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REVIEW PAPER

The Biology of Methylobacteria Capable of Degrading Halomethanes

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Abstract—Recent data on the biology of aerobic methylotrophic bacteria capable of utilizing toxic halogenated methane derivatives as sources of carbon and energy are reviewed, with particular emphasis on the taxonomic, physiological, and biochemical diversity of mono- and dihalomethane-degrading methylobacteria and the enzymatic and genetic aspects of their primary metabolism. The initial steps of chloromethane dehalogenation to formate and HCl through a methylated corrinoid and methyltetrahydrofolate are catalyzed by inducible cobalamin methyl transferase, made up of two proteins (CmuA and CmuB) encoded by the *cmuA* and *cmuB* genes. At the same time, the primary dehalogenation of dichloromethane to formaldehyde and HCl is catalyzed by cytosolic glutathione transferase with S-chloromethylglutathione as an intermediate. The latter enzyme is encoded by the structural *dcmA* gene and is under the negative control of the regulatory *dcmR* gene. In spite of considerable progress in the study of halomethane dehalogenation, some aspects concerning the structural and functional organization of this process and its regulation remain unknown, including the mechanisms of halomethane transport, the release of toxic dehalogenation products (S-chloromethylglutathione, CH_2O, and HCl) from cells, and the maintenance of intracellular pH. Of particular interest is a quantitative evaluation of the ecophysiological role of aerobic methylobacteria in the mineralization of halomethanes and the protection of the biosphere from these toxic pollutants.

Key words: halomethanes, mineralization, aerobic methylotrophic bacteria, distribution, taxonomy, metabolism, enzymes, genes.

Halomethanes are a class of methane derivatives with one or several substituent halogen atoms (Cl, Br, I, or F) in a methane molecule [1]. Among the about 30 presently known halomethanes, the most important are methyl halides (chloro- and bromomethanes) and chlorofluoromethanes (freons). Halomethanes are chemically stable nonpolar compounds with low boiling points, which are mainly synthesized chemically for use as solvents and refrigerants. The only exception is chloromethane (CH_3Cl or methyl chloride), which is synthesized biotically an order of magnitude more intensively than chemically. CH_3Cl is synthesized by marine algae, some higher plants (such as rice), wood-rotting fungi, and marsh microorganisms; it is also formed during forest fires and volcanic eruptions [2, 3]. The biotical production of CH_3Cl is about 5 million tons per year [4]) The concentration of chloromethane in the atmosphere reaches 2 g/m^3 [5]. CH_3Cl is presumably responsible for the degradation of the ozone layer in the stratosphere by 15% [6].

Bromomethane (CH_3Br or methyl bromide) is widely used in agriculture as a fumigant. The amount of bromomethane discharged into the atmosphere due to agricultural fumigation is 16 to 48 tons per year, with even greater amounts of atmospheric bromomethane being produced by phytoplankton, marine algae, fungi, and higher plants, as well as by the combustion of organic compounds [7]. Bromomethane is a major source of atmospheric Br^- ions, which are 50–60 times more active against the ozone layer than chloride ions. The appearance of the Antarctic ozone hole is considered to be 20–25% due to the reaction of chloro- and bromomethane with stratospheric ozone.

Dibromomethane (CH_2Br_2 or methylene bromide) is mainly produced by marine phytoplankton [8] and higher plants [9] and can also be synthesized chemically for use as a solvent [7]. The lifetime of CH_2Br_2 (0.3 year) is almost three times shorter than that of CH_3Br (0.8 year). In spite of this fact, the relatively short-lived molecules of dibromomethane were found in the upper troposphere and lower stratosphere, indicating that dibromomethane is likely involved in the degradation of the ozone layer [10]. The load of bromomethanes on the atmosphere is controlled by the balance between their sources (both natural and anthropogenic) and sinks (both chemical and biotic).

Dichloromethane (CH_2Cl_2 or methylene chloride) is a volatile, highly toxic, mutagenic, and carcinogenic compound widely used as a solvent and refrigerant (freon 30). The annual industrial production of dichlo-

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Aerobic methylobacteria capable of degrading mono- and dihalomethanes

Methylotrophic bacteria	Halomethane utilized	C_1-assimilation pathway	Ref.
Methylobacterium chloromethanicum CM4^T	CH_3Cl	Serine pathway (ICL$^-$)	[21–23]
Hyphomicrobium chloromethanicum CM2^T	CH_3Cl	Serine pathway (ICL$^+$)	[21–23]
Hyphomicrobium spp. PMC, SAC(N)-1, S-3(4), MAR-1	CH_3Cl	ND	[29]
Aminobacter spp. CC495, CMC, IMB-1	CH_3Br, CH_3Cl	ND	[15, 24, 25]
Leisingera methylohalidivorans MB2^T	CH_3Br, CH_3Cl, CH_3I	ND	[27]
Nocardioides sp. SAC-4	CH_3Cl	ND	[28]
Methylopila helvetica DM1^T, DM3–DM9^T	CH_2Cl_2	Serine pathway (ICL$^-$)	[49]
Hyphomicrobium sp. DM2	CH_2Cl_2	Serine pathway (ICL$^+$)	[52]
Methylobacterium dichloromethanicum DM4^T	CH_2Cl_2	Serine pathway (ICL$^-$)	[49]
Methylorhabdus multivorans DM13^T	CH_2Cl_2	Serine pathway (ICL$^-$)	[47]
Albibacter methylovorans DM10^T	CH_2Cl_2	RuBP pathway	[50]
Paracoccus methylutens DM12^T	CH_2Cl_2	RuBP pathway	[48]
Methylophilus leisingerii DM11^T	CH_2Cl_2	RuMP pathway	[45]

