



Investigations of the bacterial sink for plant emissions of chloromethane

Muhammad Farhan Ul Haque

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UNIVERSITE DE STRASBOURG

Ecole Doctorale des Sciences de la Vie et de la Santé

THÈSE présentée par
Muhammad FARHAN UL HAQUE

Investigations of the bacterial sink for plant emissions of chloromethane

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Summary

Chloromethane is the most abundant halocarbon in the environment, and responsible for substantial ozone destruction in the stratosphere. Sources and sinks of chloromethane are still poorly constrained. Although synthesized and used industrially, chloromethane is mainly produced naturally, with major emissions from vegetation and especially the phyllosphere, i.e. the aerial parts of plants. Some phyllosphere epiphytes are methylotrophic bacteria which can use single carbon compounds such as methanol and chloromethane as the sole source of carbon and energy for growth. Most chloromethane-degrading strains isolated so far utilize the *cmu* pathway for growth with chloromethane which was characterized by the team.

The main objective of this work was to investigate whether epiphytes may act as filters for plant emissions of chloromethane, by using a laboratory bipartite system consisting of the model plant *Arabidopsis thaliana*, known to produce chloromethane mainly by way of the *HOL1* gene, and the reference chloromethane-degrading bacterial strain *Methylobacterium extorquens* CM4, possessing the *cmu* pathway and of known genome sequence.

Three *A. thaliana* Col-0 variants with different levels of expression of *HOL1*, i.e. the wild-type strain, its homozygous *HOL1* knockout mutant *hol1* and an *HOL1*-OX *HOL1* overexpressor, were selected using PCR and qRT-PCR. Chloromethane-degrading strains were isolated from the *A. thaliana* phyllosphere, and shown to contain the *cmu* pathway. A plasmid-based bacterial bioreporter for chloromethane was constructed which exploits the promoter region of the conserved chloromethane dehalogenase gene *cmuA* of strain CM4. It yields rapid, highly sensitive, specific and methyl halide concentration-dependent fluorescence. Application of the bioreporter to the three *A. thaliana* variants differing in expression of *HOL1* investigated in this work suggested that they indeed synthesize different levels of chloromethane. Analysis by qPCR and qRT-PCR of metagenomic DNA from the leaf surface of these variants showed that the relative proportion and expression of *cmuA* in this environment paralleled *HOL1* gene expression.

Taken together, the results obtained indicate that even minor amounts of chloromethane produced by *A. thaliana* in the face of large emissions of methanol may provide a selective advantage for chloromethane-degrading methylotrophic bacteria in the phyllosphere environment. This suggests that chloromethane-degrading epiphytes may indeed act as filters for emissions of chloromethane from plants.

Further experiments are envisaged to further assess the adaptation mechanisms of chloromethane-degrading bacteria in the phyllosphere, building upon the comparative genomic analysis of chloromethane-degrading strains which was also performed in this work, and on the preliminary investigations using high-throughput sequencing that were initiated.

Keywords: chloromethane, methylotrophic bacteria, *Methylobacterium extorquens*, *Arabidopsis thaliana*, bacterial genomics, bioreporter, *HOL1*, *cmuA*

Résumé

Le chlorométhane est le plus abondant des composés organo-halogénés dans l'atmosphère et il est impliqué dans la destruction de l'ozone dans la stratosphère. Les sources et les puits de chlorométhane restent mal évalués. Bien que synthétisé et utilisé de manière industrielle, il est principalement produit naturellement, avec comme sources majeures les émissions provenant des végétaux et plus particulièrement de la phyllosphère, qui correspond aux parties aériennes des plantes. Certaines bactéries épiphytes de la phyllosphère sont des méthylotrophes capables d'utiliser des composés organiques sans liaison carbone-carbone comme le méthanol et le chlorométhane comme unique source de carbone et d'énergie pour leur croissance. La plupart des bactéries chlorométhane-dégradantes isolées jusqu'à présent utilisent une voie métabolique pour leur croissance sur chlorométhane appelée voie *cmu* (pour chlorométhane utilisation), caractérisée par l'équipe.

L'objectif principal de cette thèse a été de déterminer si des bactéries de la phyllosphère peuvent jouer le rôle de filtre pour l'émission de chlorométhane par les plantes. Dans ce but, un modèle de laboratoire a été mis en place, constitué de la plante *Arabidopsis thaliana* connue pour produire du chlorométhane par une réaction impliquant le gène *HOL1*, et la bactérie *Methylobacterium extorquens* CM4, souche de référence pour l'étude du métabolisme de dégradation du chlorométhane, qui possède la voie *cmu* et dont le génome complet a été séquencé et analysé.

Des variants d'*A. thaliana* avec différents niveaux d'expression du gène *HOL1* (le type sauvage, le mutant homozygote « knock-out » *hol1* et un variant *HOL1*-OX avec surexpression) ont été sélectionnés par PCR et qPCR. Des souches bactériennes chlorométhane-dégradantes ont été isolées à partir de la phyllosphère d'*A. thaliana*, dont il a été montré qu'elles possèdent la voie *cmu*. Un bio-rapporteur bactérien pour le chlorométhane a été construit à l'aide d'un plasmide exploitant la région promotrice du gène conservé de la déshalogénase (*cmuA*) de la souche *M. extorquens* CM4. Il présente une réponse fluorescente rapide, sensible, et spécifique aux méthyl-halogénés de manière concentration-dépendante. L'application du bio-rapporteur aux trois variants d'*A. thaliana* étudiés suggère des niveaux d'émissions de chlorométhane différents. L'analyse, par qPCR et qRT-PCR, de l'ADN métagénomique extrait de la surface des feuilles a montré une corrélation entre la proportion relative de bactéries portant le gène *cmuA* et l'exprimant dans cet environnement, et l'expression du gène *HOL1*. Ces résultats indiquent qu'une production de chlorométhane, même très modeste par rapport aux fortes émissions de méthanol par *A. thaliana*, confère un avantage sélectif pour les bactéries épiphytes chlorométhane-dégradantes. Ces dernières pourraient ainsi bien jouer un rôle de filtre pour les émissions de chlorométhane de la phyllosphère vers l'atmosphère.

En perspective, de nouvelles expériences complémentaires, basées sur l'analyse par génomique comparative des souches chlorométhane-dégradantes également effectuée dans le cadre du projet et sur une analyse par séquençage à haut-débit initiée dans ce travail, sont proposées pour améliorer la compréhension des mécanismes d'adaptation des bactéries chlorométhane-dégradantes dans la phyllosphère.

Mots-clés : chlorométhane, bactéries méthylotrophes, *Methylobacterium extorquens*, *Arabidopsis thaliana*, génomique bactérienne, bio-rapporteur, *HOL1*, *cmuA*.

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Abbreviations

BLAST	<u>B</u> asic <u>l</u> ocal <u>a</u> lignment <u>s</u> earch <u>t</u> ool
bp	<u>b</u> ase <u>p</u> air
C	<u>C</u> arbon
C-1	organic compounds with single carbon (or lacking C-C bonds)
CaMV	<u>C</u> auliflower <u>m</u> osaic <u>v</u> irus
CFCs	<u>C</u> hloro <u>f</u> luoro <u>c</u> arbons
<i>cmu</i>	<u>c</u> hloro <u>m</u> ethane- <u>u</u> tilization
Col-0	<u>C</u> olumbia- <u>0</u>
Ct	<u>T</u> hreshold <u>c</u> ycle
DNA	<u>D</u> eoxyribo <u>n</u> ucleic <u>a</u> cids
GC-MS	<u>G</u> as <u>c</u> hromatography- <u>m</u> ass <u>s</u> pectrometry
H ₄ F	Tetrahydrofolate (H ₄ folate)
H ₄ MPT	Tetrahydromethanopterin
HOL	<u>H</u> armless to <u>o</u> zone <u>l</u> ayer
kb	<u>k</u> ilo <u>b</u> ase pairs
LB medium	<u>L</u> uria <u>B</u> ertani medium
M3 medium	<u>M</u> inimal <u>m</u> ineral <u>m</u> edium
Mage	<u>M</u> agnifying <u>g</u> enomes, An interface of MicroScope (Génoscope)
Mb	<u>M</u> ega <u>b</u> ase pairs
MS medium	<u>M</u> urishage and <u>S</u> koog medium
OD ₆₀₀	<u>O</u> ptical <u>d</u> ensity measured spectrophotometrically at 600 nm
OTUs	<u>O</u> perational <u>t</u> axonomic <u>u</u> nits
PCR	<u>P</u> olymerase <u>c</u> hain <u>r</u> eaction
<i>p_{cmuA}</i>	<i>cmuA</i> gene promoter
Phy-R	<u>P</u> hylosphere induced <u>r</u> egulator
ppt	<u>P</u> arts per <u>t</u> rillion
qPCR	<u>q</u> uantitative PCR
qRT-PCR	<u>Q</u> uantitative <u>R</u> everse <u>t</u> ranscriptase PCR
RNA	<u>R</u> ibo <u>n</u> ucleic <u>a</u> cids
<i>syfp2</i>	The gene coding for YFP
TD-GC-MS	<u>T</u> hermal <u>D</u> esorption GC-MS
VOCs	<u>V</u> olatile <u>o</u> rganic <u>c</u> ompounds
YFP	<u>Y</u> ellow <u>f</u> luorescent protein

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Chapter 1. Introduction

Résumé

Le chlorométhane (CH_3Cl) est un composé organo-halogéné volatil, le plus abondant dans l'atmosphère. Il est responsable d'au moins 15% de la destruction de l'ozone stratosphérique causée par les composés halogénés. Les émissions mondiales de chlorométhane sont estimées entre 4,1 à 4,4 Tg par an, dont moins de 10% sont dues aux activités industrielles. Les sources naturelles de chlorométhane sont notamment les plantes, en particulier les parties aériennes des plantes (la phyllosphère), les marais salants, la combustion de la biomasse et les océans. La contribution du compartiment végétal aux émissions de chlorométhane n'a cependant pas encore été complètement évaluée. Toutefois, une méthyltransférase impliquée dans la production de chlorométhane a été isolée à partir des feuilles de *Brassica oleracea*, et chez *Arabidopsis thaliana*, l'implication d'un gène *HOL1* (harmless to ozone layer) dans la production de chlorométhane a été mise en évidence. Les puits les plus importants pour le chlorométhane incluent l'oxydation par les radicaux hydroxyles dans la troposphère, les pertes dans les eaux océaniques polaires et vers la stratosphère, ainsi que l'absorption dans le sol et la dégradation bactérienne.

Certaines bactéries méthylotrophes sont capables d'utiliser le chlorométhane comme seule source de carbone et d'énergie. Ainsi, des bactéries chlorométhane-dégradantes ont été isolées à partir de différents environnements tels que les sols, les boues activées et l'environnement marin. Ces bactéries sont phylogénétiquement variées et appartiennent à un large éventail de genres dont *Methylobacterium*, *Hyphomicrobium*, *Aminobacter*, *Roseovarius* et *Acetobacterium*. Des études biochimiques ont permis, dans un premier temps, d'élucider la voie de dégradation du chlorométhane chez la souche *Methylobacterium extorquens* CM4. Dans un deuxième temps, un ensemble des gènes essentiels à la croissance avec le chlorométhane, appelés *cmu* (chloromethane utilisation), a été identifié. L'expression spécifique de *cmuA*, induite par le chlorométhane, a été mise en évidence. Les gènes *cmu* sont très répandus parmi les bactéries chlorométhane-dégradantes, et le gène *cmuA* est le plus conservé au sein des différentes souches. De ce fait, le gène *cmuA* est utilisé comme un gène marqueur de la dégradation du chlorométhane dans différents environnements.

L'objectif général de la thèse a été d'explorer le rôle possible des bactéries chlorométhane-dégradantes de la phyllosphère comme filtre des émissions végétales de chlorométhane, en utilisant un modèle comprenant *M. extorquens* CM4 et *A. thaliana*.

1.1. Halomethanes

Halomethanes are derivatives of methane (CH_4) with one or more hydrogen atoms replaced by halogens (X): fluorine (F), chlorine (Cl), bromine (Br) or iodine (I) (Table 1.1). They are produced naturally or industrially and represent major pollutants of soil, water and air (<http://www.epa.gov>). Halomethanes and the chlorofluorocarbons (CFCs) have been amongst the commercially most important halocarbons, and have found widespread applications as cleaning agents; solvents, reactants, and coolants for refrigerators and as propellants for aerosols (Benhadid-Dib and Benzaoui, 2012). These inexpensive and chemically inert compounds were deemed ideal for industrial processes (Benhadid-Dib and Benzaoui, 2012 and references cited therein). In particular, the chlorinated methanes chloromethane (CH_3Cl), dichloromethane (CH_2Cl_2), trichloromethane (chloroform, CHCl_3) and tetrachloromethane (CCl_4) have been produced and used extensively in industry throughout the 20th century. Nevertheless; the use of tetrachloromethane is now very strictly regulated, and significant efforts to reduce the use of dichloromethane and chloroform are ongoing (<http://www.eurochlor.org>).

The extensive use and the high volatility of these compounds (chloromethane is a gas while the three other chlorinated methanes exist in the liquid state under standard conditions of temperature and pressure, Table 1.1) have led to their immoderate entry into the atmosphere. Concerns that such emissions may cause environmental problems, particularly with respect to their detrimental effects on stratospheric ozone, have given these chemicals scientific attention from the 1970s onwards (Prather and Watson, 1990). Chlorinated methanes in the stratosphere are activated by ultraviolet (UV) light and the resulting halogen radicals react with ozone to form oxygen (O_2) (Fig. 1.1). This catalytic process (it is estimated that one chlorine atom can destroy over 100,000 ozone molecules, (<http://www.epa.gov/ozone/science/process.html>) is responsible for the formation of the ozone hole over Antarctica each spring (Clerbaux *et al.*, 2007).

Table 1.1. Important properties of chlorinated methanes

Properties	Chloromethane (CH ₃ Cl) ^a	Dichloromethane (CH ₂ Cl ₂) ^b	Trichloromethane (CHCl ₃) ^c	Tetrachloromethane (CCl ₄) ^d
Physico-chemical properties				
Molecular weight	50.5	84.9	119.5	153.8
Density (101.3 kPa)	0.0023 g/cm ³ (at 0°C)	1.3255 (at 20°C)	1.48 g/cm ³ (at 25°C)	1.594 g/cm ³ (at 25°C)
Water solubility (at 25°C)	4.8 g/L	13 g/L	7.2 – 9.3 g/L	0.8 g/L
Henry's law constant (atm cm ³ per mol)	0.0102 ^e	0.0023 ^e	0.0035 ^e	0.0275 ^e
Carbon oxidation state	-2	0	2	4
Vapor pressure	575 kPa (at 25°C)	46.53 kPa (at 20°C)	21.3 kPa (at 20°C)	12.2 kPa (at 20°C)
Environmental data				
Toxic effects to humans	neurotoxicity, renal tumor and infertility	possible carcinogen, affects nervous system	possible carcinogen, liver and kidney tumors	possible carcinogen, neurological & renal damage
Genotoxicity	weak	none	very weak	none
Tolerable daily intake	-	6 µg/kg of body wt	0.015 mg/kg of body wt	1.45 g/kg of body wt
Tolerable daily inhalation	0.018 mg/m ³ of air	20 µg/m ³ of air	0.14 mg/m ³ of air	6.1 mg/m ³ of air
Atmospheric conc. (ppt)	550 ± 30 ^f	22.21 ± 6.7 ^f	7 ± 1.5 ^f	95 ± 5 ^f
Half life (years)	1.30 ^f	0.38 ^f	0.40 ^f	25 ^f

^aWHO (2000); ^bWHO (2003); ^cWHO (2004a); ^dWHO (2004b); ^eChen *et al.* (2012); ^fMontzka *et al.* (2011)

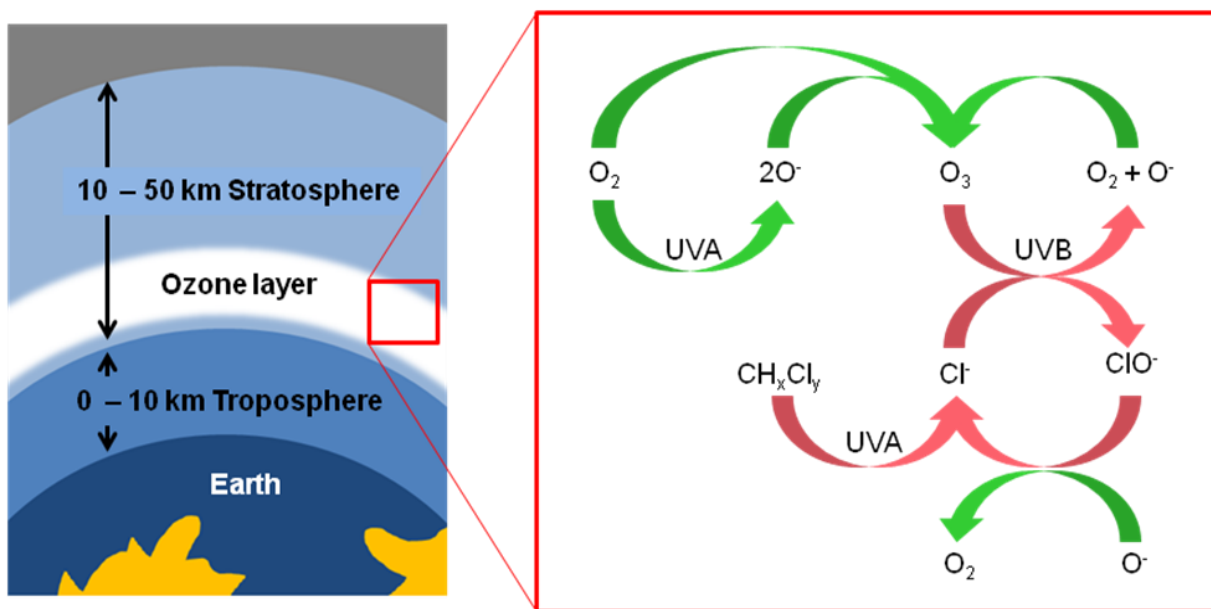


Fig. 1.1. Formation and destruction of ozone layer in the stratosphere.

Natural reactions of ozone are disturbed by halomethanes (CH_xCl_y) in presence of ultraviolet A (UVA, wavelength 315 – 400 nm) and ultraviolet B (UVB, wavelength 280 – 315) radiations. (Figure adapted from <http://www.cec.org>)

1.2. Chloromethane in the atmosphere

Chloromethane is the most abundant chlorine-containing organic compound in the atmosphere and contributes to 16% of chlorine catalyzed ozone layer destruction in the stratosphere (Table 1.1) (Montzka *et al.*, 2011). Global average concentrations of chloromethane in the atmosphere are 550 ± 30 pptv (parts per trillion by volume), with an annual increase of $2.3 - 2.7 \pm 1.2$ pptv from 2004 – 2008. The World Meteorological Organization has classified it among the long-lived gases involved in ozone depletion with a half-life of over one year (Montzka *et al.*, 2011). Chloromethane is also important in the environment due to its global warming potential that is 17 – 25 times higher than that of CO_2 (on a mass basis) (Grossman *et al.*, 1997; Schäfer *et al.*, 2007).

1.2.1. Global chloromethane budget

Evaluation of the global budget of chloromethane has been investigated in many studies, and involve in particular the use of isotopic signatures (Keppler *et al.*, 2005; Saito and Yokouchi, 2008) and 3D simulation models (Xiao *et al.*, 2010). The estimated total atmospheric burden

of chloromethane is 4 – 5 Tg (teragram = 10^{12} grams), with less than 10% from anthropogenic sources such as industries and burning (Table 1.2) (Xiao *et al.*, 2010). Natural emissions of chloromethane originate mainly from living vegetation (Saito and Yokouchi, 2008), dead plant material and senescent leaves (Hamilton *et al.*, 2003), wood rot fungi (Moore *et al.*, 2005) biomass burning (Xiao *et al.*, 2010) and oceans and coastal waters (Hu *et al.*, 2010; Xiao *et al.*, 2010).

With regard to sinks for atmospheric chloromethane, the dominant process for loss of atmospheric chloromethane is via reaction with photochemically produced OH^\cdot radicals (Table 1.2). The reaction of chloromethane with chlorine radicals in the marine boundary layer, loss to the stratosphere and polar cold oceans, and degradation by soil microorganisms are other sinks of chloromethane (Table 1.2) (Montzka *et al.*, 2011).

Current estimates of sources (1.7 to 13.5 Tg y^{-1}) and sinks (4.2 to 6.6 Tg y^{-1}) vary widely (Table 1.2). Additional sources and sinks of chloromethane may exist but remain to be identified. Currently, there is a significant shortage of sources (>1000 Gg y^{-1} , Table 1.2). While chloromethane emissions from tropical and subtropical plants, potentially estimated to contribute the major part (approx. 30 – 50%) of global emissions of chloromethane, may in fact balance or even possibly outweigh current best estimates of total sinks (Keppler *et al.*, 2005; Saito and Yokouchi, 2008). Evidence is now emerging from microbial studies (Miller *et al.*, 2004; Schäfer *et al.*, 2007) for a much larger bacterial sink for chloromethane (Keppler *et al.*, 2005). Following the large reductions of anthropogenic emissions of many ozone-depleting gases resulting from the Montreal protocol (Montzka *et al.*, 2011), halogenated gases of uncontrolled emissions from natural sources such as chloromethane have now become the major sources of active chlorine in the stratosphere. It therefore appears important to analyze natural sources and sinks in the environment more thoroughly to obtain reliable predictions of the global budget of chloromethane and its implications for stratospheric ozone.

Table 1.2. Estimated source and sink strengths for atmospheric chloromethane from observations and three-dimensional model studies

Source/sink type	Best estimates^a (Gg y⁻¹)	Range^b (Gg y⁻¹)
Sources		
Tropical plants	910	820 – 8200
Biomass burning	911	325 – 1125
Fungi	160	43 – 470
Salt marshes	170	64 – 440
Wetlands	40	48
Coal combustion	105	5 – 205
Incineration	45	15 – 75
Industrial	10	10
Rice	5	2.4 - 4.9
Oceans	600	380 – 500
Senescent or dead leaves		30 – 2500
Total known sources	2956	1743 – 13578
Sinks		
Reaction with OH in troposphere	-3180	-3800 – -4100
Loss to stratosphere	-200	-100 – -300
Reaction with Cl in marine boundary layer	-370	-180 – -550
Loss to cold ocean waters	-75	-93 – -145
Microbial degradation in soil	-890	-100 – -1600
Total sinks	-4715	-4273 – -6695
Balance of sources and sinks	-1759	

^a Best estimates of sources and sinks (Keppler *et al.*, 2005)^b Range of strengths of sources and sinks (Clerbaux *et al.*, 2007)

1.3. Vegetation as an important source of C-1 compounds

Plants release a considerable proportion of the carbon fixed by photosynthesis back into the atmosphere or into the soil. Terrestrial plants are an important source of reactive volatile organic compounds (VOCs) in the environment such as e.g. methanol, isoprene, monoterpenes, methane (Table 1.3). The source strength of these biogenic VOCs exceeds that of organic volatile emissions due to human activities by an order of magnitude (Guenther *et al.*, 2012 and references cited therein). Increasing evidence indicates that these compounds may have an important role in the adaptation of plants to changing climate, and these VOCs significantly influence atmospheric processes (Hartikainen *et al.*, 2012). Therefore, possible changes in the emissions of these volatile compounds from plants are of high importance for predicting future climate.

Table 1.3. Major volatile organic compounds released from plants

Volatile organic compound	Tg C y⁻¹	Reference
Monoterpenes	530 – 560	Guenther <i>et al.</i> (2012)
Isoprene	157 – 177	Guenther <i>et al.</i> (2012)
Methanol	100 – 105	Galbally and Kirstine (2002)
Methane	62 – 236	Keppler <i>et al.</i> (2006)
Chloromethane	0.8 – 8	Clerbaux <i>et al.</i> (2007)

The mechanisms by which chloromethane and methanol are produced by higher plants in leaf tissues in particular are described in the following.

1.3.1. Biosynthesis of chloromethane in plants

Considering the global importance of emissions of chloromethane from living vegetation, surprisingly few investigations aiming at identifying specific plant sources have been reported so far. Yokouchi *et al.* (2007) conducted a survey on an island covered with subtropical forest, and identified 33 species from a variety of families as strong chloromethane-emitting plants ($0.01 - 2.8 \mu\text{g g}^{-1} \text{ dry weight h}^{-1}$). The strongest emitters were

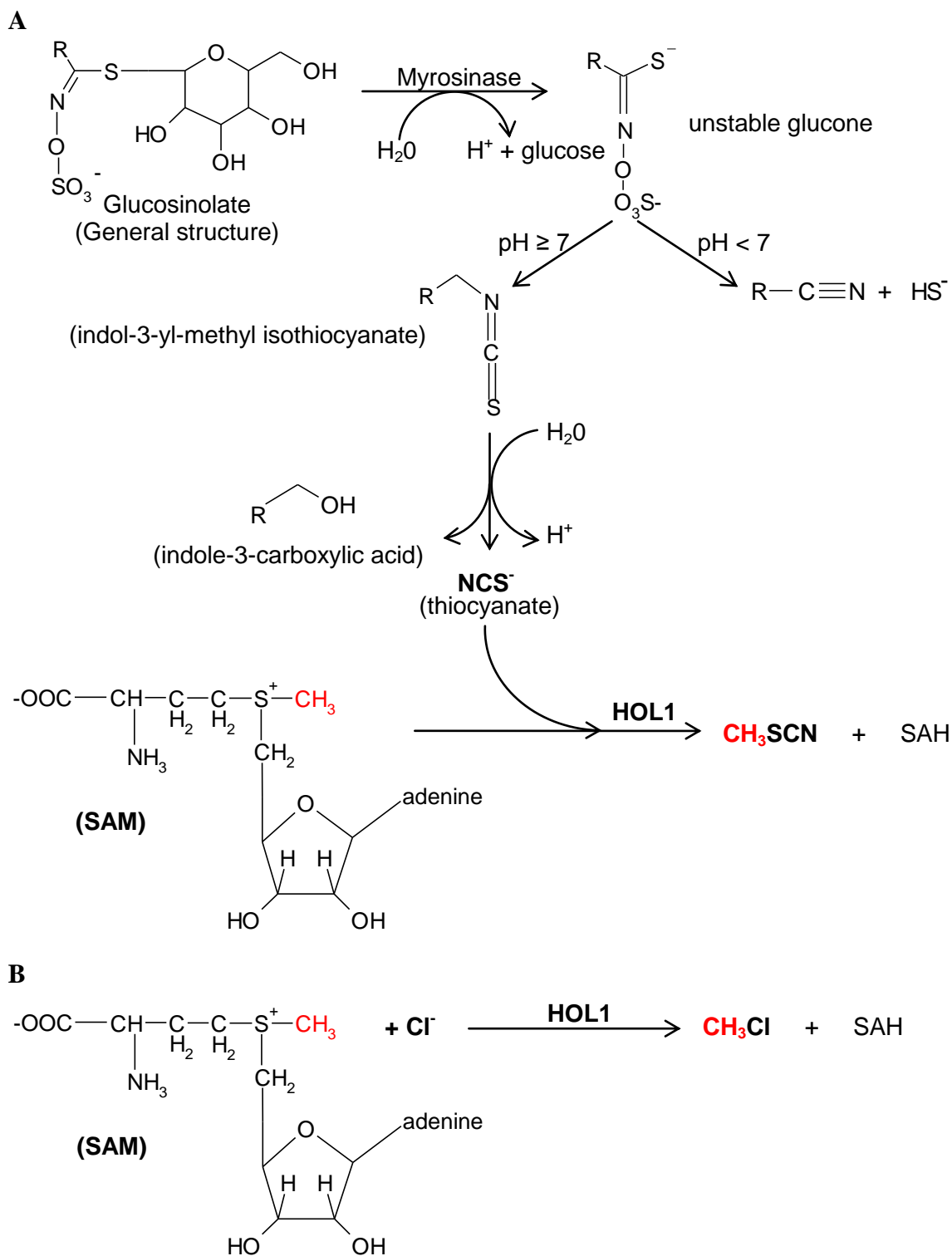


Fig. 1.2. Function of HOL1 in *A. thaliana*.

