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Serratia odorifera: analysis of volatile emission and biological impact of volatile compounds on *Arabidopsis thaliana*

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Abstract Bacteria emit a wealth of volatiles. The combination of coupled gas chromatography/mass spectrometry (GC/MS) and proton-transfer-reaction mass spectrometry (PTR-MS) analyses provided a most comprehensive profile of volatiles of the rhizobacterium *Serratia odorifera* 4Rx13. An array of compounds, highly dominated by sodorifen (approximately 50%), a bicyclic oligomethyl octadiene, could be detected. Other volatiles included components of the biogeochemical sulfur cycle such as dimethyl disulfide (DMDS), dimethyl trisulfide and methanethiol, terpenoids, 2-phenylethanol, and other aromatic compounds. The composition of the bouquet of *S. odorifera* did not change significantly during the different growth intervals. At the beginning of the stationary phase, 60 μg of volatiles per 24 h and 60 easily detectable components were released. Ammonia was also released by *S. odorifera*, while ethylene, nitric oxide (NO) and hydrogen cyanide (HCN) could not be detected. Dual culture assays proved that 20 μmol DMDS and 2.5 μmol ammonia, individually applied, represent the IC_{50} concentrations that cause negative effects on *Arabidopsis thaliana*.

Keywords Rhizobacteria · *Serratia odorifera* · Volatiles · Plant growth promotion and inhibition · Dimethyl disulfide · Sodorifen

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Introduction

Rhizobacteria comprise a diverse group of bacteria preferentially living in the soil closely associated with the roots exploiting the rich nutrient exudates (up to 40% of the plant photosynthates) that plants deliver into the rhizosphere (Lynch and Whipps 1990; Bais et al. 2006). Consequently, large microbial populations develop. Many rhizobacteria are classified as plant growth promoting rhizobacteria (PGPR) and are potentially useful for biological control because of their capability to suppress soil-borne plant diseases (Whipps 2001; Lugtenberg and Kamilova 2009). The mechanisms by which rhizobacteria/PGPR can support plant growth span from nitrogen fixation, secretion of phytohormones, solubilization of minerals or secretion of antibiotics and antifungal metabolites. Apart from these mechanisms it recently became apparent that microorganisms have developed another potential weapon against phytopathogens. They are capable of releasing functional volatile organic compounds (VOCs) (Kai et al. 2007; Vespermann et al. 2007; Kai et al. 2009). Characteristically, these compounds exhibit molecular masses below 300 Da, they are rather lipophilic and have relatively low boiling

points. Such volatiles are ideal infochemicals because they occur in the biosphere over a range of concentrations and can act over long distances (Wheatley 2002). Therefore, these compounds can have an important effect on neighboring organisms and the development of the organisms in an ecosystem. VOCs were shown to be biologically useful in numerous cases, e.g., allowing pollinators to localize flowers, to attract predators of herbivores (indirect defense), or to defeat enemies directly or to cause growth inhibition. As a result, these compounds may act inter- or intraspecifically (Alborn et al. 1997; Pare and Tumlinson 1999; Piechulla and Pott 2003).

A wealth of volatile compounds is produced and released in the microbial world (Stotzky and Schenck 1976). In 26 *Streptomyces* species, more than 120 different VOCs were identified, comprising alkanes, alkenes, alcohols, esters, ketones, sulfur compounds, and terpenoids (Schöller et al. 2002; Dickschat et al. 2005). Well known is the *Streptomyces* volatile terpenoid geosmin with its characteristic earthy and musty odor (Gust et al. 2003). In *Myxococcus xanthus* 42 volatiles were identified (Dickschat et al. 2004). Up to now, more than 400 volatiles are known to be emitted from many different bacteria (superscent, http://bioinf-services.charite.de/superscent; Schulz and Dickschat 2007). The biological significance of a few of these microbial volatiles has been investigated. Volatiles of not further characterized soil bacteria influence the growth of various fungi (Alstrom 2001; Wheatley 2002; Chuankun et al. 2004; Fernando et al. 2005). Also rhizobacterial isolates comprising *Serratia plymuthica, Serratia odorifera, Stenotrophomonas maltophilia, Stenotrophomonas rhizophila, Pseudomonas fluorescens,* and *Pseudomonas trivialis* synthesize and emit complex blends of volatiles that inhibit growth of many phytopathogenic and non-phytopathogenic fungi (Kai et al. 2007; Vespermann et al. 2007). (2*R*,3*R*)-Butanediol and acetoin, released from specific strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens*, promote growth of *Arabidopsis thaliana* seedlings (Ryu et al. 2003). Furthermore, bacterial volatiles also have an impact on protozoa, metazoa such as nematodes, and *Aedes aegypti* (reviewed in Kai et al. 2009).

Serratia species are Gram-negative γ-proteobacteria with an ubiquitous distribution. They produce a number of interesting non-volatile compounds (e.g. prodigiosin, pyrrolnitrin), which influence the growth of plant pathogenic fungi such as *Rhizoctonia solani, Sclerotinia sclerotiorum*, and *Verticillium dahliae* (Kalbe et al. 1996). The antagonistic potential is due to the release of chitinases, while the production of the hormone indole-3-acetic acid (IAA) promotes plant growth (Frankowski et al. 2001; Kalbe et al. 1996). Recently, we demonstrated that *S. odorifera* emits a substantial number of volatiles, which inhibit the growth of many fungi during co-cultivation (Kai et al. 2007; Vespermann et al. 2007).

It was the goal of the present investigtion to analyse the complex blend of volatiles released by *S. odorifera* 4Rx13 in more detail by using various analytical methods. Our interest was focussed on the qualitative and quantitative composition as well as on the timing of volatile emissions. The biological significance of some low boiling commercially available compounds was tested on the model plant *A. thaliana*.

Materials and methods

Bacterial cultures The bacteria isolate used in this investigation was obtained from the Strain Collection of Antagonistic Microorganisms (Prof. Dr. Gabriele Berg, University of Graz, Austria, Institute of Environmental Biotechnology, Petersgasse 12, A 8010 Graz). *S. odorifera* 4Rx13 was isolated from the rhizosphere of *Brassica napus*. Bacteria grew on nutrient agar II (NA II; peptone from casein 3.5 gl^{-1}, peptone from meat 2.5 gl^{-1}, peptone from gelatine 2.5 gl^{-1}, yeast extract 1.5 gl^{-1}, NaCl 5 gl^{-1}, agar-agar 15 g^{-1}, pH 7.2) or in liquid nutrient broth II (NB II: NAII without agar) at 30°C. At appropriate time points, small samples were taken and diluted to determine living cell number (cfu) and OD (at 600 nm).

