

## Review

# Reductive dechlorination in the energy metabolism of anaerobic bacteria

Christof Holliger<sup>a</sup>, Gert Wohlfarth<sup>b</sup>, Gabriele Diekert<sup>b,\*</sup>

<sup>a</sup> Swiss Federal Institute for Environmental Science and Technology (EAWAG), Limnological Research Center, CH-6047 Kastanienbaum, Switzerland

<sup>b</sup> University of Stuttgart, Institute for Microbiology, Allmandring 31, D-70569 Stuttgart, Germany

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## Abstract

Within the last few decades, several anaerobic bacteria have been isolated which are able to reductively dechlorinate chlorinated aliphatic and aromatic compounds at catabolic rates. For some of these bacteria, it has been shown that the reductive dechlorination is coupled to energy conservation, a process designated as ‘dehalorespiration’. Somewhat simple respiratory chains seem to be involved that utilize the free energy that could be gained from the exergonic dechlorination reaction quite inefficiently. With one exception, all reductive dehalogenases isolated to date contain a corrinoid and iron–sulfur clusters as cofactors. During the course of the catalytic reaction cycle, the cobalt of the corrinoid is subjected to a change in its redox state. Hence, reductive dechlorination represents a new type of biochemical reaction. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords:** Reductive dehalogenation; Dehalorespiration; Chlorophenol; Tetrachloroethene; Corrinoid; Vitamin B<sub>12</sub>

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\* Corresponding author. Tel.: +49 (711) 685-5483; Fax: +49 (711) 685-5725; E-mail: [imbgd@po.uni-stuttgart.de](mailto:imbgd@po.uni-stuttgart.de)

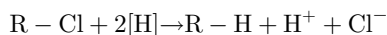
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## 1. Introduction

Over the last few decades, several bacterial cultures, both mixed and pure, have been described which are capable of reductive dechlorination of chlorinated aliphatic and aromatic hydrocarbons. These chlorinated hydrocarbons include compounds frequently encountered as environmental contaminants known to be hazardous and toxic for animals and plants, or destructive to the ozone layer. Although numerous reports and reviews on microbial reductive dechlorination activities are available [1–9], this process is not, as yet, completely understood. Many of the studies involved co-metabolic or abiotic reductive dechlorination, or dechlorination by mixed cultures, facultative anaerobes or aerobic bacteria, which will not be discussed in this communication. Nevertheless, it should be noted that several aerobes can catalyze reductive dechlorination reactions. In some aerobes, this reaction appeared to be dependent on glutathione [10], whereas in others, a requirement for NADH as an electron donor was observed [11]. The compounds reductively dechlorinated by these organisms were usually intermediates formed during the aerobic degradation of chlorinated aromatics.

Reductive dechlorination under strictly anoxic conditions was observed for a variety of anaerobes, including methanogenic and homoacetogenic bacteria [1,2,7]. In most cases, the process appeared to be mediated in cell-free systems by heat-stable compounds at low velocities. Due to the dechlorinating activity of tetrapyrrole-cofactors, such as corrinoids, iron porphyrins, and coenzyme F<sub>430</sub> in aqueous solution [3], it has been proposed that in many dechlorinating bacteria the dechlorination reaction is a co-metabolic activity of enzymes containing these cofactors as prosthetic groups. For some co-metabolically dechlorinating bacteria, it has been demonstrated that tetrapyrrole-containing enzymes are indeed responsible for the dechlorination reaction they catalyze. This type of reductive dechlorination reaction is extensively described in [1] and will not be discussed in this review.

In this communication, the description of the reductive dechlorination process in pure cultures and with purified enzymes will emphasize those bacteria which utilize the energy generated in the dechlorination process for the synthesis of ATP. This respiratory process is referred to as ‘dehalorespiration’ throughout the entire communication. In other reports, the terms ‘halorespiration’ or ‘chlororespiration’, which suggest that a halogen serves as terminal electron acceptor (in analogy to e.g. fumarate or nitrate respiration), have been used. Since this is not the case, the term ‘dehalorespiration’ is preferable, as it indicates that the dehalogenation process is coupled to ATP synthesis via a chemiosmotic mechanism. A prerequisite for the coupling of an energy conservation to reductive dechlorination is that the reaction:



is thermodynamically favorable.

It has been demonstrated that this reaction is usually exergonic, as shown for tetrachloroethene or 3-chlorobenzoate in Fig. 1. For most halogenated compounds, the standard redox potential for the couplet R-Cl/R-H lies between approximately +250 and +600 mV [8,12]. Therefore, these compounds are thermodynamically favorable as electron acceptors under anaerobic conditions. This is also true of monohalogenated compounds, some of which, for example vinyl chloride and monochlorobenzene, are often dechlorinated slowly, if at all. Anaerobes capa-

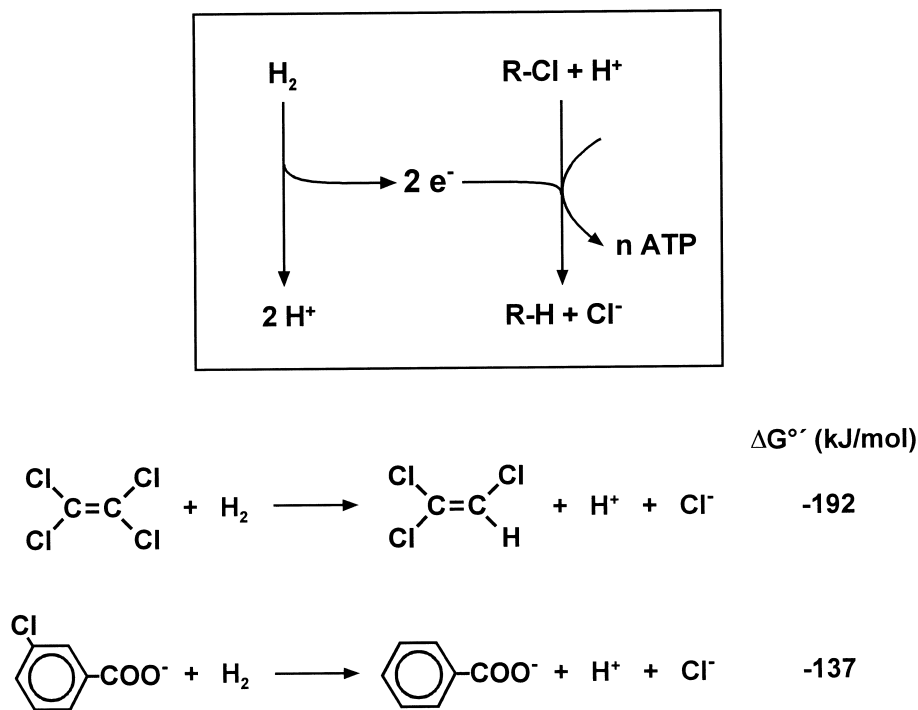


Fig. 1. Scheme of dehalorespiration with  $\text{H}_2$  as electron donor and tetrachloroethene or 3-chlorobenzoate as electron acceptor.

