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Microbes, enzymes and genes involved in dichloromethane utilization

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Abstract

Dichloromethane (DCM) is efficiently utilized as a carbon and energy source by aerobic, Gram-negative, facultative methylotrophic bacteria. It also serves as a sole carbon and energy source for a nitrate-respiring *Hyphomicrobium* sp. and for a strictly anaerobic co-culture of a DCM-fermenting bacterium and an acetogen. The first step of DCM utilization by methylotrophs is catalyzed by DCM dehalogenase which, in a glutathione-dependent substitution reaction, forms inorganic chloride and S-chloromethyl glutathione. This unstable intermediate decomposes to glutathione, inorganic chloride and formaldehyde, a central metabolite of methylotrophic growth. Genetic studies on DCM utilization are beginning to shed some light on questions pertaining to the evolution of DCM dehalogenases and on the regulation of DCM dehalogenase expression. DCM dehalogenase belongs to the glutathione S-transferase supergene family. Analysis of the amino acid sequences of two bacterial DCM dehalogenases reveals 56% identity, and comparison of these sequences to those of glutathione S-transferases indicates a closer relationship to class Theta eukaryotic glutathione S-transferases than to a number of bacterial glutathione S-transferases whose sequences have recently become available. *dcmA*, the structural gene of the highly substrate-inducible DCM dehalogenase, is carried in most DCM utilizing methylotrophs on large plasmids. In *Methylobacterium* sp. DM4 its expression is governed by *dcmR*, a regulatory gene located upstream of *dcmA*. *dcmR* encodes a *trans*-acting factor which negatively controls DCM dehalogenase formation at the transcriptional level. Our working model thus assumes that the *dcmR* product is a repressor which, in the absence of DCM, binds to the promoter region of *dcmA* and thereby inhibits initiation of transcription.

Introduction

Dichloromethane (DCM) is a major industrial solvent whose 1992 yearly production by the US Chemical Industry amounted to $1{,}6 \times 10^5$ tonnes. This represents about a 40% decrease from the 1984 figure (Anonymous 1993), indicating that DCM is being replaced by non-chlorinated solvents. DCM is chemically stable, non-polar, non-flammable and significantly soluble in water (13.2 g/l, 20 °C). Its low boiling point (40 °C) and its high vapor pressure (47,5 kPa at 20 °C) have led in the past to an efflux of DCM into the environment that has been estimated to be equivalent to the production rate of the compound (Pearson 1982). A value of 80 days is reported for its tropospheric half-life, whereas hydrolysis in water at pH 7.0 and 25°C proceeds very slowly with a half-life of 700 years (Mabey & Mill 1978). While many other halogenated methanes are produced by marine algae or white rot fungi, DCM has not been reported to be formed as a natural metabolite (Class & Ballschmiter 1988; Wever 1993).

The entry of DCM into soil, water and air is of concern since the compound has adverse effects on man. Chronic exposure to low doses is carcinogenic. DCM is hydroxylated by liver cytochrome P-450-dependent monooxygenase to produce an alcohol derivative which decomposes to formylchloride. This reactive intermediate nonenzymatically alkylates a variety of tissue molecules including DNA and there-

by induces carcinogenesis. Acute intoxication with DCM causes death by asphyxiation. The underlying mechanism is again based on the formation of formylchoride which may also undergo dehalogenation to hydrochloric acid and carbonmonoxide. High doses of DCM thus lead to lethal concentrations of carboxyhemoglobin (Anders & Pohl 1985).

In contrast to its toxicity for higher organisms, DCM serves as a growth substrate for both aerobic and anaerobic bacteria. In terms of energy generation from DCM these organisms rely either on respiration, on anaerobic respiration, or on fermentation. As shown in Table 1, aerobic, facultatively methylotrophic bacteria are the most efficient DCM degraders. DCM utilization by representatives of this group has been studied for some time, and this has led to a considerable understanding of aerobic DCM metabolism. The biochemistry, genetics and regulation of the process in these organisms are the main topics of this article and will be discussed in detail. Mechanistic aspects of the microbial metabolism of DCM and other chlorinated methanes have recently been discussed in a review by Wackett et al. (1992).

Bacterial growth with DCM under strictly anaerobic conditions has been detected only recently. The range of anaerobic organisms capable of using this substrate as well as the dehalogenation mechanisms and the degradative pathways involved are still being explored. For example, the facultative methylotroph *Hyphomicrobium* sp. DM2 has recently been shown to be capable of growth with DCM in the absence of oxygen, using nitrate as a terminal electron acceptor (Frank 1993). Judging from enzyme activity measurements, the dehalogenation enzyme used under aerobic conditions is fully expressed, but growth on DCM occurs at only about half the rate. Fermentative utilization of DCM has been observed with strictly anaerobic mixed cultures that either yield acetate (Stromeyer et al. 1991) or methane and carbon dioxide (Freedman & Gossett 1991) as final products. We have isolated from an acetogenic mixed culture a fermentative DCM-dehalogenating bacterium and an acetogenic bacterium, which as a syntrophic culture involving interspecies formate transfer, achieve the homoacetogenic fermentation of DCM (Braus-Stromeyer et al. 1993). The dehalogenation mechanism in the DCM-fermenting bacterium is as yet unresolved but appears to be fundamentally different from that in aerobic DCM-utilizers.