Bioassay Seeds of *A. thaliana* (cv. Columbia 0) were surface sterilized (2 min 70% ethanol, 5 min 5% calcium hypochlorite, rinsed four times with sterilized distilled water) and placed on Petri dishes containing half-strength Murashige–Skoog medium (MS medium; Murashige and Skoog 1962). The seeds were vernalized for 3 days at 4 °C in the absence of light, and subsequently placed in a growth chamber (16 h light/8 h darkness, light: 5 Osram L58 W/76 lamps, 84 $\mu mol m^{-2} s^{-1}$, 20°C). *A. thaliana* seedlings were transferred after 6 days from a normal Petri dish to one side of a bi-partite Petri dish. After 24 h a filter paper with various amounts of ammonia or DMDS was placed on the other side of the Petri dish. Cultivation was continued for 14 days (20°C, 84 $\mu mol m^{-2} s^{-1}$ light, 16 h/8 h light/darkness), and images of the plants were recorded (digital camera C-3030 Zoom, Camedia, Olympus, Tokyo, Japan).

Collection and GC/MS analysis of bacterial volatiles Bacteria were inoculated into 6 ml NB II and incubated at 30°C under agitation for 12–24 h (160 rpm; Bühler, Tübingen, Germany). Reaching an OD_{600} of 1.0–1.5, cultures were transferred into 100 ml NBII medium to obtain an initial inoculum of 0.005 OD_{600}. Charcoal-purified, sterile air entered the Erlenmeyer flask through an inlet with a constant flow of 1 $l min^{-1}$, which was provided by a membrane pump (Denver Gardner, Puchheim, Germany). A collection trap, containing 100 mg Super Q (Alltech Associates, Deerfield, Illinois, USA) was connected to the

outlet, and a second pump sucked the volatile-enriched air through the trap with a constant flow of 0.5 $l min^{-1}$. The excess air of 0.5 $l min^{-1}$ left the Erlenmeyer flask through a bypass. During the following incubation period, the volatiles were collected in intervals of 24 h up to 96 days. Volatiles were eluted with 200 and 100 μl hexane, and for quantitation nonyl acetate (5 ng) was added as an internal standard.

Samples were analyzed using a Shimadzu GC/MS-QP5000 system (Kyoto, Japan) equipped with a DB5-MS column (60 m×0.25 mm×0.25 μm; J&W Scientific, Folsom, California, USA). Splitless injection of 1 μl sample was performed at 200°C with solvent delay of 2 min using a CTC autosampler (CTC Analytics, Zwingen, Switzerland). The initial column temperature was set at 35°C, followed by a ramp of 10°C min^{-1} up to 280°C with a final hold for 15 min at 280°C. Helium was used as the carrier gas at a flow rate of 1.1 $ml min^{-1}$.

Mass spectra were obtained using the scan modus (70 eV, total ion count, 40–280 m/z). Confirmation of structure assignments was done by comparison of mass spectra and retention times with those of available standards and with literature data, NIST147, and Wiley library as well as by comparison of Kovats indices. Experiments were replicated five times.

Collection and PTR-MS analysis of bacterial volatiles A home built proton-transfer-reaction mass spectrometer (PTR-MS) was used in combination with a gas handling system to monitor the headspace of *S. odorifera* bacterial cultures at the department of Molecular & Laser Physics, Radboud University, Nijmegen.

Gas handling set-up For the headspace measurements, 50 ml of liquid bacterial cultures (OD 0.005) were incubated in 250 ml glass Erlenmeyer flasks with a glass top and two teflon open/close valves acting as inlet and outlet. The bacteria were kept at 30°C during the experiment in an environmental chamber (Sanyo, Sanyo Electric Co., Ltd, Osaka, Japan), and the cuvettes were continuously flushed with a flow of 1 $l h^{-1}$ of dry air (Air Liquide B.V., Eindhoven, The Netherlands) via the gas inlet (mass flow controllers from Brooks Instrument, Ede, The Netherlands). The cuvettes were continuously shaken at 160 rpm (Orbital Shaker, Sanyo Electric Co., Ltd, Osaka, Japan). During the measurement living cell number (cfu) was determined. All the sampling lines were made of teflon (Polyfluor Plastics, Oosterhout, The Netherlands) to prevent memory effects, and both the sampling lines and the drift tube were heated to 55°C for the same reason. Three experiments were performed. In each experiment five cuvettes were measured, three with *S. odorifera* bacterial cultures in liquid NBII medium and two with NBII medium alone. The cuvettes were sequentially monitored for 45 min each by switching from cuvette to cuvette using a stream selector valve (Valco Cheminert Valves, Bester BV, Amstelveen, The Netherlands).

Proton-transfer-reaction mass spectrometry measurements PTR-MS had been proven to be a great tool for sensitive online determinations of VOCs in medical research (Lindinger et al. 1998a, b; Steeghs et al. 2006, 2007), environmental monitoring (Lindinger et al. 1998a, b; de Gouw et al. 2003), and biological research (Boamfa et al. 2004) including microbial volatiles (Critchley et al. 2004; Bunge et al. 2008). PTR-MS has been described elsewhere in detail (Lindinger et al. 1998a; Boamfa et al. 2004). Briefly, in PTR-MS, trace gases with a proton affinity higher than that of water are ionized by a proton-transfer reaction with H_3O^+ ions in the drift tube, after which the produced ions are mass analyzed with a quadrupole mass spectrometer and detected by a secondary electron multiplier. Each compound is detected at the molecular mass of the substance plus the mass of the single proton m_H. There are no interferences with natural constituents of air (N_2, O_2, CO_X, NO) since their proton affinities are lower than that of water. PTR-MS is also a versatile online monitoring technique that allows the simultaneous measurement of different VOCs with a fast response and sensitivity from ppmv (parts per million volume, 10^{-6} (*v*/*v*)) to ppbv (parts per billion volume, 10^{-9} (*v*/*v*)) and even below (Lindinger et al. 1998a). The list of VOCs that can be detected with PTR-MS includes most aldehydes, ketones, alcohols, acids, and esters, as well as many unsaturated, aromatic and N or S substituted compounds.

Since PTR-MS only determines the *m/z* ratio of a molecule, a complementary technique such as gas chromatography/mass spectrometry (GC/MS) must be used for unequivocal structure elucidation. For low molecular mass compounds (such as methanol) there is not much interference from other compounds; however, with increasing molecular masses, the number of candidate compounds meeting the respective molecular mass increases. When no other technique is available, the use of natural isotopic ratios and literature search may help making an educated guess of the identity. Isotopic ratios can provide information about the composition of a molecule. Most commonly the ^{13}C isotope is used, taking into account that ^{13}C constitutes 1.1% of the carbon present, i.e., $^{13}C/^{12}C=0.011$. For example, for a compound detected with PTR-MS at *m/z*=59 with a ratio (*m/z*+1)/(*m/z*)=m60/m59=3.3%, the presence of three carbons is expected in its molecular formula. Thus, this method provides valuable information about the molecular formula of a compound, which in special cases may lead to identification. Nevertheless, care must be taken with respect to any structure assignments.

The system used in the present investigation was calibrated by diluting a mixture of six compounds (methanol, acetaldehyde, acetone, isoprene, benzene, toluene) in concentrations of 1 ppm (±5%) (Linde, Dieren, The Netherlands); this way, the calibration factor to convert ion intensity into ppb was obtained for these masses, while for the rest of the masses the calibration factor was calculated from the theory (de Gouw et al. 2003).