ble of growth on a defined medium with the chlorinated substrate as the electron acceptor and an electron donor like  $\text{H}_2$  or formate, the oxidation of which cannot be coupled to the synthesis of ATP (Fig. 1), have to gain their energy from dehalorespiration. For those organisms which require an electron donor yielding ATP via substrate level phosphorylation during oxidation, dehalorespiration is difficult to prove unambiguously, even if the organism depends on the presence of the chlorinated compound as an electron acceptor. It is feasible that the organohalogen merely serves as a favorable and/or necessary electron sink for reducing equivalents generated upon oxidation of the electron donor.

## 2. Anaerobic bacteria capable of reductive dechlorination in their energy metabolism

Chlorinated substrates reported to be reductively dehalogenated by pure cultures of anaerobes in their energy metabolism fall into two categories: chlori-

nated aromatic compounds, such as chlorinated benzoate or phenols, on the one hand and chlorinated ethenes on the other. Other organochlorines, such as polychlorinated biphenyls and chlorinated benzenes, also appear to be reductively dechlorinated in similar physiological processes [1,2,7]. Since little is known about the bacteria involved, this process will not be further discussed.

Aryl halides are converted by *Desulfomonile tiedjei* and the facultative anaerobe isolate 2CP-1, as well as by members of the newly described genus, *Desulfitobacterium*. Some species of the latter genus are also able to reduce tetrachloroethene (PCE) and trichloroethene (TCE). Other PCE reducing organisms include *Dehalobacter restrictus* and the closely related isolate TEA, as well as *Dehalospirillum multivorans*, *Desulfuromonas chloroethenica*, *Dehalococcoides ethenogenes*, and the facultative anaerobes *Enterobacter* strain MS-1 and *Enterobacter agglomerans*. All of the microorganisms capable of reductive dechlorination belong to the bacteria, several of them being related to sulfate or sulfur reducers in the  $\delta$ - and  $\epsilon$ -sub-

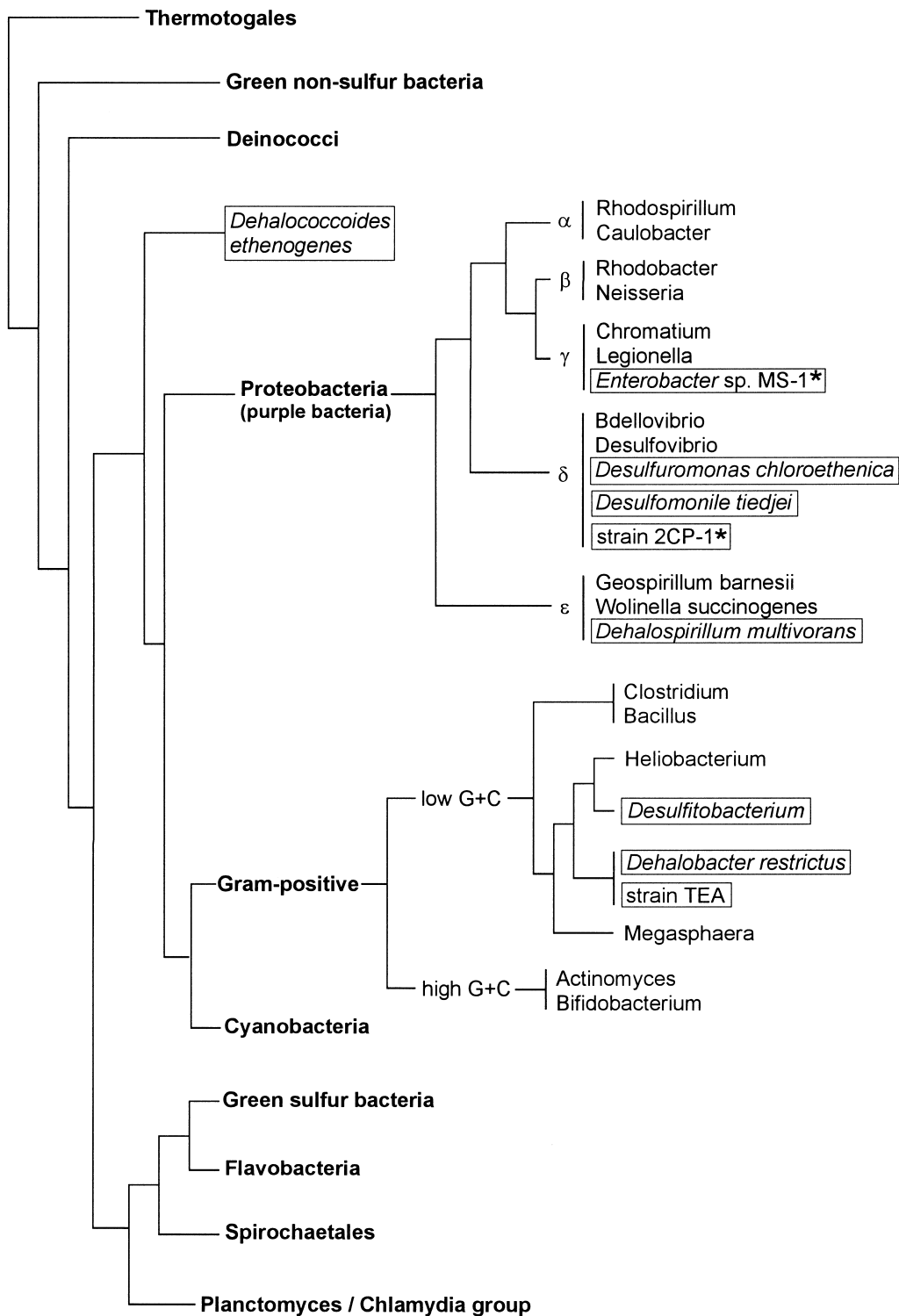


Fig. 2. Phylogenetic affiliations, based on analysis of 16S ribosomal RNA gene sequences, of bacteria capable of reductive dechlorination (framed). The facultative anaerobes are marked with an asterisk.