Note: ND stands for "no data available". ICL, RuBP, and RuMP are isocitrate lyase, ribulose bisphosphate, and ribulose monophosphate, respectively.

romethane reaches 3×10^5 tons, whereas its natural formation is insignificant [11]. This compound is the major halomethane pollutant of the atmosphere and aquatic systems, since its life time there comprises 70 days and 700 years, respectively [12]. Prolonged exposure to dichloromethane causes various occupational diseases in humans. Biological processes in bodies of water are disturbed at a dichloromethane concentration as low as 10 mg/l. This explains the interest of researchers in microorganisms capable of degrading this toxic pollutant.

Thus, the intense production and use of persistent halomethanes in various industries have led to their accumulation in the environment, where they exert mutagenic and carcinogenic action on living organisms and destroy atmospheric ozone. For these reasons, in 1992, most of the developed nations agreed to cut down on the production of hazardous halomethanes. An alternative approach to the solution of this problem may be the development of novel halomethane-degrading biotechnologies based on active microbial strains possessing dehalogenases [13]. The current and future prospects in this challenging problem are considered below with special emphasis on aerobic methylotrophic bacteria capable of degrading mono- and dihalomethanes.

Aerobic Degraders of Monohalomethanes

Distribution and taxonomy. Until recently, information available in the literature on the biodegradation of monohalomethanes (methyl halides) was fragmentary. Shorter *et al.* [14] and Miller *et al.* [15] showed that CH_3Br is actively degraded in various soils. Chloromethane can be oxidized by cells and cell extracts of methanotrophic and nitrifying bacteria [16, 17] and by the respective enzymes, methane monooxygenase [18] and ammonium monooxygenase [19]. The first isolated microorganism capable of utilizing chloromethane as a source of carbon and energy, *Hyphomicrobium* sp. MC1 [20], was thereafter lost. We have recently succeeded in isolating nine strains of aerobic methylobacteria capable of utilizing chloromethane as a source of carbon and energy from soil samples collected near a petrochemical refinery and in a dendropark [21], four of these strains were ascribed to the genus *Methylobacterium* and five, to the genus *Hyphomicrobium* [22]. Based on DNA–DNA hybridization and 16S rDNA sequence data, these two groups of isolates were identified as belonging to two new species, *Methylobacterium chloromethanicum* and *Hyphomicrobium chloromethanicum* [23] (table, Fig. 1).

The chloromethane- and bromomethane-utilizing strain IMB-1 was isolated from soil fumigated with CH_3Br over a long time period [15, 24], whereas strain CC495 was isolated from the upper layer of a forest floor. Both of these strains were ascribed to the genus *Aminobacter* [25]. The CH_3Br-utilizing marine isolate MB2 [26] was found to belong to the *Roseobacter* group and classified as *Leisingera methylohalidivorans* [27].

The enrichment cultures isolated from various habitats (soil, freshwater, and seawater) using media with chloromethane as a source of carbon and energy were dominated by bacteria of the genus *Hyphomicrobium* [28, 29]. According to 16S rDNA sequence data, strains S-3, S-4, and MAR-1 were closest to the species *H. chloromethanicum*, whereas strains PMC, SAC-1, and SAN-1 were distinct from all of the known *Hyphomicrobium* species. Strain CMC was close to strains IMB-1 and CC495 within the *Aminobacter*