Determination of ethylene emission Ethylene measurements were performed at the Radboud University Nijmegen by using a sensitive laser-based ethylene detector (type ETD-300, Sensor Sense B.V., Nijmegen, The Netherlands) in combination with a gas handling system. The ETD-300 detector is based on laser photoacoustic spectroscopy, a well-established method successfully used in many applications in Life Science where traditional methods, such as gas chromatography, cannot be employed because of lack of sensitivity and time-consuming preconcentration steps (Clarke et al. 2009; Cristescu et al. 2008; Salman et al. 2009). The detection limit of the ETD-300 is about 300 parts per trillion volume ($1{:}10^{12}$) for online ethylene within a 5 s time-scale. Gas handling was performed by a valve control box (type VC-6, Sensor Sense B.V., Nijmegen, The Netherlands) designed for measuring up to six sampling cuvettes per experiment. Emission of ethylene from *S. odorifera* was measured in real time over a 48-h period. As a source, 6 ml NBII with and without bacterial inoculation were continuously flushed with air. Through the valve control box, ethylene was transported to the ETD-300 alternately, at intervals of 30 min for each culture flask and at controlled flow rates of 2 $l h^{-1}$, thus, preventing accumulation effects. In order to obtain a good overview, the average value of ethylene production from the last 20 min out of the 30 min of sampling was displayed, indicating the ethylene emission rate every second hour.

Determination of NO emission In this study, the NO emission was measured via mid-infrared absorption-based detection technique. A quantum cascade laser-based spectrometer equipped with a multi-pass absorption cell for wavelength modulation spectroscopy on NO was used (Cristescu et al. 2008).

Hydrogen cyanide Test Whatman paper was soaked in 5 mg copper(II) ethylacetoacetate (Sigma, Aldrich, Taufkirchen, Germany) and 5 mg 4,4-methylenebis-*N*,*N*-dimethylaniline (Strem Chemicals, INC, Newburyport, USA), dissolved in 1 ml chloroform and dried in the dark (Castric and Castric 1983). This Whatman paper was transferred into an empty side of a bi-partite Petri dish. The other side was preincubated with bacteria for 24 h. The incubation was continued for 14 days. *P. fluorescens* CHA0, an isolate which was previously characterized as HCN producing strain (Voisard et al. 1989) served as positive control, whereas *P. fluorescens* CHA207 was applied as a negative control (Blumer et al. 1999).

NH_3 Test S. odorifera 4Rx13 was inoculated on NAII in one compartement of a bi-partite Petri dish. Subsequently a Quantofix® ammonium test stick (Macherey & Nagel, Düren, Germany) was inserted into the empty compartment. On this side of the Petri dish a 0.1 cm hole was punched into the side wall, which was closed with Nescofilm® to avoid contamination and exchange of air. After incubation of 24 h a microliter-syringe was injected through this hole, and 10 µl ddH_2O were applied onto the test stick to start the reaction. Thirty seconds later the reaction was stopped by dropping 10 µl of 28% NaOH onto the test stick. Images of the test sticks were then recorded using a digital camera Casio Exilim EX-Z100 (Casio Europe GmbH, Norderstedt, Germany). The same Quantofix® ammonium test sticks were used to establish a calibration curve with different concentrations of ammonia (0.5–50 µmol).

Results

GC/MS analysis of volatiles during growth of *Serratia odorifera*

S. odorifera emits a wealth of volatile organic compounds during batch cultivation on solid NAII medium (Kai et al. 2007). To correlate volatile composition to bacterial growth, *S. odorifera* was grown in liquid NBII medium. Samples were taken at appropriate times to determine the living cell number (cfu) and optical densities (OD) (Fig. 1a). The stationary phase was reached approximately 12 h after inoculation. Furthermore, headspace volatiles were collected on Super Q in intervals of 24 h (0–24 h, 24–48 h, 48–72 h and 72–96 h) and subsequently analyzed by GC/MS (Fig. 1 b, c and d). A representative GC profile of the 24–48 h interval is shown in Fig. 1b. In order to reveal more details, the profiles were magnified: compounds with retention times 8–19 min are depicted in Fig. 1c while the area of retention times 18.5–25 min is shown in Fig. 1d. The profiles demonstrate the wealth of volatiles emitted by *S. odorifera* in liquid medium; in each interval more than 40 compounds could be easily detected (Fig. 1e). The highest number of individual compounds (60) as well as the largest amounts of volatiles (ca. 62 µg during 24 h) emanated during the second interval, while in the first and third interval ca. 20–25 µg were released per day (Fig. 1f). Therefore, the beginning of the stationary phase (24–48 h) is the time when quantitatively and qualitatively highest levels of volatiles are emitted. All compounds detected