Table 1  
Anaerobic bacteria capable of reductive dechlorination in the catabolism

	Dechlorinated compounds <sup>a</sup>	Electron donor <sup>a</sup>	Remarks
<i>Desulfomonile tiedjei</i> [14]	PCE, TCEH <sub>2</sub> , 3-chlorobenzoate, pentachlorophenol	H <sub>2</sub> , formate, pyruvate	<i>meta</i> dechlorination
Isolate 2CP-1 [22]	2-chlorophenol, 2,6-dichlorophenol	acetate	facultative anaerobe; <i>ortho</i> dechlorination
<i>Desulfitobacterium chlororespirans</i> [26]	2,4,6-trichlorophenol, 3-Cl-4-OH-phenylacetate	H <sub>2</sub> , formate, pyruvate	<i>ortho</i> dechlorination
<i>Desulfitobacterium hafniense</i> [24]	3-Cl-4-OH-phenylacetate, pentachlorophenol	pyruvate, tryptophan	<i>ortho, meta</i> dechlorination
<i>Desulfitobacterium frappieri</i> [27]	2,4,6-trichlorophenol, 3-Cl-4-OH-phenylacetate	pyruvate	<i>ortho, meta, para</i> dechlorination
<i>Desulfitobacterium dehalogenans</i> [28]	PCE, 2,4,6-trichlorophenol, 3-Cl-4-OH-phenylacetate	H <sub>2</sub> , formate, pyruvate	<i>ortho</i> dechlorination
<i>Desulfitobacterium</i> strain PCE1 [25]	PCE, 2-chlorophenol, 2,4,6-trichlorophenol, 3-Cl-4-OH-phenylacetate	formate, pyruvate	minor TCE reduction; <i>ortho</i> dechlorination
<i>Desulfitobacterium</i> strain PCE-S [23]	PCE, TCE, 2,4,5-trichlorophenol, pentachlorophenol	pyruvate	<i>ortho, meta</i> dechlorination
<i>Dehalobacter restrictus</i> [32]	PCE, TCE	H <sub>2</sub>	
Isolate TEA [33]	PCE, TCE	H <sub>2</sub>	
<i>Dehalospirillum multivorans</i> [35]	PCE, TCE	H <sub>2</sub> , formate, pyruvate	
<i>Desulfuromonas chloroethenica</i> [39]	PCE, TCE	pyruvate, acetate	
<i>Dehalococcoides ethenogenes</i> strain 195 [42]	PCE, TCE, DCE, chloroethene	H <sub>2</sub>	
<i>Enterobacter</i> strain MS-1 [43]	PCE, TCE	formate, pyruvate, acetate	facultative anaerobe

PCE, tetrachloroethene; TCE, trichloroethene.

<sup>a</sup>Incomplete selection.

groups of the proteobacteria. The phylogenetic position of the bacteria capable of reductive dechlorination is depicted in Fig. 2. Table 1 summarizes some characteristics of these organisms, especially with respect to the reductive dechlorination process.

### 2.1. *Desulfomonile tiedjei*

This microorganism is a Gram-negative sulfate-reducing bacterium belonging to the  $\delta$ -subgroup of the Proteobacteria [13,14]. The bacterium contains cytochrome *c* and is dependent on 1,4-naphthoquinone as a growth factor [7]. It is able to grow with hydrogen as an electron donor and 3-chlorobenzoate as an electron acceptor [15]. Energy conservation via dehalorespiration was unambiguously demonstrated for the first time in this organism [16,17]. Cell suspensions and cell extracts were also shown to reductively dechlorinate PCE to *cis*-1,2-dichloroethene (DCE). Evidence is available that 3-chlorobenzoate and

PCE are converted by the same enzyme, as indicated by co-induction with 3-chlorobenzoate [18], and by the similar inhibition patterns of both reductive dechlorination activities [19]. The enzyme is not inducible by PCE, indicating that PCE reduction is probably a side reaction of chlorobenzoate reductive dehalogenase. Upon induction of the enzyme by 3-chlorobenzoate, the synthesis of an unusual cytochrome *c* with a negative standard redox potential of  $-342$  mV was co-induced [20]. Sulfite and thio-sulfate, rather than sulfate, inhibited reductive dechlorination in crude extracts, whereas sulfate repressed the reductive dehalogenase [21]. The 3-chlorobenzoate reductive dehalogenase has been purified and partially characterized (Table 2).

### 2.2. Isolate 2CP-1

This facultative anaerobe belongs to the same subgroup as *D. tiedjei*. It is, however, more closely re-

lated to the fruiting myxobacteria, which are obligate aerobes. Strain 2CP-1 reductively dechlorinates 2-chlorophenol with acetate as electron donor [22]. 2-Chlorophenol is only converted under strictly anoxic conditions.

### 2.3. The genus *Desulfitobacterium*

*Desulfitobacterium* is a newly described genus of Gram-positive bacteria. Some species are able to form endospores. In some of the organisms, menaquinone [23], cytochrome *b* [23], and cytochrome *c* [23–25] have been detected. *Desulfitobacterium* species are capable of reductive dehalogenation of a variety of chlorinated compounds. Alternatively, members of this genus, with one exception (see below), can utilize sulfite and thiosulfate, but not sulfate, as terminal electron acceptors. For most *Desulfitobacteria* described, dehalorespiration appears doubtful or is insufficiently documented, if documented at all. Most of the organisms utilize electron donors, such as pyruvate or grow in complex, undefined media. The reductive dehalogenases for the conversion of the aryl halides appear to be inducible, whereas the PCE reductive dehalogenases are either constitutively expressed or their inducibility not yet studied. *Desulfitobacterium chlororespirans* [26], *Desulfitobacterium hafniense* [24], and *Desulfitobacterium frappieri* [27] reduce chlorinated phenols, such as 2,4,6-trichlorophenol or 3-chloro-4-hydroxyphenylacetate (Cl-OHPA). The Cl-OHPA reductive dehalogenase has been purified from *Desulfitobacterium hafniense* and *Desulfitobacterium dehalogenans* (Table 2).

