Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Formation of chloroform and tetrachloroethene by *Sinorhizobium meliloti* strain 1021

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Significance and Impact of the Study: Volatile organohalogen compounds (VOX) strongly influence atmospheric chemistry and Earth's climate. Besides anthropogenic emissions they are naturally produced by either abiotic or biotic pathways in various environments. Particularly in soils, microbial processes drive the natural halogen cycle but the direct link to microbial VOX formation has not been studied in detail yet. In this study we provide evidence that the common and widespread soil bacterium *Sinorhizobium meliloti* strain 1021 forms chloroform and tetrachloroethene. The potential contribution of *S. meliloti* to soil VOX release could significantly influence soil and atmospheric chemistry.

Keywords
biotic halogenation, chloroform, chloroperoxidases, *Sinorhizobium meliloti*, Smc01944, tetrachloroethene, volatile organohalogen compounds.

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2015/0465: received 5 March 2015, revised 8 June 2015 and accepted 22 June 2015

doi:10.1111/lam.12462

Abstract

The mechanisms and organisms involved in the natural formation of volatile organohalogen compounds (VOX) are largely unknown. We provide evidence that the common and widespread soil bacterium *Sinorhizobium meliloti* strain 1021 is capable of producing up to 3338·6 ± 327·8 ng l^{-1} headspace volume of chloroform ($CHCl_3$) and 807·8 ± 13·5 ng l^{-1} headspace volume of tetrachloroethene (C_2Cl_4) within 1 h when grown in soil extract medium. Biotic VOX formation has been suggested to be linked to the activity of halogenating enzymes such as haloperoxidases. We tested if the observed VOX formation by *S. meliloti* can be attributed to one of its chloroperoxidases (Smc01944) that is highly expressed in the presence of H_2O_2. However, addition of 10 mmol l^{-1} H_2O_2 to the *S. meliloti* cultures decreased VOX formation by 52% for chloroform and 25% for tetrachloroethene, while viable cell numbers decreased by 23%. Interestingly, *smc01944* gene expression increased 450-fold. The quantification of extracellular chlorination activity in cell suspension experiments did not provide evidence for a role of *S. meliloti* chloroperoxidases in the observed VOX formation. This suggests that a momentarily unknown mechanism which requires no H_2O_2 might be responsible for the VOX formation by *S. meliloti*. Regardless of the underlying mechanism our results suggest that the soil bacterium *S. meliloti* might be an important source of VOX in soils.

Introduction

The formation of ozone-degrading volatile halogenated hydrocarbons (VOX) has been shown to occur in many environments like salt lake sediments, marine environments and forest soils (Albers *et al.* 2010; Ruecker *et al.* 2014, 2015; Yang *et al.* 2014). Particularly in soils, the existence of an active natural chlorine cycle has been demonstrated (Öberg 2002; Öberg and Sandén 2005). There is strong evidence that the chlorination of organic matter in soils is mainly biotic (Bastviken *et al.* 2009). *Sinorhizobium meliloti* strain 1021 is a common and

widespread soil bacterium capable of nitrogen-fixation. This microorganism also lives in symbiosis with plants of the genus *Alfalfa* (Gage 2004). *Sinorhizobium meliloti* has been the subject of extensive genetic, biochemical, and metabolic research and its genome has been sequenced previously (Galibert *et al.* 2001). Plants infected by *S. meliloti* during symbiosis react with an oxidative burst and release reactive oxygen species (ROS) to their surrounding (Santos *et al.* 2001). The release of ROS is the plants defence mechanism against the infection by microorganisms such as *S. meliloti*. These ROS cause oxidative stress and therefore *S. meliloti* needs efficient mechanisms to circumvent cell death due to oxidative stress during plant infection. Enzymes such as chloroperoxidases have been proven to be involved in oxidative stress response (Barloy-Hubler *et al.* 2004), but they might also play a role in microbial chlorination reactions in soils (Bengtson *et al.* 2013).

Therefore, the aims of this study were, (i) to quantify VOX formation in cultures of *S. meliloti* strain 1021 and (ii) to test if the chloroperoxidase triggered oxidative stress response mechanism is involved in VOX formation by *S. meliloti* strain 1021.

