A fluorescence-based bacterial bioreporter

for the specific detection of methyl halide emissions in the environment

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Running title: Chloromethane bioreporter

Keywords: chloromethane, methyl halides, bioreporter, cmuA, YFP, fluorescence,
Methylobacterium

Abbreviations used:

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Abstract

Methyl halides are volatile one-carbon compounds responsible for substantial depletion of stratospheric ozone. Among them, chloromethane (CH$_3$Cl) is the most abundant halogenated hydrocarbon in the atmosphere. Global budgets of methyl halides in the environment are still poorly understood due to uncertainties in their natural sources, mainly from vegetation, and their sinks, which include chloromethane-degrading bacteria. A bacterial bioreporter for the detection of methyl halides was developed on the basis of detailed knowledge of the physiology and genetics of Methylobacterium extorquens CM4, an aerobic Alphaproteobacterium which utilizes chloromethane as the sole source of carbon and energy. A plasmid construct with the promoter region of the chloromethane dehalogenase gene $cmuA$ fused to a promotorless yellow fluorescent protein gene cassette resulted in specific methyl halide dependent fluorescence when introduced into M. extorquens CM4. The bacterial whole-cell bioreporter allowed detection of methyl halides at femtomolar levels, and quantification at concentrations above 10 picomolar (approx. 250 ppt). As shown for the model chloromethane-producing plant Arabidopsis thaliana in particular, the bioreporter may provide an attractive alternative to analytical chemical methods to screen for natural sources of methyl halide emissions.
Introduction

Methyl halides (monohalomethanes) such as chloromethane are volatile hydrocarbons of environmental concern because of their toxicity to living organisms and their role in depletion of stratospheric ozone (1, 2). Chloromethane (CH\(_3\)Cl), a gas and the most abundant halogenated hydrocarbon in the atmosphere (currently ~550 ppt, with an approximate increase of 2.3 - 2.7 ppt annually), is considered to be responsible for over 15% of chlorine-catalyzed destruction of stratospheric ozone (2). Bromomethane (CH\(_3\)Br) also catalyzes the destruction of stratospheric ozone (2), and iodomethane (CH\(_3\)I) was shown to influence aerosol formation in the marine boundary layer (3).

Global emissions of chloromethane were recently estimated at 4.1 – 4.4 Tg (4), with industrial sources contributing to less than 10% of total emissions (5). Natural production of other methyl halides appears to weaker by one order of magnitude at least (2, 6). Natural sources of methyl halides are mainly living vegetation (7, 8), wood rot fungi (9), dead plant material (10), biomass burning, oceans, and coastal waters (11). A thiol methyltransferase involved in the production of methyl halides was first isolated from the leaves of *Brassica oleracea* (12). In *Arabidopsis thaliana*, the S-adenosylmethionine-dependent methyltransferase gene *HOL* (Harmless to Ozone Layer) was then shown to be involved in the production of methyl halides (13, 14). More recent work on cloned versions of a large series of homologs of this gene from plants, fungi and bacteria confirmed that the corresponding enzymes may produce all three methyl halides, further suggesting that methyl halide production is widespread in the living world (15). In addition, marine bacteria capable of producing methyl halides have also been isolated and characterized (16).

The global budgets of methyl halides are still poorly understood (2). This is due to large uncertainties in the sources described above, but also in the sinks of these compounds, which include oxidation by hydroxyl radicals, loss to the stratosphere and to polar ocean waters, uptake by soils, and bacterial degradation (6, 17). Current efforts to constrain the biogeochemical cycles of
methyl halides involve analytical approaches such as gas chromatography-mass spectroscopy (GC-MS), including stable isotope techniques for carbon and hydrogen elements (17, 18, 19). These methods are time- and labor-intensive, and this may constitute a drawback for screening potential sources of methyl halides in the environment. Bioreporter technology based on knowledge of gene expression and enzyme functions related to the molecules of interest represents a valuable alternative to analytical techniques in this context (20, 21).