*Desulfitobacterium dehalogenans* [25,28–30], *Desulfitobacterium* strain PCE1 [25], and *Desulfitobacterium* strain PCE-S [23] are able to convert chlorinated phenols as well as PCE. In contrast to the alkyl and aryl halide conversion carried out by *Desulfomonile tiedjei*, these processes are mediated by different enzymes with respect to their substrate spectrum (see below). PCE is converted to TCE by *Desulfitobacterium dehalogenans*, whereas traces of DCE are formed by strain PCE1. The PCE reductive dehalogenase of strain PCE-S has been purified, and shown to mediate the reduction of PCE via TCE to DCE as the end product (Table 2). The latter organism is the only *Desulfitobacterium* which is not able

to reduce sulfite or thiosulfate in its energy metabolism.

### 2.4. *Dehalobacter restrictus* and isolate TEA

*Dehalobacter restrictus* [31,32] and isolate TEA [33] are, based on 16S rDNA analysis, affiliated with the same group of Gram-positive bacteria as *Desulfitobacterium*. *Dehalobacter restrictus* contains menaquinones and cytochrome *b*. *Dehalobacter restrictus*, as well as isolate TEA, seem to be restricted to utilization of hydrogen as their electron donor and PCE or TCE as electron acceptors. Hence, these organisms derive metabolic energy from dehalorespiration [34]. The PCE reductive dehalogenase has been purified from *Dehalobacter restrictus* (Table 2), and has been found to reduce PCE to DCE.

### 2.5. *Dehalospirillum multivorans*

*Dehalospirillum multivorans* [35] is a Gram-negative bacterium belonging to the  $\epsilon$ -subgroup of the Proteobacteria. It is very closely related to *Geospirillum barnesii*, although the latter organism is not able to dechlorinate PCE (Windfuhr, Scholz-Muramatsu and Diekert, unpublished). The organism contains menaquinone and cytochromes *b* and *c*. Compared with *Dehalobacter restrictus* and strain TEA, *Dehalospirillum multivorans* is rather more versatile with respect to its substrate spectrum. Although it is able to grow with H<sub>2</sub> and PCE as energy sources, indicating energy conservation via dehalorespiration, it is also able to utilize other electron donors, like pyruvate and formate, and alternative electron acceptors, e.g. fumarate, arsenate, and selenate. Some of these alternative electron acceptors have been shown to inhibit PCE reduction in cell suspensions [36–38]. The constitutive PCE reductive dehalogenase mediates PCE reduction to DCE. It has been purified and characterized (Table 2) and the gene encoding the enzyme cloned and sequenced.

### 2.6. *Desulfuromonas chloroethenica*

This Gram-negative, sulfur-reducing bacterium utilizes pyruvate or acetate as the electron donor for the reduction of PCE or TCE to DCE [39]. It is doubtful whether the reductive dechlorination of

Table 2  
Properties of reductive dehalogenases

Enzyme	3-Cl-benzoate reductive dehalogenase [49]	Cl-OHPA reductive dehalogenase <sup>a</sup>	Cl-OHPA reductive dehalogenase [50]	PCE reductive dehalogenase [52]	PCE reductive dehalogenase [54]	PCE reductive dehalogenase [55]	PCE reductive dehalogenase [61]	TCE reductive dehalogenase [61]
Organism	<i>Desulfomonile tiedjei</i>	<i>Desulfitobacterium dehalogenans</i>	<i>Desulfitobacterium hafniense</i>	<i>Desulfitobacterium strain PCE-S</i>	<i>Dehalobacter restrictus</i>	<i>Dehalospirillum multivorans</i>	<i>Dehalococcoides ethenogenes</i>	<i>Dehalococcoides ethenogenes</i>
Substrate	3-Cl-benzoate	Cl-OHPA	Cl-OHPA	PCE, TCE	PCE, TCE	PCE, TCE	PCE	TCE, DCE, VC
Localization	membrane	membrane	membrane	membrane	membrane	cytoplasm <sup>b</sup>	membrane	membrane
Apparent molecular mass:								
Gel filtration (kDa)	n.d.	n.d.	n.d.	198	164/71 <sup>c</sup>	58	n.d.	n.d.
SDS-PAGE (kDa)	64 ( $\alpha$ ); 37 ( $\beta$ )	48	47	65	60	57	51	61
Apparent $K_m$ ( $\mu$ M)	n.d.	20 (Cl-OHPA)	n.d.	10 (PCE)	20 (PCE)	200 (PCE)	n.d.	n.d.
Specific activity in cell extracts (nkat/mg)	2.5	2.5	9.1	4	10	25	5	8
Specific activity of the purified enzyme (nkat/mg)	300	167	103	650	234	2640	350	200
Cofactors (mol/mol subunit)	heme, Fe/S: n.d.	corrinoi <sup>d</sup> , Fe/S <sup>e</sup>	1 corrinoi <sup>d</sup> , 12 Fe, 13 S	1 corrinoi <sup>d</sup> , 8 Fe, 8 S	1 corrinoi <sup>d</sup> , 8 Fe, 8 S	1 corrinoi <sup>d</sup> , 8 Fe, 8 S	corrinoi <sup>d</sup> , Fe/S: n.d.	corrinoi <sup>d</sup> , Fe/S: n.d.
Oxygen sensitivity	stable	$\approx$ 90	$\approx$ 100 <sup>f</sup>	50	280	120	n.d.	n.d.
$t_{1/2}$ (min)								

Cl-OHPA, 3-chloro-4-hydroxy-phenylacetate; n.d., not determined; PCE, tetrachloroethene; TCE, trichloroethene; DCE, *cis*-1,2-dichloroethene; VC, vinyl chloride.

<sup>a</sup>van de Pas, personal communication.

<sup>b</sup>Catalytically active subunit.

<sup>c</sup>Dependent on the detergent present.

<sup>d</sup>Indicated by light-reversible inhibition with alkyl halides.

<sup>e</sup>Determined by EPR; number of Fe and S not known.

<sup>f</sup>Determined in the membrane fraction.

PCE is coupled to energy conservation [40]. It cannot be ruled out that acetate oxidation, rather than PCE reduction, is the energy yielding pathway, assuming that acetate oxidation occurs via a modified tricarboxylic acid cycle, including a succinyl-CoA:acetate CoA-transferase and an ATP citrate lyase as described for *Desulfobacter postgatei* [41]. The organism contains cytochrome *c*.

