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# AEROBIC MICROBIAL DEGRADATION OF CHLOROMETHANE

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meinen Eltern

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## ABBREVIATIONS

| | |
|---|---|
| CoM | coenzyme M |
| $CH_3$-$H_4$folate | $N^5$-methyltetrahydrofolate |
| $H_4$folate | tetrahydrofolate |
| $H_4$MPT | tetrahydromethanopterin |
| ICP-MS | inductively coupled plasma-atomic emission mass spectrometry |
| IPTG | isopropyl thio-β-D-galactoside |
| MALDI-TOF | matrix assisted laser desorption / ionization time-of-flight mass spectrometry |

## ENZYMES

Chloromethane:corrinoid methyltransferase (EC 2.1.1.-)

Methylcobalamin:tetrahydrofolate methyltransferase (EC 2.1.1.-)

NADH-dependent methylene tetrahydrofolate reductase (EC 1.5.1.-)

## SUMMARY

The monohalomethanes $CH_3Cl$ and $CH_3Br$ originate from both natural and anthropogenic sources. They are abundantly present in the atmosphere and significantly contribute to the depletion of the ozone layer. To allow an accurate budgeting of monohalomethanes in the atmosphere the magnitude of the source and sinks has to be determined. However, only little information is available on the biological sinks for monohalomethanes. Some aerobic and anaerobic bacteria are known to degrade monohalomethanes, but detailed biochemical or genetic studies had not been carried out when this work was initiated.

*Methylobacterium chloromethanicum* CM4, an aerobic methylotrophic $\alpha$-proteobacterium, is able to grow with chloromethane as the sole carbon and energy source. Mutants of this strain, previously obtained by miniTn*5* mutagenesis, were unable to grow with chloromethane but could still grow with methanol, methylamine or formate. The transposon insertion sites in six of these mutants were mapped to two distinct DNA fragments. Sequence analysis suggested the presence of a set of genes encoding a multistep pathway for the conversion of chloromethane to formate.

Based on enzyme activity measurements in cell-free extracts of CM4 mutant strains, the *cmuA* and *cmuB* genes were found to be essential for dehalogenation of chloromethane. The CmuA protein represents a so far unique two-domain protein, combining a methyltransferase and a corrinoid binding protein. It contains a noncovalently bound corrinoid identified as vitamin $B_{12}$ and one mol zinc per mol protein. CmuB is a homodimeric protein and was shown to catalyze methyl group transfer from free methylcobalamin to tetrahydrofolate. Together, purified CmuA and CmuB proteins were sufficient to catalyze chloromethane dehalogenation. The dehalogenation mechanism proceeds via two subsequent methyl transfer reactions, which involve binding of a methyl group to the vitamin $B_{12}$ cofactor on CmuA. Thus, current data suggest that CmuA catalyzes the first methyl transfer from chloromethane onto its prosthetic vitamin $B_{12}$ group, and that the second methyl transfer reaction, from the methylated CmuA protein to tetrahydrofolate, is catalyzed by the CmuB protein.

The $C_1$ unit of methyl-tetrahydrofolate formed upon dehalogenation of chloromethane is thought to be oxidized by a set of tetrahydrofolate-dependent enzymes to formate. Sequence analysis suggested that three genes, *metF*, *folD* and *purU* located near *cmuA* and *cmuB* in the CM4 genome, encode enzymes involved in such a $C_1$ oxidation pathway.

Studies with transcriptional reporter gene fusions demonstrated chloromethane-dependent expression of these genes. Transcriptional start sites were mapped by primer extension analysis, and three promoter regions were identified that are active during growth on chloromethane. The corresponding sequences were well conserved, but differed from the *Methylobacterium* promoters described so far. This suggests that at least three coordinately regulated transcriptional units are expressed during growth of *M. chloromethanicum* CM4 with chloromethane. These units comprise *cmuA*, *cmuB* as well as the *metF*, *folD* and *purU* genes. Mutational inactivation of the *metF* and *purU* genes resulted in CM4 strains deficient in growth with chloromethane and thereby provided additional evidence for the involvement of the corresponding gene products in a specific catabolic pathway for chloromethane.

## ZUSAMMENFASSUNG

Emissionen der Monohalomethane $CH_3Cl$ and $CH_3Br$ stammen sowohl aus natürlichen wie auch aus anthropogenen Quellen. In der Atmosphäre liefern diese Gase einen signifikanten Beitrag zum Abbau der Ozonschicht. Für das Erstellen einer genauen Bilanz der atmosphärischen Monohalomethane ist es unerlässlich, den Umfang der Quellen und Senken dieser Verbindungen zu bestimmen. Bis heute ist aber erst wenig über den biologischen Abbau von Monohalomethanen bekannt. Der Abbau von Monohalomethanen wurde zwar für einige aerobe und anaerobe Bakterien gezeigt, bis zu Beginn dieser Arbeit fehlten aber detaillierte genetische und biochemische Untersuchungen.

*Methylobacterium chloromethanicum* CM4 ist ein aerobes, methylotrophes $\alpha$-Proteobakterium, welches auf Chlormethan als einziger Kohlenstoff- und Energiequelle wachsen kann. Mittels miniTn*5* Mutagenese wurden Mutanten von Stamm CM4 erhalten, welche immer noch auf Methanol, Methylamin oder Formiat, aber nicht mehr mit Chlormethan wachsen. Dies war auf Transposon-Insertionen in zwei anscheinend ungekoppelte DNA Regionen des CM4 Genoms zurückzuführen. Eine Sequenzanalyse dieser zwei Gencluster führte zur Identifizierung mehrerer Gene, welche für einen mehrstufigen Abbauweg von Chlormethan zu Formiat kodieren.

Mittels Bestimmung von Enzymaktivitäten in zellfreiem Rohextrakt der entsprechenden Mutanten des Stammes CM4 wurden die Gene *cmuA* and *cmuB* als essentiell für die Chlormethan-Dehalogenierung identifiziert. CmuA ist ein bisher einzigartiges Zwei-Domänen-Protein, welches aus einer Methyltransferase und einem Corrinoid-bindenden Teil besteht. Es zeigte sich, dass im Enzym ein nicht kovalent gebundener Vitamin $B_{12}$ Cofaktor sowie ein Zink Molekül enthalten sind. CmuB ist ein homodimeres Protein, welches den Methylgruppentransfer von freiem Methylcobalamin auf Tetrahydrofolat katalysiert. Zusammen sind die gereinigten Proteine CmuA und CmuB ausreichend für die Dehalogenierung von Chlormethan. Der Dehalogenierungmechanismus umfasst zwei aufeinanderfolgende Methyltransferreaktionen und eine intermediäre Bindung der Methylgruppe an

den Vitamin $B_{12}$ Cofaktor. Im aktuellen Modell katalysiert CmuA den ersten Methyltransfer von Chlormethan auf die prosthetische Vitamin $B_{12}$ Gruppe. Danach katalysiert CmuB den zweiten Methylgruppentransfer vom methylierten CmuA Protein auf Tetrahydrofolat.

Die an Tetrahydrofolat gebundene Methylgruppe scheint dann durch eine Reihe von Tetrahydrofolat-abhängigen Enzymen zu Formiat oxidiert zu werden. Basierend auf einer Sequenzanalyse scheinen die drei Gene *metF*, *folD* und *purU* die Enzyme eines solchen $C_1$ Abbauweges zu kodieren.

Experimente mit transkriptionellen Reportergen-Fusionen zeigten eine Chlormethan-abhängige Expression dieser Gene. Mit Hilfe von „Primer Extension"-Analyse wurden drei Promotoren identifiziert, welche während dem Wachstum auf Chlormethan spezifisch aktiviert sind. Die entsprechenden DNA-Sequenzen sind stark konserviert, haben aber keine Ähnlichkeit zu bereits bekannten Promotoren von *Methylobacterium*. Während des Wachstums von *M. chloromethanicum* CM4 auf Chlormethan ist somit die Expression von mindestens drei transkriptionellen Einheiten induziert. Diese Einheiten schliessen die Gene *cmuA*, *cmuB*, sowie *metF*, *folD* und *purU* ein. Die Inaktivierung von *metF* und *purU* durch Mutagenese führte erwartungsgemäss zu CM4 Stämmen mit defizientem Wachstum auf Chlormethan. Dies wurde als weiterer Beweis für die Funktion dieser Genprodukte in einem spezifischen Abbauweg für Chlormethan gewertet.

# Chapter 1

## General Introduction

Scientific interest in the degradation of halogenated methanes dates back to the seventies. It was initiated by concerns that emissions of chlorofluorocarbons (CFCs) to the atmosphere might pose environmental problems. It was anticipated that CFCs might make their way up to the stratosphere where halogen atoms released by ultraviolet radiation would then react with the ozone layer [1]. Along with the chloromethanes, the CFCs were the commercially most important classes of halogenated methanes (for an overview see [2]). Due to their low toxicity and inflammability, these inexpensive and chemically inert compounds were ideal for industrial processes. This included usage as liquid phase for large-scale biochemical reactions and as degreasing agents for cleaning equipment. In particular the CFCs have found widespread application, as coolants for refrigerators, later also as propellants for aerosols and in air-conditioning systems. The utilization of CFCs started in the thirties and their production increased massively during the following fifty years. The extensive use and the high volatility of these compounds caused their immoderate entry into the atmosphere. In 1985 Farman and coworkers discovered a significant depletion of the stratospheric ozone over Antarctica and provided the first clear evidence for the previously suspected negative influence of man-made CFCs on the atmosphere [3]. Since then enormous research efforts were undertaken to identify and quantify the sources and sinks of halogenated methanes and to determine their concentration in the atmosphere. Accurate budgeting of these compounds is necessary to understand their impact on the atmosphere, and to monitor a possible recovery of the stratospheric ozone concentration after constraints in their use. In fact, since 1987 the use of CFCs was gradually phased out, but due to their longevity in the atmosphere an improvement of the stratospheric ozone concentration is not expected before 2050 (European Ozone Research Coordination Unit, www.ozone-sec.ch.cam.ac.uk).

Within the scope of finding sinks for CFCs, bacteria were isolated which are able to metabolize halogenated methanes, and numerous studies addressing the enzymatic mechanisms responsible for dehalogenation were conducted (reviewed in [2,4,5]). Nevertheless, until about 5 years ago practically nothing was known about the biochemistry of microbial degradation of the structurally

simplest halogenated methanes, the monohalomethanes. The reason might be that, in contrast to man-made CFCs, emissions of both chloromethane and bromomethane to the atmosphere are for the most part of natural origin (see below). Today it is known that monohalomethanes significantly contribute to the ongoing ozone layer depletion. This implies that halogen based ozone depletion is partly a natural process, which has been brought out of balance by the extensive emissions of anthropogenic halomethanes. The natural origin of monohalomethanes further suggests that bacteria able to degrade theses compounds may be widespread in the environment (see below). These organisms are likely to represent a significant sink for monohalomethanes and might therefore play an important role in preserving the natural atmospheric equilibrium of these compounds. From a molecular point of view, it is also interesting whether enzymatic mechanisms for the degradation of naturally occurring monohalomethanes are evolutionary related to mechanisms involved in degradation of xenobiotic halomethanes. A biochemical characterization of enzymatic dehalogenation reactions in such organisms is thus both of environmental relevance and of evolutionary interest. In addition, the isolation of the genes involved in chloromethane-utilization would provide genetic markers for evaluating the environmental distribution of monohalomethane-utilizing organisms.

## 1.1 THE GLOBAL CHLOROMETHANE BUDGET

Chloromethane is a gas at ambient temperature (bp - 24°C) and it represents the most abundant source of chlorine in the atmosphere [6]. Over the past 16 years measurements of atmospheric chloromethane concentrations have been performed at many locations. The data suggest an average tropospheric concentration of 606 pptv (parts per $10^{12}$/vol). Therefore the total atmospheric burden of chloromethane is estimated to be around 5.3 million tons (Fig. 1.1, [6]). The dominant process for removal of chloromethane from the atmosphere is the reaction with tropospheric hydroxyl radicals. From the known rate of this reaction, an average global lifetime for chloromethane of about 1.3 years was estimated [7]. By dividing the total amount of chloromethane by the mean of its

lifetime in the atmosphere, the abiotic chloromethane degradation rate caused by hydroxyl radicals was calculated to be around 3.41 million tons per year. Only a relatively small part of the tropospheric chloromethane is transported to the stratosphere at an estimated rate of 280 thousand tons per year, where it is subsequently destroyed photolytically (Fig. 1.1, [7]). The chloride released to the stratosphere by this process is responsible for about 13% of the ongoing ozone depletion [8]. Only the man-made chlorofluorocarbons $CFCl_3$ (CFC-11, 18%) and $CF_2Cl_2$ (CFC-12, 23%) show greater individual chlorine-catalyzed ozone-depleting effects. Taking known atmospheric sinks into consideration, a total annual flux of 3.7 million tons of chloromethane per year is required to explain the observed constant atmospheric concentration of chloromethane [6,7].

Emissions of chloromethane mostly originate from natural sources in both marine and terrestrial environments (Fig. 1.1, [9]). The major abiotic source of chloromethane is biomass burning (900 thousand tons per year), caused by natural forest fires and slash-and-burn agriculture in the tropics [10]. Only a minor portion of chloromethane emissions originates from other burning processes, such as coal combustion and incineration of waste materials [11]. Industrially produced chloromethane is predominantly used as a methylating agent. However, only a minor fraction of the 600 thousand tons produced annually is released to the atmosphere (Fig. 1.1, [11]). The oceans with 650 thousand tons per year are another important source of chloromethane. The abiotic, nucleophilic attack of chloride in seawater on naturally produced bromomethane and iodomethane is thought to represent the major oceanic source [12].

A minor portion of the total oceanic chloromethane emissions are of biological origin and were accounted to be produced by marine algae and phytoplankton [13,14]. Another well-characterized biological source of chloromethane are polypore fungi involved in wood rotting. The ability to release chloromethane was found to be widespread in different genera of *Hymenochaetaceae* ([15]) and the magnitude of their contribution to the global annual release was estimated at 160 thousand tons per year. Recently salt

marshes were identified as an important, previously overlooked terrestrial source of both chloromethane and bromomethane [16]. These emissions are thought to originate predominantly from salt tolerant (halophytic) plants. On the basis of *in situ* measurements, a minimum worldwide production of 170 thousand tons of chloromethane per year was estimated to result from this source.

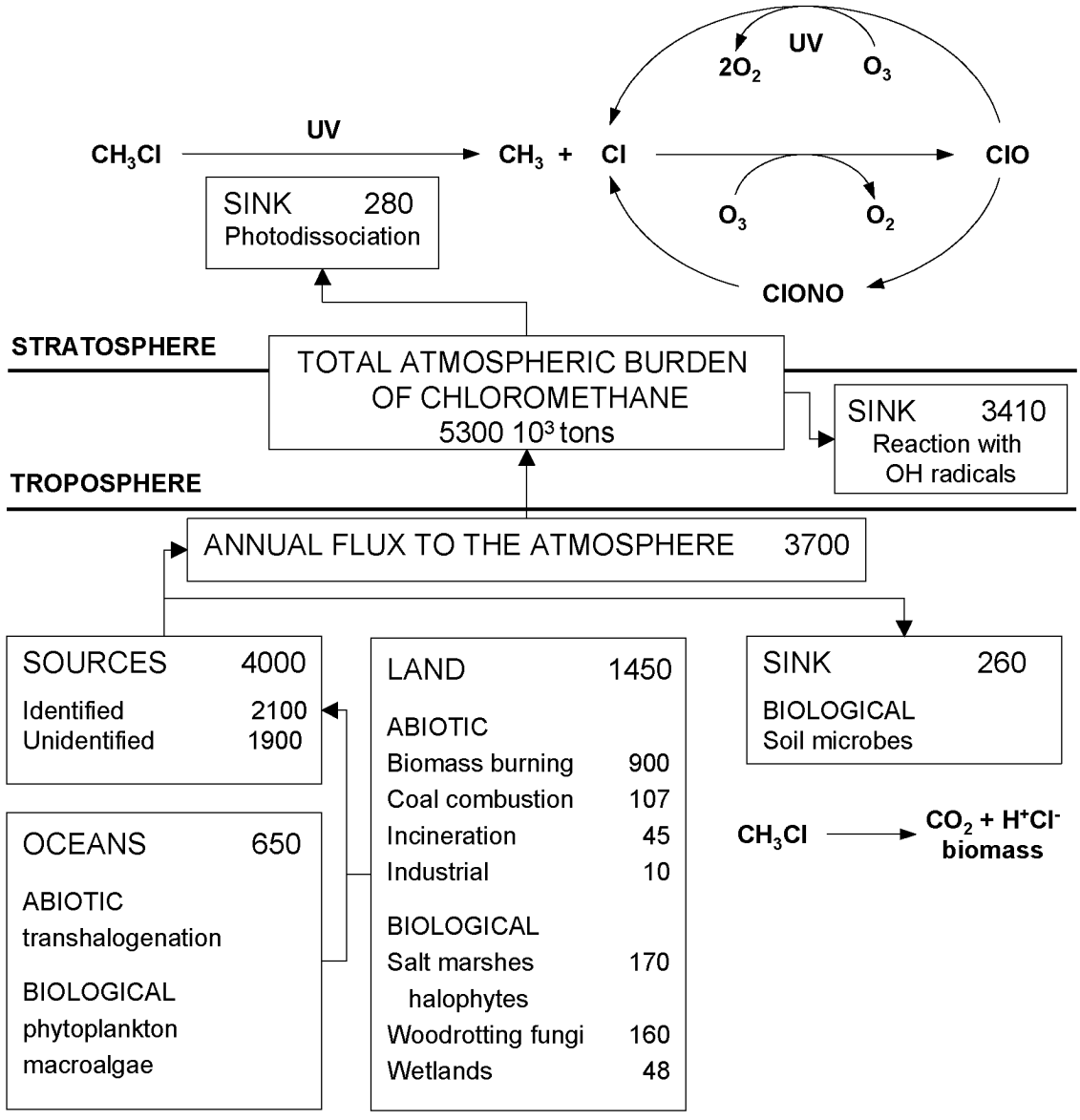


**Fig. 1.1. Schematic overview of global chloromethane fluxes.** Data were taken from [6,9,12]. With the exception of the total atmospheric burden of chloromethane, units are thousand tons per year.

Enzymatic mechanisms of halomethane formation seem to be conserved among chloromethane-emitting organisms. An enzyme able of halomethane production was first isolated from the red alga *Endocladia muricata* [13] and characterized as a S-adenosylmethionine/halide ion methyltransferase. This activity was also found in the wood-rotting fungus *Phellinus pomaceous* [17] and in several halophytic plants [13,18,19], The main function of such enzymes is thought to be the biosynthesis of secondary metabolites in fungi or in the regulation of intracellular chloride concentration in halophytes [9,18].

In addition, bromomethane is the single largest carrier of bromide to the atmosphere, where it is degraded by the same mechanisms as chloromethane. The total annual flux to the atmosphere is estimated to be around 185 thousand tons, where it accounts for about 15% of the stratospheric ozone depletion [8]. Due to its high reactivity bromine is about 50-60 fold more effective in ozone depletion than chlorine. Thus, the ozone depletion caused by bromomethane is in the same order of magnitude as that caused by chloromethane. The identified sources are essentially the same as for chloromethane. Natural sources, such as the oceans (60 thousand tons per year), biomass burning (20 thousand tons per year) and salt marshes (14 thousand tons per year, [9]) appear to dominate. However, there is also a sizeable anthropogenic flux of circa 47 thousand tons per year to the atmosphere through its use as a fumigant [20].

In contrast to the various sources presently known, sinks for halomethanes remain poorly characterized. Soils are the only significant terrestrial sink for chloromethane and bromomethane identified so far [6,21]. On the basis of soil-atmosphere exchange measurements, a total global uptake of chloromethane in the order of 260 to 500 thousand tons per year was estimated [6,7,9]. These numbers must be regarded as highly speculative until measurements from a broader selection of soil, from a larger variety of climatic zones are available.

In general, the currently identified sources of chloromethane account for only about half of the estimated annual flux to the atmosphere (Fig. 1.1). This indicates a deficiency in our current understanding of the global chloromethane cycle. There are three possible explanations that may cause this deficiency, (i)

the production from the known sources is underestimated, (ii) some major sources have not yet been identified or (iii) the atmospheric sinks for chloromethane have been greatly overestimated.

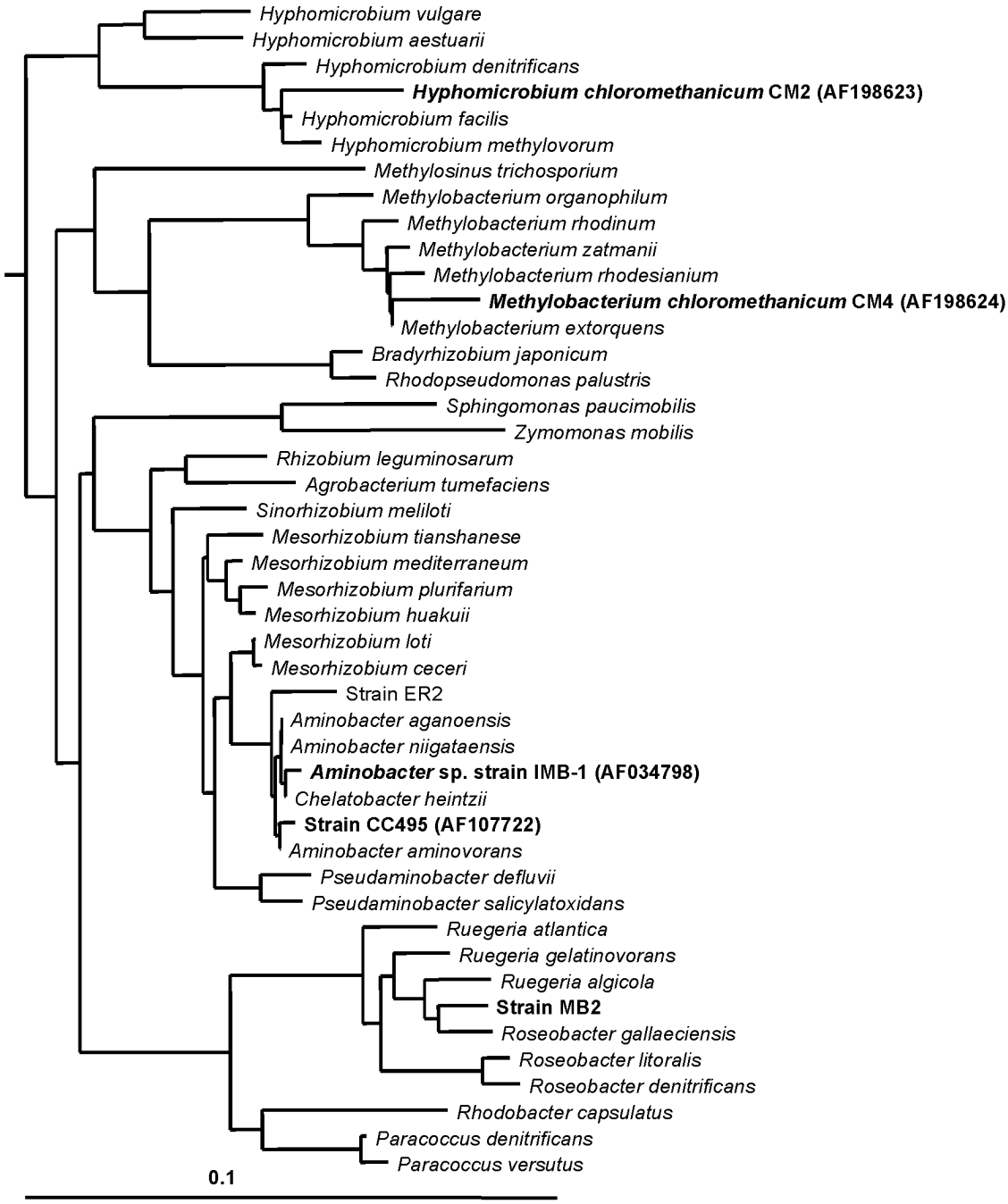


**Fig. 1.2. Phylogenetic analysis of known chloromethane degrading bacteria based on 16S rDNA analysis.** Data taken from [22]. Chloromethane-degrading strains are indicated in bold. The dendrogram was obtained using DNADIST analysis [23]. The bar insert represents 10% sequence divergence.

## 1.2 MICROBIAL DEGRADATION OF CHLOROMETHANE

In the last 5 years it became evident that bacteria are the agents responsible for the substantial degradation of monohalomethanes in soils [24,25]. In fact it has already been known for some years that a variety of microorganisms can oxidize chloromethane to formaldehyde and inorganic chloride. Until recently, however, all strains with this property were unable to use the compound as a growth substrate. This cometabolic degradation of chloromethane was demonstrated for nitrifying bacteria [26] and methanotrophs [27], and was attributed to the action of ammonium– or methane monooxygenase, respectively. It is questionable that these bacteria represent a substantial sink for chloromethane in the environment since the enzymatic activities obtained under laboratory conditions are likely to be insignificant under natural conditions. The subsequent isolation of bacteria capable of growth with chloromethane as the sole energy and carbon source thus represented a major breakthrough. Several isolates have now been characterized. They comprise a strictly anaerobic homoacetogen, *Acetobacterium dehalogenans* (formerly named strain MC, [28]), and several isolates of strictly aerobic, methylotrophic $\alpha$-proteobacteria (Fig. 1.2, [29-32]). *Hyphomicrobium* sp. strain MC1, isolated from a sewage plant in Switzerland in 1986, was the first bacterium, for which growth with chloromethane was described [32]. Unfortunately this strain is no longer available. Five other *Hyphomicrobium* and three *Methylobacterium* strains were isolated from soil samples taken from a petrochemical factory in Russia [30]. Analysis of these strains by 16S rDNA sequencing showed that these represented only two distinct species, subsequently named *Hyphomicrobium chloromethanicum* CM2 and *Methylobacterium chloromethanicum* CM4 [33]. Recently, *Aminobacter* sp. strain IMB-1, isolated from soil fumigated with bromomethane [31], was shown to grow either with chloromethane or bromomethane. In contrast to these strains, which were all isolated from polluted sites or from sewage effluent, two strains were recently isolated from a pristine environment. Strain CC495 was obtained from the litter layer of woodland soil [29] and strain MB2, which does not stem from soil but from seawater [34]. The latter strain grows with bromomethane and has so far

not been tested for growth with other halomethanes, but it seems reasonable to assume that it is also capable of growth with chloromethane. The diverse origin of chloromethane-degrading bacteria provides a first indication that such organisms may be widespread in the environment and thus represent a significant sink for monohalomethanes.

## 1.3 BIOCHEMISTRY OF METHYLOTROPHIC METABOLISM IN *METHYLOBACTERIUM*

Microorganisms capable of growth with chloromethane are per definition methylotrophic. Methylotrophy is defined as the ability to grow with compounds more reduced than $CO_2$ that lack any carbon-carbon bonds. Methylotrophic microorganisms do not represent a distinct taxonomic group but include both aerobic and anaerobic bacteria, archaea and yeasts.

Among methylotrophic bacteria, *Methylobacterium* represents a discreet genus of pink-pigmented, strictly aerobic facultative methylotrophs. All strains characterized so far grow with a limited range of substrates with carbon-carbon bonds and with several $C_1$ compounds, but not with methane (Fig. 1.3, [35]). Degradation of methanol has been investigated most extensively. Its oxidation is catalyzed by the periplasmatic pyrrolo-quinoline quinone (PQQ)-linked enzyme methanol dehydrogenase, whose genes have been cloned from *M. extorquens* AM1 and from a variety of other methylotrophs. Genetic analysis suggested that at least 24 genes were involved in the oxidation of methanol to formaldehyde in *M. extorquens* AM1. These include structural genes, regulatory genes and genes for the synthesis of PQQ [36]. Methylamine is also metabolized by a dehydrogenase, that is functionally analogous to methanol dehydrogenase, but involves a covalently bound tryptophan tryptophylquinone prosthetic group instead of PQQ. Genes encoding the enzyme and proteins involved in cofactor biosynthesis have also been described in *M. extorquens* AM1 [36].

In addition to chloromethane, dichloromethane is the only halogenated $C_1$ compound used as energy and carbon source by aerobic methylotrophs. The dehalogenase enzyme from *Methylobacterium dichloromethanicum* DM4 was

demonstrated to transform dichloromethane in a glutathione-dependent reaction into formaldehyde and inorganic chloride [37].

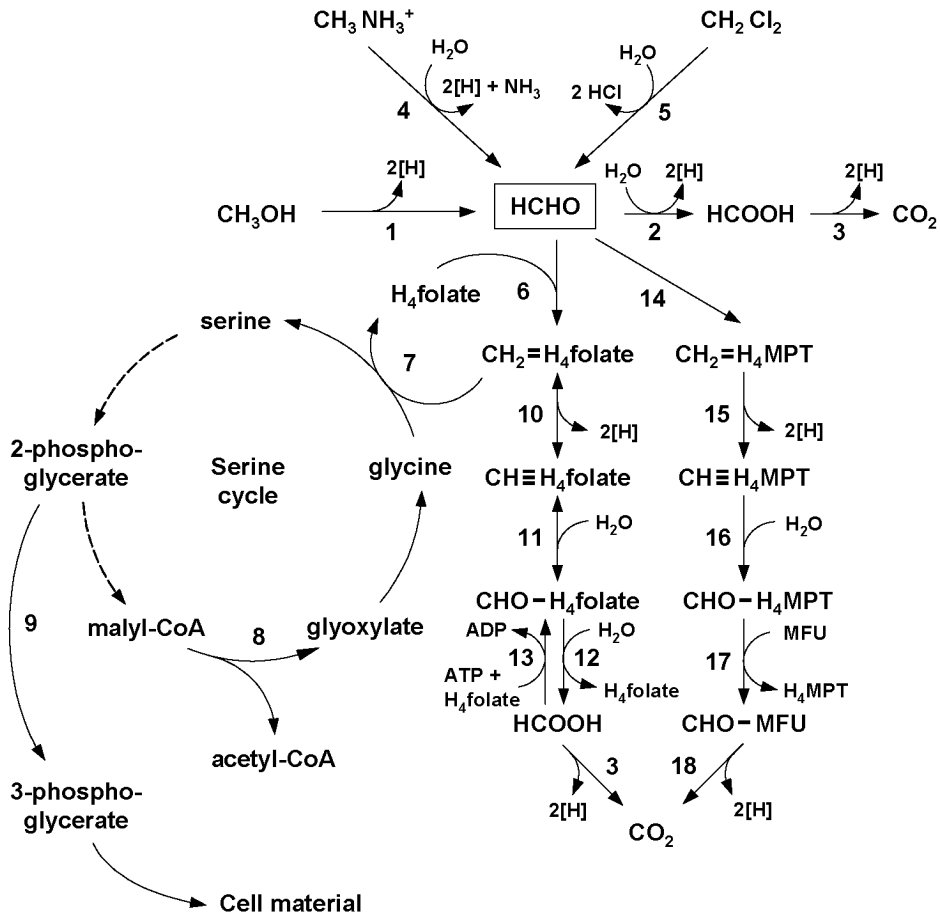


**Fig. 1.3. Metabolism of $C_1$ compounds in *Methylobacterium*.** 1, methanol dehydrogenase; 2, formaldehyde dehydrogenase; 3 formate dehydrogenase; 4, methylated amine dehydrogenase; 5, dichloromethane dehalogenase; 6, spontaneous methylene-$H_4$folate formation; 7, serine hydroxymethyltransferase; 8, malyl-CoA lyase; 9, phosphoglycerate mutase; 10, NADP-dependent methylene-$H_4$folate dehydrogenase MtdA; 11, methenyl-$H_4$folate cyclohydrolase FchA; 12, formyl-$H_4$folate synthase; 13, 10-formyl-$H_4$folate synthase; 14 formaldehyde activating enzyme Fae; 15, NAD(P)-dependent methylene-$H_4$MPT dehydrogenases MtdA and MtdB; 16, methenyl-$H_4$MPT cyclohydrolase Mch; 17 formyl-methanofuran: $H_4$MPT-formyltransferase; 18, formyl-methanofuran dehydrogenase. Dashed arrows indicate conversions involving several enzymatic steps.

In summary, it is important to stress that all $C_1$ compounds mentioned above are metabolized via formaldehyde, which is situated at a metabolic crossroad in *Methylobacterium* (Fig. 1.3). Part of the formaldehyde is assimilated into biomass and the remainder is oxidized to carbon dioxide for the gain of energy for growth. This implies that the relative flux of formaldehyde between the assimilatory and dissimilatory pathways has to be tightly regulated and depends on the growth conditions. In this context it is also important to mention that formaldehyde is toxic for bacteria and growth of *Methylobacterium* on formaldehyde is only possible at concentrations below 1 mM ([38], Vuilleumier and Kayser unpublished). Therefore, during growth with $C_1$ compounds, cells must have mechanisms to cope with the increased formation of formaldehyde. The accumulation of formaldehyde is avoided by its efficient enzymatic oxidation to carbon dioxide. So far four possible enzymatic pathways for formaldehyde degradation have been described in methylotrophic bacteria.

(i) A linear pathway involves the sequential action of formaldehyde dehydrogenase and formate dehydrogenase. Two different types of formaldehyde dehydrogenases have been described, a glutathione-dependent enzyme [39,40] and a mycothiol-dependent (previously called NAD-factor linked) enzyme [41]. These enzymes have not been reported for *Methylobacterium*. Both methanol and aldehyde dehydrogenases, are known to react non-specifically with formaldehyde and the corresponding activities were detected in cell-free extracts of *Methylobacterium*. However, the physiological relevance of these reactions *in vivo* is uncertain [42].

(ii) The oxidation and assimilation of formaldehyde via the ribulose monophosphate cycle has been observed in a variety of methylotrophs [42]. This pathway is not present in *Methylobacterium*, which uses the serine cycle for $C_1$ assimilation. The serine cycle starts with the formation of serine from glycine, a reaction catalyzed by serine hydroxymethyltransferase (Fig. 1.3, [42]). The methylene-$H_4$folate required for this reaction is formed from formaldehyde and $H_4$folate, in a reaction which is thought to occur abiotically (see [38]).

(iii) Methylene-$H_4$folate is also the starting point of a $H_4$folate-dependent formaldehyde oxidation pathway in *M. extorquens* AM1 [43]. The pathway involves two enzymes, a methylene-$H_4$folate dehydrogenase MtdA [44] and a methenyl-$H_4$folate cyclohydrolase FchA [45], both so far only described in *M. extorquens* AM1. The next step in this pathway is presumably catalyzed by a formyl $H_4$folate hydrolase, but such an enzyme has not yet been characterized in *Methylobacterium*. The formate formed by such an enzyme is then oxidized to carbon dioxide by formate dehydrogenase. A search of the *M. extorquens* AM1 genome database (Chistoserdova, pers. comm.) revealed the presence of genes potentially encoding a 10-formyl-$H_4$folate synthase and at least three putative formate dehydrogenases.

(iv) A tetrahydromethanopterin ($H_4$MPT)-dependent oxidation pathway for formaldehyde and the presence of the cofactor dephospho-$H_4$MPT were recently demonstrated in *M. extorquens* AM1 [43]. The occurrence of $H_4$MPT was previously thought to be restricted to methanogenic and sulfate-reducing archaea. A survey among 13 genera of methylotrophic proteobacteria suggested that this pathway is indeed present in many methylotrophic bacteria [46]. Based on enzyme studies and on thermodynamic considerations, the $H_4$MPT-dependent oxidation pathway is proposed to be the main pathway for energy production in *M. extorquens* AM1, whereas the $H_4$folate-dependent pathway is thought to provide one-carbon moieties for biosynthesis [43,47].

Three enzymes participating in the $H_4$MPT $C_1$ oxidation pathway in *M. extorquens* AM1 have already been biochemically characterized. In contrast to methylene-$H_4$folate, methylene-$H_4$MPT is enzymatically formed. This reaction is catalyzed by the formaldehyde-activating enzyme Fae [47]. The next step in this pathway is the transformation of methylene-$H_4$MPT to methenyl-$H_4$MPT, which is catalyzed by the MtdA paralog MtdB. MtdB does not react with methylene-$H_4$folate, whereas MtdA can use both methylene-pterins as substrates [46]. The third step in the pathway is catalyzed by methenyl-$H_4$MPT cyclohydrolase Mch, which also specifically uses $H_4$MPT as a cofactor [45]. The following steps in the pathway are presumably catalyzed by formyl methanofuran:$H_4$MPT formyltransferase and by formyl methanofuran dehydrogenase. The genes

putatively encoding these enzymes have been identified in regions of the *M. extorquens* AM1 genome closely associated with other genes of $C_1$ metabolism. However, neither of the enzymes has yet been characterized, nor has the presence of the cofactor methanofuran in *M. extorquens* AM1 been confirmed.

## 1.4 CHLOROMETHANE DEGRADATION IN *M. CHLOROMETHANICUM* CM4

*M. chloromethanicum* CM4 is an ideal candidate for a biochemical and genetic analysis of chloromethane metabolism. Its 16S rDNA sequence is 98% identical to that of *M. extorquens* AM1 [33] and the central $C_1$ metabolism is therefore expected to be similar in the two organisms (see 1.3). The results of an initial physiological characterization of *M. chloromethanicum* CM4 and other chloromethane-degraders are shown in Table 1.1. The presence of serine hydroxymethyltransferase, hydroxypyruvate reductase and malyl-CoA lyase activities in cell-free extracts suggested that *M. chloromethanicum* CM4 assimilates $C_1$ compounds via the serine cycle [30]. This is the typical assimilation pathway found in all *Methylobacterium* strains described up to now [42].

First insights into the chloromethane metabolism in strain CM4 were obtained from studies with whole cells and from the analysis of chloromethane non-utilizing mutants of the organisms, summarized in Vannelli et al. 1998 [48]. This work served as a starting point for the studies in the present thesis and will therefore be described in some detail in the following section.

