REDUCTIVE DEHALOGENATION BY ANAEROBIC BACTERIA

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Reductive Dehalogenation by Anaerobic Bacteria

Proefschrift
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Dankwoord

Een proefschrift is net een puzzel. Het bestaat uit een groot aantal deeltjes. Ook al draagt de promovendus de meeste deeltjes bij, dan is er verder een groot aantal mensen die één of meer deeltjes eraan toevoegen om het één geheel te laten worden. Als die mensen er niet geweest waren, dan zouden er stukjes van de puzzel ontbreken en dat zou natuurlijk geen gezicht zijn. Al deze mensen wil ik hierbij heel hartelijk bedanken, voor hun praktische bijdragen, voor hun adviezen, of voor een beetje oppeppen na een mislukt experiment.

Drie mensen wil ik speciaal bedanken. Alex, naast waardevolle discussies hebben je enthousiasme, je vertrouwen, en je vermogen het uiterste uit mij te halen veel ertoe bijgedragen dat het proefschrift is geworden wat het is. Hartelijk dank. Jou, Fons, wil ik bedanken voor al de leuke, leerrijke gesprekken en al de steun die je me hebt gegeven, en jou, Gosse, voor je mentale steun en je activiteiten in de achtergrond die mij veel administratieve rompslomp hebben bespaard.

STELLINGEN

1. Het weglaten van de eerder gevonden FAD-afhankelijkheid uit het model van de reductieve activering van het enzym methyl-coenzym M reductase draagt niet bij tot een beter begrijpen van dit ingewikkelde proces.

   Nagle, D. P., and R. S. Wolfe. 1983. Proc. Natl. Acad. Sci. USA **80**:2151-2155.
   Rouvière, P. E., and R. S. Wolfe. 1989. J. Bateriol. **171**:4556-4562.

2. Het onderzoek naar "interspecies" waterstof- of formiaatoverdracht bij synthrofe methanogene omzettingen van organische verbindingen in gedefinieerde systemen wordt bemoeilijkt door het feit dat er geen methanogene bacterie bekend is die alleen formiaat maar geen waterstof gebruikt.

3. Ondanks alle nijvere pogingen om door middel van recombinant DNA-technieken een "super"-micro-organisme te construeren dat een groot aantal verschillende toxische verbindingen tegelijk moet kunnen afbreken, kan men ook micro-organismen uit de natuurlijke omgeving isoleren die hetzelfde (of meer) kunnen.

   Rojo, F. ***et al.*** 1987. Science **238**:1395-1398.
   Dwyer, D. F., F. Rojo, and K. N. Timmis. 1988. *In* M. Sussmann *et al.* (eds.), The release of genetically engineered microorganisms, p. 77-88. Academic Press Inc., London.

4. Doordat het onderzoek naar de biologische beschikbaarheid van milieugevaarlijke organische verbindingen voornamelijk in moeilijk definieerbare systemen wordt uitgevoerd, worden er nauwelijks nieuwe inzichten verkregen omtrent de oplossing van dit probleem.

5. Het voorstel om op grote schaal ijzer in de zee te gooien om de algengroei te bevorderen en zo het broeikaseffect tegen te gaan door grote hoeveelheden kooldioxyde vast te leggen in de algenbiomassa, laat zien dat de mens nog steeds bereid is ecosystemen te manipuleren zonder de gevolgen ervan te kunnen overzien.

6. Het gebruik van gentechnologie en biotechnologie als synoniemen heeft tot gevolg dat het publiek al bij voorbaat een negatieve houding heeft tegenover biotechnologische projecten die niets met genetische manipulatie te maken hebben.

7. Projectvoorstellen schrijven is één zaak, projecten begeleiden een tweede.

8. Het opleiden van afgestudeerden tot zelfstandig onderzoeker en ze vervolgens managementtaken te laten verrichten is een verspilling van talent en moeite.

9. Het opvoeren van de vuilverbrandingscapaciteit in Nederland als maatregel om de immense afvalberg tegen te gaan, laat zien dat men in het algemeen nog steeds in één richting denkt en niet in termen van kringlopen.

10. De krampachtige pogingen om Europa een eenheid te laten worden staan in schril contrast met het overal opkomende nationalisme in de wereld en zullen waarschijnlijk op lange termijn weinig kans van slagen hebben.

Stellingen behorende bij het proefschrift "Reductive dehalogenation by anaerobic bacteria"

Christof Holliger Wageningen, 4 maart 1992

für Röxler, Lea und Daniela

für Mami und Dädä

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Chapter 2 has previously been published in "Biodegradation". The Chapters 3 and 4 have been submitted to "Journal of Bacteriology", Chapter 5 to "Applied and Environmental Microbiology". Part of Chapter 6 has been submitted to "Science".

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Halogenated compounds are generally regarded to be xenobiotics. These are man-made compounds which are foreign to the living world. However, the occurrence of more then 200 halogenated organic compounds produced by bacteria, algae, and sponges [90,252] leaves some doubts about the xenobiotic character of many of these compounds. Halogenated compounds of natural origin range from simple halogenated methanes to very complex molecules such as halogenated chamigrenes. These natural halogenated products appear to be readily degraded by microorganisms [90]. As a consequence they may disappear quite rapidly and can often only be detected in the vicinity of the producing organisms [90].

Large scale industrial production of synthetic halogenated compounds started about fifty years ago. They are used as solvents, intermediates, preservatives, or pesticides, to name a few examples of their widespread industrial and agricultural application. The amount of the manufactured halogenated compounds which will enter the environment depends on their principal use [208]. Halogenated pesticides such as DDT, lindane, or ethylene dibromide are by definition directly applied in natural habitats and therefore, production figures also represent the inputs into the environment. Compounds such as methylene chloride, trichloroethene, tetrachloroethene, and methyl chloroform are principally used as solvents in metal and electronic industries and dry cleaning operations. They are discharged via wastewater, or lost from storage tanks or through spills and evaporation during handling. As a consequence solvents can also be expected to enter the environment in large quantities. In case the chlorinated compound is used as an intermediate for chemical synthesis, as e.g. vinyl chloride for the production of polyvinyl chloride (PVC), the loss to the environment is only a few percent. Pearson [208] estimated that about 100% of the solvents tetra- and trichloroethene are discharged into the environment and only 2 and 10% of the intermediates chloroethene (vinyl chloride) and 1,2-dichloroethane are lost, respectively, during manufacturing processes.

Halogenated compounds are frequently detected in environments such as soils, sediments, surface and subsurface waters, and the atmosphere. This indicates that halogenated compounds persiste (bio)degradation. This persistence of synthetic organohalogen compounds and their proven or suspected harmfulness to living organisms has greatly stimulated research regarding the fate of these compounds. The fate of halogenated compounds is largely governed by transport and transformation processes. They determine whether a xenobiotic compound rapidly disappears from the environment or whether it becomes an ubiquitous pollutant which may significantly alter the quality of whole ecosystems. Therefore, these processes have obtained and still do obtain wide attention in research.

The mobility and distribution of halogenated compounds in air, water, and soil depends on their physico-chemical properties such as volatility and hydrophobicity. Halocompounds can be transported as gaseous molecules, solubilized in water, or bound to particles. The atmosphere appears to be the major "sink" for many volatile halogenated compounds. They can evaporate from production and application sites or from surface waters after their discharge [208]. Field measurements indicated that tetrachloroethene and 1,4-dichlorobenzene were predominantly eliminated from Lake Zürich, Switzerland, by evaporation [244]. Low molecular weight halogenated hydrocarbons can stay in the atmosphere and even enter the stratosphere. The effect of fluorocarbons on the ozone layer demonstrates the severe consequences of the entrance of such compounds into the atmosphere. Compounds which are regarded to be "semi-volatile" such as polychlorinated biphenyls, dibenzodioxins, and dibenzofurans were also found to be conveyed through the atmosphere [77,107,145]. Such semi-volatile compounds can be transported in the atmosphere either via the vapour phase or as particle-bound molecules [77]. Halocompounds in the atmosphere can find their way into soils or surface waters by dry or wet

deposition. The movement of organohalogen compounds in soils is very complex, especially in the water-unsaturated zones. In such zones the movement is controlled by a mixture of diffusion and mass flow processes in the gas and water phase [114,163]. The rate of these processes is influenced by sorption interactions with the heterogeneous organic and inorganic solid matrix of soils. Many halogenated compounds can be considered to be rather hydrophobic and the organic carbon fraction of the solid matrix plays an important role in hydrophobic sorption processes [114]. Adsorbed halogenated compounds can be immobilized by irreversible incorporation into soil organic matter during humification, the so called bound residue formation [23]. These polymerizations are catalyzed by enzymes (e.g. oxidoreductases), sesquioxides, or clay minerals, or the halogenated compounds react with stabilized radicals present in humic materials [23,251,294]. Downward movement of a halogenated compound through the soil profile primarily depends on infiltration of water, e.g. during and after rain falls [163]. This transport may occur in a solubilized or particle-bound form [163,181] and may lead to groundwater contamination. Besides by perculation of rain water through polluted soils, groundwater may also become contaminated with halogenated compounds via infiltration of river or lake waters. In water saturated zones the rate of travel of a compound relative to the water is strongly influences by sorption [182,242]. The mobility of a halogenated compound in aquifers with an organic-carbon content >0.1% can be estimated using the octanol/water partition coefficient (K_{ow}) [182,242]. Sorption to the inorganic fraction of the solid matrix becomes predominant with organic-carbon contents <0.001% [182]. These interactions cannot be estimated with a single constant determined in the laboratory (as e.g. K_{ow}) and can be several times that predicted based solely on the K_{ow} of the compounds and the organic carbon content of the solid matrix [174,215]. However, the retardation of the movement relative to water appeared to be quite small in such aquifers since studies with sorbents poor in organic carbon showed that some halogenated compounds were quite mobile in such media [242]. Sorption of halogenated compounds does not always lead to significant retardation or immobilization. Halogenated compounds sorbed to mobile colloids may enhance the rate of movement in soils and aquifers [181]. Colloid associated transport may play an important role in the distribution of pollutants.

Besides by transport, the fate of a halogenated compound is also controlled by degradation processes. The persistence and accumulation of synthetic halogenated chemicals in certain environmental compartments are an indication of their recalcitrance to (bio)degradation. A compound can be defined as recalcitrant in a particular environment if it maintains its identity in that environment for more than an arbitrary length of time [103]. In systems with rather undefined residence times such as soils and sediments of rivers, lakes, and oceans, a compound is considered to be recalcitrant if it resists (bio)degradation for months or even years. A molecule can be recalcitrant (i) due to its inherent resistance to degradation by virtue of its chemical structure or (ii) due to adverse environmental factors that prevent a feasible degradation [123]. Environmental factors comprise physical conditions (temperature, water potential, accessibility of a compound, etc.), chemical conditions (pH, redox, concentration of the compound, additional substrates, synergistic or antagonistic effects of other molecules, etc.), and biological conditions (presence of the right organisms or genetic information, sufficient time for adaptation, etc.). In case of (ii) recalcitrance is not a given characteristic of a certain compound but is the result of the state of the environment. Slight changes of certain parameters may alter the environment such that a persistent molecule could become readily degradable. Therefore, a compound should only be termed recalcitrant or persistent in connection with certain environmental conditions at which no (bio)degradation could be observed.

Transformations of organohalogen compounds can lead to the formation of harmless compounds but also to products which can cause an even bigger threat to living organisms than the parent compounds. Microorganisms using a halogenated compound as sole energy and carbon source (e.g. 1,4-dichlorobenzene [239]) or cooxidizing a halocompound (e.g. chloroethene oxidation by methanotrophs in soil [301]) transform the substrate into carbon dioxide, water, and halogen ions. Chemical or photochemical oxidation also leads to the production of anorganic products only [104,203,311]. The formation of ethane and ethene from halogenated C2 aliphatic hydrocarbons under anoxic conditions is another example where an environmentally acceptable product is formed [19,72,92]. Vinyl chloride production from tetrachloroethene by microbial reductive dechlorination [92,207,284] and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin from octachlorodibenzo-*p*-dioxin formation by photoreduction on soil [187] are two example where transformation led to the production of a more hazardous chemical than the parent compound.

Halogenated compounds are transformed by substitution, dehydrodehalogenation, oxidation, or reduction reactions [287]. Many of these reactions can be both abiotic and biotic. The first two reactions do not change the oxidation state of the reacting molecule and thus do not need external electron donors and acceptors. In a ***substitution*** reaction the halogen is replaced by a nucleophile. The most abundant nucleophile in nature is the hydroxyl ion OH^-. However, many halogenated compounds hydrolyse very slowly with half-lives of years [135,287]. The nucleophile bisulfide ion HS^- may be of importance in anaerobic environments [13,243]. The half-life of 1,2-dichloroethane in phosphate buffer at pH 7 was 37 years at 25°C [13]. It decreased to 6 years in the presence of 1 mM sodium sulfide [13]. Hydrolytic and thiolytic dehalogenases were shown to be involved in an overall oxidative metabolism by aerobic bacteria growing on halogenated compounds as sole carbon and energy source [130,132,262]. *Xanthobacter autotrophicus* GJ10 dechlorinates 1,2-dichloroethane first by a haloalkane dehalogenase to chloroethanol [143]. This product is oxidized to chloroacetic acid by pyrroloquinoline quinone (PQQ) and NAD- coupled reactions [131] and dechlorinated by a haloalkanoic acid dehalogenase to glycolic acid, a normal intermediate in bacterial metabolism [132]. A glutathione transferase converted dichloromethane to formaldehyde and hydrochloric acid *via* an unstable S-chloromethyl-gluthathion in *Hyphomicrobium* sp. strain DM2, an obligate methylotroph growing on this compound [149,262]. An overall substitutive transformation of tetrachloromethane to carbon dioxide catalyzed by several anaerobic bacteria [73] will be discussed in the following sections. ***Dehydrodehalogenation*** reactions consist of a removal of a halogen from one carbon atom and a concomitant (E2) or subsequent (E1) removal of a hydrogen atom from an adjacent carbon resulting in the formation of a double-bond. This reaction is documented for several compounds and seems in most cases to be a spontaneous abiotic process [47,173,221,287]. One example is the formation of 1,1-dichloroethene from 1,1,1-trichloroethane which was shown to occur in groundwater at 20°C with a rate of 0.04 yr^{-1} [285]. There are reports about a dehydrodechlorinase in DDT-resistant house flies [167,168]. For microorganisms, however, such an enzyme activity has not yet been demonstrated [159].

In contrast to substitutions and dehydrodehalogenations, oxidations and reductions require either external electron acceptors or external electron donors. ***Oxidation*** of halogenated compounds may occur by incorporation of oxygen into a carbon-hydrogen bond, by oxidation of a carbon-halogen bond, or by oxidation of a carbon-carbon double bond *via* epoxidation [287]. Examples of abiotic oxidative reactions are photooxidations [203,310] and oxidations by hydrogen peroxide, ozone, or hydroxyl radicals [245,310]. Tetrachloroethene was shown to be mineralized to carbon dioxide and hydrochloric acid by photochemical [203] as well as chemical oxidation [104]. Mono- and dioxygenases are involved in metabolic as well as cometabolic

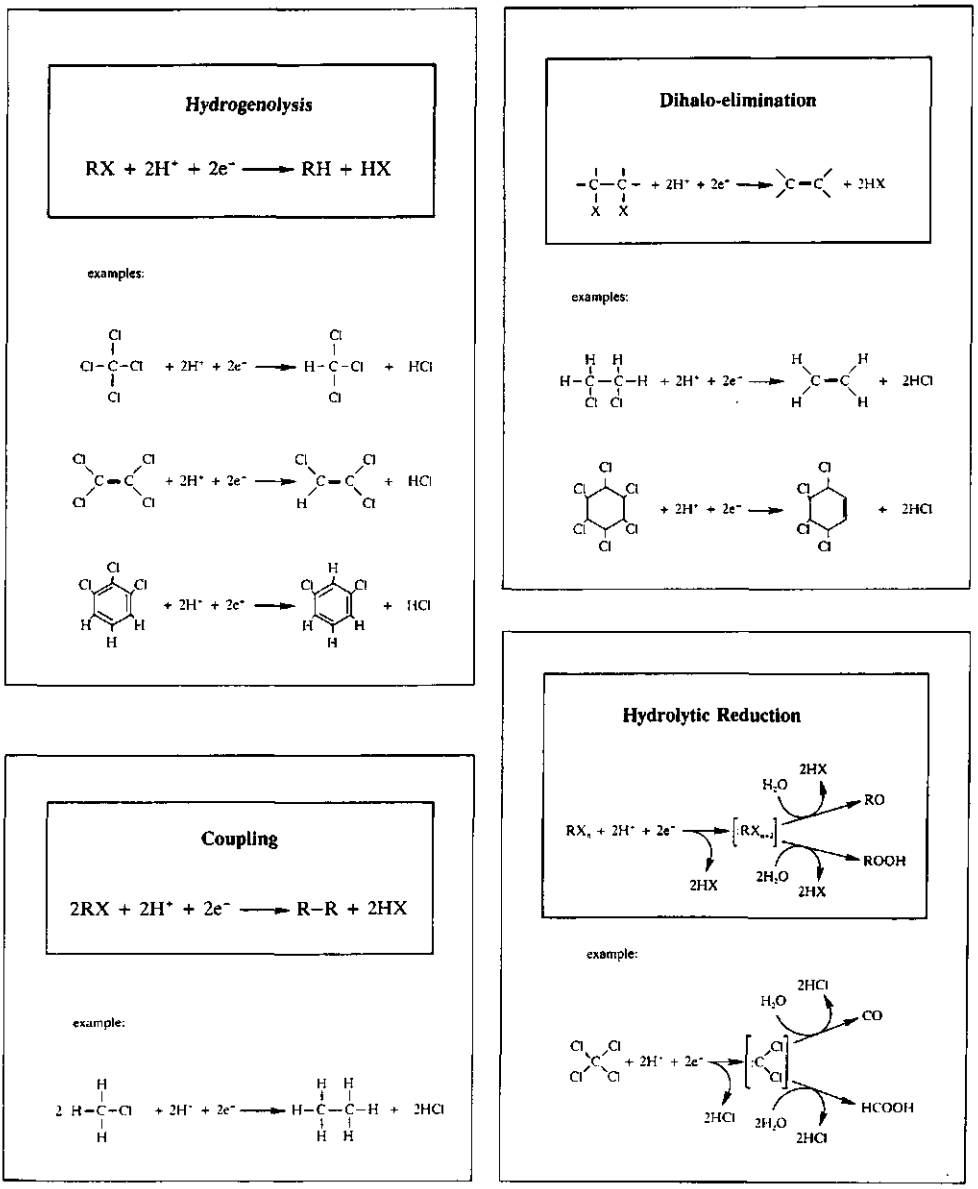

FIGURE 1.1. Reductive dehalogenation reaction types.

conversions of halogenated compounds by micro-organisms, e.g. the ring-fission of halogenated aromatics [42,46,220,274] and the cooxidation of trichloroethene by mixed and pure cultures of methanotrophs and other bacteria [83,169,197,282,313]. ***Reductive transformations*** of organo-halogen compounds are widespread and may be abiotic as well as biotic processes. Since the main objective of this study was to elucidate the physiological meaning and the biochemistry of reductive transformations of halogenated compounds catalyzed by anaerobic bacteria, these reactions will be discussed in more detail in the following sections.

1.1. Reductive dehalogenation reaction types

There are four reaction types where reductive processes are involved (Fig. 1.1). The first reaction, the hydrogenolysis, replaces a halogen substituent by a hydrogen. Hydrogenolysis is the most often encountered reductive dehalogenation reaction and is found with aliphatic and aromatic compounds. The second type is a dihalo-elimination. In this reaction two halogens are eliminated from the target compound at the same time and a double-bond is formed. It has been found with cyclic and non-cyclic aliphatic halocompounds and is restricted to compounds with more than one carbon atom. The third type is a coupling and can occur when free radicals are involved. Products resulting from a coupling reaction were only found as side-products. The fourth type is a hydrolytic reduction. The products of this reaction type are oxygenated and are formed *via* a two electron reduction of a polyhalogenated carbon to a carbenoid followed by hydrolysis. This reaction could explain the formation of CO_2 from tetrachloromethane or acetate from 1,1,1-trichloroethane [48].

1.2. Anaerobic degradation of halogenated compounds *in situ* and in microcosms from specific environments

Many investigations which were undertaken to understand transformation phenomena of halogenated compounds were done by analyzing *in situ* concentration changes and/or by following certain compounds either already present or added in samples from various environments, the so-called microcosm experiment. Such studies document the fate of a particular chemical in a particular environment. The complexity of natural samples does not allow to develop a precise understanding of the catalysts involved in the degradation. However, they provide a catalog of degradation pathways and also provide evidence about the nature of the reactions involved [173].

A great variety of halogenated compounds were and still are used as pesticides. They range from simple C2 halogenated hydrocarbons such as dibromoethane, a nematocide, to very complex molecules such as heptachlor, an alicyclic compound. Studies of the fate of halogenated compounds under anaerobic conditions were initially carried out with pesticides [3,108,121,136]. More recently, intermediates for synthetic processes and solvents were included. It would go beyond the scope of this chapter to discuss the anaerobic degradation of all halogenated compounds. In the following specific emphasis will be given to relatively simple halogenated aromatic (e.g. benzoates, phenols, benzenes) and aliphatic (e.g. methanes, ethanes, ethenes, cyclohexane) compounds. For an outstanding review about the anaerobic degradation of pesticides in soils and groundwater the reader is referred to the paper of Kuhn and Suflita [159].

1.2.1. Halogenated aromatic compounds.

Reductive dehalogenation is reported for many different aromatic compounds. The results obtained with chlorobenzoates, chlorophenols, and chlorobenzenes are summarized in Table 1.1. Reductive dehalogenation of chloroanilins [160], chlorocatechols [4], 2,4-dichloro- and 2,4,5-trichlorophenoxyacetic acid [102,184], and polychlorinated biphenyls [17,33,34,198,219,281] has also been demonstrated. Abiotic reductive dehalogenation was found in studies where the photolysis of chlorophenylacetic acids [51], pentachlorophenol [304], octachlorodibenzo-*p*-dioxin [187], or polybrominated and polychlorinated biphenyls [36,152,222,225] was investigated. Evidence was obtained in field studies that direct photoreactions can sometimes be the dominant degradation process in environments exposed to light such as surface waters and top layers of soils [311].

Microbial reductive dehalogenation of aryl halides in the laboratory was first reported by Suflita et al. [266]. A methanogenic bacterial consortium was found to be able to reductively dehalogenate several halogenated benzoates (Table 1.1). The halobenzoates were solely dehalogenated at the *meta* position by this consortium, e.g. 2,3-dichlorobenzoate was converted to 2-chlorobenzoate which was not degraded further. The dechlorination of 2-chlorobenzoate [99,100] and of 3,4-dichlorobenzoate to 3-chlorobenzoate by other enrichment cultures [101] demonstrated that bacteria exist which are able to dechlorinate chlorobenzoates at the *ortho* and

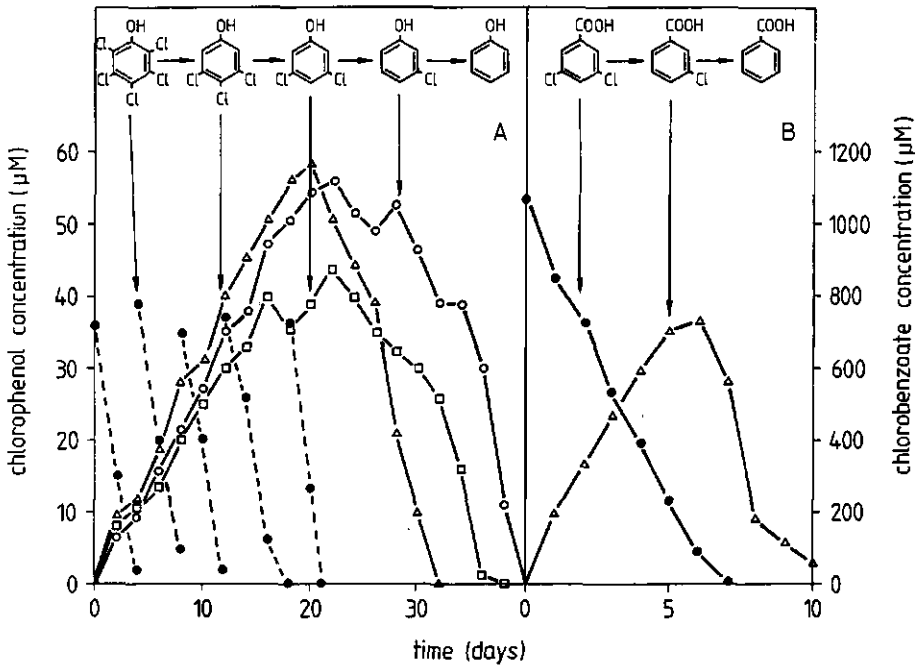

FIGURE 1.2. A. Pentachlorophenol degradation by a mixture of 2-, 3- and 4-chlorophenol acclimated sludges (redrawn from [185]). B. Pattern of dechlorination of 3,5-dichlorobenzoate exhibited by a methanogenic consortium (redrawn from [265]).

TABLE 1.1. Anaerobic biodegradation of chlorinated aromatic compounds in microcosms of specific environments and by enrichment cultures under different redox-conditions

Chlorinated aromatic compound[a]	Redox-condition[b]	Degradation[c]	First intermediary product	End product	Activity tested in:[d]	Reference(s)
Chlorobenzoates						
2-CBe	dn	+	n.d.[e]	n.d.	sed	[99]
	sr	—	—	—	sed	[99]
	me	+	benzoate	CO_2+CH_4	sed	[100]
		—	—	—	sed/ss	[226,266]
3-CBe	dn	+	n.d.	n.d.	sed	[99]
	sr	+	n.d.	n.d.	sed	[99]
		—	—	—	sed/aq	[101]
	me	+	benzoate	CO_2+CH_4	sed/aq/ss	[100,101,226,266]
4-CBe	dn	+	n.d.	n.d.	sed	[99]
	sr	—	—	—	sed/aq	[99,101]
	me	+	n.d.	n.d.	sed	[99]
		—	—	—	sed/aq/ss	[101,226,266]
2,4-, 2,6-DCBe	me	—	—	—	sed/ss	[226,266]
2,5-DCBe	me	+	—	2-CBe	sed/ss	[226,266]
3,4-DCBe	sr	—	—	—	sed/aq	[101]
	me	+	3-CBe	n.d.	sed	[101]
		+	—	4-CBe	sed/aq/ss	[101,226,266]
3,5-DCBe	sr	—	—	—	sed/aq	[101]
	me	+	3-CBe	CO_2+CH_4	sed/aq/ss	[101,226,266]
2,3,6-TCBe	me	+	—	2,6-DCBe	sed/ss	[226,266]
Chlorophenols						
2-CP	dn	+	n.d.	n.d.	sed	[99]
	sr	+	phenol	n.d.	sed	[111]
		—	—	—	sed/aq	[101]
	me	+	phenol	CO_2+CH_4	sed/aq/ss	[29,30,100,101,226]
3-CP	dn	+	n.d.	n.d.	sed	[99]
	sr	+	phenol	n.d.	sed	[111]
		—	—	—	sed/aq	[101]
	me	+	phenol	CO_2+CH_4	sed/aq/ss	[29,30,100,101,226]
4-CP	dn	+	n.d.	n.d.	sed	[99]
	sr	+	phenol	n.d.	sed	[111,150]
		—	—	—	sed/aq	[101]
	me	+	phenol	CO_2+CH_4	sed/aq/ss	[29,30,101,226,312]
2,3-DCP	me	+	2-/3-CP	n.d.	sed/ss	[29,35]
2,5-DCP	sr	—	—	—	sed/aq	[101]
	me	+	3-CP	n.d.	sed/aq/ss	[29,35,101]

TABLE 1.1 continued

Chlorinated aromatic compound	Redox-condition	Degradation	First intermediary product	End product	Activity tested in:	Reference(s)
Chlorophenols (continued)						
2,6-DCP	sr	+	2-CP	n.d.	sed	[111]
	me	+	2-CP	n.d.	sed/ss	[29,35]
3,4-DCP	sr	—	—	—	sed/aq	[101]
	me	+	3-/4-CP	n.d.	sed/aq	[35,101]
3,5-DCP	me	+	3-CP	n.d.	sed	[35]
2,4,5-TCP	sr	—	—	—	sed/aq	[101]
	me	+	2,4-DCP	4-CP	sed/aq/ss	[101,307]
2,4,6-TCP	me	+	4-CP	n.d.	ss	[184]
PCP	me	+	2,3,5,6-TeCP	phenol	sed	[35]
			2,3,4,5-TeCP	4-CP	ss	[307]
			3,4,5-TCP	CO_2+CH_4	ss	[185]
Chlorobenzenes						
CB	dn	—	—	—	ss	[26]
	me	+	n.d.	n.d.	aq	[165,299]
1,2-, 1,3-, 1,4-DCB	dn	—	—	—	sed/ss	[24,26]
	sr	+	—	CB	sed	[24]
	me	+	—	CB	sed	[24]
1,2,3-, 1,3,5-TCB	dn	—	—	—	sed	[24]
	sr	+	1,3-DCB	CB	sed	[24]
	me	+	1,3-DCB	CB	sed	[24]
1,2,4-TCB	dn	—	—	—	sed/ss	[24,26]
	sr	+	1,4-DCB	CB	sed	[24]
	me	+	1,4-DCB	CB	sed	[24]
HCB	me	+	QCB	1,3,5-TCB[f]	sed/aq/ss	[89,166,195]
				1,2,3-TCB[f]	sed/ss	[87,195]

[a] The abbreviations stand for: Be = benzoate; P = phenol or penta; B = benzene; C = chloro; D = di; T = tri, Te = tetra; Q = penta; H = hexa.

[b] dn = denitrification; sr = sulfate-reduction; me = methanogenesis. Electron acceptor present which favored the indicated redox-conditions, however, not always was tested whether the respective process took place.

[c] + = degradation observed; — = no degradation.

[d] sed = sediment; aq = aquifer material; ss = sewage sludge. It is not further specified whether experiments where carried out with slurries, continuous flow columns, or reactors.

[e] n.d. = not determined.

[f] These isomers accumulated in the experiments where HCB dechlorination was found. In some of the studies low amounts of DCB's indicated that also the accumulated TCB's were slowly dechlorinated further [89,87,166,195].

para position. Degradation of the three monochlorobenzoate isomers was also found in the presence of nitrate [99,100]. It is not known whether the initial step under denitrifying conditions was also a reductive dechlorination as it was the case under methanogenic conditions. In the presence of sulfate, chlorobenzoates were not yet found to be degraded [99,101].

The anaerobic degradation of pentachlorophenol was first demonstrated in 1972 by Ide et al. in flooded rice field soil [128]. Tetrachloro-, trichloro-, and dichlorophenol isomers were found as products. Later studies in laboratory microcosms with soils, sediments, or sewage sludge revealed that i) all chlorophenol isomers could be dechlorinated, ii) different microcosms preferentially dechlorinated an *ortho, meta,* or *para* chlorine substituent, and iii) dechlorination occurred under sulfate-reducing and methanogenic conditions but not in the presence of nitrate (Table 1.1). The *ortho* position was the easiest to dechlorinate in some systems [29,101,111,150, 184,185,307,312], the *para* substituted chlorine in another [35]. In all studies where such comparative investigations were done, the *meta* position appeared to be the most difficult to dechlorinate. A mixture of different enrichments acclimated to one of the three monochlorophenol isomers completely dechlorinated and mineralized pentachlorophenol, whereas in the separate enrichments lower chlorinated phenols accumulated as end products [185]. In Figure 1.2A the degradation pattern of pentachlorophenol as found by Mikesell and Boyd [185] is shown as an example for the degradation of a polychlorinated phenol. Characteristic is the accumulation of lower chlorinated congeners prior to their dechlorination. The same pattern was also observed with dichlorobenzoates (Fig. 1.2B). Such a pattern could best be simulated with a sequential Michaelis-Menten model where a competitive inhibition term was inserted [265].

Under methanogenic conditions chlorobenzenes were found to be dechlorinated in soil columns [24], slurries of sediments or aquifer material [166,195], an anaerobic upflow biofilm reactor [87], and batch cultures seeded with sewage sludge [89]. Hexachlorobenzene seemed to be dechlorinated by two different pathways [87,89,166,195]. The first pathway was a sequential dechlorination *via* pentachlorobenzene, 1,2,3,5-tetrachlorobenzene to 1,3,5-trichlorobenzene which was very slowly further dechlorinated to 1,3-dichlorobenzene and finally chlorobenzene [89,166,195]. In the second pathway, 1,2,3-trichlorobenzene accumulated as the major product [87,195]. Minor amounts of 1,2,4-trichlorobenzene were also found in sewage sludge [89]. In the presence of sulfate dichloro- and trichlorobenzenes were also dechlorinated in soil columns. This is the only report on chlorobenzene degradation where another major electron acceptor than carbon dioxide was present [24].

The substrate specificity of different materials and the lack of dechlorinating activity in autoclaved controls indicated that aryl reductive dechlorinations were biologically catalyzed reactions. Chlorobenzoates and chlorophenols served first as electron acceptors and subsequently, if completely dechlorinated to benzoate or phenol as electron and carbon source. Organic compounds such as short chain fatty acids or alcohols stimulated the incomplete dechlorination of 2,4,5-trichlorophenoxyacetic acid [102]. In other cases dechlorination only took place when an organic electron donor was added (e.g. with polychlorinated biphenyls [198], or chloroanilines [160]). The stimulation of the dechlorination by chemically rather stable organic electron donors support the hypothesis that aryl dehalogenations are biologically mediated processes.

TABLE 1.2. Anaerobic degradation of chlorinated aliphatic hydrocarbons in microcosms of specific environments and by enrichment cultures under different redox-conditions

Chlorinated aliphatic hydrocarbon[a]	Redox-condition[b]	Degradation[c]	First intermediary product	End product	Activity tested in:[d]	Reference(s)
CT	dn	+	CF	n.d.[e]	br	[26,27]
	sr	+	CF	n.d.	br	[27]
	me	+	CF	CO_2	br/aq	[25,27,206]
CF	dn	—	—	—	br	[26,27]
	sr	—	—	—	br	[27]
	me	+	n.d.	CO_2	br	[25,27]
1,2-DCA	me	+	n.d.	CO_2	br	[25]
1,1,1-TCA	dn	—	—	—	br	[26,27,147]
	sr	+	1,1-DCA	CA[f]	br/aq	[27,147]
	me	+	1,1-DCA	CO_2	br/aq	[25,27,147,286]
1,1,2,2-TeCA	me	+	—	1,1,2-TCA	br	[25]
HCA	ae[g]	+	—	PCE	aq	[50]
	dn	+	n.d.	n.d.	br	[27]
	sr	+	n.d.	n.d.	br	[27]
	me	+	n.d.	n.d.	br	[27]
1,1-DCE	me	+	VC	n.d.	sed/aq	[15,300]
cis-1,2-DCE	me	+	VC	n.d.	sed/aq	[15,300]
trans-1,2-DCE	me	+	CA + VC	n.d.	sed/aq	[15,300]
TCE	me	+	*cis*+*trans*-1,2-DCE	n.d.	aq	[206,207]
			1,2-DCE[h]	n.d.	soil/aq	[148,300]
PCE	sr	+	TCE	*cis*-1,2-DCE	ss	[7]
	me	+	TCE	CO_2	br	[284]
				ethene	ss	[64,92]
				cis+*trans*-1,2-DCE	aq	[247]
				cis-1,2-DCE	aq/ss	[138,237]
				n.d.	aq	[25,205,206,207]
γ-HCH	me	+	γ-TCH	benzene	soil/ss	[18,273]
α-HCH	dn	—	—	—	soil	[6]
	sr	—	—	—	soil	[6]
	me	+	n.d.	CB + CP's	soil	[6]

[a] The abbreviations stand for: CT = tetrachloromethane; CF = trichloromethane; DCA = dichloroethane; TCA = trichloroethane; CA = chloroethane; TeCA = tetrachloroethane; HCA = hexachloroethane; DCE = dichloroethene; TCE = trichloroethene; PCE = tetrachloroethene; HCH = hexachlorocyclohexane; TCH = tetrachlorocyclohexene; CB = chlorobenzene; CP = chlorophenol.

[b] ae = aerobic; dn = denitrification; sr = sulfate-reduction; me = methanogenesis. Electron acceptor present which favored the indicated redox-conditions, however, not always was tested whether the respective process took place.

[c] + = degradation observed; — = no degradation.

[d] br = biofilm reactor; sed = sediment; aq = aquifer material; ss = sewage sludge. It is not further specified whether experiments where carried out with slurries, continuous flow columns, or reactors.

[e] n.d. = not determined.

[f] Besides CA also acetate and CO_2 were formed [147].

[g] These results of an aerobic study are included because it is an example were reductive dehalogenation, a degradation reaction typical for anaerobic environments, also occurred in the presence of oxygen.

[h] The *cis* and *trans* isomer of 1,2-DCE could not be distinguished in these studies.

1.2.2. Halogenated aliphatic compounds.

Of particular interest is the fate of halogenated methanes, ethanes, and ethenes, since they represent an important class of pollutants in many environments. More than twenty years ago it was shown that the reductive dehalogenation of the soil fumigants ethylene dibromide (1,2-dibromoethene), 1,2-dibromo-3-chloropropane, and 2,3-dibromobutane in soil slurries was a biological process [39]. About a decade later the fate of chloromethanes and chloroethenes in anaerobic environments became a matter of special concern. The reasons were: both groups of compounds are major contaminants, some isomers are proven or suspect carcinogens, and the highly chlorinated congeners were persistent in the presence of oxygen. The results of investigations with microcosms and enrichments from environmental samples under anoxic conditions are summarized in Table 1.2. As in the case of halogenated aromatic compounds, the initial step in the anaerobic degradation of aliphatics was a reductive dehalogenation. Besides hydrogenolysis reactions also dihalo-eliminations were observed. Some halogenated aliphatics such as tetrachloromethane, trichloromethane, 1,2-dichloroethane, 1,1,1-trichloroethane, and tetrachloroethene were mineralized to carbon dioxide. In the case of trichloromethane and 1,2-dichloroethane it is not known whether reductive dechlorination was involved in the degradation to carbon dioxide [25]. 1,1,1-Trichloroethane and tetrachloroethene certainly were reductively dechlorinated prior to mineralization [284,286]. Abiotic as well as biotic processes were involved in anaerobic 1,1,1-trichloroethane degradation [147,286]. The stepwise reductive dechlorination to 1,1-dichloroethane and chloroethane was biologically mediated. The transformation of 1,1,1-trichloroethane by a dehydrodehalogenation to 1,1-dichloroethene was an abiotic process. 1,1-Dichloroethene was subsequently transformed *via* a hydrogenolysis reaction to chloroethene by microorganisms. Tetrachloroethene degradation was investigated in many different laboratories. In some studies, it was dechlorinated to trichloroethene, *cis*-1,2-dichloroethene, and chloroethene [206,207], in others 1,2-dichloroethene was detected, but the isomers could not be identified [148,300]. Besides the *cis*-isomer, also *trans*-1,2-dichloroethene was found to be a major or minor dechlorination product [92,206,207,247]. Total dechlorination of tetrachloroethene to ethene was also observed [64,92], however, more frequently *cis*-1,2-dichloroethene was the end product [7,138,237]. Most experiments were carried out under methanogenic conditions. However, aliphatic halocompounds were also degraded under redox conditions favoring denitrification or sulfate-reduction [7,26,27,147].

The disappearance of γ-hexachlorocyclohexane and the production of five to six chlorides per molecule γ-hexachlorocyclohexane and traces of benzene was the first convincing report of an anaerobic reductive dehalogenation reaction [3]. In later studies with soils and sewage sludge, it was found that the initial transformation step of γ-hexachlorocyclohexane was a dihalo-elimination to γ-tetrachlorocyclohexene [18,273]. A major end product of α-hexachlorocyclohexane degradation in a soil slurry under methanogenic conditions was monochlorobenzene [6]. This was probably a result of another dihalo-elimination to α-dichlorocyclohexadiene followed by a spontaneous dehydrodehalogenation [21]. In other studies benzene was found as dechlorination product of γ-hexachlorocyclohexane [18] or γ-hexachlorocyclohexane was converted to chlorinated benzenes or phenols [159]. The α-hexachlorocyclohexane isomer was not found to be degraded only very slowly in the presence of sulfate or nitrate and the ß-isomer totally resisted anaerobic degradation [6].

The reductive dechlorinations of tetrachloroethene in enrichment cultures depended on the addition of an organic electron donor [7,64,92,237,247]. This observation together with the finding that the dechlorination did not take place in autoclaved controls suggests that alkyl reductive dehalogenations are also biotic processes.

1.3. Reductive dehalogenation by pure cultures of bacteria.

1.3.1. Aryl reductive dehalogenation.

Despite the strong evidence for the involvement of biological processes in the aryl reductive dehalogenation there are only few reports about the catalysis of this type of reactions by bacterial pure cultures. One bacterium, strain DCB-1, has been isolated which was able to dehalogenate *meta*-substituted benzoic acids. After extensive physiological characterization [190,248,258,259] and 16S rRNA sequence analysis, the Gram-negative, non-motile, non-sporeforming large rod with an unusual morphological feature resembling a collar was named *Desulfomonile tiedjei* [61]. This sulfate-reducing bacterium coupled growth on formate to the reductive dechlorination of 3-chlorobenzoate [67,189]. Evidence for a chemosmotic coupling of the reductive dechlorination and ATP synthesis was obtained in experiments with respiratory inhibitors and imposed pH gradients [191]. The inhibition of 3-chlorobenzoate dechlorination by sulfite and thiosulfate was taken as a confirmation of a novel anaerobic respiration process [60]. The enzymatic character of the aryl reductive dechlorination was shown in cell extracts of *D. tiedjei* [59]. Dechlorination by cell extracts depended on the presence of reduced methyl viologen, was membrane associated and inducible. In the 3-chlorobenzoate degrading consortium *D. tiedjei* had a symbiotic relationship with the benzoate oxidizing bacterium (Fig. 1.3). Strain DCB-1 depended on the H_2 produced by the benzoate oxidizing strain whereas the benzoate oxidizing organism depended on the benzoate produced by strain DCB-1 and on a low partial pressure of H_2 maintained by strain DCB-1 and the methanogen. Dechlorination coupled growth on 3-chlorobenzoate as sole substrate was also possible in a defined biculture without the methanogen [68].

