

On-Line Monitoring of Microbial Volatile Metabolites by Proton Transfer Reaction-Mass Spectrometry[∇]

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A method for analysis of volatile organic compounds (VOCs) from microbial cultures was established using proton transfer reaction-mass spectrometry (PTR-MS). A newly developed sampling system was coupled to a PTR-MS instrument to allow on-line monitoring of VOCs in the dynamic headspaces of microbial cultures. The novel PTR-MS method was evaluated for four reference organisms: *Escherichia coli*, *Shigella flexneri*, *Salmonella enterica*, and *Candida tropicalis*. Headspace VOCs in sampling bottles containing actively growing cultures and uninoculated culture medium controls were sequentially analyzed by PTR-MS. Characteristic marker ions were found for certain microbial cultures: *C. tropicalis* could be identified by several unique markers compared with the other three organisms, and *E. coli* and *S. enterica* were distinguishable from each other and from *S. flexneri* by specific marker ions, demonstrating the potential of this method to differentiate between even closely related microorganisms. Although the temporal profiles of some VOCs were similar to the growth dynamics of the microbial cultures, most VOCs showed a different temporal profile, characterized by constant or decreasing VOC levels or by single or multiple peaks over 24 h of incubation. These findings strongly indicate that the temporal evolution of VOC emissions during growth must be considered if characterization or differentiation based on microbial VOC emissions is attempted. Our study may help to establish the analysis of VOCs by on-line PTR-MS as a routine method in microbiology and as a tool for monitoring environmental and biotechnological processes.

The diversity of volatile organic compounds (VOCs) produced in nature is enormous. VOCs from microorganisms are released mainly as metabolic products during growth, as secondary metabolites for protection against antagonists and competitors, or as signaling molecules in cell-to-cell communication (15, 20, 24, 28). Recent analytical developments have significantly improved the capability to sensitively detect microbial VOCs in a variety of matrices. For instance, the production of VOCs has been determined for ascomycetous yeast strains isolated from tropical environments (3), for the classification of endophytic fungi by their spectra of volatile antimicrobials (9), and for the detection of fungal species and one actinomycete species during growth on building materials (17). Gas chromatography-mass spectrometry (GC-MS) has been used for the identification of bacterial VOCs from cultures of cyanobacteria (14), for profiling rhizobacterial volatiles (12), and for analyzing VOCs associated with infections of *Neisseria meningitidis* (30). However, GC-MS techniques are generally disadvantaged by the requirements for preconcentration and chromatography. Hence, they are inadequate for tracking the finer temporal changes of VOC emission profiles during microbial growth, and it is therefore not surprising that most

recent studies have described VOC profiles that are based only on analyses at discrete time points.

Proton transfer reaction-mass spectrometry (PTR-MS) has the potential to overcome such difficulties. It offers the possibility of sensitive VOC detection without sample preparation or chromatography (for a review, see reference 19). PTR-MS is therefore a suitable tool for following the dynamics of VOC emissions. So far, PTR-MS has been used primarily in environmental sciences, especially in atmospheric studies (7, 29). However, the use of this novel technique has been increasingly reported for the analysis of biogenic VOCs released into the atmosphere, including wound response VOCs from leaves (10, 11), for the detection of VOCs from the tropical rainforest (5, 27) and from agricultural lands (16), and for the analysis of root exudates (26). In contrast, there are surprisingly few studies in the fields of microbiology and biotechnology. Although recent reports have underlined the suitability of PTR-MS for the analysis of microbial VOCs (4, 21, 22), previous studies have rarely addressed the temporal changes of VOC emissions. Temporal profiles of microbial VOC emissions may, however, contain important information for the detection and differentiation of microorganisms.

The objective of our study was to evaluate the use of PTR-MS as a new tool to monitor the emissions of microbial VOCs. We present the development of a sampling system coupled to a state-of-the-art PTR-MS instrument that offers the possibility for on-line detection of VOCs. Furthermore, we propose a scheme for the differentiation of microorganisms on the basis of their overall VOC patterns, their observed tempo-

