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# Bacterial Dehalogenation of Chlorinated Methanes

## Abstract

Chlorinated methanes are metabolized by various aerobic and strictly anaerobic bacteria. The highly chlorinated compounds trichloromethane and tetrachloromethane undergo fortuitous reactions with enzymes or biocatalysts and do not provide carbon or energy to the cell. Chloromethane and dichloromethane are also subject to fortuitous transformation reactions, but in addition serve as growth substrates for facultatively methylotrophic bacteria and for specialized acetogenic bacteria. These organisms use specific dehalogenases for converting chloromethane or dichloromethane to intermediates of their primary metabolism. The chloromethane dehalogenase of aerobic methylotrophs and the dichloromethane dehalogenase of an anaerobic bacterium fermenting this compound to formate and acetate are still uncharacterized. In contrast, the chloromethane dehalogenase of a homoacetogenic bacterium has been characterized in crude extracts, and extensive genetic and biochemical studies, including protein engineering, have been performed with dichloromethane dehalogenase/glutathione *S*-transferases of aerobic methylotrophs.

## Introduction

All four chlorinated methanes are industrially produced in considerable quantities, primarily for use as solvents in large scale chemical reactions and as degreasing agents. Chloromethane, and less significantly trichloromethane and tetrachloromethane are also of biogenic origin (Gribble, 1994). Because of their high volatility, the chlorinated methanes are distributed to various oxic and anoxic ecosystems where they are met by microorganisms craving for carbon and energy sources or for terminal electron acceptors respectively. In this contribution we discuss how bacteria interact with chlorinated methanes, particularly how they make use of chloromethane and dichloromethane as carbon and energy sources for growth. The subject has been addressed in an earlier communication (Leisinger *et al.*, 1993), and the mechanistic features of bacterial metabolism of chlorinated methanes have been discussed in a review by Wackett *et al.* (1992).

Microbial metabolism of the various chlorinated methanes is initiated by dehalogenation reactions. The biochemistry of these reactions, their evolutionary origin and their integration into cellular metabolism are topics of intrinsic interest. Microbial dehalogenation of chlorinated methanes is also of environmental relevance on two accounts. First, bacteria that dehalogenate these compounds are used as catalysts in the bioremediation of contaminated soil and groundwater (McCarty, 1993). Second, bacterial metabolism of chlorinated methanes in the environment may represent a sink for these compounds. For chloromethane and bromomethane it is particularly important to identify these sinks and to estimate their capacity, since both compounds are thought to make very significant contributions to the destruction of the stratospheric ozone layer (Manö & Andreae, 1994).

Table 1 gives an overview of how chlorinated methanes are metabolized by bacteria. Chloromethane and dichloromethane serve as growth and energy substrates for both aerobic and strictly anaerobic bacteria. With a view to bioremediation processes, this is clearly the preferred type of metabolism. It confers a selective advantage to the dehalogenating bacteria and thus leads to an increase of the biological catalyst when the chlorinated pollutant is present. In addition, all chlorinated methanes are subject to cometabolic transformations (Table 1). Such fortuitous reactions remain without direct benefit to the dehalogenative organisms. In biodegradation processes they are more difficult to control than dehalogenations based on growth. Dehalogenation by co-metabolic transformation thus is of interest for trichloromethane and tetrachloromethane, the chlorinated methanes not used as growth substrates.

Table 1. Metabolism of chlorinated methanes by pure cultures of aerobic and anaerobic bacteria

| Compound | aerobic | | anaerobic | |
|---|---|---|---|---|
| | Growth | Co-metabolism | Growth | Co-metabolism |
| $CH_3Cl$ | yes | yes | yes | - |
| $CH_2Cl_2$ | yes | yes | yes | yes |
| $CHCl_3$ | - | yes | - | yes |
| $CCl_4$ | - | - | - | yes |

## Co-metabolic transformation of trichloromethane and tetrachloromethane

Trichloromethane is aerobically transformed to phosgene by bacterial oxygenases with relaxed substrate specificity. Systems using methanotrophic organisms, the ammonia-oxidizing *Nitrosomonas europaea* (Wackett *et al.*, 1992), a recombinant pseudomonad expressing soluble methane monoooxygenase (Jahng & Wood, 1994), and toluene-oxidizing bacteria (McClay *et al.*, 1996) have been described. However, the formation of a reactive transformation product and the requirement for dissolved oxygen to dehalogenate the volatile chlorinated substrate impose practical limitations on these processes.

