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Correlated production and consumption of chloromethane in the Arabidopsis thaliana phyllosphere

Muhammad Farhan Ul Haque1,3, Ludovic Besaury2,4, Thierry Nadalig1, Françoise Bringel1, Jérôme Mutterer2, Hubert Schaller2 & Stéphane Vuilleumier1

Chloromethane (CH3Cl) is a toxic gas mainly produced naturally, in particular by plants, and its emissions contribute to ozone destruction in the stratosphere. Conversely, CH3Cl can be degraded and used as the sole carbon and energy source by specialised methylotrophic bacteria, isolated from a variety of environments including the phyllosphere, i.e. the aerial parts of vegetation. The potential role of phyllospheric CH3Cl-degrading bacteria as a filter for plant emissions of CH3Cl was investigated using variants of Arabidopsis thaliana with low, wild-type and high expression of HOL1 methyltransferase previously shown to be responsible for most of CH3Cl emissions by A. thaliana. Presence and expression of the bacterial chloromethane dehalogenase cmuA gene in the A. thaliana phyllosphere correlated with HOL1 genotype, as shown by qPCR and RT-qPCR. Production of CH3Cl by A. thaliana paralleled HOL1 expression, as assessed by a fluorescence-based bioreporter. The relation between plant production of CH3Cl and relative abundance of CH3Cl-degrading bacteria in the phyllosphere suggests that CH3Cl-degrading bacteria co-determine the extent of plant emissions of CH3Cl to the atmosphere.

The phyllosphere, defined as the aerial part of plants and especially leaves, is a crucial if perhaps still overlooked ecosystem, with leaf surfaces estimated to total 1 × 10⁹ km², i.e. twice the land surface area. The phyllosphere represents a major habitat for micro-organisms. Bacteria, in particular, are present at 10⁶–10⁷ cells per cm² of leaf surface, as determined by both culture-dependent and culture-independent studies1. Availability of carbon is an important factor for microbial colonisation of the phyllosphere2. Methanol (global estimates of 100 Tg/yr for plant emissions3) and photosynthates such as fructose, sucrose and glucose, are prominent carbon sources on leaves2. Methanol represents a privileged growth substrate for methylotrophic bacteria, and such micro-organisms, in particular from the Alphaproteobacterial genus Methylobacterium, are dominant in the phyllosphere4.

Chloromethane is another volatile one-carbon compound produced by plant leaves5, with global production currently estimated at approximately 1–5 Tg/yr6. Although quantitatively far less important than methanol, chloromethane has environmental significance as the most abundant halogenated hydrocarbon in the atmosphere (~550 ppt), and because it is responsible for about 15% of chlorine-catalysed destruction of stratospheric ozone6. The best characterised mechanism for chloromethane production involves a methyltransferase-catalysed reaction, first demonstrated in Brassica oleracea7. In Arabidopsis thaliana, the methyltransferase gene HOL1 (Harmless to Ozone Layer) was identified by a knock-out mutation leading to loss of chloromethane production8. A halide methyltransferase gene homolog of HOL1 in Raphanus sativus (daikon radish, another member of Brassicaceae family) was since also shown to be involved in methyl halide emissions8.

Some specialised methylotrophic bacteria utilise chloromethane as their only source of carbon and energy for growth10,11, and may thus represent natural sinks for chloromethane emissions. In this context, methylotrophic chloromethane-degrading strains from the phyllosphere of A. thaliana were isolated12. These isolates feature

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the same chloromethane utilisation pathway previously characterised in detail for *Methylobacterium extorquens* CM4\(^{13,14}\). In this pathway, chloromethane utilisation is initiated by a corrinoid- and tetrahydrofolate-dependent methyltransferase system encoded by *cmuA* and *cmuB* respectively\(^{15}\), whose expression is regulated by chloromethane\(^{14,16}\). The *cmuA* gene is strongly conserved in chloromethane-degrading strains\(^{12}\), and has been used as a biomarker gene in studies of chloromethane degradation in different environments\(^{11,17,18}\). Here, we explore the topic of the interrelationships of sources and sinks of chloromethane with the model system of the *A. thaliana* phyllosphere. We use *HOL1* and *cmuA* as biomolecular markers of chloromethane emission and consumption, respectively, to investigate the potential role of chloromethane-degrading bacteria as filters of plant emissions of chloromethane to the atmosphere.