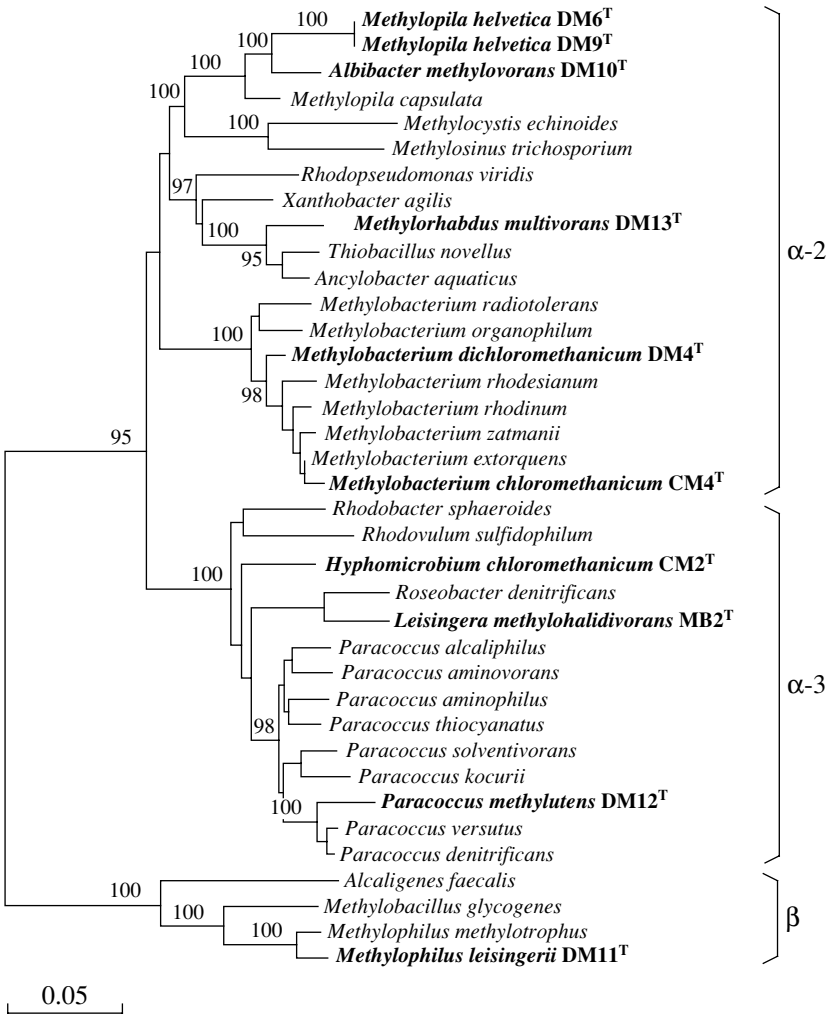

Fig. 1. A phylogenetic tree showing the position of aerobic methylobacteria capable of degrading halomethanes. Bootstrap values less than 95 are omitted. The scale bar of evolutionary distances represents 5 substitutions per 100 nucleotides.

group. Strain SAC-4 was found to belong to the genus *Nocardioides* (table). This strain is the first described gram-positive aerobic chloromethane degrader [28]. To date, aerobic monohalomethane-mineralizing methylobacteria have been isolated from many biotopes, indicating that they are ubiquitous in natural habitats [29].

The enzymatic and genetic aspects of methyl halide dehalogenation. Evidence for a specific mechanism of the aerobic dehalogenation of CH_3Cl was first obtained in culture experiments with *Hyphomicrobium* sp. MC1 [20]. When grown on chloromethane, this strain utilized chloromethane stoichiometrically with the formation and release of chloride ions into the medium. The specific growth rates of the strain on methanol, chloromethane, and formate were 0.12, 0.09, and 0.04 h^{-1}, respectively. Cells grown on chloromethane actively oxidized this substrate, while they slowly oxidized CH_3OH and failed to oxidize CH_4. All of these observations led the authors to the preliminary conclusion that methane monooxygenase is not involved in the dehalogenation of CH_3Cl. Unfortunately, work along this line was suspended because of the loss of the strain, but it received an important impetus after a decade, when new aerobic halomethane-degrading methylobacteria were isolated [21, 22] and corrinoid methyl halide transferases were discovered in anaerobes [30].

The stoichiometric formation of CH_2O and Cl^- ions from CH_3Cl was shown in our experiments with the cultivation of cultures of *M. chloromethanicum* CM4 [22]. The chloromethane-grown cells of this strain actively oxidized CH_3Cl, CH_2O, and HCOOH, while very slowly oxidizing CH_3OH. At the same time, methanol-grown cells were unable to oxidize CH_3Cl, but actively oxidized CH_3OH, CH_2O, and HCOOH. Extracts of chloromethane-grown cells catalyzed the stoichiometric formation of CH_2O and HCl from CH_3Cl in a reaction independent of $NAD(P)^+$, $NAD(P)H_2$, glutathione, and O_2, indicating that methanol is not an intermediate of CH_3Cl conversion to CH_2O

and that the respective enzyme differs from the known oxidoreductases and dehalogenases.

The biochemical and genetic investigations of Vannelli *et al.* [31, 32] allowed the mechanism of CH_3Cl dehalogenation in *M. chloromethanicum* CM4 to be discovered. The synthesis of two polypeptides with molecular masses of 67 and 35 kDa found in this strain was induced during growth on chloromethane. In this case, cells were able to dehalogenate CH_3Br and CH_3I, but not CH_2Cl_2 or other chloroalkanes (such as $CH_3CH_2Cl_2$), indicating that chloromethane dehalogenase has a high substrate specificity for monohalomethanes. Although *M. chloromethanicum* CM4 can dehalogenate CH_3Br and CH_3I, this strain is unable to grow on these substrates, presumably because of the high toxicity of Br^- and I^- ions.

Using the minitransposon Tn5 system, Vannelli *et al.* obtained *M. chloromethanicum* CM4 mutants incapable of growing on CH_3Cl. Genes with transposon insertions were cloned, sequenced, and subjected to biochemical analysis, which made it possible to reveal a new two-step pathway of CH_3Cl degradation in aerobic methylobacteria [32]. The first step of this putative pathway (Fig. 2) is catalyzed by the 67-kDa CmuA protein, which has methyl transferase and corrinoid-binding domains. The methyl transferase domain transfers the methyl group of CH_3Cl to the Co atom of the corrinoid group of the second domain (the so-called methyl transferase I activity). The second stage of this pathway is catalyzed by the 35-kDa CmuB protein, which transfers the CH_3 group further to tetrahydrofolate (THF), yielding methyl-THF (the so-called methyl transferase II activity). The methyl group of the methyl-THF is oxidized to CO_2 through formate, providing the serine cycle with reducing equivalents. Methylene-THF enters the serine cycle as well. Four genes (*cmuA*, *cmuB*, *cmuC*, and *purU*) are necessary for bacterial growth on CH_3Cl but not on other C_1-substrates [32]. In *M. chloromethanicum* CM4, these genes occur in two clusters, which also contain genes encoding the enzymes of cobalamin synthesis (*cobU*, *cobQ*, *cobD*, and *cobC*) and genes encoding the putative pterin-dependent enzymes of C_1-metabolism (*folC*, *folD*, *purU*, and *metF*).