Results and discussion

Formation of VOX by *Sinorhizobium meliloti* grown in soil extract medium

Cultivation experiments with *S. meliloti* strain 1021 grown in soil extract medium proved the biotic formation of VOX compounds such as chloroform ($CHCl_3$) and tetrachloroethene (C_2Cl_4) (Fig. 1). After 1 h of incubation at room temperature in the dark, biotic setups released 3338·6 ± 327·8 ng l^{-1} headspace volume of chloroform and 807·8 ± 13·5 ng l^{-1} headspace volume of tetrachloroethene. Concentrations of chloroform and tetrachloroethene quantified in sterile soil extract medium containing no cells were below 1 ng l^{-1}. The formation of chloroform and tetrachloroethene in the biotic setups suggest an enzymatically catalysed halogenation mechanism. Both substances are known to be emitted from soils and are likely produced by biotic reactions (Haselmann *et al.* 2000; Hoekstra *et al.* 2001). To identify potential halogenating enzymes in the proteome of *S. meliloti* strain 1021 that might be involved in VOX formation, we screened the strains proteome (Proteome ID UP000001976) in UniProt (The UniProt Consortium 2015). We found five genes possibly encoding for halogenating enzymes (Table 1). Four enzymes were classified as chloroperoxidases, one enzyme as haloperoxidase. Chloroperoxidases use H_2O_2 and a chloride ion as substrates (Vaillancourt *et al.* 2006) and are able to unspecifically chlorinate organic matter (Van Pée 1996). Chloroperoxidases are also thought to play a role in the degradation of ROS such as H_2O_2. For *S. meliloti* strain 1021 it has been demonstrated that the expression of genes encoding for an extracellular non-heme chloroperoxidase (Smc01944) is highly upregulated in the presence of H_2O_2 (Barloy-Hubler *et al.* 2004). In the presence of organic substances, e.g. organic acids (such as acetate or citrate) and glucose, it was demonstrated, that the main product of the halogenation reactions catalysed by a chloroperoxidase was chloroform (Walter and

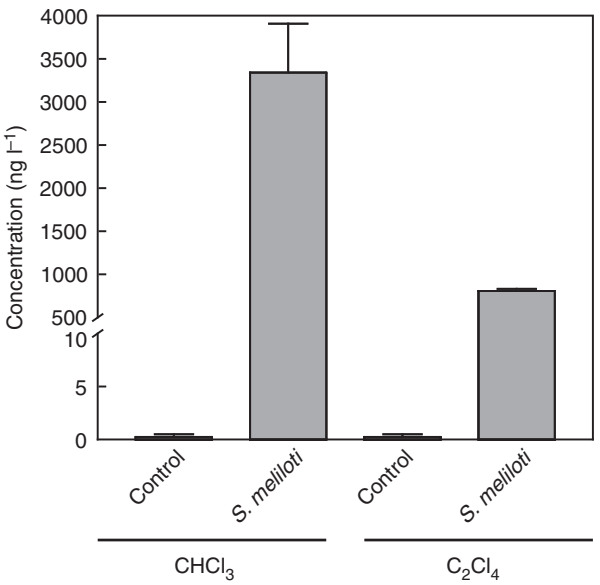

Figure 1 Formation of $CHCl_3$ and C_2Cl_4 in the cultivation experiments containing either sterile soil extract medium without cells (control) or *Sinorhizobium meliloti* cultures (OD_{600} of 1·0). Headspace concentrations were measured after 1 h of incubation. Error bars indicate the standard error from three independent replicates.