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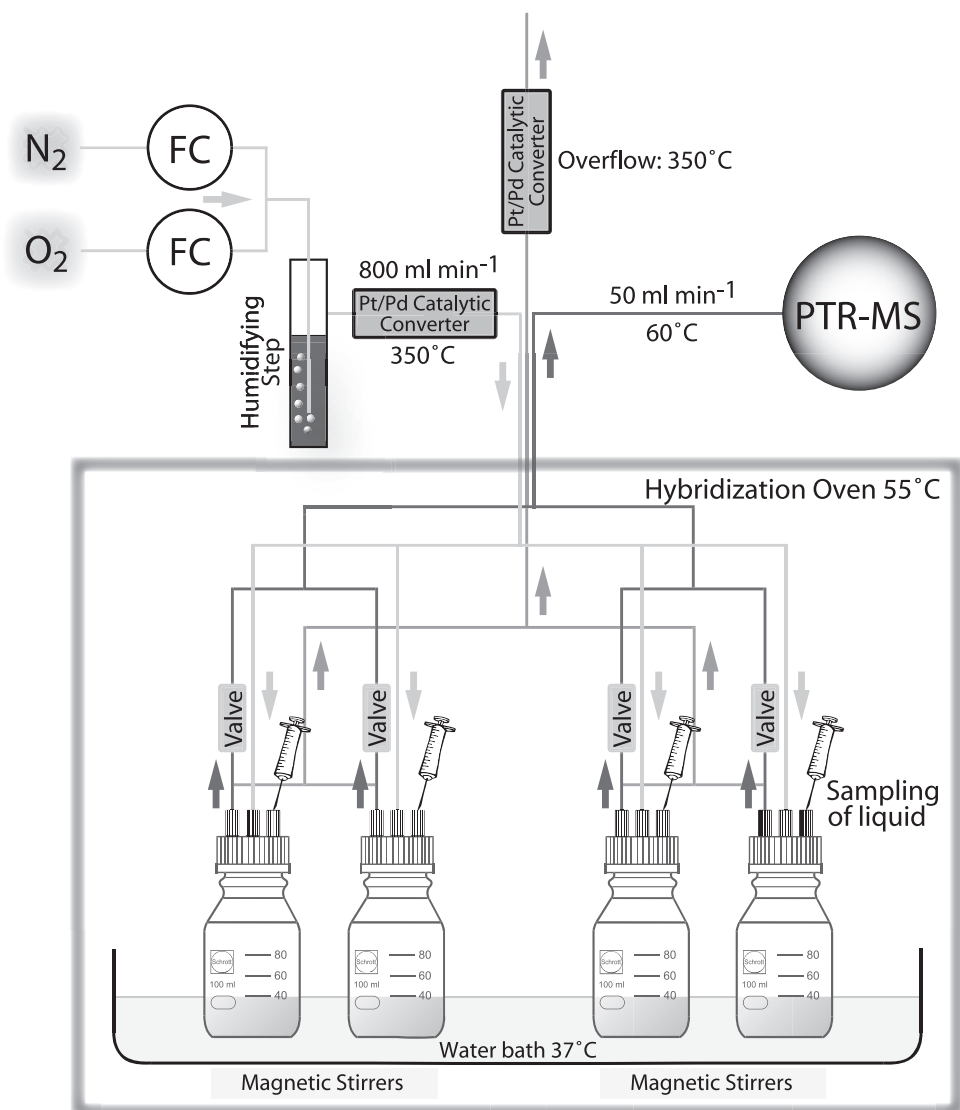


FIG. 1. Experimental setup for real-time analysis of microbial VOCs in the dynamic headspace of actively growing cultures. For experiments with *C. tropicalis*, the water bath was held at $25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$, and the hybridization oven was heated to 31°C . FC, flow controller.

ral VOC emission profiles, and the quantitative changes during bacterial growth.

MATERIALS AND METHODS

Sampling system setup. A simplified scheme of the VOC measurement system is shown in Fig. 1. For the analysis of VOCs produced by microbial cultures, common 100-ml Schott glass bottles were used, equipped with three-way polytetrafluoroethylene (PTFE [Teflon]) bottle caps, flexible PTFE tubes and fittings, and thick butyl rubber stoppers for the sampling lines through which liquid samples were withdrawn for the determination of microbial growth. An experimental setup for simultaneous headspace gas analyses of four sampling bottles was designed for the current study. The bottles were connected to the carrier gas supply through stainless steel tubes and to the PTR-MS instrument through PTFE solenoid valves, which were sequentially opened for a 12-min period during which headspace analysis was performed. With this chosen measurement time, a 36-min interval between analyses of the same sampling bottle was con-

strained, but depending on the requested time resolution, the number of sampling bottles can easily be increased or decreased.

The headspace of each sampling bottle was constantly flushed with the respective carrier gas, which was first humidified and then passed through a Pt-Pd catalyst operated at 350°C to destroy any organic or microbiological contaminants. A total carrier gas flow of 800 ml min^{-1} (at standard temperature and pressure [STP]) was used, with the flow equally divided between the four sampling bottles, i.e., the headspace of each bottle was flushed at 200 ml min^{-1} (STP). If needed, a higher headspace flow can be selected to increase the dynamic headspace dilution and to restrict headspace VOC mixing ratios to within the upper limit of linearity of the PTR-MS instrument ($\sim 10\text{ ppm per volume [ppmV]}$; $1\text{ ppmV} = 10^{-6}\text{ [vol/vol]}$). For analysis, 50 ml min^{-1} (STP) from each sampling bottle was alternately fed to the PTR-MS instrument, with the overflow discarded through a second Pt-Pd catalyst (at 350°C).