Measurements of oxygen uptake and chloride release by resting cells suggested that 1 mol chloromethane is oxidized to 1 mol carbon dioxide, producing 1 mol of hydrochloric acid and consuming 1.5 mol of oxygen. The chloromethane degrading activity appeared inducible, since it was not detected in methanol-grown cells. Chloromethane-grown cells were also capable of dehalogenating bromomethane and iodomethane, but not dichloromethane or

*n*-haloalkanes. This suggested that the enzyme(s) involved in dehalogenation are specific for monohalomethanes.

**Table 1.1.** Major characteristics of chloromethane utilizing proteobacteria. Data taken from [29-31,48]

| Characteristics | ***Hyphomicrobium chloromethanicum* CM2** | ***Methylobacterium chloromethanicum* CM4** | ***Aminobacter* sp. IMB-1** | **Strain CC495** |
|---|---|---|---|---|
| Gram stain | - | - | - | - |
| Pigmentation | - | + | - | - |
| Requirement for $O_2$ | + | + | + | + |
| Optimum growth temperature (°C) | 28-30 | 30 | 22 | 25 |
| Vitamin $B_{12}$ [b] requirement | - | - | - | for growth on CH3Cl |
| $C_1$ substrates | $CH_3Cl$<br>$CH_3OH$<br>$CH_3NH_3^+$ | $CH_3Cl$<br>$CH_3OH$<br>$CH_3NH_3^+$<br>CHOOH | $CH_3Cl$<br>$CH_3Br$<br>$CH_3I$<br>$CH_3NH_3^+$ | $CH_3Cl$<br>$CH_3Br$<br>$CH_3NH_3^+$ |
| Multi carbon substrates | Ethanol | Succinate<br>Fumarate | Acetate<br>Pyruvate<br>Glucose | Pyruvate<br>Glycerol<br>Glucose |
| $C_1$ assimilation | Serine pathway ($icl^+$) | Serine pathway ($icl^-$) | ND [a] | Serine pathway |
| $NH_4$ assimilation | Glutamate cycle | Glutamate cycle | ND [a] | ND [a] |
| G+C mol % of DNA | 60.0 | 64.4 | ND [a] | ND [a] |
| **DNA-DNA hybridization (%) with** | | | | |
| *M. extorquens* | 4.0 | 68.5 | ND [a] | ND [a] |
| *H. zavarzinii* | 29.4 | 5.2 | ND [a] | ND [a] |

[a] ND = not determined

[b] discussed in Chapter 4

Three possible mechanisms for the dehalogenation of chloromethane in strain CM4 were considered, which would all lead to the formation of formaldehyde, the central intermediate in $C_1$ metabolism of *Methylobacterium* (Fig. 1.4). (i) Monooxygenation of chloromethane would result in the formation of an unstable chlorohydrin compound, from which formaldehyde would then be spontaneously formed by abiotic elimination of chloride. This mechanism was previously proposed for chloromethane utilization in *Hyphomicrobium* strain MC1 [32]. (ii) Substitutive, hydrolytic dehalogenation would yield methanol and hydrochloric acid without a requirement of molecular oxygen. Such a mechanism was demonstrated for the haloalkane dehalogenase DhlA of *Xanthobacter autotrophicus* which grows on 1,2-dichloroethane as the sole carbon source (reviewed by [49]).

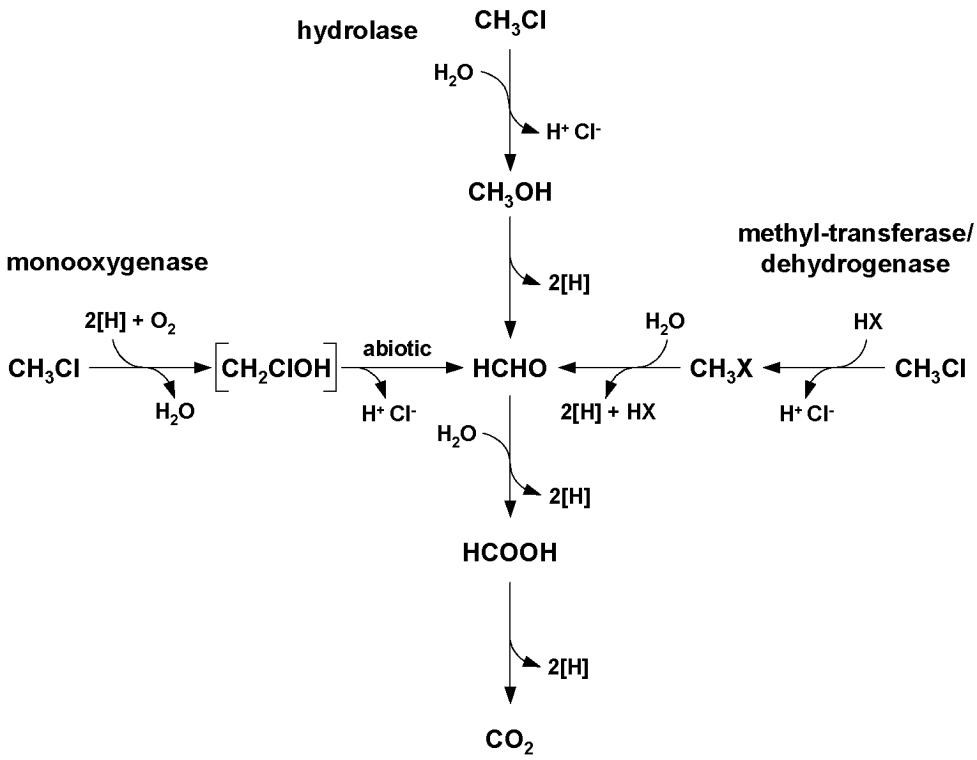


**Fig. 1.4. Possible mechanisms for the metabolism of chloromethane by methylotrophs.** Each of the pathways follows the stoichiometry of $CH_3Cl + 3/2\ O_2 \rightarrow CO_2 + H_2O + HCl$ (adapted from [48]).

(iii) In a putative methyltransferase/dehydrogenase mechanism, formaldehyde would be produced without the formation of methanol as an

intermediate. The nature of the methyl group acceptor involved in the first step of this mechanism could be either a free cofactor, such as glutathione, found as a cofactor in dichloromethane dehalogenation of *Methylobacterium dichloromethanicum* DM4 [37], or a catalytic thiol group of the protein itself.

The methyltransferase/dehydrogenase mechanism was considered to be most likely based on the following experimental evidence. (i) Growth yields strongly argued against a monooxygenase driven reaction. The growth yield of *M. chloromethanicum* CM4 with chloromethane (2.8 ± 0.1 g of protein per mol of C) was within the same range as with methanol (2.9 ± 0.1 of protein per mol of C). In contrast, with formate (0.94 ± 0.2 g of protein per mol of C) the growth yield amounted to only a third of that obtained for methanol. This is in agreement with the two electron equivalents produced from the oxidation of formate compared to the six electron equivalents gained from the oxidation of methanol (Fig. 1.4). A monooxygenase driven dehalogenation reaction would consume two reducing equivalents for the activation of chloromethane, a net yield of only two electron equivalents would be expected. In this case the growth yield with chloromethane would be expected to be of the same order of magnitude as with formate. (ii) In a hydrolytic pathway chloromethane is transformed to methanol in an oxygen-independent manner. However, resting cell assays demonstrated that in absence of oxygen no chloride is released from chloromethane, which was a first indication that a hydrolytic mechanism appeared rather unlikely. (iii) Convincing evidence against a hydrolytic mechanism came from transposon mutagenesis of *M. chloromethanicum* CM4. 4032 exconjugants were isolated and subsequently tested for growth on various $C_1$ compounds. Among these, 53 did not grow on methanol, 7 did not grow on methylamine and 2 were unable to grow on formate. 11 exconjugants could not grow on either methanol or methylamine, which probably accounts for mutations in genes encoding enzymes essential for growth on both substrates. However, most interesting in course of this study were 9 mutants which were unable to grow with chloromethane, but grew normally on all the other $C_1$ substrates tested. The fact that no mutants were isolated which could not grow on methanol and chloromethane, provided further evidence against a hydrolytic

pathway, since it indicated that methanol is not an intermediate in the metabolism of chloromethane by *M. chloromethanicum* CM4.

The nine chloromethane-utilization (cmu) mutants were subsequently divided into two phenotypically distinct groups. All mutants were unable to use chloromethane as a growth substrate. However, whereas five mutants released chloride in the presence of chloromethane, the other four mutants had lost this ability (Table 1.2). This observation suggested that the latter mutants are deficient in the dehalogenation step, whereas the other five mutants were deficient in unknown reactions required for growth with chloromethane. Analysis of chloromethane-induced proteins in mutant and wild type CM4 strains led to the identification of two proteins with estimated sizes of 35 kDa and 67 kDa. Both proteins were absent in methanol-grown cells and were also missing in some of the mutants grown with a methanol/chloromethane mixture. Southern blot analysis with probes against the transposon sequence further suggested that several different loci in the chloromethane-utilizing mutants were affected by transposon insertion (Table 1.2).

In summary, studies with cell-suspension of CM4 and transposon mutagenesis provided first evidence for a specific multistep pathway for the degradation of chloromethane in *M. chloromethanicum* CM4, which apparently involves a dehalogenation mechanism so far not described for aerobic methylotrophic bacteria.

**Table 1.2.** Phenotypes of *Methylobacterium chloromethanicum* CM4 chloromethane utilization mutants (adapted from [48])

| | | Production of chloride: | | Presence of chloromethane-induced proteins on SDS-PAGE [c]: | | DNA restriction fragments (kb) [d]: | |
|---|---|---|---|---|---|---|---|
| **Strain** | **Growth substrate [a]** | **During growth** | **In resting cells (µmol/min/mg of protein) [b]** | **67 kDa** | **35 kDa** | ***Cla*I** | ***Kpn*I** |
| wild type | CM | + | 1.16 | + | + | – | – |
| wild type | MOH | – | <0.05 | – | – | – | – |
| wild type | MOH-CM | + | 0.29 | + | + | – | – |
| **Group 1 mutants** | | | | | | | |
| 19D10 | MOH-CM | – | <0.05 | + | + | 3.9 | 7.8 |
| 22B3 | MOH-CM | – | <0.05 | – | + | 8.2 | 7.1 |
| 38A10 | MOH-CM | – | <0.05 | – | + | 8.2 | 7.1 |
| 27C10 | MOH-CM | – | <0.05 | – | + | 12.4 | 11.7 |
| **Group 2 mutants** | | | | | | | |
| 36D3 | MOH-CM | + | 0.86 | + | + | 4.2 | 7.8 |
| 30F5 | MOH-CM | + | 0.32 | + | – | 8.2 | 6.9 |
| 38G12 | MOH-CM | + | 0.37 | + | – | 8.2 | 6.9 |
| 27B11 | MOH-CM | + | 0.67 | + | + | 12.4 | 12.4 |
| 11G7 | MOH-CM | + | 0.28 | + | + | 9.9 | 15.7 |

[a] None of the mutants grew with chloromethane (CM) as the sole growth substrate. MOH, methanol.

[b] Average of triplicate runs.

[c] +, detected in MOH-CM-grown cells; –, not detectable.

[d] Using a miniTn*5*Km specific probe for detection by Southern blot hybridization.

## 1.5 OUTLINE OF THE PH.D. THESIS

In the present thesis the aerobic degradation of chloromethane was addressed at the molecular level. The previously isolated mutants of *M. chloromethanicum* CM4 unable to grow with chloromethane provided a suitable basis to investigate the biochemical mechanisms which allow this strain to grow on that compound. The main focus of the present study was to unravel the enzymatic mechanisms and to identify the essential cofactors of chloromethane dehalogenation. Chapter 2 describes the isolation of two genetic loci in *M. chloromethanicum* CM4 involved in chloromethane utilization. Sequence analysis and enzymatic measurements in cell-free extracts suggested a multistep pathway for the oxidation of chloromethane to formate. Chapters 3 and 4 are dedicated to the first step in this pathway and describe the purification and characterization of the two proteins essential for the dehalogenation reaction. The reaction involves a vitamin $B_{12}$-mediated methyl transfer from chloromethane to $H_4$folate with concomitant release of chloride. Chapter 5 summarizes expression studies and transcriptional analyses performed in *M. chloromethanicum* CM4. The data demonstrate the presence of further chloromethane-induced genes besides the genes required for dehalogenation. These experiments, along with mutational inactivation of some of the identified genes, point to a specific tetrahydrofolate-dependent $C_1$ oxidation pathway in *M. chloromethanicum* CM4.

# Chapter 2

## A corrinoid-dependent catabolic pathway for growth of a *Methylobacterium* strain with chloromethane

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## 2.1 ABSTRACT

*Methylobacterium* sp. strain CM4, an aerobic methylotrophic α-proteobacterium, is able to grow with chloromethane as carbon and energy source. Mutants of this strain that still grew with methanol, methylamine or formate but were unable to grow with chloromethane were previously obtained by miniTn*5* mutagenesis. The transposon insertion sites in six of these mutants mapped to two distinct DNA fragments. The sequences of these fragments, which extended over more than 17 kb, were determined. Sequence analysis, mutant properties, and measurements of enzyme activity in cell-free extracts allowed to define a multistep pathway for the conversion of chloromethane to formate. The methyl group of chloromethane is first transferred by the protein CmuA (cmu: chloromethane utilization) to a corrinoid protein, from where it is transferred to $H_4$folate by CmuB. Both CmuA and CmuB display sequence similarity to methyltransferases of methanogenic archaea. In its C-terminal part, CmuA is also very similar to corrinoid binding proteins, indicating that it is a bifunctional protein consisting of two domains that are expressed as separate polypeptides in methyl transfer systems of methanogens. The methyl group derived from chloromethane is then processed via pterine-linked intermediates to formate by a pathway which appears to be distinct from those already described in *Methylobacterium*. Remarkable features of this novel pathway for the catabolism of chloromethane thus include the involvement of a corrinoid-dependent methyltransferase system for dehalogenation in an aerobe, and a set of enzymes specifically involved in funnelling the $C_1$ moiety derived from chloromethane into central metabolism.

## 2.2 INTRODUCTION

Attention has been focused on chloromethane and bromomethane because of their role as sources of stratospheric chlorine and bromine, the primary agents of ozone destruction. Chloromethane ($CH_3Cl$) is the most abundant halocarbon in the atmosphere and is responsible for 15-20% of chlorine-catalyzed ozone destruction in the stratosphere [50]. It is released at an estimated global rate of 3.5-5 x $10^6$ tons per year, primarily from natural sources, and less than 1% of the global chloromethane flux is due to industrial production of the compound (reviewed in [50]). For bromomethane, current estimates of ocean emission and natural formation during combustion of vegetation fall in the range of 8 x $10^4$ tons per year, slightly more than the amount annually emitted by the use of this compound in soil fumigation [51]. On a molar basis, bromine is 40-100 times more effective than chlorine in depleting ozone [52]. Thus, chloromethane and bromomethane contribute about equally to an estimated 40% of the total global loss of stratospheric ozone.

Green plants [53] and soil bacteria [21,24,25,30,54] represent terrestrial sinks for chloromethane and bromomethane. Evidence for bacterial degradation of halogenated methanes in seawater was also reported [55]. Microbial metabolism of monohalomethanes includes oxidative [56] and hydrolytic [57] cometabolic processes as well as mineralization by methylotrophic bacteria that utilize chloromethane as a growth substrate. The homoacetogenic bacterium *Acetobacterium dehalogenans* [58] is the only known strictly anaerobic representative of the latter group [59]. Anoxic dehalogenation of chloromethane by this organism was shown to be catalyzed by enzymes that transfer the methyl group of chloromethane via a corrinoid protein to $H_4$folate to yield chloride and $CH_3$-$H_4$folate, an intermediate of the acetyl-CoA pathway [60]. In contrast, the reactions by which some recently isolated strictly aerobic methylotrophic bacteria [30] utilize chloromethane as a growth substrate have yet to be elucidated. The physiological properties of the wild type and of chloromethane utilization-negative mutants of a representative strain, *Methylobacterium* sp. CM4, led us to propose that this organism metabolizes chloromethane by initial dehalogenation via a methyl transfer reaction [48].

Here we report on the sequence of two large DNA fragments containing at least four genes essential for chloromethane metabolism in strain CM4. We present experimental evidence that this aerobic bacterium is able to catalyze transfer of the methyl moiety of chloromethane to $H_4$folate via a corrinoid intermediate to yield $CH_3$-$H_4$folate, a key intermediate of methylotrophic metabolism.

## 2.3 EXPERIMENTAL PROCEDURES

### 2.3.1 Materials

Reagents for molecular biology were obtained from Fermentas and Boehringer Mannheim. (6S)-5,6,7,8-tetrahydrofolic acid trihydrochloride ($H_4$folate) and (6S)-5-methyl tetrahydrofolate ($CH_3$-$H_4$folate) were purchased from Dr. Schircks Laboratorium (Jona, Switzerland). ATP, S-adenosyl methionine and methylcobalamin were from Sigma, and NADPH:FMN oxidoreductase was from Boehringer. All other chemicals were reagent grade or better and were purchased from Fluka.

### 2.3.2 Bacterial Strains

Bacterial strains in this study included *Methylobacterium* sp. strain CM4 that grows with chloromethane [30], and miniTn*5* [61] insertion mutants whose phenotypes have been described previously [48]. *E. coli* K12 strain DH5$\alpha$? (GIBCO/BRL-Life Technologies) was used as a host in DNA work.

### 2.3.3 DNA manipulations

Preparation of genomic DNA, restriction enzyme digestions, ligations and transformations were performed using standard procedures [62]. Plasmid pBluescript-KSII(+) (Stratagene) was used for cloning. DNA fragments from mutants of strain CM4 containing a miniTn*5* insertion were cloned by selection of transformants for kanamycin resistance (25 $\mu$g/ml).

### 2.3.4 Sequence analysis

The cloned genomic DNA was sequenced on both strands using PCR methods with fluorescent dideoxynucleotide terminators and an ABI-Prism automatic sequencer (Perkin Elmer). The precise site of insertion of the minitransposon was determined for all mutants. The sequences of cluster I (8863 nt, Acc. No. AJ011316) and cluster II (8457 nt, Acc. No. AJ011317) were assembled from sequence fragments obtained from DNA cloned from the different mutants with the GCG sequence analysis package (Version 8.1, Genetics Computer Group, Madison WI). Similarity searches were performed

using gapped BLAST and PSI-BLAST programs [63] against public protein and gene databases.

### 2.3.5 N-terminal sequencing

The 67 kDa protein induced during growth with chloromethane [48] was partially purified, and the N-terminal sequence of the corresponding protein band on SDS-PAGE was determined by Edman degradation using an Applied Biosystems 476A automatic sequencer.

### 2.3.6 Preparation of cell-free extract

*Methylobacterium* sp. strain CM4 and mutants were grown as described previously [48]. Bacteria were harvested at an $OD_{600}$ of 0.5 to 0.7 (10'000 x *g*, 15 min) and resuspended (1 g wet cells per ml) in 50 mM Tris-$SO_4$ buffer (pH 7.2) containing 5 mM DTT. Cells were disrupted by two passages through a French pressure cell (120 MPa, 4°C), and DNaseI (50 μg/ml final) was added to the suspension, which was centrifuged (17000 rpm, 45 min) to remove cell debris. The resulting supernatant was cleared from membrane components by ultracentrifugation (100'000 x g, 45 min), and the cell-free extract obtained (approx. 15 mg protein/ml) was flash-frozen in liquid nitrogen and stored at –20°C.

### 2.3.7 Activity measurements with crude extracts

Solutions were made anoxic by degassing with $N_2$ plus $H_2$ (95:5 [vol/vol]). Enzyme reactions, manipulations and measurements were performed under the same atmosphere. Assays of enzymatic activity were done at 30°C in a 3 ml volume in 12.4 ml serum flasks with gas-tight rubber stoppers. The $H_4$folate-dependent dehalogenation of chloromethane was measured by following the consumption of chloromethane by gas chromatography [64] using a Henry constant of 0.43 calculated by interpolation to 30°C of the published value for chloromethane [65]. Dehalogenation was also determined by monitoring the chloromethane-dependent formation of $CH_3$-$H_4$folate from $H_4$folate. $CH_3$-$H_4$folate was separated from the incubation mixture by HPLC [59] and detected spectrophotometrically at 320 nm. The incubation mixture contained cell-free

extract (0.8-4 mg/ml protein) in 100 mM Tris-$SO_4$ (pH 7.8) buffer, 5 mM DTT, 2.4 mM $H_4$folate (of which 1 mM was biologically available [66]), and 1 mM titanium(III)citrate. Cell-free extracts dialysed against 100 mM Tris-$SO_4$ (pH 7.8) were also used in order to check whether any endogenous cofactors in the extracts were involved in dehalogenase activity. The dehalogenation reaction was initiated by the addition of 0.5 mM chloromethane gas (based on the liquid phase volume) through the rubber stopper with a gas-tight syringe. For determination of methylcobalamin:$H_4$folate methyltransferase activity, the assay mixture contained 0.5 mM methylcobalamin instead of chloromethane. The numbers reported are from representative individual experiments which were performed at least twice and all yielded very similar results.

### 2.3.8 Determination of protein concentration

Protein was determined by the method of Bradford [67], using a commercial dye reagent (Bio-Rad) with bovine serum albumin as a standard.

## 2.4 RESULTS

### 2.4.1 Identification of genes involved in chloromethane utilization

We previously isolated miniTn*5* transposon insertion mutants of *Methylobacterium* sp. strain CM4 that were unable to grow with chloromethane [48]. Thus, nine *cmu* negative mutants were obtained that were still able to grow with methanol, methylamine, or formate. Conversely, 73 transposon mutants defective in the utilization of methanol, methylamine, methanol plus methylamine, or formate could still grow with chloromethane [48]. This suggested that chloromethane was metabolized in *Methylobacterium* sp. CM4 by reactions different from those involved in the metabolism of methanol and methylamine. The genes whose insertional inactivation caused loss of the ability to grow with chloromethane were isolated by selection of the kanamycin resistance gene present on the minitransposon [61]. The DNA fragments carrying a transposon insertion were sequenced from all *cmu* negative mutants. Transposon insertion mutants, arbitrarily labeled in order of their detection [48], were found in four apparently unlinked DNA regions that were termed cluster I (mutants 30F5, 38G12, 22B3, 38A10), cluster II (mutants 19D10, 36D3), cluster III (mutant 11G7), and cluster IV (mutant 27B11). The *cmu* negative mutant 27C10 was not analyzed in detail since it appeared to carry a partial duplication of the transposon. A schematic representation of the 14 open reading frames identified in the DNA sequences of clusters I and II is shown in Fig. 2.1.

Most of the encoded polypeptides displayed significant sequence identity to proteins of known functions (Table 2.1). With respect to their possible role in metabolism, the proteins encoded in DNA clusters I and II fell into four groups: methyltransferases, pterine-dependent enzymes, proteins associated with cobalamin biosynthesis, and proteins of unknown function. Similarity searches of the protein sequences encoded in clusters III and IV (data not shown) did not provide insights as to their association with chloromethane transformation and are not discussed further here.

**TABLE 1. Genes and open reading frames in DNA regions associated with chloromethane utilization**

| Gene (orf) | Length (aa) | calc. Mr (kDa) | Gene Begin | Gene End | Inferred function | Sequence comparison of representative hit: % protein sequence identity (Acc. No.) [a] | Identity (%) [b] |
|---|---|---|---|---|---|---|---|
| **Cluster I** | | | | | | | |
| *cobU* | 342 | 34.9 | 1502 | 474 | cobalamin biosynthesis | CobU (*P. denitrificans*, P29935) | 57 |
| *folC* | 467 | 49.8 | 2942 | 1539 | folylglutamate synthetase | FolC (*E. coli*, P08192) | 32 |
| *folD* | 306 | 32.4 | 3865 | 2945 | 5,10-methylene-$H_4$folate dehydrogenase/5,10-methenyl-$H_4$folate cyclohydrolase | FolD (*E. coli*, P24186) | 49 |
| *purU* | 287 | 32.7 | 4849 | 3986 | 10-formyl-$H_4$folate hydrolase | PurU (*Corynebacterium* sp., Q46339) | 47 |
| *orf414* | 414 | 43.7 | 5628 | 6872 | unknown | Orf (*Mycobacterium tuberculosis*, P72042) | 31 (143 aa) |
| *cmuA* | 617 | 67.0 | 6897 | 8750 | methyltransferase/ corrinoid protein | MtbA, (*Methanosarcina barkeri*, O30640)/ MtmC (*Methanosarcina barkeri*, O30641) | 24/32 [c] |
| **Cluster II** | | | | | | | |
| *cobQ* | >424 | ND | <1 | 1275 | cobalamin biosynthesis | CobQ (*P. denitrificans*, P29932) | 59 |
| *cobD* | 330 | 34.2 | 2275 | 1283 | cobalamin biosynthesis | CobD (*P. denitrificans*, P21634) | 55 |
| *orf219* | 219 | 24.9 | 3345 | 2686 | unknown | (*Synechocystis* sp., Q55963) | 32 (117 aa) |
| *metF* | 320 | 34.3 | 3507 | 4469 | 5,10-methylene-$H_4$folate reductase | Orf (*Saccharomyces cerevisiae*, P53128) | 24 (156 aa) |
| *cmuB* | 311 | 33.3 | 4703 | 5638 | methyl transfer | MtrH (*M. thermoautotrophicum*, P80187) | 30 |
| *cmuC* | 378 | 41.2 | 5635 | 6771 | methyl transfer | MtaA (*Methanosarcina barkeri*, Q48949) | 28 (104 aa) |
| *orf361* | 361 | 37.5 | 7971 | 6886 | cobalamin biosynthesis | MTH808 (*M. thermoautotrophicum*, O26899) | 35 |
| *cobC* | >162 | ND | >8456 | 7968 | cobalamin biosynthesis | CobC (*P. denitrificans*, P21633) | 38 |

ND, not determined.

[a] Accession numbers from Swissprot or Trembl databases.

[b] Sequence identity is over the entire length of the shorter of the two compared sequences, except where noted.

[c] The MtbA sequence (339 aa) can be aligned to residues 7-353 of CmuA, and the MtmC sequence (217 aa) can be aligned to residuess 401- 607 of CmuA, respectively.

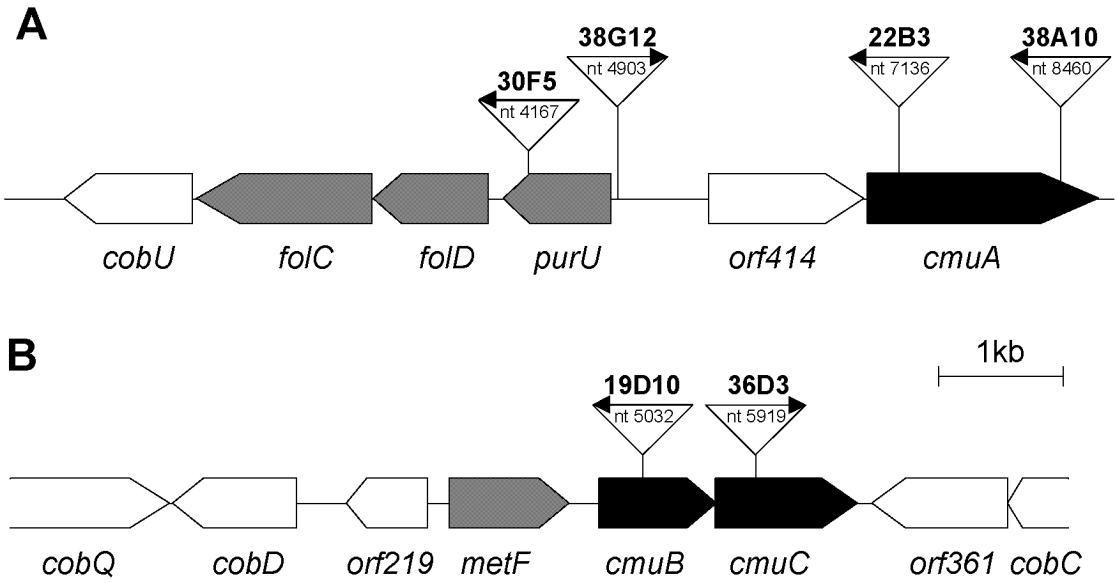


**Fig. 2.1. Schematic view of gene clusters I (A) and II (B) of *Methylobacterium* sp. CM4.** Genes encoding methyltransferases are marked in black, genes encoding putative pterine-dependent enzymes of $C_1$ metabolism are marked in grey, and genes encoding enzymes of cobalamin biosynthesis or proteins with unknown function are marked in white. The position and orientation of the transposon insertions in the genome of *cmu* negative mutants is also shown.

The products of *cmuA*, *cmuB* and *cmuC* in clusters I and II showed sequence similarity to methyltransferases or corrinoid binding proteins from archaea (Table 2.1). The C-terminal part of CmuA was found to be most similar to MtmC, the 29 kDa corrinoid protein which, when methylated by methylamine, acts as the substrate of the methyltransferase catalyzing the methylation of coenzyme M in *Methanosarcina barkeri* [68]. The C-terminal part of CmuA also showed similarity to many other corrinoid-binding proteins, including methionine synthases [69]. The N-terminal part of CmuA showed considerable sequence identity to MtbA, the 36 kDa methyltransferase that transfers the methyl group from MtmC to coenzyme M [68,70]. The similarity in sequence between the two proteins extended over the entire length of MtbA (Table 2.1). It thus appears that CmuA, whose calculated molecular weight is 67 kDa, represents an unprecedented fusion of two proteins that are expressed as separate but closely associated polypeptides in methyl transfer systems of methanogenic archaea. CmuB, the second methyltransferase-like protein suggested from sequence analysis, showed most sequence identity (30%, Table 2.1) with subunit MtrH of the membrane associated $N^5$-methyl-tetrahydromethano-

pterin:co-enzyme M methyltransferase complex from *Methanobacterium thermoautotrophicum* that catalyzes transfer of the methyl group of $N^5$-methyltetrahydromethanopterin to the corrinoid protein MtrA [71]. CmuB, unlike CmuA, also showed low but significant pairwise identity (23%) to the $CH_3$-$H_4$folate binding domain of MetH from *E. coli* (residues 337-648 in the protein sequence).

CmuC, the third methyltransferase-like putative protein, was most similar to MtaA, another corrinoid:coenzyme M methyltransferase characterized in *M. barkeri* [70] (Table 2.1). CmuC was 19% identical to the N-terminal domain of CmuA, and displayed significant but low identity (14%) over its entire length to MtbA and MtaA from *M. barkeri* and to DcuP from *E. coli* in multiple alignments.

The second group of proteins detected in clusters I and II were similar to enzymes involved in interconversion pathways of one-carbon compounds. A cmu phenotype was observed in mutant 30F5, in which the open reading frame encoding a protein with strong similarity to bacterial 10-formyl-$H_4$folate hydrolases (Table 2.1) was disrupted. Accordingly, the gene was named *purU*. Proteins similar to bacterial FolD and FolC (Table 2.1), enzymes involved in the metabolism of one-carbon compounds, are encoded by genes downstream of *purU*. It is noteworthy that cell-free extracts of mutant 38G12, in which the transposon insertion is located upstream of the *purU* gene (Fig. 2.1), lack a protein of about 35 kDa that is induced by chloromethane [48]. This protein could therefore be PurU (32.7 kDa calculated molecular mass) and perhaps also FolD (32.4 kDa; see Table 2.1). Finally, the gene tentatively named *metF* in cluster II codes for a protein similar to enzymes of the 5,10-methylene-$H_4$folate reductase family in part of its sequence. The role in chloromethane degradation of this and other open reading frames detected in the DNA sequence of clusters I and II, however, remains uncertain since no mutants are yet available in which these genes have been knocked out.

### 2.4.2 *In vitro* dehalogenation of chloromethane

Transposon insertions into genes *cmuA* and *cmuB* (Fig. 2.1) led to a dechlorination negative phenotype in the corresponding mutants, that could neither dehalogenate chloromethane nor grow with this compound. The other *cmu* negative mutants released chloride from chloromethane in a resting cell assay [48], but were unable to grow with chloromethane. The dechlorination negative phenotype of *cmuA* and *cmuB* mutants strongly indicated that the proteins encoded by these genes were directly involved in chloromethane dehalogenation.

**Table 2.2. Components required for dehalogenation of chloromethane by cell-free extracts of *Methylobacterium* sp. CM4**

| | | Maximum rate (nmol/min·mg protein) $CH_3$-$H_4$folate formation | |
|---|---|---|---|
| **Growth substrate** | **Assay mixture** [a] | **$CH_3Cl$ consumption** | **$CH_3$-$H_4$folate formation** |
| MeOH | complete | <0.1 | <0.1 |
| $CH_3Cl$ | complete | 3.9 | 3.9 |
| $CH_3Cl$ | without $CH_3Cl$ | - | <0.1 |
| $CH_3Cl$ | without $H_4$folate | 0.3 | <0.1 |
| $CH_3Cl$ | without Ti(III) citrate | 2.6 | 1.7 |

[a] See Materials and Methods

In previous work with strain CM4, chloromethane dehalogenation activity could only be detected in cell suspensions [48]. The inferred function of several open reading frames (Table 2.1) suggested that assay mixtures containing $H_4$folate and chloromethane (Table 2.2) might allow activity measurements in cell-free extracts of *Methylobacterium* sp. CM4, as previously observed in chloromethane dehalogenation in *A. dehalogenans* [59]. Indeed, chloromethane was consumed with the concomitant formation of $CH_3$-$H_4$folate from $H_4$folate by cell-free extracts of the chloromethane-grown wild type strain CM4 (Fig. 2.2) at 0.5% of the *in vivo* chloromethane degradation rate. The data presented in

Table 2.2 demonstrated that the dehalogenation activity was not present in extracts of cells grown with methanol, confirming the previously observed inducibility of chloromethane utilization in strain CM4 [48]. $CH_3$-$H_4$folate-formation was strictly dependent on chloromethane and $H_4$folate. The chloromethane dehalogenase activity converting chloromethane and $H_4$folate to $CH_3$-$H_4$folate was stimulated by the non-physiological reductant titanium(III)citrate (Table 2.2). Most notably, low molecular weight components of known corrinoid protein reactivation systems, such as ATP, as well as GTP, S-adenosyl-methionine, $FMNH_2$ and $FADH_2$ were without effect on the dehalogenase activity of *Methylobacterium* sp. CM4 (data not shown). In contrast, chloromethane dehalogenase activity in cell-free extracts of the strict anaerobe *A. dehalogenans* requires the addition of ATP, presumably in order to maintain the cobalt ion of the corrinoid cofactor in the reduced Co(I) state [59,60,72].

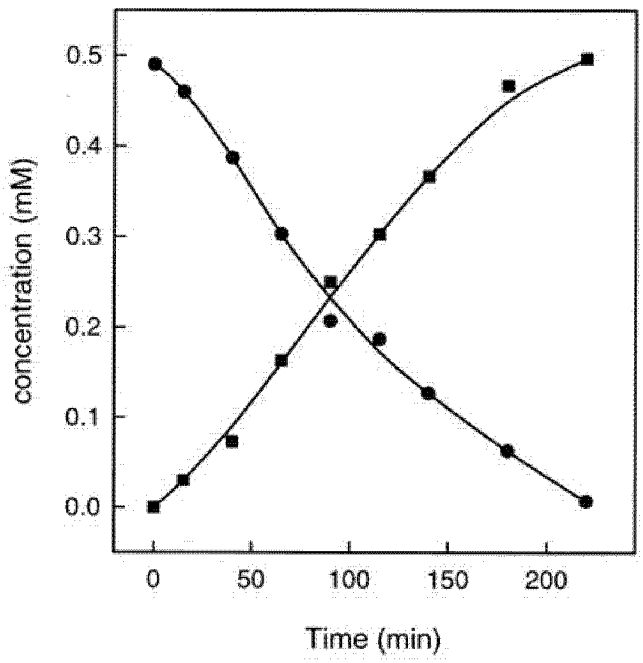


**Fig. 2.2.** Disappearance of chloromethane (circles), and formation of $CH_3$-$H_4$folate from chloromethane and $H_4$folate (squares), by cell-free extracts of chloromethane-grown *Methylobacterium* sp. CM4. Chloromethane was determined by gas chromatography and $CH_3$-$H_4$folate by HPLC (see Materials and Methods). The assay mixture contained 2.4 mg protein, 0.5 mM chloromethane, 1 mM $H_4$folate and 1 mM titanium(III) citrate.

### 2.4.3 Enzyme activities in cell-free extracts of *cmu* negative mutants

Methylcobalamin could replace chloromethane as a methyl donor in the formation of $CH_3$-$H_4$folate from $H_4$folate catalyzed by cell-free extracts of strain CM4 grown with chloromethane (Table 2.3). This suggested that the transformation of chloromethane and $H_4$folate to $CH_3$-$H_4$folate and chloride in strain CM4 resulted from two sequential methyl transfer reactions involving a methylated corrinoid intermediate (Fig. 2.3). Such sequential methyl transfer reactions were previously documented in enzyme systems of methanogens catalyzing the formation of methyl-CoM from coenzyme M and methanol or methylamine [73], and most likely also operate in the chloromethane dehalogenase of *A. dehalogenans* [60]. In these systems, methylcobalamin presumably acts as a surrogate for the physiological, protein-bound methyl-corrinoid. This may explain the about three-fold lower specific activity of the methylcobalamin:$H_4$folate methyltransferase (methyltransferase II) activity, as compared to the chloromethane dehalogenase activity representing the overall rate of the transformation of chloromethane to $CH_3$-$H_4$folate by methyltransferase I and methyltransferase II reactions (Fig. 2.3).

