Bacterial Growth with Chlorinated Methanes

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Chlorinated methanes are important industrial chemicals and significant environmental pollutants. While the highly chlorinated methanes, trichloromethane and tetrachloromethane, are not productively metabolized by bacteria, chloromethane and dichloromethane are used by both aerobic and anaerobic methylotrophic bacteria as carbon and energy sources. Some of the dehalogenation reactions involved in the utilization of the latter two compounds have been elucidated. In a strictly anaerobic acetogenic bacterium growing with chloromethane, an inducible enzyme forming methyltetrahydrofolate and chloride from chloromethane and tetrahydrofolate catalyzes dehalogenation of the growth substrate. A different mechanism for the nucleophilic displacement of chloride is observed in aerobic methylotrophic bacteria utilizing dichloromethane as the sole carbon and energy source. These organisms possess the enzyme dichloromethane dehalogenase which, in a glutathione-dependent reaction, converts dichloromethane to inorganic chloride and formaldehyde, a central metabolite of methylotrophic growth. Sequence comparisons have shown that bacterial dichloromethane dehalogenases belong to the glutathione S-transferase enzyme family, and within this family to class Theta. The dehalogenation reactions underlying aerobic utilization of chloromethane by a pure culture and anaerobic growth with dichloromethane by an acetogenic mixed culture are not known. It appears that they are based on mechanisms other than nucleophilic attack by tetrahydrofolate or glutathione.

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The incentive for exploring the microbial metabolism of chlorinated methanes is provided by their significance as environmental pollutants. Chloromethane (CH₃Cl), dichloromethane (CH₂Cl₂), trichloromethane (CHCl₃), and tetrachloromethane (CCl₄) are produced on a scale of approximately 2 million tons per year and used as intermediates in chemical synthesis and as industrial solvents. As with many other organochlorine compounds, some chlorinated methanes are also natural products. Natural formation of trace amounts of CCl₄ and CHCl₃ by marine macroalgae has been observed. The most prominent example of a natural organohalogen compound produced in large quantities is CH₂Cl, which is formed by wood-rotting fungi as well as by a variety of marine algae at an estimated rate of 5 million tons per year. This represents about 10 times the rate of the annual industrial production (1). Thus, one would assume that microorganisms have evolved that are capable of using this compound as a carbon and energy source.

This is indeed the case. Aerobic and strictly anaerobic bacteria that utilize CH₂Cl as a growth substrate have been isolated. The same holds true for CH₂Cl₂, a compound considered to be a true xenobiotic. The more highly chlorinated methanes CHCl₃ and CCl₄ are not productively metabolized, but are subject to co-metabolic transformations. CHCl₃ is gratuitously oxidized by nonspecific bacterial monooxygenases or reduced to CH₂Cl₂ by strictly anaerobic bacteria, and CCl₄ is subject to reductive dehalogenation only. In these reductive dehalogenations, CHCl₃ or CCl₄ serve as nonphysiological electron acceptors that do not provide any demonstrable advantage to the anaerobic bacteria carrying out the reactions. The biochemistry and the mechanistic background of the dehalogenation reactions involved in bacterial halomethane metabolism have recently been reviewed by Wackett et al. (2). In the following paragraphs we will focus on productive bacterial metabolism of CH₂Cl and CH₂Cl₂, i.e., on the utilization of these compounds as sole carbon and energy sources by aerobic and anaerobic bacteria. Organisms able to grow with CH₂Cl have been used in the design of biotechnological processes to remove this compound from industrial effluents and waste gases (3).

**Chloromethane**

Chloromethane is aerobically utilized by a *Hyphomicrobium* sp. that grows on this compound with a doubling time of 8 hr (4). The dehalogenation mechanism has not been elucidated, but washed cell suspensions dechlorinated CH₂Cl (MC) in an oxygen-dependent reaction. Methane was not oxidized. This suggests that MC is transformed by a monooxygenase that is not methane monooxygenase to chloromethanol, which then spontaneously decomposes to formaldehyde and chloride:

\[
\text{CH}_2\text{Cl} \rightarrow \text{H}_2\text{C}-\text{Cl} \rightarrow \text{H}_2\text{O} + \text{HCHO} + \text{CH}_3\text{OH}
\]

The use of chloromethane as a carbon source under strictly anaerobic conditions has recently been observed by Traunecker et al. (5) with a newly isolated, Gram positive, cocccoid homoacetogenic bacterium. At its optimum growth temperature of 25°C, this organism grows with 2% (v/v) MC in the gas phase with a doubling time of 30 hr. It converts 4 moles of MC plus 2 moles of carbon dioxide to 3 moles of acetate. An inducible enzyme activity catalyzing the formation of methyl--tetrahydrofolate (CH₃-THF) and chloride from MC and tetrahydrofolate (THF) has been detected in crude extracts of this acetogenic bacterium (6). This dehalogenating methyltransferase activity has not yet been purified and characterized. It suggests a novel type of dehalogenation reaction in which THF acts as a nucleophile and thereby enables the organism to feed MC directly into the acetylCoA pathway of
acetate synthesis according to the following general scheme:

We have found that all aerobic dichloromethane (DMC)-utilizing bacteria possess the enzyme DCM dehalogenase. This strongly inducible enzyme enables the organisms to transform DCM to formaldehyde, a central metabolite of methyloptrophic growth. The majority of the DCM-utilizing bacteria that have been characterized are unidentified Gram-negative facultative methylotrophs. One facultative methylotroph is a *Hyphomicrobium*, and the pink-pigmented strain DM4 was assigned to the genus *Methylomonas*. More recently a restricted facultative methylotroph growing with DCM was isolated and identified as *Methylobacterium* sp. The latter organism grows on DCM with a doubling time of 3 hr, while the other strains exhibit doubling times between 8 and 10 hr with this substrate.

In contrast to other haloalkane dehalogenases (3), nucleophilic displacement of chloride by DCM dehalogenase is not based on the direct attack by hydroxide from water, but on the thiol group of glutathione (GSH) as the initial nucleophile. Thus, the reaction catalyzed by DCM dehalogenase is thought to proceed according to the following scheme:

We have purified and characterized DCM dehalogenase from *Hyphomicrobium* sp. DM2 and found that the enzyme is a homohexamer with a subunit molecular weight of 33 kDa. It has an absolute requirement for GSH as a cofactor. The halogenated one-carbon compounds DCM, dibromomethane (CH₂Br₂), diiodomethane (CH₂I₂), and chlorobromomethane (CH₂BrCl) can all function as substrates. Among a number of chlorinated ethanes tested, only 1,1-dichloroethane was dehalogenated, although at a rate about 1000-fold lower than DCM. DCM dehalogenase from *Hyphomicrobium* sp. DM2 and the enzymes from *Methylomonas* sp. DM4, as well as from two other facultative methylotrophic bacteria, all exhibit similar properties and are designated group A enzymes. The DCM dehalogenase from the restricted facultative methylotroph *Methylobacterium* sp. DM11 differs from the group A enzymes, in its N-terminal amino acid sequence and in its catalytic properties. It also exhibits an approximately 5-fold increase of the catalytic constant kₜₐₙ and is therefore assigned to a separate group B. Accordingly, in organisms with group A enzymes DCM dehalogenase makes up for 15 to 20% of the total soluble protein, while in the group B organism *Methylobacterium* sp. DM11 the enzyme represents 7% of the total protein.

We have also studied the genes for DCM utilization. In most DCM-degrading methylotrophs, they are found to be located on large, uncharacterized plasmids with a size of about 120 kb. In general, these plasmids are maintained during growth under nonselective conditions in the laboratory. This is not the case with the 70 kb plasmid encoding the DCM utilization phenotype of *Methylobacterium* sp. DM11. After one growth cycle on a carbon source other than DCM, 70% of the cells have lost this element. In *Methylomonas* sp. DM4, the DCM utilization genes are encoded on the chromosome by a 2.8-kb fragment, the nucleotide sequence of which we have determined. This DNA fragment, the *dcm* region, contains *dcmA*, the structural gene of DCM dehalogenase, and *dcmR*, the regulatory gene responsible for inducibility of DCM dehalogenase by DCM (Figure 1). The two genes of the *dcm* system are organized in two separate, divergent transcription units. Their translational start codons are separated by a 619-bp intergenic region that contains the transcriptional start sites. The promoter regions of the two genes contain two almost identical 12-bp sequences covering positions -14 to -25 relative to the mRNA start sites. *dcmR* encodes a trans-acting factor that negatively controls DCM dehalogenase formation as well as its own synthesis at the transcriptional level. A function of *dcmR* as a regulatory gene encoding a repressor is supported by the presence of a canonical helix-turn-helix motif in the N-terminal region of its deduced amino acid sequence. These data have given rise to the model for *dcmR*-mediated regulation of DCM dehalogenase expression shown in Figure 1. It proposes that *dcmR* encodes a repressor that, in the absence of DCM, binds to recognition sites within the promoters PA and PR1 and thereby prevents initiation of transcription. DCM would abolish repressor binding and thereby relieve inhibition of transcription initiation. The model remains hypothetical since the DNA–protein interactions underlying this regulation have not yet been experimentally demonstrated.

More recently we have sequenced the *dcmA* gene of *Methylobacterium* sp. DM11 (8). Its deduced protein sequence exhibits only 56% amino acid identity with the DCM dehalogenase of *Methylomonas* sp. DM4. These data make it seem unlikely that the catalytically improved group B DCM dehalogenase of strain DM11 has, under selective pressure by pollution with DCM, recently evolved from a group A DCM dehalogenase. Rather, the data argue for an ancient divergence between these two xenobiotic-metabolizing bacterial enzymes well before the industrial use of DCM.