HOL1 catalyzes the S-adenosylmethionine (SAM) dependent methylation of thiocyanate (SCN^-) produced during glucosinolate hydrolysis, yielding methyl thiocyanate (CH_3SCN) (A), or methylation of chloride (Cl^-) to produce chloromethane

ferns *Osmunda banksiifolia*, *Cibotium balometz*, *Angiopteris palmiformis*, and halophytes *Vitex rotundifolia*, *Vitex trifolia*, and *Excoecaria agalloch*, each with chloromethane emission rates exceeding $1 \mu\text{g g}^{-1}$ dry weight h^{-1} (Yokouchi *et al.*, 2007). The capacity to emit chloromethane was shared at the genus level but not always at the higher family level (Yokouchi *et al.*, 2007). Another study (Saito and Yokouchi, 2008) reported 11 glasshouse-grown tropical plant species emitting chloromethane, and measured their stable carbon isotopic composition of chloromethane. Saito *et al.* (2008) also investigated chloromethane fluxes at leaf and canopy scale in a tropical forest and reported that 25 out of 117 plant species were chloromethane emitters.

The mechanism of chloromethane production was not investigated in these studies. It is generally assumed, however, that it may be similar to that characterized in model plants, which is described in the following.

1.3.2. *HOL1*: a gene involved in chloromethane production in *Arabidopsis thaliana*

The biochemical basis of chloromethane emissions was first described by Wuosmaa and Hager (1990) who reported a chloride methyltransferase capable of methylating chloride (Cl^-) in *Mesembryanthemum crystallinum*. Involvement of this enzyme suggested that it could be a part of salt tolerance mechanism in halophytes. Another methyltransferase purified from cabbage plant (*Brassicaceae* family) was able to catalyze the methylation of halides (X^-), sulphide (HS^-), and thiocyanate (SCN^-) on the same active site, and named halide/sulphide/thiol methyltransferase (Saini *et al.*, 1995; Attieh *et al.*, 2000). It was suggested that this enzyme might help clearance of toxic sulfur compounds resulting from glucosinolate breakdown in plants upon tissue damage (Fig. 1.2A) (Attieh *et al.*, 2000). Finally, Rhew *et al.* (2003) characterized the genetic mechanism of production of chloromethane and other methyl halides (bromomethane and iodomethane) in the small flowering plant *Arabidopsis thaliana* (mouse-ear cress), the best studied model plant at the molecular level (<http://www.arabidopsis.org/index.jsp>, The Arabidopsis Information Resource). These authors identified the gene encoding the enzyme primarily responsible for the production of chloromethane, designated *HOL* for *HARMLESS TO OZONE LAYER*, one of the consequences of loss of gene function in a *HOL* mutant. Of the three variants *HOL1*, *HOL2* and *HOL3* (also denoted as *AtHOL1*, *AtHOL2* and *AtHOL3*) found in *A. thaliana*

(Rhew *et al.*, 2003; Nagatoshi and Nakamura, 2007), *HOL1* is primarily responsible for more than 85% of methyl halide production (Rhew *et al.*, 2003). The *HOL1* gene appears quite conserved in plants, suggesting that chloromethane production by vegetation may be a quite widespread property (Rhew *et al.*, 2003; Nagatoshi and Nakamura, 2007).

Rhew *et al.* (2003) showed that the HOL1 protein belonged to a group of methyltransferases capable of catalyzing the *S*-adenosyl-L-methionine (SAM)-dependent methylation of chloride (Cl^-), bromide (Br^-), and iodide (I^-) to produce methyl halides (Fig 1.2B). They also showed that chloromethane production was largely eliminated in mutant plants with *HOL1* gene disrupted. However, the physiological advantage of chloromethane production by HOL1, if any, remains to be clarified. Recent investigations strongly suggested a physiological role for HOL1, by showing that the enzyme was highly active with thiocyanate SCN^- (a pseudohalide), resulting in methylthiocyanate (CH_3SCN) synthesis (Fig 1.2A) (Nagatoshi and Nakamura, 2009). The authors observed that substantial amounts of SCN^- were released by *A. thaliana* upon tissue damage, and that SCN^- was largely derived from myrosinase-mediated hydrolysis of glucosinolates (Fig. 1.2A). Bacterial growth assays indicated that conversion of SCN^- into CH_3SCN dramatically increased antibacterial activities against *A. thaliana* pathogens which invade damaged tissue at the wound site (Nagatoshi and Nakamura, 2009).

1.3.3. Biosynthesis of methanol in plants

Compared to methanol, however, chloromethane only represents a very minor emission from the aerial parts of plants (Table 1.3). The remodelling of cell walls during plant growth is at the origin of plant emissions of methanol (Galbally and Kirstine, 2002, and references cited therein). The main framework of the cell wall is formed by a cellulose/hemicellulose network, along with small amounts of structural proteins (Cosgrove and Jarvis, 2012). A pectin matrix acts to hold the structure together (Carpita and Gibeaut, 1993). Plasticity, rigidity and pore size of cell walls are controlled by the nature of the bonds between adjacent pectin polymers (Galbally and Kirstine, 2002). Polygalacturonic acid (PGA), a homopolymer of α -D-[1-4]-galactosyluronic acid units with different degrees of methyl esterification, mainly constitutes the basic structure of most pectins (Fig. 1.3) (Galbally and Kirstine, 2002). Along the formation of new cellulose microfibrils during cell wall elongation resulting in cell

growth, continuous adaptations of the pectin matrix are also required to increase cell wall plasticity (Galbally and Kirstine, 2002). This is mediated by differential methylation of PGA carboxylic acid moieties (Galbally and Kirstine, 2002). Once the process of cell wall extension is complete, demethylation of PGA by the action of the enzyme pectin methylesterase (PME) yields galacturonic acid residues and methanol (Fig. 1.3) (Galbally and Kirstine, 2002).

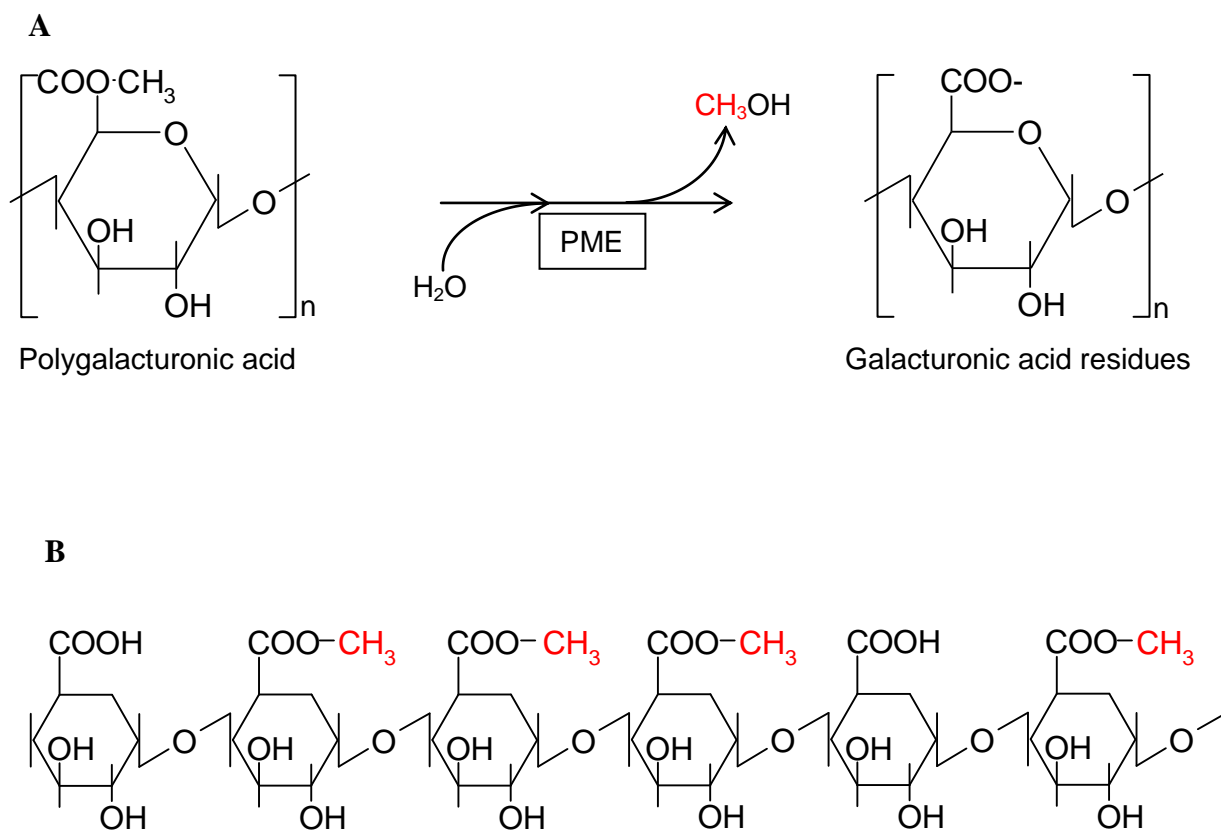


Fig. 1.3. Biosynthesis of methanol in plants.

(A) Polygalacturonic acid is demethylated by pectin methylesterase (PME) to produce methanol and galacturonic acid residues during elongation of the primary cell wall.

(B) General structure of the backbone of pectin consisting of polygalacturonic acid (a homopolymer of α -D-[1-4]-galactosyluronic acid units) with different degrees of methyl esterification (adapted from Galbally and Kirstine, 2002).

Methanol and other carbonaceous compounds such as chloromethane which are released from plants may be emitted to the atmosphere, but may also serve as the carbon source for growth of plant-associated microbes in the aerial parts of plants leaves, the phyllosphere [see **section 1.7** further below]. In the latter environment, bacteria capable of using compounds containing a single carbon atom are of particular relevance, and are presented in the next section [**Section 1.4**].

1.4. Methyлотrophy

Methyлотrophy is defined as the ability of organisms to grow with organic compounds containing a single carbon atom (C-1) or lacking C-C bonds such as methanol. Other methyлотrophic C-1 substrates include methane, formaldehyde, methylamine, methylated sulfur compounds and halogenated methanes. Methyлотrophic microorganisms are ubiquitous and can be found in soils and sediments (Kolb, 2009), fresh and marine waters (Neufeld *et al.*, 2008), roots and leaves of plants (Delmotte *et al.*, 2009; Knief *et al.*, 2012), and air (DeLeon-Rodriguez *et al.*, 2013). Methyлотrophic microbes can be found in all three domains of life, i.e. bacteria, Archaea (e.g. methyлотrophic methanogens) and Eukarya (e.g. yeasts growing with methanol), but methyлотrophic metabolism has mainly been investigated in bacteria (Chistoserdova *et al.*, 2009; Kolb, 2009; Kawaguchi *et al.*, 2011; Peyraud *et al.*, 2012). More than 150 methyлотrophic bacterial species belonging to the Alpha-, Beta-, Gamma- Proteobacteria, Verrucomicrobia, Cytophagales, Bacteroidetes, Firmicutes, and Actinobacteria, have been described so far (Kolb, 2009). These bacteria can be aerobic or anaerobic, either strictly or facultatively (Lidstrom, 2006). In terms of carbon usage, methyлотrophic bacteria are categorized into two main groups: those which can utilize methane, called methanotrophs, and those which cannot, which are often called non-methanotrophic methyлотrophs (Lidstrom, 2006). Bacteria capable of utilizing methane gas as the only source of carbon and energy possess an enzyme, methane monooxygenase (MMO), involved in the oxidation of methane to methanol (Hakemian and Rosenzweig, 2007). This enzyme exists in two different forms: soluble methane monooxygenase and membrane bounded (or particulate) methane monooxygenase (Lidstrom, 2006; Semrau *et al.*, 2011). Methanotrophs are principally obligate methyлотrophs; and only a few genera so far (e.g. *Methylocella*, *Methylocapsa*, and *Methylocystis*) can also utilize multicarbon compounds

(Dedysh *et al.*, 2005; Kolb, 2009; Semrau *et al.*, 2011). Basing on their remarkable internal membranes, carbon assimilation pathways and 16S rRNA gene sequences, methanotrophs have been classified into two groups (Hanson and Hanson, 1996; Lidstrom, 2006): type I methanotrophs which assimilate carbon through the ribulose monophosphate (RUMP) pathway and belongs to Gammaproteobacteria (e.g. *Methylomonas* and *Methylobacter*), and type II methanotrophs which utilize carbon through the serine cycle and belong to Alphaproteobacteria. During the last decade another type of methanotrophs, belonging to phylum Verrucomicrobia and oxidizing methane under extreme conditions ($\text{pH} < 1$) (Op den Camp *et al.*, 2009; Khadem *et al.*, 2012), and also capable of autotrophic CO_2 fixation through the Calvin-Benson-Bassham cycle (Khadem *et al.*, 2011), was discovered. The ribulose monophosphate and serine pathways for carbon assimilation, named after the key metabolic intermediate involved, are also found in non-methanotrophic methylotrophs [see **Section 1.4.2** below]. Non-methanotrophic methylotrophs generally lack the system of internal membranes. As methanotrophs, methylotrophs may use the RUMP and serine pathways for carbon assimilation. However, some methylotrophic bacteria use another pathway, the ribulose bis-phosphate pathway, instead (Chistoserdova *et al.*, 2009). Methylotrophic bacteria are quite often facultative methylotrophs, and may use different multi-carbon compounds for their growth (Chistoserdova *et al.*, 2009).

1.4.1. Chloromethane-degrading bacteria

While cometabolic oxidation of chloromethane was observed for nitrifying bacteria (Rasche *et al.*, 1990) and methanotrophs (Han and Semrau, 2000) and is attributed to the activity of ammonium- or methane monooxygenase, respectively, different types of methylotrophic bacteria capable of utilizing chloromethane as the only source of carbon and energy were isolated over the years (Table 1.4) (Schäfer *et al.*, 2007). The first aerobic bacterium for which growth with chloromethane was described was *Hyphomicrobium* sp. strain MC1, isolated from a sewage treatment plant in Switzerland in 1986 (Hartmans *et al.*, 1986; Vuilleumier *et al.*, 2011) and growing under aerobic conditions. In subsequent years, *Acetobacterium dehalogenans* (formerly named strain MC) (Messmer *et al.*, 1993) and *Pseudomonas aeruginosa* NB1 (Freedman *et al.*, 2004) were described to utilize chloromethane anaerobically.

Table 1.4 Chloromethane degrading strains isolated from different environments.

Bacterial strain	Origin	Gram type	Reference
<i>Hyphomicrobium</i> sp. strain MC1	industrial sewage plant (Switzerland)	negative	Hartmans <i>et al.</i> (1986)
<i>Acetobacterium dehalogenans</i> MC	activated sludge (Germany)	positive	Traunecker <i>et al.</i> (1991)
<i>Hyphomicrobium</i> sp. strain CM2	soil from a petrochemical factory (Russia)	negative	Doronina <i>et al.</i> (1996)
<i>Methylobacterium extorquens</i> sp. strain CM4 ^a	soil from a petrochemical factory (Russia)	negative	Doronina <i>et al.</i> (1996)
<i>Aminobacter ciceroni</i> IMB1	fumigated strawberry field soil (USA)	negative	Hancock <i>et al.</i> (1998)
<i>Aminobacter lissarensis</i> CC495	beech woodland soil (Ireland)	negative	Coulter <i>et al.</i> (1999)
<i>Pseudomonas aeruginosa</i> NB1	activated sludge (USA)	negative	Freedman <i>et al.</i> (2004)
<i>Leisingera methylohalidivorans</i> MB2	marine tide pool (USA)	negative	Schaefer <i>et al.</i> (2002)
<i>Roseovarius</i> sp. strain 179	coastal seawater (Scotland)	negative	Schäfer <i>et al.</i> (2005)
<i>Roseovarius</i> sp. strain 198	coastal seawater (Scotland)	negative	Schäfer <i>et al.</i> (2005)
<i>Roseovarius</i> sp. strain 217	English channel seawater (England)	negative	Schäfer <i>et al.</i> (2005)

^a *Methylobacterium extorquens* sp. CM4 was previously known as *Methylobacterium chloromethanicum* CM4 (Kato *et al.*, 2005) (McDonald *et al.*, 2001).

A collection of aerobic chloromethane-degrading bacterial strains was isolated from a petrochemical factory soil belonging to genera *Methylobacterium* and *Hyphomicrobium*. Two of these, strains CM2 and CM4, were investigated in more detail and named *Hyphomicrobium chloromethanicum* and *Methylobacterium chloromethanicum* respectively (McDonald *et al.*, 2001). In particular, strain CM4, subsequently assigned to the species *Methylobacterium extorquens* [see below, **Chapter 2**, and Marx *et al.*, 2012] became the workhorse organism to unravel the biochemistry and genetics of chloromethane utilisation [see below, **section 1.6**]. While two other strains, *Aminobacter ciceronei* CC495 and *Aminobacter lissarensis* IMB-1, were isolated from soils (McDonald *et al.*, 2005), strains *Roseovarius* sp. 179, *Roseovarius* sp. 217, *Ruegeria* sp. 198 (Schäfer *et al.*, 2005) and *Leisingeria methylohalidivorans* MB2 (Schaefer *et al.*, 2002) were obtained from marine environments (Table 1.4). Strikingly, despite the fact that terrestrial plants represent the largest source of chloromethane, chloromethane-degrading bacteria had not been isolated from plant environments until the initiation of this project.

1.4.2. Methylotrophic pathways in *Methylobacterium*

Pink-pigmented, strictly aerobic and facultative methylotrophs of the genus *Methylobacterium* are perhaps the most studied methylotrophic bacteria, and have been investigated by a wide variety of classical microbiological, genetic, proteomic, metabolomic, and genomic approaches (see e.g. (Vuilleumier *et al.*, 2009, and references cited therein; Muller *et al.*, 2011a; Schneider *et al.*, 2012; Roselli *et al.*, 2013). Three major aspects of methylotrophic metabolism can be differentiated: (i) initial oxidation of C1 compounds, (ii) assimilation of carbon into biomass and (iii) complete oxidation of carbon to CO₂ for energy production (Fig. 1.4) (Chistoserdova *et al.*, 2009). Formaldehyde (HCHO) usually constitutes the central intermediate compound in methylotrophic metabolism in *Methylobacterium* as well as in other methylotrophic microorganisms, and results from initial oxidation reactions of diverse C-1 compounds (Fig. 1.4) (Vuilleumier *et al.*, 2009, and references cited therein). In the case of methanol, pyrrolo-quinoline quinone (PQQ) dependent methanol dehydrogenase is involved (Fig. 1.4) (Lidstrom, 2006), although the importance of an alternative XoxF-dependent reaction has recently also been demonstrated (Schmidt *et al.*, 2010; Skovran *et al.*, 2011).

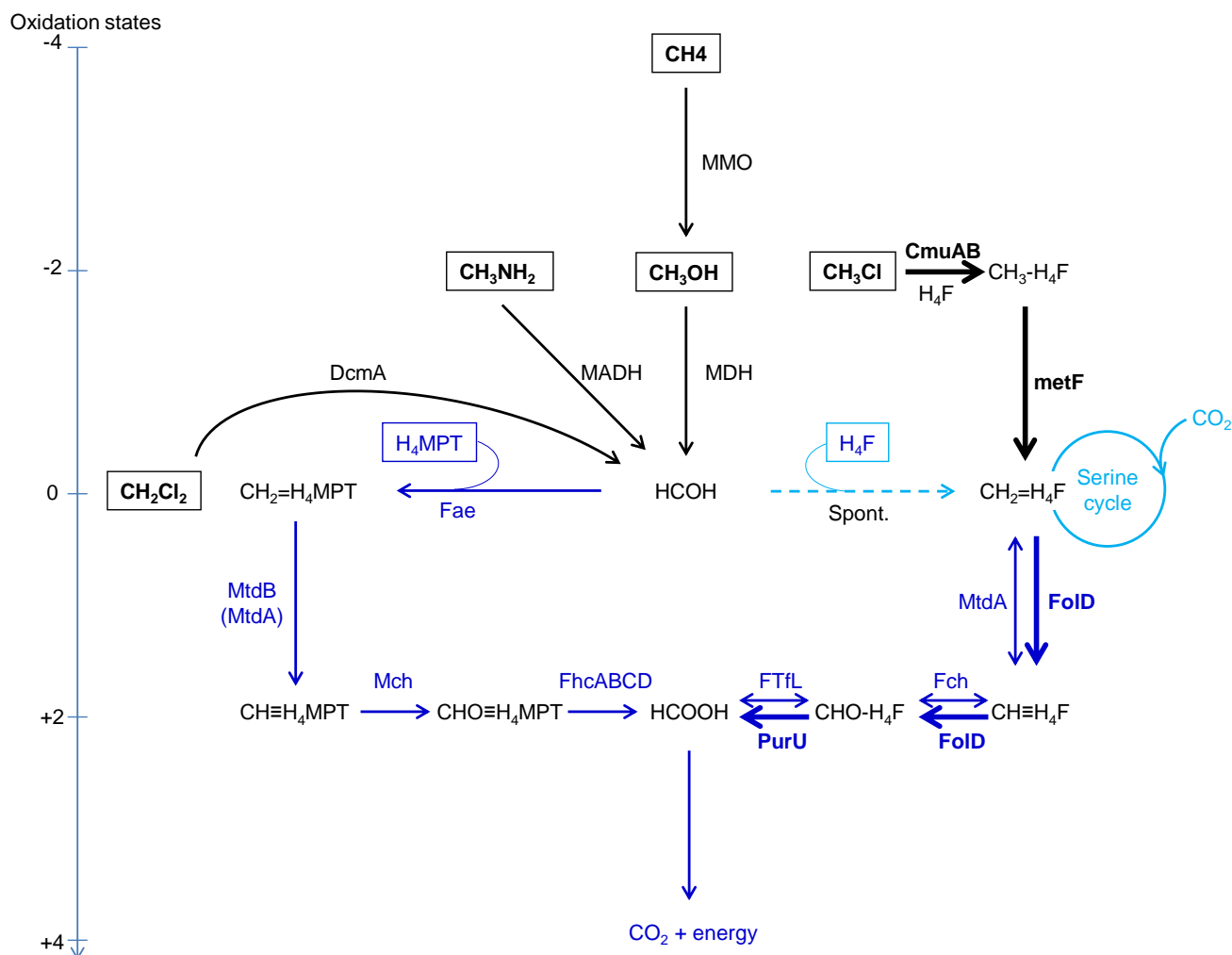


Fig. 1.4. Metabolism of C-1 compounds in *Methylobacterium extorquens*

Methylobacterium lacks the ability to transform methane or to utilize it for growth. Different C-1 substrates methanol (CH₃OH), methylamine (CH₃NH₂), dichloromethane (CH₂Cl₂) and chloromethane (CH₃Cl) are metabolized to synthesize biomass through the serine cycle (reactions in light blue) and oxidized to CO₂ to produce energy (reactions in dark blue). The specific pathway for chloromethane utilization (bold arrows) in *Methylobacterium extorquens* CM4 is different from the canonical one, which involves a tetrahydromethanopterin-dependent pathway (Vorholt, 2002; Marx *et al.*, 2012).

Enzymes for initial oxidation or dehalogenation of C-1 substrates are shown in black: dichloromethane dehydrogenase (DcmA), methylamine dehydrogenase (MADH), , methyltransferase dehalogenases (CmuAB). M. emethane monooxygenase (MMO) Reactions leading to the complete oxidation of carbon to CO₂ and energy are (dark blue): formaldehyde activation enzyme (Fae), methylene tetrahydromethanopterin dehydrogenase (MtdAB), methylene tetrahydromethanopterin cyclohydrolase (Mch), methylene tetrahydrofolate cyclohydrolase (Fch), methylene tetrahydrofolate reductase (MetF), methylene tetrahydrofolate dehydrogenase/ methylene tetrahydrofolate cyclohydrolase (FoID) and formyl tetrahydrofolate hydrolase (PurU). (Fig. adapted from Vuilleumier *et al.*, 2009)

Similarly, the oxidation of methylamine was shown to be mainly performed by TQQ-dependent methylamine dehydrogenase in some *Methylobacterium extorquens* (Lidstrom, 2006), whereas other strains appear to use an alternative enzymatic system involving γ -glutamyl-methylamide as the intermediate (Latypova *et al.*, 2010) and unpublished data from the team). For dichloromethane, dichloromethane dehydrogenase / glutathione S-transferase affords the hydrolytic transformation of dichloromethane to formaldehyde with release of hydrochloric acid (Fig. 1.4) (Muller *et al.*, 2011a).

Formaldehyde produced as a result of initial oxidations of C-1 compounds must be metabolized rapidly and efficiently because of its toxicity (Vorholt, 2002; Marx *et al.*, 2003). Part of this formaldehyde is assimilated into biomass through the serine cycle (Fig. 1.4), and the remainder is oxidized to CO₂ for energy production (Lidstrom, 2006). Two pathways have been characterized in *Methylobacterium* for oxidation of formaldehyde. One pathway involves tetrahydrofolate (H₄F, Fig. 1.5) while the other depends on tetrahydromethanopterin (H₄MPT, Fig. 1.5) and formaldehyde is oxidized to CO₂ through formic acid (HCOOH) for energy production (Fig. 1.4) (Vorholt, 2002). The pathway involving H₄F was shown to be mainly funneling carbon into the serine cycle for biomass production (Fig. 1.4) with methylene-H₄F (CH₂=H₄F) as the key intermediate (Marx *et al.*, 2005). Rather, it is the H₄MPT-dependent pathway (Fig. 1.4) which is the preferred pathway for the oxidation of formaldehyde into CO₂ and energy production in *Methylobacterium* (Vorholt *et al.*, 2000; Vorholt, 2002). In the case of chloromethane, however, the situation was shown to be quite different, in that chloromethane is directly transformed to CH₂=H₄F without intermediate formaldehyde production (Fig. 1.4) (Schäfer *et al.*, 2007, and references cited therein).as described in the following section.

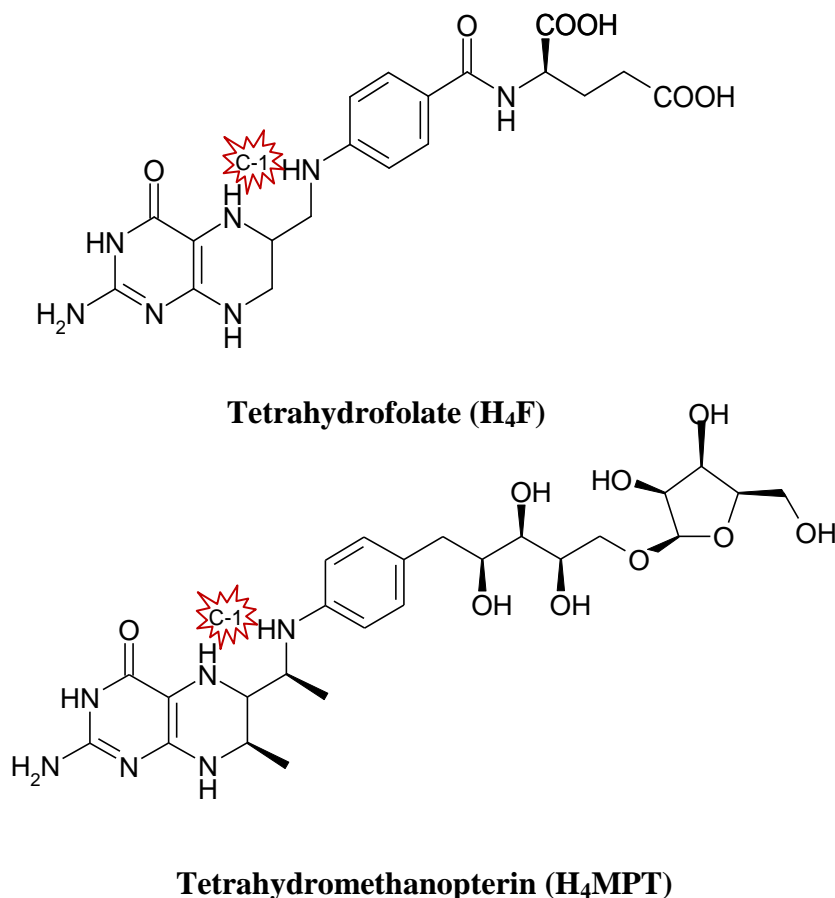


Fig. 1.5. Structures of cofactors involved in the metabolism of C-1 compounds.

