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Fast Detection of Volatile Organic Compounds from Bacterial Cultures by Secondary Electrospray Ionization-Mass Spectrometry▽†

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We propose a novel application of secondary electrospray ionization-mass spectrometry (SESI-MS) as a real-time clinical diagnostic tool for bacterial infection. It is known that volatile organic compounds (VOCs), produced in different combinations and quantities by bacteria as metabolites, generate characteristic odors for certain bacteria. These VOCs comprise a specific metabolic profile that can be used for species or serovar identification, but rapid and sensitive analytical methods are required for broad utility. In this study, the VOC profiles of five bacterial groups from four genera, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Salmonella enterica* serovar Pullorum, were characterized by SESI-MS. Thirteen compounds were identified from these bacterial cultures, and the combination of these VOCs creates a unique pattern for each genus. In addition, principal component analysis (PCA) was applied for the purpose of species or serovar discrimination. The first three principal components exhibit a clear separation between the metabolic volatile profiles of these five bacterial groups that is independent of the growth medium. As a first step toward addressing the complexity of clinical application, *in vitro* tests for mixed cultures were conducted. The results show that individual species or serovars in a mixed culture are identifiable among a biological VOC background, and the ratios of the detected volatiles reflect the proportion of each bacterium in the mixture. Our data confirm the utility of SESI-MS in real-time identification of bacterial species or serovars *in vitro*, which, in the future, may play a promising clinical role in diagnosing infections.

Bacterial infection is the root cause and/or the complicating factor of many human diseases. For example, 80% of cystic fibrosis (CF) patients will acquire chronic *Pseudomonas aeruginosa* lung infections during their lifetime, which is a primary cause of morbidity and mortality (12, 14), and uropathogenic *Escherichia coli* (UPEC) bacteria are responsible for more than 80% of urinary tract infections in the United States. (13, 26). Typical bacterial identification methods (e.g., culture, serological, and genetic methods) are time-consuming (from hours to days) and may require harsh techniques such as sputum induction or bronchoalveolar lavage for isolating samples from patients (3, 15). Therefore, an *in situ* test that is noninvasive, rapid, and sensitive, and that will facilitate timely and effective treatment decisions, is desired.

Diagnosing bacterial infections by smell has been practiced for millennia. Volatile organic compounds (VOCs), produced by bacteria as waste products or primary metabolites (e.g., acetone, ethanol, or acetic acid), or as secondary metabolites (e.g., signaling molecules), may be produced in different quantities and combinations by each bacterial species or serovar, generating characteristic odors. For instance, *P. aeruginosa* synthesizes 2-aminoacetophenone, which has a grapelike odor and can be used to detect this bacterium in culture and in burn wounds (11). Similarly, indole is responsible for the characteristic odor of *E. coli*, making this VOC a common diagnostic marker for the identification of this bacterium (30). These compounds, in combination with other VOCs, could be used as a volatile fingerprint of each bacterium.

In order to obtain and utilize VOC profile information for species or serovar identification, fast and sensitive methods are required. Detection of bacterial headspace VOCs via mass spectrometry (MS) methods has been under development for more than 3 decades. For example, gas chromatography coupled with mass spectrometry (GC-MS) has been used to detect VOCs produced by *P. aeruginosa*, and aliphatic alcohols, ketones, and alkenes from other bacterial cultures have been identified using GC-MS (17, 27, 34). However, GC-MS requires sample collection (e.g., with sorbents) and preparation (e.g., derivatization), which increases the analysis time. In more-recent studies, selected ion flow tube-MS (SIFT-MS) and proton transfer reaction-MS (PTR-MS) have overcome these limitations for VOC qualification (4, 6, 31), but the fragmentation of specific peaks, which is an important tool for compound identification in atmospheric ionization techniques, is not possible when PTR-MS or SIFT-MS is used. Alternatively, it has been shown that electrospray plumes can efficiently ionize not only analytes from the condensed phase (7, 8, 10, 28, 29) but also gaseous compounds (9, 18, 32), with the latter approach termed "secondary electrospray ionization-mass spectrometry" (SESI-MS) (22, 33). These previous studies demonstrate the possibility of real-time detection and identification of VOCs by MS, which can be applied to the characterization of biological samples (19–21) and could achieve limits of detection as low as parts per trillion (22).