A number of studies have explored the potential of DCM-utilizing bacteria for the biological treatment of industrial effluents, waste gases and groundwater. In aerobic fluidized bed reactors inoculated with DCM-utilizing methylotrophic bacteria and fed with model (Gälli & Leisinger 1985) or process wastewater (Stucki 1990), the compound was mineralized at rates of up to 1.6 g/l · h^{-1} with an efficiency of 99.9%. Waste gases containing DCM have been treated at rates of about 0.2 g/l · h^{-1} in aerobic trickling-bed reactors inoculated with DCM-degrading methylotrophs (Hartmans & Tramper 1991; Diks & Ottengraf 1991). Anaerobic systems for the degradation of DCM to innocuous natural compounds such as acetate and methane also look promising, although the biology of the process is not understood. A full-scale anaerobic fixed-bed reactor packed with charcoal removed > 99.99% of the DCM (40 mg/l) contained in anaerobic groundwater for more than three years (Stromeyer et al. 1991). In another line of applied research, the development of a biosensor based on immobilized cells of an aerobic DCM utilizing bacterium for monitoring DCM concentrations in waste streams has been reported. The system has a detection limit of 0.1 μM DCM and is specific for dihalomethanes (Henrysson & Mattiasson 1993).

Dichloromethane utilization by methylotrophic bacteria

Microbiology

Methylotrophic bacteria capable of growth with DCM as the only carbon and energy source are readily isolated from soil and groundwater that have been exposed to the compound (Leisinger et al. 1993). Enrichments from municipal wastewater and from activated sewage sludge have also been successful (La Pat-Polasko et al. 1984; Klecka 1982). Some isolates from our laboratory have been characterized with respect to their growth rates on mineral salts medium containing 10 mM DCM (Table 2). Their growth rate constants ranged between 0.05 and 0.22 h^{-1}, and generally were significantly lower than the constants observed for the same organisms on media with 10 mM methanol. Depending on the strain, this may be due to toxicity of DCM or to dehalogenation of DCM representing the growth-limiting reaction, or to both (Brunner et al. 1980; Stucki et al. 1981).

All of our isolates (Table 2) are Gram-negative facultative methylotrophic bacteria. While most of them remain unidentified, three strains chosen for more

Table 1. Microbial systems utilizing dichloromethane as a carbon and energy source.

Organism(s)	Growth conditions	Approximate Maximum degradation rate ($g/l \cdot h^{-1}$)	Reference
Various facultative methylotrophs	aerobic	1.600	Gälli & Leisinger 1985
Hyphomicrobium sp. DM2	denitrifying	0.010	Frank 1993
Mixed culture	acetogenic	0.011	Stromeyer et al. 1991
Mixed culture	methanogenic	0.007	Freedman & Gossett 1991

Table 2. Gram-negative dichloromethane utilizing methylotrophic bacteria.[1]

Strain	Organism	Growth rate with 10 mM CH_2Cl_2 (h^{-1})	Plasmids	Plasmid-encoded *dcmA* gene
DM1		0.08	120 kb	+
DM3	unidentified	0.11	120 kb	+
DM5	facultative	0.06	120 kb	+
DM6	methylotrophs	0.05	120 kb	+
DM10		0.08	120 kb	+
DM2	*Hyphomicrobium* sp.	0.07	none detected	-
DM4	*Methylobacterium* sp.	0.09	120 kb, 40 kb, 8 kb	-
DM11	*Methylophilus* sp.	0.22	70 kb	+

[1] For references of the data summarized in the Table see Leisinger et al. (1993).

detailed studies could be assigned to particular genera of the methylotrophs. These are *Hyphomicrobium* sp. DM2, *Methylobacterium* sp. DM4 and *Methylophilus* sp. DM11. 16S ribosomal RNA sequence analysis has shown that the former two organisms belong to two different clusters of strains within the α-subdivision of the purple eubacteria, whereas *Methylophilus* sp. DM11 is placed in the β-subdivision (Tsuji et al. 1990). The distinctive phylogenetic position of strain DM11 coincides with its physiological classification as a restricted facultative methylotroph growing only with methanol, DCM, glucose and galactose (Doronina & Trotsenko 1992; Green 1993).

Biochemistry

All facultative methylotrophs utilizing DCM possess the enzyme DCM dehalogenase. As shown in Fig. 1, this enzyme catalyzes the dehalogenation of DCM to formaldehyde and inorganic chloride. Formaldehyde, a key metabolite of methylotrophic growth, is generated from methanol as well as from C1-substrates containing sulfur or nitrogen. Part of it is dissimilated to carbon dioxide for energy generation, and the remainder is assimilated for biomass production. The expression of DCM dehalogenase activity in a methylotroph is essential for the bacterium to be able to utilize DCM as a growth substrate. DCM dehalogenase activity in crude cell extracts was found to be strictly dependent on glutathione (GSH) (Stucki et al. 1981), and this was later confirmed with pure enzyme preparations. The reactions of the dehalogenase-dependent dehalogenation of DCM are shown in Fig. 1. In this sequence, an S-chloromethyl glutathione conjugate is formed enzymatically which is assumed to undergo nonenzymatic hydrolysis to S-hydroxymethyl glutathione. Decomposition of the latter then leads to formaldehyde and to the regeneration of GSH. In contrast to other aliphatic dehalogenases (Leisinger & Bader 1993), nucleophilic displacement of chloride by DCM dehalogenase is not based on the direct attack by hydroxide from water or by a carboxylate at the enzyme active site, but on the thiol group of GSH. The intermediates postulated in this sequence have not been isolated but, in accordance