**Table 2.3.** Methyltransferase activities in cell-free extracts of *Methylobacterium* sp. CM4 wild-type and *cmu* negative mutants [a]

| Strain | Gene affected by miniTn*5* insertion | Initial rate of $CH_3$-$H_4$folate formation (nmol/min·mg protein) | |
|---|---|---|---|
| | | from $CH_3Cl$ | from $CH_3B_{12}$ |
| wild type | - | 2.6 [b] | 0.8 [b] |
| 30F5 | *purU* | 1.7 | 0.5 |
| 38G12 | *purU* (upstream) | 2.1 | 0.8 |
| 22B3 | *cmuA* | <0.1 | 1.0 |
| 38A10 | *cmuA* | <0.1 | 0.7 |
| 19D10 | *cmuB* | <0.1 | <0.1 |
| 36D3 | *cmuC* | 2.2 | 0.7 |

[a] grown with 20 mM methanol and 2% vol/vol $CH_3Cl$

[b] The initial rate of $CH_3$-$H_4$folate formation in extracts from wild type bacteria grown with methanol was < 0.1 nmol/min·mg

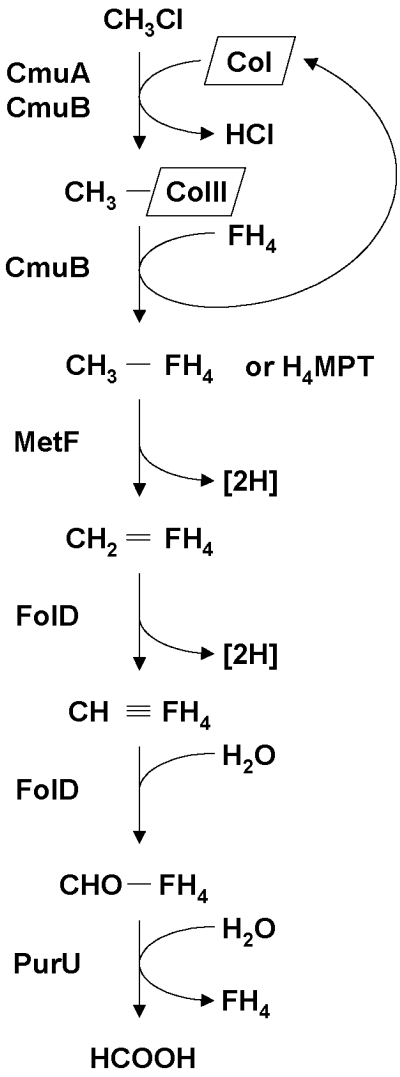


**Fig. 2.3. Proposed pathway for the oxidation of chloromethane to formate based on DNA sequence data and biochemical analysis.** CmuA, methyltransferase I; CmuB, methyltransferase II; MetF, putative 5,10-methylene-$H_4$folate reductase; FolD, putative 5,10-methylene-$H_4$folate dehydrogenase/5,10-methenyl-$H_4$folate cyclohydrolase; PurU; putative 10-formyl-$H_4$folate hydrolase. The corrinoid protein acting as the primary methyl acceptor and thought to be part of CmuA (see text) is indicated by $Co^{I}$-E.

The two mutants 22B3 and 38A10 that carried insertions in *cmuA* were defective in the dehalogenation reaction (proposed to be initiated by methyltransferase I, Fig. 2.3) but still capable to catalyze the methyltransferase II reaction (Table 2.3). This suggested that *cmuA* encoded methyltransferase I but not methyltransferase II. Moreover, the 67 kDa protein previously noted to be induced during growth with chloromethane [48] was shown to be CmuA by determination of its N-terminal sequence (XGKMTSRERMFAXTM), further suggesting an important role of CmuA in chloromethane degradation.

The inactivation of the *cmuB* gene in mutant 19D10 resulted in the loss of both dehalogenase and methyltransferase II activity (Table 2.3). Thus, CmuB appeared to be required for both methyltransferase reactions that lead from chloromethane via a putative methylated corrinoid protein to $CH_3$-$H_4$folate (Table 2.3, Fig. 2.3). Alternatively, the *cmuB* mutant may still be able to perform the initial dehalogenation reaction (catalyzed by methyltransferase I), but not the subsequent transfer of the methyl group from the corrinoid binding protein to $H_4$folate. In this case, methylated corrinoid protein would be produced in amounts stoichiometric to those of methyltransferase I in cell-free extracts, but the dehalogenation reaction would remain undetected because of the low amounts of this protein in the assay.

Mutants of *Methylobacterium* sp. CM4 disrupted in *purU* and in *cmuC* were unable to grow with chloromethane but exhibited wild type levels of both dehalogenase and methyltransferase II activity (Table 2.3). These mutants thus are unaffected in the dehalogenation reaction but are deficient in some later step of chloromethane metabolism.

## 2.5 DISCUSSION

The results presented here lead us to propose the corrinoid-dependent pathway for chloromethane catabolism shown in Fig. 2.3. This pathway implies that the dehalogenation reaction proceeds with the Co(I) of a corrinoid protein acting as primary acceptor for the methyl group of chloromethane. It requires methyltransferase I to form methylated corrinoid protein from chloromethane, and methyltransferase II for the transfer of the methyl group to the pterine cofactor.

The amino acid sequence of CmuA (Table 2.1) supports the view that this protein not only encodes methyltransferase I activity detected in cell-free extracts, but also acts as the corrinoid binding protein indicated in the model (compare Table 2.3). We thus hypothesize that the CmuA protein carrying a methylated corrinoid serves as the methyl-donating substrate for $CH_3$-$H_4$folate formation from $H_4$folate by the methyltransferase II encoded by *cmuB*.

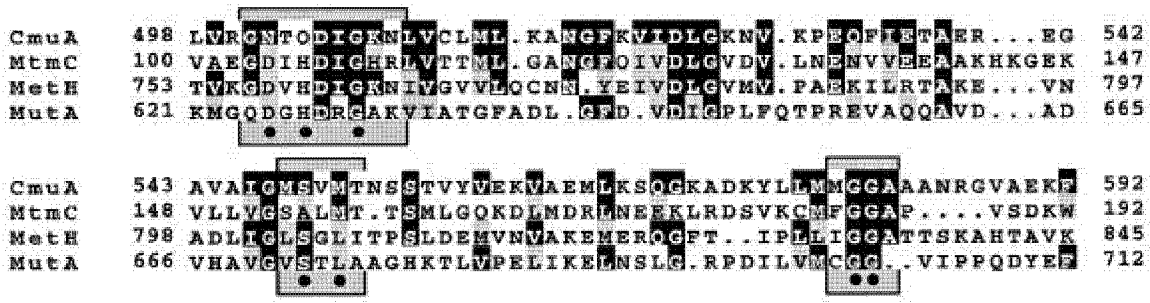

**Fig. 2.4.** Alignment of the sequences from CmuA, MtmC of *M. barkeri* (Swissprot Acc. No. O30641), MetH of *E. coli* (P13009) and MutA, the human methylmalonyl-CoA mutase (P22033), in the conserved C-terminal sequence region of CmuA around the corrinoid binding site. This site was defined from a structure-based sequence fingerprint for cobalamin dependent methionine synthases and mutases [69]. The most conserved residues in these motifs (indicated by grey boxes) are labelled by dots below the alignment. Positions in which amino acids were identical or similar to the CmuA sequence in at least two other sequences were boxed in black or grey, respectively, using the program BOXSHADE.

Sequence alignments of the C-terminal domain of the CmuA protein with that of the corrinoid binding domain of methionine synthase of *E. coli,* whose structure has been solved [74], allows one to further speculate on the properties of the CmuA protein (Fig. 2.4). The most striking feature of the CmuA sequence, when compared to the motifs which were defined for cobalamin dependent methionine synthases [69,74], is residue Gln504, equivalent to

His759 in *E. coli* (Fig. 2.4). The three residues His759, Asp757 and Ser810 in *E. coli* methionine synthase form a ligand catalytic triad with the histidine residue as the lower axial ligand of the "base-off/His-on" corrinoid [69]. His759 in *E. coli* was shown to be essential for enzyme turnover [75]. It is unclear in what way the corresponding glutamine in CmuA can be isofunctional to this residue. No other sequence in the database so far matches the corrinoid-binding motif so closely as CmuA but lacks the histidine residue. A glutamine residue, however, was recently described to be the axial ligand of the nickel porphinoid $F_{430}$ of methyl-coenzyme M reductase of *M. thermoautotrophicum* [76], and the manually aligned sequence of the AcsD corrinoid iron-sulfur protein of *Clostridium thermoaceticum* also features a Gln residue in register with His759 of methionine synthases (S. Ragsdale, personal communication). A glutamine at position 504 in CmuA is expected to contribute much weaker ligation to a corrinoid-bound cobalt than a histidine residue. As a consequence, it is also expected to render the reduction potential of the Co(II)/Co(I) couple less negative and thus to stabilize the corrinoid bound by CmuA in its Co(I) state. A reactive Co(I) species in CmuA would readily react with chloromethane, which is known to be a good corrinoid alkylating agent [77]. This could contribute to maintain the methyltransferase I in an active form by preventing oxidation of the cobalt to Co(II). In support of this idea, the ATP- and/or reductant-dependent reactivation system essential for activity of methyltransferases from anaerobes [60,78,79] and of methionine synthase [80] was not required for chloromethane dehalogenase activity in cell-free extracts of strain CM4 (Table 2.3). The sequence similarity of CmuA to *E. coli* methionine synthase does not include the C-terminal "AdoMet" domain of methionine synthase involved in reactivation of the cobalt center [80]. In addition, none of the other protein sequences deduced from the genes of cluster I or II in strain CM4 (Table 2.1) showed any detectable similarity to involved in the reductive activation of methyltransferases.

The reactions in the second part of the proposed chloromethane utilization pathway (Fig. 2.3) lead from $CH_3$-$H_4$folate to formate. Indication for a $H_4$folate-dependent pathway specific for the conversion of $CH_3$-$H_4$folate derived from chloromethane to formate is of interest in the light of recent findings on $C_1$ metabolism of *Methylobacterium extorquens* AM1 [43]. This organism possesses a dephospho-tetrahydromethanopterin-mediated $C_1$ transfer pathway that is essential for growth with $C_1$ compounds and brings about the conversion of formaldehyde to carbon dioxide. The sequences of many proteins involved in this pathway most closely resemble those of enzymes that participate in reduction of carbon dioxide to methane in methanogenic archaea. Dephospho-tetrahydromethanopterin was previously thought to be unique to methanogenic and sulfate-reducing archaea [81]. In parallel to the tetrahydromethanopterin-mediated pathway, *M. extorquens* AM1 appears to operate a $H_4$folate dependent pathway for the oxidation of formaldehyde to carbon dioxide. With the exception of the gene for NADP-dependent methylene-$H_4$folate dehydrogenase (*mtdA*), however, the genes encoding the enzymes of this pathway are still unknown [43].

The proposed pterine-dependent pathway for the conversion of chloromethane to formate in *Methylobacterium* sp. CM4 (Fig. 2.3) represents yet a third variant of the reactions for interconverting $C_1$ compounds in *Methylobacterium*. Since the *cmu* negative mutant with a disrupted copy of the *purU* gene still grew with methanol or methylamine, this pathway would be specific for processing a methylated pterine-based cofactor derived from chloromethane. While the nature of the pterin cofactor in this pathway needs to be determined, we have found that purified CmuB protein catalyses methyl transfer from methylcobalamin using $H_4$folate but not tetrahydromethanopterin as the methyl group acceptor (unpublished data).

In conclusion, our biochemical and genetic data suggest that growth of the strict aerobe *Methylobacterium* sp. CM4 with chloromethane is based on a specific catabolic pathway involving corrinoid-dependent enzymes that was hitherto unknown in organisms with an aerobic lifestyle. The similarity in sequence of CmuA and CmuB to other proteins of related function in methanogenic archaea (Table 2.1) is interesting from an evolutionary standpoint, since it extends the emerging notion that genes involved in methylotrophy and methanogenesis share a common origin [43], and that strictly anaerobic archaea and aerobic bacteria may use similar reactions to exploit $C_1$ substrates for metabolism.

# Chapter 3

## Properties of the methylcobalamin:$H_4$folate methyltransferase involved in chloromethane utilization by *Methylobacterium* sp. strain CM4

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## 3.1 ABSTRACT

*Methylobacterium* sp. strain CM4 is a strictly aerobic methylotrophic proteobacterium growing with chloromethane as the sole carbon and energy source. Genetic evidence and measurements of enzyme activity in cell-free extracts have suggested a multistep pathway for the conversion of chloromethane to formate. The postulated pathway is initiated by a corrinoid-dependent methyltransferase system involving methyltransferase I (CmuA) and methyltransferase II (CmuB) which transfer the methyl group of chloromethane onto tetrahydrofolate ($H_4$folate). We report the overexpression in *Escherichia coli* and the purification to apparent homogeneity of methyltransferase II. This homodimeric enzyme with a subunit molecular mass of 33 kDa catalyzed the conversion of methylcobalamin and $H_4$folate to cob(I)alamin and methyl-$H_4$folate with a specific activity of 22 nmol $\cdot$ $min^{-1}$ $\cdot$ (mg protein) $^{-1}$. The apparent kinetic constants for $H_4$folate were: $K_m$ = 240 μM, $V_{max}$ = 28.5 nmol $\cdot$min $^{-1}\cdot$ (mg protein) $^{-1}$. The reaction appeared first order with respect to methylcobalamin at concentrations up to 2 mM, presumably reflecting the fact that methylcobalamin is an artificial substitute for the methylated methyltransferase I, the natural substrate of the enzyme. Tetrahydromethanopterin, a coenzyme also present in *Methylobacterium*, did not serve as a methyl group acceptor for methyltransferase II. Purified methyltransferase II restored chloromethane dehalogenation by a cell free extract of a strain CM4 mutant defective in methyltransferase II.

## 3.2 INTRODUCTION

*Methylobacterium* sp. strain CM4 is a strictly aerobic methylotrophic α-proteobacterium capable of growth with chloromethane as the sole carbon and energy source [30]. To elucidate the pathway of chloromethane utilization, miniTn*5* transposon insertion mutants unable to grow with chloromethane were isolated and characterized. All cmu (chloromethane utilization) negative mutants obtained were still able to grow with methanol, methylamine, or formate, an observation suggesting that chloromethane was metabolized by reactions different from those involved in the metabolism of methanol and methylamine [48]. Sequence analysis of the DNA fragments affected by transposon insertion, mutant properties, and measurements of enzyme activity in cell-free extracts led to the proposal of a multistep pathway for the conversion of chloromethane to formate (see Chapter 2).

This pathway is thought to be initiated by a dehalogenation reaction in which the Co(I) center of a corrinoid protein acts as primary acceptor for the methyl group of chloromethane. CmuA, the protein suggested to catalyze this reaction, has a calculated molecular mass of 67 kDa and appears to represent a fusion of two proteins that are expressed as separate polypeptides in methyl transfer systems of methanogenic archaea (see Chapter 2). In its N-terminal part CmuA shows considerable sequence identity to the 36 kDa methyltransferase MtbA of *Methanosarcina barkeri* that transfers the methyl group from the 29 kDa corrinoid protein MtmC to coenzyme M [68]. The C-terminal part of CmuA was found to be similar to MtmC [68,70] as well as to many other corrinoid-binding proteins. By analogy to similar methyltransferase systems [58,82,83], this suggested that CmuA acts as both the methyltransferase I and the corrinoid binding protein in the dehalogenation of chloromethane (Fig. 3.1). Sequence analysis of *cmuB* and enzyme activity measurements in crude extracts suggest that protein CmuB is a methyltransferase II that transfers the methyl group from Co(III)-methylated CmuA onto $H_4$folate thereby yielding methyl-$H_4$folate (Fig. 3.1, see Chapter 2). The methyl-$H_4$folate derived from chloromethane is then processed to formate via pterin-linked intermediates by a pathway which is

proposed to differ from the $C_1$ transfer pathways recently described in *Methylobacterium* [44].

A dehalogenation reaction based on a corrinoid-dependent methyltransferase system is one of the striking features of the proposed pathway for chloromethane degradation in strain CM4, a strictly aerobic bacterium. A similar system for chloromethane dehalogenation has been described in the strict anaerobe *Acetobacterium dehalogenans* [60]. In contrast to chloromethane dehalogenation by *Methylobacterium* sp. CM4, it requires an ATP- and reductant-dependent reactivation system to maintain the corrinoid cobalt in the Co(I) state. To reach an understanding of Co(I)-mediated, reactivation-independent chloromethane dehalogenation in strain CM4 and to determine the nature of the cofactors involved in this process, we have set out to purify the proteins involved in the dehalogenation reaction. Here we report on the purification of CmuB and demonstrate its activity *in vitro* as a methylcobalamin:$H_4$folate methyltransferase.

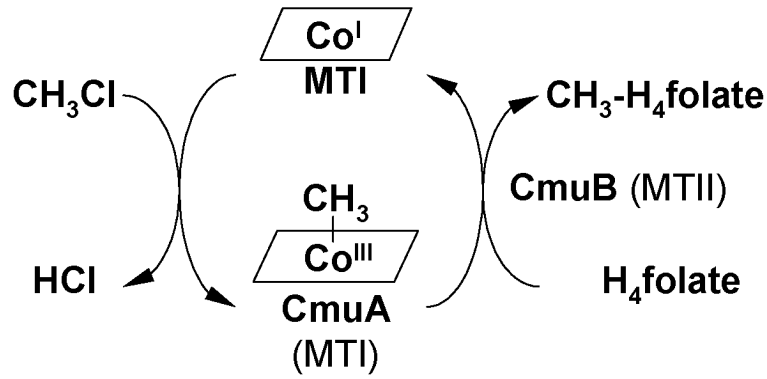


**Fig. 3.1. Postulated scheme of chloromethane dehalogenation.** The reactions catalyzed by methyltransferase I (MTI, CmuA) and methyltransferase II (MTII, CmuB) are shown.

## 3.3 EXPERIMENTAL PROCEDURES

### 3.3.1 Materials

Restriction endonucleases and T4 ligase were obtained from MBI Fermentas, *Pfu* DNA polymerase from Stratagene, methylcobalamin from Sigma, $H_4$folate and methyl-$H_4$folate from Dr. Schircks Laboratory (Jona, Switzerland). All other chemicals were of reagent grade from Fluka, Merck or Sigma.

### 3.3.2 Bacterial strains and growth conditions

*E. coli* strains XL1-Blue (Stratagene) and BL21(DE3) [84] were grown at 37° or 30°C in Luria-Bertani medium with constant shaking at 180 rpm. When required, kanamycin was added at 25 μg/ml and ampicillin at 100 μg/ml. The media and growth conditions for *Methylobacterium* sp. strain CM4 [30] and its miniTn*5* insertion mutants have been described previously [48].

### 3.3.3 DNA manipulations

Preparation of genomic DNA, restriction digests, ligations and transformations were performed using standard protocols [62]. The plasmid pBluescript-KSII(+) (Stratagene) was used for cloning.

### 3.3.4 Construction of the *cmuB* expression plasmid

A 7.5 kb *Kpn*I fragment of genomic DNA was cloned from the *Methylobacterium* sp. CM4 mutant 36D3 by selection for kanamycin resistance, yielding plasmid pME1742. The cloned fragment carried the miniTn*5* transposon plus bp 3281 to 8456 of cluster II (accession number AJ011317) which included an intact copy of the *cmuB* gene. Sequence data and mutant properties have been described previously (see Chapter 2).

The *cmuB* gene was placed under the control of the T7 RNA polymerase promoter of vector pET24a(+) (Novagene) in a two-step cloning procedure. First, the *cmuB* gene was amplified by polymerase chain reaction from pME1742. The oligonucleotide primers used were 5'-GGGAGGTTAAAT**C**A**T**ATGAATAAG-3', with a change of two bases (underlined) to introduce an N-terminal *Nde*I site (bold) and 5'-

CGATCAGCAGTCGACCGGTCGGTTGTCCC-3'. The 1012-base pair polymerase chain reaction product was cloned into the *Eco*RV site of pBluescript KSII(+) by blunt-end ligation, generating plasmid pME1747. In a second step, a 1009-base pair *Nde*I-*Hin*dIII fragment from pME1747 was cloned into pET24a(+), resulting in the expression plasmid pME1748.

### 3.3.5 Preparation of cell-free extracts

Cell extracts from *Methylobacterium* sp. strain CM4 were prepared as described previously (see Chapter 2). For the production of CmuB protein in *E. coli*, strain BL21(DE3) harboring the expression plasmid pME1748 was grown at 30°C in 5-liter Erlenmeyer flasks containing 1000 ml of LB. When the culture had reached an $A_{600}$ of 0.6, CmuB expression was induced by addition of IPTG to a final concentration of 500 μM and the culture was incubated for another 3 h to a final $A_{600}$ of about 2.0 (3.2 g wet weight). Cells were collected by centrifugation at 5000 x g for 15 min at 4°C and resuspended in 50 mM Na-phosphate buffer (pH 8.0; 0.5 g wet cells per ml). The cells were disrupted by three passages through a French pressure cell (120 mPa, 4°C) and cell debris was removed by centrifugation (14'000 x g, 30 min, 4°C). Crude cell-free extract was obtained after ultracentrifugation of the resulting supernatant (100'000 x g, 30 min, 4°C).

### 3.3.6 Determination of enzyme activity

All solutions used were made anoxic by degassing with 95% $N_2$/5% $H_2$ (v/v). Subsequent manipulations, enzymatic reactions and measurements were carried out in an anaerobic chamber under the same conditions. All reactions were performed in glass cuvettes or reaction vials with gas-tight rubber stoppers at 30°C for 40 min after addition of the enzyme. Methyl transfer from methylcobalamin to methyl-$H_4$folate was performed at 30°C with a mixture that contained 100 mM Tris/HCl (pH 8.5), 2.4 mM $H_4$folate, 0.2 mM methylcobalamin and between 20 and 40 μg CmuB protein per ml. Commercially available $H_4$folate was abiotically converted to methylene-$H_4$folate by the addition of formaldehyde, and biologically active $H_4$folate was quantified by its NADH-

dependent conversion to methyl-$H_4$folate using purified methylene-$H_4$folate reductase from *Peptostreptococcus productus* [66].

Methylcobalamin:$H_4$folate methyltransferase activity of CmuB was determined using the photometric assay described by Kreft and Schink [85], by measuring the rate of cob(I)alamin formation from methylcob(III)alamin from the absorbance decrease at 528 nm ($\Delta\ \varepsilon$ = 6.2 $mM^{-1}\ cm^{-1}$). Enzyme activity of CmuB was also determined by measuring the formation of methyl-$H_4$folate from $H_4$folate and methylcobalamin. Methyl-$H_4$folate was separated from the other components of the incubation mixture by high-performance liquid chromatography (HPLC) on a C8-reversed phase column using 0.175% $H_3PO_4$ in $H_2O$ containing 20% methanol as eluent [59]. This method was also used to monitor chloromethane dehalogenation activity in crude extracts of *Methylobacterium* sp. CM4. One unit is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of methylcobalamin to cob(I)alamin or the formation of 1 μmol of methyl-$H_4$folate per minute.

### 3.3.7 Purification of methylcobalamin:$H_4$folate methyltransferase overexpressed in *E. coli*

Ammonium sulfate was added to crude cell-free extract from *E coli* BL21(DE3)(pME1748) up to a concentration of 1.72 M and the resulting suspension was centrifuged at 45'000 x g for 30 min. The precipitate was discarded and the filtered supernatant was fractionated on a Poros PE 4.6/10 hydrophobic-interaction column with a BioCAD SPRINT apparatus (PerSeptive Biosystems Inc.) at a flow rate of 3 ml/min, using a stepwise gradient from 1.72 to 0 M in 50 mM Na-phosphate buffer pH 8.0. Active CmuB eluted at an ammonium sulfate concentration of about 0.4 M. Active fractions were pooled and subsequently desalted by repeated concentration and dilution in 50 mM Na-phosphate buffer (pH 8.0) using a Biomax 10K centrifugal filter (Millipore). The pooled fractions containing CmuB were then applied on a 1 ml ResourceQ anion-exchange column (Pharmacia) at a flow rate of 3 ml/min and eluted by a linear gradient of increasing NaCl in 50 mM Na-phosphate buffer (pH 8.0). The CmuB protein eluted at a concentration of about 270 mM NaCl.

### 3.3.8 Protein determination.

Protein was determined by the method of Bradford [67] using the BioRad reagent. Bovine serum albumin (Sigma) was used as the standard.

## 3.4 RESULTS

It was previously found that cell-free extract of *Methylobacterium* sp. CM4 grown with chloromethane catalyzed methyl transfer from chloromethane (3 mU/mg protein) onto $H_4$folate. This extract, but not extract from methanol-grown cells, also catalyzed methyl transfer from methylcobalamin to $H_4$folate at a rate of 0.8 mU/mg protein. Methyltransferase II of the chloromethane dehalogenation system thus appeared to accept methylcobalamin as an artificial methyl donor in place of methylated corrinoid protein. Cell-free extract from strain 19D10, a transposon insertion mutant that did not grow with chloromethane, lacked activity with chloromethane or methylcobalamin as a methyl donor (see Chapter 2). Since the transposon had inserted into *cmuB*, this gene was proposed to encode methyltransferase II (Fig. 3.1). The use of methylcobalamin as an artificial substrate for the CmuB protein enabled to monitor its activity. We thus have purified CmuB and characterized it as a methylcobalamin:$H_4$folate methyltransferase whose function is in accordance with the scheme proposed in Fig. 3.1.

Examination of the protein pattern of crude extracts of *Methylobacterium* sp. CM4 and of the *cmuB* mutant 19D10 by SDS/PAGE showed that the CmuB protein accounted for only about 2% of the total soluble protein. This estimation was based on the staining intensity of a 33 kDa chloromethane-induced protein which was absent from extracts of mutant 19D10 (data not shown). To obtain extract with a high concentration of methylcobalamin:$H_4$folate methyltransferase as starting material for enzyme purification, the *cmuB* gene from *Methylobacterium* sp. CM4 was expressed in *E. coli*.

### 3.4.1 Enzyme purification and molecular properties

The *cmuB* gene was amplified by the polymerase chain reaction and ligated into a pET-type expression vector [84] to yield plasmid pME1748. *E. coli* transformants carrying pME1748 produced a soluble protein with an apparent molecular mass of 33 kDa. This protein was not present in extract from untransformed recipient cells and its formation was dependent on the addition of IPTG to the growing culture of the recombinant *E. coli* strain (Fig. 3.2, lanes 1

and 2). The apparent molecular mass of the CmuB protein produced in the heterologous host was in accordance with the subunit molecular mass of 33.3 kDa calculated from the translated *cmuB* gene sequence. Crude cell-free extract of *E. coli* BL21(DE3)(pME1748) contained about 20% CmuB protein which accounted for 5.2 mU/mg protein of methylcobalamin:$H_4$folate methyltransferase. The increase in enzyme specific activity and in the concentration of CmuB thus was seven- and tenfold as compared to the values measured in crude extract of chloromethane–induced *Methylobacterium* sp. CM4. From this it appears that the specific activity of the recombinant CmuB protein from *E. coli* was similar to that of the enzyme in cell-free extracts of *Methylobacterium*.

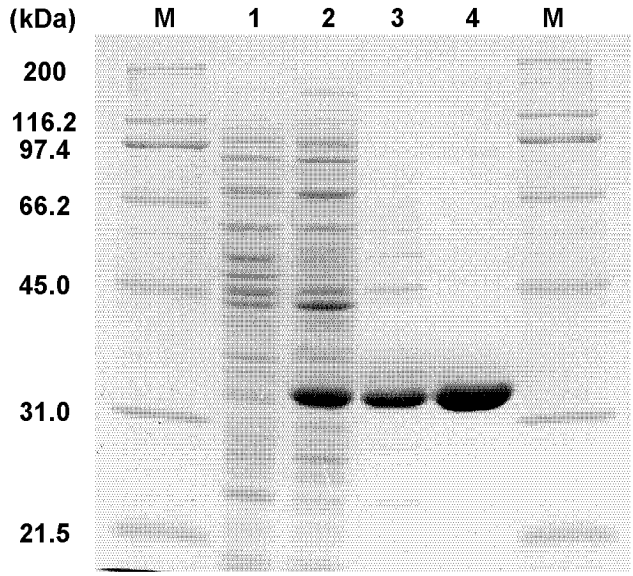


**Fig. 3.2. Purification of the *Methylobacterium* sp. CM4 methylcobalamin:tetrahydrofolate methyltransferase from cell-free extract of *E. coli* BL21(DE3) (pME1748).** Protein samples (10 μg) were analyzed at different stages of purification on a denaturing 12% SDS-polyacrylamide gel and stained by Coomassie brilliant blue. Lane M, molecular weight markers in kDa; lane 1, cell-free extract of non-induced cells; lane 2, cell-free extract of IPTG-induced cells; lane 3, fraction after PE hydrophobic interaction chromatography; lane 4, purified CmuB obtained after ResourceQ ion exchange chromatography.

Recombinant CmuB protein was purified under aerobic conditions by hydrophobic interaction and by anion-exchange chromatography. Copurification of the activity catalyzing methylcobalamin-dependent methyl-$H_4$folate formation and of the activity promoting $H_4$folate-dependent methylcobalamin consumption was observed, thus demonstrating that both of these activities are characteristic of the methylcobalamin:$H_4$folate methyltransferase activity of CmuB. Purification was 4.3-fold with 39% yield (Table 3.1). The purified enzyme retained about 80% of its activity after 5 months of storage at –20°C in buffer containing 20% (v/v) of glycerol.

**Table 3.1. Purification of the overexpressed CmuB protein from *E. coli***

| Purification step | Total protein (mg) | Total activity (mU) | Specific activity | | Purification (-fold) | Yield (%) |
|---|---|---|---|---|---|---|
| | | | methyl-$H_4$folate formation (mU/mg) | methyl-cobalamin formation (mU/mg) | | |
| **Cell-free extract** | 17.7 | 101 | 5.2 | 5.7 | 1.0 | 100 |
| **$(NH_4)_2SO_4$** | 16.5 | 106 | 6.4 | 6.8 | 1.2 | 105 |
| **HIC PE Poros** | 3.2 | 41 | 11.4 | 12.8 | 2.2 | 41 |
| **Resource Q** | 1.6 | 39 | 22.3 | 24.3 | 4.3 | 39 |

As shown by SDS/PAGE, the preparation obtained after two steps of column chromatography contained a single polypeptide (Fig. 3.2, lane 4). Its N-terminal amino acid sequence (15 amino acids) determined by Edman degradation included the N-terminal methionine and corresponded to the sequence predicted from the *cmuB* gene. The purified enzyme eluted from a Superdex 75 HR column with an apparent molecular mass of 66 kDa (data not shown), suggesting that the enzyme has a homodimeric structure. The UV-visible spectrum of the enzyme gave no indication for the presence of a chromophoric prosthetic group. Addition of EDTA (10 mM final) had no effect on the activity of the pure enzyme.

### 3.4.2 Catalytic properties

The effect of pH and temperature on enzyme activity and the substrate affinity of the purified enzyme were determined and this led to the standard assay conditions described in Materials and Methods. The enzyme activity was only weakly affected by the pH, and a broad activity optimum was observed around pH 9.2. The temperature optimum for the reaction was 48°C. When the dependence of the rate of methyl transfer on the concentrations of the substrates was examined, Michaelis-Menten kinetics were observed with respect to $H_4$folate (Fig. 3.3A). At a methylcobalamin concentration of 0.2 mM, the apparent $K_m$ for $H_4$folate was 240 ± 10 μM and the apparent $V_{max}$ was 28.5 ± 1.0 mU/mg protein.The reverse reaction, methyl transfer from $H_4$folate to cob(I)alamin also appeared to follow Michaelis-Menten kinetics Fig. 3.3B). At a cob(I)alamin concentration of 0.2 mM, the apparent $K_m$ for methyl-$H_4$folate was 12.5 ± 1 mM and the apparent $V_{max}$ was about 1200 ± 100 mU/mg protein. It was not possible to obtain an apparent $K_m$ for methylcobalamin since the rate of methyl-$H_4$folate formation was found to be first order with respect to methylcobalamin up to 2 mM (Fig. 3.3C). However, the methyl transfer reaction to $H_4$folate presumably occurs under saturation conditions *in vivo*, since the physiological cobamide mimicked by methylcobalamin *in vitro* is thought to be protein-associated.

In view of the recent discovery that *Methylobacterium*, in addition to $H_4$folate, possesses $H_4$MPT as a carrier of one-carbon units [43], it was tested whether $H_4$MPT served as a methyl group acceptor in the reaction catalyzed by CmuB. No reaction occurred when purified enzyme was provided with 1 mM $H_4$MPT in the place of $H_4$folate under standard assay conditions.

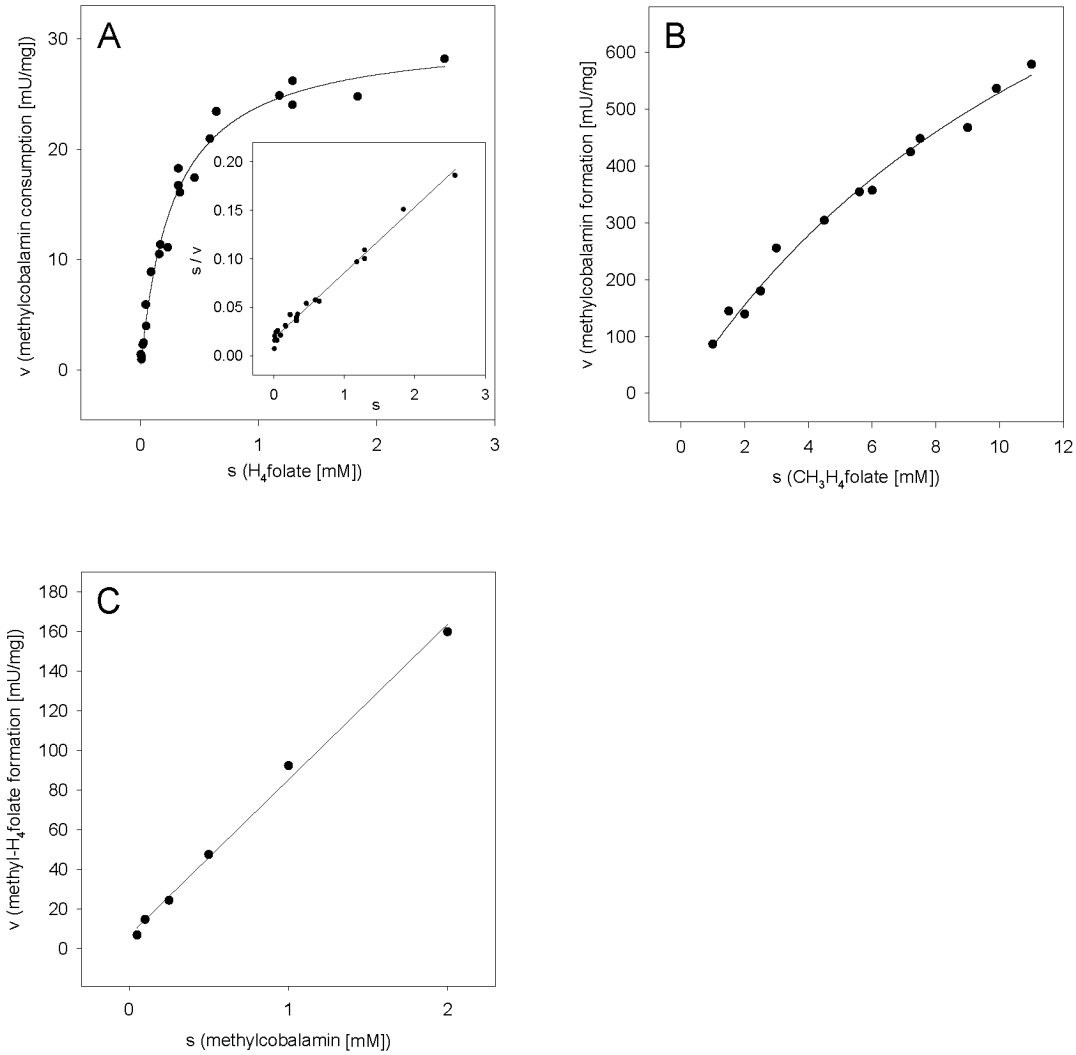


**Fig. 3.3. Dependence of methylcobalamin:tetrahydrofolate methyltransferase activity on the substrate concentration**. Assays were conducted with 5 μg of pure CmuB protein at 30°C in 100 mM Tris/HCl buffer pH 8.5. (A) Dependence of the rate of methylcobalamin consumption on the concentration of $H_4$folate. The inset shows the same data plotted according to Hanes [86]. The assay mixtures contained 0.2 mM methylcobalamin and $H_4$folate at the concentrations indicated. The rates were determined by following the absorbance change at 528 nm. (B) Dependence of the rate of conversion of cob(I)alamin to methylcob(III)alamin on the concentration of methyl-$H_4$folate. The assay mixture contained 0.2 mM cobalamin, 2.5 mM titanium(III)citrate, and methyl-$H_4$folate at the concentrations indicated. Rates were determined by following the change in absorbance at 528 nm. (C) Dependence of the rate of methyl-$H_4$folate production on the concentration of methylcobalamin. The assay mixtures contained 4 mM $H_4$folate and methylcobalamin at the concentrations indicated. The reaction rates were determined by HPLC analysis of methyl-$H_4$folate formation.

The time-dependence of the visible spectrum of the incubation mixture after addition of CmuB is shown in Fig. 3.4. A time-dependent decrease of the methylcobalamin absorbance peak at 528 nm was observed, which could be used to determine the initial rate of the reaction catalyzed by CmuB. Concomitantly to the decrease in absorbance at this wavelength, there was an increase at 388 nm where cob(I)alamin exhibits a typical absorbance peak [85]. This is strong evidence that, in accordance to the scheme proposed in Fig. 3.1, cob(I)alamin is indeed the product of the methyltransferase II reaction catalyzed by CmuB. In mixtures that were exposed to air or contained dithiothreitol, the spectrum showed absorbance characteristics typical for cob(II)alamin (not shown), indicating oxidation of Co(I) to Co(II) by oxygen or by traces of disulfide present in the DTT preparation [87].