The monomeric CmuA protein (67 kDa) contains Zn and Co atoms and vitamin B_{12} as a cofactor [33]. By analogy with the methyl transferases of methanogens, it was suggested that CmuA catalyzes the transfer of methyl groups with the involvement of a Zn-activated thioprotein. The latter methyl transferase was proved to be necessary for the transfer of methyl groups to the vitamin B_{12} cofactor of the CmuA protein. Studer *et al.* [34] also purified methyl transferase II (the CmuB protein), which catalyzes the transfer of the methyl group from methylcobalamin to THF. To obtain methyl transferase activity in vitro, the CmuB protein should be added to the reaction mixture in excess of the molar content of CmuA, suggesting that the product of the reaction catalyzed by methyl transferase I (CmuA) serves as a substrate not only for the cobalamin/THF-specific methyl transferase II, but also for a hitherto unidentified methyl transferase (putative methyl transferase III) transferring methyl groups from CmuA to tetrahydromethanopterin, whose concentration in *Methylobacterium extorquens* AM1 cells reaches 0.2 mM [35]. The CmuC protein of *M. chloromethanicum* CM4 is the most probable candidate for the role of methyl transferase III, since its amino acid sequence is very close to that of some methyl transferases, and its mutational inactivation leads to the loss of its ability to grow on chloromethane [34].

According to recent data [36], the *metF*, *folD*, and *purU* genes, which code for methylene-THF reductase, methylene-THF dehydrogenase/cyclohydrolase, and formyl-THF hydrolase, respectively, occur in the same gene clusters as the *cmuA* and *cmuB* genes. This suggests the existence of a third pathway of CH_3Cl oxidation to formate via methyl-THF in *M. chloromethanicum* CM4, since the bacterium *M. extorquens* AM1, which lacks these three genes, is unable to grow on chloromethane. In *M. chloromethanicum* CM4, the *metF*, *folD*, and *purU* genes are expressed only during growth on CH_3Cl. The nucleotide sequences of their promoters are very conservative and differ from the other known promoters of bacteria from the genus *Methylobacterium*. A mutant with inactivated *purU* and *metF* genes was unable to grow on chloromethane because of the absence of methylene-THF activity. Taken together, these data indicate that *M. chloromethanicum* CM4 has a set of specific THF-dependent enzymes responsible for the oxidation of chloromethane in a way that is independent of free formaldehyde (Fig. 2). The obtaining and comprehensive pheno- and genotypic analyses of other mutants unable to grow on chloromethane may provide insight into the molecular mechanisms of adaptation of aerobic methylobacteria to growth on monohalomethanes.

Hyphomicrobium chloromethanicum strain CM2, also has an inducible enzymatic system responsible for the utilization of CH_3Cl. This system also includes two proteins, the 67-kDa CmuA protein and the 35-kDa CmuB protein. The CmuA protein of *H. chloromethanicum* CM2 exhibits 80% homology to the CmuA protein of *M. chloromethanicum* CM4. The cluster of the *cmu* genes of *H. chloromethanicum* CM2, which is about 9.5 kb in size, contains ten ORFs and includes the *N*-terminus of the methyl transferase domain and the *C*-terminus of the corrinoid-binding domain. The CmuA and CmuB proteins of *H. chloromethanicum* CM2 possess methyl transferase activity and are very close to the methyl transferases of *M. chloromethanicum* CM4. The *folD* gene codes for methylene-THF cyclohydrolase, which is involved in the transfer of C_1-units to the serine cycle and in the formation of CO_2. The *paaE* gene codes for a putative redox enzyme [37].

The facultatively methylotrophic *Aminobacter* sp. IMB-1 likely possesses a common inducible enzymatic