Table 1 Putative halogenating enzymes identified in the proteome of *Sinorhizobium meliloti* strain 1021 based on the UniProt database. Functional prediction of the enzymes is based on sequence homology to corresponding database entries

UniProt Entry	Protein	Gene	References
Q92XA8	Chloride peroxidase	*smb20054*	Finan *et al.* (2001), Galibert *et al.* (2001)
Q92MX7	Non-heme chloroperoxidase	*smc01944*	Galibert *et al.* (2001)
Q92Y90	Non-heme haloperoxidase	*sma1809*	Barnett *et al.* (2001), Galibert *et al.* (2001)
Q92UH7	Probable non-heme chloroperoxidase	*smb20860*	Galibert *et al.* (2001)
Q92L87	Putative non-heme chloroperoxidase	*smc03810*	Capela *et al.* (2001), Galibert *et al.* (2001)

Ballschmiter 1992). Furthermore, the biotic formation of tetrachloroethene was observed in algae and even increased after addition of H_2O_2 suggesting a peroxidative formation mechanism (Abrahamsson *et al.* 1995). Tetrachloroethene was also detected in small amounts in chloroperoxidase catalysed reactions of acetic acid and acetone (Walter and Ballschmiter 1992). On the basis of this preliminary evidence, we tested if the non-heme chloroperoxidase Smc01944 is involved in the formation of chloroform and tetrachloroethene in our *S. meliloti* cultures. Therefore, we hypothesized that the addition of H_2O_2 to *S. meliloti* cultures would increase *smc01944* gene expression and also VOX formation. We added 10 mmol l^{-1} H_2O_2 to setups containing *S. meliloti* cells in soil extract medium. After 1 h of incubation we quantified $1604{\cdot}1 \pm 112{\cdot}8$ ng l^{-1} of chloroform and $611{\cdot}6 \pm 19{\cdot}0$ ng l^{-1} tetrachloroethene in the headspace (Fig. 2). This demonstrated that emissions of chloroform and tetrachloroethene after addition of 10 mmol l^{-1} H_2O_2 decreased by 52% and 25% respectively. Nevertheless, the decrease in VOX formation after addition of 10 mmol l^{-1} H_2O_2 provided additional proof that VOX formation in our culture experiments is based on a microbially catalysed process since viable cell numbers also decreased after H_2O_2 addition by approx. 23% (Fig. 3a). Interestingly, the addition of 10 mmol l^{-1} H_2O_2 increased *smc01944* gene expression by a factor of 452 (Fig. 3b), but gene expression was inversely correlated with VOX formation. All experiments were also conducted with *S. meliloti* cultures grown in Vincents minimal medium (VM medium), but in none of the setups VOX formation could be detected (data not shown). This means that a soil extract medium containing complex mixtures of organic molecules is required for the observed VOX formation. Soil extracts contain different kinds of redox-active substances such as humic substances (Piepenbrock *et al.* 2014) that could act as precursors of VOX (Asplund *et al.* 1991; Keppler *et al.* 2000). Furthermore, the redox activity of humic substances can lead to the formation of ROS, e.g. OH-radicals (Jiang *et al.* 2009; Page *et al.* 2012), which could in return stimulate chloroperoxidase activity. Since these ROS could also induce *smc01944* gene expression we tested the effect of soil extract on the *smc01944* gene expression in cultures grown in VM medium without soil extract. Our results demonstrated that the addition of soil extract does not induce *smc01944* gene expression above background levels if no additional oxidative stress through the addition of H_2O_2 is applied (Fig. 3c). Nevertheless, the *smc01944* gene is constantly expressed in our *S. meliloti* cultures with approx. $3{\cdot}1 \pm 1{\cdot}0$ transcripts per CFU but expression of a gene is not necessarily directly linked to enzyme activity (Glanemann *et al.* 2002). For *S. meliloti* strain 1021 it is known that the catalase KatB is continuously produced during growth to maintain a low H_2O_2 concentration (Sigaud *et al.* 1999). Our results suggest that *S. meliloti* strain

