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Fluorescence-Based Bacterial Bioreporter for Specific Detection of Methyl Halide Emissions in the Environment

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Methyl halides are volatile one-carbon compounds responsible for substantial depletion of stratospheric ozone. Among them, chloromethane (CH₃Cl) 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 alpha-proteobacterium 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 promoterless 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 pM (approximately 240 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.

Methyl halides (monohalomethanes) such as chloromethane are volatile hydrocarbons of environmental concern because of their toxicity to living organisms and their role in the depletion of stratospheric ozone (1, 2). Chloromethane (CH₃Cl), a gas and the most abundant halogenated hydrocarbon in the atmosphere (currently ~550 ppt, with an approximate increase of 2.3 to 2.7 ppt annually), is considered to be responsible for over 15% of the chlorine-catalyzed destruction of stratospheric ozone (2). Bromomethane (CH₃Br) also catalyzes the destruction of stratospheric ozone (2), and iodomethane (CH₃I) was shown to influence aerosol formation in the marine boundary layer (3).

Global emissions of chloromethane were recently estimated to be 4.1 to 4.4 Tg (4), with industrial sources contributing to <10% of total emissions (5). Natural production of other methyl halides appears to be weaker by 1 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 (17–19). These methods are time-consuming 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 into 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 (22–25), sludge (26–28), seawater (29), and the phyllosphere (30). The biochemistry and genetics of chloromethane degradation have been elucidated in detail for *Methylobacterium extorquens* CM4 (31–34), 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 the corrinoid methyltransferase *CmuA* and the 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, the dehaloge-

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nase 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 the 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 and 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 ultrapure water (Purelab Classic; ELGA) and sterilized by autoclaving (20 min at 121°C under 10⁵ Pa) 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. *Methylbacterium* was cultivated in chloride-free M3 mineral medium as described previously (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 with 50 ml M3 medium in Erlenmeyer flasks fitted with airtight mininert valve caps (Supelco) at 30°C with 100 rpm of agitation, with methanol (10 mM) and/or chloromethane (10 mM) as the carbon source. Growth was monitored by measuring the optical density at 600 nm (OD₆₀₀). *Escherichia coli* TOP10 (Invitrogen) was grown at 37°C on Luria-Bertani (LB) rich medium (Difco Laboratories). Kanamycin was added at a final concentration of 50 µg ml⁻¹ as required.

RNA isolation. Total RNA was extracted from samples of cultures of *M. extorquens* taken in the early, mid-, and late exponential growth phases (OD₆₀₀ of 0.06 to 0.1, 0.15 to 0.3, and >0.35, respectively) by using the NucleoSpin RNAII kit (Macherey-Nagel). RNAprotect solution (Qiagen) was used to stabilize RNA in bacterial culture samples (4 ml; OD₆₀₀ of ≥0.06), according to the manufacturer's recommendations. Cell pellets were kept frozen at -80°C until further processing. Cells were lysed by lysozyme treatment (final concentration of 2 mg/ml) in 100 ml Tris-EDTA (TE) buffer at 37°C for 15 min prior to RNA extraction according to the protocol provided by the manufacturer. RNA preparations were treated with amplification-grade DNase I (Invitrogen) for 1 h at 25°C and purified again by using the NucleoSpin RNAII kit. The RNA concentration was estimated spectrophotometrically at 260 nm (NanoDrop ND1000), and its quality was verified by its A₂₆₀/A₂₈₀ 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), according to the manufacturer's instructions. Control reactions without reverse transcriptase were performed to check for the absence of contaminating DNA. Quantitative PCR (qPCR) measurements on cDNA preparations were done with 96-well reaction PCR plates by using a GeneAmp 5700 sequence detection system (Applied Biosystems). Primers *cmuA*802F (5'-TTCAACGGCGAYATGTATCCYGG-3') (37) and *cmuA*968R (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). PCRs were carried out with a final volume of 20 µl, using 4 µl of a cDNA preparation (diluted with ultrapure molecular biology-grade water; Sigma) with a 3 µM final concentration of each primer and commercial 1× 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 by using the comparative threshold amplification cycle (C_T) method (2^{-ΔΔC_T}), as described previously (39). Gene copy numbers were determined by using known amounts of *M. extorquens* CM4 total DNA as the reference.

