omdat cytochroom c meestal membraangeassocieerd is en niet membraangebonden, is er een extra component, waarschijnlijk cytochroom b, nodig om de elektronen van de buitenkant van het membraan naar de cytoplasmatische kant van het celmembraan te transporteren. De elektronen worden dan overgedragen aan menaquinon dat 2 protonen uit het cytoplasma opneemt en deze, afhankelijk van de lokalisatie van het reductief dehalogenase, afgeeft aan de buiten- of de binnenkant van de cel (respectievelijk figuur 8.2 A en B (hoofdstuk 8)). Daarnaast is de oxidatie van cobalamine, een co-factor van chloorfenol reductief dehalogenase, aangetoond in celsuspensies na de toevoeging van Cl-OHPA. Deze waarneming laat zien dat het dehalogenase dat we hebben gekarakteriseerd, betrokken is bij *in-vivo* halorespiratie. De oriëntatie van het dehalogenase in het cytoplasmisch membraan kon niet aangetoond worden, maar dehalogenases van andere halorespirerende bacteriën zijn gelokaliseerd aan de cytoplasmatische zijde van het cytoplasmatisch membraan (Holliger *et al.*, 1999). Als het reductief dehalogenase van *D. dehalogenans* een vergelijkbare topologie zou hebben, dan zouden de groeiopbrengsten (Hoofdstuk 2) verklaard kunnen worden met model B dat te zien is in figuur 8.2. Dit model is analoog aan de modellen die zijn voorgesteld voor 3-chloorbenzoaat in *D. tiedjei* en PCE respiratie in *D. restrictus.* (Hoofdstuk 1, figuur 1.3).

De isolatie en karakterisatie van het chloorfenol reductief dehalogenase wordt beschreven in **Hoofdstuk 4.** Dit enzym werd anaëroob gezuiverd uit een Triton X-100

extract van de membraanfractie. Het gezuiverde enzym katalyseert de dechlorering van Cl-OHPA met een V_{max} van 28 units/mg eiwit en een K_m van 20 mM. Verder katalyseert het gezuiverde dehalogenase de reductieve dehalogenering van verschillende *ortho*-gechloreerde fenolen en 2-bromo-4-chlorofenol met gereduceerd methylviologeen als kunstmatige elektronendonor. De EPR analyse wijst op de aanwezigheid van één [4Fe-4S]-cluster (E_m = -440 mV), één [3Fe-4S]-cluster (E_m = 170 mV), and één cobalamine per 48-kDa monomeer. De Co^+/Co^{2+}-overgang had een E_m van -370 mV. Het corresponderende gen is geïsoleerd, gekloneerd en de DNA-sequentie ervan is bepaald. Hierbij werd de aanwezigheid van twee nauw gerelateerde genen aangetoond: (i) *cprA*, coderend voor het o-chloorfenol reductief dehalogenase, en (ii) *cprB*, coderend voor een integraal membraaneiwit dat als membraananker van het dehalogenase kan functioneren. Verder bevat *cprA* een "twin-arginine" signaalsequentie die niet aanwezig is in het gezuiverde enzym. Dit is een aanwijzing dat het enzym gelokaliseerd kan zijn aan de buitenkant van het cytoplasmatisch membraan. Praktisch alle genen die een "twin-arginine" signaalsequentie bevatten coderen voor eiwitten die aan de buitenzijde zijn gelokaliseerd (Berks *et al.*, 2000). Echter, *pceA*, het gen coderend voor het cytoplasmatisch PCE reductief dehalogenase van *Dehalospirillum multivorans,* bevat ook een "twin-arginine" signaalsequentie (Neumann *et al.*, 1998). Het is daarom mogelijk dat het TAT ("twin-arginine" transport) systeem enkel betrokken is bij de ontwikkeling van dehalogenases en niet bij het transport van deze enzymen over het cytoplasmatisch membraan.