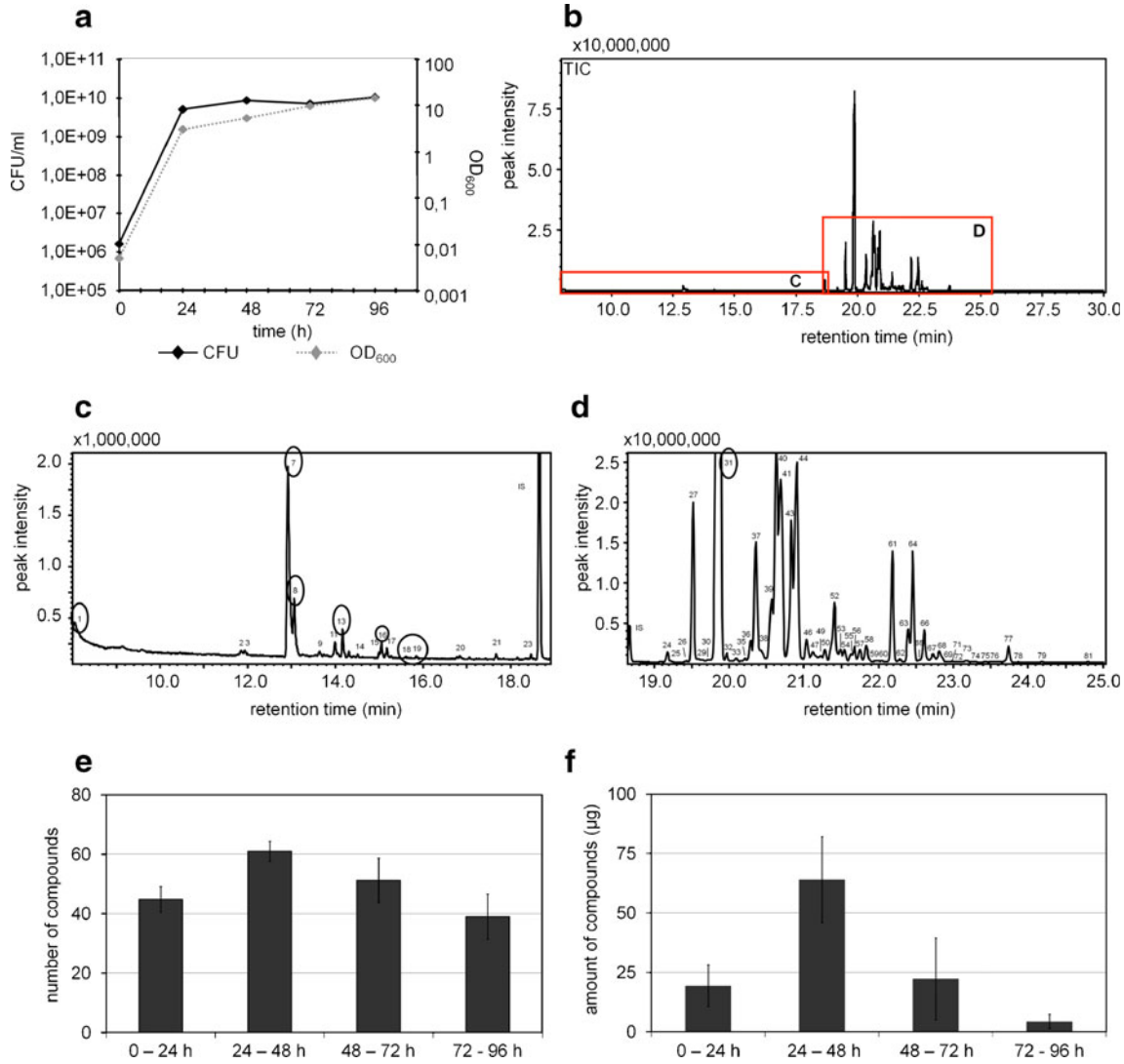

Fig. 1 GC/MS analysis of volatiles released by *Serratia odorifera*. **a** Growth of *S. odorifera* over 96 h after inoculation. Colony forming unit (cfu), optical density at 600 nm (OD_{600}). **b** GC/MS profile of headspace volatiles of *S. odorifera*. Accumulation between 24 and 48 h. **c** Magnified GC-MS profile, retention time 9–18.5 min. Peak numbers are listed in supplement Table 1. IS internal standard. **d** Magnified GC-MS profile, retention time 18.5–25 min. Peak numbers are listed in supplement Table 1. **e** Total number of easily detectable compounds emitted in each interval. n=5, SD indicated. **f** Total amount of easily detectable compounds emitted in each interval. n=5, SD indicated

were correlated with retention indices (RI), and names of unequivocally identified compounds were assigned (Electronic supplementary Table 1). DMDS, dimethyl trisulfide (DMTS), terpenoids as well as 2-phenylethanol and other aromatic compounds could be identified in the odour bouquet of *S. odorifera*. Contributions of each of the volatiles released during the four intervals were analyzed (Fig. 2). One major compound (#31=retention index RI: 1372) was released in all four intervals, comprising 40–48% of the total amount of volatiles. This major compound was the unique bicyclic oligomethyl octadiene named sodorifen (von Reuβ et al. 2010). Ten compounds (#40 (RI 1426), #41 (RI 1430), #44 (RI 1446), #43 (RI 1441), #27 (RI 1351), #39 (RI 1421), #37 (RI 1405), #64 (RI 1559), #61 (RI 1539), #52 (RI 1482)) contributed each between 2% and 10% to the bouquet (Fig. 2). The majority of ca. 70 compounds was emitted at very low levels (<1%) and comprised approximately 12% of the total amount of volatiles. Relative contributions of most compounds did not change significantly between the four growth intervals, only compound #36 (RI 1400) was reduced from 9% to 5% from the first to the fourth interval, and compounds #1 (RI 749 DMDS), # 4 (RI 943), #5 (RI 950), # 7 (RI 985 DMTS) increased particularly in the forth interval.

As a result, our analyses demonstrate that 11 compounds comprise ca. 90% of the complete bouquet of *S. odorifera*.

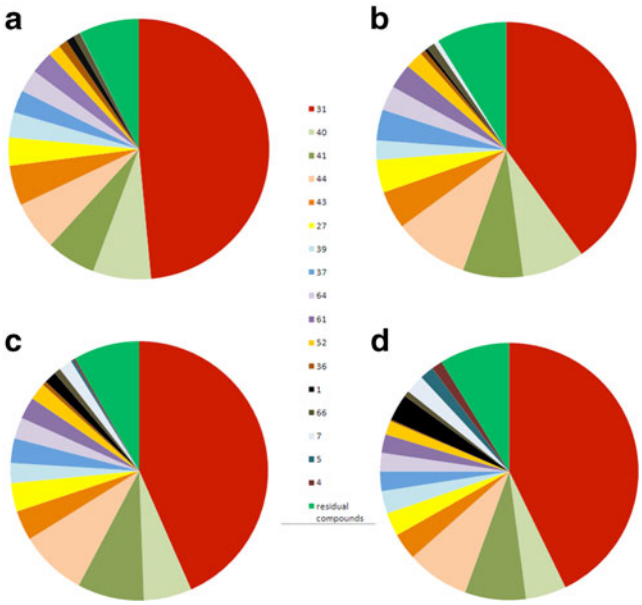

Fig. 2 Contribution of *Serratia odorifera* volatiles in different growth intervals. Contributions of each compound emitted from *S. odorifera* were quantified. **a** interval 0–24 h, **b** 24–48 h, **c** 48–72 h, **d** 72–96 h. The major compound (# 31, sodorifen) comprised 40%–48%, some compounds contributed between 2%–10% each, while ca. 70 compounds were emitted at low levels (<1%; residual compounds). Compounds are listed in electronic supplementary Table 1

PTR-MS analysis of volatiles during growth of *S. odorifera*

The solvent based GC/MS analysis of *S. odorifera* volatiles allowed a verifiable and reproducible detection of masses higher than 150. Proton-transfer-reaction mass spectrometry (PTR-MS) is an additional valuable tool to detect volatile compounds (Bunge et al. 2008). Application of PTR-MS technique has the advantage that masses smaller than 150 could also be registered. At least 25 masses between mass 33 and 149 were monitored 24 h post inoculation, corresponding to 12–15 different volatiles emitted by *S. odorifera* (Fig. 3a). Structure assignment through PTR-MS was based on the detection of protonated ions whose molecular weight corresponds to that of the neutral analyte plus 1 amu. These masses were selected after short-time headspace sampling experiments in which full scans from 20 to 160 amu were taken of the medium and the bacterial cultures (Electronic supplementary Table 2). Since PTR-MS cannot be used as an identification tool, literature based information and isotopic pattern analysis (see "Materials and methods") were used for structure assignment (GC/MS-data Electronic supplementary Table 2). For example, the compound detected at mass 49 provides natural isotopic ratios of m50/m49=2.0% (R^2=0.998) and m51/m49=4.6% (R^2=0.999). Experimentally we have found m50/m49= 1.93% and m51/m49=4.44%, respectively, supporting the assignment of m49 to methanethiol (Fig. 3b- left panel).