**Results**

A bipartite model laboratory system was set up consisting of well-studied organisms at the molecular level, *A. thaliana* for which the mechanisms of chloromethane production were identified, and *Methylobacterium extorquens* CM4, the best characterised chloromethane-degrading bacterium.

**Variants of *A. thaliana* with different expression of *HOL1***. The gene product of At2g43910 (Arabidopsis Information Resource, www.arabidopsis.org), designated *HOL1* for “Harmless to Ozone Layer”, was shown to be responsible for the major part of chloromethane emissions in *A. thaliana*. A T-DNA insertion mutant of *A. thaliana* wild-type (ecotype Col-0) causing a knock-out mutation in *HOL1* was obtained from the Salk Institute (http://signal.salk.edu) (Supplementary Information Fig. S1A). PCR genotyping of three *hol1* mutant lines (*hol1*-1, *hol1*-2, and *hol1*-3) allowed us to isolate homozygous *hol1*-1 mutant plants (Supplementary Information Fig. S1B). Two lines of *A. thaliana* overexpressing *HOL1*, termed *HOL1*-OX1 and *HOL1*-OX6\(^{19}\), were also characterised with respect to *HOL1* expression. As evaluated by RT-qPCR, *A. thaliana* hol1 plants showed significantly lower (*p < 0.0003*) expression of *HOL1* (20,000-fold). Conversely, significantly higher (*p < 0.00003*) expression of *HOL1* was observed in *HOL1*-OX1 (> 27-fold) and *HOL1*-OX6 (> 50-fold) plants compared to *A. thaliana* wild type (Supplementary Information Fig. S1C). Homozygous mutants *hol1*-1 (lowest *HOL1* expression) and *HOL1*-OX6 (highest *HOL1* expression) were chosen for further analysis, and designated as *hol1* and *HOL1*-OX, respectively.

**Quantification of *cmuA* gene content and expression in the phyllosphere of *A. thaliana* *HOL1* variants.** A quantitative PCR (qPCR) protocol targeting the chloromethane dehalogenase gene *cmuA* and the universal 16S ribosomal RNA gene in DNA obtained from washing of the leaf surface of *A. thaliana* was developed. Quantification of copy numbers of *cmuA* and 16S RNA genes was achieved, with a lower limit of 15 and 750 copies for *cmuA* and 16S RNA genes respectively (Supplementary Information Fig. S2).

The observed ratio of *cmuA*/16S rRNA gene copies was significantly higher (*p = 0.049*) in *A. thaliana* HOL1-OX plants, and significantly lower (*p = 0.004*) in *hol1* plants, as compared to wild type plants (Fig. 1A). Per weight, this corresponded to *cmuA* copy numbers per mg of fresh leaf of 13 ± 3, 71 ± 28 and 157 ± 43 on *hol1*, wild type and HOL1-OX plants respectively (Supplementary Information Table S1). In contrast, no significant differences of 16S rRNA gene copies per mg of fresh leaf (~10^6 copies per mg, *p > 0.4*) were observed for *hol1*, wild type and HOL1-OX plants.

Similar trends were observed in experiments performed with total leaf DNA, i.e. DNA extracted from both the surface and the interior of leaves (Fig. 1B). The obtained *cmuA*/16S rRNA ratio was approximately 100-fold higher with leaf surface DNA than with leaf total DNA. The number of gene copies of the 16S rRNA gene per mg
Fresh leaf was much higher in leaf total DNA (4.9 × 10⁵ for leaf surface DNA and of 6.9 × 10⁷ for leaf total DNA, Table S1), likely because of high levels of plastid DNA containing this gene in DNA preparations.