Dehalogenase activity. The 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). The concentration of chloride was measured spectrophotometrically at 340 nm, according to the method of Jörg and Bertau (40), as FeCl₂⁺ formed in acidic medium by comparison to a calibration curve of sodium chloride standards (0 to 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 an 801-bp-long PCR fragment spanning exactly the intergenic region between *purU* and *cmuC2* (see Fig. S1 in the supplemental material) by using PCR primers 5'-ATTTTGAGCTCCGA GCGATCCCCCTCGTC-3' and 5'-ATTTTGGTACCTTAGACGGCACC AGATGC-3', thereby introducing SacI and KpnI restriction sites (underlined) for subsequent cloning. PCR was performed with a total volume of 50 µl comprising 1 unit of Iproof high-fidelity polymerase (Bio-Rad), 10 µl of high-fidelity PCR buffer (5×), 0.2 µM each primer, 0.1 µM each deoxynucleoside triphosphate (dNTP), and 10 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, and 30 s of polymerization at 72°C and a final 3-min postelongation step at 72°C. The resulting PCR fragment was purified after agarose gel electrophoresis using the GeneClean Turbo kit (MP Biomedicals), digested overnight with the enzymes SacI and KpnI (Fermentas), and purified again by using the same kit. The digested PCR fragment was ligated for 24 h at 14°C with KpnI- and SacI-digested promoter probe plasmid pLM.syfp2 (see Fig. S1 in the supplemental material), which features a promoterless 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 by 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 min, washed twice in sterile ultrapure water and then in ethanol (70%), and mounted onto glass slides by using mounting oil (BacLight; Molecular Probes). Images were taken by using a Leica DM4000 fluorescence microscope (Leica Microsystems) at a ×1,000 magnification, operated with either a YFP filter cube (excitation filter, bandpass [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 OD₆₀₀ of 0.05. Cell suspensions (200 µl) were transferred onto a 96-well microtiter plate (Nunc), and the OD₆₀₀ 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 an OD₆₀₀ of 1, and expressed as a percentage of the maximum observed YFP fluorescence (see Fig. 5).

To monitor fluorescence induction after exposure to various compounds, a preculture of the reporter strain was grown in M3 medium supplemented with MeOH (20 mM) and kanamycin (50 µg/ml) until the

late exponential phase of growth (OD_{600} of ~ 0.3 to 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, and iodomethane, chloroform, and tetrachloromethane were used at 200 μ M to avoid potential toxic effects (e.g., see references 22 and 31). Samples (200 μ l) were taken after 3 h, transferred onto 96 -well microtiter plates, and subjected to fluorimetric analysis as described above.

For determination of the concentration dependence of chloromethane-induced fluorescence, serial 10 -fold dilutions of chloromethane gas, designed to yield final concentrations of chloromethane in the range of 2 fM to 20 mM, 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) by using an airtight syringe. The tube was left to equilibrate for 5 min, 1.7 ml of the gas phase was transferred into the second tube, and the procedure was repeated for each further dilution. For iodomethane, serial 10 -fold 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 to 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 a methanol-grown bioreporter cell suspension (OD_{600} of 0.2) from which 50 ml headspace gas had been removed previously. As controls, cell suspensions were exposed to chloromethane (20 mM) and methanol (20 mM) under the same conditions. After 3 h of incubation at 30°C , the fluorescence of bioreporter cell suspensions was 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 *A. thaliana* exposed to the bioreporter strain. *A. thaliana* wild-type strain Col-0 was grown in petri dishes of Murashige-Skoog medium including vitamins (Duchefa) and supplemented with 1% sugar and 0.7% Pastagar at 22°C with a 12 -h light period for 14 days in a phytotron (Plant Climatics). A cell suspension (5 ml at an OD_{600} of 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 h under a laminar flow hood. After incubation for 24 h at 22°C , leaves were removed, mounted onto microscope glass slides, and visualized for YFP fluorescence by using the $20\times$ lens of a Zeiss LSM710 confocal laser scanning microscope and the YFP filter cube (excitation filter, 488 nm). Fluorescent cells per mm^2 were counted with the "Find maxima image" 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 the *cmuA*, *syfp2*, and *rnaA* genes was performed on DNA extracted from the investigated leaf material, as described above, by 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 means with standard deviations. Data were analyzed by using Student's *t* test, with different letters in the figures indicating statistically significant differences at a *P* value of <0.05 .