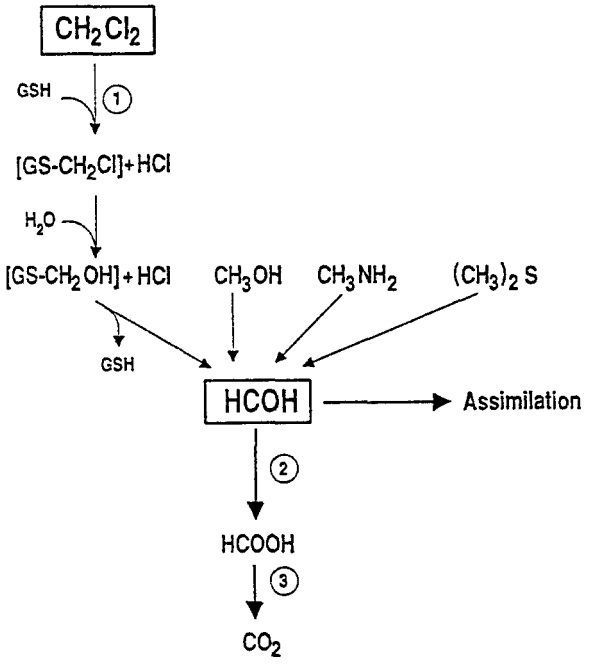

Fig. 1. Metabolism of dichloromethane by methylotrophic bacteria. 1, dichloromethane dehalogenase; 2, formaldehyde dehydrogenase; 3, formate dehydrogenase. GSH stands for reduced glutathione (γ-glutamyl-cysteinyl-glycine).

with nucleophilic displacement of chloride, CD_2Cl_2 was shown to yield dideutero-formaldehyde (Gälli et al. 1982).

DCM dehalogenases have been purified and characterized from five facultative methylotrophs isolated from different environments (Kohler-Staub & Leisinger 1985; Kohler-Staub et al. 1986; Scholtz et al. 1988). They were found to be similar with respect to subunit molecular mass, obligate requirement for GSH in catalysis and a substrate range restricted to dichloro-, dibromo- and diiodomethane. However, when other criteria such as N-terminal amino acid sequences, kinetic properties and immunological relatedness were applied, they clearly fell into two classes. One class is formed by the group A enzymes, the DCM dehalogenases from the methylotrophic strains DM1, DM2, DM4 (Table 2) and *Hyphomicrobium* sp. GJ21 (obtained from D. Janssen, Groningen). The other class consists of the group B enzyme from *Methylophilus* sp. DM11. Typical properties of a group A and a group B enzyme are listed in Table 3. The most important difference between the two groups lies in their kinetic properties. Under conditions of substrate saturation the group B enzyme is significantly faster in dechlorination than group A enzymes. This is reflected in a more than two-fold increased growth rate of *Methylophilus* sp. DM11 on DCM as compared to *Methylobacterium* sp DM4 and in a smaller fraction (7% versus 15–20%) of the total soluble protein representing DCM dehalogenase.

In the DCM utilizing methylotrophs examined so far, DCM dehalogenase was induced between 50- and 80-fold by DCM. Variations between strains were observed with respect to the range of inducers for the enzyme. 1,1-dichloroethane and 1,2-dichloroethane were effective gratuitous inducers in two *Hyphomicrobium* spp. whereas their inducing effect was marginal in strains DM1 and DM4 (Kohler-Staub et al. 1986). Induction of DCM dehalogenase by DCM is demonstrated in Fig. 2 by a comparison of immunostained thin sections prepared from methanol-grown and DCM-grown *Methylobacterium* cells. These electron micrographs also show that DCM dehalogenase is located in the cytoplasm and evenly distributed within this compartment. This contrasts with the location of methanol dehydrogenase in the periplasm of Gram-negative methylotrophs (De Vries et al. 1990). Thus, formaldehyde is produced in the periplasm during growth on methanol, whereas growth on DCM leads to the formation of this metabolite in the cytoplasm.

Genetics

The strategy used to isolate genes involved in DCM utilization genes relied on complementation of a DCM-nonutilizing (DCM^-) mutant of *Methylobacterium* sp. DM4 with a clone of a cosmid library of DM4 wild type DNA in the broad-host-range cosmid vector pVK100. Strain DM4 was chosen as an experimental system since DCM^- mutants of this organism were obtained with a yield of 99% upon growth with methanol at the supra-optimal temperature of 32 °C. A recombinant plasmid carrying a 21 kb DNA fragment complemented such a DCM^- derivative and conferred regulated expression of DCM dehalogenase as well as the ability to utilize DCM as the sole carbon and energy source on a variety of facultative methylotrophs from culture collections. Hybridization of the recombinant cosmid with a digest of genomic DNA from a DCM^- mutant produced no signal, indicating that temperature curing had led to a deletion larger than 21 kb covering the DCM utilization genes (Gälli & Leisinger 1988).

Subcloning of the 21 kb DNA fragment from strain DM4 in *E. coli* and identification of clones expressing polypeptides cross-reacting with dehalogenase anti-

Table 3. Comparison of type A and type B DCM dehalogenases.

Property	Type A (*Methylobacterium* sp. DM4)	Type B (*Methylophilus* sp. DM11)
Subunit molecular weight	33,112	31,036
Isoelectric point	6.8	5.4
N-terminal sequence	identical with other type A	different from type A
Cross-reactivity with DM4 antibody	strong	weak
k_{cat} (s^{-1})	0.87	3.3