Naast *ortho*-gechloreerde fenolen kan *D. dehalogenans* ook andere elektronenacceptoren gebruiken. Mackiewicz en Wiegel (1998) hebben aangetoond dat verschillende elektronenacceptoren, inclusief Cl-OHPA, tegelijkertijd kunnen worden gebruikt. In **Hoofdstuk 5** is de invloed van andere elektronenacceptoren op de inductie van de dechlorerende activiteit in celsuspensies en celextracten beschreven. Er is gevonden dat de dechlorerende activiteit wordt geïnduceerd door zijn substraat (Cl-OHPA) en mogelijk door fumaraat. Echter, als cellen waren geadapteerd aan het gebruik van een andere elektronenacceptor (inclusief fumaraat), dan was de inductie van dechlorering geremd in vergelijking tot de inductie van dechlorering in cellen die fermentatief gegroeid waren op pyruvaat, d.w.z. in afwezigheid van een extra elektronenacceptor. De dechloreringssnelheid van een dechlorerende cultuur daalde met 14 % als fumaraat werd toegevoegd, maar er was geen effect te zien na toevoeging van nitraat, sulfiet of thiosulfaat. In contrast daarmee had de toevoeging

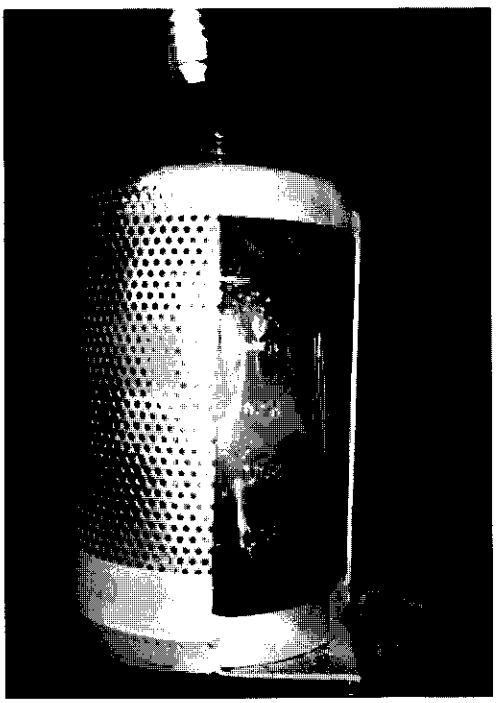
van fumaraat in celextracten geen effect, maar remden de andere elektronenacceptoren de dechlorerende activiteit. In batchcultures blijkt *D. dehalogenans* geen voorkeur te hebben voor een type elektronenacceptor, maar gebruikt hij verschillende elektronenacceptoren tegelijkertijd. Dit zou relevant kunnen zijn voor (*in-situ*) bioremediatietechnieken, omdat de aanwezigheid van meerdere elektronenacceptoren in verontreinigde sedimenten niet ongewoon is.

Terwijl *D. dehalogenans ortho*-gechloreerde fenolen als terminale elektronenacceptor voor groei kan gebruiken, kan *Desulfitobacterium* sp. stam PCE1 zowel chloorfenolen en PCE tegelijkertijd gebruiken en kan *Desulfitobacterium frappieri* stam TCE1 zowel PCE als trichlooretheen (TCE) gebruiken (Gerritse *et al.*, 1999; Gerritse *et al.*, 1996; Utkin *et al.*, 1994). We hebben de substraatspectra van celextracten van deze stammen, gegroeid met Cl-OPHA of PCE als elektronenacceptor, vergeleken in **Hoofdstuk 6**. De resultaten geven aan dat stam PCE1 verschillende enzymen voor PCE en chloorfenol dechlorering bevat. Dit werd in meer detail bekeken door de isolatie van het chloorfenol reductief dehalogenase en het PCE reductief dehalogenase van stam PCE1 en het PCE/TCE reductief dehalogenase van stam TCE1. Gebaseerd op de N-terminale sequentie, grootte en substraat spectrum van de celextracten is gevonden dat het chloorfenol reductief dehalogenase van stam PCE1 identiek is aan het dehalogenase van *D. dehalogenans.* Het PCE/TCE reductief dehalogenase van stam TCE1 heeft vergelijkbare eigenschappen als zijn beschreven voor het PCE reductief dehalogenase van *Desulfitobacterium* stam PCE-S (Miller *et al.*, 1998). Het PCE reductief dehalogenase van stam PCE1 is een nieuw type reductief dehalogenase. Het enzym katalyseert de reductie van PCE en heeft een lage activiteit met TCE. Het gezuiverde enzym had een subunit-grootte van 45 kDa op SDS-PAGE. Zowel de activiteit van dit enzym, als die van het chloorfenol reductief dehalogenase van stam PCE1 werden geremd door de toevoeging van de cobalamine remmers 1-iodopropaan en NO aan celextracten. Het is zeer waarschijnlijk dat deze enzymen, evenals de andere dehalogenases uit *Desulfitobacterium* stammen, cobalamine bevatten.