Insights on the biological transformation of methyl halides have become available from studies on the physiology and genetics of bacteria that can degrade methyl halides and utilize chloromethane as the only source of carbon and energy for growth, which have been isolated from various environments including soils (22, 23, 24, 25), sludge (26, 27, 28), seawater (29), and the phyllosphere (30). The biochemistry and genetics of chloromethane degradation have been elucidated in detail for *Methylobacterium extorquens* CM4 (31, 32, 33, 34), a strain isolated from soil of a petrochemical factory in Tatarstan (23), and the complete genome sequence of this strain was determined and analyzed (35, 36). Chloromethane dehalogenase consists of corrinoid methyltransferase CmuA and tetrahydrofolate-dependent methyltransferase CmuB (33, 34). It transforms bromomethane and iodomethane as well as chloromethane, and its expression was shown to be strongly induced by chloromethane (31). Indeed, dehalogenase proteins CmuA and CmuB were detected in strain CM4 grown in the presence of chloromethane, but not when methanol was used as the sole carbon source (36). Promoter regions and transcription start sites of chloromethane dehalogenase genes *cmuA* and *cmuB* were identified upstream of the corresponding genes (32).

In this work, a promoter-based bioreporter derivative of strain CM4 affording methyl halide-dependent production of fluorescence from a plasmid-encoded yellow fluorescent protein (YFP) was constructed, characterized in terms of the specificity and sensitivity of its response to methyl halides, and its potential for the detection of methyl halide emissions by plants was shown.
Materials and Methods

Chemicals and reagents
All chemicals and reagents (purity > 99%) were obtained from Sigma-Aldrich unless otherwise stated. Buffers, culture media and solutions were prepared in ultra-pure water (PURELAB classic, ELGA) and sterilized by autoclaving (20 min at 121°C under 1 bar) or by filtration (0.2 µm, Nalgene).

Bacterial strains, growth media and cultivation conditions
A laboratory stock of the chloromethane-degrading strain *M. extorquens* CM4 was used. *Methylobacterium* was cultivated in chloride-free mineral medium M3 as described (36). Bacterial strains were cultivated in Petri dishes on solid medium supplemented with methanol (MeOH) incubated in air-tight glass jars at 30°C. Isolated colonies were used as inocula for liquid cultures performed in 50 mL M3 medium in Erlenmeyer flasks fitted with airtight mininet valve caps (Supelco) at 30°C with 100 rpm agitation, with methanol (10 mM) and/or chloromethane (10 mM) as carbon source. Growth was monitored by measuring optical density at 600 nm (OD$_{600}$). *E. coli* TOP10 (Invitrogen) was grown at 37°C on Luria Bertani rich medium (LB, Difco laboratories). Kanamycin was added at 50 µg mL$^{-1}$ final concentration as required.

RNA isolation
Total RNA was extracted from samples of cultures of *M. extorquens* taken in early, mid and late exponential growth phase (OD$_{600}$ 0.06 - 0.1, 0.15 - 0.3 and > 0.35 respectively) using the NucleoSpin RNAII kit (Macherey-Nagel). RNAProtect (solution Qiagen) was used to stabilize RNA in bacterial culture samples (4 mL, OD$_{600}$ ≥ 0.06) as per manufacturer’s recommendations. Cell pellets were kept frozen at -80°C until further processing. Cells were lysed by lysozyme treatment (2 mg/mL final concentration) in 100 mL TE buffer at 37°C for 15 min prior to RNA
extraction following the protocol provided by the manufacturer. RNA preparations were treated with amplification grade DNase I (Invitrogen) for 1 hour at 25°C, and purified again using the NucleoSpin RNAII kit. RNA concentration was estimated spectrophotometrically at 260 nm (NanoDrop ND1000) and its quality was verified by its $A_{260}/A_{280}$ ratio.

