MICROBIOLOGICAL METHODS

Identification of *Bacillus anthracis* from Culture Using Gas Chromatographic Analysis of Fatty Acid Methyl Esters

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(Applicable to the identification of pure culture isolates of *Bacillus anthracis*.)

Caution: Reagents 1 and 4 are caustic and Reagent 2 is acidic; wear safety glasses and gloves at all times. The hexane and methyl *tert*-butyl ether (MTBE) in Reagent 3 are flammable. Extinguish all flames and heat sources before use. Handle large volumes in a chemical fume hood. Growth and harvesting of samples for the gas chromatographic fatty acid methyl ester (GC-FAME) method are to be performed in a BSL-3 containment facility. *Bacillus anthracis* must be handled according to directions in the *Biosafety in Microbiological and Biomedical Laboratories*, 4th Ed. (Centers for Disease Control and Prevention). After the saponification step of sample preparation, the organism may be killed (nonviability should be verified) and suitable for transfer from the BSL-3 to the BSL-2 area for analysis. Follow laboratory SOPs for decontamination of the sample GC vial during this transfer. The MIDI system should be contained in the BSL-2 laboratory to facilitate maintenance by technical support personnel.

A. Principle

The MIDI method requires that bacteria be grown in culture, and the fatty acids are extracted by a procedure which consists of saponification in dilute sodium hydroxide–methanol solution followed by derivatization with dilute hydrochloric acid/methanol solution to give the respective FAMEs. The FAMEs are extracted from the aqueous phase by the use of an organic solvent and the resulting solution is analyzed by GC. FAMEs are more volatile than their respective fatty acids and therefore more suitable to GC analysis. The MIDI software automates all analytical operations and uses a sophisticated pattern recognition algorithm to match the unknown FAME profile to the stored database entries for identification.

B. Apparatus

(**a**) *Identification system*.—Sherlock Microbial Identification System (MIS) software version 4.0 or higher (MIDI, Inc., Newark, DE).

(**b**) *MIDI bioterrorism library and method version BTR20 or later*.—MIDI, Inc.

(**c**) *Gas chromatograph*.—Agilent Series 6890 or 6850 or equivalent. Equipped with a flame ionization detector (FID), automatic liquid sampler, injector, controller, and sampler tray. Configured front injector to front detector. Operating conditions: injector 250°C, split ratio 100:1 (55 ± 0.5 mL/min), detector 300°C. Oven temperature ramp: 170° to 270°C at 5°C/min. GC run time: 22 min. Gases: H_2 carrier gas and N_2 makeup gas (both must be 99.999% pure); air, industrial grade, dry, <1 THC. Environmental: gas

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Due to the security-sensitive nature of the material, the collaborative study, in its entirety, shall remain confidential. This paper presents the method portion of the study. For more information, contact standards@dhs.gov.

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chromatograph will operate within temperatures of 10°–40°C (50°–104°F) and 20–80% relative humidity.

(**d**) *Capillary column*.—Agilent Ultra II, 25 m × 0.2 mm × 0.33 µm film thickness with (5%)-diphenyl-(95%)-dimethylsiloxane, or equivalent.

(**e**) *Computer system*.—The MIDI system runs on an IBM-compatible personal computer. Minimum requirements: 96 Mb RAM, 8 Gb hard drive, 300 MHz Pentium II, Windows NT, 2000 or XP, Agilent Technologies (Wilmington, DE) A.06.03x or higher ChemStation software, CD-ROM and 3.5 in. floppy drives, 1024 × 768 or higher VGA color display, LAN or GPIB instrument connection and mouse.

(**f**) *Syringe*.—23 gauge straight shank needle (Agilent Technologies or equivalent).

(**g**) *Injection port liners*.—Microbial ID, Inc. (Newark, DE).

(**h**) *Incubator*.—Capable of maintaining 35° ± 1°C. Do not use CO_2 with the incubator.

(**i**) *Water bath*.—95°–100°C and 80° ± 1°C. Do not use heating block.