The expression of cmuA gene relative to 16S rRNA gene was investigated by RT-qPCR on total RNA preparations from plant leaves. Under our experimental conditions, 16S rRNA fulfills criteria for a reference gene as described. As observed at the DNA level, differences were observed between different variants of A. thaliana (Fig. 2), with HOL1-OX plants showing significantly higher expression of cmuA (> 2-fold, p = 0.00005) compared to wild type A. thaliana, and hol1 plants showing 10 times lower (p = 0.00003) relative expression of cmuA compared to wild type A. thaliana. A significant and positive linear correlation (Pearson’s correlation coefficient = 0.998, p = 0.035 at 95% confidence interval) was found between the expression of both plant HOL1 gene for chloromethane production and the bacterial gene cmuA for chloromethane degradation (Fig. 2).

Diversity of bacteria in the A. thaliana phyllosphere. The correlation between HOL1 genotype and cmuA gene presence and expression was investigated further. In the 3 A. thaliana HOL1 variants, the composition and diversity of the phyllosphere microbiota was compared. Sequence analyses of 16S rRNA and cmuA gene fragments, obtained after PCR amplification from leaf surface DNA and pyrosequencing was performed (Tables 1 and 2, Supplementary Information Table S4). DNA yields were similar for the 3 variants, and the numbers of sequences obtained after PCR amplification were similar in all variants for the 16S rRNA gene. In contrast, numbers were different for the cmuA gene in the 3 variants, in good agreement with qPCR analysis (Supplementary Information Table S4). Based on 16S rRNA gene analysis (V1-V3 region), total microbiota was similar in the 3 variants. In total, 152 OTUs were detected with > 0.01 average abundance, 44 (28%) of which were common to...
As also found here (Table 1). Nevertheless, a predominance of surfaces of epidermal cells (Fig. 5).

Most gas exchange between plant leaves and the atmosphere takes place, as well as in the intercellular grooves and (Supplementary Information Fig. S1C). In addition, application of a chloromethane-based bioreporter provided HOL1 DNA and RNA correlate with expression levels of the gene (Fig. 1) and gene expression (Fig. 2) of the bacterial this regard. The model system introduced in this work allowed us to demonstrate that both gene copy number (77.0% sequence identity) and CM4 for OTU28 (78.2%), respectively.

Numbers of sequences recovered by pyrosequencing after PCR amplification from the same amount of leaf A to achieve21.

Real-time measurements of chloromethane production in the phyllosphere at the required sensitivity are difficult.

Direct evidence for in planta detection of chloromethane production. Detection of the amounts of chloromethane production by a bacterial fluorescent bioreporter, including on leaves of A. thaliana, was previously described (Fig. 3). This bioreporter consists of the chloromethane-degrading strain M. extorquens CM4 containing a plasmid harbouring the chloromethane dehalogenase cmuA gene promoter region fused upstream to the syfp2 gene for yellow fluorescent protein (YFP)22. Fluorescence of the bioreporter strain was only observed in the presence of methyl halides, and was proportional to their concentration21. Here, two-weeks old seedlings of wild type, hol1, and HOL1-OX A. thaliana variants were sprayed with a bioreporter cell suspension, and YFP fluorescence was observed using confocal laser scanning microscopy after 24 h incubation (Fig. 3).

No fluorescent cells were observed on leaves of hol1 plants, whereas a large number of fluorescent cells were visible on leaves of HOL1-OX and wild-type plants (Fig. 3). Quantification of fluorescent cells showed that the density of fluorescent cells on plant leaf surfaces was significantly higher for HOL1-OX plants than for wild type (> 2.6-fold, \( p = 0.02 \)) and for hol1 (> 10-fold, \( p = 0.003 \)) plants (Fig. 4). In contrast, the number of bioreporter bacteria colonising the phyllosphere, evaluated by determining copy numbers of the syfp2 on plant leaf DNA by qPCR, was not significantly different in the different plant variants (Supplementary Information Table S2). Confocal microscopy also provided preliminary evidence on the source of chloromethane emissions on plant leaves (Fig. 5). Little is yet known on the localisation of the HOL1 methyltransferase and the expression profiles of the corresponding gene23. As reported previously for the tobacco phyllosphere with a methanol-based bioreporter24, the chloromethane-based bioreporter was frequently observed to be associated with stomata, where most gas exchange between plant leaves and the atmosphere takes place, as well as in the intercellular grooves and surfaces of epidermal cells (Fig. 5).