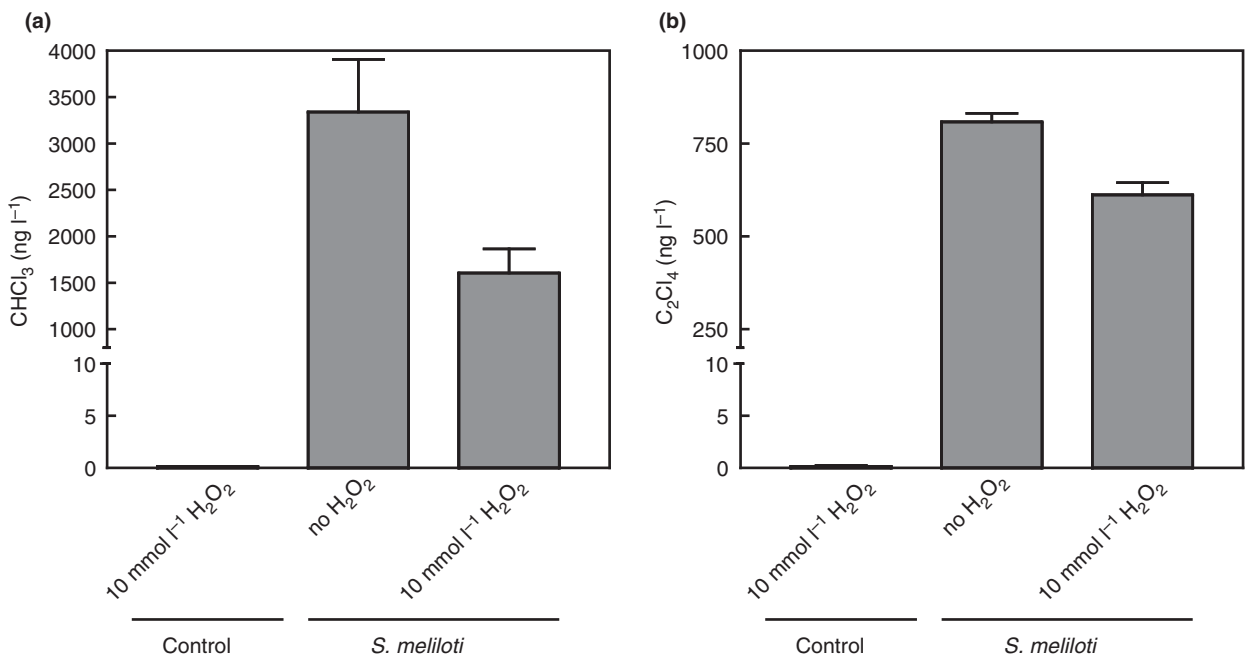

Figure 2 Formation of $CHCl_3$ (a) and C_2Cl_4 (b) in the cultivation experiments containing sterile soil extract medium without cells (control) or with *S. meliloti* cells (OD_{600} of 1·0). Measurements were taken without or after addition of 10 mmol l^{-1} H_2O_2. Headspace concentrations were measured after 1 h of incubation. Error bars indicate the standard error of three independent replicates.

