2013-06-07

# Identification and Differentiation of Tier 1 Bacterial Agents Using Gas Chromatography-Mass Spectrometry

Dan Li
*Brigham Young University - Provo*

# Identification and Differentiation of Tier 1 Bacterial Agents Using Gas Chromatography-Mass Spectrometry

Dan Li

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Milton L. Lee, Chair
H. Dennis Tolley
Richard A. Robison
David V. Dearden
Daniel E. Austin

Department of Chemistry and Biochemistry

Brigham Young University

June 2013

ABSTRACT

Identification and Differentiation of Tier 1 Bacterial Agents Using
Gas Chromatography-Mass Spectrometry

Dan Li
Department of Chemistry and Biochemistry, BYU
Doctor of Philosophy

A simple method was developed for detection and differentiation of five Tier 1 bacterial agents, including *Bacillus anthracis*, *Francisella tularensis*, *Yersinia pestis*, *Burkholderia pseudomallei* and *Burkholderia mallei* as well as their closely related near neighbors by gas chromatography-mass spectrometry (GC-MS). Generally, different classes of compounds can be used as biomarkers for biowarfare agent detection, including nucleic acids (i.e., DNA or RNA), proteins (i.e., antibodies), carbohydrates (i.e., sugars), lipopolysaccharides, lipids (i.e., fatty acids) and small molecules. One-step thermochemolysis (TCM) was developed to provide GC-MS detectable biomarker signatures, including sugars, fatty acids and small molecules. Solid phase micro-extraction (SPME) was used for biomarker extraction, concentration and introduction into the GC-MS. Statistical algorithms were constructed using a combination of biomarkers for the five agents, which were robust against different growth conditions (medium and temperature).

A general GC-MS temperature program was developed for all five Tier 1 bacteria. The total analysis time, including TCM, SPME extraction and GC-MS, is approximately 40 min. The total-ion chromatograms are very different for the five species. The final goal of this research was to develop an accurate, fast, simple, robust and automated method for field application. Therefore, an automated sample preparation system was designed, constructed and tested. The system automatically controls the movement of sample vials from one position to another, crimping of septum caps onto the vials, precise delivery of reagents and TCM reaction times and temperatures. The specific operations of introduction of sample vials, SPME sampling, injection into the GC-MS system and ejection of used vials from the system were performed manually in this study, although they can be integrated into the automated system. Manual SPME sampling is performed by following visual and audible signal prompts for inserting the fiber into and retracting it from the sampling port. A rotating carousel design allows for simultaneous sample collection, reaction, biomarker extraction and analysis of sequential samples. *Bacillus* species were used to test this autoreactor, and 96% of the samples were correctly identified using a statistical algorithm. This research applies not only to the rapid identification of Tier 1 agents after a biological attack, but should also benefit clinical diagnosis, which is essential to effective treatment.

Keywords: Tier 1 bacterial agents, detection and differentiation, biomarkers, thermochemolysis, solid phase micro extraction, gas chromatography-mass spectrometry, automated reactor

# ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to my advisor, Professor Milton L. Lee, for supporting, encouraging and guiding me during these past years at Brigham Young University. He has provided an excellent example of integrity, devotion and work ethic for not only me, but all of his students. He is the one who provided me the valuable opportunity to study in the United States, led me into the world of chromatography and gave me an interdisciplinary project to combine my microbiological background with analytical chemistry. He provided me great opportunities to participate in international conferences, which not only broadened my vision, but also helped me prepare for a subsequent career in academia or industry. I hope I can be as enthusiastic, energetic, patient and humorous as Dr. Lee, and someday succeed in life multi-dimensionally. Dr. Lee's rigorous scholarship, hard-working habits, stringent attitude for science and eagerness to share his knowledge deeply influenced me and will inspire me in my future career.

I would like to give my sincere appreciation to Dr. H. Dennis Tolley and Dr. Richard A. Robison for their scientific advice and insightful discussions about the research. Together with my advisor, Dr. Lee, they have been primary resources for receiving answers to my science questions. I sincerely thank Dr. David V. Dearden and Dr. Daniel E. Austin for their time, instruction and suggestions during my studies, especially during my progress reports.

A good support system is important for surviving in graduate school. I thank members of our project group for their help and cooperation, i.e., Aaron N. Nackos, Tai V. Truong, John R. Williams, Abhilasha Acharya, Douglas N. VanDerwerken, Cory W. Taylor, Brian C. Holt, Terri

M. Bills, Anthony D. Rands, Stephen A. Lammert, Tiffany C. Brande, Jordon K. March and Christopher E. Wilson.

My wonderful family members deserve my utmost thanks for their understanding, support and encouragement. My beloved hard-working parents provide unconditional love and care for my little brother and myself. I always have my family to count on when times are rough.

I appreciate all members of Dr. Lee's group with whom I have had the chance to work, for their help and friendship. I acknowledge Anzi Wang's great help in my research, design of TCM components, and preparation of figures for my publications and presentations. I am thankful to Jacolin A. Murray, Jesse A. Contreras and Xiaofeng Xie for their help on using and trouble-shooting the gas chromatograph-mass spectrometer. I also thank my friend, Fan Yang, for her support and encouragement during my chemistry studies.

I acknowledge BYU and the Department of Chemistry and Biochemistry for providing me an excellent high quality education, which built a strong foundation for my future career. I acknowledge Torion Technologies, Dugway Proving Ground and the Department of Homeland Security for financial support during my studies.

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AFLP | Amplified fragment length polymorphism |
| ATCC | American Type Culture Collection |
| BSL-3 | Biosafety Level 3 |
| BWA | Biowarfare agent |
| CDC | Centers for Disease Control and Prevention |
| CE | Capillary electrophoresis |
| DPA | Dipicolinic acid |
| DPAME | Dipicolinic acid methyl ester |
| DVB/CAR/PDMS | Divinylbenzene/carboxen/ polydimethylsiloxane |
| ELISA | Enzyme-linked immunosorbent assays |
| FAME | Fatty acid methyl ester |
| FFAP | Free fatty acid phase |
| FT-IR | Fourier-transform infrared |
| GC | Gas chromatography |
| GC-MS | Gas chromatography-mass spectrometry |
| ICP-AES | Inductively coupled plasma-atomic emission spectroscopy |
| ISR | Intergenic spacer region |
| KDO | Ketodeoxyoctulosonate |
| LANL | Los Alamos National Laboratory |
| LD | Leighton-Doi |
| LPS | Lipopolysaccharides |
| LSU | Louisiana State University |
| LVS | Live vaccine strain |

| | |
|---|---|
| 3-M-2-BAME | 3-methyl-2-butenoic acid |
| 3-M-3-BAME | 3-methyl-3-butenoic acid |
| MIDI | Microbial Identification Inc. |
| MS | Mass spectrometry |
| MSDS | Material safety data sheets |
| NBFAC | National Bioforensic Analysis Center |
| 3-OH-3-MBA | 3-hydroxy-3-methyl butyric acid |
| P3HB | Poly-3-hydroxybutyrate |
| P3HV | Poly-3-hydroxyvalerate |
| PCR | Polymerase chain reaction |
| PHA | Polyhydroxyalkanoate |
| PCB | Printed circuit board |
| PEI | Polyetherimide |
| poly(3HB-*co*-3HV) | poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate |
| SNPs | Single nucleotide polymorphisms |
| SPME | Solid phase micro extraction |
| ssp. | Subspecies |
| TCM | Thermochemolysis |
| UAB | University of Alabama at Birmingham |
| USDA | United States Department of Agriculture |
| VNTR | Variable number tandem repeat |

# 1 INTRODUCTION

## 1.1 OBJECTIVES OF THIS WORK

The objective of the work described in this dissertation was to develop a fast, accurate, simple and robust method for detection and differentiation of bacterial biowarfare agents (BWAs) from each other and from non-virulent, closely related species (i.e., near neighbors) using gas chromatography-mass spectrometry (GC-MS).

## 1.2 BIOWARFARE AGENTS (BWAs)

BWAs or biothreat agents are microorganisms (viruses, bacteria, fungi and protozoa) or biotoxins, which can be used to produce weapons of mass destruction.[1] Because these agents are infective in low doses, can be aerosolized, are highly contagious, can survive harsh environmental conditions, are low cost, can be easily transported and are simple to produce, they are frequently referred to as "weapons of the poor."[1] There are at least 1,400 infectious organisms that are pathogenic to humans; however, only a few of them are effective as bioweapons.[2] The most dangerous bioagents are classified today as Tier 1 by the Centers for Disease Control and Prevention (CDC). Tier 1 agents can result in high mortality rates and substantial public health impact due to easy dissemination and/or transmittance from person to person.

Most bioterrorism attack scenarios involve dispersing aerosolized pathogens in densely populated areas, sending dried endospores in the mail or other common carriers, or

contaminating food sources. *Bacillus anthracis*, *Francisella tularensis*, *Yersinia pestis*, *Burkholderia pseudomallei* and *Burkholderia mallei* the causative agents of anthrax, tularemia, the plague, melioidosis and glanders, respectively, are all classified as Tier 1 agents, and require special procedures for public health preparedness and response in case of a terrorist attack (www.cdc.gov).

## 1.3 DETECTION OF BWAs

### 1.3.1 Methods

The fear of microbes as "invisible enemies" has resulted in development of detection means for homeland security. A variety of bioanalytical methods have been used for pathogen identification, such as traditional culture-based methods, particle sizing from intrinsic fluorescence,[1-3] nucleic acid-based methods (e.g., polymerase chain reaction [PCR] and single nucleotide polymorphisms [SNPs]), antibody-based methods (e.g., enzyme-linked immunosorbent assays [ELISA] and immunofluorescence assays) and combinations of these. These tests vary greatly in sensitivity, response time, cost, availability and complexity of use. Briefly, conventional cultures for bacterial identification take 24-48 h, with additional time for confirmation. Moreover, assays involving Tier 1 bacterial agents are usually performed in a Biosafety Level 3 (BSL-3) facility by experienced specialists. These methods are time-consuming, and the results are sometimes compromised by other species closely related to the target agents.

Particle sizing from scattered and emitted fluorescence light intensity for continuous monitoring of airborne bacterial particles is attractive, but suffers from difficulty in

differentiating biological particles from non-biological particles (e.g., dust and diesel exhaust) of similar sizes.[1] Therefore, this method is highly susceptible to false alarms caused by operation in dirty environments. Improved fluorescence-based techniques can distinguish between bioaerosols and bacterial simulants, but fail in differentiating among bacteria.[4]

Although current PCR assays may be rapid, and they provide low minimum detectable quantities (i.e., femtograms or even attograms of nucleic acids),[5] they still suffer from the inability to directly classify the bacteria, especially in the case of unknown samples.[6] Other deficiencies include easy contamination, involved sample preparation, insufficient quantities of nucleic acids from target organisms to allow amplification and inefficiency of RNA amplification.[7]

Immunoassay-based methods commonly use conjugated monoclonal and polyclonal antibodies induced against target agents, and offer intermediate speed (approximately 1 h) and sensitivity (approximately $10^5$ spores).[8] However, selection and production of specific antibodies is sometimes difficult and can be a major limitation of these techniques. Moreover, many of these antibodies have shown cross-reactivity. Immunoassay-based methods are expensive and utility intensive because two monoclonal antibodies,[9] fluorescence tags[10] and refrigeration for antibody preservation are required to achieve good specificity. In addition, sample quantitation can be influenced by different sample processing methods;[11] therefore, immunoassay approaches are less compatible with fieldable methods.

The limitations of these classical biochemical methods have led to the development of modern analytical instrumentation and methods for rapid identification of microorganisms. A variety of analytical techniques have been used in microorganism analysis, including gas chromatography (GC),[12] mass spectrometry (MS), GC-MS,[13, 14] pyrolysis MS, Fourier-transform

infrared (FT-IR) spectroscopy and capillary electrophoresis (CE).[15] Procedures using GC-MS are designed to differentiate and identify biological species on the basis of detection of specific chemical biomarkers from which organisms are classified according to intrinsic differences in their GC-MS profiles or chemical fingerprints.

A particularly successful GC-based method, MIDI (commercialized by Microbial Identification Inc.), has been used to classify over 2,000 bacteria at the subspecies level using fatty acid methyl ester (FAME) profiles.[16] However, this method has several serious limitations. First, the organism must be cultured under standardized growth conditions. This requirement stems from the fact that fatty acid composition is influenced by growth medium and incubation temperature.[17] Second, sample preparation for this method includes several time-consuming and complicated steps (i.e., saponification, methylation, extraction, washing, etc.). These limitations indicate that the method is insufficiently robust and not suited for field detection.

Phage display is a fairly new and evolving technology for bacteria detection. A bacteriophage binds to a target bacterium in a unique way and, subsequently, releases a toxic compound to kill its host.[18] Selective lysis of the host by a bacteriophage can be used for identification.[19] In order to expedite the detection process, genetically engineered "bioluminescent" reporter phages were developed based on integrating genes that can encode luciferase into the phage genome.[20] Their inherent natural specificity and straightforward production make phages particularly suitable as bacteria detectors.[21] They are currently used for the detection of *B. anthracis*, *Y. pestis* and other bacteria in clinical applications. However, one of the obvious limitations is that the infectious agents (bacteriophages) are viable and their toxins typically must be inactivated prior to being used outside of the biosafety laboratory. Unfortunately, inactivation may render the bacteriophage ineffective for lysing the target cells.

Also, if a mixture of bacteria is present, false positives are possible due to interferences.[22] Phage display is technically not a biomarker approach to detection; however, it is mentioned in this review because it is a growing technique for BWA detection.

### 1.3.2 Biomarkers

Despite differences in the various chemotaxonomy methods for bacterial agent detection, the major goal for most methods is to detect unique chemical components (often called biomarkers), or to measure specific ratios of less unique biomarkers, for which these ratios are unique. Advances in whole-genome sequencing, bioinformatics and proteomics provide opportunities to define a variety of different bacterial products as biomarkers, such as nucleic acids, proteins, sugars, lipids and some unique small molecules (e.g., dipicolinic acid).

### 1.3.3 Detection of BWAs using GC-MS

This dissertation describes the detection and differentiation of bacteria using GC-MS. As a powerful identification technology, GC-MS can provide complementary information not possible by either GC or MS, which makes it possible to detect fatty acid and sugar biomarkers at low limits of detection.[23, 24] However, some degree of sample treatment is necessary to produce volatile and semi-volatile biomarkers that are amenable to GC-MS analysis.

Generally, biomarker signals produced from microorganisms are suppressed by abundant proteins or metabolites in the sample.[6] Therefore, a good sample preparation method should release the biomarkers from the microorganism, and then convert them into easily detectable forms. For GC-MS analysis, biomacromolecules must be converted to volatile compounds through controlled decomposition and derivatization.[25] These derivatized biomarkers must be less polar and more volatile than the original biomarkers. A typical sample preparation method

involves saponification and methylation of released compounds into their corresponding methyl esters.

For rapid biological agent detection, typical laboratory procedures must be modified. For example, the sample preparation time required for detection of FAMEs was reduced using online derivatization in which whole bacterial cells in the presence of strongly basic methylation reagents, were pyrolyzed in the GC injection port.[12, 26] Thermal-hydrolysis and methylation using tetramethylammonium hydroxide as a reagent has been used for GC and MS detection of *Bacillus anthracis*,[27, 28] and a micro-fabricated pyrolyzer coupled with MS was reported based on this method.[29] However, temperatures required for this micropyrolyzer were over 360 °C, which are too high to preserve many of the diagnostic biomarkers. It has been found that lower temperature thermochemolysis (TCM) preserves biomarker structures much better than high temperature pyrolysis.[30] By comparing five derivatization reagents for GC determination of fatty acids, Antolin et al. showed that sulfuric acid/methanol (i.e., hydrogen methyl sulfate, $HMeSO_4$) performed best in terms of cost, speed, safety and GC response.[31]

TCM couples biomarker generation and derivatization in one step, and it occurs at much lower temperatures than pyrolysis. This technique, originally introduced as pyrolysis with in situ methylation,[32] was developed as an alternative to overcome the limitations of conventional pyrolysis. TCM offers the advantages of improved recovery of biomarkers, no need for postextraction derivatization, minimal sample handling and reduced operator error, making it more suitable for field application.

## 1.4 BIOMARKERS FOR TIER 1 BACTERIAL AGENTS

### 1.4.1 General categories of bacterial biomarkers

Generally, nucleic acids, proteins, sugars, lipids and some unique small molecules are used as biomarkers for bacteria detection. Nucleic acid sequences can be used for discrimination of any self-replicating biological entity. Genetic markers are defined as fragments of a DNA sequence that are associated with either chromosomally or plasmid-encoded loci, which can be used to identify a particular bacterial species. General genetic markers for bacteria include genes of 16S rRNA, 23S rRNA, ropB, gyrB, dnaK, dsrAB, amoA, amoB, mip, horA, hitAM, recA, ica, frc, oxc, 16S-23S rDNA ISR and IS256.[33] However, nucleic acid-based assays may generate false negatives due to instability of their association with the pathogen(s); e.g., virulent plasmids can be lost, or near neighbor strains can harbor highly homologous chromosomal loci.[34]

The most important protein biomarkers are antibodies, because they have very high specificities and strong affinities for antigens. There are many successful applications of antibodies in the area of BWA diagnostics using biosensors or MS. There are three types of antibodies: polyclonal, monoclonal and recombinant antibodies. However, antibody selection in nature is a complex process and several important parameters must be considered for their use in biomarker applications, including sensitivity, selectivity, stability, immobilization, labeling, ease of production, cost, commercial availability and ability to engineer the desired affinity.[18]

Lipopolysaccharides (LPS) are the primary constituents of the outer membrane of Gram-negative bacteria.[35] They are key to immune stimulation via detection by host pattern recognition receptors. The basic structure of LPS is comprised of three parts: a lipid portion (Lip A), a polysaccharide core and an O antigen (or O polysaccharide).[36] Lip A is normally a

phosphorylated glucosamine disaccharide decorated with multiple fatty acids.[36] Hydrophobic fatty acid chains anchor the LPS into the bacteria membrane, and the remainder of the LPS extends out of the cell surface. The Lip A moiety is highly conserved in LPS. The core domain always contains an oligosaccharide component that attaches directly to lip A and commonly contains sugars such as heptose and 3-deoxy-D-mannoctulosonic acid (also known as keto-deoxyoctulosonate, or KDO for short).[37] The LPS cores of many bacteria also contain non-carbohydrate components, such as phosphate, amino acid and ethanolamine substituents.[37] The O antigen is attached to the core oligosaccharide and comprises the outermost domain of the LPS molecule. The composition of the O chain varies from strain to strain.

Carbohydrate (sugar) profiling has been shown to be a powerful tool for differentiating bacteria.[38] Carbohydrates are common features of bacterial cell walls (peptidoglycan), and they are present in DNA, RNA and a variety of glycoproteins. Sugar profiles generally do not depend on bacteria growth stages. Therefore, sugar biomarkers are useful in physiological status identification and in differentiation of bacterial agents.

Fatty acids have long been recognized as signatory biomarkers for chemical identification and differentiation of bacteria. They usually originate from phospholipids (essential bacterial membrane components)[39] and other complex lipid cellular components.[40] The backbone of the phospholipid molecule is glycerol with two of the three hydroxyl groups being replaced by fatty acids and the third replaced by a phosphate group. After cell death or cell lysis, cellular enzymes hydrolyze phospholipids, releasing free fatty acids. Microbial fatty acids are typically 12-24 carbons long. Typical Gram-positive bacteria are characterized by the presence of straight and methyl-branched (iso- and anteiso-) fatty acids and absence of unsaturated fatty acids.[41] Gram-negative bacteria lack branched-chain fatty acids, but contain a large proportion of cyclopropane

and unique hydroxylated fatty acids in the lipid portion of the lipopolysaccharides. For example, *F. tularensis, Burkholderia* sp. and *Yersinia* sp. are all characterized by high abundances of C16:0 and unsaturated C18:1 fatty acids.[42] The types and relative abundances of fatty acids are largely determined by an organism's genotype; however, they also can be affected by different growth conditions (type of growth medium, age of culture, incubation temperature, time of culture harvesting, etc.),[17] which lead to different "fingerprint" fatty acid profiles for the same organism.[40] Specific patterns of fatty acids can also indicate physiological or nutritional statuses of bacteria. Starvation and stationary-phase growth lead to conversion of unsaturated fatty acids (e.g., 16:1ω7c and 18:1ω7c to cyclopropane phospholipid-derived fatty acids, *cy*17:0 and *cy*19:0, respectively).[43, 44]

### 1.4.2 Specific biomarkers for Tier 1 bacterial agents

***Bacillus anthracis.*** *B. anthracis* is the causative agent of anthrax, a disease that can be acquired when *B.* anthracis is "weaponized" and used as a BWA. It is the most popular and, hence, most studied biological threat agent. *Bacillus* species are Gram-positive endospore-forming bacteria that are ubiquitous in natural environments. There are currently 65 validated *Bacillus* species, with new species continually being identified.[45] Due to their very close phenotypic and genotypic characteristics and protein composition, five of them, including *B. anthracis, B. cereus, B. thuringiensis, B. weihenstephanensis* and *B. mycoides*, are often classified as the *Bacillus cereus* group.[46] Indeed, many consider *B. anthracis, B. cereus* and *B. thuringiensis* to be the same species.[46] *B. cereus* is an opportunistic pathogen responsible for endophthalmitis, pneumonia and septicemia as well as food poisoning. [47, 48] *B. thuringiensis* is an insect pathogen with a distinguishing ability to produce a class of insecticidal proteins, known as crystallins or δ-toxins, which have been widely used in agriculture.[49, 50]

Variations in DNA sequences readily distinguish *Bacillus* organisms from their closely related near neighbors,[51-53] e.g., the *B. cereus* group from the *B. subtilis* group.[52, 53] The most direct route for nucleic acid-based detection of *B. anthracis* is the difference in 16S rRNA and 23S rRNA sequences. Evolutionary drift in 16S rRNA resulted in sequence differences in closely related *B. cereus* and *B. anthracis* species.[51] *B. mycoides* can be identified using a 16S rRNA probe,[54] and *B. anthracis* can be differentiated from *B. cereus* by differences in two nucleotide sequences of 23S rRNA.[55] Furthermore, the intergenic spacer region (ISR) DNA sequences (i.e., sequences between the 16S and 23S rRNAs),[56] located between *gyr*B and *gyr*A (gyrase subunits), were identical in the *B. anthracis* strains, but were different compared to *B. cereus* and *B. mycoides*.[57]

A more direct nucleic acid-based approach is to detect the genes that code for specific virulent factors. The *B. anthracis* genome approximately consists [16]of a 5.3-Mb chromosome and two plasmids, pXO1 (182 kb) and pXO2 (96 kb),[58] which are responsible for coding principle virulence factors. Due to the gene specificity of plasmids, some important nucleic acid biomarkers were developed based on target virulent genes located on the plasmids. Pathogenic strains of *B. anthracis* such as Ames, are detected by targeting the virulence genes *pag*, *cya* and *lef*, the biosynthetic genes for capsule synthesis on the pXO1plasmid and *cap*A and *cap*B genes associated with capsule depolymerization on the pXO2 plasmid.[59-61] Although pXO1and pXO2 are not self-transmissible, they can be transferred by conjugative plasmids and transducing phages that permit inter-species transfer of chromosomal and plasmid DNA.[62] In particular, *B. cereus* G9241 was associated with a case of severe, inhalation pneumonia, similar to that caused by *B. anthracis*,[62] because it contained a plasmid that was 99.6% identical to pXO1 and carried the lethal toxin genes of *B. anthracis,*[62] which may be caused by plasmid transfer. In addition,

two more strains of *B. cereus* from fatal pneumonias were also reported.[63] A similar situation was observed in other *B. cereus* stains isolated from great apes that died from another anthrax-like infection in Africa.[64] The function of *cap* genes (pXO2) responsible for capsule synthesis was demonstrated in *B. anthracis*; however, most of the *B. anthracis*-like strains were found to produce polysaccharide capsules.[65] It was suggested that pXO2-like plasmids were more commonly distributed in the *B. cereus* group than indicated by PCR-based screening.[66] Therefore, selections of suitable signature sequences that are stable are important for gene biomarkers.

For differentiation of subspecies of *B. anthracis*, several different nucleic acid-based methods have been employed. One of them is variable-number tandem repeats (VNTRs), which involves a polymorphism site search for variation in the number of repeating nucleotide units. One example is the observation of *vrr* genes in a large collection of *B. anthracis* isolates. This method can discriminate subspecies of *B. anthracis.*[67-69] Similarly, amplified fragment length polymorphism (AFLP) has been used for subspecies discrimination between *B. anthracis* isolates.[70] Length variations of the collagen-like region involved in the synthesis of carbohydrates (i.e., the *bclA* gene) were used as molecular biomarkers to differentiate among *B. anthracis* strains.[71-74] Additional genes that may help in *B. anthracis* differentiation include the germination operon *ger*X, the general stress transcription factor *sig*B and the gene *abr*B, a homologue of a well-characterized *B. subtilis* transition state regulator that controls growth phase-specific transcription of the toxin genes.[61]

Proteins have also been used as significant biomarkers for *B. anthracis* detection. A variety of both polyclonal and monoclonal antibodies were employed for immunological detection-based assays. *Bacillus* spores have high protein content compared to vegetative cells,[75] with up to 15% of the total protein content reported as small acid-soluble proteins.[76] Protein

biomarkers that have been explored for *Bacillus* endospore differentiation include YVRF, YDIQ, YUK [A-F], YNZA, YXIL, YOZC, YDAS and YJCB.[77, 78] However, most tests were based on non-virulent *Bacillus* strains (*B. cereus*, *B. thuringiensis, B. subtilis, B. globigii, B. mycoides* and *B. anthracis* Sterne).[79] Also, spores grown on different media appear to have different protein markers.[80] Therefore, growth conditions are also important for *Bacillus* differentiation when protein biomarkers are employed.

The collagen-like proteins show good potential for use as protein biomarkers. They are composed of Gly-Xaa-Yaa repeats, which have been identified in more than 100 prokaryotic proteins.[81] BclA, a collagen-like protein of *B. anthracis*, is a major spore surface protein[82] and is found in all members of the *B. cereus* group.[71, 74] In addition, a second collagen-like protein, BclB,[83] was also identified as a component of the *B. anthracis* exosporium. However, its distribution and structural properties have not been well characterized. Likewise, two closely related proteins, ExsH and ExsJ, contain GXY collagen-like repeats and are presumably located in the exosporium of *Bacillus* strains.[83, 84]

A class of sugar biomarkers has been extensively investigated for *Bacillus* species differentiation. The compositions of carbohydrates change from vegetative form to spore form.[85] Generally, both spores and vegetative cells contain ribose (from RNA), glucose, galactose, glucosamine, mannosamine and muramic acid, which can be used for *Bacillus* species detection.[55] Muramic acid and glucosamine are derived from peptidoglycan. Spores are differentiated from vegetative cells by the presence of high concentrations of quinovose (6-deoxyglucose, an isomer of rhamnose), rhamnose, 3-*O*-methyl rhamnose and galactosamine, and the absence of ribitol and glucuronic acid.[73] Quinovose is present in most spores of *bacilli*,[85] but not in *B. cereus*. The sugar, 3-*O*-methyl rhamnose, is quite rare in nature and is almost

exclusively present in *bacilli*.[73] Galactosamine is a major component of the cell wall of *bacilli. B. anthracis* can be differentiated typically from *B. cereus* and *B. thuringiensis* by high levels of galactose and no galactosamine.[85] Ribose, glucose, muramic acid, glucosamine and mannosamine are present in both *B. anthracis* and *B. cereus*.[85] Spores of *B. anthracis* are distinguished from *B. cereus* and *B. thuringiensis* by the absence of fucose and 2-*O*-methyl rhamnose.[85] All three species (*B. anthracis, B. cereus* and *B. thuringienesis*) contain large amounts of mannosamine, which is present in very low levels in *B. subtilis*.[73, 86] *B. subtilis* has a spore-specific carbohydrate containing 3-*O*-methyl rhamnose that resembles that found in *B. anthracis*, but is distinguished by the level of quinovose.[54] The carbohydrate composition can also reflect, to some extent, the environment under which the spores were cultured. For instance, xylose was normally not found in *B. anthracis* spores, but was detected when cultured in certain types of media.[87] Therefore, certain unique sugar biomarkers may provide useful forensic information about the growth medium used for sporulation.

Among the sugar biomarkers, a rather unique tetrasaccharide, which is located on the outermost surface of the *B. anthracis* spores, is important for *B. anthracis* detection and differentiation. It is composed of three rhamnose residues and a rare terminal sugar, 2-*O*-methyl-4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-D-glucopyranose, which has been given the common name "anthrose."[88] A number of reports have claimed that anthrose is a highly specific biomarker for *B. anthracis*.[88-90] However, recent studies have shown that anthrose is not unique to *B. anthracis*, but is also present in *B. thuringiensis* and *B. cereus*.[24, 91] Additionally, another recent study reported anthrose-deficient *B. anthracis* strains in West Africa, suggesting that anthrose may not be a perfect biomarker for all *B. anthracis* isolates.[92] Both *B. anthracis* and *B.*

*thuringiensis* Al Hakam were reported to contain high levels of anthrose;[24] therefore, two unidentified sugar biomarkers were used for their differentiation.[24]

*B. anthracis* is characterized by a high abundance of branched C15:0 (both iso- and anteiso-) and C17:0 (both iso- and anteiso-) fatty acids.[23, 93] Unique lipid profiles containing compounds such as phosphatidylethanolamine, phosphatidylgycerol and diglycosyldiacylglycerol, have been reported for *Bacillus* species and used to differentiate between them.[94] *Bacillus* is famous for producing branched-chain fatty acids and insignificant amounts of unsaturated fatty acids.[95, 96] Important fatty acid biomarkers used for detection/differentiation include C13:0 to C18:0 of different structural arrangements (i.e., *n*-, iso- and anteiso-).[97] Fatty acids such as iso C15:0, iso C17:0, anteiso C15:0 and anteiso C17:0 have been effectively used for detection of *Bacillus* species.[23, 24] The fatty acid, iso C17:1$\Delta^7$, is absent in *B. anthracis*, but is present in *B. cereus, B. thurigiensis* and *B. atropheous*.[98] As mentioned previously, growth conditions result in different lipid profiles. For *Bacillus*, fatty acid compositions were heavily dependent on the complex additives (beef/yeast extract, brain-heart solids, etc.) and protein sources (peptone, tryptone, gelatin digest or casein hydrolysate) that supply the amino acid precursors (amino acids and α-keto acids) in growth media.[99] This information confirmed that changes in fatty acid profiles from *Bacillus* species may occur when grown under different conditions.

Oleic acid (18:1 ω9c) was exclusively associated with spores grown on Columbia agar supplemented with sheep blood.[99] Similarly, *B. subtilis* produces ω-cyclopropyl, ω-cyclobutyryl, ω-cyclopentanyl, ω-cyclohexanyl and ω-cycloheptanyl fatty acids if cyclopropyl, cyclobutyryl, cyclopentanyl, cyclohexanyl and cycloheptanyl carboxylic acids, respectively, are included in the culture medium.[100] The ratio of anteiso C15:0 to iso C15:0 may change under different

growth conditions;[101] however, iso-C15:0 remains the most abundant isomer regardless of growth conditions.[42] Despite changes in growth conditions, the branched C15:0 fatty acids remain excellent biomarkers for Gram-positive typing, as most Gram-negative bacteria lack these branched fatty acids.[93]

Dipicolinic acid (2,6-pyridinedicarboxylic acid) is a spore-specific small molecule biomarker, present approximately in a 1:1 mole ratio with $Ca^{2+}$.[102] DPA levels vary from 5 to 15 wt% of the dry spore,[103] and the amount changes depending on the species and growth conditions. For example, *B. megaterium* spores are large and contain a high content of DPA.[104] Because of its uniqueness and high abundance, DPA is the most likely target for detection of Gram-positive spores at low levels. Although DPA is the most notable biomarker for the rapid detection of endospores, detection of DPA does not prove the presence of virulent *B. anthracis*. It can be used to exclude nonspore-forming bacteria.[24]

***Francisella tularensis.*** The *Francisellaceae* family is composed of a group of closely related organisms that are widespread in nature. *F. tularensis* and *F. philomiragia* are the more recognized species of the *Francisella* genus.[105, 106] *F. philomiragia* is a muskrat pathogen. *F. tularensis* subspecies (ssp.) *tularensis* is the causative agent of the zoonotic disease, tularemia or rabbit fever; it is a small and nonmotile Gram-negative coccobacillus requiring aerobic conditions.[107] It is one of the most highly infectious pathogenic bacteria known, with as few as 10 organisms sufficient to cause severe infection.[108] This nonsporulating Gram-negative organism can infect a wide variety of mammalian hosts, including wild rabbits, beavers, squirrels and muskrats.[109] It was first noticed in Californian's Tulare Country in 1911 and was, subsequently, named *Bacterium tularensis.*[110] The genus *Francisella* is subdivided into several subspecies. Formerly, it was divided into subtypes A and B; at present, four subspecies are

defined with differences based largely on virulence, geographic location and host specificity: *tularensis* (Type A), *holartica* (Type B), *mediasiatica* and *novicida*.[111, 112] Type A isolates (isolated primarily in North America) are highly virulent and cause life-threatening disease in humans and animals. Type B is relatively avirulent, produces a less severe infection and is rarely fatal in humans. A live, attenuated vaccine (live vaccine strain, LVS) was developed in 1956 from *F. tularensis* ssp. *holarctica*, although the basis for attenuation has yet to be elucidated.[113] *F. tularensis* ssp. *mediasiatica* is less virulent in humans than ssp. *tularensis* and is found predominantly in central Asia. Three of the four recognized *F. tularensis* ssp. strains are human pathogens, whereas ssp. *novicida* is generally non-pathogenic to healthy individuals, but can cause a tularemia-like illness in some.[114] *F. novicida* and *F. tularensis* are highly similar;[106] however, little is known about their cellular and molecular modes of infection, and the basis of any immune response to tularemia. The lack of adequate genetic analysis tools has hampered efforts to elucidate many questions about *F. tularensis*.

*F. tularensis* exhibits a highly conserved genomic sequence among strains of diverse origin. Both subspecies *tularensis* and *holarctica* exhibit highly species-specific regions of 16S rRNA; oligonucleotides complementary to such sequences are now used reliably for their discrimination.[105] Six partial housekeeping gene sequences (*groEL, shdA, rpoB, rpoA, pgm* and *atpA*) were used as identifiers of new isolates.[115] The substantial genetic similarities among *Francisella* subspecies makes individual strain typing difficult. All four *F. tularensis* subspecies are antigenically similar,[112] with 99.8% identity among 16S rRNA genes,[116] indicating other more specific targets are required. Thus, the highly divergent *tul4* gene (encoding 17 kDa lipoprotein) and *fopA* gene (encoding 43 kDa outer membrane protein) have been widely used for tularemia diagnosis as well as to detect *Francisella* spp.[117, 118] The two genes showed

specificity to *F. tularensis* ssp., type A *tularensis* SCHU4, type B *holarctica* and *novicida*, but not to *philomiragia*.[119] The O-antigen gene clusters in isolates SCHU S4 and LVS (live vaccine strain) of *F. tularensis* ssp. *tularensis* are identical, as compared to that of *F. novicida*, which has fewer genes in the cluster and a lower G+C ratio.[120, 121] There is an odd characteristic for *F. tularensis* ssp. *tularensis*, which does not have either of the plasmids found in LVS or *F. tularensis* ssp. *novicida*.[122-124] It is not clear whether this property is associated with the environment of the bacterium or with the specificity of its genetic apparatus.

Important protein biomarkers of *F. tularensis* include a 23-kDa cytoplasmatic protein (one of the two major virulent factors), Fop (*F. tularensis* outer membrane protein), AcpA (a 58-kDa acid phosphatase function protein), β-lactamase and the hypothetical protein FTT0484 (unique for the SCHU S4 strain).[125] AcpA has been identified in *F. tularensis* and *mediasiatica*, but not in *holarctica*, indicating its importance in the pathogenic process.[114]

Wild strains of *F. tularensis* and LVS possess a surface exopolysaccharide capsule.[126, 127] Also, an immunogenic surface capsular material on *F. tularensis* ssp. *holartica* strain 1547 has been reported.[128] The capsule extracts of SCHU S4 contain carbohydrates (including mannose, rhamnose and two unidentified dideoxy sugars), which may be used as possible biomarkers for detection.[126]

The LPS of *Francisella* ssp. are unique. The core region of *F. tularensis* LPS contains a single 3-deoxy-D-manno-octulosonic acid and lacks heptose.[129] Of critical importance is that the *Francisella* LPS lack free phosphate moieties and exhibit very low endotoxicity. The glucosamine residue of lipid A in *F. holarctica* and *F. novicida* is absent in LVS strains, which suggests a potential role in virulence[130] and can possibly be used as a biomarker for detection. The O-antigens of *F. tularensis* ssp. *tularensis* and ssp. *holarctica* are identical; however, the O-

antigen of *F. tularensis* ssp. *novicida* is distinct.[120, 129, 131] The Lipid A structure, which contains most of the fatty acid biomarkers, is unique for *F. tularensis.*[41]

The lipid concentration in the capsule and cell wall (50-70%, respectively) is unusually high for a gram-negative organism.[126] Characteristic of *F. tularensis* is the relatively large amounts of long-chain saturated and monoenoic C20-C26 fatty acids as well as alpha and beta hydroxyl fatty acids. Such long fatty acids are not usual in bacteria.[41] The fatty acid, 3-hydroxy-octadecanoate (3-OH C18:0), is present in significant amounts in *F. tularensis*.[106] Unique fatty acids reported in *F. tularensis* include saturated C10:0, C12:0, iso C14:0, C16:0, iso C16:0, C18:0, C20:0, C21:0, C22:0, C24:0 and C26:0; unsaturated C14:1ω7c, C16:1ω7c, C18:2, C18:1ω9c, C20:1ω11c, C22:1ω13c, C24:1ω15c and C26:1ω17c; and four hydroxy acids, 2-OH C10:0, 2-OH C14:0,[126] 3-OH C16:0 and 3-OH C18:0.[41, 113, 132] The amount of C16:0, C18:0 and 2-OH C10:0 are culture time-dependent.[113] The fatty acids, C20:0, C21:0, C22:0 and C24:1ω15c, are distinct biomarkers.