In **Hoofdstuk 7** is de isolatie en karakterisatie van een nieuwe *Desulfitobacterium frappieri* stam beschreven. Dit is de eerste isolaat van een *Desulfitobacterium* stam, dat geen chloorethenen of chloorfenolen kan gebruiken als terminale elektronenacceptor.

Het onderzoek dat is gepresenteerd in dit proefschrift had als doel een antwoord te geven op de vraag hoe halorespirerende bacteriën reductieve dechlorering koppelen aan energieconservering. De resultaten die zijn beschreven in hoofdstuk 2, 3 en 4 geven aanwijzingen voor het mechanisme dat is samengevat in figuur 8.2. Het verschil tussen model A en B wordt veroorzaakt door de lokalisatie van het dehalogenase. Deze lokalisatie en de rol van het TAT systeem bij de rijping van het reductief dehalogenase zijn interessante onderwerpen voor verder onderzoek. Tijdens groei in de aanwezigheid van meerdere elektronenacceptoren is gevonden dat *D. dehalogenans* reageert als een "opportunist". Het organisme schakelt niet snel over van het gebruik van de ene elektronenacceptor naar de ander. Daarnaast kan het meerdere elektronenacceptoren simultaan gebruiken als noch de elektronendonor noch de elektronenacceptor(en) limiterend zijn. In dit onderzoek is niet gekeken naar de invloed van elektronenacceptoren op dechlorering onder elektronendonor of -acceptor limiterende omstandigheden. In eerder onderzoek naar PCE dechlorering door *D. frappieri* TCE1 is echter gevonden, dat de dechlorering in dit organisme compleet geremd werd door de aanwezigheid van andere elektronenacceptoren in cellen gegroeid onder elektronendonor gelimiteerde condities, maar niet in cellen gegroeid onder elektronenacceptor gelimiteerde condities (Gerritse *et al.*, 1999). De reactie- en regulatiemechanismen zouden vergelijkbaar kunnen zijn, omdat de enzymen die de dechlorering van PCE en chloorfenol katalyseren in verschillende stammen van *Desulfitobacterium* dezelfde co-factoren bevatten. Ondanks deze overeenkomsten in de co-factoren, heeft onderzoek van PCE en chloorfenol dechlorering in *Desulfitobacterium* stam PCE1 laten zien dat de dehalogenases die met deze activiteiten geassocieerd worden, totaal verschillende substraatspecificiteiten hebben. Voor de dechlorering van verschillende gechloreerde verbindingen is zodoende een combinatie van gespecialiseerde dehalogenases nodig, die waarschijnlijk niet aanwezig zijn in één halorespirerende soort. Door de isolatie van een stam van *Desulfitobacterium frappieri*, die geen chloorethenen of chloorfenolen dechloreert, ziet men dat men voorzichtig moet zijn met het extrapoleren van de aanwezigheid van bepaalde bacteriële groepen of soorten naar de dechloreringscapaciteit van een verontreinigde omgeving (Löffler *et al.*, 2000).

Referenties: zie hoofdstuk 8



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Bram

Curriculum vitae

Bram Anton van de Pas werd geboren op 8 april 1971 te Oss. Hij groeide op in Schaijk en behaalde in 1989 zijn VWO-diploma aan het Titus Brandsma Lyceum te Oss. In september 1989 begon hij met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit Wageningen. In deze periode deed hij een zes maands afstudeervak Microbiologie betreffende zuivering van het fumaraatreductase van de mesofiele propionaat oxiderende bacterie MPOB. Na dit vak liep hij 5 maanden stage in de groep van Prof. Mirja Salkinoja-Salonen aan de Universiteit van Helsinki, waar hij participeerde in het onderzoek naar contaminerende sporenvormende bacteriën in het productieproces van kartonnen drankverpakkingen. Zijn studie sloot hij af met een afstudeervak bij de vakgroep Erfelijkheidsleer, waar hij de overdracht van dubbelstrengs RNA-virussen tussen incompatibele schimmels onderzocht. In 1995 studeerde hij af en begon met zijn promotieonderzoek waarin hij gekeken heeft naar de biochemie en fysiologie van halorespiratie door *Desulfitobacterium dehalogenans*. Dit onderzoek heeft geresulteerd in het voorliggende proefschrift. Sinds mei 2000 is hij werkzaam als projectleider bodem bij Het Milieuburo in Maasbree.