Reverse transcription and quantitative PCR

cDNA was prepared with the SuperScript III Reverse Transcriptase Kit (Invitrogen), using 50 ng of DNase-treated RNA and 250 ng of random hexamers (Roche) following manufacturer’s instructions. Control reactions without reverse transcriptase were performed to check the absence of contaminating DNA. Quantitative PCR measurements on cDNA preparations were done in 96-well reaction PCR plates using a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Primers cmuA802F (5’- TTCAACGCGAYATGTATCCYGG-3’ (37)) and cmuA968R (5’-CCRCCRTTRTAVCCVACYTC-3’, (30)) were used for amplification of the cmuA gene, and amplification of the 16S rRNA gene $rrnA$ was performed with primers BACT1369F and PROK1492R (38). PCR reactions were carried out in a final volume of 20 µL, using 4 µL of cDNA preparation (diluted with ultrapure molecular biology grade water, Sigma) with 3 µM final concentration of each primer and a commercial 1 x SYBR Green PCR mix (Eurogentec). The PCR program included a 10 min denaturation step at 95°C, followed by 40 cycles of 15 s of denaturation at 95°C and 1 min of hybridization/polymerization at 60°C. Relative gene expression levels were calculated using the comparative threshold amplification cycle (Ct) method ($2^{-\Delta\Delta Ct}$) as described (39). Gene copy numbers were determined using known amounts of $M.\ extorquens$ CM4 total DNA as the reference.

Dehalogenase activity

Chloride released by dehalogenation of chloromethane into M3 medium by strain CM4 in the presence of chloromethane was measured in culture supernatants of centrifuged samples (1 mL).
Concentration of chloride was measured spectrophotometrically at 340 nm by the method of Jörg and Bertau (40), as $(\text{FeCl})^{2+}$ formed in acidic medium by comparison to a calibration curve of sodium chloride standards (0 – 20 mM solutions) in M3 medium.

Construction of reporter plasmid pME8266

The promoter region of the *cmuA* gene of strain CM4 (p*cmuA*) (32) was amplified from promoter probe plasmid pME1791 (32) as a 801 bp-long PCR fragment spanning exactly the intergenic region between *purU* and *cmuC2* (Fig. S1) by using PCR primers 5’-ATTTTGAGCTCGAGCGATTCCCCTCGTC-3’ and 5’-ATTTTGGTACCCTAGACGGCACCAGATGC-3’, thereby introducing SacI and KpnI restriction sites (underlined) for subsequent cloning. PCR was performed in a total volume of 50 µL comprising 1 unit of Iproof high fidelity polymerase (Bio-Rad), 10 µL of high fidelity PCR buffer (5 x), 0.2 µM of each primer, 0.1 µM of each dNTPs, and 100 ng of plasmid pME1791 template.

The PCR program included a 2 min denaturation step at 95°C, followed by 30 cycles of 20 s of denaturation at 95°C, 30 s of hybridization at 62°C, 30 s of polymerization at 72°C, and a final 3 min post-elongation step at 72°C. The resulting PCR fragment was purified after agarose gel electrophoresis using the GeneClean Turbo kit (MP Biomedicals), digested overnight with enzymes SacI and KpnI (Fermentas), and purified again using the same kit. The digested PCR fragment was ligated for 24 hours at 14°C with KpnI- and SacI-digested promoter probe plasmid pLM.syfp2 (Fig. S1), which features a promotorless gene for YFP downstream of its multiple cloning site and a kanamycin resistance gene (41), and transformed into One Shot TOP10 chemically competent cells (Invitrogen) according to the manufacturer’s instructions. Kanamycin-resistant colonies were selected and plasmid pME8266 featuring the p*cmuA*-syfp2 fusion was prepared from one transformant using the NucleoSpin plasmid kit (Macherey-Nagel), after confirmation of plasmid identity by colony PCR and sequencing. Plasmid pME8266 was introduced into *M. extorquens*...
CM4 by electroporation and selection on M3-MeOH-kanamycin plates as described previously (42).