RESULTS

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

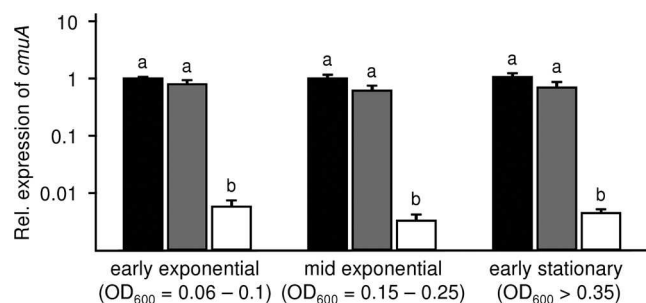


FIG 1 Relative expression levels of *cmuA* in *Methylobacterium extorquens* CM4 by qPCR in different phases of growth. RNA was extracted from bacteria grown in M3 medium with 10 mM chloromethane (black), 10 mM methanol alone (white), or both growth substrates in combination, each at 10 mM concentration (gray). Samples were taken in the early exponential (OD_{600} of 0.06 to 0.1), mid-exponential (OD_{600} of 0.15 to 0.3), and early stationary (OD_{600} of >0.35) phases of growth. The relative expression level of *cmuA* was calculated relative to that of the $16S$ rRNA gene by using the comparative threshold amplification cycle (C_T) method ($2^{-\Delta\Delta C_T}$) (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.

methane-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 by using qPCR during growth of strain CM4 under different conditions. In particular, the effects 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 were higher by 2 orders of magnitude during growth with chloromethane, in both 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 (see Fig. S2A in the supplemental material), as confirmed by measurements of chloride release into the culture medium (see Fig. S2B in the supplemental material), and the final OD_{600} in stationary phase was approximately double 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

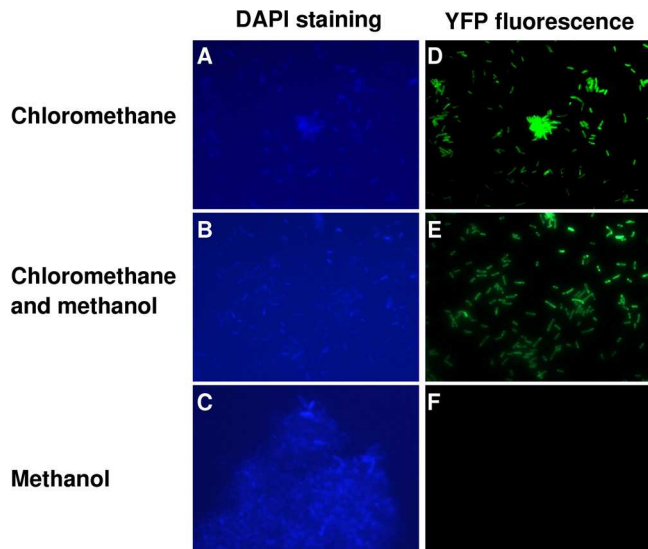


FIG 2 YFP fluorescence in *M. extorquens* CM4(pME8266). Growth of the strain was performed until the mid-exponential phase (OD_{600} of ~ 0.2) in M3 medium with either 10 mM chloromethane alone (A and D), 10 mM methanol alone (C and F), or a combination of both growth substrates, each at a 10 mM concentration (B and E). Images were taken by using a Leica DM4000 fluorescence microscope at a $\times 1,000$ magnification, using either a DAPI filter (A to C) (excitation, 360 nm [40-nm bandwidth]; dichromatic mirror, 400 nm) or a YFP filter (D to F) (excitation, 490 [20-nm bandwidth]; dichromatic mirror, 510 nm).