For *F. tularensis*, different growth media can induce the production of monounsaturated fatty acids such as C18:1 and cyclopropyl-C19:0.[42] However, the unsaturated fatty acid C24:1ω15c was present regardless of the growth media and stage;[42] therefore, it is a valuable biomarker for *F. tularensis*. The need for cysteine and enriched media for stimulating growth is useful information for identification of *F. tularensis*. However, seven *F. tularensis* strains were misidentified because they did not respond this way.[133] Fortunately, fatty acid profile analysis facilitated the correct identification of these strains.

***Yersinia pestis.*** *Y. pestis* is the etiological agent of the plague, or "Black Death," as it has been called over many centuries, usually transmitted by bites from the rat flea, *Xenopsylla cheopis.*[134] The disease can be spread through aerosols, and the fatality rate is 50-100% if

untreated.[135] It leads to outbreaks of high infectious pneumonic plague.[136] *Y. pestis* is enzootic in many countries and has the potential of being a BWA.[137, 138] There are 11 species in the *Yersinia* genus, however, only *Y. pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are considered to be pathogenic for humans.[139] *Y. pestis* causes bubonic plague, while *Y. enterocolitic* and *Y. pseudotuberculosis* cause yersiniosis, which is typically a mild diarrheal disease. It was suggested that *Y. pestis* is a clone derived from *Y. pseudotuberculosis* based on multilocus sequence typing of housekeeping genes.[140]

Most *Y. pestis* strains contain a 4.6-4.8 Mbp chromosome and several plasmids of varying size (0.06-0.12 Mb).[141, 142] *Y. pestis* shares the same plasmid pCD1 (pYV or pCad) as *Y. pseudotuberculosis* and *Y. enterocolitica*.[142] In addition, it also hosts two other well-characterized plasmids, pPCP1 (also called pPla or pPst) and pMT1 (also known as pFra), which are not carried by other *Yersinia* species.[140] The plasmid pCD1 contains a number of genes that encode for several virulence factors.[143] It governs the synthesis of a set of approximately 12 proteins called Yops ("*Yersinia* outer proteins"), which are essential for full virulence.[144] Most of the *yop* genes have been identified and sequenced, and they appear to be almost identical in the three species.[142] The plasmid pPla is responsible for protease plasminogen activator/coagulase (Pla) coding,[145-147] which is an important virulence factor found to be stable for a long time and suitable for study of plague victims from the 16$^{th}$-18$^{th}$ centuries.[148] The plasmid pMT1 codes for phospholipase D, previously characterized as *Yersinia* murine toxin (Ymt), which is responsible for the transmittance of *Y. pestis* by fleas.[134] The virulence factor gene *caf1*, encoded also by pMT1, is induced to form a protein capsule during growth above 33 °C; strains lacking this gene showed increased susceptibility to phagocytosis by macrophages in vitro.[149] Plasmid-encoded genes, *pla*, *ymt* and *caf1*, together with the chromosomal 16S rRNA genes[150] have been used as

genetic biomarkers for detection of *Y. pestis,*[145, 146, 150, 151] and they are present in all *Y. pestis* strains except for plasmid-deficient ones.[150] These plasmids are not stable and pathogenic *Y. pestis* strains exist that do not have any of these plasmids.[150, 152] Virulent genes contain a chromosomic pathogenicity island called HPI,[153] which is also used as a nucleic acid biomarker for target identification. O-antigen gene clusters (from LPS) have also been used to distinguish *Y. pseudotuberculosis* from other *Yersinia* stains.[154, 155]

Virulence protein biomarkers for *Y. pestis* mainly include Yops, F1 antigen and V-antigen (LcrV),[139] which have been found to be major defensive antigens in protecting against bubonic plague.[156] F1 antigen (capsular) is excreted by *Y. pestis* in the human body at 37 °C[157, 158] and in zoonotic vectors, and has been used as a protein biomarker for identification.[159] F1 antigen is a powerful tool for immunization against wild variants of the plague.[160] V-antigen was described as the major virulence marker of *Y. pestis*, but with less power than F1 antigen for immunization and vaccination.[161] Other promising biomarker antigens are ATP-binding cassette transporter proteins consisting of OppA, PstS, YrbD and PiuA.[162] Secretion is an essential virulence mechanism for pathogenic *Yersinia* ssp., which occurs via a syringe-like secretion structure (injectisome).[163] The abundances of injectisome proteins (YscB, YscC, YscD, YscE, YscF, YscJ, YscL, YscN, YscP and YscQ) change due to $Ca^{2+}$ removal at 37 °C.[164] Therefore, they can be used as potential biomarkers for indication of cultivation environments. LcrV, YopD, LcrH, YopE, LcrG, YopH, YopB and YopK are the most highly detectable non-injectisome protein biomarker candidates.[165] In addition to the well-characterized virulence associated proteins within plasmid pCD1, 87 hypothetical proteins were identified as potential biomarkers in one of five clusters of proteins.[165] Additional 89 chromasomal proteins, along with 4 proteins from plasmid pMT1 and 2 proteins from plasmid pPCP1, were reported as potential candidates

for further study as biomarkers.[165] Of the fluorescent antibody protein biomarkers described, however, approximately 60% exhibited cross reactivity with *Y. pseudotuberculosis*.[166]

LPS structure varieties make *Y. pestis* resistant to serum-mediated lysis and repress the proinflammatory response during transition between flea and host temperatures.[156] The number and length (i.e., 12 to 14 carbons) of fatty acid side chains of LPS lip A are diverse.[167] Several studies demonstrated that the expression and formation of LPS in *Y. pestis* can vary in different host-specific environments.[168] Surface polysaccharide poly-*N*-acetylglucosamine can be used as a biomarker for indicating growth conditions (i.e., temperature).[169]

Fatty acid compositions generally consist of C12:0, C16:0, C16:1, cyclopropane-C:17, C18:0, C18:1, OH C16:0, cyclopropane-C19:0 and C20:0. Additionally, relatively small quantities of C10:0 and C14:0 and trace quantities of OH C14:0 and OH C18:0, were also reported as biomarkers.[170] The C12:0/C14:0 ratio is lower in potentially pathogenic strains compared to non-pathogenic strains. High abundances of C16:1, C18:1$^{\Delta 42}$ and cyclopropyl-C17:0 are distinct biomarkers for *Y. pestis*.[42] Lipid profiles of *Y. pestis* were the least affected by changes in growth conditions.[42]

***Burkholderia pseudomallei and mallei.*** The *Burkholderia* (former *Pseudomonas*) genus refers to a group of gram-negative, rod-shaped bacteria, noted for its pathogenic members, *Burkholderia pseudomallei* and *Burkholderia mallei*. *Burkholderia thailandensis, Burkholderia vietnamiensis, Burkholderia multivorans* and *Burkholderiacepacia*are closely related species to *B. pseudomallei* and *B. pseudomallei,* but are different in their pathogenicity and ecological niches.[171]

*B. pseudomallei* is the causative agent of melioidosis in humans and in a wide variety of animals.[172] *B. mallei* was shown to be a clone of *B. pseudomallei,*[173] evolving through extensive

genome reduction and rearrangements.[174] It was even suggested that it should not be given separate species status based on genetic grounds.[173] It causes glanders in livestock and occasionally in humans. *B. cepacia*, *B. multivorans* and *B. vietnamiensis* are comprised of 17 closely related species called the *Burkholderia cepacia* complex,[175] which were believed to be only plant pathogens, but later emerged as important opportunistic pathogens causing chronic and life-threatening respiratory tract infections in patients with cystic fibrosis.[176] *B. thailandensis* is phenotypically similar to *B. pseudomallei* and was previously considered to be *B. pseudomallei.*[177, 178] This *B. pseudomallei*-like species was first assigned as a new species in 1998, based on differences in pathogenicity, arabinose assimilation[179, 180] and nucleotide sequences.[171, 181] *B. thailandensis* is widely considered to be nonpathogenic; however, there are some human infection reports which resemble aspects of *B. pseudomallei.*[182, 183]

Whole-genome similarity has been considered as the standard for determining bacterial taxonomy.[184] At present, the complete genome sequences of 23 *B. pseudomallei*, 4 *B. thailandensis* and 16 *B. cepacia* complex isolates have become available (http://www.ncbi.nlm.nih.gov/sits/genome). The complete genome of *B. pseudomallei* consists of two chromosomes, and it harbors multiple horizontally acquired genomic islands that are largely absent from the closely related *B. mallei*.[185] The *B. mallei* genome size is smaller than either the *B. pseudomallei* or *B. thailandensis* genomes,[185, 186] and it was suggested that *B. mallei* evolved through genome downsizing from a single clone of *B. pseudomallei*.[173, 186] Notably, more than 1,000 annotated *B. pseudomallei* genes are either missing or variant in the *B. mallei* genome. Therefore, it is not difficult to distinguish *B. mallei* from the others by their whole-genome sizes and sequences. Most genetic identification of other closely related *Burkholderia* species is carried out by sequencing the 16S rRNA gene,[187] *groEL* gene[188] and various housekeeping

genes.[173, 189] The 16S rRNA gene of the *B. cepacia* complex is significantly different from those of *B. pseudomallei*, *B. mallei* and *B. thailandensis*.[190] However, its utility within the *B. cepacia* complex strains is more limited, because similarity values are typically above 98%.[191] The BPSL1958 (*B. pseudomallei*) and BPSS1649 (*B. pseudomallei*) genes were also used as genetic markers for identification.[177, 192] Based on these genetic markers, *B. pseudomallei* can be differentiated from *B. thailandensis*, *B. cepacia* complex and *B. mallei*.[193] Since rRNA genes have limited taxonomic resolution, *recA* genes have been used for species identification in the *B. cepacia* complex.[194] The *recA* gene, a housekeeping gene, has been widely applied in bacterial identification[195] and was very useful for identification of *B. cepacia* complex species.[196, 197] The *B. pseudomallei* K96243 genome encodes a cluster of 13 genes (BPSS0417 to BPSS0429) and a cluster of 8 genes (BPSS1825 to BPSS1832) that have putative functions related to polysaccharide biosynthesis and transport.[185] However, both the polysaccharide chemical structure and the genes that encode this structure in *B. mallei* are unknown.[186]

A twin arginine translocation (Tat) domain protein (70-kDa) and a 12-kDa protein [190] were selected for differentiation of *B. pseudomallei, B. thailandensis* and the *B. cepacia* complex. The corresponding gene loci for these proteins are also potential markers for identification. Some proteins encoded by corresponding genes are present in *B. pseudomallei*, but not in *B. mallei*, such as a membrane protein MviN (BPSL0872),[198] a phospholipase C enzyme Plc-3 (BPSS0067)[199] and a lactonase family protein LfpA (BPSS2074).[200] Whole-cell protein profiles were suitable for the identification of members of the *B. cepacia* complex,[201, 202] based on the fact that closely related bacteria are likely to have highly similar protein content when grown under standard conditions. However, they failed to discriminate between *B. cepacia* complex species.

The O-antigen of LPA in *B. mallei* lacks an *O*-acetyl group at the 4' position in the talose residue within the structure →3)-β-D-glucopyranose-(1→3)-6-deoxy-α-L-talopyranose-(1→ .[203]All of the lipid A structures in the *Burkholderia* strains contain a general architecture in which the following elements are always present: two 2-amino-2-deoxy-glucopyranose (GlcN) carbohydrate backbone and an additional carbohydrate element, 4-deoxy-4-amino-arabinopyranose (Ara4N); a phosphate group; 3-OH C16:0 and 3-OH C14:0 primary fatty acids and C14:0 secondary fatty acid.[204] However, in selected strains and under particular conditions, other minor components have been found, such as C12:0 (rather than C14:0) or 2-OH C14:0 fatty acids.[17] The major differences in LPSs between the *Burkholderia* species are the amounts of fatty acids, sugars and different connecting structures.

Species-specific cellular fatty acids are important chemical markers that are frequently used in bacterial taxonomy and classification.[202] The most common saturated fatty acids that have been detected in the *Burkholderia* species include C12:0, C14:0, C15:0, C16:0, iso C17:0, C18:0 and C22:0.[17] The most common hydroxy fatty acids that have been detected include 2-OH C14:0, 3-OH C14:0, 2-OH C16:0, 3-OH C16:0 and 10-OH C18:0.[17] The 3-OH C16:0 fatty acid alone can be used for identification of the *B. cepacia* complex.[17] Unsaturated and cyclo fatty acids include C16:1ω7c, C18:1ω7c and cyclo C17:0. The fatty acid 2-OH C14:0 was found exclusively in *B. pseudomallei*.[205, 206] In fact, Novem *et al*. proposed that the 2-OH C14:0 fatty acid in *B. pseudomallei* might allow the bacterium to evade immune responses and, thus, avoid being cleared from the host.[205] In addition, they proved that C14:0, 2-OH C14:0, 3-OH C14:0 and 3-OH C16:0 fatty acids are derived from lipid A, which is located in the innermost region in the lipopolysaccharide molecule. It was previously reported that the 2-OH C16:0 fatty acid was absent in *B. thailandensis*, but present in *B. pseudomallei*.[207] The C12:0, C14:0 and C18:0

saturated fatty acids were among the most stable (i.e., independent of growth conditions) biomarkers that were observed in this study.[17] In addition, *B. vietnamiensis* had a lower C14:0 to C18:0 ratio than other species within the *B. cepacia* complex.[17] This ratio was used for differentiation of *B. vietnamiensis* from other members of the *B. cepacia* complex.

Small molecular markers, polyhydroxyalkanoates (PHAs), might also aid in the detection and differentiation of *Burkholderia* species. They represent a class of biodegradable thermoplastics that are synthesized by a wide variety of bacteria.[208, 209] Poly-3-hydroxybutyrate (P3HB), poly-3-hydroxyvalerate (P3HV) and poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate [poly(3HB-*co*-3HV)] are the most extensively studied PHAs. These compounds are typically used by bacteria as a reserve energy source during unfavorable growth conditions.[209] In addition, poly(3HB-*co*-3HV) has superior mechanical properties, which have drawn increasing interest from bio-industrial fields that require biodegradable and biocompatible materials.[210] *Burkholderia* species are capable of accumulating poly(3HB-*co*-3HV) from various carbon sources, including glucose, fructose, acetate, glycerol and lactase.

## 1.5 OVERVIEW OF THIS DISSERATION

The key to sensitive detection and identification technologies is biomarker recognition. Obviously, the most critical (and difficult) step in the development of a new biomarker detection method is discovery of the unique chemical compounds upon which detection can be based. This chapter provides reviews of previously reported methods for detection of bacterial BWAs and the current documented biomarkers (nucleic acids, proteins, fatty acids and small molecules

[Table 1.1]) for recognition of five important Tier 1 bacterial agents (*Bacillus anthracis*, *Francisella tularensis*, *Yersinia pestis, Burkholderia pseudomallei* and *Burkholderia mallei*).

Chapter 2 describes the detection and differentiation of both virulent and non-virulent *Bacillus anthracis* isolates from their closely related near neighbors, *B. thuringiensis, B. cereus, B. mycoides, B. atrophaeus* and *B. subtilis* using GC-MS. Chemical profiles that include fatty acids, sugars and DPA, were produced by one-step TCM at 140 °C for 5 min, and extracted by using solid phase micro-extraction at 80 °C for 10 min. This is the first time that actual virulent strains have been studied for differentiation.

In Chapter 3, unique biomarkers were detected and exploited using the same TCM method for detection and differentiation of *Burkholderia pseudomallei*, *Burkholderia mallei*, *Burkholderia thailandensis* and several members of the *Burkholderia cepacia complex*. Poly-3-hydroxybutyrate-*co*-hydroxyvalerate was identified for the first time as a very useful biomarker for *Burkholderia* species differentiation.

Chapter 4 describes a general GC-MS program developed for the five bacterial agents. Biomarkers used for *Yersinia pestis* and *Francisella tularensis* detection were introduced. The total analysis time was approximately 25 min. Using this program, all biomarkers used for identification and differentiation were easy to recognize.

In Chapter 5, an automated TCM sample preparation system was developed and tested for the rapid detection of Tier 1 bacterial agents for eventual use in the field. A rotating carousel design allowed for simultaneous sample collection, reaction and biomarker extraction; the total sample preparation time was less than 30 min.

Finally, Chapter 6 summarizes the conclusions from this work and outlines future studies that could be done to improve and extend this work.

**Table 1.1.** Biomarkers for four important Tier 1 bacterial species.

| | Nucleic acids (chromosome, plasmid) | | Proteins | Sugars | LPS | Fatty acids | Small molecules |
|---|---|---|---|---|---|---|---|
| *Bacillus* | 16S, 23S rRNA ISR *vrr*, *bclA*, *ger*X, *sig*B, *abr*B | *pag*, *cya* and *lef* (pXO1) *cap*A and *cap*B (pXO2) | YVRF, YDIQ, YUK [A-F], YNZA, YXIL, YOZC, YDAS, YJCB, BclA, ExsH, ExsJ | ribose, glucose, galactose, lycosamine, mannosamine, muramic acid, uinovose, rhamnose, 3-*O*-methyl rhamnose, galactosamine ribitol, glucuronic acid fucose, 2-*O*-methyl rhamnose, xylose, anthrose, two unidentified sugars | N/A | C15:0 (iso-, anteiso) C17:0 (iso-, anteiso) iso C17:1$\Delta^7$ 18:1 ω9c | DPA |
| *Francisella tularensis* | 16S rRNA *groEL, shdA, rpoB, rpoA, pgm* and *atpA fopA*, *tul4* | ssp. *tularensis* no plasmid found in LVS or novicida | 23-kDa cytoplamatic protein, Fop, AcpA, β-lactamase, FTT0484 | surface exopolysaccharide capsule (mannose, rhamnose and two unidentified didexoy sugars) | 3-deoxy-D-manno-octulosonic acid, no heptose, no phosphate, glucosamine, O-antigen | C20-26 long chain fatty acids 3-OH C18:0, C10:0, C12:0, iso C14:0, C16:0, iso C16:0, C18:0, C20:0, C21:0, C22:0, C24:0, C26:0, C14:1ω7c, C16:1ω7c, C18:2, C18:1ω9c, C20:1ω11c, C22:1ω13c, C24:1ω15c, C26:1ω17c, 2-OH C10:0, 2-OH C14:0, 3-OH C16:0 | N/A |
| *Yersinia* | 16S rRNA, HPI, O-antigen gene clusters | *yop,caf 1, pla, ymt,* | Yops, F1 antigen, LcrV, ATP-binding cassette transporter proteins (OppA, PstS, YrbD, PiuA), injectisome proteins (YscB, C, D, E, F, J, L, N, P, Q) | polysaccharide poly-*N*-acetylglucosamine | lipid A | C10:0, C12:0, C14:0, C16:0, C16:1, cyclopropane-C:17, C18:0, C18:1, OHC14:0, OHC16:0, OHC18:0, cyclopropane-C19:0, C20:0, C12:0/14:0 ratio | N/A |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Burkholderia* | 16S rRNA, whole genome sequences, *groEL,* BPSL1958, BPSS1649 *recA,* BPSS0417 to BPSS0429, BPSS1825 to BPSS1832 | | Tat, Whole-cell protein profile, MviN (BPSL0872), Plc-3 (BPSS0067), LfpA (BPSS2074) | | →3)-β-D-glucopyranose -(1→3)-6-deoxy-α-L-talopyranose-(1→ different connection of lipidA to sugars | C12:0, C14:0, C15:0, C16:0, iso C17:0, C18:0, C22:0, 2-OH C14:0, 3-OH C14:0, 2-OH C16:0, 3-OH C16:0,10-OH C18:0, C16:1ω7c, C18:1ω7c, cyclo C17:0, C14:0/C18:0 | Poly(3HB-*co*-3HV) |

## 1.6 REFERENCES


1. Pinnick, R. G.; Hill, S. C.; Nachman, P.; Videen, G.; Chen, G.; Chang, R. K., *Aerosol Sci. Tech.* **1998,** *28*, 95-104.
2. Eversole, J. D.; Hardgrove, J. J.; Cary, W. K.; Choulas, D. P.; Seaver, M., *Field Anal.Chem. Tech.* **1999,** *3*, 249-259.
3. Seaver, M.; Eversole, J. D.; Hardgrove, J. J.; Cary, W. K.; Roselle, D. C., *Aerosol Sci. Tech.* **1999,** *30*, 174-185.
4. Cheng, Y. S.; Barr, E. B.; Fan, B. J.; Hargis, P. J.; Rader, D. J.; O'Hern, T. J.; Torczynski, J. R.; Tisone, G. C.; Preppernau, B. L.; Young, S. A.; Radloff, R. J., *Aerosol Sci. Tech.* **1999,** *30*, 186-201.
5. Higgins, J. A.; Ibrahim, M. S.; Knauert, F. K.; Ludwig, G. V.; Kijek, T. M.; Ezzell, J. W.; Courtney, B. C.; Henchal, E. A., *Ann. N. Y. Acad. Sci.* **1999,** *894*, 130-148.
6. Ho, Y. P.; Reddy, P. M., *Clin. Chem.* **2010,** *56*, 525-536.
7. Gooding, J. J., *Anal. Chim. Acta* **2006,** *559*, 137-151.
8. Lim, D. V.; Simpson, J. M.; Kearns, E. A.; Kramer, M. F., *Clin. Microbiol. Rev.* **2005,** *18*, 583-607.
9. Hindson, B. J.; Brown, S. B.; Marshall, G. D.; McBride, M. T.; Makarewicz, A. J.; Gutierrez, D. M.; Wolcott, D. K.; Metz, T. R.; Madabhushi, R. S.; Dzenitis, J. M.; Colston, B. W., Jr., *Anal. Chem.* **2004,** *76*, 3492-3497.
10. Sincock, S. A.; Kulaga, H.; Cain, M.; Anderson, P.; Stopa, P. J., *Field Anal. Chem. Tech.* **1999,** *3*, 291-306.
11. Schmechel, D.; Gorny, R. L.; Simpson, J. P.; Reponen, T.; Grinshpun, S. A.; Lewis, D. M., *J. Immunol. Methods* **2003,** *283*, 235-245.
12. Dworzanski, J. P.; Berwald, L.; Meuzelaar, H. L., *Appl. Environ. Microbiol.* **1990,** *56*, 1717-1724.
13. Bohin, A.; Bouchart, F.; Richet, C.; Kol, O.; Leroy, Y.; Timmerman, P.; Huet, G.; Bohin, J. P.; Zanetta, J. P., *Anal. Biochem.* **2005,** *340*, 231-244.
14. Gardner, J. Y.; Brillhart, D. E.; Benjamin, M. M.; Dixon, L. G.; Mitchell, L. M.; Dimandja, J. M., *J. Sep. Sci.* **2011,** *34*, 176-185.
15. Price, E. P.; Matthews, M. A.; Beaudry, J. A.; Allred, J. L.; Schupp, J. M.; Birdsell, D. N.; Pearson, T.; Keim, P., *Electrophoresis* **2010,** *31*, 3881-3888.
16. *Microbial Identification System Operating Manual*. Microbial ID Inc: Newark, DE, 1993.
17. Krejci, E.; Kroppenstedt, R. M., *J. Clin. Microbiol.* **2006,** *44*, 1159-1164.
18. Conroy, P. J.; Hearty, S.; Leonard, P.; O'Kennedy, R. J., *Semin. Cell. Dev. Biol.* **2009,** *20*, 10-26.
19. Doll, J. M.; Zeitz, P. S.; Ettestad, P.; Bucholtz, A. L.; Davis, T.; Gage, K., *Am. J. Trop. Med. Hyg.* **1994,** *51*, 109-114.
20. Schofield, D. A.; Molineux, I. J.; Westwater, C., *J. Clin. Microbiol.* **2009,** *47*, 3887-3894.
21. Schofield, D. A.; Sharp, N. J.; Westwater, C., *Bacteriophage* **2012,** *2*, 105-283.
22. Jacobsson, K.; Frykberg, L., *Biotechniques* **1998,** *24*, 294-301.
23. Truong, T. V.; Nackos, A. N.; Williams, J. R.; VanDerwerken, D. N.; Kimball, J. A.; Murray, J. A.; Hawkes, J. E.; Harvey, D. J.; Tolley, H. D.; Robison, R. A.; Bartholomew, C. H.; Lee, M. L., *Anal. Methods* **2010,** *2*, 638-644.

24. Li, D.; Truong, T. V.; Bills, T. M.; Holt, B. C.; Vanderwerken, D. N.; Williams, J. R.; Acharya, A.; Robison, R. A.; Tolley, H. D.; Lee, M. L., *Anal. Chem.* **2012,** *84*, 1637-1644.
25. Hendricker, A. D.; Abbas-Hawks, C.; Basile, F.; Voorhees, K. J.; Hadfield, T. L., *Int. J. Mass Spectrom.* **1999,** *191*, 331-342.
26. Holzer, G.; Bourne, T. F.; Bertsch, W. J., *J. Chromatogr. A* **1989,** *468*, 181-190.
27. Beverly, M. B.; Basile, F.; Voorhees, K. J.; Hadfield, T. L., *Rapid Commun. Mass Spectrom.* **1996,** *10*, 455-458.
28. Kossa, W. C.; Macgee, J.; Ramachandran, S.; Webber, A. J., *J. Chromatogr. Sci.* **1979,** *17*, 177-187.
29. Havey, C. D.; Basile, F.; Mowry, C.; Voorhees, K. J., *J. Anal. Appl. Pyrol.* **2004,** *72*, 55-61.
30. Dikun, P. P.; Kostenko, L. D.; Liverovskii, A. A.; Shmulevskaia, E. I.; Romanovskaia, L. S., *Vopr. Onkol.* **1975,** *21*, 101-106.
31. Antolin, E. M.; Delange, D. M.; Canavaciolo, V. G., *J. Pharm. Biomed. Anal.* **2008,** *46*, 194.
32. Challinor, J. M., *J. Anal. Appl. Pyrolysis* **1989,** *16*, 323-333.
33. Liu, W.; Li, L.; Khan, M. A.; Zhu, F., *Mol. Gen. Mikrobiol. Virusol.* **2012**, 14-17.
34. Turingan, R. S.; Thomann, H. U.; Zolotova, A.; Tan, E.; Selden, R. F., *PLOS ONE* **2013,** *8*, e56093.
35. Raetz, C. R.; Whitfield, C., *Annu. Rev. Biochem.* **2002,** *71*, 635-700.
36. Raetz, C. R., *Annu. Rev. Biochem.* **1990,** *59*, 129-170.
37. Hershberger, C.; Binkley, S. B., *J. Biol. Chem.* **1968,** *243*, 1578-1584.
38. Wunschel, D. S.; Colburn, H. A.; Fox, A.; Fox, K. F.; Harley, W. M.; Wahl, J. H.; Wahl, K. L., *J. Microbiol. Methods* **2008,** *74*, 57-63.
39. Piotrowska-Seget, Z.; Mrozik, A., *Pol. J. Environ. Stud.* **2003,** *12*, 669-675.
40. Demirev, P. A.; Fenselau, C., *J. Mass Spectrom.* **2008,** *43*, 1441-1457.
41. Jantzen, E.; Berdal, B. P.; Omland, T., *J. Clin. Microbiol.* **1979,** *10*, 928-930.
42. Basile, F.; Beverly, M. B.; Voorhees, K. J.; Hadfield, T. L., *Trac-Trend Anal. Chem.* **1998,** *17*, 95-109.
43. Cronan, J. E., Jr., *J. Bacteriol.* **1968,** *95*, 2054-2061.
44. Guckert, J. B.; Hood, M. A.; White, D. C., *Appl. Environ. Microbiol.* **1986,** *52*, 794-801.
45. Nikerel, I. E.; Ates, O.; Oner, E. T., *Prikl. Biokhim. Mikrobiol.* **2008,** *44*, 539-544.
46. Turnbull, P. C.; Hutson, R. A.; Ward, M. J.; Jones, M. N.; Quinn, C. P.; Finnie, N. J.; Duggleby, C. J.; Kramer, J. M.; Melling, J., *J. Appl. Bacteriol.* **1992,** *72*, 21-28.
47. Drobniewski, F. A., *Clin. Microbiol. Rev.* **1993,** *6*, 324-338.
48. Vassileva, M.; Torii, K.; Oshimoto, M.; Okamoto, A.; Agata, N.; Yamada, K.; Hasegawa, T.; Ohta, M., *Microbiol. Immunol.* **2006,** *50*, 743-749.
49. Schnepf, E.; Crickmore, N.; Van Rie, J.; Lereclus, D.; Baum, J.; Feitelson, J.; Zeigler, D. R.; Dean, D. H., *Microbiol. Mol. Biol. Rev.* **1998,** *62*, 775-806.
50. Crickmore, N., *J. Appl. Microbiol.* **2006,** *101*, 616-619.
51. Ash, C.; Farrow, J. A.; Dorsch, M.; Stackebrandt, E.; Collins, M. D., *Int. J. Syst. Bacteriol.* **1991,** *41*, 343-346.
52. Johnson, Y. A.; Nagpal, M.; Krahmer, M. T.; Fox, K. F.; Fox, A., *J. Med. Microbiol.* **2000,** *40*, 241-254.

53. Lechner, S.; Mayr, R.; Francis, K. P.; Pruss, B. M.; Kaplan, T.; Wiessner-Gunkel, E.; Stewart, G. S.; Scherer, S., *Int. J. Syst. Bacteriol.* **1998,** *48 Pt 4*, 1373-1382.
54. Minich, D. M.; Vonk, R. J.; Verkade, H. J., *J. Lipid Res.* **1997,** *38*, 1709-1721.
55. Ash, C.; Collins, M. D., *FEMS Microbiol. Lett.* **1992,** *73*, 75-80.
56. Daffonchio, D.; Cherif, A.; Brusetti, L.; Rizzi, A.; Mora, D.; Boudabous, A.; Borin, S., *Appl. Environ. Microbiol.* **2003,** *69*, 5128-5137.
57. Harrell, L. J.; Andersen, G. L.; Wilson, K. H., *J. Clin. Microbiol.* **1995,** *33*, 1847-1850.
58. Rosemary S. Turingan, H.-U. T., Anna Zolotova, Eugene Tan, Richard F. Selden, *PLOS One* **2013,** *8*, 20.
59. Hanna, P., *Curr. Top. Microbiol. Immunol.* **1998,** *225*, 13-35.
60. Ramisse, V.; Patra, G.; Garrigue, H.; Guesdon, J. L.; Mock, M., *FEMS Microbiol. Lett.* **1996,** *145*, 9-16.
61. Koehler, T. M., *Curr. Top. Microbiol. Immunol.* **2002,** *271*, 143-164.
62. Hoffmaster, A. R.; Ravel, J.; Rasko, D. A.; Chapman, G. D.; Chute, M. D.; Marston, C. K.; De, B. K.; Sacchi, C. T.; Fitzgerald, C.; Mayer, L. W.; Maiden, M. C.; Priest, F. G.; Barker, M.; Jiang, L.; Cer, R. Z.; Rilstone, J.; Peterson, S. N.; Weyant, R. S.; Galloway, D. R.; Read, T. D.; Popovic, T.; Fraser, C. M., *Proc. Natl. Acad. Sci. U. S. A.* **2004,** *101*, 8449-8454.
63. Hoffmaster, A. R.; Hill, K. K.; Gee, J. E.; Marston, C. K.; De, B. K.; Popovic, T.; Sue, D.; Wilkins, P. P.; Avashia, S. B.; Drumgoole, R.; Helma, C. H.; Ticknor, L. O.; Okinaka, R. T.; Jackson, P. J., *J. Clin. Microbiol.* **2006,** *44*, 3352-3360.
64. Klee, S. R.; Ozel, M.; Appel, B.; Boesch, C.; Ellerbrok, H.; Jacob, D.; Holland, G.; Leendertz, F. H.; Pauli, G.; Grunow, R.; Nattermann, H., *J. Bacteriol.* **2006,** *188*, 5333-5344.
65. Heninger, S.; Drysdale, M.; Lovchik, J.; Hutt, J.; Lipscomb, M. F.; Koehler, T. M.; Lyons, C. R., *Infect. Immun.* **2006,** *74*, 6067-6074.
66. Cachat, E.; Barker, M.; Read, T. D.; Priest, F. G., *FEMS Microbiol. Lett.* **2008,** *285*, 220-226.
67. Keim, P.; Klevytska, A. M.; Price, L. B.; Schupp, J. M.; Zinser, G.; Smith, K. L.; Hugh-Jones, M. E.; Okinaka, R.; Hill, K. K.; Jackson, P. J., *J. Appl. Bacteriol.* **1999,** *87*, 215-217.
68. Keim, P.; Price, L. B.; Klevytska, A. M.; Smith, K. L.; Schupp, J. M.; Okinaka, R.; Jackson, P. J.; Hugh-Jones, M. E., *J. Bacteriol.* **2000,** *182*, 2928-2936.
69. Jackson, P. J.; Walthers, E. A.; Kalif, A. S.; Richmond, K. L.; Adair, D. M.; Hill, K. K.; Kuske, C. R.; Andersen, G. L.; Wilson, K. H.; Hugh-Jones, M.; Keim, P., *Appl. Environ. Microbiol.* **1997,** *63*, 1400-1405.
70. Keim, P.; Kalif, A.; Schupp, J.; Hill, K.; Travis, S. E.; Richmond, K.; Adair, D. M.; Hugh-Jones, M.; Kuske, C. R.; Jackson, P., *J. Bacteriol.* **1997,** *179*, 818-824.
71. Castanha, E. R.; Swiger, R. R.; Senior, B.; Fox, A.; Waller, L. N.; Fox, K. F., *J. Microbiol. Methods* **2006,** *64*, 27-45.
72. Sylvestre, P.; Couture-Tosi, E.; Mock, M., *J. Bacteriol.* **2003,** *185*, 1555-1563.
73. Fox, A.; Stewart, G. C.; Waller, L. N.; Fox, K. F.; Harley, W. M.; Price, R. L., *J. Med. Microbiol.* **2003,** *54*, 143-152.
74. Leski, T. A.; Caswell, C. C.; Pawlowski, M.; Klinke, D. J.; Bujnicki, J. M.; Hart, S. J.; Lukomski, S., *Appl. Environ. Microbiol.* **2009,** *75*, 7163-7172.
75. R., S.; W., F. J., *J. Bacteriol.* **1959,** *78*, 6.