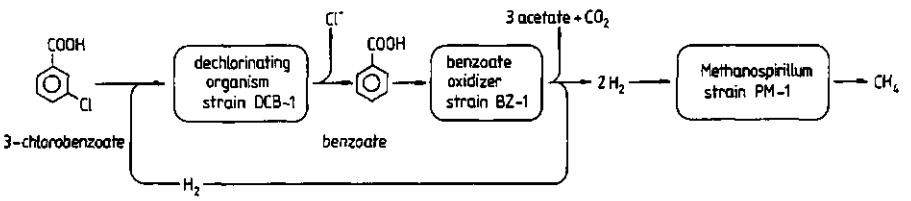

FIGURE 1.3. Hydrogen cycling in a partially circular, 3-chlorobenzoate degrading "food web" (redrawn from [69]).

A *Staphylococcus epidermidis* strain, isolated from intestinal contents of rats, dechlorinated 1,2,4-trichlorobenzene to dichlorobenzenes and chlorobenzene [272]. This reaction occurred only with hydrogen in the gas phase. In incubations with cell-free extracts of *S. epidermidis*, NADPH stimulated the dechlorination of 1,2,4-trichlorobenzene. Only trace amounts of dechlorination products were formed by whole cells, and it is not known how these findings relate to aryl reductive dehalogenation reaction observed in investigations with other environmental samples.

TABLE 1.3. Reductive dehalogenation reactions catalyzed by pure cultures of bacteria

Halogenated compound[a]	Bacteria	Products[a]	Reference(s)
CT	*Methanobacterium thermoautotrophicum*	CF → DCM + CO_2	[72,73]
	Methanosarcina barkeri	CF → DCM	[155]
	Desulfobacterium autotrophicum	CF → DCM + CO_2	[72,73,74]
	Acetobacterium woodii	CF → DCM → CM + CO_2	[73,74]
	Clostridium thermoaceticum	CF → DCM → CM + CO_2	[74]
	Clostridium sp.	CF → DCM + unidentified	[97]
	Escherichia coli	CF	[49]
CF	two *Methanosarcina* sp.	DCM → CM	[186]
Cl_3CNO_2	*Pseudomonas putida* PgG-786	Cl_2CHNO_2 → $ClCH_2NO_2$ → CH_3NO_2	[40]
1,2-DCA	several methanogens	ethene	[19,72]
1,1,1-TCA	*Methanobacterium thermoautotrophicum*	1,1-DCA	[72]
	Desulfobacterium autotrophicum		[72]
	Acetobacterium woodii		[74]
	Clostridium sp.	1,1-DCA + acetate + unidentified	[97]
BA	several methanogens	ethane	[19]
1,2-DBA	several methanogens	ethene	[19]
PCE	several methanogens	TCE	[72,86,88]
	Desulfomonile tiedjei		[88]
	Acetobacterium woodii		[74]
1,2-DBE	several methanogens	acetylene	[19]
γ-HCH	several *Clostridia*	γ-TCH →→ CB or benzene	[3,120,129,175,199]
	several *Bacilli*		[3,129]
	Citrobacter freundii		[129]
	Escherichia coli		[129]
	Enterobacter aerogenes		[129]
	Enterobacter cloacae		[129]
	Serratia marcescens		[129]
	Proteus mirabilis		[129]

[a] CT = tetrachloromethane; CF = trichloromethane; DCM = dichloromethane; CM = chloromethane; DCA = dichloroethane; TCA = trichloroethane; BA = bromoethane; DBA = dibromoethane; PCE = tetrachloroethene; TCE = trichloroethene; DBE = dibromoethene; γ-HCH = γ-hexachlorocyclohexane; γ-TCH = γ-tetrachlorocyclohexene; CB = chlorobenzene.

1.3.2. Alkyl reductive dehalogenation.

In contrast to aryl dehalogenation, there are a number of studies reporting on alkyl reductive dehalogenation by pure cultures of bacteria (Table 1.3). The bacteria involved ranged from strict anaerobic organisms such as methanogens, sulfate-reducers, and clostridia to facultative anaerobes such as *Escherichia coli* or *Pseudomonas putida*. First investigations which showed pure culture catalyzed reductive dechlorinations were carried out with pesticides [3,175]. In most studies, pure cultures were not isolated on their ability to dehalogenate but culture collection strains were screened on dehalogenating activities. The broad variety of bacteria with the property to reductively dehalogenate aliphatic hydrocarbons indicated that alkyl reductive dehalogenation is common for many bacteria. Several enzyme systems have been suggested to be involved in the alkyl dehalogenation reactions. They are protein-bound tetra-pyrrole cofactors (iron(II) porphyrins [40,41], corrinoids [74,73,154,155], or factor F_{430} [155]), flavoprotein-flavin complexes [82,93], and ferredoxins [129]. *Pseudomonas putida* induces high concentrations of the

heme protein cytochrome P-450$_{cam}$ if grown on camphor as energy and carbon source [41]. This protein was found to be involved in the reductive dechlorination of trichloronitromethane [41]. Earlier reports on reductive dehalogenating activity of iron(II) porphyrins [292], heme proteins [293], and reduced liver microsomes [303] already indicated that protein-bound iron(II) porphyrins were the catalysts of the observed dehalogenations by whole cells. Dechlorinations catalyzed by corrinoids or by factor F_{430} in buffer with excess of a reducing agent suggested that these cofactors were the catalysts of the dehalogenations by methanogens, sulfate-reducers, or homoacetogens [154,155]. The fact that only bacteria with the acetyl-CoA pathway where a corrinoid/Fe-S protein is one of the central enzymes had dechlorinating activity [74] supported this hypothesis. The dehalogenation by flavoprotein-flavin complexes was shown with *E. coli* [93] and *Ps. putida* [82]. The finding that only those bacteria that have Fe-S protein dependent fermentative H_2 evolution actively dechlorinated γ-hexachlorocyclohexane provided evidence for ferredoxin catalyzed reductive dehalogenations [129].

The production of carbon dioxide from tetrachloromethane [73,74] and acetate from 1,1,1-trichloroethane [97] are overall substitutive reactions. However, these transformations

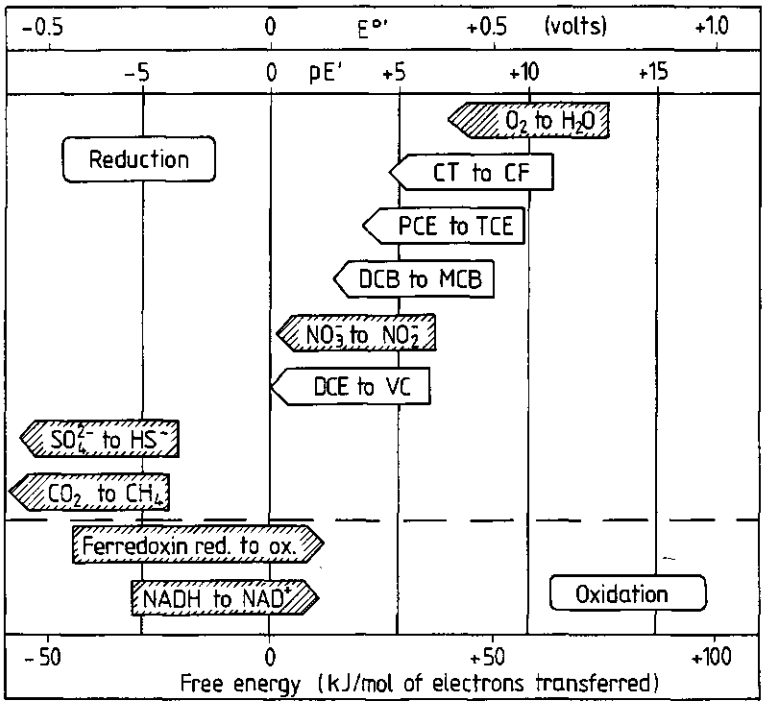

FIGURE 1.4. Half-reaction reduction potentials for reducing and oxidizing agents. Base of arrows align with potential of half-reaction shown in volts. Energy for the combination of two half-reactions can be estimated from the difference between their free energy values as indicated on the bottom axis. CT = tetrachloromethane; CF = trichloromethane; PCE = tetrachloroethene; TCE = trichloroethene, DCE = dichloroethene; VC = chloroethene; DCB = dichlorobenzene; MCB = monochlorobenzene (redrawn from [287]).

TABLE 1.4. Reductive dechlorination reactions catalyzed by hematin, cobalamin, and factor F_{430}

Catalyst	Substrate[a]	Products[a]	Electron donor	Reference(s)
hematin	CT	CF	Ti(III) citrate	[98,146]
	CF	DCM	Na_2S, cysteine	[146]
	Cl_3CNO_2	CH_2NO_2	—[b]	[41]
	1,1,1-TCA	1,1-DCA	Na_2S, cysteine	[146]
	HCA	PCE	—	[292]
	1,2-DBA	ethene	—	[292]
	PCE	TCE → *cis*-1,2-DCE	Ti(III) citrate	[98]
	γ-HCH	γ-TCH →→ CB	DTT	[180]
	HCB	QCB	Ti(III) citrate	[98]
cobalamin	CT	CF → DCM → CM → CH_4 + CO	Ti(III) citrate	[98,154,156,305]
	CFCl3	$CHFCl_2$ + CO + formate	Ti(III) citrate	[156]
	PCE	TCE → *cis*-1,2-DCE (90%) + *trans*-1,2-DCE (5%) + 1,1-DCE (5%) → VC → ethene	Ti(III) citrate	[98]
	γ-HCH	γ-TCH →→ CB	DTT	[180]
	HCB	QCB → 1,2,3,5-TeCB (20%) + 1,2,4,5-TeCB (80%)	Ti(III) citrate	[98]
	PCP	2,3,4,6-TeCP (50%) + 2,3,5,6-TeCP (50%)	Ti(III) citrate	[98]
factor F_{430}	CT	CF → DCM → CM → CH_4	Ti(III) citrate	[155]
	PCE	TCE → *cis*-1,2-DCE (97%) + *trans*-1,2-DCE (3%) → VC → ethene	Ti(III) citrate	[98]

[a] CT = tetrachloromethane; CF = trichloromethane; DCM = dichloromethane; CM = chloromethane; TCA = trichloroethane; DCA = dichloroethane; HCA = hexachloroethane; DBA = dibromoethane; PCE = tetrachloroethene; TCE = trichloroethene; DCE = dichloroethene; VC = chloroethene; γ-HCH = γ-hexachlorocyclohexane; γ-TCH = γ-tetrachlorocyclohexene; HCB = hexachlorobenzene; QCB =pentachlorobenzene; TeCB = tetrachlorobenzene; CB = chlorobenzene; PCP = pentachlorophenol; TeCP = tetrachlorophenol.

[b] In many studies with hematin, the iron(III) was reduced quantitatively to iron(II) and subsequently the oxidation by halogenated hydrocarbons was followed spectrophotometrically. In the cases where an electron donor is indicated, the reactions were carried out with excess of an electron donor and products were analyzed as prove for reductive dechlorination reactions. DTT = dithiothreitol.

could involve a two-electron reduction to a carbenoid which would be hydrolysed to form carbon monoxide and acetaldehyde [48]. These products could than be oxidized to carbon dioxide and acetate.

Apparently, all the alkyl dehalogenating strains cometabolically transformed the halocompounds, thus do not benefit from the exergonic reaction which they catalyse. Halogenated aliphatic compounds are quite strong oxidants as shown in Figure 1.4. The only indications that alkyl dehalogenating bacteria could benefit from the dechlorination reaction were obtained in studies with *Clostridium rectum* [200,201]. Cell suspensions of this strain formed about equal amounts of ATP if incubated with pyruvate as electron donor together with proline or γ-hexachlorocyclohexene as electron acceptor [200]. These results suggested a strong link between the Stickland reaction and the dechlorination.

Although many of the alkyl reductive dehalogenations found in microcosms and enrichments were catalyzed by the pure cultures listed in Table 1.3, there are still reactions to which no bacteria can as yet been assigned. One example is the degradation of tetrachloroethene. Methanogens dechlorinated tetrachloroethene very slowly to trichloroethene. Enrichments were described which completely dechlorinated tetrachloroethene to ethene at high rates [64]. Furthermore, it was shown that methanogens were not involved in the tetrachloroethene dechlorination in these enrichments. This example demonstrates that still little is known about the bacteria involved in alkyl reductive dehalogenation.

1.4. Reductive dehalogenation by tetra-pyrrole cofactors.

Some of the reductive dehalogenation reaction catalyzed by hematin, cobalamin, and factor F_{430} are summarized in Table 1.4. It is evident from these data that the tetra-pyrrole cofactors might be involved in dechlorinations found in investigations with environmental samples or with bacterial pure cultures. Many of the dechlorinations reported in studies with microcosms, enrichments, and pure cultures (Tables 1.1, 1.2 and 1.3) were also catalyzed by these cofactors.

As indicated in section 1.1. and 1.3.2., the hydrolytic reductive dechlorination could explain the transformation of tetrachloromethane to carbon dioxide found in studies with microcosms, enrichments, and pure cultures. The formation of carbon monoxide from tetrachloromethane by cobalamin indicated that a two-electron reduction to the carbenoid followed by a hydrolysis of this highly reactive species was responsible for the reaction [156]. Kinetic studies on the transformation of tetrachloromethane to carbon monoxide (75%) and methane (25%) by Cr(II) sulfate suggested a mechanism involving metal ion complexes of α-halomethyl radicals and carbenes [38]. The carbon monoxide formed by corrinoids could then be oxidized by carbon monoxide dehydrogenase, an enzyme present in all bacteria where carbon dioxide production from tetrachloromethane has been found [73].

The dechlorination of hexachlorobenzene and pentachlorophenol by cobalamin and of hexachlorobenzene by hematin was quite surprising [98]. Aryl reductive dehalogenations were considered to be catalyzed by specific dehalogenases and not to be unspecific processes. The cobalamin and hematin catalyzed aryl dechlorinations, however, indicated that also these reaction could be unspecific activities of tetra-pyrrole cofactor containing enzymes.

OUTLINE OF THIS THESIS

The aim of this thesis was to investigate the physiology and biochemistry of reductive dehalogenation reactions catalyzed by anaerobic bacteria. Alkyl reductive dehalogenations seemed to be cometabolic activities of many different bacteria. Tetra-pyrrole cofactors containing enzymes were suggested to be involved but often only indirect evidence was presented. Aryl dehalogenations were possibly catalyzed by specific dehalogenases. *Desulfomonile tiedjei* which coupled growth on formate to the reductive dechlorination of 3-chlorobenzoate demonstrated that it could even be a novel type of anaerobic respiration. Thermodynamic considerations suggest that also halogenated aliphatic hydrocarbons can act as terminal electron acceptor (Fig. 1.4). However, no evidence has yet been presented to support this suggestion.

Two approaches have been applied in this thesis to study the physiological meaning and the biochemistry of the reductive dehalogenation by anaerobic bacteria: i) characterization of the reductive dehalogenation catalyzed by methanogens and ii) enrichment and isolation of new dehalogenating bacteria. Chapter 2 describes the reductive dechlorination of 1,2-dichloroethane to ethene and chloroethane and of chloroethane to ethane by several methanogenic bacteria. The same reactions were catalyzed in buffer reduced with titanium(III) citrate by corrinoids and factor F_{430}, two cofactors present in high amounts in methanogens (chapter 3). In chapter 4 it is shown that the factor F_{430}-containing methyl-coenzyme M reductase catalyzed the reductive dechlorination of 1,2-dichloroethane to ethene and chloroethane and that the corrinoid-containing methyl-tetrahydromethanopterin: coenzyme M methyltransferase was not involved in the reductive dechlorination by cell extracts of *Methanobacterium thermoautotrophicum* ΔH. The enrichment and the properties of a 1,2,3-trichlorobenzene dechlorinating mixed culture are presented in chapter 5. Furthermore, a bacterium that grows by the reductive dechlorination of tetrachloroethene with molecular hydrogen or formate is characterized in detail (chapter 6). Finally, in chapter 7, the results obtained in preceding chapters are discussed in relation to results obtained with environmental samples. Possible treatment system applications of the reductive dehalogenation processes described in chapter 2 to 6 are also discussed.

CHAPTER 2

Reductive dechlorination of 1,2-dichloroethane and chloroethane by cell suspensions of methanogenic bacteria

Christof Holliger, Gosse Schraa, Alfons J. M. Stams,
and Alexander J. B. Zehnder

2.1. Abstract

Concentrated cell suspensions of methanogenic bacteria reductively dechlorinated 1,2-dichloroethane *via* two reaction-mechanisms: a dihalo-elimination yielding ethene and two hydrogenolysis reactions yielding chloroethane and ethane, consecutively. The transformation of chloroethane to ethane was inhibited by 1,2-dichloroethane. Stimulation of methanogenesis caused an increase in the amount of dechlorination products formed, whereas the opposite was found when methane formation was inhibited. Cells of *Methanosarcina barkeri* grown on H_2/CO_2 converted 1,2-dichloroethane and chloroethane at higher rates than acetate or methanol grown cells.

2.2. Introduction

Chlorinated aliphatic hydrocarbons are found to be biotransformed under methanogenic conditions *via* reductive dechlorination in sewage sludge, aquifers, and sediments [25,28,148,205, 284]. Reductive dechlorination by pure cultures of anaerobic bacteria was first reported for the degradation of hexachlorocyclohexane isomers by different *Clostridia* strains [129,175]. Recently, it was shown that sulfate reducing, methanogenic, and fermenting bacteria have the ability to dechlorinate 1- and 2-carbon halogenated aliphatic compounds [19,72,74,97]. The products were lower chlorinated compounds or even non-toxic products like carbon dioxide, ethene or ethane.

We report here the production of ethene and chloroethane (CA) from 1,2-dichloroethane (DCA) and ethane from CA by four strains of methanogens. Evidence is provided that the rate of dechlorination is dependent on the metabolic activity of the cells.

2.3. Materials and Methods

2.3.1. Organisms

Methanosarcina barkeri (DSM 2948) and *Methanococcus mazei* (DSM 2053) were obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, FRG). *Methanobacterium thermoautotrophicum* strain Marburg (DSM 2133) was obtained from Prof. Thauer, Marburg, FRG. *Methanothrix soehngenii* (DSM 2139) was the Opfikon strain isolated by Huser et al. [127].

2.3.2. Growth conditions

Cultivation of *M. barkeri* and *M. mazei* on acetate (100 mM) or methanol (250 mM) was performed at 37°C in 120 ml serum bottles with 60 ml of imidazol buffered medium as described by Scherer and Sahm [231], supplemented with 0.02% yeast extract. The gas phase was N_2 and the initial pH of the medium, 6.4. Subcultures on H_2/CO_2 (4/1, v/v) of *M. barkeri* and *M. thermoauto-trophicum* strain Marburg (incubated at 55°C) were made in 120 ml serum bottles with 20 ml of a phosphate/bicarbonate buffered medium described by Huser et al. [127]. *M. soehngenii* was subcultured at 37°C on the phosphate/bicarbonate buffered medium in 500 ml portions in 1-L bottles with 80 mM sodium acetate as energy substrate and a gas phase of N_2/CO_2 (4/1).

2.3.3. Dechlorination experiments

Where necessary, handlings were carried out in an anaerobic glovebox (Coy Laboratories Products, Toepffer GmbH, Göppingen, FRG). The oxygen concentration was kept low with R-20 palladium catalyst provided by BASF (Arnhem, NL).

Cells at the late log phase were harvested aseptically and anaerobically by centrifugation in sterile 300 ml stainless steel centrifugation tubes (Sorvall Instruments, Meyvis, Bergen op Zoom, NL) at 27,500 x g for 45 min at 4°C. Cells were washed twice in sterile medium without substrate, NH_4Cl or yeast extract and resuspended in the same medium. Protein concentration was measured by the modified Lowry method [193] with bovine serum albumin as standard. 5 ml portions of the cell suspension were transferred to sterile 35 ml serum bottles. The bottles were sealed with sterile 10 mm thick viton stoppers and kept on ice. The gas phase was changed with N_2, N_2/CO_2, or H_2/CO_2. Where necessary, the methanogenic substrate (methanol or acetate) and 1,2-DCA or CA dissolved in ethanol (10 µl) was added by syringe (Kloehn, Inacom Instruments B.V., Veenendaal, NL). The final ethanol concentration was 34.8 mM or 0.2%. Control experiments showed that ethanol did not lead to appreciable formation of ethene or ethane. The mesophilic cultures were incubated in a water bath at 37°C, *M. thermoautotrophicum* at 55°C. For each data point two or three cultures were sacrificed for analysis. The gas phase was analyzed for ethene, ethane, and methane. Subsequently, 5 ml hexane was added by syringe and the bottles were vigorously shaken for 1 min to extract 1,2-DCA and possible chlorinated products. The bottles were stored at 4°C until analysis of the hexane extract.

2.3.4. Analyses

C-2 gases were determined by two different methods. In the first method 200 µl headspace was injected into a 417 Packard gas chromatograph equipped with an FID connected to a Porapak T column (3m by 1/8"). Operating temperatures of the detector and the column were 120 and 60°C, respectively. Carrier gas was nitrogen at a flow rate of 30 ml/min. In the second method 500 µl headspace was injected into a 438A Chrompack Packard gas chromatograph equipped with an FID connected to a capillary column (25m by 0.32mm [inner diameter], Poraplot Q, 10 µm, Chrompack, NL) and a splitter injector (ratio 1:80). Operating temperatures of the injector, column, and detector were 250, 60, and 300°C, respectively. Carrier gas was nitrogen with an inlet pressure of 30 kPa. In most of the experiments the first method was used because it was faster. When small amounts of ethane had to be separated from large amounts of ethene the second method was applied. Methane was measured with a 417 Packard gas chromatograph equipped with TCD at 100 mA connected to a molecular sieve column (13X, 180 cm by 1/4", 60-80 mesh). Operating temperature of the detector and the column was 100°C. Carrier gas was argon at a flow rate of 30 ml/min. For 1,2-DCA and CA analysis 1 µl hexane sample was injected into a 436 Chrompack Packard gas chromatograph equipped with a ^{63}Ni-ECD connected to a capillary column (25m by 0.32mm [inner diameter], Sil 5CB, 1.22 µm, Chrompack, NL) and a splitter injector (ratio 1:50). Operating temperatures of the injector, column, and detector were 250, 90, and 300°C, respectively. Carrier gas was nitrogen with an inlet pressure of 30 kPa. Trichloroethene served as internal standard. The retention times and peak areas were determined with a Shimadzu C-3A computing integrator. CA was positively identified with GC-MS.

2.3.5. Chemicals

1,2-Dichloroethane (DCA) was purchased from Aldrich, Brussels, Belgium, 1-iodopropane and chloroethane (CA) from E. Merck, Darmstadt, FRG. All other chemicals were of analytical grade and used without further purification. Gases were purchased from Hoekloos, Schiedam, NL.

2.4. Results

1,2-DCA was reductively dechlorinated to ethene and CA by concentrated cell suspensions of four strains of methanogenic bacteria (Table 2.1). Transformation of 1,2-DCA was not only catalyzed by methanogens which solely grow on H_2/CO_2 [19,72], but also by the acetoclastic organism *M. soehngenii* and by methanogens with a broader substrate range such as *M. barkeri* and *M. mazei* grown on methanol. This shows that this dechlorination activity may be characteristic for methanogens and is not restricted to one primary substrate. Because a complete mass balance of the chlorinated substrate and products was seldom achieved (Table 2.1), it is possible that other chlorinated products were formed. If the initial step in the reductive dechlorination of 1,2-DCA is a single electron transfer, and therefore takes place *via* a radical, dimerization could occur and C-4 compounds could be formed. Peaks were never observed, however, at the positions of the GC runs where such compounds should appear. Non-volatile and non-hexane-extractable products could not be analyzed by our methods. Although abiotic dehalogenation by the medium, which could lead to the formation of alcohols and thiols [13], is not likely since the rates of such nucleophilic substitution reactions are too slow to be significant during the time-frame involved here. A decrease in 1,2-DCA concentration of 0.17% per day could be expected at 37°C with the concentrations of the nucleophilic agents OH^-, HS^-, and HPO_4^{2-} used in our experiments. This decrease is calculated with the rate constants reported by Barbash and Reinhard [13]. However, biological formation of such polar compounds cannot be excluded. Besides other chlorinated products inaccuracies in the GC measurements and

TABLE 2.1. Dechlorination of 1,2-DCA by cell suspensions of four strains of methanogenic bacteria

Strain[a]	Substrate[b]	Incubation time	(nmole/culture)			
			0 h	after Incubation		
		(h)	1,2-DCA	1,2-DCA	Ethene	CA
M. barkeri	methanol	24	329±9	271±3	4±0.2	34±2
M. mazei	methanol	24	356±5	205±17	3±0.2	107±3
M. thermoautotrophicum[c]	H_2/CO_2	48	333±5	260±11	21±2	54±4
M. soehngenii	acetate	96	246±15	168±12	9±0.3	29±3

[a] Protein content per culture was initially: *M. barkeri* 7.4 mg; *M. mazei* 5.2 mg; M. thermoautotrophicum 5.3 mg; *M. soehngenii* 2.3 mg.
[b] Substrate for growth and incubation of cell suspensions.
[c] Incubated at 55°C.

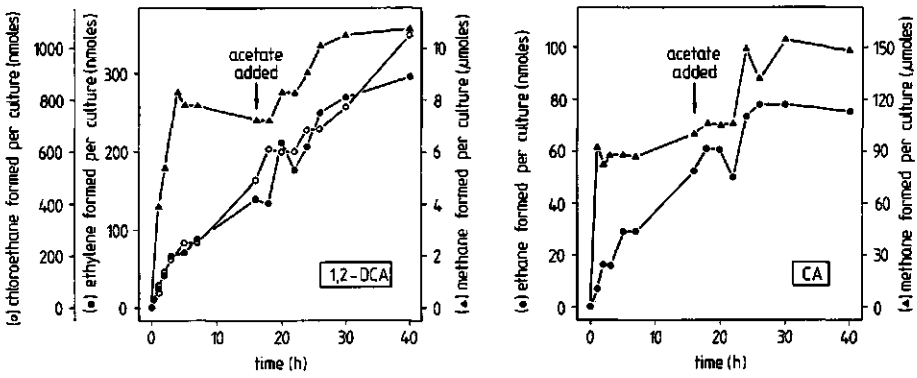

FIGURE 2.1. Dechlorination pattern of 1,2-DCA and CA by concentrated cell suspensions of *M. barkeri* grown and incubated on acetate. Protein content per culture was initially 18.5 mg. 20 mM of acetate was added after 0 and 18 h of incubation. The initial concentration of 1,2-DCA or CA was approx. 1 mM or 5 μmoles per culture, respectively.

sorption to biomass are possible additional factors which could account for the incomplete mass balance. Comparing cells grown on different substrates could give some indication what enzymes are involved in the dechlorination. Therefore, *M. barkeri* was chosen as model-organism for further study.

CA was transformed to ethane by cell suspensions of *M. barkeri*. This dechlorination reaction was inhibited 37-59% in the presence of 1,2-DCA (Table 2.2). The reductive dechlorination of 1,2-DCA and CA by cell suspensions of *M. barkeri* did not strictly follow methane formation

TABLE 2.2. Ethane production from CA in the presence and absence of 1,2-DCA by cell suspensions of *Methanosarcina barkeri* [a]

CA[b]	1,2-DCA[b]	Ethene[c]	Ethane[c]
(nmol/culture)		(nmol/culture)	
500	0	0.0	1.35±0.07
500	5000	65.2±3.3	0.85±0.07
2500	0	0.0	5.55±0.07
2500	5000	74.8±0.8	2.55±0.49
5000	0	0.0	10.40±0.85
5000	5000	64.4±7.1	4.25±2.05
0	5000	67.3±1.8	0.25±0.07

[a] The experiment was carried out with cells grown on methanol. Protein content per culture was initially 8.6 mg. Ethene and ethane were analyzed by the second method described in Materials and Methods.
[b] Added at time 0.
[c] Measured after 24 h of incubation

(Fig. 2.1). Formation of dechlorination products decreased slightly when methane production stopped, and increased with methane formation after a second addition of methanogenic substrate. Interestingly, dechlorination went on even after cease of methane production.

The dependence of dechlorination on biological activity was exhibited by stimulating or inhibiting methanogenesis. Stimulation done by adding different concentrations of methanogenic substrate, resulted in increased amounts of dechlorination products (Fig. 2.2). The amount of ethene and CA formed per mole of methane was not constant. In the presence of e.g. 2.5 mM methanol, 1.14 mmoles ethene and 1.42 mmoles CA were formed per mole of methane; whereas 250 mM methanol resulted only in 0.20 mmoles ethene and 0.42 mmoles CA per mole of methane. Inhibition of methanogenesis by 2-bromoethanesulfonic acid (BrES) or 1-iodopropane caused a decrease in dechlorination products (Fig. 2.3). BrES, as an analogue of methyl coenzyme M, is a specific inhibitor of the last step of methane formation. While it completely inhibited formation of CA, ethene was still produced even at high BrES concentrations (Fig. 2.3A). Methanogens can also form ethene from BrES [19]. To account for this, ethene formed by cultures containing only BrES was subtracted from ethene formed on 1,2-DCA and BrES together. Ethene production on BrES alone was maximum 18% of ethene formed on 1,2-DCA and BrES. 1-Iodopropane is an inhibitor of corrinoid enzyme catalyzed methyl transfer reactions [32] and was used to study the role of a corrinoid enzyme in methane formation [75]. This compound inhibited both ethene and CA production (Fig. 2.3B).

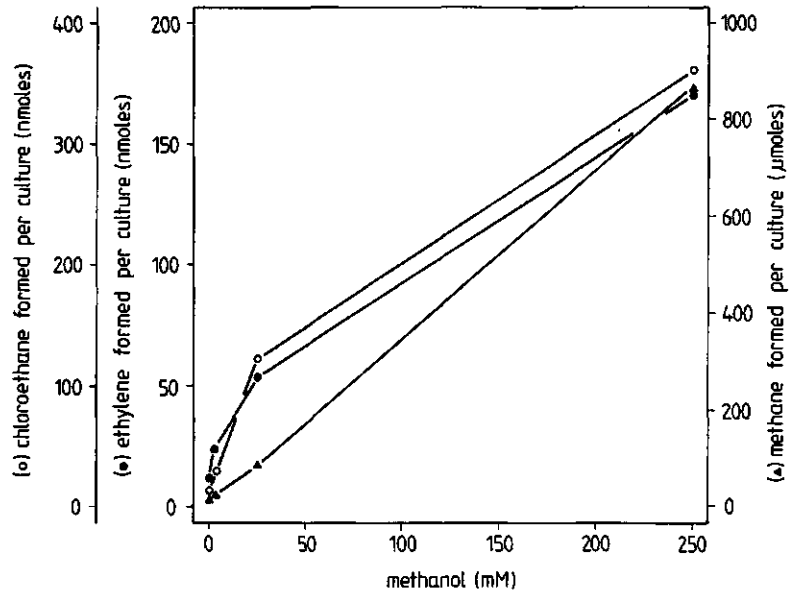

FIGURE 2.2. Effect of the stimulation of methanogenesis on dechlorination of 1,2-DCA. Different concentrations of methanol (0, 2.5, 25, and 250 mM) were added to cell suspensions of *M. barkeri* (initially 18.3 mg of protein per culture) and the products were measured after 24 h of incubation. The initial concentration of 1,2-DCA was approx. 1 mM or 5 μmoles per culture, respectively.

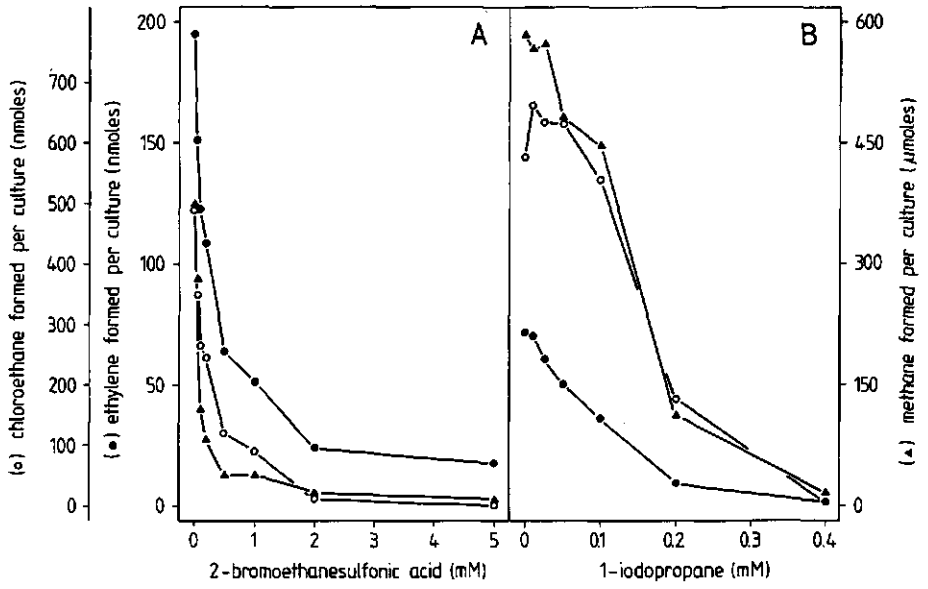

FIGURE 2.3. Effect of inhibition of methanogenesis by (A) 2-bromoethanesulfonic acid or (B) 1-iodopropane on dechlorination of 1,2-DCA by cell suspensions of *M. barkeri*. The methanogenic substrate was methanol (250 mM) and protein content of the cultures for the experiments with 2-bromoethanesulfonic acid and 1-iodopropane was initially 16.2 mg and 12.5 mg, respectively. 2-Bromoethanesulfonic acid was added by syringe from an aqueous solution, 1-iodopropane from a stock solution in ethanol. The products were measured after 24 h of incubation and the initial concentration of 1,2-DCA was approx. 1 mM or 5 μmoles per culture, respectively.

An increase in 1,2-DCA or CA concentration resulted in increasing amounts of dechlorination products formed per mole of methane (Fig. 2.4). Methane production was not affected in the concentration range used in these experiments (data not shown). Cells grown on H_2/CO_2 produced the highest amounts of ethene, ethane, and CA (Fig. 2.4). To gain insight into the enzymes responsible for the dechlorination reaction, initial production rates by cell suspensions grown and incubated with different substrates were measured (Table 2.3). The rates, in nmol per mg of protein per h, did not differ significantly among cell suspensions grown and incubated on methanol, acetate, or H_2/CO_2. Approximately the same amounts of dechlorination products were formed per mg of protein per h when CO was provided as electron donor. However, when rates were expressed in mmoles of dechlorination products per mole of methane, cells grown on H_2/CO_2 showed the highest activities. Methane formation on CO could not be quantified because the CO used was contaminated with methane.

2.5. Discussion

The transformation of 1,2-DCA to ethene, catalyzed by methanogens, was shown previously [19,72]. We demonstrate here that methanogens also form CA from 1,2-DCA and that CA is dechlorinated to ethane. In addition, we show that the ability to reductively dechlorinate

TABLE 2.3. Ethene and CA production rates from 1,2-DCA and ethane production rates from CA by cell suspensions of *Methanosarcina barkeri*

Substrate		Product formation rates[a]			
		from 1,2-DCA		from CA	
Growth	Cell suspension	Ethene		CA		Ethane	
methanol	methanol	1.64±0.20	(0.27±0.07)	3.47±0.70	(0.56±0.10)	0.40±0.10	(0.07±0.01)
	acetate	1.08±0.15	(1.72±0.46)	2.38±0.22	(3.61±0.50)	0.45±0.09	(0.58±0.10)
	H_2/CO_2	2.07±0.18	(0.83±0.16)	5.13±0.33	(2.07±0.24)	1.06±0.15	(0.44±0.10)
	CO	0.95±0.04	n.a.[b]	3.25±0.22	n.a.	0.46±0.04	n.a.
acetate	methanol	1.39±0.15	(3.28±0.42)	1.65±0.45	(3.74±0.73)	0.35±0.08	(0.57±0.12)
	acetate	1.30±0.06	(0.47±0.02)	2.15±0.10	(0.77±0.06)	0.55±0.12	(1.01±0.26)
	H_2/CO_2	1.44±0.27	(0.95±0.24)	2.25±0.30	(1.52±0.52)	0.87±0.19	(0.49±0.08)
	CO	1.33±0.18	n.a.	2.05±0.30	n.a.	0.39±0.05	n.a.
H_2/CO_2	methanol	0.97±0.38	(4.72±1.50)	4.00±0.55	(16.74±4.19)	0.47±0.08	(1.59±0.28)
	acetate	1.23±0.14	(15.62±1.86)	3.95±0.40	(47.75±5.85)	0.18±0.04	(1.28±0.26)
	H_2/CO_2	1.32±0.15	(5.52±0.68)	6.25±0.60	(26.09±3.20)	0.41±0.08	(0.83±0.39)
	CO	1.03±0.11	n.a.	4.85±0.85	n.a.	0.15±0.03	n.a.

[a] Rates are expressed in nmole.mg of protein^{-1}.h^{-1}, data in parentheses represent rates in mmole.mole of CH_4^{-1}. After 2 and 4 hours of incubation two cultures were sacrificed for analysis. Product formation was linear within this time-frame. Cultures from cells grown on methanol, acetate, and $H2/CO_2$ contained initially 26.4 mg, 10.4 mg, and 18.9 mg of protein, respectively. 250 mM of methanol, 100 mM of acetate, 1.8 bar H_2/CO_2 (80%/20%), or 1.2 bar CO/N_2 (80%/20%) were added to cell suspensions at time 0.

[b] n.a. = not applicable because CH_4 formation on CO could not be quantified.

1,2-DCA is a property of both hydrogenotrophic and acetoclastic methanogenic bacteria and that 1,2-DCA can be dechlorinated by two different reductive reaction-mechanisms (Fig. 2.5). The transformation of 1,2-DCA to ethene is a dihalo-elimination and transformation to CA a hydrogenolysis [287]. CA is dechlorinated *via* hydrogenolysis.

The inhibition of ethane production by 1,2-DCA is probably due to competition of the two chlorinated compounds for the same electrons. Such a dechlorination pattern, where the dechlorination of a lower chlorinated compound is inhibited by the higher chlorinated compound, has also been observed in mixed microbial systems like sewage sludge or soil for as well aromatic compounds [24,29,89,185,266] as aliphatic compounds [15,205,284]. These sequential dechlorination reactions in pure cultures and in mixed microbial systems indicate that the dechlorination pattern observed in complex communities could also be due to a competition for the same electrons in one kind of organism.

Dechlorination of 1,2-DCA and CA by *M. barkeri* in relation to methane formation differed from the dechlorination pattern reported for tetrachloroethene by *Methanosarcina* sp. strain DCM [86]. The transformation of tetrachloroethene to trichloroethene by this organism strongly depended on the amount of methanogenic substrate consumed. In our experiments, however, dechlorination of 1,2-DCA or CA did not strictly follow methanogenesis. This difference may be due to the different systems used. Dechlorination experiments with tetrachloroethene were performed in growing cultures whereas our experiments were carried out in concentrated cell suspensions. Reducing power could still be present in these cells and may have influenced the rates at low substrate concentrations.

Although reductive dechlorination of 1,2-DCA is catalyzed by pure cultures of methanogens,

it is not certain whether these reactions account for significant transformation in mixed cultures. Bouwer and McCarty [25] found ^{14}C-labeled carbon dioxide from [^{14}C]-1,2-DCA in methanogenic batch cultures seeded with a mixed culture from sewage sludge. Ethene and ethane, formed in the reductive dechlorination of 1,2-DCA by methanogens, are thought to be stable in methanogenic mixed cultures [232,233]. It is unlikely then, that the mineralization found by Bouwer and McCarty [25] resulted from the two C-2 gases formed by the total dechlorination of 1,2-DCA. This indicates that 1,2-DCA could also be degraded *via* reactions other than reductive dechlorination under anaerobic conditions.

Specific rates of ethene, CA, and ethane production showed that cells grown on H_2/CO_2 had the highest activity relative to methane formation. Studies of the degradation of chloromethanes by anaerobic bacteria suggest that corrinoides [74,154,305] or, in methanogens, factor F_{430} [155] are involved in the reductive dechlorination of these halocompounds. However, since *M.barkeri* grown on H_2/CO_2 did not contain the highest amounts of corrinoids or factor F_{430} [55,63,105, 157], this does not explain the highest dechlorination activities for these cells. Studies which compared the activity of oxidoreductases, hydrogenase or methyl-coenzyme M reductase in cell extracts of *M. barkeri* grown on different substrates [14,158] do not give any indications as to which enzymes could be involved in the cometabolic transformation of 1,2-DCA by methanogenic bacteria. Inhibition experiments with 1-iodopropane do not prove that only corrinoids are

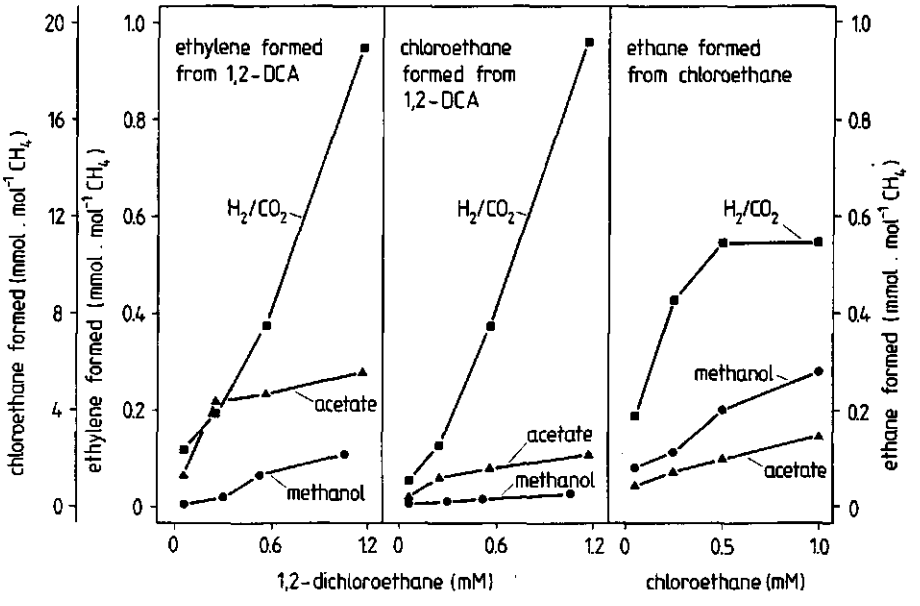

FIGURE 2.4. Effect of different concentrations of 1,2-DCA or CA on product formation rates by cell suspensions of *M. barkeri* grown and incubated on different methanogenic substrates. The cultures on methanol, acetate, and H_2/CO_2 contained initially 7.9 mg, 0.9 mg, and 5.1 mg of protein, respectively. Methanol (250 mM) and acetate (100 mM) were added by syringe from stock solutions, H_2/CO_2 (80%/20%) was present in the gasphase with a pressure of 1.8 bar.

