Differentiation Between Pure Cultures of *Streptococcus pyogenes* and *Pseudomonas aeruginosa* by FT-ICR-MS Volatile Analysis

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Abstract: Two common bacteria, *Streptococcus pyogenes* and *Pseudomonas aeruginosa,* were differentiated based on their volatile metabolic waste products. The bacteria were cultured in a closed system and the headspace above the culture medium were collected, preconcentrated, and analyzed using a gas chromatography Fourier transform ion cyclotron resonance mass spectrometer (GC/FT-ICR MS).

INTRODUCTION

For thousands of years people have noted that biota and their products may produce or have characteristic olfactory signatures (smells) that could be correlated with biota status or activity [1]. Recently, research efforts in many scientific communities have focused on establishing meaningful correlations between their volatile metabolic waste products (VMWP) and biota activities. Representative examples of contemporary [2, 3] research work on bacteria [4-6] fungi and mold [7, 8] or other pathogen VMWP profiles include: husbandry waste matter [9], tainted water [4], wine making [5, 6], cheese processing [10], putrefying matter [11, 12], halitosis [13, 14], waste water treatment [11], human physiology [15], ecology [16], human pathogens [17-20], forestry [21], sick building syndrome [7], food spoilage [22] and a number of diseases/infections [17, 23].

In this short communication, we chose two bacteria that are widely different (*S. pyogenes* [24] is gram-positive, coccus and a facultative anaerobe while *P. aeruginosa* [24] is gram-negative, rod-shaped and aerobic) to demonstrate a proof of concept for the use of GC/FT-ICR MS in the analysis of VMWP samples from each bacterium. With improvements to the GC/FT-ICR MS instrument [25], we expect to be able to differentiate between more similar bacteria and potentially identify markers unique to each bacterium.

For the analysis of volatile compounds, high resolution gas chromatography (HRGC) is unsurpassed in separation characteristics [26]. In terms of chemical analysis, FT-ICR MS has been shown to be superior to other mass spectrometers in the areas of ultra-high resolution, mass measurement accuracy (MMA), and multistage mass spectrometry [27-29]. A microscale purge and trap (MPT) preconcentrator (PC) can be used as the front-end [30, 31] for trace analysis of headspace products from bacterial culture media under controlled conditions. With this understanding, the proper interfacing of PC, GC and FT-ICR should yield a reliable and potent analytical combination [30, 31].

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Volatile organic compounds arising from bacterial infections have been proposed as diagnostic biomarkers to determine human health status [32-35]. Zechman and Labows [36] used automated headspace concentration gas chromatography to identify *Stenotrophomonas maltophilia* and were able to distinguish this bacterium from *P. aeroginosa* and others. Pavlou *et al.* [20] reported in discriminating between *Helicobacter pylori* and other bacterial gastroesophageal isolates using an odor generating system, an electronic nose, and a hybrid intelligent odor recognition system.

Frequently, biological and environmental "real world" samples are complex mixtures and their complete characterization requires numerous stages of preparation and analysis. In 2002, we reported on potential applications of GC/FT-ICR MS to analyze complex sample matrices such as automobile gasoline [37]. The GC/FT-ICR MS utilizes the separation capability of a conventional GC as well as MMA and ultra high mass resolving power of the FT-ICR MS. In this paper, we present PC/GC/FT-ICR MS results that demonstrate the advantage of MMA for biomarker identification and bacterial differentiation.

MATERIALS AND METHODS

Specimen or Sample Collection

Triplicate BD Bactec™ Plus aerobic blood culture bottles were aseptically inoculated with 0.5 ml preparations of *P. aeruginosa* (ATCC 27853) and *S. pyogenes* (ATCC 19615). The bacterial inoculum was prepared by making a direct trypticase soy broth suspension of *P. aeruginosa* and *S. pyogenes* colonies selected from an 18 to 24 hour blood agar plate. Isolated colonies were transferred to a 4-ml tube of trypticase soy broth and the suspension adjusted to visually compare to that of the 0.5 McFarland turbidity standard. The inoculated blood culture bottles and a sterile control were incubated at 35 °C for 24 hours; headspace MS analyses were conducted immediately or approximately 24 hours after storage at ~ 5 °C.

Instrumentation

Briefly, the analysis system consisted of three major components; a 3 stage Entech 7100 series Preconcentrator (PC) (Entech, Simi Valley, CA), an SRI GC system (Las

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Vegas, NV) and an IonSpec 7 tesla FT-ICR mass spectrometer (IonSpec Corp., Lake Forest, CA). Detailed descriptions of the GC/FT-ICR MS operation [37] and PC configuration [30] have been published elsewhere.