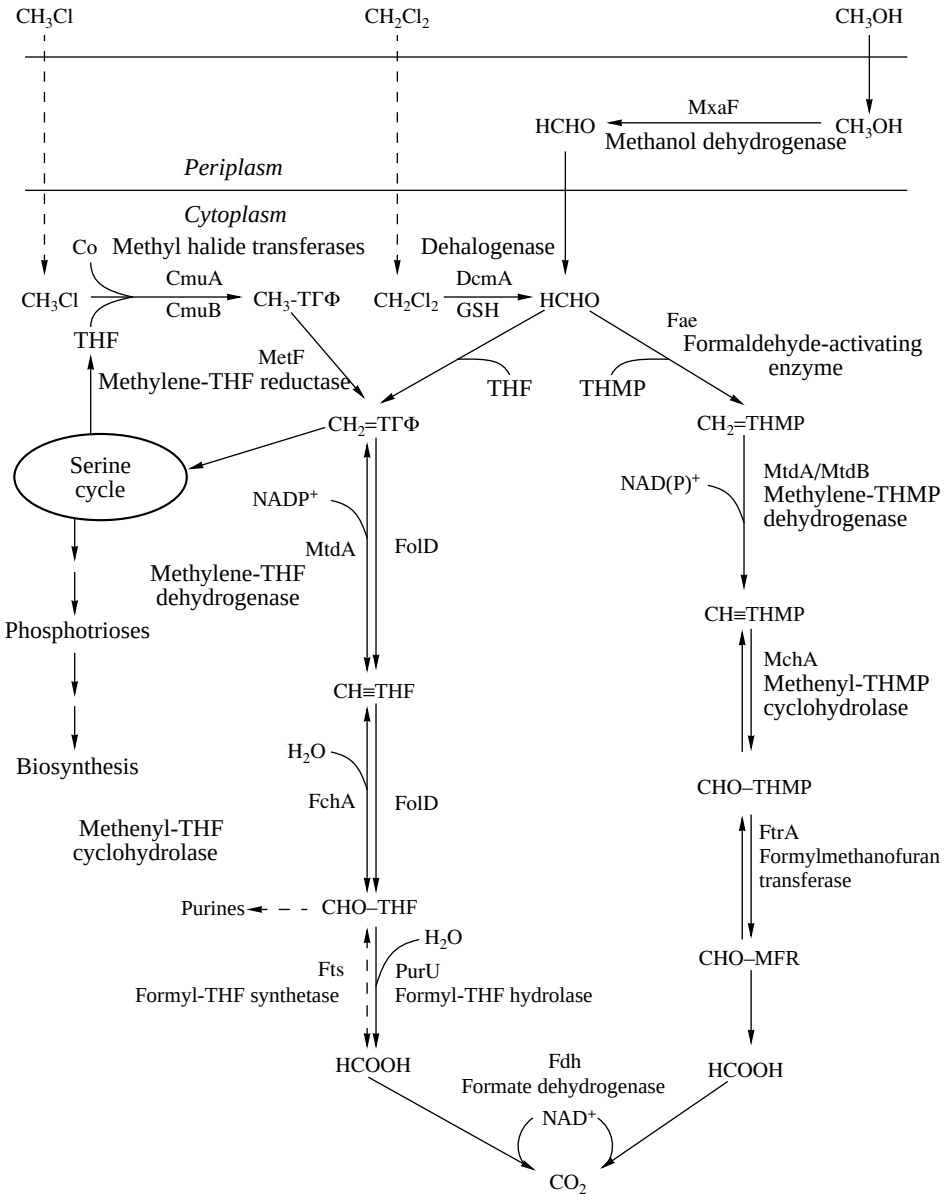

Fig. 2. Putative pathways of the primary metabolism of halomethanes in serine methylobacteria. THF, THMP, and MFR are tetrahydrofolate, tetrahydromethanopterin, and methanofuran, respectively.

system for the oxidation of CH_3Cl, CH_3Br, and CH_3I [38]. The cluster of *cmu* genes of this bacterium has six ORFs (*cmuC*, *cmuA*, *orf146*, *paaE*, *hutI*, and *metF*) [39]. The 67-kDa CmuA protein of strain IMB-1 is highly homologous (by 78%) to the known CmuA proteins of other methylobacteria and is likely involved in the dehalogenation of methyl halides. The CmuC protein of strain IMB-1 is slightly homologous (by 36%) to the CmuC protein of *M. chloromethanicum* CM4 and is presumably necessary for growth on CH_3Cl. The *cmuB* gene has not been found in the genome of this strain. The *paaE* gene encodes a reductase which is similar to the other known oxidoreductases. The PaaE protein transfers the prosthetic groups necessary for the reactivation of cobalamin. The partially cloned *metF* gene codes for methylene-THF reductase, which is necessary for the assimilation of CH_3Br as a carbon source. The *hutI* gene of the methyl halide gene cluster of strain IMB-1 codes for imidazolepropionase, which is involved in the synthesis of the imidazole ring of the nucleotide loop of cobalamin [39]. In general, the genetic analysis of *Aminobacter* sp. IMB-1 showed that this strain degrades methyl halides with the involvement of the corrinoid methyl transferase system that is close, but not identical, to the respective systems of other methylobacteria.

The growth of *Aminobacter* sp. CC495 in the presence of chloromethane was observed only when vita-

min B_{12} was added to the cultivation medium [25]. Such growth was accompanied by the inducible synthesis of two proteins 67 and 29 kDa in size. The 67-kDa protein was purified and identified as a halomethane: bisulfide/halide methyl transferase. The *N*-terminal amino acid sequence of this corrinoid enzyme, involved in methyl halide dehalogenation, was homologous to those of the CmuA proteins of *M. chloromethanicum* CM4, *H. chloromethanicum* CM2, and *Aminobacter* sp. IMB-1 by 81.3, 68.8, and 81.3%, respectively. In *M. chloromethanicum* CM4 cells, methyl halide transferase activity comprised only 5% of chloromethane: methyl-THF transferase activity [33], which casts doubt on the functional importance of methyl halide transferase in aerobic methylobacteria. Work on the cloning and sequencing of the *cmu* genes of CH_3Cl metabolism in *Aminobacter* sp. CC495 has recently been initiated.