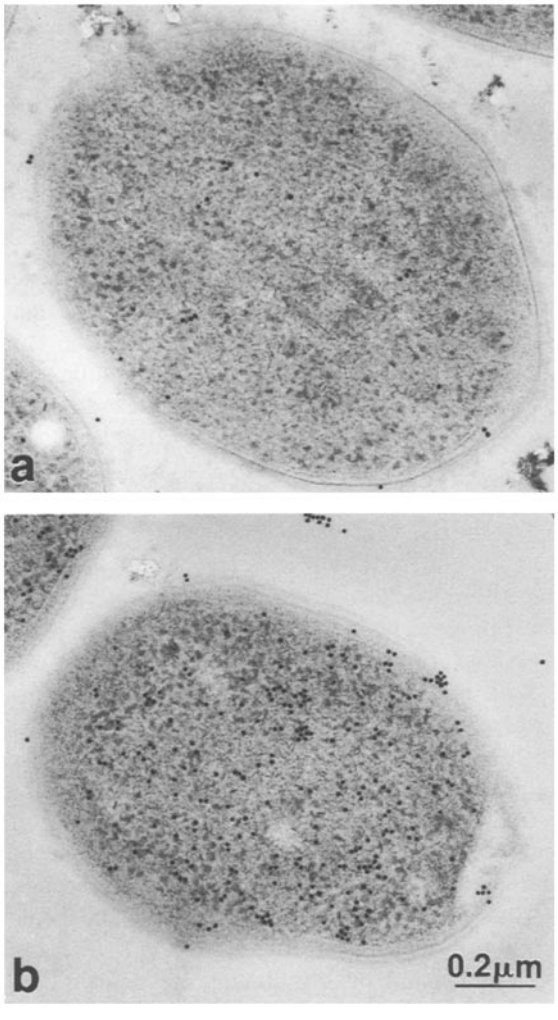

Fig. 2. Localization of DCM dehalogenase in the cytoplasm of non-induced (a) and induced (b) cells of *Methylobacterium* sp. DM4, using the protein A-gold technique. Bacteria were high-pressure frozen, freeze-substituted with ethanol containing uranyl acetate and embedded in Epon/Araldite (Hohenberg et al. 1994).

serum yielded a 2.8 kb fragment carrying the DCM utilization genes. Analysis of the nucleotide sequence of this fragment revealed two genes which were arranged in opposite direction to each other, namely *dcmA*, the structural gene of DCM dehalogenase, and *dcmR* whose product appears to be involved in regulation of dehalogenase expression (La Roche & Leisinger 1990, 1991; Fig. 3).

The deduced dehalogenase amino acid sequence demonstrated homology of this enzyme with eucaryotic GSH S-transferases, a topic which will be discussed below. To examine the evolutionary relatedness of the type A DCM dehalogenase from *Methylobacterium* sp. DM4 with the catalytically improved type B enzyme of *Methylophilus* sp. DM11, the *dcmA* gene from the latter organism was recently cloned and sequenced using a degenerate oligonucleotide derived from the N-terminal amino acid sequence of the type B enzyme (Bader & Leisinger 1993). The two DCM dehalogenases share 56% identical and 75% chemically similar amino acid residues. This indicates that the type B enzyme has probably not evolved recently, under selective pressure of an environment contaminated with DCM, from the type A enzyme. Rather, the two dehalogenases may well have diverged from each other millions of years before industrial production of DCM was initiated.

The *dcmA* genes of most DCM utilizers that have been examined are located on large plasmids (Table 2). This suggests that the DCM utilization genes represent foreign DNA which was acquired by facultative methylotrophs through plasmid transfer. In *Methylophilus* sp. DM11 such an event is very likely to have occurred since the GC-content of the plasmid encoded *dcmA* gene is 37.5% whereas genomic DNA of the organism contains 50.5 mol% GC (Bader 1994). Another indication for the horizontal transfer of DCM utilization lies in the observation that *dcm* genes are rather unstable

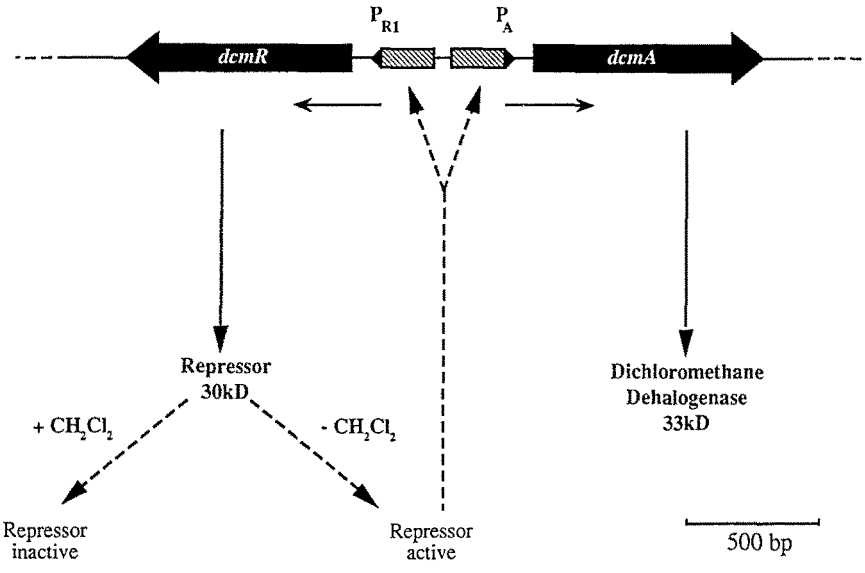

Fig. 3. Working model for the regulation of the *dcmA* and *dcmR* genes in *Methylobacterium* sp. DM4. The *dcmR* gene product codes for a putative repressor which negatively controls transcription. P_A and P_{R1} indicate the promoter regions of *dcmA* and *dcmR*, respectively.

under non-selective growth conditions. As mentioned above, a large chromosomal deletion of a region carrying the *dcm* genes occurred in strain DM4 when it was cultured 2 °C above its optimal growth temperature. Similarly, strain DM11 was recently found to lose at a high rate the 70 kb plasmid carrying the *dcmA* gene during one growth cycle with methanol or glucose as a carbon source (Bader & Leisinger 1993). In both instances, loss of DCM utilization was the only phenotypic change observed in the cured derivatives. Functions other than DCM utilization potentially encoded on the chromosome region deleted in strain DM4 or on the 70 kb plasmid of strain DM11 thus remain unknown.