(**j**) *Vortex shaker*.—Fisher Vortex Genie 2, or equivalent.

(**k**) *Inoculating loop*.—4 mm.

(**l**) *Hematology/chemical mixer-rotator*.

(**m**) *Pipeter*.—Adjustable volume, 2 and 5 mL.

(**n**) *Pasteur pipets*.—5 in. disposable.

(**o**) *Autosampler vials*.—2 mL, screw cap or crimp top cap (with crimper).

(**p**) *MIS calibration standard*.—Microbial ID, Inc.

(**q**) *QC organism, B. cereus ATCC 14579*.—American Type Culture Collection (ATCC; Manassas, VA).

C. Reagents

All reagents are good for 60 days after preparation and can be stored at room temperature in clean, brown, labeled 1 L bottles supplied with Teflon-lined caps.

(**a**) *Reagent 1*.—45 g sodium hydroxide (ACS grade), 150 mL methanol (LC grade), and 150 mL deionized H_2O.

(**b**) *Reagent 2*.—325 mL certified 6.00N hydrochloric acid (glass bottles) and 275 mL methanol (LC grade).

(**c**) *Reagent 3*.—200 mL hexane (LC grade) and 200 mL MTBE (LC grade).

(**d**) *Reagent 4*.—10.8 g sodium hydroxide (ACS grade) dissolved in 900 mL deionized or distilled H_2O.

(**e**) *Saturated NaCl*.—Dissolve 40 g ACS NaCl in 100 mL deionized H_2O.

D. Culture Media

Trypticase soy agar with 5% defibrinated sheep blood.—Use only Becton Dickenson BBL™ brand.

E. General Instructions

A few precautions are required to eliminate possible sample contamination by dirty glassware. Culture tubes and screw caps are reusable and are easily cleaned by soaking in a high quality biological cleaner, followed by a thorough rinse with deionized water. Culture tubes and reagent bottles must have Teflon®-lined caps. Four reagents are required to saponify the cells, esterify, extract, and base wash the fatty acid extract. Prepare the reagents in clean, brown, labeled 1 L bottles. Place a Teflon-coated stir bar in each bottle to aid in mixing. Only Teflon and glass should come in contact with the reagents. Reagent bottles should be cleaned and rinsed with deionized water before preparation of reagents. The bottle containing Reagent 3 (extraction solvent) must be totally dried before reagent preparation. All reagents are good for 60 days and can be stored at room temperature in bottles supplied with Teflon-lined caps.

Pipetting systems should have only Teflon or glass parts in contact with the reagents. Avoid the use of rubber pipet bulbs. The tips of disposable Pasteur pipets packaged with foam pads should be heated or flamed prior to use to eliminate contaminating peaks in the chromatographic analysis. Autopipets increase the speed of preparation but should be primed and checked for proper operation before each batch of samples. Check the calibration of the 2 or 5 mL dispensing pipets by dispensing reagents into a 10 mL graduated cylinder.

F. Preparation of Samples

(**a**) Set up laboratory according to the BSL-3 facility laboratory SOPs.

(**b**) Remove blood agar culture plates from storage and allow to equilibrate to room temperature.

(**c**) Carefully transcribe the sample ID code to the blood agar plate. A second QA reviewer must review the slants and blood agar culture plates to confirm accurate transition of sample ID codes.

(**d**) *Inoculate primary isolation plate*.—The quadrant streak pattern (*Sherlock Microbial Identification System Operating Manual*, 2002, MIDI, Inc.) is recommended for culturing cells on plates for identification by the MIDI method. The goal of this pattern is to create 4 densities of cells and to verify culture purity.

(**e**) *Incubate primary isolation plate.*—35° ± 1°C for 24 ± 2 h. Do not use CO_2 with the 35°C incubator.

(**f**) *Inoculate secondary isolation plate.*—Sterilize and cool the inoculation loop. The loop can be cooled by plunging it into the agar plate in an area without any cell colonies. Select a small portion of the bacteria from the least dense portion of the purity streak of the primary isolation plate, and streak onto the secondary isolation plate using the quadrant streak pattern.