Preconcentrator (PC)

The trapping and preconcentration of static headspace VOCs of interest was performed on an Entech 7100 PC using the MPT technique [38]. A disposable needle was connected to one of the four heated PC sampling lines by a length of plastic tubing. The disposable needle, tubing, and PC sampling line were flushed with dry N_2 prior to headspace sampling. The disposable needle was inserted through the seal on the BD Bactec™ Plus aerobic blood culture bottle and a static headspace volume (10 ml atm) containing trace VOCs was pumped through the first trap (T1) in module 1 (M1). The remainder of the PC operation was identical to the previously reported method [38].

GC Operation

The purged VOC analytes from the preconcentrator were injected onto a 60 m (0.28 mm id, 3 μm crossbonded 100% dimethyl polysiloxane stationary phase coating) MXT-1 capillary column (Restek Corporation, Bellefonte, PA) housed in an SRI model 8610C GC [37].

GC Operational Parameters

Appropriate user defined GC temperature programming allowed elution of the volatile compounds for FT-ICR MS analysis within thirty minutes [37]. The actual mass spectral acquisition duty cycle was ~ 0.11 s and 30 mass spectra were signal averaged to generate each point on the ion chromatograms. The time interval between each point on the ion chromatograms (Figs. **1-3**) was about ~ 5 s in total (including GC/mass spectral processing time). The column head pressures for the helium carrier gas (the mobile phase) was set at 24 psi. The temperature programming used consisted of, initializing at 40 °C for 2 minutes, ramping at 3 °C per minute to 70 °C, holding at 70 °C for 0 minutes, ramping at 10 °C per minute to 200 °C, and holding at 200 °C for 5 minutes.

Acquiring EI-Like Mass Spectra

We used a jet separator for the interface [37] between the GC and FT-ICR MS. The interface was operating under low flow conditions such that only an estimated 0.1% of the GC effluent was continuously flowed into the ICR cell. Mass spectra were acquired using 24 eV EI to suppress ionization of the He carrier gas. The combination of a short duty cycle (11 ms) and low analyte pressures in the ICR cell minimized self-chemical ionization processes [38]; hence, the acquired FT-ICR mass spectra closely resembled conventional EI spectra [39].

FT-ICR MS Data Processing

We have assigned the identities of the VMWP mass spectral peaks based primarily on the MMA of our FT-ICR in conjunction with the NIST online EI mass spectral database [39]. The acquired FT-ICR EI-like mass spectral patterns closely matched the VMWP mass spectra shown on the NIST online EI mass spectral database [39]. Generally, the MMA was 10 ppm with external standards and below 2 ppm when internal standards such as the background $N_2^{\cdot+}$, $O_2^{\cdot+}$, $Ar^{\cdot+}$, and $CO_2^{\cdot+}$ were used for mass calibration.

RESULTS

Various selected ion chromatograms (SICs) are shown in Figs. (**1-3**) for A) *P. aeruginosa* and B) *S. pyogenes* obtained from 10 ml headspace samples. A wide mass range, covering from m/z 29 to 95, was used in Fig. (**1**) (the background ions, *viz*., $O_2^{\cdot+}$, $Ar^{\cdot+}$, and $CO_2^{\cdot+}$ were excluded in constructing the SICs). Figs. (**2**, **3**) show narrow mass range SICs at 43.018 ± 0.002 and m/z 44.026 ± 0.002, respectively. Examination of the three figures (Figs. **1-3**) shows that the two bacteria are readily distinguishable from each other. Finally, Fig. (**4**) shows the extracted mass spectra from the SICs displayed in Fig. (**1**); based on accurate mass measurements (2 ppm or better in most cases) and their EI mass spectral appearances, the VMWP analytes were assigned as, acetaldehyde (1), methylmercaptan (2), ethanol (3) and acetone (4).

Acetaldehyde was not detectable in VMWP of *P. aeruginosa* but a significant amount of it was present in VMWP of *S. pyogenes* (RT ~ 270 s in SIC B of Figs. **1-3**). A mass spectrum corresponding to the SIC peaks at RT ~ 270 s (B ion chromatograms in Figs. **1-3**) is displayed in Fig. (**4A**) (and assigned as acetaldehyde).