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, methanol, or 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 (see Fig. S3 in the supplemental material).

Bioreporter response, specificity, and sensitivity. The response of the bioreporter to methyl halide exposure was evaluated

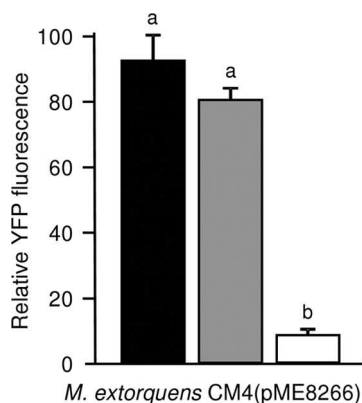


FIG 3 Expression level of *cmuA* in the *M. extorquens* CM4(pME8266) bioreporter measured by YFP fluorescence. Bacteria were grown to exponential phase (OD_{600} of 0.06 to 0.1) in M3 medium with 10 mM chloromethane (black), 10 mM methanol (white), or both carbon sources in combination, each at a concentration of 10 mM (gray). Fluorescence values are given for each condition relative to the maximal fluorescence observed (Fig. 5).

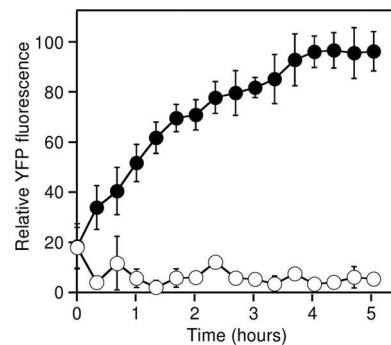


FIG 4 Kinetics of YFP fluorescence development in the CM4(pME8266) bioreporter. Cell suspensions of a methanol-grown culture of strain CM4(pME8266) resuspended in fresh M3 medium were exposed to 10 mM chloromethane (black) or methanol (white), and the fluorescence of samples was determined.

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 the background ($P = 0.009$) within only 20 min of exposure to chloromethane and stabilized after about 3 h (Fig. 4). The specificity of fluorescence development by the bacterial bioreporter was investigated by exposure to different compounds of interest, including the commercially available methyl halides chloromethane and iodomethane, the other chlorinated methanes (dichloromethane, trichloromethane, and tetrachloromethane), growth substrates of facultative methylotrophs (methanol and succinate), and chloride, a typical product of dehalogenation metabolism (Fig. 5). After 3 h of exposure, fluorescence levels above background were observed only with methyl halides. The dependence of YFP fluorescence development on the 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 was detected at a concentration of 2 fM (approximately 0.05 ppt), and concentrations of methyl halides above 10 pM (approximately 240 ppt) could be quantified. At high concentrations, the previously observed toxicity of iodomethane (22, 31) impaired the production of yellow fluorescent protein (Fig. 6) and caused a de-

