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Methylotrophs and Methylotroph Communities

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Methylotrophs and Methylotroph Populations for Chloromethane Degradation

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Françoise Bringel[1*], Ludovic Besaury[2], Pierre Amato[3], Eileen Kröber[4], Steffen Kolb[4], Frank Keppler[5,6], Stéphane Vuilleumier[1] and Thierry Nadalig[1]

[1]Université de Strasbourg UMR 7156 UNISTRA CNRS, Molecular Genetics, Genomics, Microbiology (GMGM), Strasbourg, France.
[2]Université de Reims Champagne-Ardenne, Chaire AFERE, INRA, FARE UMR A614, Reims, France.
[3] Université Clermont Auvergne, CNRS, SIGMA Clermont, ICCF, Clermont-Ferrand, France.
[4]Microbial Biogeochemistry, Research Area Landscape Functioning – Leibniz Centre for Agricultural Landscape Research – ZALF, Müncheberg, Germany.
[5]Institute of Earth Sciences, Heidelberg University, Heidelberg, Germany.
[6]Heidelberg Center for the Environment HCE, Heidelberg University, Heidelberg, Germany.

*Correspondence: francoise.bringel@unistra.fr

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Abstract

Chloromethane is a halogenated volatile organic compound, produced in large quantities by terrestrial vegetation. After its release to the troposphere and transport to the stratosphere, its photolysis contributes to the degradation of stratospheric ozone. A better knowledge of chloromethane sources (production) and sinks (degradation) is a prerequisite to estimate its atmospheric budget in the context of global warming. The degradation of chloromethane by methylotrophic communities in terrestrial environments is a major underestimated chloromethane sink. Methylotrophs isolated from soils, marine environments and more recently from the phyllosphere have been grown under laboratory conditions using chloromethane as the sole carbon source. In addition to anaerobes that degrade chloromethane, the majority of cultivated strains were isolated in aerobiosis for their ability to use chloromethane as sole carbon and energy source. Among those, the Proteobacterium *Methylobacterium* (recently reclassified as *Methylorubrum*) harbours the only characterized 'chloromethane utilization' (*cmu)* pathway, so far. This pathway may not be representative of chloromethane-utilizing populations in the environment as *cmu* genes are rare in metagenomes. Recently, combined 'omics' biological approaches with chloromethane carbon and hydrogen stable isotope fractionation measurements in microcosms, indicated that microorganisms in soils and the phyllosphere (plant aerial parts) represent major sinks of chloromethane in contrast to more recently recognized microbe-inhabited environments, such as clouds. Cultivated chloromethane-degraders lacking *cmu* genes display a singular isotope fractionation signature of chloromethane. Moreover, $^{13}CH_3Cl$ labelling of active methylotrophic communities by stable isotope probing in soils identify taxa that differ from those known for chloromethane degradation. These observations suggest that new biomarkers for detecting active microbial chloromethane-utilizers in the environment are needed to assess the contribution of microorganisms to the global chloromethane cycle.

Introduction

Chloromethane and stratospheric ozone depletion

Chloromethane (methyl chloride, CH_3Cl) is the most abundant organohalogen in the Earth atmosphere. Its global production is estimated at 4–5 megatons per year, with main sources stemming from terrestrial vegetation (Keppler, 2005). Photolytic degradation of chloromethane releases a halogen radical, which catalyses the destruction of ozone. Thus, chloromethane contributes to depletion of the stratospheric ozone layer (altitude of approximately 20 to 30 km), which constitutes the Earth's natural protective shield that absorbs the solar UVC and partially UVB radiation dangerous for living organisms. Until the 1990s, chloromethane was used as a refrigerant under the name Freon 40. Chloromethane has a stratospheric lifetime of about one year, much shorter than most other chlorofluorocarbons (CFCs), solvents and halons also banned by the international agreement of 1987 known as the Montreal Protocol on substances that deplete the ozone layer (see web resource section). There are still some uncertainties about chloromethane anthropogenic emissions (e.g. coal combustion, feedstock for chemical industries) (Li *et al.*, 2017). Chloromethane is responsible alone for approximately 16% of stratospheric chlorine-catalysed ozone destruction (Carpenter *et al.*, 2014). A detailed understanding of its sources and sinks will be essential to predict changes in atmospheric chloromethane fluxes in the context of global climate change.

Chloromethane sources and sinks

Chloromethane formation in plants and soil involves biotic and abiotic processes. Chloride ion can be alkylated during the abiotic oxidation of organic matter by an electron acceptor such as Fe(III) in soils and sediments (Keppler *et al.*, 2000). Abiotic chloromethane mainly results from the conversion of plant methoxyl groups (ether- or ester-bonded methyl groups) and their reaction with chloride ion (Keppler *et al.*, 2000; Hamilton *et al.*, 2003; Sailaukhanuly *et al.*, 2014). This process occurs in terrestrial ecosystems at ambient temperatures (Derendorp *et al.*, 2012; Keppler *et al.*, 2014), but it is much more efficient at higher temperatures such as those reached during pyrolysis and biomass burning (Hamilton *et al.*, 2003; Keppler, 2005; McRoberts *et al.*, 2015). Chloromethane release was also detected during thermal conversion of Martian soils by the Mars landers Viking (Biemann *et al.*, 1976) and Curiosity (Ming *et al.*, 2014), indicating the presence of endogenous organic matter on Mars. Chloromethane emission profiles during thermal treatment of soils sampled from other hyperarid environments hostile microbial life such as the Atacama desert were almost identical to those recorded by the Curiosity rover on Mars (Schulze-Makuch *et al.*, 2018). Furthermore, chloromethane formation was observed from thermal conversion of extraterrestrial material such as carbonaceous meteorites (Keppler *et al.*, 2014) and in protostellar environments (Fayolle *et al.*, 2017). These recent observations contributed to the emergence of an 'astronomical' fundamental interest in the understanding of chloromethane's cycle in sun-like stars and on Earth.

On Earth, plants (alive or decaying) are a major biotic source of chloromethane. Chloromethane is produced by enzymatic chloride ion methylation, as shown in higher plants affiliated to the *Brassicaceae* family (Attieh *et al.*, 1995; Rhew *et al.*, 2003) and wood-degrading fungi (Harper *et al.*, 1990). The S-adenosyl-L-methionine-dependent halide ion methyltransferase is encoded by gene *HOL1* (Harmless to Ozone Layer) in *Arabidopsis thaliana* (Nagatoshi and Nakamura, 2009). *HOL1* gene disruption correlates with decreased pathogen defence, possibly due to reduced production of methyl thiocyanate (CH_3SCN) from glucosinolate-derived thiocyanate by HOL1 (Manley, 2002; Rhew *et al.*, 2003; Nagatoshi and Nakamura, 2009). Chloromethane production is considered a by-product of plant thiocyanate metabolism. Such methyltransferases have also been detected in other crop and seaside plants (Itoh *et al.*, 2009) including marine algae (Wuosmaa and Hager, 1990; Ohsawa *et al.*, 2001; Toda and Itoh, 2011).

Identified chloromethane sinks are dominated by abiotic loss processes in the atmosphere involving reaction with OH radicals, or via chlorine radicals in the marine atmospheric boundary layer (see Web resources). The extent

of consumption of chloromethane under the control of biological processes, especially by microorganisms, constitutes one of the largest uncertainties regarding the global budget of chloromethane (Harper and Hamilton, 2003; Keppler, 2005).

Microbial degradation: an underestimated sink in the global chloromethane budget

The study of ecology and diversity of chloromethane and other methyl halide-degrading microorganisms remain a challenging field of environmental biology. Key microbial enzymes of (de)halogenation activity and active microorganisms in chlorine and other halogens (fluorine, bromine, iodine) cycling remain largely unknown (Weigold *et al.*, 2016). The global impact of microorganisms on the chloromethane sink is difficult to quantify due to concomitant processes of production and degradation of chloromethane in the environment. For instance, highly fluctuating chloromethane emissions occur in fern plants as recently discussed (Jaeger *et al.*, 2018b). Both production and degradation of chloromethane may coexist within a single organism as reported for lignin-degrading fungi. Unlike most other wood-rotting fungi, in *Phanerochaete chrysosporium*, *Phlebia radiata*, and *Coriolus versicolor,* chloromethane serves as an endogenous methyl donor in veratryl alcohol biosynthesis so that no chloromethane is emitted during lignin degradation and growth (Harper *et al.*, 1990; Coulter *et al.*, 1993). However, so far, methyl halide (CH_3X; chloromethane, bromomethane and iodomethane) cycling studies focused on chloromethane degradation by bacteria rather than by fungi (Leisinger and Braus-Stromeyer, 1995; Harper, 2000; McDonald *et al.*, 2002; Schäfer *et al.*, 2007; Cox *et al.*, 2012). While the possible implications of fungal populations on the chloromethane cycle remain to be assessed using 'omics' approaches, the aim of this review is primarily to discuss recent investigations of bacterial populations associated with chloromethane sinks in contrasting terrestrial environments, these populations potentially differing from the so far characterized microbes with known pathways for chloromethane degradation. This possibility raises questions about the composition, distribution, functioning and evolution of chloromethane-utilizing populations in response to highly fluctuating emissions of this volatile halogenated compound at the soil-plant-atmosphere interfaces.