Co-metabolic anaerobic dehalogenation of trichloromethane to $CO_2$ and dichloromethane has also been explored. This transformation is carried out by methanogenic archaea and by anaerobic bacteria. Transition metal coenzymes found in these organisms, such as coenzyme $F_{430}$, cobalamins and hematin dehalogenate trichloromethane in vitro under reduced conditions to products and with kinetics similar to the *in vivo* systems. These catalysts thus are thought to be responsible for the non-specific metabolism of the compound in anaerobic microbes (Wackett *et al.*, 1992). The potential of degrading trichloromethane in pure or mixed cultures of methanogens was found to be limited by the strong inhibitory action of the compound on methanogenesis (Bagley & Gossett, 1995).

Tetrachloromethane is not known to be transformed by microorganisms under aerobic conditions. The compound, however, is efficiently dehalogenated to trichloromethane, dichloromethane and chloromethane by the same anaerobic archaea and bacteria that react with trichloromethane. Since tetrachloromethane is metabolized exclusively by anaerobic transformation reactions, the potential of such processes for the dehalogenation of this toxic pollutant is of particular interest. Recent studies have uncovered bacteria capable of transforming tetrachloromethane to $CO_2$ without the accumulation of trichloromethane as an end product. This is achieved by *Pseudomonas* sp. strain KC, an organism that rapidly transforms tetrachloromethane under denitrifying conditions (Dybas *et al.*, 1995), and by an as yet uncharacterized anaerobic bacterium which is not a methanogen or a sulfate reducer (deBest *et al.*, 1996). In both cases the reactions involved in tetrachloromethane transformation appear to be different from the reactions catalyzed by transition metal coenzymes, and it will be interesting to observe the elucidation of the underlying biochemistry.

## Chloromethane and dichloromethane as growth substrates for acetogenic bacteria

### *Chloromethane*

Diekert and collaborators (e.g. Messmer *et al.*, 1996) have isolated a strictly anaerobic bacterium capable of growth at the expense of chloromethane as the sole energy source. The organism, strain MC, was enriched from an anaerobic sludge digester, and in its morphology it resembled the homoacetogenic bacterium *Peptostreptococcus productus*. Strain MC fermented chloromethane to acetate as the sole organic product according to the fermentation balance shown in Fig. 1A.

Analysis of the biochemistry of chloromethane utilization revealed that the compound is metabolized via the acetyl-CoA pathway operative in acetogenic bacteria. Subsequently an elaborate enzyme assay was developed which allowed the characterization of chloromethane dehalogenase activity in crude extracts of strain MC. This key enzyme introduces chloromethane into the acetyl-CoA pathway. It catalyzes methyl transfer from chloromethane to tetrahydrofolate (Fig. 1A) and, for as yet unexplained reasons, requires substoichiometric