Discussion

Bacterial chloromethane utilisation was previously investigated in several environments25, yet the phyllosphere environment, possibly the largest source of chloromethane4, had not yet been specifically explored in this regard. The model system introduced in this work allowed us to demonstrate that both gene copy number (Fig. 1) and gene expression (Fig. 2) of the bacterial cmuA gene for chloromethane dehalogenase in phyllospheric DNA and RNA correlate with expression levels of the gene HOL1 responsible for chloromethane production (Supplementary Information Fig. S1C). In addition, application of a chloromethane-based bioreporter21 provided direct evidence for in planta expression of cmuA in the phyllosphere, and confirmed that HOL1 gene variants of A. thaliana indeed emit chloromethane at different levels (Fig. 3). This finding is important because direct in situ real-time measurements of chloromethane production in the phyllosphere at the required sensitivity are difficult to achieve21.

Bacteria of the genera Methylobacterium and Sphingomonas are predominant colonisers of the phyllosphere25, as also found here (Table 1). Nevertheless, a predominance of Methylobacterium such as that observed here has not been reported so far, and may derive from controlled greenhouse conditions of our experiments compared to field conditions of previous studies. Indeed, site-specific factors were reported to strongly impact the proportion of Methylobacterium in a bacterial community26. Sphingomonas, the second most highly represented

| OTU   | Closest homolog (accession number) | Sequence identity (%) | Recovered sequencesa |
|-------|------------------------------------|-----------------------|----------------------|
| OTU1  | Methylbacterium extorquens CM4 (AF011316.1) | 99.8                  | 2454, 1, 16448       |
| OTU2  | Hyphomicrobium sp. SAC-1 (AJ871015.1) | 98.8                  | 459, 1, 3514         |
| OTU3  | Hyphomicrobium sp. MC1 (FN667867.2) | 99.8                  | 103, 1, 2100         |
| OTU4  | Aminobacter ciceronei IMB-1 (AF307143) | 89.8                  | 39, 1, 294           |
| OTU5  | Aminobacter ciceronei IMB-1 (AF307143) | 89.3                  | 30, 205, 0           |
| OTU7  | Rhodobacteraceae bacterium 198 (AJ810827.1) | 99.3                  | 21, 0, 234           |
| OTU9  | Rhodobacteraceae bacterium 179 (AJ810826.1) | 99.5                  | 0, 0, 121            |
| OTU11 | Hyphomicrobium sp. SAC-1 (AJ871015.1) | 85.9                  | 98, 0, 0             |
| OTU19 | Uncultured marine bacterium (AJ810832.1) | 91.0                  | 0, 7, 14             |
| OTU20 | Hyphomicrobium sp. SAC-1 (AJ871015.1) | 87.0                  | 0, 0, 22             |
| OTU22 | Hyphomicrobium sp. SAC-1 (AJ871015.1) | 87.0                  | 22, 0, 0             |
| OTU28 | Uncultured marine bacterium (AJ810832.1) | 89.7                  | 0, 8, 0              |
| OTU35 | Aminobacter ciceronei IMB-1 (AF307143) | 99.3                  | 0, 0, 17             |

Table 2. Diversity of the cmuA chloromethane dehalogenase gene in the phyllosphere of A. thaliana variants.

aNumbers of sequences recovered by pyrosequencing after PCR amplification from the same amount of leaf wash DNA preparation, averaged across 6 samples (technical duplicates of biological triplicates) for each A. thaliana variant. bClosest cultivable chloromethane degraders were Hyphomicrobium sp. MC1 for OTU19 (77.0% sequence identity) and Methylbacterium extorquens CM4 for OTU28 (78.2%), respectively.
genus in our experiments, was substantially lower in the phyllosphere of the hol1 variant (Table 1). Sphingomonas strains have been reported to protect A. thaliana against pathogenic bacteria27, and A. thaliana disrupted in the HOL1 gene was more prone to pathogen attack, possibly due to reduced production of methyl thiocyanate (CH₃SCN) from glucosinolate-derived thiocyanate by HOL128. Indeed, disturbances in the metabolism of glucosinolate compounds was reported to modify the bacterial diversity in the rhizosphere of A. thaliana29, and in the phyllosphere of vegetable plants30.