Assessing bacterial chloromethane sinks

Recent investigations of plant and soil samples suggest that chloromethane-degraders represent a minor fraction of microbial communities (Chaignaud *et al.*, 2018; Jaeger *et al.*, 2018a,b) that are estimated as 10^7 cells per cm^2 of leaf surface to 10^9 cells per g of soil (Vorholt, 2012). In air, microbial concentrations range from a few tens to billions per cubic metre depending on location, altitude above ground, and time of the day and year (Amato *et al.*, 2017a; DasSarma and DasSarma, 2018), representing a total of 10^{19} bacterial cells in the atmosphere (Whitman *et al.*, 1998). Microorganisms collected from aerosols, cloud and rain waters retain metabolic activity under cloud-like conditions and are able to utilize atmospheric organic compounds such as formaldehyde and formate present at relatively high concentrations in cloud droplets ($\approx$ 1–100 μM) (Amato *et al.*, 2007; Delort *et al.*, 2010; Vaïtilingom *et al.*, 2013; Šantl-Temkiv *et al.*, 2017). While cloud-borne microbial assemblages include active facultative methylotrophs (Amato *et al.*, 2017b), no isolate grew on chloromethane, no chloromethane consumption was observed (Fig. 8.1), and, consistently, no *cmuA* gene was detected using a highly sensitive capture hybridization method (see below). In contrast, viable methanotrophs (*Methylosinus* and *Methylocystis*) isolated from atmospheric samples (aerosol and rain) (Šantl-Temkiv *et al.*, 2013) were able to use methane under cloud-like physicochemical conditions at concentrations relevant for the atmospheric environment ($\approx$ 1.5 ppm). We concluded that the microbiota thriving in the investigated clouds do not represent a significant sink for chloromethane, unlike soils, aerial parts of plants (phyllosphere), or marine environments (Goodwin *et al.*, 2005; Schäfer *et al.*, 2007; Chaignaud *et al.*, 2018; Jaeger *et al.*, 2018a,b).

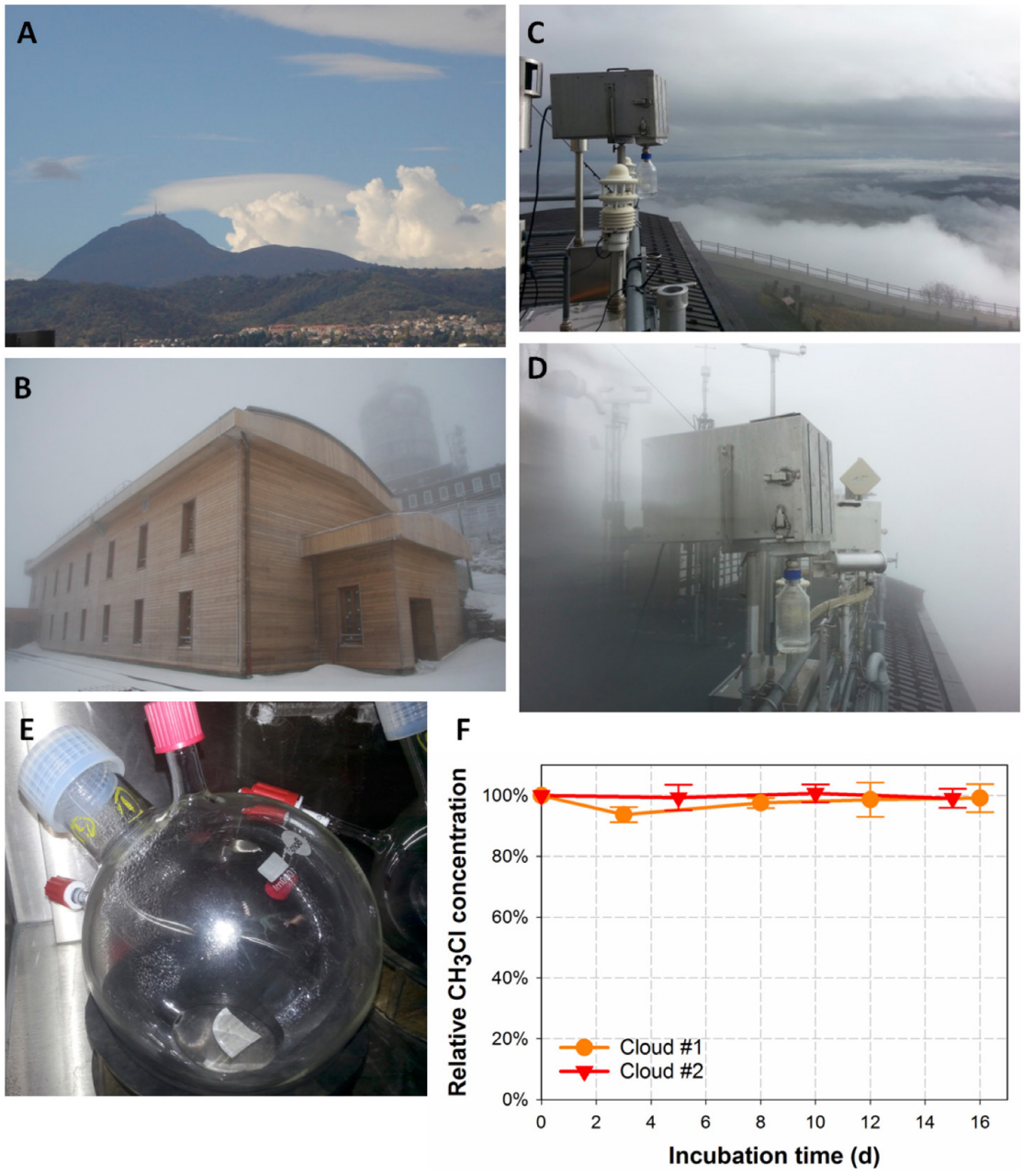

Figure 8.1 Assessing cloud water microorganisms for chloromethane degradation. Cloud water samples were collected under sterile conditions from the atmospheric station at Puy de Dôme mountain summit (1465 m above see level, France) (A) at the roof platform of a historical meteorological station (B) using cloud droplet impactors (C and D) as previously described (Amato *et al*., 2017b). Airtight microcosms used for testing chloromethane (≈10 ppm) degradation of the collected cloud water samples (E). Two independent cloud water samples (626 ml and 740 ml, respectively, containing ≈10^6 cells pre-concentrated into 20 ml) showed no detectable temporal decrease of chloromethane under the tested conditions at 17°C (F). *M. extorquens* CM4 added in sterile rainwater spiked with 10 mM of chloromethane was used as positive control (data not shown). No isolate grew on chloromethane supplied as the sole source of carbon and energy (data not shown) in enrichment cultures of cloud and rain samples, performed as previously described (Nadalig *et al*., 2011).

Cultivated chloromethane-degrading methylotrophs

Methylotrophic bacteria capable of utilizing chloromethane as the only source of carbon and/or energy have been isolated (Schaefer *et al.*, 2002; Nadalig *et al.*, 2014) from contrasting environments such as soil, plants, freshwater and marine environments (Table 8.1). Chloromethane degraders are found among facultative aerobes, obligate anaerobes, obligate and facultative methylotrophs, and conditional utilizers of chloromethane. This diversity is encompassed by organisms possessing genomes ranging in size from very small (1.3 Mb) to relatively large (6.2 Mb), G + C contents ranging from 38% to 68%, and chloromethane utilization genes can be chromosome- or plasmid-borne (Table 8.1).

Under strictly anoxic conditions, the only known pure culture that uses chloromethane as the sole energy source is the Gram-positive homoacetogenic autotrophic bacterium, *Acetobacterium dehalogenans* MC (Traunecker *et al.*, 1991). In this strain, chloromethane dehalogenation releases methyl-tetrahydrofolate (H_4F) that is further metabolized to acetate (Meßmer *et al.*, 1993, 1996). *Pseudomonas aeruginosa* NB1 uses chloromethane as the sole source of carbon and energy under either anoxic or aerobic conditions upon switching from nitrate to oxygen as the terminal electron acceptor (Freedman *et al.*, 2004). Another case of anoxic chloromethane-utilization was recently reported and provides the first example of co-utilization of two chlorinated methane substrates in a mixed culture. In the absence of dichloromethane, chloromethane was not degraded as the chloromethane-degrader partner is thought to consume H_2 generated by *Candidatus* Dichloromethanomonas elyunquensis upon dichloromethane degradation (Chen *et al.*, 2017) (Fig. 8.2A).

Under aerobic conditions, cometabolic degradation of chloromethane was observed by nitrifying bacteria (Rasche *et al.*, 1991) and methanotrophs (Han and Semrau, 2000). This is attributed to the activity of ammonium- or methane monooxygenase, respectively. When chloromethane is provided at low concentration in presence of another C_1 substrate, the growth of the facultative methanotroph *Methylomicrobium album* BG8 is enhanced (Fig. 8.2C). Another case of conditional degradation of chloromethane was found for the growth of the abundant coastal Betaproteobacterium strain HTCC2181. This obligate methylotroph utilizes chloromethane for energy only when methanol is supplied as the carbon source (Halsey *et al.*, 2012) by a yet uncharacterized methyltransferase, with possible methyl transfer from chloromethane to H_4F (Fig. 8.2B; discussed in substrate co-utilization section).

Microbial utilization of chloromethane as the sole carbon and energy source (Fig. 8.2A) was first described in *Hyphomicrobium* sp. strain MC1, isolated from a sewage treatment plant in Switzerland in 1986 (Hartmans *et al.*, 1986). Strains affiliated with other taxonomic groups were subsequently recovered from soils (*Aminobacter, Hyphomicrobium, Methylorubrum*), marine environments (*Roseovarius*, *Leisingera*) and plants (*Hyphomicrobium*) (Table 8.1). Measured doubling time during aerobic growth at 30°C with chloromethane (10–15 mM) ranges from ≈ 5 hours (*Hyphomicrobium* strains CM2, MC1 and AT2) to ≈ 19 hours (*Hyphomicrobium* strains AT3 and AT4) (Nadalig *et al.*, 2011). *M. extorquens* strain CM4 became the reference organism of chloromethane utilization (see below).