**Fluorescence microscopy**

Aliquots (5 mL) of bacterial cultures grown to mid-exponential phase in M3 medium with MeOH (10 mM) and chloromethane (10 mM), either alone or in combination, were filtered through 0.2 µm Whatman polycarbonate membrane filters. Filters were stained with 4,6-diamidino-2-phenylindole (DAPI, 1 µg/mL solution in water), placed in the dark for 15 minutes, washed twice in sterile ultra-pure water and then in ethanol (70%), and mounted on glass slides using mounting oil (BacLight, Molecular Probes). Images were taken using a Leica DM4000 fluorescence microscope (Leica Microsystems) at 1000x magnification operated with either a YFP filter cube (excitation filter: BP 490/20; dichromatic mirror 510 nm) or a DAPI filter (BP 360/40; dichromatic mirror 400 nm).

**Fluorimetric analysis**

Samples of growing cultures (1 mL) were washed and resuspended in M3 medium at a final OD$_{600}$ of 0.05. Cell suspensions (200 µL) were transferred to a 96-well microtiter plate (Nunc), and OD$_{600}$ and YFP fluorescence (excitation: 485 nm, emission: 516 nm, bandwidth 20 nm) were measured at room temperature in a microplate reader (Synergy HT, BioTek). YFP fluorescence values were corrected by subtracting background values obtained for M3 medium, normalized to OD$_{600}$ = 1, and expressed in percentage of the maximum observed YFP fluorescence (see Fig. 5).

To follow fluorescence induction after exposure to various compounds, a pre-culture of the reporter strain was grown in M3 medium supplemented with MeOH (20 mM) and kanamycin (50 µg/mL) until late exponential phase of growth (OD$_{600}$ ~ 0.3 – 0.4). Cells were centrifuged, washed and resuspended in M3 medium to a final OD$_{600}$ of 0.2. Cell suspensions (5 mL) were then exposed to compounds of interest in Hungate tubes (17 mL total volume). Chloromethane, dichloromethane, succinate, MeOH and NaCl were provided at 20 mM; iodomethane, chloroform, tetrachloromethane...
were used at 200 µM to avoid potential toxic effects (e.g. (22, 31)). Samples (200 µL) were taken after 3 hours, transferred to 96-well microtiter plates and subjected to fluorimetric analysis as described above.

For determination of concentration dependence of chloromethane-induced fluorescence, serial tenfold dilutions of chloromethane gas, designed to yield final concentrations of chloromethane in the 2 fM – 20 mM range, were prepared in airtight Hungate tubes. For the initial dilution, 2.5 mL of chloromethane gas was added to an empty airtight Hungate tube (17 mL) using an airtight syringe. The tube was left to equilibrate for 5 min and 1.7 mL of the gas phase was transferred to the second tube, and the procedure was repeated for each further dilution. For iodomethane, serial tenfold dilutions were prepared similarly, starting from a 100 mM iodomethane stock solution. Cell suspensions (5 mL) of methanol-grown bacterial reporter were then added to each tube as described above. The initial concentration of chloromethane gas was checked by measuring chloride released from chloromethane in the medium after prolonged incubation.

To screen plants for methyl halide emissions, fresh leaves (1-6 g) of living plants were collected, weighed and incubated at room temperature in 300 mL Erlenmeyer flasks fitted with airtight mininert valve caps (Supelco). After 24 h of incubation at room temperature, headspace gas (50 mL) was sampled from each flask and injected into 60 mL flasks containing 5 mL of methanol-grown bioreporter cell suspension (OD \(_{600}\) = 0.2) from which 50 mL headspace gas had previously been removed. As controls, cell suspensions were exposed to chloromethane (20 mM) and methanol (20 mM) under the same conditions. After 3 hours incubation at 30°C, fluorescence of bioreporter cell suspensions were determined as described above, and expressed as YFP fluorescence per g (fresh weight) of leaf material relative to the fluorescence intensity observed for 20 mM chloromethane.