However, isotopic pattern analysis cannot discriminate between isomeric compounds, i.e., compounds with the same molecular weight and the same atomic composition. Therefore, in some cases, an analytical technique such as GC/MS has been used for supporting information.

GC/MS analyses performed prior to PTR-MS measurements revealed the presence of dimethyl disulfide (DMDS, $C_2H_6S_2$) in the bacterial headspace and, therefore, this compound was expected to be monitored with the PTR-MS at m95 (Fig. 3a) and its most abundant isotope at m 97. The most abundant isotope is due to sulfur, with a ratio $^{34}S/^{32}S$ of 4.29%, that implies a m97/m95 ($C_2H_6(^{34}S)_2$/ $C_2H_6(^{32}S)_2$) ratio close to 8.9%. In our experiments a relative isotopic abundance of 9.48% was found, which is close to the natural relative isotopic abundance of protonated DMDS. Since GC/MS delivered DMDS as the only candidate here, we have excluded the presence of other isomeric compounds (e.g. 1,2-ethanedithiol) at m95 (Fig. 3b-right panel).

Similarly, based on GC/MS data, the compound at m123 could be identified as phenylethanol and m127 as dimethyl trisulfide. Monoterpene hydrocarbons were detected at m137.

The advantage to use PTR-MS is the ability to obtain temporal volatile emission profiles and due to its linear response it is well suited to achieve quantitative data. The dynamic of eight volatiles during bacterial growth between 0 and 96 h are shown in Fig. 3c and supplementary Fig. 1. The emission profiles of compound methanol (m33), m47 (ethanol?), methanethiol (m49), m79 (benzene?), DMDS (m95), DMTS (m127), and m137 (monoterpenes) exhibited an acute burst emission peak during logarithmic growth. After transition to the stationary phase (ca. 10 h) the emission decreased. During the stationary phase or beginning of a slow decay phase (between 25 and 50 h) another rather bulky emission peak was characteristic for these compounds. Another pattern was observed for compound m109, which also exhibited the acute peak and a continuous increase during the decay phase. The biological relevance of these characteristic emission patterns is yet not understood. The quantities of the emitted volatiles varied between three orders of magnitude (0.1 to 25 $\mu g l^{-1} h^{-1}$, Fig. 3c, supplement Fig. 1). Methanethiol (m49) emission reached peak levels of 25 $\mu g l^{-1} h^{-1}$ (Fig. 3c left), while DMDS (m95) reached 10 times less (supplement Fig. 1c).

Ethylene, NO, HCN, and NH_3 analysis during growth of *S. odorifera*

Ethylene is a typical gaseous plant hormone, and so far only a few examples exist indicating the emission of ethylene by bacteria (reviewed in Stotzky and Schenck 1976; Fukuda et al. 1993). Using the laser-based ethylene

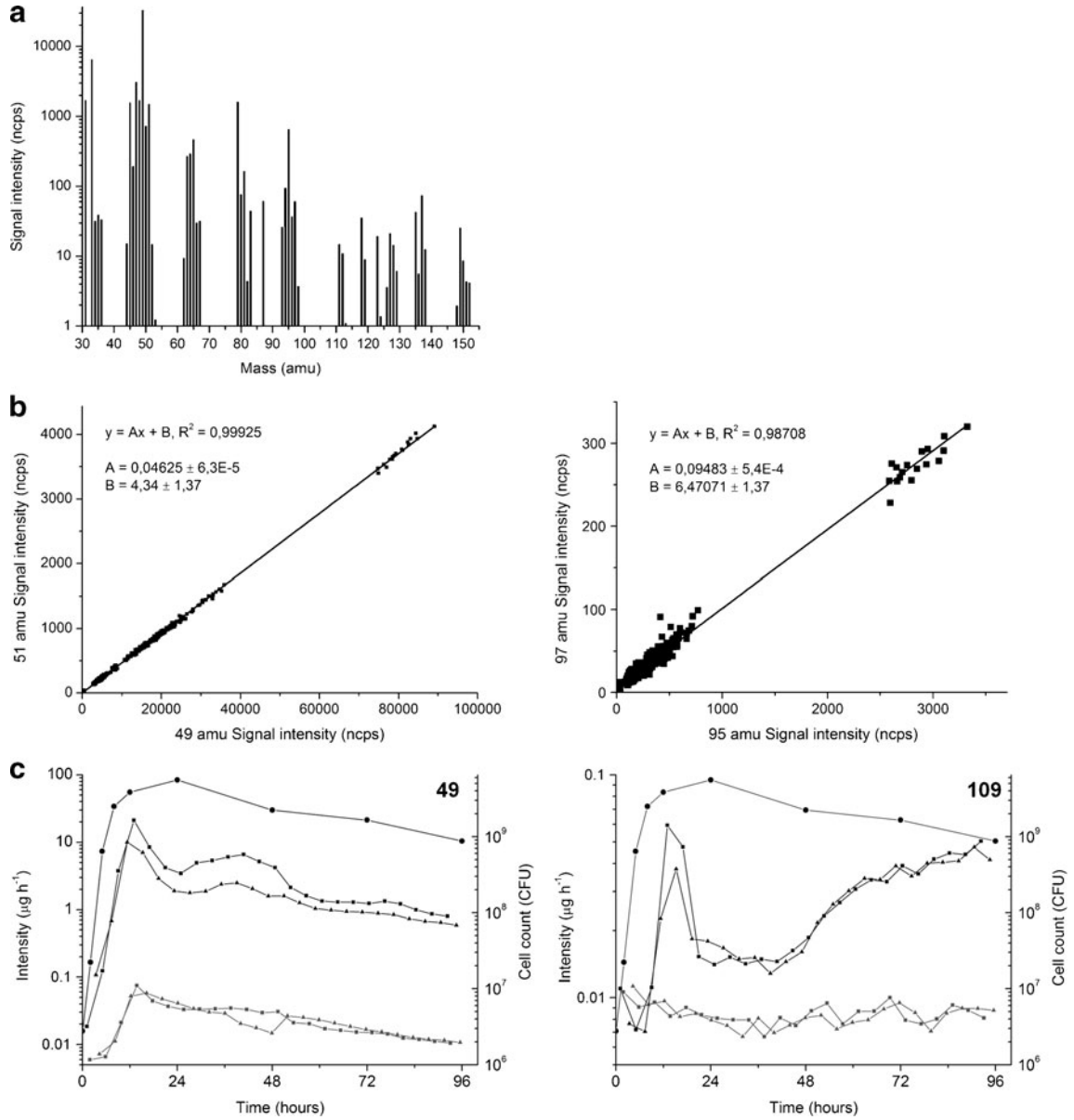

Fig. 3 PTR-MS analysis of *Serratia odorifera* volatile emission. **a** PTR-MS profile scan of headspace volatiles of *S. odorifera* with masses between m30 and m155 taken 24 h after inoculation. Identified compounds are listed in electronic supplementary Table 2. *ncps* normalized counts per second. **b** ^{34}S-isotopic ratio compound calculation (experimental value: 4.625, theoretical value: 4.4) allowed assignment of methanethiol to mass m49. *ncps* normalized counts per second. **c** ^{34}S-isotopic ratio compound calculation (experimental value, 9.483; theoretical value, 8.9) allowed assignment of dimethyl disulfide (DMDS) to mass m95. ncps: normalized counts per second. **d** Temporal volatile emission profile of methanethiol (m49) of *S. odorifera* during 96 h growth. Emission intensity ($\mu g h^{-1}$) of two independent cultures are depicted (*black lines*), emission of medium without bacteria (*gray lines*), cfu are indicated by solid dots (n=3). **e** Temporal volatile emission profile of an unknown compound m 109 during 96 h growth of *S. odorifera*. The actual molecular weight of the target is m=108. Emission intensity ($\mu g h^{-1}$) of two independent cultures are depicted (*black lines*), emission of medium without bacteria (*gray lines*), cfu are indicated by *solid dots* (n=3)