Sequence comparisons have revealed that the two bacterial DCM dehalogenases belong to the glutathione S-transferase (GST) enzyme family and, within this family, to class Theta. Soluble GSTs constitute a large superfamiliy of proteins that catalyze the nucleophilic addition of GSH to electrophilic substrates; these enzymes have been extensively studied in eukaryotes. Based on primary structure, four GST
gene classes have been recognized: Alpha, Mu, Pi, and Theta. Amino acid sequences are 60 to 80% identical within a gene class and 35 to 60% identical between gene classes. A large overlap in substrate specificity exists between representatives of the different classes, but eukaryotic class Theta GSTs exhibit a markedly restricted substrate range and an unusual efficiency in dehalogenating DCM. The association between bacterial DCM dehalogenases and eukaryotic class Theta GSTs thus reveals itself in primary structure similarities as well as at the level of substrate specificity.

Among prokaryotes, GSH production has been observed only in 2 out of 11 bacterial phyla, namely in the proteobacteria and in the cyanobacteria. Representatives of six other phyla and of the major groups of the archaea did not contain GSH, and three bacterial phyla have not been tested (9). In accordance with the distribution of this cosubstrate, bacterial GSTs have so far been reported in representatives of the proteobacteria only. They are active with 1-chloro-2,4-dinitrobenzene as a substrate, but no sequence data are available for these enzymes. DCM dehalogenases are the only prokaryotic GSTs for which an association with the GST family has been shown at the functional as well as at the structural level. The high conservation of Theta GSTs in DCM utilizing proteobacteria in maize and in the fruitfly and rat has led to the suggestion that class Theta is representative of the ancient progenitor GST, and that GSTs, first evolved for protection from oxygen toxicity, were acquired by eukaryotes by way of their mitochondrial endosymbionts (10).

**Anaerobic Growth with Dichloromethane**

Two anaerobic mixed cultures capable of growth with DCM have recently been described (11,12). They were enriched under methanogenic conditions, and they produced both methane and acetate from DCM. We have simplified the culture isolated in our laboratory to yield the acetogenic mixed culture DM. Culture DM exhibits a maximum dehalogenation rate of 0.63 ml/kg protein. It grows apparently linearly in 5 mM DCM-minimal medium to an optical density (546 nm) of about 0.1. Suspensions of resting cells of culture DM formed 1.25 mM acetate and 5.9 mM chloride as the major products from 3 mM DCM. This partial mass balance suggested the following fermentation balance:

\[
2 \text{CH}_2\text{Cl}_2 + 2 \text{H}_2\text{O} \rightarrow 2 \text{CH}_3\text{COO}^- + 4 \text{Cl}^- + 5 \text{H}^+
\]

During the conversion of DCM to acetate we observed a transient accumulation of formate that disappeared toward the end of the reaction. Hydrogen was not detected.

The degradation of \(^{14}\text{C})\text{DCM}\) was examined to test the hypothesis that formate is a major intermediate in the pathway from DCM to acetate. There was complete recovery of radioactivity, mostly in the defined products \(\text{CO}_2\) (58%), acetate (23%), and formate (11%). The radioactivity in acetate was unevenly distributed; 88% was in the methyl group at about half the specific radioactivity that was observed in the educt. These data support the transformation of DCM to formate, which gives rise to the methyl group of acetate without prior oxidation to \(\text{CO}_2\) (13).

Culture DM contained at least three different bacteria. Attempts to obtain a pure culture growing with DCM as the sole source of carbon and energy were not successful. We therefore considered the possibility of syntrophy, and we chose the formate-utilizing methanogen *Methanospirillum hungatei* as the partner for the dechlorinative organism from culture DM. This approach allowed us to isolate strain DMA, a strictly anaerobic, endospore-forming Gram-positive bacterium (Figure 2). Isolation of strain DMA via coculture with *M. hungatei* suggested that use of DCM in the acetogenic culture DM involved strain DMA and an acetogenic syntrophic partner. We enriched from culture DM the putative acetogenic syntrophic partner of strain DMA. This led to the isolation of strain DMB, a vibroid, spore-forming Gram-negative acetogenic bacterium (Figure 2).

There was no growth in liquid DCM-salts medium of mixtures of strain DMA plus *M. hungatei* or of strain DMA plus strain DMB, none of the pure cultures alone degraded DCM. However, cocultures of strain DMA with either *M. hungatei* or strain DMB in solid medium with DCM as the sole added source of carbon and energy was observed. This indicates that strain DMA is responsible for DCM degradation.

A simple pathway involving a syntrophic association of strains DMA and DMB can be deduced for the formation of acetate from DCM in the mixed culture DM (Figure 2). The dehalogenation reaction(s) in this tentative scheme leads from DCM to an intermediate at the oxidation state of formaldehyde, (CO) enzyme-bound carbonyl (Figure 2).

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