The size of the plasmids carrying the *dcm* genes (Table 2) suggests that they are self-transmissible, but this has not been tested. It was shown, however, that a 21 kb fragment of strain DM4 DNA, cloned in the broad host range vector pVK100, conferred the DCM-utilization-positive phenotype to a number of Gram-negative methylotrophic bacteria (Gälli & Leisinger 1988).

Evolutionary relationships of dichloromethane dehalogenases to glutathione S-transferases

As mentioned above, sequencing of the cloned *dcmA* genes from *Methylobacterium* sp. DM4 and *Methylophilus* sp. DM 11 revealed that DCM dehalogenases are not only functionally but also structurally related to the glutathione S-transferase (GST) enzyme family. Soluble GSTs constitute a large supergene family of proteins which catalyze the nucleophilic addition of GSH to electrophilic substrates. These dimeric (2 × 24–28 kD) enzymes have been extensively studied in eukaryotes (Tew et al. 1993). Based on primary sequence, four GST gene classes have so far been recognized: Alpha, Mu, Pi, and Theta (Meyer et al. 1991). The DM4 and DM11 enzymes can be assigned to the more recently defined Theta class on grounds of some shared properties and sequence homology (see below and Table 4, Fig. 5).

Structural information on eukaryotic GST enzymes is emerging at a rapid rate, since three-dimensional structures have been determined for GSTs of the Pi (Reinemer et al. 1991, 1992) and Mu classes (Ji et al. 1992; Johnson et al. 1993). These studies and the wealth of sequence data on GSTs already available contribute to a better understanding of the reactions catalyzed by these enzymes. Each GST subunit is folded into two domains of different structure. Domain I is involved mainly in GSH binding, and domain II has been implicated in binding the electrophilic substrate. The X-ray structures of Mu and Pi class GSTs and site-directed mutagenesis studies (Johnson et al. 1993; Kong et al. 1993; Liu et al. 1992; Manoharan et al. 1992; Meyer et al. 1993; Stenberg et al. 1991; Xia et al. 1993) yield information concerning the GSH binding site which could be of direct relevance to the bacteri-

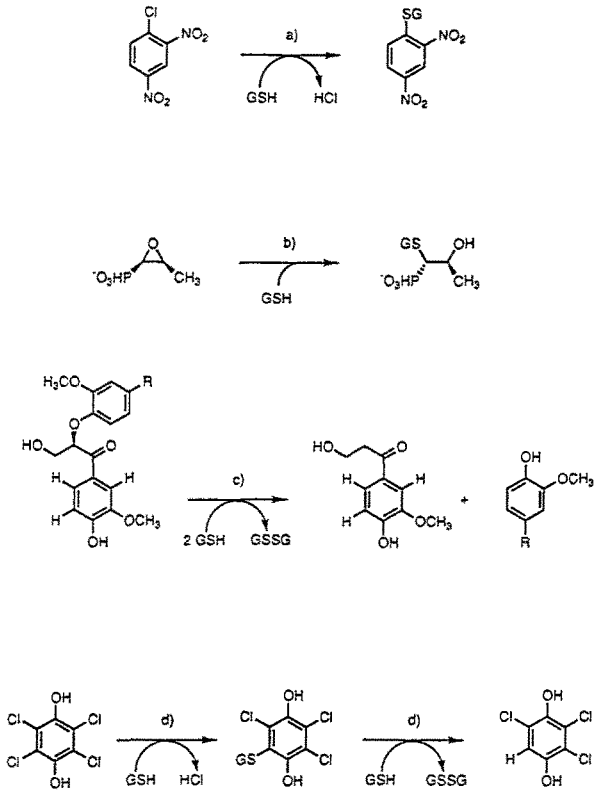

Fig. 4. Examples of reactions catalyzed by bacterial GSH S-transferases other than DCM dehalogenase. The amino acid sequences of the enzymes presented are known. (A) Nucleophilic aromatic displacement reaction catalyzed by a *Proteus mirabilis* GSH S-transferase on the common substrate 1-chloro-2,4-dinitrobenzene (CDNB) often used in the characterization of GST enzymes (Mignogna et al. 1993). (B) Epoxide ring opening reaction responsible for fosfomycin-resistance (Navas et al. 1990) catalyzed by a plasmid-encoded enzyme in *Serratia marcescens*. (C) β-Aryl ether cleavage reaction catalyzed by the membrane-associated *ligF* gene product in *Pseudomonas paucimobilis* (Masai et al. 1993). The oxidized glutathione produced in the reaction is reduced by glutathione reductase. (D) Reductive dechlorination of tetrachloro-p-hydroquinone by the *pcpC* gene product in *Flavobacterium* sp. strain AT39273 (Orser et al. 1993). The trichlorohydroquinone product can be further degraded by the same enzyme to 1,3-dichlorohydroquinone.

al DCM dehalogenases, since sequence conservation across the various classes of GSTs is more extensive in the first domain of the enzyme than in other parts of the molecule (Blocki et al. 1993). Determinants of GST substrate specificity are located in the less conserved domain II. They may prove more difficult to characterize than those involved primarily in cofactor binding, although X-ray investigations of GSTs containing bound substrate analogs (Reinemer et al. 1992; Johnson et al. 1993) as well as experiments with chimeric enzymes (Zhang et al. 1992) have given indications as to where such determinants are located.