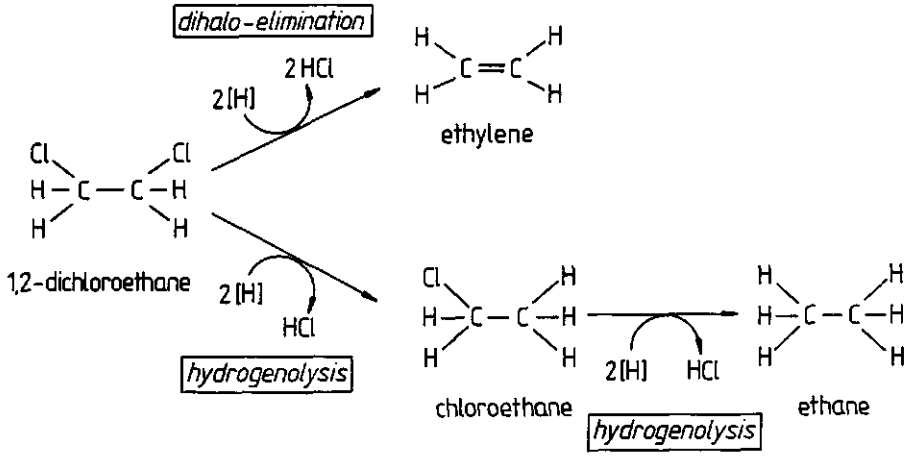

FIGURE 2.5. Degradation pathway of 1,2-DCA by cell suspensions of methanogenic bacteria.

involved in the dechlorination reactions because methane formation was inhibited as well. The dihalo-elimination of 1,2-dibromoethane to ethene by iron(II)porphyrins [292] or by low valent transition metal ions like Cr^{2+} [38] and the hydrogenolysis of 1,1,1-trichloroethane by iron(II)porphyrins [146] yielding 1,1-dichloroethane suggest that cofactors with a tetrapyrrole structure and a transition metal are involved in the cometabolic dechlorination reactions catalyzed by anaerobic bacteria. Research is underway to investigate whether this might be the case for the reductive dechlorination of 1,2-DCA and CA by methanogenic bacteria.

2.6. Conclusions

Hydrogenotrophic and acetoclastic methanogenic bacteria dechlorinate 1,2-dichloroethane (1,2-DCA) reductively by two reaction-mechanisms. Ethene is formed *via* a dihalo-elimination and chloroethane (CA) *via* a hydrogenolysis. CA is transformed to ethane *via* hydrogenolysis. This reaction is inhibited in the presence of 1,2-DCA. The rate of dechlorination is dependent on metabolic activity of the cells; *Methanosarcina barkeri* cells grown on H_2/CO_2 show higher dechlorinating activities than do cells grown on methanol or acetate.

Acknowledgments

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CHAPTER 3

Evidence for the involvement of corrinoids and factor F_{430} in the reductive dechlorination of 1,2-dichloroethane by *Methanosarcina barkeri*

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3.1. Abstract

Cobalamin and the native and diepimeric form of factor F_{430} catalyzed the reductive dechlorination of 1,2-dichloroethane (1,2-DCA) to ethene or chloroethane (CA) in a buffer with Ti(III) citrate as electron donor. Ethene was the major product in the cobalamin-catalyzed transformation and the ratio between ethene and CA formed was 25:1. Native F_{430} and 12,13-di-epi-F_{430} produced ethene and CA in a ratio of about 2:1 and 1:1, respectively. Cobalamin dechlorinated 1,2-DCA much faster than factor F_{430}. Dechlorination rates by all three catalysts showed a distinct pH-dependence, linearly correlated with catalyst concentration, and doubled with a temperature increase of 10°C. Crude and boiled cell extracts of *Methanosarcina barkeri* also dechlorinated 1,2-DCA to ethene and CA with Ti(III) citrate as reductant. The catalytic components in boiled extracts were heat- and oxygen-stable, and had a low molecular mass. Fractionation of boiled extracts by a hydrophobic interaction column revealed that part of the dechlorinating components had a hydrophilic, and part a hydrophobic character. These chemical properties of the dechlorinating components and spectral analysis of boiled extracts indicated that corrinoids or factor F_{430} were responsible for the dechlorinations. The ratio of 3:1 to 7:1 between ethene and CA formed by cell extracts suggested that both cofactors were concomitantly active.

3.2. Introduction

Chlorinated aliphatic C_1 and C_2 hydrocarbons, widespread contaminants in different environments, are found to be reductively dechlorinated by pure cultures of methanogens, sulfate-reducers, homoacetogens, and other anaerobic bacteria [19,72,74,88,97,155,186, Chapter 2]. Tetrachloromethane (CCl_4) was found to be transformed to lower chlorinated methanes and CO_2 by native or autoclaved cell suspensions of methanogens, *Acetobacterium woodii*, and *Desulfobacterium autotrophicum* [73,74,155]. These anaerobic bacteria fix CO_2 via the acetyl-CoA pathway (Wood pathway) or degrade acetate *via* a reversed acetyl-CoA pathway [95,170,268, 306]. Carbon monoxide dehydrogenase and a corrinoid/iron-sulfur protein are central enzymes of this pathway. It has been suggested that the anaerobic transformation of CCl_4 could be a cometabolic activity of Wood pathway enzymes [73,74]. Free cobalamin catalyzed reductive dechlorination of CCl_4 in a buffer reduced with Ti(III) citrate as electron donor [154]. In addition to lower chlorinated methanes, cobalamin produced CO from CCl_4 [156], a compound which is oxidized to CO_2 by carbon monoxide dehydrogenase [95,170,268,306]. In methanogenic bacteria, factor F_{430} may also be involved in reductive dechlorination. Factor F_{430} is a cofactor of methyl-CoM reductase, the enzyme which catalyses the last step in methanogensis from CH_3-S-CoM to CH_4 [78]. This cofactor reductively dechlorinated CCl_4 to lower chlorinated methanes [155]. All these results support a possible involvment of Wood pathway enzymes and/or methyl-CoM reductase in the transformation of CCl_4 by anaerobic bacteria. A recent report on reductive dechlorination of chloroethenes catalyzed by cobalamin or factor F_{430} [98] and the fact that methanogens dechlorinate tetrachloroethene to trichloroethene [86] are further evidence for a cometabolic activity of enzymes with these tetrapyrrole cofactors as prosthetic group.

Cell suspensions of methanogens reductively dechlorinated 1,2-dichloroethane (1,2-DCA) to ethene and chloroethane (CA) [Chapter 2]. We demonstrate here that the same reactions were catalyzed by cobalamin, the native (native F_{430}) and the diepimeric form (12,13-di-epi-F_{430}) of

factor F_{430}, or by crude and boiled cell extracts of *Methanosarcina barkeri* with Ti(III) citrate as reducing agent. Properties of the dechlorinating components in boiled cell extracts and spectral analysis of these extracts provide evidence that corrinoids and factor F_{430} are both involved in the dechlorination reactions catalyzed by cell extracts of *M. barkeri*.

3.3. Materials and Methods

3.3.1. Organism and growth conditions

Methanosarcina barkeri (DSM 2948) was obtained from Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). *M. barkeri* was mass cultured on 250 mM methanol in 10-l carboys, containing 8 l of imidazol buffered medium, as described by Scherer and Sahm [231], supplemented with 0.02% yeast extract. The gas phase was N_2 and the initial pH of the medium 6.4. The cultures were incubated at 37°C in the dark. Cells were harvested at the late log phase by continous centrifugation (Carl Padberg Zentrifugenbau,GmbH, Lahr/Schwarzwald, Germany).

3.3.2. Preparation of cell extracts

All procedures were carried out anaerobically unless otherwise stated. A cell paste was diluted with 100 mM TRIS/HCl pH 9 in a 1:1 ratio. The cell suspension was subjected to ultrasonic disintegration (8x30s bursts at 0°C) followed by passing through a French pressure cell at 135 MPa. Cell debris were removed by anaerobic centrifugation (11.500 x *g*, 30 min, 20°C). The supernatant is referred to as crude cell extracts. It contained 20-40 mg protein/ml as measured by the method of Bradford et al. [31] with bovine serum albumin as the standard. For preparation of boiled cell extracts, the pH of crude cell extracts was adjusted first to 5-6 with 1 M HCL. The extracts were incubated anaerobically at 100°C for 30 minutes. Denatured protein was removed by centrifugation and the pH of the supernatant was readjusted to 9 with 1 M KOH. This fraction is referred to as boiled cell extracts.

3.3.3. Fractionation of boiled cell extracts by a XAD-4 column

The method used is essentially the same as described for purification of cobamides [263]. The fractionation was carried out aerobically. A Pasteur pipet was used as column. It was filled half with XAD-4 and equilibrated with 100 mM TRIS/HCl pH 9. After application of the boiled extract (1.5 ml), the column was rinsed with 1.5 ml 100 mM TRIS/HCl pH 9 and two fractions of 1.5 ml were collected. Subsequently, the column was rinsed with 3 ml 80% methanol to elute adsorbed compounds. The methanol fraction was flash evaporated and the residue was dissolved in 1.5 ml 100 mM TRIS/HCl pH 9.

3.3.4. Dechlorination experiments

The assay was carried out in 13 ml serum bottles which were filled inside an anaerobic glovebox (Coy Laboratories Products, Toepffer GmbH, Göppingen, Germany) and sealed with viton stoppers (Maag Technic, Dübendorf, Switzerland) and aluminium crimp caps. The reaction mixture contained either 400 µl 100 mM TRIS/HCl pH 9, 50 µl 100 mM Ti(III) citrate, and 50 µl cell extract, or 445 µl 100 mM TRIS/HCl pH 9, 50 µl 100 mM Ti(III) citrate, and 5 µl 5 mM cobalamin or 5 mM factor F_{430}, unless otherwise stated. The bottles were stored at 0°C. After

the gas phase was changed with 100% N_2, 5µl 100 mM 1,2-DCA in ethanol was added by syringe. It was assured that ethanol was not transformed to ethene. The reaction was started by increasing the temperature from 0°C to 37°C. After different time intervals, bottles were sacrificed for analysis. First, 0.2 ml of the headspace was analyzed for ethene or CA production, and then, 1 ml hexane was added by syringe for extraction of 1,2-DCA.

3.3.5. Analyses

The analyses of ethene, CA, and 1,2-DCA were done gas chromatographically. Ethene was determined in the headspace sample with a 417 Packard gas chromatograph equipped with a flame ionization detector connected to a Porapak T column (3m by 1/8"). CA was quantified in the headspace sample with a 438A Chrompack Packard gas chromatograph equipped with a ^{63}Ni electron capture detector connected to a capillary column (25m by 0.32mm [inner diameter], Sil 5CB, 1.22 µm, Chrompack, The Netherlands) and a splitter injector (ratio 1:50). 1,2-DCA was analyzed by injecting 1 µl hexane extract into the same gas chromatograph.

Visible spectra were recorded with a Beckman model 25 spectrophotometer (Beckman, Amsterdam, The Netherlands). Corrinoids and factor F_{430} concentrations were quantified spectrophotometrically. For quantification of cobalamin and cobinamide with excess HCN, the molar extinction coefficients ϵ_{550} = 8700 $M^{-1}cm^{-1}$ and ϵ_{580} = 10100 $M^{-1}cm^{-1}$ were used, respectively. Extinction coefficients for factor F_{430} were ϵ_{430} = 23000 $M^{-1}cm^{-1}$ for native F_{430} and ϵ_{430} = 20300 $M^{-1}cm^{-1}$ for 12,13-di-epi-F_{430}.

3.3.6. Chemicals

1,2-Dichloroethane (1,2-DCA) was purchased from Aldrich (Brussels, Belgium), Tris(hydroxymethyl)-aminomethane (TRIS), 3-[N-Morpholino]-propanesulfonic acid (MOPS), Ti(III) chloride and 1-iodopropane from E. Merck (Amsterdam, The Netherlands), hydroxocob(III)alamin (vitamin B_{12a}) from Fluka (Perstorp Analytical, Oud-Beijerland, The Netherlands), and cobinamide dicyanide from Sigma (ICN Biomedicals, Amsterdam, The Netherlands). Gases and chloroethane (CA) were purchased from Hoekloos (Schiedam, The Netherlands). The oxygen concentration of the glovebox was kept low with R-20 palladium catalyst provided by BASF (Arnhem, The Netherlands). Factor F_{430} was isolated from *Methanobacterium thermoautotrophicum* ΔH cells by perchloric acid extraction described by Ankel-Fuchs et al. [5] (see Supplement Chapter 3). The native and diepimeric form of factor F_{430} were isolated from the crude cofactor fraction by anion exchange chromatography on QAE-Sephadex A-25 modified from a method described by Shiemke et al. [249]. This method also allowed separation of native F_{430} and 12,13-di-epi-F_{430}. *M. thermoautotrophicum* ΔH cells were a generous gift of Dr. J. T. Keltjens (University of Nijmegen, The Netherlands). All other chemicals were of analytical grade and used without further purification.

Ti(III) citrate was prepared from $TiCl_3$ and sodium citrate by a modified method described by Zehnder and Wuhrmann [309]. In the anaerobic glovebox an ampule of $TiCl_3$ (7,5 ml) was added to 25 ml of 0.6 M anaerobically prepared sodium citrate. The pH was adjusted to about 8 with solid $Na_2CO_3 \cdot 10H_2O$, and subsequently to 9 with a concentrated Na_2CO_3 solution. The volume was brought to 75 ml with anaerobic demineralized water resulting in a concentration of 100 mM Ti(III) citrate.

3.4. Results

3.4.1. Reductive dechlorination of 1,2-DCA by cobalamin and factor F_{430}

Methanogenic bacteria reductively dechlorinate 1,2-DCA to ethene and CA [Chapter 2]. Cobalamin and native F_{430} and 12,13-di-epi-F_{430} in buffer reduced with Ti(III) citrate dechlorinated 1,2-DCA to the same products as found in cell suspensions of methanogens (Fig. 3.1). The transformed 1,2-DCA was completely recovered as the two dechlorination products. 1,2-DCA was faster dechlorinated by cobalamin than by factor F_{430}, and ethene was the dominant product in cobalamin catalyzed reactions (Fig. 3.1, Table 3.1). Native F_{430} and 12,13-di-epi-F_{430} formed ethene and CA in a ratio of approximately 2:1 and 1:1, respectively. Dechlorination experiments in vials which were carefully protected from light showed the same rates, and therefore, it was concluded that photolysis did not influence product formation.

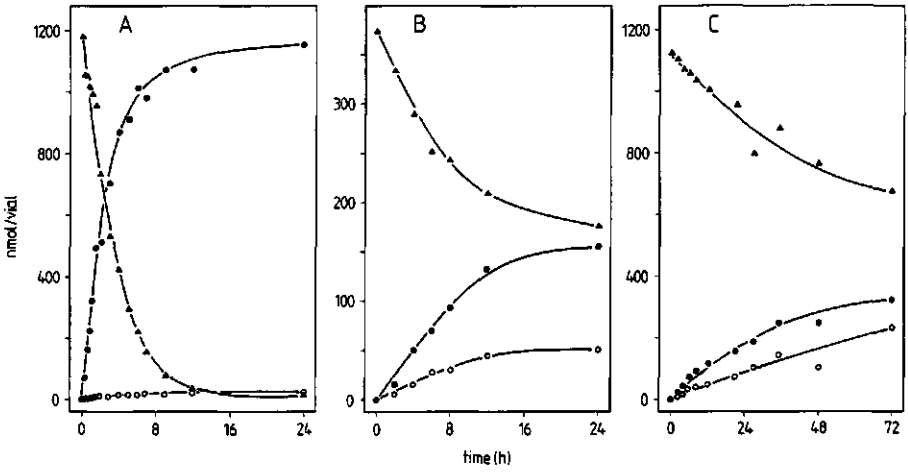

FIGURE 3.1. Reductive dechlorination of 1,2-DCA by cobalamin (A), native F_{430} (B), or 12,13-di-epi-F_{430} (C) in 90 mM TRIS/HCl pH 9 and 10 mM Ti(III) citrate. The cofactor concentration was 50 μM. Each data point represents the mean of three independent experiments. (▲) 1,2-DCA, (●) ethene, (o) CA.

Slow dechlorination by cobalamin was observed with dithiothreitol as electron donor (0.2 pmol ethene formed per min per nmol cobalamin) and no dechlorination occurred if sodium dithionite or cysteine served as reducing agents. Cobinamide, which lacks a lower axial Co-α-ligand, dechlorinated 1,2-DCA with lower rates than cobalamin (Table 3.1). A distinct pH-dependence was observed for the dechlorination of 1,2-DCA by cobalamin or factor F_{430} (Fig. 3.2 A,D,G). Ethene production rates in buffer with different pH's and different ratios of Ti(IV)/Ti(III) suggest an indirect effect of pH on dechlorination rates (Table 3.2). The midpoint potentials of cob(II)alamin/cob(I)alamin and the Ni(II)/Ni(I) couple in 12,13-di-epi-F_{430} are -610 and -618 mV *vs.* N.H.E., respectively [164; Supplement Chapter 3], and the redox potential of the Co(II)/Co(I) cobalamin couple is constant within the pH range investigated [164]. The midpoint potential of the citrate complexed Ti(IV)/Ti(III) couple in a pH range of 2-10 showed

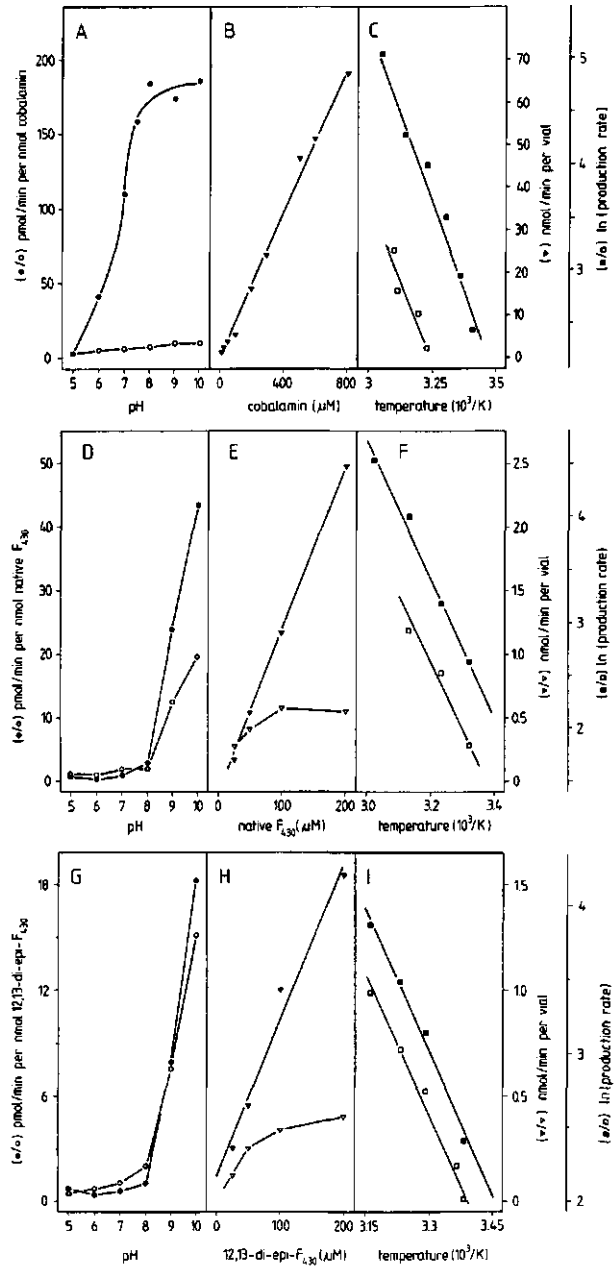

FIGURE 3.2. pH-, catalyst concentration-, and temparature-dependence of the reductive dechlorination of 1,2-DCA by cobalamin (A-C), native F_{430} (D-F), or 12,13-di-epi-F_{430} (G-I). The following buffers were applied: 100 mM acetate buffer pH 5; 100 mM MOPS/KOH pH 6 and 7; 100 mM TRIS/HCl pH 8 and 9; 50 mM TRIS/50 mM glycine pH 10.

TABLE 3.1. Initial ethene and CA production rates by cobalamin, cobinamide, native F_{430} and 12,13-di-epi-F_{430}. [a]

Catalyst	Product formation rates (pmol/min per nmol of catalyst)	
	Ethene	CA
cobalamin	192±20	7.9±0.2
cobinamide	122±9	3.5±0.7
native F_{430}	22.8±2.2	12.9±2.5
12,13-di-epi-F_{430}	8.5±1.6	6.9±1.1

[a] Product formation rates were determined within the first 20 min of incubation.

a clear pH-dependence with lower redox potentials at higher pH's [308]. From the Nernst equation it can be derived that the redox potential depends on the ratio of the oxidizing and reducing agent and therefore, the redox potential can be varied by varying the ratio of Ti(IV)/Ti(III). At pH 7 (E_H of Ti(IV)/Ti(III) = -480 mV [261]) with a Ti(IV)/Ti(III) ratio of 9:1, no ethene production could be observed (Table 3.2). The redox potential was apparentely too high to reduce significant amounts of the cofactors to their active forms. The effect of

TABLE 3.2. Ethene production rates by cobalamin and 12,13-di-epi-F_{430} in buffer with different ratios Ti(IV)/Ti(III).

Catalyst	pH[a]	Ti(IV)/Ti(III)[b] (%)	Ethene production rate (pmol/min per nmol of catalyst)
cobalamin	7	0/100	48.0±7.6
		90/10	0.0[c]
		100/0	0.0
	8	0/100	194.4±12.8
		90/10	113.2±10.8
		100/0	0.0
12,13-di-epi-F_{430}	7	0/100	1.2±0.2
		90/10	0.0[d]
		100/0	0.0
	9	0/100	7.1±2.0
		90/10	0.5±0.1
		100/0	0.0

[a] The following buffers were applied: 100 mM MOPS/KOH pH 7; 100 mM TRIS/HCl pH 8 and 9.
[b] A Ti(III) citrate solution was slowly oxydized by exposure to air to get Ti(IV) citrate.
[c] No ethene formed after 15 min incubation.
[d] No ethene formed after 60 min incubation.

different Ti(IV)/Ti(III) ratios was not as strong at pH 8 or 9 which can be explained by the lower redox potential of the Ti(IV)/Ti(III) couple at these pH's.

Ethene production correlated linearly with catalyst concentration (Fig. 3.2 B,E,H). CA production did not increase with increased concentrations of cobalamin (data not shown) and specific CA production rates by the two forms of factor F_{430} decreased with higher catalyst concentrations (Fig. 3.2 E,H). Product formation rates approximately doubled with a temperature increase of 10°C. Activation energies for ethene and CA production by cobalamin, native F_{430}, and 12,13-di-epi-F_{430} were 56 and 52 kJ/mol, 54 and 51 kJ/mol, and 55 and 55 kJ/mol, respectively. These values were calculated from *Arrhenius plots* (Fig. 3.2 C,F,I).

3.4.2. *Reductive dechlorination of 1,2-DCA by cell extracts of M. barkeri*

Crude cell extracts of *M. barkeri* dechlorinated 1,2-DCA to ethene and CA in buffer reduced with Ti(III) citrate (Table 3.3). The product formation was linear during the first 30 minutes, but declined rapidly thereafter, and stopped after about 60 minutes. This was probably due to other redox-reactions occurring in the crude cell extracts which competed for reducing equivalents of Ti(III) citrate. Denaturing the proteins by boiling cell extracts did not cause loss of activity indicating the involvement of a heat stable catalyst (Table 3.3). Addition of cobalamin or factor F_{430} to both extracts increased the product formation rates (Table 3.3). The ratio of the two dechlorination products ethene and CA varied between 3:1 and 7:1 depending on the extracts. The ratio of ethene and CA for the reaction catalyzed by cobalamin was about 25:1; for factor F_{430} this ratio was about 2:1 or 1:1, depending on the epimer (Fig. 3.1). The fact that the ratios for dechlorination by cell extracts were in between these values indicated that both cofactors were involved.

In order to get more indications about the identity of the dechlorinating components in boiled cell extracts, some chemical properties were determined and compared with cobalamin or factor F_{430} (Table 3.4). A heat treatment (20 min at 120°C) or exposure to air had no influence on

TABLE 3.3. Ethene and CA formation from 1,2-DCA by cell extracts of *Methanosarcina barkeri* [a]

Assay	Products formed from 1,2-DCA (pmol/min per vial)	
	Ethene	CA
crude extract	60±5	19±3
crude extract + cobalamin [b]	634±55	33±2
crude extract + 12,13-di-epi-F_{430} [b]	52±6	19±2
boiled extract	85±5	14±2
boiled extract + cobalamin [b]	660±28	19±5
boiled extract + 12,13-di-epi-F_{430} [b]	106±2	17±2
cobalamin	556±49	22±3
12,13-di-epi-F_{430}	21±2	14±2

[a] 50 μl cell extract added per vial (corresponding to 1.6 mg of protein before boiling).
[b] 2.5 μl 5 mM aquocob(III)alamin or 5 mM 12,13-di-epi-F_{430} added.

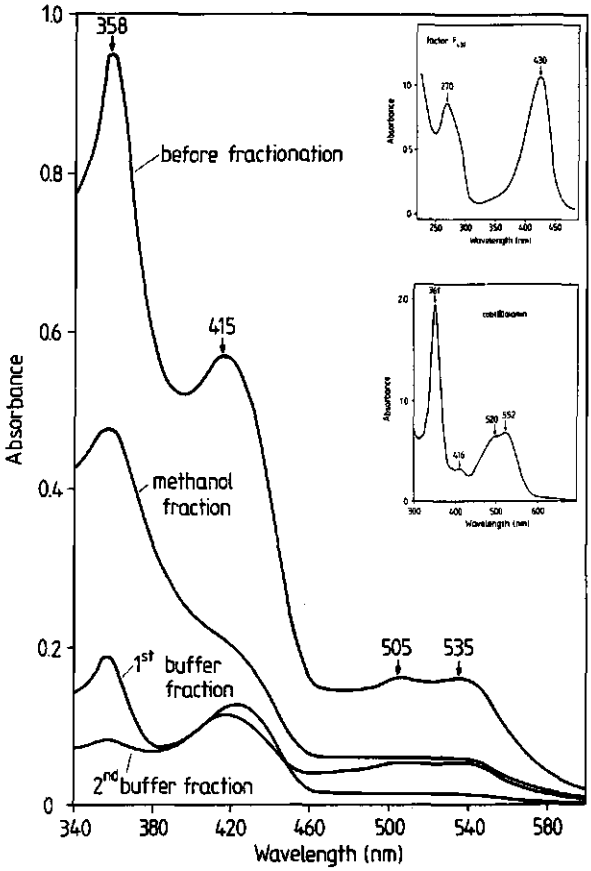

FIGURE 3.3. *Visible absorption spectra of oxidized boiled extracts before and after fractionation* with a XAD-4 column. Insets: Absorption spectra of native F_{430} (redrawn from [10]) and hydroxocob(III)alamin (redrawn from [25]).

dechlorinating activities of boiled cell extracts. Dechlorination by cobalamin or 12,13-di-epi-F_{430} was also not influenced by the heat treatment. Ethene production rates by native F_{430} after incubation at 120°C were only 50% of the rates measured in controls which were not heat-treated. This difference was probably a result of autocatalytic epimerization of native F_{430} to 12,13-di-epi-F_{430} which proceeds much faster at elavated temperatures [212,250]. Ethene production by 12,13-di-epi-F_{430} was indeed about 50% of the rate of native F_{430}. High dechlorinating activities in the fraction which passed a 10 kDa filter showed involvement of a compound with a low molecular mass in dechlorination by boiled cell extracts. Activities in fractions which were eluted from a XAD-4 column by buffer or 80% methanol revealed that part of the dechlorinating components had a hydrophilic, and part a hydrophobic character. Pure cob(III)alamin could only be removed from the XAD-4 column with 80% methanol, whereas a large portion of pure factor F_{430} eluted in the buffer fractions.

Oxidized boiled extracts had a characteristic orange color. Visible absorption spectra of oxidized boiled extracts displayed peaks at 358, 415, 505, and 535 nm (Fig. 3.3). These peaks are typical for spectra of cob(III)alamin (compare inset Fig. 3.3). However, the peak at 415 nm is too high to be explained only by absorption by corrinoids. This peak is probably a sum of absorption by corrinoids, factor F_{430}, and perhaps also factor F_{420} [43]. The shift in absorptions at 358 and 415 nm in the different fractions of the XAD-4 column correspond with elution profiles of cob(III)alamin and factor F_{430} (Table 3.4). The spectra of the first and second buffer-fraction show high absorption at 415 nm, indicating that mainly factor F_{430} is present. The methanol fraction has a spectrum which clearly resembled a cob(III)alamin spectrum.

With inhibitors it was tried to distinguish between cobamide or factor F_{430} catalyzed reactions in boiled cell extracts. 1-Iodopropane which inhibits cobamide-catalyzed reactions by alkylation of the corrinoid [32] and nitrous oxide which oxidizes cob(I)alamin to cob(II)alamin [12] were tested. Both inhibitors totally inhibited dechlorination of 1,2-DCA by boiled extracts (data not shown). However, in experiments with the pure cofactors as catalysts 1-iodopropane and nitrous oxide did not only inhibit dechlorination by cobalamin but also dechlorination by factor F_{430}. Propane was formed from 1-iodopropane in assays with factor F_{430}. In incubations with cobalamin, this product was not found. This indicates that inhibition of factor F_{430} catalyzed 1,2-DCA dechlorination by 1-iodopropane was competitive and was not a consequence of irreversible alkylation of factor F_{430} as it is reported for the inhibition of corrinoids [32].

TABLE 3.4. Comparison of chemical properties of the 1,2-DCA dechlorinating components in boiled cell extracts of *Methanosarcina barkeri* with chemical properties of cobalamin or factor F_{430}

Treatment	Product formation rates				
	(pmol/min per mg of protein)		(pmol/min per nmol of catalyst)		
	Boiled extracts [a]		Cobalamin[b]	Factor F_{430}[b]	
				Native	Diepimer
	Ethene	CA	Ethene	Ethene	Ethene
none	143±18	21±6	190±23	24.7±0.4	10.1±0.3
preincubation at 120°C for 20 min	159±9	28±5	189±14	10.1±1.2	10.3±0.9
oxidation and again reduction [c]	164±12	30±7	n.d.[d]	n.d.	n.d.
fractionation with a centricon-10 microconcentrator [e]					
fraction which passed the filter	140±0	64±1	n.d.	n.d.	n.d.
fraction which did not pass the filter	50±2	20±1	n.d.	n.d.	n.d.
fractionation with a XAD-4 column [f]					
before fractionation	146±15	34±12	197±22	24.4±0.8	10.1±0.3
first buffer-fraction	72±4	14±4	0.6±0.3	5.5±1.6	1.6±1.0
second buffer-fraction	18±1	9±2	0.3±0.1	18.6±0.6	5.7±0.0
methanol fraction	113±26	42±17	175±19	4.7±0.4	2.8±0.7

[a] Activity calculated per mg of protein present in crude cell extract.
[b] Only ethene production determined in assays with pure cofactors.
[c] Oxidation occured by exposure to air for 30 minutes.
[d] n.d. = not determined.
[e] The centricon-10 microconcentrator is a 10 kDa filter unit.
[f] Activities for cofactors calculated per nmol catalyst added before fractionation.

3.5. Discussion

In previous studies it was shown that reductive dechlorination of 1,2-DCA by cell suspensions of methanogenic bacteria depended on biological activity [Chapter 2], but, it was not possible to distinguish between a specific catabolic enzymatic activity or a cometabolic dechlorination. The dechlorination of 1,2-DCA by cobalamin and factor F_{430} or by boiled cell extracts of *M. barkeri* in buffer reduced with Ti(III)-citrate indicates that the dechlorination of 1,2-DCA to ethene and CA is a cometabolic activity of enzymes containing corrinoids or factor F_{430} as prosthetic group. Comparing ratios of the two products ethene and CA as found in incubations with cell extracts, with ratios found with pure cofactors suggested that corrinoids and factor F_{430} were both active in cell extracts. Chemical properties of the dechlorinating components in boiled cell extracts and spectral analysis of these extracts supported activity of both cofactors. We determined a corrinoid content of *M. barkeri* grown on methanol of 1.3 nmol/mg protein which is similar to reported corrinoid contents of 0.62 and 1.7 nmol/mg protein [55,105]. For the factor F_{430} content of *M. barkeri* only one concentration was reported, namely 0.4 nmol/mg protein [62]. Product formation rates per mg of protein were calculated from rates measured with pure cofactors and the measured and reported cofactor contents. This resulted in 122-329 pmol/min per mg of protein for ethene production and 8-16 pmol/min per mg of protein for CA production. Measured rates by boiled cell extracts of 54-164 pmol/min per mg of protein for ethene production and of 9-64 pmol/min per mg of protein for CA production are in the same order of magnitude as the calculated rates.

Spectral analysis of a reaction mixture with dithiothreitol showed that cob(II)alamin was the predominant form (data not shown). In the presence of Ti(III) citrate, cobalamin was completely reduced to the Co(I) form [142]. Low dechlorination rates with dithiothreitol and high rates with Ti(III) citrate indicates that cob(I)alamin is the active catalyst. Cobalamin dechlorinated 1,2-DCA mainly *via* a dihalo-elimination to ethene. CA, the product of a hydrogenolysis of 1,2-DCA, was only formed in minor amounts. This pattern can be explained by the reaction mechanism described by Scheffold et al. [230] for the cobalamin catalyzed reductive elimination of *vicinal* leaving groups (Fig. 3.4A). Cob(I)alamin, formed by Ti(III) citrate [142] reacts with 1,2-DCA *via* a classical S_N2 mechanism [240] to form an alkyl-cobalt(III)complex followed by a ß-elimination to generate ethene, two chloride ions, and cob(III)alamin. Because the reaction is carried out in a protic solvent, a net hydrogenolysis to CA occurs as a side reaction [230].

Factor F_{430} formed almost equal amounts of ethene and CA, respectively. Factor F_{430} can only carry out one-electron reductions. Possible reaction mechanisms are illustrated in Figure 3.4B. The two-electron reduction of 1,2-DCA could take place (i) *via* a alkyl-nickel(II)-intermediate ① or (ii) *via* a free radical ② which is rapidely scavenged by a second reduced factor F_{430} ③ or which abstracts a hydrogen atom from the medium ④ to form CA. The carbanion complex formed by ① or ③ can be protonated ⑤ to form CA or undergo a ß-elimination ⑥ to form ethene. Studies with factor F_{430} and iodomethane supported the first mechanism which proceeds *via* a methyl-nickel(II)-intermediate [134]. Evidence for the second mechanism is provided by studies with a macrocyclic nickel(I) complex. These studies showed that the reaction with alkyl halides proceeds *via* the formation of free radicals [9]. The relative high amount of CA formed by factor F_{430} in our study indicates that the dechlorination by factor F_{430} proceeds *via* a free radical as proposed for reductive dehalogenation reactions catalyzed by iron(II) porphyrins [292]. The exact mechanism and an explanation for the different ethene/CA ratios found with native F_{430} or 12,13-di-epi-F_{430} remains to be elucidated.

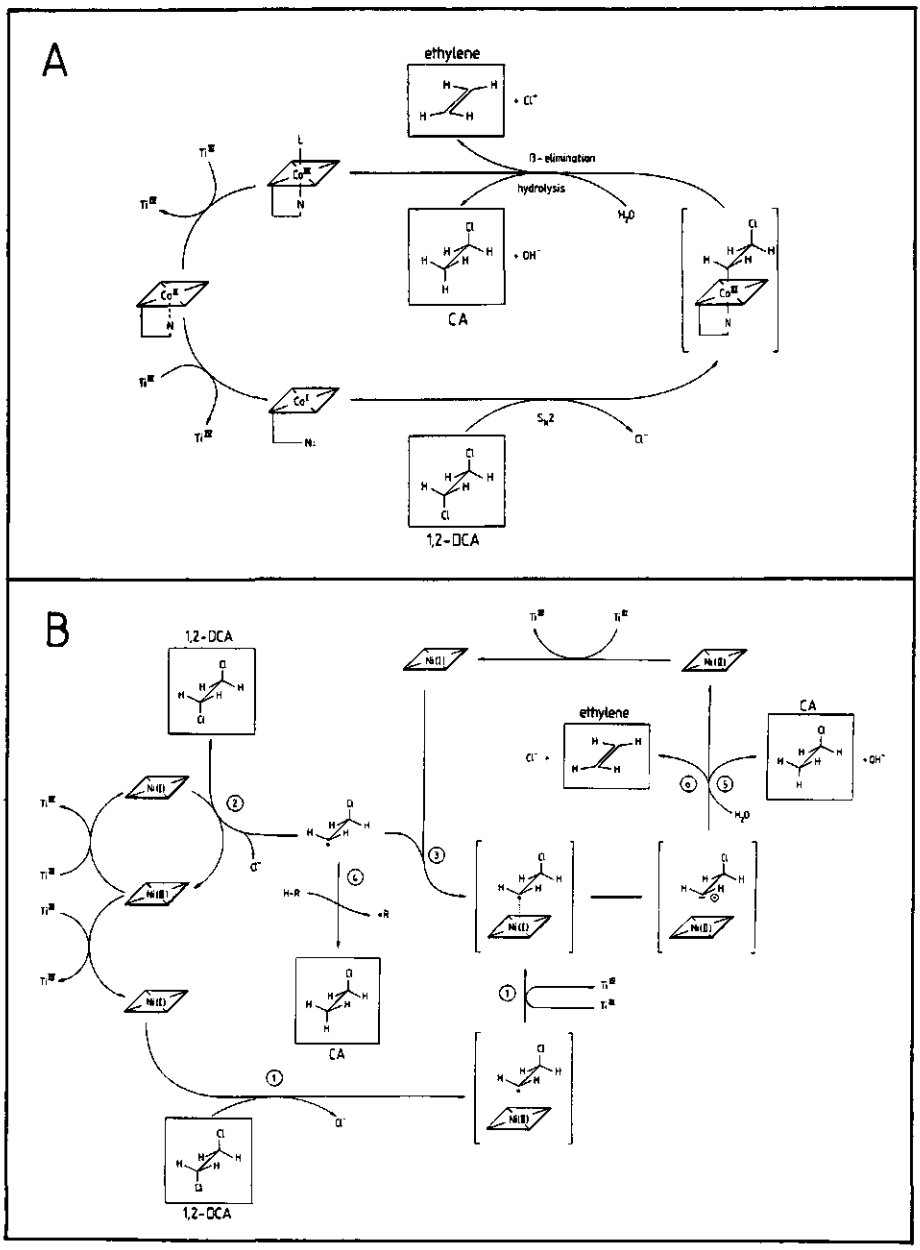

FIGURE 3.4. Proposed reaction mechanisms for the reductive dechlorination of 1,2-DCA to ethene and CA catalyzed by cobalamin (A) or factor F_{430} (B).

Cell suspensions of *M. barkeri* had the highest dechlorination rates in relation to methane formed when cultivated on H_2/CO_2 [Chapter 2]. These cells do not have the highest level of acetyl-CoA pathway enzymes [158] which indicates that in methanogens also other enzyme systems are involved in the transformation of chlorinated hydrocarbons. Possible candidates are the corrinoid-containing 5-methyl-H_4MPT : 5-hydroxy-benzimidazolyl cobamide methyltransferase [141] or the factor F_{430}-containing methyl-CoM reductase [78]. Both enzymes are involved in the last step of methanogenesis and each of these enzymes accounts for up to 5-10% of total cell protein [5,78,141]. In addition, free factor F_{430} present in cells cultivated on Ni-sufficient medium [5] could also be an *in vivo* catalyst. Which of the possible catalysts listed above are responsible for the dechlorination of 1,2-DCA *in vivo* is currently under investigation in our laboratory.

Acknowledgements

We thank N. Slotboom for the drawings and J.T. Keltjens for the generous gift of *M. thermoautotrophicum* ΔH cells and for helpful advice with the isolation and purification of factor F_{430}.

SUPPLEMENT

CHAPTER 3

Isolation of factor F_{430} and characterization of the redox chemistry of 12,13-di-epi-F_{430} by EPR and UV-visible spectroscopy

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3.6. Isolation of factor F_{430}

In the following, the method used for the isolation of factor F_{430} is described in detail. All steps of the isolation were carried out at lowest possible temperatures to avoid epimerization of native F_{430}. A batch of 275 g frozen *Methanobacterium thermoautotrophicum* ΔH cells (wet weight) were resuspended in 300 ml demineralized water and the cell suspension was cooled to 0°C. The pH of the suspension was adjusted to 2 by slow addition of 0.5 M $HClO_4$ under continuous stirring at 0°C. The suspension was stirred for another 45 min. Precipitates were removed by centrifugation for 30 min at 27,000 x *g* at 4°C. The pellet obtained was washed with 200 ml 15 mM $HClO_4$ and the suspension was again centrifuged. This step was repeated four times. The supernatants were combined and adjusted to pH 7 with 1 M $KHCO_3$. Precipitated $KClO_4$ was removed by centrifugation. The supernatant was adjusted to pH 4.5 with 5 M HCl. Cofactors were concentrated using Sep-Pak C-18 Cartridges (Millipore Corp., Milford, MA, U.S.A.). Three to four cartridges were connected in series to minimize loss of cofactors. The cartridges were activated with 10 ml 50% methanol, followed by rinsing with 10 ml demineralized water, and 10 ml 30 mM sodium acetate pH 4.5 containing 1 M NaCl. When cartridges were saturated, cofactors were eluated with 50% methanol. Before additional application of crude cofactor fraction, cartridges were again rinsed with demineralized H_2O and 30 mM sodium acetate pH 4.5 containing 1 M NaCl. The methanol fractions were combined and flash evaporated to dryness. The dry material was resuspended in 30 ml 50 mM potassium phosphate pH 6. About 8 ml of this fraction was applied (4 ml/min) to a QAE-Sephadex A-25 column (1.6 x 100 cm), equilibrated with 50 mM potassium phosphate pH 6 at 4°C. Effluent was monitored at 280 and 436 nm. Cofactors were eluted with the following NaCl gradient: 0 M (40 ml), 0-0.15 M (480 ml), 0.15 M (4320 ml), 0.15-1 M (240 ml), 1 M (480 ml). Factor III (corrinoid) eluted first, followed by flavins, methanopterins, native F_{430}, 12,13-di-epi-F_{430}, 13-epi-F_{430}, F_{560}, F_{390}, and F_{420} (Fig. 5). Native F_{430} eluted between 3000-3850 ml, 12,13-di-epi-F_{430} between 3900-4800 ml.