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In this work, we applied SESI-MS to the detection and characterization of VOCs produced by *P. aeruginosa*, *Staphylococcus aureus*, *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Salmonella enterica* serovar Pullorum, a group that represents both Gram-positive and Gram-negative genera. Each of these five bacterial groups produces a unique VOC profile by SESI-MS, demonstrating the utility of this method in the identification of bacteria, including the ability to distinguish two closely related genera (*Escherichia* and *Salmonella*) as well as groups within the same species (*S.* Typhimurium and *S.* Pullorum). Additionally, we verified that individual species in a mixed culture are identifiable and that using different growth media does not confound the correct identification of the species that we used in this study. To the best of our knowledge, this is the first time that SESI-MS has been used to characterize bacterial volatiles, and our data confirm the production of several volatile compounds previously reported for these species and serovars (2, 4). The VOC data presented here are part of a larger study focused on the development of breath and urine analyses for rapid identification of bacterial pathogens in a clinical setting.

MATERIALS AND METHODS

Bacterial strains, medium, and growth condition. The strains used in this study were *P. aeruginosa* PA14 (courtesy of Fred Ausubel, Massachusetts General Hospital), *S.* Typhimurium ST5383 (courtesy of May Kihara, Yale University), *S.* Pullorum SA1685 (courtesy of Sharon Walker, University of California Riverside), *E. coli* ATCC 25922, and *S. aureus* ATCC 25923.

Monocultures of all strains were cultured aerobically for 24 h at 37°C with shaking at 150 rpm in 50 ml of tryptic soy broth (TSB) in 100-ml sterilized glass bottles (final optical densities at 600 nm [OD_{660}] of >3.0 for all samples). PA14 was cultured in three additional media: LB-Lennox (5 g/liter NaCl), synthetic cystic fibrosis medium (SCFM; pH 6.8) (24), and MOPS (morpholinepropanesulfonic acid) (23) supplemented with 20 mM glucose and 30 mM succinate (pH 7.5). Three biological replicates, each with three technical replicates, were tested.

Mixed cultures of *S. aureus* and *P. aeruginosa* were prepared by culturing each species individually in 50 ml of TSB for 23 h at 37°C with shaking at 150 rpm (final cell densities of 1×10^9 to 2×10^9 CFU/ml for all samples). The monocultures were mixed in different proportions of *S. aureus* to *P. aeruginosa* (3:1, 1:1, and 1:3 [vol/vol]) to give a final volume of 50 ml and then incubated for an additional hour at 37°C and 150 rpm, followed by spectrum acquisition by SESI-MS as described below.

SESI-MS. The VOC mass spectra were collected using SESI-MS, with the method previously reported by Martinez-Lozano and colleagues (20). The bacterial VOCs were introduced into the mass spectrometer (API-3000; SCIEX) by flushing the culture headspace for 1 min with CO_2 (99.99%; 2 liters/min) at room temperature. Formic acid (0.1% [vol/vol]) in water was used as an electrospray solution, delivered at a flow rate of 5 nl/s through a nonconductive silica capillary (40-μm inside diameter [ID]) with a sharpened needle tip. The operation voltage was set at ~3.5 kV. Spectra were collected over 1 min as an accumulation of 40 scans in single quadrupole positive-ion mode. Tandem mass spectrometry (MS-MS) fragmentation spectra were collected at 15 to 40 eV of collision energy, with N_2 collision gas. The system was flushed with CO_2 between samples to prevent carryover. Analyst 1.4.2 software (Applied Biosystems) was used for data collection and analysis.