The *cmu* pathway for chloromethane degradation

Biochemistry and genetics of aerobic chloromethane utilization have been elucidated in detail in strain CM4 affiliated to *Methylobacterium extorquens*, recently reclassified as *Methylorubrum extorquens* (Green and Ardley, 2018). Using random mutagenesis of strain CM4 with a miniTn5 transposon, mutants unable to grow with chloromethane were used to identify chloromethane utilization (*cmu*) genes (Vannelli *et al.*, 1998, 1999) (Fig. 8.2A). The first step involves chloromethane dehalogenation with the transfer of its methyl group to the C_1 carrier tetrahydrofolate (H_4F) to produce methyl-H_4F, and one chloride and one proton. The fact that the primary product of chloromethane demethylation is methyl-H_4F rather than formaldehyde differs this pathway from C_1 oxidation pathways, including the methanol oxidation pathway (Chistoserdova, 2011; Studer *et al.*, 2002). Chloromethane dehalogenase consists of the corrinoid methyltransferase CmuA and the H_4F-dependent methyltransferase CmuB (Studer *et al.*, 1999, 2001). It transforms bromomethane

Table 8.1 Chloromethane-degrading bacteria isolated from contrasting environments

Name (taxonomical class)	Origin	Metabolism/trophic type	Genome	Comments
Acetobacterium dehalogenans MC (Clostridia)	Activated sludge (Traunecker *et a*l., 1991)	Anaerobic homoacetogenic	ns[a]	Chloromethane dehalogenation forms methyl-H_4F (Meßmer *et al*., 1996)
Acetobacterium sp. in mixed culture RM (with *Candidatus* Dichloromethanomonas elyunquensis)	Pristine river sediment (Justicia-Leon *et al*., 2012)	Anaerobic hydrogenotrophic chloromethane-degradation	ns	H_2-dependent chloromethane degradation (Chen *et al*., 2017)
Aminobacter ciceronei IMB1 (Alphaproteobacteria)	Fumigated strawberries (Hancock *et al*., 1998)	Aerobic facultative methylotroph	ns	*cmuA*[b]
Aminobacter lissarensis CC495 (Alphaproteobacteria)	Beech woodland soil (Coulter *et al*., 1999)	Aerobic facultative methylotroph	ns	*cmuA*[b]
Celeribacter indicus P73 (Alphaproteobacteria)	Deep-sea sediment (Lai *et al*., 2014)	Aerobic facultative methylotroph (genome-based)[c]	4.5 Mb, five plasmids; G + C content 66% (Cao *et al*., 2015)	Genome-based detection of *cmu* genes; 3 *folD-purU* copies (This work)
Hyphomicrobium sp. strains AT2, AT3, AT4 (Alphaproteobacteria)	Phyllosphere of *Arabidopsis thaliana* (Nadalig *et al*., 2011)	Aerobic facultative methylotroph	ns	*cmuA*[b]
Hyphomicrobium sp. MC1 (Alphaproteobacteria)	Industrial sewage plan (Hartmans *et al*., 1986)	Aerobic facultative methylotroph	4.7 Mb; G + C content 59% (Vuilleumier *et al*., 2011)	Complete *cmu* pathway
Hyphomicrobium sp. MC2 (Alphaproteobacteria)	Soil from a petrochemical factory (Doronina *et al*., 1996)	Aerobic facultative methylotroph	ns	*cmuA*[b]
Leisingera methylohalidovorans MB2 (Alphaproteobacteria)	Marine tide pool (Schaefer *et al*., 2002)	Aerobic facultative methylotroph	4.6 Mb, two plasmids; G + C content 62% (Buddruhs *et al*., 2013)	Uncharacterized *cmu*-independent chloromethane degradation
Methylomicrobium album BG8 (Gammaproteobacteria)	Freshwater (Whittenbury *et al*., 1970)	Aerobic facultative methanotroph, obligatory methylotroph	4.5 Mb, one plasmid; G + C content 56% (Kits *et al*., 2013)	Low chloromethane concentrations enhance growth on methanol (Han and Semrau, 2000). No *cmu* gene
Methylorubrum[d] *extorquens* CM4 (Alphaproteobacteria)	Soil from a petrochemical factory (Doronina *et al*., 1996)	Aerobic facultative methylotroph	6.2 Mb, two plasmids; G + C content 68% (Marx *et al*., 2012)	Plasmid pCMU01-encoded *cmu* (Roselli *et al*., 2013)
Nocardioides sp. SAC-4 (Actinobacteria)	Topsoil (McAnulla *et al*., 2001a)	Aerobic facultative methylotroph[e]	ns	
OM43 bacterioplankton clade, strain HTCC2181 (Betaproteobacteria)	Seawater (Giovannoni *et al*., 2008)	Obligate methylotroph	1.3 Mb (three contigs); G + C content 38% (Giovannoni *et al*., 2008)	*cmu*-independent chloromethane use as energy source if methanol is the C source (Halsey *et al*., 2012)

Table 8.1 Continued

Name (taxonomical class)	Origin	Metabolism/trophic type	Genome	Comments
Pseudomonas aeruginosa NB1 (Gammaproteobacteria)	Anoxic activated sludge enrichment culture (Freedman *et al*., 2004)	Aerobic facultative methylotroph	ns	Nitrate respiration, chloromethane used as C and energy source (Freedman *et al*., 2004)
Roseovarius pacificus 81-2 (Alphaproteobacteria)	Deep-sea sediment (Wang *et al*., 2009)	Aerobic facultative methylotroph[c]	4.5 Mb (52 contigs, one plasmid); G+C content 62% (Wang *et al*., 2009)	Genome-based detection of *cmu* genes (this work)
Roseovarius sp. strain 217 (Alphaproteobacteria)	Seawater (Schäfer *et al*., 2005)	Aerobic facultative methylotroph	4.6 Mb (37 contigs); G+C content 61% (Moore Foundation project NZ_AAMV00000000)	Uncharacterized *cmu*-independent chloromethane degradation pathway
Roseovarius sp. strains 179, 198 (Alphaproteobacteria)	Seawater (Schäfer *et al*., 2005)	Aerobic facultative methylotroph	ns	*cmuA*[b]

[a]ns, not sequenced; [b]gene *cmuA* was PCR amplified and sequenced; [c]chloromethane has not been tested to our knowledge; [d]*Methylobacterium* genus reclassified (Green and Ardley, 2018); [e]methylotrophic growth on chloromethane but no other tested C_1 compound (formate, methanol, methane, methylamine) (McAnulla *et al*., 2001a).

and iodomethane as well as chloromethane (Vannelli *et al*., 1998). Both proteins CmuB (Studer *et al*., 1999) and CmuA (Studer *et al*., 2001) are required for growth-supporting dehalogenation of chloromethane. CmuA acts as a bifunctional enzyme (possessing methyltransferase and the corrinoid-binding domains), and it also serves as an intermediate methyl carrier. In a second reaction, CmuB methyltransferase transfers the methyl group from CmuA to H_4F (Studer *et al*., 2001). Thus, besides H_4F, chloromethane dehalogenation requires a corrinoid cofactor, which explains an absolute need for cobalt for growth with methyl halides (Studer *et al*., 2001). This uncharacterized corrinoid cofactor possibly differs from the chromosome-encoded cobalamin compound (Roselli *et al*., 2013). In *M. extorquens* strain CM4, all genes of the *cmu* pathway and *cob* genes are present on a 380 kb plasmid called pCMU01. Comparison of complete genome sequences of strain CM4 with those of other *M. extorquens* strains unable to grow with chloromethane showed that plasmid pCMU01 harbours unique genes without homologues in the compared genomes (*bluB2, btuB, cobA, cbiD*), as well as 13 duplicated genes with homologues of chromosome-borne genes involved in cobalamin-associated biosynthesis and transport, or in H_4F-dependent metabolism (*folC2*) (Roselli *et al*., 2013). The clustering of genes coding for dehalogenase enzymes and for biosynthesis of associated cofactors suggests a history of coupled enzyme/coenzyme gene acquisition related to chloromethane utilization in strain CM4.

Coping with chloromethane upon methylotrophic growth

Bacteria that grow on chloromethane need to accommodate multiple stresses such as intracellular production of protons and chloride, adjustment of metabolism towards higher demand for dehalogenase-requiring cofactors (H_4F and cobalamin-related compounds), and the necessity to regulate their metabolism for effective chloromethane utilization (Roselli *et al*., 2013; Michener *et al*., 2016; Chaignaud *et al*., 2017). The limiting factors were inferred from experimental observations: (i) growth with chloromethane is slower than with methanol (Chaignaud *et al*., 2017); (ii) difference in growth rates under chloromethane utilization are not due to differences in chloromethane dehalogenase activity in cell-free extracts of taxonomically-related strains (Nadalig *et al*., 2011); and (iii) transfer of a *cmu* catabolic pathway gene cassette (plasmid pJM105) in 'naïve'