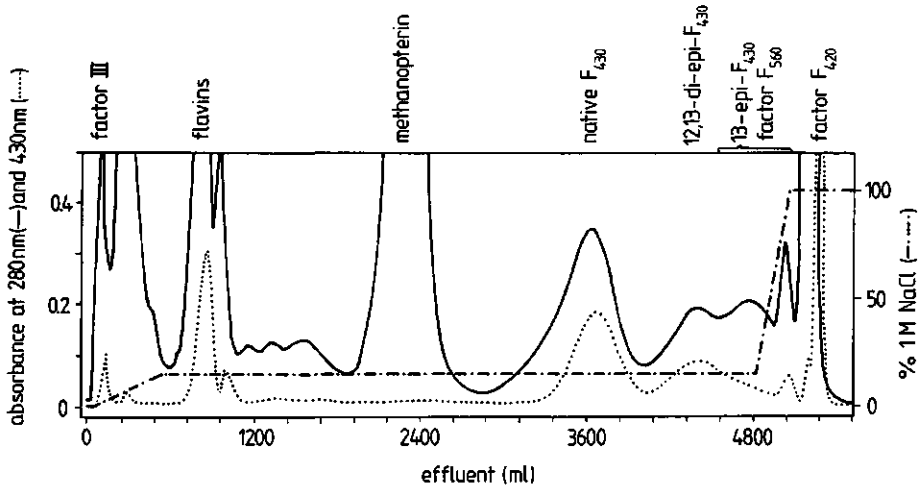

FIGURE 3.5. Elution of cofactors from the QAE-Sephadex A-25 column.

Fractions containing native F_{430} or 12,13-di-epi-F_{430} were combined, NaCl was added up to about 1 M and the pH was adjusted to 4.5. Cofactors were concentrated with Sep-Pak C-18 Cartridges. Purity was checked with UV-visible spectroscopy. Factor F_{430} was stored under N_2 at -20°C. Due to severe shrinking of the QAE-Sephadex A-25 column support by the NaCl gradient, the column had to be repacked between individual preparation runs.

3.7. Redox chemistry of 12,13-di-epi-F_{430}

The amounts of 1,2-DCA dechlorinated by factor F_{430} in buffer with Ti(III) citrate as reducing agent must have been a result of multiple turn-over cycles of factor F_{430} in the dechlorination assay. This indicated that in an aqueous system the reduction of factor F_{430} was reversible. Thus far a reversible one-electron reduction of factor F_{430} has only been reported for the pentamethyl ester of factor F_{430} (factor F_{430}M) in aprotic solvents, namely tetrahydrofuran, dimethylformamide, acetonitrile, or butyronitrile [53,96,115,133,153]. Reported reduction potentials varied between -0.29 and -0.78 V *vs.* NHE. Factor F_{430}M chemically reduced by sodium amalgam in tetrahydrofuran was characterized by EPR and UV-visible spectroscopy. Attempts to reversibly reduce the pentacarboxylic acid factor F_{430} electrochemically under protic conditions failed. The observed irreversibility was a result of a hydrogenation of the π-system [A. Pfaltz, personal communication].

In the following, the determination of the midpoint potential of 12,13-di-epi-F_{430} by EPR-spectroelectrochemical titration in basic aqueous solution is presented. In addition, the reduced 12,13-di-epi-F_{430} in aqueous solution is also characterized with UV-visible spectroscopy.

In preliminary experiments, 12,13-di-epi-F_{430} was reduced in an EPR tube. An argon-purged 250-μl syringe was filled with 175 μl buffer followed by 25 μl of 5.18 mM 12,13-di-epi-F_{430} and 50 μl of 100 mM Ti(III) citrate pH 9. The solution was then transferred to an anaerobic EPR tube and mixed. The samples were kept at room temperature for 15 min before freezing in liquid nitrogen. All buffers contained 111 μM methyl and benzyl viologen. Experiments on the kinetics of reduction by Ti(III) citrate revealed that 12,13-di-epi-F_{430} was much faster reduced in the presence of these two dyes. EPR spectra were obtained with a Bruker ER-200 D spectrometer, with peripheral instrumentation and data acquisition as described in [214].

The Ni(II) in 12,13-di-epi-F_{430} could only be reduced in basic aqueous solution (Fig. 6). At pH's >10 Ni(II) was virtually quantitatively reduced to Ni(I). The amount of Ni(I) present correlates with the ethene and CA production rates by 12,13-di-epi-F_{430} at different pH's (Fig. 2G). This supports the assumption that the observed pH-dependence was an indirect effect of higher redox potentials rather than a direct effect on the dechlorination reaction itself.

Redox-titrations were carried out at pH 10.4 with Ti(III) citrate as titrant following the method of Dutton [71]. Methyl and benzyl viologen were used as mediators (both 100 μM). The amount of Ni(I) was calculated by measuring the intensity of the features at g = 2.057 and 2.238 of the EPR spectra and correlating those with intensities of a sample where Ni(I) was quantified by double integration of the EPR spectrum. A midpoint potential of -618 mV *vs.* NHE was calculated from a Nernst plot (Fig. 7). The slope of the Nernst plot of 60 mV is in agreement with the Ni(II) single electron reduction.

The reduction of 12,13-di-epi-F_{430} was also investigated by UV-visible spectroscopy under the same conditions as employed for EPR spectroscopy. Quartz cuvettes of 1 mm path length closed with Suba-Seals were made anaerobic and filled with solutions prepared in a syringe as described for the reduction in EPR tubes. UV-visible spectra were obtained with an SLM Aminco DW-2000 spectrophotometer interfaced to an IBM computer. The spectra were digitally

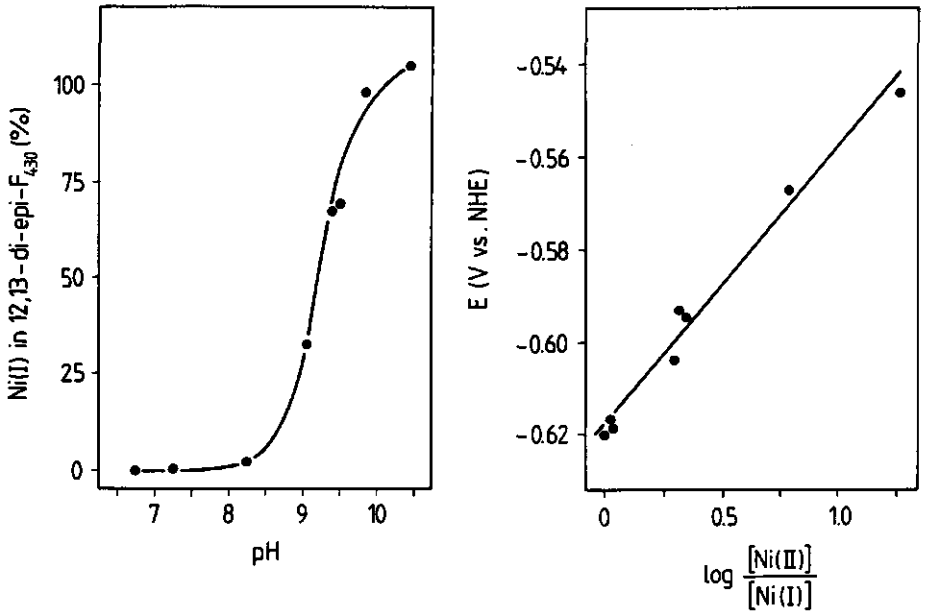

FIGURE 3.6. Ni(I) content of an aqueous 12,13-di-epi-F_{430} solution reduced by Ti(III) citrate at different pH's. The following buffers were applied: 100 mM MOPS (3-[N-Morpholino]-propanesulfonic acid) pH 6.12 and 7.02; 100 mM TRIS (Tris(hydroxymethyl)-aminomethane) pH 8.04 and 8.91; 50 mM TRIS/50 mM glycine pH 9.80; 50 mM CHES (2-[N-cyclohexylamino]-ethanesulfonic acid)/50 mM CAPS (3-[Cyclohexylamino]-1-propanesulfonic acid) pH 9.57, 10.27, and 10.97. The pH's in the figure are values of mixtures of 100 mM Ti(III) citrate pH 9 and buffer in a ratio of 1:4 (v/v) measured inside an anaerobic glovebox. Ni(I) was measured with EPR spectroscopy: microwave frequency, 9.30 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.8 mT; microwave power, 2 mW; temperature 110 K.

FIGURE 3.7. Nernst plot of a mediated redox titration of 0.259 mM aqueous 12,13-di-epi-F_{430} in 100 mM CAPS pH 10.4 with Ti(III) citrate at 25°C. The concentration of Ni(I) was determined by EPR spectroscopy (conditions identical to those for Fig. 3.6).

corrected by subtraction of the spectrum of an identical solution without 12,13-di-epi-F_{430}. An accurate determination of isosbestic points and extinction coefficients was not feasible due to substantial optical contributions of mediators and reductant. Optical monitoring of the reduction of 12,13-di-epi-F_{430} revealed bleaching of the 433 nm peak and apppearance of peaks at 376 and 710 nm. Spectra of oxidized and reduced 12,13-di-epi-F_{430} are shown in Figure 8. Both were very similar to spectra of oxidized and reduced factor $F_{430}M$ in tetrahydrofuran [133]. The main difference was a wavelength shift of the 754 nm absorbance to 710 nm in aqueous solution. The Ni(I) in 12,13-di-epi-F_{430} could be reoxidized by short exposure to air resulting in >95% restoration of the 433 nm absorbance peak (data not shown).

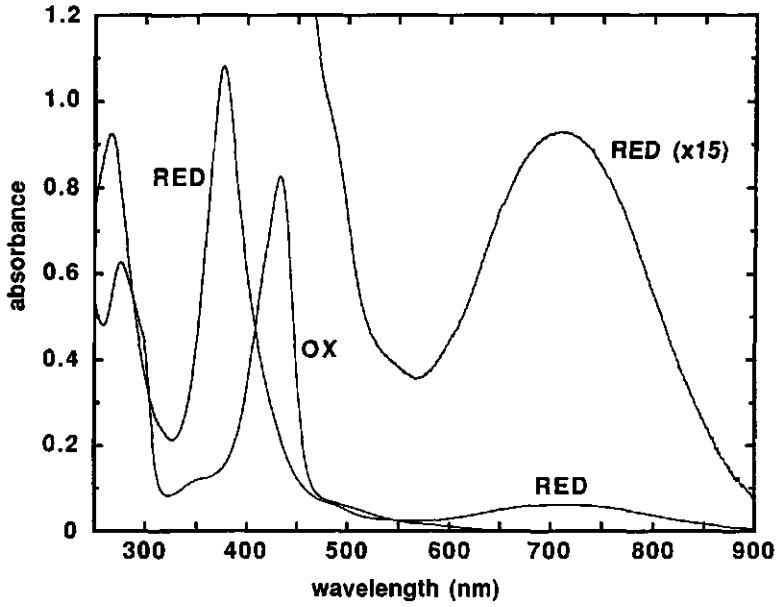

FIGURE 3.8. UV-visible spectra of oxidized and reduced 12,13-di-epi-F_{430} in 100 mM CAPS pH 10.4. Reduction was by Ti(III) citrate (final concentration 4 mM) at ambient temperature for 20 min. Quartz cuvettes of 1 mm path length were used. Spectra were corrected for contributions from blank solutions, i.e. without 12,13-di-epi-F_{430} but with mediators (100 μM methyl and benzyl viologen) and reductant.

EPR as well as UV-visible spectroscopy showed that Ni(II) in 12,13-di-epi-F_{430} can be quantitatively and reversibly reduced under protic conditions by the action of Ti(III) citrate. Preliminary EPR/UV-visible spectroscopy experiments with native F_{430} in basic aqueous solution showed that the Ni(I) form of this epimer can also be obtained by reduction with Ti(III) citrate.

CHAPTER 4

Methyl-coenzyme M reductase of *Methanobacterium thermoautotrophicum* strain ΔH catalyses the reductive dechlorination of 1,2-dichloroethane to ethene and chloroethane

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Alfons J. M. Stams, and Alexander J. B. Zehnder

4.1. Abstract

Reductive dechlorination of 1,2-dichloroethane (1,2-DCA) to ethene and chloroethane (CA) by crude cell extracts of *Methanobacterium thermoautotrophicum* strain ΔH with H_2 as electron donor was stimulated by MgATP. The heterodisulfide of coenzyme M and 7-mercaptoheptanoyl-threonine phosphate together with MgATP partially inhibited ethene production but stimulated CA production as compared to MgATP alone. The pH optimum for the dechlorination was 6.8 (at 60°C). Michaelis-Menten kinetics for initial product formation rates with different 1,2-DCA concentrations indicated the enzymatic character of the dechlorination. Apparent K_m's for 1,2-DCA of 89 and 119 μM, and V_{max}'s of 34 and 20 pmol/min per mg protein were estimated for ethene and CA production, respectively. 3-Bromopropanesulfonate, a specific inhibitor for methyl-CoM reductase, completely inhibited dechlorination of 1,2-DCA. Purified methyl-CoM reductase, together with FAD and a crude component A fraction which reduced the nickel of factor F_{430} in methyl-CoM reductase, converted 1,2-DCA to ethene and CA with H_2 as electron donor. In this system methyl-CoM reductase was also able to transform its own inhibitor 2-bromoethanesulfonate to ethene.

4.2. Introduction

Hydrogenotrophic and acetoclastic methanogenic bacteria reductively dechlorinate 1,2-dichloroethane (1,2-DCA) to ethene and chloroethane (CA) [19,72, Chapter 2]. Corrinoids or factor F_{430}, two cofactors present in high amounts in methanogens [55,63,105,157], catalyzed the same transformations in buffer with Ti(III) citrate as electron donor [Chapter 3].

Corrinoids are found in the soluble as well as the membrane fraction of methanogens [55]. Despite the high cobamide content, the role of this cofactor has not yet been fully established. Similar to already known functions of corrinoids in other organisms, cobamides in methanogens are thought to be involved mainly in methyl transfer-reactions. This role was verified for the highly purified methanol:5-hydroxybenzimidazolyl-cobamide methyltransferase of *Methanosarcina barkeri*, an enzyme involved in methanogenesis from methanol [276,277,278,279]. A possible function as a "redox-protein" was proposed for a 33 kDa purified corrinoid-containing membrane protein of *Methanobacterium thermoautotrophicum* strain Marburg [241]. A monospecific polyclonal antiserum against the 33 kDa corrinoid-containing membrane protein of strain Marburg cross-reacted with the 33 kDa and 31 kDa subunits of the corrinoid-containing 5-methyl-tetrahydromethanopterin: 5-hydroxy-benzimidazolyl cobamide methyltransferase isolated from *M. thermoautotrophicum* strain ΔH [141,264]. Based on this result a possible function as methyltransferase was suggested for the membrane protein [264]. Other processes where a corrinoid enzyme is involved are the methanogenesis from acetate and CO_2 fixation by methanogens. Both reactions are performed *via* the acetyl-CoA pathway (Wood pathway) where a corrinoid/iron-sulfur protein and CO dehydrogenase are central enzymes [95,170,268,306]. The involvement of corrinoid enzymes in the methanogenesis from acetate was shown in cell extracts of *Methanosarcina barkeri* [37,280].

Factor F_{430}, a hydrocorphinoid nickel(II) complex [213] only found in methanogens, can exist in two forms, a protein-bound and a free form [5,118]. The free form was only present under Ni-sufficient growth conditions [5]. Factor F_{430} is the chromophore of the methyl-coenzyme M reductase which catalyses the last step in methanogenesis and contains 2 mol factor F_{430}/mol enzyme [78,79,80,118,194]. Similar to the model for corrinoid-catalyzed methyl transfer reactions

[11,278], it is suggested that methyl-coenzyme M reductase is only active if the transition metal in the prosthetic group is present in the most reduced form (Ni(I) state) [2,223]. 7-Mercapto-heptanoylthreonine phosphate (component B, H-S-HTP) was found to be the electron donor for the methyl-coenzyme M (CH_3-S-CoM) reduction and the products of the reaction are a heterodisulfide (CoM-S-S-HTP) and methane [22,79]. The exact reaction mechanism, however, remains to be elucidated.

Reductive activation is a prerequisite for both methyltransferases and the methyl-CoM reductase in *in vitro* incubations [109,142,224,279,296]. The enzyme system (component A) responsible for the reductive activation of the methyl-CoM reductase (component C) was intensively studied in *M. thermoautotrophicum* strain ΔH. Component A was resolved into four different enzyme fractions [110,196,223,224]. The cofactors MgATP, FAD, and H-S-HTP were required for reconstitution of an active enzyme system [196,224], whereas coenzyme F_{420} and cobalamin stimulated methanogenic activity [196]. Enzymatic reductive activation of methyltransferases was only studied in crude cell extracts. These activations are also dependent on MgATP [142,279]. In addition to MgATP, methyl-tetrahydromethanopterin: coenzyme M methyltransferase activity can be stimulated by the heterodisulfide CoM-S-S-HTP [140].

In this study we report on the effect of MgATP and CoM-S-S-HTP on the reductive dechlorination of 1,2-DCA by crude cell extracts of *M. thermoautotrophicum* strain ΔH with H_2 as electron donor. Experiments with specific inhibitors for methyltransferase or methyl-CoM reductase activity showed that methyl-CoM reductase was responsible for the dechlorination. Purified component C, a crude component A fraction, and FAD were required for reconstitution of H_2-dependent reductive dechlorination of 1,2-DCA.

4.3. Materials and Methods

4.3.1. Materials

All chemicals were at least of analytical grade. Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) was purchased from Serva Feinbiochemica (Brunschwig Chemie, Amsterdam, The Netherlands), 1,2-dichloroethane (1,2-DCA) from Aldrich (Brussels, Belgium), and hydroxocob(III)alamin from Fluka (Perstorp Analytical, Oud-Beijerland, The Netherlands). ATP, GTP, FAD, and hexokinase were purchased from Boehringer Mannheim (Almere, The Netherlands), formaldehyde, 2-mercaptoethanesulfonic acid (H-S-CoM), Ti(III) chloride and tris(hydroxy-methyl)-aminomethane (TRIS) from E. Merck (Amsterdam, The Netherlands). 2-Bromoethane-sulfonic acid (BrES) and dithiothreitol (DTT) were from Janssen Chimica (Tilburg, The Netherlands), Q-Sepharose (fast flow) and Phenyl-Sepharose CL-4B from Pharmacia Biosystems (Woerden, The Netherlands). Gases and chloroethane (CA) were from Hoekloos (Schiedam, The Netherlands). The oxygen concentration in the anaerobic glovebox was kept low with R-20 palladium catalyst provided by BASF (Arnhem, The Netherlands). Ti(III) citrate was prepared from $TiCl_3$ and sodium citrate as previously described [Chapter 3]. CoM-S-S-HTP and H-S-HTP isolated from *M. thermoautotrophicum* strain ΔH, and CH_3-S-CoM, prepared by methylation of H-S-CoM with dimethylsulfate [139], were generous gifts of Dr J.T. Keltjens (University of Nijmegen, The Netherlands). Coenzyme F_{420}, isolated from *M. thermoautotrophicum* strain ΔH, was a generous gift of Dr B. Gruson and Dr Ph. Debeire (INRA, Villeneuve d'Ascq, France). 3-Bromopropanesulfonate (BrPS), synthesized as described by Ellermann et al. [80], was a generous gift of Prof Dr R.K. Thauer (Marburg, Germany). Factor F_{430} was isolated from *M. thermoautotrophicum* strain ΔH as described previously [Chapter 3].

4.3.2. Growth of the organism and preparation of cell extracts

Methanobacterium thermoautotrophicum strain ΔH (DSM 1053) was routinely subcultured at 65°C in 120 ml serum bottles with 20 ml of a defined phosphate/bicarbonate buffered mineral medium [238] on a rotary shaker at 200 rpm. The gas phase was H_2/CO_2 (4/1, v/v; 2 bar). The medium was reduced with 0.1 mM Ti(III) citrate and 1.5 mM cysteine/HCl, and 2 mM $Na_2S_2O_3$ was added as additional sulfur source [151]. *M. thermoautotrophicum* was mass cultured in a 10 l glass/steel fermenter containing 8 l medium with the same composition as described above. The medium was flushed with H_2/CO_2 (4/1) and vigorously stirred. Cells were harvested at the late log phase by anaerobic continuous centrifugation (Carl Padberg Zentrifugenbau GmbH, Lahr/Schwarzwald, Germany) at ambient temperature, washed in 50 mM PIPES/KOH pH 7.0 containing 1 mM DTT (buffer A) and centrifuged 30 min at 27,500 × g and 4°C. The pellet was resuspended in the same buffer containing 50 μg DNase/ml in a ratio of 1:5 (w/v). Cells were disrupted by passing twice through a French pressure cell at 135 MPa and cell debris were removed by centrifugation at 27,500 × g and 4°C. The supernatant containing 28 mg protein/ml, is referred to as crude cell extract. It was stored under 100% H_2 at -20°C.

4.3.3. Preparation of enzyme fractions

Crude cell extract was first centrifuged anaerobically at 110,000 × g and 4°C before fractionation at ambient temperature with a high resolution fast protein liquid chromatography (FPLC) system (Pharmacia Biosystems, Woerden, The Netherlands) in an anaerobic glovebox. The supernatant (20-40 ml) was applied (4 ml/min) to a Q-Sepharose (fast flow) column (2.2 × 8 cm, 30 ml) equilibrated with 50 mM TRIS/HCl pH 7.6 + 0.1 mM DTT (buffer B) containing 0.2 M NaCl. Proteins were eluted with the following linear NaCl gradient: 0.2 M (200 ml), 0.2-0.5 M (240 ml), 0.5 M (80 ml). The effluent was monitored for protein at 280 nm. The first 200 ml were pooled and concentrated to 2 ml in an Amicon Diaflo ultrafiltration cell equipped with a PM 30 filter (Grace B.V., Rotterdam, The Netherlands). The concentrated protein fraction was diluted with 50 ml buffer A and again concentrated to 3.2 ml. This protein solution served as crude component A fraction as described by Hartzell et al. [117]. Ethylene glycol was added (20%, v/v) [196] and the fraction was stored under H_2 at -20°C in the anaerobic glovebox after analyzing for component A activity. Methyl-CoM reductase, detected by its yellow colour, eluted after 384 ml in a volume of approximately 48 ml. After addition of potassium acetate (2 M) this fraction was applied (1 ml/min) to a Phenyl-Sepharose CL-4B column (2.2 × 6 cm) equilibrated with buffer B containing 2 M potassium acetate. Methyl-CoM reductase was eluted by a linear potassium acetate gradient: 2 M (96 ml), 2-1 M (30 ml), 1 M (72 ml). The enzyme eluted after 120 ml in a total volume of approximately 30 ml. This fraction was diluted with 30 ml of buffer A, concentrated to 1 ml in an Amicon Diaflo ultrafiltration cell equipped with a PM 30 filter, diluted with 60 ml buffer A and concentrated again to 3 ml. Glycerol was added (40%, v/v) [79] and the solution was stored under H_2 at -20°C. The methyl-CoM reductase solution contained 9-15 mg protein/ml.

4.3.4. Enzyme assays

The assays were performed in 9 ml serum type vials sealed with butyl rubber stoppers or with viton stoppers in the case of assaying dechlorination. All components necessary for the different assays were dissolved in anaerobic buffer and added to the reaction vial inside the anaerobic

glovebox. The specific reaction mixtures are given in legends to tables and figures. The vials were put on ice and the gas phase was changed. The gas phase was in general 100% H_2 (1.2 bar) unless otherwise stated. The reaction was started by increasing the temperature from 0°C to 60°C. At times indicated one vial was sacrificed for analysis.

Methyltransferase assay. The methyl-H_4MPT:HS-CoM methyltransferase reaction was assayed by measuring formaldehyde conversion to CH_3-S-CoM in the presence of excess H-S-CoM as described by Kengen et al. [142] with the modification that no BrES was added.

Methyl-CoM reductase assay. Methyl-CoM reductase in crude extracts was assayed in the reaction mixture used for the methyltransferase except that formaldehyde and H-S-CoM were omitted and CH_3-S-CoM was added instead. The gas phase was analyzed for CH_4 produced. Assays with purified methyl-CoM reductase (component C) were either carried out in a reaction mixture described by Ellermann et al. [79,80] or by reconstitution of the terminal step (component A, B, and C) described by the group of Wolfe [110,196,223,224]. The first reaction mixture contained: 50 mM potassium phosphate pH 7.0, 1.25 mM Ti(III) citrate, 7.5 mM DTT, 0.1 mM hydroxocob(III)alamin, 0.7 mM H-S-HTP, 10 mM CH_3-S-CoM, and 20 μl component C (0.25 mg of protein) in a volume of 500 μl. The gas phase was 100% N_2. The second reaction mixture contained: 50 mM PIPES/KOH pH 6.8 (at 60°C), 25 mM $MgCl_2$, 2.5 mM ATP, 0.5 mM CoM-S-S-HTP, 25 μM cofactor F_{420}, 25 μM FAD, 25 μM hydroxocob(III)alamin, 1.5 mM H-S-HTP, 10 mM CH_3-S-CoM, 40 μl component A (0.11 mg of protein), and component C (0.25 mg of protein) in a volume of 200 μl. The gas phase was 100% H_2.

Dechlorination assay. For assaying dechlorination by crude cell extracts formaldehyde, H-S-CoM, or CH_3-S-CoM were omitted from the reaction mixture and 1,2-DCA in isopropanol (100 mM, unless otherwise stated) was added by syringe shortly before increasing the temperature to 60°C. Dechlorination with purified methyl-CoM reductase was assayed in two different reaction mixtures. One reaction mixture contained 50 mM buffer (PIPES/KOH pH 7.0 or TRIS/HCl pH 9.0), 10 mM Ti(III) citrate, 7.5 mM DTT, 20 μl component C (0.25 mg of protein), and 1 mM 1,2-DCA in a volume of 500 μl. The gas phase was 100% N_2. The second reaction mixture is described in legends to Tables 2 and 3. H_2 served as electron donor. 5 μl 1,2-DCA in isopropanol (100 mM) was added to this reaction mixture after a preincubation of 5 min at 60°C. In all assays, the gas phase was analyzed for ethene or CA produced.

4.3.5. Other determinations

The analyses of ethene and CA were done as described previously [Chapter 3]. Methane was analyzed with the same gaschromatograph used for analysis of ethene. Protein was determined with Coomassie brillant blue G250 by the method of Bradford [31] with bovine serum albumin as standard.

4.4. Results

4.4.1. Effect of MgATP or CoM-S-S-HTP on 1,2-DCA dechlorination

For the corrinoid containing methyl-tetrahydromethanopterin: coenzyme M methyltransferase and the factor F_{430}-containing methyl-CoM reductase of *Methanobacterium thermoautotrophicum* strain ΔH a reductive MgATP-dependent preactivation is required for activity in *in vitro* systems [110,142,224]. In addition to MgATP, the heterodisulfide CoM-S-S-HTP stimulated methyl-tetrahydromethanopterin: coenzyme M methyltransferase activity in crude cell extracts [140]. In

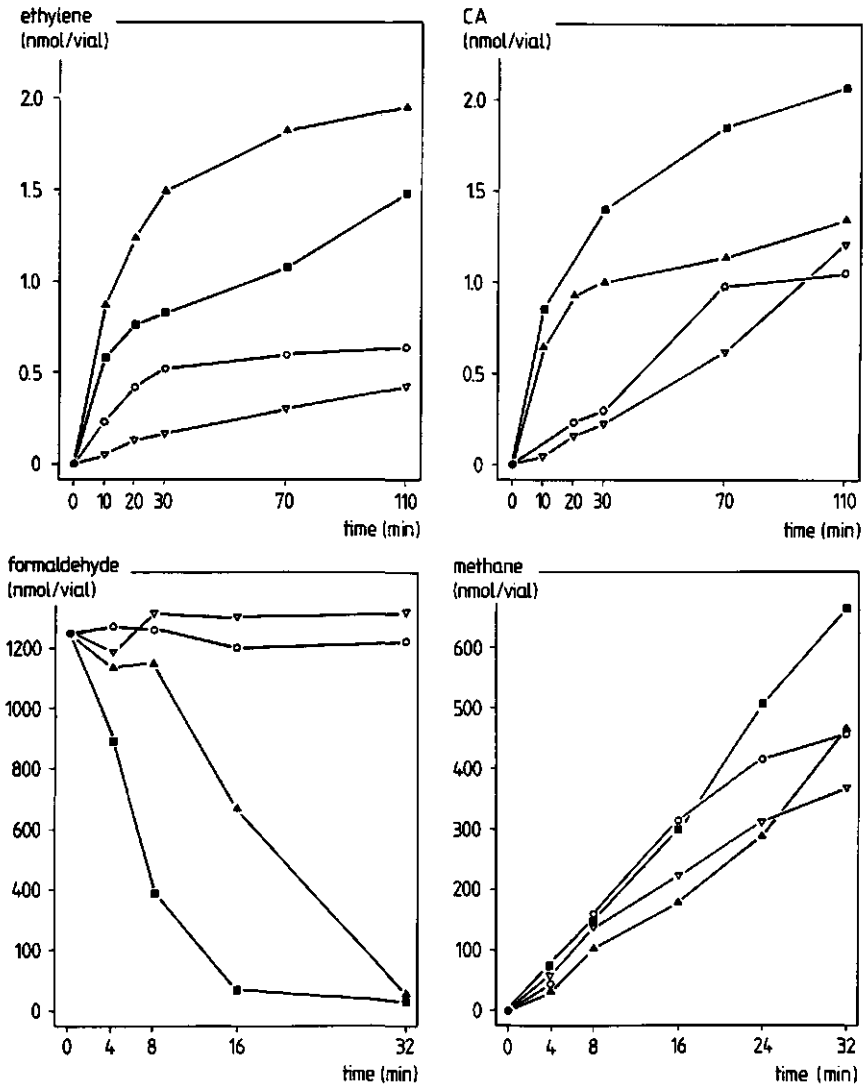

FIGURE 4.1. Effect of MgATP and CoM-S-S-HTP on the reductive dechlorination of 1,2-DCA by crude cell extracts of *M. thermoautotrophicum* strain ΔH. Reaction mixture contained: 50 mM PIPES/KOH pH 6.8 (at 60°C), 25 mM $MgCl_2$, 2.5 mM ATP, 0.5 mM CoM-S-S-HTP, and 100 μl crude cell extract (2.8 mg of protein) in a volume of 500 μl. For formaldehyde conversion the reaction mixture contained in addition 5 mM formaldehyde and 5 mM H-S-CoM, for methane formation 5 mM CH_3-S-CoM. For dechlorination experiments 5 μl of 100 mM 1,2-DCA in isopropanol were added by syringe. Assays were carried out with complete reaction mixture (■) or with reaction mixtures where either CoM-S-S-HTP (▲), $MgCl_2$ + ATP (▽), or $MgCl_2$ + ATP + CoM-S-S-HTP (○) was omitted. Omission of ATP, $MgCl_2$ + CoM-S-S-HTP, and ATP + CoM-S-S-HTP followed approximately the lines where $MgCl_2$ + ATP + CoM-S-S-HTP was not added.

order to get indications about the possible involvement of the methyl-tetrahydromethanopterin: coenzyme M methyltransferase or the methyl-CoM reductase in the reductive dechlorination of 1,2-DCA by crude cell extracts of *M. thermoautotrophicum* strain ΔH, the effect of MgATP and CoM-S-S-HTP on this reaction was investigated.

MgATP alone indeed stimulated ethene or CA production from 1,2-DCA (Fig. 4.1). However, dechlorination also occurred in the absence of MgATP though at lower rates. The effect of MgATP was clearest within the first 30 min of incubation. If incubations proceeded, ethene production remained significantly higher in the presence of MgATP, whereas CA almost reached the same level in controls where nothing was added. MgGTP gave the same stimulation as MgATP (data not shown). The effect of MgATP was also tested on formaldehyde conversion (methyltransferase activity) or methane production from CH_3-S-CoM (methyl-CoM reductase) with the same cell extracts. Just as in earlier reports [142], a decrease in formaldehyde concentration was only found in the presence of MgATP (Fig. 4.1). MgATP had no effect on methane production from CH_3-S-CoM (Fig. 4.1). This is probably due to active methyl-CoM reductase still present in these extracts [223].

CoM-S-S-HTP alone inhibited ethene production, but had no effect on CA production or methane formation (Fig. 4.1). Formaldehyde was not converted in the presence of CoM-S-S-HTP alone. When MgATP was in addition present, ethene production decreased and CA production increased as compared to MgATP alone (Fig. 4.1). There was no lag in formaldehyde conversion in the presence of CoM-S-S-HTP together with MgATP. In the reaction mixture with MgATP and CoM-S-S-HTP, all CH_3-S-CoM was converted to methane whereas methane formation ceased after 32 min in all other assays and CH_3-S-CoM was not completely converted (data not shown).

A requirement for MgATP was tested with two experiments. Preincubations of the reaction mixtures for 30 min with the ATP-trap hexokinase/glucose at 37°C or 60°C had no effect on dechlorination (data not shown). A second possibility to show MgATP-requirement was to make use of the fact that methane production from CH_3-CoM ceased after 30 min of incubation. This result indicated exhaustion of a certain component, possibly ATP. Dechlorination still occurred after preincubations of 60 min with CH_3-S-CoM at 60°C. Hence, it was not possible to show an absolute requirement of dechlorination for MgATP by these two experiments.

For methyltransferase or methyl-CoM reductase pH-optima between 7.0-7.2 or 5.6-6.0 were reported, respectively [109,142]. Dechlorination rates were highest at pH 6.8 (at 60°C). It did not matter whether the reaction was followed in PIPES/KOH or TRIS/HCl.

4.4.2. Kinetics of 1,2-DCA dechlorination

Initial ethene or CA formation rates at different 1,2-DCA concentrations nicely fitted Michaelis-Menten kinetics (Fig. 4.2). With Lineweaver-Burk plots an apparent K_m for 1,2-DCA of 89 μM for ethene production and 119 μM for CA production was estimated in a reaction mixture with MgATP but without CoM-S-S-HTP. Estimations of V_{max} were 34 and 20 pmol/min per mg protein for ethene and CA production, respectively.

4.4.3. Inhibition of 1,2-DCA dechlorination

The use of specific inhibitors for methyltransferase or methyl-CoM reductase should provide a possible instrument to distinguish between the involvement of these two enzymes in the reductive dechlorination of 1,2-DCA. After an extensive screening of several compounds (N_2O,

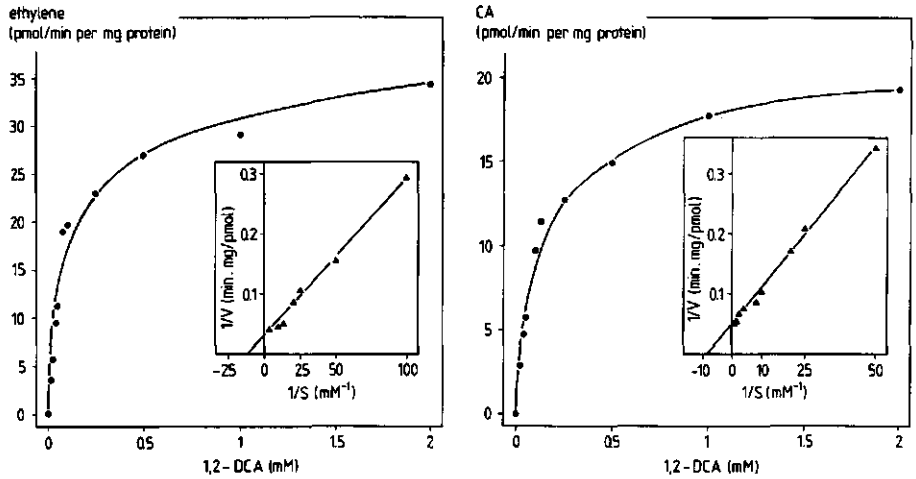

FIGURE 4.2. Kinetics of the reductive dechlorination of 1,2-DCA by crude cell extracts of *M. thermoautotrophicum* strain ΔH. Reaction mixture contained: 50 mM PIPES/KOH pH 6.8 (at 60°C), 25 mM $MgCl_2$, 2.5 mM ATP, and 100 μl crude cell extract (2.8 mg of protein) in a volume of 500 μl. The gas phase was 100% H_2. 5 μl of 1,2-DCA stock solutions in isopropanol (100 times the initial concentration) were added by syringe short before reaction was started by placing vials at 60°C. Initial rates were determined by analyzing vials after 5 and 10 min of incubation. Product formation was linear within this time frame. Insets: Lineweaver-Burk plots of the same data.

20000 ppm; NO_2^-, 1 mM; methyl viologen, 5-50 μM; cobalamin, 5-50 μM; Triton X-100, 4% (v/v); bathophenanthroline disulfonate, 1-5 mM; BrES, 50-250 μM; BrPS 100 μM), cobalamin was chosen as specific inhibitor for methyltransferase activity and BrPS as specific inhibitor for methyl-CoM reductase. All other compounds either inhibited both methyltransferase and methyl-CoM reductase activity or interfered with the measurements of dechlorination products. BrES for example, a specific inhibitor of methyl-CoM reductase and routinely used to inhibit methanogensis from CH_3-S-CoM, was also reductively transformed to ethene by crude cell extracts.

Cobalamin totally inhibited formaldehyde conversion, but methane production from CH_3-S-CoM was slightly stimulated (Table 4.1). The effect of cobalamin on dechlorination was diverse. Ethene formation was stimulated whereas CA production was partially inhibited (Table 4.1). BrPS showed a very clear inhibition pattern. Methyl-CoM reductase was completely inactivated and methyltransferase activity was not affected at all (Table 4.1). Dechlorination did not occur in the presence of this inhibitor (Table 4.1), suggesting that methyl-CoM reductase is responsible for ethene as well as CA production.

4.4.4. Dechlorination of 1,2-DCA by purified methyl-CoM reductase (component C)

Methyl-CoM reductase was purified with a yield of about 40-50%. Purity was checked with SDS/polyacrylamide gel electrophoresis. Only three major proteins were detected which represented the α, ß, and γ subunits of methyl-CoM reductase with apparent M_r of 70,000,

TABLE 4.1. Inhibition of the reductive dechlorination of 1,2-DCA by crude cell extracts of *M. thermoautotrophicum* strain ΔH compared with inhibition of formaldehyde conversion or methane production from CH_3-S-CoM. [a]

Inhibitor	Formaldehyde converted (nmol/vial)	Methane formed (nmol/vial)	Ethene formed (pmol/vial)	CA formed (pmol/vial)
none	1069±40	218±40	1102±35	799±37
cobalamin [b]	< d.l.[c]	293±14	1777±29	267±23
BrPS [d]	1084±25	< d.l.	25±2	< d.l.

[a] The reaction mixture contained: 50 mM PIPES/KOH pH 6.8 (at 60°C), 25 mM $MgCl_2$, 2.5 mM ATP, and 50 μl crude cell extract (1.4 mg of protein) in a volume of 250 μl. For formaldehyde conversion the reaction mixture contained in addition 5 mM formaldehyde and 5 mM H-S-CoM, for methane formation 5 mM CH_3-S-CoM. For dechlorination experiments 3 μl of 100 mM 1,2-DCA in isopropanol were added by syringe. The values given in the table are amounts converted or formed within 30 minutes of incubation and are means of duplicates.

[b] 50 μM hydroxocob(III)alamin added.

[c] < d.l. = below detection limit.

[d] BrPS = 3-bromopropanesulfonate, 100 μM added.

46,000, and 39,000, which are in close agreement with M_r's reported by others [78,117,118]. In a reaction mixture with Ti(III) citrate as electron donor, methane was produced from CH_3-S-CoM at a rate of 360 nmol/min per mg of component C. In a H_2-dependent enzyme system with a crude component A fraction and purified component C, methanogenic activitites of 35 nmol/min per mg of component C were obtained. No methane was formed if component A or C was omitted.

Dechlorinating activity of component C was first assayed with Ti(III) citrate as electron donor. In the presence of component C, dechlorinating activity was two times the background activity of Ti(III) citrate alone (data not shown). These initial results confirmed results obtained in inhibition experiments and showed the involvement of component C in the reductive dechlorination of 1,2-DCA.

A second possibility to assay dechlorinating activity was to use the enzyme system (component A) responsible for the activation of component C. 1,2-DCA was indeed dechlorinated in reaction mixtures where both, component A and C, were present (Table 4.2). If component A or C was omitted only small amounts of ethene were formed and CA remained below detection limit. Omission of ATP, CoM-S-S-HTP, or both had no effect on dechlorination. If boiled cell extract was omitted only slow or no dechlorination was observed. Omission of component C already indicated that component A was not able to reduce free corrinoids and factor F_{430} present in boiled cell extract which could also act as catalysts for dechlorination [Chapter 3]. Controls where additional cobalamin or factor F_{430} was added in the absence of component C showed no dechlorination at all. Interesting was that cobalamin had the same stimulating effect on ethene production in the presence of component C (Table 4.2) as was observed with crude cell extracts (Table 4.1). A partial inhibition of dechlorination by CH_3-S-CoM and of methane formation by 1,2-DCA was found (Table 4.2).

4.4.5. Cofactor requirement for 1,2-DCA dechlorination by component C

The experiments with boiled cell extract as crude cofactor fraction revealed that a component must be present in boiled cell extract needed for dechlorinating activity. According to the data in Table 4.3, FAD was the cofactor required to reconstitute a dechlorinating enzyme system out of component A and purified component C. MgATP or factor F_{420} had a stimulatory effect (Table 4.3).