In contrast to the situation in eukaryotes, data on prokaryotic GSTs are scarce. However, the amino acid sequences of a few bacterial GSTs have recently become available, and the reactions catalyzed by these enzymes are shown in Fig. 4. In Table 4 and Fig. 5 the sequences of these bacterial GSTs are compared to those of eukaryotic GSTs and bacterial DCM dehalogenases.

A GST from *Proteus mirabilis* is dimeric (2 × 24 kD), reactive with 1-chloro-2,4-dinitrobenzene (CDNB), and can be purified by affinity chromatography on GSH-derivatized matrices (Di Ilio et al. 1988). An antiserum raised against this enzyme does not react with representatives of the Alpha, Mu, or Pi GST classes, but does crossreact with crude extracts from various other proteobacteria (Piccolomini et al. 1989). The sequence of the enzyme shows 31% identity to plant GSTs (Mignogna et al. 1993), but only 22%–26% to a rat Theta enzyme or to DCM dehalogenases.

A GSH-dependent enzyme conferring resistance to fosfomycin was isolated from *Serratia marcescens* and shown to be plasmid-encoded (Navas et al. 1990). This enzyme is smaller than other GSTs (2 × 16 kD), unreactive with CDNB as a substrate, and does not bind to GSH-affinity supports. Its sequence shows no significant homology to any GST sequence nor to any other known protein sequence (Navas et al. 1990).

A gene product from *Pseudomonas paucimobilis* (LigF) appears to be a membrane-bound enzyme involved in the cleavage of an ether bond in an aromatic lignin-type compound (Masai et al. 1993). In contrast to dehalogenase GSTs, GSH is consumed in the reaction and is regenerated by glutathione reductase (Fig. 4). The LigF sequence has some similarity to Theta GSTs, but exhibits slightly closer association with sequences of GSTs from plants.

The PcpC gene product of *Flavobacterium* sp. ATCC 39723 is a constitutively expressed enzyme which catalyzes reductive dehalogenation of tetrachloro-*p*-hydroquinone (Orser et al. 1993). GSH is oxidized in this reaction as well (Fig. 4). However, the occurrence of GSH in *Flavobacterium* sp. needs to be investigated, since *Bacteroides fragilis*, the only representative of the phylum *Bacteroides/Flavobacterium* tested to date, did not contain the compound. Fahey & Sundquist (1991) thus have tentatively concluded that GSH is absent from this phylum. The PcpC pro-

Table 4. Pairwise sequence identity relationships (in percent) between dichloromethane dehalogenases and other GSH transferases.[1]

	DM4	DM11	Pm	LigF	PepC	Fos	GT32	rGSTT1	rGSTT2	rGSTA2	rGSTM1	pGSTP1
DM4	100											
DM11	56	100										
Pm	26	25	100									
LigF	21	17	31	100								
PcpC	22	23	20	18	100							
Fos	16	14	13	12	17	100						
GT32	26	25	31	29	26	21	100					
rGSTT1	26	26	22	28	23	12	23	100				
rGSTT2	29	27	25	25	22	18	28	51	100			
rGSTA	22	23	27	19	17	20	22	23	20	100		
rGSTM1	23	25	18	15	27	16	20	18	22	23	100	
pGSTP1	24	23	20	21	22	20	23	20	17	33	33	100

[1] Pairwise identities were determined using the program GAP (GCG package, University of Wisconsin). The GSH S-transferase sequences compared are: *Dichloromethane dehalogenases*: DM4 from *Methylobacterium sp.* DM4 (La Roche & Leisinger 1990); DM11, from *Methylophilus sp.* DM11 (Bader & Leisinger 1993); *Bacterial GSTs*: Pm, from *Proteus mirabilis* (Mignogna et al. 1993); LigF, from *Pseudomonas paucimobilis* (Masai et al. 1993); PcpC, from a *Falvobacterium sp.* (Orser et al. 1993), and Fos from *Serratia marcescens* (Navas et al. 1990); *Plant GST*: GT32 from maize (Grove et al. 1988); *Theta class rat GSTs*: rGSTT1 (Pemble & Taylor 1992), rGSTT2 (Ogura et al. 1991); GSTs from other classes: rGSTA (alpha (rat); Telakowski-Hopkins et al. 1985); rGSTM1 (mu (rat), Lai et al. 1986); pGSTP1 (pi (pig), Reinemer et al. 1991).

tein has only little similarity to class Theta GSTs and again shows slightly greater sequence identity to plant enzymes.

Overall, the comparison of bacterial GST sequences shows that DCM dehalogenases are more closely related to eukaryotic class Theta enzymes than to other bacterial GSTs described so far (Table 4). Similarly, the *Proteus mirabilis* GST and the *ligF*-encoded β-etherase are more closely associated with class Theta enzymes, including the DCM dehalogenases, than with GSTs of the Alpha, Mu and Pi classes. This may indicate that class Theta, or class Theta-related enzymes are more widely distributed among bacteria than GSTs of the other classes.

Eukaryotic class Theta GSTs are slightly larger (240–290 amino acids) and present in the cell in smaller amounts than GST enzymes of other classes. They usually lack reactivity towards the commonly used substrate CDNB, and they are not retained on GSH-derivatized affinity supports. With the exception of the *Proteus mirabilis* enzyme, these properties are shared by all bacterial GST enzymes sequenced so far. This may explain why GSTs have rarely been reported in bacteria, since detection of GST activity most often relied on the isolation of proteins from crude extracts which bind to GSH affinity matrices or on the demonstration of enzymic activity with CDNB as a substrate.