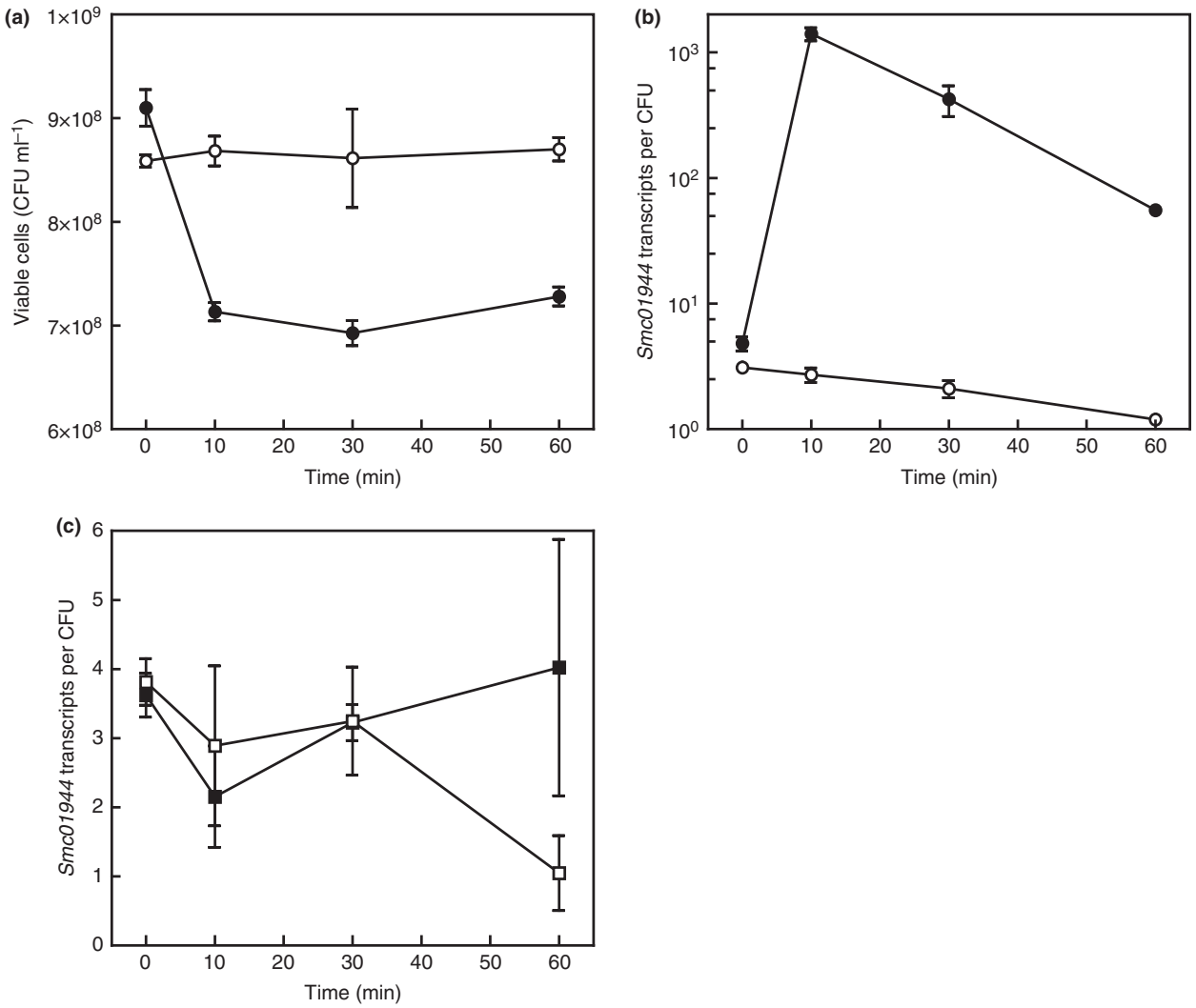

Figure 3 Viable cell counts (CFU) (a) and gene expression of the *smc01944* gene (b) in the absence (○) and presence (●) of 10 mmol l^{-1} H_2O_2 for *S. meliloti* cultures (OD_{600} of 1·0) grown on soil extract medium. (c) Gene expression after addition of soil extract to *S. meliloti* cultures (OD_{600} of 1·0) grown on VM medium. No soil extract (□), 20% (v/v) soil extract (■). Error bars represent the standard error of three independent cultures.

1021 also constantly expresses its Smc01944-type chloroperoxidase to immediately react to ROS while gene expression is highly upregulated under oxidative stress.

Since the chloroperoxidase Smc01944 is an extracellular enzyme (Barloy-Hubler *et al.* 2004), we tested if we can detect extracellular chlorination activity in cell suspension experiments applying an improved version of the monochlorodimedone assay (Hager *et al.* 1966). The assay quantifies the chlorination of monochlorodimedone (MCD) by chloroperoxidase activity to dichlorodimedone (DCD). For these experiments we used cell suspensions of *S. meliloti* in 0·9% NaCl to obtain higher amounts of enzymes in the culture supernatant and to avoid chlorination of medium compounds other than MCD. In *S. meliloti* cell suspensions treated with 10 mmol l^{-1} H_2O_2 no chlorination of MCD to DCD could be detected (Fig. 4). We furthermore tested cell suspensions at pH 4 and supernatants of *S. meliloti* cultures grown in VM medium and soil extract medium at pH 4 and pH 7 but in none of the setups DCD was formed after H_2O_2 addition (data not shown). However, formation of DCD was observed in positive control experiments when the chloroperoxidase of *Caldariomyces fumago* was added to the respective media (Fig. 4). This demonstrated that the improved MCD assay in principle worked. Nonetheless, the obtained results raised the question whether Smc01944 has chlorination activity. Smc01944 was classified as a non-heme chloroperoxidase based on *in silico*

