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Characterization of the *Clostridium difficile* volatile metabolome using comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry

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ABSTRACT

Clostridium difficile is a bacterial pathogen capable of causing life-threatening infections of the gastrointestinal tract characterized by severe diarrhea. Exposure to certain classes of antibiotics, advanced age, and prolonged hospitalizations are known risk factors for infection by this organism. Anecdotally, healthcare providers have reported that they can smell *C. difficile* infections in their patients, and several studies have suggested that there may indeed be an olfactory signal associated with *C. difficile*-associated diarrhea.

In this study, we sought to characterize the volatile molecules produced by an epidemic strain of *C. difficile* (R20291) using headspace solid-phase microextraction (HS-SPME) followed by two-dimensional gas chromatography time-of-flight mass spectrometry (GC×GC-TOFMS). We report on a set of 77 volatile compounds, of which 59 have not previously been associated with *C. difficile* growth *in vitro*. Amongst these reported compounds, we detect both straight-chain and branched-chain carboxylic acids, as well as *p*-cresol, which have been the primary foci of *C. difficile* volatile metabolomic studies to-date. We additionally report on novel sulfur-containing and carbonyl-containing molecules that have not previously been reported for *C. difficile*. With the identification of these novel *C. difficile*-associated volatile compounds, we demonstrate the superior resolution and sensitivity of GC×GC-TOFMS relative to traditional GC–MS.

1. Introduction

Clostridium difficile is a bacterial pathogen that causes a severe gastrointestinal disease known as *Clostridium difficile*-associated diarrhea (CDAD). Major risk factors for the development of CDAD include advanced age, prior antibiotic use, and prior hospital admission [1,2]. It is estimated that the 500,000 infections caused by *C. difficile* each year cost the United States health care system greater than $3.2 billion [3,4], and can result in a 30-day mortality that exceeds 25% amongst critically-ill patients [5–9]. Current diagnosis of CDAD relies on the identification of *C. difficile*-associated toxins or toxin-encoding genes in patient stool, using a variety of techniques including polymerase chain reaction (PCR) and enzyme immunoassay (EIA). Detection assays used in combination have the best test performance, with sensitivities ranging from 0.68 to 1.00 and specificities ranging from 0.92 to 1.00 [10].

Healthcare providers have reported that they can smell a *C. difficile* infection based on the odor of patient diarrhea. Several studies involving both humans and dogs suggest that there may indeed be some olfactory signal associated with CDAD. Two studies using humans have reported sensitivities ranging from 0.55 to 0.84 and specificities ranging from 0.77 to 0.83 [11,12] for the detection of CDAD, while two studies involving a trained dog have reported sensitivities ranging from 0.83 to 0.86 and specificities ranging from 0.97 to 0.98 [13,14]. The findings from these studies suggest that there is a volatile molecular signature associated with CDAD.

Straight-chain and branched-chain carboxylic acids, as well as the aromatic alcohol *p*-cresol, have been identified in the extrac-

Abbreviations: BHIS, brain heart infusion supplemented broth; CDAD, *Clostridium difficile* associated diarrhea; GC×GC-TOFMS, two-dimensional gas chromatography time-of-flight mass spectrometry; HS-SPME, headspace solid-phase microextraction; RI, retention index; RT, retention time.

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tants of either *C. difficile* cultures [15–22] or patient stool [23–25] using GC coupled to either a flame ionization detector (GC-FID) [16–25] or thermal conductivity detector (GC-TCD) [15]. Alternatively, headspace volatiles from *in vitro C. difficile* cultures have been analyzed via proton-transfer-reaction time-of-flight mass spectrometry (PTR-TOF-MS) [26], GC-FID [20,27–29], and GC coupled to mass spectrometry (GC–MS) [29,30], while those from fecal samples have been analyzed using only GC–MS [30–32]. Studies that have analyzed *C. difficile* headspace volatiles have reported on acids, alcohols, amines, esters, and sulfides, while those that have focused on volatile compounds present in the extractants of *C. difficile* cultures have predominantly reported on only carboxylic acids.

Two-dimensional gas chromatography time-of-flight mass spectrometry (GC × GC-TOFMS) utilizes two capillary columns of dissimilar (often orthogonal) stationary phase connected in series, via an on-column cryogenic modulator that sequentially transfers effluent from the first column onto the second [33]. The additional resolution provided by the second dimension improves spectral purity and facilitates the detection and identification of low abundance peaks that might otherwise co-elute with more abundant or chemically similar analytes in the first dimension [34]. GC × GC-TOFMS's improved sensitivity and resolution relative to traditional GC–MS makes it particularly well-suited for the analysis of complex mixtures containing thousands of analytes, including petrochemicals [35] and breath [36]. Recently, one application of GC × GC-TOFMS has been the analysis of bacterially-derived volatile metabolites produced *in vitro* [34,37,38]. This novel application has resulted in the identification of volatile compounds that had not previously been detected using other analytical techniques and has drastically increased the number of volatile molecules associated with both *Pseudomonas aeruginosa* [34] and *Klebsiella pneumoniae* [37].