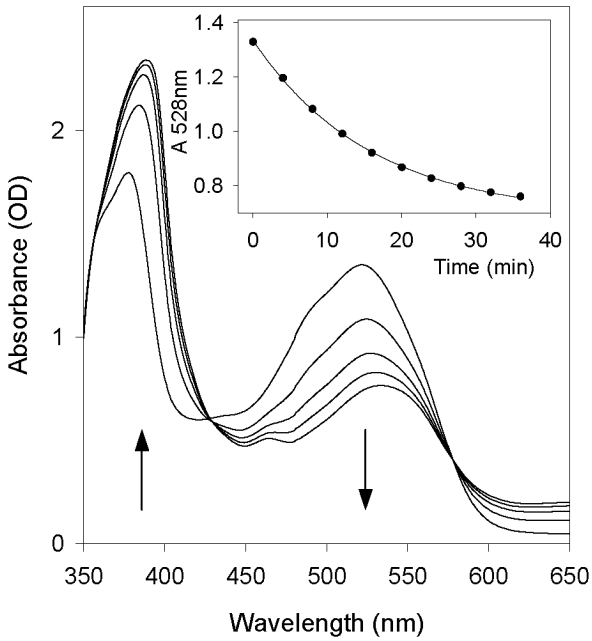


**Fig. 3.4. Formation of cob(I)alamin by purified methylcobalamin:tetrahydrofolate methyltransferase.** The reaction was carried out under anaerobic conditions at 40°C in 100 mM Tris/HCl buffer pH 8.5 containing 0.2 mM methylcobalamin and 4 mM $H_4$folate. The reaction was started by the addition of 50μg of purified CmuB protein. The spectra plotted were taken at different times after addition of the enzyme. The increase of absorbance at 388 nm is characteristic for the formation of the cob(I)alamin species [85].

### 3.4.3 Restoration of dehalogenation activity in cell extract of a *cmuB* mutant

Cell-free extract from *Methylobacterium* wild type cells was able to dehalogenate chloromethane, i.e. to transfer the methyl group onto $H_4$folate by the concerted action of methyltransferase I and methyltransferase II. Because of its deficiency in methyltransferase II, extract from the *cmuB* mutant 19D10 was unable to carry out this reaction. As shown in Fig. 3.5, the ability to convert chloromethane to methyl-$H_4$folate was restored to wild type level by the addition to the mutant extract of purified CmuB protein in threefold molar excess over CmuA. This suggests that the heterologously expressed and purified CmuB protein catalyzed not only the methylcobalamin:$H_4$folate methyltransferase reaction but also the physiological methyl transfer from methylated CmuA protein onto $H_4$folate. It also indicates that the proteins possessing methyltransferase I and methyltransferase II activities need not be coexpressed for chloromethane dehalogenation activity to be obtained.

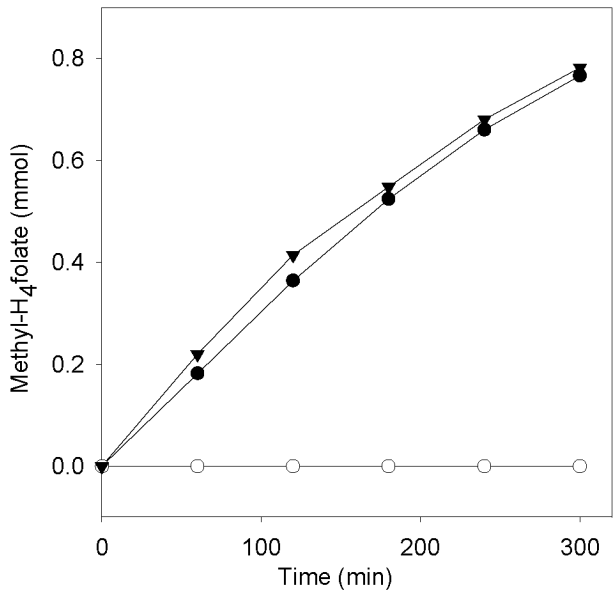


**Fig. 3.5. In vitro complementation of chloromethane dehalogenation by purified methylcobalamin:tetrahydrofolate methyltransferase.** Formation of methyl-$H_4$folate from chloromethane was measured in a 2 ml assay mixture containing 50 mM Tris/HCl pH 8.5, 4 mM $H_4$folate and 2 mM chloromethane in the gas-phase, and either (●) 400μg cell-free extract from *Methylobacterium* sp. CM4 wild type, or (○) 400μg cell-free extract from the *cmuB* mutant 19D10, or (▼) a mixture of 400μg cell-free extract from the *cmuB* mutant 19D10 and 50μg purified CmuB protein.

## 3.5 DISCUSSION

The results reported here support the concept that chloromethane dehalogenation by *Methylobacterium* sp. CM4 proceeds by two sequential methyltransferase reactions. In the first reaction, the bifunctional protein CmuA acts as a methyltransferase I and at the same time provides a cobalamin prosthetic group which serves as an intermediate methyl carrier. In the second reaction, protein CmuB acts as a methyltransferase II and transfers the methyl group from CmuA onto $H_4$folate (Fig. 3.1). Mutational loss of either CmuA or CmuB activity has previously been shown to abolish the liberation of chloride from chloromethane as well as the conversion of chloromethane to methyl-$H_4$folate (see Chapter 2, [48]). Addition of purified methyltransferase II reconstituted the *in vitro* transformation of chloromethane to methyl-$H_4$folate by a cell-free extract of a mutant devoid of CmuB activity, thus demonstrating the expected interaction of methyltransferase II with methylated methyltransferase I (Fig. 3.5).

Based on its reaction with methylcobalamin, methyltransferase II of *Methylobacterium* sp. CM4 was purified and characterized as methylcobalamin:$H_4$folate methyltransferase. Non-physiological methyl transfer from free methylcobalamin has previously been reported for corrinoid-dependent methyltransferases involved in the metabolism of one-carbon compounds by anaerobic Archaea and Bacteria. This applies for example to the methyltransferases involved in the formation of methyl-coenzyme M from methanol and coenzyme M by *Methanosarcina barkeri*. Protein MtaB, the methyltransferase I of this system, catalyzes the hydrolysis of methylcobalamin to cob(I)alamin plus methanol [83] and protein MtaA, the methyltransferase II, can use free methylcobalamin as the methyl donor for the formation of methyl-coenzyme M [88]. Similarly, the methyltransferase II of the *Halophaga foetida* enzyme system for O-demethylation of methoxylated aromatic compounds catalyzed the methylation of $H_4$folate with methylcobalamin as an artificial substrate [85]. These enzymes exhibited either a very high apparent $K_m$ value for methylcobalamin [88] or, similar to methyltransferase II of *Methylobacterium*

sp. CM4, displayed first order kinetics with respect to the concentration of methylcobalamin [83,85].

The CmuB sequence is most similar to the sequence of subunit H of the methyl-$H_4$MPT: coenzyme M methyltransferase from methanogens (see Chapter 2). It can best be aligned to domain 9948 of the ProDom database [89] that was defined on the basis of the sequences of the MtrH protein from *Methanobacterium thermoautotrophicum* [90] and related proteins. This is somewhat intriguing given that in CmuB, methyltransferase activity was only detected with $H_4$folate as the methyl acceptor. Modest similarity of MtrH to the putative $H_4$folate binding domain of cobalamin-dependent methionine synthase from *E. coli* (MetH), however, was noted previously [91]. A more detailed analysis of the CmuB sequence using pattern-initiated BLAST (PHI-BLAST, [92]) with the short conserved motif D-[FY]-[IVL]-X-[FY]-G-[PL]-[IV]-[ED] common to CmuB and sequences in the MtrH-based Prodom domain yields that only MtrH- and MetH-like sequences as significant hits. The ProDom domain 9873 corresponding to the putative $H_4$folate binding domain in cobalamin-dependent methionine synthases [93] also includes protein AcsE of *Clostridium thermoaceticum*, which is involved in methyl transfer from methyl-$H_4$folate to an iron/sulfur corrinoid protein in acetate synthesis from $CO_2$ [94]. Overall, the level of sequence similarity between MtrH, MetH, and CmuB is quite low (Fig. 3.6). A sequence motif can be defined that is both common and unique to all these proteins (see Fig. 3.6, legend). However, in the absence of knowledge on the residues involved in catalysis or cofactor binding in any of these proteins, the significance of this motif remains unclear.

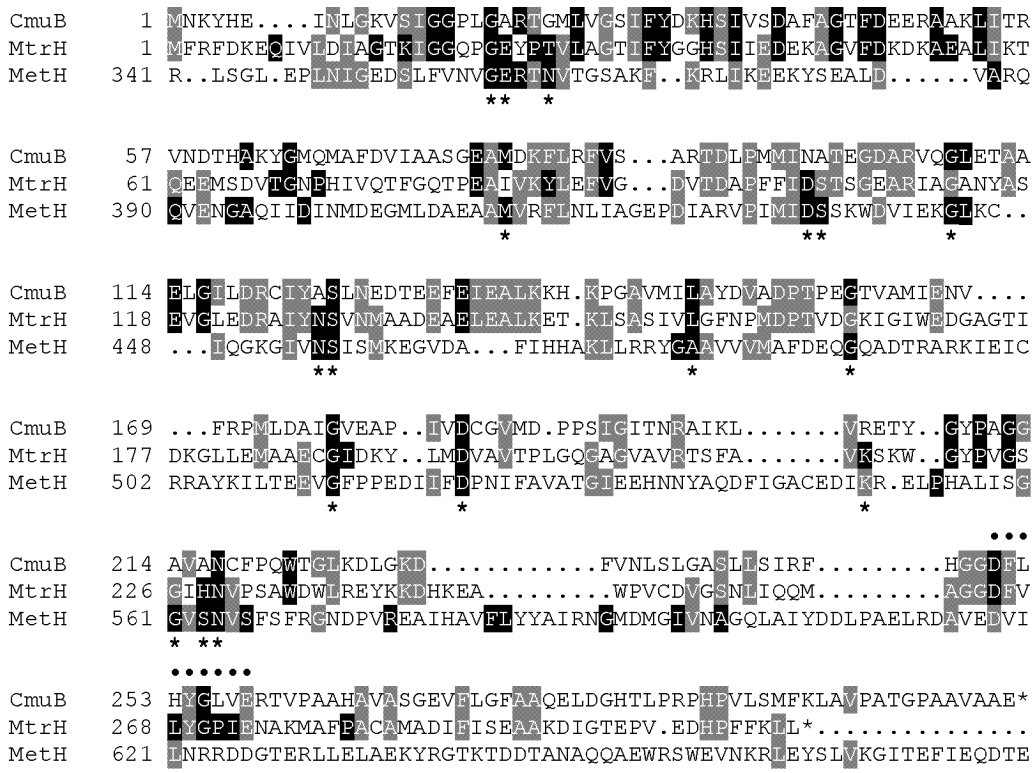


**Fig. 3.6. Sequence analysis of the CmuB protein.** The alignment shows sequence similarity of CmuB to the H subunit of the $N^5$-methyltetrahydromethanopterin:coenzyme M methyltransferase complex of *Methanobacterium thermoautotrophicum* (MtrH [90], 30% identity) and to the putative tetrahydrofolate binding domain in *E. coli* cobalamin-dependent methionine synthase (MetH [93], 13% identity). The short ad hoc sequence pattern (PROSITE format [95]) D-[FY]-[IVL]-X-[FY]-G-[PL]-[IV]-[ED] (dots) unique to CmuB and MtrH-like sequences was used in the pattern-hit initiated sequence analysis [92] of CmuB (see text). Absolutely conserved residues in MtrH-like sequences (ProDom domain 9948; release 99.1, [89]), or in MetH-like sequences (domain 9873) including the AcsE protein from *Clostridum thermoaceticum* [94], are boxed in black. Other residues identical in two or more sequences in the alignment are boxed in grey. The asterisks indicate a sequence pattern, G-[EA]-X(2)-[TNG]-X(47,56)-[LIM]-X(16,20)-[DN]- [RSA]- X(8) - [GA] - X (10,15) - [NA] - S-X(19,24) -[AL]- X(10,18)-[GA]- X(9,23) -G- X(6,8)-D-X(19,27)-[KRA]-X(8,10)-[GA]-X-[HAS]-N, that is present in all 8 MtrH- and in 9 MetH-related sequences only (Swissprot release 38; Embl release 55).

The physiological reaction catalyzed by CmuB is suggested to be the transfer of a methyl group from a corrinoid protein to $H_4$folate. The corresponding methyl transfer reactions in the methyl-$H_4$folate: corrinoid/iron-sulfur protein methyltransferase from *Clostridium thermoaceticum* [96], in methyl-$H_4$MPT: coenzyme M methyltransferases from methanogens [97], or in methionine synthases [93], proceed in the reverse direction *in vivo*. In these cases, methyl-$H_4$folate or methyl-$H_4$MPT serves as the alkylating agent for a corrinoid protein with cobalt in the Co(I) state. The absorption maxima and isobestic points that arise in the CmuB-catalyzed reaction of methylcobalamin in the presence of $H_4$folate are characteristic of Co(I) (Fig. 3.4) and suggest a $S_N2$ type reaction

involving formal transfer of 2 electrons rather than a radical reaction [96]. Most notably, cob(I)alamin is produced by CmuB under conditions (95% $N_2$/5% $H_2$) that cannot elicit the regeneration of cob(I)alamin from cob(II)alamin. CmuB was found to have a much lower apparent $K_m$ for $H_4$folate (250 μM; Fig. 3.3A) than for methyl-$H_4$folate (12.5 mM; Fig. 3.4B), in contrast to what was observed previously for the AcsE methyltransferase from *Clostridium thermoaceticum* [96].

Active CmuB protein purified from *E. coli* is now helping to isolate and characterize the other protein(s) comprising the chloromethane dehalogenase of *Methylobacterium* sp. CM4. Coupling of methyltransferase I to purified methyltransferase II provides an activity-based assay for methyltransferase I that is now being exploited for the purification and characterization of the CmuA protein. By affording the physiological, protein-associated methylcobamide methyl donor, purified CmuA protein should allow investigation of the kinetic properties of the CmuB protein in catalyzing the transfer of a cobalt-bound methyl group to $H_4$folate.

# Chapter 4

## Chloromethane:tetrahydrofolate methyl transfer by two proteins from *Methylobacterium chloromethanicum* strain CM4

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## 4.1 ABSTRACT

The *cmuA* and *cmuB* genes are required for growth of *Methylobacterium chloromethanicum* strain CM4 with chloromethane as the sole carbon source. While CmuB was previously shown to possess methylcobalamin:tetrahydrofolate methyltransferase activity, sequence analysis indicated that CmuA represented a novel and so far unique two-domain methyltransferase/corrinoid-binding protein involved in methyl transfer from chloromethane to a corrin moiety. CmuA was purified from wild-type *M. chloromethanicum* strain CM4 and characterized as a monomeric, cobalt and zinc containing enzyme of a molecular mass of 67 kDa with a bound vitamin $B_{12}$ cofactor. In combination, CmuA and CmuB proteins catalyze the *in vitro* transfer of the methyl group of chloromethane to tetrahydrofolate, thus affording a direct link between chloromethane dehalogenation and core $C_1$ metabolism of *Methylobacterium*. Chloromethane dehalogenase activity *in vitro* is limited by CmuB, as formation of methyltetrahydrofolate from chloromethane displays apparent Michaelis-Menten kinetics with respect to methylated CmuA, with an apparent $K_m$ of 0.27 μM and a $V_{max}$ of 0.45 U $mg^{-1}$. This contrasts with sequence-related systems for methyl transfer from methanogens, which involve methyltransferase and corrinoid protein components in well-defined stoichiometric ratios.

## 4.2 INTRODUCTION

Chloromethane, a compound which to a large extent is naturally produced, is the most abundant halogenated compound in the atmosphere and accounts for about 15% of the destruction of the ozone layer by halogenated chemicals [8,9]. Characterization of the sources and sinks of chloromethane in the environment is thus of topical interest. While the global budget for chloromethane presents a significant deficit in known sources of the compound [8], additional sinks of bacterial origin have recently emerged [29,30,59,98]. The strictly aerobic, facultatively methylotrophic $\alpha$-proteobacterium *Methylobacterium chloromethanicum* strain CM4 [30,33,48] is capable of growth with chloromethane as the sole source of carbon and energy. Physiological and genetic studies with this organism have shown that the products of the *cmuA* and *cmuB* genes are essential for the dehalogenation of chloromethane and growth with this compound, and have further suggested that chloromethane is degraded to formate by a multistep pathway (Fig. 4.1; see Chapter 2, [48]).

The *cmuA* gene encodes a 67 kDa protein with unique features. CmuA shows similarity to known methyltransferases as well as to their cognate corrinoid protein binding proteins in the N-terminal and C-terminal part of its sequence, respectively (see Chapter 2). In analogy to the scheme proposed for the chloromethane dehalogenase of anaerobic bacteria [60], the CmuA protein was suggested to catalyze the first step of the chloromethane degradation pathway, comprising the dehalogenation of chloromethane and the methylation of an associated cobalt corrin moiety. The second step of the pathway likely involves CmuB, which was shown to be a homodimeric enzyme of 33 kDa subunits capable of using methylcobalamin as a substitute for methylated CmuA protein as a methyl donor to generate methyltetrahydrofolate ($CH_3$-$H_4$folate) from tetrahydrofolate ($H_4$folate, see Chapter 3). Later steps in the chloromethane degradation pathway (Fig. 4.1) remain to be investigated in detail. However, genes *metF, folD* and *purU* encode proteins similar to enzymes involved in the oxidation of pterin-linked one-carbon units to formate. These genes were found nearby the *cmuA* and *cmuB* genes in *M. chloromethanicum*

strain CM4, and mutational inactivation of the operon containing *purU* and *folD* genes prevented growth of this strain with chloromethane (see Chapter 2). The chloromethane degradation pathway postulated for *M. chloromethanicum* strain CM4 may be widespread among methylotrophic bacteria that grow with this compound. Indeed, *cmuA* and *cmuB* genes were also identified in *Hyphomicrobium chloromethanicum* strain CM2 [99], and a protein of similar size and 81% identical N-terminal sequence to CmuA was described from the chloromethane degrading strain CC495 [29].

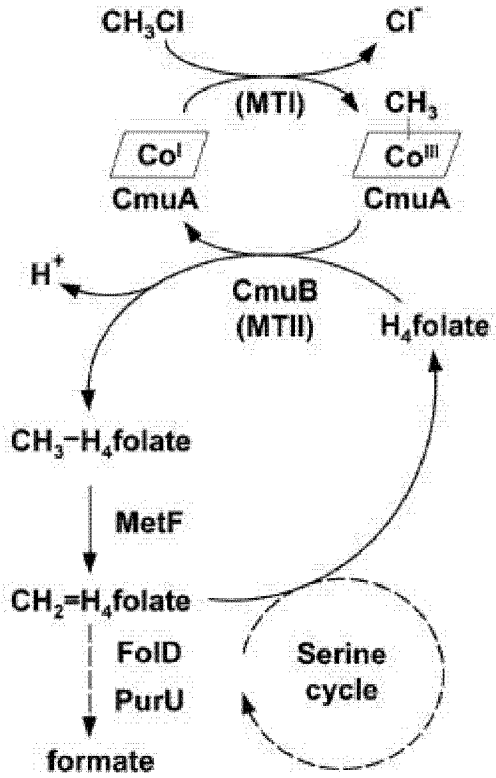


**Fig. 4.1. Postulated scheme for chloromethane utilization in *M. chloromethanicum* strain CM4.** Transformations involving single enzymes are indicated by continuous arrows and multistep enzymatic pathways by broken arrows. MetF (5,10-methylene-$H_4$folate reductase), FolD (5,10-methylene-$H_4$folate dehydrogenase / 5,10-methenyl-$H_4$folate-cyclohydrolase), PurU (10-formyl-$H_4$folate hydrolase), proteins involved in the catabolic pathway basing on previous sequence analysis (see Chapter 2).

In the present study, we report on the reconstitution *in vitro* of the novel dehalogenation and methyl transfer reaction catalyzed in the two initial steps of the pathway (Fig. 4.1). We present the purification of the CmuA protein from *M. chloromethanicum* strain CM4 and identify vitamin $B_{12}$ as a prosthetic group of the enzyme. It is shown that purified CmuA displays the properties inferred from sequence analysis in that it functions both as a chloromethane methyltransferase and as the substrate of the CmuB protein, which transfers the corrinoid-bound methyl group derived from chloromethane to $H_4$folate.

## 4.3 EXPERIMENTAL PROCEDURES

### 4.3.1 Materials

$H_4$folate and $CH_3$-$H_4$folate were purchased from Dr. Schircks Laboratory (Jona, Switzerland). Protein markers for SDS-PAGE came from New England Biolabs, and reference proteins for molecular mass determination from Pharmacia. N-ethylmaleimide, *o*-phenanthroline and EDTA were from Fluka. All other chemicals were reagent grade and obtained from Fluka, Merck or Sigma.

### 4.3.2 Growth conditions and preparation of cell-free extract

*Methylobacterium chloromethanicum* strain CM4 is deposited in the All-Russian Collection of Microorganisms as VKM B-2223. Media and the growth conditions for the organism have been described previously [48]. Bacteria were harvested from a chloromethane-grown culture (12 l) at late exponential phase ($OD_{600}$ 0.8 - 0.9) by centrifugation for 30 min at 10,000 g. The cell pellet (14.5 g wet weight) was resuspended in 40 ml of 50 mM sodium phosphate buffer (pH 8.0), disrupted by four passages through a French pressure cell (120 MPa, 4°C) and cell debris removed by centrifugation (14,000 g, 30 min, 4°C). Crude cell-free extract was obtained after ultracentrifugation of the resulting supernatant (100,000 g, 1 h, 4°C).

### 4.3.3 Isolation of corrinoids from *M. chloromethanicum* strain CM4

Bacteria were grown on chloromethane in the presence of 0,1 μM [$^{57}$Co] (Amersham Buchler) cobalt chloride (275 $Bq \cdot nmol^{-1}$), harvested during exponential phase (8.3 g wet weight) and resuspended in 50 ml 100 mM sodium acetate buffer pH 5.0. Corrinoids were isolated using a method adapted from [100]. After addition of potassium cyanide (final concentration 0.1 mM) and boiling for 20 min, the resulting suspension was centrifuged (10,000 g, 10 min) after cooling and the pellet reextracted with 100 mM sodium acetate buffer pH 5.0 containing 0.1 mM KCN (26 ml). Supernatants were pooled and loaded onto a XAD-4 column 10/3 (Serva). The column was washed with 60 ml $H_2O$ and the corrinoids subsequently eluted with 10 ml methanol. The methanol fraction was flash-evaporated at 30°C, the residue redissolved in 1 ml $H_2O$ and analyzed by

reversed phase HPLC (C18 Nucleosil 120-10, 3.9 mm x 21 cm column) equilibrated in a mixture of 17 mM aqueous acetic acid and methanol in a 23/77 (vol/vol) ratio. Corrinoids were eluted (12 ml/min) with a linear gradient of the same solvent components from 23/77 to 40/60 in 30 minutes. The elution was monitored spectrophotometrically at 254 and 546 nm and 0.5 ml fractions were collected, which were subsequently analyzed in a Packard Minaxi auto-gamma 5000 gamma counter.

### 4.3.4 CmuA purification

Ammonium sulfate (final concentration, 1.64 M) was added to crude cell-free extract of *M. chloromethanicum* strain CM4 and the resulting suspension centrifuged at 45,000 g for 60 min. After further ammonium sulfate addition to the resulting supernatant (final concentration 2.46 M), the precipitate was harvested by centrifugation (45,000 g, 60 min) and treated with 20 ml 1.64 M ammonium sulfate in 50 mM sodium phosphate buffer (pH 8.0). After clearing by centrifugation (45,000 g, 60 min), the supernatant was filtered through a 0.45 μm filter disk (Millipore) and loaded on a Source 15ISO hydrophobic interaction column 10/10 (Pharmacia) connected to a BioCAD SPRINT apparatus (PerSeptive Biosystems). Protein was fractionated at a flow rate of 1.5 ml $min^{-1}$ with a stepwise gradient from 1.64 M to 0.82 M ammonium sulfate in 50 mM sodium phosphate buffer, pH 8.0. Fractions eluting between 1.25 and 1.0 M ammonium sulfate were pooled, concentrated using a Biomax 50 K centrifugal filter (Millipore), and eluted with 50 mM Tris chloride pH 8.0 through a PD10 size exclusion cartridge (Pharmacia) equilibrated in the same buffer. The protein fraction was then applied to a MonoQ anion exchange column 5/5 (Pharmacia) and eluted at a flow rate of 1.5 ml $min^{-1}$ in a gradient of 0-200 mM NaCl in 50 mM Tris chloride, pH 8.0. Purified CmuA protein eluted at a concentration of approximately 125 mM NaCl.

### 4.3.5 Molecular mass determination

The apparent molecular mass of the CmuA protein was determined by analytical gel filtration on a Superdex 200 column (Pharmacia) equilibrated and eluted in 50 mM Tris chloride (pH 8.0) containing 200 mM NaCl. The column

was calibrated using ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and RNAse (13.7 kDa) as reference proteins.

### 4.3.6 Other protein chemical methods

Protein was determined by the method of Bradford [67] using a commercial reagent (BioRad) and bovine serum albumin (Sigma) as the standard. Protein purification was followed by SDS-PAGE (12% acrylamide) and Coomassie blue staining. UV-visible absorbance spectra were recorded on a Hitachi U3300 spectrophotometer (1 nm interval, 300 nm $min^{-1}$), at room temperature in quartz cuvettes of 1 cm pathlength. MALDI-TOF analysis was performed on a Voyager-DE Elite (Perseptive Biosystems) mass spectrometer operated in the reflection mode, using 2,5-dihydroxybenzoic acid as the matrix. Two-dimensional gel electrophoresis was carried out as previously described [101] using the broad pI calibration kit (Pharmacia) for pI determination. ICP analysis was performed in metal-free 5 mM Tris chloride buffer pH 8.0 on a SCIEX ELAN 6100 DRC ICP-MS (Perkin Elmer) in the Laboratory of Inorganic Chemistry ETH Zürich (Prof. D. Günther). The N-terminal sequence of the CmuA protein was determined by Edman degradation (Applied Biosystems 476A) after electrophoresis of the purified protein by 8% SDS-PAGE and electroblotting of the protein band onto a PVDF membrane (Immobilon-P, Millipore) as described by the manufacturer.

### 4.3.7 Enzyme assays

Methyl transfer from chloromethane catalyzed by the CmuA protein was determined by following $CH_3$-$H_4$folate formation in a coupled assay with CmuB protein purified from *E. coli* (see Chapter 3). Methyl-$H_4$folate formation was quantified by HPLC with spectrophotometric detection at 320 nm as described previously (see Chapter 2). Solutions were made anoxic by degassing with $N_2/H_2$ (95/5 [vol/vol]) and enzyme assays were performed under the same atmosphere. Reactions (500 µl liquid volume) were performed at 30°C in 9 ml crimp vials sealed with gas-tight butyl rubber stoppers in 100 mM Tris sulfate buffer (pH 8.7) containing 2.4 mM $H_4$folate (of which 50% was biologically

available, [66]), 2 mM titanium(III)citrate, 1.82 μM purified CmuB protein and CmuA containing protein fraction (4-12 $\mu g \cdot ml^{-1}$). The enzymatic reaction was started by addition, through the rubber stopper with a gas-tight syringe, of 200 μl (8.9 μmol) chloromethane corresponding to an initial concentration of 2.1 mM in the liquid phase assuming a Henry constant of 0.43 [65]. One unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol $CH_3$-$H_4$folate per minute.

The halomethane:halide exchange activity of the CmuA protein was measured by a previously described method [29] in a total reaction volume of 1 ml comprising 50 mM sodium phosphate buffer (pH 7.0), 5 mM DTT and 2 mg cell-free extract or 50 μg (0.75 μM) purified CmuA protein using the same vials as above. Chloromethane (0.5 mM based on the liquid phase volume) was added through a gas-tight syringe and the sealed vial incubated at 30°C for 1 h, after which it was opened and allowed to stand in air for 1 h. Chloromethane was again added, followed by potassium iodide (3 mM) to initiate the halide exchange reaction. The vial was sealed again, incubated at 30°C, and chloromethane consumption and iodomethane production were quantified by gas chromatography using flame ionization detection (Hewlett Packard 8700 gas chromatograph). Headspace samples (200 μl) were injected on a 180 x 0.2 cm Poropack P (80/100 mesh) column (Supelco) at an oven temperature of 150°C, with $N_2$ (30 ml $min^{-1}$) as the carrier gas. Under these conditions, chloromethane and iodomethane eluted from the column at retention times of 58 s and 91 s, respectively.

## 4.4 RESULTS

### 4.4.1 Cobalt requirement of *M. chloromethanicum* strain CM4

Sequence analysis of the DNA comprising the *cmuA* and *cmuB* genes essential for chloromethane dehalogenation and growth with chloromethane by *M. chloromethanicum* strain CM4 revealed the presence of several open reading frames encoding homologs of enzymes involved in cobalamin biosynthesis (see Chapter 2). The *cmuA* gene appeared to encode a corrinoid binding domain in its C-terminal half (see Chapter 2). We thus investigated the cobalt requirement and the corrinoid content of *M. chloromethanicum* strain CM4 growing with chloromethane. Growth of this bacterium on a mineral salts medium with chloromethane as the only carbon and energy source showed a strict dependence on the presence of cobalt in the cultivation medium (Fig. 4.2), whereas no such requirement was observed with methylamine (Fig. 4.2) or methanol (not shown).

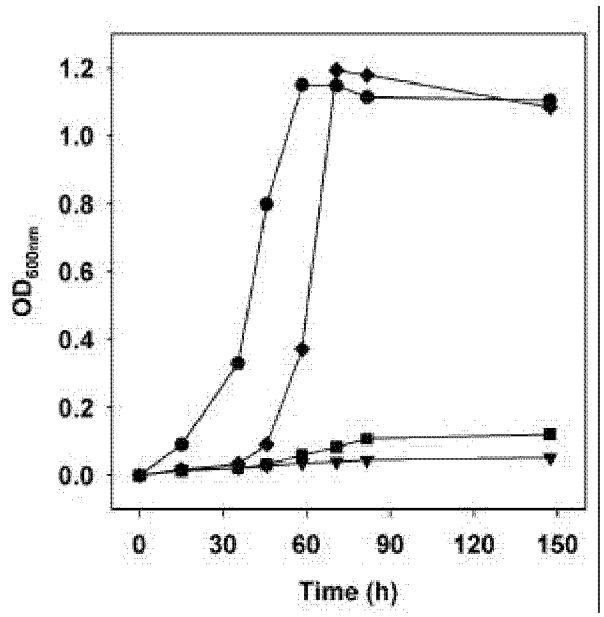


**Fig. 4.2. Cobalt dependence of growth of *M. chloromethanicum* strain CM4 with chloromethane.** Minimal medium (100 ml) devoid of $Co(NO_3)_2 \cdot 6H_2O$ was inoculated to a final $OD_{600}$ of 0.01 with a preculture grown with 50 mM methylamine and supplemented with 5 % (v/v) chloromethane together with either 86 nM (♦), 8.6 nM (n) or no $Co(NO_3)_2 \cdot 6H_2O$ (? ), or with 50 mM methylamine without $Co(NO_3)_2 \cdot 6H_2O$ (1) as a control.

HPLC analysis of the cyano-corrinoids extracted from *M. chloromethanicum* strain CM4 grown with chloromethane in the presence of radiolabelled [$^{57}$Co]-cobalt chloride then showed that the major radioactive fraction (53% of the total radioactivity) co-chromatographed with authentic vitamin $B_{12}$, suggesting that *M. chloromethanicum* strain CM4 requires vitamin $B_{12}$ for growth with chloromethane, and that it can synthesize it *de novo*. In contrast, the chloromethane degrading strain CC495 depended on supplementation with cyanocobalamin for growth with chloromethane [29]. The estimated vitamin $B_{12}$ content of *M. chloromethanicum* strain CM4 was about 4 nmol/g dry weight, at the low end of the range reported for both aerobic and anaerobic prokaryotes [102,103].

### 4.4.2 Purification of the CmuA protein

Initial fractionation of cell-free extract by anion exchange chromatography led to complete loss of chloromethane:$H_4$folate methyltransferase activity, suggesting that proteins involved in this reaction were being separated from each other in this process. Addition of purified CmuB protein with methylcobalamin:$H_4$folate methyltransferase activity (see Chapter 3) to the collected fractions restored $CH_3$-$H_4$folate formation from chloromethane in some fractions. This observation was exploited for the purification to apparent homogeneity of a 67 kDa protein required for chloromethane dehalogenase activity in addition to CmuB, by a combination of ammonium sulfate fractionation, hydrophobic-interaction chromatography and anion-exchange chromatography under aerobic conditions (Fig. 4.3, Table 4.1). The purified protein was of the size expected for CmuA, and Edman degradation yielded a 10-residue N-terminal sequence corresponding to that predicted for CmuA from the sequence of the corresponding gene.

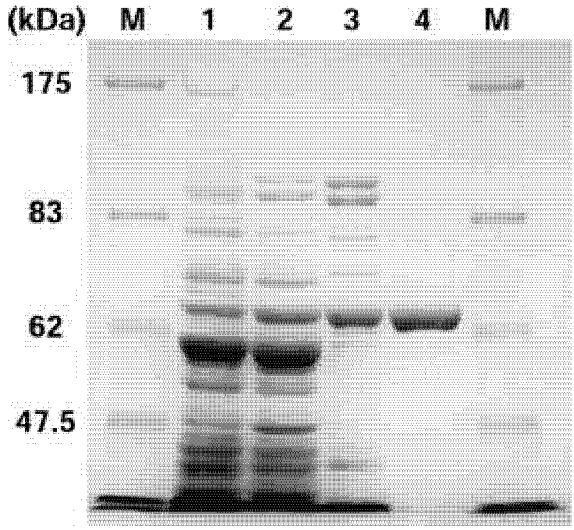


**Fig. 4.3. CmuA purification.** Protein samples (15 μg) were analyzed at different stages of purification on a 8 % SDS-PAGE gel stained with Coomassie Brilliant Blue. Lane M, molecular mass markers in kDa; lane 1, cell-free extract of chloromethane grown cells; lane 2, ammonium sulfate fraction (40-60% saturation); lane 3, Source 15ISO hydrophobic interaction chromatography fraction; lane 4, purified CmuA protein obtained after MonoQ anion exchange chromatography.

**Table 4.1. Purification of the CmuA protein from *M. chloromethanicum* strain CM4**

| Purification step | Total protein | Total activity | | Specific activity | Purification |
|---|---|---|---|---|---|
| | (mg) | (U) | (%) | (mU mg-1) | (-fold) |
| **Cell-free extract** | 1449.0 | 33.6 | 100.0 | 23.2 | 1.0 |
| **$(NH_4)_2SO_4$** | 468.9 | 34.5 | 102.6 | 73.6 | 3.2 |
| **Source 15ISO** | 39.0 | 9.5 | 28.3 | 248.9 | 10.7 |
| **MonoQ** | 1.9 | 1.1 | 3.7 | 577.3 | 28.7 |

### 4.4.3 Molecular properties of the CmuA protein

Purified CmuA eluted as a symmetrical peak with a molecular mass of 67 kDa upon gel filtration on Superdex 200 (data not shown), in good agreement with the apparent molecular mass of 66 kDa estimated by SDS-PAGE (Fig. 4.3) and the relative molecular mass of 66988 Da calculated from the gene sequence, suggesting that the CmuA protein is monomeric. In two-dimensional SDS-PAGE, the CmuA protein migrated to an isolectric point of 4.8 ± 0.1, compared to a value of 5.3 calculated from the CmuA amino acid sequence. A solution of purified CmuA protein was of light orange color. As isolated, the CmuA protein displayed an UV-Vis spectrum similar to that of the vitamin $B_{12}$-containing methionine synthase [104], with an absorption peak at 470 nm (Fig. 4.4) typical of the spectrum of free cobalamin in the Co(II) form [105]. Upon reduction of the protein with titanium(III)citrate, the apparition of a sharp absorption peak at 390 nm signalled a change to the Co(I) state (Fig. 4.4, [105]).

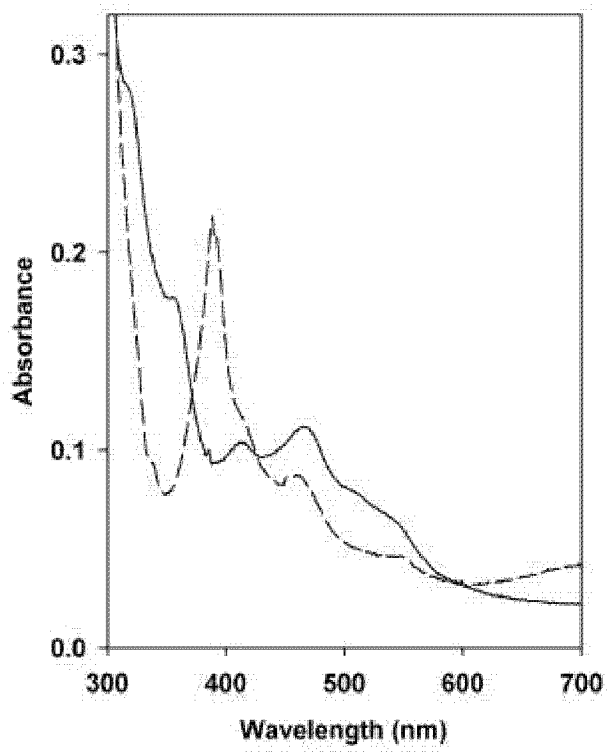


**Fig. 4.4. UV-visible spectra of purified CmuA protein in the oxidized and reduced states.** Purified CmuA protein (0.24 mg in 100 µl 50 mM Tris chloride (pH 8.0) containing 10 % glycerol) was analyzed as isolated in air after MonoQ ion exchange chromatography (continuous line), and after reduction with 0.5 mM titanium(III)citrate (dotted line).