### 2.7. *Dehalococcoides ethenogenes*

This organism is a eubacterium without close affiliation to any known group [42]. It is the only anaerobe isolated so far which is able to completely dechlorinate PCE via TCE, DCE, and vinyl chloride (VC) to ethene with hydrogen as electron donor, indicating energy conservation via dehalorespiration. Alternative electron donors and acceptors tested were found not to be utilized. Two reductive dehalogenases, which have since been purified (Table 2), mediate the reduction of PCE to TCE and of TCE to ethene via DCE and VC.

### 2.8. *Enterobacter strain MS-1 and E. agglomerans*

These Gram-negative bacteria, which belong to the  $\gamma$ -subgroup of the Proteobacteria, are the only facultative anaerobes reported so far now to reductively dechlorinate PCE, thereby converting it to DCE. This occurs only under strictly anoxic conditions [43]. A variety of electron donors were utilized for PCE reduction. The coupling of energy conservation with reductive dehalogenation could not be demonstrated.

### 2.9. DCE dechlorinating mixed cultures

Several mixed cultures have been described which are able to completely dechlorinate PCE to ethene. The dichloroethene-utilizing organisms in these mixed cultures are obviously difficult to isolate and/or cultivate. It should be noted that DCE reduction to ethene in such cultures was found to be inhibited by bromoethanesulfonate (BES) in the absence of methanogenesis. BES was formerly thought to be a specific inhibitor of methanogenesis. Thus, inhibition of reductive dechlorination by BES is not indicative of the involvement of methanogens [44].

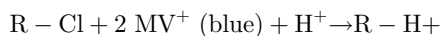
### 2.10. Reductive dechlorination in syntrophic associations

Chlorinated compounds may also be reductively dechlorinated in defined or undefined syntrophic consortia. Experiments were performed with a defined consortium consisting of *Desulfovibrio desulfuricans*, producing hydrogen upon oxidation of ethanol to acetate in the absence of sulfate concentrations greater than 5 mM, *Dehalospirillum multivorans* as a H<sub>2</sub> and PCE utilizing bacterium, and *Methanobacterium formicicum* as a bacterium competing for the use of the hydrogen. Under the experimental conditions imposed, the dechlorinating organism outcompeted the methanogen, demonstrating the high affinity of this organism for H<sub>2</sub> [45]. Earlier studies on mixed cultures [46–48] led to the conclusion that at least some dechlorinators must have a higher affinity for H<sub>2</sub> than methanogens.

A stable consortium consisting of *Desulfomonile tiedjei*, a benzoate-utilizing bacterium BZ-2, and a *Methanospirillum* species was found to convert 3-chlorobenzoate to acetate and methane [7]. In this consortium *Desulfomonile tiedjei* produced benzoate from 3-chlorobenzoate plus H<sub>2</sub>. Benzoate was converted to acetate, H<sub>2</sub> and CO<sub>2</sub> by the syntrophic organism BZ-2. The methanogen also utilized the hydrogen formed upon benzoate oxidation and provided vitamins for *Desulfomonile tiedjei*. In this consortium, none of the H<sub>2</sub>-consuming bacteria outcompeted any others.

## 3. Biochemistry and molecular biology of reductive dehalogenases

The reductive dehalogenases studied up until now have mediated the in vitro reduction of the chlorinated compounds with methyl viologen (MV;  $E^{\circ} = -446 \text{ mV}$ ;  $\epsilon_{578} = 9.7 \text{ mM}^{-1} \text{ cm}^{-1}$ ) as an artificial electron donor according to the following equation:



This simple reaction allowed for the spectrophoto-



metric recording of the reaction and was also essential for the purification of these enzymes. Due to the low redox potential of the reduced MV, this reaction had to be measured in the absence of molecular oxygen. The characteristics of the reductive dehalogenases which have so far been purified are summarized in Table 2.

### 3.1. 3-Chlorobenzoate reductive dehalogenase of *Desulfomonile tiedjei*

The first reductive dehalogenase reported to have been purified was the membrane-bound 3-chlorobenzoate converting enzyme from *Desulfomonile tiedjei* [49]. This oxygen-stable enzyme consists of two subunits with apparent molecular masses of 64 and 37 kDa, respectively. The purified protein exhibited UV-visible spectra, suggesting the presence of a heme cofactor for the protein; however, the cofactor content was not determined. The co-induction of the enzyme by a cytochrome *c* of a negative redox potential indicates the involvement of this cytochrome in 3-chlorobenzoate respiration [20].

All other reductive dehalogenases appeared to contain a corrinoid as a cofactor. This was surprising since corrinoids had not previously been known to catalyze any reactions involving electron transfer.

### 3.2. Ortho-chlorophenol reductive dehalogenases of *Desulfitobacteria*

The reductive dehalogenases converting 3-chloro-4-hydroxy-phenylacetate (Cl-OHPA) have recently been purified from *Desulfitobacterium dehalogenans* (van de Pas, personal communication) and *Desulfitobacterium hafniense* [50]. The membrane-bound enzymes consist of a single subunit with apparent molecular masses of 48 and 47 kDa, respectively. The enzyme of *Desulfitobacterium dehalogenans*, which also dehalogenated 2,3-dichlorophenol (van de Pas, personal communication), was light-reversibly inactivated by propyl iodide, indicating the presence of a corrinoid cofactor. The presence of a corrinoid was confirmed by preliminary EPR experiments that also revealed the presence of iron-sulfur clusters (van de Pas, personal communication). The enzyme of *Desulfitobacterium hafniense* contains one corrinoid and

about 12 Fe and 13 acid-labile sulfur per subunit [50].

An oxygen-stable 2,3-dichlorophenol-reducing enzyme system was found to be localized in the cytoplasmic membrane of *Desulfitobacterium chlororespirans* [51]. The reductive dehalogenase in crude membrane fractions was shown to be inhibited by sulfite, which also acted as an inhibitor for other reductive dehalogenases. Methyl viologen, rather than benzyl viologen, served as the artificial electron donor for the dechlorination reaction.