76. Hathout, Y.; Setlow, B.; Cabrera-Martinez, R.; Fenselau, C.; Setlow, P., *Appl. Environ. Microbiol.* **2003,** *69*, 7.
77. Hathout, Y.; Ho, Y. P.; Ryzhov, V.; Demirev, P.; Fenselau, C., *J. Nat. Prod.* **2000,** *63*, 1492-1496.
78. Ryzhov, V.; Bundy, J. L.; Fenselau, C.; Taranenko, N.; Doroshenko, V.; Prasad, C. R., *Rapid Commun. Mass Spectrom.* **2000,** *14*, 1701-1706.
79. Ryzhov, V.; Hathout, Y.; Fenselau, C., *Appl. Environ. Microbiol.* **2000,** *66*, 3828-3834.
80. Wunschel, D.; Wahl, J.; Willse, A.; Valentine, N.; Wahl, K., *J. Chromatogr. B* **2006,** *843*, 25-33.
81. Rasmussen, M.; Jacobsson, M.; Bjorck, L., *J. Biol. Chem.* **2003,** *278*, 32313-32316.
82. Sylvestre, P.; Couture-Tosi, E.; Mock, M., *Mol. Microbiol.* **2002,** *45*, 169-178.
83. Waller, L. N.; Stump, M. J.; Fox, K. F.; Harley, W. M.; Fox, A.; Stewart, G. C.; Shahgholi, M., *J. Bacteriol.* **2005,** *187*, 4592-4597.
84. Todd, S. J.; Moir, A. J.; Johnson, M. J.; Moir, A., *J. Bacteriol.* **2003,** *185*, 3373-3378.
85. Fox, A.; Black, G. E.; Fox, K.; Rostovtseva, S., *J. Clin. Microbiol.* **1993,** *31*, 887-894.
86. Wunschel, D.; Fox, K. F.; Black, G. E.; Fox, A., *Syst. Appl. Microbiol.* **1995,** *17*, 625-635.
87. Colburn, H. A.; Wunschel, D. S.; Antolick, K. C.; Melville, A. M.; Valentine, N. B., *J. Microbiol. Methods* **2011,** *85*, 183-189.
88. Daubenspeck, J. M.; Zeng, H.; Chen, P.; Dong, S.; Steichen, C. T.; Krishna, N. R.; Pritchard, D. G.; Turnbough, C. L., Jr., *J. Biol. Chem.* **2004,** *279*, 30945-30953.
89. Dhenin, S. G.; Moreau, V.; Morel, N.; Nevers, M. C.; Volland, H.; Creminon, C.; Djedaini-Pilard, F., *Carbohydr. Res.* **2008,** *343*, 2101-2110.
90. Saksena, R.; Adamo, R.; Kovac, P., *Carbohydr. Res.* **2005,** *340*, 1591-1600.
91. Dong, S.; McPherson, S. A.; Tan, L.; Chesnokova, O. N.; Turnbough, C. L., Jr.; Pritchard, D. G., *J. Bacteriol.* **2008,** *190*, 2350-2359.
92. Tamborrini, M.; Holzer, M.; Seeberger, P. H.; Schurch, N.; Pluschke, G., *Clin. Vaccine Immunol.* **2010,** *17*, 1446-1451.
93. Basile, F.; Voorhees, K. J.; Hadfield, T. L., *Appl. Environ. Microbiol.* **1995,** *61*, 1534-1539.
94. Shu, X.; Liang, M.; Yang, B.; Li, Y.; Liu, C.; Wang, Y.; Shu, J., *Anal. Methods* **2012,** *4*, 3111-3117.
95. Kaneda, T., *J. Biol. Chem.* **1963,** *238*, 1222-1228.
96. Kaneda, T., *Bacteriol. Rev.* **1977,** *41*, 391-418.
97. Kaneda, T., *Microbiol. Rev.* **1991,** *55*, 288-302.
98. Whittaker, P.; Fry, F. S.; Curtis, S. K.; Al-Khaldi, S. F.; Mossoba, M. M.; Yurawecz, M. P.; Dunkel, V. C., *J. Agric. Food Chem.* **2005,** *53*, 3735-3742.
99. Ehrhardt, C. J.; Chu, V.; Brown, T.; Simmons, T. L.; Swan, B. K.; Bannan, J.; Robertson, J. M., *Appl. Environ. Microbiol.* **2010,** *76*, 1902-1912.
100. Dreher, R.; Poralla, K.; Konig, W. A., *J. Bacteriol.* **1976,** *127*, 1136-1140.
101. Nickerson, K. W.; Bulla, L. A., Jr.; Mounts, T. L., *J. Bacteriol.* **1975,** *124*, 1256-1262.
102. Chung, L.; Rajan, K. S.; Merdinger, E.; Grecz, N., *Biophys. J.* **1971,** *11*, 469-482.
103. Tabor, M. W.; MacGee, J.; Holland, J. W., *Appl. Environ. Microbiol.* **1976,** *31*, 25-28.
104. Nackos, A. N.; Truong, T. V.; Pulsipher, T. C.; Kimball, J. A.; Tolley, H. D.; Robison, R. A.; Bartholomew, C. H.; Lee, M. L., *Anal. Methods* **2011,** *3*, 245-258.
105. Forsman, M.; Sandstrom, G.; Sjostedt, A., *Int. J. Syst. Bacteriol.* **1994,** *44*, 38-46.

106. Hollis, D. G.; Weaver, R. E.; Steigerwalt, A. G.; Wenger, J. D.; Moss, C. W.; Brenner, D. J., *J. Clin. Microbiol.* **1989,** *27*, 1601-1608.
107. Pohanka, M.; Skladal, P., *Folia Microbiol.* **2009,** *54*, 263-272.
108. Dennis, D. T.; Inglesby, T. V.; Henderson, D. A.; Bartlett, J. G.; Ascher, M. S.; Eitzen, E.; Fine, A. D.; Friedlander, A. M.; Hauer, J.; Layton, M.; Lillibridge, S. R.; McDade, J. E.; Osterholm, M. T.; O'Toole, T.; Parker, G.; Perl, T. M.; Russell, P. K.; Tonat, K., *JAMA* **2001,** *285*, 2763-2773.
109. Hopla, C. E., A. K. Hopla, Tularemia. In *Handbook of zoonoses*, CRC Press: Boca Raton, Fla, 1994; pp 113-126.
110. Francis, E., *JAMA* **1983,** *250*, 3216-3224.
111. Svensson, K.; Larsson, P.; Johansson, D.; Bystrom, M.; Forsman, M.; Johansson, A., *J. Bacteriol.* **2005,** *187*, 3903-3908.
112. Sjostedt, Family XVII, Francisellaceae. Genus I, Francisella. In *Bergey's manual of systematic bacteriology*, 2 ed.; Springer-Verlag: New York, N.Y., 2003; Vol. 2, pp 111-113.
113. Whitfaker, P.; Day, J. B.; Sherill, K.; Fry, F. S., *J. AOAC Int.* **2007,** *90*, 4.
114. Hubalek, M.; Hernychova, L.; Brychta, M.; Lenco, J.; Zechovska, J.; Stulik, J., *Proteomics* **2004,** *4*, 3048-3060.
115. Mikalsen, J.; Olsen, A. B.; Tengs, T.; Colquhoun, D. J., *Int. J. Syst. Bacteriol.* **2007,** *57*, 1960-1965.
116. Titball, R. W.; Johansson, A.; Forsman, M., *Trends Microbiol.* **2003,** *11*, 118-123.
117. Michelet, L.; Bonnet, S.; Madani, N.; Moutailler, S., *Vet. Microbiol.* **2013,** *163*, 399-403.
118. Versage, J. L.; Severin, D. D.; Chu, M. C.; Petersen, J. M., *J. Clin. Microbiol.* **2003,** *41*, 5492-5499.
119. Emanuel, P. A.; Bell, R.; Dang, J. L.; McClanahan, R.; David, J. C.; Burgess, R. J.; Thompson, J.; Collins, L.; Hadfield, T., *J. Clin. Microbiol.* **2003,** *41*, 689-693.
120. Prior, J. L.; Prior, R. G.; Hitchen, P. G.; Diaper, H.; Griffin, K. F.; Morris, H. R.; Dell, A.; Titball, R. W., *J. Med. Microbiol.* **2003,** *52*, 845-851.
121. Thomas, R. M.; Titball, R. W.; Oyston, P. C.; Griffin, K.; Waters, E.; Hitchen, P. G.; Michell, S. L.; Grice, I. D.; Wilson, J. C.; Prior, J. L., *Infect. Immun.* **2007,** *75*, 371-378.
122. Larsson, P.; Oyston, P. C.; Chain, P.; Chu, M. C.; Duffield, M.; Fuxelius, H. H.; Garcia, E.; Halltorp, G.; Johansson, D.; Isherwood, K. E.; Karp, P. D.; Larsson, E.; Liu, Y.; Michell, S.; Prior, J.; Prior, R.; Malfatti, S.; Sjostedt, A.; Svensson, K.; Thompson, N.; Vergez, L.; Wagg, J. K.; Wren, B. W.; Lindler, L. E.; Andersson, S. G.; Forsman, M.; Titball, R. W., *Nat. Genet.* **2005,** *37*, 153-159.
123. Pavlov, V. M.; Mokrievich, A. N.; Volkovoy, K., *FEMS Immunol. Med. Microbiol.* **1996,** *13*, 253-256.
124. Pomerantsev, A. P.; Obuchi, M.; Ohara, Y., *Plasmid* **2001,** *46*, 86-94.
125. Konecna, K.; Hernychova, L.; Reichelova, M.; Lenco, J.; Klimentova, J.; Stulik, J.; Macela, A.; Alefantis, T.; Delvecchio, V. G., *Proteomics* **2010,** *10*, 4501-4511.
126. Hood, A. M., *J. Hyg.* **1977,** *79*, 47-60.
127. Sorokin, V. M.; Pavlovich, N. V.; Prozorova, L. A., *FEMS Immunol. Med. Microbiol.* **1996,** *13*, 249-252.
128. McLendon, M. K.; Apicella, M. A.; Allen, L. A., *Annu. Rev. Microbiol.* **2006,** *60*, 167-185.
129. Vinogradov, E.; Perry, M. B., *Carbohydr. Res.* **2004,** *339*, 1643-1648.

130. Schmitt, P.; Splettstosser, W.; Porsch-Ozcurumez, M.; Finke, E. J.; Grunow, R., *Epidemiol. Infect.* **2005,** *133*, 759-766.
131. Thirumalapura, N. R.; Goad, D. W.; Mort, A.; Morton, R. J.; Clarke, J.; Malayer, J., *J. Med. Microbiol.* **2005,** *54*, 693-695.
132. Nichols, P. D.; Mayberry, W. R.; Antworth, C. P.; White, D. C., *J. Clin. Microbiol.* **1985,** *21*, 738-740.
133. Bernard, K.; Tessier, S.; Winstanley, J.; Chang, D.; Borczyk, A., *J. Clin. Microbiol.* **1994,** *32*, 551-553.
134. Hinnebusch, B. J.; Rudolph, A. E.; Cherepanov, P.; Dixon, J. E.; Schwan, T. G.; Forsberg, A., *Science* **2002,** *296*, 733-735.
135. Brubaker, R. R., *Clin. Microbiol. Rev.* **1991,** *4*, 309-324.
136. Brubaker, *Clin. Microbiol. Rev.* **1991,** *4*, 16.
137. Henderson, D. A., *Science* **1999,** *283*, 1279-1282.
138. Kortepeter, M. G.; Parker, G. W., *Emerg. Infect. Dis.* **1999,** *5*, 523-527.
139. Perry, R. D.; Fetherston, J. D., *Clin. Microbiol. Rev.* **1997,** *10*, 35-66.
140. Amedei, A.; Niccolai, E.; Marino, L.; D'Elios, M. M., *J. Infect. Dev. Ctries.* **2011,** *5*, 628-639.
141. Brubaker, *Curr. Top. Microbiol. Immunol.* **1972,** *57*, 8.
142. Cornelis, G. R.; Boland, A.; Boyd, A. P.; Geuijen, C.; Iriarte, M.; Neyt, C.; Sory, M. P.; Stainier, I., *Microbiol. Mol. Biol. Rev.* **1998,** *62*, 1315-1352.
143. Hu, P.; Elliott, J.; McCready, P.; Skowronski, E.; Garnes, J.; Kobayashi, A.; Brubaker, R. R.; Garcia, E., *J. Bacteriol.* **1998,** *180*, 5192-5202.
144. Hinnebusch, J.; Cherepanov, P.; Du, Y.; Rudolph, A.; Dixon, J. D.; Schwan, T.; Forsberg, A., *Int. J. Med. Microbiol.* **2000,** *290*, 483-487.
145. Norkina, O. V.; Kulichenko, A. N.; Gintsburg, A. L.; Tuchkov, I. V.; Popov Yu, A.; Aksenov, M. U.; Drosdov, I. G., *J. Appl. Bacteriol.* **1994,** *76*, 240-245.
146. Rahalison, L.; Vololonirina, E.; Ratsitorahina, M.; Chanteau, S., *J. Clin. Microbiol.* **2000,** *38*, 260-263.
147. Lathem, W. W.; Price, P. A.; Miller, V. L.; Goldman, W. E., *Science* **2007,** *315*, 509-513.
148. Bianucci, R.; Rahalison, L.; Massa, E. R.; Peluso, A.; Ferroglio, E.; Signoli, M., *Am. J. Phys. Anthropol.* **2008,** *136*, 361-367.
149. Du, Y.; Rosqvist, R.; Forsberg, A., *Infect. Immun.* **2002,** *70*, 1453-1460.
150. Tomaso, H.; Reisinger, E. C.; Al Dahouk, S.; Frangoulidis, D.; Rakin, A.; Landt, O.; Neubauer, H., *FEMS Immunol. Med. Microbiol.* **2003,** *38*, 117-126.
151. McDonough, K. A.; Schwan, T. G.; Thomas, R. E.; Falkow, S., *J. Clin. Microbiol.* **1988,** *26*, 2515-2519.
152. Winter, C. C.; Cherry, W. B.; Moody, M. D., *Bull. World Health Organ.* **1960,** *23*, 408-409.
153. Leal, N. C.; Almeida, A. M., *Rev. Inst. Med. Trop. Sao. Paulo.* **1999,** *41*, 339-342.
154. Bogdanovich, T.; Carniel, E.; Fukushima, H.; Skurnik, M., *J. Clin. Microbiol.* **2003,** *41*, 5103-5112.
155. Matero, P.; Pasanen, T.; Laukkanen, R.; Tissari, P.; Tarkka, E.; Vaara, M.; Skurnik, M., *APMIS* **2009,** *117*, 34-44.
156. Amedei, A.; Niccolai, E.; Marino, L.; D'Elios, M. M., *J. Infect. Dev. Ctries* **2011,** *5*, 628-639.

157. Williams, J. E.; Gentry, M. K.; Braden, C. A.; Leister, F.; Yolken, R. H., *Bull. World Health Organ.* **1984,** *62*, 463-466.
158. Chanteau, S.; Rabarijaona, L.; O'Brien, T.; Rahalison, L.; Hager, J.; Boisier, P.; Burans, J.; Rasolomaharo, M., *Trans. R. Soc. Trop. Med. Hyg.* **1998,** *92*, 572-573.
159. Kilonzo, B. S.; Mbise, T. J.; Mwalimu, D. C.; Kindamba, L., *Tanzan. Health Res. Bull.* **2006,** *8*, 1-6.
160. Wang, T.; Qi, Z.; Wu, B.; Zhu, Z.; Yang, Y.; Cui, B.; Dai, R.; Zhang, Q.; Qiu, Y.; Wang, Z.; Wang, H.; Guo, Z.; Wang, X.; Yang, R., *Protein Expr. Purif.* **2008,** *61*, 7-12.
161. Santi, L.; Giritch, A.; Roy, C. J.; Marillonnet, S.; Klimyuk, V.; Gleba, Y.; Webb, R.; Arntzen, C. J.; Mason, H. S., *Proc. Natl. Acad. Sci. U. S. A.* **2006,** *103*, 861-866.
162. Tanabe, M.; Atkins, H. S.; Harland, D. N.; Elvin, S. J.; Stagg, A. J.; Mirza, O.; Titball, R. W.; Byrne, B.; Brown, K. A., *Infect. Immun.* **2006,** *74*, 3687-3691.
163. Cornelis, G. R., *J. Cell. Biol.* **2002,** *158*, 401-408.
164. Motin, V. L.; Georgescu, A. M.; Fitch, J. P.; Gu, P. P.; Nelson, D. O.; Mabery, S. L.; Garnham, J. B.; Sokhansanj, B. A.; Ott, L. L.; Coleman, M. A.; Elliott, J. M.; Kegelmeyer, L. M.; Wyrobek, A. J.; Slezak, T. R.; Brubaker, R. R.; Garcia, E., *J. Bacteriol.* **2004,** *186*, 6298-6305.
165. Hixson, K. K.; Adkins, J. N.; Baker, S. E.; Moore, R. J.; Chromy, B. A.; Smith, R. D.; McCutchen-Maloney, S. L.; Lipton, M. S., *J. Proteome. Res.* **2006,** *5*, 3008-3017.
166. Devdariani, Z. L.; Verenkov, M. S.; Feodorova, V. A.; Solodovnicov, N. S.; Belov, L. G., *FEMS Immunol. Med. Microbiol.* **1993,** *6*, 31-35.
167. Miller, S. I.; Ernst, R. K.; Bader, M. W., *Nat. Rev. Microbiol.* **2005,** *3*, 36-46.
168. Montminy, S. W.; Khan, N.; McGrath, S.; Walkowicz, M. J.; Sharp, F.; Conlon, J. E.; Fukase, K.; Kusumoto, S.; Sweet, C.; Miyake, K.; Akira, S.; Cotter, R. J.; Goguen, J. D.; Lien, E., *Nat. Immunol.* **2006,** *7*, 1066-1073.
169. Yoong, P.; Cywes-Bentley, C.; Pier, G. B., *mBio* **2012,** *3*, e00217-00212.
170. Tornabene, T. G., *Biochim. Biophys.* **1973,** *306*, 173-185.
171. Brett, P. J.; DeShazer, D.; Woods, D. E., *Int. J. Syst. Bacteriol.* **1998,** *48 Pt 1*, 317-320.
172. Chaowagul, W.; White, N. J.; Dance, D. A.; Wattanagoon, Y.; Naigowit, P.; Davis, T. M.; Looareesuwan, S.; Pitakwatchara, N., *J. Infect. Dis.* **1989,** *159*, 890-899.
173. Godoy, D.; Randle, G.; Simpson, A. J.; Aanensen, D. M.; Pitt, T. L.; Kinoshita, R.; Spratt, B. G., *J. Clin. Microbiol.* **2003,** *41*, 2068-2079.
174. Lin, C. H.; Bourque, G.; Tan, P., *Mol. Biol. Evol.* **2008,** *25*, 549-558.
175. Vandamme, P.; Dawyndt, P., *Syst. Appl. Microbiol.* **2011,** *34*, 87-95.
176. Lipuma, J. J., *Curr. Opin. Pulm. Med.* **2005,** *11*, 528.
177. Kim, H. S.; Schell, M. A.; Yu, Y.; Ulrich, R. L.; Sarria, S. H.; Nierman, W. C.; DeShazer, D., *BMC Genomics* **2005,** *6*, 174.
178. Wuthiekanun, V.; Smith, M. D.; Dance, D. A.; Walsh, A. L.; Pitt, T. L.; White, N. J., *J. Med. Microbiol.* **1996,** *45*, 408-412.
179. Smith, M. D.; Angus, B. J.; Wuthiekanun, V.; White, N. J., *Infect. Immun.* **1997,** *65*, 4319-4321.
180. Trakulsomboon, S.; Dance, D. A.; Smith, M. D.; White, N. J.; Pitt, T. L., *J. Med. Microbiol.* **1997,** *46*, 565-570.
181. Brett, P. J.; Deshazer, D.; Woods, D. E., *Epidemiol. Infect.* **1997,** *118*, 137-148.
182. Glass, M. B.; Gee, J. E.; Steigerwalt, A. G.; Cavuoti, D.; Barton, T.; Hardy, R. D.; Godoy, D.; Spratt, B. G.; Clark, T. A.; Wilkins, P. P., *J. Clin. Microbiol.* **2006,** *44*, 4601.

183. Lertpatanasuwan, N.; Sermsri, K.; Petkaseam, A.; Trakulsomboon, S.; Thamlikitkul, V.; Suputtamongkol, Y., *Clin. Infect. Dis.* **1999,** *28*, 927.
184. Vanlaere, E.; Baldwin, A.; Gevers, D.; Henry, D.; De Brandt, E.; LiPuma, J. J.; Mahenthiralingam, E.; Speert, D. P.; Dowson, C.; Vandamme, P., *Int. J. Syst. Bacteriol.* **2009,** *59*, 102-111.
185. Holden, M. T.; Titball, R. W.; Peacock, S. J.; Cerdeno-Tarraga, A. M.; Atkins, T.; Crossman, L. C.; Pitt, T.; Churcher, C.; Mungall, K.; Bentley, S. D.; Sebaihia, M.; Thomson, N. R.; Bason, N.; Beacham, I. R.; Brooks, K.; Brown, K. A.; Brown, N. F.; Challis, G. L.; Cherevach, I.; Chillingworth, T.; Cronin, A.; Crossett, B.; Davis, P.; DeShazer, D.; Feltwell, T.; Fraser, A.; Hance, Z.; Hauser, H.; Holroyd, S.; Jagels, K.; Keith, K. E.; Maddison, M.; Moule, S.; Price, C.; Quail, M. A.; Rabbinowitsch, E.; Rutherford, K.; Sanders, M.; Simmonds, M.; Songsivilai, S.; Stevens, K.; Tumapa, S.; Vesaratchavest, M.; Whitehead, S.; Yeats, C.; Barrell, B. G.; Oyston, P. C.; Parkhill, J., *Proc. Natl. Acad. Sci. U. S. A.* **2004,** *101*, 14240-14245.
186. Nierman, W. C.; DeShazer, D.; Kim, H. S.; Tettelin, H.; Nelson, K. E.; Feldblyum, T.; Ulrich, R. L.; Ronning, C. M.; Brinkac, L. M.; Daugherty, S. C.; Davidsen, T. D.; Deboy, R. T.; Dimitrov, G.; Dodson, R. J.; Durkin, A. S.; Gwinn, M. L.; Haft, D. H.; Khouri, H.; Kolonay, J. F.; Madupu, R.; Mohammoud, Y.; Nelson, W. C.; Radune, D.; Romero, C. M.; Sarria, S.; Selengut, J.; Shamblin, C.; Sullivan, S. A.; White, O.; Yu, Y.; Zafar, N.; Zhou, L.; Fraser, C. M., *Proc. Natl. Acad. Sci. U. S. A.* **2004,** *101*, 14246-14251.
187. Gee, J. E.; Sacchi, C. T.; Glass, M. B.; De, B. K.; Weyant, R. S.; Levett, P. N.; Whitney, A. M.; Hoffmaster, A. R.; Popovic, T., *J. Clin. Microbiol.* **2003,** *41*, 4647-4654.
188. Woo, P. C.; Woo, G. K.; Lau, S. K.; Wong, S. S.; Yuen, K., *Diagn. Microbiol. Infect. Dis.* **2002,** *44*, 143-149.
189. Spilker, T.; Baldwin, A.; Bumford, A.; Dowson, C. G.; Mahenthiralingam, E.; LiPuma, J. J., *J. Clin. Microbiol.* **2009,** *47*, 2607-2610.
190. Ho, C. C.; Lau, C. C.; Martelli, P.; Chan, S. Y.; Tse, C. W.; Wu, A. K.; Yuen, K. Y.; Lau, S. K.; Woo, P. C., *J. Clin. Microbiol.* **2011,** *49*, 814-821.
191. Vanlaere, E.; Lipuma, J. J.; Baldwin, A.; Henry, D.; De Brandt, E.; Mahenthiralingam, E.; Speert, D.; Dowson, C.; Vandamme, P., *Int. J. Syst. Bacteriol.* **2008,** *58*, 1580-1590.
192. Wongtrakoongate, P.; Mongkoldhumrongkul, N.; Chaijan, S.; Kamchonwongpaisan, S.; Tungpradabkul, S., *Mol. Cell. Probes* **2007,** *21*, 81.
193. Koh, S. F.; Tay, S. T.; Sermswan, R.; Wongratanacheewin, S.; Chua, K. H.; Puthucheary, S. D., *J. Med. Microbiol.* **2012**.
194. McDowell, A.; Mahenthiralingam, E.; Moore, J. E.; Dunbar, K. E.; Webb, A. K.; Dodd, M. E.; Martin, S. L.; Millar, B. C.; Scott, C. J.; Crowe, M.; Elborn, J. S., *J. Clin. Microbiol.* **2001,** *39*, 4247-4255.
195. Karlin, S.; Weinstock, G. M.; Brendel, V., *J. Bacteriol.* **1995,** *177*, 6881-6893.
196. Mahenthiralingam, E.; Bischof, J.; Byrne, S. K.; Radomski, C.; Davies, J. E.; Av-Gay, Y.; Vandamme, P., *J. Clin. Microbiol.* **2000,** *38*, 3165.
197. Payne, G. W.; Vandamme, P.; Morgan, S. H.; Lipuma, J. J.; Coenye, T.; Weightman, A. J.; Jones, T. H.; Mahenthiralingam, E., *Appl. Environ. Microbiol.* **2005,** *71*, 3917-3927.
198. Ling, J. M.; Moore, R. A.; Surette, M. G.; Woods, D. E., *Can. J. Microbiol.* **2006,** *52*, 831-842.
199. Korbsrisate, S.; Tomaras, A. P.; Damnin, S.; Ckumdee, J.; Srinon, V.; Lengwehasatit, I.; Vasil, M. L.; Suparak, S., *Microbiology* **2007,** *153*, 1907-1915.

200. Boddey, J. A.; Day, C. J.; Flegg, C. P.; Ulrich, R. L.; Stephens, S. R.; Beacham, I. R.; Morrison, N. A.; Peak, I. R., *Cell Microbiol.* **2007,** *9*, 514-531.
201. Vandamme, P.; Holmes, B.; Vancanneyt, M.; Coenye, T.; Hoste, B.; Coopman, R.; Revets, H.; Lauwers, S.; Gillis, M.; Kersters, K.; Govan, J. R., *Int. J. Syst. Bacteriol.* **1997,** *47*, 1188-1200.
202. Vandamme, P.; Pot, B.; Gillis, M.; de Vos, P.; Kersters, K.; Swings, J., *Microbiol. Rev.* **1996,** *60*, 407.
203. Knirel, Y. A.; Paramonov, N. A.; Shashkov, A. S.; Kochetkov, N. K.; Yarullin, R. G.; Farber, S. M.; Efremenko, V. I., *Carbohydr. Res.* **1992,** *233*, 185-193.
204. De Soyza, A.; Silipo, A.; Lanzetta, R.; Govan, J. R.; Molinaro, A., *Innate. Immun.* **2008,** *14*, 127-144.
205. Novem, V.; Shui, G.; Wang, D.; Bendt, A. K.; Sim, S. H.; Liu, Y.; Thong, T. W.; Sivalingam, S. P.; Ooi, E. E.; Wenk, M. R.; Tan, G., *Clin. Vaccine Immunol.* **2009,** *16*, 1420.
206. Inglis, T. J.; Aravena-Roman, M.; Ching, S.; Croft, K.; Wuthiekanun, V.; Mee, B. J., *J. Clin. Microbiol.* **2003,** *41*, 4812.
207. Levy, A.; Merritt, A. J.; Aravena-Roman, M.; Hodge, M. M.; Inglis, T. J., *Am. J. Trop. Med. Hyg.* **2008,** *78*, 599.
208. Steinbuchel, A., *Acta Biotechnol.* **1991,** *11*, 419.
209. Steinbuchel, A.; Valentine, H. E., *FEMS Microbiol. Lett.* **1995,** *128*, 219.
210. Kim do, Y.; Park, D. S.; Kwon, S. B.; Chung, M. G.; Bae, K. S.; Park, H. Y.; Rhee, Y. H., *J. Microbiol.* **2009,** *47*, 651.

# 2 SIMPLE GC-MS METHOD FOR POSITIVE DETECTION OF *BACILLUS ANTHRACIS* ENDOSPORES

## 2.1 INTRODUCTION

Reliable identification of the spore-forming bacterium *Bacillus anthracis* is challenging, due to the similarities of bacteria within the *Bacillus cereus* group, which includes six closely related species: *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis*. In fact, some have postulated that *B. cereus*, *B. anthracis* and *B. thuringiensis* should be included in the lineage of *B. cereus*.[1,2] They share a high degree of similarity (>99%) as demonstrated by their 16S rRNA nucleotide sequences.[3] The major distinguishing feature of *B. anthracis* from *B. cereus* and *B. thuringiensis* is the presence of the two virulence plasmids, pXO1 and pXO2, which encode for the toxins and capsule, respectively.[4,5] Loss of either plasmid results in strain attenuation. The *B. anthracis* Ames strain possesses both essential virulence plasmids and is capable of causing the disease known as anthrax. While both plasmids are generally not present together in species other than *B. anthracis*, a notable exception, called "*Bacillus anthracis*-like isolate," caused an anthrax-like disease in African apes.[6] Despite their similarities, *B. anthracis*, *B. cereus*, and *B. thuringiensis* have some genetic and phenotypic differences, which can be used for classification.[7–9] In this study, we included *B. atrophaeus* (formerly *Bacillus subtilis* var. *globigii*), which although not closely related to *B. anthracis*, has historically been a model organism and nonpathogenic surrogate for *B. anthracis* in bioweapons research.

Due to the efficacy of *B. anthracis* as a bioweapon, a number of techniques have been developed to detect it. Molecular diagnostic tools have been adapted using DNA-based and

antibody-based approaches. These tests vary greatly in sensitivity, response time, cost, availability and complexity of use. These methods are time-consuming, and the results are sometimes compromised by other species closely related to *B. anthracis*.[10] Most of the DNA-based assays detect target sequences on the *B. anthracis* virulence plasmids pXO1 and pXO2.[11] However, research has shown that the pXO1 and pXO2 genes are not always specific to *B. anthracis*.[12–15] In addition to the virulence plasmids, chromosomal markers can provide further discriminatory information.[16,17] However, some of the chromosomal markers for *B. anthracis* are also present in other species in the *B. cereus* group.[18] So far, many SNPs and PCR assays have failed to totally discriminate between *B. anthracis* and other *B. cereus* group members when used separately. However, a recent method based on PCR amplification of an original chromosomal marker harboring SNPs can discriminate *B. anthracis* from non-*B. anthracis* strains.[18]

Recent bioterrorism events have emphasized the need for rapid and accurate detection and identification of *B. anthracis*. Biomarkers for *B. anthracis* recognition have been summarized in Chapter 1. This chapter demonstrates the use of TCM with $HMeSO_4$ for release and derivatization of biomarkers, including anthrose, fatty acids, dipicolinic acid and two unidentified sugars, followed by GC-MS analysis. A statistical algorithm was constructed from GC-MS data for *B. anthracis* and other *B. cereus* group organisms grown at two different temperatures in two different growth media.

## 2.2 EXPERIMENTAL

### 2.2.1 Materials and reagents

HPLC grade methanol (MeOH) and 4,6-dideoxy-4-(3-hydroxy-3-methylbutanamido)-2-*O*-methyl-D-glucopyranose (anthrose) were purchased from Sigma Chemical (St. Louis, MO, USA). Hydrogen methyl sulfate ($HMeSO_4$) was prepared as a 10% solution of $H_2SO_4$ in methanol (v/v), which was left at room temperature for approximately 8 days. Phosphate buffer (1 M, pH 6.5) was made by mixing 1 M potassium phosphate monobasic solution and 1 M potassium phosphate dibasic solution until the pH reached 6.5 (both reagents were purchased from Sigma Chemical, St. Louis, MO, USA).

Leighton-Doi sporulation medium was prepared by adding 4.8 g of Difco nutrient broth (Becton-Dickinson, Franklin Lakes, NJ, USA) and 8 g of agar (EMD Chemicals, Rockland, MA, USA) to 600 mL of distilled water. This mixture was brought to a boil and autoclaved at 120 °C for 40 min. Various salt solutions were prepared by dissolving 5.0 g of magnesium sulfate heptahydrate (Spectrum Chemical, Gardena, CA, USA), 0.4 g of manganese sulfate monohydrate (Spectrum), and 0.06 g of ferrous sulfate heptahydrate (Columbus Chemical, Columbus, WI, USA), each separately in 100 mL of distilled water. A fourth solution was prepared by adding 1 mL of each of the three salt solutions, 3.8 g of potassium chloride (Mallinckrodt, St. Louis, MO, USA), 0.6 g of calcium chloride dihydrate (Columbus Chemical), and 1.8 g of dextrose (VWR, Radnor, PA, USA) to 200 mL of additional distilled water and filtered using a Millipore vacuum filter (0.2 mm, Millipore, Billerica, MA, USA). The autoclaved medium was cooled to 50 °C using a Lauda water bath (Series RM 20, Lauda-Brinkman, Delran, NJ, USA), and a 60 mL portion of the salt solution was added to the cooled medium, which was then mixed and poured

into Petri dishes. Columbia agar was made using Difco Columbia agar base (26.4 g in 600 mL), which was heated to boiling temperature, autoclaved and cooled to 50 °C before pouring into Petri dishes.

### 2.2.2 Growth and harvesting of endospores

All virulent endospore suspensions, as well as *B. anthracis* Pasteur, were prepared in a CDC licensed BSL-3 facility located on the campus of Brigham Young University. Fifteen *Bacillus* species were selected for this study (Table 2.1). Each organism was first cultured on Columbia agar at 37 °C for verification of purity by gram staining and inspection by light microscopy (Zeiss Axioskop 2, Göttingen, Germany). The Leighton-Doi and Columbia media were inoculated for confluent growth with an isolated colony from the isolation plates and were grown at two temperatures (32 and 37 °C). *B. mycoides*, *B. cereus* and *B. thuringiensis* Israelensis were also grown at 28 °C due to poor sporulation at 32 and 37 °C. All plates were bagged to ensure that the media would not dry out and were incubated for approximately 10 days at 32 or 37 °C. Additional inspection using wet mounts and phase-contrast microscopy ensured 90−100% sporulation before the spores were harvested. All spores were harvested using sterile HPLC water (Sigma-Aldrich, St. Louis, MO, USA) and heat-shocked at 65 °C for 30 min. The harvested suspensions were then washed twice by centrifugation at 3220*g* for 30 min in a swinging bucket rotor. The spores were resuspended in 35 mL of cold (4 °C) sterile HPLC water. The supernatant was decanted after each centrifugation. The remaining spore pellet was washed twice more by resuspending in 10 mL of cold, sterile HPLC water, storing overnight at 4 °C, diluting with an additional 25 mL of cold, sterile HPLC water, centrifuging and decanting the supernatant. Endospore concentrations were determined by Petroff-Houser counting (Bright-Line

**Table 2.1.** *Bacillus* strains and characteristics.

| species name | identifiers | pXO1 + | pXO2 + | group | source[a] |
|---|---|---|---|---|---|
| *B. anthracis* | A0231/K3677/#83 | yes | yes | *B. anthracis* | LSU |
| *B. anthracis* | A0264/K7948/14 | yes | yes | *B. anthracis* | LSU |
| *B. anthracis* | A0300/K2284 | yes | yes | *B. anthracis* | LSU |
| *B. anthracis* | A2084/Ancestral Ames | yes | yes | *B. anthracis* | LANL |
| *B. anthracis* | 1043/Sterne | yes | no | *B. anthracis* | LANL |
| *B. anthracis* | 6602/Pasteur | no | yes | *B. anthracis* | LANL |
| *B. thuringiensis* | Kurstaki | no | no | *B. thuringiensis* | NBFAC |
| *B. thuringiensis* | Al Hakam | no | no | *B. thuringiensis* | UAB |
| *B. thuringiensis* | ATCC 19269 | no | no | *B. thuringiensis* | ATCC |
| *B. thuringiensis* | ATCC 19270 | no | no | *B. thuringiensis* | ATCC |
| *B. thuringiensis* | Israelensis/ATCC 35646 | no | no | *B. thuringiensis* | ATCC |
| *B. cereus* | ATCC 14579 | no | no | *B. cereus* | ATCC |
| *B. atrophaeus* | ATCC 51189 | no | no | *B. subtilis* | ATCC |
| *B. subtilis* | Spizizenii/ATCC 6633 | no | no | *B. subtilis* | ATCC |
| *B. mycoides* | ATCC 6462 | no | no | *B. mycoides* | ATCC |

[a]LSU = Louisiana State University, LANL = Los Alamos National Laboratory, NBFAC = National Bioforensic Analysis Center, UAB = University of Alabama at Birmingham, ATCC = American Type Culture Collection.

hemacytometer, Horsham, PA, USA). Spore pellets were then created by centrifuging the solution and decanting the supernatant.

### 2.2.3 Experimental design

The experimental design for training the algorithm and estimating parameters was a three factor factorial design: species (6), temperature (3) and growth medium (2). For each combination, we replicated the experiment from 1 to 6 times. We did not perform an equal number of replications of each species for every combination of temperature and growth medium because of inadequate sporulation. A second experiment was undertaken to confirm the method and algorithm but with only 2 to 3 replications per combination. The algorithm was applied to these data in a blind manner. The classification of each tested vial was made by the computer algorithm using these data without knowledge of what species or how many of each were in the data set. Operation of the algorithm was done without input from the experimenters.

### 2.2.4 TCM of endospores

Methanol was added to the spore pellet to give a final concentration of ~$10^6$ spores/$\mu$L. A 20 $\mu$L volume of the suspension and 20 $\mu$L of $HMeSO_4$ (10% $H_2SO_4$ in methanol, v/v) were combined in a 1.5 mL microcentrifuge tube (Axygen, Union City, CA, USA). These solutions were pipet-mixed, and 20 $\mu$L of solution were added to a clear flatbottom crimp vial (7 × 40 mm, National Scientific, TN, USA). The vial was then capped with a crimp top seal (8 mm, clear PTFE/red rubber, National Scientific) using a crimping tool. A custom machined heating block with slots that accommodate 4 glass vials and a digital mini temperature CSC 32 controller (Omega, Stamford, CT, USA) was used to heat the vials to 140 °C for 5 min. All BSL-3 organisms were treated by TCM in duplicate, with one of the two vials subjected to a viability check (see below) prior to removal from the BSL-3 facility. Neutralizing aqueous phosphate

buffer (1 M, pH 6.5) was then added into the vial through a septum using a 1 mL syringe (Becton-Dickinson, Franklin Lakes, NJ, USA) equipped with a 261/2-gauge needle (Becton-Dickinson). A 2 cm divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS) solid phase micro-extraction (SPME) fiber from Supelco (Bellefonte, PA, USA) was inserted into the vial with half of the fiber in the liquid and half exposed to the vapor, and the biomarkers were extracted for 10 min at 80 °C. The SPME fiber was then introduced into the GC injection port, the GC program was started and the fiber was left in the injection port for 2 min.

### 2.2.5 Viability testing for organisms treated in the BSL-3 facility

After the TCM reaction, all vials containing virulent *B. anthracis* endospores were refrigerated overnight at 4 °C. One of the two samples processed was chosen at random for viability testing. Using a 1-mL syringe (Becton-Dickinson, Franklin Lakes, NJ, USA) equipped with a 221/2-gauge needle (Becton-Dickinson), 200 $\mu$L of filter-sterilized water were added to each vial, which was then vortexed vigorously for 5 s (S/P Votex Mixer, Baxter Diagnostics, Deerfield, IL, USA). The entire content of each vial was plated onto two Columbia agar plates containing 5% sheep's blood (100 $\mu$L aliquots each). The vials were discarded, and the plates were incubated for 5 days at 37 °C. If no growth was observed, sterility was confirmed and the duplicate vials were brought out of the BSL-3 facility for GC-MS analysis. Each virulent *B. anthracis* isolate was originally tested for viability prior to any samples being removed from the BSL-3 laboratory. Forty vials of each isolate were treated and plated according to this procedure. No growth was observed on any of the viability culture plates, thus verifying that the TCM treatment rendered the spores nonviable.