The purified CmuA protein was analyzed by MALDI-TOF to determine the size of its corrinoid cofactor. An intense signal was obtained at a molecular mass of 1329.3 ± 0.5, corresponding to the mass of vitamin $B_{12}$ (1329.4). Further, ICP spectrometry demonstrated the presence of 0.68 mol Co as well as 0.89 mol Zn per mol purified CmuA protein, suggesting partial loss of CmuA-bound vitamin $B_{12}$ cofactor during purification.

### 4.4.4 Chloromethane:$H_4$folate transferase activity

Purified CmuA and CmuB (see Chapter 3) proteins in combination were sufficient to catalyze methyl-group transfer from chloromethane to $H_4$folate (Fig. 4.5), but neither CmuA nor CmuB protein was able to do so by itself (Fig. 4.5).

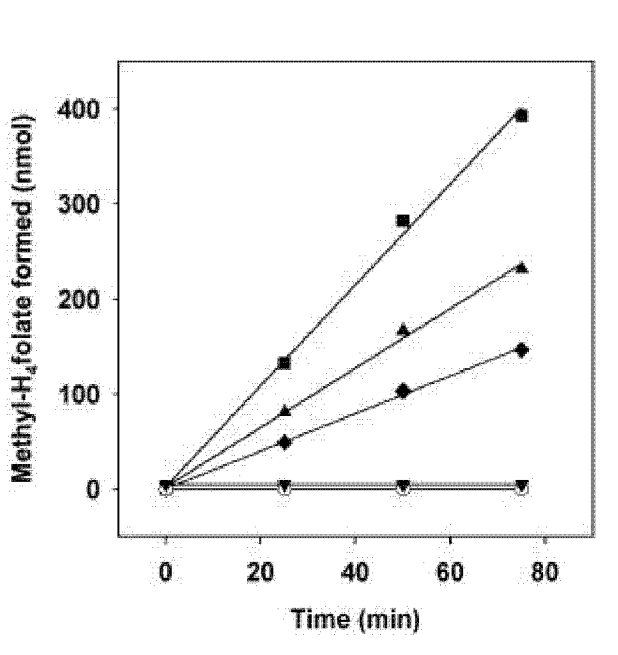


**Fig. 4.5. *In vitro* reconstitution of chloromethane:$H_4$folate methyltransferase activity.** Time course of $CH_3$-$H_4$folate formation in 500 µl 100 mM Tris sulfate (pH 8.7) containing 30.2 µg (0.91 nmol) purified CmuB methylcobalamin:$H_4$folate methyltransferase (see Chapter 3), 2.4 mM $H_4$folate, 2 mM titanium(III)citrate, 2.1 mM chloromethane (in the liquid phase) and different amounts of purified CmuA protein: no CmuA added (○), 1.65 µg (25 pmol) CmuA (♦), 3.3 µg (50 pmol) CmuA (? ), and 6.6 µg (100 pmol) CmuA (■); (? ), 5 µg (75 pmol) CmuA in the absence of CmuB protein.

Notably, however, the rate of $CH_3$-$H_4$folate formation measured under standard assay conditions (Fig. 4.5) was not proportional to the amount of CmuA protein in the reaction mixture (see below and Fig. 4.6B). The effect of pH on enzyme activity was examined over a range from pH 5.5 to pH 9.5, and

maximal chloromethane:$H_4$folate methyltransferase activity was found at pH 8.7 in Tris sulfate buffer. Exposure of CmuA to oxygen led to complete inactivation. Activity was restored under anoxic conditions by addition of the low-potential electron donor titanium(III)citrate. There was no indication for the requirement of an ATP- and reductant-dependent reactivation system [60] to maintain the corrinoid bound by CmuA in its active Co(I) state. The purified CmuA enzyme retained full activity after two months of storage at -80°C in 50 mM Tris sulfate buffer (pH 8.0) containing 10 % glycerol.

In view of the sequence similarity of CmuA to methylcobamide:CoM methyltransferases from Archaea in its N-terminal half (see Chapter 2), the possibility of coenzyme M (CoM) being an alternative methyl acceptor in chloromethane dehalogenation was investigated. However, inclusion of coenzyme M up to 14 mM showed no effect on chloromethane:$H_4$folate methyltransferase activity under standard assay conditions.

Chloromethane:$H_4$folate methyltransferase activity was also determined in the presence of various potential inhibitors. Metal chelating agents appeared to show little effect, as EDTA had no effect on enzyme activity up to a concentration of 20 mM, and 50 % inhibition by *o*-phenanthroline required a 2 mM concentration of this compound. Similar findings were reported for the zinc-dependent methionine synthase MetE of *E. coli* with EDTA [106]. In contrast, methylcobamide:CoM methyltransferase activity of *M.barkeri* was shown to be reduced by half upon addition of 5 μM EDTA only [88]. The thiol active agent N-ethylmaleimide (NEM), in contrast, caused 50 % inhibition at 0.6 mM and complete inhibition at 1 mM. Inhibition by NEM was not reversible by dilution and independent of the presence of titanium(III)citrate during preincubation.

### 4.4.5 Dependence of the chloromethane:$H_4$folate methyltransferase activity on the ratio of CmuA to CmuB

The overall rate of the reaction catalyzed by CmuA and CmuB, as measured by $CH_3$-$H_4$folate production, increased linearly with the amount of CmuB protein up to at least a 35-fold molar excess of CmuB over CmuA (Fig. 4.6A). In the presence of saturating chloromethane (2.1 mM in liquid phase), it reached 1.6 μmol $min^{-1}$ $mg^{-1}$ CmuA protein. In contrast, the reaction followed apparent

Michaelis-Menten kinetics when CmuB was kept constant and CmuA was varied (Fig. 4.6B), allowing an apparent $K_m$ of 0.27 μM with respect to CmuA and a $V_{max}$ of 0.45 U $mg^{-1}$ to be determined.

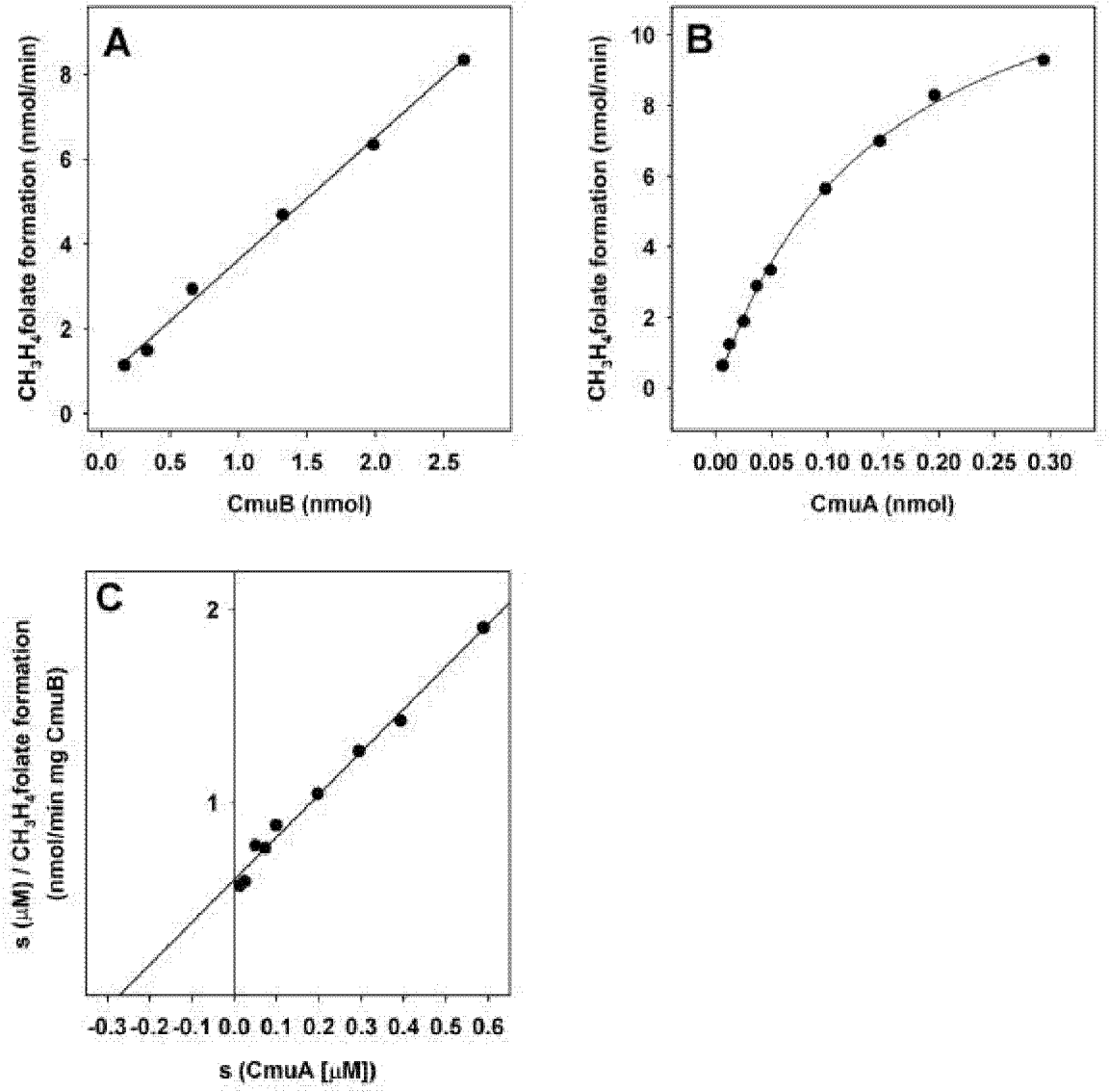


**Fig. 4.6. Dependence of chloromethane:$H_4$folate methyltransferase activity on the ratio of CmuA to CmuB in the reaction mixture.** Reactions were performed as described (Fig. 4.5, legend) with variable amounts of CmuA and CmuB proteins. (A) Rate of $CH_3$-$H_4$folate formation from chloromethane in the presence of purified CmuA (5 μg, 75 pmol) and variable amounts of purified CmuB protein. (B) Rate of $CH_3$-$H_4$folate formation from chloromethane in the presence of purified CmuB (30.2 μg, 0.91 nmol) and variable amounts of purified CmuA protein. (C) Hanes plot of the data from Fig. 4.6B.

The specificity of the interaction between CmuA and CmuB proteins in this reaction is emphasized when these parameters are compared with the lack of saturation observed previously for the CmuB protein with methylcobalamin as the methyl donor, where the observed rate of $CH_3$-$H_4$folate formation was proportional to the concentration of the methylcobalamin substrate up to 2 mM (see Chapter 3). In other words, it appears that methylated CmuA is the specific physiological substrate for CmuB which, under the chosen assay conditions, is rate-limiting for the chloromethane:$H_4$folate methyltransferase reaction.

### 4.4.6 Chloromethane:halide methyltransferase activity

The molecular mass and the N-terminal sequence that were reported for the cobalt-dependent methyltransferase protein purified from the chloromethane-degrading strain CC495 [29] are similar to those of CmuA. The protein from strain CC495, however, was characterized as a halomethane:bisulphide/halide ion methyltransferase catalyzing the interconversion of methyl halides, with a specific activity of 58.9 nmol $min^{-1}$ $mg^{-1}$ for the conversion of chloromethane to iodomethane in the presence of iodide. This activity was also measured for CmuA from *M. chloromethanicum* strain CM4, albeit at a lower rate (16.3 $\pm$ 0.5 nmol $min^{-1}$ $mg^{-1}$). This rate corresponded to an approximately 20-fold increase in specific activity from cell-free extract (0.8 $\pm$ 0.1 nmol $min^{-1}$ $mg^{-1}$), in reasonable agreement with the increase in chloromethane:$H_4$folate methyltransferase activity observed for the same purified preparation of CmuA (Table 4.1). Notably, however, the chloromethane:halide methyltransferase activity of CmuA amounted to at most 5% of the measured chloromethane:$H_4$folate methyltransferase activity (Fig. 4.6A, lowest point).

## 4.5 DISCUSSION

The work described here confirms that the initial dehalogenation step in the *M. chloromethanicum* strain CM4 pathway for chloromethane utilization requires two proteins, CmuA and CmuB, which are functionally similar to components of the *Methanosarcina barkeri* systems for the utilization of methylamines and methanol as growth substrates [107,108]. The latter archaeal pathways for methanogenesis from monomethylamine [109], dimethylamine [108], trimethylamine [110] or methanol [83] all involve three proteins. These are a pathway-specific methyltransferase I, a cognate corrinoid protein, and a methyltransferase II that transfers the methyl group from the corrinoid protein to CoM. The interaction of the methyltransferases I with the corresponding corrinoid proteins is specific, whereas a single methyltransferase II (MtbA) demethylates the three corrinoid proteins carrying methyl groups originating from mono,- di- or trimethylamine, and another methyltransferase II (MtaA) preferentially demethylates the corrinoid protein involved in the methanol utilization pathway [82]. For methanogenesis from dimethylsulfide in *M. barkeri* only two proteins are required [111]. The same protein (MtsA) methylates and demethylates its cognate corrinoid protein (MtsB) and thus exhibits both methyltransferase I and II activity [112]. In some cases, the interaction of methyltransferase I with its matching corrinoid protein involves the formation of a stable complex between the two components [108,112]. In the chloromethane utilization system of *M. chloromethanicum* strain CM4, the association between a methyltransferase I and its cognate corrinoid protein appears to have reached an extreme in that the two corresponding homologs are linked in the single polypeptide CmuA, resulting in a methyl transfer pathway that involves the fusion protein CmuA and another methyltransferase, protein CmuB.

Whereas the analogy between the chloromethane methyl transfer system of *M. chloromethanicum* and the *M. barkeri* systems holds at the level of overall organization of these proteins, it does not when the cosubstrates used by the different systems are compared. In the chloromethane system, the metabolic methyl group acceptor is $H_4$folate, in the archaeal systems it is CoM. The

archaeal methyltransferases II thus catalyze methyl transfer onto the thiol group of CoM whereas CmuB methylates $H_4$folate. This difference is reflected by sequence comparisons. The archaeal representatives MtaA, MtbA, and MtsA are about 50% identical [70,88,113], but unrelated to CmuB, whose sequence is more similar to that of subunit H of the methyl-$H_4$MPT:CoM methyltransferase from methanogens (see Chapter 3). MtrH is thought to catalyze the transfer of a methyl group from $H_4$MPT to a cognate corrinoid protein [91], which represents a reversal of the physiological reaction proposed for CmuB in the aerobic chloromethane degradation pathway.

In contrast to CmuB, the archaeal methyltransferases II also contain a conserved zinc binding motif (H-X-C $X_n$-C) typical of enzymes that catalyze the alkylation of a thiol group, such as the cobalamin-independent methionine synthases of various organisms [114] and the epoxyalkane:CoM methyltransferase of *Xanthobacter* strain Py2 [115]. Indeed, MtaA, the methyltransferases II involved in methanol utilization, and MtsA, the bifunctional methyltransferase involved in dimethylsulfide utilization by *M. barkeri*, have been shown to contain 1 mol of zinc per mol of enzyme [88,116]. Zinc was found to be essential for catalysis, presumably acting in the activation of the thiol group of CoM for nucleophilic attack of the corrinoid-bound methyl group [107]. The N-terminal part of CmuA displays evident similarity to both MtaA and MtsA of *M. barkeri* (see Chapter 2), and also features the corresponding zinc-binding motif [114]. In the present work, we have determined that purified CmuA contains 0.9 mol of zinc per mol of protein. Thus, by analogy to the reaction catalyzed by its methanoarchaeal counterpart, the N-terminal domain of CmuA may catalyze methyl transfer by means of a zinc-activated protein thiol. The inhibition of chloromethane:$H_4$folate methyltransferase activity by NEM observed here, while rather weak, constitutes preliminary experimental evidence in favor of such a hypothesis. Another possibility is that zinc is capable of activating chloromethane to facilitate nucleophilic attack by the corrinoid as previously demonstrated for MtaB in methanol utilization [83]. However such a role of zinc seems questionable since vitamin $B_{12}$ in the Co(I) state is a powerful nucleophile and readily reacts with chloromethane in aqueous solution with a

pseudo-first-order rate constant in the order of 30 $M^{-1}$ $s^{-1}$ [77]. It thus remains to be investigated whether a CmuA-encoded methyltransferase activity is actually required for methyl transfer to CmuA-bound vitamin $B_{12}$.

The specific activity of chloromethane:$H_4$folate methyltransferase in crude extracts of *M. chloromethanicum* was 23 nmol·$min^{-1}$$mg^{-1}$ protein under standard assay conditions (Table 4.1), a value amounting to only 3% of the specific activity observed in bacteria growing with chloromethane at a rate of 0.12 $h^{-1}$ [48]. With purified CmuA and CmuB high specific chloromethane:$H_4$folate methyltransferase activities are only achieved when CmuB is present in large excess to CmuA (Fig. 4.6A). This is surprising given that an approximately equimolar ratio of protein components yielded maximal activity in other methyltransferase systems [108,117]. The difference in the concentrations of CmuA and CmuB needed for maximum activity of chloromethane $H_4$folate methyltransferase *in vitro* cannot be explained by the relative content of these proteins in the cell, estimated at a few percent of the total soluble protein in both cases (see Chapter 2 and Table 4.1). It is tempting to speculate that CmuA, in addition to serving as a substrate for the $H_4$folate-specific CmuB methyltransferase, also is a substrate for other yet uncharacterized methyltransferases. *Methylobacterium extorquens* AM1 was shown to possess a tetrahydromethanopterin-dependent pathway, which is likely to be essential for the oxidation of $C_1$ units to $CO_2$, in addition to the usual $H_4$folate-dependent pathway of $C_1$-metabolism [44]. Hence, the presence in strain CM4 of another methyltransferase able to catalyze the transfer of a methyl group from CmuA to tetrahydromethanopterin is an attractive hypothesis. The CmuC protein encoded directly downstream of the *cmuB* gene in *M. chloromethanicum* strain CM4 is a possible candidate for such a methyltransferase. Its sequence shows similarity to some methyltransferases, and its mutational inactivation leads to lack of growth with chloromethane (see Chapter 2).

# Chapter 5

## Chloromethane induced genes that encode a third $C_1$ oxidation pathway in *Methylobacterium chloromethanicum* CM4

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Thomas LEISINGER and Stéphane VUILLEUMIER

## 5.1 ABSTRACT

*Methylobacterium chloromethanicum* CM4 is an aerobic α–proteobacterium which is capable of growth on chloromethane as sole energy and carbon source. The proteins CmuA and CmuB were previously shown to catalyze the dehalogenation of chloromethane by a vitamin $B_{12}$ mediated methyl group transfer to tetrahydrofolate. A set of genes, designated *metF*, *folD* and *purU,* located nearby the *cmuA* and *cmuB* genes suggested the presence of an oxidation pathway from methyl-tetrahydrofolate to formate. Southern blot analysis indicates that these genes are distinct for CM4 and are not present in other *Methylobacterium* strains. Studies with transcriptional *xylE* fusions demonstrated chloromethane-dependent expression of these genes. Transcriptional start sites mapped by primer extension led to the identification of three promoter regions specifically active during growth with chloromethane. The promoters display a high degree of conservation and are structurally different from the *Methylobacterium* promoters described so far. Mutational inactivation of the *metF* and *purU* genes resulted in strains deficient in growth with chloromethane. Complementation of these mutants and the expression patterns observed with transcriptional *xylE* fusions suggest the presence of at least three transcriptional units, each of them comprising several genes. Taken together this is evidence that *M. chloromethanicum* CM4 requires a set of tetrahydrofolate-dependent enzymes for growth with chloromethane.

## 5.2 INTRODUCTION

Aerobic methylotrophic $\alpha$-proteobacteria of the genus *Methylobacterium* are capable of growth with methanol and methylamine as sole carbon and energy source [42]. These substrates are oxidized via formaldehyde, a central intermediate in methylotrophic metabolism [118]. Formaldehyde is then either assimilated into cell material by means of the serine cycle, or completely oxidized to carbon dioxide [42]. Historically, the oxidation of formaldehyde was thought to proceed via a linear pathway, involving the sequential action of a formaldehyde dehydrogenase and formate dehydrogenase. However, the formaldehyde dehydrogenases described for *Methylobacterium* are non-specific aldehyde dehydrogenases with often low specific activities with formaldehyde [119]. It was therefore considered questionable that this pathway is responsible for growth of *Methylobacterium* with $C_1$ compounds [42,119]. An alternative hypothesis indicated that formaldehyde oxidation proceeds via pterin-dependent intermediates [120]. Indeed, two pterin-dependent pathways have recently been shown to be essential for growth with methanol in *Methylobacterium extorquens* AM1 (Fig. 5.1, [43,121]). One of these pathways is tetrahydrofolate ($H_4$folate)-dependent and it has so far been described only in *M. extorquens* AM1. The other pathway is tetrahydromethanopterin ($H_4$MPT)-dependent and appears to be present in most methylotrophic bacteria [46]. It was proposed that the $H_4$MPT-dependent pathway predominantly operates in the oxidative direction, whereas the $H_4$folate-dependent pathway works in either direction, depending upon the cellular pools of $C_1$ intermediates available for biosynthesis or energy generation [43-45].

With 16S rDNA sequence identity of 98%, *Methylobacterium chloromethanicum* CM4 is closely related to *M. extorquens* AM1 [33]. Central $C_1$ metabolism is thus expected to be similar in these bacteria. *M. chloromethanicum* CM4, however, is distinct from all other *Methylobacterium* species described so far in its ability to grow with chloromethane as sole carbon and energy source [30]. Physiological and genetic studies demonstrated that the *cmuA* and *cmuB* genes are essential for growth on chloromethane and that

they encode the proteins responsible for the dehalogenation of the compound (see Chapter 2, [48]). These two proteins were recently purified and were demonstrated to catalyze the dehalogenation of chloromethane *in vitro* (see Chapter 3 and 4).

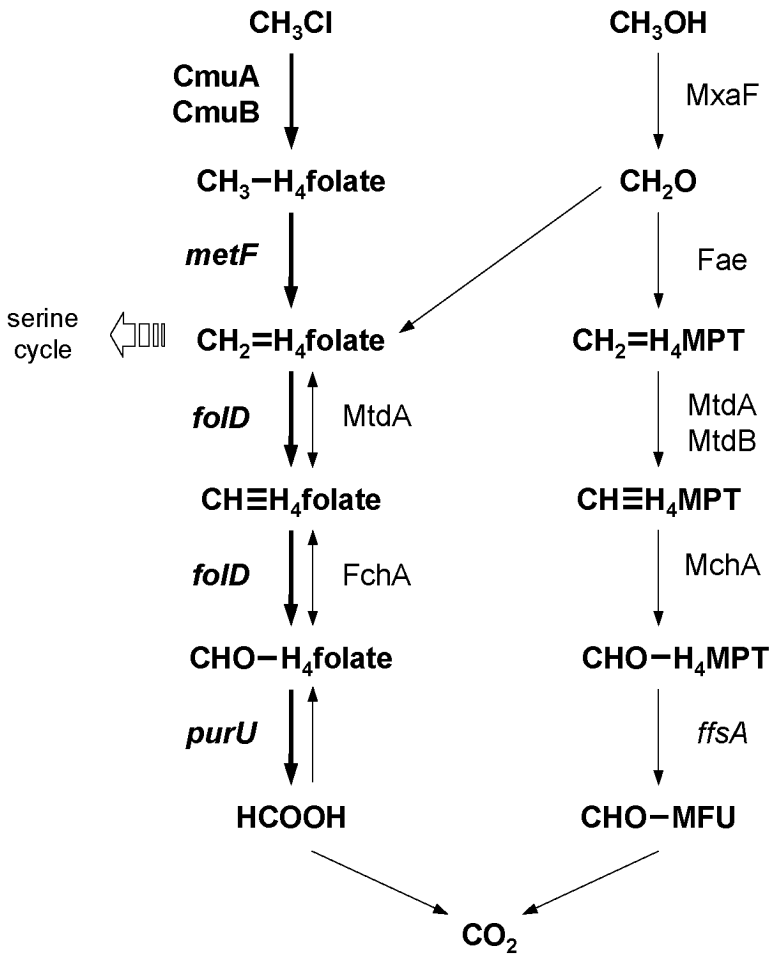


**Fig. 5.1. Pterin-dependent $C_1$ metabolism in *M. chloromethanicum* CM4.** Transformations shown by light arrows indicate reactions common to methylotrophs and previously demonstrated to be essential for growth with methanol of *M. extorquens* AM1. Transformations shown by fat arrows are specific for *M. chloromethanicum* CM4. CmuA, chloromethane:corrinoid methyltransferase; CmuB, methylcobalamin:$H_4$folate methyltransferase; *metF,* methyl-$H_4$folate reductase; *folD*, bifunctional methylene-$H_4$folate dehydrogenase/cyclohydrolase; *purU*, 10-formyl-$H_4$folate hydrolase; MtdA, NADP-dependent methylene-$H_4$folate dehydrogenase; FchA, methenyl-$H_4$folate cyclohydrolase; Fae, formaldehyde activating enzyme; MtdB, NAD(P)-dependent methylene-$H_4$MPT dehydrogenase; MchA, methenyl-$H_4$MPT cyclohydrolase; *ffsA*, formylmethanofuran:$H_4$MPT formyltransferase.

Dehalogenation occurs by a methyl group transfer from chloromethane onto a vitamin $B_{12}$ cofactor bound to the protein CmuA. The methyl group is then transferred from CmuA to $H_4$folate by CmuB, an enzyme specific for $H_4$folate and unable to catalyze methyl-transfer to $H_4$MPT (see Chapter 3). The formation of methyl-$H_4$folate suggests that the degradation of chloromethane does not involve formaldehyde as an intermediate, as it is the case for other $C_1$ substrates in *Methylobacterium* [118]. In fact, it appears more likely that the methyl-$H_4$folate derived from chloromethane is oxidized to formate via $H_4$folate-linked intermediates in *M. chloromethanicum* CM4 (Fig. 5.1). The presence of genes *metF*, *folD* and *purU* encoding proteins similar to $H_4$folate-dependent enzymes nearby *cmuA* and *cmuB* in the CM4 genome is supportive of a $H_4$folate-dependent oxidation pathway (see Chapter 2). This pathway, however, would be distinct from the $H_4$folate-dependent oxidation pathway already known in *M. extorquens* AM1 [43,121]. Indeed, the latter involves two proteins, the methylene-$H_4$folate dehydrogenase MtdA [44] and methenyl-$H_4$folate cyclohydrolase FchA [45], which are different from the bifunctional methylene-$H_4$folate dehydrogenase/cyclohydrolase potentially encoded by *folD* in strain CM4.

It is not obvious why *M. chloromethanicum* CM4 would need yet a third formaldehyde oxidation pathway in addition to the two already described. In this contribution we apply transcriptional reporter gene fusions, analysis of transcription start sites and mutational inactivation to explore whether the genes encoding this pathway are specific for growth with chloromethane.

## 5.3 EXPERIMENTAL PROCEDURES

### 5.3.1 Materials

Restriction endonucleases, T4 ligase and T4 polymerase were obtained from MBI Fermentas. *Pfu* DNA polymerase was from Stratagene. All other chemicals were from Fluka, Sigma or Merck.

### 5.3.2 Bacterial strains and plasmids

The bacterial strains and plasmids used in this work are listed in Table 4.1. Conjugation of plasmids into *M. chloromethanicum* CM4 from *Escherichia coli* donor strains S17-1 or S17-1(λpir) and selection of transconjugants were performed as described previously [122].

### 5.3.3 Media and growth conditions

Luria-Bertani medium [123] was used for growth of *E. coli*. *M. chloromethanicum* was grown in 500 ml rubber-stoppered serum bottles filled with 100 ml minimal medium [48]. Chloromethane gas was added with a syringe through the rubber stopper to a final concentration of 4.5 mM (2% [vol/vol]), corresponding to an initial concentration of 1.4 mM in the liquid phase assuming a Henry constant of 0.43 [65]. Methanol, methylamine and formate were added to a concentration of 20-40 mM from sterile filtered solutions. Where required, the following concentrations (μg per ml) of antibiotics were used: ampicillin, 100; kanamycin, 25; and tetracycline 25.

### 5.3.4 DNA manipulations

Preparation of total and plasmid DNA, recombinant DNA work and Southern analysis were performed according to standard protocols [62].

**Table 4.1. Bacterial strains and plasmids used in this study.**

| Strains or plasmids | Relevant phenotype or genotype | Source or reference |
|---|---|---|
| ***E. coli*** | | |
| DH5α | *sup* E44 Δ*lac*U169 (Φ80*lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 | BRL |
| CC118(λ*pir*) | Δ(*ara-leu*), *araD*, Δ*lacX*74, *galE*, *galK*, *phoA*20, *thi*-1, *rpsE*, *rpoB*, *argE*, *recA* 1, λ *pir* lysogen | [124] |
| S17-1 | *hsd*R (RP4-2 Km$^r$::Tn*5* Tc::MU chromosomally integrated) | [125] |
| S17-1(λ*pir*) | λpir lysogen of *E. coli* S17-1 | |
| ***M. chloromethanicum*** | | |
| CM4 | wild type | [30] |
| 30F5 | *purU*::miniTn*5*-Km | [48] |
| 19D10 | *cmuB*::miniTn*5*-Km | [48] |
| 22B3 | *cmuA*::miniTn*5*-Km | [48] |
| PK1 | *metF*::pME1781 | This work |
| **Plasmids** | | |
| pBLS KS II$^+$ | Ap$^r$, cloning vector | Stratagene |
| pKNOCK-Km | Kan$^r$, broad-host-range suicide vector | [126] |
| pCM62 | Tet$^r$, broad-host range vector | [127] |
| pCM130 | Tet$^r$, *xylE* promoter-probe vector | [127] |
| pCM131 | Tet$^r$, *mxaF::xylE* fusion in pCM130 | [127] |
| pME1703 | *M. chloromethanicum* 1.9 kb *Hin*dIII fragment from *cmuB* mutant 19D10 in pBLS KS II$^+$ | this work |
| pME1742 | *M. chloromethanicum* 7.5 kb *Kpn*I fragment from *cmuC* mutant 36D3 in pBLS KS II$^+$ | this work |
| pME1712 | 8.0 kb *Cla*I chromosomal fragment in pBLS KS II$^+$ | this work |
| pME1754 | 7.0 kb *Sac*I chromosomal fragment in pBLS KS II$^+$; *purU folD folC cobU* genes | this work |
| pME1776 | 2.8 kb *Bam*HI/*Sac*I fragment from pME1754 in pCM62; contains *purU* | this work |
| pME1781 | 0.6 kb *Sma*I/*Cla*I fragment in pKNOCK-Km | this work |
| pME1789 | 2.8 kb *Hin*dIII/*Fsp*I pME1742 fragment in pCM62; contains *metF* | |
| pME1790 | 779 bp PCR fragment in pCM130; *purU::xylE* fusion | this work |
| pME1791 | 779 bp PCR fragment in pCM130; *orf414::xylE* fusion | this work |
| pME1796 | 159 bp PCR fragment in pCM130; *orf219::xylE* fusion | this work |
| pME1797 | 159 bp PCR fragment in pCM130; *metF::xylE* fusion | this work |
| pME1798 | 1.4 kb *Eco*RI chromosomal fragment in pBLS KS II$^+$; contains *purU* | this work |
| pME1799 | 1.7 kb *Hin*dIII/*Xho*I chromosomal fragment in pCM130; *metF cmuB::xylE* fusion | this work |
| pME8250 | 0.6 kb *Cla*I/*Xho*I chromosomal fragment in pCM130; *cmuB::xylE* fusion | this work |
| pME8251 | 1.4 kb *Eco*RI chromosomal fragment cloned as *Hin*dIII/*Bam*HI fragment from pME1798 into pCM130; *purU folD::xylE* fusion | this work |
| pME8252 | 0.7 kb *Xho*I/*Eco*RI chromosomal fragment cloned as *Xho*I/*Bam*HI fragment from pME1798 into pCM130; *purU::xylE* fusion | this work |
| pME8253 | 0.7 kb *EcoRI/XhoI* chromosomal fragment cloned as *Hin*dIII/*Xho*I fragment from pME1798 fragment in pCM130; *folD::xylE* fusion | this work |

### 5.3.5 Construction of *M. chloromethanicum* CM4 *metF* mutant strain

The *metF* gene was mutagenized by integration of the 2666 bp plasmid pME1781 in a single crossover event (Fig. 5.2). Plasmid pME1781 is a pKNOCK-Km [126] derivative containing the 597 bp *Sma*I/*Cla*I internal fragment of the *M. chloromethanicum* CM4 *metF* gene cloned from plasmid pME1703 (Table 5.1).

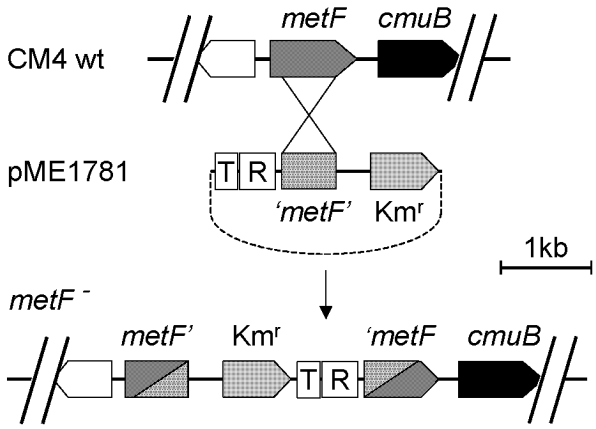


**Fig. 5.2. Construction of metF mutant RB1.** Abbreviations: T, RP4 plasmid oriT region; R, R6K γ-origin of replication; Km$^r$, Kanamycin resistance gene [126].

### 5.3.6 Construction of plasmids containing transcriptional *xylE* fusions

Transcriptional *xylE* fusions of the *folD* and *purU* genes were obtained by the insertion of different fragments from plasmid pME1742 into the *xylE* reporter plasmid pCM130 (Fig. 5.3, [127]). Plasmid pME1742 harbors a 1.4 kb genomic *Eco*RI fragment. (i) A 1.4kb *Hin*dIII/*Bam*HI fragment of plasmid pME1742 was cloned into pCM130 digested with the same restriction enzymes, yielding plasmid pME8251. (ii) A 0.7 kb *Hin*dIII/*Xho*I fragment of pME1742 with the *Xho*I site filled in was cloned into pCM130 sequentially digested with *Pst*I blunted and digested with *Hin*dIII to yield plasmid pME8252. (iii) Plasmid pME8253 harbors a 0.7 kb *Xho*I/*Bam*HI fragment and was obtained by digestion of plasmid pME8251 with *Hin*dIII and *Xho*I, filling in and religation.

The *purU-orf414* promoter region was inserted into pCM130 as a PCR fragment from pME1712 generated with primers ast26 (5'-TTCCGCCA**TCTAGA**GATTCC-3'; nucleotide position 4841-4860 in AJ011316) with a change of two bases (underlined) containing a *Xba*I site (bold) and ast25

(5'-GGCGA**CATATG**ACGGCACC-3'; nucleotide position 5635-5617 in AJ011316) with a change of two bases from the wild type CM4 sequence (underlined) to introduce a *Nde*I site. This PCR fragment was digested with *Xba*I and *Nde*I, its ends were filled in, and then cloned into the unique *Hin*dIII site of pCM130 by blunt-end ligation, yielding plasmids pME1790 (*purU::xylE* fusion) and pME1791 (*orf414::xylE* fusion).

The transcriptional *cmuB::xylE* fusions of plasmids pME1799 and pME8250 were constructed by cloning a 1.7 kb *Hin*dIII/*Xho*I genomic fragment and a 0.65 kb *Cla*I/*Xho*I genomic fragment into vector pCM130, respectively. Cohesive ends were filled in and the fragments were cloned into the unique *Hin*dIII site on pCM130 by blunt-end ligation.

The *orf219-metF* promoter region was PCR-amplified from plasmid pME1742 using the primers ast30 (5'-GCTTTC**GGATCC**ATCAGACG-3'; nucleotide position 3332-3351 in AJ011317) and ast31 (5'-CCACATATGC**GGATCC**$GTC_{3494}$-3'; nucleotide position 3512-3494 in AJ011317). A BamHI site (bold) was introduced in primer ast30 and changes to the wild type CM4 sequence are underlined. The PCR fragment was then cloned as a *BamHI* fragment into pCM130 digested with the same enzyme, resulting in plasmid pME1796 (*orf219::xylE* fusion) and pME1797 (*metF::xylE* fusion, opposite orientation of insert).

### 5.3.7 Catechol-2,3-dioxygenase activity

*M. chloromethanicum* CM4 cultures were grown at 30°C and harvested in the exponential phase ($A_{600}$ 0.6-0.7). Samples (1.5 ml) were centrifuged and resuspended in 0.2 ml cold catechol-2,3-dioxygenase (C23O) assay buffer (50 mM potassium phosphate pH 7.5, 10% acetone). A 50 µl aliquot was added to 1 ml of C23O buffer, the reaction was initiated by the addition of 10 µl of 0.1 M catechol-1,2-dihydroxybenzene and the change in absorbance at 375 nm was recorded at 25°C. A molar extinction coefficient ($\varepsilon_{375}$) of $4.4 \times 10^4$ was used for calculating the specific activity of C23O [128], which was expressed as nmol product liberated per min per $10^8$ cells assayed. An $A_{600}$ of 1 was shown to correspond $4 \times 10^8$ cells per ml (M. Kayser, unpublished data).