The asterisk with C-1 shows the attachment position of methyl, methylene, methenyl and formyl groups.

1.5. Biochemistry and genetics of chloromethane degradation in *Methylobacterium extorquens* CM4

At the end of the 1990s, biochemical and genetic studies were carried out to elucidate the utilization of chloromethane in *Methylobacterium extorquens* CM4 and suggested the existence of a novel and metabolic pathway (Fig. 1.6) (Vannelli *et al.*, 1998; Vannelli *et al.*, 1999). Vannelli *et al.* (1998) first observed that *Methylobacterium extorquens* CM4 metabolized chloromethane as the only source of carbon and energy, releasing stoichiometric amounts of chloride in the medium during growth. It was observed that 1 mole chloromethane was oxidized to 1 mole carbon dioxide, producing 1 mole of hydrochloric acid and consuming 1.5 moles of oxygen. Proteomic analysis by one-dimensional SDS-PAGE showed that two proteins of 67 kDa and 36 kDa were induced in cells of CM4 grown

with chloromethane, and were not detected in methanol-grown cells (Vannelli *et al.*, 1998). Random mutagenesis of strain CM4 with a miniTn5 transposon was then performed and led to identify mutants unable to grow with chloromethane (Fig. 1.6) (Vannelli *et al.*, 1998).

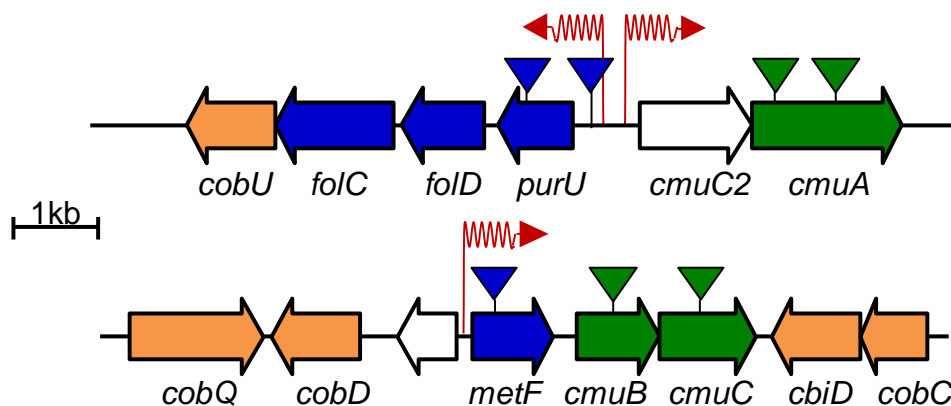


Fig. 1.6. Chloromethane-utilization (*cmu*) genes in *Methylobacterium extorquens* CM4.

Genes responsible for dehalogenation of chloromethane (green), for growth with chloromethane (blue), and genes involved in the metabolism of cobalamine (orange) required for the dehalogenation, are localized in two clusters, subsequently found to be closely associated on megaplasmid pCMU01 (380 kb) of strain CM4 (cf. Chapter 2 below and Marx *et al.*, 2012). Triangles indicate the insertion sites of miniTn5 transposon in characterized mutants and red flashes indicate the chloromethane-induced promoter regions that were subsequently identified (Studer *et al.* 2002). (Fig. adapted from Vannelli *et al.*, 1999)

Mutants unable to grow with chloromethane were able to utilize other C-1 substrates such as methanol, methylamine and formate, while mutants defective in the utilization of these compounds could still grow normally with chloromethane (Vannelli *et al.*, 1998). This suggested that chloromethane was metabolized in strain CM4 strain by reactions different from those involved in the metabolism of other C-1 compounds (Fig. 1.4). Further analysis of mutants unable to grow with chloromethane allowed the identification of chloromethane-utilization (*cmu*) genes which mapped to two different approx. 10 kb DNA fragments (Fig. 1.6), and to define a specific pathway for chloromethane utilization (Fig. 1.4) (Vannelli *et al.*, 1999). Sequence analysis showed that two of the genes essential for chloromethane utilization corresponded to the previously identified 67 kDa and 36 kDa proteins induced by chloromethane (Vannelli *et al.*, 1998). These genes were predicted to encode two different types of methyltransferases and were named *cmuA* and *cmuB* respectively (Vannelli *et al.*, 1999). The corresponding protein CmuA is a two-domain protein; with an N-terminal

methyltransferase domain at and a C-terminal corrinoid-binding domain, similar to methyl transfer systems of methylotrophic archaeal methanogens (Burke *et al.*, 1998; Vannelli *et al.*, 1999). Protein CmuB showed methyltransferase activity with tetrahydrofolate as suggested from sequence similarity searches (Studer *et al.*, 1999). Both proteins CmuB (Studer *et al.*, 1999) and CmuA (Studer *et al.*, 2001) were purified to homogeneity and allowed to demonstrate that chloromethane dehalogenase of strain CM4 required two subsequent reactions involving these two proteins (Studer *et al.*, 2001) (Fig. 1.7).

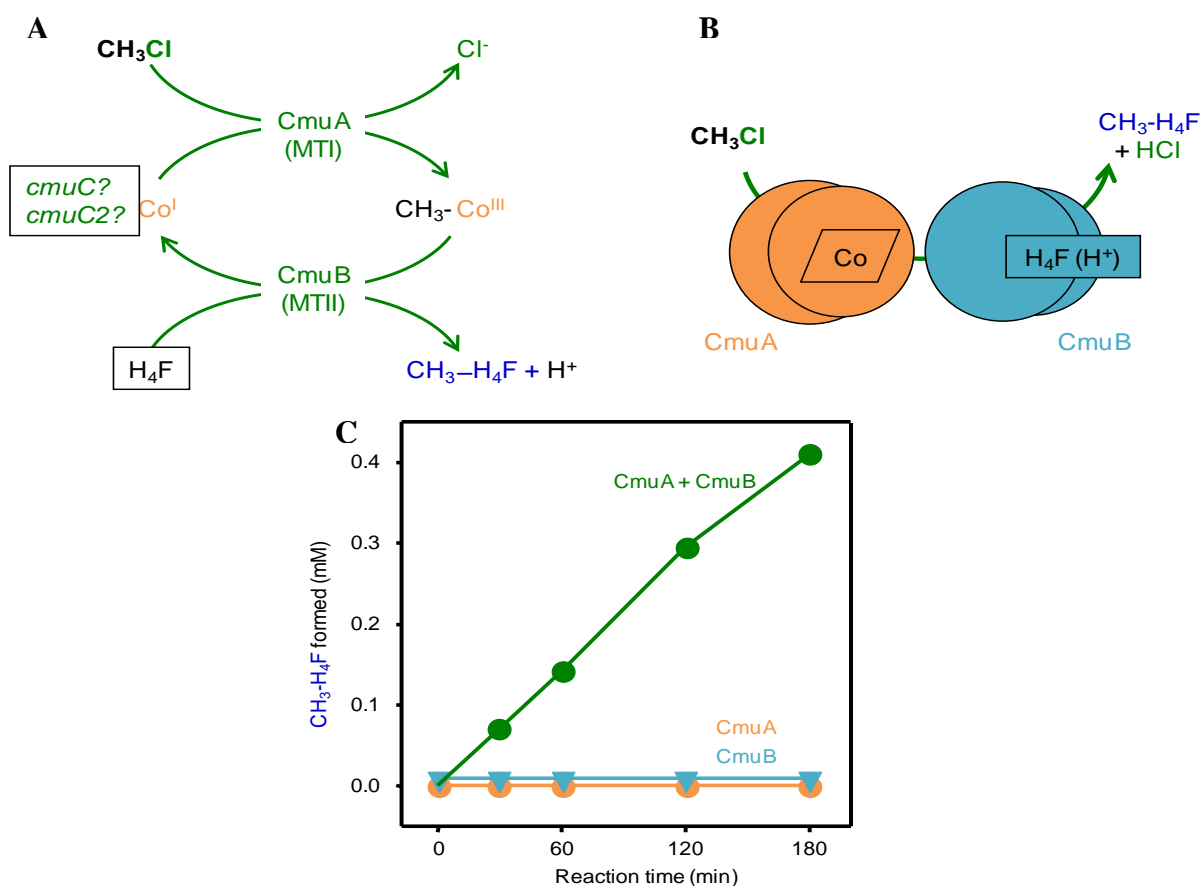


Fig. 1.7. Dehalogenation of chloromethane by CmuA and CmuB proteins.

(A & B) CmuA acting as a bifunctional protein (methyltransferase I and corrinoid-binding domains) and serves as an intermediate methyl carrier. In a second reaction, CmuB methyltransferase II transfers the methyl group ($-\text{CH}_3$) from CmuA to tetrahydrofolate (H_4F). (C) In combination; CmuA and CmuB proteins catalyze the *in vitro* transfer of the methyl group of chloromethane to tetrahydrofolate to form methyltetrahydrofolate ($\text{CH}_3\text{-H}_4\text{F}$). (Fig. adapted from Studer *et al.*, 2001). The function of *cmuC*, a gene essential for growth with chloromethane (Fig. 1.6), and of its close homolog *cmuC2* located upstream of *cmuA*, are still unknown.

The resulting $\text{CH}_3\text{-H}_4\text{F}$ is then transferred to formate by a tetrahydrofolate-dependent pathway that appears distinct from those found in *Methylobacterium* strains (Fig. 1.4) [Section 1.4.2]. This was inferred from the phenotype of minitransposon mutants of strain CM4 in *metF*, *folD* and *purU* genes (Fig. 1.6) coding for homologs of methylene- H_4F reductase, methylene- H_4F dehydrogenase/methenyl- H_4F cyclohydrolase and formyl- H_4F hydrolase, respectively (Vannelli *et al.*, 1999). These genes were found nearby *cmuA* or *cmuB* genes (Fig. 1.6) and the mutants of these genes were also unable to grow with chloromethane, but exhibited wild-type levels of chloromethane dehalogenase activity (Vannelli *et al.*, 1999). As shown in Fig. 1.4, the chloromethane-utilization pathway is similar to H_4F dependent oxidation of formaldehyde to CO_2 defined by other enzymes methylene- H_4F dehydrogenase (MtdA) (Vorholt *et al.*, 1998) and methenyl- H_4F cyclohydrolase (FchA) (Pomper *et al.*, 1999). In addition, gene fusion studies showed chloromethane-dependent expression of *metF*, *folD*, *purU* genes (Studer *et al.*, 2002).

Following these studies elucidating the detailed metabolic pathway of chloromethane, *Methylobacterium extorquens* CM4 became the model organism for the study for bacterial degradation of chloromethane. Its genome sequence was subsequently determined (Marx *et al.*, 2012) and analyzed, allowing global proteomic (Roselli *et al.*, 2013) and transcriptomic (Bringel *et al.*, work in progress), investigations of chloromethane utilization in *Methylobacterium*, as well as application of strain CM4 to address this topic in the context of the most relevant niche with regard to the chloromethane budget, the phyllosphere

1.6. The phyllosphere as a microbial niche

The parts of the plants in immediate contact with air (mainly leaves) are defined as the phyllosphere (Fig. 1.8) (Vorholt, 2012). Due to the presence of various carbon compounds including methanol but also sugars leaching from the interior of the plant (Mercier and Lindow, 2000; Lindow and Brandl, 2003), the phyllosphere is a hospitable habitat for microorganisms. Estimated at $1 \times 10^9 \text{ km}^2$ globally, leaf surfaces represent a very important surface on earth, i.e. approximately twice the land surface area (Vorholt, 2012). Global bacterial population in the phyllosphere habitat is estimated to be 10^{26} (Vorholt, 2012, and references cited therein). Bacteria are the most abundant microbes in the phyllosphere at 10^6 - 10^7 per cm^2 of leaf surface (Lindow and Brandl, 2003), although Archaea, filamentous fungi

and yeasts are also observed (Whipps *et al.*, 2008). Microbes can be found on the plant surface as epiphytes and also within plant tissues as endophytes (Whipps *et al.*, 2008; Vorholt, 2012).

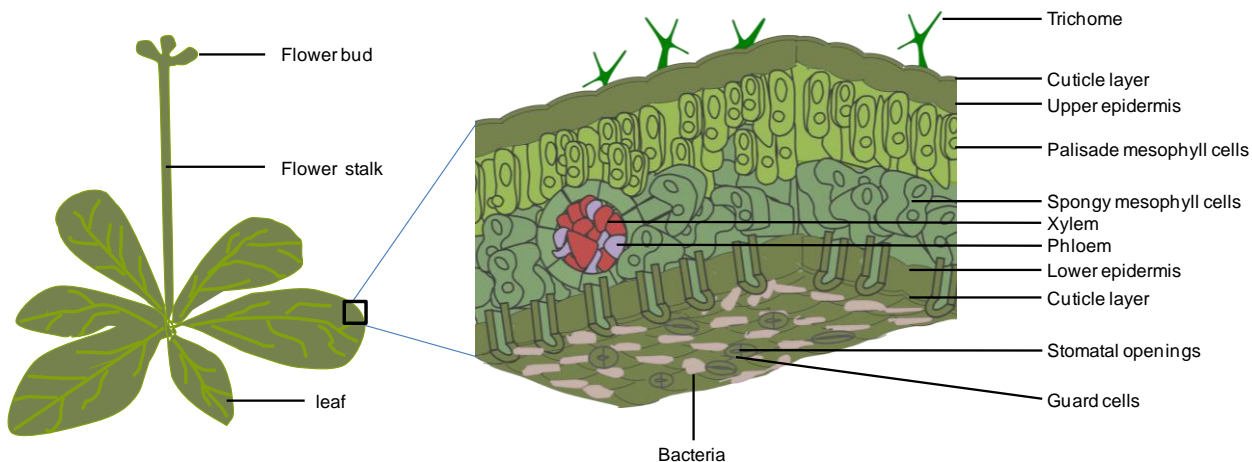


Fig. 1.8. The phyllosphere of *Arabidopsis thaliana*.

The left panel drawing shows a rosette of an *A. thaliana* plant. The right panel shows a schematic representation of a magnified cross-section of an *A. thaliana* leaf. Bacteria are found on both leaf surfaces (epiphytes) as well as within leaf tissues (endophytes). Fig. adapted from Vorholt (2012).

1.6.1. Bacterial diversity of the phyllosphere

Phyllosphere bacteria have been explored in a number of culture-dependent (Enya *et al.*, 2007; Ceballos *et al.*, 2012) and culture-independent (Delmotte *et al.*, 2009; Knief *et al.*, 2012) studies, revealing the abundance of Proteobacteria in the phyllosphere. Studies on culturable epiphytes and endophytes of *Oryza sativa* (rice) showed that Alphaproteobacteria made up more than 70 % of the microbial compartment (Elbeltagy *et al.*, 2000; Mano *et al.*, 2007). It was reported that culturable epiphytic and endophytic isolates from the rice phyllosphere mainly belonged to *Sphingomonas* (28% of endophytes, 27% of epiphytes), *Methylobacterium* (19% of endophytes, 12% of epiphytes), and *Bacillus* (13% of endophytes, 12% of epiphytes) (Mano *et al.*, 2007). Yang *et al.* (2001) were the first to use culture-independent methods to analyze phyllosphere communities and revealed that most dominant phyllosphere organisms were not detected by conventional culture-based methods. More recently, Delmotte *et al.* (2009) and Knief *et al.* (2012) reported detailed metaproteogenomic studies of the phyllosphere of *Glycine max* (soybean), *Trifolium repens* (clover), *Arabidopsis*

thaliana and rice plants. They also observed the dominance of Proteobacteria phylum. Alphaproteobacteria was the most dominant class accounting for more than 70% abundance of the bacterial community in all samples investigated (Delmotte *et al.*, 2009; Knief *et al.*, 2012; Vorholt, 2012). Bacteroidetes was the second most abundant phylum in the soybean, clover and *A. thaliana* communities (Delmotte *et al.*, 2009) while in the phyllosphere of rice plant, the class Actinobacteria was the most represented class (Knief *et al.*, 2012). *Agrobacterium*, *Burkholderia*, *Methylobacterium*, *Mycobacterium*, *Pseudomonas*, *Sphingobium*, *Sphingomonas* and *Xanthomonas* were of the most abundantly colonizing genera in all phyllosphere samples (Delmotte *et al.*, 2009; Knief *et al.*, 2012; Vorholt, 2012). Knief *et al.* (2010b) evaluated the effects of geographical site, host plant species, time and the presence of other phyllosphere bacteria on *Methylobacterium* community composition. They observed different *Methylobacterium* communities in the phyllosphere of the same plants from different locations, and also observed a similar *Methylobacterium* community on different plants growing at the same site, suggesting that site-specific factors had a stronger impact on the *Methylobacterium* community composition (Knief *et al.*, 2010b). In contrast, (Redford *et al.*, 2010) observed more differences in phyllosphere communities on different tree species from the same site, than those of the same tree species from different locations. Although the species/site dependent bacterial diversity in the phyllosphere is substantial, it appears to be lower as compared to the bacterial diversity in the rhizosphere or bulk soil (Vorholt, 2012, and references cited therein).

1.6.2. Plant-bacterial relationships in the phyllosphere

Bacteria inhabiting the phyllosphere can have beneficial, neutral or pathogenic effects on host plants (Kutschera, 2007). During phyllosphere colonization, *Methylobacterium* bacteria benefit from the abundance of methanol (as carbon and energy source) released from plant leaves, while *Sphingomonas* (due to the presence of TonB-dependent receptors) can utilize various carbohydrates and sugars leached from plant leaves (Delmotte *et al.*, 2009). It is believed that plant-bacteria interactions in the phyllosphere are often mutually advantageous. Plants provide the carbon and other nutrients to bacteria, while bacteria impart beneficial effects on plant growth through one or more mechanisms directly or indirectly. For example, experiments showed that the growth of axenically cultured protonemal filaments of a moss

plant was promoted by methylobacteria (Hornschuh *et al.*, 2002); This effect was attributed to the production of cytokinins excreted by the bacteria into the culture medium and taken up by the plant cells. An auxin (indole-3-acetic acid) based plant-growth promoting effect of *Methylobacterium* was also observed (Hornschuh *et al.*, 2006). Other beneficial aspects of phyllosphere bacteria to plants include nitrogen fixation (Furnkranz *et al.*, 2008), regulation of ethylene through 1-aminocyclopropane-1-carboxylate deaminase activity in higher plants (Chinnadurai *et al.*, 2009), production of siderophores (Idris *et al.*, 2004; Idris *et al.*, 2006), and reduction of phytotoxicity of heavy metals (Baldi *et al.*, 1995). On the other hand, citrus canker, bacterial blight, bacterial spot on pepper and tomato, bacterial blight of rice, black rot of crucifers, brown spot of beans and crown-gall tumor are some of the diseases caused by phyllosphere-colonizing strains of *Xanthomonas* (Xu *et al.*, 2008), *Pseudomonas* (Hirano and Upper, 2000) and *Agrobacterium* (Hu *et al.*, 2013). Several isolates of *Sphingomonas* from phyllosphere have been reported to have a remarkable plant-protective effect by suppressing disease symptoms and reducing plant pathogens growth in *A. thaliana* (Innerebner *et al.*, 2011). A model system comprising of *Sphingomonas* spp., *Pseudomonas syringae* and *Arabidopsis thaliana*, was proposed to study plant protection affect of phyllosphere bacteria against palnt pathogens (Innerebner *et al.*, 2011). Recently, mini-Tn5 transposon insertional mutants of *Sphingomonas* sp. Fr1 screened on the basis of their plant protective and plant colonizing traits suggested the possibility of nutrient competition along with other mechanisms to be further investigated, in the biocontrol activity of phyllosphere bacteria (Vogel *et al.*, 2012).

1.6.3. Adaptation of bacteria to the phyllospheric environment

Bacteria on the surface of leaves are exposed to highly variable micro- and macro-environments, resulting in irregular distribution and uneven growth rates (Vorholt, 2012). Various factors such as climatic conditions, ultraviolet radiation from sunlight, the waxy characteristics of the leaf cuticle layer and other leaf features (e.g. veins, trichomes and hydathodes) which affect water and nutrient availability, and plant metabolites and antimicrobial compounds will influence the structure and the growth dynamics of bacterial communities in the phyllosphere (Lindow and Brandl, 2003; Vorholt, 2012). Plant genotypes may also affect bacterial colonization of the phyllosphere (Whipps *et al.*, 2008). It thus seems

clear that phyllospheric bacteria will have adaptive mechanisms to counteract the various stresses associated with life on aerial plant surfaces. For instance, formation of aggregates on leaf surfaces may provide bacteria with the ability to modify their local environment (Whipps *et al.*, 2008). Production of extracellular polysaccharides may also increase nutrient availability, protection against desiccation of epiphytic bacteria, and shield cells from antimicrobial compounds through the formation of biofilms (Morris and Monier, 2003; Rigano *et al.*, 2007). Release of plant growth regulators (e.g. indole-3-acetic acid) associated with enhanced nutrient leakage from plants is another widespread adaptive mechanism in phyllosphere colonization (Brandl and Lindow, 1998). Biosurfactants released by phyllosphere bacteria may also enhance nutrient availability by increasing wettability and substrate leaching (Mousivand *et al.*, 2012, and references cited therein), and production of pigments afford protection from ultraviolet radiations (Jacobs *et al.*, 2005). Further, the existence of bacteria capable of harvesting sunlight through bacteriorhodopsins (Atamna-Ismaeel *et al.*, 2012b) and anoxygenic photosynthesis in the phyllosphere has been reported recently, suggesting yet another mechanism of adaptation to the phyllosphere under nutrient limited conditions (Atamna-Ismaeel *et al.*, 2012a). In recent years, proteomic and transcriptomic studies have helped to better understand adaptive mechanisms of phyllosphere bacteria. For example, Gourion *et al.* (2006) identified 40 proteins which were up-regulated in *M. extorquens* specifically during phyllosphere colonization. These proteins include enzymes involved in C-1 metabolism, general stress proteins e.g.; heat-shock proteins, enzymes involved in DNA repair, and catalases and superoxide dismutases. In addition, a novel two-component response regulator named PhyR (phyllosphere induced regulator) was also discovered. PhyR was demonstrated to be essential for phyllosphere colonization and is responsible for the induction of several proteins specifically expressed during epiphytic growth (Gourion *et al.*, 2006; Gourion *et al.*, 2008).

1.7. Objectives of the PhD project

The degree to which the biosphere contributes to the atmospheric burden of certain climate-relevant trace gases such as chloromethane is poorly understood. On the one hand, plant leaves possibly represent the quantitatively most important source of chloromethane produced in the biosphere [discussed in **Sections 1.2** and **1.3** above], but this has not yet been investigated comprehensively. On the other hand, the importance of bacteria capable of utilizing chloromethane as the only source of biomass and energy [see **Sections 1.4 - 1.6** above] has not been addressed in this context. Since several carbon compounds including methanol are released from plants on the surface of leaves, making the phyllosphere an important habitat for bacteria and especially methylotrophic bacteria [see **Section 1.6** above], phyllospheric bacteria may therefore also constitute a significant sink for chloromethane. In

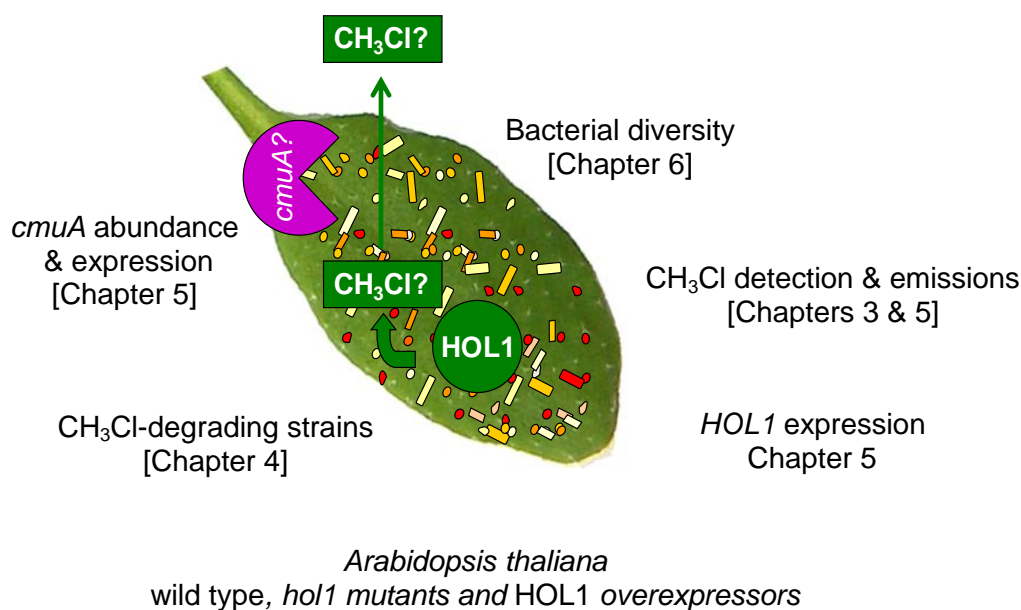


Fig. 1.9. Objectives and questions of research project.

The objectives of the research project were to develop tools to detect chloromethane emissions [**Chapter 3**] based on the sequencing data of genomes of chloromethane-degrading strains [**Chapter 2**], especially to detect chloromethane emissions from the model plant *A. thaliana* and its mutants with varying expression of *HOL1*, the gene responsible for chloromethane emissions [**Chapter 5**], to detect and isolate chloromethane-degrading strains from the phyllosphere of *A. thaliana* [**Chapter 4**], to investigate if chloromethane emissions from plants provide selective advantage to bacteria for phyllosphere colonization [**Chapter 5**] and to evaluate the possible influence of chloromethane emissions on the phyllosphere bacterial diversity [**Chapter 6**], in order to address the question whether phyllosphere bacteria can act as filters for plant emissions of chloromethane

this project, the topic of bacterial chloromethane degradation in the phyllosphere was explored using an experimental plant-bacterial model system involving two reference organisms, *Arabidopsis thaliana* which was demonstrated to emit chloromethane [see **Section 1.3** above], and *Methylobacterium extorquens* CM4 [see **Section 1.6** above]. Both organisms have well-annotated sequenced genomes, and are currently the best studied organisms in respect to chloromethane metabolism [see **Sections 1.3 & 1.5** above].

The general question at the basis of the project (Fig. 1.9) was whether epiphytic chloromethane-degrading bacteria in the phyllosphere may act as a filter for emissions of chloromethane by plants.

To address this question, the following three major experimental objectives were defined in this project:

- 1) Develop molecular tools for the detection of chloromethane-related processes in the phyllosphere of *A. thaliana*
- 2) Determine if bacteria can be isolated from the phyllosphere that degrade chloromethane
- 3) Explore the diversity of bacteria residing in the phyllosphere in relation to chloromethane emissions from plants, and investigate in particular if chloromethane emissions from plants provide a selective advantage to chloromethane-degrading bacteria for phyllosphere colonization

The results of the work performed in this PhD project to address these objectives are presented in the following Chapters of the thesis manuscript.

In the next chapter [**Chapter 2**], an overview of comparative genomic analysis of chloromethane-degrading strains, which has resulted in a clearer picture of the *cmu* pathway for chloromethane utilisation at the basis of the development of robust molecular tools to detect chloromethane-degrading bacteria in the phyllosphere, is first presented.