Data processing and analysis. The spectra of the monocultures in this study represent the average values for nine spectra for each bacterial species or serovar (three technical replicates of three biological replicates), which have been blank subtracted (the blank spectrum is the volatile signature of the medium) and normalized to the peak of the greatest intensity from each individual spectrum. Compound identification was aided by Seven Golden Rules software (16) and Massbank (http://www.massbank.jp/). The probable compound identities for ethanol, acetone, 2-pentanone, 2-aminoacetophenone, acetic acid, 1-butanol, pyrimidine, ethylene glycol, 3-methyl-1-butanol, and indole were further validated by comparing the MS-MS fragmentation spectra of standards (American Chemical Society [ACS] or high-performance-liquid-chromatography [HPLC] grade) to the sample VOC fragmentation spectra. To differentiate the correlations among bacterial VOC patterns, the peaks with mass-to-charge ratios (*m*/*z*) between 40 and 150 and relative intensity thresholds greater than 1% after blank subtraction were used as variables for principal component analysis (PCA; SAS version 9.2); all experimental replicates were used as observations.

The spectra of the mixed cultures of *S. aureus* and *P. aeruginosa* represent the average values for three biological replicates. The predicted spectra for the mixed cultures (3:1, 1:1, and 1:3 [vol/vol] *S. aureus* to *P. aeruginosa*) were calculated by scaling the spectra of the monocultures of *S. aureus* and *P. aeruginosa* relative to their proportion in the mixture and then adding the scaled spectra.

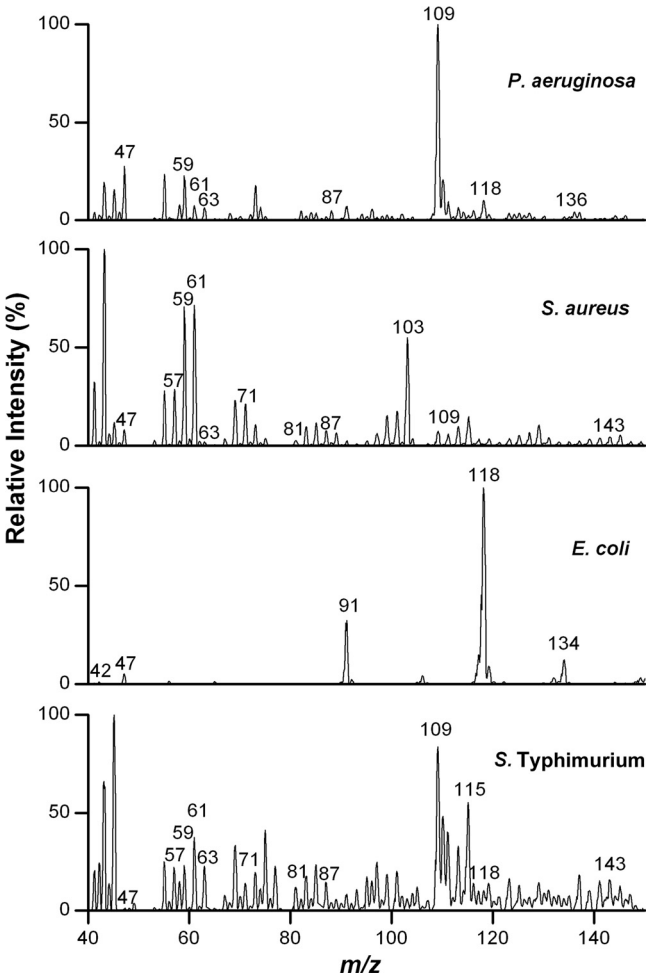

FIG. 1. Positive-ion-mode full-scan spectra (*m*/*z* of 40 to 150) of bacterial culture headspace for *P. aeruginosa*, *S. aureus*, *E. coli*, and *S.* Typhimurium grown aerobically in TSB at 37°C for 24 h. Every spectrum represents an average of spectrum values for nine samples (three biological replicates, each with three technical replicates), with the media blank subtracted and normalization to the peak of greatest intensity.