Theta class enzymes also appear to exhibit a more restricted substrate range than other GSTs and particularly an unusual efficiency in conjugating epoxides and degrading DCM (Pemble & Taylor 1992), but it is unknown whether the ability to use DCM as a substrate is a general property of this group. A report on the presence in human blood of a Theta-like GST acting on DCM has recently appeared (Hallier et al. 1993). Strikingly, the specific activity of purified subunit 5 rat liver enzyme with DCM is about ten times that of DCM dehalogenase from *Methylobacterium* sp. DM4 (Meyer et al. 1991), although, due to the large amounts of enzyme in the bacterium, the activity per mg of total cellular protein in *Methylobacterium* is approximately 300 times that of liver (Banger et al. 1993).

The high conservation of Theta GSTs in *Methylobacterium* (a proteobacterium), maize, the fruitfly and rat, has led to the hypothesis that this class is representative of an ancient progenitor GST acquired by eukaryotes by way of the proteobacterial endosymbionts that have led to the mitochondria (Pemble & Taylor 1992). Compatible with such a scenario, GSH has been observed in only two out of the eleven bacterial phyla, the proteobacteria and the cyanobacteria (Fahey & Sundquist 1991).

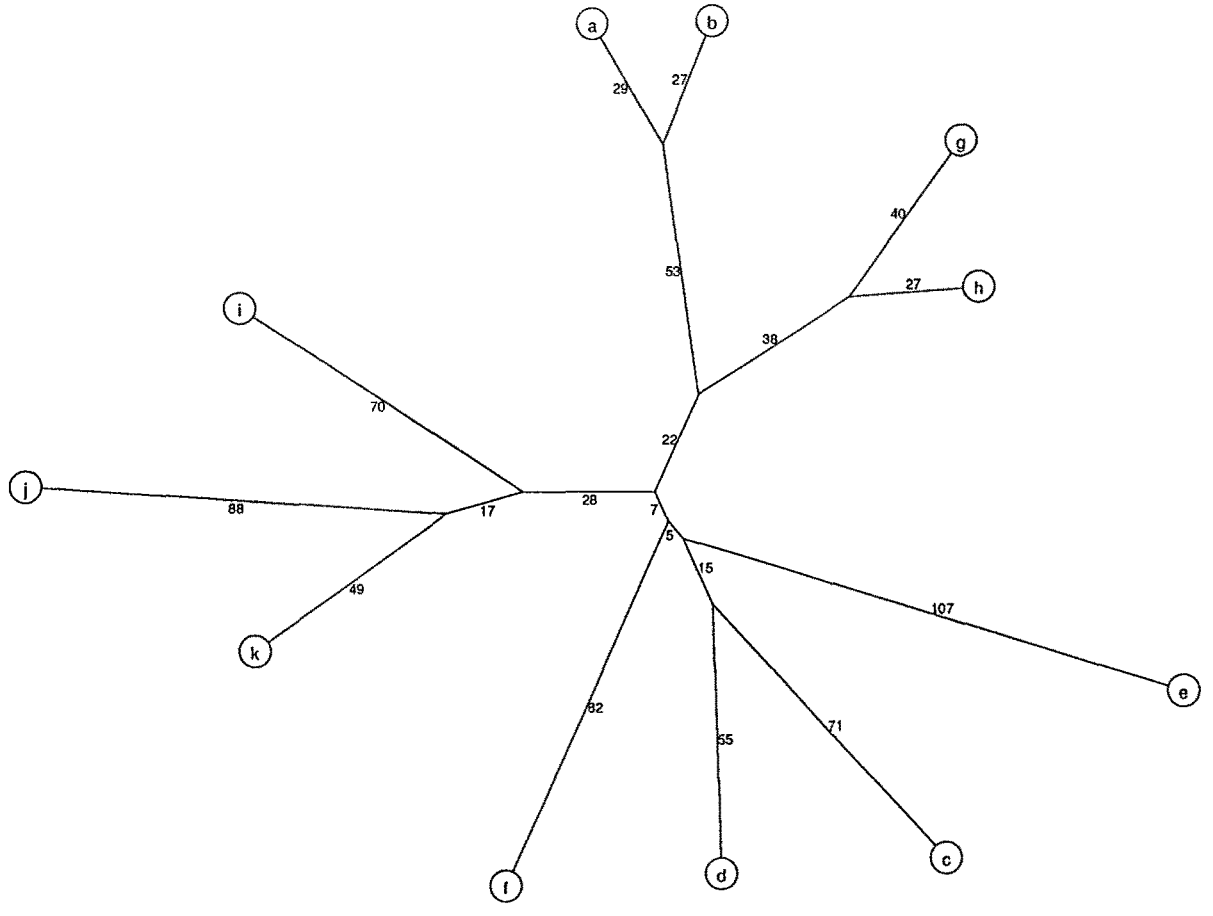

Fig. 5. Unrooted tree describing the relationship between the sequences listed in Table 4, created using the Darwin program (Gonnet 1992). The tree was constructed from a least-square fit of the estimated pairwise PAM distances and their variances. The evolutionary distance between the nodes is given in PAM units under each segment. Different branches are obtained for DCM dehalogenases and Theta-class GSTs (top, right), Alpha-, Mu- and Pi-GSTs (left), and bacterial and plant GSTs (bottom). The GST sequences are (a) *Methylobacterium* sp. DM4, (b) Methylophilus sp. DM11, (c) Pm from *Proteus mirabilis*, (d) LigF from *Pseudomonas paucimobilis*, (e), PcpC from *Flavobacterium* sp., (f) GT32 from maize, (g) Theta GSTT1 from rat, (h) Theta GSTT2 from rat, (i) Class Alpha from rat, (j), Class Mu from rat, (k) Class Pi from pig. A long branch to Fos, the unrelated GST from *Serratia marcescens* (Table 4) was omitted for clarity.