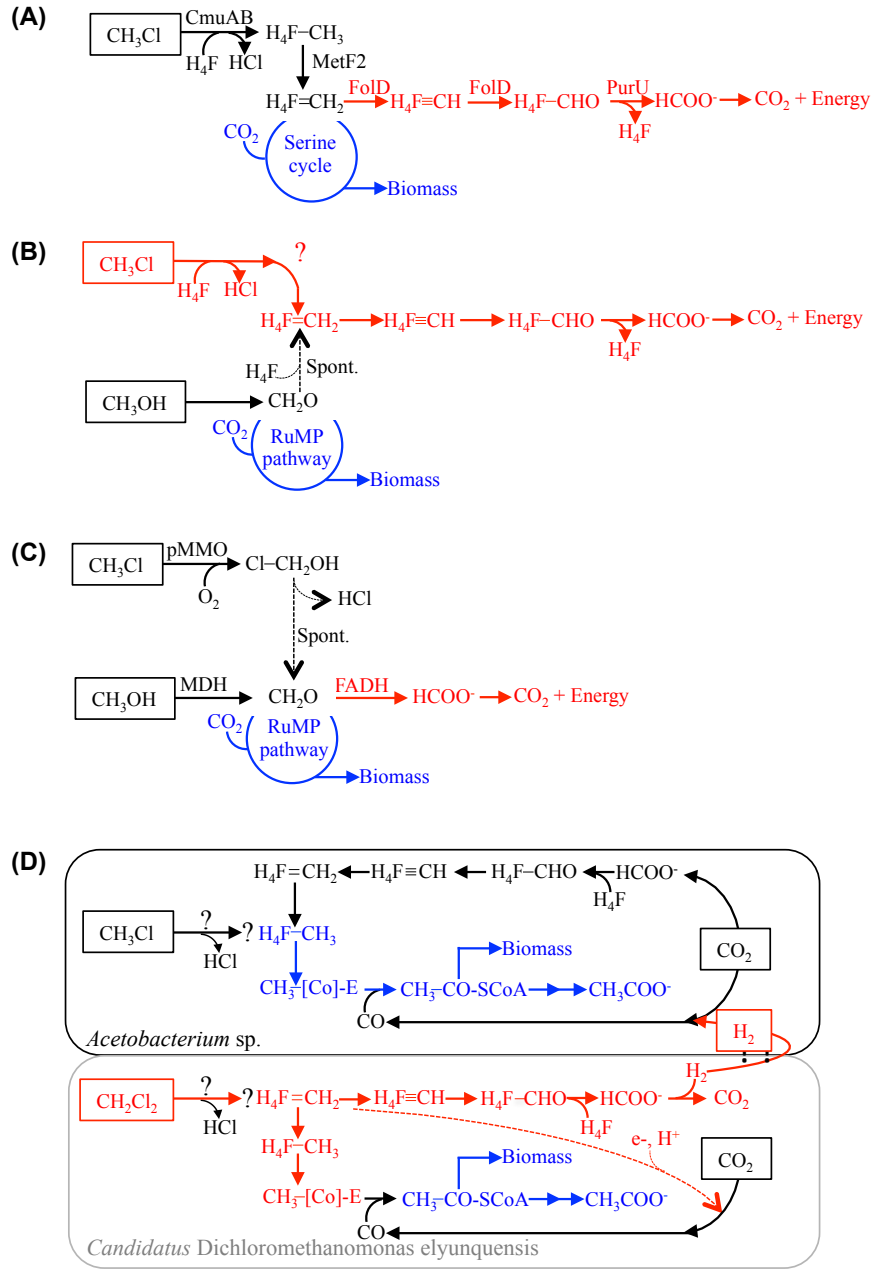

Figure 8.2 Examples of methylotrophic pathways for chloromethane dissimilation (source of energy, in red) and assimilation (source of carbon for biomass, in blue) in cultivated strains and mixed cultures. Bacteria cultivated under aerobic (A to C) and anaerobic (D) conditions. Abbreviations: FADH, formaldehyde dehydrogenase; H_4F, tetrahydrofolate; MDH, methanol dehydrogenase; pMMO, particulate methane monooxygenase; RuMP, ribulose monophosphate pathway. (A) The *cmu* pathway characterized in details in *M. extorquens* CM4 and *Hyphomicrobium* strains, adapted from (Vannelli *et al*., 1999; McAnulla *et al*., 2001b); (B) Bacterioplankton HTCC2181, adapted from (Giovannoni *et al*., 2008); (C) *Methylomicrobium album* BG8, adapted from (Han and Semrau, 2000); and (D) Culture RM, adapted from (Chen *et al*., 2017). Chloromethane and dichloromethane are each degraded by a different organism in a mutualism interaction. Chloromethane could not substitute for dichloromethane as the sole source of energy. It remains unknown how chloromethane enters the C_1 cycle of the chloromethane-degrading homoacetogen strain performing H_2/CO_2 reductive acetogenesis (Kleindienst *et al*., 2017). For *Candidatus* Dichloromethanomonas elyunquensis, dichloromethane is a source of energy. No dichloromethane degradation occurs in medium without CO_2 (bicarbonate) supplementation (Kleindienst *et al*., 2017). Enzymes involved in dichloromethane degradation and CO_2 assimilation have not been characterized.

Methylorubrum strains conferred poor growth ability on chloromethane supplied as the sole energy and carbon source (Michener *et al.*, 2016). Among potential limiting factors, intracellular release of protons and chloride ions is likely, since dehalogenation of chloromethane, as of dichloromethane, initiates growth-supporting degradation. Nonetheless, each of these chlorinated methanes has specific adaptive responses for chloride export. For example, in *Methylorubrum,* the chromosome-encoded proton/chloride antiporter ClcA had a significant fitness impact for growth with dichloromethane but not with chloromethane (Michener *et al.*, 2014a, 2016). Adaptive responses to growth with chloromethane compared with growth with methanol were detected using complementary approaches of random mutagenesis (Vannelli *et al.*, 1999), genome sequencing (Marx *et al.*, 2012), comparative genomics, proteomics and transcriptomics (Roselli *et al.*, 2013; Chaignaud *et al.*, 2017), and experimental evolution (Michener *et al.*, 2016). A summary of the global response to growth with chloromethane in *M. extorquens* strain CM4 is proposed in Fig. 8.3.

According to this model, the adaptive response to chloromethane is complex and includes general oxidative stress response functions (Fig. 8.3) such as molecular chaperones, DNA repair and reactive oxygen species (ROS) removal functions, as well as a dehalogenation-specific response to intracellular hydrochloric acid production (membrane-bound proton translocating pyrophosphatase HppA and a putative chloride/proton antiporter ClcA2 encoded by plasmid pCMU01). The *pnt* gene cluster encoding a NADH/NADPH transhydrogenase with cross-membrane proton translocation activity, most likely plays a role in maintaining both the intracellular pools of NAD^+ and $NADP^+$, and the internal cellular pH with the efflux of protons during chloromethane dehalogenation. Higher expression of genes (enzymes and transporters) for biosynthesis of methyltransferase cofactors were detected, especially *bluB2* (sharing 38% amino acid identity with the chromosomal-encoded *bluB* gene). BluB

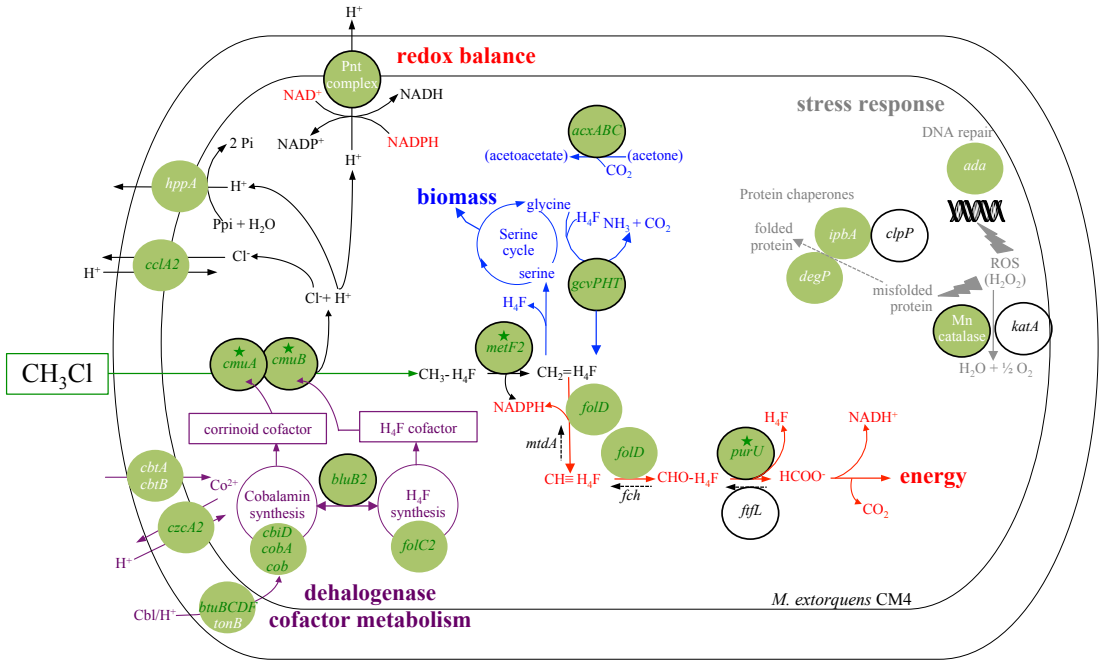

Figure 8.3 Coping with chloromethane upon methylotrophic growth. In *M. extorquens* CM4, the *cmu* pathway-encoding genes and associated genes are plasmid pCMU01-borne (gene names in green). In cultures grown on chloromethane compared with methanol, more abundant RNAs (green bullets) (Chaignaud *et al.*, 2017) and proteins (black-circled bullets) (Roselli *et al.*, 2013) were found. Essential genes in chloromethane assimilation or dechlorination are marked with a star (Vannelli *et al.*, 1999; Michener *et al.*, 2016). For the putative role of the acetone carboxylase components encoded by the plasmid-borne *acxABC* gene cluster chloromethane assimilation, refer to Roselli *et al.* (2013).

catalyses both the production of a lower ligand of cobalamin (5,6-dimethylbenzimidazole) and of a H_4F precursor (erythrose 4P), thus linking together the biosynthesis of the two cofactors of chloromethane dehalogenation (Roselli *et al.*, 2013). In conclusion, the ability to utilize chloromethane likely involves adaptation to oxidative stress, production of reducing equivalents, conversion of H_4F-bound C_1 units, and central metabolism. The adaptive response to growth with chloromethane has little overlap with other toxic halogenated compounds (Roselli *et al.*, 2013; Michener *et al.*, 2014b, 2016; Chaignaud *et al.*, 2017), and possibly with chloromethane degradation by *cmu*-independent pathways.