### 5.3.8 RNA isolation

Total RNA was isolated from *M. chloromethanicum* CM4 cells grown to $A_{600}$ of 0.6-0.7 according to Völker et al. [129]. A culture aliquot (25 ml) was added to 20 ml of frozen and crushed 20 mM Tris chloride buffer pH 7.5 containing 5 mM $MgCl_2$ and 20 mM $NaN_3$. Cells were harvested by centrifugation (20000 g, 15 min), resuspended in 2.5 ml ice cold 1 mM EDTA in 20 mM sodium acetate pH 5.5, and the aqueous phase extracted with 2.5 ml of prewarmed (65°C) acidic phenol (pH 5.5) containing 0.5% sodium dodecyl sulfate (wt/vol). The aqueous phase was further extracted with 2.5 ml of phenol:chloroform:isoamylalcohol (49.5:49.5:1) and then with 2.5 ml of dichloromethane. The RNA was precipitated with 2.5 volumes of ethanol and dissolved in diethyl pyrocarbonate-treated $H_2O$ (0.1%).

### 5.3.9 Mapping of transcriptional start sites

*M. chloromethanicum* CM4 was grown on either 40 mM methanol or 5% chloromethane, and RNA was isolated as described above. Approximately 15-20 μg of RNA and 2 – 3 x $10^5$ cpm of radiolabeled primer were used for primer extension experiments, which were performed as previously described [130]. Extension products were purified by phenol extraction followed by ethanol precipitation, before separation on 6% denaturing polyacrylamide gels. The primers used were ast27 (5'-CGCACCTGAAACGGCAGCGACGATGC-3'; nucleotide position 4775-4800 in AJ011316) for *purU*, ast32 (5'-CGACAGACCCGAACCTCGCCATTGG–3'; nucleotide position 5509-5533 in AJ011316) for *orf414* and ast33 (5'- GGGAGACCTCCAATGACAGATCGCG–3'; nucleotide position 3627-3603 in AJ011317) for *metF*. Sequencing reactions were carried out with the same primers and a suitable plasmid as template, using the fmol cycle sequencing kit (Promega).

## 5.4 RESULTS

### 5.4.1 Identification of genes encoding pterin-dependent $C_1$ oxidation enzymes in *Methylobacterium*

Measurements of enzymes involved in the interconversion of $H_4$folate derivatives in cell-free extracts from wild type *M. chloromethanicum* CM4 grown on chloromethane or methanol did not lead to conclusive results (data not shown). This might reflect the fact that different pterin-dependent enzymes are active during growth of *M. chloromethanicum* CM4 on different $C_1$ sources (Fig. 5.1). Southern blot analyses with genomic DNA from *M. extorquens* AM1 and from the dichloromethane-degrading strain *M. dichloromethanicum* DM4 using specific probes against the *purU*, *folD* and *metF* genes of *M. chloromethanicum* CM4 were performed. No signals were detected, suggesting that these genes are present in strain CM4 but not in the other two strains (data not shown). This finding is supported by an analysis of the database of the *M. extorquens* AM1 sequencing project (Chistoserdova, pers. comm.), which showed that neither *purU* nor *folD* is present in strain AM1. A putative *metF* homologue was found in the genome sequence of strain AM1, but the gene had only low similarity to *metF* from strain CM4, which might be the reason why it was not detected by Southern hybridization.

Hybridization analysis with probes against the genes *mtdA*, *fae* and *mchA* of *M. extorquens* AM1 (Fig. 5.1) indicated that these genes are present in CM4 (Kayser, Ucurum and Vuilleumier unpublished).

### 5.4.2 Expression analysis of plasmid-borne transcriptional *xylE* fusions

In order to gain insight into the chloromethane-dependent regulation in *M. chloromethanicum* CM4, plasmids harboring transcriptional *xylE* fusions with *metF*, *folD, purU, cmuB and orf414* were introduced into the wild type strain (Fig. 5.3).

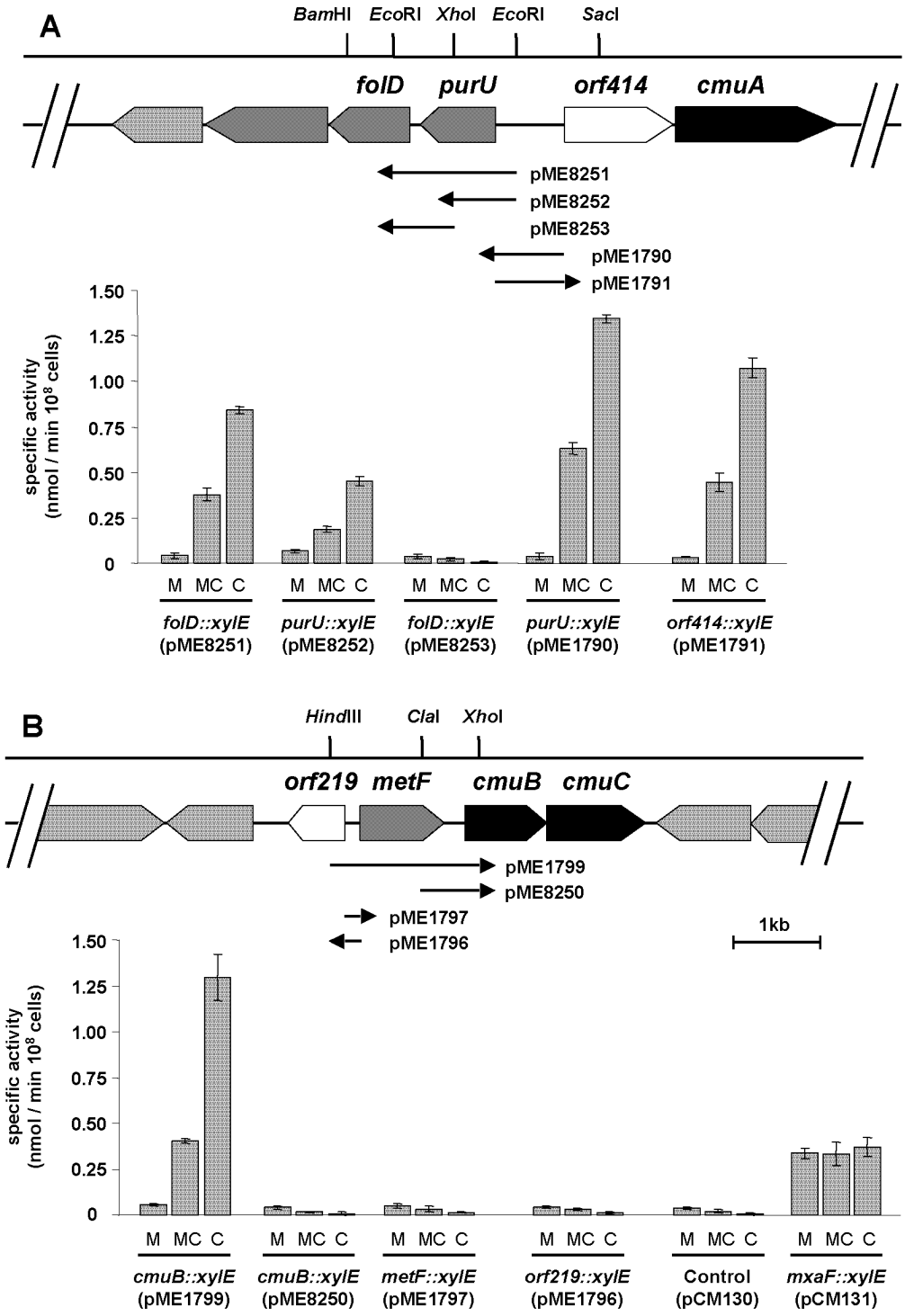


**Fig. 5.3. Expression of plasmid-borne *xylE* fusions in *Methylobacterium chloromethanicum* CM4.** Genetic organization of the gene clusters I (A) and II (B) involved in chloromethane utilization in *M. chloromethanicum* CM4. Genes encoding methyltransferases are shown in black, genes encoding putative $H_4$folate-dependent enzymes in $C_1$ metabolism are in dark gray, vitamin $B_{12}$ biosynthesis genes in light gray and genes of unknown functions in white. Bar diagrams show catechol dioxygenase activity in transconjugants of wild type *M. chloromethanicum* CM4 with different putative intragenic regions fused to the the promoterless *xylE* gene in vector pCM130 [127]. Plasmid constructs are discussed in the text and schematically reproduced below the sequence, The orientation of the *xylE* gene is indicated by a black arrowhead. M, methanol; MC, methanol and chloromethane; C; chloromethane.

Transconjugant strains were grown with methanol, with chloromethane or with a mixture of both $C_1$ sources. Catechol oxygenase activity was measured in exponentially growing bacteria. Both the *folD* and *purU* gene from cluster I were induced in the presence of chloromethane (Fig. 5.3A). This suggests that the two genes are co-expressed, and that the promoter is located upstream of the *purU* gene (Fig. 5.3A, plasmids pME8251 and 8252). Construct pME8253 misses this region and showed no chloromethane-induced catechol dioxygenase activity. Further, a DNA fragment PCR-amplified between the *purU* and *orf414* genes showed high chloromethane-induced promoter activities in the direction of *purU* (Fig. 5.3A, pME1790).

Construct pME1791 contains the same insert as pME1790 in the inverse orientation and thus represents a promoter probe vector for *orf414* and *cmuA.* Catechol dioxygenase activities of the same magnitude were measured as *folD* and *purU*. This suggests the presence of two divergent chloromethane-dependent promoters in cluster I.

As for the genes located in cluster II, *cmuB* seems to be expressed from a promoter upstream of the *metF* gene and not from a promoter in the non-coding region between the two genes (Fig. 5.3B, plasmids pME1799 and 8250). However, the short fragment between the start sites of the *metF* gene and *orf219* is not itself sufficient to promote chloromethane-induced *xylE* reporter activity in either direction (Fig. 5.3B, plasmids pME1796 and 1797). The controls used in these experiments were *M. chloromethanicum* CM4 cells harboring the xylE-expression vector pCM130 without insert and pCM131 with the *xylE* gene under the control of part of the *mxaF* promotor. The *mxaF* gene encodes the large subunit of methanol dehydrogenase of *M. extorquens* AM1 and was shown to be highly expressed during growth on $C_1$ compounds (Fig. 5.3B, [127]). In summary, expression studies suggest the presence of at least three chloromethane-inducible promoters, which are located upstream of *purU*, *metF* and *orf414*, respectively.

### 5.4.3 Determination of chloromethane induced transcription initiation sites in *M. chloromethanicum* CM4

The transcriptional start sites were mapped by primer extension using RNA isolated from wild type *M. chloromethanicum* CM4 grown on either chloromethane or methanol. Specific elongation products were observed in reactions performed with RNA from chloromethane-grown cells. Their 3'-ends were located upstream of *purU*, *metF* and *orf414* (Fig. 5.4). In contrast, no extension products were obtained using primers designed to detect transcriptional start sites upstream of *cmuA*, *folD* and *orf219* (data not shown). The promoters upstream of the chloromethane-induced transcription start sites in strain CM4 exhibited significant sequence conservation (Fig. 5.5). Their –35 region is identical to the minus –35 region of the dichloromethane dehalogenase promoter of *Methylobacterium dichloromethanicum* DM4 [131]. Conservation is less extensive in the –10 region, and the deduced consensus has no similarity to the –10 promoter regions identified in *Methylobacterium* (Fig. 5.5). A transcriptional start site was observed 340 nucleotides upstream of the translational start of *orf414*. Detailed analysis of the sequence upstream of *orf414* indicated the presence of a previously unidentified open reading frame encompassing 168 nucleotides, termed *orf55*, which appears to be translationally coupled to *orf414* (Fig. 5.4).

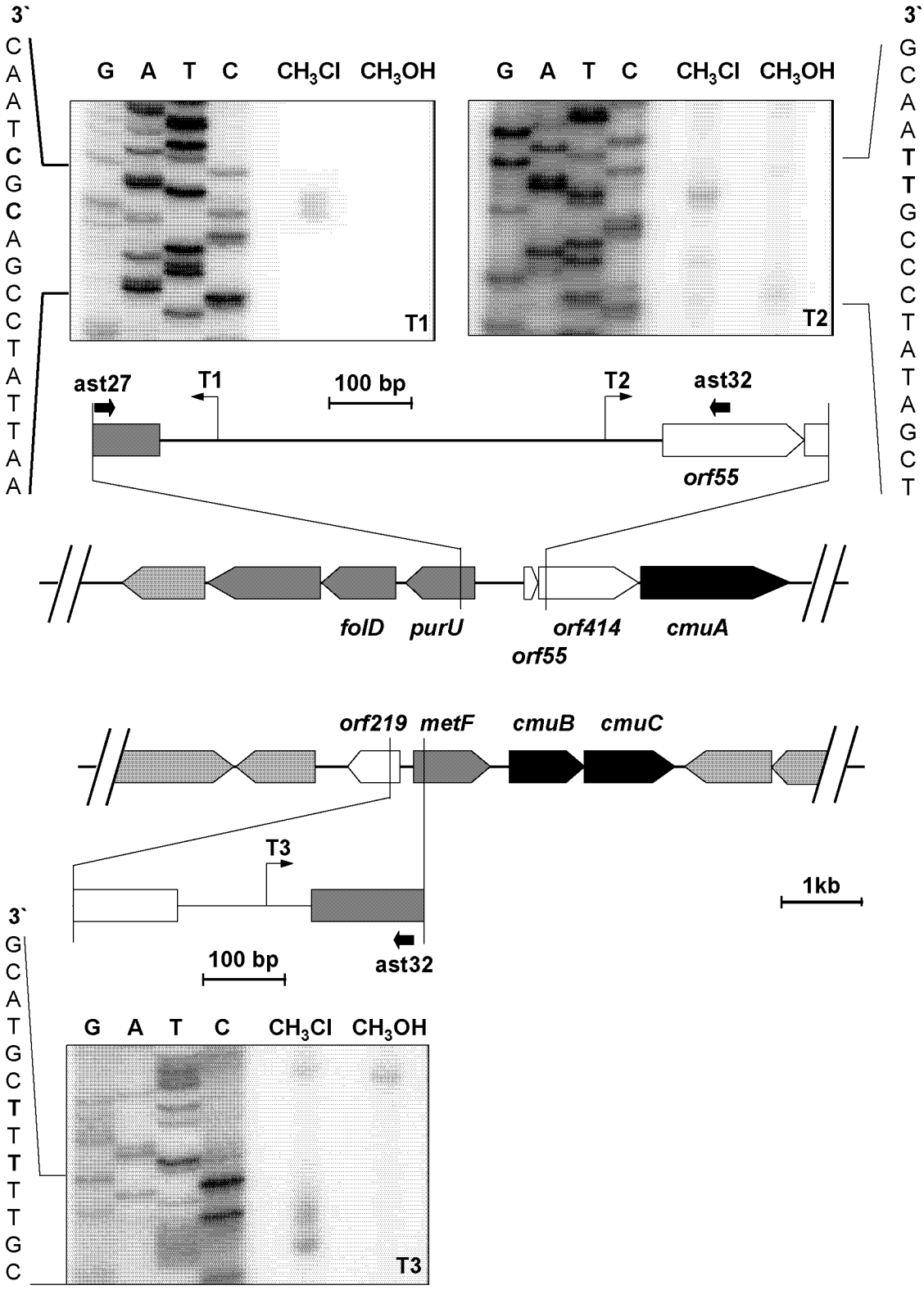


**Fig. 5.4. Transcriptional start sites of chloromethane induced genes in *Methylobacterium chloromethanicum* CM4.** RNA was isolated from *M. chloromethanicum* CM4 grown on chloromethane or methanol and reverse transcribed using primers indicated by black arrows on the schematic view of the two gene clusters (see legend Fig. 5.3). Lanes A,T,C and G show sequencing ladders obtained using the same primers. The transcriptional start sites (T1-T3, hooked arrows) identified are marked in bold in the sequence.

```
                  -35                      -10
orf414            GATTGACAGCCTTACCCATAAATGGAATCGTACGTTAA..
purU              GCTTGACAAAGCGAGCGG-CACATTAATCATACGTTAGCG
metF              CATTGACATTCAAAAATCACACCTTAA-CGTACGAAAAA.
consensus           TTGACA                  AA C TACG
dcmA(DM4)         GCTTGACAGAGATGCATAGCCTTGTATAGAACTAGCCC..
mxaF(AM1)         TAAAGACATCGCGTCCAATCAAAGCCTAGAAAATATAGG.
mxaF(XX)          TAAAGACATCTCCTTCAATCAACGCCTAGAAACGATA...
σ70(E.coli)       ..TTGACA.......N17±1......TATAAT.....-...
```

**Fig. 5.5. Alignment of chloromethane specific promoter regions. in *Methylobacterium chloromethanicum* CM4.** Putative –35 and –10 regions are indicated in boldface, and experimentally determined transcription initiation sites are underlined. A likely consensus promoter is shown. Promoter regions of previously identified *Methylobacterium* genes involved in one-carbon metabolism are also shown for comparison. *dcmA*, dichlormethane dehalogenase promoter of *M. dichloromethanicum* [131]; *mxaF*(AM1) methanol dehydrogenase promoter of *M. extorquens* AM1 [132]; *mxaF*(XX), methanol dehydrogenase promoter of *M. organophilum* XX [133]. The *E. coli* σ70 consensus promoter sequence is also given [134].

### 5.4.4 Growth characteristics of *metF* and *purU* mutants

The previously obtained *purU* mutant (30F5) [48] was found to be unable to grow with chloromethane, but exhibited wild-type dehalogenase activity (see Chapter 2). In contrast, the growth yield of the *purU* mutant on methanol was in wild type range (Table 5.2). Considering the inability of the *purU* mutant to grow with chloromethane alone, growth yields in the range of the chloromethane dehalogenase mutant 22B3 had been expected (Table 5.2). This mutant was previously shown to contain a transposon insertion in the *cmuA* gene (see Chapter 2). The growth yield of the *cmuA* mutant with a mixture of methanol/chloromethane was only two thirds of that of the wild type CM4. This appeared reasonable since the *cmuA* mutant metabolizes the methanol but not the chloromethane present in the medium (Table 5.2). Interestingly however, even though the *purU* mutant did not grow with chloromethane, it obviously metabolized chloromethane in the presence of methanol. Thus, growth yields determined for wild type CM4 and for the *purU* mutant with a mixture of methanol and chloromethane were of the same order as those determined for wild type CM4 (Table 5.2).

Growth of the *purU* mutant with chloromethane was restored upon complementation with plasmid pME1776. This plasmid harbors a 2.85 *Bam*HI/*Sac*I genomic fragment comprising the entire *purU* gene flanked by part

of the *folD* gene and part of *orf414*. Southern blot analysis showed that this plasmid was stably maintained (data not shown).

A *metF* mutant was constructed in order to investigate whether this gene had a function in chloromethane metabolism. The gene was disrupted by insertional mutagenesis using pKNOCK-Km [126]. Southern blot analysis demonstrated that the pKNOCK derivative pME1781 inserted into the CM4 genome as expected (Fig. 5.2), causing a disruption of *metF* (data not shown). The growth rate of the *metF* mutant on methanol was about the same as that of the wild type. In contrast, no growth was observed on chloromethane and more interestingly, on a mixture of chloromethane and methanol (see below).

Chloromethane utilization was restored in the *metF* mutant by introducing plasmid pME1789, which contains a short 1.3 kb genomic *Kpn*I/*Fsp*I fragment with an intact copy of the *metF* gene into the *metF* mutant. Subsequent Southern blot analysis revealed that a recombination event had occurred and that plasmid pME1789 was not stably maintained in the presence of chloromethane (data not shown). Nevertheless this suggested that the observed growth phenotype of the *metF* mutant was indeed caused by disruption of the *metF* gene.

**Table 5.2. Growth yields of *Methylobacterium chloromethanicum* CM4 chloromethane utilization mutants [a]**

| CM4 strain | MOH [b] | MOH-CM [b] | CM [b] |
|---|---|---|---|
| wild type | 10.0 ± 0.2 | 9.5 ± 0.2 | 10.6 ± 0.7 |
| *purU*⁻ (30F5) | 9.9 ± 0.3 | 9.6 ± 0.2 | NG[c] |
| *cmuA*⁻ (22B3) | 9.7 ± 0.3 | 6.2 ± 0.2 | NG |

[a] Average of triplicate runs in g dry weight per mol C-source, standard deviations of three measurement are given

[b] MOH, 20 mM methanol; MOH-CM, 20 mM methanol and 10 mM chloromethane; CM 10 mM chloromethane

[c] NG, no growth

## 5.5 DISCUSSION

Expression analysis of transcriptional reporter gene fusions suggests the presence of at least three transcriptional units for chloromethane utilization in two gene clusters identified in *M. chloromethanicum* CM4 (Fig. 5.5). Mapping of the respective transcriptional start sites upstream of *purU*, *metF* and *orf414* supports this hypothesis. The three promoter regions identified show a significant degree of conservation and are different from any other *Methylobacterium* promoter described until now. This suggests that genes *cmuA* and *cmuB* as well as *metF*, *purU* and *folD* are regulated by similar mechanisms at the level of transcription initiation. A database search using the promoter consensus proposed in Fig. 5.6 as a pattern did not reveal the presence of further similar promoters in both gene clusters of *M. chloromethanicum* CM4 (AJ011316 and AJ011317). Moreover, the same analysis performed with the sequences identified in the chloromethane degraders *Aminobacter* sp. strain IMB-1 (AF281260, [135]) and *Hyphomicrobium chloromethanicum* CM2 (AF281259, [99]) did not reveal any similar promoter regions. This indicates that the identified promoter consensus in strain CM4 does not represent an ubiquitous motif for the regulation of chloromethane genes in methylotrophic bacteria and that regulation of the *cmu* genes is probably genus-specific.

The analysis of gene induction and the phenotypic properties of mutants strongly support the operation of $C_1$ oxidation pathway from methyl-$H_4$folate to formate that is specific for growth of strain CM4 with chloromethane. Expression studies suggest that *purU* and *folD* are co-expressed from a promoter identified upstream of *purU* (Fig. 5.5). Since it is very likely that the transposon insertion in the *purU* mutant 30F5 has a polar effect on the *folD* gene, the question arose as to whether both genes or just *purU* or *folD* are involved in chloromethane degradation. Complementation of the chloromethane minus phenotype of the *purU* mutant 30F5 by providing an intact copy of the *purU* gene in *trans* using plasmid pME1776 (Table 5.1) indicated that *folD* is not essential for growth on chloromethane. It is conceivable that the isofunctional enzyme pair of methylene-$H_4$folate dehydrogenase MtdA [44] and methenyl-$H_4$folate

cyclohydrolase FchA [45] are able to compensate for the lack of FolD. Indeed a recently obtained *folD* mutant does not have a chloromethane minus growth phenotype (McAnulla unpublished). FolD thus appears to be non-essential for growth with chloromethane, but might allow efficient oxidation of methylene-$H_4$folate during growth with chloromethane. The fact that a *folD* homologue is present near the chloromethane-dehalogenase gene *cmuA* in *Aminobacter* strain sp. IMB-1 [135] supports such a hypothesis.

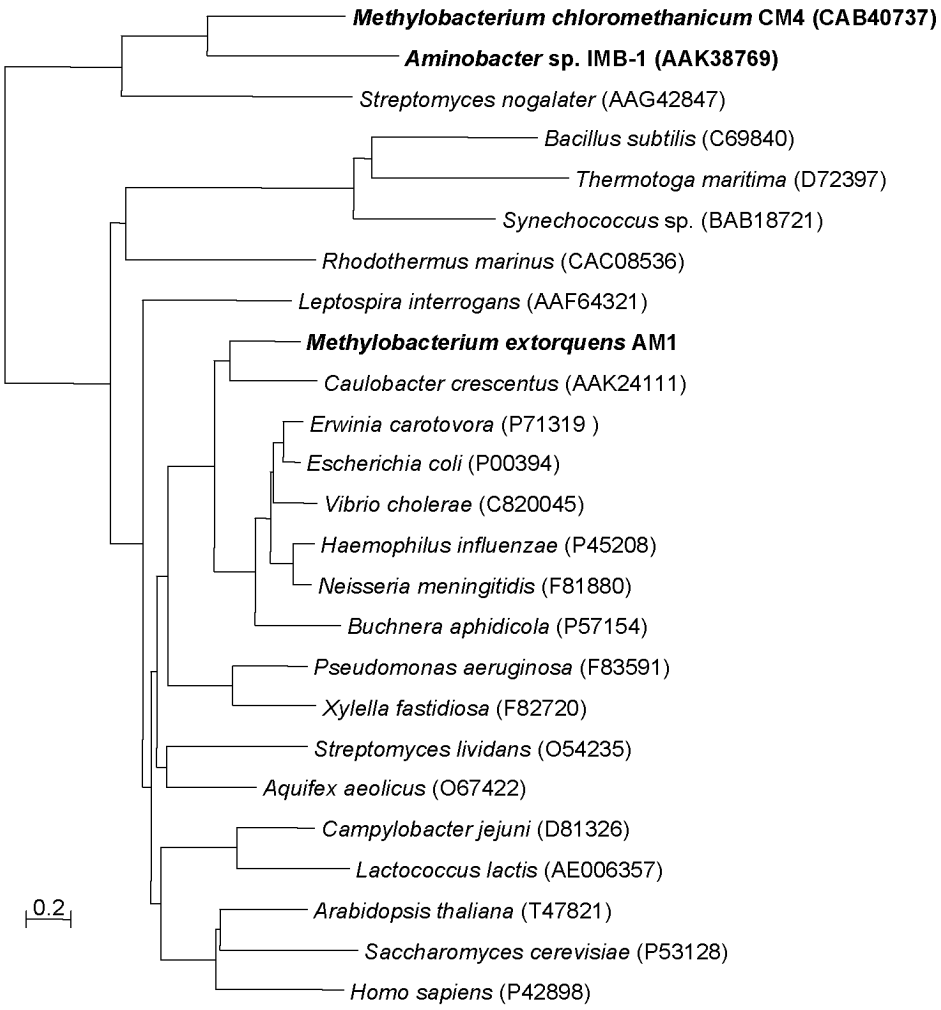


**Fig. 5.6**. **Phylogenetic analysis of MetF proteins from bacteria.** A multiple alignment of MetF sequences (see text) obtained with T_Coffee [136] was used to generate a phylogenetic tree with FITCH from PHYLIP [23]. The tree was drawn with NJPlot [137]. Accession numbers are given in brackets.

Proteins related to PurU, the putative 10-formyl-$H_4$folate hydrolase, are present in a variety of bacteria. The protein from *Escherichia coli* is 37% identical in its amino acid sequence with PurU from *M. chloromethanicum* CM4. The *E. coli* PurU catalyzes the hydrolysis of 10-formyl-$H_4$folate to $H_4$folate and formate, and was shown to be important for setting the ratio of alkylated to free $H_4$folate in the cell [138]. The observed phenotype of the *M. chloromethanicum* CM4 *purU* mutant is interesting in this context. The higher growth yield observed with a mixture of methanol and chloromethane than with methanol alone suggested that chloromethane is utilized for biomass formation in the presence of methanol as a co-substrate. It is possible that a yet unknown enzyme(s) that is only induced in the presence of methanol can complement for the lack of 10-formyl-$H_4$folate hydrolase in the *purU* mutant. Whatever the case may be, it is likely that a lack of PurU negatively affects $H_4$folate metabolism in strain CM4.

In contrast to FolD and PurU, MetF of strain CM4 displays only low sequence similarity to its mammalian and bacterial counterparts (see Chapter 2). In *E. coli,* MetF catalyzes the reduction of methylene-$H_4$folate to methyl-$H_4$folate. This commits the one-carbon precursor for use in the synthesis of methionine from homocysteine [139]. The low sequence similarity of the CM4 MetF protein might be due to the fact that in *M. chloromethanicum* CM4 MetF is supposed to catalyze the reverse reaction to perform its proposed physiological role (Fig. 5.1). Indeed, the halomethane-utilizer *Aminobacter* sp. IMB-1 contains a *metF* gene with the so far highest sequence similarity to MetF from CM4 (Fig. 5.6). These two proteins, together with an unknown gene product from *Streptomyces nogalater* form a subgroup of MetF homologues whose members are different from the MetF proteins usually found in bacteria (Fig. 5.6). In contrast, a gene encoding a putative MetF protein identified in the *M. extorquens* AM1 genome was more related to the *E. coli* protein. This implies that strain CM4 might have a second, so far not identified *metF* gene.

Interestingly, the *metF* mutant of strain CM4 was not only unable to grow with chloromethane, but was also sensitive to the presence of chloromethane during

growth with methanol. A physiological explanation for this phenomenon might be that the cofactor is trapped by the methylation catalyzed by chloromethane dehalogenase, at the expense of all $H_4$folate derivatives in the cell. Other biosynthetic pathways relying on $C_1$ moieties provided by $H_4$folate might thereby be abolished. This is reminiscent of the situation prevailing in human patients with pernicious anemia. According to the currently favored “methyl trap” model developed to account for the observed clinical picture, a disfunctional methionine synthase leads to the accumulation of methyl-$H_4$folate [140]. The consequence is poor availability of the $H_4$folate cofactor for other important physiological processes.

# Chapter 6

## General Discussion

This study addressed chloromethane degradation in aerobic bacteria at both genetic and biochemical levels. Genes involved in chloromethane utilization by *Methylobacterium chloromethanicum* CM4 were isolated and their expression was demonstrated to be regulated at the level of transcription. The mechanism of chloromethane dehalogenation was investigated in more detail, which required the purification and characterization of the responsible proteins. This Chapter discusses results and current ideas concerning chloromethane utilization in strain CM4 to provide a stimulus for future work on this topic.

## 6.1 GENES INVOLVED IN THE METABOLISM OF CHLOROMETHANE

As described in Chapter 2, a genetic approach was instrumental in the discovery of the chloromethane utilization pathway of *M. chloromethanicum* CM4. In the following, I discuss the genes that have been proven or are suspected to be involved in the metabolism of chloromethane by this organism.

Such genes fall into 3 classes (Fig. 5.1). The first class comprises genes whose roles in chloromethane metabolism have been elucidated by biochemical and/or genetic experiments as described in the previous Chapters. The second class is formed by genes which encode proteins with a possible function in chloromethane metabolism, some of which were previously analyzed in Chapter 2. Others will be presented in some detail below. Finally, a third class comprises genes which are not, or only indirectly, involved in chloromethane utilization. These include genes of clusters I and II encoding proteins involved in cobalamin biosynthesis in strain CM4 (see Chapter 2), and genes located downstream of transposon insertions in clusters III and IV which were isolated from so far uncharacterized *cmu* minus mutants (Fig. 5.1).

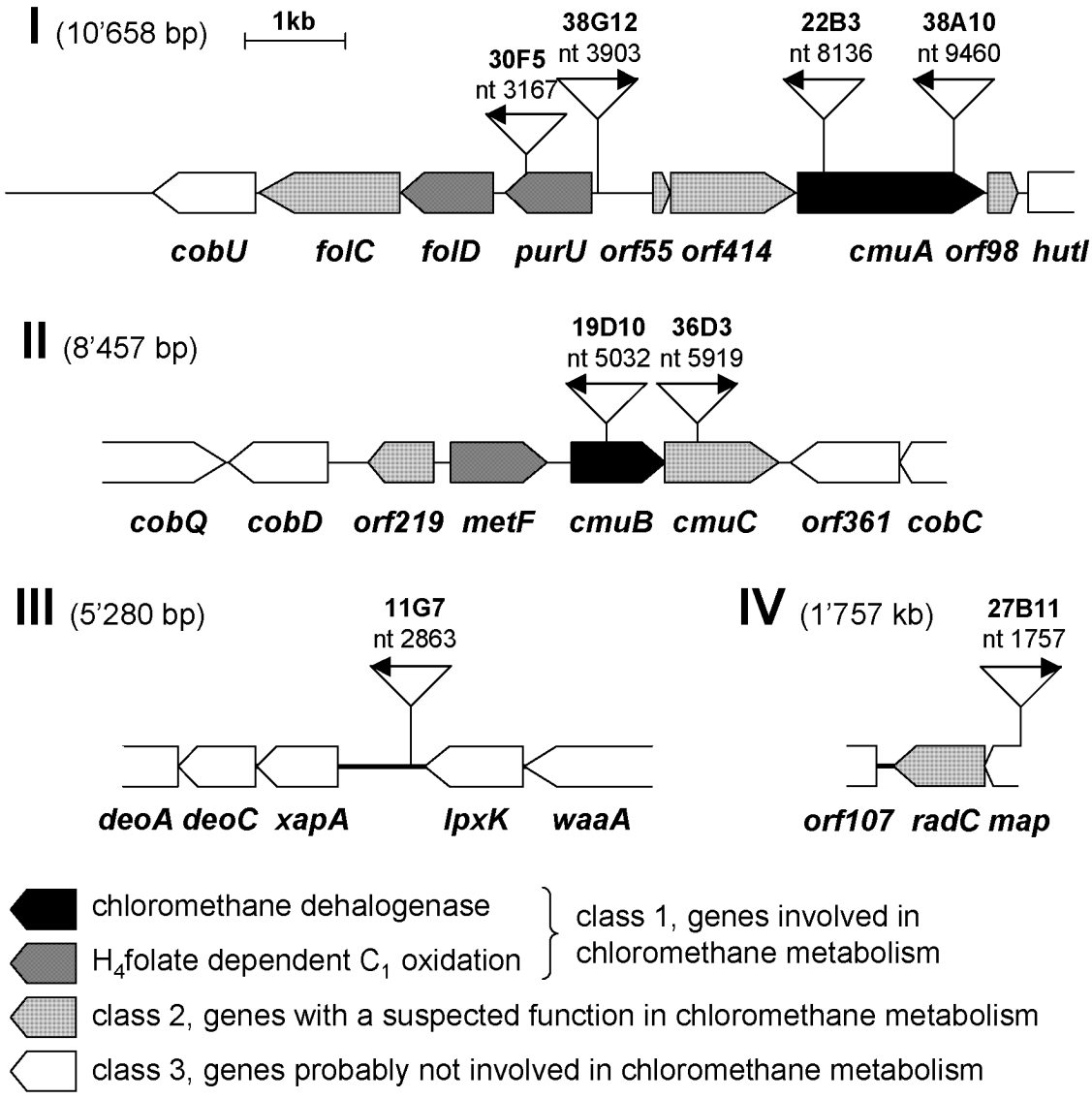


**Fig. 6.1. Schematic view of gene clusters I - IV of *Methylobacterium chloromethanicum* CM4.** The position and orientation of the transposon insertions in the genome of the *cmu* minus mutants are shown. Genes in cluster III and IV were named according to Table 5.1, genes in clusters I (AJ011316) and II (AJ011317) are discussed in Chapter 2 and in the present Chapter.

### 6.1.1 Comparative sequence analysis of the *cmu* genes in chloromethane utilizing methylotrophic bacteria

Gene clusters I and II of *M. chloromethanicum* CM4 were previously described in detail (Fig. 6.1, see Chapter 2). In accordance with the properties of the corresponding mutants (see Chapter 1, [48]), the gene products of *cmuA* and *cmuB* were demonstrated to catalyze the formation of methyl-$H_4$folate from chloromethane (see Chapters 3 and 4). Further, the *metF*, *folD* and *purU* genes

were shown to be specifically expressed during growth on chloromethane and thus are likely to encode enzymes of a $H_4$folate-dependent $C_1$ oxidation pathway (see Chapter 5).

The *cmuA* and *cmuB* genes from *M. chloromethanicum* CM4 were used as probes to identify homologues in *Hyphomicrobium chloromethanicum* CM2 [99]. This led to the isolation of a 9.5 kb gene fragment comprising the genes *cmuB*, *cmuC* and *cmuA* (Fig. 6.2). These three genes were clustered in the CM2 genome and their products exhibited 57%, 36% and 80% amino acid sequence identity to their CM4 homologues, respectively. Recently, a *cmu* gene cluster was isolated from *Aminobacter* sp. IMB-1 and part of *cmuC* and the entire *cmuA* were sequenced (Fig. 6.2, [135]). With 36% and 78% amino acid identity, the corresponding translation products displayed significant similarity to their CM4 counterparts (Fig. 6.2). The *cmuB* gene is located immediately upstream of *cmuC* in strains CM2 and CM4, but this DNA region has not yet been sequenced in strain IMB-1. Taken together, such findings suggest that the proteins involved in the cobalamin dependent dehalogenation of chloromethane are conserved among aerobic bacteria capable of growth with this substrate.

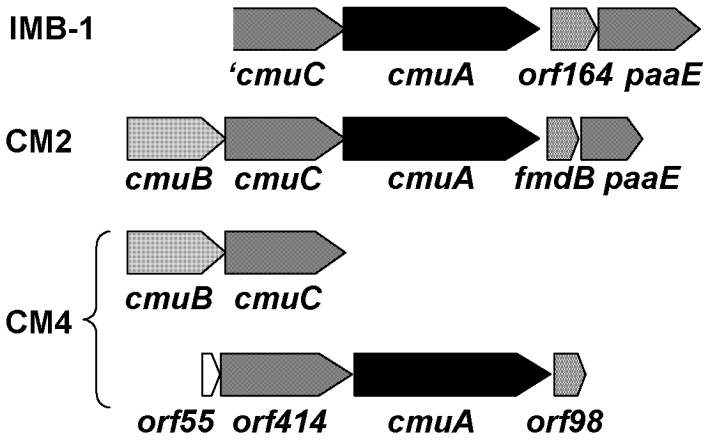


**Fig. 6.2. Schematic representation of the arrangement of *cmu* genes in chloromethane utilizing strains.** IMB-1, *Aminobacter* sp. IMB-1 (Acc. No. AF281260); CM2, *Hyphomicrobium chloromethanicum* CM2 (AF281259) and CM4, *M. chloromethanicum* CM4 (AJ011316, AJ011317). Homologous genes have the same shading pattern.