detector, the headspace of *S. odorifera* and culture medium without bacterial inoculation was real-time monitored. There was no difference between the emission of ethylene of the background of the medium (quantified at 0.1 $nl h^{-1}$) and during 96 h of bacterial growth, verifying that *S. odorifera* did not emit ethylene under these growth conditions.

Using the infrared absorption-based NO detection technique, the headspace of *S. odorifera* and culture medium without bacterial inoculation was real-time monitored. There was no difference between the emission rate of NO from the medium and the bacterial culture during 96 h of growth, verifying, that *S. odorifera* did not emit NO under these growth conditions.

Using *Pseudomonas* bacteria, known to release HCN (reviewed in Stotzky and Schenck 1976), *S. odorifera* was tested for HCN emission. *S. odorifera* as well as *P. fluorescens* wildtype (CHA0–HCN emitter) and a mutant (CHA207–HCN minus mutant) were cultivated in the presence of a suitable indicator (copper(II) ethylacetoacetate and 4,4-methylenebis-*N*,*N*-dimethylaniline - Fig. 4). *P. fluorescens* wt volatiles turned the test paper into dark blue, indicating the emission of HCN, while the *P. fluorescens* mutant and *S. odorifera* did not produce HCN.

Other bacteria such as *Xanthomonas* produce ammonia (Stall et al. 1972, Weise and Piechulla unpublished results). Using Nessler's reagent, which allows the detection of ammonia and amides, *S. odorifera* was tested positive (Fig. 5a). According to the dilution series between 0.5 and 50 µmol of commercially obtained ammonia (Fig. 5b), *S. odorifera* emits 0.5–1 µmol ammonia (or low boiling amines) under the test conditions.

Testing individual volatiles with *A. thaliana*

As a result of the analyses presented above, it was obvious that *S. odorifera* emits at least 100 different compounds, of which less than ca. 10% could be identified (supplement Tables 1 and 2). Co-cultivation of *A. thaliana* with *S. odorifera* in bi-partite Petri dishes, which only allowed volatiles to diffuse from one side to the other, resulted in dramatic growth inhibition of plants (Vespermann et al. 2007). However, hexane extract of headspace collected volatiles did not show bioactivity on *A. thaliana* (data not shown). Application of the main component, sodorifen, in amounts comparable to that present in the headspace of *S. odorifera* 4Rx13 did not alter growth of *A. thaliana* (data not shown). Another compound among the *S. odorifera* volatiles was dimethyl disulfide (DMDS) (supplement Tables 1 and 2). DMDS was tested in concentrations ranging from 5 nmol up to 0.5 mmol (Fig. 6). High amounts of DMDS (50 and 500 µmol) inhibited growth of *A. thaliana* by 90%, 5 µmol, and 500 nmol revealed growth reduction of 20–30%, while 50 and 5 nmol were almost ineffective. The IC50 (concentration that leads to 50% inhibition) was approximately 20 µmol under these test conditions. From PTR-MS measurements it became apparent that *S. odorifera* emitted only 5 nmol DMDS within 6 days under similar growth conditions. Therefore, the contribution of DMDS to observed growth inhibition with *A. thaliana* seems to be less pronounced. Ammonia might be another candidate acting as an inhibitory compound in the bi-partite Petri dish. *S. odorifera* emitted ammonia at concentrations of <1 µmol, while concentrations of 2.5 µmol and higher inhibited growth of the plants (Fig. 5c). Therefore, DMDS, ammonia, and most likely other compounds may act additively or synergistically during co-cultivation of *A. thaliana* and *S. odorifera* (Vespermann et al. 2007). Additional information on the chemistry of *S. odorifera* volatiles is required to understand the complex phenomena associated with these compounds.

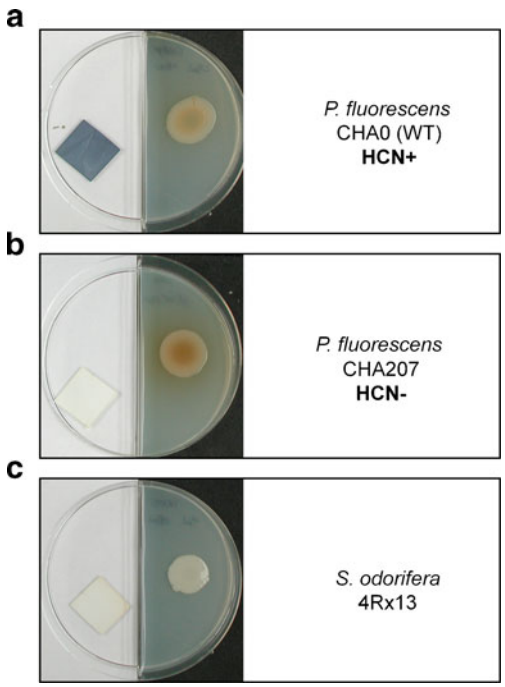

Fig. 4 HCN emission. In one compartment of a bi-partite Petri dish, the test paper was placed; the other compartment was inoculated with 7×10^6 cfu bacteria. **a** Blue coloration of test paper indicates that *Pseudomonas fluorescens* wildtype CHA0 emits HCN. **b** Test paper without coloration indicates that *P. fluorescens* HCN-negative mutant CHA207 does not emit HCN. **c** Test paper without coloration indicates that *S. odorifera* does not emit HCN

Discussion

S. odorifera belongs to the γ-proteobacteria (Enterobacteriaceae) and was defined as a new species due to DNA sequence comparisons (Grimont et al. 1978). Early it was recognized to release a characteristic odor (Gallois and Grimont 1985). Most strains were isolated from clinical specimens, however, *S. odorifera* 4Rx13 was originally obtained from the rhizosphere of *B. napus* (Berg et al. 2002).