The proportion of chloromethane-degrading bacteria in the phyllosphere microbiota inferred from qPCR of the cmuA gene have some important implications. First, the total size of the bacterial community (as deduced from 16S rRNA gene copies) is similar for all three A. thaliana variants, and is higher by several orders of magnitude than those of chloromethane-degrading bacteria containing the cmuA gene (Supplementary Information Table S1). This is in keeping with well-established knowledge that carbon compounds such as methanol and sugars are released from plant leaves in much larger quantities than chloromethane30. Second, if one considers
a single cmuA copy per chloromethane-degrading bacterium as usually observed so far\textsuperscript{31}, the average number of chloromethane-degrading bacteria in the phyllosphere of wild-type \emph{A. thaliana} may be estimated at approximately 71 ± 28 per mg of fresh leaf (Supplementary Information Table S1). Based on a chloromethane dehalogenase activity of 1.670 µmol per day per mg protein by \emph{M. extorquens} CM4\textsuperscript{32}, and on a value of approx. 165 fg protein per bacterial cell\textsuperscript{33}, this number of bacteria could transform about 20 pmol chloromethane per day per mg of fresh leaf. By comparison, total emissions of chloromethane from wild type \emph{A. thaliana} were reported at 0.6 pmol per day per mg of fresh leaf\textsuperscript{8}, i.e. approximately an order of magnitude lower. This suggests not only that the phyllosphere microbiota can serve as an efficient filter for plant emissions of chloromethane, but also that chloromethane emissions from plants reported in the literature reflect the difference between chloromethane emissions from vegetation and degradation of chloromethane by vegetation-associated bacteria, rather than total chloromethane production by vegetation.

Despite widely available methanol, the modest amounts of chloromethane produced by \emph{A. thaliana} are associated with an increased presence of chloromethane-degrading methylotrophic bacteria containing the cmuA gene in the phyllosphere. Whether this is solely due to chloromethane-dependent growth or to another as yet unknown phenomenon is still unknown, and deserves further investigation.

In conclusion, the biomolecular tools developed here to quantify gene signatures for chloromethane production and utilisation, as well as to detect and potentially quantify chloromethane production, may eventually contribute to better estimates of the overall budget of chloromethane crucial to environmental issues associated with destruction of stratospheric ozone. This, however, will require a better understanding of bacterial chloromethane utilisation in the environment. An important open question in this context is whether pathways for utilisation of
chloromethane other than the cmu pathway, the only microbial pathway for chloromethane degradation char-
acterised so far, are also relevant in defining the bacterial sink for natural emissions of chloromethane to the
atmosphere, and from the phyllosphere in particular.

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 clas-
ic, ELGA) and sterilisation was done by autoclaving (20 min at 121 °C under 2 bars) or by filtration (0.2 μm,
Nalgene).

Bacterial strains and growth conditions. Chloromethane-degrading Methylobacterium extorquens
CM4 (NCIMB 13688, VKM B-2223) and the bioreporter strain were laboratory stocks conserved at –80 °C in
10% glycerol in minimal mineral medium (M3)14. Liquid cultures were performed in 50 mL M3 medium in
Erlenmeyer flasks (250 mL) fitted with scaled miniblunt valve caps (Supelco) and incubated at 30 °C with agitation
at 100 rpm. Liquid M3 medium was supplemented with 20 mM methanol and/or chloromethane as described
previously12. Growth of liquid cultures was monitored by measuring optical density at 600 nm.