### 3.3. Dehalogenases reducing chlorinated ethenes

The PCE reductive dehalogenase of *Desulfitobacterium* strain PCE-S is membrane-associated [52]. This enzyme has been purified from cells grown with pyruvate and fumarate as energy sources, indicating its constitutive expression [52]. The enzyme, which mediates the reduction of PCE and TCE to DCE, consists of a single subunit with an apparent molecular mass of 65 kDa. It contains one corrinoid and around 8 Fe and 8 acid-labile sulfur as cofactors. Chlorinated aromatic compounds like Cl-OHPA were not converted by the enzyme. The enzyme was found to be inhibited by cyanide and sulfite, an observation which indirectly indicated the involvement of a cob(III)alamin in the catalytic cycle of the dechlorination reaction [52,53]. Although the standard redox potentials ( $E^{\circ}$ ) of the couples PCE/TCE (+580 mV) and TCE/DCE (+540 mV) are very positive (see above), it was found that PCE reduction in vitro required electron donors with redox potentials  $< -360$  mV, indicating a reduction of cob(II)alamin to cob(I)alamin in the catalytic cycle of the reaction (enzyme-bound cob(II)/cob(I)alamins:  $E^{\circ}$  between  $-350$  and  $-600$  mV).

A PCE reductive dehalogenase with properties similar to those of the strain PCE-S enzyme has been purified from *Dehalobacter restrictus* [54]. This enzyme, which catalyzes the reductive dechlorination of PCE to DCE, appears to be an integral membrane protein. The single subunit (60 kDa) contains a corrinoid with a standard redox potential of  $-350$  mV, as determined by EPR spectroscopy. In addition, the EPR studies performed showed that the cobalt is not coordinated by either a base or a histidine, and that it must hence be present in the base-off and his-off

form. In addition, the presence of 2  $\text{Fe}_4\text{S}_4$  clusters with redox potentials around  $-480$  mV, which are probably involved in the electron transfer, was reported. The amino acid sequence of the N-terminus of the enzyme [52] showed a high degree of similarity to that of the PCE reductive dehalogenase of *Desulfotobacterium* strain PCE-S (19 out of 20 amino acids were identical; Schumacher and Holliger, unpublished results).

The PCE reductive dehalogenase of *Dehalospirillum multivorans*, which is also known to reduce PCE to DCE, was recovered in the soluble fraction and was the only reductive dehalogenase purified in the absence of detergents [55]. This was surprising, since this enzyme is involved in a respiratory process and would thus be expected to be membrane-associated. This enzyme was the first PCE reducing dehalogenase purified and characterized. The soluble protein fraction consists of a single subunit with an apparent molecular mass of 58 kDa. The enzyme contains one corrinoid and about 8 Fe and 8 acid-labile sulfur per subunit. The requirement for a low potential electron donor ( $E^\circ' < -360$  mV) as well as this enzyme's light-reversible inactivation by alkyl halides, provided the first indication of the involvement of a corrinoid cofactor in reductive dechlorination. Cyanide and sulfite were found to inhibit enzyme activity [53,55], while ammonium ions were found to stimulate this activity. This effect has yet to be explained, and has so far only been observed for this specific PCE reductive dehalogenase.

The gene coding for the enzyme of *Dehalospirillum multivorans* was cloned in *Escherichia coli* and subsequently sequenced [56]. An operon was found, containing two open reading frames (ORFs), one of which (*pceA*) encoded the soluble PCE reductive dehalogenase. Downstream of *pceA* a second ORF, *pceB* (222 bp), was found, which coded for a hydrophobic protein, PceB (8.4 kDa), containing two transmembrane helices. This protein did not contain either histidine or cysteine, indicating the absence of a bound Fe–S cluster or a heme prosthetic group. It was suggested that this protein served as a 'membrane anchor' for the soluble, catalytic subunit of the PCE reductive dehalogenase PceA. This role for PceB would be in accordance with the function of PceA in respiratory processes.

The ORF *pceA* was comprised of 1503 bp and contained the binding motifs for two iron–sulfur clusters. These binding motifs corresponded to the typical consensus sequence for the binding of two  $\text{Fe}_4\text{S}_4$  clusters [57], with the exception that the first cysteine of the second cluster required for the binding of one of the Fe atoms was replaced by glycine. As a result, it cannot be excluded that one of the iron–sulfur clusters may contain 3 Fe rather than 4. The typical  $\text{B}_{12}$  binding motif (DXHXXG) [58] was not found. At the 5' terminus of *pceA*, a signal sequence containing the consensus sequence RRXFXXK followed by a hydrophobic stretch [59], was detected. This type of leader sequence is usually found in periplasmic proteins containing redox-active cofactors, where it is responsible for the excretion of the native holoenzyme. Aside from the PCE reductive dehalogenase of *Dehalospirillum multivorans*, only one other cytoplasmic protein has been reported to contain this leader sequence, namely the dimethylsulfoxide reductase of *Escherichia coli*. This latter protein contains a small hydrophobic subunit, the role of which is to prevent the enzyme from being exported into the periplasm [60]. It is feasible that the small hydrophobic 'subunit', PceB, of the reductive PCE dehalogenase of *Dehalospirillum multivorans* fulfils a similar role in this enzyme.

With the exception of the leader sequence and the consensus sequences for the binding of the Fe/S clusters, no similarities between the *pceA* and *pceB* and any other known proteins in the gene databases were found. In addition, the N-terminus of the processed, as well as of the unprocessed protein did not exhibit any similarities to the N-termini of the PCE reductive dehalogenases of *Dehalobacter restrictus* and strain PCE-S.

Two membrane-bound reductive dehalogenases have been purified via gel electrophoresis from a mixed enrichment culture containing *Dehalococcoides ethenogenes* [61]. The PCE reductive dehalogenase (51 kDa) mediates PCE reduction to TCE, and the TCE reductive dehalogenase (61 kDa) TCE reduction to ethene. Both enzymes probably contain a corrinoid, as indicated by light-reversible inhibition by alkyl halides. Cyanide and sulfite inhibit the activity of both enzymes.

#### 4. Reaction mechanism of corrinoid-dependent reductive dehalogenases

Based on the finding that low-potential electron donors ( $< -360$  mV) are required for corrinoid-dependent reductive dechlorination, it can be concluded that an enzyme-bound cob(I)alamin is involved in the reaction. Two reaction mechanisms have been proposed for the reductive dechlorination of PCE: (1) the addition of cob(I)alamin to one of the carbons with subsequent  $\beta$ -elimination of the chlorine substituent [9]; and (2) a one-electron transfer from the cob(I)alamin to PCE followed by an elimination of a chloride anion, yielding a trichloroethyl radical that combines with a H-radical to yield TCE [62]. The first mechanism is based purely on theoretical considerations, but there is experimental evidence for the second [54,62–64]. The PCE reductive dehalogenases of *Dehalospirillum multivorans* and of *Desulfitobacterium* strain PCE-S are also able to enzymatically dechlorinate chlorinated propenes [63,64]. *Trans*-1,3-dichloropropene is dechlorinated to 1-chloropropene. The fact that elimination of the chlorine substituent occurs from the  $sp^3$  hybridized carbon rather than from the  $sp^2$  hybridized C-1 clearly argues against an addition at C-1/elimination at C-2 reaction, since this mechanism would yield vinyl chloride plus methyl chloride as the reaction

products. The dechlorination at C-3 is also contradictory to an addition/elimination at C-2, which would result in the formation of 3-chloropropene. From these findings it may be deduced that the dechlorination at C-3 must be due either to a radical (= one-electron transfer) mechanism or to a substitution reaction.