One of the several genes known to be involved in the degradation of CH_3Cl, namely, *cmuA*, was chosen as a functional marker of chloromethane degradation, since it is the CmuA protein that catalyzes the primary binding and dehalogenation of chloromethane molecules [39]. The unique structure of this protein was taken into account when developing the respective primer system, with the forward 929f primer (AACTAGCTGCTGAGGTGGCTAYAAYGGNGG) corresponding to the 5'-region of the methyl transferase domain and the reverse 1669r primer (CAACGTATACGGTGGAGGAGTTNGTCATNAC) corresponding to the 3'-region of the corrinoid-binding domain. The analysis of these primers for the specificity of amplification showed that they yield a proper product, 741 bp in size, with the DNA of the CH_3Cl-degrading type strains and new gram-negative isolates. The correspondence of PCR products to the *cmuA* gene was confirmed by sequence analysis. The primers were then used to amplify the *cmuA* sequences of DNA samples obtained from soil enrichment cultures. The cloning and sequencing of PCR products revealed three *cmuA* sequences, two of which were identical to those of the *Hyphomicrobium* sp. S-4 and *Aminobacter* sp. IMB-1, whereas the third sequence turned out to be a new sequence, although it was highly homologous (by 91%) to that of *H. chloromethanicum* CM2 [29].

In spite of significant progress in the study of the aerobic metabolism of methyl halides, there are still some open questions concerning the mechanisms of methyl halide transport and the release of the toxic dehalogenation products H^+ and Cl^- into the medium. Furthermore, it still remains unclear whether or not all of the products of the *cmuA*, *cmuB*, and *cmuC* genes are necessary for the degradation of methyl halides by gram-negative and gram-positive aerobic methylobacteria and whether or not CH_3Cl and CH_3Br are oxidized by the same or different methyl transferases. The genetic analysis of mutants obtained by marker exchange mutagenesis and the comprehensive kinetic analysis of enzymes involved in the primary oxidation of methyl halides in bacteria may answer these questions.

Experiments with strains amenable to mutagenesis and genetic transformation can provide insight into the regulation of methyl halide metabolism in aerobic methylobacteria and the inhibition, repression, and induction mechanisms of the CmuA protein. The role of aerobic methylobacteria in the degradation of methyl halides in soil and aquatic biotopes is studied by conventional microbiological methods using enrichment and pure cultures, as well as by the modern methods of molecular ecology with the use of functional genetic probes based on the *cmuA* and other key genes of methyl halide metabolism. The transcriptional organization of these genes and their coexpression are studied using a wide range of primers, northern-blotting, and PCR analysis. The crystallization and structural investigation of the CmuA protein may also contribute to a better understanding of the molecular mechanisms of methyl halide degradation in methylobacteria [29].

Aerobic Dihalomethane-degrading Bacteria

Distribution and taxonomy. The bacterial oxidation of $^{14}CH_2Br_2$ was revealed in water samples taken from various biotopes with salinities ranging from 0 to 77 g/l, the most active oxidation being in seawater [26]. The cooxidation of dihalomethanes was observed not only for methanotrophic and nitrifying bacteria, but also for their enzymes (ethane monooxygenase and ammonium monooxygenase, respectively) [19, 41]. The strong carbon–chlorine bond of dichloromethane (DCM) is difficult to hydrolyze or cleave abiotically [42], whereas the biodegradation of DCM was observed in soils, bodies of water [43], and activated sludges [44]. In collaboration with the research group of Leisinger, we isolated eleven DCM-degrading aerobic bacteria, characterized them taxonomically, and studied their primary and central C_1-metabolism [45–50]. These isolates turned out to belong to ten new taxa of aerobic methylobacteria, including three new genera, *Methylorhabdus*, *Methylopila*, and *Albibacter* (see table). The 16S rDNA gene sequencing (Fig. 1) confirmed the phylogenetic diversity of aerobic DCM-degrading bacteria [51].

The enzymatic and genetic aspects of dichloromethane dehalogenation. The stoichiometric conversion of DCM in the presence of reduced glutathione (GSH) was first observed in experiments with cell-free extracts of *Hyphomicrobium* sp. DM2 [52]:

$$CH_2Cl_2 + GSH \longrightarrow GS\text{-}CH_2Cl + HCl$$

$$\longrightarrow GS\text{-}CH_2OH + HCl \longrightarrow GSH + CH_2O.$$

The intermediate S-chloromethylglutathione converts spontaneously to S-hydroxymethylglutathione, which is degraded to formaldehyde and GSH [53].

Some aspects of CH_2Cl_2 dehalogenation in methylobacteria are still poorly understood. The formation of

the putative intermediate S-chloromethylglutathione has not yet been proved experimentally. The chemically synthesized model compound S-(chloromethyl)-*N*-acetyl-cysteine has a half-life of 4 s at 0°C at pH 7.0 [54]. Another model compound, S-fluoromethylglutathione, which is less reactive, has a half-life of 5.8 min in D_2O. In a reaction mixture containing GSH, CH_2ClF, and the dehalogenase of *M. leisingerii* DM11, Blocki *et al.* detected a ^{19}F-NMR signal close to that of S-fluoromethylglutathione [55]. The chemically synthesized S-chloromethylglutathione was found capable of alkylating DNA bases in vitro, causing DNA damage. Presumably, S-chloromethylglutathione possesses mutagenic activity and may impair DNA replication in vivo.