RESULTS AND DISCUSSION

Using SESI-MS, we collected the mass spectra of the headspace volatiles produced by four genera of bacteria, represented by *P. aeruginosa*, *S. aureus*, *E. coli*, and *S.* Typhimurium (Fig. 1). Qualitatively, these spectra are all unique, possessing distinctive features that can be used to distinguish these bacterial groups from one another strictly by their volatile profiles. The mass spectrum captures more information about the bacterial volatiles than just the smell of the culture. In some

TABLE 1. Identified compounds from *P. aeruginosa*, *S. aureus*, *E. coli*, and *S.* Typhimurium SESI-MS spectra in positive-ion mode

m/z	Identity[a]	Relative signal intensity[b]			
		P. aeruginosa	*S. aureus*	*E. coli*	*S.* Typhimurium
42	Acetonitrile	+	+	+	++
47	Ethanol	+++	++	++	+
57	Butanol	−	+++	−	++
59	Acetone	++	+++	−	++
61	Acetic acid	++	+++	−	+++
63	Ethylene glycol	++	+	−	++
71	Isopentanol	−	++	−	++
81	Pyrimidine	−	+	−	++
87	*2-Pentanone*	+	++	−	++
109	*4-Methylphenol*	+++	+	−	+++
118	Indole	++	−	+++	++
136	2-Aminoacetophenone	+	−	−	−
143	*2-Nonanone*	−	+	−	++

[a] Compounds indicated in italics were tentatively identified with a reasonable degree of certainty based on MS-MS spectrum interpretation. All other compounds were verified by comparison of their fragmentation patterns to a standard.
[b] +++, 25% to 100%; ++, 5% to 25%; +, 1% to 5%; −, below the 1% threshold.

instances, the compound responsible for the characteristic odor of the bacterial culture is the dominant feature of the spectrum, as in the case of indole (30) at an *m/z* of 118 in the *E. coli* spectrum (Fig. 1). In other cases, the most recognizable odorant is only a minor feature of the spectrum. In the spectrum for *P. aeruginosa*, for example, the dominant compound is 4-methylphenol (*m/z* = 109), but the compound that gives *P. aeruginosa* its characteristic grapelike odor is 2-aminoacetophenone at an *m/z* of 136 and a relative intensity of less than 5% compared to the level for 4-methylphenol (11).

A total of 13 VOCs from these four bacterial genera were identified, which are listed in Table 1 with the relative peak intensities observed for each species (the relative intensities for all peaks with *m/z* of 40 to 150 are listed in Table S1 in the supplemental material). The majority of these compounds were observed as the protonated species in the mass spectra, as the spectra were collected in positive-ion mode. However, a few compounds, such as butanol and isopentanol, were observed as $[M-17]^+$, indicating the loss of a water molecule during sample analysis, which is a common occurrence for primary alcohols in gas phase ion-molecule reactions (31). Some of the compounds that are present in the headspace of these bacterial cultures give rise to more than one peak in the mass spectrum, either due to dimer formation ($[2M+1]^+$, e.g., an *m/z* of 93 in the *P. aeruginosa* spectrum) or due to fragmentation during ionization (e.g., an *m/z* of 91 in the *E. coli* spectrum). These additional peaks are reproducible under a given set of instrumental parameters and are therefore robust components of the unique spectra produced by these species. Several compounds, such as ethanol (*m/z* = 47), acetone (*m/z* = 59), and acetic acid (*m/z* = 61), are produced through central metabolic pathways and are commonly observed for many species of bacteria as well as in human breath (1, 2, 4, 5). However, the relative intensities of these central metabolites can be distinctive for some species (Table 1), and in combination with the intensities of other peaks in the spectra (identified or not), a volatile profile unique to a species or serovar can be built.

While the relative intensities of the 13 identified compounds from the headspace of *P. aeruginosa*, *S. aureus*, *E. coli*, and *S.* Typhimurium are sufficient information to distinguish between the four genera that these bacterial groups represent, there is a substantial quantity of additional information in the unidentified compounds of the spectra. We have used principal component analysis (PCA), without peak identification information, to demonstrate the ability to discriminate species or serovars by their volatile profiles (Fig. 2). PCA is a mathematical approach for cluster separation as well as outlier checking. It is typically applied when the measurements have a number of observed variables (i.e., *m/z* from the mass spectra) and the investigators cannot distinguish the differences among observations (i.e., bacterial samples). In this study, we have used