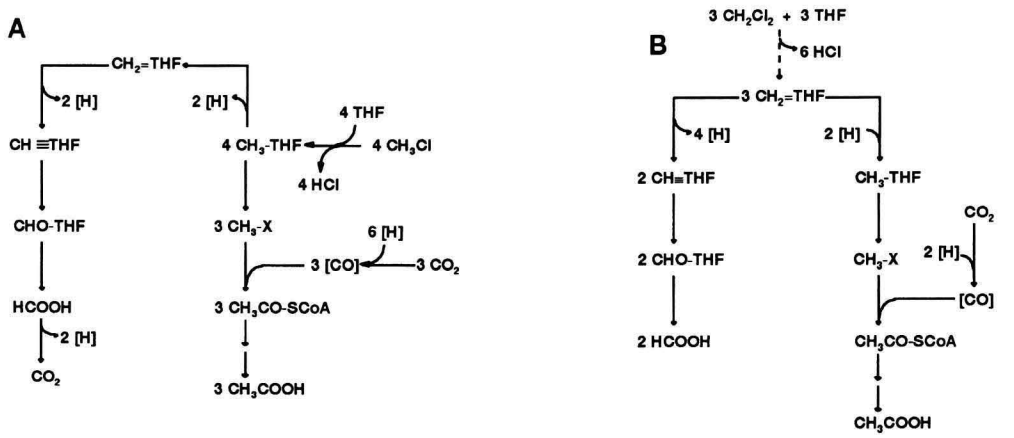



Fig. 1. Metabolism of chloromethane and dichloromethane by strictly anaerobic acetogenic bacteria.
A: Chloromethane utilization by the homoacetogenic bacterium strain MC (modif. from Messmer *et al.*, 1996). Fermentation balance: $4CH_3Cl + 3CO_2 + 2H_2O \rightarrow 3CH_3COOH + CO_2 + 4HCl$.
B: Hypothetical pathway for dichloromethane utilization by *Dehalobacterium formicoaceticum* strain DMC (Mägli *et al.*, 1996) in accordance with the observed fermentation balance: $3CH_2Cl_2 + CO_2 + 4H_2O \rightarrow 2HCOOH + 3CH_3COOH + 6HCl$.
THF, tetrahydrofolic acid; $CH_3$-X, methylated corrinoid-iron/sulfur protein.

amounts of ATP. It is induced by its substrate and is not identical with O-demethylase, an enzyme transferring the methyl group from methoxylated aromatics onto tetrahydrofolate and thereby enabling acetogenic growth of the organism on these compounds. Purification and characterization of chloromethane dehalogenase is under way (Messmer *et al.*, 1996). It is expected to provide information on the number and the properties of the protein components involved in the reaction and to clarify whether a reduced corrinoid or a thiol is involved as a nucleophile in this novel dehalogenation reaction.

*Dichloromethane*

Two anaerobic methanogenic enrichment cultures capable of growth with dichloromethane as the sole carbon and energy source have been described (Leisinger *et al.*, 1996). From one of these we have recently isolated a strictly anaerobic bacterium which converts dichloromethane to formate and acetate in a molar ratio of 2:1 and to biomass and traces of pyruvate. The fermentation balance observed for this organism is shown in Fig. 1B. Phylogenetic analysis of the 16S ribosomal DNA sequence placed the dichloromethane utilizing anaerobe in the *Clostridium-Bacillus* subphylum of the gram positive bacteria. The lack of sequence identity above 90% with any previously described taxon indicated that it was a representative of a new genus, and the organism was named *Dehalobacterium formicoaceticum* (Mägli *et al.*, 1996). More than fifty potential substrates

and combinations of substrates, including methoxylated aromatics and various C1 compounds were tested, but only dichloromethane supported growth.

It thus appears that *D.formicoaceticum* is specialized for the utilization of an anthropogenic compound. A metabolic explanation for this trait is at present not available. NMR-analysis of the products formed by resting cells from $^{13}$C-dichloromethane showed that formate and the methyl group, but not the carboxyl group of acetate are derived from the labelled substrate (A. Mägli, unpublished). This observation and the presence of key enzymes of the acetyl-CoA pathway in cell extracts have led us to propose the pathway shown in Fig. 1B for the degradation of dichloromethane by *D.formicoaceticum*. The first step in this scheme, the conversion of dichloromethane plus tetrahydrofolate to methylene tetrahydrofolate plus chloride, is purely hypothetical. The development of a sensitive assay for this reaction therefore is a prerequisite for analysis of the dehalogenation enzyme in this system.

## Chloromethane and dichloromethane as growth substrates for aerobic facultative methylotrophic bacteria

### *Chloromethane*

Aerobic mineralization of chloromethane has previously been observed with a *Hyphomicrobium* sp. that utilizes the compound as a carbon and energy source (Hartmans *et al.*, 1986). More recently, further facultative methylotrophs capable of growth with chloromethane were isolated from contaminated environments (Doronina *et al.* 1996). With one of these, *Methylobacterium* sp. CM4, we have initiated studies to elucidate the metabolism of chloromethane (T.Vannelli and A.Sokolov, unpublished). Efforts to measure chloromethane dehalogenation in cell-free extracts have so far been unsuccessful, but a preliminary view of chloromethane metabolism by the organism has been obtained from studies with whole cells.

Oxygen uptake studies with resting cells indicated that chloromethane dehalogenation is inducible (Doronina *et al.*, 1996). As shown in Fig. 2, there are at least three possible pathways for the metabolism of chlormethane to carbon dioxide and hydrochloric acid by *Methylobacterium* CM4. They all generate formaldehyde but differ in the enzymatic step(s) that lead to this intermediate. In the hydrolytic mechanism, methanol and hydrochloric acid are produced without oxygen being required. The methanol produced would then be oxidized to formaldehyde, generating two electrons in an oxygen-dependent manner by methanol dehydrogenase. In the dehydrogenase mechanism, formaldehyde is produced without methanol as an intermediate, again generating two electrons in an oxygen-dependent manner. In the monooxygenase mechanism, formaldehyde is produced by an oxygen-dependent reaction, but two electrons are consumed.