Probing the diversity of chloromethane-degrading bacteria by comparative genomics and isotopic fractionation

The strategy of combining stable isotope approaches (Compound-Specific Isotope Analysis; CSIA) with comparative genomics helps to explore the diversity of microbial degradation of chloromethane (Nadalig *et al.*, 2014). CSIA is a chemical method to determine the ratio of isotopes of different elements (e.g. C, N, H, O, Cl) in environmental samples (Nijenhuis and Richnow, 2016). This ratio can change during the course of microbial degradation. For example, carbon large fractionation shifts ($\delta\ ^{13}C$ from an initial value of –60‰ to –30‰) were observed when 90% of the chloromethane was degraded by high-cell-density suspensions of methylotrophic bacteria (Miller *et al.*, 2001). Combined measurements of carbon and hydrogen isotope fractionation of the remaining untransformed chloromethane provide further evidence of biotic degradation and clues about the metabolic pathway and enzymes involved (Nadalig *et al.*, 2013) (Table 8.2). When genomes of experimentally validated chloromethane-degrading methylotrophs were searched for the presence of *cmuA*, two groups of bacteria were detected: (i) *M. extorquens* CM4 and *Hyphomicrobium* sp. MC1 isolated from soils share the *cmu* pathway genes; (ii) *Leisingera methylohalidovorans* MB2 and *Roseovarius*

Table 8.2 Degradation of chloromethane and its isotopic enrichment factor for carbon (ε_C) and hydrogen (ε_H) of pure cultures and environmental samples

	Stable isotope enrichment factor for chloromethane upon bacterial degradation[a]			
	Test condition	ε_C (‰)	ε_H (‰)	Reference
Pure bacterial cultures (*cmu* pathway)				
Aminobacter ciceronei IMB1	Resting cells, 26°C	–47 ± 4	Nd[b]	Miller *et al*. (2001)
Aminobacter lissarensis CC495	Resting cells, 26°C	–42 ± 2	Nd	Miller *et al*. (2001)
Methylorubrum extorquens CM4	Dividing cells, 30°C	–42	–39	Nadalig *et al*. (2014)
	Resting cells, 30°C	–41 ± 5	–29 ± 6	Nadalig *et al*. (2013)
Hyphomicrobium sp. MC1	Dividing cells, 30°C	–54	–51	Nadalig *et al*. (2014)
	Resting cells, 30°C	–38 ± 3	–27 ± 10	Nadalig *et al*. (2013)
Pure bacterial cultures (*cmu*-independent pathway)				
Leisingera methylohalidovorans MB2	Dividing cells, 30°C	–76	0	Nadalig *et al*. (2014)
	Resting cells, 26°C	–44 ± 4	Nd	Miller *et al*. (2001)
Environmental samples				
Phyllosphere (fern: *Cyathea cooperi*)	Microcosm, 20–30°C	–43 ± 12	–8 ± 19	Jaeger *et al*. (2018b)
Soils (agricultural, grassland, forest soils)	Microcosm, 20–30°C	–38 to –11 ± 3	–50 ± 19	Jaeger *et al*. (2018a)

[a]Increased carbon isotope fractionation compared with hydrogen isotope fractionation most likely results from the primary isotope effect in cleavage of the carbon-halogen bound during chloromethane dehalogenation, as previously suggested (Elsner *et al*., 2005); [b]Nd, not determined.

sp. 217 isolated from marine environments harbour no *cmu* genes and degrade chloromethane by a yet uncharacterized mechanism (Table 8.1). Cultures of *L. methylohalidovorans* MB2 displayed clear differences of both carbon and hydrogen isotope signatures upon chloromethane dehalogenation compared with *cmu*-containing strain cultures of *Aminobacter, Hyphomicrobium* and *Methylorubrum* (Nadalig *et al.*, 2014).

The *cmu* gene organization is unique in strain CM4 (Roselli *et al.*, 2013), where the genes are plasmid-borne and located in two distant regions (Vannelli *et al.*, 1998). In other strains, when detected by comparative genomics and targeted PCR amplifications, *cmu* genes (Fig. 8.4) were conserved on a single chromosomal cluster (McAnulla *et al.*, 2001b; Nadalig *et al.*, 2014). In particular, a trio of adjacent genes (*cmuBCA*) is conserved in genomes of aerobic chloromethane-degraders isolated from soils and plants, genomes of Alphaproteobacteria isolated from deep-sea (*Celeribacter indicus* P73 and *Roseovarius pacificus* 81-2), as well as of obligate anaerobes (*Desulfobacula* sp. TS; *Desulfomonile tiedjei* DSM 6799, *Desulfurispora thermophila* DSM 16022, *Desulfotomaculum alcoholivorax* DSM 16058, *Thermincola potens* JR and *Thermosediminibacter oceani* DSM 16646). These anaerobic strains harbour *cmuA* homologues closely related to each other (identities at the protein level between 84–93%) (Nadalig *et al.*, 2014) but none of these strains has yet been reported to degrade chloromethane. The overall conserved *cmuBCA* trio suggests a functional link between the encoded CmuA, CmuB and CmuC. While CmuA and CmuB are two methyltransferases essential for chloromethane dehalogenation (Studer *et al.*, 2001), the function of CmuC remains uncharacterized. CmuC, a putative third methyltransferase-like protein most similar to MtaA (corrinoid:coenzyme M methyltransferase), is dispensable for dehalogenation but needed for chloromethane assimilation (Vannelli *et al.*, 1999). Mobile genetic elements (black arrows in Fig. 8.4) in the vicinity of conserved *cmu* clusters potentially suggest their acquisition by horizontal gene transfer. Duplicated *cmu* genes found in strain CM4 (two homologous *cmuC* copies adjacent to either *cmuB* or *cmuA*) and in *D. tiedjei* DSM 6799 (a *cmuAB* copy in addition to the conserved *cmuBCA*) suggest specific strain adaptation. An 'orphan' *cmuA*-like gene (encoding for a protein sharing 43% amino acid identity with *M. extorquens* CM4 CmuA) with no other detected genes of the *cmu* pathway in its genome, raises the question of whether the marine Gammaproteobacterium *Vibrio orientalis* CIP 102891 in able to degrade chloromethane. No chloromethane degradation was observed for *V. orientalis* CIP 102891 resting cells (Fig. 8.5) or by actively growing cell cultures supplied with chloromethane in addition to another carbon source (data not shown). This suggests that the *V. orientalis* CmuA-like homolog might play a yet unknown function, possibly different from chloromethane dehalogenation.

Methylotrophic populations of the chloromethane sink

Chloromethane-degrading populations reduce chloromethane emissions to the atmosphere

In contrast to live soils, sterilized soils or plants do not degrade chloromethane (Chaignaud *et al.*, 2017; Jaeger *et al.*, 2018a,b) suggesting a microbial sink of chloromethane. Compared to chloromethane degrading methylotrophs (Table 8.2), the carbon isotope enrichment factor (ε_C) of remaining chloromethane was similar for fern leaves (mean of -43 ± 12‰) and lower for soils (ranging from –38 to 11 ±3‰ in agricultural, grassland and forest soils, respectively). Stable isotope hydrogen fractionation (ε_H) observed during degradation experiments with fern leaves (-8 ± 19‰), and soils (-50 ± 19‰) was similar to reported ε_H for chloromethane-degrading bacterial strains (Table 8.2). No change in stable isotope fractionation signature was observed when the investigated environmental samples were sterilized, suggesting a biotic microbially driven process (Chaignaud *et al.*, 2017; Jaeger *et al.*, 2018a,b). This hypothesis is in agreement with the recent finding that the occurrence and the diversity of bacterial chloromethane degradation (*cmu* gene expression) is correlated with differential expression of the plant gene *HOL1* involved in the production of chloromethane (Farhan Ul Haque *et al.*, 2017). Thus, a relation was established between chloromethane production by plants and relative abundance of leave-associated chloromethane-degrading bacteria. This suggests that chloromethane-degrading bacteria co-determine the extent of chloromethane

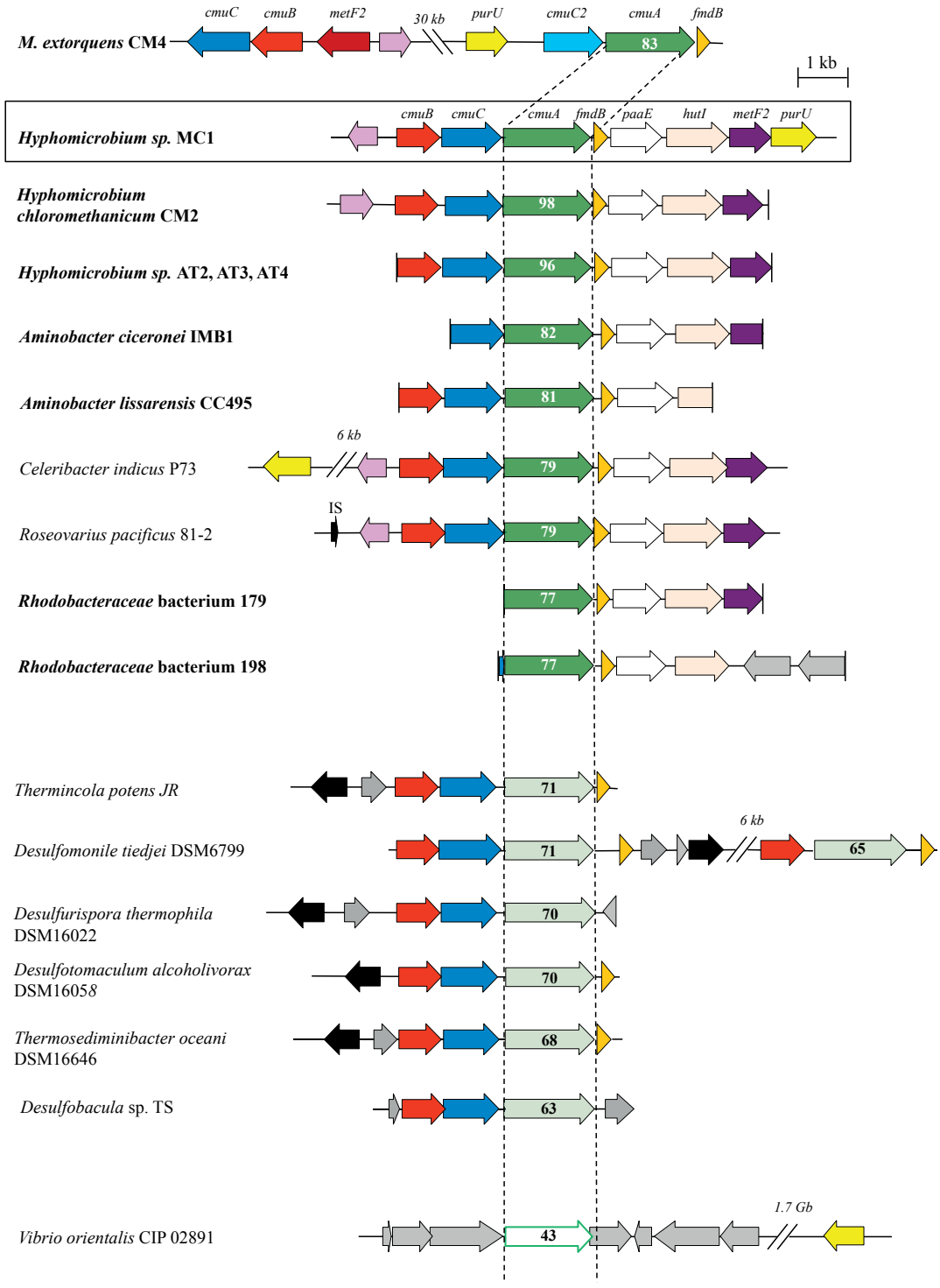

Figure 8.4 Organisation of *cmu* genes in bacteria. Names of experimentally validated chloromethane-degrading bacteria are written in bold. Arrows represent protein-coding genes, and annotations above arrows indicate gene names. Homologous genes are given with identical colour except for arrows in black, pink and grey, which indicate mobile elements, transcriptional regulators, and genes not involved in chloromethane degradation, respectively. The percentage of amino acid sequence identity with the reference *Hyphomicrobium* sp. MC1 CmuA protein is indicated. Gene clusters are drawn to scale.