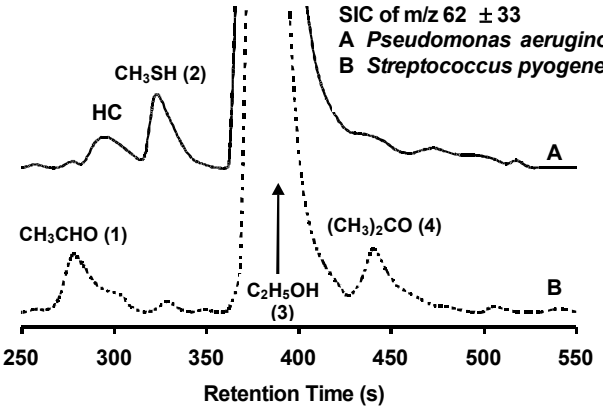

Fig. (1). Wide mass range GC/FT-ICR MS SICs from m/z 29 to 95 range (excluding background oxygen, argon and carbon dioxide contributions) for **A**) *P. aeruginosa* and **B**) *S. pyogenes*. Both SICs A and B have common Y-axis scaling but **A** has been offset for visual comparison. The peak labeled HC denotes a hydrocarbon species. The shoulder observed on the ethanol peak for *P. aeruginosa* (**A**) is due to tailing resulting from the large ethanol injection onto the GC column.

Conversely, the observed concentration of methylmercaptan (RT ~ 330 s in Fig. **1**) in *P. aeruginosa* VMWP (SIC A, Fig. **1**) was about 6 times higher than that of *S. pyogenes* VMWP (SIC B, Fig. **1**); a mass spectrum for corresponding to RT ~ 330 s is shown in Fig. (**4B**) and is assigned as methylmercaptan. Acetone (RT ~ 440 s in SIC B, Figs. **1**, **2**) was only detectable from *S. pyogenes* VMWP samples; a mass spectrum corresponding to RT ~ 440 s of Figs. (**1**, **2**) is shown in Fig. (**4D**) (assigned as acetone).

The SICs of m/z = 43.018 ± 0.002 (shown in Fig. **2**) demonstrate that *P. aeruginosa* and *S. pyogenes* can be readily differentiated from each other. The *P. aeruginosa* SIC has only 1 major peak at retention time (RT) of ~ 380 s (an

ethanol fragment ion at m/z ~ 43), whereas the *S. pyogenes* SIC has 3 major peaks at RT of ~ 270 s (acetaldehyde fragment ion, $[M - H]^+$), ~ 380 s (an ethanol fragment ion at m/z ~ 43), and ~ 440 s (an acetone fragment ion, $[M - CH_3]^+$). Similarly, the SICs of m/z = 44.026 ± 0.002 (shown in Fig. **3**) demonstrate that the two bacteria can also be distinguished unambiguously using this narrow mass range SIC.

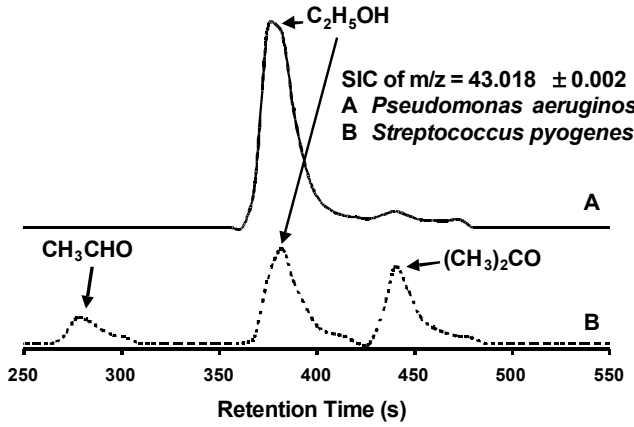

Fig. (2). Narrow mass range GC/FT-ICR MS SICs: m/z = 43.018 ± 0.002 for **A**) *P. aeruginosa* and **B**) *S. pyogenes*. Both SICs **A** and **B** have common Y-axis scaling but A has been offset for visual comparison.

DISCUSSION

Our headspace results for *P. aeruginosa* showed many of the same VMWP detected by SIFT-MS in the headspace of BacT/Alert FA blood culture bottles containing *P. aeruginosa* incubated for 24 h [40]. One major difference was that acetic acid and ammonia were not detected in the present work. The difference in the VMWP profiles could arise from variations of culturing media. Our observations are consistent with GC/MS results of Labows and co-workers [18]; they reported that 10 ml static HS samples (analyzed by a GC equipped with a sulfur detector) of *P. aeruginosa* cultured on Trypticase soy agar for 24 hours at 37 ^oC contained only one major component, methylmercaptan, and a few other components. Dynamic headspace sampling of *P. aeruginosa* revealed a number of ketones (including acetone), dimethyl disulfide, and dimethyl trisulfide but no methylmercaptan was observed [18]. Comparisons between the SIFT-MS [40], GC/MS [18] and the present work suggest that differences in culturing, temporal factors, sampling techniques, and analysis methods may lead to potential variations in the *P. aeruginosa* VMWP profiles.