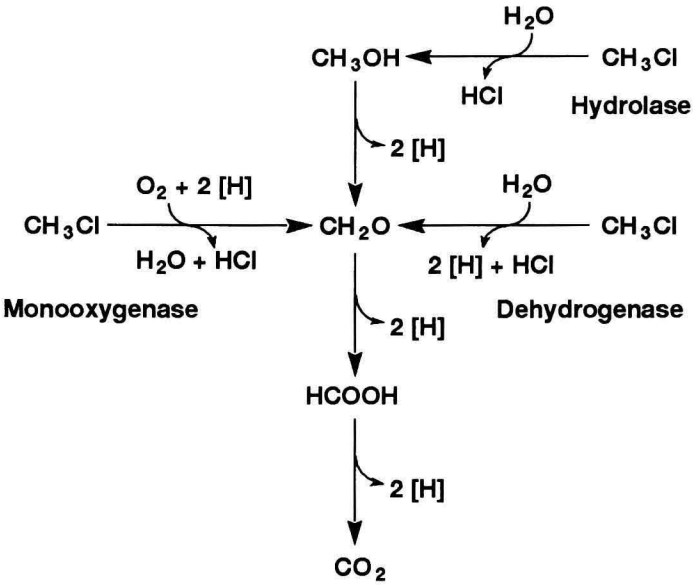


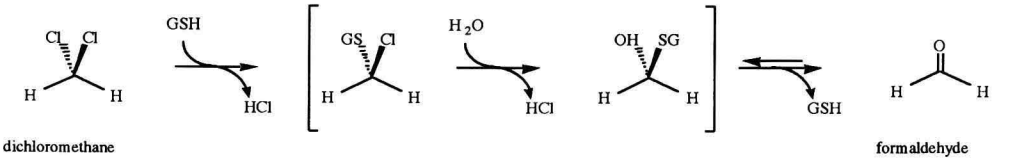

Fig. 2. Possible reactions for the dehalogenation of chloromethane by *Methylobacterium* sp. strain CM4

Based on the following observations we consider that a dehydrogenase mechanism is likely to be involved in chloromethane dehalogenation: i) The growth yields of *Methylobacterium* CM4 on chloromethane or methanol were both 3 times higher than when cells were grown on formate. Since two electrons are generated from the oxidation of formate and six from methanol, these growth yields suggest that six electrons were also generated from chloromethane and thereby argue against the monooxygenase mechanism (Fig. 2). ii) When cells were incubated anaerobically with chloromethane, no chloride was produced, but when oxygen was subsequently introduced, release of chloride was initiated. Since chloride production by a hydrolase is not dependent on oxygen, such a mechanism appears unlikely. Further, when the cells were incubated aerobically with EDTA, methanol oxidation was inhibited, but chloromethane oxidation was not, indicating that methanol was not an intermediate in the metabolism of chloromethane.

*Dichloromethane*

Dichloromethane degradation by methylotrophic proteobacteria that use this compound as the sole source of carbon and energy for growth has been investigated both at the biochemical and the genetic level in our laboratory (Leisinger *et al.*,1996). These bacteria contain an inducible enzyme, dichloromethane dehalogenase/glutathione *S*-transferase, which requires glutathione as a cofactor to hydrolyse dichloromethane to formaldehyde and hydrochloric acid

(Table 2). Dichloromethane dehalogenases are atypical enzymes among glutathione S-transferases (GST). In contrast to most enzymes of the GST superfamily, their substrate range is very narrow and restricted to dihalomethanes. Further, the tripeptide glutathione is not consumed in an initial conjugation with the electrophilic substrate, but regenerated in the course of the dehalogenation reaction:




dichloromethane formaldehyde

Although the function of dichloromethane dehalogenase enzymes in metabolism is reminiscent of glutathione-dependent dehydrogenases, no significant sequence similarity can be detected between such proteins and dichloromethane dehalogenases. At the protein sequence level, dichloromethane dehalogenases are distantly but unequivocally related to GST enzymes of the Theta-class. Database sequences most closely related to dichloromethane dehalogenases are GSTT1 and GSTT2 enzymes from human, rat and mouse (25–28% identity) rather than bacterial GST enzymes (13–23% identity). Interestingly, it appears from the recent literature that GSTT1 enzymes are active with dichloromethane whereas GSTT2 enzymes are not, although the level of sequence identity of dichloromethane dehalogenase is about the same with GSTT1 and GSTT2 enzymes. However, no sequence regions or residues unique to both dichloromethane dehalogenase and GSTT1 sequences are readily apparent from sequence alignments. Thus, only low sequence identity is required to generate enzymatic activity with dihalomethanes within the glutathione *S*-transferase scaffold. This suggests that organisms as far apart as mammals and bacteria may have independently evolved GST enzymes with dichloromethane dehalogenase activity, well before the onset of anthropogenic contamination with dihalomethanes. Indeed, the sequence divergence of 44% between dichloromethane dehalogenase

Table 2. Kinetic properties of dichloromethane dehalogenases / glutathione *S*-transferases

| Dichloromethane dehalogenase from | $K_m^{DCM}$ ($\mu$M) | $K_s^{DCM}$ ($\mu$M) | $K_m^{GSH}$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) |
|---|---|---|---|---|
| *Hyphomicrobium* sp. strain DM2[a] | 11 | ND | 741 | 0.6 |
| *Methylobacterium* sp. strain DM4[a] | 9 | 0.5 | 2075 | 0.6 |
| *Methylophilus* sp. strain DM11[b] | 59 | 5.6 | 66[c] | 3.3 |
| rat liver Theta GSTT1 (GST5-5)[d] | 300 | ND | 4200-5900 | 10 |

[a] Helga Sorribas, unpublished results.
[b] Data from Vuilleumier & Leisinger (1996)
[c] $S_{0.5}^{GSH}$ value (sigmoid saturation curve)
[d] Data from Blocki *et al.* (1994), and Meyer (1993)

from *Methylophilus* sp. DM11 and its closest known relative from *Methylobacterium* sp. DM4 indicates that the latest common ancestor of these enzymes may have existed as long as hundreds of millions years ago (Vuilleumier & Leisinger, 1996).

In the absence of a three-dimensional structure for dichloromethane dehalogenase or closely related GST enzymes, we are following several experimental approaches to elucidate the molecular determinants of catalytic efficiency and specificity of dichloromethane dehalogenase enzymes. First, protein engineering studies on the enzyme from strain DM11 have pinpointed several key residues required for activity and specificity (Vuilleumier & Leisinger, 1996) or glutathione affinity (Vuilleumier *et al.*, unpublished). Further, we have recently used PCR methods to amplify and sequence dichloromethane dehalogenase genes from previously isolated strains. Most variations in dichloromethane dehalogenase sequences observed so far are base substitutions leading to changes in the corresponding protein sequence. Whether these amino acid exchanges represent random drift or functional adaptation of the enzymes to the various host environments is currently under investigation.

Finally, we performed chemostat studies to determine the effect of dehalogenase properties on the growth behaviour of the model organism *Methylobacterium* sp. strain DM4. Transconjugants of this strain that carry a plasmid-encoded, constitutively expressed copy of a given dichloromethane dehalogenase or GST gene under the control of the promoter from the strain DM4 dehalogenase gene are being characterized with respect to $\mu_{max}$ and $K_s$, the Monod kinetic constants of the organism. For example, at low steady state concentrations of dichloromethane, a transconjugant expressing the catalytically superior enzyme from strain DM11 was outcompeted by an otherwise identical strain carrying the dichloromethane dehalogenase gene from strain DM4, due to the much higher affinity of the strain DM4 enzyme for dichloromethane (Table 2). These results show that dehalogenase characteristics are directly reflected in the growth properties of the host. They may contribute to explain our observations that methylotrophs expressing enzymes closely related to the strain DM4 dichloromethane dehalogenase are often more abundant in natural samples. Our recent results, however, also indicate that under conditions where dichloromethane is plentiful, growth of the strain with the better dehalogenase from strain DM11 occurs at a higher rate. Moreover, both strain DM4 and strain DM11 types of dichloromethane dehalogenase genes can be detected in sludge from some wastewater treatment plants. This suggests that, depending on the rate of dichloromethane supply, organisms with either strain DM4 or strain DM11 type genes may prevail in these environments.

## Acknowledgments

Work in the authors' laboratory was supported by grants from the Federal Institute of Technology and by the Swiss National Research Foundation, grant No. 5002-037905 of the Biotechnology Priority Programme.

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