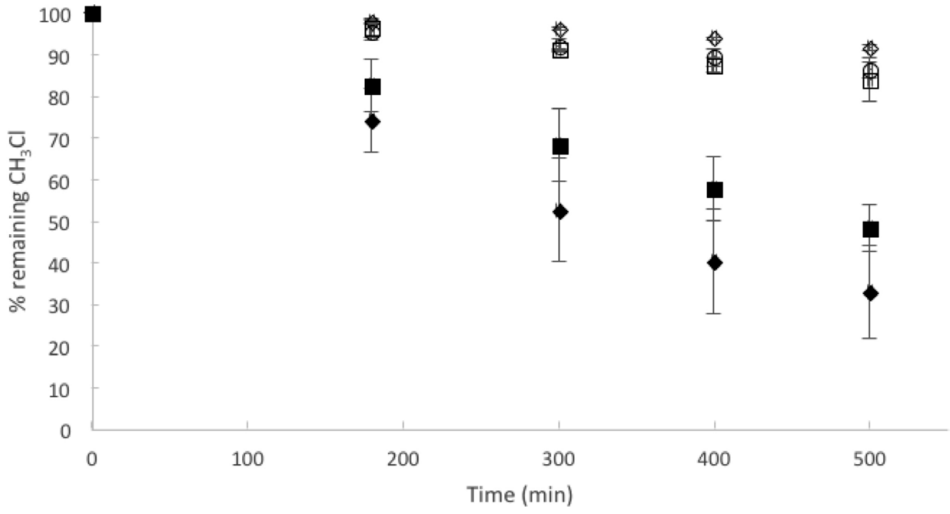

Figure 8.5 Assessing the ability of *Vibrio orientalis* CIP 102891 to degrade chloromethane. Triplicates of resting cells were exposed to chloromethane in gastight flasks at room temperature. The remaining chloromethane was quantified by gas chromatography (GC-FID) at different time points. Cell suspensions of *V. orientalis* CIP 102891 (empty square) were harvested from exponential-grown phase as described for *L. methylohalidovorans* MB2 saline cultures (Nadalig *et al*., 2014). For *V. orientalis* CIP 102891, the amount of chloromethane remaining was similar to that of the negative controls without cells (empty circle, saline phosphate buffer; empty diamond, phosphate buffer) but significantly different from the positive degraders *M. extorquens* CM4 in (black diamond) and *L. methylohalidovorans* MB2 (black square).

emissions by plants to the atmosphere, and that methylotrophic microorganisms are involved in the terrestrial chloromethane sink.

Rare occurrence of *cmuA* in the environment

Of the genes of the *cmu* pathway, the *cmuA* gene is the most conserved (Farhan Ul Haque *et al*., 2017; Nadalig *et al*., 2011). Thus, *cmuA* has been used to study the diversity of methyl halide-degrading organisms in the environment (Miller *et al*., 2004; Schäfer *et al*., 2005; Nadalig *et al*., 2011; Cox *et al*., 2012; Chaignaud *et al*., 2018). With increasing numbers of *cmuA* sequences in databases, oligonucleotide primers have been modified for more efficient PCR amplification and improved detection of *cmuA* (see details in Chaignaud *et al*., 2018, supplemental material). No PCR products were retrieved from environmental DNA prior to enrichment steps of forest soil (Chaignaud *et al*., 2018) and plant leaves (Jaeger *et al*., 2018b). When amplified, a low diversity of *cmuA* sequences was found in samples of soils, plant leaves and large volumes of oceanic and coastal waters (Cox *et al*., 2012; Farhan Ul Haque *et al*., 2017; Chaignaud *et al*., 2018). This suggested that *cmuA*-harbouring bacteria might represent a small component of the investigated environmental microbiota.

Recently, an extremely sensitive molecular tool for *cmuA* detection by 'targeted gene capture' was developed (Gasc *et al*., 2016; Ribière *et al*., 2016). Its detection limit of 0.00006% makes it particularly adapted to investigate rare genes and biological functions in the environment (Gasc and Peyret, 2018). It consists of utilizing metal beads for recovering the sequences of interest within a community (or within nucleic acid extracts) by hybridization. The beads are coated with biotinylated RNA sequences of ≈ 80 bp, complementary to the targeted gene sequence and potential orthologs. Using KASpOD software (Parisot *et al*., 2016), such 80-mer sequences have been successfully used to design specific probes based on large sequence datasets to reveal microbial diversity missed using current profiling methods. Based on known *cmuA* gene sequences, gene capture method was attempted in different environments [e.g. soil (Jaeger *et al*., 2018a), plant leaves (Jaeger *et al*., 2018b) and cloud microbial communities (L. Besaury, P. Amato, P. Peyret, C. Gasc, unpublished data)] with no success. When homologous sequences were searched in metagenomic databases in a wider range of

Table 8.3 Occurrence of *cmuA* sequences in metagenomes available from public databases

Database	Environment	Metagenome numbers	Total size (Mb)[a]	*cmuA* hit[b]
IMG	**Engineered**	**2332**	**254,327**	
	Bioreactor	108	35,565	0
	Bioremediation	68	12,879	0
	Biotransformation	24	5411	0
	Build environment	1231	31,275	0
	Food production	3	6,600	0
	Lab enrichment	218	11,002	0
	Lab synthesis	4	401	0
	Modelled	65	2804	0
	Solid waste	69	22,575	0
	Wastewater	542	132,349	0
	Aquatic	**9322**	**1,968,340**	
	Freshwater	3912	755,498	0
	Marine	3034	1,049,257	1 (wetland)
	Non-marine saline and alkaline	702	86,625	0
	Sediment	271	34,754	0
	Thermal spring	1403	42,206	0
	Terrestrial	**2915**	**1,508,938**	
	Deep subsurface	183	13,202	0
	Geologic	4	170	0
	Oil reservoir	3	283	0
	Peat	56	10,558	0
	Plant litter	47	2663	0
	Rock-dwelling	8	3220	0
	Soil	2609	1,481,714	0
	Volcanic	5	26	0
	Host-associated	**4106**	**876,365**	
	Algae	64	44,092	0
	Animal	25	6426	0
	Annelida	91	43,120	0
	Arthropoda	147	73,213	0
	Birds	18	5505	0
	Cnidaria	36	723	0
	Fish	4	489	0
	Fungi	102	19,978	0
	Human	2489	139,540	0
	Insecta	34	3153	0
	Invertebrate	15	8056	0
	Mammal	267	104,135	0
	Microbial	24	2132	0

Table 8.3 Continued

Database	Environment	Metagenome numbers	Total size (Mb)[a]	*cmuA* hit[b]
	Mollusca	11	3247	0
	Plants	761	581,931	3 (*Arabidopsis thaliana*)
	Porifera	10	1642	0
	Tunicates	8	3377	0
NCBI	**Diverse**	Metagenome BLAST database		
	Marine		81,542	7 (sediment)
	Freshwater, hydrothermal vent, human gut		94,265	0

[a]Total of 4,783,777 Mb publicly available in May 2018; [b]Blast searches were performed using a *cmuA* consensus template from 122 nucleic acid sequences downloaded from GenBank. Hits displaying an E-value lower than 10^{-5} were kept, and the corresponding proteins aligned with characterized CmuA sequences to only retain members closely related to one of three clades shown in Figure 8.6B.

environments (Table 8.3), only 11 *cmuA* sequences were retrieved, of which three originated from the rhizosphere of the plant *A. thaliana*, and the others from marine environments (Table 8.3). Basing on these results, *cmuA* is estimated to represent around one DNA sequence out of 3 billion, and can be considered as a rare gene (Kislyuk *et al.*, 2011). It may be necessary for future sampling campaigns to assess if environmental niche partitioning of microbial minorities consuming chloromethane is found as for methane (Bodelier *et al.*, 2013).

Using Blast searches, *cmuA* DNA sequences were retrieved from environmental metagenomes, translated to proteins and aligned to reference '*sensu stricto*' proteins of functionally characterized CmuA proteins of Alphaproteobacteria cultivated under aerobic conditions, to uncharacterized proteins of the clade called 'CmuA-anaerobe' found in strict anaerobes and of '*CmuA*-like' proteins found in bacteria lacking *cmuBC* (as defined in Fig. 8.6). Of the 11 metagenomic *cmuA* sequences, one was closely related to the '*sensu stricto* CmuA' clade, one to the 'CmuA-anaerobe', and the remaining belonged to the most-distant CmuA-like clade. Remarkably, chloromethane dehalogenation by '*sensu stricto* CmuA' does not require aerobic conditions and is even sensitive to oxygen (Studer *et al.*, 2001). Thus, anaerobic bacteria with *cmu* genes may be able to use chloromethane as a source of carbon and energy, as previously discussed (Nadalig *et al.*, 2014). Nonetheless, the ability to degrade methyl halides under environmental conditions remains to be confirmed for bacterial populations coding for 'CmuA-anaerobe' and CmuA-like proteins.