In Chapter 3, the development of a bacterial bioreporter based on *M. extorquens* CM4 for the detection of chloromethane is presented.

The work resulting in the first isolation of chloromethane-degrading bacteria from the phyllosphere of *A. thaliana* is presented in Chapter 4.

A detailed investigation on the abundance and expression of *cmuA* in different variants of *A. thaliana*, chosen for emitting different amounts of chloromethane as verified using the bioreporter developed in Chapter 3, is presented in Chapter 5.

An exploratory preliminary study of the bacterial diversity of the *A. thaliana* phyllosphere in different *HOLI* gene variants of *A. thaliana* evaluated by 16S pyrosequencing is reported in Chapter 6.

The conclusions of the work performed are summarised in a final Chapter 7, in which the perspectives arising from this work are also presented.

Chapter 2. Comparative genomics of chloromethane-degrading strains

This Chapter also includes two published articles:

1) Complete genome sequence of the chloromethane-degrading strain *Hyphomicrobium* sp. strain MC1.

Vuilleumier S., Nadalig T. , **Farhan Ul Haque M.**, Magdelenat G., Lajus A, Roselli S., Muller E.E.L., Gruffaz C., Barbe V., Médigue C., and Bringel F. (2011). *J. Bacteriol.* **193**, 5035-5036.

2) Complete genome sequences of six strains of the genus *Methylobacterium*. *J. Bacteriol.* **194**, 4746-4748.

Marx C.J., Bringel F., Chistoserdova L., Moulin L., **Farhan Ul Haque M.**, Fleischman D.E., Gruffaz C., Jourand P., Knief C., Lee M.-C., Muller E.E.L., Nadalig T., Peyraud R., Roselli S., Russ L., Goodwin L.A., Ivanova N., Kyrpides N., Lajus A., Land M.L., Médigue C., Mikhailova N., Nolan M., Woyke T., Stolyar S., Vorholt J.A., Vuilleumier S. (2012).

Résumé

Ce chapitre présente les résultats de génomique comparative obtenus par l'analyse de génomes séquencés des souches utilisant le chlorométhane. Le but principal de cette étude a été de définir les gènes spécifiques des souches dégradant le chlorométhane, ou qui leur sont communs, et d'évaluer si la seule voie d'utilisation du chlorométhane décrite à ce jour est conservée parmi les différentes souches capables d'utiliser le chlorométhane. Ce travail a bénéficié des programmes de séquençage et d'assemblage des génomes de deux souches étudiées au laboratoire, *Methylobacterium extorquens* CM4 et *Hyphomicrobium* sp. MC1, ainsi que d'autres souches des mêmes genres *Methylobacterium* et *Hyphomicrobium* menés au Génoscope (France) et au Joint Genomic Institute (USA) durant la période de thèse.

L'analyse par génomique comparative a confirmé que les gènes *cmuABC* codant les protéines impliqués dans la déshalogénéation et les gènes encodant les protéines nécessaires à l'assimilation du carbone et pour l'oxydation complète du chlorométhane en CO₂ pour produire de l'énergie (*metF*, *fold* et *purU*) sont fortement conservés chez les souches CM4 et MC1. L'exploration comparative des génomes bactériens disponibles à ce jour a aussi permis de retrouver les gènes *cmu* dans la séquence génomique complète de trois bactéries anaérobies et dont l'utilisation du chlorométhane n'a pas encore été décrite, à savoir *Desulfomonile tiedjei* DSM 6799, *Thermosediminacter oceani* DSM 16646 et *Thermincola potens* JR. En revanche, les gènes *cmu* ne sont pas conservés chez les autres souches chlorométhane-dégradantes *Leisingera methylohalidivorans* DSM 14336 et *Roseovarius* sp. 217 dont la séquence génomique a aussi été déterminée, confirmant ainsi que d'autres voies d'utilisation bactériennes du chlorométhane que la voie *cmu* existent, et qu'elles restent encore à découvrir.

Par ailleurs, les outils de la plateforme MicroScope du Génoscope ont permis de définir l'ensemble des gènes communs et uniques aux souches chlorométhane-dégradantes *M. extorquens* CM4 et *Hyphomicrobium* sp. MC1 par comparaison aux génomes d'autres souches de *Methylobacterium* et *Hyphomicrobium* déjà séquencées. En plus des gènes-clés impliqués dans l'utilisation du chlorométhane, d'autres gènes, notamment ceux impliqués dans l'utilisation de l'acétone, ont été mis en évidence.

2.1. Introduction

The comparison of structural and functional characteristics of different organisms is a primordial theme in the field of biological research. Since the sequencing of the first complete genome of *Haemophilus influenzae* in 1995 (Fleischmann *et al.*, 1995) rapidly developing sequencing techniques have been used to determine the genome sequence of numerous organisms, and especially bacteria with their relatively small genomes. Indeed, as of March 2013, 2,175 finished and 14,643 ongoing projects for sequencing bacterial genomes are now publically inventoried (<http://www.genomesonline.org>). These advances have led to the emergence of a new discipline, comparative genomics (Koonin and Aravind, 2009), whereby the genomes of different organisms of interest are compared to identify the genes or proteins that are conserved among organisms, and which confer to each organism its features and functions. Comparative genomics also provides a powerful tool to obtain information about the structure and plasticity of genomes, the evolution of different species, and the adaptation mechanisms of organisms to diverse physico-chemical constraints in the environment.

This chapter presents a summary of the main results of comparative genomic analyses performed during the thesis period that focused on genomes of strains capable of degrading chloromethane. The main objectives of these analyses were to:

- i) determine and analyse the genome sequences of two reference chloromethane-degrading methylotrophs investigated by our group;
- ii) check for the occurrence of the chloromethane dehalogenase *cmuA* gene in complete bacterial genomes found in sequence databases;
- iii) identify genes common and/or unique to methylotrophs able to utilize chloromethane as the sole carbon and energy source;
- iv) assess the diversity of chloromethane utilization pathways within genomes of chloromethane-degrading bacteria beyond the Proteobacteria phylum.

This analysis was also expected to yield valuable insights for future experimental investigations of bacterial chloromethane utilization.

2.2. Sequencing and genome analysis of chloromethane-degrading strains

The genome sequence of the chloromethane-degrading strain mainly investigated in our laboratory, *Methylobacterium extorquens* CM4, was determined as part of a collaborative project initiated in 2005 by Prof. Chris J. Marx (Harvard University) leading an international consortium of labs from Switzerland, USA and France, and aiming at

obtaining the complete genome sequence of six *Methylobacterium* strains at the Joint Genomic Institute (JGI) (USA). Subsequently, the genome of another chloromethane-degrading strain, *Hyphomicrobium* sp. MC1, the first chloromethane-degrading strain to be reported (Hartmans *et al.*, 1986), was sequenced at Génoscope (France) following a find of original laboratory stocks of this strain in Strasbourg. I became co-author of the two published genome announcement articles describing the genomes of these two strains, on the basis of my contributions to the preparation of cultures and DNA extraction for sequencing purposes [see Appendix of Chapter 2], and to subsequent manual gene annotation and genome analysis of these strains using the Microscope platform at Genoscope [see following section].

The two genome announcement publications are reproduced in the following.

2.2.1. Complete genome sequences of six strains of the genus *Methylobacterium*

Journal of Bacteriology, volume 193, number 18, pages 5035-5036

2.2.2. Complete genome sequence of the chloromethane-degrading *Hyphomicrobium* sp. strain MC1

Journal of Bacteriology, volume 194, number 17, pages 4746-4748

Complete Genome Sequences of Six Strains of the Genus *Methylobacterium*

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The complete and assembled genome sequences were determined for six strains of the alphaproteobacterial genus *Methylobacterium*, chosen for their key adaptations to different plant-associated niches and environmental constraints.

Genomic and metagenomic investigations have highlighted the prevalent role of methylotrophic microorganisms in a variety of marine, freshwater, and terrestrial environments (3–4, 6). These data have propelled new understanding of the molecular intricacies of microbial methylotrophic metabolism (1) and have sparked continued interest in their potential for biotechnological applications (15). In this work, the assembled complete genome sequences of six strains of the alphaproteobacterial genus *Methylobacterium* were determined. The selected strains were chosen for key characteristics, in terms of ecology, physiology, and metabolism (Table 1), in order to investigate how such adaptive features are reflected at the level of genome composition and architecture.

Genomes were sequenced at the Joint Genome Institute (JGI) using combinations of small to medium DNA libraries (3, 6, and 8 kb), as well as fosmid libraries (35 and 40 kb), with Sanger sequencing (7.3 to 9.6× coverage) completed with 454 pyrosequencing (20× coverage). All general aspects of library construction and sequencing can be found at http://www.jgi.doe.gov/sequencing/protocols/protos_production.html. Draft assemblies and quality assessment were obtained using the Phred/Phrap/Consed software package. Possible misassemblies were corrected with Dupfinisher (8), PCR amplification, and transposon bombing of bridging clones (Epicentre Biotechnologies, Madison, WI). Gaps between contigs were closed by editing in Consed, custom primer walking, and PCR amplification. A final assembly (7.5 to 10.5× coverage) was obtained for all 6 genomes (Table 1), and automatic annotation was performed using the JGI-Oak Ridge National Laboratory annotation pipeline (12). Additional automatic and manual sequence annotations, as well as comparative

genome analysis, were performed using the MicroScope platform at Genoscope (16).

The six *Methylobacterium* strains show significant variation in chromosome size and plasmid content (Table 1), and each possesses several conserved gene clusters known to be involved in methylotrophy in *Methylobacterium* (2, 18). Five of the strains possess conserved clusters of genes associated with photosynthesis, including genes encoding the light-harvesting complex and the reaction center, and genes involved in biosynthesis of bacteriochlorophyll and carotenoids. Further analyses of these six genomes will include comparisons to the two *Methylobacterium* genomes already reported (18), i.e., *M. extorquens* AM1, a major model strain in studies of methylotrophy (2) and genome evolution (5), and the dichloromethane-degrading strain *M. extorquens* DM4 (14). This will define both core- and strain-specific features of *Methylobacterium* strains and provide new insights into the metabolic flexibility of these facultative methylotrophs and into the modes of bacterial adaptation to specific ecological niches.

Nucleotide sequence accession numbers. GenBank accession numbers for all the chromosomes and plasmids sequenced in this study are shown in Table 1.

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TABLE 1 Characteristics of the six complete *Methylobacterium* genomes sequenced in this study

Organism	Key characteristic(s)	Genome analysis					GenBank accession no.	Reference
		Size (Mb)	% GC	No. of <i>rrn</i> operons	No. of tRNAs	No. of CDS ^a		
<i>M. extorquens</i> strain PA1	<i>Arabidopsis thaliana</i> epiphyte	5.471	68.2	5	58	5,410	NC_010172	11
<i>M. extorquens</i> strain CM4	Chloromethane degrader	5.778	68.2	5	61	3,112	NC_011757	13
		0.380	66.3			388	NC_011758	
		0.023	63.9			44	NC_011760	
<i>M. extorquens</i> strain BJ001 ^b	<i>Populus deltoides</i> x <i>nigra</i> DN34 endophyte	5.800	69.4	5	58	6,017	NC_010725	17
		0.025	64.9			30	NC_010727	
		0.023	66.8			31	NC_010721	
<i>M. radiotolerans</i> strain JCM 2831	Radioresistant strain	6.078	71.5	4	56	6,325	NC_010505	9
		0.586	69.6			650	NC_010510	
		0.047	62.5	1	1	66	NC_010509	
		0.043	63.2			75	NC_010514	
		0.038	63.7	1	1	60	NC_010517	
		0.036	62.0			64	NC_010518	
		0.028	61.0	1	1	45	NC_010502	
		0.022	61.1			38	NC_010504	
		0.021	65.1			33	NC_010507	
<i>Methylobacterium</i> sp. strain 4-46	<i>Lotononis bainesi</i> nodulating, photosynthetic	7.659	71.6	6	63	8,337	NC_010511	7
		0.058	65.1			108	NC_010373	
		0.020	59.2			34	NC_010374	
<i>M. nodulans</i> strain ORS 2060	Nonpigmented, nitrogen fixing, <i>Crotalaria</i> nodulating	7.772	68.9	7	71	8,879	NC_011894	10
		0.488	65.9			630	NC_011892	
		0.458	65.7	2	2	609	NC_011887	
		0.040	64.2			84	NC_011893	
		0.038	61.6	1	1	66	NC_011895	
		0.020	61.4			30	NC_011888	
		0.013	60.5			16	NC_011889	
		0.010	67.2	1	1	14	NC_011890	

^a Number of annotated protein-coding sequences in MicroScope (16).

^b This strain, originally reported as *M. populi* strain BJ001 (17), was assigned to the species *M. extorquens* based on 16S rRNA gene identity (99.3%) and overall genome similarity with the four other sequenced *M. extorquens* strains (~80% identity over 75% of its genome sequence) (also see reference 18).

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Complete Genome Sequence of the Chloromethane-Degrading *Hyphomicrobium* sp. Strain MC1

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***Hyphomicrobium* sp. strain MC1 is an aerobic methylotroph originally isolated from industrial sewage. This prosthecate bacterium was the first strain reported to grow with chloromethane as the sole carbon and energy source. Its genome, consisting of a single 4.76-Mb chromosome, is the first for a chloromethane-degrading bacterium to be formally reported.**

Strains of the genus *Hyphomicrobium* originally attracted interest for their distinctive prosthecae and atypical complex growth cycle (9). Strains of this genus are ubiquitous but were detected in wastewater treatment plants in particular, and often under denitrifying conditions (3, 8). Following the description of strain MC1 (6), several chloromethane-degrading *Hyphomicrobium* strains were isolated from various aquatic and soil environments, and most recently also from the surfaces of plant leaves (11). Strain MC1 features the consecutive *cmuBCA* gene arrangement; these genes encode chloromethane dehalogenase, which has been found in all aerobic chloromethane-degrading bacteria characterized so far with the exception of *Methylobacterium extorquens* CM4, from which chloromethane dehalogenase was purified (13) and in which regulation of chloromethane dehalogenase expression was investigated (14).

The assembled genome sequence of *Hyphomicrobium* sp. strain MC1 was obtained using a mix of sequencing technologies. A mate-paired 454 library with an 8-kb insert size was constructed (version Titanium), and 559,691 reads (173,407,941 bp; approximately 36× coverage) were assembled using Newbler (version 2.3, release 091027_1459). Assembly validation was made via Consed (www.phrap.org), and 129 PCRs between contigs were performed and sequenced for gap closure. For quality assessment, a total of 25,299,825 Illumina 36-bp reads were mapped onto the whole genome sequence using SOAP (http://soap.genomics.org.cn) (2), allowing us to correct potential base errors and confirming the final closed circular 4,757,528-bp assembly. Sequence annotation and comparative genome analysis are under way using the MicroScope platform at Genoscope (15).

Based on the 16S rRNA sequence of its single rRNA operon, strain MC1 appears to be most closely related to *Hyphomicrobium facile* subspecies type strains. Of its 4,679

predicted open reading frames (ORFs), 947 (20%) have close homologs (>80% amino acid identity over >80% of protein length, almost all of them in synteny) in *Hyphomicrobium denitrificans* ATCC 51888, whose genome sequence has been determined (NC_014313) (4).

Genes encoding enzymes and proteins for oxidation of methanol (*mx*) and methylamine (*mgs* and *mgd* [5, 7] but not *mau* genes) were identified, together with proteins and enzymes involved in pyrroloquinoline quinone synthesis and tetrahydrofolate- and tetrahydromethanopterin-linked pathways. Genes for complete serine and ethylmalonyl coenzyme A pathways (1) for carbon assimilation were identified. The genome of strain MC1 also encodes a complete glycolysis pathway and a closed tricarboxylic acid cycle, but no genes for the glyoxylate shunt (isocitrate lyase and malate synthase) were detected. Six terminal oxidases of different types were identified. Unlike in *H. denitrificans* ATCC 51888, genes for N₂ fixation, a complete uptake hydrogenase gene cluster, and gene systems for both assimilative (*nas*) and dissimilative (*nar*) reduction of nitrate, ammonia and nitrate/nitrite transport, a putative nitrate-inducible formate dehydrogenase, an alkane sulfonate oxidation and transport system, and an acetone carboxylase (*acxRABC*) cluster (12) were detected. In contrast, the dichloromethane dehalogenation genes (*dcmRABC*) (10) allowing *H. denitrificans* ATCC 51888 to grow on DCM were not found in strain MC1.

Hyphomicrobium sp. strain MC1 thus emerges as a promising model for investigating the degradation of halogenated pollutants in the context of methylotrophic metabolism using genomic information (16) and for studies of morphological and metabolic features supporting bacterial growth under nutrient-scarce conditions.

Nucleic acid sequence accession number. The *Hyphomicrobium* sp. strain MC1 genome sequence was deposited in GenBank/EMBL under accession number FQ859181.

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2.3. Comparative genomics of chloromethane-degrading strains

2.3.1. Comparative genomics using the Microscope platform of Genoscope

MicroScope (<http://www.genoscope.cns.fr/agc/microscope>) is a platform developed by Genoscope (Evry, France) which provides easy-to-use tools to study, annotate and perform comparative genomic analysis of bacterial genomes (Fig. 2.1) (Vallenet *et al.*, 2013). This platform comprises three major components: (i) a resource providing a relational database for completed and ongoing genome projects named PkGDB (prokaryotes genome database), which stores information on organisms, sequences and genomic objects; (ii) a panel of bioinformatics tools linked to the PkGDB database to provide results of syntactic and functional annotation pipelines as well as metabolic pathway tools (e.g. KEGG, MicroCyc) analysis specific to each genome; and (iii) an interface, MaGe (Magnifying genomes), allowing manual annotations and comparative genomic analyses (Vallenet *et al.*, 2006; Vallenet *et al.*, 2009). The MaGe interface combines graphical interfaces and analysis of synteny (i.e., conservation of organization of genes among different genomes), together with genome data sources and metabolic pathway tools to assist in data evaluation in order to manually assign the best possible annotation to a given gene product (Fig. 2.1) (Vallenet *et al.*, 2006; Vallenet *et al.*, 2009). It also offers useful tools and functionalities to explore genome data according to the specifications and requests of the annotator. MicroScope can be used as an open access resource (for publicly available genomes) or as a restricted access resource (where access can be restricted to a limited group of annotators defined by the project leader). MicroScope resource currently contains data for more than 1600 microbial genomes from 129 different projects (Vallenet *et al.*, 2013). According to the latest report of MicroScope, there are several projects related to the study of genomes of bacteria of environmental relevance and capable of utilizing a large variety of compounds of high industrial and environmental interest (Vallenet *et al.*, 2013). Our laboratory coordinates several Microscope annotation projects, notably Methyloscope, Methanoscope, and Hyphoscope, which include both public and newly determined private genome sequences in the field of methylotrophic and methanotrophic bacterial metabolism. These projects have already led to the publication of 10 genome announcements so far, including the two presented in the previous section (Vuilleumier *et al.*, 2011; Marx *et al.*, 2012), and have also provided the basis for more detailed bioinformatics and experimental studies (Muller *et al.*, 2011a; Muller *et al.*, 2011b; Nadalig *et al.*, 2011; Roselli *et al.*, 2013).

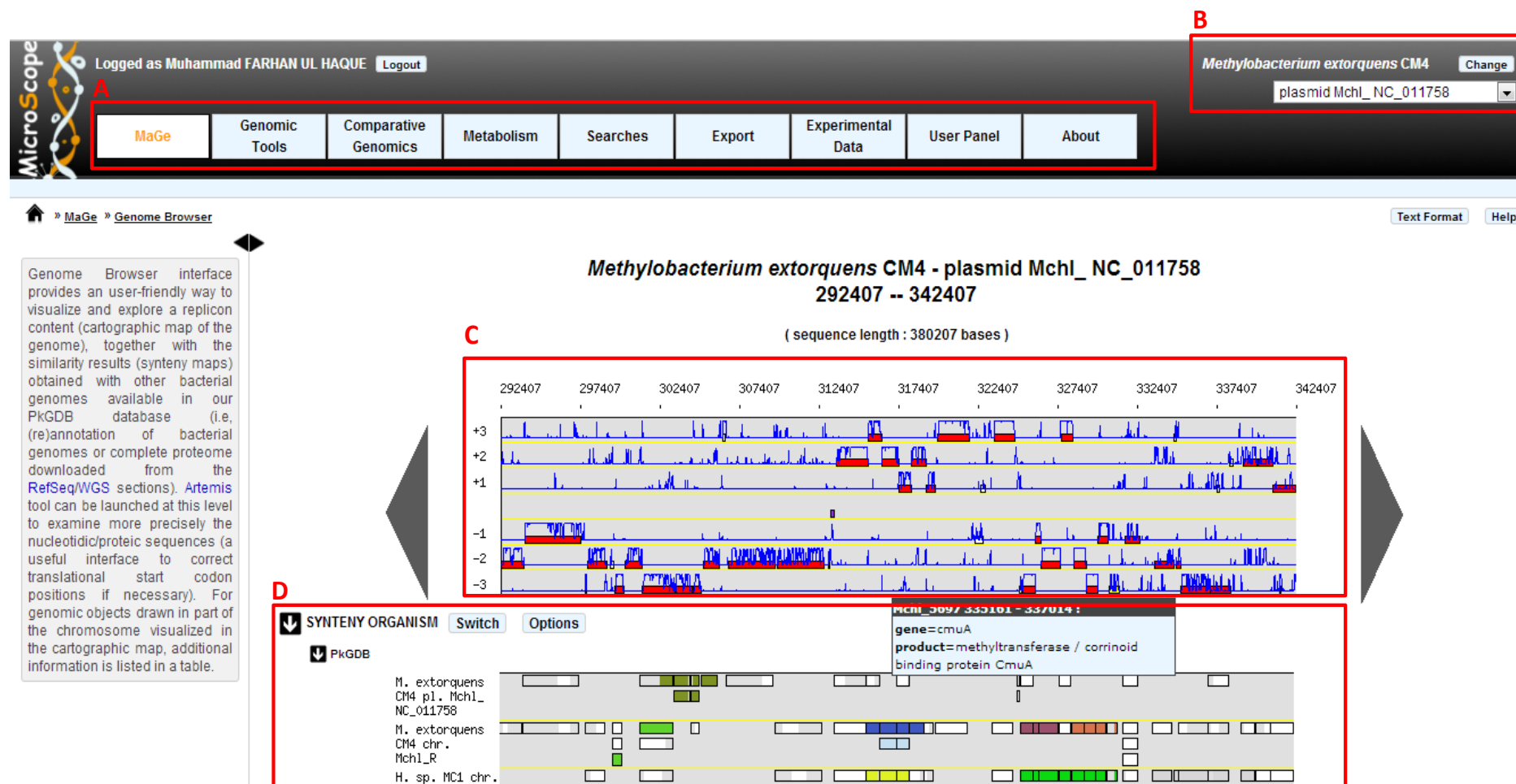


Fig. 2.1. A screen capture of the “MicroScope” web platform.

The screenshot was taken during analysis of the genome of *Methylobacterium extorquens* CM4 in the “Genome Browser” window. Several tools (A) can be used to explore the genome of the selected (pivot) organism (B). Genomic objects (predicted genes in the 6 frames) (C) are displayed as rectangles, along with (D) genes in synteny in other organisms in the MicroScope database (PkGDB, <http://www.genoscope.cns.fr/agc/microscope>) and publically available genome sequences (Refseq).

2.3.2. Complete genome sequences of chloromethane-degrading strains and *cmu* gene containing strains

To date, 7 complete genome sequences are directly relevant to the analysis of bacterial chloromethane utilization. They include those of strains known to grow with chloromethane, and those containing close homologs of *cmu* genes known to be involved in chloromethane dehalogenation [**Chapter 1**]. They are briefly described in the following, and their key properties are described in Table 2.1.

- *M. extorquens* CM4 is the strain for which the only known metabolic pathway of aerobic chloromethane utilization so far, the *cmu* pathway, has been studied in detail (Fig. 2.2) (Vannelli *et al.*, 1999; Studer *et al.*, 2002). The genome of this strain has been published (Marx *et al.*, 2012), and a detailed study of its megaplasmid harboring *cmu* has been recently reported by Roselli *et al.* (2013).
- *Hyphomicrobium* sp. MC1 is the first strain for which growth with chloromethane was described (Hartmans *et al.*, 1986). Its genome sequence was determined at Genoscope, and published by our group (Vuilleumier *et al.*, 2011).
- *Roseovarius* sp. 217 is a chloromethane-degrading strain isolated from marine waters (Schäfer *et al.*, 2005). Its sequence, determined relatively early on in the sequencing age, was not completely assembled, but it is publically available as a set of 37 contigs (<http://img.jgi.doe.gov/cgi-bin/w/main.cgi>).
- *Leisingera methylohalidivorans* DSM 14336 is another aerobic chloromethane-degrading strain isolated from marine waters (Schaefer *et al.*, 2002). Its genome sequence, fully determined but not yet published, is publically available on the IMG platform of the Joint Genomic Institute (<http://img.jgi.doe.gov/cgi-bin/w/main.cgi>).
- *Desulfomonile tiedjei* DSM 6799 is an anaerobic bacterium involved in the reductive dehalogenation of chlorophenol and chlorobenzoate (Villemur *et al.*, 2006) (<http://img.jgi.doe.gov/cgi-bin/w/main.cgi>).
- *Thermosediminibacter oceani* DSM 16646, isolated from a core sample of deep sea sediment, uses thiosulfate, elemental sulfur and MnO₂ as electron acceptors, and grows at 68°C (Pitluck *et al.*, 2010)..
- *Thermincola potens* JR, an electricity-producing bacterium isolated from a thermophilic microbial fuel cell, is capable of using carbon monoxide as its only

source of carbon and energy and thus also is a methylotrophic bacterium (Byrne-Bailey *et al.*, 2010).

The first five of these strains belong to the phylum Proteobacteria, while the last two belong to the Firmicutes. The last three strains contain homologs of *cmuA* and *cmuB* chloromethane dehalogenase. Unlike other chloromethane-degrading strains with the *cmu* pathway, however, they have an anaerobic lifestyle and utilization of chloromethane has not been reported for these strains. (Table 2.1).

In addition, available complete genome sequences of other methylotrophic bacteria unable to degrade chloromethane for growth and belonging to the genera *Methylobacterium* and *Hyphomicrobium* were used in comparative genomic analysis. These included the sequences of *Methylobacterium extorquens* AM1 (Vuilleumier *et al.*, 2009), *M. extorquens* DM4 (Vuilleumier *et al.*, 2009; Muller *et al.*, 2011a), *M. extorquens* PA1 (Marx *et al.*, 2012), *M. extorquens* BJ001 (Marx *et al.*, 2012), *M. nodulans* ORS 2060 (Marx *et al.*, 2012), *Methylobacterium* sp. 4-46 (Marx *et al.*, 2012), *M. radiotolerans* JCM 2831 (Marx *et al.*, 2012), *Hyphomicrobium denitrificans* ATCC 51888 (Brown *et al.*, 2011), *Hyphomicrobium* sp. GJ21 (unpublished genome sequence of the DCM-degrading strain (see e.g. (Vuilleumier *et al.*, 2001)) and *Hyphomicrobium* sp. VS (Pol *et al.*, 1994).

2.4. Comparative genomics analysis of sequenced strains featuring *cmu* genes

The main objective of the analysis was to investigate if the genes involved in the *cmu* pathway (Fig. 2.2) (Studer *et al.*, 2002) [see Section 1.5, **Chapter 1** for details], are conserved among the strains capable of utilizing chloromethane. The *cmu* pathway involves three main aspects: (i) dehalogenation; (ii) assimilation of carbon for biomass production; (iii) generation of energy through carbon mineralization to CO₂ (Fig. 2.2). Genes conserved in chloromethane-degrading strains include genes essential for dehalogenation of chloromethane (*cmuA*, *cmuB*, and *cmuC*), genes essential for growth with chloromethane as the sole carbon (*metF2*) and energy source (*folD*, *purU*), and genes found in the vicinity of *cmuA*, *cmuB* and *cmuC* in methylotrophic chloromethane-degrading strains (*fmdB*, *hutI*) (Table 2.1) (Roselli *et al.*, 2013).

