CHARACTERIZATION OF VOLATILE ORGANIC COMPOUND PROFILES OF BACTERIAL THREAT AGENTS

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ABSTRACT

Volatile organic compound (VOC) profiles are potentially an underutilized class of threat agent signatures that may be exploited in the identification of threat agents. In the present study we first focused on determining if VOC profiles collected from liquid culture headspace could be utilized to differentiate between bacterium of different genus, in this case *Bacillus* and *Yersinia*. The second focus of this study was to determine is VOC profiles could effectively differentiate between species of the same genus.

1. INTRODUCTION

Current protocols for field detection and identification of pathogenic bacteria include a two step process. A presumptive identification is first made based upon the results of polymerase chain reaction (PCR) assays or immunological methods (such as ECL, ELISA or hand-held assays). The results are then confirmed by laboratory culture, which is time-consuming. PCR and immunochromatographic (hand-held) assays can be performed in less than an hour, ELISA requires a few hours, and laboratory culture often requires 12-24 hours or longer. Reagentless methods are currently sought by DOD because of the reduced logistical support required relative to devices that employ "wet biochemistry" methods. The current technology is intended to be robust in its ability to rapidly identify threat agents when grown under optimal/suboptimal conditions as may be the case in a battlefield. Rapid, positive identification of a possible threat will allow commanders to take appropriate action in a significantly shorter time frame than that allowed by current technology.

A number of studies have shown that bacteria may be identified by an analysis of the volatile organic compounds contained in the headspace of bacterial cultures (Aathithan et al., 2001, Bunge et al., 2008, Casalinuovo et al., 2006, Lechner et al., 2005, Probert et al., 2004, Shnayderman et al., 2005). Such analyses, generally using gas chromatography / mass spectroscopy (GC-MS), generate a profile of the chemical compounds and relative concentrations of VOCs generated by a bacterial culture during growth. Such profiles have even been used to "diagnose" infections in humans and plants. Probert et al. (2004) identified the type of bacterial or viral species causing gastrointestinal infection in humans. Other researchers have used GC-MS to identify VOCs collected from potato tubers that were inoculated with fungi (de Lacy, Costello, et al. 2001) citing the results that some VOCs were specific to each pathogen. Similar methods could be developed to identify threat agent bacterial species in liquid culture using commercially available equipment and a customized software database.

2. MATERIALS AND METHODS

2.1 Bacterial strains and culture methods

The strains used in this study were obtained from in-house culture collections at ECBC; most non-select agent strains are available from American Type Culture Collection (ATCC, Manassas, VA) or the Bacillus Genetic Stock Center (The Ohio State University, Columbus, OH). Strains of *Bacillus* spp. and *Yersinia pestis* (Table 1) were grown in liquid Luria Broth (Difco), prepared according to the manufacturer's instructions. Bacteria were grown overnight at 30°C (*Bacillus*) and 37°C (*Yersinia*) in a shaking incubator at 200 rpm or on solid media. Glycerol stocks were made for each strain and were stored in LB broth and 10% glycerol at -80°C.

Table 1. Bacterial strains used in preliminary studies.

Bacillus anthracis VNR1-Δ1
Bacillus thuringiensis kurstaki
Bacillus subtilis V.niger
Bacillus cereus 1122
Bacillus cereus 13824
Bacillus myoides 6462
Bacillus megaterium 14581
Yersinia pestis EV76

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2.2 Sampling and analysis procedure

For headspace analysis of the culture one end of a multi-bed sorbent tube (Fig. 1) is attached to a 500mL büchner flask and the other end is attached to a vacuum. Using a mass flow controller set at 100ml/min collection volume, a known amount of the headspace culture is collected on an absorbent tube packed with Tenax TA, Carboxen and Carbosieve sorbents that will collect the VOCs of interest.