PTR-MS. VOCs were measured by PTR-MS. PTR-MS (19) is a chemical ionization technique that enables measurements of individual VOCs ranging from pptV ($10^{-12}\text{ [vol/vol]}$) to ppmV (10^{-6} [vol/vol]) levels. VOCs are ionized via proton transfer reactions from H_3O^+ ions and are mass spectrometrically

detected one atomic mass unit (amu) higher ($M + 1$) than the relative molecular weight (M_r) of the neutral compounds. A commercially available PTR-MS instrument (Standard PTR-MS; Ionimed Analytik, Innsbruck, Austria) was used for all measurements. Mass scans were performed from 18 to 150 amu, using a dwell time of 1 s per amu. PTR-MS operating conditions were as follows: drift tube voltage, 600 V; drift tube pressure, 0.20 ± 0.005 kPa; drift tube temperature, 60°C; E/N , 140 Townsends (Td) (where E is the electric field strength in the drift tube and N is the gas number density in the drift tube; $1 \text{ Td} = 10^{-17} \text{ cm}^2 \text{ V molecule}^{-1}$); $\text{O}_2^+:\text{H}_3\text{O}^+$ ratio, $\leq 3\%$; inlet flow, 50 ml min^{-1} (STP); inlet temperature, 60°C. Measurements were carried out continuously for 24 h. Memory effects (due to the sequential analysis of different samples) were reduced by the application of elevated inlet and drift tube temperatures (60°C) and the use of Silcosteel inert material for the inlet tubing.

The PTR-MS instrument was calibrated using a dynamically diluted VOC standard (Apel-Riemer Environmental Inc., Denver, CO) containing approximately 1 ppmV (accuracy, less than $\pm 5\%$) of a series of hydrocarbons including the target compounds acetaldehyde, ethanol, and acetone. As previously reported (6), the variability of PTR-MS calibration factors is in the order of 15 to 40% for hydrocarbons containing heteroatoms O and N. The signals at 73, 89, and 118 amu were calibrated using the response factor for acetone as a proxy value. The associated uncertainty is estimated to be less than $\pm 40\%$.

Microbial strains and culture conditions for growth. The following strains were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ): *Escherichia coli* DSMZ 30083, *Shigella flexneri* DSMZ 4782, *Salmonella enterica* subsp. *enterica* DSMZ 9274, and *Candida tropicalis* DSMZ 11953. Cultivation was performed using complex media and culture conditions, as instructed by the DSMZ (www.dsmz.de). The enterobacteria were grown in DSMZ medium no. 1 or no. 681 (*S. enterica*); a universal yeast medium was used for the cultivation of *C. tropicalis* (DSMZ medium no. 186).

All open connections of the sampling bottles were wrapped with aluminum foil, and bottles were autoclaved for 25 min at 121°C and subsequently stored at 105°C in a drying oven for at least 2 h. Manipulations, including filling with culture medium and inoculating with material of precultures (16 h), were performed by using sterile microbiological techniques. The culture bottles (40-ml total liquid volume) were inoculated to an initial optical density at 660 nm (OD_{660}) of 0.01 and were transported immediately to the PTR-MS laboratory.

After bottles were connected to the PTR-MS instrument and conditioned at 37°C (25°C for *C. tropicalis*) for 60 min, PTR-MS analysis was started. The cultures were mixed with magnetic stirrers, and their headspaces were flushed with a sterile and constant carrier gas flow of 200 ml min^{-1} (STP). Humidified synthetic air ($\text{N}_2\text{-O}_2$ in a ratio of 80:20) was used for routine cultivation of the reference strains. Although anaerobic and microaerophilic conditions for cultivation were not employed in the current study, these conditions can be easily achieved by mixing the headspace gases in the desired ratios.

Prior to their connection to the culture vessels, the stainless steel connectors downstream of the Pt-Pd catalyst were flame sterilized for 30 s at 300°C using a hot-air gun. Constant temperature in the culture bottles during analysis was ensured with temperature-controlled water baths set to $37.0^\circ\text{C} \pm 0.5^\circ\text{C}$ ($25.0^\circ\text{C} \pm 0.5^\circ\text{C}$ for *C. tropicalis*). To avoid condensation, the whole setup was kept in a hybridization oven at 55°C (31°C for *C. tropicalis*). Heat transfer from the headspace resulted in a temperature increase of 1°C in the culture bottles.

One-milliliter samples were withdrawn at predetermined time intervals, using aseptic sampling techniques and sterile single-use syringes and needles. These samples were used to routinely determine the OD_{660} , the total cell numbers over the incubation time, and the CFU on growth plates using 1.5% (wt/vol) agar as the solidifying component for the respective medium. Usually, one sampling bottle remained uninoculated, for the estimation of background VOC emissions from the culture medium, and served as a sterile control for the culture medium and headspace gas, respectively.

Data analysis. PTR-MS ion signals were normalized to 10^6 counts per second (cps) primary ions [determined from $\text{H}_3\text{O}^+ + \text{H}_3\text{O}^+ \cdot \text{H}_2\text{O} + \text{H}_3\text{O}^+ \cdot (\text{H}_2\text{O})_2$]. Only ion signals of more than 10 normalized cps (corresponding to approximately 0.3 ppbV) were used for further statistical analysis. To avoid equilibration effects and carryover artifacts between individual samples, data from the first 5 min (after opening the respective PTFE valves) were not included in the analysis.