### 2.2.6 GC-MS analysis

All GC-MS experiments were conducted using an Agilent 6890 gas chromatograph (Agilent, San Jose, CA, USA) equipped with a split/splitless injector containing a 79 mm long × 1.2 mm i.d. × 6.3 mm o.d. deactivated fused silica liner (Restek, Bellefonte, PA, USA) and a 30 m × 0.25 mm i.d. × 0.25 $\mu$m Zebron-FFAP column from Phenomenex (Torrance, CA, USA). The GC injection port was set at 260 °C for all experiments, and the inlet was operated in the splitless mode at a constant helium pressure of 24 psi. The temperature was programmed from 50 °C (1 min initial hold), increased at 8 °C/min to 110 °C (1 min hold), then 5 °C/min to 150 °C (1 min hold), then 5 °C/min to 190 °C (1 min hold), and finally 15 °C/min to 250 °C (4 min hold). The GC was coupled to an Agilent 5793 MS with electron ionization source and quadrupole mass analyzer set to scan over a 33−550 *m/z* range. The transfer line to the mass spectrometer was maintained at 260 °C. A blank run (using SPME without sample extraction) was performed before each sample run. Cleaning the SPME fiber between runs was achieved by placing the fiber in deionized water with agitation for approximately 20−30 min and then introducing it into the GC-MS injection port for 1 min to remove any possible contamination or carryover from previous analyses. Chemstation software package was used for GC-MS data analysis.

### 2.2.7 Safety considerations

Sulfuric acid is corrosive. It can cause severe skin burns and eye damage, and it is harmful or fatal if swallowed. When handling sulfuric acid, appropriate personal protection equipment (especially safety goggles) should be worn.

The high methylating activity of $HMeSO_4$ salts poses a possible danger. Although not a carcinogen, $HMeSO_4$ salts are generally given toxin and irritant status on material safety data sheets (MSDSs). Dimethyl sulfate ($Me_2SO_4$) is anticipated to be carcinogenic. Applications of

high temperatures to $HMeSO_4$ could promote the formation of $Me_2SO_4$.[19] Production of and exposure to $Me_2SO_4$ may be avoided or minimized using a dilute, nonagitated solution of $H_2SO_4$ in MeOH.

All virulent endospore suspensions, as well as *B. anthracis* Pasteur, should be prepared and handled in a CDC licensed BSL-3 facility. The BSL-3 facility used in this work has been inspected and approved by the CDC and abides by all federal guidelines and mandates with respect to activities involving select biological agents.

## 2.3 RESULTS AND DISCUSSION

### 2.3.1 TCM reaction and GC-MS analysis

Biomarkers present in organisms are usually integrated in larger structural units, chemically bound within nucleic acids, enzymes, lipids and various polymers. Thus, chemical, mechanical, and/or thermal treatments are necessary to release them. Subsequently, large biomarker molecules may be fragmented and derivatized to produce smaller, less polar and more volatile biomarkers. In our TCM procedure, multiple reactions can be accomplished in a single step. Sulfuric acid self-methylates in methanol to form $HMeSO_4$, which breaks or permeabilizes the endospores at an elevated temperature (140 °C) to release the biomarkers. Additionally, it serves as a strong methylation reagent to convert the biomarkers into their methyl esters, which can be easily analyzed by GC-MS. Yields of 3-methyl-2-butenoic acid methyl ester (3-M-2-BAME) and DPAME were measured at 5 different reaction temperatures from 110 to 150 °C. For 3-M-2-BAME, the higher temperatures gave the best yield; however, for DPAME, 130 °C was the optimum temperature. Since the DPAME abundance was usually much higher than 3-M-

2-BAME, we selected 140 °C as the best overall reaction temperature. Other parameters, including reaction time and the amount and concentration of reagents, were optimized (data not shown). The procedure was finalized as described in the Experimental Section. For fatty acids, TCM produced saturated and unsaturated compounds from C8 to C18 and branched iso and anteiso C15:0 and C17:0 compounds, which had previously been used as *B. anthracis* endospore biomarkers.[20] For sugars, a series of permethylated deoxy aldonic acids were produced by TCM.

### 2.3.2 SPME sampling

SPME was used in this work as a simple and fast solventless extraction tool for sample preparation.[21] After comparison of extraction results for four commercial fibers, it was found that DVB/CAR/PDMS provided the best extraction efficiencies for our target analytes. DVB provided good extraction of 3-M-2-BAME and DPAME due to induced dipole interactions; PDMS provided significant van der Waals interactions with long chain FAMEs, and CAR strongly absorbed 3-M-2-BAME. SPME was performed by exposing half of the fiber coating to the liquid sample and the other half to the headspace above the sample (called half−half extraction). The half−half mode improved the overall extraction efficiencies for the biomarkers; 3-M-2-BAME and 3-methyl-3-butenoic acid methyl ester (3-M-3-BAME) had higher relative intensities than if only liquid extraction was done, and DPAME and FAMEs appeared in greater abundances than if only headspace sampling was performed.

The effect of extraction time on peak intensities of the biomarker derivatives was investigated. The slope of peak intensity vs. extraction time was different for the different target compounds; however, longer extraction time resulted in higher peak intensity before reaching equilibrium, which occurred in approximately 1 h. The results indicated that short extraction time (~1 min) could be applied to detect $>10^8$ *B. anthracis* endospores. However, with lower

endospore concentrations, longer SPME extraction was necessary. In most cases, achieving equilibrium was not required for either identification or quantitation; therefore, we finally chose 10 min as the extraction time. TCM combined with half−half SPME sampling provided a fast, simple method compared to the method reported by Dong[22] for detection of anthrose, which involved the determination of monosaccharides by suspending spores in methanolic−HCl in a heating block at 80 °C for 16 h, followed by drying and derivatization.

### 2.3.3 Identification of biomarkers

The three key spore biomarker types obtained by TCM in this study were (1) DPAME, (2) FAMEs and (3) derivatized sugars, which were determined by selected-ion chromatograms of *m/z* 137 for DPAME, *m/z* 74 for FAMEs and 83/88 for target sugar derivatives (Figure 2.1). DPAME and FAME profiles from GC-MS analyses of TCM products were reported for detecting and differenting *B. anthracis* endospores from other *Bacillus* species.[20] DPAME is unique to endospore-forming bacteria; however, it is not diagnostic beyond indicating the general presence of endospores. Fatty acids constitute an important class of biomarkers since they are generally present in all bacteria and exhibit considerable diversity. Typical FAMEs, such as iso C15:0 and C17:0 and anteiso C15:0 and C17:0 were used to differentiate *Bacillus* from *Clostridium*.[20] In addition, the presence and absence of certain FAMEs (i.e., C16:1 $\Delta^9$, iso C17:1 and anteiso C17:1) allowed the differentiation of endospores from the four main *Bacillus* species, i.e., *B. anthracis*, *B. atrophaeus*, *B. cereus* and *B. thuringiensis*.[20] However, the limitation of this method was that the FAMEs were easily affected by growth conditions and, therefore, were not as robust as sugar biomarkers. In our study, we used one fatty acid methyl ester (an unidentified C13:0 FAME) for differentiation. In addition to DPAME and FAMEs,

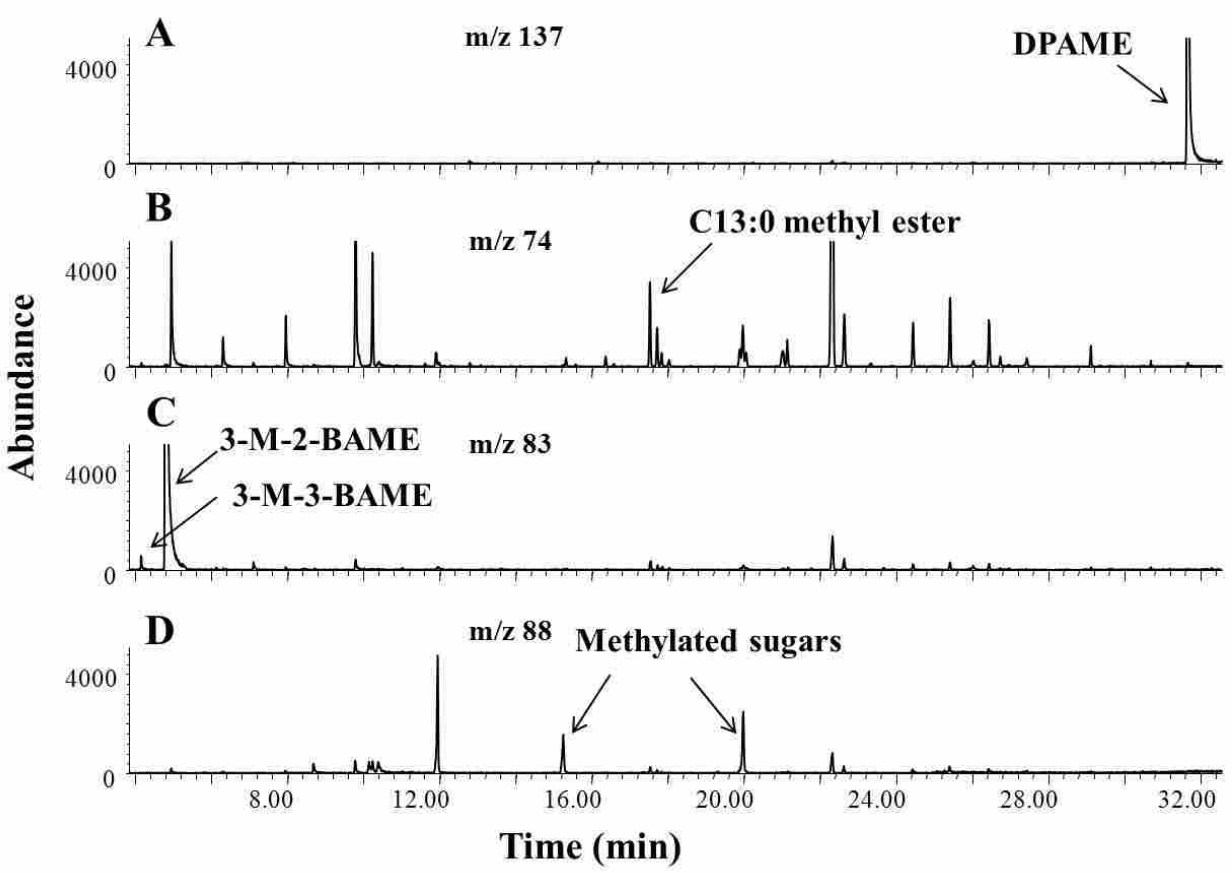



**Figure 2.1.** Extracted-ion chromatograms of target biomarkers for B. anthracis differentiation (B. thuringiensis Al Hakam, cultured at 37 ºC on Leighton-Doi medium). Conditions: see Section 2.2.

sugar derivatives (as sugar fragments and methylated monosaccharides) were found and investigated for *B. anthracis* detection and differentiation.

### 2.3.4 Determination of anthrose via its TCM by-product derivative, 3-M-2-BAME

A major objective in this work was to detect anthrose (see structure in Figure 2.2). During TCM, the amide group in anthrose was hydrolyzed to a carboxylic acid and an amine by acid catalysis ($HMeSO_4$) at high temperature. The mechanism of the conversion followed a series of reactions, including cleavage of the tetrasaccharide, nucleophilic acyl substitution of anthrose and methylation. An intermediate TCM product via nucleophilic acyl substitution was 3-hydroxy-3-methyl butyric acid (3-OH-3-MBA), which was dehydrated and methylated, forming two methyl derivatives, 3-M-2-BAME and 3-M-3-BAME. According to this mechanism, 3-M-2-BAME should be the main product. The detection of 3-M-2-BAME was advantageous over detection of 3-OH-3-MBA because of its high volatility, easy separation by conventional GC and characteristic base peak (*m/z* 83), which was the selected ion chosen for MS detection of the biomarker. Dong[22] reported that anthrose was detected in other *Bacillus* species, such as *B. thuringiensis* Al Hakam, *B. thuringiensis* Kurstaki and *B. cereus*, and we also verified the existence of this biomarker in *B. mycoides* and other *B. thuringiensis Bacillus* species. Although anthrose alone might not be a definitive biomarker for detection and differentiation of *B. anthracis* from other *Bacillus* species, false positive detection should be reduced using this biomarker along with other selected biomarkers such as DPAME, C13:0 FAME and certain sugar derivatives. Other sugar derivatives were detected after heat treatment in methanolic $H_2SO_4$. Figure 2.1D shows a chromatogram of two unique sugar derivatives obtained using selected ion *m/z* 88, which indicates that TCM with $HMeSO_4$ converted most of the hydroxyl groups into methoxy groups. However, it is impossible to know whether a methoxy group of a

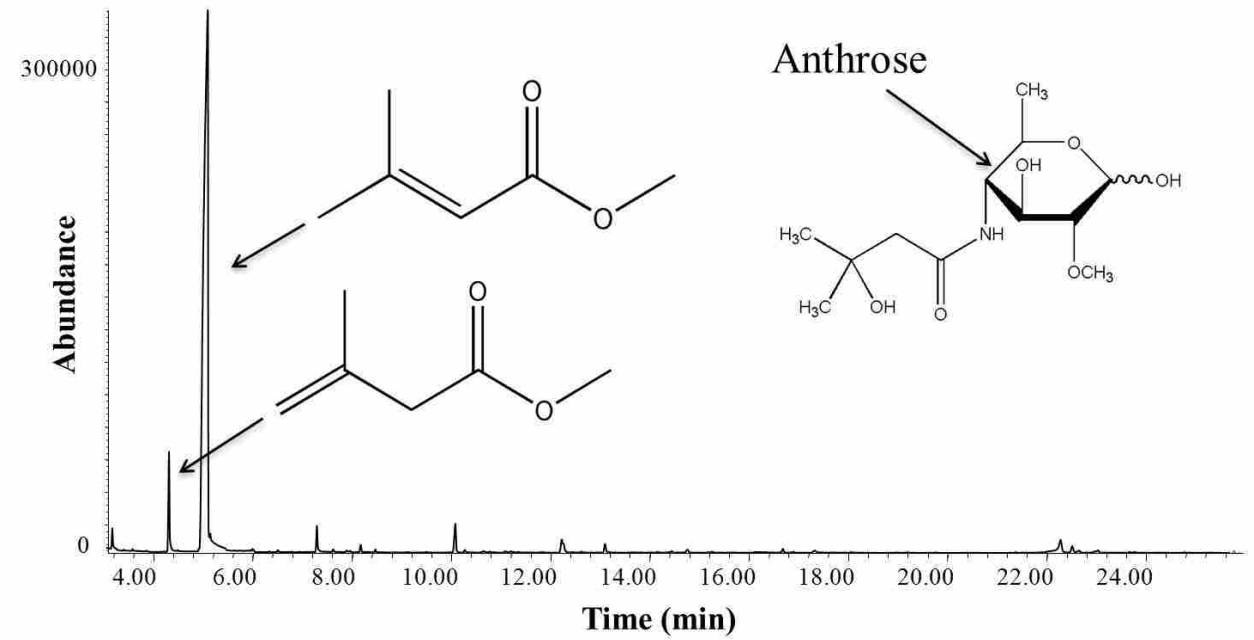



**Figure 2.2**. Total-ion chromatogram of standard anthrose after TCM treatment. Conditions: see Section 2.2.

sugar derivative was a methylated form of a sugar OH group or an original methoxy group in the sugar. The structures of the sugars were predicted by searching the NIST mass spectral library and choosing structures with the highest matching score. Unfortunately, we were not able to identify the exact names or structures of the two unique sugars. We applied TCM to some standard common bacterial sugars, such as rhamnose, fucose, glucose, galactose, manose and arabinose; however, none of the standard monosaccharides matched the chromatograms of the two unique sugars we used for differentiation. We suspect that the sugars we detected were cleaved from unique di- or multisaccharides and then methylated. These two sugars were detected with high intensities in *B. thuringiensis* Al Hakam and were important for differentiating most of the non-*B. anthracis* anthrose-producing *Bacillus* species from *B. anthracis*.

### 2.3.5 Confirmation of biomarkers derived from anthrose

An anthrose standard was treated according to the TCM procedure and analyzed by GC-MS in the same manner used for the endospores. From the resultant GC-MS total-ion chromatogram, the two derivatized biomarker peaks, 3-M-2-BAME and 3-M-3-BAME, were detected in chromatograms of *B. anthracis* and several other *Bacillus* species in approximately the same ratio (Figures 2.1C and 2.2). According to the Saytzeff rule,[23] 3-M-2-BAME was expected to be much more abundant than 3-M-3-BAME, which corresponds to the experimental results (see Figure 2.1C).

### 2.3.6 Construction of the detection algorithm

The statistical software package "R" was used for construction of the differentiation algorithm, which entailed two steps. In the first step, potential biomarkers generated by TCM

were identified using nonvirulent *B. anthracis* Sterne and other nonvirulent near neighbors. Because none of the isolates were virulent, this step was not performed in the BSL-3 facility.

After initial biomarkers were identified, the second step was to test these biomarkers for differentiation of virulent *B. anthracis* from nonvirulent *B. anthracis*. In this step, data were included from TCM of virulent *B. anthracis* as well as *B. anthracis* Sterne and *B. anthracis* Pasteur endospore suspensions. Because wide ranges were observed in the amounts of DPAME, 3-M-2-BAME and the sugar biomarkers across the strains of *B. anthracis*, we rescaled the DPAME data by taking the natural logarithm of the DPAME peak areas and then used these values to normalize the values of the other biomarkers. Biomarkers initially identified were able to discriminate between *B. anthracis* and its near neighbors, except for *B. mycoides*. It was found that *B. mycoides* could be distinguished from *B. anthracis* using an additional biomarker, C13:0 FAME. The C13:0 FAME peak areas for virulent *B. anthracis* were approximately 1 order of magnitude smaller than for *B. mycoides*. For the detection algorithm, the C13:0 FAME peak area was normalized relative to the natural logarithm of the DPAME peak area, as was done with the other biomarkers. A schematic of the final detection algorithm is shown in Figure 2.3. Using this decision tree, we could exclude the nonsporulating cells by the absence of DPAME, narrow down the detection to anthrose-producing *Bacillus* species by the presence of 3-M-2-BAME, and finalize the differentiation of *B. anthracis* by the absence of either of the unique sugars (Figure 2.1D) and the C13:0 FAME biomarkers.

The results of the algorithm for all endospore preparations are summarized in Table 2.2. The *B. anthracis* samples that were labeled non-*B. anthracis* instead of *B. anthracis* were primarily *B. anthracis* 264. This strain contained a particularly low level of anthrose. With the

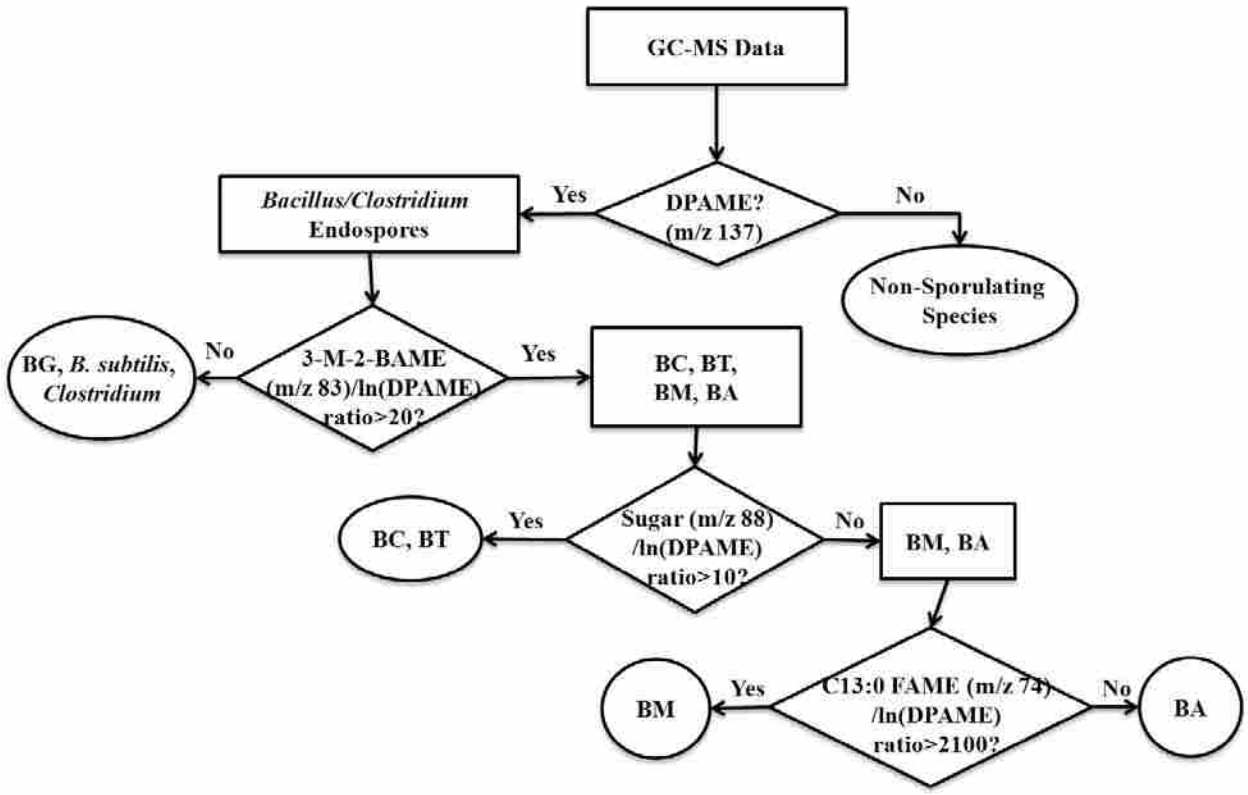



**Figure 2.3.** *B. anthracis* identification decision tree.

**Table 2.2.** Results from the automated *B. anthracis* detection algorithm.

| bacteria samples | number in study | number correct | percent correct |
|---|---|---|---|
| *B. anthracis* | 124 | 113 | 91% |
| non-*B. anthracis* | 205 | 182 | 89% |
| total | 329 | 295 | 90% |

exception of *B. anthracis* 264, the results indicate that the uniqueness of the biomarker profiles was consistent across different growth conditions and sample pretreatments.

Table 2.3 lists the average ratios (biomarker peak area to natural logarithm DPAME peak area) for all of the data obtained in this study. Any peak that was less than 3 standard deviations above the background was set to zero. The high level of variability in the measured levels of the biomarkers is evident from these data. However, even in the presence of this variability, the algorithm correctly discriminated between *B. anthracis* and non-*B. anthracis* approximately 90% of the time.

### 2.3.7 Limits of detection

The detection limits were investigated by decreasing the number of endospores introduced into the vial for TCM until the signal-to-noise ratio of a biomarker peak height of interest in its respective selected-ion plot was between 3 and 10 (using Chemstation software). The detection limits found for *B. anthracis* Sterne were 30, 000 and 50, 000 endospores for DPAME and 3-M-2-BAME, respectively.

The detection limit for *B. thuringiensis* Al Hakam endospores was 400, 000 endospores, since differentiation also required two sugar biomarkers. These results were obtained using clean endospores and may not be applicable for a real sample involving a complex matrix. The detection limits can be greatly improved (decreased) using a longer SPME extraction time. However, in field applications, analysis time often must be short.

### 2.3.8 Occurrence of anthrose in *Bacillus* species

Anthrose was previously reported to be a sugar specific to *B. anthracis*. However, we found anthrose in many *B. anthracis* near-neighbors, i.e., *B. thuringiensis*, *B. cereus* and *B.*

**Table 2.3.** Normalized ratios[a] and standard deviations (SD) of biomarker peak areas for different bacteria species.

| strain | n | conditions (medium,[c] temp., °C) | average sugar1[b]/ ln(DPAME) (SD) | average sugar2[b]/ ln(DPAME) (SD) | average C13:0 FAME/ ln(DPAME) (SD) | average 3-M-2-BAME/ ln(DPAME) (SD) | ln(DPAME) (SD) | missed |
|---|---|---|---|---|---|---|---|---|
| *B. anthracis* 231 | 3 | C 32 | 0.0 (0.0) | 0.0 (0.0) | 23.4 (21.7) | 681.1 (533.7) | 12.3 (1.1) | 0 |
| *B. anthracis* 231 | 6 | C 37 | 0.0 (0.0) | 0.0 (0.0) | 54.4 (43.4) | 361.2 (232.1) | 10.4 (1.2) | 0 |
| *B. anthracis* 231 | 7 | LD 32 | 0.0 (0.0) | 0.0 (0.0) | 45.8 (52.0) | 206.8 (178.7) | 10.0 (1.5) | 0 |
| *B. anthracis* 231 | 7 | LD 37 | 0.0 (0.0) | 0.0 (0.0) | 86.0 (46.8) | 282.5 (340.1) | 10.9 (1.5) | 2 |
| *B. anthracis* 264 | 3 | C 32 | 0.0 (0.0) | 0.0 (0.0) | 312.5 (63.1) | 104.0 (76.0) | 11.2 (0.1) | 1 |
| *B. anthracis* 264 | 5 | C 37 | 0.0 (0.0) | 0.0 (0.0) | 434.3 (398.2) | 103.1 (45.3) | 10.1 (1.8) | 0 |
| *B. anthracis* 264 | 2 | LD 32 | 0.0 (0.0) | 0.0 (0.0) | 263.0 (14.6) | 0.0 (0.0) | 11.2 (0.3) | 2 |
| *B. anthracis* 264 | 2 | LD 37 | 0.0 (0.0) | 0.0 (0.0) | 36.4 (44.3) | 0.0 (0.0) | 8.2 (0.0) | 2 |
| *B. anthracis* 300 | 4 | C 32 | 0.0 (0.0) | 0.0 (0.0) | 1126.4 (1177.6) | 2847.2 (3152.8) | 11.2 (2.1) | 1 |
| *B. anthracis* 300 | 7 | C 37 | 0.0 (0.0) | 0.0 (0.0) | 497.0 (193.4) | 843.2 (968.7) | 10.4 (0.8) | 0 |
| *B. anthracis* 300 | 5 | LD 32 | 0.0 (0.0) | 0.0 (0.0) | 461.3 (221.5) | 299.5 (177.6) | 9.8 (1.4) | 0 |
| *B. anthracis* 300 | 5 | LD 37 | 0.0 (0.0) | 0.0 (0.0) | 331.9 (268.6) | 1954.7 (2369.4) | 10.4 (2.6) | 0 |
| *B. anthracis* Ames | 5 | C 32 | 0.0 (0.0) | 0.0 (0.0) | 604.8 (349.9) | 603.5 (726.6) | 9.5 (0.6) | 0 |
| *B. anthracis* Ames | 5 | C 37 | 0.0 (0.0) | 0.0 (0.0) | 328.5 (215.6) | 681.0 (785.5) | 9.3 (1.0) | 0 |
| *B. anthracis* Ames | 8 | LD 32 | 0.0 (0.0) | 0.0 (0.0) | 395.4 (365.8) | 389.7 (333.3) | 11.5 (1.3) | 0 |
| *B. anthracis* Ames | 6 | LD 37 | 0.0 (0.0) | 0.0 (0.0) | 398.8 (227.2) | 260.4 (327.8) | 11.1 (1.1) | 0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *B. anthracis* Pasteur | 2 | C 32 | 0.0 (0.0) | 0.0 (0.0) | 341.0 (18.1) | 83.1 (20.1) | 9.3 (0.3) | 0 |
| *B. anthracis* Pasteur | 5 | C 37 | 0.0 (0.0) | 0.0 (0.0) | 352.4 (94.9) | 25.8 (12.0) | 7.9 (0.5) | 2 |
| *B. anthracis* Pasteur | 1 | LD 32 | 0.0 (0.0) | 0.0 (0.0) | 729.3 (0.0) | 28.3 (0.0) | 8.2 (0.0) | 0 |
| *B. anthracis* Pasteur | 3 | LD 37 | 0.0 (0.0) | 0.0 (0.0) | 303.0 (316.7) | 80.3 (38.9) | 7.8 (0.5) | 0 |
| *B. anthracis* Sterne | 7 | C 32 | 0.0 (0.0) | 0.0 (0.0) | 579.5 (568.8) | 2915.9 (5413.2) | 11.8 (0.7) | 0 |
| *B. anthracis* Sterne | 7 | C 37 | 0.0 (0.0) | 0.0 (0.0) | 345.7 (334.1) | 7925.5 (3354.0) | 13.3 (0.6) | 0 |
| *B. anthracis* Sterne | 11 | LD 32 | 0.0 (0.0) | 0.0 (0.0) | 981.0 (987.8) | 6342.1 (5562.0) | 13.0 (1.8) | 1 |
| *B. anthracis* Sterne | 8 | LD 37 | 0.0 (0.0) | 0.0 (0.0) | 158.7 (84.1) | 3993.4 (2121.9) | 12.7 (0.7) | 0 |
| *B. cereus* | 1 | C 32 | 0.0 (0.0) | 0.0 (0.0) | 1392.9 (0.0) | 25.3 (0.0) | 7.7 (0.0) | 1 |
| *B. cereus* | 2 | LD 28 | 17.3 (24.4) | 18.4 (26.0) | 954.4 (995.4) | 106.0 (78.0) | 9.2 (0.2) | 0 |
| *B. cereus* | 12 | LD 32 | 16.3 (26.1) | 4.9 (11.6) | 649.2 (488.9) | 16.6 (26.5) | 9.4 (1.5) | 2 |
| *B. cereus* | 9 | LD 37 | 0.0 (0.0) | 3.0 (8.9) | 1355.2 (1063.4) | 9.0 (14.9) | 9.0 (1.4) | 1 |
| *B. atrophaeus* | 7 | C 32 | 0.0 (0.0) | 0.0 (0.0) | 5.9 (3.7) | 0.0 (0.0) | 11.4 (0.7) | 0 |
| *B. atrophaeus* | 7 | C 37 | 0.0 (0.0) | 0.0 (0.0) | 19.4 (5.6) | 0.0 (0.0) | 13.0 (0.8) | 0 |
| *B. atrophaeus* | 7 | LD 32 | 0.0 (0.0) | 0.0 (0.0) | 15.9 (7.6) | 0.0 (0.0) | 11.0 (0.5) | 0 |
| *B. atrophaeus* | 6 | LD 37 | 0.0 (0.0) | 0.0 (0.0) | 35.7 (19.9) | 0.0 (0.0) | 12.6 (0.4) | 0 |
| *B. mycoides* | 1 | C 28 | 0.0 (0.0) | 0.0 (0.0) | 3365.2 (0.0) | 61.0 (0.0) | 9.3 (0.0) | 0 |
| *B. mycoides* | 2 | C 32 | 0.0 (0.0) | 0.0 (0.0) | 3450.6 (981.0) | 76.9 (14.2) | 8.3 (0.3) | 0 |
| *B. mycoides* | 2 | C 37 | 0.0 (0.0) | 0.0 (0.0) | 5081.0 (884.8) | 155.7 (7.0) | 9.1 (0.1) | 0 |
| *B. mycoides* | 4 | LD 28 | 0.0 (0.0) | 0.0 (0.0) | 1004.7 (733.0) | 0.0 (0.0) | 7.0 (1.5) | 0 |
| *B. mycoides* | 2 | LD 32 | 0.0 (0.0) | 0.0 (0.0) | 4069.6 (528.0) | 57.9 (14.7) | 8.6 (0.6) | 0 |
| *B. mycoides* | 2 | LD 37 | 0.0 (0.0) | 0.0 (0.0) | 3463.6 (1643.7) | 137.8 (5.8) | 9.0 (0.0) | 0 |
| *B. subtilis* | 7 | C 32 | 0.0 (0.0) | 0.0 (0.0) | 12.1 (9.0) | 0.0 (0.0) | 12.9 (0.3) | 0 |
| *B. subtilis* | 6 | C 37 | 0.0 (0.0) | 0.0 (0.0) | 15.4 (14.4) | 0.0 (0.0) | 13.1 (0.4) | 0 |
| *B. subtilis* | 8 | LD 32 | 0.0 (0.0) | 0.0 (0.0) | 17.9 (10.4) | 0.0 (0.0) | 12.3 (0.6) | 0 |
| *B. subtilis* | 6 | LD 37 | 0.0 (0.0) | 0.0 (0.0) | 18.4 (7.3) | 0.0 (0.0) | 12.9 (0.8) | 0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *B. thuringiensis* 19269 | 7 | C 32 | 72.8 (61.5) | 1.6 (4.2) | 2686.8 (2013.2) | 208.2 (128.4) | 11.1 (0.3) | 1 |
| *B. thuringiensis* 19269 | 7 | C 37 | 55.6 (37.3) | 50.6 (52.4) | 1364.7 (612.1) | 349.8 (306.8) | 11.7 (0.3) | 1 |
| *B. thuringiensis* 19269 | 6 | LD 32 | 259 (213.1) | 74.3 (67.7) | 1836.3 (829.2) | 336.8 (220.4) | 11.6 (0.3) | 0 |
| *B. thuringiensis* 19269 | 7 | LD 37 | 257.9 (235.9) | 145.7 (149.0) | 1542.0 (1375.8) | 345.4 (199.3) | 12.3 (1.1) | 1 |
| *B. thuringiensis* 19270 | 6 | C 32 | 0.0 (0.0) | 0.0 (0.0) | 3671.2 (3730.9) | 239.2 (303.1) | 10.9 (1.5) | 1 |
| *B. thuringiensis* 19270 | 1 | C 37 | 0.0 (0.0) | 0.0 (0.0) | 513.3 (0.0) | 158.8 (0.0) | 8.8 (0.0) | 1 |
| *B. thuringiensis* 19270 | 7 | LD 32 | 310 (258.7) | 209.0 (219.5) | 855.5 (512.7) | 632.4 (229.4) | 12.7 (0.4) | 1 |
| *B. thuringiensis* 19270 | 6 | LD 37 | 107.1 (121.2) | 221.9 (269.2) | 405.0 (360.3) | 645.3 (438.3) | 11.9 (1.0) | 1 |
| *B. thuringiensis* Kurstaki | 5 | C 32 | 5.0 (11.3) | 34.4 (58.0) | 477.0 (379.9) | 21.4 (21.0) | 11.7 (0.6) | 2 |
| *B. thuringiensis* Kurstaki | 4 | C 37 | 0.0 (0.0) | 0.0 (0.0) | 618.3 (475.6) | 31.6 (22.6) | 10.9 (0.5) | 3 |
| *B. thuringiensis* Kurstaki | 7 | LD 32 | 18.4 (23.7) | 159.2 (124.9) | 701.0 (590.3) | 31.7 (43.4) | 12.6 (0.4) | 1 |
| *B. thuringiensis* Kurstaki | 7 | LD 37 | 0.0 (0.0) | 0.0 (187.3) | 998.5 (783.6) | 3.0 (7.9) | 10.4 (0.2) | 1 |
| *B. thuringiensis* Al Hakam | 6 | C 32 | 182.7 (179.4) | 179.2 (221.0) | 1433.7 (813.7) | 4781.9 (3441.5) | 12.9 (0.4) | 0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *B. thuringiensis* Al Hakam | 7 | C 37 | 334.3 (310.0) | 438.6 (427.8) | 928.1 (966.4) | 5073.8 (2342.8) | 13.4 (0.3) | 1 |
| *B. thuringiensis* Al Hakam | 10 | LD 32 | 229.2 (220.4) | 525.3 (556.2) | 638.6 (477.9) | 5027.2 (3665.7) | 11.7 (0.7) | 0 |
| *B. thuringiensis* Al Hakam | 8 | LD 37 | 242.2 (145.0) | 359.6 (298.7) | 600.5 (198.5) | 4690.7 (2242.2) | 12.1 (0.9) | 1 |
| *B. thuringiensis* Israelensis | 2 | C 28 | 27.0 (38.1) | 0.0 (0.0) | 492.8 (395.0) | 571.8 (808.7) | 11.0 (0.4) | 0 |
| *B. thuringiensis* Israelensis | 2 | C 32 | 44.2 (12.8) | 0.0 (0.0) | 967.3 (411.9) | 936.4 (285.7) | 11.4 (0.2) | 0 |
| *B. thuringiensis* Israelensis | 2 | C 37 | 0.0 (0.0) | 5.4 (7.6) | 2029.0 (52.8) | 22.2 (31.4) | 10.7 (0.9) | 0 |
| *B. thuringiensis* Israelensis | 2 | LD 28 | 152 (14.7) | 19.3 (27.3) | 677.6 (99.6) | 476.4 (96.6) | 12.4 (0.0) | 0 |
| *B. thuringiensis* Israelensis | 3 | LD 32 | 0.0 (0.0) | 0.0 (0.0) | 383.6 (173.1) | 305.0 (59.1) | 11.0 (0.3) | 3 |
| *B. thuringiensis* Israelensis | 2 | LD 37 | 50.7 (5.5) | 2.6 (3.7) | 338.6 (474.3) | 16.5 (3.6) | 11.2 (0.6) | 0 |
| Total | 329 | | | | | | | 34 |

[a]Normalized to natural logarithm of DPAME peak areas

[b]Two unique sugars (Figure 2.1D)

[c]C = Columbia, LD = Leighton-Doi

*mycoides* (Table 2.3). This is not surprising since *B. anthracis*, *B. thuringiensis*, *B. cereus* and *B. mycoides* belong in the same *B. cereus* group and, therefore, are closer genetically compared to *B. subtilis* or *B. atrophaeus*. We report here, for the first time, that while anthrose can be detected in all of the virulent and nonvirulent *B. anthracis* endospores studied so far, it is present in much lower levels in many fully virulent strains compared to those in *B. anthracis* Sterne. Tamborrini et al. published results similar to this, explaining that the spread of *B. anthracis* strains containing no anthrose may be due to the widespread use of the anthrose-containing Sterne strain as an animal vaccine in many African regions, thereby selecting for virulent nonanthrose-containing escape mutants.[24] In contrast, we and others observed that some non-*B. anthracis* endospores (e.g., *B. thuringiensis* Al Hakam) can contain much higher levels of anthrose than many virulent *B. anthracis* isolates.