Once the sample is collected onto the tube, it is thermally desorbed using an ACEM 900 (Dynatherm) which is coupled to a GC-MS from Agilent. As molecules elute from the GC column, they are separated from the mobile phase carrier gas and enter the mass spectrometer. The mass spectrometer scans a defined mass range (from 45-300 amu in this application) enabling detection of the individually charged fragments, which are virtually unique for every molecule. Positive identification of target analyses is achieved by; (1) comparing eluted GC peak retention times in the total ion to those contained in the three-point calibration, and (2) by examining the mass spectral pattern of the eluted peaks. We have been using a standard method that uses very slow temperature ramp to establish and separate as many compounds as possible. Once the sample was completely desorbed from the multi-bed sorbent tube, the GC-MS chromatograms were compared to the chromatogram from un-inoculated media samples to determine unique peaks. Once we found unique peaks with identification quality greater than 50% we would compare those peaks to those from a different species of bacteria.

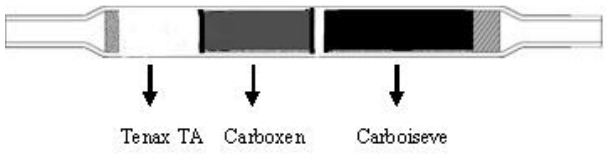

Figure 1. Multi-bed sorbent tube. Sorbent tubes are used for the collection and sampling of gases and vapors. The tubes we used were made of glass and contain 3 types of solid adsorbent materials, Tenax TA, Carboxen and Carboiseve. These materials make it possible to trap and retain compounds of interest.

3.0 RESULTS

3.1 Determination of Different Genus

The first task was to examine bacteria of different genus to determine if they produced unique spectra and differing concentrations of volatile compounds. For this experiment, *Bacillus anthracis* VNR-1Δ1 and *Yersinia pestis* EV-76 GC peak retention times and mass spectral patterns were examined. Diethyl ester decanedioic acid and toluene were particularly prevalent in the *Yersinia pestis* EV-76 samples while tetrahydro-3-methyl-furan and methyl cyclopentane were the two major components of *Bacillus anthracis* VNR1-Δ1 (Figure 2a & 2b). The data collected from the two samples provided sufficient evidence that the GC-MS is capable of differentiating between two different genus based on their VOC profiles.

3.2 Differences between Bacillus species

After determining that VOCs from the genus Yersinia and Bacillus could be differentiated visually by their chromatograms and by the major peaks detected by the GC-MS, the next step was to determine if unique VOCs from several different species of Bacillus could be determined. The following Bacillus species were utilized in this task, *Bacillus anthracis* VNR1-Δ1, *Bacillus thuringiensis* kurstaki, *Bacillus subtilis* V.niger, *Bacillus megaterium* (ATCC 14581), *Bacillus mycoides* (ATCC 6462), *Bacillus cereus* (ATCC 13824) and *Bacillus cereus* (ATCC 1122). Preliminary results indicate that GC-MS analysis is capable of differentiating bacterial species by their unique VOC fingerprints (Table 2, Figure 2b, 2c, 2d).

Table 2. Summary of unique VOC peaks identified for each bacterium tested.

Bacteria	Volatile Class	Volatile Component
YP EV-76	Acid	Decanedioic acid, diethyl ester
	Aromatic hydrocarbon	Toluene
BA VNR1-Δ1	Furan	Furan, tetrahydro-3-methyl
	Alicyclic hydrocarbon	Cyclopentane, methyl-
BT kurstaki	Aromatic hydrocarbon	Naphthalene
	Sulfide	Disulfide, dimethyl
	Aromatic hydrocarbon	Toluene
	Aromatic hydrocarbon	2,2'-Dimethylbiphenyl
BS V.niger	Aromatic hydrocarbon	Naphthalene
	Acid	Benzenecarboxylic acid
BC 1122	Sulfide	Disulfide, dimethyl
	Aldehyde	Nonanal/Decanal
BC 13824	Sulfide	Disulfide, dimethyl
	Acid	1,2 Benzenedicarboxylic acid
BM 6462	Sulfide	Disulfide, dimethyl
	Aromatic ketone	Acetophenone
BM 14581	Acid	Benzenecarboxylic acid
	Aldehyde	Decanal