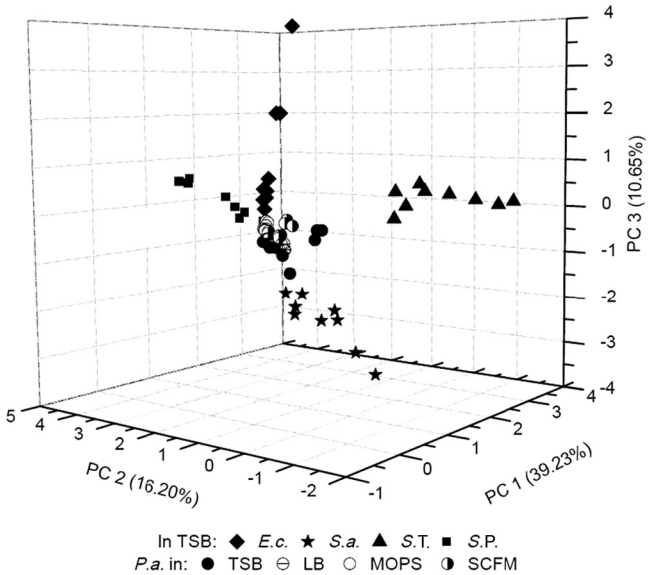

FIG. 2. Principal component analysis of the absolute intensities for all peaks (*m/z* of 40 to 150) of mass spectra of the headspace volatiles of *E. coli* (*E.c.*), *S. aureus* (*S.a.*), *S.* Typhimurium (*S.*T.), *S.* Pullorum (*S.*P.), and *P. aeruginosa* (*P.a.*) grown in TSB (closed symbols) as well as *P. aeruginosa* grown in LB-Lennox, MOPS-glucose-succinate, and synthetic cystic fibrosis medium (SCFM). All cultures were grown aerobically at 37°C for 24 h. Each point represents one sample; a total of 72 samples are included.

PCA to generate a graphical representation of the similarities and differences between species or serovars that are contained in the blank-subtracted mass spectral data. The first three principal components (PCs) accounted for 66.08% of the variance from all the variables (*m*/*z* values). Figure 2 shows a clear separation between the mass spectra of volatiles produced by *P. aeruginosa*, *E. coli*, *S. aureus*, and *S.* Typhimurium. Furthermore, in order to examine the discrimination ability of SESI-MS in regard to identifying two groups within the same species, we tested two *Salmonella enterica* serovars, *S.* Typhimurium and *S.* Pullorum (see Fig. S1 in the supplemental material). The results obtained after PCA show that not only are these two serovars clearly separated based on their VOC data but they are also separated from the other three species that were tested (Fig. 2).

As we argue that SESI-MS has great potential for clinical application, it must be noted that different bacterial growth conditions, for example, medium and oxygen levels, could result in the production of different volatile species as well as different quantities of characterized VOCs. To establish the influence of growth medium on the ability to identify bacteria by their VOC profile, *P. aeruginosa* was cultured aerobically in a variety of rich media: TSB, LB-Lennox, and synthetic cystic fibrosis medium (SCFM) as well as the defined medium MOPS, containing glucose and succinate as carbon sources. These media represent two standard laboratory media (TSB and LB), one medium that is modeled after a host environment (SCFM, modeled after the CF lung environment) (24), and one medium that is used to directly study pathogen nutrient utilization in clinical samples (MOPS) (23, 25). After 24 h of growth at 37°C, the SESI-MS spectra were collected and, using PCA, were compared to each other and to the spectra of the other four bacterial groups grown in TSB (Fig. 2). The spectra of *P. aeruginosa* grown in all four media cluster together at the center of the PCA plot and do not overlap with any of the other four bacterial groups grown in TSB. These data demonstrate that SESI-MS could be used to accurately identify a species grown aerobically on different substrates. The effect of oxygen tension on bacterial volatiles and their SESI-MS spectra will be addressed in future experiments.