Key genes of chloromethane utilization in the context of the *cmu* pathway were compared in all sequenced strains reported to utilize chloromethane for growth or shown to harbor homologs of the chloromethane dehalogenase gene *cmuA* (Table 2.1).

Table 2.1. Characteristics of bacterial genomes harboring homologs of the *cmuA* gene^a

Strains	<i>Methylobacterium extorquens</i> CM4	<i>Hyphomicrobium</i> sp. MC1	<i>Roseovarius</i> sp. 217	<i>Leisingera methylohalidivorans</i> DSM 14336	<i>Desulfomonile tiedjei</i> DSM 6799	<i>Thermosediminibacter oceani</i> DSM 16646	<i>Thermincola potens</i> JR
Affiliation	Alphaproteobacteria	Alphaproteobacteria	Alphaproteobacteria	Alphaproteobacteria	Deltaproteobacteria	Clostridia	Clostridia
Halomethane utilized	CH ₃ Cl, CH ₃ Br, CH ₃ I	CH ₃ Cl	CH ₃ Cl, CH ₃ Br	CH ₃ Cl, CH ₃ Br, CH ₃ I	unknown	unknown	unknown
Key genes related of the <i>cmu</i> pathway^b							
<i>cmuA</i>	MchI_5697	HYPMCv2_2273 (84%) ^c	ROS217_19562 (34%) ^h	Leime_2531 (34%) ^h	Desti_5437 (64%)	Toce_1533 (66%) ^c	TherJR_0143 (68%) ^c
<i>cmuB</i>	MchI_5727	HYPMCv2_2275 (64%) ^c	nd ^d	nd	Desti_5438 (60%)	Toce_1535 (62%) ^c	TherJR_0145 (62%) ^c
<i>cmuC</i> (<i>cmuC2</i>)	MchI_5728, MchI_5698	HYPMCv2_2274 (38%) ^c	nd nd	nd nd	nd nd	Toce_1534 (34%) ^c nd	TherJR_0144 (36%) ^c nd
<i>fndB</i>	MchI_5696	HYPMCv2_2271 (47%) ^c	nd	nd	Desti_1975 (27%)	Toce_1532 (37%) ^c	TherJR_0142 (42%) ^c
<i>folD</i>	MchI_5700	HYPMCv2_2266 (47%)	ROS217_08129 (54%)	Leime_4077 (51%) Leime_3180 (51%)	Desti_2379 (46%)	Toce_0805 (40%)	TherJR_1709 (41%)
<i>hutI</i>	MchI_5694	HYPMCv2_2269 (53%)	ROS217_11786 (37%)	Leime_0109 (39%)	nd	Toce_1473 (33%)	TherJR_0722 (25%)
<i>metF2</i> (<i>metF</i>)	MchI_5726 MchI_1881 (26%)	HYPMCv2_2268 (31%) nd	ROS217_18837 (39%) nd	Leime_2159 (34%) Leime_2796 (30%)	Desti_2947 (29%) nd	nd Toce_1081	nd TherJR_2661
<i>purU</i>	MchI_5699	HYPMCv2_2267 (62%)	ROS217_19582 (38%) ^h	Leime_2536 (36%) ^h	Desti_5520 (28%) ^h	Toce_1499 (33%) ^h	TherJR_0829 (33%) ^h
Genome size (Mb) ^e	6.2	4.8	4.8	4.6	6.5	2.3	3.2
GC (%) ^f	66.1	59.2	60.8	62.3	50.1	46.8	45.9
rRNA operon	5	1	2	5	1	3	3
Total CDS	6035	4955	4772	4608	5664	2460	3343
Sequence status	complete	complete	37 contigs	complete	complete	complete	complete
Plasmids	pCMU01 (380 kb) ^g pMCHL02 (22 kb)	0	unknown	p1 (220 kb) p2 (285 kb)	pDESTI01 (27 kb)	0	0
Accession numbers	NC_011757 NC_011758 NC_011760	NC_015717	NZ_AAMV01	PRJNA74371	NC_018025 NC_018026	NC_014152	NC_014152
Sequencing platform	JGI (USA)	Génoscope (France)	J Craig Venter (USA)	JGI (USA)	JGI (USA)	JGI (USA)	JGI (USA)

^a Data from the MicroScope microbial genome annotation and analysis platform of Génoscope (<https://www.genoscope.cns.fr/agc/microscope/mage/>) , Integrated Microbial Genomes platform of the US American Joint Genomic Institute (<http://img.jgi.doe.gov/cgi-bin/w/main.cgi>), and from the NCBI website compiling available information on sequenced bacterial genomes (<http://www.ncbi.nlm.nih.gov/genome>).

^b Locus tag identifier and percentage protein identity of identified homologs of key genes of the *cmu* pathway in *M. extorquens* CM4

^c Organization and orientation of *cmu* genes was the same as in other chloromethane-degrading *Hyphomicrobium* strains (Nadalig *et al.*, 2011)

^d No homolog detected

^e Total genome size including chromosome and plasmids

^f Average GC% of chromosome and plasmids

^g Megaplasmid of strain *M. extorquens* CM4 harboring *cmu* genes

^h Protein identity less than 50% to the *M. extorquens* CM4 reference

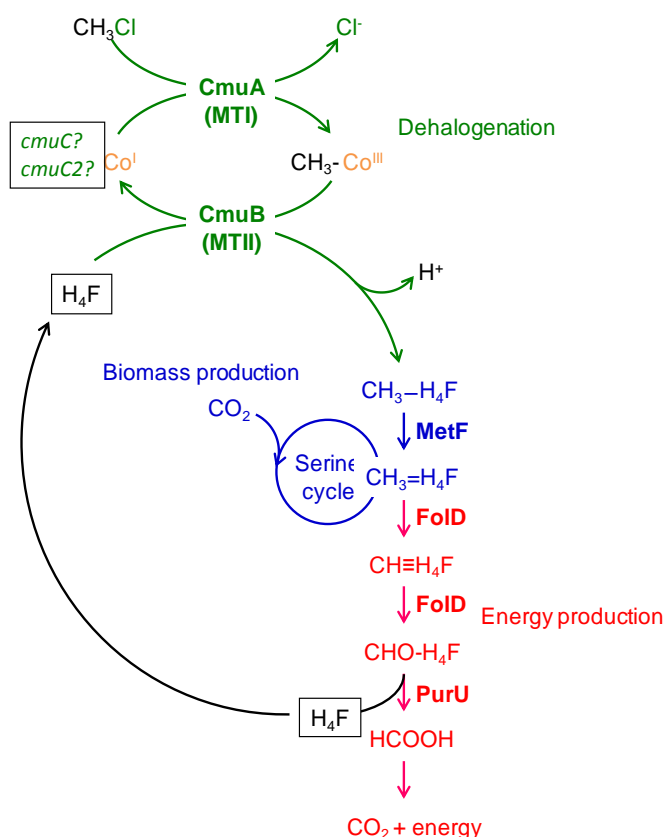


Fig. 2.2 Pathway for chloromethane-utilization (*cmu*) in *Methylobacterium extorquens* CM4.

Chloromethane (CH_3Cl) is dehalogenated for use as carbon source mediated by the serine cycle, and for energy production through tetrahydrofolate (H_4F) dependent pathway.

CmuA (methyltransferase I, MTI), CmuB (methyltransferase II, MTII), MetF (5,10-methylene- H_4folate reductase), FoID (putative 5,10- methylene- H_4folate dehydrogenase / 5,10- methenyl- H_4folate cyclohydrolase), PurU (10-formyl- H_4folate hydrolase).

The corrinoid acting as the primary methyl acceptor and thought to be part of CmuA is indicated by Co^{I} and Co^{III} . (adapted from Studer *et al.*, 2002)

2.4.1. Chloromethane dehalogenase genes

The *cmuA* gene encodes the CmuA protein, a two-domain protein with an N-terminal methyltransferase domain and a C-terminal corrinoid-binding domain (Fig. 2.3) (Vannelli et al., 1999). The *cmuA* gene has been found in all the aerobic chloromethane-degrading strains isolated from different environments (Nadalig *et al.*, 2011). A majority of the chloromethane-degrading strains compared here (Table 2.1) have predicted *cmuA* homologs with over 64% protein identity to CmuA of demonstrated function of strain CM4, with the exception of the two marine strains of the *Rhodobacteraceae* family, i.e. *Roseovarius* sp. 217 and *Leisingera methylohalidivorans* DSM 14336, which only contain a predicted protein with 34% protein identity to the C-terminal domain of CmuA of strain CM4 (Table 2.1) (Fig. 2.3).

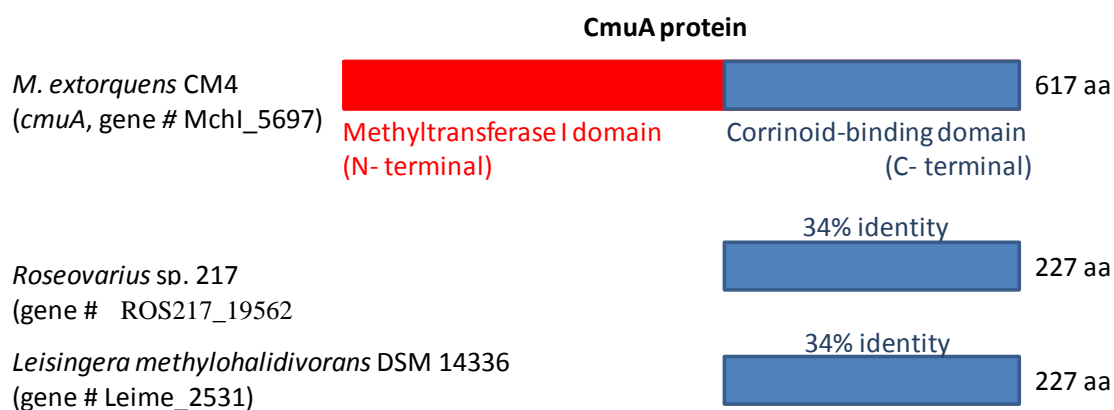


Fig. 2.3. Comparison of CmuA of *Methylobacterium extorquens* CM4 and its homologs in *Roseovarius* sp. 217 and *Leisingera methylohalidivorans*

Similarly, two other methyltransferase proteins involved in chloromethane-dehalogenation (i.e. CmuB and CmuC encoded by *cmuB* and *cmuC* genes) conserved in chloromethane-degrading strains with the *cmu* pathway, lack a corresponding homolog in *Roseovarius* sp. 217 and *Leisingera methylohalidivorans* DSM 14336 (Table 2.1). Taken together, the lack of homologs of *cmuB* and *cmuC*, and the only distant *cmuA* homolog in strains *Roseovarius* sp. 217 and *Leisingera methylohalidivorans* DSM 14336 (Fig. 2.3, Table 2.1), suggest that an alternative pathway for dehalogenation of chloromethane is used in these strains. However, it should be noted that other closely related chloromethane-utilizing strains from the same family (*Rhodobacteraceae*), i.e. *Roseovarius* sp. 179 and *Ruegeria* sp. 198, whose sequences are not yet available, were

reported to contain *cmuABC* genes and to utilize chloromethane through the *cmu* pathway (Schäfer *et al.*, 2005).

Surprisingly, homologs of *cmu* genes for chloromethane dehalogenase CmuAB with over 60% identity at the protein level were also detected in the recently reported genome sequences of *Desulfomonile tiedjei* DSM 6799, *Thermosediminibacter oceani* DSM 16646 (Pitluck *et al.*, 2010) and *Thermincola potens* JR (Byrne-Bailey *et al.*, 2010) (Table 2.1), which suggest that they may be capable of chloromethane dehalogenation. Interestingly, *Desulfomonile tiedjei* DSM 6799 was isolated for its capacity to dehalogenate multicarbon halogenated compounds (e.g. chlorobenzoate) through reductive dehalogenation in an energy yielding process (Villemur *et al.*, 2006; Ding and He, 2012). However, none of these three strains were described to perform methylotrophic metabolism, and all feature an anaerobic lifestyle, unlike all chloromethane-degrading strains containing the *cmu* pathway so far.

2.4.2. Genes associated to carbon assimilation and energy production

The gene *metF2* encoding methylene tetrahydrofolate reductase in strain CM4 and responsible for funnelling the methyl group of chloromethane into the serine cycle for carbon assimilation (Fig. 2.2) features homologs in all chloromethane-utilizing strains compared here (Table 2.1). The corresponding protein is thought to convert $\text{CH}_3\text{-H}_4\text{F}$ produced after dehalogenation of chloromethane to $\text{CH}_2\text{=H}_4\text{F}$, which then enters the serine cycle (Fig. 2.2). In *Thermosediminibacter oceani* DSM 16646 and *Thermincola potens* JR, the detected homologs of methylene tetrahydrofolate reductase were closer to the chromosomal paralog of *metF* in *M. extorquens* CM4 which, unlike its megaplasmid homolog *metF2*, has no proven role in growth with chloromethane (Vannelli *et al.*, 1999; Studer *et al.*, 2002; Roselli *et al.*, 2013). It is actually more closely homologous to *metF* of *E. coli* (Sheppard *et al.*, 1999) and *Thermus thermophilus* HB8 (Igari *et al.*, 2011), which are actually involved in the reverse reaction from $\text{CH}_2\text{=H}_4\text{F}$ to $\text{CH}_3\text{-H}_4\text{F}$ during methionine synthesis (Sheppard *et al.*, 1999).

Similarly, comparative analysis of *folD* and *purU* genes essential for growth with chloromethane in strain CM4 (Studer *et al.*, 2002) (Fig. 2.2) were also conserved in the different strains considered in this analysis, but to a lesser extent than genes *cmuA* and *cmuB* (Table 2.1). At the protein level, levels of identity of all considered homologs of *FolD* exceed 40%, whereas homologs of *PurU*, highly identical between *M. extorquens* CM4 and *Hyphomicrobium* sp. MC1, are much more divergent and only cover 30-40% of

the PurU protein length in chloromethane-degrading strains *Roseovarius* sp. 217 and *Leisingera methylohalidivorans* (Table 2.1).

2.4.3. Organization of *cmu* genes

The organization of genes in gene clusters and operons may be used to infer their role in a metabolic pathway. In *M. extorquens* CM4, *cmu* genes are located in two clusters on the megaplasmid pCMU01, which are separated by a 27 kb DNA region containing genes involved in cobalamin (vitamin B12) metabolism, and vitamin B12 is indeed required for chloromethane utilization (Fig. 2.4) (Roselli *et al.*, 2013). In *Hyphomicrobium* sp. MC1, *cmu* genes are located in a single cluster, presumably as an operon, and on a putative genomic island. The organization of *cmu* in *Hyphomicrobium* sp. MC1 is conserved in other chloromethane-degrading strains isolated from different environments (Nadalig *et al.*, 2011) [see **Chapter 4**]. In *Roseovarius* sp. 217 and *Leisingera methylohalidivorans* DSM 14336, in contrast, homologs of *cmu* genes are not clustered as in strains CM4 and MC1, confirming the suggestion that such distant gene homologs to *bona fide cmu* gene counterparts in strains CM4 and MC1 may actually not be involved in chloromethane utilization. The reverse situation applies to strains *Thermosediminibacter oceani* DSM 16646, *Desulfomonile tiedjei* DSM 6799, and *Thermincola potens* JR, in which *cmu* gene homologs were found, but which are not known to dehalogenate chloromethane or to use it as a growth substrate. In *Thermosediminibacter oceani* DSM 16646 and *Thermincola potens* JR, the detected homologs of chloromethane dehalogenation genes *cmuABC*, along with another gene coding for the putative regulatory protein FmdB, were found in the same putatively operonic organisation as in strain MC1. Interestingly, in *Desulfomonile tiedjei* DSM 6799, no *cmuC* homolog was detected between *cmuA* and *cmuB* or elsewhere in the genome (Table 2.1) (Fig. 2.4). This is actually in fair agreement with the fact that the *cmuC* gene is the least conserved among chloromethane-degrading strains with the *cmu* pathway (Nadalig *et al.*, 2011), and confirms the elusive role of *cmuC* in chloromethane utilisation despite its essential character in strain CM4 (Roselli, 2006 - 2009) The capacity for chloromethane dehalogenation of these three strains will need to be addressed. Investigations along these lines are all the more interesting given the fact that these strains have an anaerobic lifestyle which was not yet encountered in a chloromethane-degrading bacterium possessing the *cmu* pathway.

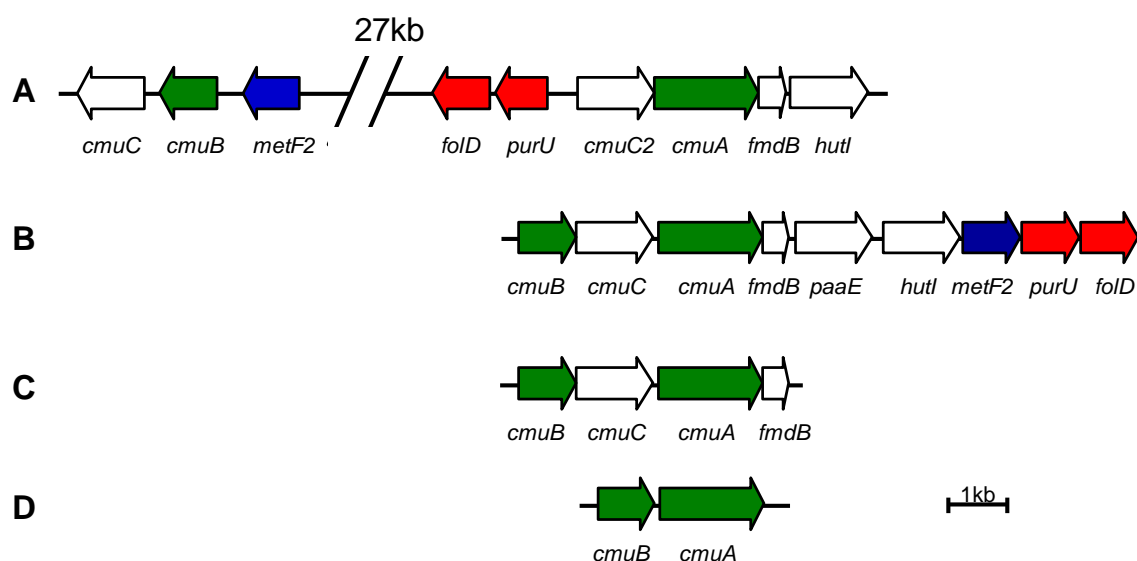


Fig. 2.1. Comparison of *cmu* gene organization in different genomes.

Arrows represents *cmu* genes associated with chloromethane dehalogenation (green), genes essential for assimilation of carbon into biomass (blue), genes involved in the oxidation of chloromethane to CO₂ (red), and genes of unknown function (white). These genes are located in distinct but close gene clusters on the megaplasmid of *M. extorquens* CM4 (A) (Roselli *et al.*, 2013) and on a putative unique operon in the chromosome of *Hyphomicrobium* MC1 and in other chloromethane-degrading strains with the *cmu* pathway (B) (Nadalig *et al.*, 2011), *Thermosediminibacter oceani* DSM 16646 and *Thermincola potens* JR (C), and *Desulfomonile tiedjei* DSM 6799 (D). In *Roseovarius* sp. 217 and *Leisingera methylohalidivorans* DSM 14336, *cmu* genes for dehalogenation were absent, and identified homologs of accessory genes (Table 2.1) were not colocalized.

Table 2.2. Common and unique genes found in the genomes of *Methylobacterium extorquens* CM4 and *Hyphomicrobium* sp. MC1^{a, b}

CM4	Gene name	Annotaion	Homolog in MC1 (% protein identity)
Mchl_4782 ^c	–	conserved protein of unknown function	HYPMCv2_1808 (42%) HYPMCv2_1839 (44%)
Mchl_5521	<i>acxC</i>	acetone carboxylase gamma subunit	HYPMCv2_0473 (79%)
Mchl_5522	<i>acxB</i>	acetone carboxylase alpha subunit	HYPMCv2_0474 (87%)
Mchl_5523	<i>acxA</i>	acetone carboxylase beta subunit	HYPMCv2_0475 (89%)
Mchl_5524	<i>acxR</i>	acetone carboxylase transcriptional regulator	HYPMCv2_0476 (58%)
Mchl_5696	–	putative regulatory protein FmdB	HYPMCv2_2271 (45%)
Mchl_5697	<i>cmuA</i>	methyltransferase / corrinoid binding protein CmuA	HYPMCv2_2273 (84%)
Mchl_5699	<i>purU</i>	formyltetrahydrofolate hydrolase	HYPMCv2_2267 (62%)
Mchl_5719	–	putative membrane protein, putative transport protein	HYPMCv2_2263 (37%)
Mchl_5727	<i>cmuB</i>	CmuB protein, methylcobalamin:H4folate methyltransferase	HYPMCv2_2275 (64%)
Mchl_5728	<i>cmuC</i>	CmuC protein, putative methyltransferase	HYPMCv2_2274 (38%) HYPMCv2_2287 (52%)
Mchl_5731	<i>cobA</i>	uroporphyrinogen-III C-methyltransferase	HYPMCv2_3968 (42%)

^a Analysis performed using the MicroScope platform at Génoscope (Vallenet *et al.*, 2009; Muller *et al.*, 2011b; Vallenet *et al.*, 2013).

^b Proteins common to *M. extorquens* CM4 and *Hyphomicrobium* sp. MC1 without close homologs in any of the other completely sequenced *Methylobacterium* and *Hyphomicrobium* strains, i.e. *Methylobacterium extorquens* strains AM1 (6531 proteins, accession number NC_012808), *M. extorquens* DM4 (5773 proteins, accession number NC_012988), *M. extorquens* PA1 (5357 proteins, accession number NC_01017), *M. extorquens* BJ001 (6027 proteins, accession number NC_010725), *M. nodulans* ORS 2060 (10161 proteins, accession number NC_011894), *Methylobacterium* sp. 4-46 (8356 proteins, accession number NC_010511), *M. radiotolerans* JCM 2831 (7293 proteins, accession number NC_010505), *Hyphomicrobium denitrificans* ATCC 51888 (3948 proteins, accession number NC_014313), *Hyphomicrobium* sp. GJ21(4055 proteins, accession number: not public) and *Hyphomicrobium* sp. VS (3598 proteins, accession number: not public)

Threshold for common proteins: over 35% identity on over 75% of the protein sequences compared.

Threshold for excluded proteins: any homolog with over 35% identity over 75% of the protein sequence in any of the considered genomes.

^c Located on the chromosome of *M. extorquens* CM4

2.4.4. The chloromethane-specific gene set in Alphaproteobacteria

M. extorquens CM4 and *Hyphomicrobium* sp. MC1 are the only sequenced methylotrophic strains using the *cmu* pathway (Table 2.1) for utilization of chloromethane. The genomes of these two strains were compared to ten other publically available genome sequences of *Methylobacterium* and *Hyphomicrobium* strains that do not grow with chloromethane (Table 2.2), in order to try to define a set of proteins that is common and unique to chloromethane-degrading strains with the *cmu* pathway.

For this analysis, the comparative genomic tool ‘Gene Phyloprofile’ of the MicroScope platform of Génoscope was used. This yielded a short list (Table 2.2) of 12 proteins common to *M. extorquens* CM4 and *Hyphomicrobium* sp. MC1, but lacking close homologs in any of the other completely sequenced *Methylobacterium* and *Hyphomicrobium* strains (excluded proteins with over 35% identity over 75% of the protein sequence in any of the considered genomes). All of these proteins except one (Mchl_4782 of unknown function) are encoded by genes located on the megaplasmid of *M. extorquens* CM4, on which *cmu* genes are located (Roselli *et al.*, 2013). Along with the key genes involved in chloromethane metabolism already described (i.e. *cmuABC* and *purU*, Table 2.1), *cobA* and a gene coding for putative regulatory protein (FmdB) were found (Table 2.2). Protein CobA is involved in the synthesis of cobalamin (also known as vitamin B12), an essential cofactor for the function of the CmuAB dehalogenase (Fig. 2.2) (Studer *et al.*, 1999; Roselli *et al.*, 2013). The gene encoding for a putative regulatory protein FmdB adjacent to *cmuA* in both MC1 and CM4 strains is located close to *cmu* genes, and may represent the still unknown regulator of chloromethane dehalogenase. Further experiments involving mutation of this gene in CM4 or MC1 may be performed to confirm its involvement in chloromethane utilization.

A few other genes highly conserved and unique in both CM4 and MC1 strains were detected in addition to *cmu* genes involved in chloromethane dehalogenation. Interestingly, these genes define a complete acetone-utilizing gene cluster encoding the acetone carboxylase subunits (*acxABC* genes) and its transcriptional activator (*acxR* gene) (Table 2.2), and thereby also involve transformation of another key C1 compound, carbon dioxide. Acetone carboxylase is the key enzyme of bacterial acetone metabolism, catalyzing the ATP-dependent carboxylation of acetone to form acetoacetate (Sluis *et al.*, 2002). In preliminary experiments designed to address the question of the presence of *acx* genes together with *cmu*

genes on the pCMU01 megaplasmid, Roselli *et al.* (2013) recently showed that *M. extorquens* CM4, a facultative methylotroph growing with several multicarbon compounds including acetate, ethanol and succinate (Anthony, 2011; Peyraud *et al.*, 2012), is indeed also able to grow and utilize acetone as the sole source of carbon of energy. The comparative genome analysis presented here (Table 2.2) suggests that *Hyphomicrobium* sp. MC1 may also degrade acetone and grow with this compound.

2.5. Conclusions and perspectives

Summarizing, comparative genomic analysis of chloromethane-degrading strains performed during this PhD project showed that

- i) The *cmu* pathway is conserved and consists of closely colocalized genes and gene clusters in the genomes of chloromethane-degrading *Methylobacterium* and *Hyphomicrobium* strains, and other Proteobacteria for which the entire genome sequence is unknown (Nadalig *et al.*, 2011);
- ii) Genes *cmuABC* are absent in marine chloromethane-degrading strains *Roseovarius* sp. 217 and *Leisingera methylohalidivorans* DSM 14336, suggesting the existence of at least one alternative pathway for bacterial growth with chloromethane. In future experiments, *Roseovarius* sp. 217 and *Leisingera methylohalidivorans* DSM 14336 could be grown with C¹³-labelled chloromethane to discover new genes and enzymes expressed during growth with chloromethane and involved in chloromethane utilization.
- iii) Some strains of known genome sequence contain *cmu* genes but have not been reported to utilize chloromethane, e.g. *Desulfomonile tiedjei* DSM 6799, *Thermosediminibacter oceani* DSM 16646 and *Thermincola potens* JR. These strains also harbor homologs of genes involved in the serine cycle for assimilation of C-1 compounds (notably *glyA* and *eno*) (Kalyuzhnaya and Lidstrom, 2005; Vuilleumier *et al.*, 2009), suggesting that they may also be capable of growing with C1 compounds
- iii) Strains CM4 and MC1 not only showed conservation of *cmu* genes but also of acetone utilization genes. Growth of *Hyphomicrobium* sp. MC1 with acetone could be tested. Acetone is the second most abundant carbonyl carbon in the atmosphere after formaldehyde (Fischer *et al.*, 2012). Bacterial utilization of chloromethane and acetone may be correlated considering that sinks and sources of these compounds are quite

similar, with largest emissions from plants and oceans for both compounds (Montzka *et al.*, 2011; Fischer *et al.*, 2012).

iv) The *cmuA* gene is the most conserved of all *cmu* genes in chloromethane-degrading strains (Nadalig *et al.*, 2011), including in the three anaerobic strains discussed here for the first time in the context of chloromethane utilization. This strengthens its status as a biomolecular tool of choice in experimental investigations of chloromethane utilization, including those presented in the following Chapters of this manuscript.