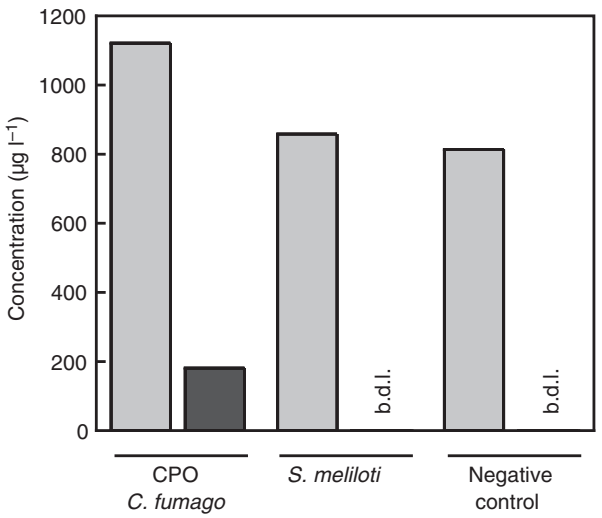

Figure 4 Concentrations of monochlorodimedone (substrate, light grey) and dichlorodimedone (product, dark grey) after 1 h of incubation. The chloroperoxidase of *Caldariomyces fumago* was used as a positive control, sterile NaCl solution (0·9%) as negative control. In negative controls and setups with *S. meliloti* dichlorodimedone concentrations were below the detection limit (b.d.l.).

predictions and is 74% identical to the chloroperoxidase PrxC (*cpoF* gene) of *Pseudomonas fluorescens* (Barloy-Hubler *et al.* 2004). Peroxidase activity has been shown for the supernatant of *S. meliloti* cultures treated with H_2O_2 (Barloy-Hubler *et al.* 2004) but to directly link any chlorination activity to Smc01944, the enzyme would need to be purified and evaluated for its chlorination activity using e.g. the MCD assay.

Experimental evidence collected in this study revealed that *S. meliloti* strain 1021 produces chloroform and tetrachloroethene when grown in a soil extract medium. However, our experiments also suggested that the chloroperoxidase Smc01944 is most likely not responsible for the observed formation of chloroform and tetrachloroethene under the here tested conditions. Chloroperoxidases need H_2O_2 as substrate (Vaillancourt *et al.* 2006), but we detected VOX formation in the absence of H_2O_2. In the presence of H_2O_2, VOX formation even decreased. The higher VOX formation in the absence of H_2O_2 also suggests that it is unlikely that any of the other potential chloroperoxidases present in the *S. meliloti* proteome (Table 1) are involved in the VOX formation if their expression is induced under the prevailing growth conditions. The proteome of *S. meliloti* contains no other halogenases and the products chloroform and tetrachloroethene are unlikely to be formed by other halogenating enzymes than haloperoxidases since e.g. flavin- or alpha-ketoglutarate dependent halogenases are substrate specific, regioselective and involved in the halogenation of large molecules e.g. antibiotics (Blasiak and Drennan 2003). Abiotic processes such as desorption of chloroform or tetrachloroethene from other organic molecules for example humic substances can be excluded because the soil extract was autoclaved which should have led to the volatilization of all sorbed VOX (Chen and Dural 2002; Krasner and Wright 2005). Further cultivation experiments with *S. meliloti* combined with stable chlorine isotope analysis might be necessary to identify the enzymatic halogenation mechanism of *S. meliloti*. Pulse chase experiments in combination with compound-specific stable isotope analysis have previously been successfully applied in forest soils (Aeppli *et al.* 2013) or to elucidate the $CHCl_3$ formation mechanism of a chloroperoxidase (Breider and Hunkeler 2014). Furthermore, transcriptomic and proteomic studies could help to identify new gene targets and enzymes that are expressed and active during VOX formation by *S. meliloti*.