Indirect evidence for a one-electron transfer is available from EPR data of the purified PCE reductive dehalogenase of *Dehalobacter restrictus*, despite the fact that a radical signal indicating the formation of a radical intermediate was not observed [54]. Further studies will be required to elucidate the mechanism of this unusual reaction. A tentative scheme for a radical mechanism possibly involved in reductive dechlorination is outlined in Fig. 3. From all experimental data available, it is obvious that the cobalamin undergoes a change in its redox state during the catalytic cycle of the reaction. Hence, reductive dechlorination represents a new type of corrinoid-dependent reaction.

To date, only a small amount of information is available about the reductive dehalogenases which convert halogenated aromatic compounds as substrates. The finding that at least some of the reductive dehalogenases dechlorinating aryl halides also are corrinoid-dependent indicates that the presence of a corrinoid as a prosthetic group is a common

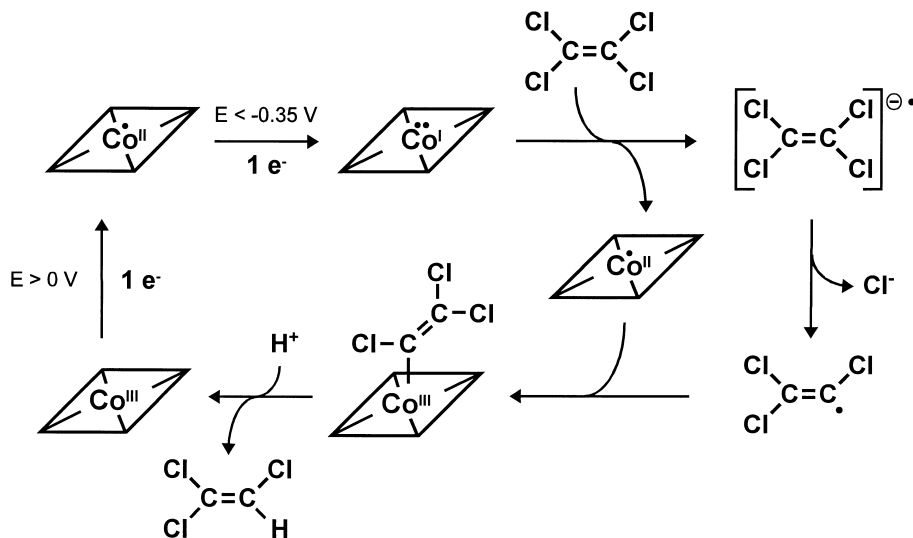


Fig. 3. Tentative scheme of the reductive dechlorination reaction mechanism involving a one-electron transfer (radical mechanism).  $\text{Co}^{\text{I-III}}$  within the rhombus represents the corrinoid with cobalt in the respective oxidation state.

feature of reductive dehalogenases. Aryl halides are also abiotically converted by cobalamins [3]. So far, nothing is known about the mechanism of 3-chlorobenzoate dechlorination mediated by the heme-containing 3-chlorobenzoate reductive dehalogenase. Although hemes have been reported to mediate the abiotic reductive dechlorination of alkyl halides [3], heme-dependent, enzyme-mediated aryl halide dechlorination of aromatic compounds must also be considered as a new type of biochemical reaction.

### 5. Energy conservation via dehalorespiration

The coupling of hydrogen and formate oxidation to reductive dechlorination of chlorinated compounds implies energy conservation via electron transport phosphorylation. The first dissimilatory dechlorinating bacterium, *Desulfomonile tiedjei*, was isolated and shown to utilize pyruvate as an electron donor; respiratory growth was not unambiguously proven [13]. After extensive characterization and testing of different media, *Desulfomonile tiedjei* was successfully cultivated with hydrogen and formate as electron donors, thus providing the first evidence for growth via dehalorespiration [16,65]. Experiments with uncouplers, protonophores, and oxidant pulses in cell suspensions of *Desulfomonile tiedjei* further indicated chemiosmotic coupling of dechlorination and ATP synthesis [17]. Many of the bacteria listed in Table 1 utilize hydrogen and formate as electron donors, indicating the dissimilatory character of the catalyzed dechlorination reaction.

Electron transfer phosphorylation requires an association between the proteins involved and the cytoplasmic membrane. This was the case for the majority of the reductive dehalogenases described so far, with the exception of the PCE reductive dehalogenase of *Dehalospirillum multivorans*, which was always recovered in the soluble fraction (Table 2). It is thought, however, that this enzyme may well be anchored to the membrane by a hydrophobic protein PceB, for which the gene *pceB* has been located in the same operon as the gene *pceA* that encoded the soluble PCE reductive dehalogenase [56]. The localization and arrangement of the two major enzymes involved in the hydrogen-dependent dehalorespiration, the hydrogenase and the reductive dehaloge-

nase, has been investigated for three PCE-dechlorinating bacteria [6,34,38,53]. In all cases, the hydrogenase was membrane-associated and faced the outside of the cytoplasmic membrane. The PCE reductive dehalogenases were all found to face the inside of the cytoplasmic membrane.