At least 100 volatile compounds are released by *S. odorifera* 4Rx13. To comprise the whole spectrum of emitted volatiles, various methods were applied, such as PTR-MS, GC/MS, photoacustic spectroscopy, infrared absorption as well as analytical test kits. In contrast to the high number of emitted volatiles, a surprisingly small number of compounds (less than 10%) could be identified so far. Volatiles of *S. odorifera* comprises sodorifen, methanol, ethanol, methanethiol, DMDS, DMTS, monoterpene hydrocarbons, 2-phenylethanol, and other aromatic compounds, as

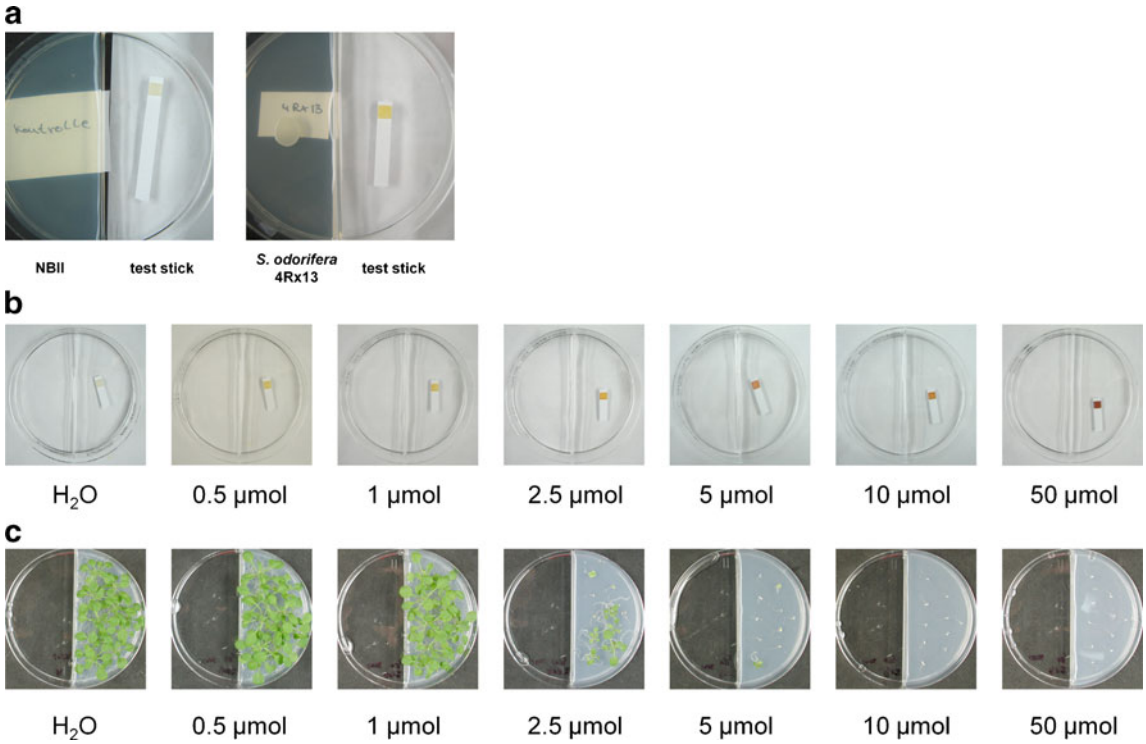

Fig. 5 NH_3 emission. **a** In one compartment of a bi-partite Petri dish test sticks were placed, the other compartment was incoculated with 7×10^6 cfu *S. odorifera*. Application of Nessler's reagent to Quantofix® ammonium test sticks indicates the emission of NH_3 by *Serratia odorifera* (*right*); control without bacteria (*left*). **b** A dilution series of 0.5 to 50 µmol ammonia solution was applied to Whatman paper. The reaction was stopped after 30 s and coloration was documented. **c** Left compartment of bi-partite Petri dish was filled with water or respective ammonia solution, ten *Arabidopsis thaliana* plants were placed into the right compartment. Cultivation time, 14 days

well as ammonia. Mass spectrometry proved a plethora of volatiles, eluting between 19 and 23 min to be isomers of sodorifen. Whether these play a defined role as components of the bouquet or whether they are just by-products of the biogenesis remains to be investigated. In a separate investigation, CO_2 emission was also documented for *S. odorifera* (Kai and Piechulla 2009).

The present analyses of *S. odorifera* volatiles are, to the best of our knowledge, the first performed in such detail. The number of emitted compounds from *S. odorifera*

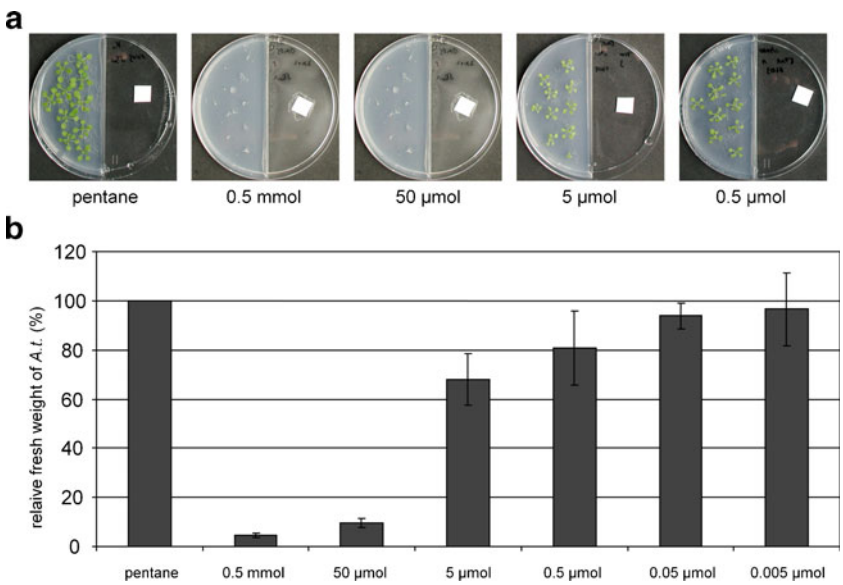

Fig. 6 DMDS effects on *Arabidopsis thaliana*. **a** To the right compartment of a bi-partite Petri dish, 50 µl of pentane was applied or a solution of dimethyl disulfide in amounts as indicated in the Figure. In the left compartment ten *A. thaliana* plants were placed. Phenotypes of *A. thaliana* were recorded after 14 days of cultivation with DMDS. **b** Fresh weight of *A. thaliana* compared with the control with solvent alone (100%) was determined at day 14

4Rx13 varied in the different growth intervals between 40 and 60 easily detectable compounds (Fig. 1e), while the largest amount of compounds was released at the beginning of the stationary phase. The relative contribution of the major individual compounds remained very similar (Fig. 2). The increased number of recorded compounds as compared to our earlier investigation (Kai et al. 2007) is most likely due to (1) additionally applied detection methods and higher sensitivity as well as (2) varied growth conditions. Threefold more compounds were released when *S. odorifera* 4Rx13 was grown in liquid medium. Fiddaman and Rossall (1994) had shown that variation of the medium (e.g., addition of glucose, peptone, tryptone) altered the volatile bouquet of *B. subtilis* resulting in different growth capacities of *R. solani*. Also the volatile profiles of *Streptomyces* species varied in structural diversity and quantity (Dickschat et al. 2005).