Taking advantage of the high MMA and mass resolving power of GC/FT-ICR MS, the narrow mass range SICs [37] for m/z = 43.018 ± 0.002 and m/z = 44.026 ± 0.002 are displayed in Figs. (**2**, **3**), respectively. Displaying the narrow m/z range of 43.018 ± 0.002 allows to separate acetyl cation (CH_3CO^+ at m/z = 43.01784) from a low level background ion ($C_3H_7^+$) at m/z = 43.05423. Similarly,the background $CO_2^{\cdot+}$ (m/z = 43.98928) can be completely removed from the VMWP, $C_2H_4O^{\cdot+}$ (m/z = 44.02567) in the SIC at 44.026 ± 0.002 shown in Fig. (**3**). The VMWP species, $C_2H_4O^{\cdot+}$ (m/z = 44.02567), is either the molecular ion of acetaldehyde or an EI fragment ion of ethanol.

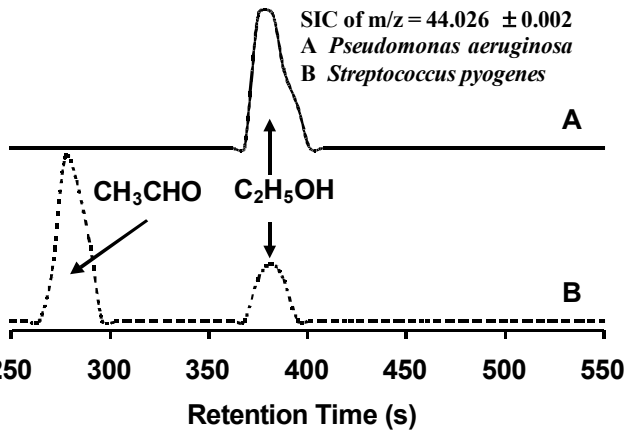

Fig. (3). Narrow mass range GC/FT-ICR MS SICs: m/z = 44.026 ± 0.002 for **A**) *P. aeruginosa* and **B**) *S. pyogenes*. Both SICs **A** and **B** have common Y-axis scaling but A has been offset for visual comparison.

Accurate mass measurements and high mass resolving power can reduce the number of chemical formula candidates ideally to one [41, 42]. For example, a restricted search using double bond equivalent (DBE) range of -1.5 to 3 (elemental composition calculator: version 2.0.0, 2000-2005, IonSpec Corp., Lake Forest, CA) for possible chemical formulae within the m/z range of 44 ± 0.1 yielded 14 possible hits; the selected elements for this search included C, H, Cl, F, N, O, P, S, or Si. A selection of reasonable elemental compositions for ions at m/z range 44.0 ± 0.1 include $CO_2^{\cdot+}$ (m/z 43.9893), $N_2O^{\cdot+}$ (m/z = 44.0005), $C_2H_4O^{\cdot+}$ (m/z 44.0257), $CH_4N_2^{\cdot+}$ (m/z 44.0369), and $C_3H_6^{\cdot+}$ (m/z 44.0464). However, within a ± 45 ppm SIC narrow mass window (i.e., m/z range of 44.026 ± 0.002 in Fig. (**3**), which is within our MMA of ± 10 ppm), the only candidate is $C_2H_4O^{\cdot+}$; all other reasonable candidate ions differ in mass by at least 250 ppm. Similar arguments were used to assign the chemical composition for ions in the SIC at m/z 43.018 ± 0.002 as $C_2H_3O^+$, an EI fragment ion of acetaldehyde, ethanol, and acetone.

Mass spectra extracted from the PC/GC/FT-ICR MS selected ion chromatograms in Fig. (**1**) were ascribed to acetaldehyde, methylmercaptan, ethanol, and acetone are shown in Figs. (**4A-D**), respectively. The MMAs of below 10 ppm in conjunction with the NIST mass spectral database [39] were used to positively assign the analyte identities shown in Fig. (**4**).