TABLE 4.2. Reductive dechlorination of 1,2-DCA by methyl-CoM reductase (component C) of *M. thermoautotrophicum* strain ΔH with boiled cell extractas cofactor fraction. [a]

Reaction mixture		Products formed from 1,2-DCA (pmol/vial)	
Omissions	Additions	Ethene	CA
none	none	450	656
ATP	none	534	295
CoM-S-S-HTP	none	531	455
BCE	none	194	329
ATP/CoM-S-S-HTP	none	575	621
ATP/BCE	none	19	< d.l.[b]
CoM-S-S-HTP/BCE	none	12	< d.l.
ATP/CoM-S-S-HTP/BCE	none	59	< d.l.
component A	none	12	< d.l.
component C	none	22	< d.l.
none	cobalamin [c]	1062	382
component C	cobalamin	50	< d.l.
none	factor F_{430} [d]	537	334
component C	factor F_{430}	25	< d.l.
none	CH_3-S-CoM [e]	250	414

[a] The complete reaction mixture contained: 50 mM PIPES/KOH pH 6.8 (at 60°C), 25 mM $MgCl_2$, 2.5 mM ATP, 0.25 mM CoM-S-S-HTP, 50 μl protein-free boiled cell extract (BCE, 28 mg of protein · ml^{-1} before boiling), 200 μl component A (0.57 mg of protein), and 100 μl component C (1.24 mg of protein) in a volume of 500 μl. The values given in the table are amounts formed within the 120 minutes of incubation.

[b] < d.l. = below detection limit.

[c] 50 μM hydoxocob(III)alamin.

[d] 50 μM 12,13-di-epi-F_{430}.

[e] 5 mM CH_3-S-CoM added; 128 nmol CH_4 were formed in the presence of 1,2-DCA, 286 nmol in the absence of 1,2-DCA.

TABLE 4.3. Cofactor requirements for the reductive dechlorination of 1,2-DCA by methyl-CoM reductase (component C) of *M. thermoautotrophicum* strain ΔH. [a]

Reaction mixture		Products formed (pmol/vial)	
Omissions	Additions	Ethene	CA
none	none	1797	1282
ATP	none	962	905
FAD	none	28	< d.l.[b]
factor F_{420}	none	787	867
component A	none	40	< d.l.
component C	none	34	< d.l.
H_2	none	0	< d.l.
1,2-DCA	BrES [c]	1199	—[d]
1,2-DCA/component C	BrES	0	—

[a] The reaction mixture contained: 50 mM PIPES/KOH pH 6.8 (at 60°C), 25 mM $MgCl_2$, 2.5 mM ATP, 25 μM FAD, 25 μM factor F_{420}, 150 μl component A (0.43 mg of protein), and 150 μl component C (2.16 mg of protein) in a volume of 500 μl. The gas phase was 100% H_2, unless otherwise stated. The values given in the table are amounts formed within the 120 minutes of incubation.

[b] < d.l. = below detection limit.

[c] 2 mM BrES added by syringe from a 200 mM stock in 50 mM PIPES/KOH pH 6.8 (at 60°C).

[d] Not formed in this reaction mixture.

4.4.6. Ethene production from BrES catalyzed by cobalamin, factor F_{430} or methyl-CoM reductase

BrES was transformed to ethene by crude cell extracts of *M. thermoautotrophicum* strain ΔH as observed in inhibition studies described above. BrES is structurally similar to 1,2-DCA, a C2-compound with two leaving groups, one on each carbon. BrES was rapidly transformed to ethene in buffer reduced with Ti(III) citrate and cobalamin or factor F_{430} as catalysts (Fig. 4.3). In contrast to results with 1,2-DCA where dechlorination by cobalamin and factor F_{430} took place with different rates [Chapter 3], ethene formation from BrES occurred with about the same rate (500 pmol/min per nmol catalyst) with either catalyst. Purified component C was also able to transform its own inhibitor BrES to ethene (Table 4.3).

4.5. Discussion

The reductive dechlorinating activity of methyl-CoM reductase substantiates the hypothesis that factor F_{430} is involved in reductive dechlorination reactions catalyzed by whole cells of methanogens [Chapter 3]. First evidence for this hypothesis was obtained in model systems with

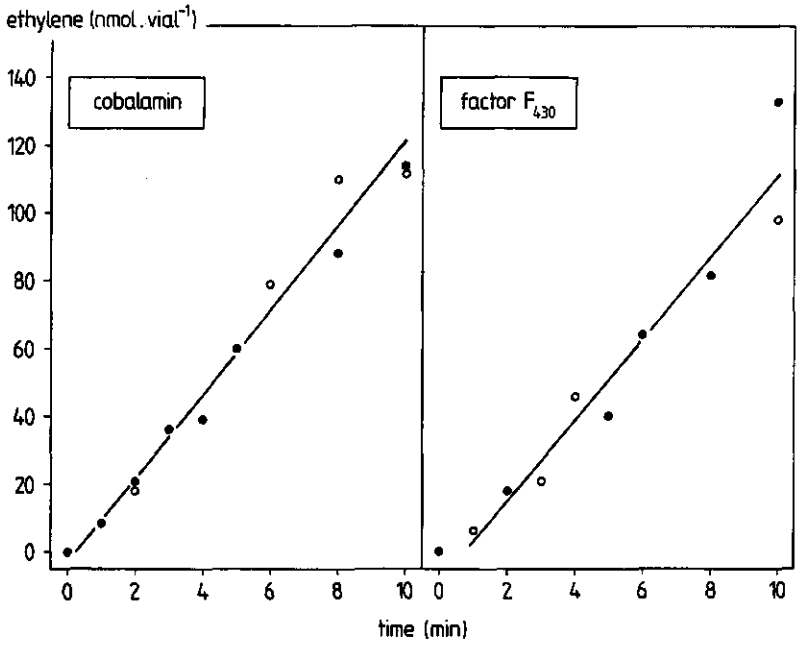

FIGURE 4.3. Ethene production from 2-bromoethanesulfonate (BrES) in buffer reduced with Ti(III) citrate and with cobalamin or factor F_{430} as catalysts. Reaction mixture contained: 790 mM TRIS/HCl pH 9, 20 mM Ti(III) citrate, 50 μM hydroxo-cob(III)alamin or 12,13-di-epi-F_{430}, and 2 mM BrES in a volume of 500 μl. The gas phase was 100% N_2. (●) and (○) are data of two independent experiments.

purified factor F_{430} and Ti(III) citrate as electron donor [98,155, Chapter 3]. Because factor F_{430} is present in a protein-bound and free form in cells of methanogens when cultivated in a nickel-sufficient medium, the question was whether free or protein-bound factor F_{430}, or both are responsible for the dechlorination. Stimulation of dechlorination by MgATP and the Michaelis-Menten type kinetics in crude cell extracts, shown here, indicated that enzymatic catalyzed reactions were responsible for dechlorination by crude cell extracts with hydrogen as electron source. The complete inhibition of dechlorination by BrPS and the dependence of dechlorinating activity on purified component C unequivocally proved protein-bound factor F_{430} to be the catalyst. These results excluded not only free factor F_{430} as the *in vivo* catalyst, but also an involvement of corrinoid-enzymes. This was surprising since reductive dechlorinating activity of other anaerobes [49,74,97], which do not contain factor F_{430}, demonstrates that also other enzymes are able to catalyze these reactions. A possible involvement of a corrinoid/iron-sulfur enzyme of the acetyl-CoA pathway present in some of these organisms remains to be proven [74]. Reductive dechlorination by a corrinoid-enzyme has been shown for N^5-methyl tetrahydrofolate-homocysteine transmethylase [305].

The reductive dechlorination of 1,2-DCA by methyl-CoM reductase of *M. thermoautotrophicum* strain ΔH could be incorporated in the model of Rouvière and Wolfe for the methyl-CoM reductase system [223] as shown in Figure 4.4. In this model, inactive component C, with the

nickel in factor F_{430} in the Ni(II) state, is reduced to the active Ni(I) form by the action of component A3a and A2. MgATP is required as cofactor. The electrons are derived from H_2 by component A3b and transferred by an unknown electron carrier. If 1,2-DCA is added to the assay instead of CH_3-S-CoM and HS-HTP or CoM-S-S-HTP, the reduced nickel in active component C is most probably oxidized, and ethene or CA are formed. Non-enzymatic dechlorination by free factor F_{430} with Ti(III) citrate as electron donor [Chapter 3] supports such a mechanism. Since the dihalo-elimination to ethene and the hydrogenolysis to CA require a two-electron reduction, one may envisage that both molecules of factor F_{430}, present in one component C molecule, take part in this reaction. The oxidation of Ni(I) to Ni(II) short-circuits the activation process and component C has to be reduced again.

According to the model, the reductive activation of component C would require MgATP as an activator. However, the results obtained with MgATP are completely contradictory to the mechanism suggested above. MgATP was not required for the reconstitution of a H_2-dependent dechlorinating system, whereas it was shown that methanogenesis did not proceed in the absence of MgATP [224]. Possibly, in our study, enough MgATP was still present in the crude component A fraction. Puzzling is, however, that also in crude cell extracts absolute requirement for MgATP could not be shown. This is in contrast with results found for methyltransferase as well as methyl-CoM reductase activity in crude systems where the hexokinase/glucose system worked perfectly as ATP-trap [142,279,296].

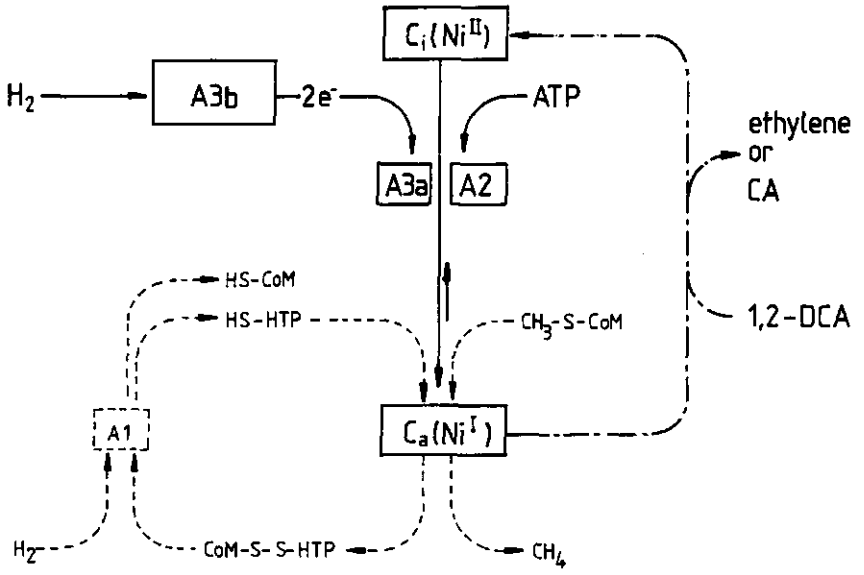

FIGURE 4.4. Model for the reductive dechlorination of 1,2-DCA by methyl-CoM reductase adapted from the model for the functioning of the methylreductase system presented by Rouvière and Wolfe [223]. (——) reductive activation of inactive component C, (– – –) methanogenesis from CH_3-S-CoM and H-S-HTP catalyzed by active component C, (–·–·–) oxidation of activated component C by reductive dechlorination of 1,2-DCA to ethene or CA.

The absolute requirement for FAD for dechlorination and the stimulatory effect of cobalamin and factor F_{420} are in agreement with results obtained for methane production from CH_3-S-CoM by this enzyme system [196]. The role of FAD in the methyl-CoM reducing enzyme system has not yet been fully established. Whereas FAD was specifically required for methanogenesis in a study with protein components A1, A2, A3, and C [196], it had no effect in a later study in a reaction mixture with protein components A1, A2, A3a, A3b, and C [223]. FAD is found in the heterodisulfide reductase of *M.thermoautotrophicum* strain Marburg where it could be involved in the disulfide reduction [119]. The FAD-dependent dechlorination suggests that this cofactor could also be involved in the electron transfer from component A3b to A3a.

An interesting observation is the transformation of BrES to ethene by crude cell extracts or purified methyl-CoM reductase. Cultures of methanogens amended with BrES are known to recover from BrES inhibition [19,254]. These observations might have been the result of degradation of BrES to ethene by its target enzyme component C. However, results obtained with BrES-resistant cells indicated that spontaneous mutagenesis of the transport system for H-S-CoM [10] was responsible for resistance rather than the degradation of BrES [144,254]. Characterization of BrES resistant strains of *Methanosarcina barkeri* strain 227 supported the evidence for a resistance due to an altered cell permeability, since component C of BrES-resistant mutants was equally sensitive to BrES as the wild type methyl-CoM reductase [253].

All studies of the methyl-CoM reductase activating enzyme system were assayed by methane formation from CH_3-S-CoM [109,196,223,224]. However, interpretation of the data was complicated by the uncertainty whether a component was indeed directly involved in the activation or rather in the catalytic process. If the dechlorination of 1,2-DCA by component C really functions as indicated in the model, it could provide an instrument to assay the activation of this enzyme directly. Possible additional effects of a certain compound on other catalytic functions in the multi-component enzyme system (e.g. reduction of the heterodisulfide CoM-S-S-HTP) would then be excluded in this assay.

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CHAPTER 5

Enrichment and properties of an anaerobic mixed culture reductively dechlorinating 1,2,3-trichlorobenzene to 1,3-dichlorobenzene

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5.1. Abstract

Hexachlorobenzene (HCB), pentachlorobenzene (QCB), all three isomers of tetrachlorobenzene (TeCB), 1,2,3-trichlorobenzene (1,2,3-TCB), and 1,2,4-trichloro-benzene (1,2,4-TCB) were reductively dechlorinated by enrichment cultures in the presence of lactate, glucose, ethanol, or isopropanol as electron donors. Enrichments originated from percolation columns filled with river Rhine sediment in which dechlorination of tri- and dichlorobenzenes occurred. A stable consortium obtained by transferring on lactate as energy and carbon source in the presence of 1,2,3-TCB dechlorinated this isomer stoichiometrically to 1,3-DCB. Dechlorinating activity could only be maintained when an electron donor was added. Lactate, ethanol, and hydrogen appeared to be the best suited substrates. Optimal temperature and pH for dechlorination were 30°C and 7.2, respectively. The specificity of the enrichment on lactate and 1,2,3-TCB was tested after approximately sixty transfers (after two and a half years). HCB and QCB were stoichiometrically dechlorinated to 1,3,5-TCB and minor amounts of 1,2,4-TCB. 1,3,5-TCB was the sole product formed from 1,2,3,5-TeCB, while 1,2,3,4-TeCB and 1,2,4,5-TeCB were converted to 1,2,4-TCB. 1,2,4-TCB, 1,3,5-TCB, and the three isomers of dichlorobenzene were not dechlorinated during four weeks of incubation. For further enrichment of the 1,2,3-TCB dechlorinating bacteria, a two-liquid-phase (hexadecane/water) system was used with hydrogen as electron donor and 1,2,3-TCB or CO_2 as electron acceptor. Methanogens and acetogens were the major substrate-competing (H_2/CO_2) microorganisms in the two-liquid-phase system. Inhibition of methanogenesis by 2-bromoethanesulfonic acid did not influence dechlorination, and acetogens which were isolated from the enrichment did not have dechlorinating activity. These results indicated that bacteria were present using 1,2,3-TCB as terminal electron acceptor. Although dechlorination was found in dilutions down to 10^{-6} from the two-liquid-phase system, attempts to isolate a bacterium in pure culture able to use 1,2,3-TCB as terminal electron acceptor failed.

5.2. Introduction

Chlorinated benzenes are widely used as solvents, intermediates, odorisers, insect repellents, and fungicides. Therefore, they became almost ubiquitous pollutants in surface waters, groundwater, sediments, soils, and sewage [202,209,244,283]. In the absence of light, biodegradation is the only possibility to transform these chemically stable chloroaromatics into harmless compounds. Aerobic mineralization is well documented for chlorobenzenes with up to four chlorine substituents in microcosms and by pure cultures [57,113,179,204,228,239,255,275]. Bacteria described in these studies used chlorobenzenes as sole energy and carbon source. Reports on anaerobic biotransformation of chlorobenzenes are very scarce. Laboratory studies with sediment columns, biofilm reactors, and batch cultures inoculated with sewage sludge or aquifer material revealed that chlorobenzenes are biologically transformed by reductive dechlorination [24,87,89,123,166,195]. In most cases, a stoichiometric conversion to lower chlorinated isomers, but no mineralization was observed. One exception is the $^{14}CO_2$ production from ^{14}C-monochlorobenzene (MCB) in batch cultures inoculated with a ferulic acid degrading methanogenic consortium or creosote-contaminated aquifer material [165]. However, only a few percent of the added ^{14}C-MCB was recovered as $^{14}CO_2$. It was suggested that reductive dechlorination of chlorobenzenes also takes place in nature, for example in dune infiltration sites or in lake sediments [8,24,202].

Reductive dechlorination is described for a wide variety of chloroaromatics [for reviews, see

16,159,269]. However, until now only little is known about the bacteria involved in the reductive dechlorination of chlorinated aromatic compounds. Shelton and Tiedje isolated a bacterium able to dechlorinate *meta*-halogenated benzoates [248]. Further physiological characterization revealed that this organism appeared to be a sulfidogenic bacterium [258], and it was classified as *Desulfomonile tiedjei* [61]. *D. tiedjei* used 3-chlorobenzoate as terminal electron acceptor with hydrogen or formate as electron donor in a process which led to conservation of metabolic energy [67,189]. Besides this fascinating organism, no other pure culture has as yet been isolated able to reductively dechlorinate chloroaromatics.

The present investigation was undertaken to learn more about the microbiology of the reductive dechlorination of chlorobenzenes. The enrichment of organisms catalyzing these reactions is made difficult because an electron donor is needed, and chlorobenzenes are toxic. We report here that reductive dechlorination of 1,2,3-trichlorobenzene (1,2,3-TCB) to 1,3-dichlorobenzene (1,3-DCB) can be enriched and maintained by cultivation in a two-liquid-phase system (water-/organic-phase) with hydrogen as electron donor. Evidence is presented that 1,2,3-TCB was used as electron acceptor. The stable consortium was also able to dechlorinate hexa- (HCB), penta- (QCB), and all three tetrachlorobenzene (TeCB) isomers.

5.3. Materials and Methods

5.3.1. Materials

All chemicals were at least of analytical grade. Hexachlorobenzene (HCB), 1,2,3,5-tetrachlorobenzene (1,2,3,5-TeCB), 1,2,4,5-tetrachlorobenzene (1,2,4,5-TeCB), and 1,3,5-trichlorobenzene (1,3,5-TCB) were obtained from Aldrich Chemie N.V., Brussels, Belgium. Pentachlorobenzene (QCB), 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB), 1,2,4-trichlorobenzene (1,2,4-TCB), 1,2-dichlorobenzene (1,2-DCB), 1,3-dichlorobenzene (1,3-DCB), 1,4-dichlorobenzene (1,4-DCB), and chlorobenzene (MCB) were purchased from E. Merck, Darmstadt, FRG. 1,2,3-Trichlorobenzene (1,2,3-TCB) was from Janssen Chimica, Beerse, Belgium. Gases were from Hoekloos (Schiedam, NL). Where necessary, experiments were carried out in an anaerobic glovebox (Coy Laboratories Products, Toepffer GmbH, Göppingen, FRG). The oxygen concentration in the anaerobic glovebox was kept low with R-20 palladium catalyst provided by BASF (Arnhem, NL).

5.3.2. Source of Inoculum

The inoculum was material of columns in which all isomers of tri- and dichlorobenzenes were reductively dechlorinated [24]. These columns were wet-packed with anaerobic sediment from the river Rhine near Wageningen, The Netherlands [24,275] and were percolated continuously with an anaerobic mineral medium resembling the mineral composition of Rhine water [275]. Samples for inoculation were taken after 9 and 12 months of operation of the columns. Initial experiments were also carried out with granular anaerobic sludge [211] of a sugar refinery (Centrale Suiker Maatschappij, Breda, NL) as inoculation material.

5.3.3. Anaerobic media

Three different media were used. Medium 1 and 3 were phosphate/bicarbonate-buffered and had a low chloride concentration. Medium 2 was phosphate-buffered and was used for incubations in completely filled bottles. The composition of the media was as follows (in g/l of

demineralized water, unless otherwise stated): Medium 1: KH_2PO_4, 0.41; $Na_2HPO_4 \cdot 2H_2O$, 0.53; NH_4HCO_3, 0.42; $CaCl_2 \cdot 2H_2O$, 0.12; $MgSO_4 \cdot 7H_2O$, 0.13; $NaHCO_3$, 4.2; $Na_2S \cdot 9H_2O$, 0.24; resazurin 0.0005; 1 ml trace element solution (according to Houwen et al. [125]); 1 ml vitamin solution (containing in mg/l: biotin, 2; folic acid, 2; pyridoxine, 10; riboflavin, 5; thiamine, 5; cyanocobalamin, 5; nicotinamide, 5; *p*-aminobenzoic acid, 5; lipoic acid, 5; pantothenic acid, 5). Medium 2: KH_2PO_4, 1.77; $Na_2HPO_4 \cdot 2H_2O$, 1.6; NH_4Cl, 0.3; $CaCl_2 \cdot 2H_2O$, 0.11; $MgCl_2 \cdot 6H_2O$, 0.1; $NaHCO_3$, 0.8; NaCl, 0.3; $Na_2S \cdot 9H_2O$, 0.24; resazurin 0.0005; 1 ml trace element solution [125]; 1 ml vitamin solution (see above). Medium 3: K_2HPO_4, 0.63; $NaH_2PO_4 \cdot 2H_2O$, 0.19; NH_4HCO_3, 0.45; $CaCl_2 \cdot 2H_2O$, 0.11; $Mg(CH_3COO)_2$, 0.11; $NaHCO_3$, 3.81; $Na_2S \cdot 9H_2O$, 0.24; resazurin 0.0005; 1 ml trace element solution [125]; 10 ml vitamin solution (see above). For cultivation in medium 1 and 3 the atmosphere was either N_2/CO_2 or H_2/CO_2 (4/1, v/v) at 1.5 bar. The pH of all media was 7.0-7.2. Bottles were sealed with butyl rubber stoppers (Rubber B.V., Hilversum, NL), viton septa, or viton stoppers (Maag Technic AG, Dübendorf, CH).

5.3.4. Enrichments on chlorobenzenes

Serum bottles (117 ml) with 40 ml of medium 1 were inoculated with approximately 0.5 g sediment material or 1 ml granular sludge. Chlorobenzenes were added as crystals or as a few drops of the pure chemical. At day 0 and after 1, 2, 3, and 4 weeks lactate, ethanol, isopropanol, and glucose were added as electron donors to a final concentration of 20 mM. These cultures are referred to as "first generation enrichments". Bottles were incubated stationary at 20°C in the dark. Dechlorination was followed by measuring chloride concentration and by analyzing water samples with GC or HPLC. After 280 days of incubation 10% (v/v) was transferred into fresh medium ("second generation enrichments").

5.3.5. Enrichment of reductive dechlorination of 1,2,3-TCB

Screw-cap bottles totally filled with medium 2 and sealed with viton septa were used for the enrichment of 1,2,3-TCB dechlorinating activity. 1,2,3-TCB was added by replacing part of the medium with 1,2,3-TCB saturated medium (for an initial concentration of x μM 1,2,3-TCB x-times 1.5% (v/v) had to be replaced; solubility of 1,2,3-TCB in water at 22°C: 66 μM [283]). Samples were taken from opened bottles with sterile pipettes and immediately extracted with hexane. When 1,2,3-TCB was depleted part of the culture was replaced with 1,2,3-TCB saturated medium. Initial cultures (37 ml bottles) were inoculated with approximately 0.5 g sediment material. After three additions of 1,2,3-TCB, 10% (v/v) of the culture was transferred into fresh medium amended with electron donors (1. transfer). After another three additions of 1,2,3-TCB, where the initial 1,2,3-TCB concentration was stepwise increased, enrichment was continued in bottles of 134 ml (2. transfer). Subsequently, the mixed population on lactate and 1,2,3-TCB was routinely subcultured in 1 l screw-cap bottles with lactate or ethanol (1, 5, or 20 mM) and 1,2,3-TCB (20 μM). These cultures served as inoculum for experiments. Initially, cultures were incubated stationary at 20°C in the dark. After determination of the optimal temperature, incubations were stationary at 30°C.

5.3.6. Dechlorination of other chlorobenzene isomers

Because of different solubilities of different chlorobenzene isomers (solubilities at 22°C, in μM: HCB, 0.4; QCB, 1.0; 1,2,3,4-TeCB, 16; 1,2,3,5-TeCB, 11; 1,2,4,5-TeCB, 1.4; 1,2,4-TCB, 105;

1,3,5-TCB, 32; 1,2-DCB, 680; 1,3-DCB, 469; 1,4-DCB, 333 [283]) two experimental set-ups were applied. For both experimental set-ups, tubes filled with sterile medium served as controls.

"Soluble" isomers (1,2,3,4-TeCB, 1,2,3,5-TeCB, TCB's and DCB's): Chlorobenzenes dissolved in ethanol were added to 500 ml fresh medium 2 resulting in a final concentration of chlorobenzenes slightly below solubility (TeCB's) or not higher than 20 μM (TCB's and DCB's) and of 10 mM ethanol. The medium was inoculated with 10% (v/v) of a 1 l culture and immediately after inoculation, the fresh culture was dispensed into sterile cultivation tubes (25 ml). The tubes were totally filled and sealed with teflon lined septa. At day 0 and after times indicated three tubes per isomer were sacrificed for analysis. The tubes were opened and samples of 1 ml were extracted with hexane or mixed with 1 ml acetonitrile. Samples were stored at 4°C until analysis.

"Insoluble" isomers (HCB, QCB, and 1,2,4,5-TeCB): 1 ml of stock solutions in pentane were added to sterile cultivation tubes. The tubes were flushed with nitrogen to evaporate the pentane. Fresh medium with 10 mM ethanol as electron donor was inoculated (10%, v/v), and immediately thereafter 10 ml were dispensed into each tubes. The tubes were sealed with teflon lined septa. At day 0 and after times indicated three tubes per isomer were sacrificed for analysis. 5 ml hexane or 10 ml acetonitrile was added by syringe and the tubes were vigorously shaken for 1 min. Samples were stored at 4°C until analysis.

5.3.7. Enrichment of 1,2,3-TCB dechlorinating activity in a two-liquid-phase system

To further enrich for 1,2,3-TCB dechlorinating activity a two-liquid-phase system was used. Cultures were cultivated in bottles partially filled with medium 3 and sealed with viton stoppers. Cells of a 1 l culture in medium 2 were anaerobically centrifuged, resuspended in medium 3 and served as first inoculum. 1,2,3-TCB dissolved in hexadecane (0.2 M) was added to these cultures (50 ml/l medium). With this amount an increase in chloride of 10 mM could be expected if all of the 1,2,3-TCB was dechlorinated. The log of the partition coefficients k_{HW} (partition between hexadecane and water) of 1,2,3-TCB and 1,3-DCB were 4.04 and 3.56, respectively. Dechlorination was followed by measuring chloride concentration and by qualitatively checking for the presence of 1,3-DCB in the water phase.

5.3.8. Analytical methods

Chlorobenzenes were analyzed by both capillary gas chromatography (GC) and high pressure liquid chromatography (HPLC).

For GC analysis culture fluid was extracted with hexane (1/1, v/v). 1 μl hexane was injected into a 438A Chrompack Packard gas chromatograph equipped with an injection splitter (split ratio 80:1) and a ^{63}Ni electron capture detector connected to a capillary column (10m x 250μm [inner diameter], Sil 5CB, 0.11μm, Chrompack, The Netherlands). Carrier gas was nitrogen with an inlet pressure of 30 kPa. Operating temperature of the injector and detector was 250 and 300°C, respectively. The column was operated with the following temperature program: initial column temperature 80°C (1 min), followed by an increase of 25°C/min to a final temperature of 160°C (3 min). If only 1,2,3-TCB and 1,3-DCB were present in the sample, the operating column temperature was 120°C.

For HPLC analysis the sample was diluted with acetonitrile (1/1, v/v) and filtered through a 0.2 μm filter (Millipore, GVWP 01300). 50 μl was injected into a high pressure liquid chromatography system (LKB 2150 pump and 2152 controller; Ultra Pac column [250 x 4 mm]

filled with Lichrosorb RP-8 and RP-18 [10 μm diameter]) connected to an LKB 2140 Rapid Spectral Detector, operated at a wavelength scanned between 200 and 220 nm, with a time average of 0.2 seconds. The mobile phase was acetonitrile-water (3/2, v/v) at a flow rate of 0.6 ml/min.

Retention times and peak areas of both analyses were determined with a Shimadzu C-R3A computing integrator. Peaks were identified and quantified by comparing injections with authentic standards prepared in hexane or 50% acetonitrile. HPLC analysis enabled the detection of MCB which was not possible by GC in the configuration used in this study. In addition, peak maxima of 1,2,3,5-TeCB and 1,2,4,5-TeCB which coeluted from the GC column could be separated by HPLC.

Lactate and acetate were analyzed by HPLC as previously described [126].

Chloride ion concentrations were determined with a Marius Micro-chlor-o-counter (Marius, Utrecht, NL) with an NaCl solution as the standard. Prior to analysis, 0.5 ml samples were acidified with 10 μl sulfuric acid and purged with nitrogen for 5 minutes to eliminate sulfide ions which interfered with the chloride measurements.

Growth was determined by measuring the protein concentration by a modified Lowry method [193] with bovine serum albumin as the standard.

5.4. Results

5.4.1. Reductive dechlorination of HCB, QCB, TeCB's, and TCB's

Anaerobic degradation of HCB, QCB, TeCB's and TCB's was tested in mineral medium supplemented with lactate, glucose, ethanol, or isopropanol as electron donor. TeCB's and TCB's were added combined to first generation enrichments. Table 5.1 summarizes results obtained with first and second generation enrichments amended with lactate and inoculated with column material. Cultures amended with either glucose, ethanol, or isopropanol showed similar results. First generation enrichments with granular sludge as inoculum did not show any dechlorination at all. After lag-phases of one to three months an increase in chloride ion concentration could be observed in first generation enrichments inoculated with column material. No increase in chloride could be observed in autoclaved controls or in controls without inoculum or where no electron donor had been added (data not shown). Chloride production rates were highest on QCB and the lag-phase was also shortest on this isomer. First generation enrichments on lactate and chlorobenzenes were transferred into fresh medium (10%, v/v) after 280 days of incubation. TeCB's and TCB's were incubated separately in second generation enrichments. The lag phase was shortened, but dechlorination rates did not increase (Table 5.1). Dechlorinating activity was found on all three tetrachlorobenzene isomers. 1,2,3-TCB was the only trichlorobenzene isomer on which dechlorinating activity could be observed after 138 days of incubation. A final check after about a year of incubation revealed that also on 1,2,4-TCB chloride was produced.

1,3,5-TCB was the only product found in samples of enrichments on HCB, QCB, or 1,2,3,5-TeCB. 1,2,4-TCB was observed where 1,2,3,4-TeCB or 1,2,4,5-TeCB were present. 1,3-DCB was formed from 1,2,3-TCB, and 1,3-DCB and 1,4-DCB from 1,2,4-TCB. MCB was not detected in any of these samples. The addition of not precisely defined amounts of chlorobenzenes did not allow to perform a mass balance. However, the determination of lower chlorinated benzenes suggests that reductive dechlorination was responsible for the increase of chloride.

TABLE 5.1. Chloride production in anaerobic batch cultures amended with lactate and chlorobenzenes and inoculated with river Rhine sediment adapted to trichlorobenzenes[a]

Chlorobenzene isomer	Lag-phase[b] (d)	Chloride produced[c] (mM)	Highest chloride production rate (μmol/l per d)	Products[d]
1st generation enrichments				
HCB	73	1.56	13.2 (73-148)[e]	1,3,5-TCB
QCB	36	2.57	17.8 (73-148)	1,3,5-TCB
TeCB combined	73	0.89	6.9 (73-148)	1,2,4-TCB 1,3,5-TCB
TCB combined	100	1.06	6.1 (100-148)	1,3-DCB
2nd generation enrichments[f]				
HCB	27	0.70±0.11	21.5 (47-60)	1,3,5-TCB
QCB	27	0.61±0.15	22.3 (47-60)	1,2,3,5-TeCB 1,3,5-TCB
1,2,3,4-TeCB	47	0.26±0.03	9.2 (47-60)	1,2,4-TCB
1,2,3,5-TeCB	47	0.25±0.04	10.0 (47-60)	1,3,5-TCB
1,2,4,5-TeCB	47	0.51±0.06	12.3 (47-60)	1,2,4-TCB
1,2,3-TCB	27	0.48±0.12	9.2 (47-60)	1,3-DCB
1,2,4-TCB	>138	1.20±0.13[g]	—	1,3-DCB[h] 1,4-DCB
1,3,5-TCB	>365	0	—	—

[a] Enrichemnts were set in as described in Materials and Methods for enrichments on chlorobenzenes. Chlorobenzenes were added in amounts above the solubility of the compounds.

[b] With lag-phase the period is meant after which an increase in chloride ion concentration could be observed.

[c] Chloride produced during 280 days of incubation for first generation enrichments and 138 days for second generation enrichments.

[d] Samples were analyzed with GC and HPLC, first generation enrichments at day 280, second generation enrichments at day 138.

[e] In parenthesis the period of highest chloride production is given.

[f] 10% (v/v) of first generation enrichments was transferred into fresh medium on day 280.

[g] Chloride produced during about a year of incubation.

[h] This sample was analyzed after about a year of incubation.

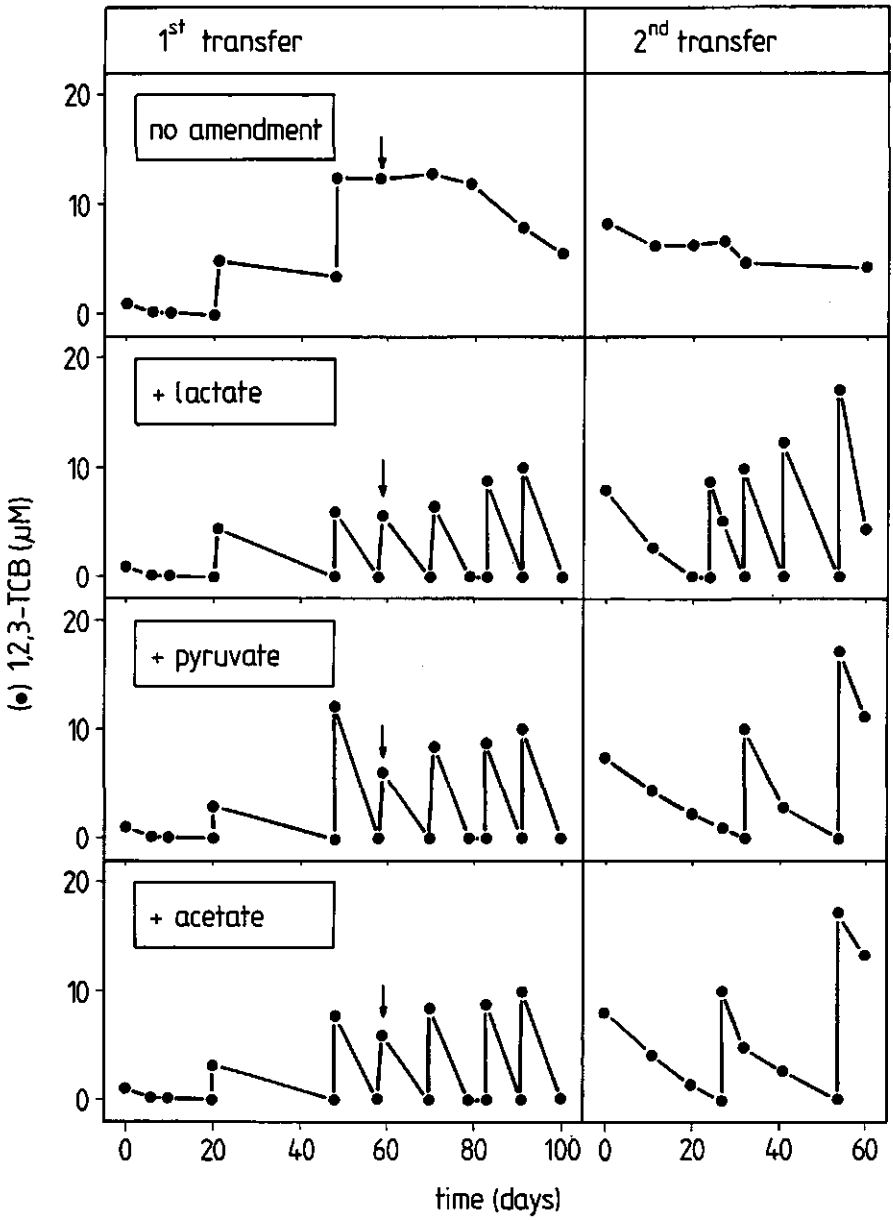

FIGURE 5.1. Effect of electron donor addition on reductive dechlorination of 1,2,3-TCB after a first and second transfer. Electron donors (20 mM) were only added at day 0. 1,2,3-TCB was added each time depletion was observed. (↓) At day 59, 10% of the first transfer cultures was transferred into fresh medium. Cultures were incubated stationary at 20°C in the dark.

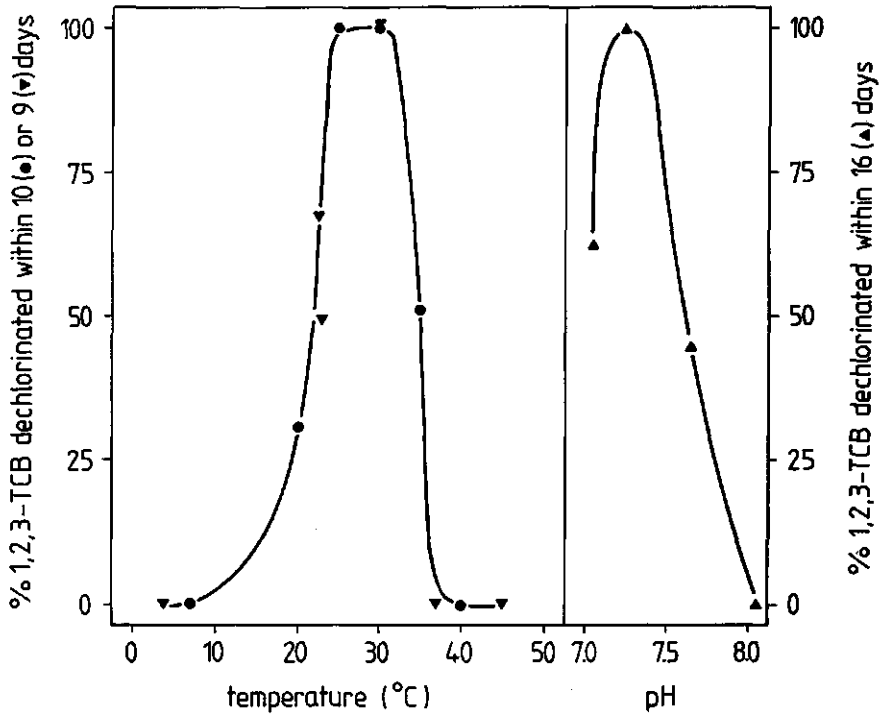

FIGURE 5.2. Temperature and pH dependence of the reductive dechlorination of 1,2,3-TCB. Both dependences were determined in medium 2 in totally filled 137 ml bottles. The temperature optimum was determined with an inoculum grown at 20°C (●), and one grown at 30°C (▼). The pH of the medium was varried by using different ratios of $H_2PO_4^-/HPO_4^{2-}$. The concentrations of sodium or potassium ions added were kept constant. Incubation was stationary at 30°C.

5.4.2. Reductive dechlorination of 1,2,3-TCB to 1,3-DCB

Parallel to the enrichments described above, enrichments with defined amounts of 1,2,3-TCB were started. This isomer was the first one transformed in sediment columns [24]. Again, material from the sediment columns served as inoculum. After 110 days the first amendment of 6 μM of 1,2,3-TCB had disappeared and simultaneously the same amount of 1,3-DCB was formed (data not shown). Two further additions of 1,2,3-TCB were transformed within 20 and 10 days, respectively and again a stoichiometric increase in 1,3-DCB could be observed. These cultures were transferred into fresh medium (1st transfer) containing besides 1,2,3-TCB either lactate, pyruvate, acetate, succinate, propionate, formate, ethanol, methanol, or isopropanol (all 20 mM) as electron donor. Every time 1,2,3-TCB was depleted, new 1,2,3-TCB was added (Fig. 5.1). Already in this first transfer, dechlorinating activity was lost when an electron donor was omitted (Fig. 5.1). The cultures were transferred into fresh medium (2nd transfer) on day 59 (Fig. 5.1), amendment with 1,2,3-TCB, however, was continued in first transfer cultures. Dechlorination rates were highest in second transfer cultures grown on lactate and therefore, this substrate was used for further enrichment. The following experiments were performed with stable enrichment cultures subcultured with lactate and 1,2,3-TCB as substrates.

Initially, enrichment cultures were, just like the sediment columns [24], incubated at 20°C. The

temperature optimum of the dechlorination, however, was found to be between 25 and 30°C (Fig. 5.2). At 37°C dechlorination ceased. Subsequently, all experiments were carried out at 30°C. The dechlorination had a very narrow pH optimum around pH 7.2 (Fig. 5.2).

A typical dechlorination pattern in the presence of lactate is shown in Figure 5.3A. The culture was grown on 15 mM lactate and dechlorinated 20 μM 1,2,3-TCB within ten days under concomitant formation of a stoichiometric amount of 1,3-DCB. New additions of 1,2,3-TCB to the same culture were increasingly faster degraded. The dechlorination of 1,2,3-TCB was paralleled by lactate degradation and growth of the mixed culture (Fig. 5.3B). Acetate was found to be the only organic acid formed during lactate degradation in these cultures.

A screening of organic electron donors revealed that in addition to lactate, also ethanol was a suitable substrate. In the presence of pyruvate, propionate, formate, succinate, or isopropanol no dechlorination was observed. Dechlorinating activity was successfully maintained if a 10% inoculum was used with 5 mM electron donor or if a 50% inoculum was used with 1 mM electron donor. Transfers of only 10% with 1 mM electron donor resulted in a loss of dechlorinating activity.

Hydrogen was found to support dechlorination of 1,2,3-TCB as well (Fig. 5.4). If hydrogen was replaced by nitrogen, lactate had to be added to obtain dechlorination.