Based on this arrangement of the enzymes involved, and considerations of the presence of menaquinone and cytochrome *b*, a tentative model for the respiration can be proposed (Fig. 4). This model is very similar to the respiratory chain proposed for fumarate respiration in *Wolinella succinogenes* [66]. In *Dehalobacter restrictus*, it was found that cytochrome *b* can be reduced by the hydrogenase (Schu-

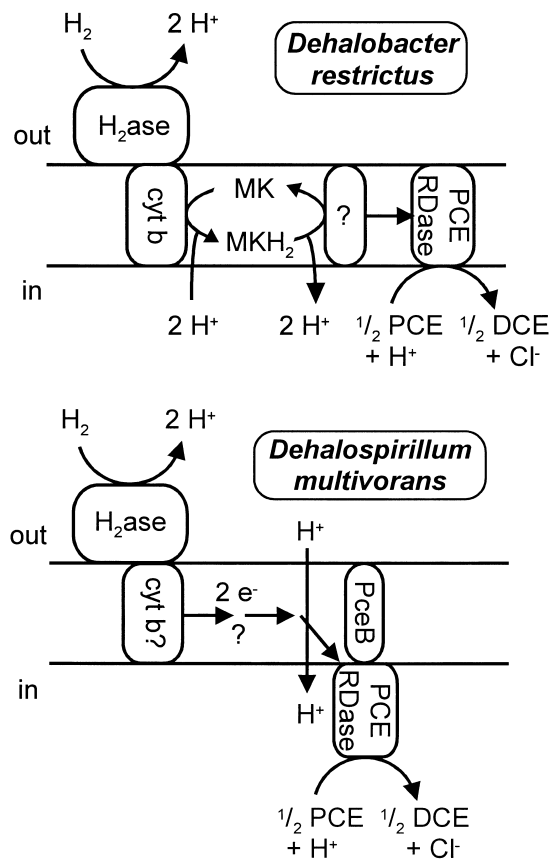


Fig. 4. Tentative scheme of dehalorespiration in *Dehalobacter restrictus* and *Dehalospirillum multivorans*. MK( $\text{H}_2$ ), menaquinone in the oxidized or reduced state; *cyt b*, cytochrome *b*; PCE RDase, PCE reductive dehalogenase; PceB, membrane anchor protein.

macher and Holliger, unpublished). Redox difference spectra of membrane fractions, and experiments with the menaquinone analog 2,3-dimethyl-1,4-naphthoquinone (DMN) and the inhibitor 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide provided evidence for the involvement of menaquinone in the electron transfer [34]. The involvement of a further component in the transfer of electrons from menaquinone to the PCE reductive dehalogenase is proposed based on experiments with reduced DMN. DMNH<sub>2</sub> was observed to act as an electron donor for the PCE reductive dehalogenase in membrane fractions, but not in membrane extracts and not for the purified enzyme (Schumacher and Holliger, unpublished). This indicated that the respiration chain had to be intact and that menaquinone was therefore not the direct electron donor for the PCE reductive dehalogenase.

Without the involvement of a proton pump during the transfer of the electrons from the hydrogenase to the reductive dehalogenase, a proton motive force with a H<sup>+</sup>/e<sup>-</sup> ratio of 1.0 can be established. Oxidant pulse experiments with cell suspensions of *Dehalobacter restrictus* generated an extrapolated H<sup>+</sup>/e<sup>-</sup> ratio of 1.25 ± 0.2, indicating that a proton pump is involved [34]. Menaquinone that could possibly function as proton translocating coenzyme during electron transfer was shown to take up and release the protons on the cytoplasmic side of the membrane during the redox reaction [34]. Hence, menaquinone does not function as a proton translocating compound in *Dehalobacter restrictus*. For *Dehalospirillum multivorans*, an involvement of menaquinone in electron transfer is doubtful [38].

The free energy ( $\Delta G^{\circ}$ ) available from PCE dechlorination to DCE with hydrogen as the electron donor is 189 kJ per mol H<sub>2</sub>. This amount of energy could theoretically yield approximately 2.5 ATP, assuming an energy requirement ( $\Delta G'$ ) of 70 kJ per mol for the formation of 1 ATP from 1 ADP and 1 P<sub>i</sub> [67]. The proposed respiratory chain generates a proton gradient of 2 H<sup>+</sup> per mol H<sub>2</sub>, which corresponds to two-thirds of an ATP, taking into account that three protons cross the membrane per ATP formed [67]. The low cell yields found for different PCE dechlorinating bacteria [32,35,42] indicate that not even 1 mol ATP is gained per chloride removed, which fits in nicely with the proposed respiration mechanism. In addition, evidence of a reversed elec-

tron transport during dechlorination, which would consume part of the proton gradient formed, was obtained for *Dehalospirillum multivorans* [38]. This could explain the low ATP and biomass yields. However, no indications for a reversed electron transport have been found in *Dehalobacter restrictus*, as dechlorination still occurred in the presence of the protonophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP) [34].

## 6. Conclusions and outlook

In the past decades, the chemical industry has developed a large variety of different chlorinated compounds to be used as intermediates for chemical synthesis, as cooling agents, solvents, pesticides, and many other applications. These chlorinated compounds can be reductively dechlorinated by anaerobic bacteria. The diversity of reductively dechlorinating bacteria is large, almost equaling the diversity of chlorinated compounds synthesized. However, many new genera and species of dechlorinating bacteria remain undiscovered. The dechlorinating organisms known at present belong, phylogenetically, to different groups of bacteria and range in their physiology from facultative anaerobes, nitrate, iron, and sulfoxo anion reducers, fermentative bacteria, and methanogens to strict reducers of chlorinated compounds. Some, such as acetogens and methanogens, reductively dechlorinate the chlorinated compounds in a co-metabolic reaction, while others utilize the chlorinated compounds as electron acceptors in their energy metabolism. For some of the latter organisms, it has been unambiguously proven that they dechlorinate the chlorinated compound in a process known as dehalorespiration. Dehalorespiration has best been characterized with PCE as the electron acceptor. Rather simple respiratory chains seem to be involved, which use the free energy that could be gained from the exergonic dechlorination reaction quite inefficiently. All of the reductive dehalogenases of PCE as well as aromatic compounds studied so far are membrane-associated enzymes, most of which contain a corrinoid, and possibly iron-sulfur clusters, as cofactors. The catalysis of dechlorination reactions that involves electron transfer is a new type of corrinoid-dependent reaction, but can also be cat-

alyzed by reductases not containing a corrinoid cofactor. Although there is some experimental evidence in support of a single-electron transfer in the dechlorination of PCE, additional research is required to fully understand the reaction mechanism. So far, nothing is known about the respiratory chain and reaction mechanisms involved in the dehalogenation of aryl halides. The recent advances in investigating dissimilatory dehalogenation reactions have opened up a field that is of great interest and importance for fundamental as well as applied research. The big challenge now is to transfer the knowledge gained on the bacteria and biochemistry involved to applied situations, in order to realize the goal of being able to remediate polluted sites.

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