In this exploratory analysis of the *C. difficile* volatile metabolome, we utilized headspace solid-phase microextraction (HS-SPME) followed by GC × GC-TOFMS for the analysis of volatile molecules produced by cultures of *C. difficile* grown in brain-heart infusion supplemented (BHIS) media. We identified 77 volatile molecules associated with the *in vitro* growth of *C. difficile*, of which 59 had not previously been reported. In total, these molecules increase the total number of *C. difficile*-associated volatile molecules by more than three-fold.

2. Experimental

2.1. Bacterial strains, culture conditions, and sample preparation

Clostridium difficile strain R20291 (isolated from a 2006 outbreak in the United Kingdom [39]) was grown in brain heart infusion-supplemented (BHIS) broth (37 g Bacto™ Brain Heart Infusion (Becton Dickinson (BD), Franklin Lakes, NJ, USA), 5 g yeast extract (Fisher Scientific, Hampton, NH, USA), and 10 mL 10% cysteine (w/v) per liter) [40] overnight, diluted 1:50 into fresh media, and grown to mid-exponential (4 h) and stationary (7 h) phases of growth under anaerobic conditions. Cultures were centrifuged at 7000 rpm for 5 min, and supernatants were transferred to 15 mL conical flasks and stored at −20 °C. Immediately prior to analysis, 4 mL of culture supernatants were transferred to 20 mL headspace vials containing a magnetic stir bar.

2.2. HS-SPME and GC × GC-TOFMS parameters

Headspace volatiles were concentrated using a 2 cm triphasic Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) solid-phase microextraction (SPME) fiber (Supelco, Bellefonte, PA, USA) (30 min, 45 °C, 250 rpm). The GC × GC-TOFMS (Pegasus 4D, LECO Corporation, St. Joseph, MI, USA) was equipped with a rail autosampler (MPS, Gerstel, Linthicum Heights, MD, USA) and fitted with a two-dimensional column set consisting of an Rxi®-624Sil (60 m × 250 μm ID × 1.4 μm d_f) first column (Restek, Bellefonte, PA, USA), followed by a Stabilwax (Crossbond Carbowax polyethylene glycol; 1 m × 250 μm ID × 0.5 μm d_f) second column (Restek). The main oven containing column 1 was held at 35 °C for 30 s, and ramped at 7.0 °C/min from 35 °C to 230 °C. The secondary oven containing column 2, and quad-jet modulator (2 s modulation period, 0.5 s alternating hot and cold pulses), were heated in step with the primary oven with +5 °C and +25 °C offset relative to the primary oven, respectively. The helium carrier gas flow rate was 2 mL/min (constant flow mode). A splitless injection was used, with a 180 s desorption time. The inlet and transfer line temperatures were set to 270 °C and 250 °C, respectively. Mass spectra were acquired over the range of 30 to 500 *m/z*, with an acquisition rate of 200 spectra/s. Detector voltage offset was set at 50 V. Data acquisition and analysis was performed using ChromaTOF software, version 4.50 (LECO Corporation).

2.3. Determination of retention indices

Retention indices (RIs) were calculated using external alkane standards (C_6–C_{14}). The SPME fiber was exposed to a vial containing a pure retention index mixture (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at 50 °C and desorbed at a 30:1 split. Experimental RIs are between the literature values for polar and non-polar column sets, due to the midpolarity of the Rxi®-624Sil stationary phase. Retention indices less than 600 or greater than 1400 (corresponding to C_6 and C_{14}, respectively) were not extrapolated.

2.4. Data processing and chromatographic alignment

Chromatographic data was processed and aligned using ChromaTOF. For peak identification, a signal-to-noise (S/N) threshold was set at 100:1, and resulting peaks were identified by a forward search of the NIST 2011 library. A forward match score of at least 700 was required for putative compound identification. For the alignment of peaks across chromatograms, maximum first and second dimension retention time deviations were set at 6 s and 0.2 s, respectively, and the inter-chromatogram spectral match threshold was set at 600. For peak identification, a S/N ratio of 100:1 was required in at least 1 chromatogram, and a minimum of 10:1 S/N ratio in all others.

2.5. Statistical analysis and compound selection

The relative abundance of compounds across chromatograms was first normalized using Probabilistic Quotient Normalization [41]. All reported compounds fulfilled the following six criteria:

1) A S/N threshold of greater than 100:1 in one chromatogram from stationary and/or mid-exponential phase cultures, and at least 10:1 in all others;
2) Detection only in *C. difficile* cultures (not detected in sterile BHIS) *or* 5-fold or greater relative abundance (total ion current (TIC)) in *C. difficile* cultures relative to sterile BHIS;
3) Detection in all biological replicates ($n = 5$ per sampling time) from stationary phase and/or mid-exponential phase cultures;
4) Average peak area (TIC) in *C. difficile* cultures minus one standard deviation was greater than the average peak area in sterile BHIS plus one standard deviation, *or*, for compounds not detected in sterile BHIS, average peak area in *C. difficile* cultures minus one standard deviation was > 0;