Confocal microscopy of \textit{A. thaliana} exposed to the bioreporter strain
A. thaliana wild-type Col-0 was grown in Petri dishes of Murashige and Skoog medium including vitamins (Duchefa) supplemented with 1% sugar and 0.7% pastagar, at 22°C with 12 hours light period for 14-days in a phytotron (Plant Climatics). A cell suspension (5 mL OD$_{600}$ = 0.2 per Petri dish containing 20 plants) of either bioreporter strain CM4(pME8266) or control strain CM4(PLM.syf2), grown to mid-exponential phase in M3 medium with MeOH as the sole source of carbon and energy, was overlaid uniformly on leaves and left to evaporate for 1 hour under a laminar flow hood. After incubation for 24 h at 22°C, leaves were removed, mounted on microscope glass slides and visualized for YFP fluorescence using the 20x lens of a Zeiss LSM710 confocal laser scanning microscope and the YFP filter cube (excitation filter 488 nm). Fluorescent cell per mm$^2$ were counted with the ‘Find maxima imageJ’ tool of ImageJ software (http://rsbweb.nih.gov/ij/index.html), using a value of 50 for the noise setting. In order to check for strain and plasmid content, qPCR analysis of genes cmuA, syfp2 and rrlA was performed on DNA extracted from the investigated leaf material, as described above and using primers ACAAGCAGAAGAACGGCATC and GCTTGGACTGGTAGCTCAGG for the syfp2 gene.

**Statistical analysis**

Experiments were performed in at least two biological replicates, with technical repeats for each biological replicate. Data are presented as the mean with standard deviation. Data were analyzed using Student’s t-test, with different letters in the Figures indicating statistically significant differences at p < 0.05.
Results

Chloromethane-dependent induction of chloromethane dehalogenase in *Methylobacterium extorquens* CM4

Strong chloromethane-dependent gene expression in *M. extorquens* CM4 was demonstrated, in previous work with *xylE* fusion constructs of upstream regions of chloromethane dehalogenase genes (32), suggesting the potential of the corresponding sequences for the development of a bacterial bioreporter for detection of chloromethane. This was investigated further in this work, and the chloromethane-dependent transcription profile of *cmuA* was determined using qPCR during growth of strain CM4 under different conditions. In particular, the effect of methanol, a major substrate for methylotrophic growth, and of the growth phase, were investigated. Total RNA was extracted from cultures grown with chloromethane alone, methanol alone or a mixture of both compounds (Fig. 1). Transcript levels of the *cmuA* gene relative to those of the 16S rRNA gene was higher by two orders of magnitude during growth with chloromethane, both in the absence and the presence of methanol, than during growth with methanol alone. This effect was observed throughout growth and in all phases of growth. No diauxic behavior was observed in cultures to which both chloromethane and methanol were provided together as carbon and energy sources at 10 mM each (Suppl. Fig. S2A), as confirmed by measurements of chloride release into the culture medium (supplementary Fig. S2B), and the final OD$_{600}$ in stationary phase was approximately double of that with 10 mM chloromethane or methanol alone. Taken together, these findings showed that strain CM4 expresses *cmuA* in a specific and chloromethane-dependent manner, throughout growth (Fig. 1), and independently of the presence of methanol.

Development of a bacterial bioreporter for detection of chloromethane

The 801 bp intergenic region upstream of the gene cluster including *cmuA* contains an experimentally characterized start site of chloromethane-induced transcription (32). The
corresponding sequence was cloned upstream of the promoterless gene for YFP in promoter probe vector pLM.syfp2 (41), yielding plasmid pME8266. Strain M. extorquens CM4(pME8266) was fluorescent when grown on chloromethane (Fig. 2) and showed no significant differences in the levels of chloromethane-induced fluorescence in the presence or absence of methanol. As expected, no fluorescence was observed when the bioreporter strain was grown with methanol as the sole carbon and energy source (Fig 2). Relative levels of fluorescence were quantified in microplate readings of cell suspensions of the bioreporter strain grown with chloromethane, with methanol or with a mixture of both compounds (Fig. 3). Control experiments showed that neither strain CM4 in which the original vector pLM.syfp2 was introduced, nor the wild-type strain, produced fluorescence under the investigated growth conditions (supplementary Fig. S3).