CONCLUSIONS

In the present work, only static HS analyses were performed to simplify and minimize sample collection procedures to demonstrate our minimalist noninvasive approach to identify biomarkers. Unambiguous identification of biomarkers is a vital step for designing small detectors such as biomedical devices and or environmental monitoring tools. The PC/GC/FT-ICR MS allowed us to assign molecular compositions for unknown peaks at a high level of confidence. Our ongoing activities to enhance instrumental sensitivity and sample collection methods [28] should permit detection of additional minor components in VMWP and construction of detailed bacterial-prints for identification and characterization of biomarkers. Small devices can be de-

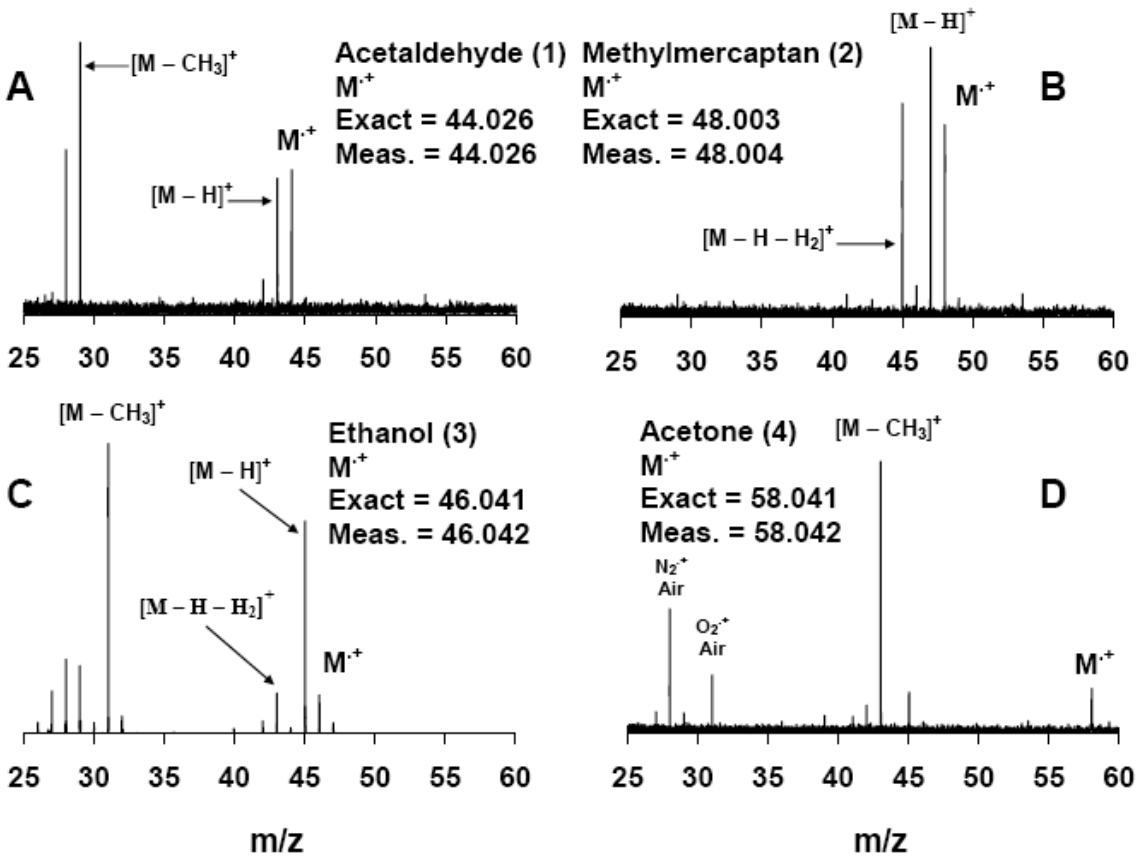

Fig. (4). Mass spectra extracted from the PC/GC/FT-ICR MS selected ion chromatograms for acetaldehyde (**1**), methylmercaptan (**2**), ethanol (**3**), and acetone (**4**) are shown in **A**, **B**, **C**, and **D**, respectively.

signed and fabricated to test for the presence of specific biomarkers in either static HS or near site air "sniffing" sample volumes.

ACKNOWLEDGEMENTS

This material is based in part upon work supported by the National Science Foundation under Grant No. CHE-0228971, Defense Advanced Research Projects Agency (Grant #: DARPA-N65236-98-1-5415, and Department of Defense (CDMRP-OC060322 – Award Number: W81XWH-07-1-0472). The views and conclusions contained herein are those of the authors' and should not be interpreted as necessarily represent the official policies, or endorsements, either expressed or implied of the DARPA, DOD, and U.S. Government.

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Received: February 11, 2008 Revised: September 10, 2008 Accepted: January 13, 2009