Regulation of dichloromethane dehalogenase expression

As it is the case for many haloalkanoic acid and haloalkane dehalogenases, DCM dehalogenases in methylotrophic bacteria are strongly induced by their substrate. Bacterial GSH S-transferases on the other hand are reported to be formed constitutively (Sysoev et al. 1990; Orser et al. 1993) or their inducibility by the substrate has not been investigated (Mignogna et al. 1993; Masai et al. 1993). We have initiated studies to define the elements involved in regulation of expression of the *Methylobacterium* sp. DM4 DCM dehalogenase. This system is of interest not only because the enzyme is both a dehalogenase and a GSH S-transferase, but also because it exemplifies that, to result in efficient utilization of a novel substrate, acquisition of a catabolic enzyme must be accompanied by the acquisition of a corresponding regulatory system.

Nuclease S1 mapping has indicated the start points of mRNA synthesis from the *dcm* region. A single transcript was identified for *dcmA*. Transcription of an open reading frame (*dcmR*) arranged in the opposite direction to *dcmA* initiated at a major site (P_{R1}) 64 bp upstream of the translation start. Three weaker transcripts running into *dcmR* initiated further upstream of this site, but it is not known whether they originate from initiation at independent promoters or from processing of a larger transcript. The signals correspond-

Table 5. Putative promoter sequences of *dcm* genes from *Methylobacterium* sp. DM4 (La Roche & Leisinger 1990, 1991) and *Methylophilus* sp. DM11 (Bader 1994).

Strain(s)	Gene	Sequence[1]				
DM4	*dcmA*	*TTGACA*	16 bp	*TATA*GA	5 bp	start
DM4	*dcmR*	*TTG*CGG	17 bp	*TA*ACTA	9 bp	start
DM11	*dcmA*	*TTGAC*C	17 bp	*TATAGT*	6 bp	start
various[2]	*moxF*	AAGACA	16-18 bp	TAGAAA	5-7 bp	start

[1] Bases of the putative dcm promoters corresponding to the *E. coli* σ^{70} consensus promoter are in italics.
[2] Putative consensus promoter for the *moxF* genes of various methylotrophs (Lidstrom & Sterling 1990).

ing to both the *dcmA* and the *dcmR* transcripts where much weaker in RNA extracted from methanol-grown cells than in RNA from DCM-grown cells, indicating that expression of the *dcm* genes is regulated at the transcriptional level. At positions −15 to −26 relative to the transcriptional start site, both the *dcmA* and the *dcmR* (P_{R1}) promoter regions contain nearly identical (11 of 12bp) 12bp sequences whose significance is not known (La Roche & Leisinger 1990, 1991). The putative promoter regions upstream of the *dcm* genes from *Methylobacterium* sp. DM4 and of the *dcm*A gene of *Methylophilus* sp. DM11 are shown in Table 5. Since the DCM dehalogenases represent up to 20% of the total cellular protein, the two putative *dcmA* promoters would be highly effective in methylotrophs. They bear more resemblance to the *E. coli* σ^{70} consensus promoter than to the putative consensus promoter proposed for the *moxF* genes of several methylotrophs (Lidstrom & Sterling 1990). Clearly, the significance of the putative promoters in the *dcm* system needs to be explored by mutagenesis studies.

The *dcmR* nucleotide sequence predicts a 30.1 kD protein (DcmR) with a typical helix-turn-helix motif near the N-terminus, but with no significant homology to known proteins or gene sequences. Experiments with *dcmR*'-'*lacZ* fusions indicated that *dcmR* expression was markedly autoregulated at the level of transcription and less so at the protein level. These observations suggested that *dcmR* encodes a regulatory protein involved in governing DCM dehalogenase expression, a possibility that was further examined by complementation analysis. Integration of *dcmA* and 230 bp of its upstream region into the chromosome of a strain DM4 mutant deleted for the *dcm* region resulted in constitutive synthesis of DCM dehalogenase at the induced level. This deletion derivative offered the possibility to test for restoration of regulation by providing various fragments of the *dcmR* region in *trans*. Complementation of regulation was possible with plasmids carrying the intact *dcmR* gene plus 253bp of its upstream region. However, a plasmid encoding for a DcmR protein truncated of 66 amino acids at its C-terminus was ineffective (La Roche & Leisinger 1991).

A working model compatible with the data summarized above postulates that both *dcmA* and *dcmR* expression are negatively controlled at the transcriptional level by the DcmR protein. It assumes that DcmR is a repressor which, in the absence of DCM, binds to recognition sites in the promoter regions P_A of *dcmA* and P_{R1} of *dcmR* and thereby hinders initiation of transcription. DCM abolishes in some way repressor binding and thus relieves inhibition of transcription (Fig. 3). It must be emphasized, however, that the molecular events assumed by the model remain hypothetical. To validate the model it is essential to demonstrate specific binding of the DcmR protein to the P_A and P_{R1} DNA-regions. Efforts towards this goal, using crude extracts of strain DM4 or *E. coli* extracts overexpressing *dcmR* as a protein source, have so far remained unsuccessful. Another putative event requiring investigation concerns the inactivation of the DcmR protein by the presence of DCM in the growth medium. This process may be based on direct interaction or on a regulatory cascade. It is thus essential to unequivocally establish whether all elements involved in regulation of DCM dehalogenase expression are encoded on the *dcm* region or whether regulatory components encoded on the host chromosome participate in the process.

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