Bioreporter response, specificity and sensitivity

Response of the bioreporter to methyl halides exposure was evaluated in more detail in cell suspensions of the strain grown with methanol to mid-exponential phase. YFP fluorescence was observed at a significant level over background ($p = 0.009$) within only twenty minutes of exposure to chloromethane and stabilized after about 3 hours (Fig. 4). The specificity of fluorescence development by the bacterial bioreporter was investigated by exposure to different compounds of interest, including commercially available methyl halides chloromethane and iodomethane, the other chlorinated methanes (dichloromethane, trichloromethane, tetrachloromethane), growth substrates of facultative methylotrophs (methanol and succinate), and chloride, a typical product of dehalogenation metabolism (Fig. 5). After 3 hours of exposure, fluorescence levels above background were only observed with methyl halides. The dependence of YFP fluorescence development on methyl halide concentration was characterized, and found to be qualitatively and quantitatively similar for both chloromethane and iodomethane (Fig. 6). A fluorescence signal above background fluorescence was detected at a concentration of 2 fM (approx. 0.05 ppt), and concentrations of methyl halides above 10 pM (approx. 250 ppt) could be quantified. At high
concentrations, the previously observed toxicity of iodomethane (22, 31) impaired production of yellow fluorescent protein (Fig. 6), and caused a decrease in OD$_{600}$ of the bioreporter suggestive of cell lysis. The linear relationship of YFP fluorescence with chloromethane or iodomethane concentration was indistinguishable for both methyl halides in the range between picomolar and millimolar concentrations, underlining the high sensitivity and biological specificity of the bioreporter, and its potential for the detection and quantification of methyl halides.

**Application of the bioreporter to detect plant emissions of methyl halides**

To visualize the potential of the bioreporter to detect natural emissions of methyl halides, a cell suspension of methanol-grown, non-fluorescent bioreporter strain CM4(pME8266) was applied to leaf surfaces of two-weeks old seedlings of the model plant *A. thaliana*, reported to produce chloromethane in low amounts (0.6 nmol per g fresh weight per day, (14)). Confocal laser scanning microscopy after $24\text{h}$ incubation revealed a large number of fluorescent cells on leaves, whereas no fluorescence was visible with the control isogenic strain with the original promotorless YFP plasmid (Fig. 7). Fluorescence was evaluated quantitatively *in planta* (Fig. 7, legend) by counting the number of fluorescent cells per square mm$^2$ relative to the copy number of the *yfp* gene for yellow fluorescent protein present on leaves determined by qPCR.

To further demonstrate the applicability of the bioreporter for the screening of potential sources of natural emissions of methyl halides, selected plants previously reported either not to produce chloromethane or to produce chloromethane (14, 43) were investigated with the bioreporter (Table 1). After incubation of fresh leaves in airtight flasks for 24 h, samples of headspace gas were transferred to airtight flasks containing cell suspensions of the bioreporter which were analyzed as described above. The obtained results confirm previous reports (14, 43), and suggest that the bioreporter has potential to help screen for emissions of methyl halides from a variety of biological materials and environmental sources.
Discussion

The rapid response (Fig. 4) and sensitivity (Fig. 6) of the bacterial bioreporter described here compares favorably with several reports on the development of bioreporters in the recent literature. For example, a bioreporter assay for detection of various alkanes, based on production of an enhanced green fluorescent protein, was reported to require between 6 hours and 5 days (44). Similarly, known bioreporters for arsenic are either rapid with moderate sensitive ranges, or slower with high sensitive limits (45). The linearity of response of the methyl halide bioreporter over a wide range of concentrations (Fig. 6) is also noteworthy. For chloromethane, the threshold concentration for quantification is similar to the detection limit of analytical GC/MS spectrometry methods (100 ppt, i.e. approx. 4 pM), which most often involve sample pre-concentration (see e.g. (14)). For iodomethane, however, analytical chemical methods are more sensitive (approx. 6 ppt (about 0.25 pM), see e.g. (14, 46)).