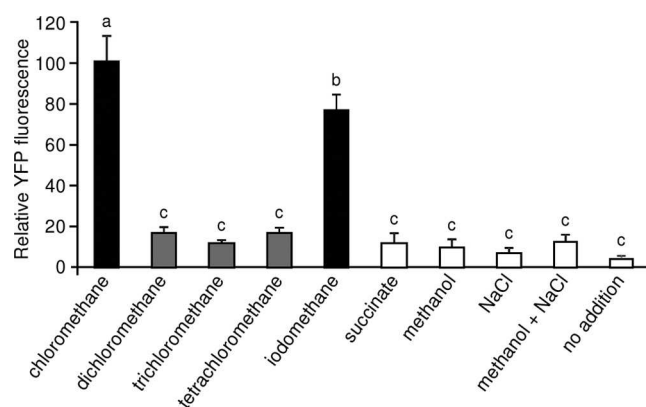


FIG 5 Specificity of the bioreporter response. Cell suspensions (5 ml) of strain CM4(pME8266) were grown with methanol to mid-exponential phase, resuspended in fresh M3 medium at final OD_{600} of 0.2 in 17-ml Hungate tubes, and exposed to different potential inducers for 3 h (see Materials and Methods).

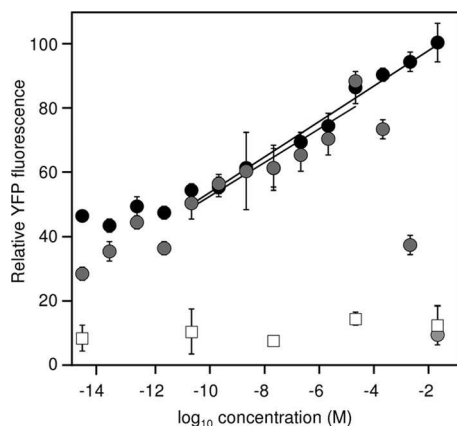


FIG 6 Concentration dependence of YFP fluorescence development. Cell suspensions of a methanol-grown culture of strain CM4(pME8266) were exposed to different concentrations of chloromethane (black), iodomethane (gray), and methanol as a control (white). Fluorescence (F) values for the samples were measured after 3 h. The linear relationships of the methyl halide concentration ($[c]$) with fluorescence were as follows: $F = 108.66 + 5.53 [c]$ ($R^2 = 0.99$) for chloromethane and $F = 104.71 + 5.25 [c]$ ($R^2 = 0.93$) for iodomethane.

crease in the OD_{600} of the bioreporter, suggestive of cell lysis. The linear relationship of YFP fluorescence with the 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, nonfluorescent bioreporter strain CM4(pME8266) was applied to leaf surfaces of 2-week-old seedlings of the model plant *A. thaliana*, reported to produce chloromethane in small amounts (0.6 nmol per g [fresh weight] per day [14]). Confocal laser scanning microscopy after 24 h of incubation revealed a large number of fluorescent cells on leaves, whereas no fluorescence was visible with the control isogenic strain with the original promoterless YFP plasmid (Fig. 7). Fluorescence was evaluated quantitatively *in planta* (Fig. 7) by counting the number of fluorescent cells per 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 into 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 compare favorably with several reports