Furthermore, the genetic and physiological background of a given bacterial species and population determines which and how many volatiles are synthesized and emitted. *S. plymuthica*, *P. trivialis*, *P. fluorescens*, *Xanthomonas phaseoli, Streptomyces* spp., and *Myxococcus* spp. emitted rich patterns of volatiles, while, e.g., *Escherichia coli*, *S. maltophilia*, *Staphylococcus epidermidis*, and *Xanthomonas campestris* release only a small number of compounds (Schöller et al. 2002; Dickschat et al. 2004; Kai et al. 2007, Scholz and Piechulla unpublished results). Often one or a few compounds dominate the bacterial blends, such as indole of *E. coli*, and acetoin and butanediol of bacilli. *S. odorifera* 4Rx13 emits a characteristic major compound (#31, RI 1374, sodorifen), accomplishing approximately 45% of the total volatile mixture. Pyrazines, which are otherwise typical for the potatolike smell of *Serratia* and *Cedecea* strains (Gallois and Grimont 1985) were not detected in the blend of *S. odorifera* 4Rx13. Consistent with other reports is the absence of sesquiterpenes in the headspace of *S. odorifera* (Minerdi et al. 2009).

Stotzky and Schenck (1976) summarized emission and biological functions of bacterial volatiles. In most cases, the responding organisms were fungi of which, e.g., mycel growth and sporulation was reduced or spore germination inhibited upon exposure to bacterial volatiles. For a long time the physiological potential of volatile emissions of bacteria, and the multitude of activities of bacterial volatiles was largely underestimated. Recently, awareness on this subject increased, but little is known about the multiple roles that volatiles may play for the producing organism and/or the perceiving organisms/populations living in the same community. For the producer volatile end products of biosynthetic pathways may be advantageous waste compounds because due to their volatility they do not accumulate to higher concentrations in the immediate vicinity of the producer. This might be beneficial when the volatile end products are of low-energetic value and/or toxic on organisms of the ecosystem. Examples for such waste volatiles are CO_2, NH_3, or HCN. Ammonia has been known to be toxic for many photosynthetic organisms for a long time, since it destroys the oxygen-evolving complex of photosystem II (Stall et al. 1972; Drath et al. 2008). Here we demonstrated that in dual cultures 2.5 μmol of NH_3 were toxic for *A. thaliana*. Bacterial CO_2 could be a carbon source for autotrophic organisms such as chemolithoautotrophic microorganisms and plants, and HCN is not emitted by *S. odorifera*. In contrast, it was demonstrated that CO_2 is definitely released by *S. odorifera* 4Rx13 during co-cultivation and can promote the growth of *A. thaliana* (Kai and Piechulla 2009).

Sodorifen, emitted by *S. odorifera*, is a polymethylated hydrocarbon (von Reuβ et al. 2010) that is released in surprisingly large amounts. Each of the eight carbons comprising the parent bicyclic system carries an additional carbon. Any biosynthetic pathways leading to this unique compound are unknown, and supportive physiological experiments are presently missing. Further investigations using labeled possible precursors and synthetic reference samples will be necessary to establish the biosynthesis and the biological significance of sodorifen.

Volatiles may also be regarded as secondary metabolites that act as growth promoting or inhibiting substances on organisms of the community. If volatiles emitted by *S. odorifera* 4Rx13 possess such roles, it has to be considered that the bacteria originate from the rhizosphere. Recently, evidence increased that volatiles also play a major role in the belowground community (Wenke et al. 2010). Volatiles can diffuse through the air-filled pores and depending on the type of mineral, texture and particle architecture, they may in fact be trapped in the soil (Aochi and Farmer 2005). It is a quite new finding that volatiles besides other compounds may be involved in the action of fungistasis and suppressive soils (Zou et al. 2007). Volatiles may travel short and long distances belowground, and soil-dwelling organisms and roots were shown to synthesize, excrete and perceive volatiles (reviewed in Wenke et al. 2010). These volatiles have to be considered for a better understanding of the entire integrity of an ecosystem.

As shown above, the volatile blend released by *S. odorifera* comprised at least 100 individual compounds, and it can be envisioned that such complex mixtures of bacterial volatiles affect other organisms. To unravel the biological significance of such a bouquet, it is and will be important to study individual compounds or defined combinations of bioactive volatiles. After structure elucidation and syntheses of relevant compounds from *S. odorifera*, functional assignments will be possible. The emission of methanethiol and DMDS by *S. odorifera* was

quite substantial. Methanethiol, an important compound of the global sulfur cycle, was released by *S. odorifera* in large amounts of 25 $\mu g h^{-1}$. The compound was described to act negatively on *Candida albicans* (Lewis 1985). Oxic and anoxic bacteria can convert methanethiol to dimethyl sulfide, and certain microbes use it as substrate for methanogenesis (Rimbault et al. 1988). DMDS exerts insecticidal activity via inhibition of cytochrome oxidase in the mitochondrial electron transport system and potassium channel blockage (Dugravot et al. 2003; Gautier et al. 2008). In this study we could show that the IC 50 value of DMDS to *A. thaliana* was ca. 20 µmol. Another compound emitted by *S. odorifera* is 2-phenylethanol, which destroys biological membranes and is, therefore, another potential inhibitory compound acting additively or synergistically on the test organisms during co-cultivation (Etschmann et al. 2002). Apart from these compounds it is of interest to determine the bioactivity of other components of the volatile mixture released by *S. odorifera*. The substances that were trapped on Super Q and extracted with hexane, however, displayed no growth effects on *A. thaliana* in the dual culture system (data not shown). It could be that (1) the bioactive principle was not extracted with this method or (2) the bioassay was not sufficient. Since a certain volatile collection technique only depicts a certain range of the real emission profile of an organism, further studies must include other collection techniques.

To understand why bacteria such as *S. odorifera* might produce such a wealth of organic volatiles it is also important to consider the time course of emission. Particularly PTR-MS analysis allowed quantitative measurements in 45 min intervals resulting in detailed emission profiles (Fig. 3 and supplement Fig. 1). During the first 12 h after inoculation volatile emission correlated with logarithmic growth, indicating that (1) emitted volatiles did not (accumulate and) inhibit the growth of the bacteria, (2) release of the carbon rich compound sodorifen did not deplete the carbon source for cellular maintenance and development, and (3) release of several compounds appeared simultaneously and not consecutively. At the beginning of the stationary phase, which usually starts with nutrient limitation and/or accumulation of toxic compounds, volatile emission is drastically reduced. Interestingly, continued incubation resulted in slightly elevated volatile emission during stationary/beginning of decay phase. The physiological relevance of this intermittent volatile emission is not understood. It can be speculated that after the consumption of the primary nutrients and after a short period of adaptation, released compounds (like in bacilli) or other nutrients of the medium were made accessible for *S. odorifera* and volatiles are released subsequently. Further physiological studies are necessary to understand this complex pattern of biosyntheses.

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