The robustness of the developed methyl halide biosensor was supported by the demonstration, following up on earlier studies (31, 32), of chloromethane-dependent transcription of the cmuA gene and expression of chloromethane dehalogenase throughout growth in strain CM4 (Fig. 1, Fig. S2). The underlying mechanism for this methyl halide-specific regulation remains unknown, and the implication of putative regulator genes found in the vicinity of cmu genes (30) still needs to be experimentally investigated. Nevertheless, the observed lack of discrimination of the bioreporter CM4(pME8266) between different methyl halides was expected, since chloromethane dehalogenase transforms the higher molecular weight methyl halides bromomethane and iodomethane as well as chloromethane (31). Nevertheless, global sources of chloromethane (4.1-4.4 Tg yr⁻¹ (4)) are larger by at least an order of magnitude than those of iodomethane (approx. 550 Gg yr⁻¹) and bromomethane (approx. 110 Gg yr⁻¹) (2, 6). Thus, use of the bioreporter in screening for methyl halide emissions in natural environments will mainly inform on emissions of chloromethane as the major methyl halide produced. In particular, terrestrial biomes contribute little to the global budget of CH₃I (33 Gg yr⁻¹, (2)), with terrestrial plants alone generating 2200 Gg yr⁻¹ chloromethane (2, 4).
As suggested by initial data of the present study (Table 1), the laboratory screening of plants and various types of vegetation for which emissions of chloromethane are not yet known represents an attractive application for the bioreporter. Only few studies so far have identified plants which emit chloromethane (43, 47), despite the fact that vegetation is likely the main contributor to global emissions of chloromethane to the atmosphere (2). Given that methanol is the most important carbonaceous compound emitted by vegetation (approx. 100 Tg yr\(^{-1}\) (48, 49), i.e. 10- to 100-fold those of chloromethane), the fact that bioreporter fluorescence is not affected by methanol appears most valuable in this respect. Moreover, envisaged applications of the bioreporter foresee its use for experiments in a laboratory setting and on a short time-scale (Table 1). Since the antibiotic kanamycin is required for long-term stability of the reporter plasmid, use of this system in environmental settings may require further developments, e.g. by markerless chromosomal integration of the reporter system in strain CM4 using methodology established for *Methylobacterium* (50).

In conclusion, the bioreporter developed in the present work may represent a useful laboratory tool to increase our knowledge of natural sources of methyl halides, and thereby contribute to consolidate corresponding global budgets.
Acknowledgments

We thank Lisa Metzger and Julia Vorholt (ETH Zürich) for providing plasmid pLM.syfp2, Jérôme Mutterer (Institut de Biologie Moléculaire des Plantes, Strasbourg) for help with confocal microscopy, and Gisèle Haan-Archipoff (Herbarium, Université de Strasbourg) and Philippe Obliger (botanical garden, Université de Strasbourg) for plant material. Support from the Higher Education Commission of Pakistan, in form of a PhD fellowship to M.F.U.H, from the CNRS EC2CO program (2010-2011), and from REALISE, the Alsace Network of Laboratories in Environmental Sciences and Engineering (http://realise.unistra.fr), is gratefully acknowledged.
Table 1. Identification of methyl halide-emitting plants using the bioreporter

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Relative bioreporter fluorescence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reported CH₃Cl emissions&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>([g fresh weight]&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>(ng [g dry weight]&lt;sup&gt;−1&lt;/sup&gt; h&lt;sup&gt;−1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Vitex rotundifolia</td>
<td>52</td>
<td>2800&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Hoya carnosa</td>
<td>10</td>
<td>negative&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Codiaeum variegatum</td>
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<td>negative&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>24</td>
<td>12.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
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<td></td>
</tr>
<tr>
<td>20 mM CH₃Cl</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>20 mM methanol</td>
<td>11</td>
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</tbody>
</table>

<sup>a</sup> Relative to the fluorescence (set to a value of 100) observed after exposure of the bioreporter to 20 mM CH₃Cl (see Materials and Methods for details). Data from a representative experiment are shown.

<sup>b</sup> Data taken from (43). Plants were scored negative for emissions below 10 ng [g dry weight]<sup>−1</sup> h<sup>−1</sup>.