Correlation and covariance analyses were performed (i) to identify related masses (e.g., fragments and hydrated ions), (ii) to identify ions which are produced by more than one neutral compound, and (iii) to test isotopic patterns for sum formula determination. For each experiment, the two-dimensional data (normalized ion signals versus time) were reduced to one dimension by creating an unfolded temporal profile (2), i.e., the profiles of all normalized ion signals between 40 and 150 amu were sequentially inserted into a single-vector column. Unfolded temporal profiles were created for data from *Escherichia coli* (three

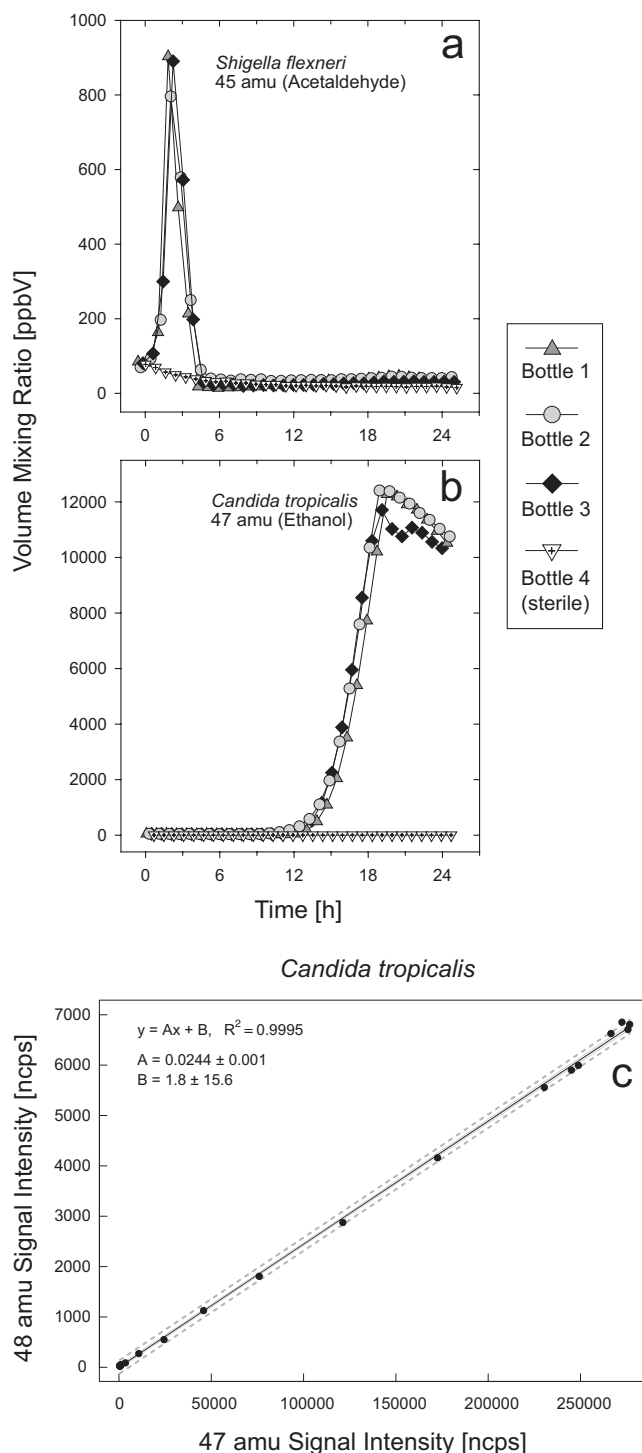


FIG. 2. Exemplary temporal profiles of acetaldehyde (a) and ethanol (b) in cultures of *Shigella flexneri* and *Candida tropicalis*, demonstrating the high reproducibility of results between the three replicates. The small shifts along the time axis are caused by the sequential analysis of the culture bottles. (c) ^{13}C isotope analysis of the PTR-MS signal detected at 47 amu. The isotopic abundance seen at 48 amu closely resembles the natural relative isotopic ^{13}C abundance of protonated ethanol (2.3%).