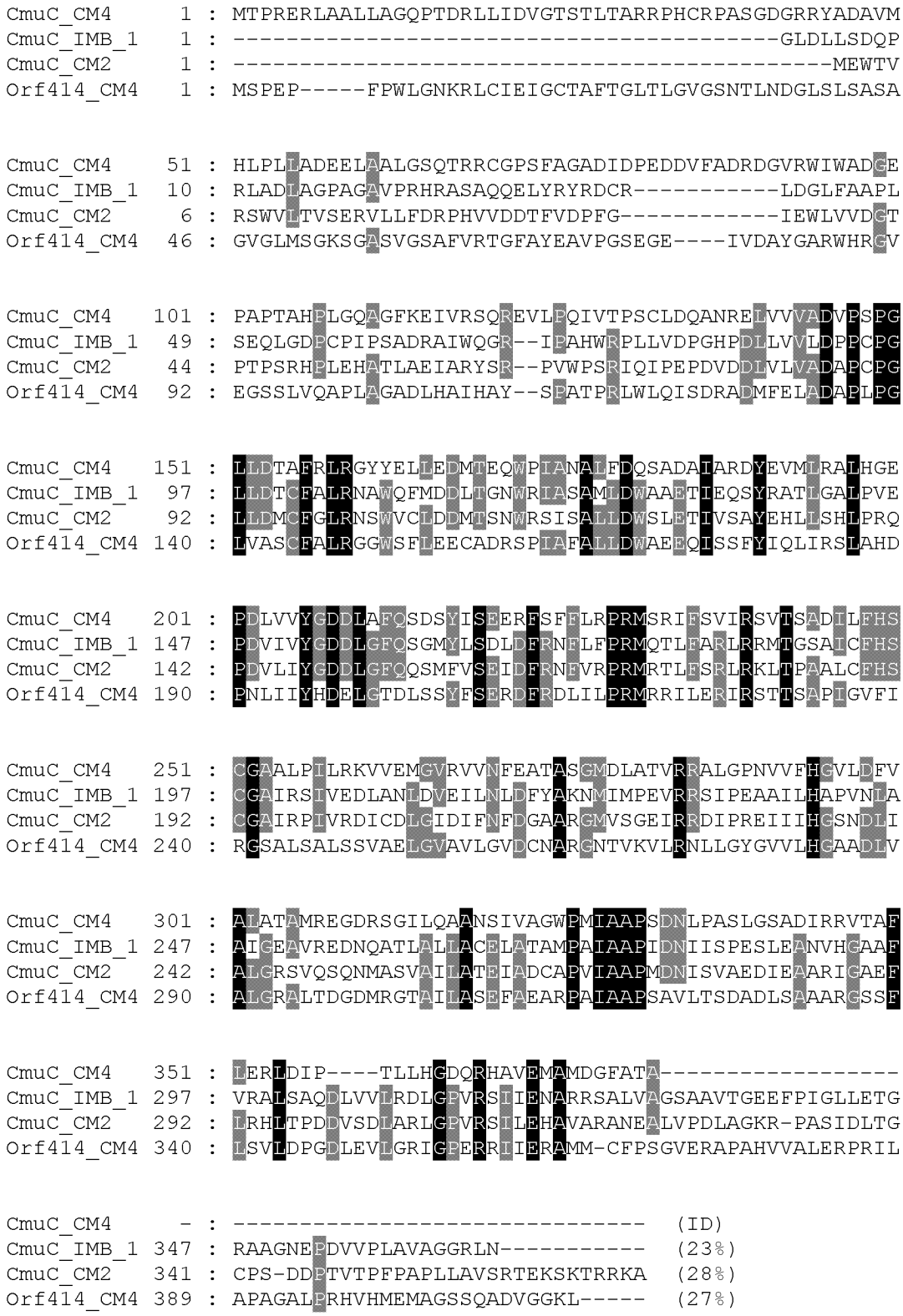


**Fig. 6.3.** Protein sequence alignment of CmuC and Orf414 of *Methylobacterium chloromethanicum* CM4 with CmuC from *Hyphomicrobium chloromethanicum* CM2 (Q9APJ9) and *Aminobacter* sp. IMB-1 (AAK38765). Identical positions in all four proteins are indicated as white letters on black background; identities in only three of the four proteins are shown as white letters on gray background. Percent identity (ID) of the individual protein sequence to CmuC of strain CM4 is indicated.

In contrast to strains IMB-1 and CM2, the *cmu* genes of *M. chloromethanicum* CM4 are found at two separate loci (Fig. 6.2). Since the *cmu* genes of both clusters are surrounded by genes putatively involved in cobalamin biosynthesis, it is possible that the *cmuA* and *cmuBC* genes are arranged in close proximity to each other on the CM4 genome.

Of interest with respect to future studies is the striking sequence similarity of *cmuC* and *orf414* (Fig. 6.3). The arrangement of *cmuC* immediately upstream of *cmuA* in *H. chloromethanicum* CM2 and in *Aminobacter* sp. IMB-1 suggests that a gene duplication event had occured which, combined with a translocation, has led to the separation of the *cmu* genes in *M. chloromethanicum* CM4 (Fig. 6.2). Mutant studies demonstrated that in strain CM4 the *cmuC* gene product performs an essential but so far unknown function in chloromethane metabolism (see Chapter 1). In contrast, it is not known whether *orf414* is essential for growth on chloromethane, since an *orf414* mutant is not yet available. However, the intact *orf414* gene is not sufficient to allow growth on chloromethane in the *cmuC* mutant, so that the two genes are clearly not isofunctional.

### 6.1.2 Genes encoding possible chloromethane responsive regulatory proteins

Three promoter regions with significant similarity to each other were identified in clusters I and II and expression of the genes under their control was specifically induced during growth on chloromethane (see Chapter 5). Regulatory protein(s) or a conserved operator region potentially involved in chloromethane dependent transcriptional regulation have yet to be identified in strain CM4. A possible candidate for a regulatory protein is encoded by *orf219* in cluster II (Fig. 6.1). *Orf219* is predicted to encode a 219 amino acid protein similar to proteins annotated as members of the family of MerR response regulators (Fig. 6.4). These regulatory proteins occur in a range of bacteria and respond to a wide variety of external stimuli. The prototype of this family is the mercury resistance regulator MerR, present in both Gram negative and Gram positive bacteria [141]. The MerR family includes mainly metal-responsive regulators of gene expression which, for example, respond to cobalt in

*Synechocystis* PCC 6803 (CoaR; [142]), cadmium in *Pseudomonas putida* (CadR; [143]), copper (CueR, [144]) and zinc in *E. coli* (ZntR; [145]).

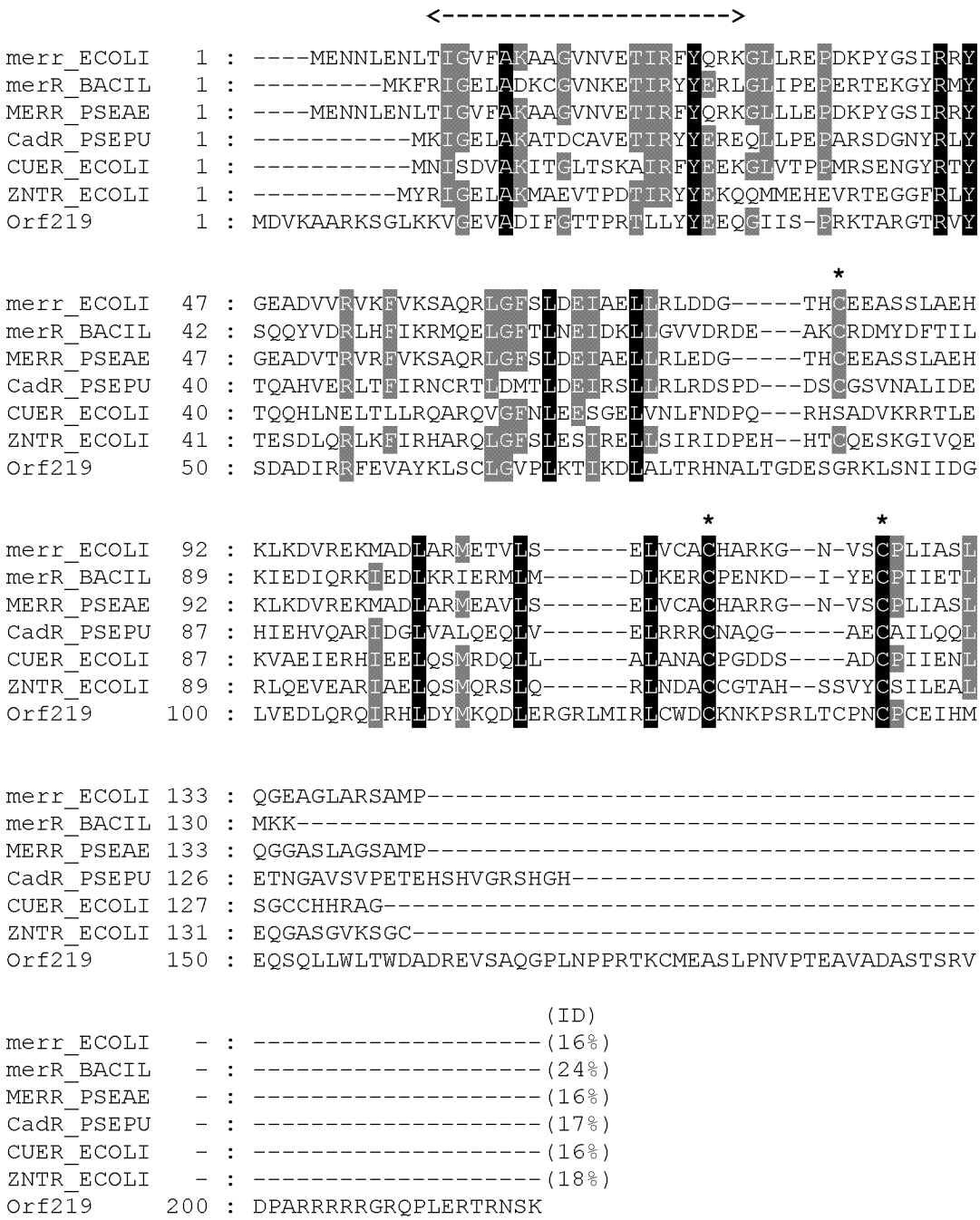


**Fig. 6.4.** Amino acid sequence alignment of Orf219 of *Methylobacterium chloromethanicum* CM4 with members of the MerR-type family of transcriptional activators, including MerR of *E. coli* (AAC33922), *Pseudomonas aeruginosa* (P06688) and *Bacillus subtilis* (P22853), as well as ZntA of *E. coli* (P36676), CadR of *Pseudomonas putida* (AAK48830) and CueR of *E. coli* (P77565). Amino acids are indicated as white letters on black background when conserved in all seven proteins and as white letters on gray background when conserved in five of the seven proteins. The identities (ID) of the individual amino acid sequences to Orf219 of strain CM4 are indicated. *, conserved cysteine residues; <--->, helix-turn-helix motif.

In addition, MerR-type regulatory proteins are involved in the response to superoxide stress in *E. coli*, in thiostrepton resistance in *Streptomyces*, and in regulation of nodulation in *Bradyrhizobium* and of multidrug efflux protein synthesis in *Bacillus* [141]. MerR type regulatory proteins display stretches of conserved sequence in their amino-terminal domain which includes a helix-turn-helix DNA-binding motif (Fig. 6.4, [141]). The C-terminal domain is less conserved and thought be involved in sensing of specific effectors. Specifically, two cysteines (Cys131 and Cys143), of the three cysteine residues required for mercury-binding and promoter activation in MerR, are conserved in Orf219 (Fig. 6.4, [146]).

Currently, the role of Orf219 as a regulator in chloromethane-utilization is purely hypothetical. It is based on the location of *orf219* adjacent to *metF* and their divergent direction of transcription, which are features typical of MerR-type regulators and their target genes [141]. However, the conserved inverted repeat and the extended spacer region between the –35 and –10 regions typical of known MerR-type regulators [146,147] were not found in chloromethane-induced promoters of strain CM4 (see Chapter 5).

Another candidate for a regulatory protein in chloromethane degradation is Orf98 in cluster I. Originally, the *orf98* gene was overlooked until now due to the lack of significant similarity of Orf98 to any gene product in the database. With the availability of the *cmu* gene clusters of *Aminobacter* sp. IMB-1 and *H. chloromethanicum* CM2 [99,135], however, it has become evident that all three organisms have an *orf98*–like open reading frame located immediately downstream of *cmuA* (Fig. 6.2). Further, all three *orf98*–like proteins have a conserved $CX_2CX_{19}CX_2C$ motif at their amino-terminal end (Fig. 6.5), which is typical of metal-binding sites in zinc-finger domains [148]. Interestingly, all three gene products show some low level of similarity (<19% identity) to the protein FmdB, a likely positive transcriptional regulator for the expression of formamidase in *Methylophilus methylotrophus* [149].

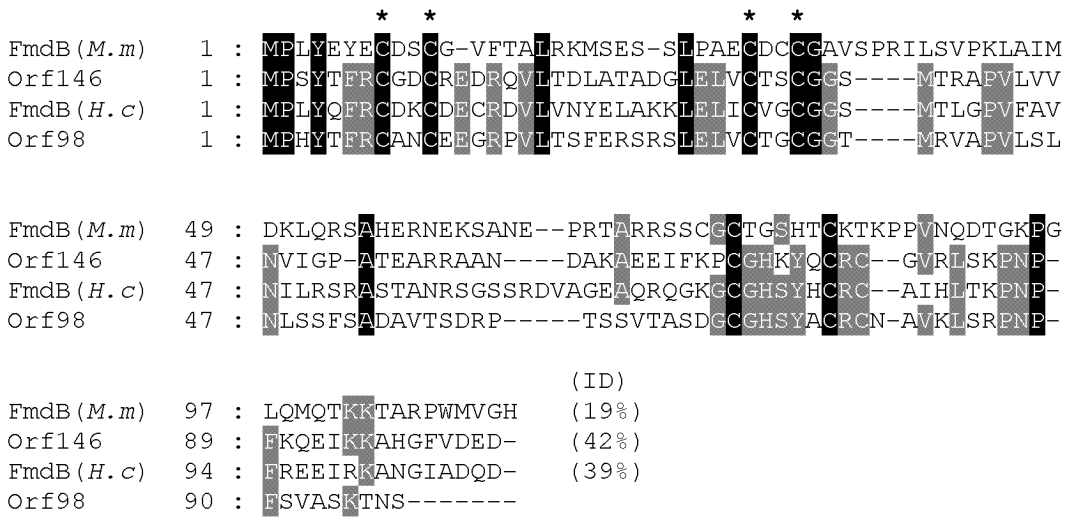


**Fig. 6.5.** Sequence alignment of Orf98 of *Methylobacterium chloromethanicum* CM4 with FmdB from *Hyphomicrobium chloromethanicum* CM2 (AAK01348), the C-terminal part of Orf146 from *Aminobacter* sp. IMB-1 (AAK38766) and FmdB from *Methylophilus methylotrophus* S74214. Amino acids are indicated as white letters on black background when conserved in all four proteins and as white letters on gray background when conserved in three of the four protein sequences. Cysteine residues in the "CXXC" motifs are marked by asterisks. Identity (ID) of the individual sequences to Orf98 of strain CM4 are indicated.

Clearly, in the case of expanded regulation studies in strain CM4, both *orf219* and *orf98* would be interesting targets for mutational inactivation.

### 6.1.3 Clusters III and IV contain genes whose functions in chloromethane metabolism are not known

Only six transposon mutants displaying a chloromethane utilization negative phenotype have been studied in some detail. In these cases, the transposon insertion was found in clusters I or II (Fig. 6.1). However, three other mutants with a *cmu* minus phenotype, mutants 11G7, 27B11 and 27C10 were isolated (Fig. 6.1, see Chapter 1, [48]). In the latter mutant, *orf414* was affected by insertion of the transposon, but some uncharacterized additional genetic rearrangement had occurred in this mutant, which was therefore not characterized further. The DNA regions affected by transposon insertions in strains 11G7 and 27B11, termed clusters III and IV in the following, appeared unrelated to each other or to clusters I and II (Fig. 6.1).

A 5.3 kb region covering both sides of the transposon insertion site in mutant 11G7 was sequenced. The insertion mapped to a DNA sequence of *M. chloromethanicum* CM4 that either is non-coding or encodes a protein with no similarity to any of the proteins stored in the database (Fig. 6.1). The reason for the *cmu* minus phenotype of mutant 11G7 is thus not obvious. Moreover, the putative genes identified near by the transposon insertion site have no immediate relation to chloromethane metabolism and showed similarity to proteins involved in nucleotide degradation or lipid biosynthesis (Table 6.1).

A 1.75 kb region on one side of the miniTn*5* insertion of a 17 kb *Cla*I fragment cloned from mutant 27B11 (in plasmid pME1733) was also sequenced. The insertion mapped to an open reading frame encoding a putative gene product with similarity to methionine aminopeptidase (Map) from a variety of bacteria [150]. This protein is responsible for the removal of the N-terminal methionine after translation. In this case as well, a link between the putative function of the disrupted gene in mutant 27B11 and chloromethane metabolism is difficult to envisage. Immediately downstream of *map,* however, lies *radC*, which presumably encodes a protein involved in the repair of UV-induced DNA damage [151]. It can be envisaged that the transposon insertion in mutant 27B11 has a polar effect on the expression of *radC* which leads to an impairment of the DNA repair machinery. That such a transposon insertion would correlate with a *cmu* minus phenotype is of interest, considering the known systemic methylating potency of monohalomethanes, which can lead to DNA damage [152]. Indeed, related observations were recently made with *Methylobacterium dichloromethanicum* DM4. In this organism the *polA* gene, which encodes DNA polymerase I, an enzyme with a well-known role in DNA repair [151], was found to be essential for growth with dichloromethane [122]. Growth with dichloromethane was also impaired in an *uvrA* mutant of *M. dichloromethanicum* DM4 (Kayser and Vuilleumier unpublished), a gene likely to encode a protein involved in nucleotide excision repair [151]. These observations suggest that due to the reactivity of halogenated methanes, *Methylobacterium* needs an efficient DNA repair machinery for growth with such compounds.

**TABLE 6.1. Genes and open reading frames in clusters III and IV.**

| Gene orf | Length aa | Gene Begin | Gene End | Inferred function | Representative hit [a] | Identity % |
|---|---|---|---|---|---|---|
| **Cluster III** | | | | | | |
| *deoA* | >183 | 551 | <1 | thymidine phosphorylase (nucleotide catabolism) | DeoA, P07650 (*E. coli*) | 62 |
| *deoC* | 256 | 1321 | 551 | deoxyribose-phosphate aldolase (nucleotide catabolism) | DeoC, P00882 (*E. coli*) | 48 |
| *xapA* | 229 | 2143 | 1334 | purine nucleoside phosphorylase (purine nucleoside salvage) | XapA, P45563 (*E. coli*) | 34 |
| *lpxK* | 326 | 3981 | 3001 | tetraacyldisaccharide 4'-kinase (lipid biosynthesis) | LpxK, P27300 (*E. coli*) | 34 |
| *waaA* | >429 | >5280 | 3992 | 3-deoxy-D-manno-octulosonic-acid transferase (lipopolysaccharide biosynthesis) | WaaA, P23282 (*E. coli*) | 35 |
| **Cluster IV** | | | | | | |
| *orf107* | >107 | 321 | <1 | Unknown | Orf, PA1730 (*P. aeruginosa*) | 44 |
| *radC* | 302 | 1432 | 524 | DNA repair protein | RadC, P25531 (*E. coli*) | 33 |
| *map* | >118 | >1757 | 1401 | methionine aminopeptidase | Map, P07906 (*E. coli*) | 53 |

[a] Accession numbers from Swissprot or Trembl databases.

## 6.2 THE COBALAMIN-DEPENDENT CHLOROMETHANE DEHALOGENATION REACTION

The main features and components of chloromethane dehalogenation were characterized in the course of this study (see Chapter 3 and 4). The reaction involves a cobalamin-dependent methyl transfer from chloromethane to $H_4$folate. This finding came as a surprise, since catabolic reactions based on a cobalamin-dependent methyl transfer had so far been observed only in strictly anaerobic bacteria and archaea [60,153].

**Table 6.2. Classes of cobalamin-dependent methyltransferases**

| Methyltransferase | proteins [a] | reaction | |
|---|---|---|---|
| | | donor ($CH_3$-X) [d] | acceptor (Y) [e] |
| methinone synthase | MetH | $CH_3$-$H_4$folate | homocysteine |
| $CH_3$X:CoM methyltransferase | MtaA,B,C | methanol | HSCoM |
| | MtbA, MtmB,C | monomethylamine | HSCoM |
| | MtbA,B,C | dimethylamine | HSCoM |
| | MtbA, MttB,C | trimethylamine | HSCoM |
| | MtsA,B | methanethiol | HSCoM |
| methyl-$H_4$MPT:CoM methyltransferase | MtrA-E | $CH_3$-$H_4$MPT | HSCoM |
| acetyl coenzyme A synthase | AcsD, E | $CH_3$-$H_4$folate | carbon monoxide dehydrogenase |
| aromatic *O*-demethylase | OmdA [c] | phenylmethylethers | $H_4$folate |
| | MtvA,B,C [b] | | |
| chloromethane dehalogenase | CmuA, CmuB | chloromethane | $H_4$folate |

[a] Biochemically characterized proteins for which the corresponding genes were sequenced

[b] The aromatic *O*-demethylase of *Acetobacterium dehalogenans* [58] consists of three components of which only the gene encoding the corrinoid binding protein was sequenced so far.

[c] Aromatic *O*-demethylase of *Moorella thermoacetica* [154] of unknown sequence

[d,e] Abbreviations according to Fig. 6.6.

Several classes of cobalamin-dependent methyl transfer reactions have been described (Table 6.2), which all feature a common reaction mechanism (Fig. 6.6). The crucial step in the reaction takes place at the corrinoid-binding protein (E, Fig. 6.6), which is methylated by a methyltransferase I (MTI) exhibiting binding affinity for the methyl group donor ($CH_3$-X). The methylated corrinoid protein is demethylated by another methyltransferase (MTII) with affinity to the methyl group acceptor (Fig. 6.6). In cobalamin dependent methyl transfer reactions, the methyl-cobalt bond is cleaved heterolytically. Both bonding electrons stay on the cobalt and the reaction thus formally corresponds to a transfer of a methyl carbocation. The cob(I)alamin form of the protein formed in such a reaction is a powerful nucleophile able to demethylate various substrates (Table 6.2). It is therefore essential for catalytic activity. However, during turnover the cob(I)alamin can occasionally be oxidized into its inactive cob(II)alamin form (Fig. 6.6). The reductive reactivation of the protein to the active cob(I)alamin form requires a fourth enzymatic reaction (AE). Reactivation mechanisms differ in various corrinoid dependent methyltransferases and are discussed in some detail below.

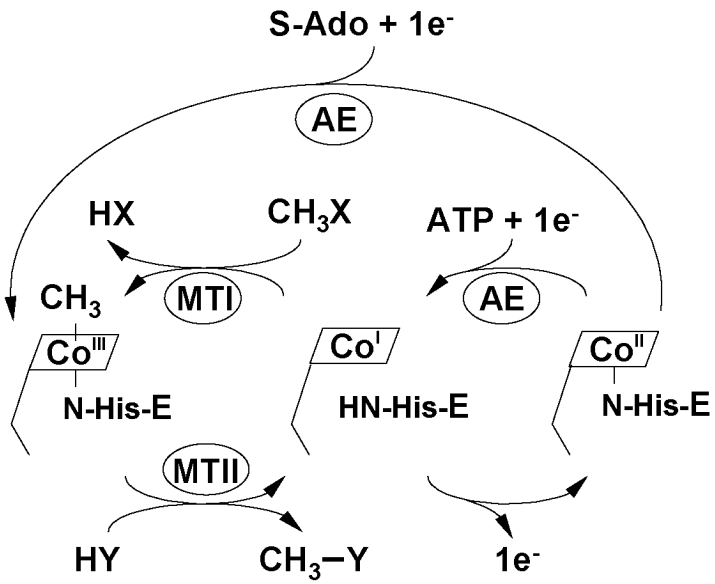


**Fig. 6.6. General scheme of corrinoid-dependent methyl transfer reactions.** MT, methyltransferase; AE, possible reactivation enzyme; N-His-E, protein histidine residue (see text). $CH_3X$ represents the methyl group donor and Y the methyl group acceptor (see Table 6.2).

Generally speaking, the different enzymatic activities associated with methyl transfer reactions are reflected in the modular structure of cobalamin-dependent methyltransferases. In some cobalamin dependent methyltransferases all activities are combined on a single polypeptide, but are found on separate polypeptides in other enzymes. The cobalamin dependent methionine synthase MetH from *E. coli* represents one extreme of this spectrum. It is a monomeric enzyme of 136 kDa which consists of distinct structural domains. Ligand and activity studies conducted with the protein dissected by limited proteolysis led to the identification of four functional domains (Fig. 6.7): two methyltransferase domains, one specific for the methyl-group acceptor homocysteine and one for the methyl-group donor methyl-$H_4$folate, a corrinoid binding domain and a domain involved in the reactivation [153]. On the other hand, all four activities are expressed as separate polypeptides in CoM methyltransferases of methanogens (discussed in Chapter 4) or in aromatic *O*-demethylases [154,155].

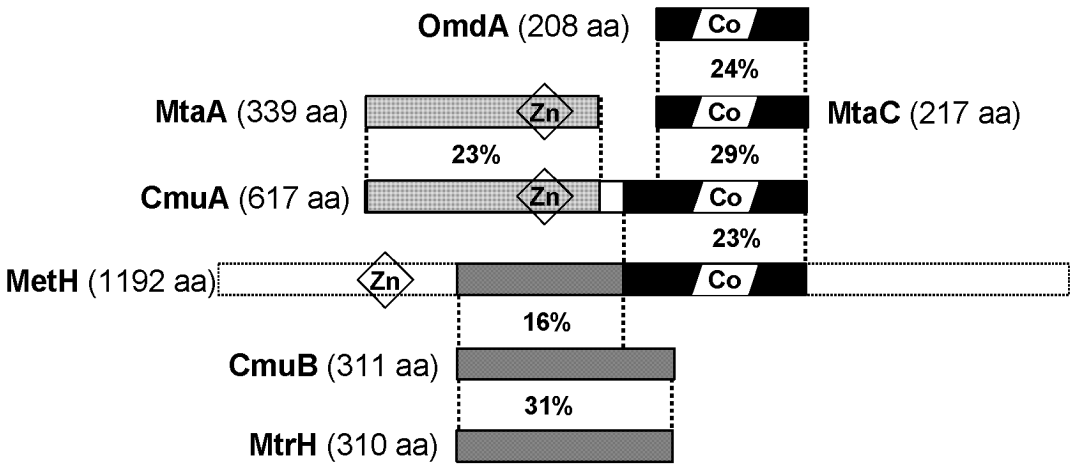


**Fig. 6.7. Schematic representation of proteins similar to CmuA and CmuB involved in cobalamin dependent methyl transfer.** OmdA, corrinoid-binding protein of *O*-demethylase of *Acetobacterium dehalogenans* (O87603); MtaA and MtaC, methylcobamide:CoM methyltransferase and its cognate corrinoid binding protein from *Methanosarcina barkeri* (Q48927, P94920); MetH, cobalamin-dependent methionine synthase from *E. coli* (P13009); MtrH, $H_4$MPT binding subunit of $H_4$MPT:CoM methyltransferase (P80187). Corrinoid binding proteins are indicated in black. Zinc-binding methyltransferases are indicated in light gray and pterin-binding methyltransferases in dark gray.

The chloromethane dehalogenase of *M. chloromethanicum* CM4 represents an intermediate case since the methyltransferase specific for chloromethane and its cognate corrinoid binding protein are fused to a single polypeptide CmuA. The methyltransferase II CmuB represents a separate protein which uses methylated CmuA as a substrate for methyl transfer onto $H_4$folate. Nevertheless, domains with similar function often exhibit a significant degree of conservation between different classes of cobalamin-dependent methyltransferases. Sequence similarities of CmuA and CmuB to other cobalamin-dependent methyltransferases discussed in Chapter 2 are shown schematically in Fig. 6.7.

The properties of the different domains in the Cmu proteins are discussed in relation to similar proteins of other cobalamin-dependent methyltransferases in the following. The main emphasis is put on the CmuA protein, which catalyzes the dehalogenation of chloromethane and represents the only known enzyme to combine the methyltransferase I and the corrinoid binding domain. Finally, the last part of the Chapter will describe the reactivation mechanisms in corrinoid dependent methyltransferases, since the reactivation system in strain CM4 is not yet known.

### 6.2.1 The corrinoid binding domain of CmuA

As discussed in detail in Chapter 4, CmuA is a bifunctional protein containing a vitamin $B_{12}$ cofactor bound in a non covalent manner. In free methylcobalamin, the cobalt is in the +3 oxidation state and coordinated to six ligands [153]. These are the four nitrogens of the corrin ring, termed the equatorial ligands, a methyl group as upper ligand and the dimethylbenzimidazole moiety of the cofactor as lower ligand. In contrast, the unmethylated cob(I)alamin form of the cofactor (oxidation state +1) is only tetrahedrally coordinated by the equatorial nitrogen ligands of the corrin ring with the dimethylbenzimidazole residue dissociated. The two free electrons in the orbital perpendicular to the plane of the corrin ring impart cob(I)alamin its strong nucleophilic character [156].

In corrinoid proteins, the axial ligand is replaced by a histidine residue of the protein, which modulates the reactivity of the corrinoid. A conserved sequence motif D-X-H-X(2)-G-X(41)-S-X-L-X(26)-G-G was proposed to be involved in the binding of the cobalamin cofactor [157]. This motif is evident in sequence alignments (Fig. 6.8) and present in most corrinoid binding proteins, but only poorly conserved in CmuA.

Determination of the three-dimensional structure of the corrinoid binding domain of methionine synthase MetH from *E. coli* confirmed the involvement of residues in this sequence motif in cofactor binding [74]. Residues D757, H759 and S810 were identified as members of a ligand triad important for catalytic activity, in which the histidine residue replaces the dimethylbenzimidazole tail as lower axial ligand to the cobalt of the corrinoid cofactor. Further, the three conserved glycine residues at amino acid positions 762, 833 and 834 were shown to provide the necessary space for the dissociated dimethylbenzimidazole tail of the prosthetic cobalamin cofactor [74,153]. H759 is thought to be instrumental in stabilizing the cob(I)alamin and methylcobalamin states of the cofactor by dissociation and association, and thereby to facilitate catalysis of methyl group transfer [153,156]. Similar results were obtained for the MtaC protein from *Methanosarcina barkeri*, where, using site directed mutagenesis, a histidine was also identified as the essential axial ligand of the corrinoid [117].

Although the C-terminal region of the CmuA protein from *M. chloromethanicum* CM4 showed considerable sequence similarity with other corrinoid-binding proteins involved in methyl transfer, the cobalamin-binding motif is only partially conserved (Fig. 6.8, see Chapter 2). The sequence alignment suggests that in CmuA the histidine is replaced by a glutamine residue as lower axial ligand (Fig. 6.8). This change in the corrinoid binding motif is conserved among all CmuA sequences obtained so far [99,135]. It is not clear whether a glutamine residue is able to act as the lower axial ligand of cobalamin and if so, whether it can exert the same function as a histidine residue. The potential impact of such an amino acid change was briefly discussed in Chapter 2.

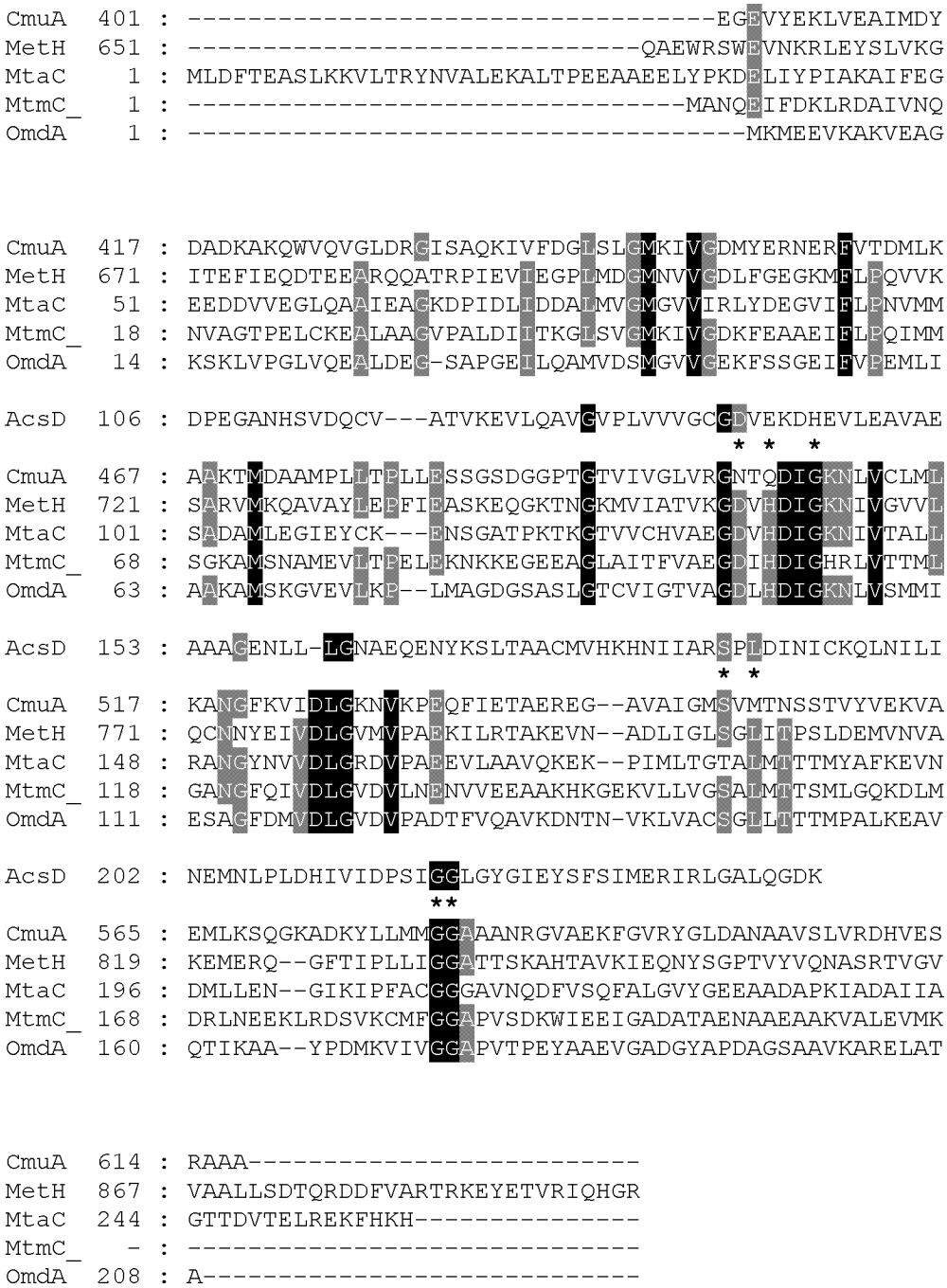


**Fig. 6.8. Amino acid sequence alignment of the corrinoid-binding domain of CmuA with other corrinoid proteins.** MetH from *E. coli* (P13009), MtaC (P94920) and MtmC (O30641) from *Methanosarcina barkeri* and OmdA from *Acetobacterium dehalogenans* (O87603). Amino-acids are indicated as white letters on black background when conserved in all five proteins and as white letters on gray background when conserved in four of the five protein sequences. The corrinoid binding motif [157] is marked by asterisks. Part of the corrinoid binding protein AcsD from *Clostridium thermoaceticum* (Q07341) was aligned separately and shaded according to the alignment.

Even though histidine was found to be essential as a lower axial ligand in both MetH and MtaC, such a structural arrangement does not seem to be universal for catalysis of cobalamin-dependent methyl transfer. For example, the corrinoid/iron-sulfur protein AcsD involved in acetyl coenzyme A synthesis in *Clostridium thermoaceticum* has neither the dimethylbenzimidazole nucleotide nor a nitrogenous amino acid ligand from the protein coordinated to the cobalt [158]. Part of the AcsD protein sequence aligned with other corrinoid-binding proteins suggests little relatedness to the corrinoid binding motif (Fig. 6.8).

### 6.2.2 The zinc-binding methyltransferase domain of CmuA

Purified CmuA protein was found to contain 1 mol of zinc per mol protein (see Chapter 4). The transition metal zinc has been identified as a ligand in various types of proteins and enzymes, where it serves both structural and catalytic roles [159]. Zinc is usually 4-coordinated, the ligands being nitrogen or sulfur atoms from His, Cys, Asp and Glu residues. In zinc binding sites involved in catalysis, two of the amino acids participating are often separated by only 1 to 3 residues in the protein sequence, while a third residue is found at a larger distance [159]. Interestingly, such a H-X-C-$X_n$-C zinc-binding motif is also found in the CmuA protein (Fig. 6.9, see Chapter 4). Two other methyltransferases are known which contain one equivalent of zinc per mol protein and display the same zinc-binding motif. These are the cobalamin-independent methionine synthase MetE from *E. coli* [114,160] and the methylcobamide:CoM methyltransferase MtaA from *Methanosarcina barkerii* [88,116].