Plant material and growth conditions. All plants used in this study belonged to the Arabidopsis thaliana
Columbia (Col-0) background. The Salk T-DNA lines of hol1 mutant plants (SALK_005204, hol1-1, hol1-2, and
hol1-3) were obtained from the Salk Institute (http://signal.salk.edu/cgi-bin/tdnaexpress), and seeds of the A.
thaliana lines HOL1-OX1 and HOL1-OX6 (expressing HOL1 under the control of the CaMV 35 S promoter) were
obtained from Drs. Yukari Nagatoshi and Tatsuo Nakamura (Yokohama National University, Yokohama, Japan).
Plants were grown at 22 °C with a 12 hours light period in standard horticultural soil in a plant growth chamber
(PGC-15.5HILX) (Percival Scientific, USA). Relative humidity was 60% to 80%, and photon fluence rate from
white fluorescent tubes was 60 μmol m−2 s−1 at the level of rosettes14.

Wild-type (Col-0) and HOL1 gene mutants of A. thaliana were screened by PCR reactions (Supplementary
Information Fig. S1).

DNA extraction from plants. For leaf total DNA, fresh green leaves (2–3 leaves, 100–230 mg) were plucked
from plants just before flower bolting stage. Total DNA from leaves was extracted using the FAST DNA spin kit
(MP Biomedicals, Santa Ana, CA) as described35. Leaf material was transferred to lysing matrix A tubes and
macerated in 200 μL of phosphate buffer (120 mM, pH 8). After addition of 600 μL CLS-VF buffer and 200 μL PPS
buffer from the kit, leaf material was lysed using a Mikro-Dismembrator (Sartorius Stedim Biotech, France) with
3 treatments of 1 min each at 3000 min−1. DNA was then purified from the lysate according to the manufacturer's
protocol. Final elution of purified DNA was performed in 100 μL of DNase-free sterile water. DNA was quantified
by NanoDrop and purity was checked by gel electrophoresis on agarose gel (1%).

Leaf surface bacterial DNA was extracted from A. thaliana leaf washings using a protocol adapted from the lit-
erature86,37. Briefly, 10–15 leaves of A. thaliana plants (1–2 g fresh weight) were washed in 50 mL sterile tubes con-
taining 30 mL TE buffer (pH 7.5) with 0.2% Silwet L-77 (GE Bayer Silicones). Bacteria were dislodged from the
leaf surface by shaking (350 rpm, 10 min) and sonication (25% amplitude, 30 seconds, VibraCell 75042, Bioblock
Scientific), followed by vortexing for 30 seconds. The resulting suspensions were then filtered (Nylon net filters
180 μm; Millipore) to separate bacterial material from cell suspensions. Percoll was added to the cell suspensions,
and centrifuged at 12'000 g for 10 min. Supernatant was removed, and the obtained cell pellet was washed in TE
buffer (pH 7.5), resuspended in 200 μL of TE buffer (pH 7.5), and transferred to lysing matrix A tubes for DNA
 extraction using the Fast DNA spin kit (MP Biomedicals, Santa Ana, CA). Cells lysis was performed in 800 μL
of CLS-VC reagent from the kit using Mikro-Dismembrator (Sartorius Stedim Biotech, France) with three treat-
ments of 45 s each at 3000 min−1. DNA was extracted from the lysate using the protocol as described above for
leaf total DNA.

Total RNA extraction from plants. Total RNA from plant leaves was extracted from 8–10 weeks old
wild-type, hol1 and HOL1-OX6 plants of A. thaliana. Leaf samples (100 mg) were transferred to 1.5 mL
RNase-free tube and immediately flash frozen in liquid nitrogen. The leaf material was lysed under liquid nitro-
gen-in lysing matrix A tubes (MP Biomedicals, Santa Ana, CA) using Mikro-Dismembrator (Sartorius Stedim
Biotech, France) with 3 treatments of 1 min each at 3000 min−1. Total RNA was extracted using the NucleoSpin
RNA plant kit (Macherey-Nagel, France), according to the manufacturer's protocol. RNA was quantified
by NanoDrop, and purity was checked by gel electrophoresis (1% agarose gel). Absence of DNA contamination in
the RNA was checked by PCR with ACT2 gene primers (Supplementary Information Table S3).