The DCM dehalogenases isolated from the facultative methylobacteria *M. dichloromethanicum* DM4 and *Hyphomicrobium* sp. DM2 are similar catalytically and immunologically and have identical N-terminal amino acid sequences and a hexameric structure with a molecular mass of monomers equal to 33 kDa [56]. Both enzymes belong to group A dehalogenases, which are only induced by DCM and have low catalytic activity. When these methylobacteria grow in the presence of DCM, the amount of DCM dehalogenase in cells may reach 20% of their total protein content during batch cultivation and 50% during limited chemostat cultivation.

The DCM dehalogenase isolated from *M. leisingerii* DM11 was attributed to group B dehalogenases. This dehalogenase is 5.6 times more active with CH_2Cl_2 than group A dehalogenases [56]. The dehalogenases of groups A and B differ immunologically, kinetically, and in their *N*-terminal amino acid sequences. Nevertheless, they are obviously related evolutionarily, as is evident from the results of hybridization of their *dcmA* genes, the identical requirements of these two kinds of dehalogenases for GSH, and the equal molecular masses of their subunits [57].

The sequencing of the *dcmA* genes from *M. dichloromethanicum* DM4 [58] and *M. leisingerii* DM11 [59] confirmed the fact that the respective dehalogenases belong to the theta-class of glutathione transferases. These bacterial enzymes are highly specific and active, so that DCM is degraded about 10^8 times faster biotically than abiotically [55]. The *dcmA* genes mentioned are very similar in their nucleotide sequences, but the encoded DCM dehalogenases are homologous in their amino acid sequences by only 56%. Furthermore, they exhibit only 15–25% identical amino acid sequences with the other known glutathione transferases of the theta-class [60] and have virtually no homology with other protein families. The partial mapping of the *dcmA* gene of *M. dichloromethanicum* DM4 [61, 62] showed that this gene is controlled by the neighboring *dcmR* gene, which is transcribed in the opposite direction. In the presence of CH_2Cl_2, the *dcmR* gene acts as a transcriptional repressor of the *dcmA* gene. If the *dcmR* gene is absent, the *dcmA* gene is expressed constitutively.

As in rhizobia [63], GSH in methylobacteria is involved not only in dehalogenation but also in the maintenance of intracellular pH and ionic homeostasis. The H^+ and Cl^- ions formed intracellularly by DCM-utilizing methylobacteria are partially released into the medium and partially remain in cells. The resultant acidification of the cytoplasm may explain the lower biomass yield of methylobacteria with respect to the CH_2Cl_2 consumed than with respect to the CH_3OH consumed [53, 57]. Presumably, the cytotoxic effects of formaldehyde in these two cases are also different, since methanol dehydrogenase is a periplasmic enzyme, whereas DCM dehalogenase is localized in the cytoplasm (Fig. 2) [64]. The primary metabolism of CH_2Cl_2 and CH_3OH in methylobacteria depends on the intracellular pool of GSH and is associated with the accumulation of H^+, Cl^-, S-chloromethylglutathione, and CH_2O in the cytoplasm. These reactive intermediates may exert a lethal effect on cells if they do not have efficient protective mechanisms [53, 65].

The similarity of most DCM dehalogenases from group A suggests that the structural and regulatory genes of these enzymes may be transferred horizontally with the aid of transposable elements [56]. In methylotrophs, these genes are amplified with the primers corresponding to the flanking regions of the *dcmA* gene of *M. dichloromethanicum* DM4 [57]. The observed mutation rates and the similarity of the *dcm* genes of various methylobacteria allow one to suggest that these genes evolved billions of years ago from a common precursor [66]. Ancient DCM dehalogenases were likely able to degrade various natural substrates close in structure to CH_2Cl_2, CH_2Br_2, and CH_3Cl.

Based on plasmid pJB3K_m1, Gisi *et al.* [67] have constructed plasmids bearing the *dcmA* genes of *M. dichloromethanicum* DM4 and *M. leisingerii* DM11 under the control of the P_A promoter. The *dcmA* gene–expressing transformant *M. extorquens* AM1 was found incapable of growing on DCM, suggesting that the dehalogenation of DCM requires not only the *dcmA* gene product but also some other proteins. The minitransposon (Tn5) mutant of *M. dichloromethanicum* DM4 that was unable to excrete Cl^- ions into the medium could grow only at low concentrations of CH_2Cl_2 in the cultivation medium, indicating the important role of ionic homeostasis in the growth of methylobacteria on halomethanes. Another Tn5 mutant (DM4-z1445), which has the same DCM dehalogenase activity as the parent strain DM4, did not exhibit DNA polymerase I activity and could not grow on CH_2Cl_2 [68]. The cloning and sequencing of the DNA polymerase I gene *polA* of strain DM4 showed the presence of only one copy of this gene in this strain. The transfer of the *polA* gene to the DM4-z1445 mutant with the aid of the plasmid pME8112 restored in it the ability to grow on DCM. This finding allowed Kayser *et al.* [68] to suggest that DNA polymerase I is essential for bacterial growth in the presence of DCM, since this enzyme is involved in

the repair of the DNA damaged by S-chloromethylglutathione.