Material and methods

Cultivation

Sinorhizobium meliloti strain 1021 was either cultivated in soil extract medium (1 g l^{-1} yeast extract, 10 g l^{-1} mannitol, 20% (v/v) soil extract, pH 7) or Vincents minimal medium (VM medium) (Bastiat *et al.* 2012) with 20 mmol l^{-1} succinate as carbon source at 28°C and 100 rev min^{-1} to an OD_{600} of 1·0. Soil extract for soil extract medium was prepared by mixing 80 g forest soil (vertic cambisol (WRB), total organic carbon content: 301 g kg^{-1} dry soil) with 200 ml water and 0·2 g Na_2CO_3. The soil suspension was autoclaved for 1 h at 121°C. Autoclaved soil suspension was centrifuged at 7000 ***g*** for 10 min and the supernatant was adjusted to pH 7·2. For counts of colony forming units (CFU), cells were incubated on agar plates with soil extract medium containing 15 g l^{-1} agar at 28°C for 48 h.

Setup of VOX formation experiments and quantification of volatile halogenated hydrocarbons

VOX formation experiments with *S. meliloti* strain 1021 were prepared in triplicates in 20 ml GC headspace vials. Each vial was filled with 10 ml sterile medium or a *S. meliloti* culture pregrown to an OD_{600} of 1·0. The initial concentration of H_2O_2 in each setups was adjusted to 10 mmol l^{-1} to induce an oxidative stress response in *S. meliloti* (Barloy-Hubler *et al.* 2004). Vials were closed with PTFE (polytetraflouroethylene) silicone septa and incubated for 60 min in the dark under oxic conditions at room temperature. Volatile halogenated hydrocarbons were quantified in the gaseous headspace using a Trace GC Ultra coupled to a DSQ II single quadrupole mass

spectrometer (Thermo Fisher Scientific, Waltham, MA) at the University of Duisburg-Essen. Details of the in tube extraction method (ITEX), the GC program and MS parameters were published before (Laaks *et al.* 2010; Ruecker *et al.* 2014). All standards and solutions were prepared with water from an analytical water purification system (PURELAB Ultra, ELGA LabWater, Celle, Germany). EPA 624 standard calibration mix (100 μg ml^{-1} of each component in methanol) as well as trichloromethane were purchased from Sigma-Aldrich (Saint Louis, MO). Standards were prepared in soil extract medium which was also used for culturing *S. meliloti* strain 1021. We did not observe any matrix effects.

Sinorhizobium meliloti proteome analysis

The proteome of *S. meliloti* strain 1021 (UniProt Proteome ID UP000001976) containing 6169 proteins was screened in the UniProt database (The UniProt Consortium 2015) for halogenating enzymes by their corresponding EC numbers.

Setup of chloroperoxidase gene expression experiments

Gene expression experiments were set up in triplicates using the *S. meliloti* cultures grown in soil extract medium. About 10 mmol l^{-1} H_2O_2 were added to induce oxidative stress response. In the negative controls the same volume of sterile water was added. Samples for RNA extraction and cell counts were taken before H_2O_2 addition and 10, 30 and 60 min thereafter. For RNA extraction, 1 ml of culture was centrifuged (1 min, 6000 ***g***) and the pellet was immediately frozen in liquid nitrogen. In parallel, a sample for counts of CFU was taken, diluted in sterile phosphate buffered saline (PBS) buffer (pH 7) and plated on agar plates containing soil extract medium. To evaluate whether soil extract induces chloroperoxidase gene expression the *S. meliloti* cultures grown in VM medium were mixed with sterile soil extract in a ratio of 4 : 1. Negative controls were mixed with VM medium in the same ratio. Samples for RNA extraction and cell counts were retrieved as described above.