## 2.4 CONCLUSIONS

A rapid, sensitive and specific method for identification of *B. anthracis* endospores was developed. On the basis of a review of the literature and experimental data, we constructed a decision tree that relied on five key biomarkers: DPAME, anthrose-derived 3-M-2-BAME, two unidentified methylated sugars and a fatty acid (C13:0) methyl ester. An automated discrimination algorithm was constructed that differentiates nonvirulent *B. anthracis* from their genetic near-neighbors. This is the first report of the analysis of anthrose levels in a variety of virulent *B. anthracis* strains. The anthrose levels in these virulent *B. anthracis* isolates are much lower than in the *B. anthracis* Sterne strain; however, anthrose is still useful as a biomarker for *B. anthracis* detection. Future research will focus on extending these results to a larger number of

virulent *B. anthracis* strains, as well as to a greater number of near-neighbors. In addition, other biomarkers will be sought and the detection algorithm will be refined to reduce the occurrence of false positives and false negatives.

## 2.5 REFERENCES


1. Helgason, E.; Okstad, O. A.; Caugant, D. A.; Johansen, H. A.; Fouet, A.; Mock, M.; Hegna, I.; Kolsto, A. B., *Appl. Environ. Microbiol.* **2000,** *66*, 2627-2630.
2. Daffonchio, D.; Cherif, A.; Borin, S., *Appl. Environ. Microbiol.* **2000,** *66*, 5460-5468.
3. Ash, C.; Farrow, J. A.; Dorsch, M.; Stackebrandt, E.; Collins, M. D., *Int. J. Syst. Bacteriol.* **1991,** *41*, 343-346.
4. Green, B. D.; Battisti, L.; Koehler, T. M.; Thorne, C. B.; Ivins, B. E., *Infect. Immun.* **1985,** *49*, 291-297.
5. Mikesell, P.; Ivins, B. E.; Ristroph, J. D.; Dreier, T. M., *Infect. Immun.* **1983,** *39*, 371-376.
6. Klee, S. R.; Ozel, M.; Appel, B.; Boesch, C.; Ellerbrok, H.; Jacob, D.; Holland, G.; Leendertz, F. H.; Pauli, G.; Grunow, R.; Nattermann, H., *J. Bacteriol.* **2006,** *188*, 5333-5344.
7. Radnedge, L.; Agron, P. G.; Hill, K. K.; Jackson, P. J.; Ticknor, L. O.; Keim, P.; Andersen, G. L., *Appl. Environ. Microbiol.* **2003,** *69*, 2755-2764.
8. Chang, Y. H.; Shangkuan, Y. H.; Lin, H. C.; Liu, H. W., *Appl. Environ. Microbiol.* **2003,** *69*, 4502-4510.
9. Keim, P.; Kalif, A.; Schupp, J.; Hill, K.; Travis, S. E.; Richmond, K.; Adair, D. M.; Hugh-Jones, M.; Kuske, C. R.; Jackson, P., *J. Bacteriol.* **1997,** *179*, 818-824.
10. Klee, S. R.; Nattermann, H.; Becker, S.; Urban-Schriefer, M.; Franz, T.; Jacob, D.; Appel, B., *J. Appl. Microbiol.* **2006,** *100*, 673-681.
11. Hu, X.; Swiecicka, I.; Timmery, S.; Mahillon, J., *FEMS Microbiol. Ecol.* **2009,** *70*, 344-355.
12. Hoffmaster, A. R.; Hill, K. K.; Gee, J. E.; Marston, C. K.; De, B. K.; Popovic, T.; Sue, D.; Wilkins, P. P.; Avashia, S. B.; Drumgoole, R.; Helma, C. H.; Ticknor, L. O.; Okinaka, R. T.; Jackson, P. J., *J. Clin. Microbiol.* **2006,** *44*, 3352-3360.
13. Hoffmaster, A. R.; Ravel, J.; Rasko, D. A.; Chapman, G. D.; Chute, M. D.; Marston, C. K.; De, B. K.; Sacchi, C. T.; Fitzgerald, C.; Mayer, L. W.; Maiden, M. C.; Priest, F. G.; Barker, M.; Jiang, L.; Cer, R. Z.; Rilstone, J.; Peterson, S. N.; Weyant, R. S.; Galloway, D. R.; Read, T. D.; Popovic, T.; Fraser, C. M., *Proc. Natl. Acad. Sci. USA.* **2004,** *101*, 8449-8454.
14. Pannucci, J.; Okinaka, R. T.; Sabin, R.; Kuske, C. R., *J. Bacteriol.* **2002,** *184*, 134-141.
15. Pannucci, J.; Okinaka, R. T.; Williams, E.; Sabin, R.; Ticknor, L. O.; Kuske, C. R., *BMC Genomics* **2002,** *3*, 34.

16. Easterday, W. R.; Van Ert, M. N.; Zanecki, S.; Keim, P., *Bio.Techniques* **2005,** *38*, 731-735.
17. Antwerpen, M. H.; Zimmermann, P.; Bewley, K.; Frangoulidis, D.; Meyer, H., *Mol. Cell. Probes* **2008,** *22*, 313-315.
18. Irenge, L. M.; Durant, J. F.; Tomaso, H.; Pilo, P.; Olsen, J. S.; Ramisse, V.; Mahillon, J.; Gala, J. L., *Appl. Microbiol. Biotechnol.* **2010**.
19. Kaman, W. E.; Hulst, A. G.; van Alphen, P. T.; Roffel, S.; van der Schans, M. J.; Merkel, T.; van Belkum, A.; Bikker, F. J., *Anal. Chem.* **2011,** *83*, 2511-2517.
20. Truong, T. V.; Nackos, A. N.; Williams, J. R.; VanDerwerken, D. N.; Kimball, J. A.; Murray, J. A.; Hawkes, J. E.; Harvey, D. J.; Tolley, H. D.; Robison, R. A.; Bartholomew, C. H.; Lee, M. L., *Anal. Methods* **2010,** *2*, 638-644.
21. Pawliszyn, J., Solid Phase Microextraction: Theory and Practice, 1 ed.; Wiley-VCH: New York, 1997; p 247.
22. Dong, S.; McPherson, S. A.; Tan, L.; Chesnokova, O. N.; Turnbough, C. L., Jr.; Pritchard, D. G., *J. Bacteriol.* **2008,** *190*, 2350-2359.
23. Vollhardt, K. P. C.; Schore, N. E., Organic Chemistry: Structure and Function, 3 ed.; W. H. Freeman and Company: New York, 1998; p 463.
24. Tamborrini, M.; Bauer, M.; Bolz, M.; Maho, A.; Oberli, M. A.; Werz, D. B.; Schelling, E.; Zinsstag, J.; Seeberger, P. H.; Frey, J.; Pluschke, G., *J. Bacteriol.* **2011,** *193*, 3506-3511.

# 3 GC-MS METHOD FOR RAPID IDENTIFICATION AND DIFFERENTIATION OF *BURKHOLDERIA PSEUDOMALLEI*, *BURKHOLDERIA MALLEI*, *BURKHOLDERIA THAILANDENSIS* AND SEVERAL MEMBERS OF THE *BURKHOLDERIA CEPACIA* COMPLEX

## 3.1 INTRODUCTION

Several species within the genus *Burkholderia* are classified as human pathogens. Perhaps the most well-known of these pathogenic species are *Burkholderia pseudomallei*, *Burkholderia mallei* and several members of the *Burkholderia cepacia* complex. *B. pseudomallei* is a soil saprophyte that is indigenous to Southeast Asia, Northern Australia and tropical regions near the equator.[1, 2] It is also the causative agent of melioidosis, a human disease with symptoms that range from localized abscesses to acute septicemia and pneumonia. Melioidosis has a mortality rate of 40% in Northern Thailand, 19% in Northern Australia and 39% in Singapore.[3-5] In fact, during the first 48 h of hospital admittance, untreated cases of septicemia have mortality rates as high as 80-90%.[6, 7] Additionally, in northeast Thailand melioidosis accounts for 20% of community-acquired bacteremia and is the third most common cause of death by an infectious disease, following AIDS and tuberculosis.[5, 8] The number of cases of melioidosis is also increasing in other populated countries such as China, Brazil and India.[9-13]

*B. mallei* is the causative agent of glanders, an abscess-forming infection predominantly found in the equine population. Although rare, it can cause serious disease in humans. Veterinarians, laboratory workers, equine handlers and slaughterhouse workers are at risk due to the possibility of repeated exposure to the microorganism. Humans infected with *B. mallei* experience fever, rigors, malaise, diaphoresis, pneumonia, bacteremia, pustules and abscesses.[14,

[15] The disease has a 95% case fatality rate for untreated septicemia and a 50% case fatality rate in antibiotic-treated patients.[7, 15] Without antibiotic treatment, death typically occurs in 7-10 d.[14, 15]

*B. pseudomallei* and *B. mallei* are very closely related to each another. This has been demonstrated in part by comparing their cellular lipid and fatty acid compositions.[16-18] In addition, it has been shown that these organisms have a high degree of genetic similarity, which has created difficulties in developing accurate molecular-based differentiation assays.[19-21] Both organisms are recognized by the CDC as possible bioterrorism agents based on their low infectious dose and their potential to cause widespread disease.

*Burkholderia thailandensis* is also closely related to *B. pseudomallei* and was only recently classified as a new species based on differences in 16s rRNA sequences, some biochemical properties and a much lower virulence in humans.[22] There are very few cases of human infection by *B. thailandensis*, and those presumably resulted from a high infectious dose.[23-25] Although *B. thailandensis* holds very little clinical significance, it does share several virulence homologs with *B. pseudomallei* and *B. mallei*, and is thus considered by many to be a model organism for studying the pathogenesis of various *Burkholderia* species. In addition, *B. thailandensis* is known to co-localize with *B. pseudomallei* in the environment and has similar phenotypic characteristics to that of *B. pseudomallei* by routine diagnostic tests.[26]

*Burkholderia cepacia*, *Burkholderia multivorans, Burkholderia vietnamiensis* and several other closely related bacterial species make up the *Burkholderia cepacia* complex.[27] These organisms were originally believed to be only plant pathogens, but later emerged as important opportunistic pathogens causing chronic and life-threatening respiratory tract infections in patients with cystic fibrosis.[28] These members of the *B. cepacia* complex share many genetic similarities with *B. pseudomallei*, *B. mallei* and *B. thailandensis*.

The similarities between these different *Burkholderia* species have complicated the design and development of detection and differentiation assays. Nonetheless, several methods have been developed for the identification and discrimination of various combinations of the *Burkholderia* species that were described above. These methods include serological tests, biochemical tests, microscopic methods, PCR assays and the GC analysis of cellular fatty acids.[26, 29-47] However, there are limitations associated with each of these assay types.

In endemic areas, serological tests for *B. pseudomallei* are unreliable due to the frequent seroconversion of individuals previously exposed to the organism.[48] Therefore, these serological tests have low sensitivity and specificity in areas of endemicity, but may prove useful in non-endemic areas.[42, 49] Biochemical assays have frequently misidentified *B. pseudomallei* as *Pseudomonas* spp.*, B. vietnamiensis, Stenotrophomonas maltophilia* and *Chromobacterium violaceum*.[30, 37, 39] Commercial biochemical tests have also misidentified members of the *B. cepacia* complex as *Burkholderia gladioli, Ralstonia pickettii, Alcaligenes* spp., *S. maltophilia, Flavobacterium* spp. and *Chryseobacterium* spp.[50, 51] In addition, many of these detection methods require the organism to be cultured prior to testing, which may take up to 7 days. Furthermore, similar colony morphologies and biochemical functions make it difficult to differentiate between the different species of *Burkholderia*.[52, 53]

PCR has revolutionized microbial detection due its accuracy, sensitivity and speed. PCR-based assays are usually designed around a well conserved gene. However, it is possible, especially in emerging pathogens, for mutations to occur in the gene of interest which can compromise the assay.[54] Restricting bacterial detection to a single target is another limitation of PCR-based assays. This issue can be overcome by developing multiplex PCR assays; however, such assays are difficult to implement due to the high degree of optimization that is required. The

high sensitivity of PCR-based assays is also a limitation. False positives can arise from background contamination from external sources of DNA, such as the "carry-over" products from earlier PCR reactions.[55, 56] Conversely, false negatives can occur due to inadequate removal of PCR inhibitors. Although PCR-based assays have the potential to provide high-throughput, the limitations associated with possible gene mutations, false positives, false negatives, sample processing and the need to validate with other established assays decreases the overall throughput of the entire PCR process.

Species-specific cellular fatty acids are important chemical markers that are frequently used in bacterial taxonomy and classification.[57] A GC-based commercial microbial identification system was developed by MIDI (Newark, DE, USA) and provides a database of bacterial cellular fatty acid profiles. Using this commercial method, Krejci and Kroppenstedt[58] described certain fatty acids that are unique to the *Burkholderia* species and that could potentially be used for their detection and differentiation. For example, they proposed that the 3-OH C16:0 fatty acid could be used for the identification of the *B. cepacia* complex.

Poly(3-hydroxyalkanoates) (P3HAs) might also aid in the detection and differentiation of *Burkholderia* species. They represent a class of biodegradable thermoplastics that are synthesized by a wide variety of bacteria.[59, 60] Poly-3-hydroxybutyrate (P3HB), poly-3-hydroxyvalerate (P3HV), and poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate [poly(3HB-*co*-3HV)] are the most extensively studied polyhydroxyalkanoate (PHA) compounds. These compounds are typically used by bacteria as a reserve carbon and energy source during unfavorable growth conditions.[60] In addition, poly(3HB-*co*-3HV) has superior mechanical properties which have drawn increasing interest from bio-industrial fields that require biodegradable and biocompatible materials.[61] *Burkholderia* species are capable of accumulating

poly(3HB-*co*-3HV) from various carbon sources, including glucose, fructose, acetate, glycerol and lactase.[61] We hypothesized that P3HB might be a suitable identification target for discrimination between the different species of *Burkholderia*.

In this study, we developed a simple method for the rapid identification and differentiation of *B. pseudomallei*, *B. mallei*, *B. thailandensis* and several members of the *B. cepacia* complex (*B. cepacia*, *B. multivorans* and *B. vietnamiensis*) using a GC-MS system. The method relied on several cellular fatty acids and poly(3HBA-*co*-3HVA) derivatives as biomarkers. These biomarkers were released from the bacterial cells and derivatized into compounds that were more amenable to GC analysis via a single-step TCM procedure. The TCM procedure was a modified version of the protocol that was used to generate biomarkers from the spores of several *Bacillus* species.[62] A statistical discrimination algorithm was then constructed using a combination of biomarkers, and the identities of the different species of *Burkholderia* were confirmed in a statistically designed test using the algorithm. These results demonstrated that the algorithm was robust against different growth conditions (i.e., medium and temperature).

## 3.2 EXPERIMENTAL

### 3.2.1 Chemical reagents

HPLC grade methanol (MeOH) and sulfuric acid ($H_2SO_4$) were purchased from Sigma Chemical (St. Louis, MO, USA). Hydrogen methyl sulfate ($HMeSO_4$) was chosen as the derivatization agent because, as shown in past studies, it performed best in terms of cost, speed, safety and GC response.[63] It was prepared as a 20% solution of $H_2SO_4$ in methanol (v/v). The solution was allowed to rest at room temperature for approximately 8 d prior to use, which gave

the reagents enough time to react. Phosphate buffer (1 M, pH 6.5) was made by mixing 1 M potassium phosphate monobasic solution and 1 M potassium phosphate dibasic solution until the pH reached 6.5; both reagents were purchased from Sigma Chemical.

### 3.2.2 Bacterial strains

The bacterial strains used in this study are described in Table 3.1. The purity of each strain was verified by Gram stain and subsequent inspection using a light microscope. The identities of the *B. mallei* and *B. pseudomallei* strains were further confirmed by the GC analysis of cellular fatty acids using an Agilent 6890 Series Gas Chromatograph (Santa Clara, CA, USA) and software purchased from MIDI (Newark, DE, USA). This method of identification was also attempted in conjunction with the other species of *Burkholderia*. Furthermore, a real-time PCR assay was used to definitively confirm the identity of each *B. mallei* and *B. pseudomallei* strain.[64] The assay was conducted as described by U'Ren et. al.[64] and using primers from Integrated DNA Technologies (Coralville, IA, USA) and TaqMan® probes from Applied Biosystems (Foster City, CA, USA).

### 3.2.3 Laboratory conditions

Procedures involving *B. mallei* and *B. pseudomallei* were performed under BSL-3 operating conditions. All other procedures were conducted under BSL-2 operating conditions.

### 3.2.4 Growth conditions

Each organism was initially cultured on Blood Agar, which contained a Columbia Blood Agar Base (Becton, Dickinson and Company, Sparks, MD, USA) and 5% (v/v) sheep blood (Hema Resource and Supply, Aurora, OR, USA). After incubating for approximately 2-3 d at 37 °C, isolated colonies were spread over Blood Agar and Brain Heart Infusion Agar (Becton,

**Table 3.1.** *Burkholderia* strains used in this study.

| species | strain | country | origin | source[a] |
|---|---|---|---|---|
| *Burkholderia pseudomallei* | 20 | Thailand | Human blood | PHLS |
| | 72 | Malasia | Human | PHLS |
| | 83 | Australia | Soil | PHLS |
| | 99/SID 4349 | United Kingdom | Human | PHLS |
| *Burkholderia mallei* | 10229 | Turkey | Human | NCTC |
| | 10248 | Hungary | - | NCTC |
| | 10399 | China | Horse lung | ATCC |
| | Turkey #1 | Turkey | - | CDC |
| *Burkholderia thailandensis* | 700388 | Thailand | Soil | ATCC |
| *Burkholderia cepacia* | 10856 | - | - | ATCC |
| *Burkholderia multivorans* | BAA-247 | Belgium | Human | ATCC |
| *Burkholderia vietnamiensis* | BAA-248 | VietNam | Rhizosphere | ATCC |

[a] PHLS, Public Health Laboratory Service, UK; NCTC, National Collection of Type Cultures, UK; ATCC, American Type Culture Collection, USA; CDC, Center for Disease Control and Prevention, USA.

Dickinson and Company). All organisms were cultured on both types of growth medium and grown at two different temperatures (32 °C and 37 °C) for approximately 2-3 d.

### 3.2.5 Harvesting techniques

The confluent bacterial growth was transferred to 5 mL of physiological saline solution and pelleted via centrifugation at 3200*g* for 5 min at room temperature. Subsequently, the pellet was suspended in 5 mL sterile HPLC water (Sigma Chemical). An aliquot (1 mL) of this cell suspension was then transferred to a 1.7 mL micro-centrifuge tube (BioExpress, Kaysville, UT, USA) and pelleted by centrifugation at 20,000*g* for 5 min at room temperature.

### 3.2.6 Thermochemolysis

The cell pellet was suspended in methanol to give a final concentration of approximately 1 x $10^7$ cells $mL^{-1}$. A 20 $\mu$L aliquot of the cell suspension was then transferred to a 1.7 mL micro-centrifuge tube and combined with 20 $\mu$L of $HMeSO_4$. After thoroughly mixing the solution with a pipette, aliquots of 20 $\mu$L were transferred to clear flat-bottom crimp vials (7 × 40 mm, National Scientific, TN, USA). The vials were capped with crimp top seals (8 mm, clear PTFE/red rubber, National Scientific) and sealed using a crimping tool. A custom machined heating block and a digital mini temperature CSC 32 controller (Omega, Stamford, CT, USA) were used to heat the vials to 140 °C for 5 min. The heating block had enough slots to accommodate four glass vials at one time. After returning the vials to room temperature, they were stored at -20°C until the viability testing or GC-MS analysis was conducted. Two vials were prepared for every sample that originated in the BSL-2. Alternatively, four vials were prepared for every sample that originated in the BSL-3, two of which were subjected to a viability testing.

### 3.2.7 Viability testing

Vials were injected with 200 $\mu$L of phosphate buffer (1 M, pH 6.5) to neutralize the acidic solution. This was done by using 1 mL syringes and 22 gauge 1 1/2 inch needles that were purchased from Becton, Dickinson and Company. The vials were then mixed vigorously with a vortex mixer for 5 s. The entire contents of the vials were cultured on Blood Agar plates. Subsequently, the vials were discarded, and the plates were incubated for 5 d at 37 °C. If no growth was observed, then sterility was confirmed and the remaining vials were removed from the BSL-3 facility for GC-MS analysis.

### 3.2.8 GC-MS analysis

Vials were injected with 400 $\mu$L phosphate buffer (1 M, pH 6.5) to neutralize the acidic solution. This was done by using 1 mL syringes and 26 gauge 1 1/2 inch needles that were purchased from Becton, Dickinson and Company. Biomarkers were extracted using a 2 cm DVB/CAR/PDMS SPME fiber that was purchased from Supelco (Bellefonte, PA, USA). Roughly half of the fiber was submerged in the liquid phase and half was exposed to the vapor phase. The extraction was conducted at 80 °C for 10 min. The SPME fiber was then placed in the GC injection port for 2 min for desorption.

An Agilent 6890 GC was used in the analysis of all samples. The GC was equipped with a split/splitless injector containing a 79 mm long × 1.2 mm i.d. × 6.3 mm o.d. deactivated fused silica liner (SGE, Austin, TX, USA) and a 30 m × 0.25 mm i.d. × 0.25 $\mu$m Zebron-FFAP column from Phenomenex (Torrance, CA, USA). The injection port was set at 260 °C for all samples and the inlet was operated in the splitless mode at a constant helium pressure of 24 psi. The temperature program was set from 60 °C (1 min initial hold), increased at 10 °C $min^{-1}$ to 170 °C (1 min hold), then 5 °C $min^{-1}$ to 210 °C (1 min hold) and finally 8 °C $min^{-1}$ to 250 °C (5 min

hold). The GC was coupled to an Agilent 5973 MS with electron ionization source and quadrupole mass analyzer set to scan over a 33-550 *m/z* range. The transfer line to the MS was maintained at 230 °C. Blanks (SPME fiber without sample extraction) were performed at the beginning of each day and periodically during the analyses. Cleaning the SPME fiber in between runs was achieved by placing the fiber in deionized water with agitation for approximately 20-30 min. The SPME fiber was then inserted into the GC-MS injection port for 1 min to remove any possible contamination or carry-over from previous samples. Chemstation software package was used for GC-MS data analysis.

### 3.2.9 Experimental design

In total, 304 samples from 3 different batches were prepared and processed in this study. The first batch was used for constructing and training the differentiation algorithm. It included the results from 32 samples of *B. pseudomallei*, 32 samples of *B. mallei* and 48 samples of the near neighbors. Each strain of *B. pseudomallei* and *B. mallei* was cultured under the four growth conditions (see above) and processed in duplicate. Each of the near neighbors was also cultured under the four growth conditions; however, they were processed in triplicate. Construction of the algorithm was done using "R", a statistical software package. The second and third batches were used for validating and testing the algorithm, respectively. They each contained 32 samples of *B. pseudomallei*, 32 samples of *B. mallei* and 32 samples of the near neighbors. All organisms were cultured under the four growth conditions and were processed in duplicate. The algorithm was applied to the test data set in a blind manner. The classification of each tested vial was made by the computer algorithm using these data without knowledge of what species or how many samples of each species were in the data set. Operation of the algorithm was done without input from the experimenters. The test data set was never used in algorithm construction.

### 3.2.10 Biomarker identification and confirmation

Fatty acid methyl esters (FAMEs) were named and their structures predicted by searching the Mass Spectral Library that is available through the *National Institute of Standards and Technology* (NIST). The majority of the FAMEs were previously described in the literature, which allowed us to further confirm their names and structures.[58, 65, 66] In addition, the identities of the biomarkers generated from poly(3HBA-*co*-3HVA) were confirmed by subjecting a poly(3HBA-*co*-3HVA) standard (Sarchem Laboratories, Farmingdale, NJ, USA) to the TCM procedure and subsequent GC-MS analysis.

### 3.2.11 Detection limit

A suspension of *B. thailandensis* cells in methanol was prepared as described above. The concentration of the bacterial cells in the suspension was determined by using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA, USA). The suspension was then serially diluted in methanol to extinction. Subsequently, two samples from each dilution were prepared, processed and analyzed as described above. This procedure was repeated on four different occasions. *B. thailandensis* was used to estimate the detection limit because it displayed very low abundance of a key biomarker (2-BAME).

## 3.3 RESULTS AND DISCUSSION

### 3.3.1 Biomarker identification and confirmation

The TCM procedure generated biomarkers from the cellular fatty acids and the poly(3HBA-*co*-3HVA) polymers that were found in the different species of *Burkholderia*. The cellular fatty acids were converted to their respective fatty acid methyl esters (FAMEs).

Saturated, unsaturated, hydroxyl and cyclo FAMEs from C4 to C18 were observed. The most common saturated fatty acids that were detected in the *Burkholderia* species include C12:0, C14:0, C15:0, C16:0, iso C17:0, C18:0 and C22:0. The most common hydroxy fatty acids that were detected include 2-OH C14:0, 3-OH C14:0, 2-OH C16:0, 3-OH C16:0 and 10-OH C18:0. The unsaturated and cyclo fatty acids were C16:1ω7c, C18:1ω7c and cyclo C17:0.

The poly(3HBA-*co*-3HVA) polymers were fragmented into six important biomarkers. The biomarkers were identified as 3-butyric acid methyl ester (3-BAME), 2-butyric acid methyl ester (2-BAME), 3-valeric acid methyl ester (3-VAME), 2-valeric acid methyl ester(2-VAME), 3-hydroxy butyric acid methyl ester (3-HBAME) and 3-hydroxy valeric acid methyl ester (3-HVAME). The six peaks associated with these biomarkers are shown in the total ion chromatogram (TIC) of *B. pseudomallei* PHLS 72 which was cultured on Blood Agar at 37 °C (Figure 3.1). The source of these different peaks was confirmed by subjecting a poly(3HBA-*co*-3HVA) standard to the TCM procedure (see above). The TIC of the poly(3HBA-*co*-3HVA) standard after the TCM procedure is shown in Figure 3.2.

The different fragments that were generated by exposing the poly(3HBA-*co*-3HVA) standard to the TCM procedure revealed information regarding the reactions that occur during the procedure itself. Initially, the C-O bond between the acyl group and the oxygen was cleaved and hydrolyzed to the parent carboxylic acids, 3-hydroxy butyric acid (3-HBA) and 3-hydroxy valeric acid (3-HVA). These carboxylic acids were then methylated into their corresponding methyl esters (3-HBAME and 3-HVAME). After dehydration and methylation, each of the acids yielded two additional compounds, 2-BAME and 3-BAME or 2-VAME and 3-VAME (Figure 3.2). The biomarkers derived from 3-HBA (3-BAME, 2-BAME and 3-HBAME) were more

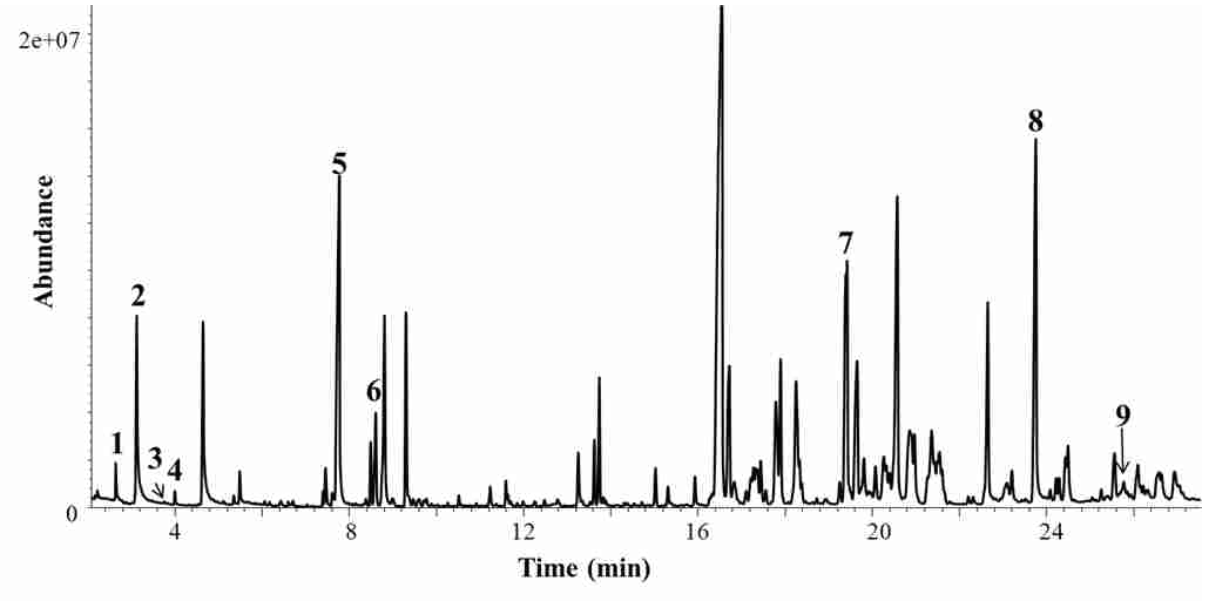



**Figure 3.1.** Total-ion chromatogram (TIC) of *Burkholderia pseudomallei* PHLS 72. This specific sample was cultured at 37 °C on Blood Agar. The target biomarkers for the differentiation of the various *Burkholderia* species are labeled 1-9. Peak 1, 3-BAME (*m/z* 69); peak 2, 2-BAME (*m/z* 69); peak 3, 3-VAME (*m/z* 55); peak 4, 2-VAME (*m/z* 83); peak 5, 3-HBAME (*m/z* 43); peak 6, 3-HVAME (*m/z* 43); peak 7, 2-OH C14:0 (*m/z* 199); peak 8, 3-OH C16:0 (*m/z* 103); peak 9, unidentified unsaturated aldehyde (*m/z* 55).

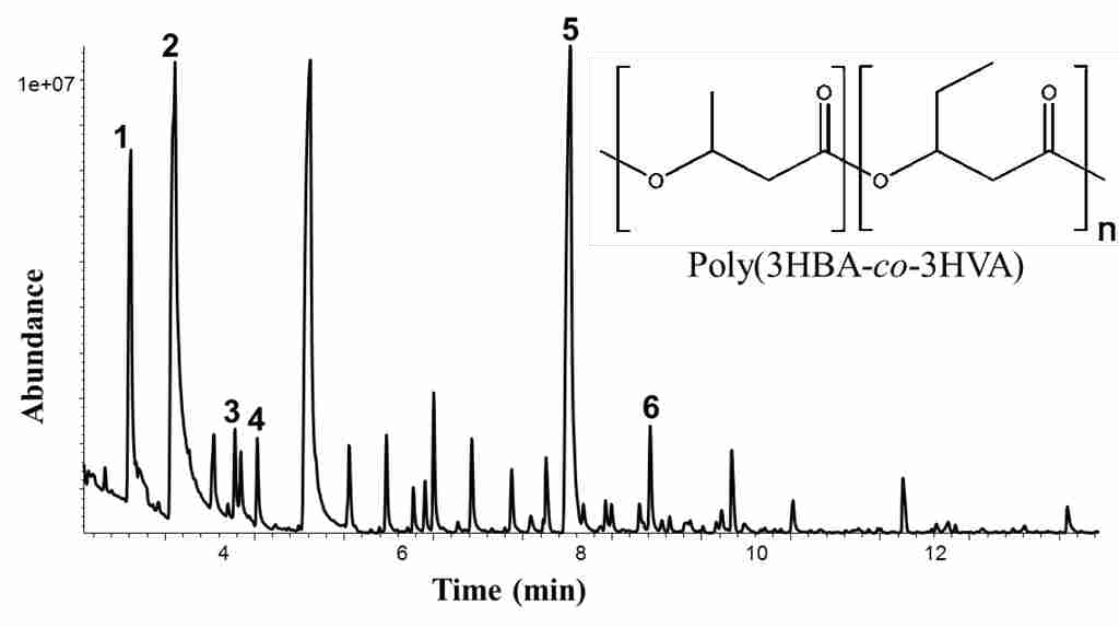



**Figure 3.2.** Total-ion chromatogram (TIC) of the poly(3HBA-*co*-3HVA) standard following the TCM procedure. Peaks 1-6 are 3-BAME, 2-BAME, 3-VAME, 2-VAME, 3-HBAME and 3-HVAME, respectively.

abundant than the biomarkers from 3-HVA (3-VAME, 2-VAME and 3-HVAME). This resulted from the standard polymer containing more 3-HBA than 3-HVA.

### 3.3.2 Algorithm construction and testing

The biomarkers were tested to see if they could be used to differentiate between the select agents and the other *Burkholderia* species. The 2-OH C14:0 biomarker was found exclusively in *B. pseudomallei*, which allowed us to easily distinguish *B. pseudomallei* from the other species. The six biomarkers that resulted from fragmenting the poly(3HBA-*co*-3HVA) polymers via the TCM procedure were present in all of the *Burkholderia* species. Nevertheless, these biomarkers were found to be useful in differentiating between *B. mallei* and near neighbors. An unidentified unsaturated aldehyde peak and the ratio of C18:0 to C14:0 were also found to be useful in differentiating *B. mallei* from the near neighbors. The unidentified aldehyde and the six fragments of poly(3HBA-*co*-3HVA) were more abundant in the near neighbors than in *B. mallei*.

The differentiation algorithm was used to predict the identities of all samples that were prepared in this study; a schematic of the final differentiation algorithm is shown in Figure 3.3. The results from applying the algorithm to the samples are summarized in Table 3.2. Of the 25 samples that were misidentified, 13 were *B. vietnamiensis*, 11 were *B. cepacia* and only one was *B. multivorans*. All of these samples were incorrectly identified as *B. mallei* because they displayed an unusually low abundance of the six biomarkers that were generated from poly(3HBA-*co*-3HVA). In addition, the 13 samples of *B. vietnamiensis* exhibited large differences in the abundance of important biomarkers. The variation in these samples made it difficult to effectively use the ratio of C18:0 to C14:0, which is typically higher in *B. vietnamiensis*, to identify these samples. However, this ratio was generally higher among *B. vietnamiensis* samples when compared to samples with roughly equal abundance. The peak area

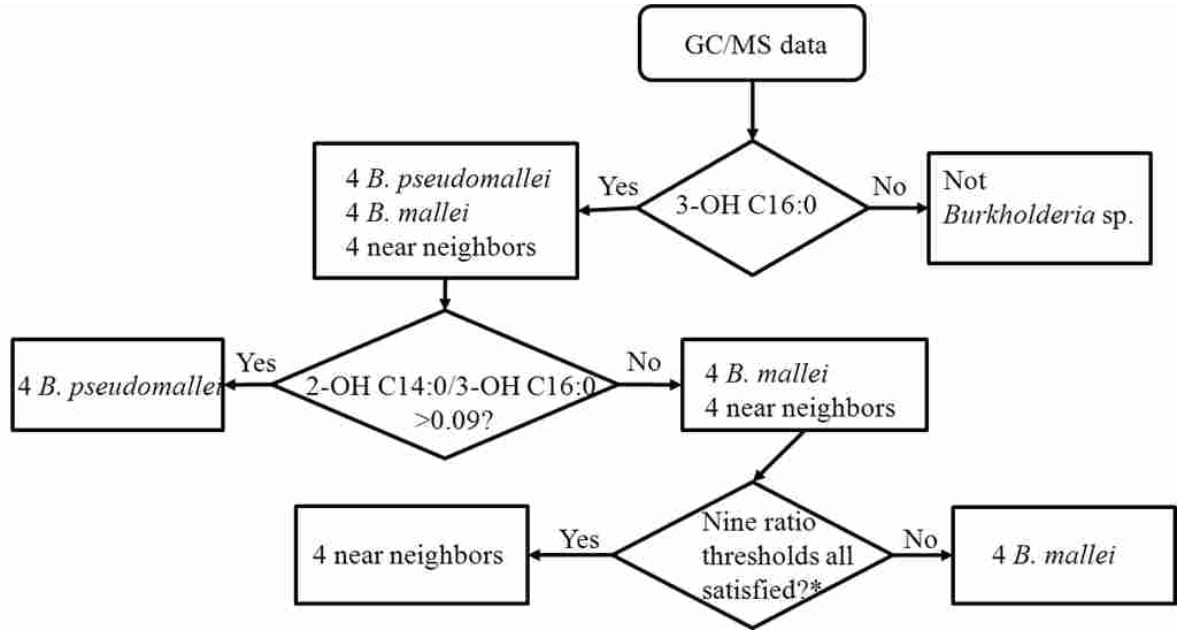



**Figure 3.3.** Decision tree for differentiation of the *Burkholderia* species. *The nine ratios are as follows: 2-BAME/ 3-OH C16:0 > 0.8; 3-BAME/ 3-OH C16:0 > 0.024; 2-VAME/ 3-OH C16:0> 0.005; 3-VAME/ 3-OH C16:0 > 0.009; 3-HVAME/ 3-OH C16:0 > 0.0015; 3-HBAME/ 3-OH C16:0 > 0.5; butane, 1,1,3-tri-methoxy- / 3-OH C16:0> 0.5; unidentified unsaturated aldehyde/ 3-OH C16:0 < 0.036; C18:0/C14:0 > 3.