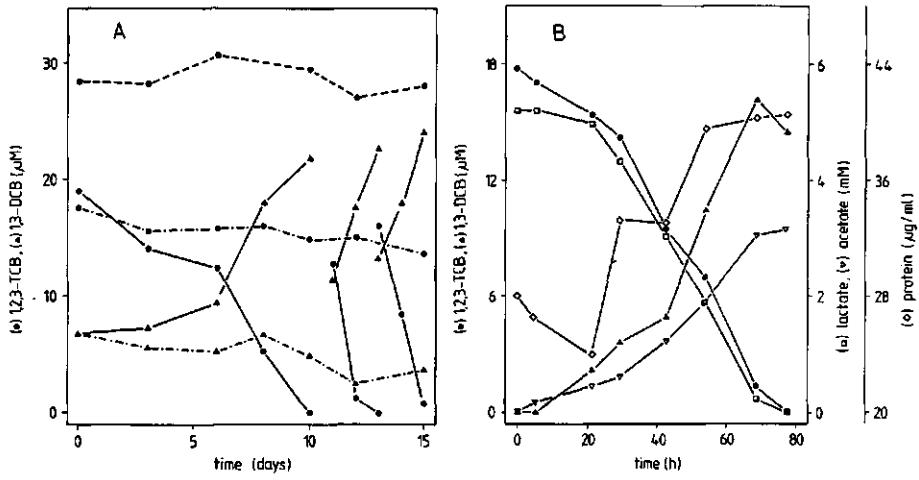

FIGURE 5.3. (A) Pattern of reductive dechlorination of 1,2,3-TCB to 1,3-DCB. At day 0, 10% was transferred into fresh medium. Incubation was stationary at 30°C. On day 11 and 13, 1,2,3-TCB was added. (●) 1,2,3-TCB, (▲) 1,3-DCB, (– –) not inoculated control, (-·-) autoclaved control, (—) active cultures.

(B) Dechlorination of 1,2,3-TCB followed in time simultaneously with lactate degradation and growth of the 1,2,3-TCB dechlorinating anaerobic mixed culture. The experimental set-up as described in Materials and Methods for the dechlorination of "soluble" chlorobenzene isomers was applied. Cells of the inoculum were centrifuged and washed prior to inoculation to avoid the presence of 1,3-DCB at the beginning of the experiment. The amount of cells inoculated corresponds to a transfer of 50% (v/v). Incubation was stationary at 30°C. (●) 1,2,3-TCB, (▲) 1,3-DCB, (□) lactate, (▽) acetate, (◇) protein.

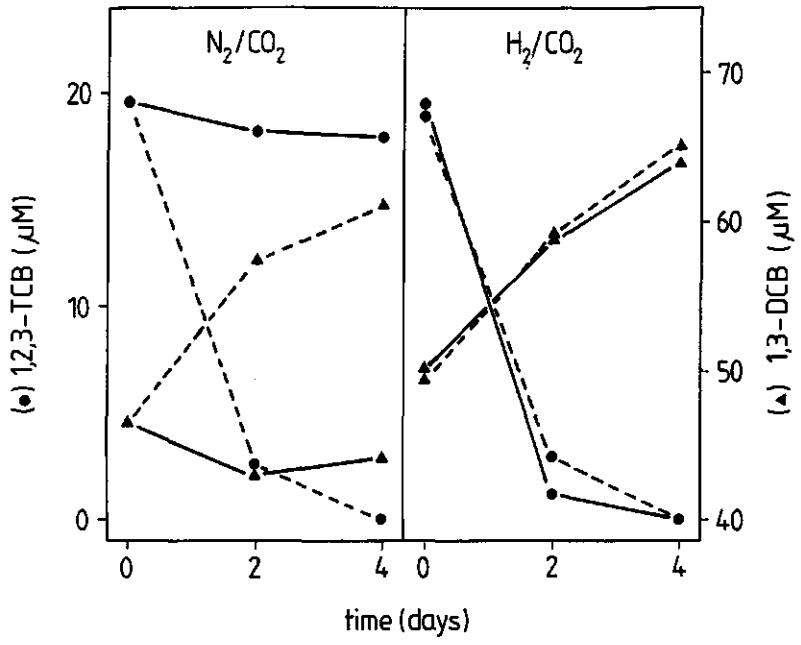

FIGURE 5.4. Reductive dechlorination of 1,2,3-TCB with hydrogen as electron donor. 20 ml of a culture already containing 1,2,3-TCB and, where indicated, lactate (5 mM) were immediately after inoculation dispensed into sterile 117 ml serum bottles. The bottles were sealed with viton stoppers and the gas phase was changed to N_2/CO_2 or H_2/CO_2 (4/1, v/v; 1.5 bar). After changing the gas phase, time 0 samples were withdrawn from the cultures by syringe. Incubation was stationary at 30°C. (—) without lactate, (- - -) with lactate.

5.4.3. Specificity of the enrichment culture

After approximately sixty transfers (after two and a half years) on lactate and 1,2,3-TCB, the specificity of dechlorination was tested. Besides 1,2,3-TCB, also HCB, QCB, and all three isomers of tetrachlorobenzene were dechlorinated (Fig. 5.5). 1,2,4-TCB, 1,3,5-TCB, and all isomers of dichlorobenzene remained unchanged during the four weeks of incubation (data not shown). HCB was sequentially dechlorinated to 1,3,5-TCB and minor amounts of 1,2,4-TCB via QCB, 1,2,3,5-TeCB, and 1,2,4,5-TeCB. With QCB the same products were observed as with HCB. 1,2,3,4-TeCB and 1,2,4,5-TeCB were dechlorinated to 1,2,4-TCB, and 1,2,3,5-TeCB to 1,3,5-TCB. Controls with sterile medium showed a loss of the parent compounds (Fig. 5.5). Formation of lower chlorinated isomers, however, was never observed in these samples.

5.4.4. Enrichment of dechlorinating activity in a two-liquid-phase system

No dechlorination was observed above 40 μM of 1,2,3-TCB or 70 μM of 1,3-DCB (data not shown). Therefore, 1,2,3-TCB saturated medium could not be used for the enrichment of 1,2,3-TCB dechlorinating activity. If 1,2,3-TCB can serve as terminal electron acceptor for

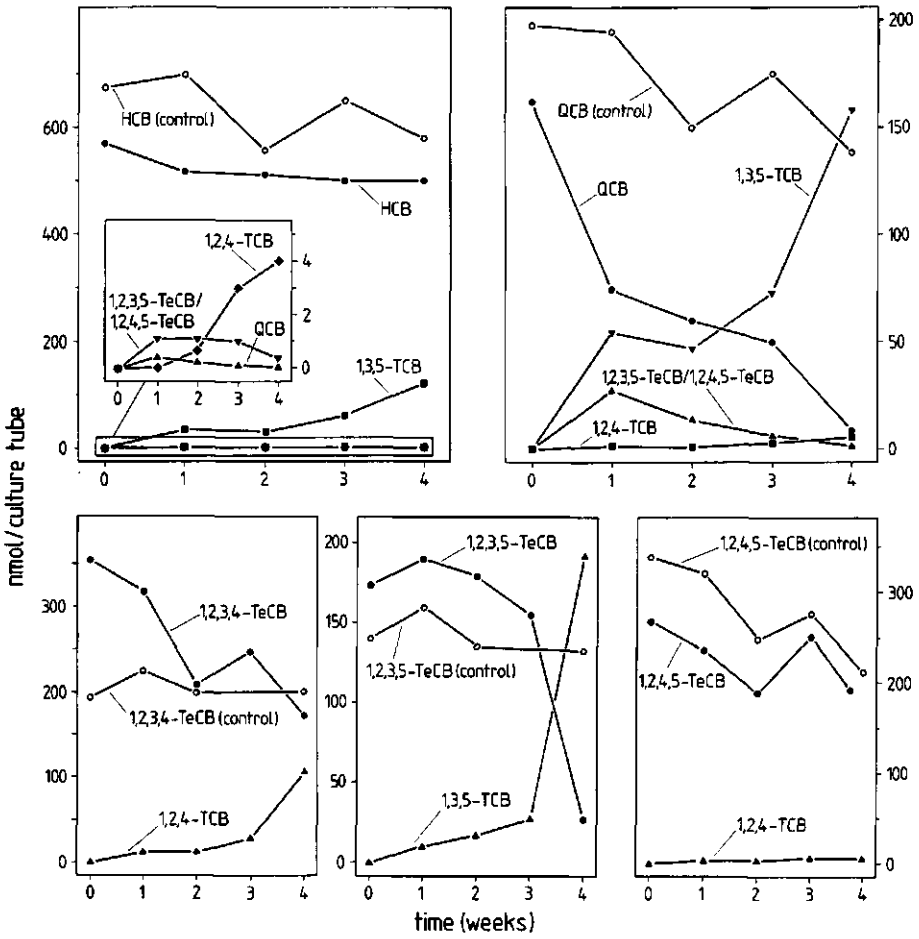

FIGURE 5.5. Dechlorination of HCB, QCB, and the three isomers of TeCB by the 1,2,3-TCB dechlorinating anaerobic mixed culture. Not inoculated tubes served as controls. Incubation was stationary at 30°C. Note that different amounts of chlorobenzenes were added.

microorganisms, a limiting factor for the creation of selective advantages for these bacteria was the low amount of 1,2,3-TCB which could be added to the cultures. This problem was circumvented by using a two-liquid-phase system where 1,2,3-TCB was dissolved in an organic phase (hexadecane). The concentration of 1,2,3-TCB and 1,3-DCB in the water phase was kept low due to the advantageous partition coefficients k_{HW} (partition between hexadecane and water) of 1,2,3-TCB and 1,3-DCB, and a considerable amount of 1,2,3-TCB could be added to the enrichment culture.

Hexadecane was chosen as organic phase. Slow anaerobic degradation of this saturated hydrocarbon has been reported only for a sulfate-reducing bacterium [298], but was not found in methanogenic systems [233]. Therefore, enrichment for hexadecane degrading organisms was

TABLE 5.2. Chloride production on 1,2,3-TCB by the 1,2,3-TCB dechlorinating anaerobic mixed culture incubated in a two-liquid-phase system with 1,2,3-TCB dissolved in hexadecane and with different electron donors. [a]

Electron donor	Added per day (mM)	Shaking (+/–)	Chloride produced within 30 days (mM)	1,3-DCB in waterphase (+/–)
lactate	0.1	–	0.58	+
	0.1	+	0	–
ethanol	0.1	–	0.38	+
	0.1	+	0	–
H_2	—	–	1.20	+
	—	+	0	–

[a] Lactate and ethanol were added daily from concentrated stock solutions and in these cultures the gasphase was N_2/CO_2 (4/1, v/v) at 1.5 bar. For cultures with hydrogen as electron donor, the gas phase was H_2/CO_2 (4/1, v/v) at 1.5 bar. Cultures were incubated stationary or horizontally on a rotary shaker at 100 rpm at 30°C. The presence of 1,3-DCB was checked by analyzing water samples gas chromatographically.

very unlikely. Lactate, ethanol, and hydrogen were used as electron donors for the first enrichments in such a two-liquid-phase system. Lactate and ethanol were added daily in low amounts. An increase in chloride ion concentration of 0.38 to 1.20 mM and the presence of 1,3-DCB in the water phase after 30 days of incubation showed that such a two-liquid-phase system was indeed suitable for cultivation of the 1,2,3-TCB dechlorinating mixed culture (Table 5.2). Dechlorination was found with all three electron donors. The shaking of such two-liquid-phase cultures resulted in a loss of dechlorinating activity.

With hydrogen as electron donor, only methanogenesis and acetogenesis compete with dechlorination. Therefore, hydrogen was chosen as substrate for further studies. The addition of 200 mg/l yeast extract enhanced chloride production and 2-bromoethane-sulfonic acid (BrES), a specific inhibitor of methanogenic bacteria, did not reduce dechlorinating activity (data not shown). Acetate was formed in BrES inhibited cultures, but acetate as sole electron donor did not support dechlorination in the two-liquid-phase system (data not shown). The increase in chloride corresponded stoichiometrically to the decrease in 1,2,3-TCB and the increase in 1,3-DCB (Fig. 5.6). Dechlorination took place under growing conditions. Dechlorination rates increased in the two-liquid-phase system to about 200 μmol/l per d. In dilution series dechlorination was found in dilutions down to 10^{-8}. MPN-counts where growth on H_2/CO_2 was compared with the presence of dechlorination revealed that 0.2-23% of the bacteria growing in these cultures were dechlorinating organisms. Although dechlorination was found in dilutions of 10^{-8}, we were not yet able to obtain pure cultures responsible for the dechlorination of 1,2,3-TCB.

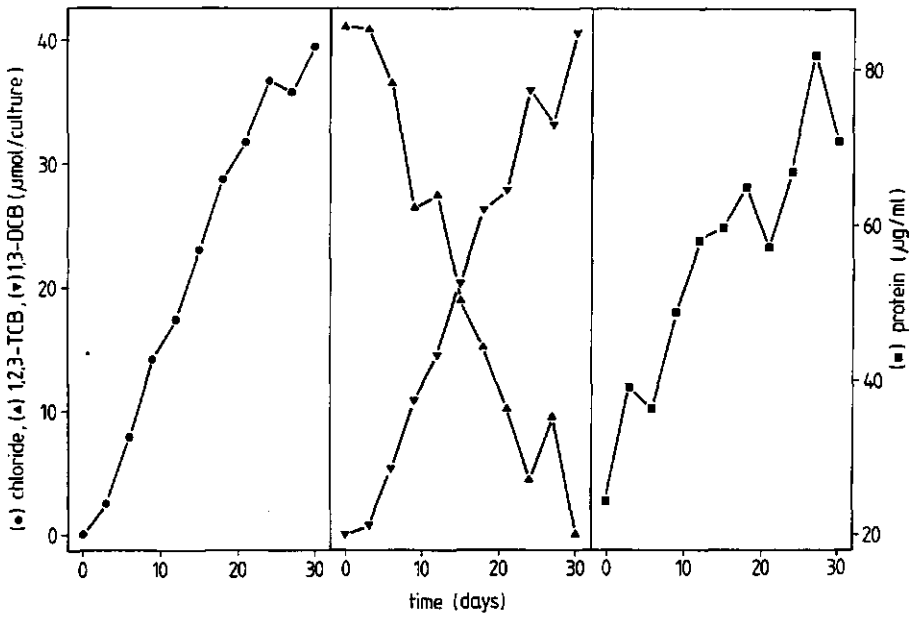

FIGURE 5.6. Stoichiometry of the dechlorination of 1,2,3-TCB and growth of the 1,2,3-TCB dechlorinating anaerobic mixed culture in a two-liquid-phase system. The medium was amended with 0.02% yeast extract and 5 mM 2-bromoethanesulfonic acid. The gas phase was H_2/CO_2 (4/1, v/v) at 1.5 bar. Incubation was stationary at 30°C. At day 0 and every third day four cultures were sacrificed for analysis. Two cultures were extracted with 5 ml hexane for analysis of chlorobenzenes, the water phase of the other two was analyzed for chloride and protein content. (●) chloride, (▲) 1,2,3-TCB, (▼) 1,3-DCB, (■) protein.

5.5. Discussion

The reductive dechlorination of HCB, QCB, and all three isomers of TeCB in enrichments inoculated with river Rhine sediment shows that indigenous microorganisms in this sediment are able not only to transform trichloro- and dichlorobenzenes [24], but also highly chlorinated benzenes. These results support the comment of Bailey [8] on results presented by Oliver and Nicol [202] that reductive dechlorination of chlorobenzenes could have taken place in the Great Lakes sediments.

The anaerobic mixed culture on lactate and 1,2,3-TCB was enriched from material of a sediment column which dechlorinated all trichloro- and dichlorobenzene isomers. However, the enrichment on 1,2,3-TCB did not transform 1,2,4-TCB, 1,3,5-TCB, and all dichlorobenzene isomers. This substrate specificity of the enriched culture suggests that different bacteria are present in the sediment columns catalyzing different dechlorinations. Substrate specificity is also described for chlorobenzoates and chlorophenols. *D. tiedjei* was only able to dechlorinate *meta*-halogenated benzoates [59,266]. Enrichments able to dechlorinate 2-chloro- or 3-chlorobenzoate did not dechlorinate 3-chloro- and 4-chlorobenzoate or 2-chloro- and

4-chlorobenzoate, respectively [100]. Monochlorophenol degrading methanogenic consortia enriched from sewage sludge or sediment acclimated to one isomer also showed specificity to a certain extent [29,100]. A mix of acclimated sludges was able to completely dechlorinate pentachlorophenol, whereas in individual sludges dichloro-, trichloro-, and tetrachlorophenols accumulated [185]. All these results support the hypothesis that different microorganisms are involved in the different dechlorinations.

The specificity was only true for lower chlorinated benzenes. HCB, QCB, and the TeCB's were dechlorinated by both, enrichments inoculated with column material and the 1,2,3-TCB dechlorinating enrichment after two and a half years of subculturing on this isomer. The dechlorination pathway for HCB in our enrichments is identical with the one proposed by Fathepure et al. [89] (Fig. 5.7). The specific dechlorination of the tetrachlorobenzene isomers shows that the small amounts of 1,2,4-TCB formed from HCB or QCB were a product of 1,2,4,5-TeCB as suggested by Fathepure et al. [89]. 1,2,3,4-TeCB is not considered as possible precursor of 1,2,4-TCB because this isomer was never found in cultures amended with either HCB or QCB. Electrochemical reduction of chlorobenzenes was suggested as a model system to predict dechlorination pathways in the environment [84]. Results obtained electrochemically, however, do not correspond with pathways observed in enrichments as presented here or in other studies where 1,3,5-TCB or 1,2,3-TCB was the major product of HCB and where 1,2,3-TCB was stoichiometrically transformed to 1,3-DCB [24,87,89,166,195]. Electrochemical reduction would predict 1,2,4-TCB as major intermediate of HCB degradation and 60% of 1,2,3-TCB should be recovered as 1,2-DCB [84]. A quantitative structure-activity relationship (QSAR) study predicted the dechlorination of HCB to 1,3,5-TCB [210]. Nevertheless, HCB degradation to 1,2,3-TCB as major product [87,195] revealed that the use of QSAR's has also

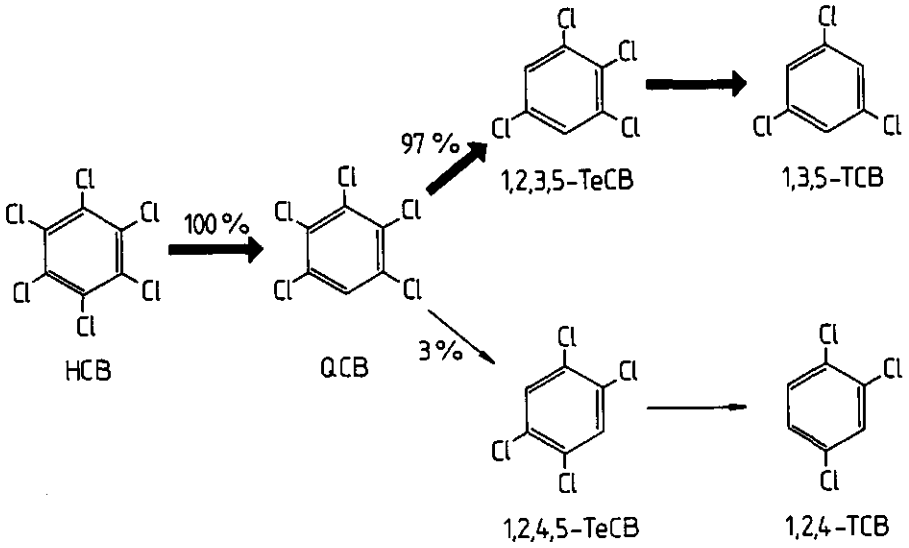

FIGURE 5.7. Reductive dechlorination pathway of HCB catalyzed by the 1,2,3-TCB dechlorinating anaerobic mixed culture.

its limitations to predict microbial mediated dechlorinations.

Dechlorination patterns of HCB or QCB differ from patterns observed with other polychlorinated aromatics. During degradation of 3,5-dichlorobenzoate, pentachlorophenol, or 2,4-dichlorophenol less chlorinated intermediates accumulated and only after the parent substrates were almost depleted, degradation of the accumulated intermediates started [185,265,312]. Suflita et al. [265] found that the best simulation of such a dechlorination pattern could be achieved by the use of a sequential Michaelis-Menten model where a competitive inhibition term was inserted. QCB nor 1,2,3,5-TeCB did accumulate to significant high amounts during the transformation of HCB to 1,3,5-TCB in our enrichments. Apparently, inhibition did not occur and dechlorinations of QCB and 1,2,3,5-TeCB were not the rate limiting steps.

Reductive dechlorination of chlorobenzenes only took place if an electron donor was added. Transformation of perchloroethene, chloroanilines, and polychlorinated biphenyls are further examples where it is shown that an electron donor is needed for dechlorinating activity [7,92,160,198,247]. Hence, the chlorinated compounds acted only as an electron sink. Calculations of half-reaction reduction potentials illustrate that chlorinated hydrocarbons are stronger oxidants than nitrate [287]. The novel type of anaerobic respiration of *D. tiedjei* with 3-chlorobenzoate as terminal electron acceptor [67,189] demonstrates that bacteria do exist which conserve energy for growth from reductive dechlorination reactions. Hydrogen, formate, acetate, or pyruvate serve as electron donors for the dechlorination of 3-chlorobenzoate by *D. tiedjei* [60,61,67,189] and sulfate, sulfite, or thiosulfate could replace 3-chlorobenzoate as electron acceptor [60,61,258]. Sulfate reducers isolated from our mixed culture did not show dechlorinating activity. Methanogenesis and acetogenesis are the most important processes which compete for H_2/CO_2 in the two-liquid-phase system. Elimination of substrate-competing organisms only partially succeeded. Inhibition of methanogenesis by 2-bromoethanesulfonic acid did eliminate this group of bacteria. A specific inhibitor for acetogens is unfortunately not known. Pure cultures of acetogens isolated from the 1,2,3-TCB dechlorinating enrichment had no dechlorinating activity which indicates that also this group of bacteria is not involved in the dechlorination of 1,2,3-TCB. All these findings suggest that bacteria are present in the 1,2,3-TCB dechlorinating mixed culture which use 1,2,3-TCB as electron acceptor and H_2 as electron donor.

Acknowledgements

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Chapter 6

Dehalobacter restrictus gen. nov. sp. nov., a highly purified bacterium which coupled growth to the reductive dechlorination of tetrachloroethene

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6.1. Abstract

A microscopically pure culture, "PER-K23", was enriched from material of an anaerobic packed-bed column which reductively transformed tetrachloroethene (PCE) to ethane via trichloroethene (TCE), *cis*-1,2-dichloroethene (*cis*-1,2-DCE), chloroethene (VC), and ethene. PER-K23 catalyzed the dechlorination of PCE via TCE to *cis*-1,2-DCE and coupled this reductive dechlorination to growth. H_2/CO_2 or formate were the only electron donors which supported growth with PCE or TCE as electron acceptors. The culture did not grow in the absence of PCE or TCE. Nor O_2, NO_3^-, NO_2^-, SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, S, fumarate, or CO_2 could replace PCE or TCE as electron acceptor with H_2 as electron donor. PER-K23 was not able to grow fermentatively on any of the organic compounds tested. Electron balances showed that all electrons derived from H_2 or formate consumption could be recovered in dechlorination products and biomass formed. $^{14}CO_2$-experiments revealed that CO_2 was only incorporated *via* heterotrophic CO_2 fixation under the growth conditions tested. The presence of low activities of CO dehydrogenase, formate dehydrogenase, and 5,10-methylene tetrahydrofolate reductase in cell extracts of PER-K23 indicated the presence of the acetyl-CoA pathway in PER-K23. Preliminary experiments showed alkyl reductive dehalogenase activity in crude cell extracts of PER-K23. Difference spectra of reduced minus oxidized cell extracts excluded the presence of cytochromes. A menaquinone has been found but was not further characterized. PER-K23 possesses major amounts of saturated iso- and anteiso-methyl branched long-chain fatty acids with 14-16 carbon atoms. The cell wall of PER-K23 contained a type A3γ peptidoglycan with LL-diaminopimelic acid at position 3 of the peptide and a glycine interpeptide bridge between position 3 and 4. PER-K23 is a Gram-negative rod with one lateral flagellum. The DNA of PER-K23 has a G+C-content of 45.3±0.3 mol%. Analysis of the 16S ribosomal RNA of PER-K23 demonstrated that this bacterium belongs to the subdivision of species with Gram-negative cell walls within the phylum of Gram-positive bacteria. Based on physiological and molecular properties of the isolate, we propose *Dehalobacter* as the name of the genus of this newly described reductive dehalogenating bacterium. The type species, *Dehalobacter restrictus* sp. nov., is named after the restricted spectra of electron donors and acceptors utilized.

6.2. Introduction

Tetrachloroethene (PCE) is one of the most often encountered contaminants in different environments [208,283,295], and is the only chloroethene isomer which persists aerobic biodegradation. The lower chlorinated chloroethene isomers can be cometabolically mineralized by different aerobic microorganisms [83,169,197,282,289, 291]. Monooxygenases with a rather broad substrate range were responsible for the observed degradation. However, cooxidizers inactivated themselves by the formation of highly reactive intermediates, possibly epoxides [169] and transformation rates were rather low. Two isoprene utilizing bacteria were the only trichloroethene (TCE) degraders described which did not suffer from inactivation during TCE degradation [83]. For chloroethene (better known as vinyl chloride, VC), bacteria are described which use this isomer as sole energy and carbon source [116].

Under anoxic conditions, PCE is found to be reductively dechlorinated *via* stepwise hydrogenolysis reactions. First indications of such biotransformations of PCE were obtained in batch experiments seeded with a methanogenic mixed culture from a laboratory-scale digester [25] or muck from a pristine site in the Everglades [207]. Further studies revealed that PCE

could even be mineralized to CO_2 [284]. In most cases, however, VC accumulated from PCE, a product even more toxic than the parent compound [15,205,284]. PCE could also be completely dechlorinated to ethene [58,64,92]. Only the packed-bed column of de Bruin et al. [58] performed complete transformation to ethene and eventually ethane with no residual VC. All these data show that anaerobic mixed cultures have the potential to detoxify PCE. The often observed incomplete transformation emphasizes the need of a better understanding of the underlying microbiological aspects involved.

Methanogens are able to dechlorinate PCE to TCE [85,86,88]. However, dechlorination was incomplete and rates were very low. Corrinoids and factor F_{430}, two tetra-pyrrole cofactors present in high amounts in methanogens [55,63], catalyzed the same reactions [98] which indicated that PCE transformation by methanogens is an unspecific activity of enzymes with corrinoids or factor F_{430} as prosthetic group. Enrichments on methanol and PCE [64] or benzoate and PCE [237] showed that bacteria others than methanogens also dechlorinate PCE. The high amounts of electrons recovered in dechlorination products (e.g. 31%) suggested that PCE served as electron acceptor in enrichments with methanol or benzoate plus PCE [64,237]. It was hypothesized that chlorinated hydrocarbons could act as terminal electron acceptor in a respiration process [70,123]. Thermodynamic calculations demonstrate that chlorinated compounds are even stronger oxidants than nitrate [287]. *Desulfomonile tiedjei* [61] dechlorinates 3-chlorobenzoate and couples this reductive dechlorination reaction to growth on formate [67,189]. Up to now this is the only bacterium in pure culture which uses reductive dechlorination as novel type of anaerobic respiration.

We obtained a microscopically pure culture, PER-K23, from a packed-bed column with H_2/CO_2 as energy and carbon source and with PCE as electron acceptor. The motile, Gram-negative, small rod did not grow in the absence of PCE. Physiological characterization together with 16S ribosomal RNA analysis suggests that PER-K23 belongs to a novel genus of bacteria.

6.3. Materials and Methods

6.3.1. Materials

All chemicals were of analytical grade and used without further purification. Tetrachloroethene (PCE), trichloroethene (TCE), 3-[N-Morpholino]-propanesulfonic acid (MOPS), dithiothreitol (DTT), dithioerythritol (DTE), and tris[hydroxymethyl]amino-methane (TRIS) were from E. Merck, Darmstadt, Germany. 1,1-Dichloroethene, *cis*-1,2-dichloroethene (*cis*-1,2-DCE), and *trans*-1,2-dichloroethene (*trans*-1,2-DCE) were obtained from Aldrich Chemie N.V., Brussels, Belgium. Chloroethene (VC), ethene, and all other gases were from Hoekloos, Schiedam, The Netherlands. Where necessary, experiments were carried out in an anaerobic glovebox (Coy Laboratories Products, Toepffer GmbH, Göppingen, Germany). The oxygen concentration in the anaerobic glovebox was kept low with R-20 palladium catalyst provided by BASF (Arnhem, The Netherlands).

6.3.2. Source of Inoculum

The inoculum was material of a packed-bed column which transformed PCE to ethane [58]. The packed-bed column was wet-packed with anaerobic sediment from the river Rhine near Wageningen, The Netherlands and with grounded granular anaerobic sludge [211] of a sugar refinery (Centrale Suiker Maatschappij, Breda, The Netherlands). The reactor was percolated

continuously with an anaerobic mineral medium [58]. Samples for inoculation were taken after 14 months of operation.

6.3.3. Anaerobic media

The medium used was phosphate/bicarbonate-buffered and had a low chloride concentration. The composition of the medium was as follows (in g/l of demineralized water, unless otherwise stated): K_2HPO_4, 0.65; $NaH_2PO_4 \cdot 2H_2O$, 0.20; NH_4HCO_3, 0.44; $CaCl_2 \cdot 2H_2O$, 0.11; $MgCl_2 \cdot 6H_2O$, 0.10; $NaHCO_3$, 3.73; $Na_2S \cdot 9H_2O$, 0.24; resazurin 0.0005; 1 ml trace element solution (containing in mg/l: $FeCl_2 \cdot 4H_2O$, 2000; $MnCl_2 \cdot 4H_2O$, 100; $CoCl_2 \cdot 6H_2O$, 190; $ZnCl_2$, 70; $CuCl_2$, 2; $AlCl_3 \cdot 6H_2O$, 10; H_3BO_3, 6; Na_2MoO_4, 36; $NiCl_2 \cdot 6H_2O$, 24; ethylenediaminetetraacetate, 500; 1 ml of concentrated HCl); vitamins (mg/l medium: biotin, 0.05; folic acid, 0.02; pyridoxine, 0.1; riboflavin, 0.05; thiamine, 0.1; cyanocobalamin, 0.1; nicotinamide, 0.55; *p*-aminobenzoic acid, 0.25; lipoic acid, 0.05; pantothenic acid, 0.05); 10 ml of a fermented yeast extract solution (4%, w/v). Vitamins were stored in 10^3-fold concentrated, filter-sterilized stock solutions at 4°C. Separate solutions of cyanocobalamin, riboflavine, and thiamine were made. The rest of the vitamins was combined in one stock solution. Anaerobic yeast extract solutions (4%; w/v) were fermented with anaerobic granular sludge of a sugar refinery (Centrale Suiker Maatschappij, Breda, The Netherlands) for 3 weeks at 37°C. Cells were removed by centrifugation (27.000 x *g*, 30 min, 4°C) and the supernatant was sterilized by filtration (0.22 μm) or by autoclaving (121°C, 20 min). Media were prepared as follows: Aliquots of stock solutions of K_2HPO_4, NaH_2PO_4, and resazurin were made up with demineralized H_2O to 90% of the final volume of the medium. Bottles were sealed with viton stoppers (Maag Technic AG, Dübendorf, Switzerland). The atmosphere was changed with either N_2/CO_2 or H_2/CO_2 (4/1, v/v) and brought to 1.5 bar. The bottles were sterilized at 121°C. The rest of the components were added aseptically by syringe from four filter-sterilized stock solutions (A-D). Solution A contained 1 ml trace element solution, 1 ml of each vitamin stock solution and 20 ml demineralized H_2O. Solution B contained NH_4HCO_3, $NaHCO_3$, and Na_2S, 20-fold the final concentration. Solution C contained $CaCl_2$ and $MgCl_2$, 40-fold the final concentration. Solution D was the fermented yeast extract solution. 25 ml from solution A and C were added per liter medium, 50 ml from solution B, and 10 ml from solution D. The pH of the medium was 7.0-7.2.

6.3.4. Enrichment and cultivation

The culture was enriched and cultivated in a two-liquid-phase system as described previously [Chapter 5]. Initial enrichment in 500 ml bottles was started by inoculating 200 ml medium with approximately 0.5 g material from a PCE dechlorinating packed-bed column. The gas phase was either N_2/CO_2 or H_2/CO_2. In case N_2/CO_2 was the gas phase, lactate was added as electron donor supplied daily to a final concentration in the enrichment of 1 mM. 5 ml PCE dissolved in hexadecane (0.2 M) was added by syringe. The log of K_{HW} (partition coefficient between hexadecane and water) of PCE is 3.7. Hexadecane was sterilized by heat (121°C, 60 min) and PCE was filter-sterilized through 0.22 μm membrane-filters (type GVWP, Millipore, Etten-Leur, The Netherlands). Cultures were incubated stationary at 30°C in the dark. Dechlorination was monitored by measuring the chloride ion concentration. If an increase of Cl^- was found, the gas phase was analyzed for the presence of dechlorination products. Initial enrichments were transferred into fresh medium after 30 days of incubation. In parallel the initial enrichment was diluted to 10^{-8}. The highest dilution showing dechlorination was diluted again to 10^{-8}. The stable

culture obtained by this procedure, in the following called "PER-K23", was routinely subcultured (1% [v/v] transfers) in 117 ml serum bottles containing 20 ml medium. For the cultivation of higher amounts of cells, several 500 ml bottles were used containing 200 ml medium. Best growth was obtained if cultures were incubated stationary for 2-3 days, followed by an incubation while shaking horizontally at 120 rpm on a rotary shaker. Cells were harvested in the late log phase. Cultures were poured into a separating funnel inside the anaerobic glovebox and the two phases were allowed to separate. The water phase with the cells was centrifuged at 27.000 *x g* at 4°C for 30 min. The pellet was resuspended in 100 ml 50 mM MOPS pH 7.0 containing 2 mM DTT and 0.2 mM $Na_2S_2O_4$. Cell suspensions were again centrifuged and the pellet was stored at -20°C under 100% hydrogen or immediately used for enzyme measurements.

Purity of PER-K23 was checked microscopically or in a medium containing 13 g of Wilkins-Chalgren anaerobe broth (Oxoid, Basingstoke, Great Britain) per liter demineralized water. The gas phase was 100% N_2.

6.3.5. Nutritional requirement

Thousand fold concentrated stock solutions of different growth factors were prepared according to Widdel [297]. Where indicated growth factors were solubilized in a buffer instead of demineralized water. Solutions were filter-sterilized (f) or autoclaved (a). The following stock solutions were prepared (mg/l): 4-aminobenzoate, 12.5 (a); biotin, 2.5 (heated up to 65°C for better solubilization; a); lipoic acid, 2.5 (50 mM sodium phosphate, pH 7; a); nicotinic acid, 25 (a); riboflavin 50 (20 mM acetic acid; a); thiamine, 5 (200 mM sodium phosphate, pH 3.4; a); nicotinamide, 25 (a); cyanocobalamin, 5 (a); cholin chloride, 100 (f); folic acid, 1 (f); *myo*-inositol, 75 (f); menadione sodium disulfite, 5 (f); pantothenic acid, 2.5 (f); pyridoxal, 2 (f); pyridoxol, 2.5 (f); pyridoxamine, 7.5 (f); 1,4-naphthoquinone, 10 (first dissolved in 20 ml acetone, then filled up to 1 l with demineralized water; f); hematin, 50 (50 mM NaOH; f). $Na_2SeO_3 \cdot 5H_2O$ (3 mg/l) and $Na_2WO_4 \cdot 2H_2O$ (4 mg/l) were dissolved in 12.5 mM NaOH and filter-sterilized. A solution of different fatty acids was filter-sterilized and contained (mM): propionate, 100; butyrate, 100; isobutyrate, 100; methylbutyrate, 20; valerate, 100; isovalerate, 20; methylvalerate, 20. An aliquot of 1 ml of each solution was added aseptically by syringe to a sterile serum bottle containing 5 ml demineralized water. Immediately thereafter, 25 ml/l of this growth factor mixture was added to the medium replacing fermented yeast extract.

6.3.6. Electron donor and acceptor utilization

Tests for utilization of various compounds as electron donors with and without PCE as electron acceptor were performed in 117 ml serum bottles containing 20 ml medium. The final concentration of the substrates was 20 mM. Formate and methanol were added to a final concentration of 50 mM, CO was tested at a partial pressure of 0.5 bar. Electron acceptors were tested at a final concentration of 10 mM with H_2 as electron donor. Chlorinated compounds others than PCE and TCE were added dissolved in hexadecane (0.2 M; 1 ml/vial), except for chloroethane and chloroethene which were added as gaseous compounds (5 ml/vial). Duplicates were inoculated (10%, v/v), and the increase in turbidity was taken as a measure for growth.

6.3.7. Effect of temperature and pH

Cultures in 117 ml serum bottles containing 20 ml medium and inoculated with 1% (v/v) were

incubated at different temperatures (15, 20, 25, 30, 35, and 37°C), stationary in the dark. They were assayed daily for chloride production.

The effect of pH was tested with cultures growing on formate and PCE. The pH of the medium was varied by using different partial pressures of CO_2 in the gas phase. Culture vials contained 40 ml medium and were inoculated with 1% (v/v). Samples of 1.5 ml were withdrawn daily by syringe and analyzed for pH and chloride concentration.

6.3.8. Electron balances

Electron balances were performed in 117 ml serum bottles containing 20 ml or 40 ml medium. The gas phase consisted of $N_2/H_2/CO_2$ (16/4/5; v/v/v) at 1.5 bar in the case of H_2 as electron donor and N_2/CO_2 (4/1) in the case of formate. Bottles were inoculated with 1% (v/v) and incubated stationary at 30°C in the dark. At time 0 and after 14 days of incubation H_2, formate, chloroethenes, chloride, and protein were determined. For the calculation of the hydrogen and formate consumption by biomass production, the following assumptions were used: dry weight of biomass contains 60% protein; $<C_5H_7O_2N>$ is the formula for biomass with a MW 113; biomass is formed heterotrophically from organic material (CH_2O) and CO_2 according to $^{10}/_3H_2 + {}^{10}/_3CH_2O + {}^{5}/_3CO_2 + NH_3 \longrightarrow <C_5H_7O_2N> + {}^{14}/_3H_2O$ or $^{10}/_3HCOOH + {}^{10}/_3CH_2O + NH_3 \longrightarrow <C_5H_7O_2N> + {}^{5}/_3CO_2 + {}^{10}/_3H_2O$.

6.3.9. $^{14}CO_2$ fixation experiment

$^{14}CO_2$ fixation experiments were performed in 117 ml serum bottles containing 40 ml medium. The gas phase was H_2/CO_2 (4/1) at 1.5 bar. Only 90% of the routinely used $NaHCO_3$ was added by solution B. The other 10% was added as follows: 97 μl 500 mM $NaH^{14}CO_3$ (100 μCi/mmol) was added to 900 μl 111 mM $NaHCO_3$ and this solution was added to cultures by syringe shortly after inoculation (1%, v/v). Cultures were incubated in a shaking waterbath at 30°C. CO_2/HCO_3^- content of the cultures was determined from cultures to which only unlabelled bicarbonate was added. After analyzing the headspace for CO_2 content, 5 ml samples were transferred into sealed 117 ml serum bottles containing 1 ml 5 M HCl. The headspace of these bottles was analyzed for CO_2 and related to standards prepared with 5 ml $NaHCO_3$ solutions of different concentrations and 1 ml 5 M HCl. For the determination of total radioactivity per culture the same amount of $NaH^{14}CO_3$ was added to viton stopper sealed 35 ml serum bottles containing 20 ml of 1 M NaOH. Samples of 50 μl were transferred to scintillation vials containing 4 ml Aqualuma scintillation cocktail. Biomass content of ^{14}C-containing cultures was analyzed by measuring protein. Total organic carbon (TOC) together with protein content was determined of cultures cultivated on unlabelled compounds. This resulted in a conversion factor for protein content into carbon content of the biomass of 0.81. To measure the radioactivity incorporated into biomass, samples of 5 ml of the cultures were filtered through a 0.22-μm membrane filter (Millipore, Etten-Leur, The Netherlands) and the filters were washed with 20 ml of 100 mM $NaHCO_3$. Air-dryed filters were exposed to HCl vapors in a desiccator for 1 h. Then, filters were transferred to scintillation vials containing 4 ml aqualuma scintillation cocktail and after several days at ambient temperature radioactivity was measured. Filters were totally dissolved at the time of measurement. The radioactivity was counted with a LKB Wallac 1211 Rackbeta liquid scintillation counter. Quench corrections were made by the channels-ratio method.

6.3.10. Electron microscopy

For preparation of thin sections, cells were washed and centrifuged. The cell pellet was fixed in 3% (v/v) glutaraldehyde and 1% OsO_4 (w/v) in 0.1 M sodium cacodylate buffer pH 7.2 for 1 h at 0°C. The cell pellet was dehydrated in graded ethanol and impregnated with epoxypropane. Subsequently, the cell pellet was embedded in Epon 812. Ultra-thin sections of 50-70 nm were cut on a Reichert Ultracut E microtome. After staining with uranyl acetate and lead citrate, sections were examined with a Philips 201 transmission electron microscope.

For negative staining cells were fixed in 3% (v/v) glutaraldehyde and 2% paraformaldehyde (w/v) in 10 mM sodium phosphate buffer pH 7.0. A formvar coated copper grid (400 mesh, ⌀ 3 mm, reinforced with a 5 nm carbon layer) was placed on a drop of cell suspension for 5 min. After washing the grid with distilled water, cells were contrasted with uranyl acetate and the grid was air-dryed. The samples were examined with a Philips CM 12 transmission electron microscope or a Zeiss EM 109 Turbo transmission electron microscope.

For shadow-casting with palladium a formvar coated copper grid (150 mesh, ⌀ 3 mm) was laid on a drop of cell suspension of unfixed cells for 5 min. The air-dryed grid was coated with a 1-2 nm thick layer of palladium (amount vaporized 18 mg) in a Balzers Coater BAE 80T at $5 \cdot 10^{-5}$ mbar with a fixed angle of 30° from a distance of 15 cm. The samples were examined with a Philips CM 12 transmission electron microscope.