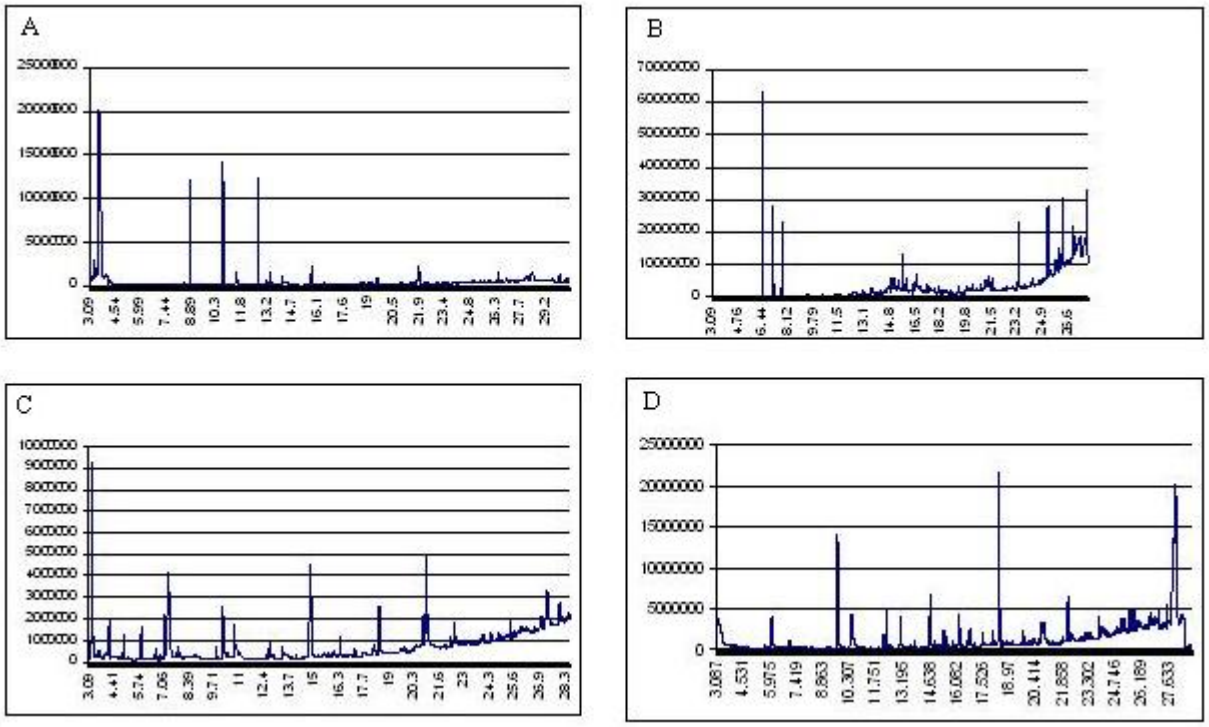

Figure 2. Typical examples of a gas chromatography-mass spectroscopy (GC-MS) chromatogram for (A) *Yersinia pestis* EV-76, (B) *Bacillus anthracis* VNR1-Δ1, (C) *Bacillus thuringiensis* kurstaki and (D) *Bacillus subtilis* V.niger. Data not shown for *Bacillus cereus* (ATCC 1122), *Bacillus cereus* (ATCC 13824), *Bacillus mycoides* (ATCC 6462) and *Bacillus megaterium* (ATCC 14581).

CONCLUSIONS

Future studies are planned to determine if this technology can specifically identify bacterial species at the strain level. Different culture media and cultivation conditions will also be investigated to determine which VOC fingerprints are uniquely characteristic of the microbial metabolism of each strain, regardless to the culturing process. If this is successful, a searchable database could be built that would allow for VOC analysis in the field to yield rapid and reliable identification of bacterial species grown in liquid culture.

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