**Table 3.2.** Evaluation of the automated differentiation algorithm against samples from various *Burkholderia* species.

| sample type | number in study | number correct | percent correct |
|---|---|---|---|
| *Burkholderia pseudomallei* | 96 | 96 | 100% |
| *Burkholderia mallei* | 96 | 96 | 100% |
| Near neighbors | 208 | 183 | 88% |
| Total | 304 | 279 | 92% |

of each biomarker was divided by the peak area of 3-OH C16:0, which is a biomarker that displayed approximately equal relative abundance in all strains. This step was taken to make all biomarkers invariant to abundance.

While testing the algorithm against the various samples that are described above, we also investigated the detection limit of this new method. We determined that the limit of detection for *B. thailandensis* is approximately 4,000 cells when using the 2-BAME biomarker. In actuality, the abundance of 2-BAME is lower in *B. thailandensis* than in *B. pseudomallei*. Therefore, we would expect the detection limit to be lower for *B. pseudomallei.*

### 3.3.3 MIDI detection method

As stated previously, the MIDI method was used to confirm the identities of the various *Burkholderia* species that were used in this study. This method correctly identified all of the *B. pseudomallei* and *B. mallei* strains. However, it was unable to correctly differentiate the near neighbors from *B. pseudomallei* and *B. mallei*. For example, *B. thailandensis* was incorrectly identified as *Escherichia coli*, *Shigella sonnei*, *Shigella flexneri*, *B. pseudomallei* and *B. mallei*. Similarly, *B. cepacia* and *B. vietnamiensis* were misidentified as *B. gladioli* and *B. mallei*. Additionally, the method was simply unable to find a match for *B. multivorans.*

### 3.3.4 Discussion

In this study, we were able to differentiate *B. pseudomallei* from *B. mallei* and the near neighbors based on the presence of 2-OH C14:0. This biomarker was found exclusively in *B. pseudomallei*, which is in agreement with several published studies.[38, 66, 67] In fact, Novem et al.[67] proposed that the 2-OH C14:0 fatty acid in *B. pseudomallei* might allow the bacterium to evade immune responses and thus avoid being cleared from the host. In addition, they proved that the

C14:0, 2-OH C14:0, 3-OH C14:0 and 3-OH C16:0 fatty acids are derived from lipid A, which is the innermost region of the lipopolysaccharide molecule.

We investigated the possibility of using the 2-OH C16:0 fatty acid to aid in the differentiation of *B. pseudomallei* from the other species of *Burkholderia*. It was previously reported that the 2-OH C16:0 fatty acid was absent in *B. thailandensis*, but present in *B. pseudomallei*.[66] However, we detected this biomarker in both of these species. Given this information, we chose not to use the 2-OH C16:0 fatty acid in our differentiation assay.

The C12:0, C14:0 and C18:0 saturated fatty acids were some of the most stable (i.e., independent of growth conditions) biomarkers that were observed in this study. In 2006, Krejci and Kroppenstedt[58] reported similar observations. In addition, they reported that *B. vietnamiensis* had a lower ratio of C14:0 to C18:0 than other species within the *B. cepacia* complex. They used this ratio to differentiate *B. vietnamiensis* from other members of the *B. cepacia* complex. We used the inverse of this ratio to assist in the differentiation of *B. mallei* from the near neighbors.

*Burkholderia* species are often considered promising candidates for the biosynthesis of substantial amounts of poly(3-HBA-*co*-3HVA). However, we observed that different amounts of poly(3HB-*co*-3HV) were formed by each *Burkholderia* species. For example, the near neighbors displayed very low abundances of poly(3HBA-*co*-3HVA) and *B. mallei* displayed even less. The low levels of expression in these species of *Burkholderia* required us to use the C18:0 to C14:0 ratio, an unidentified unsaturated aldehyde peak (*m/z* 55), and the six biomarkers that were generated from poly(3HBA-*co*-3HVA) to differentiate *B. mallei* from the near neighbors.

We are currently developing statistical methods to identify other biomarkers that might aid in differentiation; several biomarkers have already been identified. Preliminary results indicate that these biomarkers will allow us to fully differentiate *B. cepacia*, *B. multivorans* and

*B. vietnamiensis* from each other and from *B. mallei*. We are also investigating the ability of this method to detect and differentiate each of the *Burkholderia* species from environmental samples and mixed samples.

Despite the continuing research, the method described in this study represents a novel approach for the differentiation of *B. pseudomallei*, *B. mallei*, *B. thailandensis* and several members of the *B. cepacia* complex (*B. cepacia*, *B. multivorans* and *B. vietnamiensis*) using a GC-MS system. The correct identification of these microbes from the environment is essential to research involving disease transmission and epidemiology in endemic areas. The results of this study indicate that the method is fast, acurate and simple to use. In addition, the the results show that the algorithm is robust against differernt growth conditions (medium and temperature). This assay may also prove beneficial in a clinical diagnostic setting, where the rapid identification of *B. pseudomallei* and *B. mallei* is essential to effective treatment. In addition, this method could be easily employed after a suspected biological attack to confirm the presence of either *B. pseudomallei* or *B. mallei*.

## 3.4 CONCLUSIONS

We have developed a simple GC-MS method for the detection and differentiation of *Burkholderia pseudomallei*, *Burkholderia mallei*, *Burkholderia thailandensis* and several members of the *Burkholderia cepacia* complex. Biomarkers were generated by one-step TCM and analyzed using a GC-MS system. Fragments of poly(3HBA-*co*-3HVA) produced by TCM were useful biomarkers. Several cellular fatty acid methyl esters were important in differentiating the various *Burkholderia* species. A statistical discrimination algorithm was

constructed using a combination of biomarkers. The identities of four *B. pseudomallei* strains, four *B. mallei* strains and one strain of each near neighbor were confirmed in a statistically designed test using the algorithm. The detection limit for this method was found to be approximately 4000 cells. The method is fast, acurate and easy to use. The algorithm is robust against different growth conditions (medium and temperature). This assay may prove beneficial in a clinical diagnostic setting, where the rapid identification of *B. pseudomallei* is essential to effective treatment. This method could also be easily employed after a biological attack to confirm the presence of either *B. pseudomallei* or *B. mallei*.

## 3.5 REFERENCES


1. Howe, C.; Sampath, A.; Spotnitz, M., *J. Infect. Dis.* **1971,** *124*, 598.
2. Chaowagul, W.; White, N. J.; Dance, D. A.; Wattanagoon, Y.; Naigowit, P.; Davis, T. M.; Looareesuwan, S.; Pitakwatchara, N., *J. Infect. Dis.* **1989,** *159*, 890-899.
3. Currie, B. J.; Fisher, D. A.; Howard, D. M.; Burrow, J. N.; Lo, D.; Selva-Nayagam, S.; Anstey, N. M.; Huffam, S. E.; Snelling, P. L.; Marks, P. J.; Stephens, D. P.; Lum, G. D.; Jacups, S. P.; Krause, V. L., *Clin. Infect. Dis.* **2000,** *31*, 981.
4. Cheng, A. C.; Currie, B. J., *Clin.Microbiol. Rev.* **2005,** *18*, 383.
5. Limmathurotsakul, D.; Wongratanacheewin, S.; Teerawattanasook, N.; Wongsuvan, G.; Chaisuksant, S.; Chetchotisakd, P.; Chaowagul, W.; Day, N. P.; Peacock, S. J., *Am. J. Trop. Med. Hyg.* **2010,** *82*, 1113.
6. White, N. J.; Dance, D. A.; Chaowagul, W.; Wattanagoon, Y.; Wuthiekanun, V.; Pitakwatchara, N., *Lancet* **1989,** *2*, 697.
7. Sanford, J. P., Pseudomonas Species (Including Melioidosis and Glanders). In *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*, Mandell, G. L.; Bennett, J. E.; Dolin, R., Eds. Chruchill Livingstone: New York, 1995; p 2003.
8. Suputtamongkol, Y.; Hall, A. J.; Dance, D. A.; Chaowagul, W.; Rajchanuvong, A.; Smith, M. D.; White, N. J., *Int. J. Epidemiol.* **1994,** *23*, 1082.
9. John, T. J.; Jesudason, M. V.; Lalitha, M. K.; Ganesh, A.; Mohandas, V.; Cherian, T.; Mathai, D.; Chandy, M. J., *Indian J. Med. Res.* **1996,** *103*, 62.
10. Yang, S., *Acta Trop.* **2000,** *77*, 157.
11. Currie, B. J.; Dance, D. A.; Cheng, A. C., *Trans. R. Soc. Trop. Med. Hyg.* **2008,** *102 Suppl 1*, S1.
12. Chen, Y. S.; Lin, H. H.; Mu, J. J.; Chiang, C. S.; Chen, C. H.; Buu, L. M.; Lin, Y. E.; Chen, Y. L., *J. Clin. Microbiol.* **2010,** *48*, 1432.

13. Brilhante, R. S.; Bandeira, T. J.; Cordeiro, R. A.; Grangeiro, T. B.; Lima, R. A.; Ribeiro, J. F.; Castelo-Branco, D. S.; Rodrigues, J. L.; Coelho, I. C.; Magalhaes, F. G.; Rocha, M. F.; Sidrim, J. J., *J. Clin. Microbiol.* **2012,** *50*, 3349.
14. Gregory, B. C.; Waag, D. M., Glanders. In *Military Medicine*, Dembek, Z. F., Ed. Department of Defense Office of The Surgeon General US Army Borden Institute: Washington, D.C., 2007; p 121.
15. Whitlock, G. C.; Estes, D. M.; Torres, A. G., *FEMS Microbiol. Lett.* **2007,** *277*, 115.
16. Rogul, M.; Brendle, J. J.; Haapala, D. K.; Alexander, A. D., *J. Bacteriol.* **1970,** *101*, 827.
17. Yabuuchi, E.; Kosako, Y.; Oyaizu, H.; Yano, I.; Hotta, H.; Hashimoto, Y.; Ezaki, T.; Arakawa, M., *Microbiol. Immunol.* **1992,** *36*, 1251.
18. Godoy, D.; Randle, G.; Simpson, A. J.; Aanensen, D. M.; Pitt, T. L.; Kinoshita, R.; Spratt, B. G., *J. Clin. Microbiol.* **2003,** *41*, 2068-2079.
19. Sprague, L. D.; Zysk, G.; Hagen, R. M.; Meyer, H.; Ellis, J.; Anuntagool, N.; Gauthier, Y.; Neubauer, H., *FEMS Immunol. Med. Microbiol.* **2002,** *34*, 231.
20. Gee, J. E.; Sacchi, C. T.; Glass, M. B.; De, B. K.; Weyant, R. S.; Levett, P. N.; Whitney, A. M.; Hoffmaster, A. R.; Popovic, T., *J. Clin. Microbiol.* **2003,** *41*, 4647-4654.
21. Lee, M. A.; Wang, D.; Yap, E. H., *FEMS Immunol. Med. Microbiol.* **2005,** *43*, 413.
22. Brett, P. J.; DeShazer, D.; Woods, D. E., *Int. J. Syst. Bacteriol.* **1998,** *48 Pt 1*, 317-320.
23. Dharakul, T.; Tassaneetrithep, B.; Trakulsomboon, S.; Songsivilai, S., *J. Clin. Microbiol.* **1999,** *37*, 1906.
24. Lertpatanasuwan, N.; Sermsri, K.; Petkaseam, A.; Trakulsomboon, S.; Thamlikitkul, V.; Suputtamongkol, Y., *Clin. Infect. Dis.* **1999,** *28*, 927.
25. Glass, M. B.; Gee, J. E.; Steigerwalt, A. G.; Cavuoti, D.; Barton, T.; Hardy, R. D.; Godoy, D.; Spratt, B. G.; Clark, T. A.; Wilkins, P. P., *J. Clin. Microbiol.* **2006,** *44*, 4601.
26. Thibault, F. M.; Valade, E.; Vidal, D. R., *J. Clin. Microbiol.* **2004,** *42*, 5871.
27. Vandamme, P.; Dawyndt, P., *Syst. Appl. Microbiol.* **2011,** *34*, 87-95.
28. Lipuma, J. J., *Curr. Opin. Pulm. Med.* **2005,** *11*, 528.
29. Walsh, A. L.; Smith, M. D.; Wuthiekanun, V.; Suputtamongkol, Y.; Desakorn, V.; Chaowagul, W.; White, N. J., *J. Clin. Pathol.* **1994,** *47*, 377.
30. Inglis, T. J.; Chiang, D.; Lee, G. S.; Chor-Kiang, L., *Pathology* **1998,** *30*, 62.
31. Bauernfeind, A.; Schneider, I.; Jungwirth, R.; Roller, C., *J. Clin. Microbiol.* **1999,** *37*, 1335.
32. Samosornsuk, N.; Lulitanond, A.; Saenla, N.; Anuntagool, N.; Wongratanacheewin, S.; Sirisinha, S., *Am. J. Trop. Med. Hyg.* **1999,** *61*, 735.
33. Steinmetz, I.; Reganzerowski, A.; Brenneke, B.; Haussler, S.; Simpson, A.; White, N. J., *J. Clin. Microbiol.* **1999,** *37*, 225.
34. Anuntagool, N.; Naigowit, P.; Petkanchanapong, V.; Aramsri, P.; Panichakul, T.; Sirisinha, S., *J. Med. Microbiol.* **2000,** *49*, 1075.
35. Mahenthiralingam, E.; Bischof, J.; Byrne, S. K.; Radomski, C.; Davies, J. E.; Av-Gay, Y.; Vandamme, P., *J. Clin. Microbiol.* **2000,** *38*, 3165.
36. Chenthamarakshan, V.; Vadivelu, J.; Puthucheary, S. D., *Diagn. Microbiol. Infect. Dis.* **2001,** *39*, 1.
37. Lowe, P.; Engler, C.; Norton, R., *J. Clin. Microbiol.* **2002,** *40*, 4625.
38. Inglis, T. J.; Aravena-Roman, M.; Ching, S.; Croft, K.; Wuthiekanun, V.; Mee, B. J., *J. Clin. Microbiol.* **2003,** *41*, 4812.
39. Glass, M. B.; Popovic, T., *J. Clin. Microbiol.* **2005,** *43*, 479.

40. Inglis, T. J.; Merritt, A.; Chidlow, G.; Aravena-Roman, M.; Harnett, G., *J. Clin. Microbiol.* **2005,** *43*, 2201.
41. Wuthiekanun, V.; Desakorn, V.; Wongsuvan, G.; Amornchai, P.; Cheng, A. C.; Maharjan, B.; Limmathurotsakul, D.; Chierakul, W.; White, N. J.; Day, N. P.; Peacock, S. J., *Clin. Diagn. Lab. Immunol.* **2005,** *12*, 555.
42. Cheng, A. C.; O'Brien, M.; Freeman, K.; Lum, G.; Currie, B. J., *Am. J. Trop. Med. Hyg.* **2006,** *74*, 330.
43. Novak, R. T.; Glass, M. B.; Gee, J. E.; Gal, D.; Mayo, M. J.; Currie, B. J.; Wilkins, P. P., *J. Clin. Microbiol.* **2006,** *44*, 85.
44. Amornchai, P.; Chierakul, W.; Wuthiekanun, V.; Mahakhunkijcharoen, Y.; Phetsouvanh, R.; Currie, B. J.; Newton, P. N.; van Vinh Chau, N.; Wongratanacheewin, S.; Day, N. P.; Peacock, S. J., *J. Clin. Microbiol.* **2007,** *45*, 3774.
45. Bowers, J. R.; Engelthaler, D. M.; Ginther, J. L.; Pearson, T.; Peacock, S. J.; Tuanyok, A.; Wagner, D. M.; Currie, B. J.; Keim, P. S., *PLOS ONE* **2010,** *5*, e15413.
46. Hagen, R. M.; Frickmann, H.; Elschner, M.; Melzer, F.; Neubauer, H.; Gauthier, Y. P.; Racz, P.; Poppert, S., *Int. J. Med. Microbiol.* **2011,** *301*, 585.
47. Puthucheary, S. D.; Puah, S. M.; Chai, H. C.; Thong, K. L.; Chua, K. H., *J. Mol. Microbiol. Biotechnol.* **2012,** *22*, 198.
48. White, N. J., *Lancet* **2003,** *361*, 1715-1722.
49. Wuthiekanun, V.; Amornchai, P.; Chierakul, W.; Cheng, A. C.; White, N. J.; Peacock, S. J.; Day, N. P., *J. Clin. Microbiol.* **2004,** *42*, 3435.
50. Kiska, D. L.; Kerr, A.; Jones, M. C.; Caracciolo, J. A.; Eskridge, B.; Jordan, M.; Miller, S.; Hughes, D.; King, N.; Gilligan, P. H., *J. Clin. Microbiol.* **1996,** *34*, 886.
51. McMenamin, J. D.; Zaccone, T. M.; Coenye, T.; Vandamme, P.; LiPuma, J. J., *Chest* **2000,** *117*, 1661.
52. Wongtrakoongate, P.; Mongkoldhumrongkul, N.; Chaijan, S.; Kamchonwongpaisan, S.; Tungpradabkul, S., *Mol. Cell. Probes* **2007,** *21*, 81.
53. Chantratita, N.; Meumann, E.; Thanwisai, A.; Limmathurotsakul, D.; Wuthiekanun, V.; Wannapasni, S.; Tumapa, S.; Day, N. P.; Peacock, S. J., *J. Clin. Microbiol.* **2008,** *46*, 568.
54. Klein, D., *Trends Mol. Med.* **2002,** *8*, 257.
55. Fredricks, D. N.; Relman, D. A., *Clin. Infect. Dis.* **1999,** *29*, 475.
56. Yang, S.; Rothman, R. E., *Lancet Infect. Dis.* **2004,** *4*, 337.
57. Vandamme, P.; Pot, B.; Gillis, M.; de Vos, P.; Kersters, K.; Swings, J., *Microbiol. Rev.* **1996,** *60*, 407.
58. Krejci, E.; Kroppenstedt, R. M., *J. Clin. Microbiol.* **2006,** *44*, 1159-1164.
59. Steinbuchel, A., *Acta Biotechnol.* **1991,** *11*, 419.
60. Steinbuchel, A.; Valentine, H. E., *FEMS Microbiol. Lett.* **1995,** *128*, 219.
61. Kim do, Y.; Park, D. S.; Kwon, S. B.; Chung, M. G.; Bae, K. S.; Park, H. Y.; Rhee, Y. H., *J. Microbiol.* **2009,** *47*, 651.
62. Li, D.; Truong, T. V.; Bills, T. M.; Holt, B. C.; Vanderwerken, D. N.; Williams, J. R.; Acharya, A.; Robison, R. A.; Tolley, H. D.; Lee, M. L., *Anal. Chem.* **2012,** *84*, 1637-1644.
63. Antolin, E. M.; Delange, D. M.; Canavaciolo, V. G., *J. Pharm. Biomed. Anal.* **2008,** *46*, 194.

64. U'Ren, J. M.; Van Ert, M. N.; Schupp, J. M.; Easterday, W. R.; Simonson, T. S.; Okinaka, R. T.; Pearson, T.; Keim, P., *J. Clin. Microbiol.* **2005,** *43*, 5771.
65. Phung, L. V.; Tran, T. B.; Hotta, H.; Yabuuchi, E.; Yano, I., *Microbiol. Immunol.* **1995,** *39*, 105.
66. Levy, A.; Merritt, A. J.; Aravena-Roman, M.; Hodge, M. M.; Inglis, T. J., *Am. J. Trop. Med. Hyg.* **2008,** *78*, 599.
67. Novem, V.; Shui, G.; Wang, D.; Bendt, A. K.; Sim, S. H.; Liu, Y.; Thong, T. W.; Sivalingam, S. P.; Ooi, E. E.; Wenk, M. R.; Tan, G., *Clin. Vaccine Immunol.* **2009,** *16*, 1420.

# 4 GENERAL PROCEDURE FOR GC-MS DETECTION OF PRIMARY TIER 1 BACTERIAL AGENTS

## 4.1 INTRODUCTION

Tier 1 biological select agents and toxins include 13 microorganisms (Table 4.1) according to an October 2012 revision of the select agent regulations.[1] There are six important bacterial Tier 1 agents, including *Bacillus anthracis, Francisella tularensis, Yersinia pestis, Burkholderia mallei, Burkholderia pseudomallei* and *Clostridium*.

The prospect of using microorganisms for terrorism has made methods for Tier 1 agent detection of high priority. Ideally, detection platforms should be capable of rapidly detecting and confirming biothreat agents directly from their closely related nonpathogenic near neighbors. Furthermore, such assays must be sensitive, fast, specific and capable of detecting a variety of biothreat agents using the same procedure, because suspect unknown samples may contain different types of bioagents or interfering analytes. For known biothreat agents, different detection assays can be designed according to their characteristics. However, it is difficult to develop universal methods (both sample preparation and sample analysis) for all of the biothreat agents. For this reason, suspect target analytes usually must be isolated or purified prior to analysis and identification. These steps can add hours or days to detection protocols and often cannot be performed in the field.

This chapter describes the development of a general procedure for detection of primary Tier 1 bacterial agents, including *Bacillus anthracis, Burkholderia mallei, Burkholderia*

**Table 4.1.** Tier 1 biological select agents and toxins.

| agents | disease | recommended Biosafety levels |
|---|---|---|
| Ebola virus | Ebola hemorrhagic fever | 4 |
| Marburg virus | Marburg hemorrhagic fever | 4 |
| Variola major virus | Smallpox | 4 |
| Variola minor virus | Smallpox | 4 |
| Botulinum neurotoxin | Botulism | 2 |
| Botulinum neurotoxin producing species of *Clostridium* | Botulism | 2 |
| ***Bacillus anthracis*** | **Anthrax** | **2 or 3** |
| ***Francisella tularensis*** | **Tularenmia** | **2 or 3** |
| ***Yersinia pestis*** | **Plague** | **2 or 3** |
| ***Burkholderia mallei*** | **Glanders** | **2 or 3** |
| ***Burkholderia pseudomallei*** | **Melioidosis** | **2 or 3** |
| Foot and Mouth Disease virus | FMDV | 3 |
| Rinderpest virus | Cattle plague | 3 |

*pseudomallei, Yersinia pestis and Francisella tularensis* incorporating TCM and GC-MS analysis. The total analysis time is approximately 25 min.

## 4.2 EXPERIMENTAL

The bacterial strains used in this study are listed in Table 4.2. The growth conditions for *Bacillus* and *Burkholderia* species have been described previously (Chapters 2 and 3).[2] *Yersinia* and near-neighbor isolates were cultured on two media types, Columbia agar with 5% sheep blood and Brain Heart Infusion (BHI) agar and at two temperatures, 28 and 37 °C. Plates containing microorganisms cultured under each of the four conditions were bathed in a $CO_2$ incubator with 5% $CO_2$ for 72 h. The bacteria were then harvested using Physiological Saline Solution (PSS), the suspension was washed by centrifugation, and the pellet was re-suspended in sterile HPLC water. *Francisella* isolates were cultured on two growth media, Mueller Hinton *Francisella* agar and BHI *Francisella* agar, at 32 and 37 °C. Plates were inoculated using an isolated colony and incubated for 96 h. Bacteria were harvested using PSS, and then washed and centrifuged. The resultant bacteria pellet was re-suspended in sterile HPLC water until TCM treatment. Procedures involving virulent isolates were performed according to BSL-3 requirements, while other procedures were conducted under BSL-2 conditions.

In this chapter, the TCM procedures for *Yersinia*, *Francisella* and *Bacillus* strains were the same as for *Burkholderia* species, which was previously described in detail (Chapter 3). Viability tests were also performed according to *Yersinia* and *Francisella* culture conditions, respectively. SPME extraction procedures were also previously described. A new GC temperature program was developed for analysis of all bacteria samples. A 30 m × 0.25 mm i.d.

**Table 4.2.** Tier 1 bacteria strains tested.

| species | strain | source* |
|---|---|---|
| *Bacillus anthracis* | A0231/K3677/#83 | LSU |
| | A0264/K7948/14 | LSU |
| | A0300/K2284 | LSU |
| | A2084/Ancestral Ames | LANL |
| | 1043/Sterne | LANL |
| | 6602/Pasteur | LANL |
| *Bacillus thuringiensis* | Kurstaki | NBFAC |
| | Al Hakam | UAB |
| | ATCC 19269 | ATCC |
| | ATCC 19270 | ATCC |
| | Israelensis/ATCC 35646 | ATCC |
| *Bacillus cereus* | ATCC 14579 | ATCC |
| *Bacillus atrophaeus* | ATCC 51189 | ATCC |
| *Bacillus subtilis* | Apizizenii/ATCC 6633 | ATCC |
| *Bacillus mycoides* | ATCC 6462 | ATCC |
| *Burkholderia pseudomallei* | 20 | PHLS |
| | 72 | PHLS |
| | 83 | PHLS |
| | 99/SID 4349 | PHLS |
| *Burkholderia mallei* | 10229 | NCTC |
| | 10248 | NCTC |
| | 10399 | ATCC |
| | Turkey #1 | CDC |
| *Burkholderia thailandensis* | 700388 | ATCC |
| *Burkholderia cepacia* | 10856 | ATCC |
| *Burkholderia multivorans* | BAA-247 | ATCC |
| *Burkholderia vietnamiensis* | BAA-248 | ATCC |

| | | |
|---|---|---|
| *Yersinia pestis* | TS | MSU |
| | CO-92 | DPG |
| | KIM D27 | MSU |
| | Yokohama (D15) | MSU |
| *Yersinia pseudotuberculosis* | ATCC 29833 | ATCC |
| | ATCC 13980 | LANL |
| *Yersinia enterocolitica* | ATCC 9610 | ATCC |
| *Yersinia frederiksenii* | Y61 | WUSTL |
| *Francisella tularensis* | SCHU S4 | Rocky Mountain Lab |
| | 1741 | New Mexico |
| | 80606987 | Utah |
| | 80501131 | Utah |
| | LVS | Austrilia |
| *Francisella novicida* | U112 | Dugway, Utah |
| | 6168 | University of Victoria |
| *Francisella philomiragia* | ATCC 25015 | Utah |

*LSU, Louisiana State University; LANL, Los Alamos National Laboratory; NBFAC, National Bioforensic Analysis Center; UAB, University of Alabama at Birmingham; ATCC, American Type Culture Collection; PHLS, Public Health Laboratory Service, UK; NCC, National Collection of Type Cultures; CDC, Center for Disease Control and Prevention, USA; MSU, Michigan State University, USA; DPG, Dugway Proving Ground, USA.

× 0.25 $\mu$m film thickness Zebron-FFAP column from Phenomenex (Torrance, CA, USA) was used for all of the GC-MS analyses. The injection port was 260 °C and the helium carrier gas pressure was 24 psi in the splitless mode.

The initial GC temperature program used for analysis of *Yersinia* species was 80 °C to 190 °C at 8 °C $min^{-1}$, 3 °C $min^{-1}$ to 210 °C (1-min hold), and finally 10 °C $min^{-1}$ to 240 °C. The initial GC-MS program for analysis of *Francisella* species was 80 °C to 180 °C at 15 °C $min^{-1}$, then 5 °C $min^{-1}$ to 190 °C (1-min hold), and finally 12 °C $min^{-1}$ to 250 °C (10-min hold).

The final temperature program used for all bacteria samples was 70 °C (1-min initial hold), to 180 °C at 15 °C $min^{-1}$, then 3 °C $min^{-1}$ to 210 °C, and finally 15 °C $min^{-1}$ to 250 °C (3-min hold). The total GC-MS analysis time was less than 24 min.

## 4.3 RESULTS AND DISCUSSION

Cellular fatty acids and sugars generated by TCM were used for differentiation of the *Yersinia* species. The most common fatty acid methyl esters observed were C12:0, iso-C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C14:1, C16:1, C18:1, cyclo C17:0, 3-OH C14:0, and 3-OH C18:0. Of these, C16:1, C18:1 and cyclo-C17:0 were reported to be distinct, highly abundant biomarkers for *Y. pestis*.[3] The *Yersinia* species could be identified by the fatty acid of 3-OH C14:0. An unidentified sugar derivative was found in 3 of the near neighbors (*Y. enterocolitica 9610*, *Y. fredericksenii Y61* and *Y. pseudotuberculosis 13980*), but not in *Y. pseudotuberculosis* 29833 and all four virulent isolates (Figure 4.1). The *Yersinia* species could be further differentiated by 2-OH C12:0 fatty acid, which was only present in *Y. fredericksenii* Y61 (Figure 4.2).

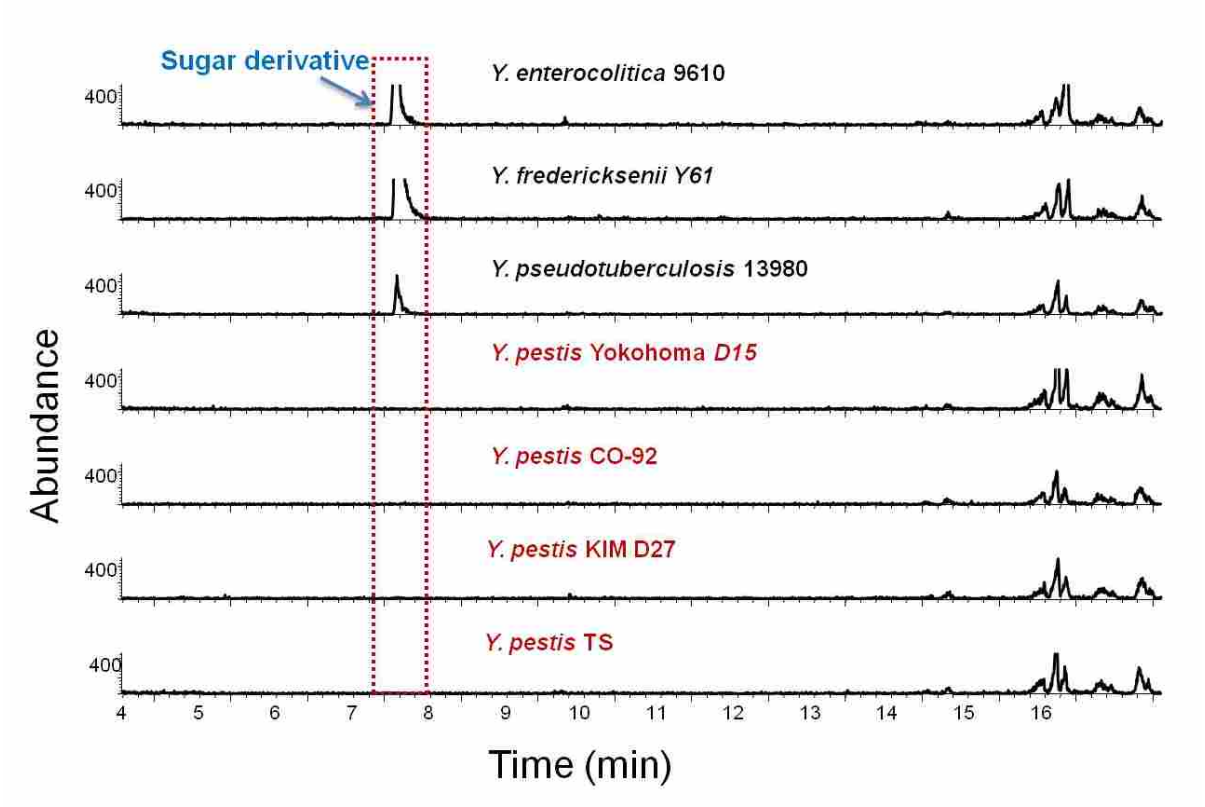



**Figure 4.1.** Extracted-ion chromatograms (*m/z* 110) for *Yersinia* species cultured on BHI agar at 28 °C.

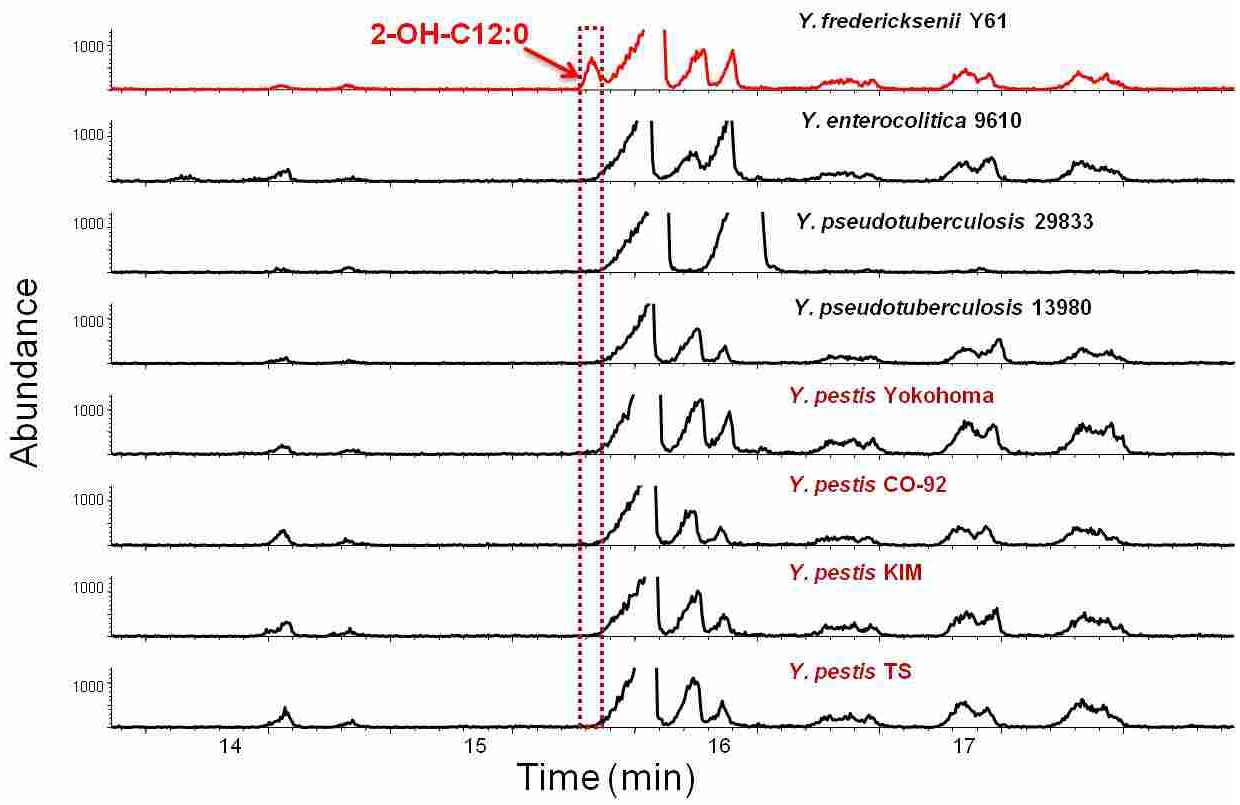



**Figure 4.2.** Extracted-ion chromatograms (*m/z* 97) of *Yersinia* species cultured on BHI agar at 28 °C.

As seen from the algorithm (Figure 4.3) for the *Yersinia* species, by using the biomarkers of 3-OH C14:0, 2-OH C12:0 and the unidentified sugar derivative, most of the virulent isolates could be differentiated from the non-virulent isolates, except *Y. pseudotuberculosis* 29833. The average correct identification percentage obtained in this study was 86% (Table 4.3). Most of the misidentified samples were *Y. pseudotuberculosis* 29833.

Fatty acids detected in high abundance from GC-MS analysis of the *Francisella* species are listed in Figure 4.4, and most of these are characteristic biomarkers reported in the literature.[4-7] Fatty acid C24:1$^{\Delta 15}$ was unique to the *Francisella* species and, therefore, was used to identify this species. The fatty acid 2-OH C10:0 was absent in *F. philomiragia* 015 (Figure 4.5). Methyl nicotinate, a small molecule biomarker, was detected in the four virulent *F. tularensis* species and *F. novicida* U112 (Figure 4.6). This is the first report of use of this compound as a biomarker for differentiation of *Francisella*. The algorithm for identification and differentiation of *Francisella* species is shown in Figure 4.7, and uses the biomarkers C24:1$^{\Delta 15}$, 2-OH C10:0 and methyl nicotinate. However, *F. novicida* Utah 112 could not be differentiated from the virulent *F. tularensis* isolates.