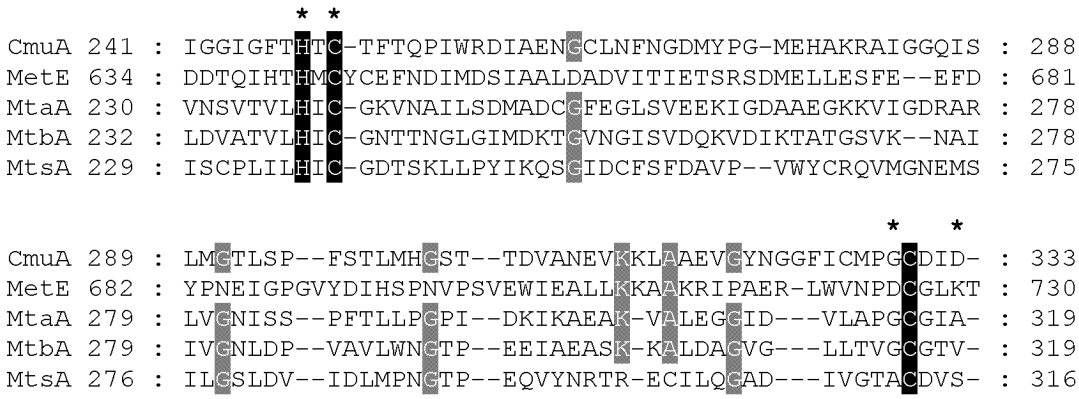


**Fig. 6.9. Amino acid sequence alignment of the zinc-binding motif of CmuA with similar methyltransferases.** MetE from *E. coli* (P25665), MtaA (Q48927), MtbA (O30640) and MtsA (Q48924) from *Methanosarcina barkeri*. Amino acids are indicated as white letters on black background when conserved in all four proteins and as white letters on gray background when conserved in three of the four protein sequences. Zinc-binding residues discussed in text are marked by asterisks.

MtaA and related methylcobamide:CoM methyltransferases from methanogenic archaea show highest similarity to the N-terminal domain of CmuA (Fig. 6.7, see Chapter 2). Zinc was recently shown to be involved in CoM activation in MtaA, and to be essential for methyl group transfer from methylcobalamin to CoM [107]. The zinc atom was therefore postulated to activate the thiol group of CoM for nucleophilic attack on the corrinoid-bound methyl group (Fig. 6.10B, [161]). This hypothesis is based on observations formulated previously for the activation of homocysteine by the cobalamin independent methionine synthase MetE from *E. coli* [160,162]. In the latter case, homocysteine was identified as the fourth ligand of zinc, in addition to three amino acid ligands of the protein. Zinc was proposed to stabilize the thiolate of the homocysteine substrate at neutral pH and thereby to activate the thiol for nucleophilic attack on methyl-$H_4$folate (Fig. 6.10A).

Such a thiol activation mechanism was first described for the Ada protein of *E. coli*, which is the transcriptional regulator in the adaptive response to DNA alkylation [163]. The Ada protein is automethylated by DNA methylphosphotriesters at a specific cysteine residue which is also one of four conserved cysteines involved in zinc-binding (Fig. 6.10C). Upon methylation, Ada undergoes a conformational change and acquires the ability to bind to

operator sites on the DNA. This leads to the transcription of genes that confer resistance to methylating agents [163]. It is thus tempting to speculate that chloromethane-responsive gene regulation in *M. chloromethanicum* CM4 could be based on a mechanism similar to that of the Ada protein from *E. coli* (Fig. 6.10C). Indeed, alignments of Orf219 and Orf98 suggest the presence of possible zinc-binding sites in both cases. As pointed out above, both proteins are candidate regulatory proteins in chloromethane metabolism in strain CM4. In such a scenario, methylation of one or both of these proteins by chloromethane might enable them to act as transcriptional activators.

Given the presence of zinc and a zinc binding motif in the sequence of CmuA (Fig. 6.9), it is conceivable that methyl transfer in chloromethane dehalogenation is based on a mechanism similar to that of Mta, MetE or Ada (Fig. 6.10A-C). Such a putative reaction mechanism would theoretically involve four zinc ligands, instead of three zinc ligands as for MetE or MtaA. Cys326 in CmuA could possibly play this role (Fig. 6.9). In this case, the zinc binding site in CmuA would be reminiscent of that of Ada. Alternatively, a low molecular weight thiol compound, such as CoM or methanethiol, might be involved in zinc binding of CmuA. However, no evidence has been obtained so far for the requirement of such a cofactor in chloromethane degradation.

Should a catalytic thiol group in CmuA indeed be involved in corrinoid dependent chloromethane dehalogenation, two possible mechanisms are conceivable a priori (Fig. 6.10D). Either chloromethane is dehalogenated by thiol conjugation prior to methyl transfer onto the corrinoid cofactor (Fig. 6.10D1), or the methyl group of chloromethane is transferred from the corrinoid to the thiol to facilitate nucleophilic attack by $H_4$folate (Fig. 6.10D2).

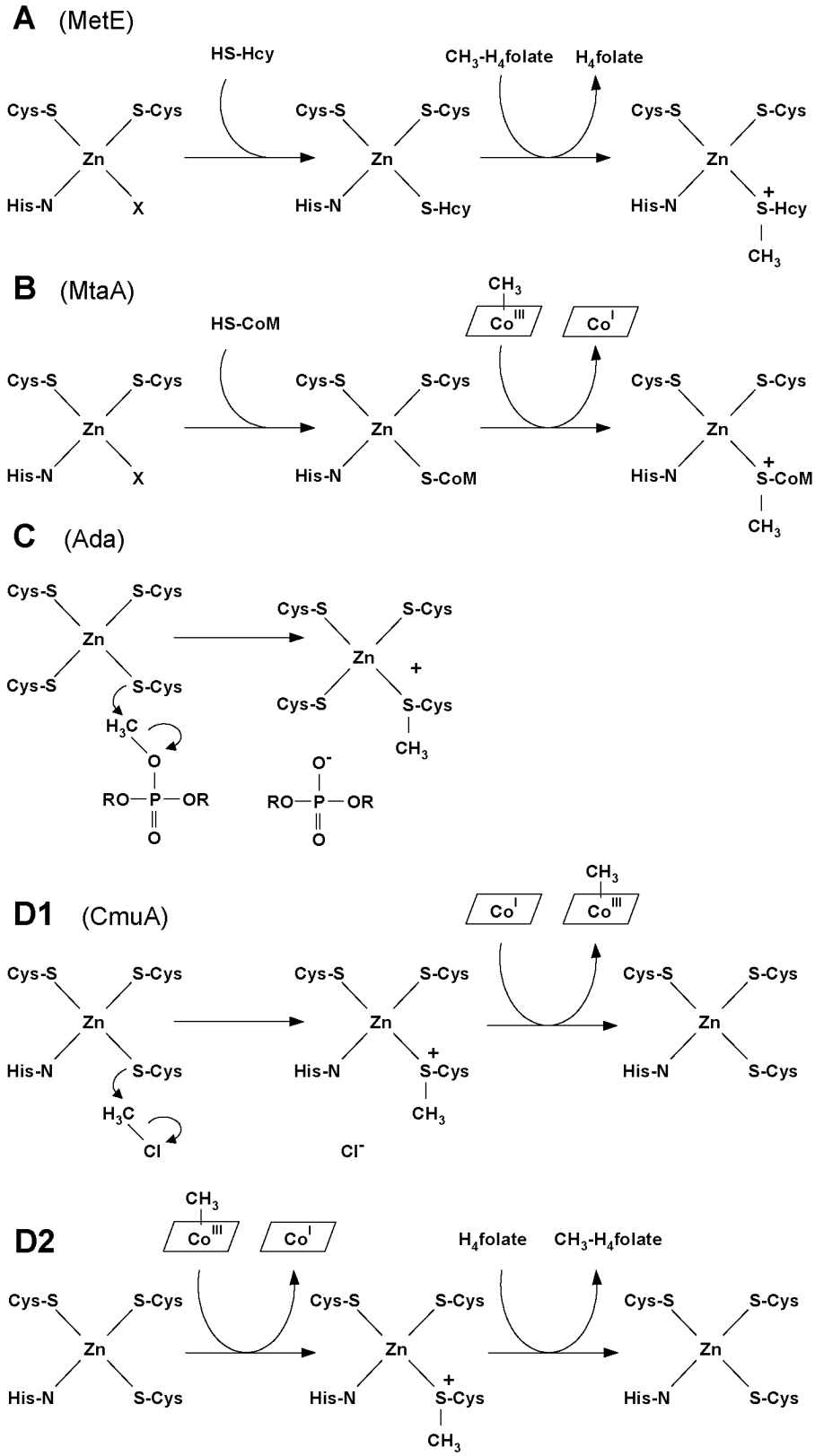


**Fig. 6.10. Zinc-dependent methyl transfer reactions.** (A) Mechanism for homocysteine activation in MetE (adapted from [160]) (B) Mechanism for activation of CoM proposed for MtaA [161] (C) Mechanism of the methylation of the Ada protein [163] (D) Proposed role of zinc in CmuA in chloromethane dehalogenation by the thiol (D1) or in facilitating methyl transfer onto $H_4$folate (D2). HS-Hcy, homocysteine; X, unknown ligand.

Concerning the first mechanism, it should be remembered that there is in principle no need for enzymatic dehalogenation since chloromethane readily reacts abiotically with cob(I)alamin [77]. On the other hand it appears likely that the CmuA protein should offer a certain protection of the corrinoid cofactor against oxidative inactivation so that dehalogenation of chloromethane by CmuA-bound cobalamin may not proceed as readily as the chemical reaction in solution. The second mechanism (Fig. 6.10D2), in contrast, may provide a means to facilitate nucleophilic attack of $H_4$folate onto the methyl group derived from chloromethane. Future biochemical studies, perhaps involving selected site-directed mutants of CmuA and CmuB proteins, will no doubt allow to distinguish between these different possibilities and to elucidate mechanisms of chloromethane dehalogenation in more detail.

### 6.2.3 How many methyltransferases are needed for growth with chloromethane ?

CmuA and CmuB were sufficient to catalyze chloromethane dehalogenation *in vitro* (see Chapter 4). CmuA exhibits methyltransferase I activity and CmuB catalyzes the methyltransferase II reaction. Based on sequence, the conserved *cmuC* gene (Fig. 6.2) seems to encode yet another methyltransferase essential for growth on chloromethane besides CmuA and CmuB (see Chapter 2). One may thus speculate that CmuC exhibits a function similar to CmuB and may also use CmuA as a substrate for methyl group transfer onto an alternative, so far unidentified cofactor. The existence of $H_4$MPT-dependent $C_1$ oxidation in *M. extorquens* AM1 (see Chapter 1, [43]) and the identification of the cofactor $H_4$MPT in strain CM4 (Studer and Keltjens unpublished) in particular are indications for a possible methyl group transfer from CmuA to $H_4$MPT catalyzed by CmuC (discussed in Chapter 4).

This hypothesis is in accordance with the phenotypes of *cmuB* and *cmuC* mutants [48]. Both mutants were unable to grow with chloromethane, but in contrast to the *cmuB* mutant, the *cmuC* mutant was still able to release chloride in the presence of chloromethane. This observation could be explained by the polar effect of the miniTn*5* insertion in *cmuB* on the expression of the *cmuC* gene. Such an effect would inactivate both methyltransferases in the *cmuB* mutant, whereas in the *cmuC* mutant one methyltransferase (CmuB) would still be active. However, the recent complementation of the two mutants with the individual genes did not support the hypothesis of CmuC acting in chloromethane dehalogenation (McAnulla unpublished). The *cmuC* mutant was complemented in presence of the *cmuC* gene, whereas the *cmuB* mutant was not complemented with only *cmuB* present. This is consistent with the polar effect in the *cmuB* mutant on *cmuC*. However, the *cmuB* mutant complemented with the *cmuC* gene alone did not show any chloride release in the presence of chloromethane (McAnulla unpublished), which demonstrates that CmuC is not directly involved in the dehalogenation of chloromethane. Therefore the essential function of CmuC in chloromethane metabolism of strain CM4 remains to be elucidated.

### 6.2.4 A reactivation mechanism for the CmuA corrinoid protein

One problem associated with corrinoid-dependent dehalogenation is the low midpoint potential of the cob(I)alamin/cob(II)alamin couple, which is – 606 mV for the free cofactor [164]. Bound to an enzyme, the potential is usually 100 to 150 mV higher, still below that of many natural electron donors, especially in aerobic organisms [165]. Corrinoid-dependent methyltransferase are therefore prone to inactivation by oxidation and the cell requires an efficient mechanism to maintain these proteins in an active form. Such reactivation systems are functionally diverse among different organisms. However, they share the requirement for a low potential electron donor and for a high energy compound (Fig. 6.6). For example, methanol:CoM methyltransferase activation requires an ATP-dependent methyltransferase-activating protein and involves a ferredoxin, a hydrogenase and molecular hydrogen as electron donor[165]. In contrast, MetH, the inactive cob(II)alamine enzyme is reactivated by a reductive methylation that involves a reduced flavodoxin and adenosylmethionine [166,167].

The physiological reactivation system is not known and the midpoint potential of the cob(I)alamin/cob(II)alamin couple has not been determined for chloromethane dehalogenase. Thus, activity of the enzyme could so far only be maintained under anoxic conditions using the artificial low-potential electron donor titanium(III)citrate. This suggests that the midpoint potential is in the range of other corrinoid dependent methyltransferases. Recently, a gene encoding a putative oxidoreductase was identified immediately downstream of CmuA in *H. chloromethanicum* CM2 and *Aminobacter* sp. IMB-1 (Fig. 6.2), which might be part of a reductive activation system for chloromethane dehalogenase in these organisms [135]. However, a similar gene is not present in strain CM4 judging Southern analysis (McAnulla unpublished). Therefore, the nature of the corrinoid reactivation system remains elusive in strain CM4.

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# APPENDIX

## Bacterial strains

| strains | Relevant phenotype or genotype | Source or reference |
|---|---|---|
| ***E. coli*** | | |
| DH5α | *sup* E44 Δ*lac*U169 (Φ80*lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 | BRL |
| BL21(DE3) | *hsdS gal* (λcl*ts*857 *ind*1 Sam7 *nin*5 *lac*UV5-T7 gene *1*) | [86] |
| CC118(λ*pir*) | Δ(*ara-leu*), *araD*, Δ*lacX*74, *galE*, *galK*, *phoA*20, *thi*-1, *rpsE*, *rpoB*, *argE*, *recA* 1, λ *pir* lysogen | [126] |
| S17-1 | *hsd*R (RP4-2 Km$^r$::Tn*5* Tc::MU chromosomally integrated) | [127] |
| S17-1(λ*pir*) | λpir lysogen of *E. coli* S17-1 | |
| ***M. chloromethanicum* CM4** | | |
| CM4 | wild type | [30] |
| 30F5 | *purU*::miniTn*5*-Km | [50] |
| 38G12 | *purU*::miniTn*5*-Km | [50] |
| 27C10 | *orf414*::miniTn*5*-Km, with unknown genomic rearrangement | [50] |
| 22B3 | *cmuA*::miniTn*5*-Km | [50] |
| 38A10 | *cmuA*::miniTn*5*-Km | [50] |
| 19D10 | *cmuB*::miniTn*5*-Km | [50] |
| 36D3 | *cmuC*::miniTn*5*-Km | [50] |
| 11G7 | miniTn*5*-Km insertion in unknown region | [50] |
| 27B11 | miniTn*5*-Km insertion in unknown region | [50] |
| PK1 | *metF*::pME1781 cointegrate | This work |

## Plasmids

| plasmid | description |
|---|---|
| **pME1700** | $Ap^R$, $Km^R$, 3.6 kb *Cla*I chromosomal fragment from mini-Tn*5* mutant 19D10 in pBLS KS $II^+$ |
| **pME1701** | $Ap^R$, $Km^R$, 7.7 kb *Kpn*I chromosomal fragment from mini-Tn*5* mutant 19D10 in pBLS KS $II^+$ |
| **pME1702** | $Ap^R$, 0.8 kb chromosomal fragment in pBLS KS $II^+$, after removal of *Hin*dIII fragment from pME1700 |
| **pME1703** | $Ap^R$, 1.8 kb chromosomal fragment in pBLS KS $II^+$, after removal of *Hin*dIII fragment from pME1701 |
| **pME1704** | $Ap^R$, 3.7 kb *Hin*dIII fragment from pME1701 in pBLS KS $II^+$ |
| **pME1705** | $Ap^R$, $Km^R$, 3.7 kb *Sac*I chromosomal fragment from mini-Tn*5* mutant 22B3 in pBLS KS $II^+$ |
| **pME1706** | $Ap^R$, $Km^R$, 3.9 kb *Sac*I chromosomal fragment from mini-Tn*5* mutant 38A10 in pBLS KS $II^+$ |
| **pME1707** | $Ap^R$, 1.4 kb chromosomal fragment in pBLS KS $II^+$, after removal of *Avr*II fragment from pME1705 |
| **pME1708** | $Ap^R$, 1.6 kb chromosomal fragment in pBLS KS $II^+$, after removal of *Sfi*I fragment from pME1706 |
| **pME1709** | $Ap^R$, 1.4 kb chromosomal fragment in pBLS KS $II^+$, after removal of *Acc*I fragment from pME1704 |
| **pME1710** | $Ap^R$, 2.0 kb chromosomal fragment in pBLS KS $II^+$, after removal of *Eco*O109I fragment from pME1704 |
| **pME1711** | $Ap^R$, 2.0 kb chromosomal fragment in pBLS KS $II^+$, after removal of *Pst*I fragment from pME1704 |
| **pME1712** | $Ap^R$, $Km^R$, 8.0 kb *Cla*I chromosomal fragment from mini-Tn*5* mutant 22B3 in pBLS KS $II^+$ |
| **pME1713** | $Ap^R$, $Km^R$, 8.3 kb *Cla*I chromosomal fragment from mini-Tn*5* mutant 30F5 in pBLS KS $II^+$ |
| **pME1714** | $Ap^R$, $Km^R$, 8.1 kb *Cla*I chromosomal fragment from mini-Tn*5* mutant 38A10 in pBLS KS $II^+$ |
| **pME1715** | $Ap^R$, $Km^R$, 11.0 kb *Sac*I chromosomal fragment from mini-Tn*5* mutant 19D10 in pBLS KS $II^+$ |
| **pME1716** | $Ap^R$, 6.2 kb chromosomal fragment in pBLS KS $II^+$, after removal of *Sfi*I fragment from pME1712 |
| **pME1717** | $Ap^R$, 6.5 kb chromosomal fragment in pBLS KS $II^+$, after removal of *Sfi*I fragment from pME1714 |
| **pME1718** | $Ap^R$, $Km^R$, 6.6 kb *Not*I chromosomal fragment from mini-Tn*5* mutant 22B3 in pBLS KS $II^+$ |

| plasmid | description |
|---|---|
| **pME1719** | $Ap^R$, $Km^R$, 7.7 kb *Not*I chromosomal fragment from mini-Tn*5* mutant 38A10 in pBLS KS $II^+$ |
| **pME1720** | $Ap^R$, 1.5 kb chromosomal fragment in pBLS KS $II^+$, after *Kpn*I digest of pME1716 |
| **pME1721** | 4.7 kb chromosomal fragment in pBLS KS $II^+$, after *Not*I digest of pME1716 |
| **pME1722** | $Ap^R$, $Km^R$, 7.6 kb *Not*I chromosomal fragment from mini-Tn*5* mutant 19D10 |
| **pME1723** | $Ap^R$, 2.3 kb chromosomal fragment in pBLS KS $II^+$, after *Pst*I digest of pME1713 |
| **pME1724** | $Ap^R$, 1.2 kb *Hin*dIII fragment from pME1719 in pBLS KS $II^+$ |
| **pME1725** | $Ap^R$, 9.0 kb *Eco*RI fragment from pME1715 in pBLS KS $II^+$ |
| **pME1726** | $Ap^R$, 5.5 kb chromosomal fragment in pBLS KS $II^+$, after *Pst*I digest of pME1725 |
| **pME1727** | $Ap^R$, $Km^R$, 8.7 kb *Xba*I chromosomal fragment from mini-Tn5 mutant 27C10 in pBLS KS $II^+$ |
| **pME1728** | $Ap^R$, $Km^R$, 9.0 kb *Sac*I chromosomal fragment from mini-Tn5 mutant 30F5 in pBLS KS $II^+$ |
| **pME1729** | $Ap^R$, 4.0 kb chromosomal fragment in pBLS KS $II^+$, after *Hin*dIII digest of pME1728 |
| **pME1730** | $Ap^R$, 3.5 kb chromosomal fragment in pBLS KS $II^+$, after Hind*III* digest of pME1727 |
| **pME1731** | $Ap^R$, 3.0 kb *Hin*dIII fragment from pME1727 in pBLS KS $II^+$ |
| **pME1732** | $Ap^R$, $Km^R$, 10.8 kb *Cla*I chromosomal fragment from mini-Tn*5* mutant 11G7 in pBLS KS $II^+$ |
| **pME1733** | $Ap^R$, $Km^R$, 17.0 kb *Cla*I chromosomal fragment from mini-Tn*5* mutant 27B11 in pBLS KS $II^+$ |
| **pME1734** | $Ap^R$, $Km^R$, 9.0 kb *Not*I chromosomal fragment from mini-Tn*5* mutant 27C10 in pBLS KS $II^+$ |
| **pME1735** | $Ap^R$, $Km^R$, 3.6 kb *Not*I/*Eco*RV chromosomal fragment from mini-Tn*5* mutant 27C10 in pBLS KS $II^+$ |
| **pME1736** | $Ap^R$, 3.2 kb chromosomal fragment in pBLS KS $II^+$, after *Hin*dIII cut digest of pME1732 |
| **pME1737** | $Ap^R$, 2.6 *Hin*dIII fragment from pME1732 in pBLS KS $II^+$ |
| **pME1738** | $Ap^R$, $Km^R$, 4.6 kb *Cla*I chromosomal fragment from mini-Tn*5* mutant 36D3 in pBLS KS $II^+$ |
| **pME1739** | $Ap^R$, $Km^R$, 4.5 kb chromosomal fragment in pBLS KS $II^+$, after *Eco*RV digest of pME1733 |
| **pME1740** | $Ap^R$, $Km^R$, 10.0 kb *Cla*I chromosomal fragment from mini-Tn*5* mutant 38G12 in pBLS KS $II^+$ |
| **pME1741** | $Ap^R$, 1.4 kb *Hin*dIII fragment of pME1735 |

| plasmid | description |
|---|---|
| **pME1742** | $Ap^R$, $Km^R$, 7.5kb *Kpn*I chromosomal fragment from mini-Tn*5* mutant 36D3 in pBLS KS $II^+$ |
| **pME1743** | $Ap^R$, 1.4kb *Cla*I/*Kpn*I fragment from pME1740 in pBLS KS $II^+$ |
| **pME1744** | $Ap^R$, $Km^R$, 2.3kb *Hind*III fragment from pME1740 with miniTn5 cassette |
| **pME1745** | $Ap^R$, 1.5kb *Cla*I fragment from pME1742 in pBLS KS $II^+$ |
| **pME1746** | $Cm^R$, 1.5kb *Cla*I fragment from pME1745 in pBBRMCS-1 |
| **pME1747** | $Ap^R$, 1kb Pfu amplification of *cmuB* with primers ast10 and ast11 cloned blunt end in pBLS KS $II^+$ |
| **pME1748** | $Km^R$, 1kb *Nde*I/*Hin*dIII fragment from pME1747 in pET24a(+), CmuB expression vector |
| **pME1749** | $Ap^R$, 4.3 kB *Eco*RI chromosomal fragment from wt CM4 in pBLS KS $II^+$, comprising *orf414* and *cmuA* |
| **pME1750** | $Ap^R$, 1.85 kb *pfu* amplification of *cmuA* with primers ast8 and ast9 cloned blunt end in pBLS KS $II^+$ |
| **pME1751** | $Km^R$, 1.85 kb *Nde*I/*Hin*dIII fragment from pME1750 in pET24a(+), CmuA expression vector |
| **pME1752** | $Cm^R$, 4,3kb *Hin*dIII/*Xba*I fragment from pME1749 in pMMB207 containing *orf414* and *cmuA* under the control of their natural promoter |
| **pME1753** | $Cm^R$, 4,3kb *Hin*dIII/*Xba*I fragment from pME1749 in pBBRMCS-1 containing *orf414* and *cmuA* under the control of their natural promoter |
| **pME1754** | $Ap^R$, 4.0 kb *Sac*I/*Hin*dIII fragment from pME1723 and 3.0 kb *Hin*dIII/*Sac*I fragment from pME 1712 ligated into *Sac*I site of pBLS KS $II^+$ |
| **pME1755** | $Ap^R$, 2.5 kB *Eco*RI/*Sma*I fragment from pME1701 in pBLS KS $II^+$ |
| **pME1756** | $Ap^R$, 0.8 kB *Eco*RI/*Sma*I fragment from pME1742 in pUC29 |
| **pME1757** | $Ap^R$, 3.3kB *Sal*I/*Eco*RI fragment from pME1756 cloned into pME1757 opened *Sal*I/*Eco*RI, restores *cmuB cmuC* |
| **pME1758** | $Tet^R$, 3.3kB *Kpn*I/*Xba*I fragment of pME1756 in pJBTc19 |
| **pME1761** | $Ap^R$, 1.1 kB *pfu* amplification of *cmuC* cloned bluntend into *Eco*RV site of pBLS KS $II^+$ |
| **pME1762** | $Km^R$, 1.1 kB *Nde*I/*Bam*HI fragment from pME1761 in pET24a(+), CmuC expression vector |
| **pME1764** | $Tet^R$, $Km^R$, 4.3kB *Eco*RI fragment from pME1749 in pCM52; *orf414 cmuA* |
| **pME1765** | $Tet^R$, $Km^R$, 3,7 kB *Pst*I/*Eco*RI fragment from pME1749 in pCM52; *orf414 cmuA* without natural promotor |
| **pME1766** | $Tet^R$, 3,7 kB *Pst*I/*Eco*RI fragment from pME1749 in pCM62; *orf414 cmuA* without natural promotor |
| **pME1767** | $Tet^R$, 3,7 kB *Pst*I/*Eco*RI fragment from pME1749 in pCM52; *orf414 cmuA* without natural promotor |

| plasmid | description |
|---|---|
| **pME1768** | $Tet^R$, 3,7 kB *Pst*I/*Eco*RI fragment from pME1749 in pCM52; *orf414 cmuA* without natural promotor |
| **pME1769** | $Tet^R$, 4.3kB *Eco*RI fragment from pME1749 in pCM52; *orf414 cmuA* |
| **pME1770** | $Ap^R$, 0.7 kb Pfu amplification of *purU orf414* intragenic region with primer ast25 and ast26 *Xba*I/*Nde*I digested and cloned into pME1750; fusion of natural promoter region directly to *cmuA* |
| **pME1771** | $Tet^R$, 2,6 kb *Xba*I/*Hin*dIII fragment from pME1770 in pCM62 |
| **pME1772** | $Tet^R$, 1.9 kb *Pst*I/*Bam*HI fragment from pME1750 in pCM62; *cmuA* under *lac*P promoter |
| **pME1773** | $Ap^R$, 0.5 kb *Eco*RI/*Bam*HI fragment from pME1716 in pBLS KS $II^+$ |
| **pME1774** | $Km^R$, 0.5 kb *Not*I/*Xho*I fragment from pME1773 in pKNOCK-Km; internal *folD* fragment |
| **pME1775** | $Tet^R$, 5.8 bp *Xba*I/*Sac*I fragment from pME1754 in CM62; *purU folD folC cobU* |
| **pME1776** | $Tet^R$, 2.8 kb *Bam*HI/*Sac*I fragment from pME1754 in pCM62; *purU* |
| **pME1777** | $Tet^R$, 3.1 kb *Kpn*I(blunted)/*Sac*I fragment from pME1754 in CM62 opened *Hin*dIII(blunted)/*Sac*I |
| **pME1778** | $Tet^R$, 4.8 kb *Nco*I(blunted)/*Sac*I fragment from pME1754 in CM62 *Hin*dIII(blunted)/*Sac*I |
| **pME1780** | $Km^R$, 592bp *Bam*HI/*Hinc*II fragment of pME1749 containing *orf414* part |
| **pME1781** | $Km^R$, 0.6 kb *Sma*I/*Cla*I internal *metF* fragment in pKNOCK-Km |
| **pME1782** | $Ap^R$, $Km^R$, 1.2 kb *Mlu*I fragment of pKNOCK-Km ($Km^R$ gene) in pME1749 *Mlu*I opened |
| **pME1784** | $Tet^R$, $Km^R$, 3.4kb *Xba*I fragment from pME1757 in pCM52; *cmuB cmuC* |
| **pME1785** | $Tet^R$, $Km^R$, 3.4kb *Xba*I fragment from pME1757 in pCM62; *cmuB cmuC* |
| **pME1786** | $Tet^R$, $Km^R$, 3.4kb *Xba*I fragment from pME1757 in pCM80; *cmuB cmuC* |
| **pME1787** | $Tet^R$, $Km^R$, 3.2 kb *Kpn*I fragment of pME1782 (*orf414* with $Km^R$ gene) in pWM41 |
| **pME1788** | $Tet^R$, $Km^R$, 3.2 kb *Kpn*I fragment of pME1782 (orf414 with $Km^R$ gene) in pWM41 |
| **pME1789** | $Tet^R$, $Km^R$, 1.2 kb *Hin*dIII/*Fsp*I fragment from pME1742 in pWM41; *metF* |
| **pME1790** | 779 bp PCR fragment in pCM130; *purU::xylE* fusion |
| **pME1791** | 779 bp PCR fragment in pCM130; *orf414::xylE* fusion |
| **pME1792** | $Ap^R$, 0.3 kb blunted *Nde*I/*Xho*I internal *orf219* fragment from pME1726 in pBLS KS $II^+$ *Eco*RV opened |
| **pME1793** | $Tet^R$, 1.7 kb *Hin*dIII/*Xho*I fragment from pME1726 in pME 1785 *Hin*dIII/*Xho*I opened; *metF cmuB cmuC* under control of natural promoter region |

| plasmid | description |
|---|---|
| **pME 1794** | $Ap^R$, 0.2 kB Pfu amplification of *orf219*/*metF* intragenic region with primer ast30 and ast31 ligated blunted into *Eco*RV opened pBLS KS $II^+$ |
| **pME 1795** | $Ap^R$, 0.2 kB Pfu amplification of *orf219*/*metF* intragenic region with primer ast30 and ast31 ligated blunted into *Eco*RV opened pBLS KS $II^+$ |
| **pME 1796** | $Tet^R$, 159 bp *Bam*HI fragment from pME1795 in pCM130; *orf219::xylE* fusion (checked by sequencing) |
| **pME 1797** | $Tet^R$, 159 bp *Bam*HI fragment from pME1795 in pCM130; *metF::xylE* fusion (checked by sequencing) |
| **pME1798** | $Ap^R$, 1.4 kb *Eco*RI fragment from pME1716 in pBLS KS $II^+$; *purU folD'* |
| **pME1799** | $Tet^R$, 1.7 kb *Hin*dIII/*Xho*I fragment from pME1793 in pCM130; *metF cmuB::xylE* fusion |
| **pME8250** | $Tet^R$, 0.6 kb *Cla*I/*Xho*I fragment from pME1793 in pCM130; *cmuB::xylE* fusion |
| **pME8251** | $Tet^R$, 1.4 kb *Hin*dIII/*Bam*HI from pME1798 fragment in pCM130; *Eco*RI chromosomal fragment with *purU folD::xylE* fusion |
| **pME8252** | $Tet^R$, 0.7 kb *Xho*I/*Bam*HI pME1798 fragment in pCM130; *Xho*I/*Eco*RI chromosomal fragment with *purU::xylE* fusion |
| **pME8253** | $Tet^R$, 0.7 kb *Hin*dIII/*Xho*I fragment in pCM130 with *folD::xylE* fusion, obtained after removal of a *Xho*I/*Bam*HI fragment from pME8251 and religation of the blunted plasmid |
| **pBLS II $KS^+$** | $Ap^r$, cloning vector, Stratagene |
| **pKNOCK-Km** | $Km^r$, broad-host-range suicide vector, $ori_{R6K}$, Stratagene |
| **pET24a(+)** | $Km^r$,expression vector |
| **pCM52** | $Tet^r$, $Km^r$, broad-host range vector |
| **pCM62** | $Tet^r$, broad-host range vector |
| **pCM80** | $Tet^r$, broad-host range vector |
| **pCM130** | $Tet^r$, *xylE* promoter-probe vector |
| **pCM131** | $Tet^r$, *mxaF::xylE* fusion in pCM130 |

## Oligonucleotides

| name | nucleotide sequence | description |
|---|---|---|
| ast1 | AACAATCTAGCGAGGGCTTGG | primer facing out of miniTn*5* (pos 194-214) |
| ast2 | AAGGGCTCCAAGGATCGGGCC | primer facing out of miniTn*5* (pos 2070-2090) |
| ast3 | TTCAAAAGGTCATCCACCGG | primer facing inward in miniTn*5* (pos 165-184) |
| ast4 | CCTGCTATTGCTGCCCCGTCC | forward primer (pos 6570-6590, AJ011316) |
| ast5 | CCAGCGCGTACTCCGGATCC | reverse primer (pos 7421-7440, AJ011316) |
| ast6 | CCAGACTCGAACATAAGTCG | reverse primer (pos 7482-7501, AJ011316) |
| ast8 | GACTGGAACATATGGCTGCACAAAGCGG | forward primer to introduce *Nde*I site at translational start site of *cmuA* (pos 6886-6913, AJ011316) |
| ast9 | GGACGGATCCCGAACGAAAGGCGC | reverse primer to introduce *Bam*HI site downstream of *cmuA* (pos 8750-8773, AJ011316) |
| ast10 | GGGAGGTTAAATCATATGAATAAG | forward primer to introduce *Nde*I site at translational start site of *cmuB* (pos 4688-4711, AJ011317) |
| ast11 | CGATCAGCAGTCGACCGGTCGGTTGTCCC | reverse primer to introduce *Sal*I site site downstream of *cmuB* (pos 5670-5698, AJ011317) |
| ast12 | CCGTCGCGGCCATATGACC | forward primer to introduce *Nde*I site at translational start site of *cmuC* (pos 5622-5640, AJ011317) |
| ast13 | GCCAGCAAGTGTCACGCCG | reverse primer at the N-terminal side of *cmuC* (pos 6764-6782, AJ011317) |
| ast14 | TTCCCTTGTCCAGATAGCC | primer facing into MCS of plasmid pKNOCK-Km |
| ast15 | TCGGCTCGTATAATGTGTGG | primer facing into MCS of plasmid pCM52 |
| ast16 | CTTCTCTCATCCGCCAAAAC | primer facing into MCS of plasmid pCM52 |
| ast17 | GAGCATCTTCCAGCATCC | forward primer (pos 5563-5580, AJ011316) |
| ast18 | GCACCATGCAATACTACGC | reverse primer (pos 6463-6481, AJ011316) |
| ast19 | CGATATCTGCAACCAAAGC | reverse primer (pos 5981-5999, AJ011316) |
| ast20 | CAAGAAGCTGTCTCCTACGC | forward primer (pos 3527-3546, AJ011317) |

| name | nucleotide sequence | description |
|---|---|---|
| ast21 | GCTCGCGAAACTATATCTGC | reverse primer (pos 4417-4436, AJ011316) |
| ast22 | GCAACTAGGAGGATCTCACG | reverse primer (pos 3852-3871, AJ011316) |
| ast25 | GGCGACATATGACGGCACC | reverse primer to introduce *Nde*I at translational start site of *orf414* (pos 5617-5635, AJ011316) |
| ast26 | TTCCGCCATCTAGAGATTCC | forward primer to introduce *Xba*I site at translational start site of *purU* (pos 4841-4860, AJ011316) |
| ast27 | CGCACCTGAAACGGCAGCGACGATGC | forward primer for primer extension analysis of *purU* (pos 4775-4800, AJ011316) |
| ast28 | CCGAGCCACGGAAACGGTTCAGGCG | reverse primer for primer extension analysis of *orf414* (pos 5632-5656, AJ011316) |
| ast29 | CGAACATCCGCTCACGGCTCGTCATC | reverse primer for primer extension analysis of *cmuA* (pos 6917-6942, AJ011316) |
| ast30 | GCTTTCGGATCCATCAGACG | forward primer to introduce *Bam*HI site at translational start site of *orf219* (pos 3332-3351, AJ011317) |
| ast31 | CCACATATGCGGATCCGTC | reverse primer to introduce *Nde*I at translational start site of *metF* (pos 3494-3512, AJ011317) |
| ast32 | CGACAGACCCGAACCTCGCCATTGG | reverse primer for primer extension analysis of *orf414* (pos 5509-5533, AJ011316) |
| ast33 | GGGAGACCTCCAATGACAGATCGCG | reverse primer for primer extension analysis of *metF* (pos 3603-3627, AJ011317) |
| ast34 | CCCATCTATCACTTGTGCAATCATG | forward primer for primer extension analysis of *folD* (pos 3842-3866, AJ011316) |
| ast35 | CGGAGAAATGATGCCTTGCTCCTCA | forward primer for primer extension analysis of *orf219* (pos 3229-3253 AJ011317) |
| ast36 | CCTCGATCTCTCAATCCCTGG | forward primer (pos 3360-3380, AJ011317) |
| ast37 | GAATGTCAATGGGATCGTGCG | reverse primer (pos 3440-3460, AJ011317) |
| ast38 | CAGATACGGTAAACTAGCCTCG | primer facing into MCS of plasmid pCM130 |

## CURRICULUM VITAE

Alexander Studer
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| | |
|---|---|
| **1978 – 1982** | Primary education in Pratteln (BL), Switzerland |
| **1982 – 1986** | Secondary education (Progymnasium) in Pratteln (BL), Switzerland |
| **1986 – 1990** | Secondary education (Gymnasium) in Muttenz (BL), Switzerland<br>Graduation: Matura C |
| **1992 – 1995** | Studies of Molecular Biology and Biochemistry at the University of Basel, Biozentrum, Switzerland |
| **1995 – 1996** | Masters degree (Diploma) in Microbiology: "Type IC restriction-modification system *Eco*R124" at the University of Basel, Biozentrum, Switzerland |
| **1997 – 2001** | Research assistant at the Swiss Federal Institute of Technology, Institute of Microbiology, Zürich, Switzerland |
| **2001** | Ph.D. thesis at the Swiss Federal Institute of Technology, Institute of Microbiology, Zürich, Switzerland |

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