Primer design. Primers used in this study are listed in Supplementary Information Table S3. Primers for
qPCR of HOL1 plant gene were designed using PrimerQuest tool (Integrated DNA Technologies), and their
specificity was verified with a BLAST analysis at NCBI. Primers for qPCR of cmuA were designed on the basis
of alignments of cmuA sequences from reference chloromethane-degrading strains. Already described forward
primer cmuA802F17 and a newly designed reverse primer cmuA968R with identity > 80% over the entire length
of primer with all cmuA sequences in the NCBI database, were used.

Quantitative PCR (qPCR). Evaluation of cmuA and 16S rRNA gene copy number was performed by qPCR
using GeneAmp 5700 Sequence Detection System (Applied Biosystems). Analyses were carried out in a 96 well
reaction PCR plate (Applied Biosystems), using gene specific primers (Supplementary Information Table S3 for
sequences and references of all primers) in a total reaction volume of 20 μL, as described previously12. Conditions
for qPCR reaction consisted of initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/elongation at 60 °C for 60 s. Negative controls without DNA and control reactions to check for absence of inhibition of PCR amplification in each DNA sample were performed by spiking known quantities of M. extorquens CM4 DNA. Specificity of amplification was determined after qPCR cycles from dissociation curves obtained by increasing 1 °C per 30 s from 60 °C to 90 °C, or after gel electrophoresis of PCR products on 2% agarose. The gene copy number of cmuA and 16S rDNA per µg of phyllosphere DNA were determined using calibration curves obtained from qPCR of tenfold dilution series of DNA standards (Supplementary Information Fig. S2). Analyses were performed with at least three biological replicates of each plant type and three technical repeats for each biological sample.

**Quantitative reverse transcriptase – PCR (RT-qPCR).** Expression of HOL1 gene in plant leaves was quantified using ACT2 as a reference gene in RT-qPCR analysis. Superscript III Reverse Transcriptase Kit (Invitrogen) was used to synthesize cDNA from plant total RNA (500 ng) using random primers (Roche) according to the manufacturer’s protocol. Expression of bacterial cmuA gene in plant leaves was also quantified by RT-qPCR analysis, using 16S rRNA as a reference gene. Phyllosphere total RNA (500 ng) was used to synthesize cDNA using Superscript III Reverse Transcriptase Kit (Invitrogen), and gene specific primers (cmuA968R and PROK1492R for cmuA and 16S rDNA respectively) according to the manufacturer’s protocol. After the RT reaction, the cDNA was diluted 2.5 times, and 5µL used as template for qPCR. Real-time qPCR measurements were performed as described above for DNA samples. Control reactions without RT enzyme were performed for each RNA extraction to check for absence of contaminating DNA. The relative level of cmuA and HOL1 gene expression was estimated using the comparative threshold amplification cycle method (2^(-ΔΔCt)) as described35. Analysis was performed with three to five biological replicates of each plant type, with three technical repeats for each biological replicate.

**Pyrosequencing analysis.** DNA was extracted as described above. Bacterial tag-encoded FLX 454 pyrosequencing of phyllosphere DNA (bTEFAP) was performed by Research and Testing Laboratory (Lubbock, TX, USA) as described previously38–40, with a Genome Sequencer FLX instrument and Titanium protocols and reagents (Roche Applied Science, Indianapolis, IN). Primers Gray28F and Gray519R (Supplementary Information Table S4) spanning the variable regions V1–V3 in the 16S rRNA gene38,39 were used in a single step PCR reaction (35 cycles) using HotStar HiFidelity Polymerase (Qiagen, Valencia, CA). Primer cmuA802F 17 was used together with primer cmuA1244R (Supplementary Information Table S4) for amplification of a 443 bp cmuA amplicon under the same conditions.