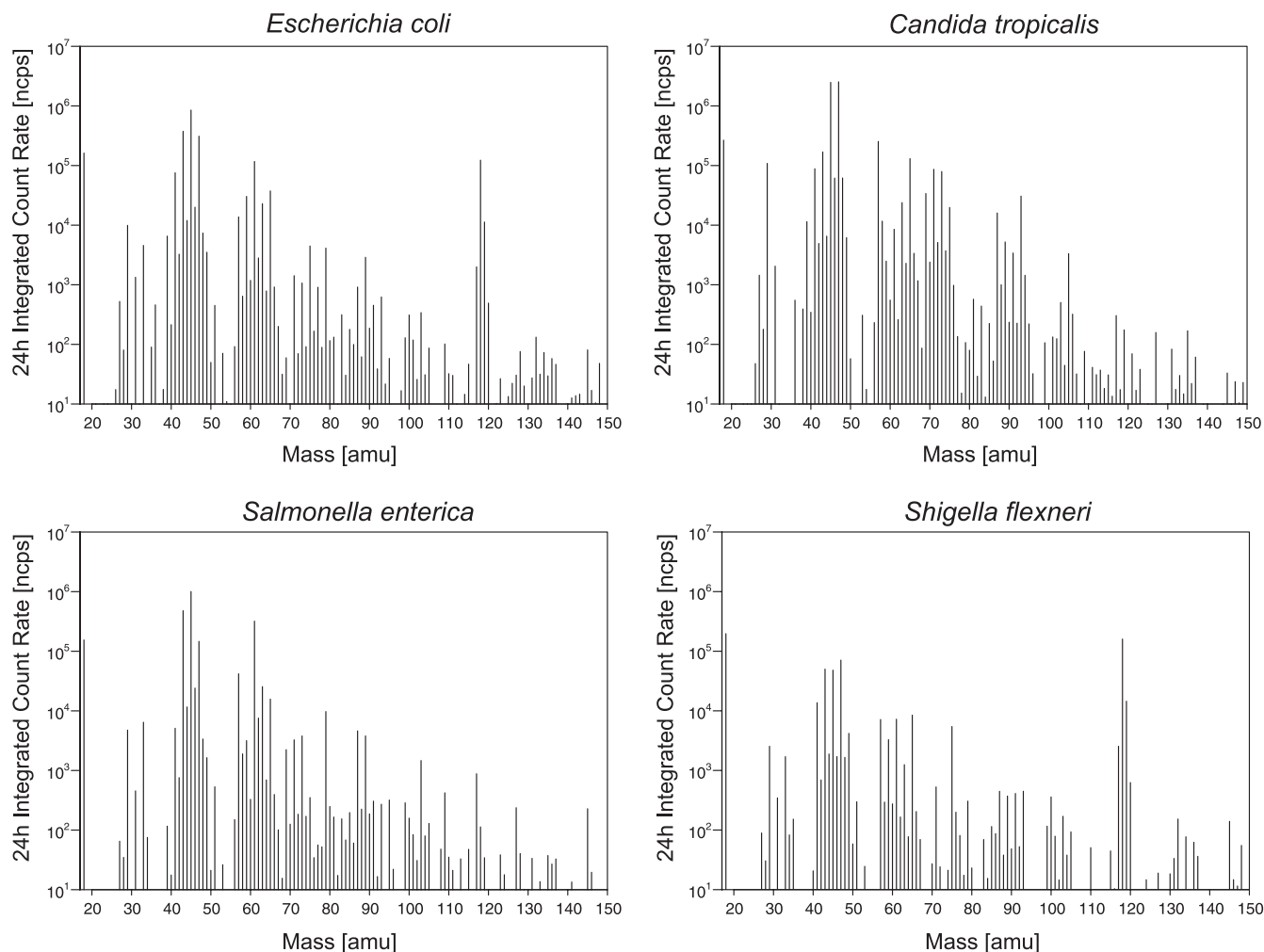


FIG. 3. Twenty-four-hour-integrated PTR-MS mass spectra from the dynamic headspace of cultures of *Escherichia coli*, *Candida tropicalis*, *Salmonella enterica*, and *Shigella flexneri*.

replicates), *Shigella flexneri* (three replicates), *Candida tropicalis* (four replicates), and *Salmonella enterica* (three replicates), producing a 13-column matrix which was subsequently subjected to principal component analysis (23). The singular value decomposition method (13) was used for the simultaneous calculation of eigenvectors and singular values. Singular value decomposition is generally accepted to be a numerically accurate and stable technique for calculating the principal components of a data matrix.

RESULTS AND DISCUSSION

Method validation. Extensive tests were performed to ensure robustness and reliability of the proposed standardized PTR-MS measurement protocol for dynamic headspace analysis of microbial VOCs (see Materials and Methods). Both quantitative changes and emission dynamics were highly comparable between the sampling bottles. As an example, the temporal emission behavior of acetaldehyde ($M + 1 = 45$ amu, *Shigella flexneri*) and ethanol ($M + 1 = 47$ amu, *Candida tropicalis*), as observed for the headspace of the four separate sampling bottles, is shown in Fig. 2a and b, respectively. Temporal profiles of both acetaldehyde and ethanol were reproducible between the three replicates. Neither acetaldehyde nor ethanol production was observed with the sterile controls. A

similar degree of reproducibility was obtained for all detected PTR-MS signals (see below).

Compound identification. Compound identification by PTR-MS is based on the detection of protonated ions whose molecular weight corresponds to that of the neutral analyte molecule plus 1 amu ($M + 1$). This implies that PTR-MS a priori cannot differentiate between compounds with the same molecular weight. Fragmentation and clustering of product ions further complicate the qualitative interpretation of the obtained mass spectra and require detailed correlation analyses of the observed signals.