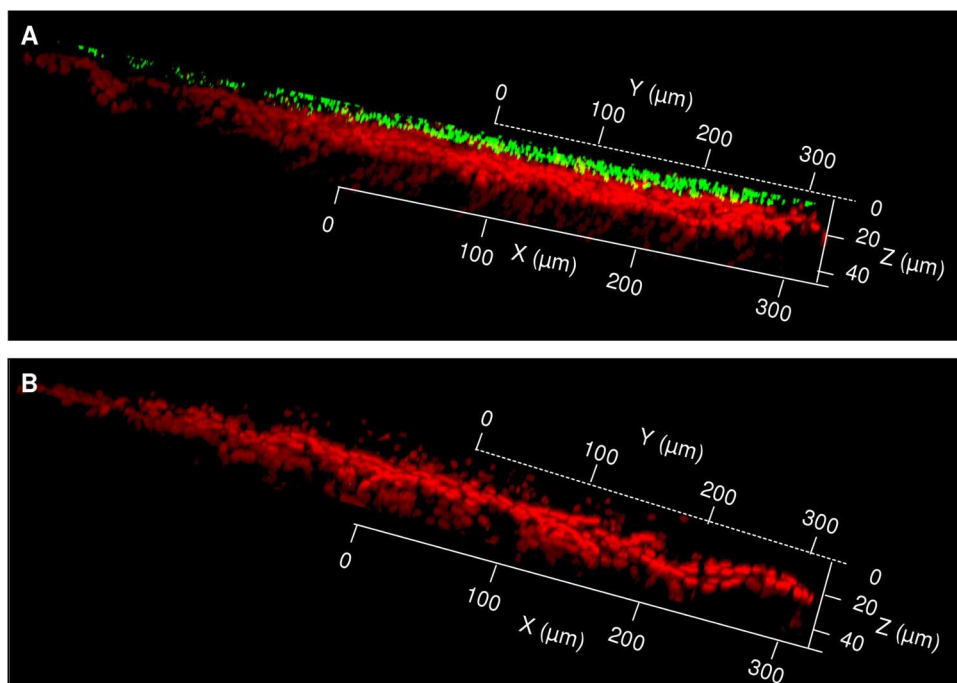


FIG 7 Confocal laser microscopy of *in planta* methyl halide production of *A. thaliana*. Leaves of 2-week-old *A. thaliana* Col-0 seedlings were overlaid with equal volumes of cell suspensions of the bioreporter strain CM4(pME8266) (A) and of the control strain CM4(pLM.syfp2) (B) at the same cell density, revealing fluorescent bioreporter bacteria (green) overlaying the plant leaf (red autofluorescence is from chlorophyll). Copy numbers of the YFP and *cmuA* genes determined by qPCR were closely similar for both bacterial strains (see Table 1 in the supplemental material), whereas numbers of fluorescent cells per mm^2 determined by ImageJ analysis were $2,410 \pm 970$ and 150 ± 120 for the bioreporter and control strains, respectively.

TABLE 1 Identification of methyl halide-emitting plants using the bioreporter

Plant species or control	Relative bioreporter fluorescence ^a ([g {fresh wt}] ⁻¹)	Reported CH ₃ Cl emission (ng [g {dry wt}] ⁻¹ h ⁻¹)
Plant species		
<i>Vitex rotundifolia</i>	52	2,800 ^b
<i>Hoya carnosa</i>	10	Negative ^b
<i>Codiaeum variegatum</i>	8	Negative ^b
<i>Arabidopsis thaliana</i>	24	12.6 ^c
Controls		
20 mM CH ₃ Cl	100	
20 mM methanol	11	

^a 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.

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

^c Data derived from reference 14, converted from a reported value of 0.6 nmol [g (fresh weight)]⁻¹ day⁻¹, assuming that dry weight represents 10% of the fresh weight.

on the development of bioreporters in recent literature. For example, a bioreporter assay for detection of various alkanes, based on the production of an enhanced green fluorescent protein, was reported to require between 6 h and 5 days (44). Similarly, known bioreporters for arsenic are either rapid with moderate sensitivity ranges or slower with high sensitivity limits (45). The linearity of the 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 methods (100 ppt, i.e., approximately 4 pM), which most often involve sample preconcentration (see, e.g., reference 14). For iodomethane, however, analytical chemical methods are more sensitive (approximately 6 ppt [about 0.25 pM] [see, e.g., references 14 and 46]).

The robustness of the developed methyl halide biosensor was supported by the demonstration, following up on previous studies (31, 32), of chloromethane-dependent transcription of the *cmuA* gene and expression of chloromethane dehalogenase throughout growth in strain CM4 (Fig. 1; see also Fig. S2 in the supplemental material). 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 bioreporter strain 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). Global sources of chloromethane (4.1 to 4.4 Tg year⁻¹ [4]) are larger by at least an order of magnitude than those of iodomethane (approximately 550 Gg year⁻¹) and bromomethane (approximately 110 Gg year⁻¹) (2, 6). Thus, the 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 year⁻¹ [2]), with terrestrial plants alone generating 2,200 Gg year⁻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 (approximately 100 Tg year⁻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. 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, the 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 consolidating corresponding global budgets.

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