(**g**) *Incubate secondary isolation plate.*—Incubate at 35° ± 1°C for 24 ± 2 h. Do not use CO_2 with the 35°C incubator.

(**h**) *Harvesting.*—Transfer approximately 40 mg cells (one heaping 4 mm loopful). At 24 h growth time, colonies should be harvested from the most dilute quadrant(s) exhibiting confluent growth along the streaking axis (log phase). For the MIDI assay, the cells are typically harvested from Quadrant 3, but for fast-growing *Bacillus* species, the harvesting may need to be done from Quadrant 3 and/or Quadrant 4. Transfer to a clean, dry, labeled 13 × 100 mm screw-cap glass culture tube. Wipe the cells off the loop and onto the lower inner surface of the culture tube.

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G. Preparation of FAMEs from Samples

(*Note*: Follow all steps exactly as described. Any deviation may result in an incorrect match.)

(**a**) *Saponification*.—Use a boiling, optionally circulating water bath. Pipet 1.0 ± 0.1 mL Reagent 1, the methanolic base, into each of the culture tubes in the bath. Close caps tightly. After a total of 30 min of saponification in the water bath, remove and set the rack of tubes in a pan of cold tap water to cool. After this step, the organism has been killed.

(**b**) *Methylation*.—Add 2.0 ± 0.1 mL Reagent 2, the methylation reagent, to each tube. Mix the solution on a Vortex mixer for 5–10 s. Close caps tightly. Heat the tubes in an 80° ± 1°C water bath for 10 ± 1 min. Remove and cool to room temperature by placing tubes in a tray of room temperature tap water.

(**c**) *Extraction*.—Add 1.25 ± 0.1 mL Reagent 3, the extraction solvent, to each tube. Close caps tightly. Place tubes in a laboratory rotator and gently mix end-over-end for 10 min. Using a clean Pasteur pipet for each sample, remove and discard the aqueous (lower) phase.

(**d**) *Base wash*.—Add 3.0 ± 0.1 mL Reagent 4, the base wash, to each tube. Close caps tightly. Gently rotate the tubes end-over-end for 5 min. A few drops of a saturated ACS grade NaCl–water solution can be added to the tube to aid in breaking the emulsion. Hold the tube vertically and roll it rapidly between the palms of the hands, and allow it to settle for a few minutes. Alternatively, brief centrifugation (3 min at 2000 rpm × *g*) is recommended to clarify the interface between the phases when an emulsion is present (this step is rarely needed).

(**e**) *Transfer of extract to sample vial.*—Label the autosampler vials for extract identification. Using a clean Pasteur pipet for each sample, transfer about 2/3 of the organic (upper) phase from the tube and transfer to a clean GC sample vial. Crimp a cap (with a PTFE septa) onto the sampler vial.

(**f**) *Delay during sample preparation.*—After extraction and removal of the aqueous phase, the organic phase can be held, refrigerated, overnight. Samples will degrade in contact with the base wash (Reagent 4). Remove the top phase promptly. The completed extract, in the crimped-top GC vial, can either be stored at room temperature for up to 7 days or refrigerated (2°–8°C) for up to 7 days before GC analysis. Following GC analysis, the extracts can be stored refrigerated (2°–8°C) for up to 7 days.

H. Calibration Standard

The MIDI method requires an external calibration standard, a mixture of the straight chain saturated fatty acids from 9 to 20 carbons in length and 5 hydroxy acids. The hydroxy compounds are especially sensitive to changes in pressure/temperature relationships and to contamination of the injection port liner. As a result, these compounds function as quality control checks for the system. Retention time data obtained injecting the calibration standard is converted to Equivalent Chain Length (ECL) data for bacterial fatty acid naming. The ECL value for each fatty acid can be derived as a function of its elution time in relation to the elution time of a known series of straight chain fatty acids. The GC and column allow windows to be set at 0.010 ECL units, giving great precision in resolution of isomers. The software compares the ECL values for the most stable series (e.g., saturated straight chain or branched chain acids) to the peak naming table's theoretically perfect values and may recalibrate internally if sufficient differences are detected [Sasser, M. (2001) Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids, MIDI Technical Note #101, www.midi-inc.com/media/pdfs/TechNote_101.pdf].