Literature information for microbial VOC production can be used to elaborate educated guesses and to provide tentative peak assignments. Whereas no interfering compounds are known for low-molecular-weight compounds, such as methanol and acetaldehyde, isotopic pattern analysis must be used for other abundant signals to discriminate between compounds with identical masses but different atomic compositions (isobaric compounds). Such data analysis was performed to reliably identify key metabolites. For example, ethanol (C_2H_5OH) and formic acid ($HCOOH$) are both detected at an $M + 1$

TABLE 1. Ion signals in the dynamic headspace of reference cultures produced over 24 h of incubation

Mass (amu) ^a	Reference organism			
	<i>E. coli</i>	<i>S. flexneri</i>	<i>S. enterica</i>	<i>C. tropicalis</i>
33 (methanol)	+	+	+	+
39	+			+
41	+	+		+
43	+	+	+	+
45 (acetaldehyde)	+	+	+	+
47 (ethanol)	+	+	+	+
49 (methanethiol)	+	+	+	+
53	+			+
57 (1-butanol) ^b	+	+	+	+
59 (acetone)	+	+		+
61 (acetic acid)	+	+	+	+
69			+	+
71	+	+	+	+
73 (2-butanone) ^c			+	+
75	+	+		+
87	+	+	+	+
89 (2-methyl-1-butanol) ^d	+	+	+	+
91	+			+
93	+			+
95				+
103	+		+	+
105				+
109			+	+
117	+	+	+	+
118 (indole)	+	+		+
119	+	+		+
127			+	+
132	+			+
135				+
145	+		+	

^a Tentative assignments to specific VOCs are given in parentheses.
^b Or fragments of 3-methyl-1-propanol (21).
^c Or a fragment of 3-hydroxy-2-butanone.
^d Or 3-methyl-1-butanol (8).

value of 47 amu. The natural relative isotopic ¹³C abundance of protonated ethanol is 2.3% (with two C atoms present), while for protonated formic acid, it is only 1.2% (one C atom present). In the *Candida tropicalis* experiments, a relative isotopic ¹³C abundance of 2.4% was observed (Fig. 2c), indicating that ethanol was produced. It should be pointed out that isotopic pattern analysis cannot be used to discriminate between isomeric compounds, i.e., compounds with the same molecular weight and the same sum formula. Dimethyl ether (CH₃OCH₃) is isomeric to ethanol and cannot be distinguished from the alcohol by PTR-MS. The microbial production of this ether in large quantities, however, can be excluded with high confidence.

Time-integrated mass spectra and marker VOCs for particular microorganisms. An inherent feature of PTR-MS is the linear instrument response in the pptV-to-ppmV range. PTR-MS is therefore well suited to achieve quantitative mass spectra. Figure 3 compares 24-h-integrated mass spectra obtained from the dynamic headspace of the investigated cultures of *Escherichia coli*, *Candida tropicalis*, *Salmonella enterica*, and *Shigella flexneri*. Our measurements revealed complex mass spectra in the investigated range between 18 and 150 amu; preliminary results had shown that no signals could be detected in the 150- to 300-amu range using the experimental setup as