6.3.11. Determination of G+C content

Pelleted cells were resuspended in 10 mM TRIS/HCl pH 8.0 containing 1 mM EDTA, 0.5% SDS and 1 g/l proteinase K. After 20 min incubation at 55°C, phenol was added (35%, v/v) and incubation was continued for 15 min. The suspension was thoroughly deproteinized by several phenol/chloroform extractions followed by a chloroform extraction. Nucleic acids were precipitated with 40% isopropanol and washed with 70% ethanol. DNA was dried and resuspended in 10 mM TRIS/HCl containing 1 mM EDTA. After hydrolyzation with P1 nuclease and dephosphorylation of the nucleotides with bovine alkaline phosphatase, deoxyribonucleosides were analyzed by HPLC [183].

6.3.12. Preparation of cell extracts

All procedures were carried out anaerobically. 0.5 g wet cells were suspended in 1.5 ml 50 mM MOPS pH 7.5 containing 2 mM DTT, 0.2 mM NaS_2O_4, and 50 μg/ml DNase. The cell suspension was subjected to ultrasonic disintegration (6 times 30s at 0°C). Cell debris were removed by centrifugation at 27.000 x g at 4°C for 30 min. The supernatant (cell extract) containing 8-16 mg of protein/ml as measured by the method of Bradford et al. [31] was stored in 8.5 ml serum vials under 100% N_2 at 0°C.

6.3.13. Enzyme assays

Enzyme assays were performed in quartz glass cuvettes (d=1 cm) at 30°C filled with 1 ml of reaction mixture and closed with butyl rubber stoppers, unless otherwise stated. One unit of enzyme activity is defined as 1 μmol substrate consumed or product formed per min (in the case of methyl viologen 1 U = 2 μmol reduced or oxidized per min).

Hydrogenase, formate dehydrogenase, pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and CO dehydrogenase were assayed in 50 mM TRIS/HCl pH 8.0 containing 1 mM methyl viologen and 0.1 mM DTE. Gas phase was either 100% N_2, H_2, or CO. For formate dehydrogenase the reaction mixture contained in addition 10 mM sodium formate, for pyruvate dehydrogenase 10 mM sodium pyruvate and 0.2 mM HSCoA, and for 2-oxoglutarate dehydrogenase 10 mM sodium 2-oxoglutarate and 0.2 mM HSCoA. Before starting the reaction with 80-160 μg extract protein, a sodium dithionite solution (20 mM) was added until a slightly blue colour was observed. Reduction of methyl viologen was followed at 578 nm (ϵ_{578} = 9.7 $mM^{-1} \cdot cm^{-1}$).

5,10-Methylenetetrahydrofolate reductase was assayed according to Spormann and Thauer [256]. Methyltetrahydrofolate (CH_3-FH_4) oxidation to methylenetetrahydrofolate was followed by measuring methylene blue reduction at 578 nm (ϵ_{578} = 17.1 $mM^{-1} \cdot cm^{-1}$.). The reaction mixture contained: 50 mM MOPS/KOH pH 7.5, 0.1 mM methylene blue, and 50-200 μg extract protein. The reaction was started by adding 0.13 mM CH_3-FH_4.

Reductive dehalogenase was assayed by measuring oxidation of reduced methyl viologen or by measuring the formation of dechlorination products in 10 ml serum vials with hydrogen as electron donor. The reaction mixture contained: 100 mM TRIS/HCl pH 8.0, 1 mM methyl viologen, and 0.1 mM DTE. In cuvettes the gas phase was 100% N_2 and methyl viologen was reduced with sodium dithionite until an absorbance A_{578} of about 2 was reached. The reaction was started either by the addition of 50-200 μg extract protein or the addition of 10 μl 100 mM PCE in ethanol. In experiments with 10 ml serum vials with hydrogen and PCE cell extracts of *Clostridium granularum* [217] served as control.

6.3.14. Nucleic acid isolation

Total DNA extraction from PER-K23 cultures grown on H_2/CO_2 plus PER as well as from PER-K23 cultures grown on Wilkins-Chalgren anaerobe broth medium (in the following called PER-K23-rich) was performed using the method described for the determination of the G+C content. Ribosomal RNA from both cultures as well as from *E. coli* was obtained after mild sonication in 7.5 M guanidine-hydrochloride/ 1 M TRIS/HCl, pH 7, precipitation with ethanol and phenol/ chloroform extraction [112].

6.3.15. Oligonucleotides

For PCR amplification, the eubacterial 16S rRNA-targeted primer 1510 [81] was synthesized with an additional restriction site (*Pst*I, 5'GTG*CTGCAG*GG-TTACCTTGTTACGACT). The second primer was complementary to primer 124 [81] equipped with an additional *Bam*HI site (5'CAC*GGATCC*GGACGGGTGAGTAACACG). The universal eubacterial primers 530, 5'GTATTACCGCGGCTGCTG; 704, 5'TCTGCGCATTCCAC; 900, 5'CCGTCAATTCATTTGA-GTTT; 1115, 5'AGGGTTGCGCTCGTTG and 1390, 5'GTGAAGCTTCGGTGTGTACAAGG-CCC commonly used in reverse transcriptase sequencing of 16S rRNA [81,161] were used in sequence analysis or hybridization studies. Probe PER-K23 (5'TCTCAACTTTCCCCGAAG) was designed after sequence alignment and comparison of the two sequences obtained from PER-K23 and PER-K23-rich with those of related organisms obtained from the EMBL database.

6.3.16. PCR amplification, cloning and sequencing

PCR was performed in a total volume of 100 µl containing 10 µl 10 x PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM Tris/HCl pH 8.4, 1 mg/ml gelatine, 0.001 % NP-40), 1 µl dNTPs (each 10 mM in 10 mM Tris/HCl, pH 7,5), 0.2 µl Taq polymerase (BRL, 5 U/ µl), 1 µl of both primers (100 ng/ µl) and 1 µl DNA preparation. Thirty rounds of temperature cycling (95°C for 1 minute, 50°C for 2 minutes and 72°C for 3 minutes) were followed by a final seven minute incubation at 72°C.

Prior to cleavage with restriction enzymes (*Bam*HI and *Pst*I, BRL) and cloning, the amplification products were treated with proteinase K (BRL, final concentration 60 ng/100 µl) at 37°C for 15 minutes [52], phenol/chloroform extracted and precipitated with ethanol. The DNA-fragments were obtained by TAE-agarose gel electrophoresis followed by excision of the fragments and subsequent purification by the Gene-Clean procedure (Bio 101, La Jolla, California). Cloning of the amplification products into M13mp18/19 was done by standard methods [178].

Cloned amplification products were sequenced using the T7 DNA polymerase kit (Sequenase, U.S.Biochemicals) following the manufacturer's instructions.

6.3.17. Filter hybridization

Dot blot hybridization experiments were performed on GeneScreen filters (DuPont). Ribosomal RNA (100 ng/ dot) was applied with a Hybri.Dot manifold (BRL), immobilized by UV light and hybridized according to Church and Gilbert [44]. Oligonucleotide probes were 5'-labelled using phage T_4 polynucleotide kinase (BRL) and 10-20 µCi of [γ-^{32}P]adenosine-5'-triphosphate (3000 Ci/mmol; Amersham) [178].

6.3.18. Sequence alignment and phylogenetic tree

The partial nucleotide sequence was aligned to those of other eubacteria taking into account both, sequence similarity and higher order structure. Evolutionary distance values (Knuc) were calculated according to Hori and Osawa [124]. The unrooted phylogenetic tree was constructed using the neighbor joining method of Saitou and Nei [227] as implemented in the program NEIGHBOR in the program package (PHYLIP version 3.4) developed by Felsenstein [91]. The topology of the tree was evaluated performing a bootstrapped parsimony analysis using the program DNABOOT of Felsenstein.

6.3.19. Analyses

Chloroethenes, H_2, and CO_2 were determined gas chromatographically. PCE, TCE, and *cis*-1,2-DCE were determined in a headspace sample with a 438A Chrompack Packard gas chromatograph equipped with a flame ionization detector connected to a capillary column (25m by 0.32mm [inner diameter], Sil 5CB, 1.22 µm, Chrompack, NL) and a splitter injector (ratio 1:50). Operating temperatures of the injector, column, and detector were 250, 90, and 300°C, respectively. Carrier gas was nitrogen with an inlet pressure of 30 kPa. Standards were prepared in 117 ml serum bottles containing 20 ml water and 200 µl hexadecane, and sealed with viton stoppers. PCE, TCE, and *cis*-1,2-DCE dissolved in ethanol was added to these bottles. The retention times and peak areas were determined with a Shimadzu C-3A computing integrator.

H_2 was measured with a 417 Packard gas chromatograph equipped with a thermal conductivity detector at 100 mA connected to a molecular sieve column (13X, 180 cm by 1/4", 60-80 mesh). Operating temperature of the detector and the column was 100°C. Carrier gas was argon at a flow rate of 30 ml/min. CO_2 was measured with a 406 Packard gas chromatograph equipped with a thermal conductivity detector at 100 mA connected to a packed column (Porapak Q, 600 cm by 1/8", 80-100 mesh). Operating temperature of the detector and the column was 100°C. Carrier gas was argon at a flow rate of 30 ml/min. Standards were prepared by adding different amounts 100% CO_2 to sealed 117 ml serum bottles or by adding sodium carbonate solution to sealed 117 ml serum bottles containing 1 ml 5 M HCl.

Chloride was analyzed as described previously [Chapter 5]. Formate was analyzed by HPLC [126]. Protein of whole cells was measured by a modified Lowry method [193]. TOC was determined with an Ionics 555 carbon analyzer (Thermal Instruments, Etten-Leur, The Netherlands).

6.3.20. Other methods

Gram staining was performed by standard procedures [65]. In addition, the Gram type was checked by the KOH method [106]. Fatty acid analysis was carried out as described by Mirza et al. [188]. Preparation of the cell walls and determination of the peptidoglycan type were carried out as described by Schleifer and Kandler [234]. Analyses of quinones were performed as described by Collins et al. [45].

6.4. Results

6.4.1. Enrichment and isolation

Initial enrichments were performed with lactate or H_2/CO_2 as electron donor, PCE as electron acceptor, and were inoculated with material from a PCE dechlorinating packed-bed column. After 33 days of incubation the chloride ion concentration had increased by 3.4-4.6 mM. Analysis of the gas phase revealed that dechlorination products like TCE, *cis*-1,2-DCE, VC, and ethene were formed. Ethane, the end product of the PCE degradation in the packed-bed column, was not found. Enrichment was continued with H_2/CO_2 only. In the first transfer, methanogenic activity was inhibited by 2-bromoethanesulfonate (5 mM) which had no effect on dechlorination. Already after several serial dilutions, methanogens were not present anymore and the routine addition of 2-bromoethanesulfonate was stopped. Two morphologically different bacteria were predominant in the PCE dechlorinating culture, a small thin rod and a larger thick rod. In the absence of PCE, only the thick rod grew on H_2/CO_2. The formation of up to 20 mM acetate from H_2/CO_2 indicated that the thick rod was a homoacetogen. In initial enrichments and subsequent dilution series, the medium did not contain fermented yeast extract, but selenium and tungsten were present as trace elements. Amendment of the medium with fermented yeast extract increased dechlorination rates. Omission of selenium and tungsten resulted in the loss of the homoacetogen. With additional four serial dilutions, a microscopically homogeneous culture was obtained consisting of the small thin rod. Growth was only observed in the presence of PCE. Nor methane nor acetate was found in the presence of H_2/CO_2. In the following, the dechlorinating small thin rod will be referred to as "PER-K23".

Purity-tests on Wilkins-Chalgren anaerobe broth medium indicated that the dechlorinating culture was still contaminated. If the rod-shaped bacterium growing on this complex medium

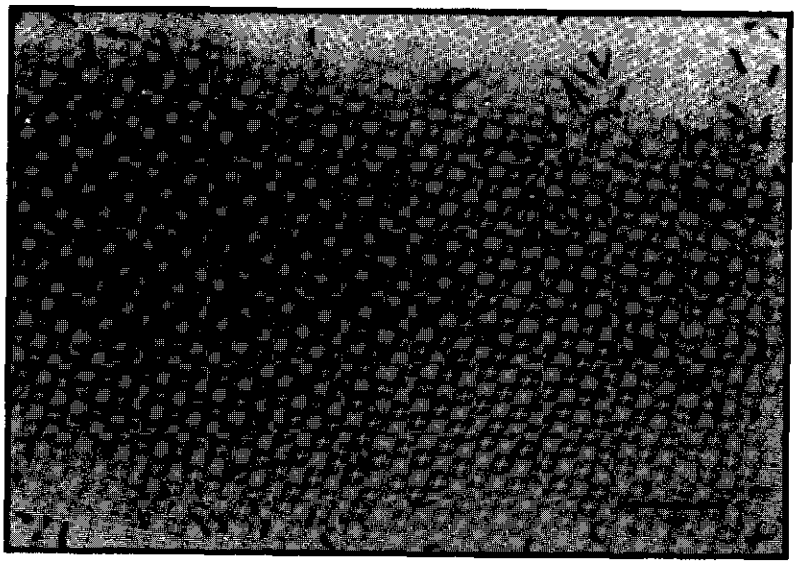

FIGURE 6.1. Bright-light micrograph of methylene blue colored cells of PER-K23; bar indicates 10 μm.

was isolated by dilution series or from single colonies on agar-containing complex medium and transferred into medium with H_2/CO_2 and PCE no dechlorination was observed. Comparing partial sequences of the 16S rRNA indicated that PER-K23 and the bacterium growing on the complex medium were indeed two different organisms (data not shown). In the following, the bacterium growing on the complex medium will be referred to as "PER-K23-rich".

6.4.2. Morphology and cytological characterization

PER-K23 was a motile, straight rod with tapered ends (Fig. 6.2 and 6.3). In starved cultures, no spores have been observed. The Gram stain was negative. The length of the cells varied depending on the incubation conditions. Cells were up to 8 μm long in stationary incubated cultures. In shaken cultures all cells had about the same length of 2-3 μm and occurred single or in pairs (Fig. 6.1 and 6.3A-C). The diameter was 0.3-0.5 μm. PER-K23 had one lateral flagellum which was about 6 μm long (Fig. 6.3A-C). Long cells from non-shaken cultures had several lateral flagella regularly distributed lengthways the cell (not shown). Ultrathin sections showed irregular structures in the cytoplasm (Fig. 6.2) and in some cells an electron-dense granule was present (Fig. 6.3B). A typical feature in non-shaken cultures was the formation of a small daughter cell (Fig. 6.3D+E). Reduced minus oxidized difference spectra of cell extracts showed a distinct peak at 397 nm and did not indicate the presence of cytochromes (Fig. 6.4). Menaquinone(s) were detected in PER-K23, however, amounts were too low for a further characterization. The DNA base composition of PER-K23 was 45.3±0.3 mol% G+C.

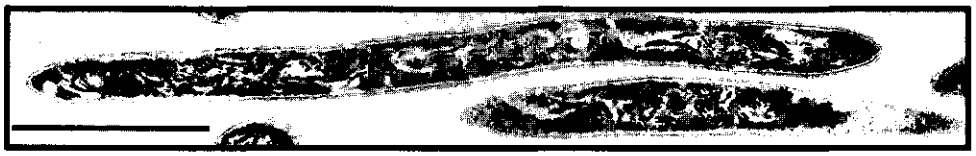

FIGURE 6.2. Electron micrograph of a thin section of PER-K23; bar indicates 1 μm.

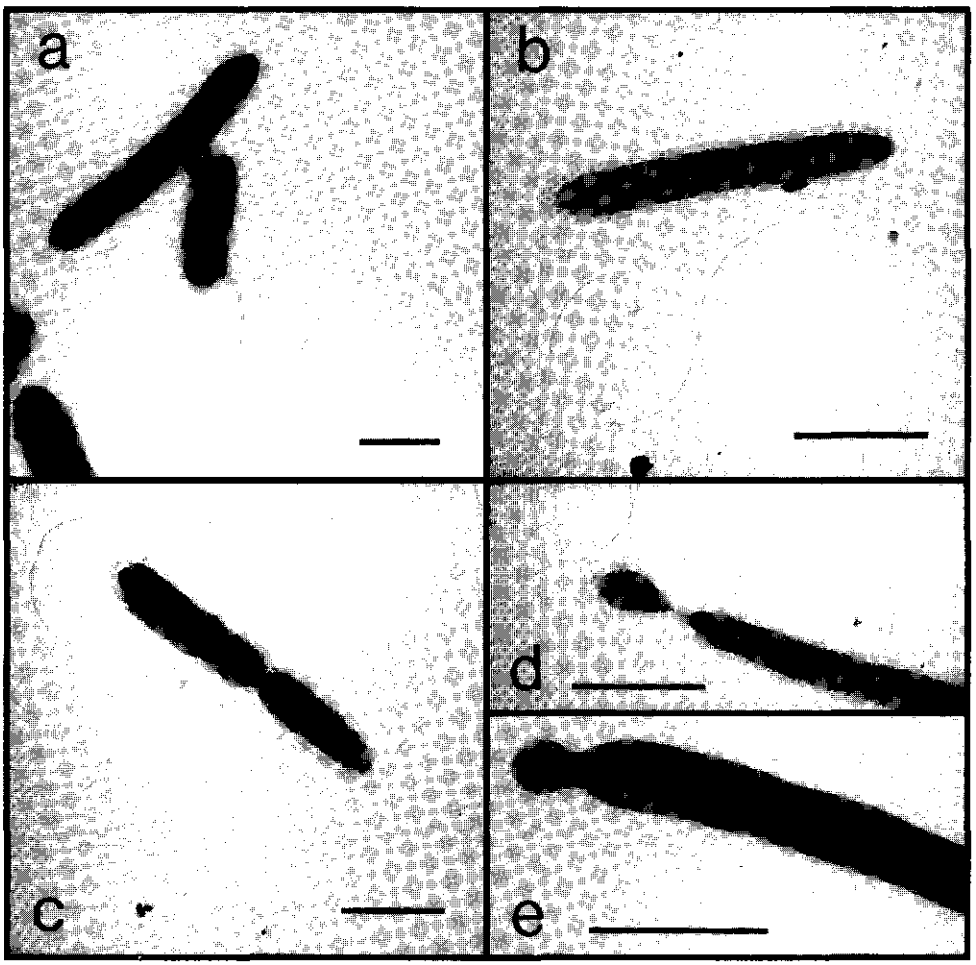

FIGURE 6.3. Electron micrographs of negatively stained (a,e) or shadow-casted (b-d) cells of PER-K23 demonstrating the lateral flagellum, cells in pairs, the formation of small daughter cells, and the presence of an electron dense particle; bars indicate 1 μm.

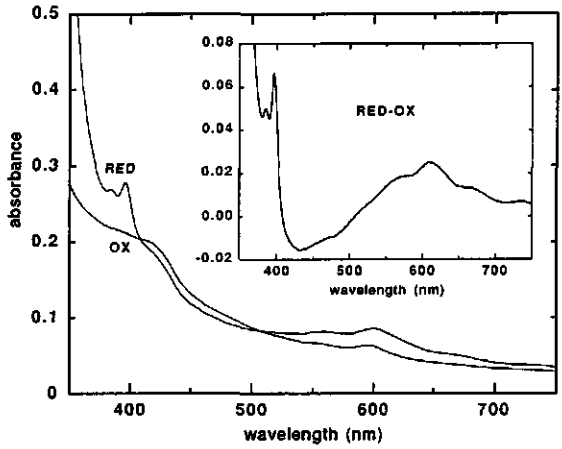

FIGURE 6.4. Spectra of oxidized (OX) and dithionite reduced (RED) cell extracts of PER-K23, obtained with an SLM Aminco DW-2000 spectrophotometer interfaced to an IBM computer. Inset: Difference spectra RED-OX obtained by subtraction of the spectra digitally.

6.4.3. Nutritional requirements, physiology and growth conditions

PER-K23 grew only in mineral media supplemented with 1% (v/v) of a fermented yeast extract solution (40 g per liter). Addition of a mixture of different growth factors to media did not substitute for fermented yeast extract. Attempts to replace fermented yeast extract by supernatant of a grown PER-K23 culture or of a culture of PER-K23-rich grown on medium amended with 1% yeast extract, or by crude or boiled cell-extracts of PER-K23-rich grown on Wilkins-Chalgren anaerobe broth failed.

PER-K23 had a very narrow substrate spectrum. The sole electron donors which supported growth were H_2 and formate, and that only in the presence of PCE or TCE as electron acceptors (Table 6.1). PCE or TCE could not be replaced by any of the tested chlorinated C-2 hydrocarbons nor by inorganic electron acceptors used by other anaerobic bacteria (Table 6.1).

PER-K23 grew optimally between 25 and 35°C (Fig. 6.5A). At 37°C no growth was observed. Cultures incubated at 10°C did not grow. Dechlorinating activity in the packed-bed column incubated at 10°C indicated that dechlorination can take place at such low temperatures typical

TABLE 6.1. Electron donors and electron acceptors tested for growth with PER-K23

Electron donors utilized with PCE as electron acceptor:[a]
H_2 and formate
Electron donors not utilized with and without PCE:
lactate, pyruvate, propionate, butyrate, acetate, succinate, fumarate, glycine, alanine, aspartate, glutamate, methanol, ethanol, propanol, glucose, fructose, xylose, glycerol, acetoin, CO
Electron acceptors not utilized with H_2 as electron donor:
NO_3^-, NO_2^-, SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, S, and CO_2
acetoin, acetol, dimethylsulfoxide, fumarate, glycine, 2-oxoglutarate, pyruvate, trimethylamine *N*-oxide
all chloroethane isomers, 1,1-DCE, *trans*- and *cis*-1,2-DCE, VC, hexachloro-1,3-butadiene

[a] No growth obtained in the absence of PCE as electron acceptor.

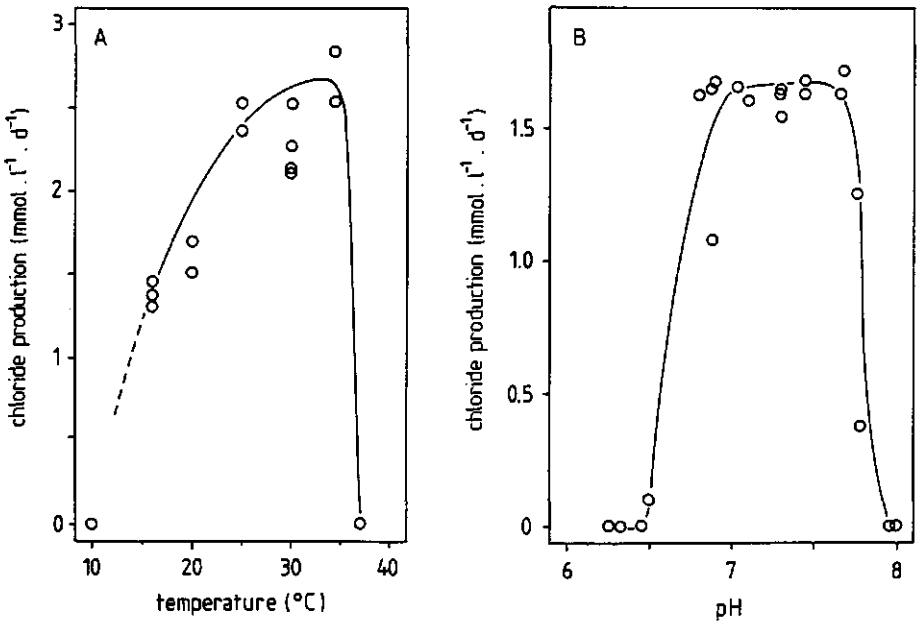

FIGURE 6.5. (A) Temperature- and (B) pH-dependence of the chloride production rate on PCE

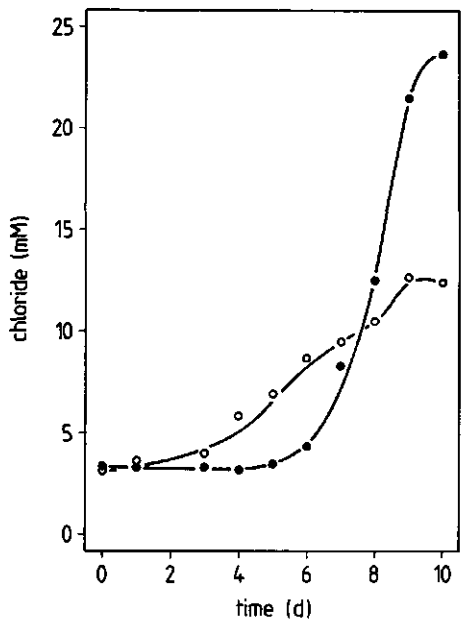

FIGURE 6.6. Chloride production on PCE by PER-K23 during (o) stationary incubation or (●) incubation on a rotary shaker at 120 rpm.

for sediments and aquifers in the Netherlands [58]. Dechlorination rates remained constant between pH 6.8 and 7.6 (Fig. 6.5B). No growth was observed below pH 6.4 and above pH 8.0. Maximum PCE or TCE concentrations in hexadecane for growth were 1 M and 0.5 M, respectively (data not shown). Higher concentrations were apparently toxic for PER-K23.

Chloride production in stationary incubated cultures became linear after a short exponential phase (Fig. 6.6) indicating growth limitation caused by too slow mass transfer of H_2, CO_2, or PCE. Gently shaking cultures of PER-K23 indeed extended the exponential phase (Fig. 6.6). A maximal growth rate of 0.024 h^{-1} (t_d = 29 h) was estimated from the chloride production data.

6.4.4. H_2 and PCE metabolism during growth, electron balances and growth yields

H_2 consumption was paralleled by chloride production, PCE degradation, *cis*-1,2-DCE formation, and growth of PER-K23 (Fig. 6.7). TCE was only found in minor amounts and did not accumulate. In electron balances determined from PER-K23 cultures grown on H_2/CO_2 plus PCE or formate plus PCE, all electrons derived from H_2 or formate oxidation were completely recovered in dechlorination products and biomass (Table 6.2). This showed that no other electron consuming process took place in these cultures. The growth yield was 2.1 g protein/mol Cl^-. However, this might be an underestimation since the hexadecane phase was present as an emulsion after growth of PER-K23 indicating that possibly significant amounts of biomass could have been adsorbed to the organic phase.

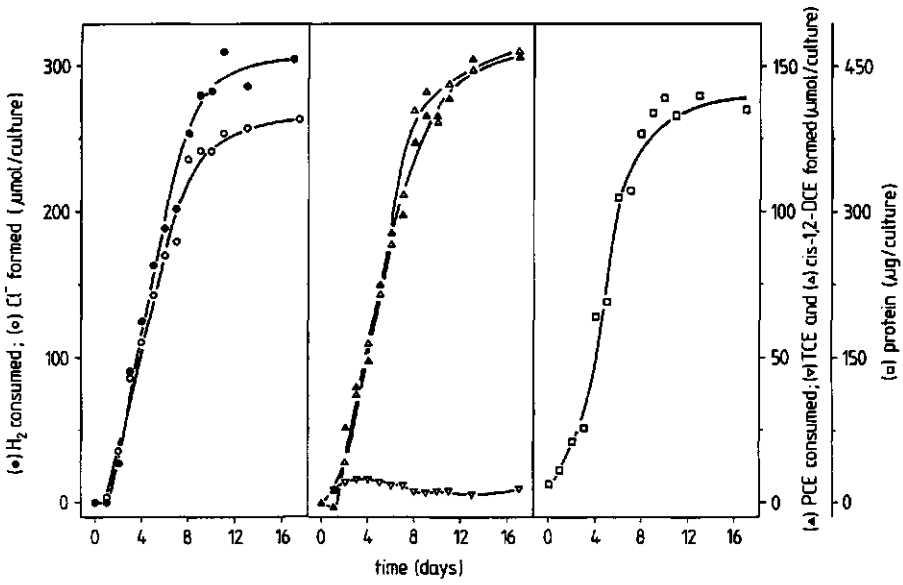

FIGURE 6.7. H_2 and PCE consumption and product formation during growth of PER-K23.

TABLE 6.2. Electron balances of the reductive dechlorination of PCE to *cis*-1,2-DCE by PER-K23 with hydrogen or formate as electron donors.

Electron donor [a]	H_2 or formate consumed	PCE consumed	TCE formed	*cis*-1,2-DCE formed	Chloride formed	Protein formed	Hydrogen or formate consumed			
							Measured	Calculated from		
								Chloride +biomass	DCE-TCE +biomass	PCE-TCE +biomass
	(μmol/culture)					(μg/culture)	(μmol/culture)	(μmol/culture) (% recovery ± SD) [b]		
H_2	282	145	5	134	248	315	282	263 (93±3)	288 (99±7)	300 (107±9)
formate	600	294	3	302	502	752	600	539 (92±11)	644 (109±5)	622 (96±8)

[a] Per electron donor an electron balance of one culture is given.
[b] Values in parenthesis are percentages of the electrons derived from electron donor consumption recovered in dechlorination products and biomass and are means of 5 independent cultures with standard deviation (SD).

6.4.5. $^{14}CO_2$ incorporation into biomass

The specific radioactivity of the carbon fraction in the biomass was only about one third of the specific radioactivity in the CO_2/HCO_3^- pool (Table 6.3). This indicated that CO_2 has been incorporated into biomass by heterotrophic CO_2-fixation (e.g. pyruvate synthase) and autotrophic growth did not occur in the employed medium. Apparently, still enough assimilable organic compounds were present in the fermented yeast extract to account for the rest of the biomass formed. Normal activities of pyruvate synthase and low activities of CO dehydrogenase, formate dehydrogenase, and 5,10-methylenetetrahydrofolate reductase in cell extracts of PER-K23 also suggested that CO_2 was only heterotrophically fixed (Table 6.4). However, the latter enzyme activities indicated the presence of the acetyl-CoA pathway. The absence of 2-oxoglutarate dehydrogenase excluded the presence of a complete tricarboxylic acid cycle.

TABLE 6.3. $^{14}CO_2$ incorporation into biomass during growth of PER-K23 on H_2/CO_2 and PCE [a]

Fraction	Carbon content	Radioactivity	Specific Radioactivity	
	(μmol/culture)	(dpm/culture)	(dpm/μmol C)	(%)
CO_2/HCO_3^-	3164	$1.071 \cdot 10^7$	3385	100
biomass	90	$1.034 \cdot 10^5$	1149	34

[a] The values given are means of three independent cultures.

TABLE 6.4. Enzyme activities in cell extracts of PER-K23

Enzyme	Specific activity (nmol/min per mg protein)
hydrogenase	77-352
CO dehydrogenase	8-25
formate dehydrogenase	16-61
5,10-methylenetetrahydrofolate reductase	5-9
pyruvate synthase	116-304
2-oxoglutarate dehydrogenase	0

6.4.6. Alkyl reductive dehalogenase

In preliminary qualitative experiments the presence of an alkyl reductive dehalogenase activity was tested. The two dechlorination products TCE and *cis*-1,2-DCE were formed in 10 ml serum vial with 1 ml reaction mixture, extract protein from PER-K23 (200 μg), and H_2 in the gas phase. Controls with extract protein of *Clostridium granularum* which had high hydrogenase activity showed no dechlorinating activity. Assays where the oxidation of dithionite reduced methyl viologen was followed spectrophotometrically showed alkyl reductive dehalogenase activities of 72±11 nmol/min per mg protein. The activity was destroyed by heat but was not affected by short exposure of cell extracts to air (30 min). Analysis of a headspace sample of a cuvette where methyl viologen oxidation was observed showed that TCE and *cis*-1,2-DCE were formed.

6.4.7. Fatty acid composition

The fatty acid analysis revealed that five major fatty acids (14:0 iso, 15:0 iso, 15:0 anteiso, 16:0 iso, and the unknown 16:? iso OH) accounted for approximately 80% of the total fatty acids detected in PER-K23 (Table 6.5). The same fatty acids only comprise 21% of the total fatty acids of PER-K23-rich. Differences can be expected if a bacterium is cultivated in two different media, but such a shift in fatty acid composition is unlikely. Therefore, these results were additional evidence that PER-K23 and PER-K23-rich were two different organisms. Characteristic for the fatty acid composition of PER-K23 is the absence of unsaturated fatty acids and predominance of branched 14-16 carbon fatty acids.

6.4.8. Peptidoglycan amino acids

Cell walls of PER-K23 contained the peptidoglycan type A3γ (Fig. 6.8) [234]. The peptidoglycan amino acids were alanine, glutamic acid, glycine, and LL-diaminopimelic acid. Glycine formed an interpeptide bridge between position 3 and 4 of the oligopeptides L-Ala-D-glu-LL-Dpm-D-Ala.

TABLE 6.5. Fatty acid composition of PER-K23 and PER-K23-rich [a]

Fatty acid	PER-K23 (%)	PER-K23-rich (%)
Saturated		
12:0 [b]	—	1.2
14:0	2.2	2.1
16:0	3.6	6.8
Unsaturated		
16:1 cis9	—	1.9
Branched		
13:0 iso	—	4.1
13:0 anteiso	—	3.2
14:0 iso	11.6	2.6
15:0 iso	10.3	11.2
15:0 anteiso	24.3	6.6
16:0 iso	10.7	—
17:0 iso	1.1	0.8
17:0 anteiso	1.5	—
Hydroxy		
14:0 OH	0.4	1.4
15:0 iso OH	1.0	3.3
15:0 anteiso OH	1.3	0.5
16:0 OH	8.2	26.7
17:0 iso OH	2.3	2.8
17:0 anteiso OH	3.7	0.4
Aldehyde	1.9	14.5
16:0 alde		
Unknown	14.7	1.0
16:? iso OH		

[a] Fatty acids detected in amounts <1% in only PER-K23, PER-K23-rich, or both cultures were not included in the table.

[b] Number of carbon atoms in fatty acids: Number of double bonds per molecule.

6.4.9. Phylogeny

The 16S rRNA sequence of PER-K23 was determined by cloning and sequencing a PCR product (1419 b) isolated from a PER-K23 culture grown on H_2/CO_2 plus PCE. A dot blot hybridization experiment provided strong evidence that the obtained 16S rRNA sequence originated from the PCE dechlorinating organism and was not amplified ribosomal DNA of the contaminant (PER-K23-rich). Ribosomal RNA isolated from PER-K23 cells or from PER-K23-rich cells was hybridized with a universal eubacterial probe or probe PER-K23. Hybridization signals with probe PER-K23 were only found with rRNA of PER-K23 whereas the universal probe hybridized with both rRNA isolations (data not shown).

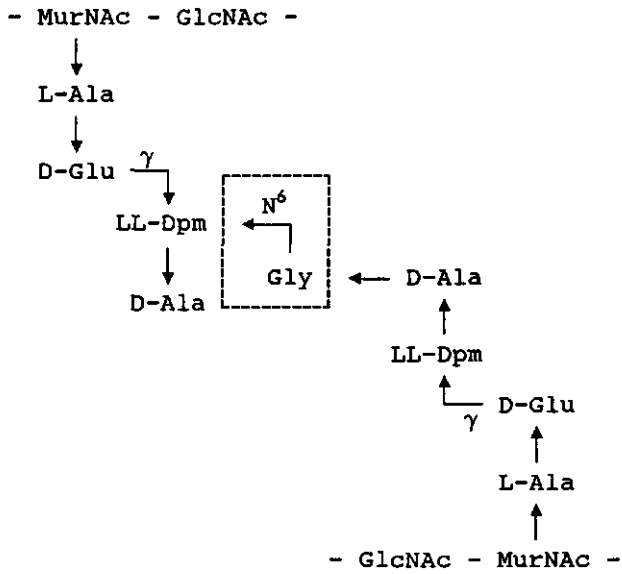

FIGURE 6.8. Fragment of the primary structure of the peptidoglycan of PER-K23. The interpeptide bridge is marked by a dashed frame.

The phylogenetic relationship of PER-K23 to other eubacteria is depicted in Fig. 6.9. The 16S rRNA sequence analysis indicated that PER-K23 belongs to the subdivision of species with Gram-negative cell walls within the phylum of Gram-positive bacteria [302]. These sequence data support the results of the physiological characterization where PER-K23 could not simply be assigned to an already known group of bacteria.

6.5. Discussion

PER-K23 is the first bacterium described which completely depends on a chlorinated hydrocarbon as an electron acceptor. *D. tiedjei* was the first organism in pure culture which coupled growth on formate, hydrogen, or acetate to a reductive dechlorination reaction, namely of the reductive dechlorination of 3-chlorobenzoate [67,189]. After extensive physiological characterization and 16S rRNA analysis *D. tiedjei* was classified as a new species of a new genus of sulfate-reducers [61]. PER-K23 could not be affiliated to an already known group of bacteria, nor on basis of physiological characteristics, nor on basis of 16S rRNA sequence analysis. A particular feature of PER-K23 is the narrow spectrum of electron donors utilized. Other anaerobic H_2 oxidizing eubacteria like sulfate-reducers or homoacetogens are more versatile [66,298]. Methanogens are the only group of bacteria with a comparable limited number of substrates [288]. However, 16S rRNA analysis revealed that PER-K23 does not belong to the archaebacteria, the primary kingdom that includes methanogens [302]. Most surprisingly, PER-K23 utilized only PCE and TCE as electron acceptors. Chlorinated ethenes have no natural origin and were not present in the environment before starting large-quantity industrial applications about fifty years ago. It is not yet known what the physiological properties of

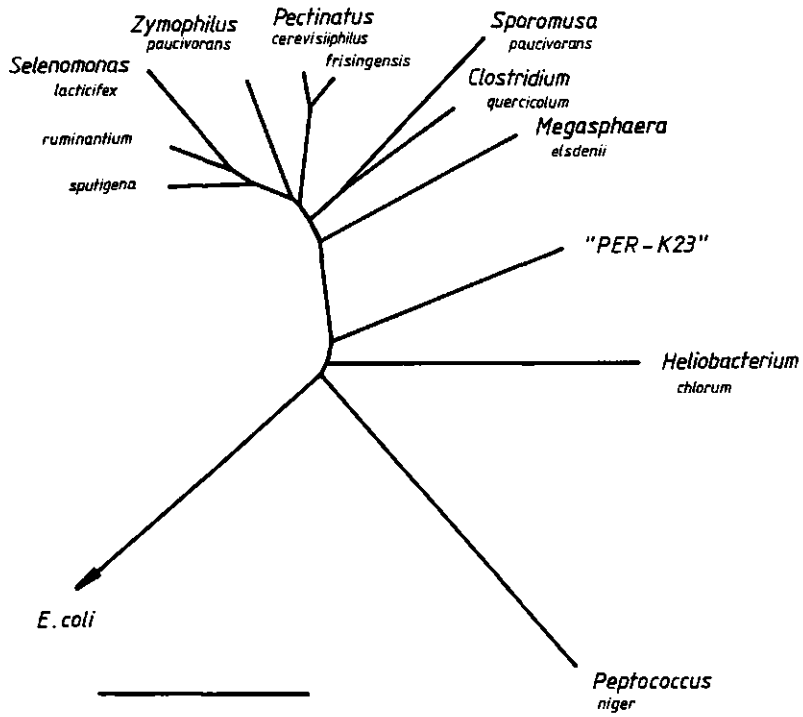

FIGURE 6.9. A distance matrix phylogenetic tree based on comparative sequence analysis of 16S rRNAs of PER-K23 and other eubacteria. Only positions which are invariant in at least 50% of the entire set of sequences were used for the calculation of the distance values. The bar indicates 0.05 Knuc.

PER-K23 were before PCE was present as a contamination in the environment. PER-K23 indicates that within a few decades a new type of bacteria has evolved. Further, it demonstrates the tremendous capacity and imaginativeness of the bacterial world to adapt to new anthropogenically altered environmental conditions.

Reductive dechlorination of PCE to *cis*-1,2-DCE is a quite exergonic reaction:

$$PCE + 2H_2 \longrightarrow \textit{cis}\text{-}1,2\text{-}DCE + 2H^+ + 2Cl^-$$

$$\Delta G^{\circ\prime} = -377.5 \text{ kJ/mol PCE.}$$

This would be enough energy to synthesize approximately 5 ATP taking into account that an energy difference of 70 kJ is needed for the formation of one ATP in an irreversible reaction under physiological conditions [267]. Since ATP formation by substrate-level phosphorylation is unlikely to occur upon H_2 oxidation, electron transport phosphorylation is probably responsible for ATP synthesis in PER-K23. Redox difference spectra of cell extracts did not indicate the presence of cytochromes, coenzymes often involved in respiratory reactions. The

exact process of energy conservation and the efficiency of this process remains to be elucidated.

The affinity of PER-K23 for PCE is apparently very high. Initial enrichment and subsequent subculturing occurred with 40 μM and 200 μM PCE in the water phase. From an energetical point of view, reductive dechlorination is more favourable than acetogenesis or methanogenesis, two processes competing for the electron donor in enrichment cultures with H_2/CO_2. However, nothing is known yet about the affinity of PER-K23 for H_2. To predict the competitiveness of PER-K23 with other H_2 or formate consuming bacteria in natural environments these kinetic data are needed. Results obtained with initial enrichments in this study and in enrichments from digested sludge [64] suggest that PCE-dechlorinating organisms can easily outcompete methanogens but not acetogens. It is not known whether winning the competition over methanogens is based on a higher substrate affinity or the toxic effects of PCE. Investigations showing the inhibitory effect of chlorinated ethenes on methanogens suggest the latter to be true [19].

Incorporation of $^{14}CO_2$ indicated heterotrophic growth of PER-K23. Autotrophic anaerobic bacteria fix CO_2 mainly by three different pathways: the reductive pentose cycle (Calvin cycle), the reductive tricarboxylic acid cycle (or reverse Krebs cycle), or the acetyl-CoA pathway (Wood pathway) [306]. The presence of activities of key enzymes of the acetyl-CoA pathway in cell extracts of PER-K23 suggested that PER-K23 could autotrophically grow by this CO_2-fixation mechanism. The measured activities could almost account for the specific growth rate of PER-K23: Given (a) the specific growth rate as $\mu = 4 \cdot 10^{-4}$ min^{-1}, (b) the carbon content of the cell material X as 50%, (c) the protein content as 60%, and (d) the portion of cell carbon ultimately derived from acetyl-CoA via the Wood pathway as two third, then the specific rate of autotrophic acetyl-CoA synthesis follows from $dX/dt = \mu \cdot X$ as approximately 18 nmol acetyl-CoA formed from CO_2/min per mg protein [229] which is about the activity measured. For *Methanobacterium thermoautotrophicum*, an autotroph assimilating CO_2 *via* the acetyl-CoA pathway and growing with a $\mu = 5.8 \cdot 10^{-3}$ min^{-1}, CO dehydrogenase activities of 200-350 nmol/min per mg protein were reported [56,76]. This is in agreement with the activity of 260 nmol/min per mg protein needed for this specific growth rate. Only the development of a defined medium can answer the question whether PER-K23 is indeed able to grow autotrophically or not.