I. System Suitability

A performance qualification (PQ) table needs to be filled out with each batch of sample runs. The PQ table is a record of optimal operating parameters for the MIDI method. Each sample batch will contain 2 calibration runs, an ATCC strain of *Bacillus cereus* (14579) as a positive control and a reagent blank (containing no bacteria) as a negative control. These 2 quality control (QC) samples need to be analyzed after the calibrations and before any other samples in the batch. The positive control data is diagnostic for problems with the sample preparation procedure and is required to be above a Similarity Index (SI) of 0.600 for successful operation. An SI of at least 0.600, which demonstrates a high quality match against this well-known strain, ensures proper laboratory technique. The negative control data are diagnostic for contaminants. The second calibration analysis data are diagnostic for problems with the hardware.

J. Determination

For each new sample batch, use the following procedure:

(**a**) Inject MIDI calibration standard twice.

(**b**) Inject the QC blank.

(**c**) Inject QC *Bacillus cereus* (ATCC 14579).

(**d**) Fill out the parameters in the MIDI PQ table immediately after the 2 QC samples are run.

(**e**) If any of the parameters are out of range, stop processing samples and take corrective action.

(**f**) If the MIDI PQ table passes, proceed with the sample runs and inject each sample extract.

(**g**) The system will automatically inject an additional calibration standard after every 11th sample run.

K. Interpretation of Results

MIDI bacterial identifications are based on an SI. The SI in the MIDI system is a numerical value in the sample report, which expresses how closely the fatty acid composition of the unknown sample compares with the fatty acid composition of the BTR20 library entries. The library search presents the best matches in the BTR20 library to the unknown sample and the associated SI values for each match. An exact match would give an SI = 1.000. The culture is identified as *B. anthracis* when this is the first choice match against the database and the SI exceeds 0.400. This cutoff was selected using information gained in the inclusivity/exclusivity study.

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The MIDI system automatically checks samples for a variety of quality controls including sufficient total GC response and GC peaks that are correctly formed. Samples that do not meet the quality specifications are *flagged* by the system with a message describing the problem. After following the instructions to correct the problem for that sample, a flagged sample is re-run. If it is still flagged, then that sample is invalid. A flagged sample is always considered to be invalid.

(**a**) *Determination of Bacillus anthracis positive or negative*.—In order to determine if a sample is *B. anthracis* positive, one should apply the following rules:

(*1*) If the sample is flagged, then the sample is invalid.

(*2*) If BA SI < 0.400 or BA SI < NN SI, then the sample is *Bacillus anthracis* negative.

(*3*) If BA SI ≥ 0.400 and BA SI ≥ NN SI, then the sample is *Bacillus anthracis* positive.

BA SI is the highest similarity index reported to the MIDI system for *B. anthracis*. NN SI is the highest similarity index reported to the MIDI system for a nearest neighbor entry.

The 0.400 SI cutoff for *B. anthracis* is designed to ensure that the match against the database reflects a definitive species-level match.

(**b**) *Sensitivity rate*.—The sensitivity rate is the probability that the method will classify a test sample as positive, given that a test sample is a known positive. An estimate of sensitivity (p_+) is obtained by counting the number of known positives for the test method (a_i) out of the *B. anthracis* test samples (n_i) for the ith laboratory:

$$p_+ = \frac{\sum_1^L a_i}{\sum_1^L n_i}$$

(**c**) *Specificity rate*.—The specificity rate is the probability that the method will classify a test sample as negative, given that a test sample is a known negative. An estimate of specificity (p_-) is obtained by counting the number of known negatives for the test method (b_i) out of the near neighbor test samples (m_i) for the ith laboratory:

$$p_- = \frac{\sum_1^L b_i}{\sum_1^L m_i}$$

Ref.: *J. AOAC Int*. **88**, 178–181(2005)

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