<sup>c</sup> Data derived from (14), converted from a reported value of 0.6 nmol [g fresh weight]<sup>−1</sup> d<sup>−1</sup>, assuming that dry weight represents 10% of fresh weight.
Figure Legends

Fig. 1. Relative expression of cmuA in *Methylobacterium extorquens* CM4 by qPCR in different phases of growth. RNA was extracted from bacteria grown in M3 medium with 10 mM chloromethane (black), 10 mM methanol alone (white), or with both growth substrates in combination, each at 10 mM concentration (grey). Samples were taken in early exponential (OD$_{600}$ = 0.06 - 0.1), mid exponential (OD$_{600}$ = 0.15 - 0.3) and early stationary (OD$_{600}$ > 0.35) phase of growth. Relative expression of cmuA was calculated relative to that of the 16S rRNA gene using the comparative threshold amplification cycle (Ct) method (2$^{-\Delta\Delta\text{Ct}}$) (39), and normalized to a value of 1 defined for the average of the three biological replicates of chloromethane-grown cultures in early exponential phase.

Fig. 2. YFP fluorescence in *M. extorquens* CM4(pME8266). Growth of the strain was performed until mid-exponential phase (OD$_{600}$ ~0.2) in M3 medium with either 10 mM chloromethane alone (A, D), 10 mM methanol alone (C,F), or a combination of both growth substrates, each at 10 mM concentration (B,E). Images were taken using a Leica DM4000 fluorescence microscope at 1000x magnification, using either a DAPI filter (A - C) (excitation 360 nm (40 nm bandwidth); dichromatic mirror 400 nm) or a YFP filter (D - F) (excitation 490 (20 nm bandwidth); dichromatic mirror 510 nm).

Fig. 3. Expression of cmuA in the *M. extorquens* CM4(pME8266) bioreporter as measured by YFP fluorescence. Bacteria were grown to exponential phase (OD$_{600}$ = 0.06 - 0.1) in M3 medium with 10 mM chloromethane (black), 10 mM methanol (white), or with both carbon sources in combination, each at a concentration of 10 mM (grey). Fluorescence values are given for each condition relative to the maximal fluorescence observed (see Fig. 5).
Fig. 4. Kinetics of YFP fluorescence development in the CM4(pME8266) bioreporter. Cell suspensions of a methanol-grown culture of strain CM4(pME8266) resuspended in fresh M3 medium were exposed to 10 mM of chloromethane (black) or methanol (white), and the fluorescence of samples was determined.

Fig. 5. Specificity of the bioreporter response. Cell suspensions (5 mL) of strain CM4(pME8266) were grown with methanol to mid-exponential phase, resuspended in fresh M3 medium at final OD<sub>600</sub> = 0.2 in 17 mL Hungate tubes, and exposed to different potential inducers for 3 hours (see Materials and Methods).

Fig. 6. Concentration dependence of YFP fluorescence development. Cell suspensions of a methanol-grown culture of strain CM4(pME8266) were exposed to different concentrations of chloromethane (black), iodomethane (grey), and methanol as a control (white). Fluorescence (F) in the samples was measured after 3 hours. The linear relationship of methyl halide concentration ([c]) with fluorescence was F = 108.66 + 5.53 [c] (R² = 0.99) for chloromethane and F = 104.71 + 5.25 [c] (R² = 0.93) for iodomethane.

Fig. 7. Confocal laser microscopy of in planta methyl halide production of A. thaliana. Leaves of 2-weeks old A. thaliana Col-0 seedlings were overlaid with equal volumes of cell suspensions of the bioreporter strain CM4(pME8266) (A) and of the control strain CM4(pLM.syfp2) (B) at the same cell density, revealing fluorescent bioreporter bacteria (green), overlaying the plant leaf (red autofluorescence from chlorophyll). Copy numbers of yfp and cmuA genes determined by qPCR were closely similar for both bacterial strains (Suppl. Table 1), whereas numbers of fluorescent cells per mm<sup>2</sup> as determined by ImageJ analysis were 2410 ± 970 and 150 ± 120 for bioreporter and control strains, respectively.
References