Substrate co-utilization as a strategy to sustain growth and resilience of chloromethane-utilizing methylotrophic populations

In the troposphere, chloromethane is a trace gas with a global mean concentration of ≈ 500 to 600 parts per trillion by volume (pptv). In environments with low nutrient levels, methylotroph populations growing on different C_1 substrates (Neufeld *et al.*, 2008; Gifford *et al.*, 2016) would benefit from simultaneous utilization of several C_1 compounds as a strategy to enhance their growth. In coastal ecosystems, methanol is released during phytoplankton blooms (Heikes *et al.*, 2002). When methanol is present in seawater, the methanol-assimilating methylotroph HTCC 2181 uses chloromethane as source of energy (Halsey *et al.*, 2012). Its genome, one of the smallest known for bacteria, lacks the tetrahydromethanopterin (H_4MPT) C_1 transfer module, so C_1 transfer can only rely on H_4F (Giovannoni *et al.*, 2008). Strain HTCC 2181 may have evolved by reducing its C_1 abilities (loss of the genes for the C_1 carrier, H_4MPT synthesis) and thus reducing its metabolic versatility in favour of specialization on carbon compounds at low amounts (Giovannoni *et al.*, 2008).

To our knowledge, no data are available on chloromethane concentration in upper soil of terrestrial

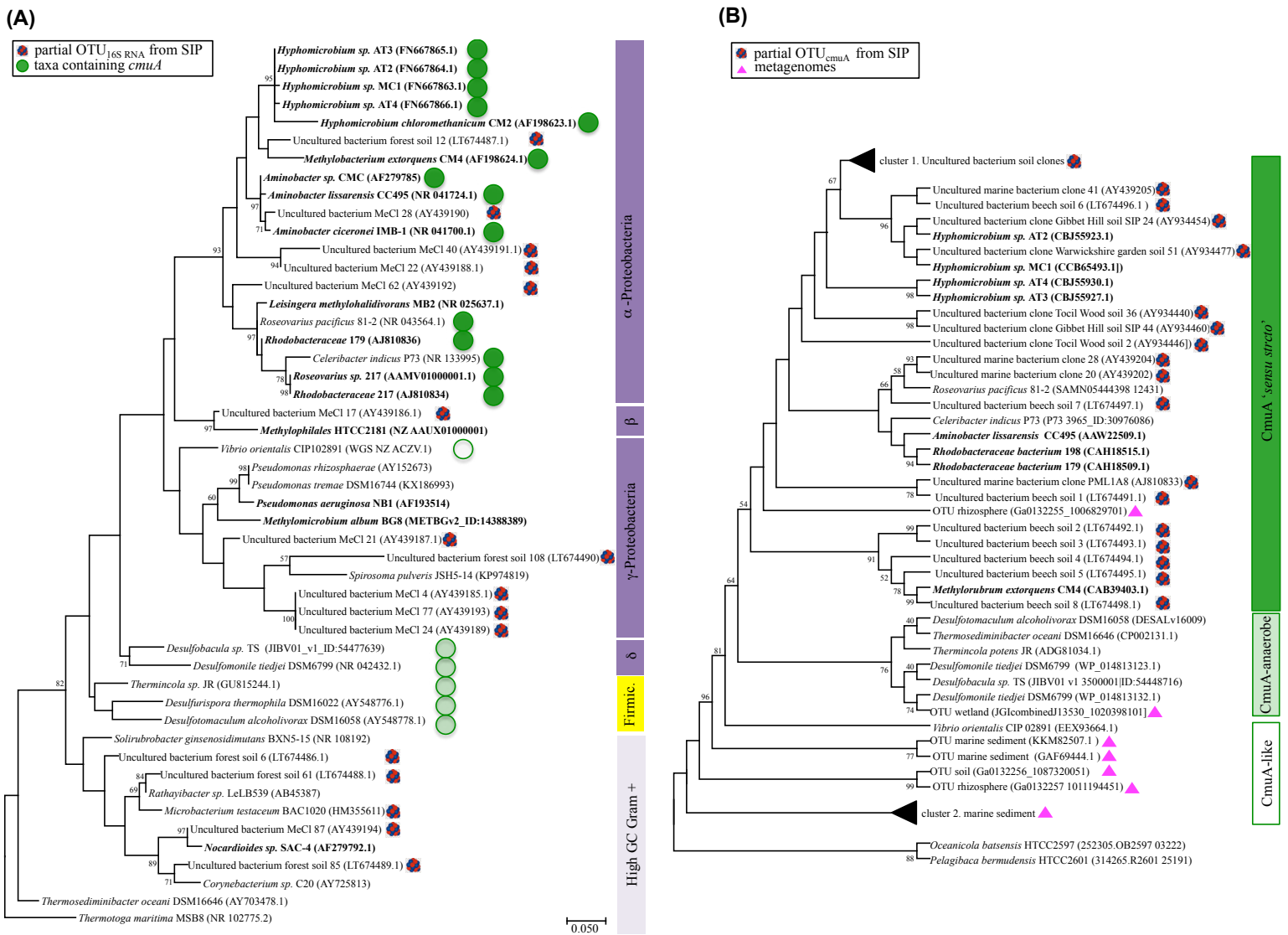
(A)
partial OTU16S RNA from SIP
taxa containing cmuA
Hyphomicrobium sp. AT3 (FN667865.1)
Hyphomicrobium sp. AT2 (FN667864.1)
Hyphomicrobium sp. MC1 (FN667863.1)
Hyphomicrobium sp. AT4 (FN667866.1)
Hyphomicrobium chloromethanicum CM2 (AF198623.1)
Uncultured bacterium forest soil 12 (LT674487.1)
Methylobacterium extorquens CM4 (AF198624.1)
Aminobacter sp. CMC (AF279785)
Aminobacter lissarensis CC495 (NR 041724.1)
Uncultured bacterium MeCl 28 (AY439190)
Aminobacter ciceronei IMB-1 (NR 041700.1)
Uncultured bacterium MeCl 40 (AY439191.1)
Uncultured bacterium MeCl 22 (AY439188.1)
Uncultured bacterium MeCl 62 (AY439192)
Leisingera methylohalidivorans MB2 (NR 025637.1)
Roseovarius pacificus 81-2 (NR 043564.1)
Rhodobacteraceae 179 (AJ810836)
Celeribacter indicus P73 (NR 133995)
Roseovarius sp. 217 (AAMV01000001.1)
Rhodobacteraceae 217 (AJ810834)
Uncultured bacterium MeCl 17 (AY439186.1)
Methylophilales HTCC2181 (NZ AAUX01000001)
Vibrio orientalis CIP102891 (WGS NZ ACZV.1)
Pseudomonas rhizosphaerae (AY152673)
Pseudomonas tremae DSM16744 (KX186993)
Pseudomonas aeruginosa NB1 (AF193514)
Methylomicrobium album BG8 (METBGv2_ID:14388389)
Uncultured bacterium MeCl 21 (AY439187.1)
Uncultured bacterium forest soil 108 (LT674490)
Spirosoma pulveris JSH5-14 (KP974819)
Uncultured bacterium MeCl 4 (AY439185.1)
Uncultured bacterium MeCl 77 (AY439193)
Uncultured bacterium MeCl 24 (AY439189)
Desulfobacula sp. TS (JIBV01_v1_ID:54477639)
Desulfomonile tiedjei DSM6799 (NR 042432.1)
Thermincola sp. JR (GU815244.1)
Desulfurispora thermophila DSM16022 (AY548776.1)
Desulfotomaculum alcoholivorax DSM16058 (AY548778.1)
Solirubrobacter ginsenosidimutans BXN5-15 (NR 108192)
Uncultured bacterium forest soil 6 (LT674486.1)
Uncultured bacterium forest soil 61 (LT674488.1)
Rathayibacter sp. LeLB539 (AB45387)
Microbacterium testaceum BAC1020 (HM355611)
Uncultured bacterium MeCl 87 (AY439194)
Nocardioides sp. SAC-4 (AF279792.1)
Uncultured bacterium forest soil 85 (LT674489.1)
Corynebacterium sp. C20 (AY725813)
Thermosediminibacter oceani DSM16646 (AY703478.1)
Thermotoga maritima MSB8 (NR 102775.2)
0.050
α -Proteobacteria
β
γ-Proteobacteria
δ
Firmic.
High GC Gram +
(B)
partial OTUcmuA from SIP
metagenomes
cluster 1. Uncultured bacterium soil clones
Uncultured marine bacterium clone 41 (AY439205)
Uncultured bacterium beech soil 6 (LT674496.1)
Uncultured bacterium clone Gibbet Hill soil SIP 24 (AY934454)
Hyphomicrobium sp. AT2 (CBJ55923.1)
Uncultured bacterium clone Warwickshire garden soil 51 (AY934477)
Hyphomicrobium sp. MC1 (CCB65493.1])
Hyphomicrobium sp. AT4 (CBJ55930.1)
Hyphomicrobium sp. AT3 (CBJ55927.1)
Uncultured bacterium clone Tocil Wood soil 36 (AY934440)
Uncultured bacterium clone Gibbet Hill soil SIP 44 (AY934460)
Uncultured bacterium clone Tocil Wood soil 2 (AY934446])
Uncultured marine bacterium clone 28 (AY439204)
Uncultured marine bacterium clone 20 (AY439202)
Roseovarius pacificus 81-2 (SAMN05444398 12431)
Uncultured bacterium beech soil 7 (LT674497.1)
Celeribacter indicus P73 (P73 3965_ID:30976086)
Aminobacter lissarensis CC495 (AAW22509.1)
Rhodobacteraceae bacterium 198 (CAH18515.1)
Rhodobacteraceae bacterium 179 (CAH18509.1)
Uncultured marine bacterium clone PML1A8 (AJ810833)
Uncultured bacterium beech soil 1 (LT674491.1)
OTU rhizosphere (Ga0132255_1006829701)
Uncultured bacterium beech soil 2 (LT674492.1)
Uncultured bacterium beech soil 3 (LT674493.1)
Uncultured bacterium beech soil 4 (LT674494.1)
Uncultured bacterium beech soil 5 (LT674495.1)
Methylorubrum extorquens CM4 (CAB39403.1)
Uncultured bacterium beech soil 8 (LT674498.1)
Desulfotomaculum alcoholivorax DSM16058 (DESALv16009)
Thermosediminibacter oceani DSM16646 (CP002131.1)
Thermincola potens JR (ADG81034.1)
Desulfomonile tiedjei DSM6799 (WP_014813123.1)
Desulfobacula sp. TS (JIBV01 v1 3500001|ID:54448716)
Desulfomonile tiedjei DSM6799 (WP_014813132.1)
OTU wetland (JGIcombinedJ13530_1020398101]
Vibrio orientalis CIP 02891 (EEX93664.1)
OTU marine sediment (KKM82507.1)
OTU marine sediment (GAF69444.1)
OTU soil (Ga0132256_1087320051)
OTU rhizosphere (Ga0132257 1011194451)
cluster 2. marine sediment
Oceanicola batsensis HTCC2597 (252305.OB2597 03222)
Pelagibaca bermudensis HTCC2601 (314265.R2601 25191)
CmuA 'sensu strcto'
CmuA-anaerobe
CmuA-like