Differentiation of members of the *Bacillus*, *Burkholderia*, *Yersinia* and *Francisella* species was first accomplished using specific, optimized GC temperature programs for each species type. Since the species type(s) is typically not known in an actual terrorist attack, a single GC program that is applicable to all species is desirable. Therefore, a general GC-MS temperature program was developed. Using this program, all of the biomarkers used for identification and differentiation had different retention times and were easy to recognize using an automated statistical algorithm. As shown in Figure 4.8, total-ion chromatograms of the four

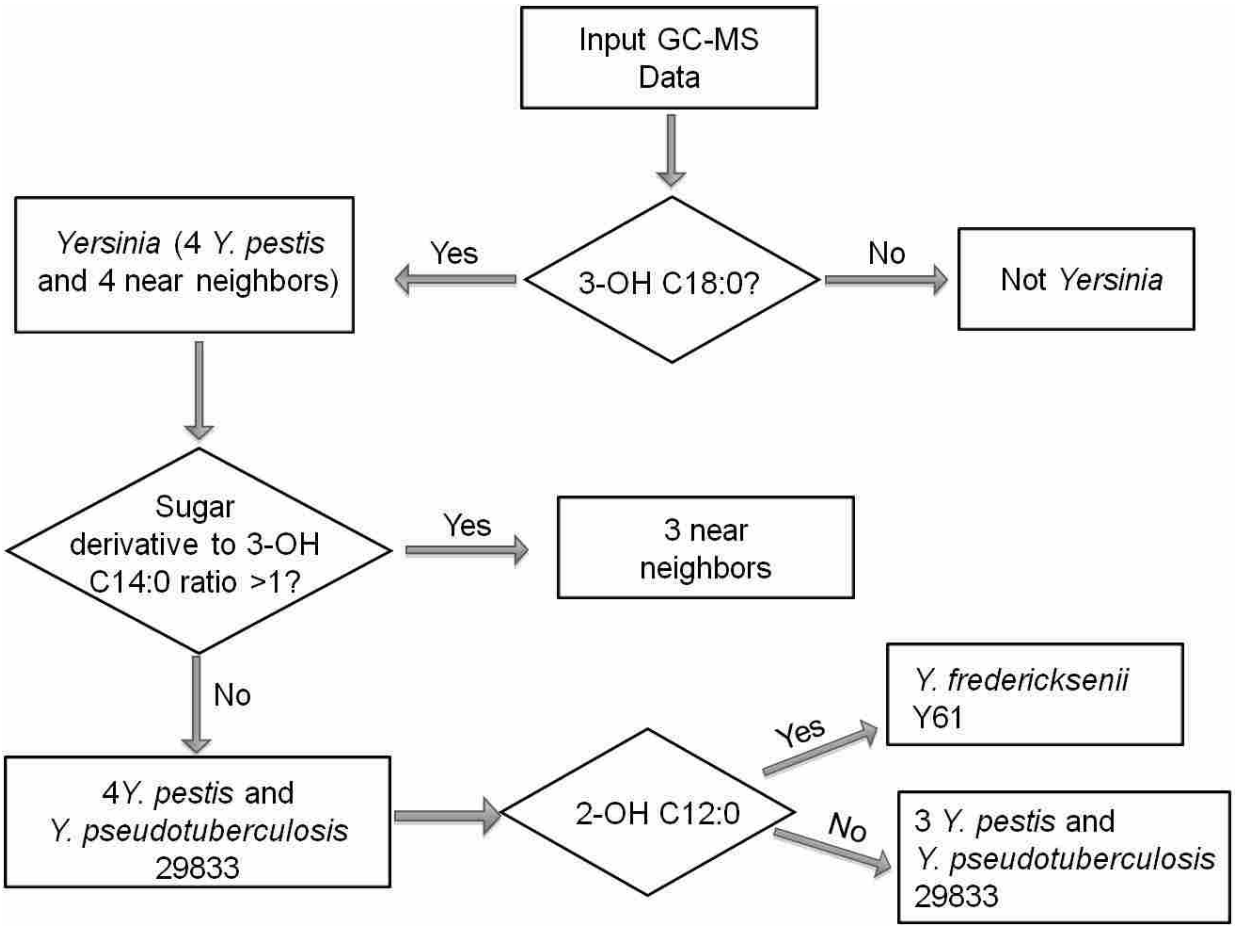
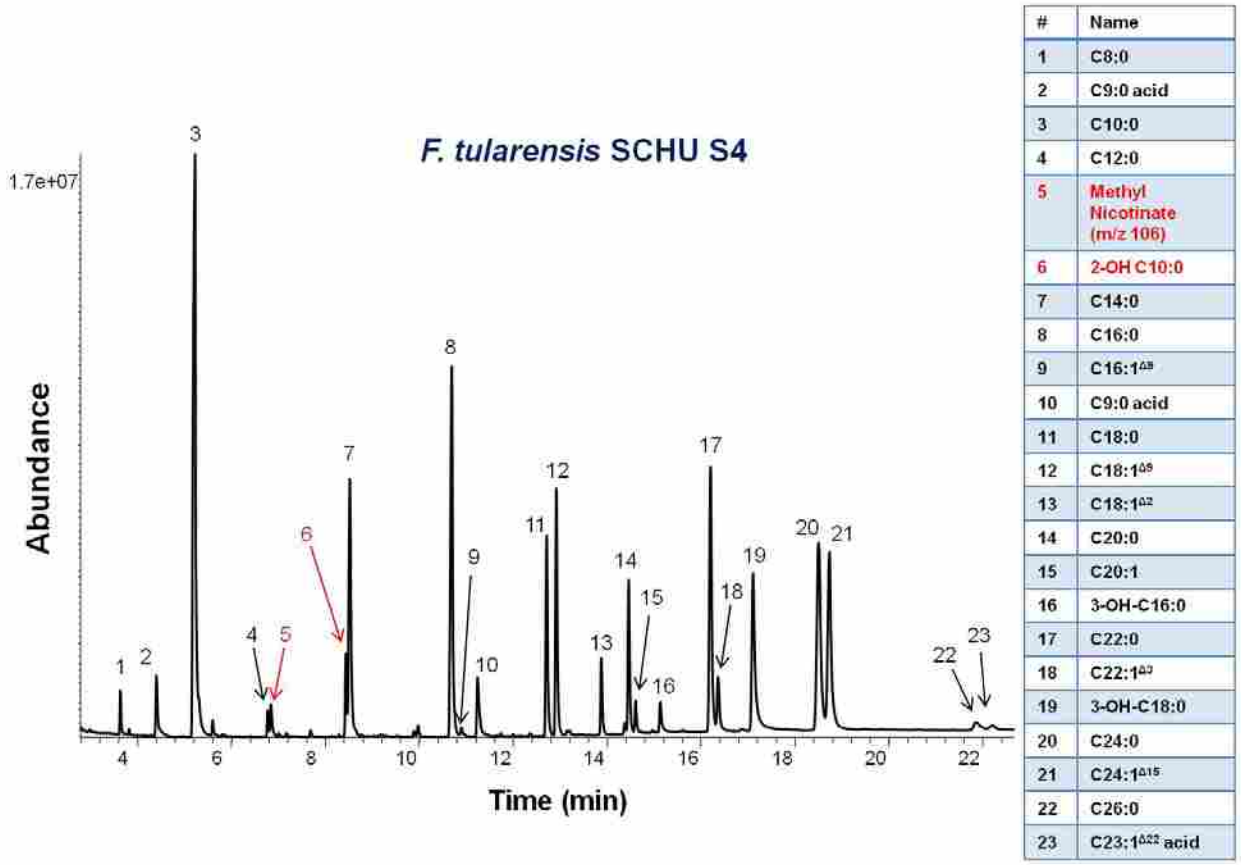



**Figure 4.3.** Decision tree for differentiation of *Yersinia* species.

**Table 4.3.** *Yersinia* differentiation results.

| | number in study | number correct | percent correct |
|---|---|---|---|
| *Y. pestis* | 114 | 112 | 98% |
| not *Y. pestis* | 120 | 89 | 74%* |
| total | 234 | 201 | 86% |

Note: *Y. pseudotuberculosis* 29833 samples could not be differentiated from virulent *Y. pestis.*




| # | Name |
|---|---|
| 1 | C8:0 |
| 2 | C9:0 acid |
| 3 | C10:0 |
| 4 | C12:0 |
| 5 | Methyl Nicotinate (m/z 106) |
| 6 | 2-OH C10:0 |
| 7 | C14:0 |
| 8 | C16:0 |
| 9 | $C16:1^{\Delta 9}$ |
| 10 | C9:0 acid |
| 11 | C18:0 |
| 12 | $C18:1^{\Delta 9}$ |
| 13 | $C18:1^{\Delta 2}$ |
| 14 | C20:0 |
| 15 | C20:1 |
| 16 | 3-OH-C16:0 |
| 17 | C22:0 |
| 18 | $C22:1^{\Delta 2}$ |
| 19 | 3-OH-C18:0 |
| 20 | C24:0 |
| 21 | $C24:1^{\Delta 15}$ |
| 22 | C26:0 |
| 23 | $C23:1^{\Delta 22}$ acid |

**Figure 4.4.** Total-ion chromatogram of *F. tularensis* SCHU S4 cultured on BHI agar at 37 °C.

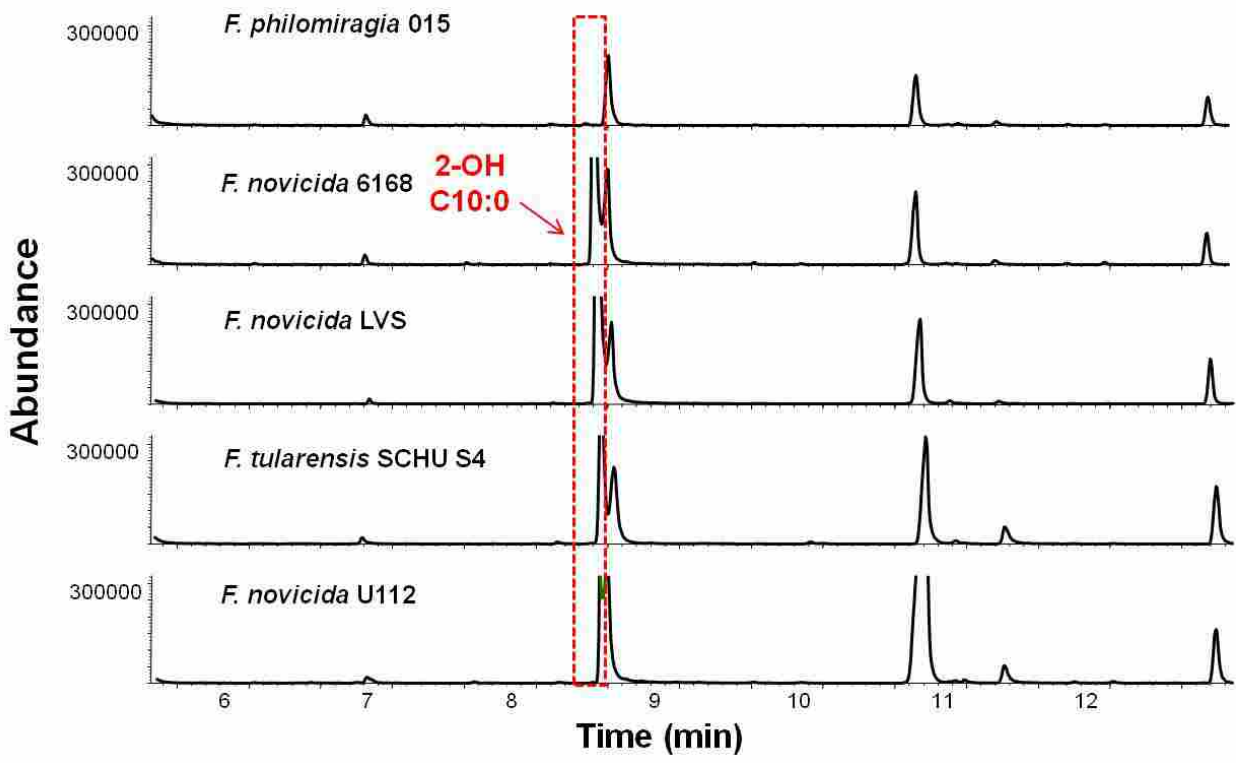



**Figure 4.5.** Extracted-ion chromatograms (*m/z* 69) of *F. tularensis* species cultured on BHI agar at 37 °C.

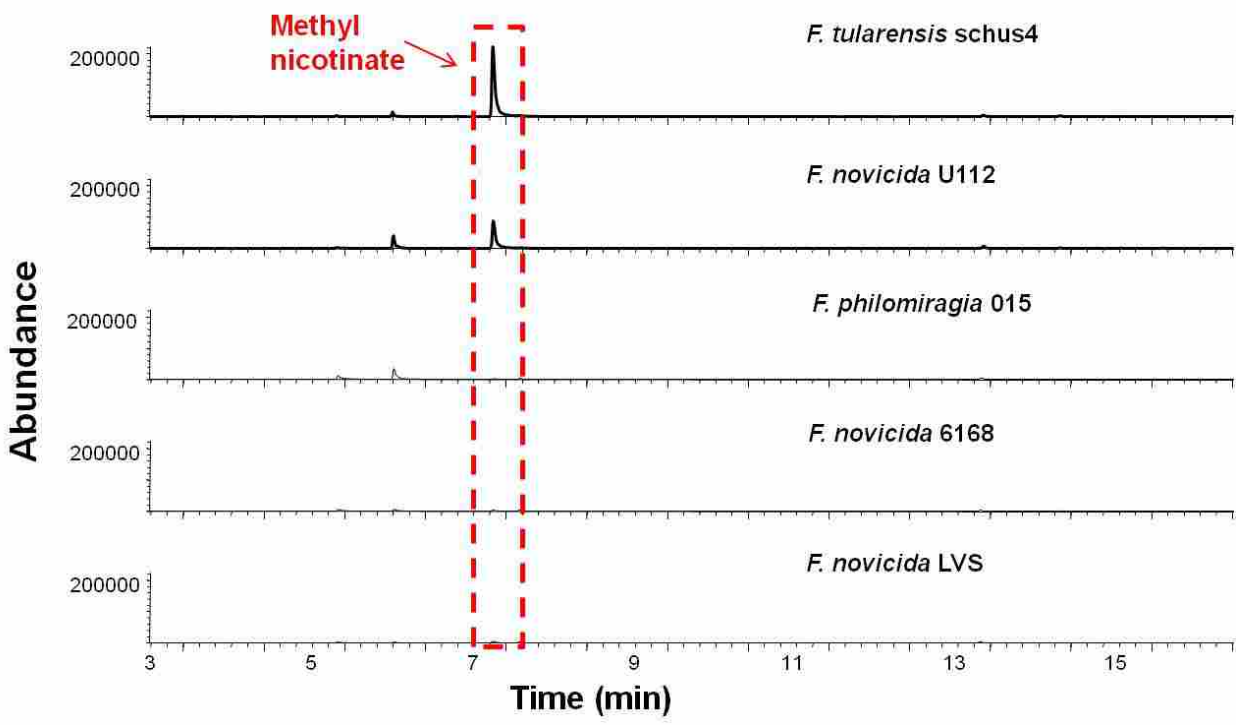



**Figure 4.6.** Selected-ion chromatograms (*m/z* 106) of *F. tularensis* species cultured on BHI agar at 37 °C.

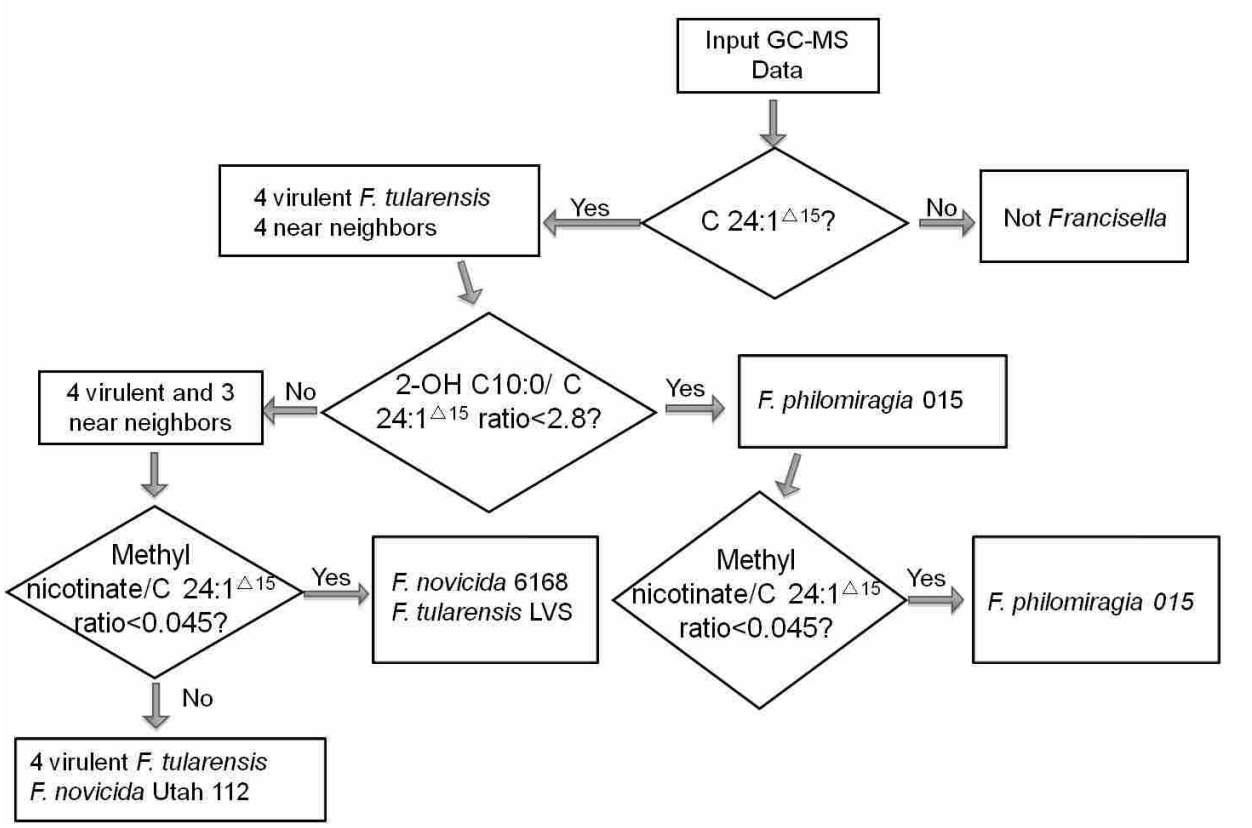



**Figure 4.7.** Decision tree for differentiation of *Francisella* species.

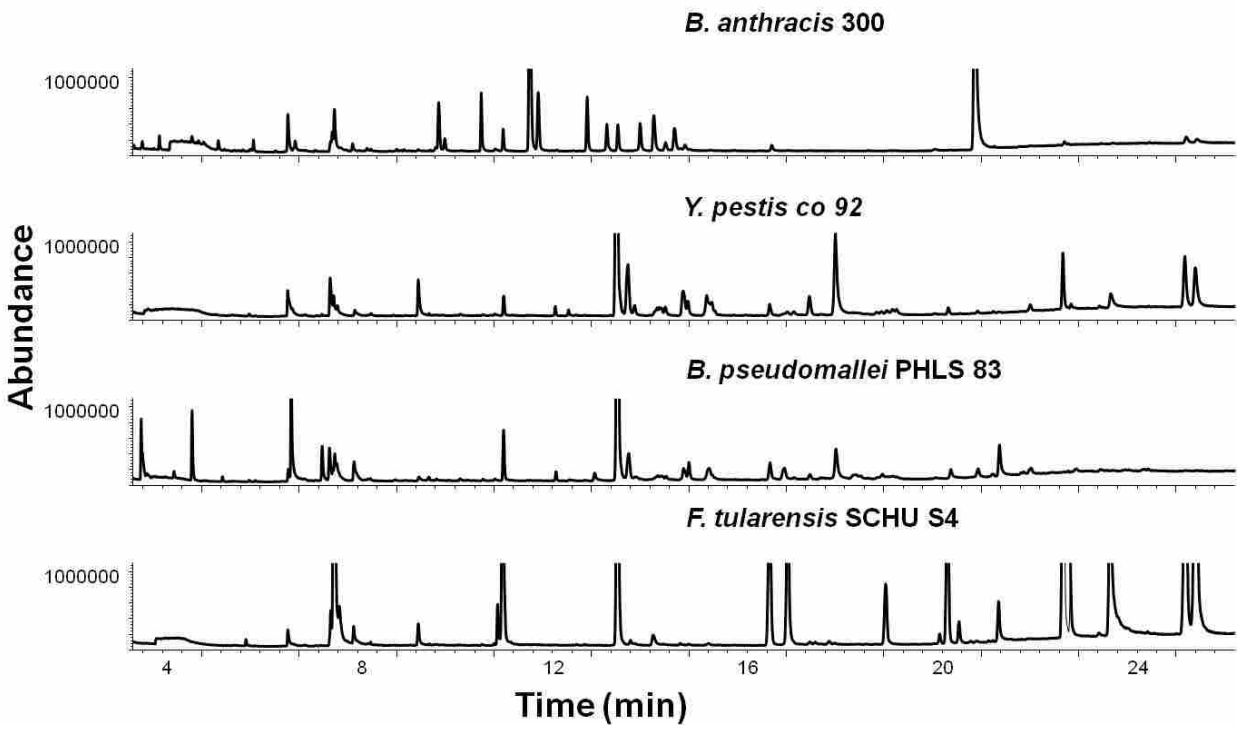



**Figure 4.8.** Total-ion chromatograms of 4 different Tier 1 bacterial species using the general GC-MS program.

species have very different profiles, which can be easily used to differentiate them from each other. The total correct identification percentage of *Francisella* species was 85% (Table 4.4).

## 4.4 CONCLUSIONS

The TCM procedure is an easy and rapid biomarker generation method for Tier 1 bacterial agents. Virulent *Y. pestis* isolates can be differentiated from their near neighbors by the biomarkers 3-OH C14:0 and 2-OH C12:0 and an unidentified sugar derivative, except for *Y. pseudotuberculosis* 29833. Biomarkers C24:$1^{\Delta 15}$, 2-OH C10:0 and methyl nicotinate can be used for differentiation of the *Francisella* species. *F. novicida* Utah 112 could not be differentiated from the virulent *F. tularensis* isolates. The correct identification percentages for *Yersinia* and *Francisella* species were 86% and 85%, respectively. A general GC-MS temperature program was developed for differentiation of all five Tier 1 bacterial agents.

**Table 4.4.** *Francisella* differentiation results.

| | number in study | number correct | percent correct |
|---|---|---|---|
| *F. tularensis* | 96 | 84 | 88% |
| not *F. tularensis* | 96 | 79 | 82% |
| total | 192 | 163 | 85% |

## 4.5 REFERENCES


1. Select Agents and Toxins List. www.selectagents.gov.
2. Li, D.; Truong, T. V.; Bills, T. M.; Holt, B. C.; VanDerwerken, D. N.; Williams, J. R.; Acharya, A.; Robison, R. A.; Tolley, H. D.; Lee, M. L., *Anal. Chem.* **2012,** *84*, 1637-44.
3. Basile, F.; Beverly, M. B.; Voorhees, K. J.; Hadfield, T. L., *Trac-Trends Anal. Chem.* **1998,** *17*, 95-109.
4. Jantzen, E.; Berdal, B. P.; Omland, T., *J. Clin. Microbiol.* **1979,** *10*, 928-30.
5. Whitfaker, P.; Day, J. B.; Sherill, K.; Fry, F. S., *J. AOAC Int.* **2007,** *90*, 4.
6. Nichols, P. D.; Mayberry, W. R.; Antworth, C. P.; White, D. C., *J. Clin. Microbiol.* **1985,** *21*, 738-40.
7. Hood, A. M., *J. Hyg. (Lond)* **1977,** *79*, 47-60.

# 5 AUTOMATED THERMOCHEMOLYSIS REACTOR FOR DETECTION OF *BACILLUS ANTHRACIS* ENDOSPORES BY GC-MS

## 5.1 INTRODUCTION

The well-known 2001 *Bacillus anthracis* letter mailing incident created a global awareness that rapid and reliable identification of BWAs was necessary,[1] emphasizing the critical need for fieldable systems for real-time detection of BWAs. Rapid and reliable identification of microorganisms without extensive sample preparation is a major goal in field detection. Many factors should be considered in evaluating a fieldable detection system, such as selectivity, sensitivity, detection time, autonomy, portability, power requirements and reagent consumption.[2] Extremely important in BWA detection are consistency and reproducibility in sampling, sample preparation and analysis, which are seldom possible in manual operations. However, preparing biological samples for analysis is always challenging, due to their great diversity and complexity.[3]

The general BWA detection methods have been summarized in Chapter 1. For field detection, the primary techniques include particle sizing from intrinsic fluorescence,[4-6] immunoassay-based biochip or biosensor techniques,[7] real-time PCR-based analysis[8,9] and combinations of these.[10-13] Such technologies have been employed to different degrees in autonomous systems, and they meet some of the criteria listed above. An autonomous pathogen detection system was developed by the Lawrence Livermore National Laboratory, which integrated both immunoassays and PCR for detection.[12] By using two biological assays, the possibility of false alarms was greatly reduced. However, improvements are still needed for this

unit, such as continuous operation, rapid detection (< 60 min intervals for immunoassays), less frequent maintenance and less expensive operation. GC-MS draws on the strengths of both techniques, and can be designed to satisfy the requirements of robustness, simple operation, rapid analysis and portability.[14]

Preliminary results using a low temperature TCM method were recently reported by us (Chapter 2) for positive detection of *Bacillus anthracis* endospores.[15] This method is simple (only one step) and fast (5 min). TCM reaction, SPME and GC-MS analysis provides an efficient, specific, rapid and sensitive method for identification of bacteria.[15] Automation of the sample preparation steps in a small, portable system would greatly facilitate field detection using this approach.

In this study, we designed, constructed, and tested an automated bioagent TCM preparation system: an autoreactor capable of sequentially processing samples for detection of chemical biomarkers of biological threat agents. While collection of bacterial endospores from the air and SPME sampling of TCM products for GC-MS analysis were not automated operations in this system, it is evident that a completely autonomous sampling/sample preparation/analysis system for use in the field could be developed in the future without too much difficulty.

## 5.2 EXPERIMENTAL

### 5.2.1 Chemicals and supplies

Hydrogen methyl sulfate ($HMeSO_4$) was made by mixing $H_2SO_4$ and methanol (Sigma Chemical, St. Louis, MO, USA) in a ratio of 1:9 (v/v) at room temperature, and this solution was

left for approximately 8 days before use. Phosphate buffer (1 M, pH 6.5) and Leighton-Doi or Columbia growth media were prepared as previously described.[15] All of the *Bacillus* strains (Table 5.1) were cultured at 32 and 37 °C on Leighton-Doi or Columbia agar in a BSL- 2 facility.[15] The endospore culture method was described previously.[15]

### 5.2.2 GC-MS analysis

While a portable GC-MS system would obviously be appropriate for detection of bacteria using this approach in the field, a laboratory GC-MS system was used in this study because a portable system was not available. The same operation and results are expected whether using a laboratory or portable system. GC-MS analyses using the same instrumentation and operating conditions for both the GC and MS were conducted as optimized previously.[15] The GC-MS system was an Agilent 6890 gas chromatograph-5973 mass spectrometer (Agilent, San Jose, CA, USA), and the GC column was a 30-m × 0.25-mm i.d. × 0.25-μm Zebron-FFAP column (Phenomenex, Torrance, CA, USA). The temperature program for all runs was 50 °C (1 min initial hold), then 8 °C/min to 110 °C (1min hold), then 5 °C/min to 150 °C (1 min hold), then 5 °C/min to 190 °C (1 min hold), and finally 15 °C/min to 250 °C (4 min hold). A 2-cm DVB/CAR/PDMS SPME fiber (Sigma Chemical, St. Louis, MO, USA) was conditioned at 250 °C in the GC injection port for approximately 1 h, and then for an additional 30 min at 260 °C, before being used to extract and transfer analytes from the autoreactor to the GC-MS system for analysis.

### 5.2.3 Autoreactor instrumentation

The central feature of the autoreactor (Figure 5.1) is a carousel, which is positioned just under the top plate shown in Figures 5.1 and 5.2. Vials move through six positions (labeled **1** to **6** in Figures 5.1 and 5.2) while rotating in a circle, allowing for continuous consecutive sample

**Table 5.1.** *Bacillus* strains and sources.

| species name | identifiers | group | source[a] |
|---|---|---|---|
| *B. anthracis* | 1043/Sterne | BA | LANL |
| *B. thuringiensis* | Al Hakam | BT | UAB |
| *B. thuringiensis* | ATCC 19270 | BT | ATCC |
| *B. thuringiensis* | Kurstaki | BT | NBFAC |

[a]LANL = Los Alamos National Laboratory, UAB = University of Alabama at Birmingham, ATCC = American Type Culture Collection, NBFAC = National Bioforensic Analysis Center

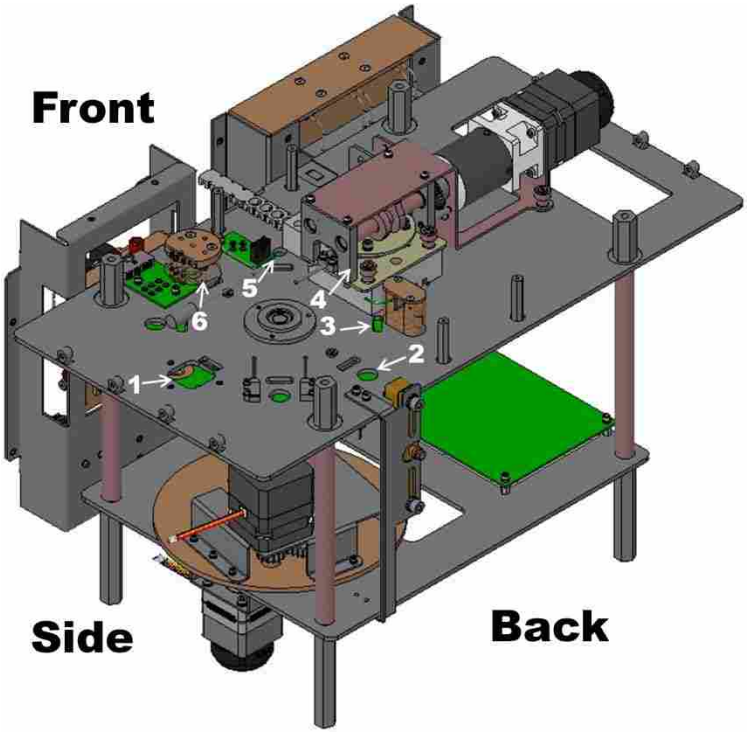



**Figure 5.1.** Diagram of the autoreactor setup, indicating the six automated sample handling and TCM process positions.

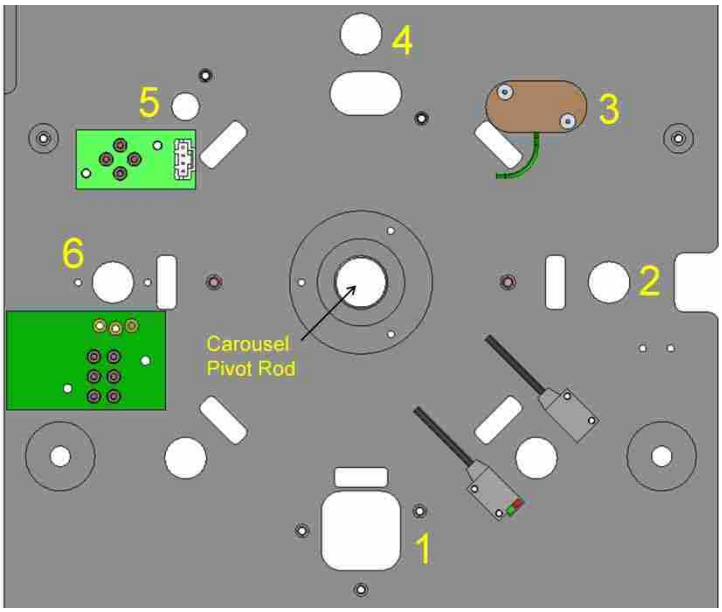



**Figure 5.2.** Diagram of the autoreactor top plate, indicating the six automated sample handling and TCM process positions. The sample vials are placed in a carousel (attached to the carousel pivot rod) located under this top plate. The pivot rod rotates the vials stepwise to the different processing positions, and moves them vertically up and down for cap crimping and heating operations.

preparation. The system accepts 1 mL (7-mm diameter × 40-mm tall) clear flat-bottom quartz vials with crimp caps containing 8-mm PTFE septum caps (National Scientific, Rockwood, TN, USA). Two of the positions have a heater mounted underneath. The carousel has the ability to move on two axes, vertical and rotational on a central pivot rod. A series of three guide pins provide necessary realignment accuracy according to the precision required for vial placement. Each vial passes through the 6 positions: (**1**) vial introduction/removal, (**2**) sample introduction, (**3**) reagent dispensing, (**4**) vial cap crimping, (**5**) reagent heating and (**6**) sample extraction. The vial introduction position (**1**) is where the user inserts new vials and removes used vials. Each vial moves sequentially through all of the positions and returns to the original position where the user removes it and inserts a new vial. A following position (**2**) was designed to eventually capture bacterial particles from the air by accepting intake air flow, exhausting the major flow, and directing a minor flow through the vial (i.e., a concentrating virtual impactor system). During this process, particulates with an effective diameter between 1 and 10 μm would be collected in the vial. To date, the collection of particulates from air has not been implemented. In this study, endospore samples were directly introduced into the vials before they were inserted into the autoreactor at the first position.

The $HMeSO_4$ reagent (10 μL) is introduced into the vial at position **3** through a reagent dispensing assembly. A dispensing diaphragm metering pump (KNF Neuberger, Trenton, NJ, USA) directs air through a series of small dead volume solenoid valves and then into a reagent storage vial. The resulting pressure pushes the reagent liquid into a sample loop of predetermined volume. Waste reagent from this process is collected in a separate storage vial for disposal. Finally, the air flow is switched to push the reagent from the sample loop into the vial. This pump has the ability to pump 10 μL per stroke of gas or liquid at 20 strokes per second.

The components of the crimping assembly at position **4** include the crimping jaws, vial ejection piston, crimper anvil and storage magazine containing vial caps (Figure 5.3). Four jaws positioned in a circular fashion similar to the four quadrants of the unit circle, surround the anvil. These jaws have a lip at the lowest part that provides the inward bending of the vial cap lip. To accept a vial for crimping, four compressed springs push the anvil and anvil plate into the "up" position (Figure 5.4A and B), which forces the Garter spring to open the jaws. As the vial is pushed up by the carousel, it pushes through the vial cap storage magazine. The piston breaks the beam of an electro-reflective sensor. The 17Y3 stepper motor (Anaheim Automation, Anaheim, CA, USA) rotates a series of cams that push the anvil in between the jaws, which forces them to close and seal the vial. As the cams continue to rotate, a second set of cams activate a second closing of the jaws, thus, providing a secondary sealing of the vial. To eject the vial, a special cam, offset from the sealing cams, pushes down on the piston. This motion restores the sensor beam, and the carousel immediately starts to descend. After the ejection piston has completed its cycle, the assembly returns to its default ready state.

Position **5** is one of two using a heating block (1/8" OD, Sun Electric Heater, Salem, MA, USA). The lower half of the vial height is in direct contact with the heating block (Figure 5.5). Since the sample volume occupies less than one third of the vial, the whole sample is in the heated zone. In order to initiate heating, the vial/heating block assembly is raised by the carousel pivot rod until the electrical heater contact pins make contact with connection pads on the assembly (Figure 5.5A and B). The temperature is controlled for 5 min at 140 °C for TCM. Prior to introduction of the buffer, the temperature of the vial must be reduced to approximately 70–80 °C. A ventilation fan was added to help remove heat from the heating block during cool-down.

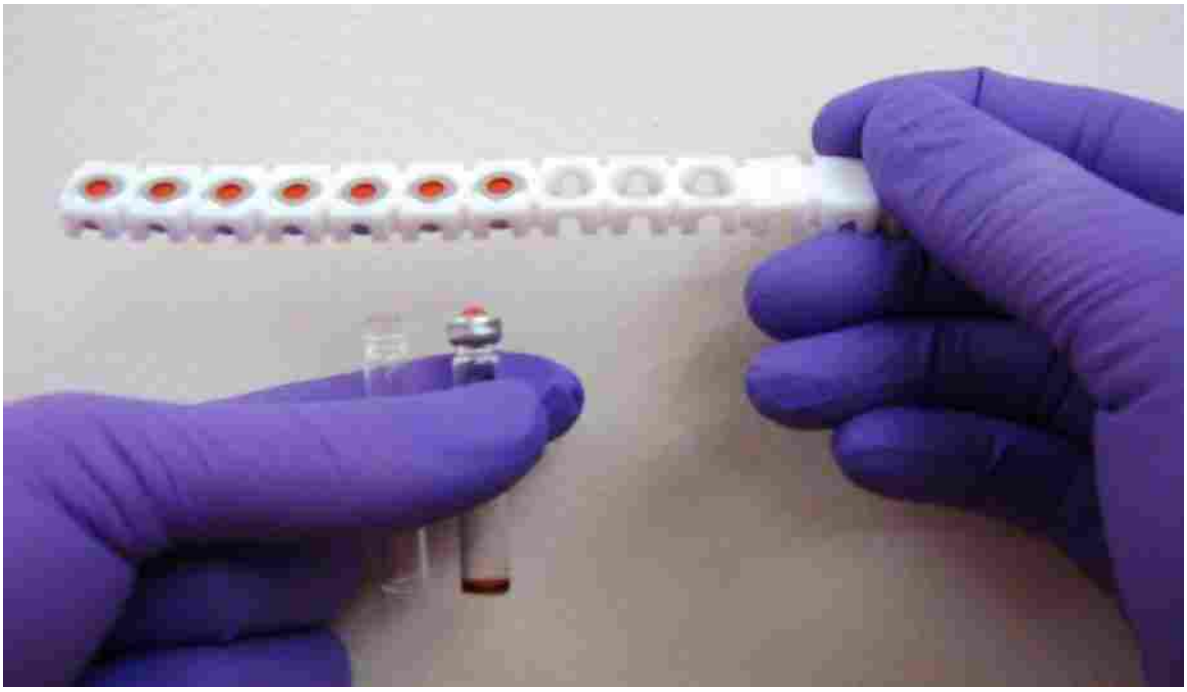


**Figure 5.3** Photograph of gloved hands holding two vials (one empty and the other with sample and crimped cap) and a magazine containing new vial caps.