2.6. Appendix Chapter 2

2.6.1. Protocol : Total DNA extraction from large volume bacterial cultures for genome sequencing

Bacterial strain: *Hyphomicrobium* MC1 grown in M3 medium (Roselli *et al.*, 2013), with an initial unique 10 mM portion chloromethane as the sole carbon source

Kit: Wizard Genomic DNA Purification Kit

1. Centrifuge 500 mL of grown culture (OD₆₀₀ approx. 0.3) using Sorvall Evolution/F10 rotor (8000 rpm, 15 min). Discard supernatant.
2. Resuspend and wash the cell pellet in 15 mL fresh M3 medium; transfer to a single sterile SS34 rotor tube. Centrifuge (Sorvall Evolution/SS34 rotor) for 5 minutes at 12000 rpm (17000 g) and discard supernatant.
3. Resuspend in 13 mL of Nuclei Lysis Solution from the kit. Add 250 µl of DNase-free Proteinase K (20 mg/mL) and 0.5 mL of 0.5M EDTA solution. Incubate for 5 min at 80°C to lyse cells and cool to room temperature.
4. Add 50 µl of DNase-free RNase (from the kit). Invert the tube 2-5 times for homogeneisation. Incubate at 37°C for 30 min, at 80°C for 5 min and cool to room temperature.
5. Add 13 mL of protein precipitation solution (from the kit). Mix thoroughly by repeated inversion, but **without** vortexing. Centrifuge at 15000 rpm (Sorvall Evolution/SS34 rotor) for 15 min at 4°C.
6. Transfer the supernatant (16 mL) very carefully (**without any fines**) to a fresh sterile SS34 tube containing 12 mL of -20°C isopropanol. Homogenize the mixture by inverting the tube very gently until appearance of turbidity.
7. Centrifuge at 15000 rpm (Sorvall Evolution/SS34 rotor) for 15 min at 4°C.
8. Mark the position of the pellet on the outside of the tube with a permanent marker. Carefully remove the supernatant using a 5 mL pipetman (taking care not to dislodge the DNA pellet), carefully invert the tube and dry on absorbing paper in a laminar flow hood.

9. Carefully add 10 mL cold 70% ethanol (kept at -20°C) and incubate at room temperature for 15 minutes.
10. Centrifuge again at 15000 rpm (Sorvall Evolution/SS34 rotor) for 15 min at 4°C; take care to centrifuge with the tube in exactly the same orientation as in the first centrifugation (i.e. with the pellet facing outwards).
11. Carefully remove the supernatant with a 1 mL pipetman. Dry the pellet in a laminar flowhood.
12. Add 1 mL of DNA Rehydration Solution (from the kit). Incubate at 65°C for 1 hour and/or at 4°C overnight. Measure the DNA concentration by NanoDrop, agarose gel electrophoresis (and PicoGreen dsDNA quantification kit Molecular Probes if available) (Typical yield: 2.5 μ g/mL; 260/280 ratio 2.0-2.1; 260/230 ratio 1.75-1.85).
13. Store at -20°C until further use.

Chapter 3. A fluorescence-based bioreporter for the specific detection of methyl halides

Manuscript by **Farhan Ul Haque M.**, Nadalig T., Bringel F., and Vuilleumier S., submitted to *Applied and Environmental Microbiology* in April 2013.

Résumé

Les monohalométhane (halogénures de méthyle) sont des composés organiques volatils qui jouent un rôle important dans l'environnement, tant en raison de leur toxicité pour les organismes vivants que pour leur implication dans le fonctionnement de l'atmosphère. Ainsi, le chlorométhane et le bromométhane sont responsables de la destruction d'ozone stratosphérique, alors que l'iodométhane influe sur la formation d'aérosols à l'interface océan-atmosphère. Le bilan atmosphérique global de ces halométhane, en particulier le chlorométhane, est encore mal évalué en raison d'un grand nombre d'incertitudes dans les estimations et la non prise en compte possible de certaines sources et puits en raison des difficultés de détection et de quantification du chlorométhane. L'objectif principal de cette étude était de développer un bio-rapporteur pour la détection spécifique du chlorométhane et des autres mono-halométhane. *Methylobacterium extorquens* CM4, une souche qui peut utiliser le chlorométhane comme seule source de carbone et d'énergie, a été sélectionnée pour cette étude. Le gène *cmuA* a été choisi en tant que gène marqueur et l'induction de son expression spécifique par le chlorométhane a été vérifiée par qRT-PCR. Un plasmide, comportant une cassette d'expression du gène codant la protéine fluorescente jaune (YFP) placée sous le contrôle du promoteur du gène *cmuA*, a été construit. En présence de chlorométhane, une émission de fluorescence, proportionnelle à la concentration de chlorométhane sur une large gamme de valeurs (2 pM - 20 mM), a été quantifiée chez *M. extorquens* CM4. Le temps nécessaire pour détecter la production de fluorescence est de 20 minutes environ, et une concentration seuil de chlorométhane correspondant à seulement 60 molécules de chlorométhane par cellule de bio-rapporteur a été déterminée. Ce rapporteur biologique pourrait être utilisé comme méthode de détection sensible des émissions de chlorométhane provenant de différents milieux (par exemple les sols et les plantes) au laboratoire ainsi que *in situ*.

3.1. Abstract

Methyl halides are volatile one-carbon compounds which play an important role in the functioning of the atmosphere. Global budgets of methyl halides are still poorly understood due to uncertainties in estimations of their natural sources and sinks. Among them, chloromethane (CH_3Cl) is the most abundant halogenated hydrocarbon in the atmosphere, and responsible for substantial destruction of stratospheric ozone. A bacterial bioreporter for the detection of methyl halides was developed on the basis of detailed knowledge of the physiology and genetics of the Alphaproteobacterium *Methylobacterium extorquens* CM4, which utilizes chloromethane as the sole source of carbon and energy for growth. A plasmid construct with the promoter region of the chloromethane dehalogenase gene *cmuA* fused to a promoterless yellow fluorescent protein gene cassette resulted in methyl halide dependent fluorescence when introduced into *M. extorquens* CM4. Fluorescence production of the bacterial bioreporter to methyl halides was shown to be rapid, specific and highly sensitive. This bioreporter may provide an attractive alternative to analytical chemical methods to screen for methyl halide emissions from different environments, including plants

Chapter 4. Detection and isolation of chloromethane-degrading bacteria from the *Arabidopsis thaliana* phyllosphere, and characterization of chloromethane utilization genes

Article by Nadalig T., **Farhan Ul Haque M.**, Roselli ., Schaller H., Bringel F., Vuilleumier S.

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Résumé

Le chlorométhane est un composé volatil produit naturellement dans la phyllosphère, le compartiment défini comme les parties aériennes des plantes et qui abrite une flore bactérienne riche. Le chlorométhane peut servir comme substrat pour la croissance des bactéries méthylotrophes aérobies qui possèdent les gènes *cmu*. Ainsi, des bactéries ont été isolées à partir de sols et d'eaux douce ou marine mais aucune n'a été décrite provenant de la phyllosphère. Dans notre étude, nous avons mis en évidence la présence de bactéries dégradant le chlorométhane sur la surface des feuilles d'*Arabidopsis thaliana*, par PCR quantitative spécifique du gène *cmuA* codant la protéine chlorométhane déhalogénase. A partir de cultures d'enrichissement contenant du chlorométhane comme seule source de carbone et inoculés avec des feuilles d'*Arabidopsis thaliana*, des souches bactériennes ont été isolées. Le génotypage basé sur l'analyse des produits PCR du gène *cmuA* après digestion par une enzyme de restriction (DdeI) a été utilisé pour évaluer la diversité des bactéries dégradant le chlorométhane pendant l'enrichissement et après l'isolement des souches. Sur la base des séquences des gènes de l'ARNr 16S, les isolats obtenus ont été affiliés au genre *Hyphomicrobium* dont la présence caractéristique d'hyphe a pu être observée au microscope. Les gènes *cmu* de ces isolats ont été analysés à l'aide de nouvelles amorces PCR conçues au laboratoire, et leurs séquences ont été comparées avec celles des souches aérobies dégradant le chlorométhane précédemment décrites. Les trois isolats présentaient un arrangement colinéaire des gènes *cmuBCA*, similaire à ceux de toutes les autres souches précédemment caractérisées, à l'exception de *Methylobacterium extorquens* CM4. Cette étude est la première à décrire des souches bactériennes dégradant le chlorométhane et isolées de la phyllosphère.

4.1. Article

Detection and isolation of chloromethane-degrading bacteria from the *Arabidopsis thaliana* phyllosphere, and characterization of chloromethane utilization genes

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Keywords

chloromethane; phyllosphere; *cmu* genes; methyl halide metabolism.

Abstract

Chloromethane gas is produced naturally in the phyllosphere, the compartment defined as the aboveground parts of vegetation, which hosts a rich bacterial flora. Chloromethane may serve as a growth substrate for specialized aerobic methylotrophic bacteria, which have been isolated from soil and water environments, and use *cmu* genes for chloromethane utilization. Evidence for the presence of chloromethane-degrading bacteria on the leaf surfaces of *Arabidopsis thaliana* was obtained by specific quantitative PCR of the *cmuA* gene encoding the two-domain methyltransferase corrinoid protein of chloromethane dehalogenase. Bacterial strains were isolated on a solid mineral medium with chloromethane as the sole carbon source from liquid mineral medium enrichment cultures inoculated with leaves of *A. thaliana*. Restriction analysis-based genotyping of *cmuA* PCR products was used to evaluate the diversity of chloromethane-degrading bacteria during enrichment and after strain isolation. The isolates obtained, affiliated to the genus *Hyphomicrobium* based on their 16S rRNA gene sequence and the presence of characteristic hyphae, dehalogenate chloromethane, and grow in a liquid culture with chloromethane as the sole carbon and energy source. The *cmu* genes of these isolates were analysed using new PCR primers, and their sequences were compared with those of previously reported aerobic chloromethane-degrading strains. The three isolates featured a colinear *cmuBCA* gene arrangement similar to that of all previously characterized strains, except *Methylobacterium extorquens* CM4 of known genome sequence.

Introduction

Chloromethane, the most abundant volatile halocarbon in the atmosphere (600 p.p.t.), may be responsible for about 15% of chlorine-catalysed ozone destruction in the stratosphere (Clerbaux *et al.*, 2007). The global chloromethane budget is uncertain, with estimated known sources not fully accounting for identified sinks. The major sink for chloromethane likely involves reactions with hydroxyl radicals in the troposphere (Yoshida *et al.*, 2004). Industrial emissions of the compound estimates range from < 10% (Yoshida *et al.*, 2004) to 60% (Trudinger *et al.*, 2004) of total known emissions. Natural sources of chloromethane emissions include higher plants, grasslands, salt marshes, peatlands, wood-rotting fungi, senescent leaves, biomass

burning and oceans, with vegetation possibly representing the major biotic source (Saito & Yokouchi, 2008). A methyltransferase responsible for chloromethane production was purified from leaves of *Brassica oleracea* (Attieh *et al.*, 1995, 2000). In *Arabidopsis thaliana*, a protein encoded by the gene *HOL* (harmless to ozone layer) catalyses S-adenosyl-L-methionine-dependent methylation of chloride (Rhew *et al.*, 2003; Nagatoshi & Nakamura, 2009). This enzyme is involved in the transformation of thiocyanate produced upon the degradation of glucosinolate, and its product methylisothiocyanate appears to play a role in the resistance of *A. thaliana* to bacterial infection (Nagatoshi & Nakamura, 2009). A physiological role for enzyme-produced chloromethane remains to be demonstrated.

Table 1. Growth of chloromethane-degrading strains and dehalogenase activity of resting cells with chloromethane

Strains	Doubling time (h)	Specific activity (nmol min ⁻¹ mg ⁻¹ protein)*
<i>Methylobacterium extorquens</i> CM4	7.3 ± 1.1	26.1 ± 5.8
<i>Hyphomicrobium chloromethanicum</i> CM2	4.9 ± 0.3	24.8 ± 2.2
<i>Hyphomicrobium</i> sp. strain MC1	5.1 ± 0.3	29.5 ± 4.0
<i>Hyphomicrobium</i> sp. strain AT2	5.9 ± 0.7	28.0 ± 0.3
<i>Hyphomicrobium</i> sp. strain AT3	18.1 ± 0.5	22.8 ± 2.0
<i>Hyphomicrobium</i> sp. strain AT4	19.8 ± 0.5	21.6 ± 0.2

*Control experiments in buffer without cells yielded an abiotic degradation rate under the same conditions of 0.054 ± 0.008 nmol min⁻¹ corresponding to < 0.1% the observed biotic rates. Similarly, the observed rate for cell suspensions of the nondechlorinating strain *Methylobacterium extorquens* AM1 (Vuilleumier *et al.*, 2009) was 0.0093 ± 0.0018 nmol min⁻¹ mg⁻¹ protein.

Certain methylotrophic bacteria are able to use chloromethane as the sole carbon and energy source for growth (McDonald *et al.*, 2002; Trotsenko & Doronina, 2003; reviewed in Schäfer *et al.*, 2007; Table 1). Chloromethane-degrading bacteria have been isolated from diverse environments such as soils (Doronina *et al.*, 1996; Miller *et al.*, 1997; Coulter *et al.*, 1999; McAnulla *et al.*, 2001a), activated sludge (Hartmans *et al.*, 1986; Traunecker *et al.*, 1991; Freedman *et al.*, 2004), freshwater (McAnulla *et al.*, 2001a), and seawater (Schäfer *et al.*, 2005), and include representatives affiliated to a wide range of genera including *Aminobacter*, *Hyphomicrobium*, *Leisingera*, *Methylobacterium*, *Roseovarius* (*Alphaproteobacteria*), *Pseudomonas* (*Gammaproteobacteria*) and *Acetobacterium* (*Actinobacteria*). However, the association of such bacteria with plants has not yet been investigated.

The only pathway for chloromethane catabolism by aerobic bacteria characterized so far was investigated in detail for strain *Methylobacterium* sp. CM4 (Doronina *et al.*, 1996). This strain may now be affiliated to the species *Methylobacterium extorquens* on the basis of its complete genome sequence, including revised 16S rRNA gene sequences (GenBank NC_011757). A set of genes essential for growth on chloromethane (Vannelli *et al.*, 1998, 1999), termed *cmu* genes for chloromethane utilization, was identified by minitransposon mutagenesis of strain CM4. These genes and the corresponding enzymes defined a specific pathway for chloromethane utilization in methylotrophic metabolism that depends on corrinoide and folate cofactors (Vannelli *et al.*, 1999). The chloromethane dehalogenase enzyme, consisting of a corrinoide methyltransferase protein encoded by *cmuA* and of a tetrahydrofolate-dependent methyltransferase encoded by *cmuB*, was purified and characterized (Studer *et al.*, 1999, 2001). The role in chloromethane utilization of *cmuC*, encoding another puta-

tive methyltransferase essential for chloromethane utilization (Vannelli *et al.*, 1999), remains to be elucidated. Chloromethane-dependent expression of *cmu* genes was also demonstrated (Studer *et al.*, 2002).

Several other chloromethane-degrading strains were subsequently reported (Woodall *et al.*, 2001; McAnulla *et al.*, 2001b; Schäfer *et al.*, 2005; Warner *et al.*, 2005) and also contained *cmu* genes. The *cmuA* gene has been used as a molecular biomarker of bacterial methyl halide metabolism in a large variety of environments (McAnulla *et al.*, 2001a; Miller *et al.*, 2004; Borodina *et al.*, 2005; Schäfer *et al.*, 2005). The plant environment, however, has not yet been explored in the context of bacterial chloromethane metabolism, despite the fact that some of the genera featuring representatives of chloromethane-degrading bacteria, notably *Hyphomicrobium*, *Methylobacterium* and *Pseudomonas*, are known to be efficient colonizers of the phyllosphere (Kinkel, 1997; Andrews & Harris, 2000; Trotsenko *et al.*, 2001; Knief *et al.*, 2008, 2010; Raja *et al.*, 2008; Delmotte *et al.*, 2009).

To investigate the occurrence of chloromethane-degrading bacteria in the phyllosphere, bacterial enrichments from leaves of *A. thaliana*, which is the best-characterized chloromethane-emitting plant at the molecular level (Rhew *et al.*, 2003; Nagatoshi & Nakamura, 2009), were performed with the aim of isolating new bacterial strains able to grow with chloromethane as the sole carbon source, and to develop new molecular tools to efficiently detect and characterize gene signatures for chloromethane utilization in isolated strains and environmental DNA, in particular in the phyllosphere environment.

Materials and methods

DNA and bacterial strains

Strains *M. extorquens* CM4 and *Hyphomicrobium chloromethanicum* CM2, isolated in Russia from petrochemical factory soil (Doronina *et al.*, 1996), and *Hyphomicrobium* sp. strain MC1, isolated from industrial sewage sludge (Hartmans *et al.*, 1986), were laboratory stocks. Genomic DNA extraction of bacterial strains was performed using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Total DNA of *Aminobacter lissarensis* CC495, *Aminobacter ciceronei* IMB-1, *Rhodobacteraceae* 179 and *Rhodobacteraceae* 198 was kindly provided by H. Schäfer (University of Warwick, UK).

Growth media

Liquid cultures for enrichment and growth were performed in a mineral medium for methylotrophic bacteria (M3) containing (L⁻¹ of distilled water) KH₂PO₄ (6.8 g), (NH₄)₂SO₄ (0.2 g), NaOH (5 M) (5.85 mL), yielding a final pH of 7.2. After autoclaving, 1 mL L⁻¹ medium each of

calcium nitrate solution (25 g L^{-1}) and of trace elements solution containing (mg L^{-1}) $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (100), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (100), ZnSO_4 (29.5), $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (25), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (25), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (25), NH_4VO_3 (14.4), $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ (10), H_3BO_3 (10) and 0.5 mL L^{-1} of H_2SO_4 (95%) were added.

Bacterial strains were cultivated and isolated on a solid medium containing (L^{-1} of distilled water) K_2HPO_4 (1.04 g), $(\text{NH}_4)_2\text{SO}_4$ (0.2 g), $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (0.65 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 g), bromothymol blue (0.1 g) and agar (15 g) at a pH of 7.2. Calcium nitrate and trace elements were added after autoclaving as described for liquid M3 medium.

Enrichment culture and strain isolation from plant leaves

Enrichment cultures were set up by adding one leaf of greenhouse-grown *A. thaliana* Columbia (Col-0, ~40 mg) to a 300-mL vial fitted with a sealed mininert valve cap (Sigma) and containing 50 mL of M3 medium. Following the addition of 12 mL (approximately 10 mM) chloromethane gas (Fluka, approximately 5 atm) to the headspace, enrichment cultures were incubated under shaking (100 r.p.m.) at 30°C . After 24 h, leaf material was removed and 12 mL of chloromethane gas was again added. Enrichment cultures obtained after three successive subcultures were spread onto mineral agar plates and incubated in sealed, gas-tight jars containing 1.3% chloromethane (v/v). Chloromethane dehalogenation was indicated by the development of yellow colour on a green background around dehalogenating colonies, which were selected and purified on fresh solid M3 medium.

Dehalogenation activity

Resting cell suspensions were prepared from exponential-phase cultures (50 mL, $\text{OD}_{600 \text{ nm}} < 0.3$), obtained with chloromethane as the sole carbon source. After centrifugation at $14\,700 \text{ g}$ for 10 min, cells were washed twice in 50 mM chloride-free phosphate buffer pH 7.0, and the cell pellet was resuspended in the same buffer (6 mL final volume). Protein determination was performed with 1 mL of cell suspension using the bicinchoninic acid assay and a commercial kit (Pierce). For activity measurements, 5 mL of cell suspensions of chloromethane-degrading strains were added to 17-mL Hungate vial capped with a gas-tight mininert valve (Sigma). Chloromethane gas (10 mL) was added in excess and the vial was incubated at 30°C . At different times, aliquots (0.5 mL) were sampled through the valve with a 1-mL syringe, transferred to Eppendorf tubes on ice, centrifuged immediately, and the resulting supernatants were transferred to fresh Eppendorf tubes and kept frozen until further use. Control experiments with phosphate buffer and with cell suspensions of the nondechlorinating

strain *M. extorquens* AM1 (Vuilleumier *et al.*, 2009) were performed to evaluate the nonenzymatic degradation of chloromethane.

Chloride concentration was determined spectrophotometrically as $[\text{FeCl}]^{2+}$ ($\lambda_{\text{max}} = 340 \text{ nm}$) formed in a highly acidic medium using the method of Jörg & Bertau (2004). Chloride concentration was determined by comparison with a calibration curve (0–5 mM) obtained with a sodium chloride solution in the same buffer, and dehalogenase activity was expressed as $\text{nmol min}^{-1} \text{ mg}^{-1}$ protein.

DNA extraction from bacterial cultures and environmental samples

Genomic DNA extraction from enrichment cultures (10 mL at $\text{OD}_{600 \text{ nm}} = 0.6$) was performed using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's recommendations. DNA was extracted from epiphytic bacteria on the surface of *A. thaliana* leaves. Briefly, 10–15 leaves of *A. thaliana* plants (360–860 mg fresh weight) were washed as described previously (Delmotte *et al.*, 2009), with 30 mL TE buffer, pH 7.5, containing 0.1% Silwet L-77 (GE Bayer Silicones). After centrifugation, total DNA was prepared using the FastDNA spin kit (MP Biomedicals, Santa Ana, CA), as described by Knief *et al.* (2008). Cell lysis was performed using a Mikro-Dismembrator S (Sartorius Stedim Biotech, France) by three consecutive 1-min treatments at 3000 min^{-1} .

Quantitative PCR (qPCR)

CmuA and 16S rRNA gene copy numbers were evaluated through qPCR using an ABI PRISM 5700 sequence detection system (Applied Biosystem, Foster City, CA). qPCR analysis was carried out in triplicate using phyllospheric DNA (5–10 ng) in 96-well plates, using the primer pairs *cmuA802f* and MF2 (5'-CCRCCTTTRTAVCCVACYTC) for the *cmuA* gene and BACT1369F and PROK1492R (Suzuki *et al.*, 2000) for the 16S rRNA gene, respectively. The PCR reaction mix contained $1 \times$ qPCR Mastermix Plus for SYBR Green I (Eurogentec S.A., Belgium), 0.3 and $12.8 \mu\text{M}$ of *cmuA802f* and MF2 primers, respectively, for *cmuA* amplification, or $0.4 \mu\text{M}$ of each primer BACT1369F and PROK1492R for 16S rRNA gene amplification. Reaction conditions were 10 min at 95°C , followed by 45 cycles of denaturation at 95°C for 15 s, followed by annealing and elongation at 60°C for 60 s. Calibration curves were obtained using genomic DNA from *M. extorquens* CM4 for both *cmuA* and 16S rRNA gene analysis.

PCR and RFLP analysis

The primers used in this study are listed in Table 2. Reactions were performed in 0.2-mL microcentrifuge tube

Table 2. Primers used in this study

Target gene	Primer*	Sequence (5'–3')†	Position‡	References
<i>cmuA</i>	cmuA802f	TTCAACGGCGAYATGTATCCYGG	7404–7426	Miller <i>et al.</i> (2004)
	cmuAfor2§	CAAGAACGTAAAGCCTGAGCA	8180–8200	This study
	cmuA1609R	TCTCGATGAAGTCTCRGGCT	8212–8190	Miller <i>et al.</i> (2004)
	cmuArev§	GGCATGTCGGTGATGACMAAYTC	8263–8241	This study
	cmuArev2§	ATGCACGGATGGACGACGGA	7519–7500	This study
	cmuA1802r	TTVGCRCTCRAGVCCGTA	8404–8388	This study
<i>cmuB</i>	cmuBfor	GGCRTGCARATGGCGTTTCGACG	4630–4651	This study
	cmuBfor3§	TTCCCCAAGTGGACGG	5094–5109	This study
	cmuBfor4§	GCGAATGGGTCA	5128–5139	This study
	cmuBrev	CTACGCTTCGTCGCGAGGAAC	5273–5252	This study
	cmuBrev2§	ATGATCAACGCATCAGAGGC	4742–4723	This study
	cmuCfor§	GGCGACGACCTGGCTTTCAG	5954–5974	This study
<i>cmuC</i>	cmuCfor2§	TCCGAAATTGATTTTC	5980–5995	This study
	cmuCfor3§	GCATGTTCTGTCCGAAAT	5969–5987	This study
	cmuCfor4§	GCGCCGATGGACAATATTC	6313–6332	This study
	cmuCrev2§	ACGCCGGACGATGT	6401–6388	This study
	paaErev1	TSTCGTCGAARTCGAT	9865–9850	This study
<i>hutI</i>	hutIrev2	TCVTCRCARHAVRCYCDAC	10 655–10 635	This study

*Annealing temperatures for PCR: 67 °C, cmuA802f-cmuArev; 55 °C, cmuA802f-cmuA1609R and cmuA802f-cmuA1802r; 61 °C, cmuA802f-paaErev1; 65 °C, cmuA802f-hutIrev2; 62 °C, cmuBfor-cmuBrev; 67 °C, cmuBfor-cmuArev.

†IUPAC-code (M = A/C, R = A/G, W = A/T, Y = C/T, S = C/G, K = G/T, H = A/C/T, V = A/C/G, D = A/G/T, B = C/G/T, N = A/C/G/T).

‡Positions correspond to the chloromethane utilization cluster of *Hyphomicrobium chloromethanicum* strain CM2 GenEMBL accession no. AF281259.

§Used for sequencing only.

using a thermal cycler (Mastercycler Personal, Eppendorf, Germany). Each PCR reaction mixture consisted of 2.5 µL of PCR buffer (New England Biolabs), 0.25 µL of dNTPs (200 µM), 1 µL of each forward and reverse primers (20 µM), 17.9 µL of distilled water, 0.3 µL of *Taq* polymerase (5 U µL⁻¹, New England Biolabs), 0.05 µL of *Pfu* polymerase (3 U, Promega) and 2 µL of template DNA solution (25 ng). After initial denaturation (94 °C, 3 min), DNA amplification was performed by 30 cycles of 45-s denaturation at 94 °C, annealing for 1 min (at 52 °C for the 16S rRNA gene and between 61 and 67 °C for *cmu* genes; see Table 2 for details), extension for 1–4.5 min (depending of fragment length) at 72 °C and a final extension step of 7 min at 72 °C.

A two-step semi-degenerate PCR strategy (Jacobs *et al.*, 2003) was used to access the 5'-upstream region of the *cmuB* gene fragments obtained. In the first PCR, primer cmuBrev was used with a mix of the three semi-degenerate primers cekg2A, cekg2B and cekg2C (Jacobs *et al.*, 2003). The second PCR involved using the reverse primer cmuBrev2 and the primer cekg4 targeting the tail of the semi-degenerate primers used in the first PCR (Jacobs *et al.*, 2003).

Amplified *cmuA* fragments from each strain and from enrichment cultures were purified using the 'GENECLEAN Turbo' kit (MP Biomedicals Europe, France). Purified products (2 µg) were digested with the enzyme DdeI (30 U; Fermentas, France) and the appropriate buffer at 37 °C for 20 h. Digested products were size fractionated on 2% agarose/Nusieve (3/1) gels.

Sequencing

PCR products were purified with ExoSAP-IT reagent (USB Corporation) according to the manufacturer's recommendations; DNA sequences were obtained from PCR products with appropriate primers (Table 2) on an ABI Prism 3130 XL Genetic Analyzer (Applied Biosystems, UK). The assembled sequences for the *cmuBCA* clusters of *Hyphomicrobium* strains MC1, AT2, AT3 and AT4 obtained were deposited in the EMBL database under numbers FN667867, FN667868, FN667869 and FN667870 respectively, together with the corresponding partial rRNA gene sequences (FN667863, FN667864, FN667865 and FN667866, respectively).