As infections are frequently polymicrobial in nature, there is increasing interest in developing methods for rapid detection and characterization of a more complicated mixed-culture situation. In a first pass at addressing this important issue, we made a simple mixed-culture system of different proportions of *S. aureus* and *P. aeruginosa* (3:1, 1:1, and 1:3 [vol/vol]) to demonstrate the utility of SESI-MS in differentiating the production of volatiles from multiple bacterial groups. The SESI-MS data show that as the proportion of *S. aureus* decreased in mixed culture, the VOC profile transferred from *S. aureus*-like to *P. aeruginosa*-like in a linear fashion (Fig. 3). For example, an *m*/*z* of 109 and an *m*/*z* of 118, which are present in much higher proportions in *P. aeruginosa* spectra than in *S. aureus* spectra, decreased in absolute intensity with a proportional increase in *S. aureus* ($R^2 = 0.95$ and 0.97, respectively) (see Fig. S2 in the supplemental material). Likewise, an *m*/*z* of 59, which is present in higher proportions in *S. aureus* headspace spectra, increased with increasing proportions of *S. aureus* ($R^2 = 0.94$) (Fig. S2).

The linear relationship of the SESI-MS peaks to the proportion of each species in the mixed culture demonstrates that the VOCs of the mixture are within the linear range of the detector and that the quantitation of individual VOC signals is not being influenced by a more complex volatile mixture. These two factors indicate that a SESI-MS spectrum of a mixed culture of unknown proportions can be used to determine the relative amount of each species or serovar. To demonstrate this, we generated predicted spectra of the mixed *S. aureus* and *P. aeruginosa* cultures by scaling the SESI-MS spectra of the two monocultures to match the proportions of the cultures used in the experiment (3:1, 1:1, and 1:3 *S. aureus* to *P. aeruginosa* or 75%, 50%, and 25% *S. aureus*) (Fig. 3). The predicted spectra match the measured spectra, indicating that the SESI-MS spectra of a mixed culture contain information about the proportions of the species in the mixture. We also applied

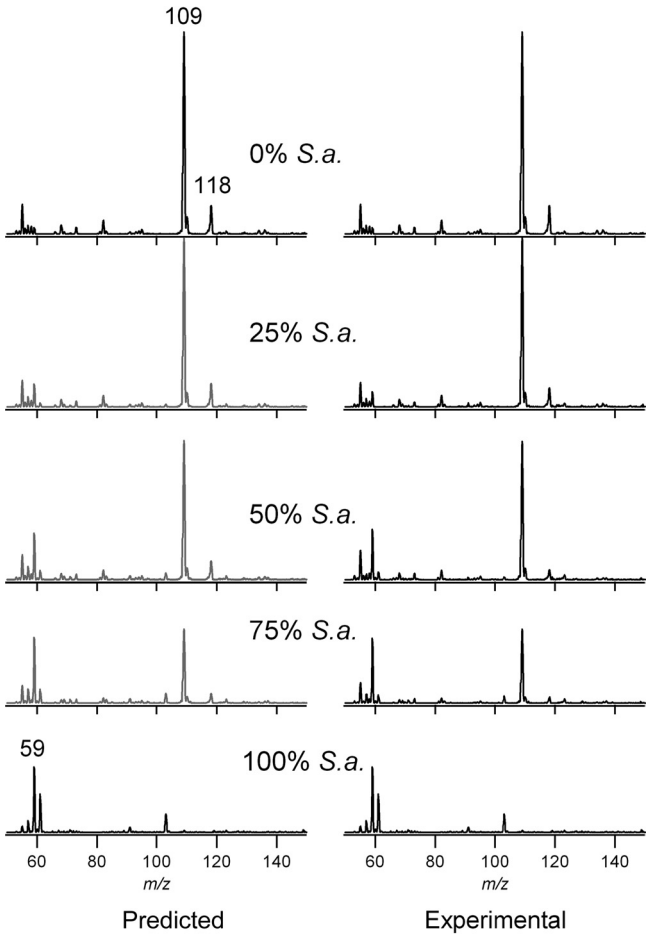

FIG. 3. Predicted (gray) and experimental (black) SESI-MS spectra for mixed cultures of *S. aureus* (*S.a.*) and *P. aeruginosa* in TSB. The predicted spectra for the mixed cultures were calculated by scaling the spectra of the monocultures of *S. aureus* and *P. aeruginosa* relative to their proportions in the mixture and then adding the two scaled spectra. For the sake of clarity, only the proportion of *S. aureus* in the mixture is labeled; the remaining percentage of the culture is represented by *P. aeruginosa*. All of the experimental spectra are plotted on the same scale (*y* axis; 0 to 9×10^7 cps); the predicted spectra are scaled to the same signal intensity as those of their respective experimental spectra. The trends for the labeled peaks are shown in Fig. S2 in the supplemental material.