The transfer (with the aid of plasmids pME8220 and pME8221 and the shuttle vector pCM62) of the *dcm A* gene of *M. dichloromethanicum* DM4 to *M. chloromethanicum* CM4 and *M. extorquens* AM1, which are unable to grow in the presence of CH_2Cl_2, led to the obtaining of two transconjugants [69]. Both transconjugants possessed DCM dehalogenase activity, but only the transconjugant of *M. chloromethanicum* CM4 could grow in the presence of CH_2Cl_2. The DCM dehalogenase activity of the methanol-grown transconjugants bearing plasmid pME8221 with the *dcm A* gene and its negative transcriptional regulator *dcm R* depended on the presence of CH_2Cl_2 in the medium. At the same time, in methanol-grown pME8220 transconjugants lacking the regulatory gene *dcm R*, the *dcmA* gene was expressed constitutively, irrespective of the presence of CH_2Cl_2 in the medium. It remains unclear why the *M. extorquens* AM1 transconjugants did not acquire an ability to grow on DCM. The most likely reason for this is the low tolerance of such transconjugants to intracellularly formed S-chloromethylglutathione, CH_2O, and Cl^- ions. To compare the functional genomics of methylobacteria capable and incapable of growing in the presence of DCM, it is necessary to develop specific systems of genetic manipulation. The possibility cannot be excluded that methylobacteria implementing different pathways of primary C_1-metabolism detoxify CH_2Cl_2 intermediates by different mechanisms.

The investigation of DCM mineralization by aerobic methylobacteria over the last two decades has provided essential information on the distribution and taxonomy of DCM-degrading bacteria and their specific metabolic pathways. Nevertheless, the molecular mechanisms of DCM mineralization remain unclear. It should be noted in this regard that the kinetic properties of the DCM dehalogenases/glutathione transferases of groups A and B considerably differ and, hence, their comparative structural study may provide insight into the mechanisms of their action. On the other hand, the limited diversity of methylobacterial DCM dehalogenases has, thus far, not advanced our understanding of the mechanism of action of these enzymes.

Recent studies showed that not only dehalogenases but also some additional enzymes and genes are necessary for the bacterial mineralization of DCM. The complete sequencing of the *M. extorquens* AM1 genome and the high-resolution analysis of gene expression may lead to the determination of all genes and proteins involved in the mineralization of halomethanes. This will provide deeper insight into the mineralization mechanisms implemented by aerobic methylobacteria grown on DCM as a source of carbon and energy. Knowledge of these mechanisms will promote the development of more efficient relevant biotechnologies. The maximum rate of aerobic degradation of DCM achieved in the already created bioreactors (12 kg DCM per m^3 per day) [70–73] indicates the expediency of employing DCM-mineralizing bacterial strains for the treatment of DCM-containing industrial sewage.

CONCLUSION

Investigations during the last three decades have demonstrated a great taxonomic diversity and metabolic versatility of aerobic methylotrophic bacteria growing on relatively simple C_1-compounds. In particular, aerobic methylobacteria can utilize mono- and dihalomethanes with the involvement of corrinoid methyl transferases (in the case of methyl halides) or glutathione transferases (in the case of dihalomethanes). There are grounds to believe that aerobic methylobacteria acquired and modified the corrinoid methyl transferase systems of anaerobic methylotrophs through the horizontal transfer of the *cmuA*, *cmuB*, and *cmuC* genes. Actually, this is the second example of the presence of elements of anaerobic metabolism in strict aerobes, the first being the tetrahydromethanopterin-dependent pathway of formaldehyde oxidation to formate and CO_2 [35], which heretofore was only attributed to anaerobic methylotrophic archaea. These two examples indicate that, in the past, aerobic and anaerobic methylotrophic prokaryotes were closely related both trophically and genetically. Also of interest is the fact that gram-positive methylobacteria oxidize formaldehyde with the involvement of a new cofactor, mycothiol [74]. Altogether this calls for the comparative study of the molecular phylogeny of halomethane-mineralizing bacteria.

Some important structural and regulatory aspects of halomethane metabolism in aerobic methylobacteria, such as transport, protective, and osmoregulation mechanisms, are still poorly understood. It is not clear why methylotrophic yeasts possess glutathione transferase but cannot grow on DCM. It should be noted that naturally occurring aerobic bacterial degraders of tri- and tetrahalomethanes have not yet been found, but, taking into account the great number of known halomethanes (about 30) and their ever-increasing selective pressure on microbiotas, it is obvious that such degraders will be found in the near future. The application of genetic and enzymatic engineering approaches may reduce the time necessary for the obtaining of new efficient halomethane-mineralizing bacterial strains and relevant enzymes.

The role of aerobic methylobacteria in the mineralization of highly toxic and persistent halomethanes, which degrade the ozone layer of the Earth's atmosphere, remains to be evaluated. The fact that this role is very important follows from biogeochemical data and from the great biodiversity and wide distribution of halomethane-mineralizing methylobacteria [29, 75].

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