Phylogenetic analysis

DNA sequences were compared with databases by online BLAST searches (<http://www.ebi.ac.uk/tools/blast>). DNA and protein sequences of interest were analysed using the Mobyle platform (<http://mobyle.pasteur.fr>). Multiple alignments were obtained using CLUSTALW, and manually adjusted using JALVIEW, as implemented on the Myhits online portal (<http://myhits.isb-sib.ch>). Multiple alignments were analysed using the PHYLIP suite of programs, and in particular the DNADIST, NEIGHBOR, SEQBOOT and CONSENSE, as implemented on the Mobyle platform. Bootstrap analysis was performed with 100 replicates.

Results

Quantification of 16S rRNA and *cmuA* genes on the leaf surfaces of *A. thaliana*

Total DNA was extracted from the leaf surface of leaves *A. thaliana*, yielding approximately 0.4–1.2 ng DNA mg⁻¹ fresh weight of leaves (range 30–1000 ng DNA per leaf). The *cmuA* gene was detected in all samples, with a copy number of 8.0 ± 0.8 copies ng⁻¹ DNA. The 16S rRNA gene was detected in the same samples at a copy number of $10\,100 \pm 2000$ copies ng⁻¹ DNA. Assuming a single copy of 16S rRNA gene per bacterial genome, this suggested that on average, < 1 in 1000 bacterial cells on leaf surfaces carried the *cmuA* gene and may be capable of dehalogenating chloromethane.

Isolation, characterization and identification of chloromethane-utilizing bacteria

Enrichment cultures were set up with *A. thaliana* leaves as the inoculum in a chloride-free mineral medium with chloromethane as the sole carbon and energy source (1.3%, v/v). Chloromethane-dependent growth with concomitant chloride production was observed in enrichment cultures.

Three chloromethane-degrading bacterial strains termed AT2, AT3 and AT4 (AT in reference to *A. thaliana*) were obtained from such enrichment cultures, as single colonies on a solid mineral medium with chloromethane as the sole carbon and energy source. These isolates grew aerobically on both liquid and solid mineral medium with chloromethane, methanol or succinate as the sole carbon source, indicating that they were facultative methylotrophs. All three strains displayed characteristic hyphae, indicative of the genus *Hyphomicrobium* (Moore, 1981). Taxonomical affiliation to cluster II (Rainey *et al.*, 1998) was confirmed using 16S rRNA gene sequence analysis (Fig. 1). The ability of strains AT2, AT3 and AT4 to transform chloromethane and to use it as the sole carbon and energy source for growth was compared with reference strains *M. extorquens* CM4 and *H. chloromethanicum* CM2 (Table 1). *Hyphomicrobium* strains AT2 grew with chloromethane with similar doubling times as the previously described strains CM2 and MC1 ($t_d \sim 5$ h). The growth of *M. extorquens* CM4 was slightly slower (t_d 7.3 h), whereas newly isolated *Hyphomicrobium* strains AT3 and AT4 were the slowest growing (t_d over than 18 h). In contrast, specific chloromethane dehalogenation activities inferred from measurements of chloride concentration in the supernatants of cell suspensions were similar for all strains (Table 1).

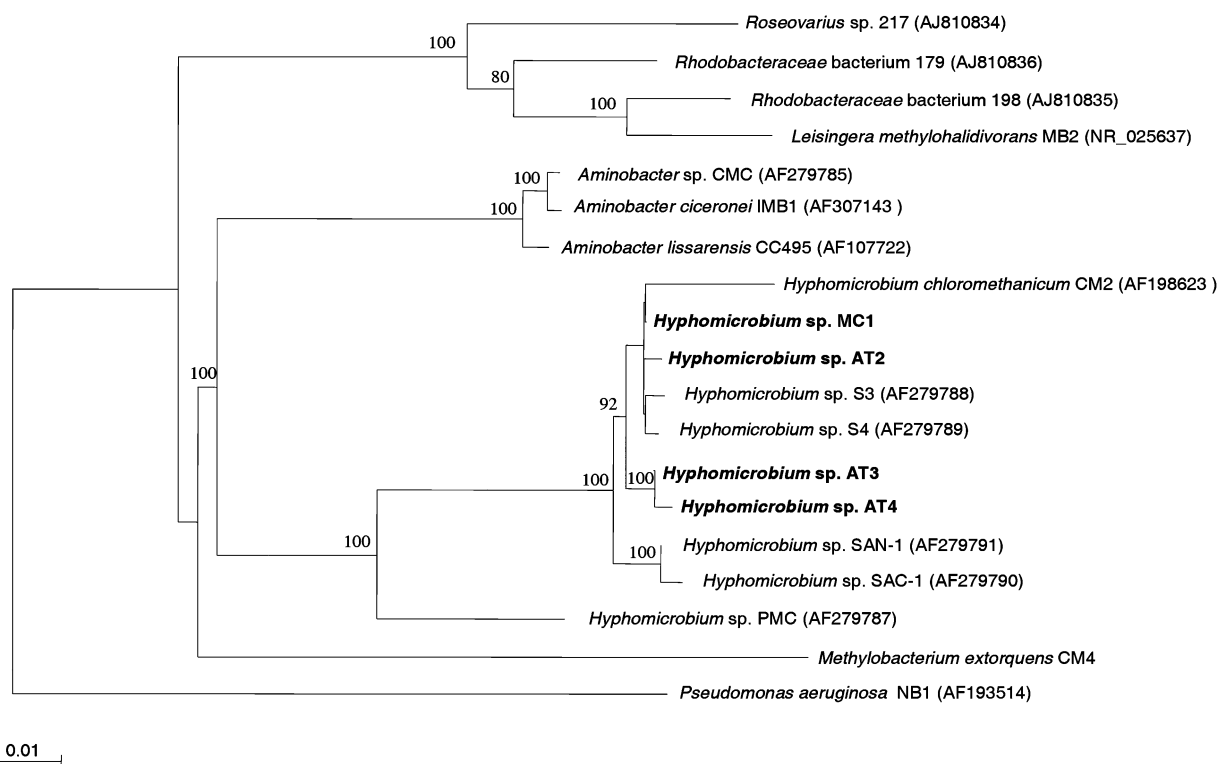


Fig. 1. Taxonomic affiliation of alphaproteobacterial chloromethane-degrading bacteria based on 16S rRNA gene sequences. Strains isolated in this study are shown in bold. The gammaproteobacterium *Pseudomonas aeruginosa*, which is unable to degrade chloromethane, was used as the outgroup. Multiple sequence alignments (1403 bp) were obtained with CLUSTALW and analysed with PHYLIP (see Materials and methods). Bootstrap analysis was performed on 100 replicate trees, and the values of nodes recovered in more than 75% of cases are shown. Scale bar = 1% sequence divergence.

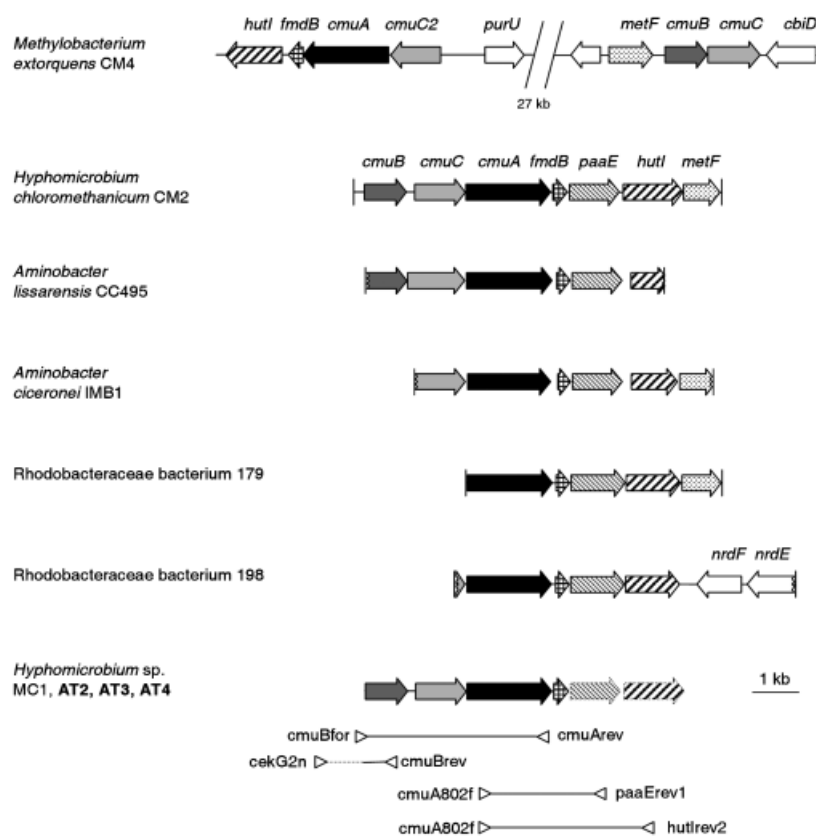


Fig. 2. Comparisons of *cmu* gene organization in chloromethane-degrading bacteria. Arrows represent protein-coding genes, and homologous genes are given with identical shading. Annotations above arrows indicate the name of the gene. Chloromethane degradation genes are part of two different clusters in *Methylobacterium extorquens* CM4. Amplified PCR products used to characterize the new isolates (highlighted in bold), as well as the primers used, are indicated. Dotted lines refer to products of the 3' end of the *cmu* gene cluster obtained by two-stage semi-degenerate PCR (Jacobs *et al.*, 2003). Gene clusters are drawn to scale.

Organization and diversity of *cmu* genes in chloromethane-degrading strains

Genes *cmuA*, *cmuB* and *cmuC* could be amplified and sequenced from total DNA of chloromethane-degrading phyllosphere isolates using both previously described primers (McAnulla *et al.*, 2001b; Miller *et al.*, 2004) and primers newly designed from the better conserved sequence regions in previously reported *cmu* gene clusters (Table 2). A two-step PCR strategy (Jacobs *et al.*, 2003) afforded access to the unknown sequence region upstream of amplified *cmuB* gene fragments yielding complete sequences of the *cmuB* gene for the phyllosphere isolates. The sequences and organization of *cmu* genes in strains isolated from *A. thaliana* were compared with those of previously described strains (Figs 2 and 3, Supporting Information, Fig. S1).

Phylogenetic analysis of partial *cmuA* gene sequences from phyllosphere isolates obtained using the primer pair *cmuA802f*-*cmuA1609R* (Miller *et al.*, 2004) was compared with those of previously reported chloromethane-degrading strains, and from selected *cmuA* gene fragments available in sequence databases and obtained from environmental DNA of different origins (Miller *et al.*, 2004; Borodina *et al.*, 2005; Schäfer *et al.*, 2005) (Fig. 3). This analysis yielded a picture

congruent with that obtained for the analysis of the 16S rRNA gene (Fig. 1). It also suggested that phyllosphere isolates, together with strain MC1, belong to a clade that includes sequences from woodland soil covered with leaf-litter and garden soils (Borodina *et al.*, 2005). The levels of sequence identity between *cmuA* gene fragments were 75–80% between *Hyphomicrobium* strains and either *M. extorquens* CM4 or *Aminobacter* strains. *cmuA* amplicons of strains AT3 and AT4 showed identical sequences, differing from those of strains AT2, CM2 and MC1 which, with over 99% pairwise identity, clustered tightly together (Fig. 3).

In contrast with *cmuA*, only a few partial or full *cmuB* and *cmuC* gene sequences are available so far, all obtained from cultivated and isolated strains (Fig. 2). The new degenerate primer pairs developed in this work allowed the detection and retrieval of *cmuB* and *cmuC* gene sequences from the newly isolated chloromethane-degrading strains reported here, and comparison with previously described *cmu* genes (Table 2). Overall, the sequences for *cmuB* gene fragments (Fig. S2a) showed high levels of identity, but *cmuB* sequences of *Hyphomicrobium* strains were only about 60% identical to that of *M. extorquens* CM4. Regarding *cmuC* (Fig. S2b), sequence analysis of amplicons again showed that sequences from AT3 and AT4 were most closely related

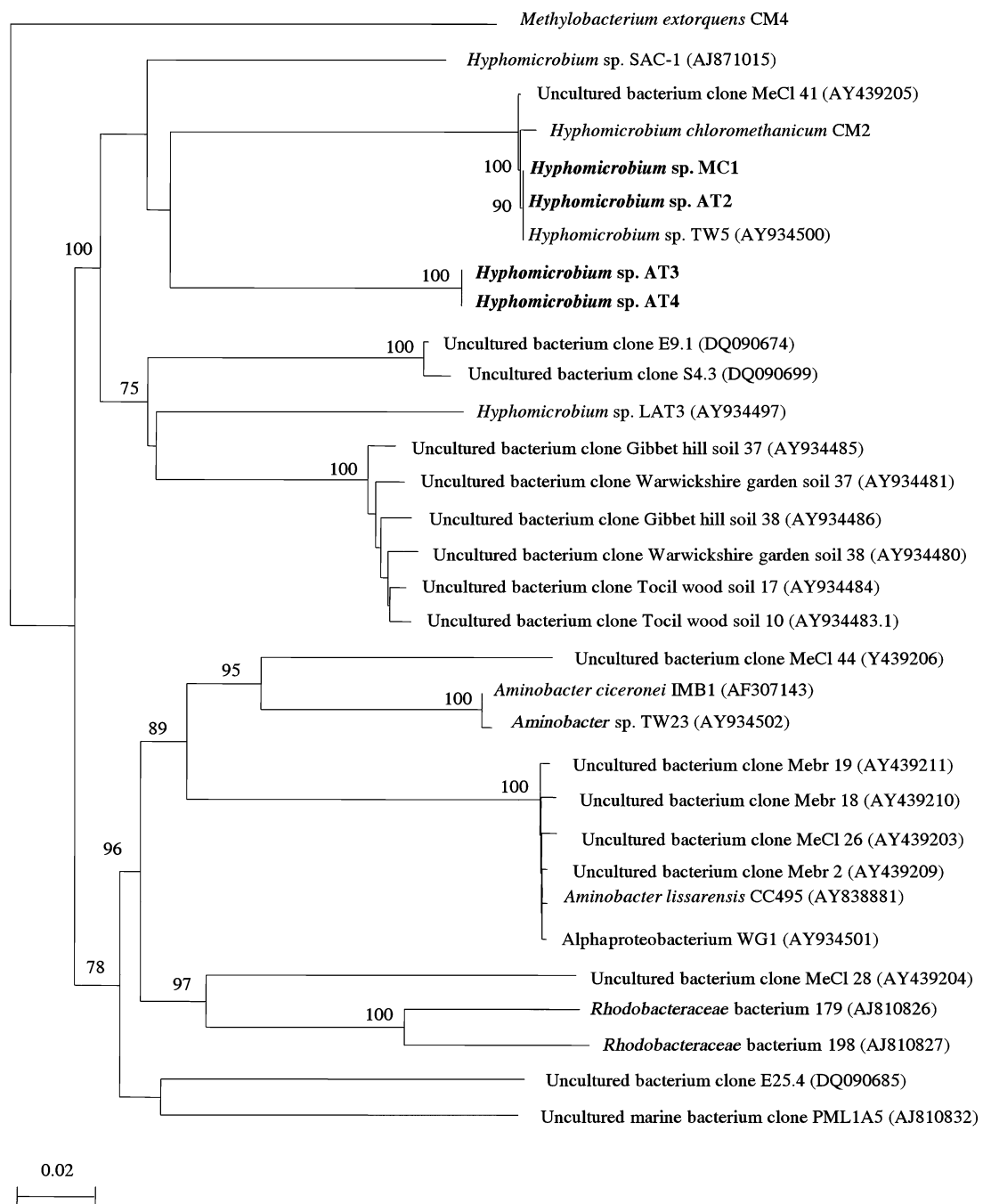


Fig. 3. Phylogenetic analysis of characterized *cmuA* genes from chloromethane-degrading strains. Strains characterized in this study are shown in bold. Bootstrap analysis of the multiple alignments of *cmuA* gene fragments (765 nt) obtained with CLUSTALW was performed on 100 replicate trees, and the values of nodes recovered in more than 75% of the cases are shown. Scale bar = 2% sequence divergence.

(97.9% identity), and that sequences from strains MC1, AT2 and CM2 formed a closely related cluster (> 91% identity). Notably, *cmuC* sequences of *Hyphomicrobium* strains including the new isolates were equally distant (~47% identity) to *cmuC* and to *cmuC2* of unknown function found immediately upstream of *cmuA*, of strain CM4. This

emphasizes the lesser degree of conservation of *cmuC* despite it being essential for growth with chloromethane in strain CM4 (Vannelli et al., 1999).

The *cmuBCA* cluster organization of *cmu* genes for *Hyphomicrobium* strains isolated from *A. thaliana* leaves and for strain MC1 was the same as that found previously

for *H. chloromethanicum* CM2 and all other previously isolated strains, with the exception of *M. extorquens* CM4 (Fig. 2). In all cases where it was characterized, this single-cluster *cmu* gene arrangement also featured genes *paaE* and *hutI*, encoding a putative oxidoreductase and a putative imidazolone hydrolase, respectively. In this work, PCR reactions with *cmuA802f*-*paaE*rev1 and *cmuA802f*-*hutI*rev2 primer pairs (Table 2, and data not shown) provided evidence that *paaE* and *hutI* genes were also present in the three new strains reported here and in the same arrangement as in strain *H. chloromethanicum* CM2 (McAnulla *et al.*, 2001b).

New PCR primers for *cmuA* analysis

The published reverse primer *cmuA1609R* used for the detection of *cmuA* sequences in environmental samples (Miller *et al.*, 2004) allowed to accommodate the shorter *cmuA* sequence of *A. ciceronei*. In this work, a frameshift in the *cmuA* sequence originally reported for *A. ciceronei* IMB1 (AF307143) sequence was detected and corrected, extending its predicted *cmuA* gene from 1704 to 1851 nt. Amplification using the primer pair *cmuA802f* together with *cmuA1802r* newly designed in this work yields a larger *cmuA* gene PCR fragment of approximately 1 kb (Table 2). Similar sensitivity was achieved with the newly designed *cmuA* primers and with the previously described primers (see Fig. S1). However, the new primer *cmuA1802r* may allow the detection of a wider diversity of *cmuA* sequences, because it could be successfully used to amplify *cmuA* from marine strain *Rhodobacteraceae* 198, unlike primer *cmuA1609R* (Schäfer *et al.*, 2005) (Fig. 4). Also, the 193-bp 3'-end *cmuA* sequence also amplified with this newly defined primer pair is slightly less conserved than the sequence between primers

cmuA802f and *cmuA1609R* (see Table S1), thus potentially allowing better discrimination of *cmuA* sequences retrieved from environmental DNA.

Restriction fragment profiling of PCR-amplified *cmuA* in chloromethane-degrading enrichment cultures obtained from plant leaves

A protocol for monitoring chloromethane-degrading enrichment cultures obtained from leaves of *A. thaliana* as inocula was developed using restriction digestion of PCR-amplified *cmuA* gene fragments (Fig. 4). At the timepoint chosen to isolate chloromethane-degrading strains by plating out of the liquid enrichment culture on a solid selective mineral medium ($OD_{600\text{ nm}} = 0.6$, 8 days), the detection limits using primer pair *cmuA802f*-*cmuA1802r* developed here were typically 0.5 and 10 pg of the DNA template for the reference strain *M. extorquens* CM4 and for the enrichment culture, respectively (Fig. S1). This suggested that the chloromethane-degrading bacterial subpopulation in enrichment cultures represented about 5% of the total bacteria present in the cultures at that stage.

The amplicons obtained were digested with the restriction enzyme DdeI, which cuts one to three times and at variable positions in the *cmuA* sequence of previously characterized chloromethane-degrading strains (Schäfer *et al.*, 2005) (Fig. 4). The digestion patterns of amplicons in enrichment cultures were distinct from all reference strains, except for *H. chloromethanicum* CM2 and *Hyphomicrobium* sp. MC1. Strain AT2 showed the same pattern as the enrichment culture from which it was isolated (Fig. 4), and the same situation was found for strains AT3 and AT4 and the corresponding enrichment culture from which these strains originated (data not shown). The restriction profiling method

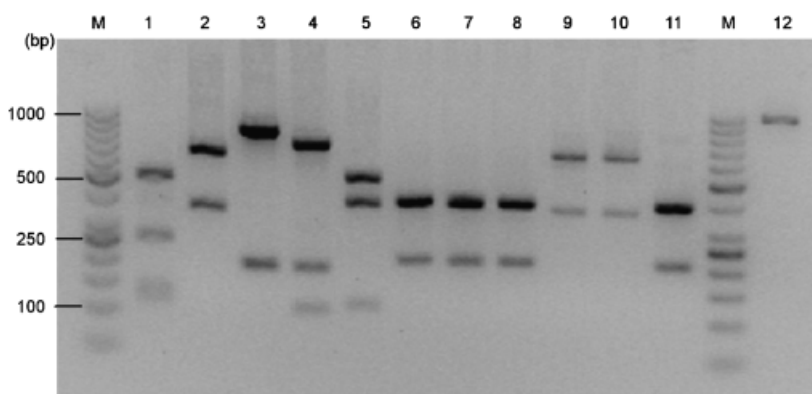


Fig. 4. DdeI restriction digestion patterns of PCR-amplified *cmuA* fragments obtained with the primers *cmuA802f* and *cmuA1802r*. M, lanes with molecular mass marker 1 kb ladder (Fermentas); lane 1, *Aminobacter* sp. IMB1; lane 2, *Aminobacter* sp. CC495; lane 3, *Rhodobacteraceae* 179; lane 4, *Rhodobacteraceae* 198; lane 5, *Methylobacterium extorquens* CM4; lane 6, *Hyphomicrobium chloromethanicum* CM2; lane 7, *Hyphomicrobium* strain MC1; lane 8, *Hyphomicrobium* strain AT2; lane 9, *Hyphomicrobium* strain AT3; lane 10, *Hyphomicrobium* strain AT4; lane 11, representative chloromethane-degrading enrichment culture with chloromethane as the sole additional carbon source ($OD_{600\text{ nm}} = 0.6$, 8 days) obtained after inoculation with a single leaf of *Arabidopsis thaliana*; lane 12, undigested PCR-amplified *cmuA* fragment of the same enrichment culture.

applied here to amplicons of 1000 nucleotides thus holds promise for a focused, time-saving exploration of chloromethane utilization genes aiming at discovering new, more divergent *cmu* gene sequences in environmental samples, and at characterizing the corresponding bacteria.

Discussion

Bacteria growing aerobically with chloromethane as the sole source of carbon and energy had previously been isolated from a variety of environments, and so far, all feature the *cmu* pathway for chloromethane utilization (Studer *et al.*, 2002; Schäfer *et al.*, 2007). However, the phyllosphere compartment of vegetation, possibly the quantitatively most important source of chloromethane (Clerbaux *et al.*, 2007), had not yet been investigated in this respect. In this work, the key gene *cmuA* involved in dehalogenation of chloromethane was detected and quantified in DNA from the leaf surface of the model plant *A. thaliana*, and three chloromethane-degrading *Hyphomicrobium* strains were isolated from enrichment cultures originating from leaves of *A. thaliana* grown with chloromethane as the sole carbon source. In addition, several degenerate primer pairs and an associated genotyping approach were developed for the detection and characterization of *cmu* genes in enrichment cultures and isolated strains.

The *Hyphomicrobium* chloromethane-degrading strains isolated in this work possessed *cmu* genes in the same arrangement as in most previously isolated strains from other ecosystems, confirming the dominant status of the *cmu* pathway in the bacterial degradation of chloromethane. However, the isolation of strains belonging to the *Hyphomicrobium* genus was unexpected, inasmuch as *Methylobacterium* strains were recently shown to be efficient leaf colonizers and predominant in the *A. thaliana* phyllosphere, with *Hyphomicrobium* likely representing only a minor contribution (Delmotte *et al.*, 2009; Knief *et al.*, 2010). Indeed, enrichment cultures obtained from plant leaves in the same medium, but with methanol as the sole carbon and energy source led to the enrichment of strains belonging to the genus *Methylobacterium* (data not shown). However, chloromethane-degrading *Hyphomicrobium* isolates also grew well with methanol as the carbon source, suggesting that *Hyphomicrobium* strains may be better adapted to growth with chloromethane than *Methylobacterium* strains, albeit in an as yet unknown way. Other aspects of *Hyphomicrobium* metabolism require further investigation, in particular, the fact that similar chloromethane dehalogenase activity was detected in cell-free extracts of all chloromethane-degrading strains despite differences in the growth rates of the strains with chloromethane (Table 1).

The demonstration of chloromethane-degrading bacteria at the surface of *A. thaliana* leaves is of relevance for the overall budget of chloromethane in the environment in the

light of current estimates for chloromethane emissions above plant areas ($\sim 1.8 \text{ Tg Cl year}^{-1}$) (Yoshida *et al.*, 2006). If indeed, as suggested from this work, some phyllosphere bacteria function as a filter for emissions of chloromethane from plants, then measurements and estimates of chloromethane emissions above plant areas will actually tend to reflect the difference between total chloromethane emissions from vegetation and bacterial degradation of chloromethane in the phyllosphere, rather than the total chloromethane potential from plants. Whether this may contribute towards explaining the deficit in identified sources of chloromethane (Clerbaux *et al.*, 2007) is a topic for further investigation. Clearly, assessing the importance of chloromethane degradation by specialized methylo-trophic bacteria in the phyllosphere will require further work, especially considering that plant emissions of methanol, itself a growth substrate for most methylo-trophic bacteria, exceed those of chloromethane by over three orders of magnitude (Nemecek-Marshall *et al.*, 1995; Rhew *et al.*, 2003).

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Analysis of PCR-amplified fragments of *cmuA* obtained with primers cmuA802f and cmuA1802r.

Fig. S2. Phylogenetic analysis of *cmuB* and *cmuC* genes in chloromethane-degrading strains.

Table S1. Sequence identity of amplified *cmuA* gene fragments.