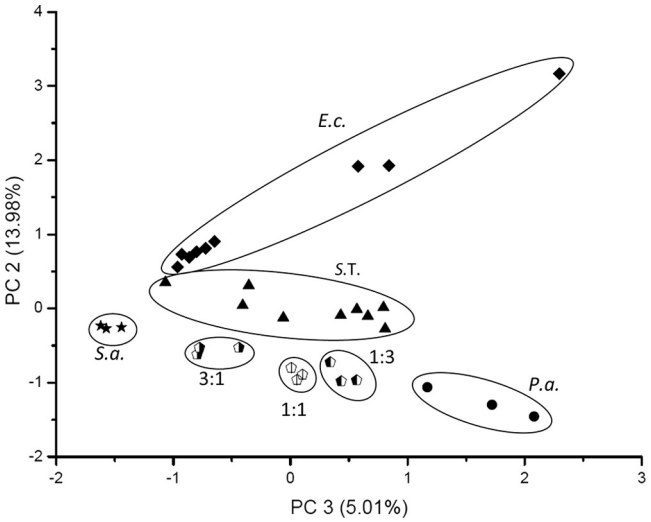

FIG. 4. PCA (PC 2 versus PC 3) of SESI-MS peaks collected in positive mode (*m/z* of 40 to 150) for pure culture of *S. aureus* (*S.a.*), *P. aeruginosa* (*P.a.*), *E. coli* (*E.c.*), and *S.* Typhimurium (*S.*T.) as well as mixed cultures of *S. aureus* and *P. aeruginosa*, all grown for a total of 24 h at 37°C in TSB. Mixed cultures of *S. aureus* and *P. aeruginosa* were prepared by culturing each species individually in 50 ml of TSB for 23 h at 37°C, followed by mixing in different proportions of *S. aureus* to *P. aeruginosa* (3:1, 1:1, and 1:3 [vol/vol]) to give a final volume of 50 ml, and incubating the culture for an additional hour. The three-dimensional (3D) PCA plot is provided in Fig. S3 in the supplemental material.

PCA to the data for the mixed cultures in the context of the four genera of bacteria that we have tested (Fig. 4). The mixed cultures are distributed between the two monocultures of *S. aureus* and *P. aeruginosa*, are ordered by their volume proportions, and do not overlap with the spectra obtained for *E. coli* and *S.* Typhimurium. Together, these data can be taken as a good example that an unknown mixture of bacteria could be identified in species or serovar and proportion by the SESI-MS spectra of their VOCs. These results are the first indication that bacterial volatiles may be detectable and quantifiable among the natural volatiles of human clinical samples, such as breath or urine.

The data from our SESI-MS analysis of the headspace volatiles produced by *P. aeruginosa*, *E. coli*, *S. aureus*, *S.* Typhimurium, and *S.* Pullorum demonstrate that SESI-MS, combined with a statistical analysis method, has great potential for bacterial identification *in vitro*. Using SESI-MS, we are able to discriminate genera, species or serovar, and mixed cultures, and it is a robust technique that is capable of making these differentiations when the bacteria are grown in different nutritive environments. It is also notable that the mass spectrometer scan of VOCs from each bacterial culture headspace was completed within 60 s, without any sample preparation, emphasizing the potential utility of this method for rapid detection of bacterial infection in a clinical setting. We will continue to explore bacterial headspace VOCs *in vitro* under a variety of growth conditions, developing this technique into a clinical diagnostic tool for detecting bacterial infections *in vivo*.

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AUTHOR'S CORRECTION

Fast Detection of Volatile Organic Compounds from Bacterial Cultures by Secondary Electrospray Ionization-Mass Spectrometry

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Volume 48, no. 12, p. 4426–4431, 2010. Page 4430: The following sentence was inadvertently omitted from the end of the first paragraph of the Acknowledgments: "This study was also supported by CF RPD grant STANTO07R0."