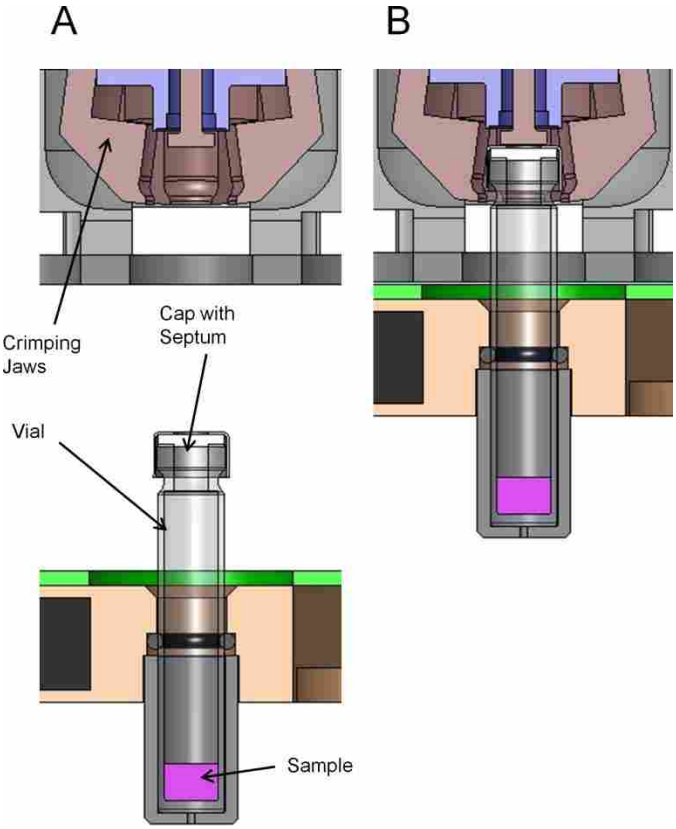



**Figure 5.4.** Diagrams of the cap crimping stage **4** (A) before and (B) after the carousel is raised to position the cap and vial lip within the crimping jaws.

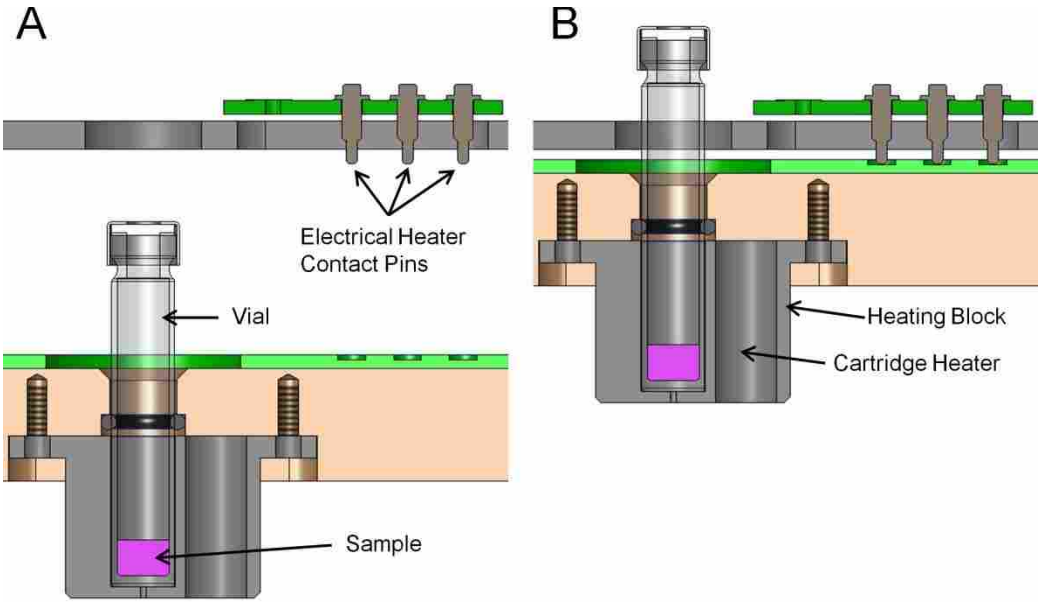



**Figure 5.5.** Diagrams of stage **5** (A) before and (B) after the carousel is raised to make electrical contact for activating the heating block cartridge.

Several processes occur at position **6**. While the top of the vial is sealed, two needles puncture the cap through the septum without losing vaporized biomarkers (evidenced by the pressure that is created by, and maintained after, TCM in the vials). One needle is connected to a syringe that adds 400 μL of neutralizing phosphate buffer (pH 6.5, 1 M) to the vial through a dispensing pump, and the other is an SPME syringe needle. A 2-cm SPME fiber is used for extraction of biomarkers. An important feature that assists biomarker extraction is an encapsulated micro vibration motor (Jinlong Machinary, Wenzhou, Zhejiang, China) attached to the heating block, which agitates the vial at 11,000 rpm during SPME. The height of the SPME fiber in the vial can be adjusted, which allows the fiber to be positioned equally between the headspace and the liquid (i.e., the fiber exposure ratio is 50:50). In this way, both liquid and headspace vapor phases of the sample can be extracted simultaneously.[15] During insertion of the SPME needle into the sample vial, the conductive surface of the SPME hub contacts two electric plunger pins and triggers the vibration motor and an internal clock. The SPME fiber is then extended from the syringe-like fiber holder (Torion Technologies, American Fork, UT, USA) by pushing the ball-point-pen-like button on the top, and extraction begins. The syringe-like SPME fiber holder looks and operates like a ball-point-pen to extend the fiber from, and retract it back into, the fiber needle. This fiber holder was designed for one-hand use, which is convenient for field sampling.

During the extraction process, the heating block maintains the temperature at 80 °C and, after 10 min, the SPME fiber is retracted into the protective needle and is removed from the autoreactor. A sensor provides SPME needle position feedback to the controller. In addition, an LED (light-emitting diode) light on the exterior of the autoreactor reminds the user to insert the

SPME needle, and a buzzer tells the user to remove the needle. The SPME syringe is then inserted into the GC injection port for 2-min desorption at 260 °C followed by GC-MS analysis.

Two reagent vessels ($HMeSO_4$ and phosphate buffer) and one waste storage vessel constructed of 40 mL clear glass vials with PTFE/silicone septa and polypropylene caps (National Scientific, Rockwood, TN, USA) were used. The storage cabinet was constructed of polyetherimide (PEI), which is resistant to sulfuric acid. In the event of leakage of reagent, a PTFE catch basin is located directly below the storage vials. These 40 mL vials are inserted into position by opening the PEI lined cabinet door and placing the vials into the vial shuttle. Then, the vial is lifted up the shuttle while the vial septum is pierced with the two needles. The two reagent vials require two needles, while the waste vial only requires a single needle. The shorter needle pressurizes the vial, which in turn forces liquid into the longer needle by the dispensing pump. The dispensing pump operates for a predetermined amount of time to allow for pressurized chemicals to fill the sample loop. By design, the sample loop produces excess chemicals that must be collected by a waste storage vial. When the waste fills to two-thirds of its capacity, it is replaced with a fresh vial. For the amounts of liquid required to perform a single experiment, it is estimated that a single vial of methyl sulfate could last for up to 200 uses, while the buffer could last for up to 50 treatments. The dispensing concept utilizes two separate manifolds. The material used for both manifolds is PEI. This prevents corrosion due to prolonged exposure to $HMeSO_4$.

The controller printed circuit board (PCB) provides the following components: (1) control logic circuits, (2) sensor and feedback input, (3) stepper motor control and feedback, (4) heater control and temperature feedback, (5) vibration control, (6) solenoid control, (7) pump control, (8) external RJ45 port for computer access to command and control module and (9) user

interface button control. The user can control the autoreactor through a custom program. With this program, all parameters of the device can be altered, such as type and amount of reagents, heating temperature, heating time, speed of motor, activation of solenoid, etc. Therefore, this system can be used for a variety of automated bio-reactor applications.

### 5.2.4 Portability

The autoreactor was envisioned to eventually become field-portable, which requires the unit to be miniaturized further and to be self-powered. The current weight of the system is 15.9 kg. The circuit architecture of the controller PCB was designed to add battery and battery charging circuits. In this manner, the unit could be used with a rechargeable battery in addition to 110-V ac line power.

### 5.2.5 Safety considerations

Methyl sulfate ($HMeSO_4$) salts are considered to be toxic and can cause skin irritation. Dimethyl sulfate ($Me_2SO_4$) is anticipated to be carcinogenic, and application of high temperature to $HMeSO_4$ could promote the formation of $Me_2SO_4$. In this autoreactor design, the reagents are stored in sealed vials with appropriate precautions to prevent leakage.

Brigham Young University has well-equipped BSL facilities (i.e., BSL-2 and BSL-3). The BSL-3 facility is registered with both the United States Department of Agriculture (USDA) and the CDC. All federal guidelines and mandates are strictly followed with respect to activities involving various biological agents, including operation, security and access. While the *Bacillus* strains used in this study are not considered to be human pathogens, all safety precautions for BSL-2 agents were followed to ensure a comfortable margin of safety.

## 5.3 RESULTS AND DISCUSSION

### 5.3.1 Comparison of the performance of the autoreactor with manual TCM

For testing, a 10-μL suspension of endospores (~$10^6$) in methanol was added into an empty vial, which was then inserted into the autoreactor at position **1** (Figures 5.1 and 5.2). The autoreactor began to operate automatically, after an operator pushed the "start" button. After approximately 15 min, when the LED light began to blink, the operator inserted the SPME needle into the extraction position and pushed the plunger button to expose the fiber for biomarker extraction. The buzzer informed the operator when to remove the SPME plunger and perform an injection into the GC-MS. The operator then removed the old vial from the introduction/removal position and introduced a new vial to start a new cycle. To remove a reagent vial, the user simply rotated the retention bar down and pulled the vial straight down the shuttle path until the insertion needles were clear. Also, the user could view the liquid level within each vial by simply looking through the provided slits. The time for one rotation (i.e., sample treatment) was approximately 30 min; however, this could be efficiently shortened by starting samples one after another and allowing them to proceed through each stage in sequence. In this case, the effective sample treatment rate would be 10 min/sample.

The current capabilities of the autoreactor include vial movement, reagent introduction, cap crimping, vial heating, reagent agitation and timing of biomarker extraction. The system performance was evaluated and compared with manual TCM by detecting 3-M-2-BAME, DPAME and two methylated sugars, which are key biomarkers for detecting *Bacillus anthracis* endospores.[17] Figure 5.6 shows reconstructed-ion chromatograms of 3-M-2-BAME, DPAME and FAME biomarkers from both the autoreactor and manual TCM. Comparisons were

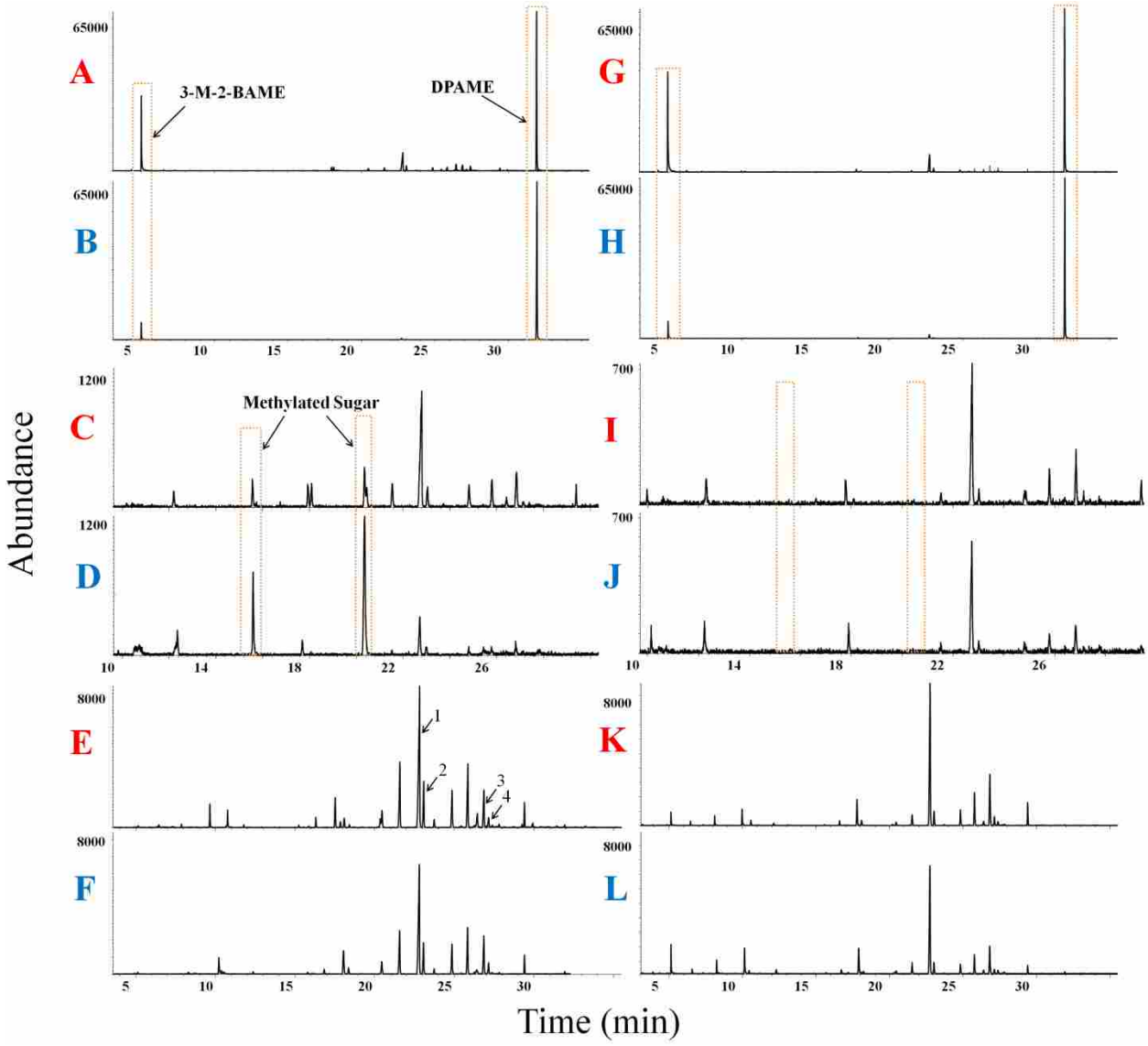



**Figure 5.6.** Reconstructed-ion chromatograms of target biomarkers (m/z 83 for 3-M-2-BAME, m/z 137 for DPAME, m/z 88 for methylated sugars and m/z 74 for FAMEs). (A), (C), (E), (G), (I) and (K) were obtained using the autoreactor; (B), (D), (F), (H), (J) and (L) were obtained from manual TCM. (A)-(F) are from *B. thuringiensis* Al Hakam, cultured at 32 ◦C on Columbia agar; (G)-(L) are from *B. anthracis* Sterne, also cultured at 32 °C on Columbia agar. In Figure 5.6E, numbers 1–4 represent iso C15:0, anteiso C15:0, iso C17:0 and anteiso C17:0, respectively.

conducted with the same amounts from the same batches of *B. thuringiensis* Al Hakam (Figure 5.6A to F) and *B. anthracis* Sterne (Figure 5.6G to L) endospores. In Figure 5.6A and B, it can be observed that the DPAME levels were approximately the same; however, the 3-M-2-BAME peak showed significantly greater abundance from the autoreactor than from manual TCM. The same results were found in *B. anthracis* Sterne (Figure 5.6G and H). This is mainly because of the vibration imposed by the autoreactor heating block, which facilitates mass transport to the fiber. Usually, a magnetic stir bar is used in SPME; however, the rotational speed is not as effective or controllable.[16] In our case, the diameter of the vial is only 8 mm, which is not large enough for a spinning magnetic stir bar; therefore, it was not included in the manual TCM heating device. Since vibration facilitates volatile biomarker expulsion from the liquid, volatile biomarkers have faster mass transport rates than semi-volatiles, and are extracted more rapidly.

Levels of the two methylated sugars from manual TCM (Figure 5.6D) were slightly higher than from the autoreactor (Figure 5.6C). FAMEs from *B. thuringiensis* Al Hakam (Figure 5.6E and F) were similar for both. Similar trends can be found in the FAME profiles for *B. anthracis* Sterne (Figure 5.6K and L). Typical FAMEs in *Bacillus* strains, such as iso C15:0, anteiso C15:0, iso C17:0 and anteiso C17:0, are indicated in Figure 5.6E (peaks labeled 1–4). The absence of the two methylated sugar biomarkers in *B. anthracis* Sterne (Figure 5.6I and J) provided clear differentiation between *B. anthracis* and *B. thuringiensis* Al Hakam.[15]

### 5.3.2 Statistical analysis

The performance of the instrument was evaluated by identifying *Bacillus* endospore samples in a statistically designed algorithm. A total of 25 viable endospore samples were analyzed using the autoreactor. Of these, 5 were selected and analyzed manually as well.

Statistical comparison of the 5 matched analyses showed that the relative peak areas were statistically higher for the autoreactor. Relative peak areas for each of the peaks used in the discrimination algorithm showed large variation from sample to sample with RSD values ranging from 50% to 98%. However, when the biomarker peaks are used collectively for discrimination, the correct classification rate is 96%. The individual classification rates are given in Table 5.2. The correct classification rates here are numerically larger than those obtained by manual analyses published previously,[15] although not statistically significantly higher. Lack of statistical significance here could be due to the possibility that the effect size (i.e., the difference between the autoreactor results and the manual analysis results) is nearly zero, or that the small number of samples analyzed did not have sufficient power to detect a difference for a small effect size.

The classification was done (without operator input) using our previously published differentiation algorithm.[15] This algorithm was developed with the statistical software package "R" using biomarkers identified previously. Data were included from TCM of *B. anthracis* Sterne samples as well as several near neighbors (i.e., *B. thuringiensis* Al Hakam, *B. thuringiensis* Kurstaki and *B. thuringiensis* 19270) cultured under four conditions (two growth temperatures and two growth media). The biomarkers were effective in discriminating between *B. anthracis* Sterne and all other strains when the abundances were high. The misidentified sample had marginal DPAME level, indicating that the endospores in that sample may have begun to degrade. Any biomarker peak that was less than 3 standard deviations above the background was assigned a value of zero.

**Table 5.2.** Results from the automated *Bacillus* detection system.

| species | total in study | total identified correctly | percentage identified correctly |
|---|---|---|---|
| *B. anthracis* | 8 | 8 | 100% |
| not *B. anthracis* | 17 | 16 | 94% |
| overall | 25 | 24 | 96% |

## 5.4 CONCLUSIONS

We have developed and evaluated an automated sample preparation system that can be used to perform TCM on *Bacillus* endospores. The system is capable of continuous operation, and the total preparation time for one sample is less than 30 min. Compared with manual TCM, the autoreactor appears to show improved performance for volatile biomarker detection as measured by the number of samples correctly classified, although not statistically significantly better. In total, 96% of 25 endospore-forming *Bacillus* species were correctly identified using a statistically designed test. This new autoreactor device provides a number of advantages in simplicity, ease of operation and flexibility. The autoreactor is capable of monitoring other biothreat agents using the same TCM procedure. Future improvements will include smaller size, lighter weight, and simpler operation and automation of air particulate collection, SPME sampling and sample introduction into the GC-MS.

## 5.5 REFERENCES


1. Borio, L.; Frank, D.; Mani, V.; Chiriboga, C.; Pollanen, M.; Ripple, M.; Ali, S.; DiAngelo, C.; Lee, J.; Arden, J.; Titus, J.; Fowler, D.; O'Toole, T.; Masur, H.; Bartlett, J.; Inglesby, T., *J. Am. Med. Assoc.* **2001,** *286*, 2554-9.
2. Stachowiak, J. C.; Shugard, E. E.; Mosier, B. P.; Renzi, R. F.; Caton, P. F.; Ferko, S. M.; Van de Vreugde, J. L.; Yee, D. D.; Haroldsen, B. L.; VanderNoot, V. A., *Anal. Chem.* **2007,** *79*, 5763-70.
3. Demirev, P. A.; Fenselau, C., *Annu. Rev. Anal. Chem.* **2008,** *1*, 71-93.
4. Seaver, M.; Eversole, J. D.; Hardgrove, J. J.; Cary, W. K.; Roselle, D. C., *Aerosol Sci. Tech.* **1999,** *30*, 174-85.
5. Pinnick, R. G.; Hill, S. C.; Nachman, P.; Videen, G.; Chen, G.; Chang, R. K., *Aerosol Sci. Tech.* **1998,** *28*, 95-104.
6. Eversole, J. D.; Hardgrove, J. J.; Cary, W. K.; Choulas, D. P.; Seaver, M., *Field Anal. Chem. Tech.* **1999,** *3*, 249-59.

7. Stratis-Cullum, D. N.; Griffin, G. D.; Mobley, J.; Vass, A. A.; Vo-Dinh, T., *Anal. Chem.* **2003,** *75*, 275-80.
8. Makino, S. I.; Cheun, H. I.; Watarai, M.; Uchida, I.; Takeshi, K., *Lett. Appl. Microbiol.* **2001,** *33*, 237-40.
9. Regan, J. F.; Makarewicz, A. J.; Hindson, B. J.; Metz, T. R.; Gutierrez, D. M.; Corzett, T. H.; Hadley, D. R.; Mahnke, R. C.; Henderer, B. D.; Breneman, J. W. t.; Weisgraber, T. H.; Dzenitis, J. M., *Anal. Chem.* **2008,** *80*, 7422-9.
10. Anderson, G. P.; King, K. D.; Cuttino, D. S.; Whelan, J. P.; Ligler, F. S.; MacKrell, J. F.; Bovais, C. S.; Indyke, D. K.; Foch, R. J., *Field Anal. Chem. Tech.* **1999,** *3*, 307-14.
11. McBride, M. T.; Masquelier, D.; Hindson, B. J.; Makarewicz, A. J.; Brown, S.; Burris, K.; Metz, T.; Langlois, R. G.; Tsang, K. W.; Bryan, R.; Anderson, D. A.; Venkateswaran, K. S.; Milanovich, F. P.; Colston, B. W., *Anal. Chem.* **2003,** *75*, 5293-9.
12. Hindson, B. J.; Makarewicz, A. J.; Setlur, U. S.; Henderer, B. D.; McBride, M. T.; Dzenitis, J. M., *Biosens. Bioelectron.* **2005,** *20*, 1925-31.
13. Hindson, B. J.; Brown, S. B.; Marshall, G. D.; McBride, M. T.; Makarewicz, A. J.; Gutierrez, D. M.; Wolcott, D. K.; Metz, T. R.; Madabhushi, R. S.; Dzenitis, J. M.; Colston, B. W., Jr., *Anal. Chem.* **2004,** *76*, 3492-7.
14. Contreras, J. A.; Murray, J. A.; Tolley, S. E.; Oliphant, J. L.; Tolley, H. D.; Lammert, S. A.; Lee, E. D.; Later, D. W.; Lee, M. L., *J. Am. Soc. Mass Spectrom.* **2008,** *19*, 1425-34.
15. Li, D.; Truong, T. V.; Bills, T. M.; Holt, B. C.; VanDerwerken, D. N.; Williams, J. R.; Acharya, A.; Robison, R. A.; Tolley, H. D.; Lee, M. L., *Anal. Chem.* **2012,** *84*, 1637-44.
16. Pawliszyn, J., *Solid Phase Microextraction: Theory and Practice*. 1st ed.; Wiley: New York, 1997.

# 6 CONCLUSIONS AND FUTURE WORK

## 6.1 CONCLUSIONS

A one-step TCM procedure was developed and optimized to generate chemical profiles of bacteria, especially for Tier 1 bacterial agents. This TCM method is fast (5 min), easy to perform (one step), robust and sensitive; it has been successfully applied to five different Tier 1 bacterial agents: *Bacillus anthracis, Francisella tularensis, Yersinia pestis, Burkholderia pseudomallei* and *Burkholderia mallei*. A variety of biomarkers, including lipids (i.e., fatty acids), carbohydrates (i.e., sugars) and some small specific biomarkers (e.g., DPA and poly-3-hydroxybutyrate-*co*-hydroxyvalerate), were investigated. Moreover, a general GC temperature program was developed for detection and differentiation of all five agents. The final goal of this study was to develop a fast, simple, fieldable detection method for BWAs. Therefore, an automated TCM reactor was developed for routine use in the field. This reactor is capable of automatically processing suspected biothreat agents by TCM to release and derivatize chemical biomarkers.

### 6.1.1 *Bacillus anthracis* detection and differentiation

A statistical algorithm for *B. anthracis* discrimination was developed using five key biomarkers: DPA, anthrose, two unidentified methylated sugars and a fatty acid (C13:0). Fifteen endospore-forming *Bacillus* species were studied, including six *B. anthracis* strains (four virulent isolates), five *B. thuringiensis* isolates, *B. mycoides, B. atrophaeus, B. cereus* and *B. subtilis.* Virulent *B. anthracis* strains (~90%) were correctly differentiated from non-virulent isolates and

near neighbors. The detection limit was found to be 50,000 endospores, on the basis of an anthrose-derived biomarker.

### 6.1.2 Detection and differentiation of *Burkholderia pseudomallei* and *Burkholderia mallei*

*B. pseudomallei* and *B. mallei* are Tier 1 agents that are closely related to each other. Several biomarkers, including poly(3HBA-*co*-3HVA), 2-OH C14:0, 3-OH C16:0 and C18:0, were generated from TCM for differentiation of *B. pseudomallei*, *B. mallei*, *B. thailandensis* and several members of the *B. cepacia* complex (i.e., *B. cepacia*, *B. multivorans* and *B. vietnamiensis*). The detection limit for this method was found to be approximately 4,000 cells. The correct identification percentages for *B. pseudomallei*, *B. mallei* and near neighbors were 100%, 100% and 88%, respectively.

### 6.1.3 Automated TCM reactor

TCM can be automatically performed using an autoreactor designed for field sample preparation. The reactor performs six steps, including vial introduction/removal, sample introduction, reagent dispensing, vial cap crimping, reagent heating and sample extraction. It is capable of continuous operation, and the total preparation time for one sample is less than 30 min. This autoreactor is simple, easy to operate and rapidly prepares samples for detection by GC-MS.

### 6.1.4 General GC temperature program for Tier 1 agent detection

*Yersinia pestis* can be detected and differentiated from its near neighbors using three biomarkers, including 3-OH C14:0, 2-OH C12:0 and an unidentified sugar derivative. The only exception is *Y. pseudotuberculosis* 29833, which could not be differentiated from *Yersinia pestis*. The biomarkers C24:$1^{\Delta 15}$, 2-OH C10:0 and methyl nicotinate can be used for differentiation of the *Francisella* species. *F. novicida* Utah 112 could not be differentiated from the virulent *F.*

*tularensis* isolates. The correct identification percentages for *Yersinia* and *Francisella* species were 86% and 85%, respectively. A general GC-MS temperature program was developed for detection and differentiation of all five Tier 1 agents.

## 6.2 RECOMMENDATIONS FOR FUTURE WORK

Although five of the Tier 1 bacterial agents have been identified and differentiated using specific biomarkers, there still remain some tests that should be conducted to further evaluate and optimize the method. Therefore, I recommend the following future work.

### 6.2.1 Evaluation of matrix effects

Detection assays must be sensitive, specific and capable of detecting low concentrations of target agents without interference from background materials. To date, only pure bacteria cultures have been evaluated. Real samples can exist in a number of matrices, including powders, food, water, human body fluids (e.g., blood, sputum, urine and cerebral spinal fluid) and air samples containing dust, pollen, soil and other chemical pollutants. Food samples with high concentrations of sugars and lipids, and indigenous bacteria in water and air pose a challenge for target bacteria detection. Therefore, matrix factors (ratios of biomarker detection limits with and without matrix) should be calculated to determine the extent of interference by sample background materials.

### 6.2.2 Quantitation

Quantitative determination of bacterial populations is often required in biological/clinical studies. Traditional methods for determining the number of bacteria are plate count and spectrophotometric (turbidimetric) analysis. The standard plate count method consists of diluting

a sample with sterile saline or phosphate buffer diluents until the bacteria are diluted enough to count accurately (30-300 colonies). However, this method is laborious, time consuming and only useful for counting live bacteria. Turbidity, measured by a spectrophotometer, is another index of bacterial growth and cell numbers (biomass). Although this method is faster than plate counting, it relies on liquid nutrient culture and its sensitivity is restricted to bacterial suspensions of $10^7$ cells or greater. A hemocytometer, a chamber with specific dimensions, is widely used for cell number determination. Calculation of concentration is based on the chamber volume and the cell number counted using a microscope, which is faster than culture-based methods. However, since this device is filled through capillary action, it is not suitable when volatile solvents are used (e.g., methanol). Moreover, it is not accurate for particles larger than 100 μm. In our study, the endospores or cells were re-suspended in methanol; therefore, it was not accurate for use in our work. Flow cytometry is a laser-based real-time cell counting method. This method is fast and capable of measuring both live and dead cells. Therefore, I recommend using a flow cytometer for bacteria quantitation.

In order to correlate spore/cell concentrations to biomarker concentrations, it is important to find a stable internal standard. A stable isotope-labeled fatty acid was used as an internal standard in our work, and was added to the bacteria samples before TCM. The amount of this methyl ester was measured to estimate the TCM reaction efficiency. The ratio of biomarkers to internal standard can be calculated for quantitative work. However, previous results showed that ratios using the same biomarker in the same strain under the same growth conditions were not consistent. This may be caused by inaccurate addition of internal standard or spores/cells, inaccurate spore/cell number calculation; or genetic changes in biomarker production. Because all endospores/cells and internal standard were suspended or dissolved in

methanol, traditional air displacement pipettes are not accurate for transfer of organic liquids due to liquid leakage, which is the main cause for inaccurate transfer of both internal standard and cells in methanol. Therefore, I suggest using positive displacement pipettes, which have a piston that moves in a tip in direct contact with the liquid. Actually, the ideal way for quantitation is to find an internal standard that is present in all bacterial strains within a species and stable under different cultivation conditions. However, so far we have not found any components that can be used as internal standards. I suggest employing a statistical tool, such as "boosting", to exploit possible standards. The amount of standard should reflect the concentration of bacterial cells used.

### 6.2.3 Relationship between DPA and $Ca^{2+}$

DPAME is an important and sensitive indicator for the presence of *Bacillus* endospores. However, during the study of *Bacillus* biomarkers, we observed an unusual phenomenon. In some cases, DPAME was at a relatively low level compared to other biomarkers such as fatty acids in fully sporulated endospores. Calcium dipicolinate, which is believed to stabilize the DNA, is responsible for up to 20% of the dry weight of the endospores. It is responsible for the heat resistance of spores and may aid in resistance to oxidizing agents.[1] However, mutants resistant to heat, but lacking DPA, have been reported, suggesting that some isolates with low levels of DPA also exist.[1] In the future, it will be beneficial to determine the concentration of calcium ions as well as DPAME to further evaluate the production of calcium dipicolinate in different isolates, which may be used to identify certain endospores. Therefore, I suggest using inductively coupled plasma-atomic emission spectroscopy (ICP-AES) analysis for determination of calcium ion concentration, which has been reported previously.[2] In this method, 0.1% $HNO_3$ was used to wash endospores, and 70% $HNO_3$ was applied to treat endospores at 90 °C overnight

to release calcium ions. Before ICP-AES analysis, the resulting material was diluted to the desired appropriate concentration with deionized water.

### 6.2.4 Systematic study of anthrose for *Bacillus anthracis* strains

It is interesting that different species of *Bacillus* or even different isolates from the same species contain different levels of anthrose as shown from our *B. anthracis* study. The virulent isolates contain much less anthrose than the Sterne strain. Even *B. thuringiensis* Al Hakam yields a higher level of anthrose than most of the virulent *B. anthracis* isolates. There are no studies that report and compare anthrose levels in virulent isolates. It would be worthwhile to relate the anthrose levels to gene operon, gene expression and protein production levels, as well as to investigate the possible causes for the observed variations in anthrose level. In the BYU BSL-3, there are 314 isolates of *B. anthracis*. It would be useful to provide information on the anthrose levels in all of the *B. anthracis* isolates to assist BWAs detection.

### 6.2.5 Field analysis using the TCM autoreactor and portable GC-MS

The final goal of this study has been to develop a fast, accurate and fieldable detection method for onsite analysis. Currently, all detection and differentiation results for all five Tier 1 agents are based on traditional benchtop GC-MS. Therefore, the next step is to test this method using portable GC-MS. With proper method optimization, a 3 m × 0.1 mm i.d. column could provide much faster analysis with minimum carrier gas and power consumption. Regarding the autoreactor, a virtual impactor should be added for collecting aerosolized standard bacteria and real endospore powders from air. Further needed improvements in the autoreactor include minimizing the size and weight, making it more robust and automating the SPME sampling and GC-MS sample introduction operations to achieve total automation for field analysis. The

autoreactor should be employed in combination with a portable GC-MS to evaluate the robustness of the method in the field.

### 6.2.6 SPME autosampler for GC-MS

Agilent Technologies has marketed an autosampler for SPME injection (CombiPAL GC-MS sample injector) for which the sample holder can be heated and vibrated to facilitate the extraction process. If this system could be employed for our analysis, it would not only increase the productivity but also improve the accuracy by reducing the bias caused by different operators. I suggest developing a similar versatile injector for portable GC-MS to assist liquid, headspace and SPME injections, which would be more efficient and less operator-dependent.

### 6.2.7 Online extraction system

The sampling process in biodetection consists of sample collection, sample transport, biomarker generation, biomarker extraction and sample injection. The whole process can be performed by the autoreactor in combination with an SPME autosampler. However, this process is not entirely operator-independent, and the required virtual impactor and SPME are not currently incorporated in the system. Therefore, I propose a simple online extraction system for field analysis, which contains three components (Figure 6.1): a reaction chamber, a switchable flow path with adsorbent and an air pump. Initially, the air sample is pumped into the reaction chamber to which the required amount of $HMeSO_4$ is added, and the reactor is heated to 140 °C. Volatile biomarkers generated from the reaction chamber flow to the extraction section, which is packed with adsorbent material for biomarker extraction and concentration. Once a desired amount of sample is collected, valves 1 and 2 can be switched to introduce the extracted sample into the GC-MS with helium carrier gas flow. The adsorbent section can also be heated to assist biomarker desorption. This system automates the whole process of sample collection, TCM

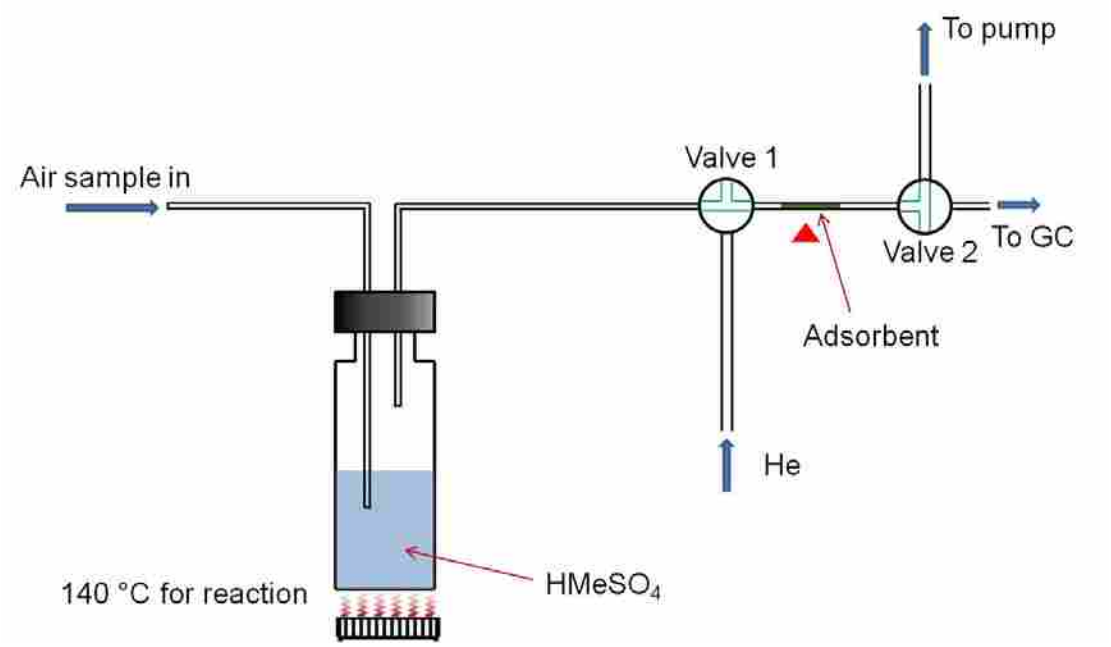



**Figure 6.1.** Proposed online extraction system.

reaction, biomarker extraction and concentration, sample desorption and injection into the GC-MS. It is continuous, simple, small, operator-independent and low cost. One disadvantage is that only volatile or semi-volatile biomarkers can be transferred for extraction, while less volatile biomarkers dissolved in the liquid phase would not reach the GC-MS. However, so far in our studies, most of the biomarkers used for Tier 1 agent detection are volatile. This system can be designed to be a small, portable accessory for field applications.

## 6.3 REFERENCES


1 L. Prescott, *Microbiology*, Wm. C. Brown Publishers, 1993.

2 H. Liu, N. H. Bergman, B. Thomason, S. Shallom, A. Hazen, J. Crossno, D. A. Rasko, J. Ravel, T. D. Read, S. N. Peterson, J. Yates, 3rd and P. C. Hanna, *J. Bacteriol.* 2004, **186**, 164-178.