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Figure sup. 1

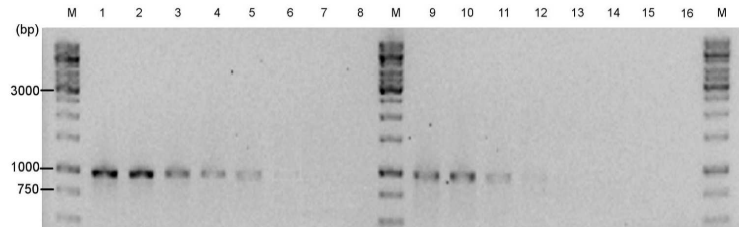


Figure S1. Analysis of PCR-amplified fragments of *cmuA* obtained with primers *cmuA802f* and *cmuA1802r*. PCR products separated by agarose gel electrophoresis were obtained from *Methylobacterium extorquens* CM4 (lanes 1 to 8) and from an enrichment culture obtained after incubation with a single *A. thaliana* leaf (lanes 9 to 16). M, lanes with molecular mass marker 1 kb ladder (Fermentas); lanes 1 and 9, 500 pg of DNA template; lanes 2 and 10, 100 pg of DNA template; lanes 3 and 11, 50 pg of DNA template; lanes 4 and 12, 10 pg of DNA template; lanes 5 and 13, 1 pg of DNA template; lanes 6 and 14, 0.5 pg of DNA template; lanes 7 and 15, 0.1 pg of DNA template; lanes 8 and 16, no DNA (water).
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Figure sup. 2

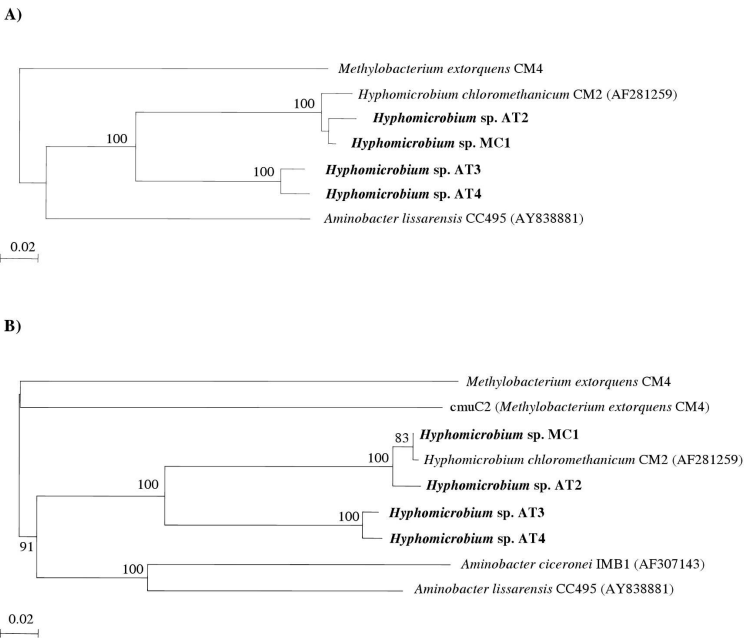


Figure S2 Phylogenetic analysis of cmuB and cmuC genes in chloromethane-degrading strains. Strains characterised in this study are shown in bold. Phylogenetic trees were constructed from multiple alignments of cmuB (A, 683 nt), and cmuC (B, 957 nt) as described in Methods. Bootstrap analysis was performed on 100 replicate trees, and values of nodes recovered in more than 75% of cases are shown. Scale bar, 2% DNA sequence divergence.
163x233mm (300 x 300 DPI)

Table S1. Sequence identity of amplified *cmuA* gene fragments.

strain n°	species or strain	sequence identity (%) ^a							
		1	2	3	4	5	6	7	8
1	<i>Hyphomicrobium chloromethanicum</i> CM2		76	78	80	99	99	84	84
2	<i>Methylobacterium extorquens</i> CM4	74		75	76	76	76	79	79
3	<i>Aminobacter lissarensis</i> CC495	70	76		85	78	78	80	80
4	<i>Aminobacter ciceronei</i> IMB1	76	76	84		81	81	81	81
5	<i>Hyphomicrobium</i> sp. strain AT2	100	74	70	76		99	84	84
6	<i>Hyphomicrobium</i> sp. strain MC1	100	74	70	76	100		84	84
7	<i>Hyphomicrobium</i> sp. strain AT3	82	72	75	75	82	82		100
8	<i>Hyphomicrobium</i> sp. strain AT4	82	72	75	75	82	82	100	

^a Upper right panel, percentage identity in sequences of the *cmuA* gene fragment between cmuA802f and cmuA1609R primers (808 bp, Miller *et al.*, 2004). Lower left panel, percentage identity in the 193 bp 3'-end region of the *cmuA* gene fragment accessible with the new reverse PCR primer cmuA1802r proposed for PCR detection of the *cmuA* gene.

**Chapter 5. Biomolecular evidence for correlated
production and consumption of chloromethane in the
phyllosphere of *Arabidopsis thaliana***

Manuscript by **Farhan Ul Haque M.**, Nadalig T., Bringel F., and Vuilleumier S.,
in preparation for *Environmental Microbiology*.

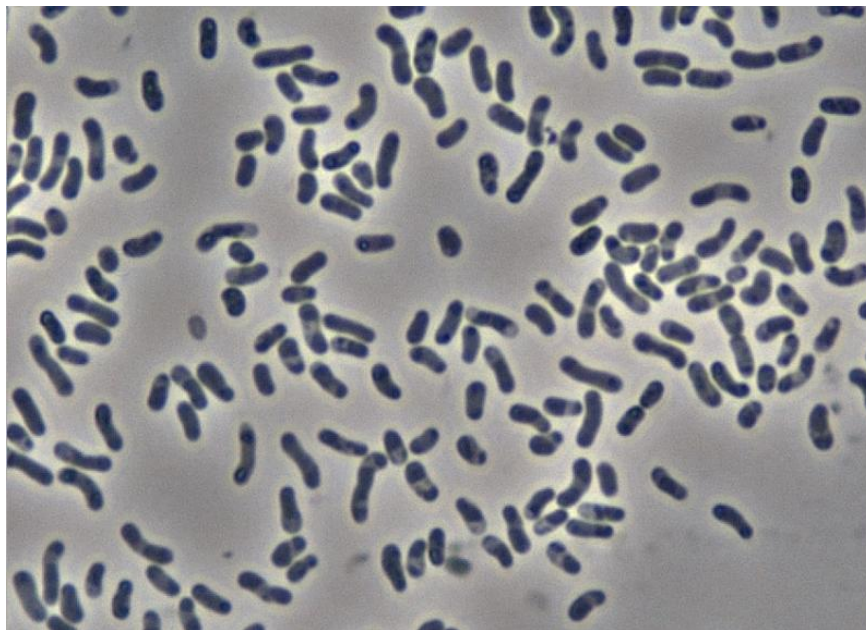
**Chapter 6. Bacterial community composition in the
phyllosphere of *A. thaliana* *HOL1* gene variants**

Chapter 7.

Conclusions and perspectives



Arabidopsis thaliana plant (picture taken by Myhammad FARHAN UL HAQUE)



Methylobacterium extorquens CM4 cells (1000x) (picture taken by Sandro ROSELLI)

The degree to which the biosphere contributes to the atmospheric burden of climate-relevant trace gases is still not well understood. Novel observations and measurements have confirmed not only the important role of the plant biosphere in emissions of one-carbon volatile organic compounds, which play a role in global warming and ozone depletion (Harren and Cristescu, 2013) , but also of the microbial compartment associated with plants in mitigating such emissions. Plants and bacteria are thought to play a key role in the global cycling of chloromethane (Clerbaux et al., 2007), but have not yet been studied together in the context of chloromethane metabolism.

This context provided the incentive to propose a PhD thesis project aimed at exploring the topic of bacterial chloromethane degradation in the phyllosphere. A laboratory model system comprising *Arabidopsis thaliana*, a well-characterized chloromethane-emitting plant (Nagatoshi and Nakamura 2009), and the most studied chloromethane degrading bacterial strain, *Methylobacterium extorquens* CM4 (Studer *et al.*, 2002), using bacterial chloromethane dehalogenase gene *cmuA* and the gene *HOL1* involved in the production of chloromethane in *A. thaliana* as molecular biomarkers.

Taken together, the results obtained in the PhD work using biomolecular approaches indirectly but conclusively confirmed that different *HOL1* variants of *A. thaliana* emit chloromethane to different extents, and that these emissions, although quite minor in comparison to emissions of methanol, affect the phyllosphere microflora, and methyl halide degrading bacteria in particular. A synthetic summary of the main conclusions of this work is given in the following, together with a brief presentation of work in progress, and an outline of proposed future experiments.

7.1. Biomolecular tools to study chloromethane-associated processes in the phyllosphere

New tools were developed to efficiently detect and estimate chloromethane emissions in the phyllosphere environment, and to follow their effect on the phyllosphere microflora, with particular emphasis on bacteria involved in chloromethane degradation. Relative expression of the *HOL1* gene responsible for chloromethane emissions in *A. thaliana* was quantified using qRT-PCR in different variants of the plant [Chapter 5]. On the other hand, basing on knowledge acquired during this work on genome sequences of both dechlorinating and non-dechlorinating Alphaproteobacteria [Chapter 2], degenerate oligonucleotide primers were developed for quantitative PCR analysis at both DNA and RNA levels that targeted the chloromethane dehalogenase gene *cmuA* of chloromethane-degrading bacteria (Nadalig *et al.*, 2011) [Chapter 4 & 5].

In addition, a highly sensitive bacterial bioreporter was developed to detect and quantify methyl halides and chloromethane in particular [Chapter 3]. A plasmid-based transcriptional reporter construct featuring a promoterless YFP gene cassette fused downstream of the *cmuA* gene promoter region afforded halomethane-dependent production of fluorescence in *Methylobacterium extorquens* CM4. The fluorescence signal was proportional to the presence of halomethane over a wide range of concentrations (pM – mM). The time required to detect onset of YFP fluorescence production was as low as 20 minutes, and the threshold concentration was approximately 2 pM (about 60 molecules of chloromethane per bioreporter cell). The characteristics of this bioreporter for detection of methyl halides are attractive, considering the high sensitivity to evaluate low level natural emissions of chloromethane from plants and other natural sources using gas chromatography. Such analytical sensitivity is out of reach for most laboratories today, and thus represents a major bottleneck on the way to establish a reliable global budget of chloromethane and other methyl halides.

7.2. Chloromethane-degrading bacteria as filters of chloromethane emissions to the atmosphere

New insights obtained in this work supported our initial working hypothesis, i.e. that chloromethane-degrading bacteria are present in the phyllosphere, and that they may act to filter plant emissions of chloromethane to the atmosphere.

First, chloromethane-degrading bacteria were detected in the phyllosphere of *A. thaliana* using both culture-dependent and culture-independent methods (Nadalig *et al.*, 2011) [Chapter 4]. Chloromethane-degrading strains were isolated from enrichment cultures of leaves of *A. thaliana*. They possessed *cmu* genes in the same arrangement as most previously described chloromethane-degrading strains isolated from other environments (Schäfer *et al.*, 2007), confirming the dominant status of the *cmu* pathway in the bacterial degradation of chloromethane.

In parallel, the abundance and expression of *cmuA* was investigated, and showed that the proportion of chloromethane-degrading bacteria on the surface of leaves of *A. thaliana*, although relatively low (<1‰), was sufficient to significantly affect net *A. thaliana* emissions of chloromethane on the basis of previously published values of chloromethane dehalogenase activity (Vannelli *et al.*, 1998) and *A. thaliana* emissions of chloromethane (Rhew *et al.*, 2003) [Chapter 5]. These observations also suggested that available values for emissions of chloromethane may actually reflect the difference between total chloromethane emissions from vegetation and bacterial degradation of chloromethane in the phyllosphere, rather than the total potential of plants for emissions of chloromethane

7.3. *HOL1*-dependent bacterial diversity and activity in the *A. thaliana* phyllosphere

Preliminary data obtained in this work suggest that *HOL1* genotype and expression may modulate bacterial diversity and activity in the *A. thaliana* phyllosphere. Analysis of the bacterial compartment of the phyllosphere of different *HOL1* variants of *A. thaliana* using 454 pyrosequencing of PCR amplicons of the variable region of the 16S rRNA gene [Chapter 6] indicated minor differences in community structure in *A. thaliana* *HOL1* variants. Methylophilic bacteria belonging to the genus *Methylobacterium* were highly dominant irrespectively of the variant genotype in the performed experiments, yet abundance of *Sphingomonas*, a genus of which several phyllosphere-isolated strains had been shown to

be involved in plant defense against pathogens (Innerebner *et al.*, 2011; Vogel *et al.*, 2012), varied significantly among the three *HOL1* gene variants investigated.

However, the observed slight differences in bacterial community structure [Chapter 6] of the *HOL1* variants might not only be due to differences in chloromethane emissions, but could follow from production of other volatile compounds (such as methyl thiocyanate) or the metabolism of glucosinolate metabolic products previously reported to affect the bacterial population and community composition in the phyllosphere (Ruppel *et al.*, 2008; Schreiner *et al.*, 2009), and also dependent on the activity of the HOL1 enzyme [see Chapter 1].

Indeed, *in planta* visualization by confocal laser scanning microscopy of the phyllosphere of *HOL1* variants of *A. thaliana* exposed to the chloromethane-induced bioreporter developed in this work [Chapter 3], confirmed our working hypothesis that different *HOL1* gene variants of *A. thaliana* emitted different amounts of chloromethane [Chapter 5]. Further, evidence obtained through qPCR analyses of the three investigated *A. thaliana* variants (i.e wild type, *hol1-1* knock-out mutant plants and HOL1-OX6 plants overexpressing *HOL1* gene) strongly suggested that *HOL1* gene expression in plants closely correlated with bacterial chloromethane metabolism, and specifically with the relative expression and abundance of the chloromethane dehalogenase *cmuA* gene [Chapter 5], which was also confirmed in this work to be accompanied by dehalogenation of chloromethane [Chapter 3]. Taken together, these data strongly suggest that plant emissions of chloromethane, however minor compared to those of methanol for example (Clerbaux *et al.*, 2007; Bracho-Nunez *et al.*, 2011) (Table 1.3), may provide a selective advantage for growth of chloromethane-degrading bacteria in the phyllosphere, for reasons which need to be understood in more detail in future work.

7.4. Perspectives for future work

The insights obtained in the different aspects of this project suggest many avenues for further research in order to gain a better understanding of chloromethane degradation by bacteria in the phyllosphere.

7.4.1. Sensitive detection and quantification of chloromethane and other volatile metabolites emitted in the phyllosphere

Direct measurements of chloromethane emissions from the phyllosphere are clearly still required to complete the experiments performed, and to confirm the evidence gained by biomolecular tools for differential emissions of chloromethane in the different *HOLI* variants of *A. thaliana* investigated. However, the amounts of chloromethane emitted by *A. thaliana* may in some cases be below the detection or quantification limits of analytical instruments, at least when no pre-concentration step is used before analysis. For example the sensitive limits for chloromethane detection based on GC/MS methods described previously are 1 - 100 ppt (μM range) or even higher (McClenny *et al.*, 2002; Rhew *et al.*, 2003; Blei and Heal, 2011; Derendorp *et al.*, 2011). While the bioreporter developed in this work may help in this context, the use of stable isotope analysis will also be considered. Stable isotope analysis is a powerful tool to constrain fluxes of chloromethane between various environmental compartments (Greule *et al.*, 2012). In collaboration with the team of Frank Keppler of the Max Planck Institute of Chemistry in Mainz, Germany, our group has measured hydrogen and carbon isotope fractionation of the remaining untransformed chloromethane following its degradation by *Methylobacterium extorquens* CM4 (Nadalig *et al.*, submitted) Use of this technique may help to further demonstrate the existence of both chloromethane emissions by *A. thaliana* and its consumption in the phyllosphere.

In addition, the emissions from the phyllosphere with respect to differential *HOLI* expression in *A. thaliana* variants also need to be investigated further. Plant metabolites emitted by *A. thaliana* may be detected by gas chromatography coupled to mass spectrometry (GC-MS) (Brodersen *et al.*, 2012). For these experiments, the phyllosphere of *HOLI* gene variant plants grown from sterile seeds, under axenic conditions on MS medium in closed jars using well-established laboratory procedures, will be analyzed. Targeted volatile compounds to be monitored will include chloromethane and other volatile chemicals such as C-1 compounds (CO_2 , methanol, and formaldehyde), plant leaf reference metabolites such as phytohormones (indole-3-acetic acid, cytokinin), monoterpenes and ethylene. Other metabolites associated with the *HOLI* gene, e.g. thiocyanate and methylthiocyanate (Nagatoshi and Nakamura, 2009) will also be followed. Gases will be captured and extracted using a gas flux column for given periods of time to help concentrate and circumvent temporal fluctuations of plant-

emitted volatile compounds. Headspace volatile organic compounds can be collected using glass jars or using Teflon bags and measured by thermodesorption-GC-MS (TD-GC-MS) (Houshyani *et al.*, 2013; Pearse *et al.*, 2013). This work, planned in collaboration with IBMP, CNRS Strasbourg, will require substantial further funding, which has been applied for in the context of the ANR BIOADAPT program.

7.4.2. Co-localization of expression of *HOL1* and *cmuA* gene

Confocal microscopy has strongly suggested a stomatal localization of the chloromethane-degrading bacteria expressing the *cmuA* gene [Chapter 5]. However, the precise localization at the cellular level of *HOL1* expression, and hence of chloromethane production, remains to be investigated. A first version of a reporter construct consisting of the *HOL1* gene fused at its C-terminal to the gene for green fluorescent protein (GFP). Constitutive expression of the cDNA encoding the HOL1-GFP fusion is driven by the 35S promoter. The functionality of the HOL1-GFP construct currently at hand will be assayed in a transient expression platform system using *Nicotiana benthamiana* and confocal microscopic observation (Kanagarajan *et al.*, 2012). Using this construct, transgenic *A. thaliana* plants will be developed to visualize the HOL1 protein distribution *in planta*. The potential co-localization of *HOL1* gene expression and chloromethane-induced bacterial colonizers will be investigated. As a further potential development, different *A. thaliana* transgenic lines harboring the engineered HOL1-GFP reporter, potentially expressed in a controlled fashion, could be inoculated with bacterial CM4 wild-type bioreporter expressing another type of fluorescence (mCherry) under the control of the *cmuA* promoter induced by chloromethane. In this way, the localization of bacterial mCherry- and plant GFP-tagged fluorescent proteins may be detected independently and simultaneously to provide new information on the spatial relationships between chloromethane production and consumption in the phyllosphere environment and thereby better evaluate the adaptive response of chloromethane degrading bacteria in this habitat. This strategy may also allow to address the question of a potential selective advantage provided by the chloromethane degrading ability of bacteria in colonization of the phyllosphere. Alternatively, this question may also be investigated by competition experiments such as those developed by the Vorhjolt group (Knief *et al.*, 2010a; Innerebner *et al.*, 2011). For such experiments, we envisage to inoculate *A. thaliana* plants with varying

expression of *HOL1* gene (wild type, *HOL1* overexpressor, and *hol1* knockout mutant plants) with *M. extorquens* CM4 wild-type, and mutants thereof unable to grow with chloromethane and/or methanol, and containing plasmids encoding fluorescent proteins with the corresponding genes under the control of either constitutive or chloromethane-induced promoters. In this case as well, the colonization patterns of these strains in the phyllosphere of different *A. thaliana* plants may be evaluated both by confocal observations and by qPCR analysis, as described in this work.

7.4.3. Functional diversity of chloromethane degrading bacteria

Several approaches can be used to investigate the adaptive response of epiphytic communities to chloromethane in the phyllosphere of *A. thaliana* plants with different emissions of chloromethane. Ongoing rapid developments in sequencing technology (Wang *et al.*, 2012b) allow to envisage further experiment basing on our laboratory bipartitemodel, under either laboratory or environmental conditions of chloromethane concentration, and at both DNA and RNA levels (Fig. 7.1).

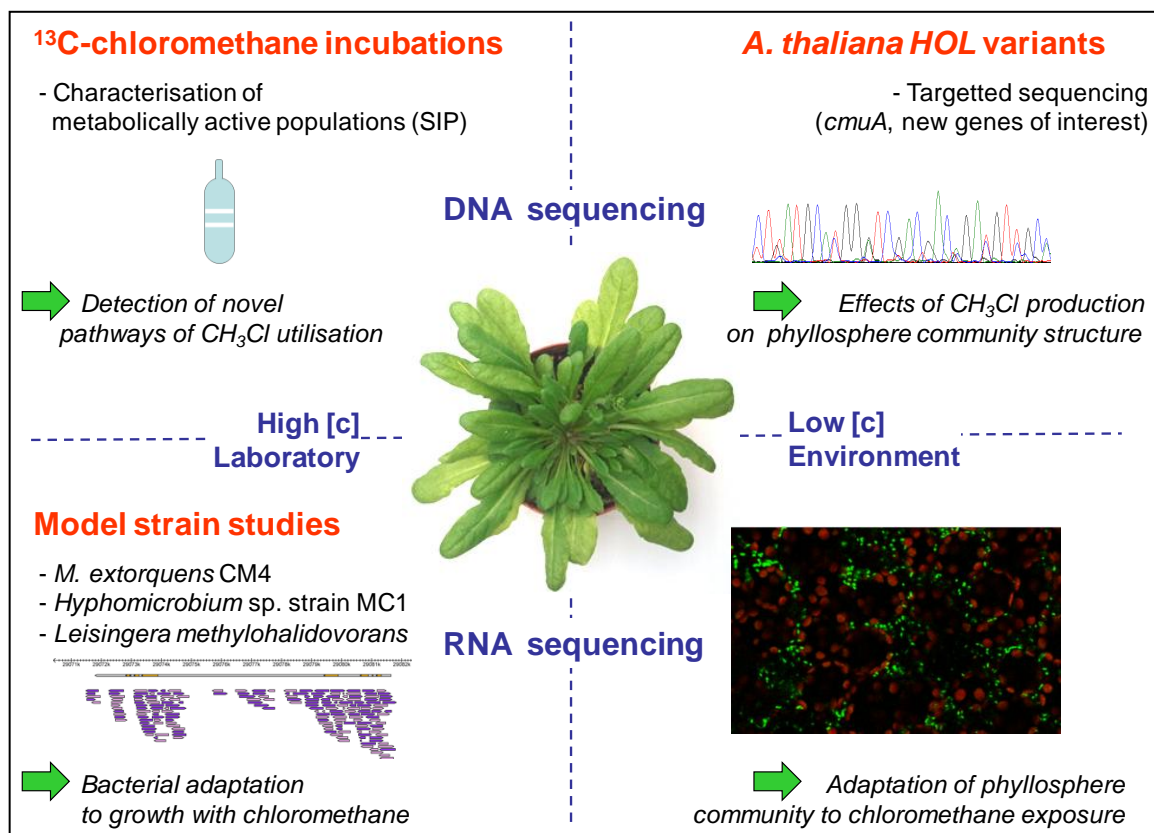


Fig. 7.1. Current perspectives for the *A. thaliana*-based model developed in the project. Ongoing rapid developments in sequencing technology allow to envisage further work on the developed model under methyl halide concentration regimes typical of laboratory conditions (left) or environmental conditions (right), and at the DNA level (top) as well as at the RNA level (bottom).

An immediate and already ongoing approach (top right, see Chapter 6) will consist in high-throughput sequencing of PCR amplicons of *cmuA* obtained from total DNA from leaf surfaces of different HOL variants using 454 pyrosequencing technology, as well as random shotgun sequencing of this DNA using Illumina technology.

Other genes of interest, identified by ongoing RNAseq studies of model strains designed to characterise the adaptations involved in metabolism of chloromethane (bottom left), may also be investigated by this approach.

Further, stable isotope probing approaches involving exposure of *A. thaliana* to artificially high concentrations of ¹³C-labelled chloromethane (top left), by affording the isolation by ultracentrifugation of “heavy” ¹³C-labelled DNA enriched in genetic material from metabolically active chloromethane-degrading populations, may uncover novel pathways for utilisation of chloromethane beyond the *cmu* pathway characterised in model chloromethane-degrading strains such as *M. extorquens* CM4 and *Hyphomicrobium* sp. strain MC1.

The outcome of these different approaches will feed in the ultimate and most demanding long-term objective of this work (bottom right), i.e. the characterisation of the functional adaptation of bacterial communities in the phyllosphere to exposure to ambient concentrations of chloromethane.

One immediately accessible approach will be to develop the pyrosequencing experiments initiated on the structural diversity of the phyllosphere based on the 16S rRNA gene [Chapter 6], by focussing on amplification of the chloromethane dehalogenase *cmuA* gene from nucleic acids extracted from the phyllosphere of the three *HOLI* variants of *A. thaliana*. In order to identify further functional genes of interest in the context of chloromethane metabolism, the team has recently embarked on applying the RNA-seq approach for global transcriptomic analysis of chloromethane utilization by strain *M. extorquens* CM4 (project led by Françoise Bringel and funded by IBiSA Génoscope, France). In the RNASeq approach, purified total mRNAs are transcribed into cDNA, and subjected to high-throughput deep sequencing. The obtained sequence reads are mapped onto a reference genome sequence to infer expressions of transcripts in the investigated samples (Haas *et al.*, 2012; Wang *et al.*, 2012a; Westermann *et al.*, 2012). This project is expected to define the global adaptive response of bacteria to chloromethane through the discovery of transcriptional units specifically expressed in chloromethane-grown versus methanol-grown cultures.

Knowledge of *cmu* genes identified in this RNA-seq project will be useful in metagenomics studies of the *A. thaliana* phyllosphere. However, chloromethane-degrading strains exist that do not have *cmu* genes [Chapter 2], and which metabolize chloromethane by yet unknown pathways. To detect such bacteria in the environment, and characterize the pathways of corresponding strains which have been isolated, stable isotope probing DNA-SIP may be used, a method in which the growth substrate is labelled by a stable isotope and leads to incorporation of the label in the biomass (including DNA and proteins) of metabolically active bacteria. Today, this approach is applied in combination with metagenomics in particular (Chen *et al.*, 2011; Uhlik *et al.*, 2013). (Chen *et al.*, 2011; Henneberger *et al.*, 2013). In the context of the present project, enrichment of epiphytic communities capable of using ^{13}C -labelled chloromethane may help uncover the phyllosphere chloromethane degradome, defined as the metagenome of chloromethane-utilizing epiphytic communities, and thereby investigate the diversity of chloromethane-utilization pathways associated with the phyllosphere beyond the *cmu* pathway. Funding for this work has also been applied for in the context of the ANR BIOADAPT program.

The different approaches outlined in Fig. 7.1 have the potential to achieve the long-term objective of the team of characterizing the functional adaptation of bacteria and microbial communities to chloromethane. Clearly, the work reported in this manuscript only represented a first attempt to unravel chloromethane-related metabolism at the low ambient concentrations encountered in the environment. The tools developed in this project will now also be used to follow chloromethane emissions and its degradation in different environments. For example, a PhD project (“thèse en cotutelle” of Pauline Chaignaud, in collaboration with University of Bayreuth, codirected by Françoise Bringel and Steffen Kolb and cofunded by the German DFG and Région Alsace), was recently initiated in the lab to explore bacterial chloromethane metabolism in forest soils, basing on studies of global gene expression in reference dehalogenating strains. This project is expected to contribute to the team’s overall objectives of developing effective bacterial bioindicators and bioreporters to answer ecological questions of adaptation and response to environmental stress specifically addressing bacterial dehalogenative metabolism of chlorinated methanes in particular, but also of chlorinated pollutants in general, in natural environments.

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Scientific communications

Peer-reviewed publications

1. Nadalig T, **Farhan Ul Haque M**, Roselli S, Schaller H, Bringel F, Vuilleumier S. 2011. Detection and isolation of chloromethane-degrading bacteria from the *Arabidopsis thaliana* phyllosphere, and characterization of chloromethane utilisation genes. *FEMS Microbiol. Ecol.* **77**:438-448.
2. Vuilleumier S, Nadalig T, **Farhan Ul Haque M**, Magdelenat G, Lajus A, Roselli S, Muller EEL, Gruffaz C, Barbe V, Médigue C, Bringel F (2011) Complete genome sequence of the chloromethane-degrading strain *Hyphomicrobium* sp. strain MC1. *J. Bacteriol.* 193: 5035-5036.
3. Bois P, Huguenot D, Norini MP, **Farhan Ul Haque M**, Vuilleumier S, Lebeau T (2011) Herbicide degradation and copper complexation by bacterial mixed cultures from a vineyard stormwater basin. *J. Soils Sedim.* 11: 860-873.
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5. **Farhan Ul Haque M**, Nadalig T, Bringel F, Vuilleumier S. A fluorescence-based bioreporter for the specific detection of methyl halides. Submitted to *Appl. Environ. Microbiol.*
6. **Farhan Ul Haque M**, Nadalig T, Bringel F, Mutterer J, Schaller H, Vuilleumier S. Biomolecular evidence for correlated production and consumption of chloromethane

in the phyllosphere of *Arabidopsis thaliana* (in prep. Target journal *Environ. Microbiol.*)

Oral communications

1. **Farhan Ul Haque M**, Nadalig T, Schaller H, Bringel F, Vuilleumier S (2012) Is there a bacterial sink for plant emissions of chloromethane? Séminaire de Microbiologie de Strasbourg 2012, March 15, Strasbourg, France.

Poster presentations

1. **Farhan Ul Haque M**, Nadalig T, Schaller H, Bringel F, Vuilleumier S (2012) Development of a bioreporter assay for the detection of methyl halides. ISME14 (14th International symposium on microbial ecology), August 19-24, Copenhagen, Denmark.
2. **Farhan Ul Haque M**, Nadalig T, Schaller H, Bringel F, Vuilleumier S (2012) Is there a bacterial sink for plant emissions of chloromethane? Gordon conference on 'The molecular basis of microbial one carbon metabolism', August 4-9, Bates College, Lewiston, Maine, USA.
3. **Farhan Ul Haque M**, Schaller H, Nadalig T, Bringel F, Vuilleumier S (2012) Towards detection and quantification of bacterial chloromethane utilization in the *Arabidopsis thaliana* phyllosphere. Forum Réalise, March 29, Strasbourg, France.