**Sequence processing and analysis.** For amplicons of the 16S rRNA gene, obtained sequences were screened, failed sequence reads, low quality sequence ends, tags and primers were removed, and non-bacterial ribosome sequences, chimeras detected using black box chimera check software B2C241, and short reads (<250 bp) were discarded as described previously38–40. Operational taxonomic units (OTUs) were assigned at 5% dissimilarity, commonly used to represent the genus level33, validated using taxonomic distance methods, and data reduction analysis performed as described previously38,40.

For cmuA amplicon analysis, Mothur was used to extract flow grams from sff files. Flow grams were de-noised and translated to DNA sequences, and reads with errors in the barcode or primer region, ambiguous bases, or homopolymer runs >6 bp were removed. Quality filtering and sizing of reads (between 400 and 450 bp), conversion to fasta format, read dereplication, abundance sorting and removal of singletons was done with USEARCH44. Chimeras were filtered out using UCHIME within USEARCH. Iterative clustering of OTUs was carried out with UPARSE within USEARCH to a cutoff of 85%. The most abundant sequence within each OTU was chosen as representative of the OTU, and taxonomic affiliation was performed by Blast comparisons to the Genbank database.

**Inoculation of bioreporter to plant leaves.** The bacterial bioreporter strain constructed based on plasmid harbouring the syfp2 gene coding for yellow fluorescent protein (YFP) fused with the promoter region of cmuA gene was grown until mid-exponential phase (OD_600 ~0.4) in M3 medium supplemented with MeOH (20 mM). Cells were harvested after centrifugation at 4500 x g, washed and resuspended in M3 medium at a final OD_600 of 0.2.

A. thaliana wild type, hol1 and HOL1-OX plants were grown in Petri dishes on Murashige and Skoog (MS) medium including vitamins (Duchefa) supplemented with 1% sugar and 0.7% pastagar. Plant seeds were surface sterilised (with 25% commercial solution of sodium hypochlorite for 10 minutes and then rinsed five times with sterile water) before sowing to avoid undesired bacteria on plant leaves. Plants were grown at 22 °C with 12 hours light period for 14-days in a plant growth room. Cells suspensions (5 mL per Petri dish containing 20 plants) were sprinkled uniformly on leaves and left to dry for 1 hour under a laminar flow hood. Petri dishes were then further incubated for 24 hours under the same plant growth conditions before observation under confocal microscopy.

**Confocal microscopy and image analysis.** Leaves of similar sizes were cut from plants treated with the bioreporter, mounted on microscope glass slides and visualised for YFP fluorescence (YFP filter, 488 nm) under the 20x lens of confocal laser scanning microscope (Zeiss LSM710), and images were recorded. Three images from different locations per plant, and three plants for each genotype were randomly selected. Images were analysed to quantify YFP fluorescence on leaves using ImageJ software (http://rsbweb.nih.gov/ij/index.html). Fluorescence intensity was used to discriminate bacteria from the background. Each fluorescent spot identified by ‘Find maxima imageJ’ tool with noise value set at 50 was considered as a fluorescent bacterial bioreporter cell, and...
the number of fluorescent cells per mm² was measured. After confocal observation, total DNA was also extracted from inoculated leaves to confirm the equal abundance of bioreporter bacteria on each type of plant by qPCR of the yfp-2 gene.

**Statistical analysis.** Experiments were performed in at least three biological replicates, with two to three technical repeats for each biological replicate. For statistical significance, data were analysed using Student’s t-test, with different letters in figures indicating statistically significant differences at p < 0.05. Pearson’s correlation coefficient R for average relative expression levels of HOL1 versus the average relative expression levels of cmuA (Fig. 2), and propagated errors, were calculated from errors associated with values of cmuA and HOL1 expression based on ± 1 standard deviation of the mean of 3 replicate measurements.

**Data Availability.** The sequence data generated in this study were deposited to Genbank under accession numbers SANN07314135-SANN07314170 (Bioproject PRJNA393055).

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**Author Contributions**

M.F.U.H., H.S. and S.V. conceived the work and designed experiments, and M.F.U.H., J.M. and H.S. performed the experiments. All co-authors contributed to analyse the data, write the manuscript, and take public responsibility for the manuscript content.

**Additional Information**

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