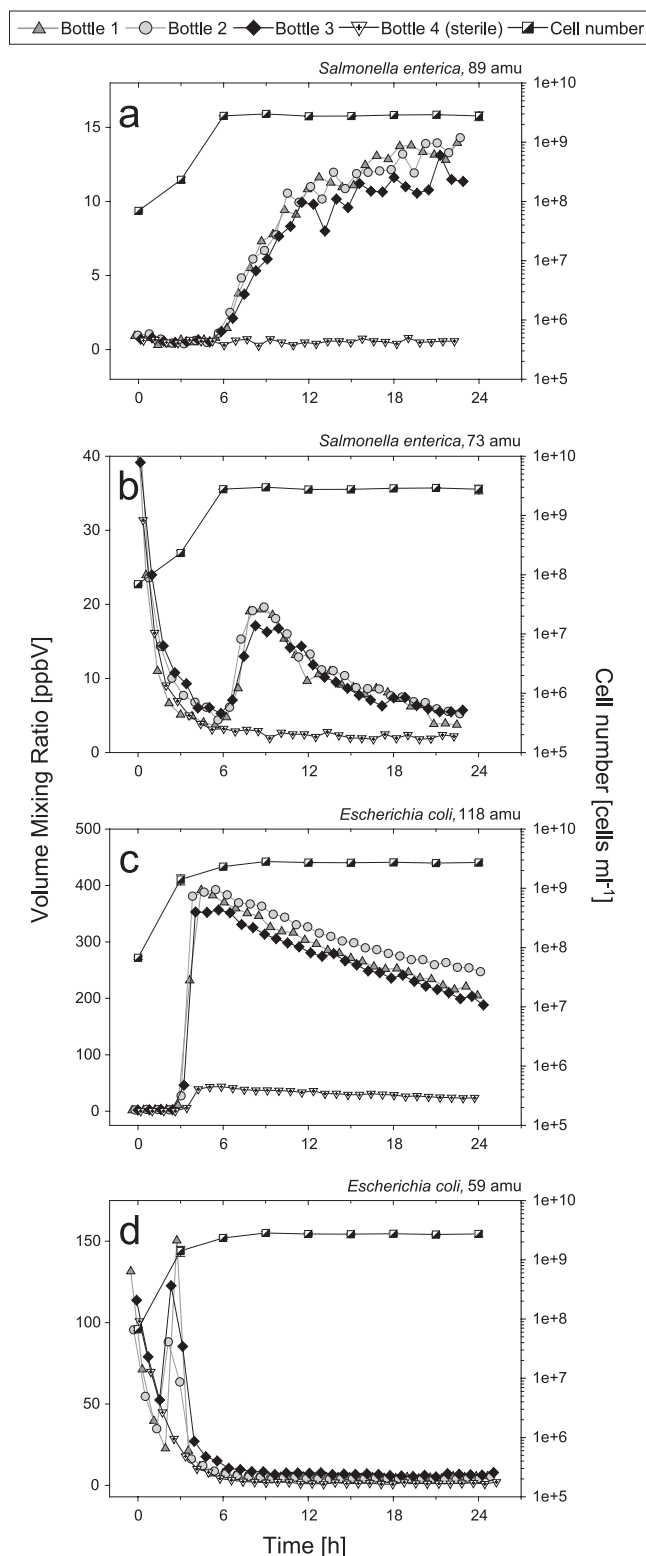


FIG. 4. Temporal evolution of VOCs in the dynamic headspace of microbial cultures over 24 h of growth. Data are shown for three actively growing cultures and one uninoculated control.

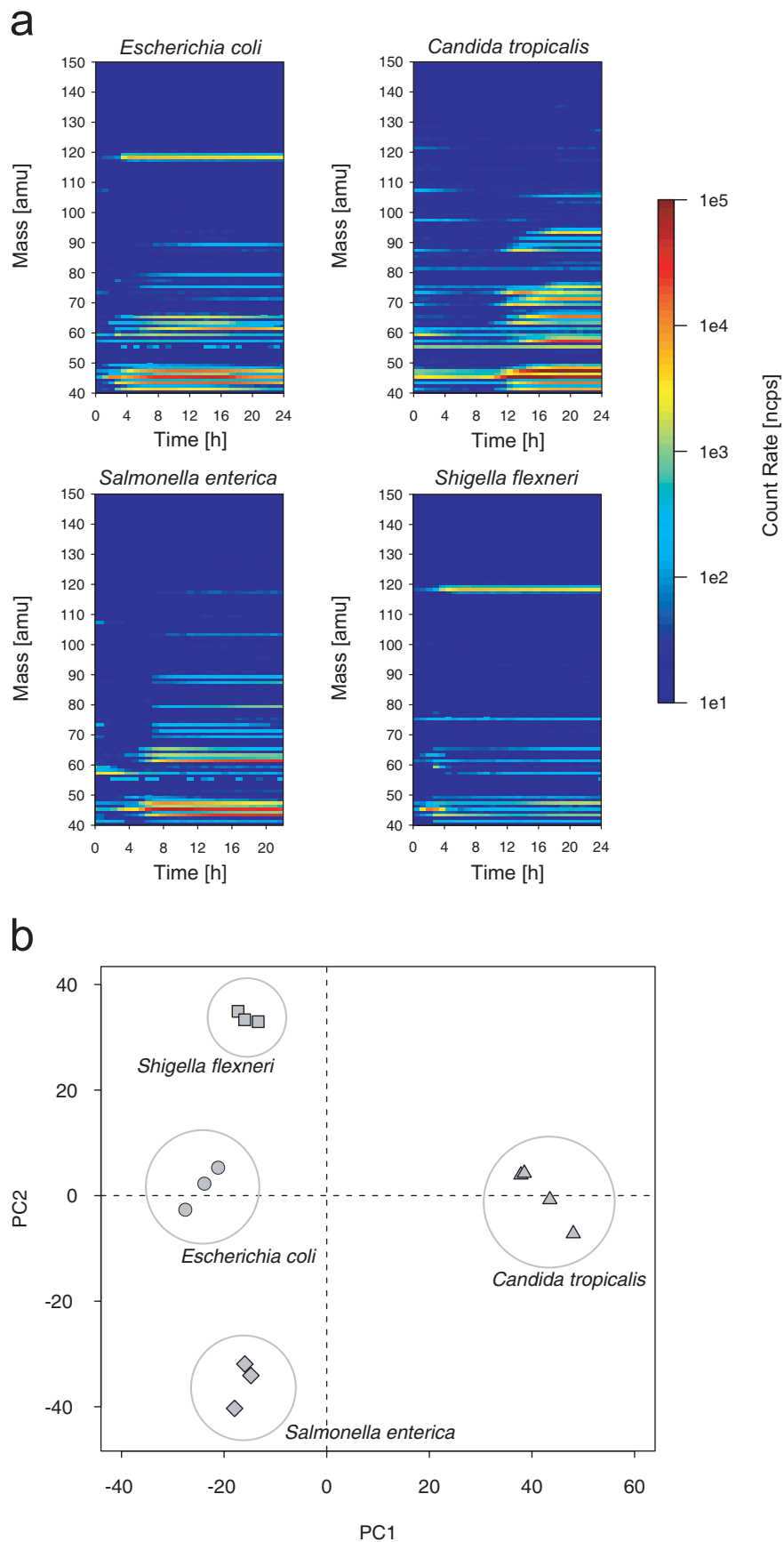


FIG. 5. Contour plots show the temporal evolution of all ion signals detected in the 40- to 150-amu range (y axis) during the 24-h incubation period (x axis) (a). The signal intensity (count rate given as normalized count rates) is color coded. Warm colors indicate high signal intensities. Scatter plots of the scores for the first (PC1) versus the second principal component (PC2) are shown (b). Unfolded temporal profiles (see Materials and Methods) were used for principal component analysis.

described. Yet, within the limited mass range, the PTR-MS instrument detected a large number of signals that were produced by the investigated strains with different relative abundances ranging over more than 6 orders of magnitude.