PER-K23 completely depended on the addition of fermented yeast extract. The nature of the essential growth factor present in fermented yeast extract is not known. The contaminant PER-K23-rich was found in every dilution where growth occurred. Based on this observation, one might even postulate a certain symbiotic relationship between PER-K23 and PER-K23-rich.

Phylogenetic studies based on 16S rRNA analysis revealed a relationship of PER-K23 with *Selenomonas, Sporomusa, Megashera, Pectinatus, Zymophilus, and Heliobacterium*, all strict anaerobic bacteria. A common feature of all these strains is the Gram-negative cell wall on the one hand and the distinct although remote phylogenetic relationship to the 16S rRNA cluster of Gram-positive eubacteria on the other hand [236,257]. PER-K23 had morphologically and physiologically most in common with *Sporomusa* which has several lateral flagella, a DNA G+C content of 42-47%, and is a chemoautotroph fixing CO_2 via the Wood pathway [95,192]. Cell wall as well as lipoquinone analysis, however, did not provide additional evidence for the relationship with strains of the fourth subdivision of Gram-positive bacteria. Strains of this subdivision have direct cross-linked *meso*-diaminopimelic acid containing peptidoglycan [236,257], a completely different type of peptidoglycan than found in PER-K23. The only anaerobic bacteria which are known to have the same peptidoglycan type like PER-K23 are several Gram-positive *Propionibacterium* strains [137,234,235, N. Weiss, personal communication]. Cell walls of PER-K23 did also not contain covalently bound diamines like putrescin or cadaverin as found in cell walls of *Selenomonas, Sporomusa, Megashera* and others [236,257]. A

characteristic cellular compound of strains of the fourth subdivision of Gram-positive bacteria is lipid F, a lipoquinone of unknown structure [236,257]. PER-K23 did not contain lipid F but a menaquinone instead. The fatty acid composition did also not provide evidence for a relationship with these strains [257].

Based on the physiological and phylogenetic characteristics of PER-K23, we describe here a new genus and one new species.

6.6. Description

Dehalobacter restrictus gen. nov. et sp. nov. De.ha.lo.bac'ter. L. pref. de, off, from; Gr. n. hal salt, referring to halogens; Gr. fem. n. bacteria rod or staff; M. L. masc. n. Dehalobacter a halogen-removing rod-shaped bacterium, re.stric'tus L. adj. restrictus limited, confined, referring to the limited substrate range utilized.

A combined generic and specific description follows.

Rod-shaped, motile cells with tapered ends, 0.3-0.5 x 2-3 μm. Cells appear single or in pairs. Forms long cells upon stationary incubation. No spores formed. Gram-negative. One lateral flagellum per cell. Cell wall peptidoglycans contain L-alanine, D-glutaric acid, LL-diaminopimelic acid, and glycine and are cross-linked between position 3 and 4 with a glycine interpeptide bridge (type A3γ). A menaquinone was present but no cytochromes. Fatty acids consisted mainly of saturated 14-16 carbon atoms-containing iso- and anteiso-branched isomers.

Strict anaerobe. Chemoheterotroph. Only H_2 and formate are used as electron donor with PCE or TCE as terminal electron acceptor which are dechlorinated to *cis*-1,2-DCE. Nitrate, nitrite, fumarate, sulfate, sulfite, thiosulfate and sulfur are not reduced. Growth depends on the addition of fermented yeast extract to a mineral salts medium containing vitamins and trace elements.

Grows at temperatures between 15 and 35°C with an optimum between 25 and 35°C, and at pH values between 6.5 and 7.8, with an optimum between pH 6.8-7.6.

The G+C content of the DNA is 45.3±0.3 mol%.

Comparative analysis of 16S rRNA sequences revealed a distinct relationship with genera from the fourth subdivision of Gram-positive eubacteria.

The type strain *Dehalobacter restrictus* PER-K23 was isolated from a PCE dechlorinating packed-bed column which was filled with river Rhine sediment and grounded anaerobic granular sludge from a sugar refinery. The organism was enriched on H_2/CO_2 and PCE.

Acknowledgements

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CHAPTER 7

GENERAL DISCUSSION

7.1. Metabolic versus co-metabolic processes

In the aerobic metabolism of microorganisms, organic compounds can either serve as electron donor, carbon source, or both, or they can be transformed in a co-metabolic reaction. Co-metabolism is the fortuitous modification of a compound by one or several enzymes which normally catalyse other reactions. Microorganisms do not benefit from co-metabolic transformations. The first two metabolic functions lead to growth of the microorganisms, hence, the energy of these exergonic reactions is used to produce biomass. The aerobic bacterium *Hyphomicrobium* sp. strain DM2 grows on dichloromethane as sole carbon and energy source [262]. The same is true for *Xanthobacter autotrophicus* strain GJ10 and 1,2-dichloroethane [130]. Both organisms convert these compounds to harmless products such as carbon dioxide, water, chloride, and biomass. On the contrary, trichloroethene, one of the most frequently detected contaminants in the environment, was only found to be co-metabolically transformed. It was co-oxidized by monooxygenases of different bacteria [83,169,282,291] and a dioxygenase from a pseudomonad involved in the degradation of aromatic compounds [197,289,313]. The co-metabolism of trichloroethene produces an epoxide as intermediate which reacts with the cellular components and causes an inactivation of the organisms [290].

Just as under oxic conditions similar reactions types exist for the degradation of organic compounds by anaerobic microorganisms. However, in addition organic compounds can act as a terminal electron acceptor (e.g. fumarate) in anaerobic systems. Studies with aromatic compounds and anaerobic mixed cultures indicated that the haloaromatic compound had first to be completely dehalogenated before use of the aromatic ring as electron donor [29,185,266]. The 3-chlorobenzoate degrading consortium described by Tiedje and coworkers [69,248] showed that the dechlorinated aromatic compound only indirectly served as electron donor for the dehalogenating bacterium *Desulfomonile tiedjei* (Fig. 1.3). *D. tiedjei* obtained the reducing power for growth and dechlorination *via* inter-species hydrogen transfer from the benzoate-oxidizing bacterium. The fascinating feature of *D. tiedjei* was the ability to use 3-chlorobenzoate as terminal electron acceptor in novel anaerobic respiration process [67,189]. A similar type of respiration was found for *Dehalobacter restrictus*, a bacterium which coupled growth on hydrogen or formate to the reductive dechlorination of tetrachloroethene (PCE) [Chapter 6]. Specific methyl viologen-dependent dehalogenases were shown to be responsible for the transformation *in vitro* [59, Chapter 6].

Recently a homoacetogen has been isolated which used dichloromethane for growth [270]. This was the first report which showed that a halogenated compound can serve as sole energy and carbon source for anaerobic bacteria.

Methanogenic bacteria appeared to co-metabolically dehalogenate halogenated aliphatic compounds. The dechlorinating activity correlated with the amount of methane formed, hence, depended on metabolic active organisms [86,186, Chapter 2]. The dechlorination of 1,2-dichloroethane to ethene and chloroethane was catalyzed by the methyl-CoM reductase, an enzyme with a rather different metabolic function [Chapter 4]. Homoacetogens and other strict anaerobes also seem to co-metabolically transform haloaliphatics (Table 1.3). The biochemistry involved in these reactions remains to be elucidated.

Dechlorination rates of metabolic and co-metabolic reactions can differ by several orders of magnitude as illustrated in Table 7.1. Only the co-metabolic dechlorination of tetrachloromethane by *Acetobacterium woodii* with rates of 21 nmol/min per mg protein occurs at similar rates as the metabolic dechlorination of PCE by *D. restrictus* [74]. Besides slow rates,

TABLE 7.1. Tetrachloroethene dechlorination rates by different anaerobic bacteria

Organism	Metabolic (me)/ cometabolic (co)	Product	Dechlorination rate (*nmol/min per mg protein*)	Reference
Dehalobacter restrictus	me	*cis*-1,2-DCE[a]	$3.3 \cdot 10^{2}$ [b]	[Chapter 6]
Acetobacterium woodii	co	TCE	$<6 \cdot 10^{-2}$	[74]
Methanosarcina sp.	co	TCE	$5.8 \cdot 10^{-4}$	[88]
Methanosarcina mazei	co	TCE	$3.3 \cdot 10^{-4}$	[88]
Desulfomonile tiedjei	co	TCE	$1.6 \cdot 10^{-3}$	[88]

[a] *cis*-1,2-DCE = *cis*-1,2-dichloroethene; TCE = trichloroethene.
[b] Estimated from chloride production and cell yields.

considerable amounts of substrate have to be metabolized for the co-metabolic reduction of haloaliphatic compounds. In methanogens, only 0.005 to 1.6% of the electrons generated from substrate consumption were used for the dehalogenation compared with the amount of methane produced [86,186, Chapter 2]. In case of PCE dechlorination by *D. restrictus*, all electrons derived from hydrogen consumption were recovered in dechlorination products and biomass [Chapter 6].

7.2. Microbially catalyzed versus abiotic reactions in the environment

Anaerobic bacteria transform halogenated compounds predominantly *via* reduction reactions [Chapter 1]. The production of carbon dioxide from tetrachloromethane by *A. woodii* [74] and of acetate from 1,1,1-trichloroethane by *Clostridium* sp. [97] are two examples where a halogenated compound was transformed by an overall substitutive process. However, as pointed out in Chapter 1, these products could be a result of a two-electron reduction to a carbenoid followed by hydrolysis. Other substitution reactions or dehydrodehalogenations with the evidence to be microbially catalyzed are not yet reported. Three types of reactions may occur abiotically under anoxic conditions: nucleophilic substitution, dehydrodehalogenation, and reduction.

Abiotic nucleophilic substitutions reactions are in general very slow. Hydrolysis of tetrachloromethane, 1,2-dichloroethane, and PCE at 25°C resulted in half-lives of 40.5, 72, and $9.9 \cdot 10^{8}$ years [135]. The half-life of 37 years of 1,2-dichloroethane in 50 mM phosphate buffer at pH 7 was decreased to 6.1 years in the presence of 0.67 mM Na_2S [13]. Dehydrodehalogenations seem to be exclusively abiotic reactions. However, there are not many reports where intermediates or end products indicate that such reactions are major transformation processes in the environment. One of this few examples is the dehydrodechlorination of 1,1,1-trichloroethane to 1,1-dichloroethene. A rate of 0.04 $year^{-1}$ (half-life = 17.3 years) was determined for the 1,1-dichloroethene formation in groundwater at 20°C [285]. 1,1,1-trichloroethane would biologically be transformed to 1,1-dichloroethane under the same conditions [97,147,286]. Acetate, another product of 1,1,1-trichloroethane degradation, can

be a product of abiotic as well as biotic reactions [97,147]. In a study with samples from an aquifer 1,1,1-trichloroethane was transformed to 1,1-dichloroethane, acetate, 1,1-dichloroethene, and carbon dioxide under methanogenic and sulfate-reducing conditions [147]. Only acetate, 1,1-dichloroethene, and carbon dioxide were formed under oxic and denitrifying conditions. In controls poisoned with $HgCl_2$ the same three products were detected under all redox conditions tested. Pseudo-first order rates for 1,1,1-trichloroethane under methanogenic and sulfate-reducing conditions were 0.0039-0.0167 day^{-1}. Under oxic and denitrifying conditions the rates were 0.0005 day^{-1}, the same rates as found in controls poisoned with $HgCl_2$. These results indicated that in the presence of oxygen or nitrate as electron acceptors only abiotic transformation of 1,1,1-trichloroethane occurred. Although abiotic substitution and dehydrodehalogenase reactions are slow, they might be of importance in environments where microbial activity is low or where environmental conditions are such that the microbial populations able to transform the compound in question are not active.

Abiotic reductive dehalogenations have been shown in model systems with reducing agents relevant for the environment. They were ferrous iron or hydrogen sulfide. The reactions proceeded faster in the presence of a catalyst. The catalyst was either a microbial product (e.g. tetra-pyrrole cofactors and quinoid-type compounds), minerals or humic compounds. Hexachloroethane and tetrachloromethane were shown to be dechlorinated by hydrogen sulfide or ferrous iron in the presence of humic acids at 50°C with rates of $2.7 \cdot 10^{-2}$ and $8.9 \cdot 10^{-3}$ h^{-1}, respectively [54]. Dechlorination rates with the reducing agent alone were only $3.74 \cdot 10^{-3}$ and $5.9 \cdot 10^{-4}$ h^{-1}, respectively. Hydroquinone groups present in humic acids were proposed to be responsible for at least some of the catalytic activity of this complex organic material. The presence of minerals such as biotite, vermiculite, and zeolite also increased the dehalogenation rates by about one order of magnitude [54]. Results summarized in Table 1.4 [Chapter 1] show that tetra-pyrrole cofactors catalyse a whole range of reductive dehalogenation reactions. All these investigations demonstrate that reductive dehalogenations can besides biotically catalyzed also be the result of abiotic processes.

Data on the kinetics of such abiotic reductions are too sketchy to draw any conclusions about the significance of either abiotic or biotic reactions in the environment based on kinetic parameters. The reducing agents used in abiotic reductive dehalogenation studies were hydrogen sulfide and ferrous iron. Both electron donors are predominantly products of microbial processes. Therefore, it was suggested that the loss of dehalogenating activity by different sterilization techniques could also have been the result of killing microorganisms which produce these inorganic electron donors and not of killing metabolically or co-metabolically dechlorinating bacteria [16,173]. Macalady et al. [173] have further pointed out that autoclaving samples from the environment only yield ambiguous results. A severe heat-treatment as done during autoclaving can also destroy possible abiotic catalysts and therefore not only excludes biotic processes. However, there is quite some experimental evidence that reductive dehalogenation of several halogenated compounds is exclusively a microbially mediated process. Enrichments described in Chapters 5 and 6 contradict the indirect involvement of bacteria in the dechlorination of chlorobenzenes and PCE. Initial enrichments were set in by adding original sediment material to autoclaved medium containing 1 mM Na_2S. Only enrichment cultures where lactate, ethanol, or glucose was added performed dechlorination. In the absence of such, at ambient conditions, chemically rather stable electron donors no dechlorination occurred although hydrogen sulfide and intact sediment material was present. In a study with hexachloroethane as model compound, humic acids or minerals as catalysts, and ferrous iron or hydrogen sulfide as electron donor, PCE was

formed as end product [54]. There were no indications that PCE could be further dechlorinated in these systems. The absence of PCE dechlorinating activity in abiotic model systems and the presence of dechlorination products in sites where PCE was spilled [177,207] indicate that biologically mediated dechlorination reactions are responsible for PCE conversion in anaerobic environments.

Regardless of the difficulties to clearly distinguish between abiotic and biotic processes in environmental samples, it can be concluded from the present knowledge that the reductive dehalogenations of hexachloroethane and tetrachloromethane observed in the environment presumably are the result of a mixture of abiotic and biotic reactions. PCE and haloaromatic dehalogenations, on the other hand, appear to be exclusively biological processes. Microorganisms play a crucial role in both type of reactions, either indirectly by producing the reducing agents or directly by metabolically or co-metabolically dehalogenating halogenated compounds.

7.3. Applied aspects

Besides investigating transport and transformation processes to predict the fate of halogenated compounds in the environment, research in the past also concentrated on treatment processes to decontaminate groundwater of heavily polluted sites. A halogenated compound can be removed from the environment by physico-chemical, chemical, or biological processes. In the following PCE shall be used as model compound to discuss possible decontamination techniques.

There exists a variety of physico-chemical techniques for the cleanup of sites contaminated with volatile halogenated compounds such as PCE. At present the two best available technologies are air stripping and granular activated carbon (GAC) filtration. A combination of the two methods is often required since the halocompound-containing stripper off-gases would otherwise cause contamination of the atmosphere. Both methods merely transfer the problem from one medium (water) to another (air or GAC). Therefore, technologies which lead to environmentally acceptable products are preferable.

Laboratory studies and pilot-scale evaluations have shown that PCE can be chemically mineralized to carbon dioxide and chloride [1,104]. PCE was oxidized by ozone with half-lives of 25-50 min which were reduced to 13 min by the addition of hydrogen peroxide [104]. Hydroxyl radicals were suggested to be responsible for the oxidation. Calculating annual treatment costs indicated that the process is competitive with conventional techniques such as air stripping and certainly is much cheaper than air stripping combined with GAC filtration [1]. Another abiotic process to mineralize PCE is heterogeneous photocatalysis [203]. In such a process an oxide semiconductor, e.g. titanium dioxide, is illuminated producing hydroxyl radicals and other highly oxidizing products which react with the oxidizable contaminant. Although the potential exists to mineralize contaminants by photocatalysis the stage of scaling-up and the demonstration of an economic feasibility have to be done before this process can be applied in water treatment systems.

Another possibility to chemically detoxify PCE might be to design a system with electrochemically reduced cobalamin as a catalyst. Cobalamin is able to dechlorinate PCE to ethene and ethane, environmentally acceptable products [98, unpublished results]. Transformation to ethene in buffer with 27 mM titanium(III) citrate as electron donor and 46 μM cobalamin at 22°C would be completed after approximately 250 days as shown by experimentally determined dechlorination rates and computer simulations [98]. Cobalamin is a very heat-stable

compound [94] and therefore, these transformations might also be carried out at 80 to 90°C. Assuming the same temperature effect as found for the rate of 1,2-dichloroethane dechlorination [Chapter 3], PCE transformation to ethene could be completed within approximately 2-4 days at these elevated temperatures. Increasing the concentration of cobalamin would in addition considerably enhance the transformation rates [Chapter 3]. Scheffold et al. [230] have pointed out that cobalamin-mediated electrochemical reactions could be interesting for the production of certain fine chemicals despite the relatively high costs of cobalamin (3-20 US$ per gram). A treatment plant of contaminated waters based on the catalytic potential of cobalamin may be too prohibitive.

Two possibilities for a biorestoration of PCE contaminated groundwater are an anaerobic or a sequential anaerobic/aerobic treatment. As shown by de Bruin et al. [58] PCE can be very efficiently transformed to ethane by an anaerobic process in a packed-bed column. Even at 10°C an influent concentration of 8 μM was completely dechlorinated to ethene after a residence time of 2.4 h which resulted in a transformation rate of 0.55 g/m^3 per h. The major problem for the scaling-up of this system is the biologically active material. The packed-bed column was packed with river Rhine sediment and grounded anaerobic granular sludge. Reproducing the complete transformation of PCE in such systems appeared to be a difficult task to achieve [Wil de Bruin, personal communication]. Either long lag-phases preceded complete transformation or only incomplete dechlorination to *cis*-1,2-dichloroethene and chloroethene was observed. Therefore, the challenge for the microbiologists is to get a stable mixed population which could be used to inoculate a fixed-bed bioreactor. The isolation of *D. restrictus* was the first step in this direction.

The second possibility, a sequential aerobic/anaerobic treatment, is feasible but less attractive because of a much more sophisticated technology needed for such a process. PCE which completely resisted aerobic biodegradation could be anaerobically transformed to lower chlorinated isomers, e.g. *cis*-1,2-dichloroethene by *D. restrictus*. Subsequently, these isomers could be further degraded by aerobic bacteria. Trichloroethene, dichloroethenes, and chloroethene were shown to be co-metabolically mineralized by many different bacteria [83,169,197,282,289,291]. A trickle fixed-film, packed-bed bioreactor containing a methanotrophic consortium with methane as primary substrate transformed trichloroethene and *trans*-1,2-dichloroethene at rates of 9 and 30 μM/h, respectively, at ambient temperatures of 22-24°C [260]. However, still 50% of the trichloroethene was present in the effluent and recycling was necessary to lower this concentration. Other studies showed that also the *cis*-1,2-dichloroethene isomer, the end product of the *D. restrictus* catalyzed PCE dechlorination, can be cometabolized by aerobic bacteria [83,282,289,291]. These results demonstrate that the microbial degradation potential for such a sequential anaerobic/aerobic treatment of PCE contaminated waters is present and it is now up to engineers in collaboration with microbiologists to design a suitable treatment system.

A third possibility for a biorestoration of a PCE contaminated site is to stimulate certain microbial populations *in situ*. The advantage of such a process is that the contaminated waters, soils, or sediments do not have to be pumped or transported. The disadvantage is the difficulty to assess whether a process actually takes place, to what extend the contaminant is transformed, and what the products are of the degradation [176]. The most promising *in situ* process for PCE decontamination is to first stimulate anaerobic, methanogenic bacteria, followed by creating oxic conditions for methanotrophs. In such a sequence the products of an incomplete anaerobic dechlorination, e.g. dichloroethenes and chloroethene, could be oxidized by co-metabolic activities of methanotrophs. Whether such a process is really achievable and could be recommended is a matter of future investigations.

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SUMMARY

The understanding of the fate of synthetic halogenated hydrocarbons became a matter of major interest over the last two decades. Halogenated compounds may threaten ecosystems due to their biocide properties. The degradability of halocompounds determines whether they will accumulate in a certain environment or whether they will be transformed to harmless products. A whole range of anthropogenic organohalogen compounds was detected in soils, sediments, surface and subsurface waters, and the atmosphere. Explanations for these accumulations could be that the halocompound is not degradable, that the transformation reactions are too slow, or that adverse environmental conditions prevent degradation. Aerobic bacteria are able to mineralize many halogented compounds under optimal conditions in the laboratory. However, polyhalogenated congeners often persisted aerobic biodegradation. This observation led to an intensification of biodegradation studies under anoxic conditions. Halogenated hydrocarbons were transformed in methanogenic microcosms of soils, sediments, aquifer material, or sewage sludge by reductive dehalogenation. Many of these microcosm studies indicated that the observed reductive dehalogenation reactions were biologically mediated. Nevertheless, only little was known about the micro-organisms which catalyzed these reactions. The aim of this thesis was to investigate the kind of bacteria involved in the reductive dehalogenation and to elucidate the physiological meaning and the biochemistry of the process. The results presented in this thesis showed that the physiological meaning of reductive dechlorination reactions catalyzed by anaerobic bacteria can be two-fold: i) a cometabolic activity and ii) a novel type of anaerobic respiration.

Methanogenic bacteria were known to reductively dechlorinated aliphatic C1 and C2 hydrocarbons. The dechlorination of 1,2-dichloroethane (1,2-DCA) by these organisms was characterized in detail in Chapters 2 to 4. Concentrated cell suspensions of methanogenic bacteria reductively dechlorinated 1,2-DCA *via* two reaction mechanisms: a dihalo-elimination yielding ethene and two hydrogenolysis reactions yielding chloroethane (CA) and ethane, consecutively (Chapter 2). These reactions were catalyzed by hydrogenotrophic as well as acetoclastic methanogens. Stimulation of methanogenesis caused an increase in the amount of dechlorination products formed, whereas the opposite was found when methane formation was inhibited. The observation that the dechlorination occurred independently from the primary substrate metabolized indicated that an enzyme system present in all methanogens was involved in the dechlorination reactions. Possible catalysts of the dechlorination of 1,2-DCA were corrinoids or factor F_{430}, two tetra-pyrrole cofactors present in high amounts in methanogens.

Cobalamin and the native and diepimeric form of factor F_{430} indeed catalyzed the reductive dechlorination of 1,2-DCA to ethene or CA in a buffer with Ti(III) citrate as electron donor (Chapter 3). Ethene was the major product in the cobalamin-catalyzed transformation and the ratio between ethene and CA formed was 25:1. Native F_{430} and 12,13-di-epi-F_{430} produced ethene and CA in a ratio of about 2:1 and 1:1, respectively. Crude and boiled cell extracts of *Methanosarcina barkeri* also dechlorinated 1,2-DCA to ethene and CA with Ti(III) citrate as reductant. The catalytic components in boiled extracts were heat- and oxygen-stable, and had a low molecular mass. Fractionation of boiled extracts by a hydrophobic interaction column revealed that part of the dechlorinating components had a hydrophilic, and part a hydrophobic character. These chemical properties of the dechlorinating components and spectroscopic analysis of boiled extracts indicated that corrinoids or factor F_{430} were responsible for the dechlorinations. The ratio of 3:1 to 7:1 between ethene and CA formed by cell extracts suggested that both cofactors were concomitantly active.

Reductive dechlorination of 1,2-DCA could also be performed in cell extracts of methanogens with the physiological electron donor H_2. Experiments with crude cell extracts of *Methanobacterium thermoautotrophicum* strain ΔH were carried out to get indications about the involvemnet of protein-bound corrinoids and factor F_{430} in the 1,2-DCA dechlorination (Chapter 4). First the effect of MgATP and CoM-S-S-HTP, the heterodisulfide of coenzyme M and 7-mercaptoheptanoylthreonine phosphate, was investigated. Other studies demonstrated that the corrinoid-containing methyl-tetrahydromethanopterin: coenzyme M methyltransferase and the factor F_{430}-containing methyl-coenzyme M reductase required an ATP-dependent reductive activation in *in vitro* systems of *M. thermoautotrophicum* strain ΔH. The methyltransferase could in addition be activated by CoM-S-S-HTP. The dechlorination of 1,2-DCA to ethene and CA by crude cell extracts of *M. thermoautotrophicum* strain ΔH with H_2 as electron donor was stimulated by MgATP. CoM-S-S-HTP together with MgATP partially inhibited ethene production but stimulated CA production as compared to MgATP alone. Michaelis-Menten kinetics for initial product formation rates with different 1,2-DCA concentrations indicated the enzymatic character of the dechlorination. Apparent K_m's for 1,2-DCA of 89 and 119 μM, and V_{max}'s of 34 and 20 pmol/min per mg protein were estimated for ethene and CA production, respectively. 3-Bromopropanesulfonate, a specific inhibitor for methyl-CoM reductase, completely inhibited dechlorination of 1,2-DCA. Purified methyl-CoM reductase, together with FAD and a crude fraction of component A, an enzyme system which reduces the nickel of factor F_{430} in methyl-CoM reductase, converted 1,2-DCA to ethene and CA with H_2 as electron donor. These results showed that, at least partially, the *in vivo* dechlorination was based on the activity of the methyl-CoM reductase.

The enrichment and characterization of bacteria which possibly use the reductive dechlorination as a novel type of anaerobic respiration is described in Chapter 5 and 6. Trichloro- and dichlorobenzenes were reductively dechlorinated in columns packed with river Rhine sediment. Enrichments inoculated with material from these percolation columns reductively dechlorinated hexachlorobenzene, pentachlorobenzene, all three isomers of tetrachlorobenzene, 1,2,3-trichlorobenzene (1,2,3-TCB), and 1,2,4-trichlorobenzene in the presence of lactate, glucose, ethanol, or isopropanol as electron donors (Chapter 5). A stable consortium grown on lactate as energy and carbon source in the presence of 1,2,3-TCB dechlorinated this isomer stoichiometrically to 1,3-dichlorobenzene. Dechlorinating activity could only be maintained when an electron donor was added. Lactate, ethanol, and hydrogen appeared to be the best suited substrates. For further enrichment of the 1,2,3-TCB dechlorinating bacteria, a two-liquid-phase (hexadecane/water) system was used with hydrogen as electron donor and 1,2,3-TCB or CO_2 as electron acceptor. Methanogens and acetogens were the major substrate-competing (H_2/CO_2) microorganisms in the two-liquid-phase system. Inhibition of methanogenesis by 2-bromoethanesulfonic acid did not influence dechlorination, and acetogens which were isolated from the enrichment did not have dechlorinating activity. These results indicated that bacteria were present using 1,2,3-TCB as terminal electron acceptor. Although dechlorination was found in dilutions down to 10^{-8} from the two-liquid-phase system, attempts to isolate a bacterium in pure culture able to use 1,2,3-TCB as terminal electron acceptor failed.

A microscopically pure culture, "PER-K23", was enriched from material of an anaerobic packed-bed column which reductively transformed tetrachloroethene (PCE) to ethane via trichloroethene (TCE), *cis*-1,2-dichloroethene (*cis*-1,2-DCE), chloroethene, and ethene (Chapter 6). PER-K23 catalyzed the dechlorination of PCE via TCE to *cis*-1,2-DCE and

coupled this reductive dechlorination to growth. H_2 or formate were the only energy sources which supported growth with PCE or TCE as electron acceptors. In the absence of PCE or TCE, no growth occurred. Nor O_2, NO_3^-, NO_2^-, SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, S, fumarate, or CO_2 could replaced PCE or TCE as electron acceptors with H_2 as electron donor. PER-K23 was also not able to grow fermentatively on any of the organic compounds tested. Electron balances showed that all electrons derived from H_2 or formate consumption could be recovered in dechlorination products and biomass formed. PER-K23 is a Gram-negative rod with one lateral flagellum. Analysis of the 16S ribosomal RNA of PER-K23 demonstrated that this bacterium belongs to the subdivision of species with Gram-negative cell walls within the phylum of Gram-positive bacteria. Based on physiological and molecular properties of the isolate, we propose *Dehalobacter* as the name of the genus of this newly described reductive dehalogenating bacterium. The type species, *Dehalobacter restrictus* sp. nov., is named after the restricted spectra of electron donors and acceptors utilized.

SAMENVATTING

De afgelopen twintig jaar was het van groot belang om kennis omtrent het lot van synthetische gehalogeneerde koolwaterstoffen in het milieu te verkrijgen. Gehalogeneerde verbindingen vormen door hun biocide karakter een bedreiging voor de kwaliteit van ecosystemen. Een hele reeks van synthetische gehalogeneerde stoffen werd inmiddels aangetoond in verschillende milieucompartimenten zoals grond, sedimenten, oppervlakte- en grondwater en de lucht. De aanwezigheid van die stoffen in het milieu wijst erop dat ze niet of onvoldoende kunnen worden afgebroken. Dit kan zijn oorzaak vinden in de volgende factoren: i) de verbinding is niet afbreekbaar, ii) de afbraak is op zich heel langzaam, of iii) ongunstige milieuomstandigheden belemmeren de afbraak. Studies aan de biodegradatie, die uitgevoerd werden onder optimale laboratoriumomstandigheden, hebben laten zien dat veel van de gehalogeneerde verbindingen in aanwezigheid van zuurstof kunnen worden gemineraliseerd door aërobe bacteriën. Echter, meervoudig gehalogeneerde verbindingen konden niet of slechts zeer langzaam worden afgebroken. Onderzoek naar de afbreekbaarheid van gehalogeneerde verbindingen onder anoxische omstandigheden gaf te zien dat deze condities wel leiden tot de afbraak van meervoudig gehalogeneerde verbindingen. Bijvoorbeeld, gehalogeneerde koolwaterstoffen werden omgezet in methanogene systemen die geënt waren met grond, sediment, of zuiveringsslib via een proces van reductieve dehalogenering. Hoewel uit die onderzoeken bleek dat de reductieve dehalogenering biologisch van aard was bleef er weinig bekend over de micro-organismen die bij dit proces betrokken waren. Het doel van dit onderzoek was om erachter te komen wat voor bacteriën verantwoordelijk zijn voor de reductieve dehalogenering en de fysiologische betekenis en de biochemie ervan op te helderen. De resultaten, die in dit proefschrift gepresenteerd worden, laten zien dat de reductieve dehalogenering fysiologisch gezien uit twee processen bestaat: i) een cometabolische activiteit en ii) een nieuw soort ademhaling.

Uit ander onderzoek was reeds bekend dat methaanbacteriën gehalogeneerde verbindingen die een of twee koolstofatomen bevatten reductief kunnen dehalogeneren. In dit proefschrift werd de reductieve dehalogenering door methaanbacteriën gekarakteriseerd aan de dechlorering van 1,2-dichloorethaan (1,2-DCA) (hoofdstukken 2 tot 4). In geconcentreerde celsuspensies van methaanbacteriën werden twee verschillende reacties vastgesteld waarmee 1,2-DCA reductief werd omgezet. Op de eerste plaats kan dit verlopen via een dihaloeliminatie wat de vorming van etheen uit 1,2-DCA opleverde, en op de tweede plaats twee achtereenvolgende hydrogenolysen waardoor chloorethaan (CA) en vervolgens ethaan gevormd werden (hoofdstuk 2). Deze reacties werden zowel door hydrogenotrofe als door acetoclastische methaanbacteriën gekatalyseerd. Stimulering van de methaanvorming veroorzaakte een toename in de hoeveelheid gevormde dechloreringsproducten, terwijl omgekeerd de dechlorering afnam wanneer de methaanvorming werd geremd. Het feit dat de dechlorering door alle soorten methaanbacteriën werd gekatalyseerd wees erop dat een algemeen voorkomend enzymsysteem in die bacteriën verantwoordelijk was voor de omzettingen. Mogelijke katalysatoren van de dechloreringsreacties konden twee tetrapyrrol cofaktoren zijn die in grote hoeveelheden in methaanbacteriën voorkomen, namelijk een corrinoid en factor F_{430}.

Uit verschillende expertimenten bleek inderdaad dat cobalamine en de twee epimeren van factor F_{430}, de natieve vorm en het 12,13-di-epimeer, de reductieve dechlorering van 1,2-DCA naar etheen en CA in een buffer met Ti(III)citraat als elektronendonor konden katalyseren (hoofdstuk 3). De reactie die door cobalamine werd gekatalyseerd leidde vooral tot de vorming van etheen. De verhouding tussen de twee dechloreringsproducten, etheen en

CA, was 25:1. Reacties uitgevoerd met natief F_{430} of 12,13-di-epi-F_{430} leidden tot etheen en CA in een verhouding van, respectievelijk, 2:1 en 1:1. De dechlorering van 1,2-DCA naar etheen en CA kon eveneens worden aangetoond in ruwe en gekookte celvrije extracten van *Methanosarcina barkeri* met Ti(III)citraat als elektronendonor. De katalyserende bestanddelen in gekookt celvrij extract bleken zeer hittestabiel, zuurstofongevoelig, en hadden een lage moleculaire massa. Scheiding van componenten van gekookt celvrij extract op een hydrofobe interactie kolom liet zien, dat de dechlorerende activiteit deels in een hydrofiele, deels in een hydrofobe fractie terecht kwam. Deze resultaten samen met de spectroscopische analyse van het gekookt celvrij extract wezen erop dat de corrinoid en factor F_{430} verantwoordelijk waren voor de dechloreringsactiviteit in celvrije extracten. De verhouding tussen de mogelijke producten etheen en CA lag in reacties die uitgevoerd werden met celvrije extracten tussen de 3:1 tot 7:1, hetgeen suggereert dat beide cofactoren actief waren.

De reductieve dechlorering van 1,2-DCA kon ook worden aangetoond in celvrije extracten van methaanbacteriën met de fysiologische elektronendonor waterstof. Om aanwijzingen te krijgen over de mogelijke betrokkenheid van de enzymgebonden corrinoid en factor F_{430} bij de dechlorering van 1,2-DCA met de fysiologische elektronendonor waterstof werden experimenten met celvrije extracten van *Methanobacterium thermoautotrophicum* stam ΔH uitgevoerd. Eerst werd het effect van MgATP en CoM-S-S-HTP, het heterodisulfide van coenzym M en 7-mercaptoheptanoylthreonine fosfaat, nader bestudeerd. Uit ander onderzoek was namelijk bekend dat het corrinoid-bevattende enzym methyl-tetrahydromethanopterin: coenzym M methyltransferase en het factor F_{430}-bevattende enzym methyl-coenzym M reductase een ATP-afhankelijke reductieve activering nodig hebben in *in vitro* systemen van *M. thermoautotrophicum* stam ΔH. Het methyltransferase kon verder ook door CoM-S-S-HTP geactiveerd worden. De omzetting van 1,2-DCA naar etheen en CA door celvrije extracten van *M. thermoautotrophicum* met waterstof als elektronendonor werd door MgATP gestimuleerd. CoM-S-S-HTP in aanwezigheid van MgATP remde gedeeltelijk de etheenproductie maar stimuleerde de vorming van CA vergeleken met MgATP alleen. De initiële productiesnelheden bij verschillende beginconcentraties 1,2-DCA konden beschreven worden met Michaelis-Menten kinetische parameters, hetgeen wees op het enzymatische karakter van de dechlorering. Voor respectievelijk de etheen- en CA-vorming werden waarden geschat voor de K_m voor 1,2-DCA van 89 en 119 μM, en voor de V_{max} van 34 en 20 pmol/min per mg eiwit. De dechlorering van 1,2-DCA kon volledig geremd worden door 3-broompropaansulfonaat, een specifieke remmer van het methyl-CoM reductase. In een reactie van gezuiverd methyl-CoM reductase, FAD, en een ruwe fractie van componente A, een enzymsyteem dat het nikkel in factor F_{430} van methyl-CoM reductase reduceert, werd 1,2-DCA naar etheen en CA omgezet met waterstof als elektronendonor. Deze resultaten gaven duidelijk aan dat de *in vivo* dechlorering tenminste gedeeltelijk gebaseerd is op de activiteit van de methyl-CoM reductase.

De ophoping en de karakterisering van bacteriën die de reductieve dechlorering mogelijk gebruiken als een nieuw soort ademhaling is beschreven in hoofdstuk 5 en 6. In kolommen gepakt met Rijnsediment werd de reductieve dechlorering van trichloor- en dichloorbenzenen waargenomen. Ophopingen van bacteriën uit materiaal van die kolommen dechloreerden hexachloorbenzeen, pentachloorbenzeen, alle tetrachloorbenzeen isomeren, 1,2,3-trichloorbenzeen (1,2,3-TCB) en 1,2,4-trichloorbenzeen in aanwezigheid van lactaat, glucose, ethanol, of isopropanol als elektronendonor. Een stabiele mengcultuur die gekweekt werd op lactaat als energie- en koolstofbron in aanwezigheid van 1,2,3-TCB dechloreerde deze verbinding stoichiometrisch naar 1,3-dichloorbenzeen. Dechloreringsactiviteit kon alleen worden

gehandhaafd wanneer er een elektronendonor werd toegevoegd. Hiervoor bleken lactaat, ethanol en moleculaire waterstof de meest geschikte substraten te zijn. Om het dechlorerende bacterie verder op te hopen werd gebruik gemaakt van een kweeksysteem met twee vloeistoffasen, een organische fase (hexadecaan) en een waterige fase (het kweekmedium). Als elektronendonor voor de ophopingscultuur werd waterstof gebruikt. Omdat het medium bicarbonaat gebufferd was, was er ook CO_2 als elektronenacceptor aanwezig. Methanogene en homoacetogene bacteriën waren de meest belangrijke organismen die om het substraat (H_2/CO_2) concurreerden in het twee-fasen systeem. Het specifiek remmen van methaanbacteriën door het toevoegen van de remmer 2-broomethaansulfonaat had geen invloed op de waargenomen dechloreringsactiviteit. Verder vertoonden acetogene bacteriën die uit de cultuur konden worden geïsoleerd, geen dechlorerende activiteit. Deze resultaten gaven aan dat er mogelijk andere bacteriesoorten aanwezig waren in de ophopingscultuur die 1,2,3--TCB als elektronenacceptor gebruikten. De dechloreringsactiviteit kon nog gevonden worden in verdunningen van de originele ophopingscultuur tot 10^{-8}; pogingen om een reincultuur te verkrijgen van een 1,2,3-TCB dechlorerende bacterie zijn niet gelukt.

Uit een vast bed kolom waarin reductieve dechlorering van tetrachlooretheen (PCE) naar ethaan via trichlooretheen (TCE), *cis*-1,2-dichlooretheen (*cis*-1,2-DCE), chlooretheen, en etheen werd waargenomen, kon een microscopisch reine cultuur, "PER-K23", geïsoleerd worden (hoofdstuk 6). PER-K23 katalyseerde de dechlorering van PCE naar *cis*-1,2-DCE en koppelde de reductieve dechlorering aan groei. Waterstof en formiaat waren de enige elektronendonoren die groei met PCE of TCE als elektronenacceptor mogelijk maakten; in afwezigheid van PCE of TCE als elektronenacceptor werd geen groei waargenomen. Geen enkele van de andere geteste elektronenacceptoren (zoals O_2, NO_3^-, NO_2^-, SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, S, fumaraat, of CO_2) kon PCE of TCE vervangen. PER-K23 was tevens niet in staat om fermentatief te groeien op diverse organische substraten. Wanneer een elektronenbalans werd opgemaakt, bleek dat alle elektronen, die afkomstig waren van consumptie van waterstof of formiaat, teruggevonden werden in de dechloreringsprodukten en in gevormde biomassa. Determinatie van PER-K23 liet zien dat het een Gram-negatief staafje was met een laterale flagel. Analyse van het 16S ribosomale RNA van PER-K23 gaf aan dat de bacterie een nieuwe soort was in de subdivisie van bacteriën met een Gram-negatieve celwand in het phylum van Gram-positieve bacteriën. Gebaseerd op de fysiologische eigenschappen en de 16S rRNA sequentie van het isolaat hebben wij het geslacht van deze nieuwe reductief dechlorerende bacterie *Dehalobacter* genoemd. Als soortnaam van de geïsoleerde bacterie hebben we *Dehalobacter restrictus* sp. nov. voorgesteld, een naam die betrekking heeft op het zeer beperkte spectrum van elektronendonoren en elektronenacceptoren die door de bacterie gebruikt kunnen worden voor groei.

Curriculum Vitae

Op 7 november 1959 werd ik geboren als Hans Christof Holliger in Zürich, Zwitserland. Ik groeide op in Adliswil, een stadje vlak bij Zürich. Hier heb ik de eerste acht jaar van mijn schooltijd doorgebracht. Aansluitend bezocht ik het "mathematisch-naturwissenschaftliche Gymnasium" in Zürich, dat ik in 1979 met een "Matura Typus C" heb beëindigd. Na een jaar militaire dienst begon ik in 1980 met mijn studie biologie aan de "Eidgenössische Technische Hochschule (ETH)" in Zürich. Het diploma ("Naturwissenschaftler, Richtung Biologie") behaalde ik in 1984 met de eindexamenvaken microbiologie, toxikologie, plantenfysiologie en -pathologie, limnologie en bodemkunde. Na een jaar reizen in Amerika en een jaar werken in een kantoor begon ik eind 1986 met mijn promotie-onderzoek aan de vakgroep Microbiologie van de Landbouwuniversiteit Wageningen. De resultaten van het onderzoek zijn beschreven in dit proefschrift.