Figure 8.6 (see p.164) Phylogenetic trees of partial 16S rRNA and partial protein CmuA sequences associated with chloromethane-degradation of cultivated strains and environmental samples. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones *et al*., 1992). Bootstrap values were calculated from 1000 replicates, % values ≥ 50 are presented. Analyses were conducted using the software MEGA7 (Kumar *et al*., 2016). Sequences of experimentally validated chloromethane-degraders are written in bold. Sequences marked with a ^{13}C icon result from DNA-SIP ($^{13}CH_3Cl$) studies in soil microcosms from a deciduous German forest dominated by beech (16S RNA: LT674486.1 to LT674490.1; CmuA: LT674491.1 to LT674498.1 in Chaignaud *et al*. (2018)), clone libraries from Californian agricultural soils (16S RNA: AY439185.1 to AY439194.1; CmuA: AY439202 to AY439204 in Miller *et al*. (2004)), and CmuA from clone libraries from English woodland soil (AY934428 to AY934484) (Borodina *et al*., 2005). (A) 16S rRNA tree based on 197 nucleotide sequences. *Thermotoga maritima* sequence served as outgroup. (B) CmuA tree based on 60 amino acid sequences. *Oceanicola batsensis* HTCC2597 and *Pelagibaca bermudensis* HTCC2601 sequences served as outgroup. Pink triangles indicate sequences retrieved from metagenome JGI database. Condensed branch of 11 sequences in cluster 1 (uncultured bacterium clone Warwickshire enrichment 3 (AY934432), 25 (AY934433), 36 (AY934430) and 42 (AY934428); Uncultured bacterium clone Warwickshire garden soil 37 (AY934481); Uncultured bacterium clone Gibbet Hill soil 9 (AY934448), 16 (AY934452), and 37 (AY93448); Uncultured bacterium clone Tocil Wood soil 17 (AY934484), 25 (AY934444), and 38 (AY934439)). Condensed branch of 4 marine sediment metagenome sequences in cluster 2 (GAF77597.1, GAF84365.1, GAG35382.1, GAG79179.1 and GAI71391.1).

ecosystems, which are highly active sinks (Borodina *et al*., 2005; Chaignaud *et al*., 2018). In the laboratory, high chloromethane ratios (in the percent range) have been used to isolate and grow pure chloromethane-degrading cultures that originated from soil (Schäfer *et al*., 2007) in order to obtain enough biomass for growth and chloromethane degradation studies. The identification of natural microbial populations actively assimilating carbon from chloromethane was first achieved using stable isotope probing (DNA SIP) (Miller *et al*., 2004). Studies in soil revealed that bacterial population members of soil microbial communities take up carbon from amended chloromethane (Miller *et al*., 2004; Borodina *et al*., 2005; Chaignaud *et al*., 2017). Moreover, it has been demonstrated that methanotrophs, as investigated with a pure culture of *Methylomicrobium album* BG8, can assimilate carbon from chloromethane when a surplus of methanol occurs, although chloromethane alone was not assimilated (Han and Semrau, 2000). These findings suggest a possible co-metabolic lifestyle of some methanotrophs regarding chloromethane degradation. In a forest soil, the rate of chloromethane degradation was recently shown to be enhanced when methanol was present (Chaignaud *et al*., 2018). In this study, *Beijerinckiceae* (i.e. phylotypes affiliating closely with *Methylovirgula ligni*), assimilated carbon from methanol or from chloromethane as suggested by SIP experiments of soil microcosms amended with labelled or unlabelled methanol, chloromethane or both simultaneously (Chaignaud *et al*., 2017; Morawe *et al*., 2017). Methylotrophic co-utilization of chloromethane with other C_1 compounds such as methanol, which is found in larger amounts in the environment and utilized by most methylotrophs, may thus represent an important component of chloromethane sink activity. The utilization of methanol as an alternate substrate to chloromethane may favour the persistence of chloromethane-degrading populations in the context of shortage of chloromethane in natural habitats. The presence of pollutants such as toluene in ocean water was found to inhibit chloromethane and bromomethane uptake, thereby challenging the persistence of methyl halide-degrading populations and subsequent methyl halide oceanic uptake, which would in turn disturb an important component of the biogeochemical cycle of these ozone-depleting compounds (Goodwin *et al*., 2005).

Future trends

Low- and high-affinity degraders

One of the key aspects of future explorations of the methylotrophic chloromethane sink is the need to unravel the enzymes and pathways that define the global sink of halocarbons not only in bacteria but also in fungi. Future research needs to grasp the diversity of chloromethane-degrading-associated metabolisms and taxa associated with the microbial chloromethane sink. Much effort has focused on molecular processes for methylotrophic growth under laboratory conditions. Owing to technical

limitations, so far biological chloromethane sinks have been assessed using higher than natural concentrations. Future investigations should focus on environmentally relevant chloromethane levels (picomolar). High variations in chloromethane flux emissions have been reported (Jaeger *et al*., 2018) so it can be postulated that low and high affinity chloromethane-degrading populations may be found in the environment, as for methane (Maxfield *et al*., 2009).

Unknowns of the chloromethane cycle

How plant chloromethane emissions vary during their developmental stage or their physiological states (chloride stress, pathogen invasion, exposure to chlorinated pesticides), and the role of chloromethane production by plants, is unknown (Bringel and Couée, 2018). Host-associated microbiota may elicit a response that modulates the interactive interplay between host/microbial cells/insects as found for other plant-produced VOCs (review by Bringel and Couée, 2015). For example, chloromethane regenerates veratryl alcohol during degradation of lignin by some white-rot fungi (Harper *et al*., 1990), and one may speculate that associated bacterial communities may interfere with this process. Similarly, methylotroph populations may interfere with chlorine output through volatilization via chloromethane emissions, as white-rot fungi and higher plants methylate chloride (Oberg, 2002).

On the bacterial side, even with *M. extorquens* strain CM4, in the *in-labo* best-characterized chloromethane degrader, our current understanding is not sufficient to predict its behaviour under environmental conditions. Key actors remain uncharacterized: the regulator of the *cmu* pathway, the chemical nature of the native corrinoid cofactor of the CmuA methyltransferase from chloromethane to H_4F, many genes involved in corrinoid cofactor metabolism (Roselli *et al*., 2013), and conserved genes adjacent to *cmuA* such as *cmuC* (Fig. 8.4) (Michener *et al*., 2016). In fact, all the details of the dehalogenation reactions and carbon funnelling to central metabolism remain to be investigated physiologically, biochemically on wild-type and mutants. Unfortunately, reverse genetics for site-directed mutagenesis has not been successful so far in *M. extorquens* strain CM4 (Michener *et al*., 2016) and *Hyphomicrobium* sp. Nonetheless, complementary 'omics' technologies such metabolomics, fluxomics and experimental evolution are available for *Methylorubrum* studies (Ochsner *et al*., 2015).

Biomarkers of chloromethane sinks

Gene *cmuA* is the only known biomarker for bacterial chloromethane utilization. It is rarely found in metagenomes (Table 8.2) (Jaeger *et al*., 2018a,b), and is absent in the genome of some chloromethane utilizers (Buddruhs *et al*., 2013) (Table 8.1). Together, these observations reveal the need to define *cmuA*-independent biomarkers of the chloromethane microbial sinks. In open oceans (Goodwin *et al*., 2005) and soils (Rasche *et al*., 1991; Chaignaud *et al*., 2018), co-substrate consumption may be a major driver of chloromethane microbial sinks. Thus, new strategies should be explored for investigating microbial *cmu*-independent chloromethane-degrading pathways, such as experiments on co-consumption of chloromethane with mixtures of other environmentally relevant carbon sources, co-cultures of mixed populations (Nai and Meyer, 2018), and cultivation-independent approaches using single-cell labelling and identification, such as microfluidic cell sorting combined with 'omics' methods and Raman-FISH (Musat *et al*., 2012; Hol and Dekker, 2014). This quest for new biomarkers will help explore the dynamics and diversity of chloromethane-degrading microorganisms active *in situ* in natural and anthropogenic environments.

Web resources

Data reporting on production and consumption of ozone depleting substances

The Montreal Protocol on Substances that Deplete the Ozone Layer:
http://ozone.unep.org/en/treaties-and-decisions/montreal-protocol-substances-deplete-ozone-layer
Scientific Assessments of Ozone Depletion conducted under the auspices of the World Meteorological Organization (WMO) and the United Nations Environment Programme (UNEP): https://www.esrl.noaa.gov/csd/assessments/ozone/and http://www.wmo.int/pages/prog/arep/gaw/ozone/

Genome of chloromethane-degrading strains

http://www.genoscope.cns.fr/agc/microscope opens the MicroScope platform that hosts *M. extorquens* CM4 genome and provides annotation results relative to BLAST similarities, COG assignations, enzymatic function prediction (PRIAM software), TMHMM and SignalP predictions, and synteny conservation (Syntonizer software). Complete *Hyphomicrobium* sp. MC1 and *Vibrio orientalis* CIP 102891 genomes available at https://www.ncbi.nlm.nih.gov/nuccore/FQ859181 (Vuilleumier *et al.*, 2011) and (http://www.ebi.ac.uk/ena/data/view/Project:40487) (Yang *et al.*, 1983), respectively. Comparative RNA-Seq data of *M. extorquens* CM4 grown with chloromethane or with methanol available at: https://www.genoscope.cns.fr/agc/microscope/transcriptomic/NGSProjectRNAseq.php?projType=RNAseq

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