The microbes investigated in this study were screened for the release of characteristic marker ions. Our measurements revealed three specific signals for *Candida tropicalis* at 95, 105, and 135 amu (all unidentified; see Table 1). Using the PTR-MS setup described here and with the limited set of organisms investigated in our study, we were able to find key masses that were produced only by particular bacterial strains. Compared to the other bacteria, *Salmonella enterica* produced four characteristic peaks (Table 1, 69, 73, 109, and 127 amu) and could therefore be distinguished from the strains of *Shigella flexneri* and *E. coli*. Characteristic signals also could be detected in the headspace of *E. coli*; in comparison to *Shigella flexneri* and *Salmonella enterica*, *E. coli* produced unique ions at 39, 53, 91, 93, and 132 amu. These data suggest that in some cases, characteristic markers may be found for the identification and differentiation of microbes, also between closely related species. The identification of specific markers for particular bacteria will possibly allow other physiological groups of microorganisms to be distinguished, even without full identification of the VOCs produced. The findings are in agreement with recent studies that have demonstrated the usefulness of VOCs emission patterns for the classification of different microbial species (3, 8, 9).

Temporal VOC emission patterns and microbial growth dynamics. Our analysis of time-integrated mass spectra did not take into consideration the temporal variability of VOC emissions. It is worth mentioning that temporal changes of VOC emissions have rarely been addressed in previous studies of microbial systems. Since PTR-MS offers the possibility of on-line VOC monitoring, we have routinely used this feature to follow the dynamics of microbial VOC emissions. To identify possible correlations with the PTR-MS signals, microbiological growth data were compared to the VOC emission dynamics of the respective microorganisms and uninoculated media controls. Only a few signals, e.g., the unidentified signal at 89 amu in cultures of *Salmonella enterica* (Fig. 4a), correlated with bacterial cell numbers. For most signals, a more complex time-dependent behavior was observed. Figure 4b illustrates a peak at the 73 amu signal (tentatively identified as 2-butanone) occurring not until 9 h of incubation. A few compounds showed a rapid temporal change (in Fig. 2a, production of an acetaldehyde peak by *Shigella flexneri*), and some VOCs could be detected even before any signs of microbial growth were visible. In cultures of *Escherichia coli*, the signal at 118 amu (which can be assigned to indole with high confidence) showed a sharp increase of 3 orders of magnitude after 3 to 4 h of incubation, followed by a slow decrease over the remaining incubation period (Fig. 4c). Even more intriguingly, some VOCs were only transiently produced. Figure 4d shows the evolution of the signal at 59 amu (tentatively identified as acetone), which was only detectable within a time period of about 100 min. The temporal behavior of all PTR-MS signals in the 40- to 150-amu range is best shown as a contour plot, as displayed in Fig. 5a. These data indicate that off-line single-point VOC analysis, either by conventional gas chromatography, by selected ion flow tube MS (as recently used for bacte-

rially infected blood cultures [1, 25]), or by PTR-MS, as used for the detection of VOC emissions from meat spoilage (21) and biowaste (22), is not appropriate for monitoring more dynamic microbial systems. Furthermore, our results clearly challenge the use of nontemporal VOC patterns for the identification of bacteria, as recently suggested by Lechner et al. (18) and Scotter et al. (25). It is immediately evident from our results that headspace analysis of microbial VOCs must include data about the dynamics of VOC production. In an attempt to use this additional information for microbial discrimination, we performed principal component analysis using unfolded temporal profiles of 111 masses (40 to 150 amu) from 13 independent cultures. The results revealed a clear separation of the VOC emission signatures for the respective strains (Fig. 5b).

Our study demonstrates the potential of this novel technique for on-line monitoring of VOCs in the dynamic headspace of microbial cultures. The findings indicate that the temporal evolution of microbial VOC emissions during growth must be considered if real-time detection and identification of microbial processes based on VOC emissions are to be attempted.

Future work should address the influences of different culture media and cultivation conditions on microbial VOC emissions patterns and should assess the use of VOC profiles as indicators for the status of microbial metabolism. Since microbial VOC emission profiles are directly linked to the physiological state of the bacteria and the underlying metabolic processes, our sampling system connected to PTR-MS paves the way for establishing a direct monitoring method for microbial systems, with cultivation, sampling, and VOC analysis taking place simultaneously. The real-time identification of VOCs produced under substrate- or O₂-limiting conditions will help to develop a continuously regulated alarm system for bioprocess control and may rapidly convey the innovative technology into routine applications.

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