RNA extraction, DNA digestion and reverse transcription

RNA was extracted from the frozen cell pellets with the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). DNA was digested using the Ambion TURBO DNA-free™ Kit (Life Technologies, Carlsbad, CA). Successful DNA removal was confirmed by PCR using the purified RNA as template and the *smc01944* gene specific primers Smc01944-A-F (5′-GGATTTCACGGAGGACTTGA-3′) and Smc01944-A-R (5′-CTGAGGGACCAGCTTCTTG-3′), amplifying a 118 bp fragment, with the following conditions: 2 min at 95°C, 40 cycles with 20 s at 95°C, 25 s at 59°C and 30 s at 72°C followed by a final elongation step of 2 min at 72°C. RNA extracts were used for reverse transcription when no PCR products were obtained. The reaction mix for the reverse transcription (total volume 13 μl) contained 5 μl RNA (~0·5 to 1 μg), 8 μl DEPC-treated water, 2 mmol l^{-1} dNTP mix (New England Biolabs, Ipswitch, MA) and 5 ng μl^{-1} random primer (Invitrogen, Life Technologies, Carlsbad, CA). The reaction mix was incubated for 5 min at 65°C before 1× First Strand buffer (Invitrogen, Life Technologies), 5 mmol l^{-1} DTT (Invitrogen, Life Technologies), 2 U RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, Life Technologies) and 10 U SuperScript® III Reverse Transcriptase (Invitrogen, Life Technologies) were added. The reaction mix was then incubated for 5 min at 25°C, 60 min at 50°C and 15 min at 70°C in a S1000 thermal cycler (Bio-Rad Laboratories GmbH, Munich, Germany).

Quantitative PCR targeting the *Smc01944* gene

Quantitative PCR (qPCR) targeting the Smc01944 gene of *S. meliloti* was performed using an iQ5 real-time PCR detection system (iQ5 Optical System Software, ver. 2.0, Bio-Rad). Primers Smc01944-A-F and Smc01944-A-R were used with a fragment of the *smc01944* gene of *S. meliloti* cloned in a pEX-A plasmid vector (Eurofins Genomics, Ebersberg, Germany) as standard. Ten microlitre reaction volumes contained 1× Sso Advanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH), 1 μl of 10-fold diluted cDNA and 250 nmol l^{-1} of each primer. The qPCR conditions were 2 min at 98°C and 40 cycles with 15 s at 98°C and 20 s at 59°C. The protocol was followed by a melting curve analysis. All qPCR experiments were designed, performed and analysed according to the MIQE-guidelines (Bustin *et al.* 2009).

Chloroperoxidase enzyme activity

Chloroperoxidase activity was measured via the monochlorodimedone (MCD) assay (Hager *et al.* 1966). Due to the drawbacks of the assay (Wagner *et al.* 2008), we quantified monochlorodimedone and dichlorodimedone with an Agilent 6490 triple quadrupole mass spectrometer coupled to an Agilent 1260 HPLC (Agilent, Santa Clara, CA). Selected ion monitoring (SIM) was used in negative ionization mode to record the abundance of the $[M\text{-}H]^-$ ions at *m/z* 173 and *m/z* 207 for monochlorodimedone and dichlorodimedone respectively. Water with 0·1% formic acid and acetonitrile with 0·1% formic acid were used as eluents. For separation of the

analytes we used a Synergi™ Polar-RP column (3 × 150 mm, 4 μm particles, Phenomenex, Torrance, CA) with a Polar-RP guard cartridge (4 × 2 mm, Phenomenex Torrance, CA). For cell suspensions, *S. meliloti* was grown in VM medium to an OD_{600} of 1·0. Cultures were centrifuged (6000 ***g***, 1 min) and washed three times in 0·9% NaCl (pH7) and finally resuspended in 0·9% NaCl containing 1 mg l^{-1} monochlorodimedone. Sixty minutes after the addition of 10 mmol l^{-1} H_2O_2 to induce chloroperoxidase gene expression, 1 ml sample was centrifuged (6000 ***g***, 1 min) and filtered through a 0·22 μm mixed cellulose ester filter (Fisher Scientific GmbH, Schwerte, Germany) prior to LC-MS measurement. As a positive control the MCD assay was set up as described previously (Morris and Hager 1966) using a 0·9% NaCl solution (pH 3) with sodium phosphate instead of citric acid buffer. The chloroperoxidase of *Caldariomyces fumago* (Sigma-Aldrich) was used in a final concentration of 0·5 U ml^{-1}. Negative controls contained 0·9% NaCl solution with 1 mg l^{-1} monochlorodimedone and 10 mmol l^{-1} H_2O_2.

Acknowledgements

The authors thank Karin Stoegerer for assistance with the qPCR experiments and Jens Laaks for assistance with the GC measurements. This study was supported by the research unit 763 'Natural Halogenation Processes in the Environment – Atmosphere and Soil' funded by the German Research Foundation (DFG).

Conflict of Interest

The authors have no conflicts of interest to declare.

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